MONSANTO

Forage Genetics

Petition for Determination of Nonregulated Status:
Roundup Ready® Alfalfa (*Medicago sativa* L.)
Events J101 and J163

The undersigned submits this petition under 7 CFR Part 340.6 to request that the Administrator make a determination that the article should not be regulated under 7 CFR part 340.

Submitted by:

Glen Rogan
Regulatory Affairs Manager
Monsanto Company
800 N. Lindbergh Blvd.
St. Louis, MO 63167

And

Sharie Fitzpatrick
Director Regulatory Affairs
Forage Genetics International
N5292 S. Gills Coulee RD
West Salem, WI 54669

Submitted April 16, 2004

Petition # 04-AL-116U

OECD Unique Identifiers:
Event J101 - MON-ØØ1Ø1-8
Event J163 - MON-ØØ163-7

Prepared by:
Glen Rogan and Sharie Fitzpatrick

List of Contributors:
Karu Karunanandaa, Daniel Kendrick, Melinda McCann, Todd Pester, Elizabeth Petersen, Jon Watson

No CBI

Release of Information

Monsanto and Forage Genetics are submitting the information in this petition for review by the USDA as part of the regulatory process. By submitting this information, Monsanto and Forage Genetics do not authorize its release to any third party. In the event the USDA receives a Freedom of Information Act request, pursuant to 5 U.S.C., § 552, and 7 CFR Part 1, covering all or some of this information, Monsanto and Forage Genetics expect that, in advance of the release of the document(s), USDA will provide Monsanto and Forage Genetics with a copy of the material proposed to be released and the opportunity to object to the release of any information based on appropriate legal grounds, e.g., responsiveness, confidentiality, and/or competitive concerns. Monsanto and Forage Genetics understand that a copy of this information may be made available to the public in a reading room and by individual request, as part of a public comment period. Except in accordance with the foregoing, Monsanto and Forage Genetics do not authorize the release, publication or other distribution of this information (including website posting) without Monsanto and Forage Genetics' prior notice and consent.

Petition For Determination of Nonregulated Status for Roundup Ready Alfalfa (*Medicago sativa* L.) Events J101 and J163

Summary

The Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA) has responsibility, under the Plant Protection Act (7 CFR U.S.C. sections 7701-7772), to prevent the introduction and/or dissemination of plant pests into the United States or interstate introduction and/or dissemination. The APHIS regulations, at 7 C.F.R. § 340.6, provide that an applicant may petition APHIS to evaluate submitted data to determine that a particular regulated article does not present a plant pest risk and should no longer be regulated. If APHIS determines that the regulated article does not present a plant pest risk, the petition is granted, thereby allowing unrestricted introduction of the article.

Monsanto Company and Forage Genetics International are submitting this request for a determination of nonregulated status to APHIS for Roundup Ready® alfalfa (*Medicago sativa* L.) events which are tolerant to Roundup, the active ingredient in Roundup® agricultural herbicides. The glyphosate tolerance of Roundup Ready alfalfa events J101 and J163 was imparted by the insertion of a 5-enolpyruvylshikimate-3-phosphate synthase (epsps) gene from *Agrobacterium* sp. strain CP4 (*cp4 epsps*) into the alfalfa genome. When alfalfa plants containing the inserted gene are treated with Roundup herbicide, the plants are unaffected since the continued action of the expressed tolerant CP4 EPSPS enzyme provides the plant's need for aromatic amino acids (OECD, 1999; Padgette et al., 1996).

Alfalfa (*Medicago sativa* L.) is a perennial plant species and is one of the most important forage crops in the world. Forage Genetics International (FGI) and Monsanto Company are jointly developing Roundup Ready alfalfa technology for weed control in alfalfa. Roundup Ready alfalfa varieties will be commercialized using a combination of two different *cp4 epsps* insertion events combined through a conventional breeding process.

Roundup Ready alfalfa events were produced using an *Agrobacterium*-based plant transformation system. The transformation vector PV-MSHT4 contains a single copy of the *cp4 epsps* gene. The *cp4 epsps* gene was derived from the donor organism *Agrobacterium* sp. strain CP4. Characterization of the inserts present in the Roundup Ready alfalfa events showed that each insert contained a single intact copy of the *cp4 epsps* gene. No elements present in the transformation vector other than the expected *cp4 epsps* gene cassette and border sequences were detected in the alfalfa genome. The CP4 EPSPS protein expressed in Roundup Ready alfalfa events J101 and J163 has been characterized and is homologous to plant and microbial EPSPS proteins which have an established history of safe use.

Segregation analysis of the glyphosate-tolerant phenotype confirmed that glyphosate tolerance is inherited as a single Mendelian trait. Southern blot analysis confirmed the stability of each insert when combined through conventional breeding. The mean level of

® Roundup and Roundup Ready are registered trademarks of Monsanto Technology LLC.

CP4 EPSPS protein across two seasons and from multiple cuttings of forage was 257 and 270 micrograms/gram on a tissue fresh weight basis for alfalfa plants that contained events J101 and J163, respectively.

Phenotypic data and other information presented in this Petition demonstrate that it is no more likely that the regulated articles will present a plant pest risk than conventional alfalfa for the following reasons: 1) no biologically meaningful agronomic or phenotypic differences in alfalfa populations containing either event J101 or J163 were detected over multiple years and locations in comparison to control and conventional alfalfa populations, indicating that there were no increased weediness or competiveness in alfalfa populations containing either of the Roundup Ready events; 2) alfalfa populations containing either of the transformation events were no more susceptible to disease or insect pests than conventional alfalfa populations; 3) the composition and quality of forage derived from alfalfa populations containing the Roundup Ready alfalfa events were comparable to the composition and quality of forage derived from control and conventional alfalfa varieties, and 4) alfalfa has no relatives present in North America for hybridization, and gene flow can only occur to other cultivated or feral alfalfa. As such, the *cp4 epsps* gene and the CP4 EPSPS protein do not confer plant pest characteristics to alfalfa.

The environmental consequences of the introduction of Roundup Ready alfalfa events J101 and J163 were considered and there is no reason to believe that these events would have an adverse impact on organisms beneficial to plants or to nontarget organisms, including threatened or endangered organisms, based on the following: 1) the impact on crop rotational practices will be minimal because there are numerous options available for control of volunteer alfalfa; 2) the Roundup Ready trait does not confer a biologically meaningful fitness advantage to alfalfa and, while the trait may eventually move through gene flow to feral alfalfa populations, glyphosate is not used to any extent to control feral alfalfa populations; 3) the EPSPS family of proteins, and specifically CP4 EPSPS as produced in a number of Roundup Ready crops, has been shown to be comparable to the EPSPS proteins present in other food crops and common microbes; and 4) there has been no adverse environmental impact from the commercial planting of other Roundup Ready crops which contain the CP4 EPSPS protein.

The use of a Roundup Ready alfalfa weed control system can benefit current agronomic practices in alfalfa forage and seed production by: (1) offering the producer a wide-spectrum weed control option that will enhance the effectiveness of stand establishment and increase alfalfa forage and seed purity through better weed control of most of the weeds that impact forage and seed production; (2) increasing flexibility to treat weeds on an as-needed basis; (3) allowing alfalfa production on marginal land with severe weed infestations; and (4) providing growers with a weed control system that has a reduced risk profile for the environment.

Data and information in this request demonstrate that events J101 and J163 do not represent a unique plant pest risk. Therefore, Monsanto Company and Forage Genetics International request a determination from APHIS that events J101 and J163 and any progenies derived from conventional breeding of these events, no longer be considered regulated articles under regulations in 7 CFR part 340.

Abbreviations, Definitions and Acronyms[1]

~	Approximately
§	Section
3'	The distal, or growing end, of an mRNA transcript; the end nearest to or containing the polyA sites
5'	The proximal, or start end, of an mRNA transcript; the end nearest to or containing the P-eFMV promoter
A	Adenine
aad	Bacterial promoter and gene encoding an aminoglycoside-modifying endonuclease, 3'(9)-O-nucleotidyltransferase from the transposon Tn7
a.e.	Acid equivalent
a.i./A/yr	Active ingredient per Acre per year
BCA	Bicinchoninic acid
bp	Nucleotide base pairs
C	Cytosine
ca.	Approximately
CaMV	Cauliflower mosaic virus
Ci	Curie
cp4 epsps	Coding sequence for the native EPSPS protein from *Agrobacterium sp.* strain CP4
CP4 EPSPS	EPSPS protein from *Agrobacterium sp.* strain CP4
CsCl	Cesium chloride
CTAB	Cetyltrimethylammonium bromide
CTP2	Chloroplast transit peptide sequence from *Arabidopsis*
ctp2	DNA sequence coding for CTP variant 2
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
Da	Daltons
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
dTTP	Deoxythymidine triphosphate
E. coli	*Escherichia coli*
E9 3'	A 3' nontranslated region of the pea ribulose-1,5-bisphosphate carboxylase, small subunit (*rbc*) E9 gene
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPSP	5-Enolpyruvylshikimate-3-phosphate
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase protein from *Agrobacterium sp.* strain CP4
EtOH	Ethanol
g	Gram
g	Gravity
G	Guanine

Abbreviations, Definitions and Acronyms continued

HCl	Hydrochloric acid
HSP70	Petunia heat shock protein 70 5' untranslated leader sequence
Kb	Nucleotide kilobase pairs
kDa	KiloDalton
LB	Left border
lb/A	Pounds per Acre
M	Molar
MBC	Modified backcross
min	Minutes
$MgCl_2$	Magnesium chloride
ml	Milliliter
mM	Millimolar
mRNA	Messenger RNA
MW	Molecular weight
Na_2HPO_4	Disodium phosphate
NaCl	Sodium chloride
NaOAc	Sodium acetate
NaOH	Sodium hydroxide
NDF	Neutral Detergent Fiber
NH_4OAc	Ammonium acetate
NOS	Nopaline synthase
NPTII	Neomycin phosphotransferase II
OECD	Organization for Economic Co-operation and Development
ori	Origin of replication
ori-322	Origin of replication for *E. coli*
ori-V	Bacterial origin of replication from RK2 plasmid
PCR	Polymerase chain reaction
P-eFMV	Promoter from figwort mosaic virus
PEP	Phosphoenolpyruvate
PVP	Polyvinylpyrolidone
PV-MSHT4	Plasmid vector used for transformation of alfalfa
RB	Right border
rop	Repressor of primer
rpm	Revolutions per minute
RR	Roundup Ready
RT	Room temperature
s	Seconds
S3P	Shikimate-3-phosphate
SDS	Sodium dodecyl sulfate
sp.	Species
spp	Subspecies
SSC	Saline-sodium citrate buffer (20X SSC is 3 M sodium chloride, 0.3 M)
Syn 1,2,3	Synthetic alfalfa populations

Abbreviations, Definitions and Acronyms (continued)

T	Thymine
T-DNA	Transfer DNA
TE	Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)
TFW	Tissue Fresh Weight
TMB	(3,3',5,5' Tetramethylbenzidene) peroxidase substrate
Tris-HCl	Tris(hydroxymethyl)aminomethane
tRNA	Transfer ribonucleic acid
U	Units
U.S.C.	United States Code
UV	Ultraviolet
µM	micro Molar
µCi	micro Curie
µg	micro gram
µL	micro Liter
V	Volts

[1]Standard abbreviations, e.g., units of measure, will be used according to the format described in 'Instructions to Authors' in the *Journal of Biological Chemistry*

Certification

The undersigned certifies that, to the best knowledge and belief of the undersigned, this petition includes all information and views on which to base a determination, and that it includes all relevant data and information known to the petitioners, which are unfavorable to the petition.

Glen Rogan
Regulatory Affairs Manager
Monsanto Company
800 N. Lindbergh Blvd.
St. Louis, MO 63167

Sharie Fitzpatrick
Director Regulatory Affairs
Forage Genetics International
N5292 S. Gills Coulee RD
West Salem, WI 54669

Table of Contents

List of Tables and Figures
Table number, title and page

Figure number, title and page:

I. Rationale for Submission of Request for Determination of Nonregulated Status

A. Basis for Request for a Determination of Nonregulated Status under 7 CFR Part 340.6

The Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture (USDA) has responsibility, under the Plant Protection Act (7 U.S.C. § 7701-7772) and the Plant Quarantine Act (7 U.S.C. § 151-167) to prevent the introduction and dissemination of plant pests into the United States. The APHIS regulations 7 CFR 430.6 provide that an applicant may petition APHIS to evaluate submitted data to determine that a particular regulated article does not present a plant pest risk and should no longer be regulated. If APHIS determines that the regulated article does not present a plant pest risk, the petition is granted, thereby allowing unrestricted introduction of the article.

B. Roundup Ready Alfalfa Events J101 and J163

Monsanto Company and Forage Genetics International (FGI) have developed Roundup Ready® alfalfa events J101 and J163 that are tolerant to glyphosate, the active ingredient in the Roundup® family of agricultural herbicides. In accordance with the Organisation for Economic Co-operation and Development's (OECD) "Guidance for the Designation of a Unique Identifier for Transgenic Plants," J101 has been assigned the unique identifier MON-ØØ1Ø1-8 and J163 has been assigned the unique identifier MON-ØØ163-7. These events were developed using *Agrobacterium*-mediated transformation to stably incorporate the *cp4 epsps* coding sequence derived from the native soil microorganism, *Agrobacterium* sp. strain CP4, into the alfalfa genome. The 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme is functionally similar to plant EPSPS enzymes but has a greatly reduced affinity for glyphosate (Padgette et al., 1996). In plants, glyphosate binds to the plant EPSPS enzyme and blocks the biosynthesis of aromatic amino acids, thereby preventing plant production of these essential compounds (Steinrucken and Amrhein, 1980; Padgette et al., 1996). In Roundup Ready alfalfa, the biosynthesis of aromatic amino acids is maintained by the continued action of the CP4 EPSPS enzyme in the presence of glyphosate.

Roundup Ready alfalfa will enable the use of Roundup agricultural herbicides to provide effective weed control during forage and seed production. Roundup agricultural herbicides are highly effective against the majority of annual and perennial weeds common to alfalfa seed and forage production. Roundup herbicides also have excellent environmental safety features, such as rapid soil binding (making them resistant to leaching), as well as low toxicity to mammals, birds and fish. In addition, glyphosate is one of the few herbicidal active ingredients classified as Category E by the Environmental Protection Agency (EPA) (evidence of noncarcinogenicity for humans) (57 FR 8739).

Events J101 and J163 will be combined through conventional breeding to produce commercial Roundup Ready alfalfa seed. Roundup Ready alfalfa varieties will consist of a segregating population of individuals containing either event J101, event J163, a

® Roundup and Roundup Ready are registered trademarks of Monsanto Technology LLC.

combination of J101 and J163 or neither event. The breeding process for Roundup Ready alfalfa is described by Samac and Temple (in press).

C. Benefits of Roundup Ready Alfalfa Events J101 and J163

Roundup Ready alfalfa will offer growers a weed control tool to address many of their problems and concerns. The product concept will allow for glyphosate applications at preplant, preemergence, and/or postemergence timings in both seedling and established Roundup Ready alfalfa, thus offering control of weeds when most appropriate.

Current weed control programs in alfalfa production have significant practical limitations for growers, including:

- application timing restrictions;
- need for cultivation or irrigation for effective weed control;
- application restrictions related to air temperature, soil type and water;
- narrow weed control spectrum - tank mixing or application of multiple products is often necessary;
- significant risk of crop injury;
- carryover concerns and rotation restrictions;
- substantial pre-harvest interval restrictions;
- water contamination issues;
- endangered species concerns;
- incomplete control of some weeds; and
- variability in product performance.

Key benefits of the Roundup Ready alfalfa system will include:

Excellent broad-spectrum control and flexibility for season-long control. Using the Roundup Ready alfalfa system, growers can control troublesome perennial weeds such as quackgrass, Johnsongrass and nutsedge. Many products currently in use have a narrow window of application based on specific weed size or crop stage. In-crop Roundup herbicide applications can be made from crop emergence up to five days before cutting regardless of weed size. This flexibility will allow the grower a wide window of application, and allow application timing based on weed pressure, not on crop or weed stages.

Control weeds that are poisonous to livestock. Using the Roundup Ready alfalfa system, growers can control weeds such as fiddleneck, starthistle, and groundsel.

Control of noxious parasitic weeds. Using the Roundup Ready alfalfa system, growers can control parasitic weeds like dodder in forage and seed production.

Greater success in stand establishment. Weed competition for moisture, nutrients and light is detrimental to the establishment of new alfalfa stands and to the subsequent quality and yield of hay. Limitations of currently available products often force growers to use alternative approaches such as: 1) seeding with a cover or companion crop which may suppress weeds but also competes with alfalfa; 2) delaying first cutting for a minimum of 60 days, thus sacrificing hay quality to allow surviving alfalfa to get ahead of the competing weeds; or 3) delaying seeding of new stands until late summer or early fall when there is less weed competition. All of these alternative approaches result in

considerable loss in a grower's first-year production that can reach $300 per acre. The flexibility and ease of use of Roundup agricultural herbicides will allow growers to seed new stands and eliminate weeds early with little risk of stand, yield, or quality loss.

Improved forage quality and higher yields. Weed infestations are a major limiting factor in the production of high-quality alfalfa forage. The value per ton of alfalfa forage to both commercial hay and livestock producers is based largely on forage quality. A weed infestation of as little as 10% of the field can result in as much as a 15% decrease in quality and a significant decrease in the dollar per ton value; high-quality forage commands a higher price per ton. It is anticipated that the superior weed control performance of Roundup agricultural herbicides with Roundup Ready alfalfa will lead to healthier and more vigorous stands that will generate higher quality forage and higher yields (more tonnage of alfalfa per acre).

Potential for reduced cultivation. The environmental benefits of reduced tillage, such as erosion control, reduced use of fossil fuels, and improved soil quality, are well known to growers. Many of the available weed control products used in alfalfa production, require immediate soil incorporation and thorough tillage is usually recommended. Such added cultivations would be eliminated with the use of Roundup agricultural herbicides in Roundup Ready alfalfa.

Improved weed control flexibility in established stands. Because alfalfa is a perennial crop, perennial weeds often reestablish themselves each season, competing for water and nutrients and affecting the quality of harvested forage. If not controlled, perennial weeds compete with the alfalfa and effectively shorten the productive life of the stand. Winter annual weeds are also a serious problem where non-dormant alfalfa varieties are grown and harvested all year long. These weeds tend to flourish during the cooler winter months and current products are limited in their effectiveness in controlling such weeds during this time period. The use of Roundup agricultural herbicides will give growers much greater flexibility in controlling tough perennial weeds and winter annual weeds in established stands.

Alternate mode of action. The in-crop use of Roundup agricultural herbicides in alfalfa will provide growers with an additional, unique mode of action for managing herbicide resistant weeds.

Excellent crop safety. Other herbicides labeled for use in alfalfa can cause crop injury, particularly when applied at the incorrect crop stage under stressful environment conditions. Roundup Ready alfalfa has demonstrated outstanding crop safety in field tests. This reduction in the potential of herbicide crop injury, along with greater weed control, will give growers the opportunity to maximize yield.

D. Submissions to Other Regulatory Agencies

D.1. Submission to FDA

Roundup Ready alfalfa events J101 and J163 are within the scope of the FDA policy statement concerning regulation of products derived from new plant varieties, including those developed through biotechnology, which was published in the Federal Register on May 29, 1992. In compliance with this policy, Monsanto submitted to FDA a food and

feed safety and nutritional assessment summary for events J101 and J163 in October of 2003.

D.2. Submission to EPA

The EPA has authority over the use of pesticidal substances, under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA), as amended (7 U.S.C. § 136[u]). The submission of glyphosate residue data and proposed labeling for the use of Roundup UltraMAX® herbicide (EPA Reg. No. 524-512) over the top of Roundup Ready alfalfa was made to the EPA on March 28, 2002. A subsequent petition for Reduced Risk status was submitted to the EPA on June 27, 2002, and the EPA granted Reduced Risk status for review of the data on July 23, 2002. The proposed use of Roundup UltraMAX herbicide on Roundup Ready alfalfa will not require an increase in the glyphosate residue tolerance of 400 ppm in the animal feed, non-grass group. A new glyphosate tolerance for alfalfa seed of 0.5 ppm has been proposed.

Pursuant to section 408(d) of the Federal Food Drug and Cosmetic Act (FFDCA), 21 U.S.C. 346 a(d), the EPA has previously reviewed and established an exemption from the requirement for a tolerance for the CP4 EPSPS protein and the genetic material necessary for the production of this protein in or on all raw agricultural commodities (40 CFR 180.1174).

D.3. Submissions to Foreign Governments

Regulatory submissions for import and production approvals have been made to several countries, and additional submission will be made to other countries that import U.S. alfalfa forage and have regulatory approval processes in place. Submissions were made in December of 2003 to Health Canada and the Canadian Food Inspection Agency (CFIA) as well as to Mexico's Health Ministry. Submissions are planned for the Japanese Ministry of Health, Labor and Welfare (MHLW) and Ministry of Agriculture Forestry and Fisheries (MAFF). As appropriate, notifications of import will be made to importing countries that do not have a formal approval process.

® Roundup UltraMAX is a registered trademark of Monsanto Technology LLC.

II. The Alfalfa Family

A number of excellent references provide an extensive review of the alfalfa family including *Alfalfa and Alfalfa Improvement* (Hanson et al., 1988). In accordance with Section 99-3 of the USDA's "Guide for Preparing and Submitting a Petition for Genetically Engineered Plants," this reference is cited as a broad review of the family. Also pertinent to this petition is an OECD consensus document on the genes and their enzymes that confer tolerance to Roundup, the active ingredient in Roundup agricultural herbicides (OECD, 1999). The following sections provide a brief review of the origin, use, biology, taxonomy, genetics, related species and history of development of alfalfa.

A. Alfalfa as a Crop

Alfalfa (*Medicago sativa* L.) is recognized as the oldest plant grown solely for forage. Alfalfa is the most important forage crop species in the United States and Canada and is recognized as the most widely adapted agronomic crop. Alfalfa forage is valued at over $60B dollars annually. Although alfalfa is grown in all continental states, the crop value is highest in the West, Plains and upper Midwest. The United States has over 23 million acres of alfalfa under cultivation. Forty percent of these acres are pure stand, 35 percent are planted with a cover (nurse) crop and the remaining 25 percent are planted with a companion crop, usually a grass. This total acreage has been quite stable over the past ten years.

Alfalfa is highly valued for animal feed because of its high protein content, high intake potential, and digestibility. Alfalfa can provide the sole plant component in many livestock feeding programs when supplemented with the proper minerals.

B. Taxonomy and Genetics of Alfalfa

Medicago sativa L. belongs in the order Fabales, family Fabaceae, tribe Trifolieae, genus *Medicago*. The genus *Medicago* is very extensive, consisting of more than 60 different species; two thirds of the species are annuals and one third are perennials (Quiros and Bauchan, 1988).

Commercially cultivated alfalfa properly belongs to the *M. sativa* complex, a group of closely related subspecies that are interfertile and share the same karyotype. The most commonly cultivated alfalfa in the world is *M. sativa* subsp. *sativa*, but subspecies *falcata* is also cultivated on a limited basis, primarily under rangeland conditions and in colder regions (e.g., Canada and Siberia). Other subspecies in the complex include subsp. *glutinosa*, subsp. *coerulea*, subsp. x *tunetana*, subsp. x *varia*, subsp. x *polychroa*, and subsp. x *hemicycla* (Quiros and Bauchan, 1988). Two other closely related species, *M. prostrata* and *M. glomerata*, can be considered capable of limited natural hybridization with alfalfa (Quiros and Bauchan, 1988). *M. prostrata* and *M. glomerata* do not occur naturally in North America (Table II-1). *M. glomerata* is generally listed as one parent of subsp. x *tunetana*, which occurs in North Africa (Lesins and Lesins, 1979).

Cultivated alfalfa, *M. sativa* subsp. *sativa*, is a tetrasomic tetraploid (2n = 4x = 32), characterized by purple flowers and coiled pods (Quiros and Bauchan, 1988). Subsp. *falcata* occurs both as tetraploid and diploid (2n = 2x = 16) accessions and has yellow flowers and straight to sickle-shaped pods. Purple-flowered *M. sativa* ssp. *coerulea* is a

diploid form of *M. sativa* ssp. *sativa*. Interploidy gene flow is possible through the production of unreduced (2n) gametes (McCoy and Bingham, 1988). All other members of the *M. sativa* complex readily cross-pollinate with cultivated alfalfa; subsp. x *varia* is actually the hybrid of subsp. *sativa* and *falcata*.

C. Geographic Origin of Alfalfa and Historical Development

Alfalfa, including both cultivated alfalfa and closely related subspecies, originated in Asia Minor, Transcaucasia, Turkmenistan, and Iran, and is endemic throughout the Mediterranean region, North Africa, the Middle East, most of Europe, Siberia, northern India, and China (Ivanov, 1988; Michaud et al., 1988; Quiros and Bauchan, 1988). The following history of development of alfalfa was taken from Michaud et al. (1988). Alfalfa has been cultivated before recorded history and is now found growing wild in Asia, Europe, and North Africa. It has become acclimatized in South Africa, Australia, New Zealand, and North and South America. The oldest reference to alfalfa's use as a forage dates to 3000 B.C.E. Archeologists discovered a reference to alfalfa's use as an animal feed during the winter months on Hittite (1400-1200 B.C.E.) brick tablets excavated from an archeological site in the Corum/Alacahöyük region of Turkey. Other historical references to alfalfa are from Turkey (1300 B.C.E.) and Babylonia (700 B.C.E.). The development of alfalfa is closely tied with the spread of civilization because of its importance as an animal feed. There is widespread historical evidence of alfalfa's use in Media (northwestern Persia) in the first millennium B.C.E. Theophrastus described how invading Median armies brought alfalfa to Greece to feed their chariot horses in the fourth century B.C.E. Later, writers such as Aristophanes (440-380 B.C.E.) and Aristotle (384-322 B.C.E.) either mentioned or discussed alfalfa at length.

The Romans acquired alfalfa in the second century B.C.E. and the crop thrived and spread throughout Italy. The Romans are credited as being the fathers of forage cultivation because of their development of forage management systems. With the arrival of alfalfa into Italy, the crop also began to spread eastward into China. In 126 B.C.E. the Chinese Emperor Wu dispatched an expedition into the Russian Turkenistan area to secure prized Iranian horses. While collecting the horses, seed from the alfalfa forage used to feed the horses was also collected. Soon after this, alfalfa appeared in the gardens of the Imperial palaces throughout China. Alfalfa then became established throughout northern China as an important forage crop.

During the period of the Roman Empire (27 B.C.E.-395 C.E.), Roman colonists established alfalfa in newly acquired provinces. Separate establishments of alfalfa in Spain may have been planted by the Muslims through North Africa during the Moorish invasions. With the fall of the Roman Empire, alfalfa declined and virtually disappeared from Italy and was reintroduced in the 16th century from Spain. From the mid 15th century to the 18th century, alfalfa spread to France, Belgium, Holland, England, Germany, Austria, Sweden and Russia. In the 18th century, alfalfa was taken from Europe to the New World, Australia and New Zealand.

Spanish explorers introduced alfalfa into Mexico and Peru during the 16th century and its cultivation had spread throughout North and South America by the late 1800s. Alfalfa also was brought to South Africa, Australia, and New Zealand during the 19th century. Today, alfalfa is grown to some extent on all continents (except Antarctica), though most

extensively in temperate climates. In the United States, over 23 million acres of alfalfa or alfalfa-grass mixtures were grown for hay in 1997, and although some alfalfa was grown in most states, the twelve states with more than 700,000 acres each were California, Colorado, Iowa, Kansas, Michigan, Minnesota, Montana, Nebraska, North Dakota, Pennsylvania, South Dakota and Wisconsin (USDA, 1998).

D. Growth and Reproductive Characteristics of Alfalfa

Alfalfa (*Medicago sativa* L.) is a perennial herbaceous legume (Lesins and Lesins, 1979). Its general morphology was studied by Teuber and Brick (1988) and Barnes and Sheaffer (1995). The mature alfalfa plant is characterized by a strong taproot. This taproot can be twenty or more feet in length with several to many lateral roots connected at the crown. The crown, a complex structure near the soil surface, has perennial meristem activity, producing buds that develop into stems. Tri- or multi-foliolate leaves form alternately on the stem, and secondary and tertiary stems can develop from leaf axils. A plant in a typical forage production field has between five and 25 stems and can reach nearly three feet tall. Following harvest, regrowth occurs either directly from crown-produced buds, or from auxiliary buds developed in the remaining stubble. Flowers, borne in clusters in a raceme and attached to the central rachis, develop in leaf axils at stem apices. Stems are indeterminate so that vegetative and reproductive growth occurs simultaneously. Flowering will continue for several weeks until either the plant is harvested or the stem becomes senescent.

Alfalfa is exclusively an insect-pollinated crop that, unlike other insect-pollinated crops, is pollinated by a small number of insect species, primarily bees. Alfalfa flowers have a tripping mechanism, which is triggered by bees visiting the flower to collect nectar or pollen. After it is tripped, the stigma of the flower becomes lodged into the groove of the standard petal of the flower. Tripped flowers cannot be fertilized again. Because of the nonreversible tripping mechanism within the alfalfa flower, each alfalfa bloom may be pollinated only a single time, by a single pollinating insect. Flowers do not shed pollen to the wind. After pollination, alfalfa seed requires four to six weeks of adequate growing conditions to ripen. Rainfall during the ripening period will cause poor seed quality and decrease seed yield. Commercial production of the alfalfa seed crop, therefore, is largely confined to the regions where late season rain is unlikely and irrigation is used. Alfalfa seed is used primarily as planting stock for forage production stands and is not used as a grain for feed or food consumption.

E. Related Species

Medicago is in the tribe Trifolieae, which also includes *Trifolium* (true clovers), *Melilotus* (sweetclover), and *Trigonella* (fenugreek). *Medicago* does not hybridize with any of these (or other) genera. Within *Medicago*, at least 56 species are recognized. Small and Jomphe (1989) described 83 species in their review, the most recent complete taxonomic study. Of these species, roughly two-thirds have an annual life cycle; the others are perennial and include cultivated alfalfa, *Medicago sativa* L. Because annual x perennial hybrids cannot be produced artificially, and no evidence exists for their occurrence in nature, the annual species are not considered further. Although one report of a hybrid was published (Sangduen et al., 1982) it did not produce seed, no further research on the

hybrid was reported, and the experiment has never been repeated. *M. sativa* is the only cultivated perennial species in the genus.

The *M. sativa* complex has been successfully hybridized with 12 other perennial species (McCoy and Bingham, 1988), summarized in Table II-1. However, many of these interspecific hybrids have only been successful by using embryo culture of the hybrid (McCoy and Smith, 1986) making them highly unlikely to occur in nature. Not included in the 12 successful hybridizations are putative hybrids with *M. lupulina*. According to Lesins and Lesins (1979), and Turkington and Cavers (1979), considerable doubt exists as to whether these plants were actually of hybrid origin as the hybrid has not been confirmed or repeated (Lesins and Gillies, 1972; See Section VII-E.1 and Appendix 4). In addition to these, a hybrid between *M. sativa* and *M. arborea* was developed through protoplast fusion (Nenz et al., 1996). Thus, the realistic extent of natural gene flow from cultivated alfalfa is only to other members of the species complex and to the closely related *M. prostrata* and *M. glomerata*.

Table II-1. *Medicago* Species Hybridized to Alfalfa and their Distribution

Species	Distribution	Hybridization Method	Result[1]
M. glomerata	Southern Europe to North Africa (Quiros and Bauchan, 1988)	Hand-pollination Natural	Successful Putative ancestor to subsp. x *tunetana*
M. prostrata	Eastern Austria and Italy, eastern Adriatic coast to Greece	Hand-pollination	Successful, especially when *prostrata* is female
M. cancellata	Southeastern European Russia, north of Caucasus	Hand-pollination	Successful, but ploidy may interfere in crosses of certain genotypes because *cancellata* is a hexaploid.
M. rhodopea	Mountain ranges of Bulgaria	Hand-pollination Ovule/embryo culture	Successful, but aberrant ploidies in progeny. Successful with normal chromosome complements.
M. rupestris	Crimean mountains	Hand-pollination Ovule/embryo culture	Not successful. Successful, but F_1 plants had very low fertility and backcross progeny were only produced using ovule/embryo culture.

Table II-1. *Medicago* Species Hybridized to Alfalfa and their Distribution (continued)

Species	Distribution	Hybridization Method	Result[1]
M. saxatilis	Crimean mountains	Hand-pollination	Successful, particularly when alfalfa was maternal parent.
M. daghestanica	Mid-mountain zone of Daghestan, Russia	Hand-pollination Ovule/embryo culture Hand-pollination using trispecies bridge	No seed produced. Successful. Alfalfa was hand crossed to a *daghestanica x pironae* hybrid that had been colchicine doubled to a tetraploid; resulted in hybrid seed.
M. pironae	Eastern Alps in northeast Italy	Ovule/embryo culture; Trispecies bridges	As for *daghestanica*, *viz*. ovole/embryo culture worked directly, but for hand-pollination, a trispecies bridge was required.
M. papillosa	Pontus mountains of north-eastern Anatolia to adjacent Caucasus mountains	Hand-pollination	Successful when using uneven ploidy levels.
M. dzhawakhetica	Mountains of Transcauscasia	Hand-pollination	Successful when using uneven ploidy levels. F_1 were triploid and produced nonviable pollen. Backcrosses to alfalfa possible.
M. marina	Mediterranean and Black Sea shores, Atlantic coast of Iberia and France	Hand-pollination Ovule/embryo culture	Unsuccessful. Weak hybrids that did not produce flowers.
M. hybrida	Corbier mountains. and east Pyrenees	Ovule/embryo culture	Successful, no other data.
M. lupulina	Europe, most of Asia, North Africa, North America	Hand-pollination	Some reported hybrids, but contemporary experts contend they were selfed [also see Turkington and Cavers (1979)].

Table II-1. *Medicago* Species Hybridized to Alfalfa and their Distribution (continued)

Species	Distribution	Hybridization Method	Result[1]
M. arborea	Southern Europe from Canary Islands to Greece	Protoplast fusion	Viable hybrids formed between these sexually incompatible species (Nenz et al., 1996).
M. rugosa	Mediterranean Region	Hand-pollination with embryo rescue	Single sterile plant only, no progeny produced (Piccirilli and Arcioni, 1992)
M. scutellata	Mediterranean region	Hand-pollination	Single plant only, no progeny produced; never replicated (Sangduen et al., 1982).

[1]All data is taken from Lesins and Lesins (1979) or McCoy and Bingham (1988) unless otherwise noted. Table excludes all references to natural cross-pollination among subspecies in the *M. sativa* complex (see main text for further information).

F. Geographic Location of Compatible Species

No perennial *Medicago* species are present naturally in the Americas, Australia, New Zealand, or South Africa. Therefore, no risk for interspecific hybridization exists, but natural cross-pollination to the scattered naturalized populations of *M. sativa* would be possible.

In other areas of the world, particularly Europe, Asia, the Middle East, and North Africa, native populations of various members in the *M. sativa* complex, as well as other perennial *Medicago* species, are present (Sinskaya, 1961; Lesins and Lesins, 1979; Ivanov, 1988). Recently, Jenczewski et al. (1999) have shown that gene flow occurs naturally between cultivated and wild alfalfa populations, using both isozyme markers and analysis of quantitative traits. This study shows that cross-pollination between cultivated alfalfa and wild *M. sativa* is possible, particularly in regions with abundant native or naturalized populations.

G. Potential of Over-Wintering/Survivability/Weediness and Seed Dormancy

Alfalfa is a perennial that can survive winter temperatures as low as -20ºC (McKenzie et al., 1988). Alfalfa cultivars are bred to possess different cold tolerances depending upon the intended geographic region for growth of the cultivars. Alfalfa undergoes biochemical changes in the fall of the year that increase tolerance to low temperature stresses. While all alfalfa tissues are capable of attaining some degree of cold tolerance, crown buds are generally the most cold tolerant. The crown, a complex structure near the soil surface, has perennial meristem activity, producing buds that develop into stems. Cold tolerance is controlled by genetic and environmental factors such as temperature, photoperiod and soil environment. The degree of winter hardiness associated with an alfalfa variety is dependent upon the source of germplasm from which it was derived, with more cold tolerant varieties having germplasm that originated from colder northern

sources. Environmental factors such as decreasing photoperiod, reduced temperature in the fall, and reduced soil moisture serve to initiate the cold tolerance response and impact the survivability throughout the winter months. Ability to survive through the winter is strongly correlated to the cultivar's reaction to shortened photoperiod. Highly fall-dormant plants dramatically reduce foliar growth in the early fall, whereas extremely non-dormant plants continue to produce growth during short winter days. Fall dormancy is measured on a scale of 1 (very fall dormant) to 11 (very non-dormant).

Mature alfalfa seed often has an impermeable seed coat that prevents the uptake of water; these seeds are referred to as hard seeds (Bass et al., 1988). Both the production environment and the genotype effect of different cultivars affect the percentage of hard seeds; 40-50% hard seed is common in seed produced in Washington, Oregon, and Idaho, but seed from the southwestern U.S. is usually <30% hard (Bass et al., 1988). The genetic effect is smaller than the environmental effect on hard seed percentage (Acharya et al., 1999). To reduce the percentage of hard seed to acceptable levels (typically <10%), commercial alfalfa seed is mechanically scarified to slightly scratch the seed coat, allowing the seed to imbibe water; unscarified seed can lie dormant in the soil for many years (Bass et al., 1988). The size of the alfalfa seed bank or length of time seeds survive in soil are unclear; no citations related to the alfalfa seed bank were present in the Agricola database on August 30, 1999.

Alfalfa is not a noxious weed in continental North America (Skinner, et al., 2000; USDA-APHIS, 2000; USDA-APHIS, 2002; USDA-ARS, 2003; USDA-NRCS, 2003).

III. Description of the Transformation System

A. Description of the Transformation System

Agrobacterium-mediated transformation was carried out using a two-step procedure adapted from methods described by Walker and Sato (1981) and Austin et al. (1995). Plasmid PV-MSHT4 (Figure III-1) was used to generate Roundup Ready alfalfa events J101 and J163. *Agrobacterium tumefaciens* binary strain (ABI) is an unregistered Monsanto proprietary *Agrobacterium* strain that contains the required transacting functional region, trfA, that in the presence of an introduced plasmid with the ori-V origin of replication allows plasmid replication and maintenance in *A. tumefaciens*. The ABI strain also contains additional genes that facilitate transfer of the T-DNA of interest, contained on plasmid PV-MSHT4, into the recipient plant. Plasmid PV-MSHT4 contains the *cp4 epsps* coding region under the control of a constitutive promoter. The recipient for transformation was an alfalfa clone R2336. Line R2336 was selected from an elite, high-yielding, fall-dormant FGI alfalfa breeding population using a tissue culture screen for callus formation and somatic embryo induction.

Each resultant callus from the *Agrobacterium*-mediated transformation was initially selected for the Roundup Ready trait through the addition of glyphosate to the plant culture media. Following somatic embryo induction, the glyphosate was removed and the embryos were allowed to develop. The resulting plantlets were transferred to soil pots as the T_0 generation. Rooted stem cuttings from the T_0 plants were selected for vegetative tolerance to glyphosate through a 3.0 lb a.e./acre application of Roundup Ultra herbicide. The subsequent F1 and MBC1 generations of Roundup Ready alfalfa plants were treated with Roundup Ultra herbicide (3.0 lb a.e./acre) at the two to three trifoliate stages. Roundup Ready alfalfa events J101 and J163 were determined to be hemizygous for the trait and displayed superior vegetative and reproductive tolerance in field studies with three sequential 128 oz/acre applications of Roundup Ultra herbicide. Each application was two times the expected commercial treatment rate of Roundup Ultra herbicide on Roundup Ready alfalfa. Introgression of Roundup Ready alfalfa events J101 and J163 into new alfalfa varieties was done using FGI's breeding process. The flow diagram shown in Figure III-2 illustrates the steps in the development of the Roundup Ready alfalfa varieties.

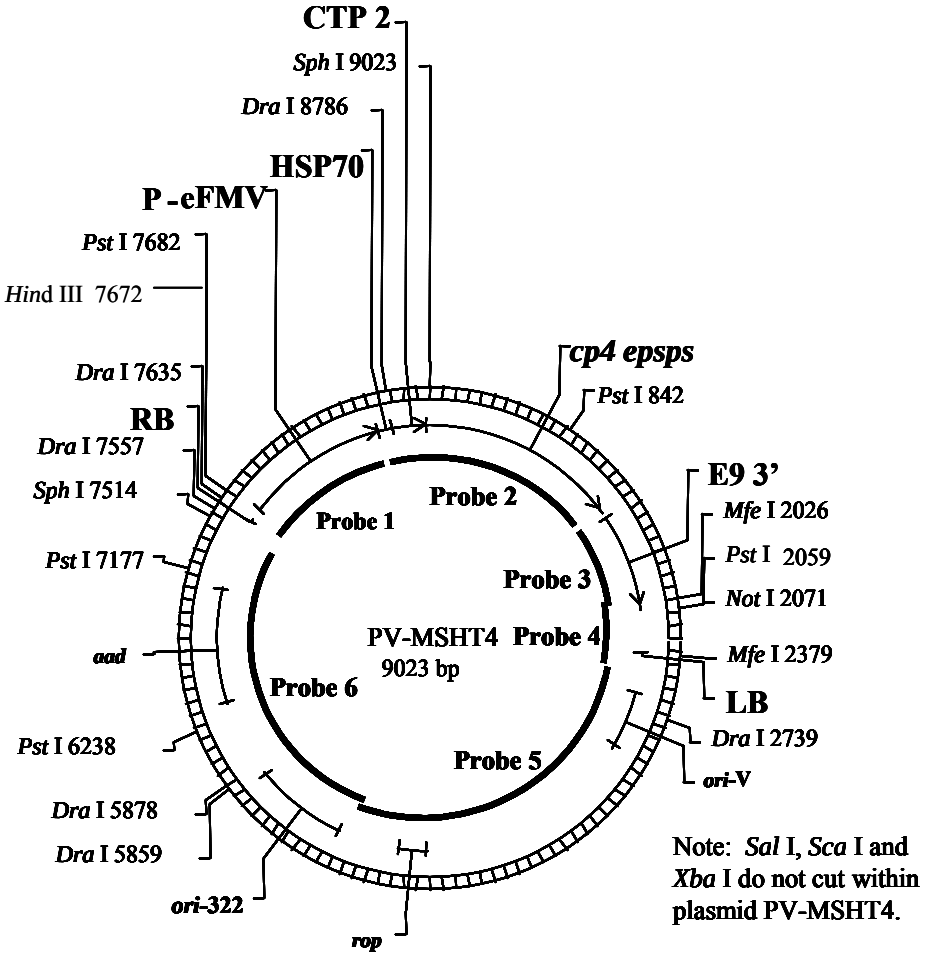


Figure III-1. Plasmid Map of PV-MSHT4

A circular map of the plasmid vector PV-MSHT4 used in the transformation events J101 and J163 with genetic elements annotated is shown above. The portion of the plasmid transferred to the plant genome begins near the right border, extends through the *cp4 epsps* coding region, and ends near the left border.

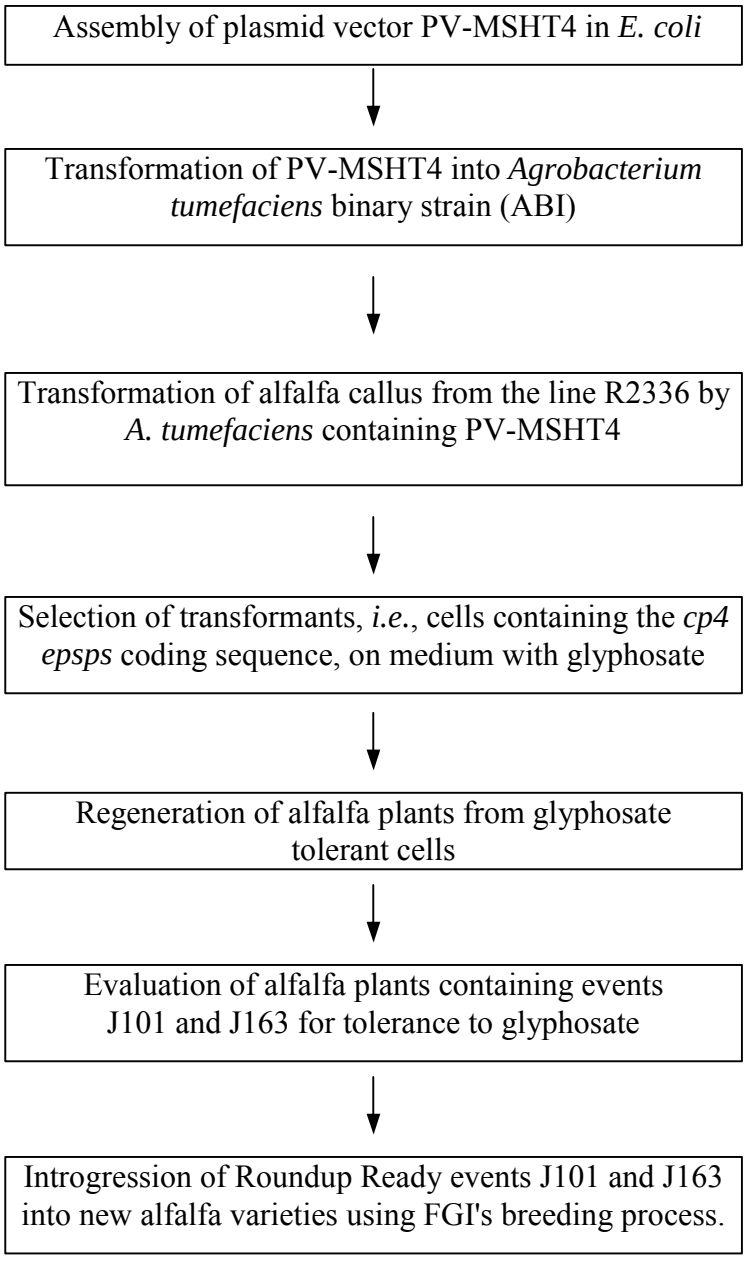


Figure III-2. Development of Roundup Ready Alfalfa Varieties.

The flow diagram illustrates the steps in the development of the Roundup Ready alfalfa varieties.

IV. Donor Genes and Regulatory Sequences

A. Vector PV-MSHT4

Events J101 and J163 were developed through *Agrobacterium*-mediated transformation of alfalfa callus (from line R2336) using the double border, binary vector PV-MSHT4 shown in Figure III-1. This vector contains a region of DNA (T-DNA), which has one *cp4 epsps* expression cassette flanked by left and right border sequences. This sequence, of approximately 3.8 Kb, was transferred into the alfalfa genome by *Agrobacterium tumefaciens* during the transformation process. The *cp4 epsps* expression cassette contains the *cp4 epsps* coding sequence under the regulation of the 35S promoter, a heat shock protein intron (HSP70), a chloroplast transit peptide (*CTP2*) sequence and a E9 3' polyadenylation sequence. The *ctp2 cp4 epsps* coding region used to produce events J101 and J163 is the same as that employed in several other Roundup Ready crops such as soybean, which have been previously reviewed and granted nonregulated status by the USDA. A description of all the elements in the PV-MSHT4 is provided in Table IV-1.

B. The *cp4 epsps* Gene and CP4 EPSPS Protein

The *cp4 epsps* coding sequence has shown the potential to provide high levels of tolerance to glyphosate when introduced into plants (Padgette et al., 1996; OECD, 1999). Glyphosate binds to and blocks the activity of its target enzyme, EPSPS, an enzyme of the aromatic amino acid biosynthetic pathway. In plants, the EPSPS enzyme is located within the chloroplast; thus, in the construction of PV-MSHT4, a chloroplast transit peptide coding sequence was joined to the *cp4 epsps* coding sequence to provide transport to the alfalfa chloroplast. The CP4 EPSPS protein has been completely sequenced and encodes a 47.6 kDa protein consisting of a single polypeptide of 455 amino acids (Padgette et al., 1996). The CP4 EPSPS protein with its CTP2 of 76 amino acids is approximately 56 kDa in size. The deduced amino acid sequence of the CP4 EPSPS protein is shown in Figure IV-1.

The CP4 EPSPS protein is one of many EPSPSs found in nature (Schulz et al., 1985). CP4 EPSPS is naturally highly tolerant to inhibition by glyphosate and has high catalytic efficiency, compared to most EPSPSs (Barry et al., 1992; Padgette et al., 1996). Plant cells producing the CP4 EPSPS protein are tolerant to glyphosate because the continued EPSPS enzyme activity meets the needs for production of aromatic compounds. The bacterial isolate, CP4, was identified by the American Type Culture Collection as an *Agrobacterium* species. The CP4 EPSPS and native alfalfa EPSPS enzymes are functionally equivalent, except for their affinity for glyphosate.

C. The *Arabidopsis thaliana* EPSPS Transit Peptide (*CTP2*)

In the plant gene expression cassette, the *cp4 epsps* coding sequence is joined to a chloroplast transit peptide sequence (designated CTP2) isolated from the *Arabidopsis thaliana epsps* gene (Klee et al., 1987). This transit peptide directs the CP4 EPSPS protein to the chloroplast, the location of EPSPS in plants and the site of aromatic amino acid biosynthesis (Kishore and Shah, 1988). Transit peptides are typically cleaved from the mature protein following delivery to the plastid (Della-Cioppa et al., 1986).

D. Regulatory Sequences

The *cp4 epsps* cassette contains the *ctp2-cp4 epsps* coding sequence under the control of the enhanced figwort mosaic virus sequence (P-eFMV), which consist of the FMV promoter with a duplicated enhancer region (Richins et al., 1987). Located between the P-eFMV and the *ctp2-cp4 epsps* sequence is the 5' untranslated leader sequence from the petunia heat shock protein (HSP70), which is present to increase the levels of gene transcription (Rochester et al., 1986). In the cassette, the *cp4 epsps* sequence is joined to the *3'* nontranslated region of the pea ribulose-1-5-biphosphate carboxylase, small subunit (*rbc*) E9 gene (Corruzzi et al., 1984), which functions to terminate transcription and direct polyadenlyation of the *cp4 epsps* mRNA.

E. T-DNA Borders

Plasmid vector PV-MSHT4 contains DNA sequences that are necessary for transfer of T-DNA into the plant cell. These are termed the Right Border and Left Border Regions, where each region contains a 25 to 26 bp sequence that defines the extent of DNA that should be transferred into the plant genome. The Right Border Region present in PV-MSHT4 is a 26 bp nucleotide sequence that was originally isolated from *A. tumefaciens* plasmid pTiT37 (Depicker et al., 1982). The Left Border Region present in PV-MSHT4 is a 25 bp nucleotide sequence that was originally isolated from *A. tumefaciens* plasmid pTI15955, a derivative of plasmid pTiA6 (Barker et al., 1983).

F. Genetic Elements Outside the T-DNA Borders

The elements described below are present on plasmid vector PV-MSHT4 but are outside the borders of the T-DNA. Hence, they were not expected to be transferred into the alfalfa genome, and their absence has been confirmed by data presented in Section V of this petition.

ori-V: A 395 bp DNA segment, derived from plasmid RK2 (Stalker et al., 1981) that contains a vegetative origin of DNA replication, allowing maintenance of PV-MSHT4 in *Agrobacterium*.

ori-322: A 627 bp DNA segment containing an additional origin of DNA replication from the plasmid pBR322 for the maintenance of the PV-MSHT4 plasmid in other bacteria, such as *E. coli* (Sutcliffe, 1978).

rop: A 193 bp DNA segment, containing the coding sequence for repressor of the primer protein for maintenance of plasmid copy number in *E. coli* (Giza and Huang, 1989).

aad: A 790 bp DNA segment from the bacterial transposon Tn7, containing the gene that codes for the enzyme streptomycin adenyltransferase and allows selection of bacteria on culture media containing streptomycin or spectinomycin (Fling et al., 1985).

Table IV-1. Summary of the Genetic Elements

Genetic Element	Position in Figure III-1	Function (reference)
cp4 epsps	9021-9023 1-1365	Coding sequence for the synthetic CP4 EPSPS protein from *Agrobacterium sp*. strain CP4 (Padgette et al., 1996).
Intervening Sequence	1366-1407	Synthetic sequence, polylinker
E9 3'	1408-2040	A 3' nontranslated region of the pea ribulose-1,5-bisphosphate carboxylase, small subunit (rbc) E9 gene (Corruzzi et al., 1984), which functions to terminate transcription and direct polyadenlyation of the *cp4 epsps* mRNA.
Intervening Sequence	2041-2097	Synthetic sequence, polylinker
Left Border Region	2098-2373	DNA sequences derived from *Agrobacterium* (Barker et al., 1983).
LB	2374-2397	Left border sequence essential for transfer of T-DNA derived from *Agrobacterium* (Barker et al., 1983).
Intervening Sequence	2398-2553	DNA sequences derived from *Agrobacterium* (Barker et al., 1983).
Intervening Sequence	2554-2646	Synthetic sequences and DNA derived from *E. coli.* (Stalker et al., 1981).
ori-V	2647-3040	Origin of replication for *Agrobacterium* derived from the broad host range plasmid RK2 (Stalker et al., 1981).
Intervening Sequence	3041-3281	Synthetic sequences and DNA derived from *E. coli.* (Stalker et al., 1981).
Intervening Sequence	3282-4544	Portion of the plasmid pBR322 (Sutcliffe, 1978).
ROP	4545-4736	Coding sequence for repressor of primer protein for maintenance of plasmid copy number in *E. coli* (Giza and Huang, 1989).

Table IV-1. Summary of the Genetic Elements (continued)

Genetic Element	Position in Figure III-1	Function (reference)
Intervening Sequence	4737-5153	Portion of the plasmid pBR322 (Sutcliffe, 1978).
ori-322	5154-5779	Origin of replication from pBR322 for maintenance of plasmid in *E. coli* (Sutcliffe, 1978).
Intervening Sequence	6239-6321	Derived from *E. coli* and synthetic sequences (Fling et al., 1985).
aad	6322-7110	Bacterial promoter and coding sequence for an aminoglycoside-modifying endonuclease, 3'(9)-O-nucleotidyltransferase from the transposon Tn7 (Fling et al., 1985).
Intervening Sequence	7111-7595	Derived from *E. coli* and synthetic sequences (Fling et al., 1985).
RB	7596-7620	Right border sequence essential for transfer of T-DNA derived from *Agrobacterium* (Depicker et al., 1982).
Intervening Sequence	7621-7703	DNA sequences derived from *E. coli,* synthetic sequences and polylinker (Depicker et al., 1982).
P-eFMV	7704-8684	The 35S promoter (Figwort Mosaic Virus) with duplicated enhancer region (Richins et al., 1987).
HSP70-Leader	8685-8790	The petunia heat shock protein 70 5' untranslated leader sequence (Rochester et al., 1986).
Intervening Sequence	8791-8792	Synthetic sequence, polylinker.
CTP2	8793-9020	Chloroplast transit peptide, isolated from *Arabidopsis thaliana* EPSPS, present to direct the CP4 EPSPS protein to the chloroplast, the site of aromatic amino acid synthesis (Klee et al., 1987).

```
  1     MLHGASSRPA TARKSSGLSG TVRIPGDKSI SHRSFMFGGL ASGETRITGL
 51     LEGEDVINTG KAMQAMGARI RKEGDTWIID GVGNGGLLAP EAPLDFGNAA
101     TGCRLTMGLV GVYDFDSTFI GDASLTKRPM GRVLNPLREM GVQVKSEDGD
151     RLPVTLRGPK TPTPITYRVP MASAQVKSAV LLAGLNTPGI TTVIEPIMTR
201     DHTEKMLQGF GANLTVETDA DGVRTIRLEG RGKLTGQVID VPGDPSSTAF
251     PLVAALLVPG SDVTILNVLM NPTRTGLILT LQEMGADIEV INPRLAGGED
301     VADLRVRSST LKGVTVPEDR APSMIDEYPI LAVAAAFAEG ATVMNGLEEL
351     RVKESDRLSA VANGLKLNGV DCDEGETSLV VRGRPDGKGL GNASGAAVAT
401     HLDHRIAMSF LVMGLVSENP VTVDDATMIA TSFPEFMDLM AGLGAKIELS
451     DTKAA
```

Figure IV-1. Deduced amino Acid Sequence for the CP4 EPSPS Protein in Roundup Ready Alfalfa Events J101 and J163.
The amino acid sequence of the plant-produced CP4 EPSPS protein was deduced from the coding region of the full-length *cp4 epsps* gene present in Roundup Ready alfalfa events J101 and J163.

V. Product Characterization of Roundup Ready events J101 and J163

A. Molecular Characterization

Molecular analysis was performed to characterize the integrated DNA in Roundup Ready alfalfa events J101 and J163. Southern blot analysis was used to determine insert number, copy number, cassette intactness and to demonstrate the stability of the T-DNA for each event during conventional breeding. The genomic DNA sequences flanking the 5' and 3' ends of each transformation event were confirmed using PCR and DNA sequencing methodologies.

A.1. Materials and Methods

Test Substances. The test substances for characterization were the Roundup Ready alfalfa events J101 and J163. Leaf tissue was harvested periodically from T_0 plants (the original plants that were generated during transformation) grown in a growth chamber and used in this study. The lot numbers assigned to this material were as follows: J101, lot RDR-0201-11983-S; J163, lot RDR-0201-11987-S. Stability of the gene insertion was assessed in the dihomogenic Syn 1 (advanced breeding population) generation that was produced through conventional breeding. Leaf tissue for the stability analysis was obtained from Forage Genetics International (West Salem, WI) for the following dihomogenic Syn 1 generation: J101XJ163, lot GLP-0206-12762-S.

Control Substance. The control substance was the conventional (nontransgenic) alfalfa cultivar R2336 (lot REF-0201-11980-S). Leaf tissue was produced in a growth chamber and harvested periodically throughout the study.

Reference Substances. The reference substances included the plasmid PV-MSHT4 that was used to create Roundup Ready alfalfa events J101 and J163, as well as gel-purified restriction fragments from this plasmid. For Southern blot analyses, picogram amounts of the plasmid PV-MSHT4 (approximately 0.5 and/or 1 genome copy equivalents) were spiked into digested DNA from alfalfa cultivar R2336. Additionally, molecular size markers from Roche [DNA Molecular Weight Marker II (23.1 kb-0.6 kb), DNA Molecular Weight Marker IX (1.4 kb-0.1 kb)] and Invitrogen [High Molecular Weight DNA Marker (48.5 kb-8.3 kb)] were used for size estimations on Southern blots. The 100 bp DNA ladder (2.1 kb-0.1 kb) from Gibco BRL was used for size estimations in the PCR analyses.

Test, Control, and Reference Substance Characterization. The identities of the T_0 test substances and the control substance were confirmed by Southern blot event-specific fingerprint analysis. The identity of the plasmid PV-MSHT4 was confirmed by digestion of the plasmid with a variety of restriction enzymes.

Genomic DNA Purification. Three methods of DNA purification were used for the molecular characterization of events J101 and J163.

Method 1. DNeasy Plant Maxi Kit (QIAgen):

Genomic DNA from the test and control substances was extracted from alfalfa leaf tissue using the Dneasy Plant Maxi Kit from QIAgen. The manufacturer's protocol

was followed with the following exception: prior to lysis, lyophilized leaf tissue was ground in a paint shaker in the presence of ~5 ml of 3 mm glass beads for ~3 min.

Method 2. CTAB DNA Purification:

Approximately 5-7 ml of glass beads were placed in a 50 ml conical tube with 400 mg of lyophilized alfalfa leaf tissue. Approximately 17 ml of CTAB extraction buffer (0.1 M Tris pH 7.5, 0.7 M NaCl, 4 mM EDTA, 1% CTAB) were added to the tube and mixed by gentle inversion. Samples were incubated at ~55°C for ~2 hours. RNase A was then added to the solution and incubated for ~30 min. Lysates were then separated into two portions and transferred to two new 50 ml conical tubes. Approximately 8.5 ml of chloroform/isoamyl alcohol (24:1) were added to the glass beads to rescue the remaining lysate, mixed, and the chloroform/lysate mixture distributed between the two new tubes. Samples were mixed by inversion for ~10 min. Samples were then centrifuged at ~1000 x *g* for ~20 min. The upper aqueous layer was removed to a new 50 ml conical tube and 7 ml of isopropanol were added to each tube and mixed by gentle inversion. DNA was spooled out with a glass hook and placed in a 15 ml conical tube. Samples were washed with 5 ml of a 75% EtOH solution containing 0.2 M NaOAc for ~ 20 min, then rinsed again with a 75% EtOH solution containing 10 mM NH_4OAc for 5 min. Spooled DNA samples were removed, gently dried on the side of the conical tube, blotted on Whatman 3M paper to remove excess ethanol, and resuspended in TE.

Method 3. Cesium Chloride Gradient Purification:

In some instances DNA extracted according to either method 1 or method 2 (above) was further purified via a cesium chloride gradient. DNA samples were brought to a final volume of approximately 20 ml with TE. Samples were then weighed and an equal weight of cesium chloride (CsCl) was added. Samples were gently mixed until the CsCl was dissolved. The density of each sample was adjusted to 1.50-1.55 g/ml by the addition of either TE or CsCl. Fifty microliters of EtBr [10 mg/ml (w/v)] were added to each sample, the samples were mixed by inversion, and protected from light. Samples were centrifuged at ~50,000 rpm using a Sorvall ultracentrifuge with a Ti80 rotor for ~18 hours at ~24°C. The DNA band was rescued with a 3 ml syringe and 18-gauge needle. Sample volume was then adjusted to ~2 ml with TE. An equal volume of isoamyl alcohol was added, the sample was mixed by inversion, centrifuged for ~5 min at 365 x *g*, and the lower, aqueous phase was transferred to a new tube. The isoamyl alcohol extraction procedure was repeated until no pink coloration was visible. Three volumes of water (relative to the final volume) were added to the sample, followed by 0.1 volume of 3M NaOAc and 2 volumes of 100% EtOH (relative to the final volume). The DNA was spooled out and blotted onto Whatman paper. The pellet was washed with 70% EtOH and resuspended in TE.

Quantitation of Genomic DNA. Quantitation of DNA samples was performed using a Hoefer DyNA Quant 200 Fluorometer or a SpectraMAX Gemini Fluorescence microplate reader. All readings were taken using DNA Molecular Weight Marker IX (Roche) as a DNA calibration standard.

Restriction Endonuclease Digestion of Genomic DNA. Approximately 10 µg or 20 µg of genomic DNA from the test substances and 10 µg of genomic DNA from the control

substance were used for restriction endonuclease digestions. Whole plasmid PV-MSHT4, when used as a positive hybridization control, was spiked into the control substance sample prior to incubation. Alternatively, for some Southern blot analyses, restriction fragments of the plasmid PV-MSHT4 were spiked into the control sample immediately prior to loading the gel to serve as the positive hybridization control. Overnight digests were performed at 37°C in a total volume of 500 µl using 100 units (each) of the appropriate restriction endonuclease(s). After digestion, the samples were precipitated by adding 1/10 volume (~50 µl) of 3 M NaOAc, pH 5.2, and 2 volumes relative to the original digest volume (~1 ml) of 100% ethanol, followed by incubation in a -20°C freezer for at least 30 minutes. The digested DNA was pelleted in a microcentrifuge at maximum speed, washed with 70% ethanol, dried, and redissolved in TE. In those cases where 20 µg of DNA were digested, the redissolved sample was divided evenly between lanes 3 and 6 on the gel, so that each lane contained 10 µg of test sample DNA.

DNA Restriction Fragment/Positive Hybridization Control Preparation. Positive hybridization controls were prepared by digestion of the plasmid PV-MSHT4 using appropriate restriction endonucleases. Approximately 20-500 ng of the plasmid vector were used for the restriction endonuclease digestions. Digests were performed at 37°C for 1-2 hours in a total volume of 20 or 50 µl using 10 units of the appropriate restriction endonuclease. Restriction fragments were then separated by electrophoresis using a 0.8% or 1.0% agarose gel. The gels were electrophoresed at 90-100 V for 1-2 hours. Gels were photographed and the appropriate bands were excised from the gel and purified according to the manufacturer's instructions using the QIAquick Gel Extraction Kit from Qiagen.

DNA Probe Preparation for Southern Blot Analyses. DNA probe templates were prepared by PCR using a restriction fragment from plasmid PV-MSHT4 as a template. Approximately 25-50 ng of Probes 1, 2, 4, 5, and 6 (Figure V-1a) were labeled with α^{32}P-dCTP (6000 Ci/mmol) by a random priming method (RadPrime DNA Labeling System, Gibco BRL). Probes 3 and 4 (Figure V-1a) were labeled with α^{32}P-dCTP (6000 Ci/mmol) using PCR with DNA probe template (25-50 ng); sense and antisense primers specific to the template (0.25 µM each); 1.5 mM $MgCl_2$; 3 µM each of dATP, dGTP and dTTP; 100 µCi of α^{32}-P-dCTP; and 2.5 Units of *Taq* DNA polymerase in a final volume of 20µl. The cycling conditions were as follows: 1 cycle at 94°C for 3 minutes; 2 cycles at 94°C for 45 seconds, 52°C for 30 seconds, and 72°C for 2 minutes; 1 cycle at 72°C for 10 minutes. All radiolabeled probes were purified using a Sephadex G-50 column (Roche).

Southern Blot Analyses of Genomic DNA. Southern blot analyses (Southern, 1975) were performed by digesting the DNA samples with restriction endonucleases and separating by electrophoresis using a 0.6% agarose gel in which a long run and a short run were performed. The long run enabled greater separation of higher molecular weight DNA fragments while the short run allowed smaller molecular weight DNA fragments to be retained on the gel. The long-run samples were loaded onto the gel and typically electrophoresed overnight at 20-40 volts. The short-run samples were loaded in adjacent lanes on the same gel the following day and the gel was typically electrophoresed for 2-6 additional hours at 60-100 volts. The stability analysis was not performed using long and short runs. Instead, samples were electrophoresed on a 0.8% agarose gel at 30 V overnight and 45 V the following day for ~1 hour. After electrophoresis, gels were stained in ethidium bromide for 5-15 minutes and photographed. After photographing, the gels were

placed in a depurination solution (0.125 N HCl) for ~10 minutes, followed by a denaturing solution (0.5 M NaOH, 1.5 M NaCl) for ~30 minutes and then a neutralizing solution (0.5 M Tris-HCl pH 7.0, 1.5 M NaCl) for ~30 minutes. The gels were then placed in 20X SSC for 5-30 minutes. DNA from the agarose gels was transferred to Hybond-N nylon membranes (Amersham) using a Turboblotter (Schleicher & Schuell). The DNA was allowed to transfer overnight (using 20X SSC as the transfer buffer) and covalently cross-linked to the membrane with a UV Stratalinker 1800 (Stratagene), using the auto crosslink setting. Blots were prehybridized for 0.5-10 hours at 60-65°C in an aqueous solution of 250 mM $Na_2HPO_4 \bullet 7H_2O$, 7% SDS, and 0.1 mg/ml tRNA. Hybridization with the radiolabeled probe was performed in fresh prehybridization solution for 13-22 hours at 60 or 65°C. Membranes were washed four times in an aqueous solution of 0.1% SDS and 0.1× SSC for ~15-20 minutes at 60 or 65°C using fresh solution for each wash. Multiple exposures of the blots were then generated using Kodak Biomax MS film in conjunction with one Kodak Biomax MS intensifying screen in a -80°C freezer. The blot containing the stability analysis was exposed to film only at room temperature.

A.2. Results and Discussion – Molecular Characterization

Genomic DNA from Roundup Ready alfalfa events J101 and J163 was digested with a variety of restriction endonucleases and subjected to Southern blot analyses (Figure V-1B). Each Roundup Ready alfalfa event was assessed for the number of inserts present within the plant genome, the number of copies of the gene cassette present at each locus of integration, the integrity of the T-DNA, and the absence of plasmid backbone. A map of plasmid PV-MSHT4 annotated with the probes used in the Southern blot analyses is presented in Figure V-1A. A schematic representation of the T-DNA from the transformation vector PV-MSHT4 (Figure V-1B) predicts the sizes of restriction fragments generated in Southern blot analyses for Roundup Ready alfalfa events J101 and J163. In some of the Southern blot analyses, areas of nonspecific hybridization in the form of round dots, ellipses, or lines were observed; however, none of these areas of nonspecific hybridization affected the analysis or interpretation of any of the Southern blot data.

A.2.a. Results for Roundup Ready alfalfa event J101

Insert Number Analysis for Roundup Ready Alfalfa Event J101. The number of inserts (the number of integration sites of T-DNA in the genome) in Roundup Ready alfalfa event J101 was evaluated by digesting test and control genomic DNA with the restriction endonucleases *Sal* I, *Sca* I and *Xba* I, all of which do not cleave within the plasmid PV-MSHT4. Control DNA was spiked with the two gel-purified *Sph* I restriction fragments of the vector PV-MSHT4 to serve as positive hybridization controls. The blot was probed with Probe 1 and Probe 2 (Figure V-1A). If a single insert were present within the plant genome, this restriction endonuclease and probe combination would yield a single restriction fragment containing the inserted T-DNA and adjacent plant genomic DNA. The number of restriction fragments observed would reflect the number of inserts present containing the elements encompassed by the probes. The results of the insert number analysis for Roundup Ready alfalfa event J101 are shown in Figure V-2. Lane 2 containing digested DNA from the control line R2336 yielded no signal, as expected. *Sph* I restriction fragments of the plasmid PV-MSHT4, spiked into a matrix of previously digested R2336 control DNA (lanes 4 and 5), produced the predicted size bands of approximately 1.5 kb

and 7.5 kb (Figure V-1A). Roundup Ready alfalfa event J101 yielded a single hybridization signal of ~9.0 kb in both the long and short runs (lanes 3 and 6). These results, in conjunction with the results from the E9 3' polyadenylation signal and left border region Southern blots described below, establish that Roundup Ready alfalfa event J101 contains a single insert.

Copy Number Analysis for Roundup Ready Alfalfa Event J101. The number of copies of the T-DNA within the single insertion was assessed for Roundup Ready alfalfa event J101 by digesting test and control genomic DNA with the restriction endonuclease *Sph* I, which cleaves once within the T-DNA (Figure V-1B). The blot was probed with Probes 1 and 2 (Figure V-1A). Digested control DNA was spiked with the two gel-purified *Sph* I restriction fragments from the plasmid PV-MSHT4 (Figure V-1A) to serve as positive hybridization controls. If a single copy of the T-DNA was present at the locus of integration, the *Sph* I restriction endonuclease would yield two border fragments, each containing a portion of the inserted T-DNA along with the associated alfalfa genomic DNA flanking the insert. When probed with Probe 1 and Probe 2, the detection of two hybridization signals indicates the presence of a single copy of the T-DNA, containing the elements encompassed by the probes, at the site of integration. Results of the analysis are shown in Figure V-3. Digested DNA from the conventional control line R2336 yielded no signal, as expected (lane 2). *Sph* I restriction fragments of the plasmid PV-MSHT4 spiked into a matrix of digested R2336 control DNA (lanes 4 and 5) produced the predicted size bands of approximately 1.5 kb and 7.5 kb. The long and short runs of Roundup Ready alfalfa event J101 digested DNA yielded bands of approximately 13.0 kb and 6.5 kb (lanes 3 and 6). Based on these results, in conjunction with the results from the E9 3' polyadenylation signal and left border region Southern blots discussed below, it is concluded that the Roundup Ready alfalfa event J101 contains a single copy of the T-DNA at the site of integration.

Cassette Intactness for Roundup Ready Alfalfa Event J101. The integrity of the transformation cassette in Roundup Ready alfalfa event J101 was determined by digesting test and control genomic DNA with the restriction endonuclease *Pst* I. This restriction endonuclease cleaves three times within the T-DNA: at the beginning of the P-eFMV promoter region, in the middle of the *HSP70-ctp2-cp4 epsps* coding region, and again at the 3' end of the E9 3' polyadenylation sequence (Figure V-1B). Use of this restriction endonuclease would produce two restriction fragments of ~1.2 kb and ~2.2 kb from an intact copy of the T-DNA from plasmid PV-MSHT4. To serve as positive hybridization controls, control DNA was spiked with the two gel-purified *Pst* I restriction fragments of the plasmid PV-MSHT4 that exist between the right and left borders (Figure V-1A). Individual Southern blots were probed separately with the P-eFMV promoter region (Probe 1), the *HSP70-ctp2-cp4 epsps* coding region (Probe 2), and the E9 3' polyadenylation sequence (Probe 3). The following results would be expected if the *cp4 epsps* gene cassette were intact: 1) probing the *Pst* I digested Roundup Ready alfalfa genomic DNA with the P-eFMV genetic element (Probe 1) would yield an ~2.2 kb hybridization signal, 2) probing the *Pst* I digested Roundup Ready alfalfa genomic DNA with the *HSP70-ctp2-cp4 epsps* genetic element (Probe 2) would yield two hybridization signals of ~1.2 kb and ~2.2 kb, and 3) probing the *Pst* I digested Roundup Ready alfalfa genomic DNA with the E9 3' genetic element (Probe 3) would yield an ~1.2 kb hybridization signal.

Probe 1 for Roundup Ready Alfalfa Event J101. Results of the analysis for Roundup Ready alfalfa event J101 are shown in Figure V-4. R2336 control DNA (lane 2) showed no detectable hybridization signals, as expected for the negative control. *Pst* I restriction fragments of the plasmid PV-MSHT4 mixed with R2336 control DNA in lanes 4 and 5 yielded the expected ~2.2 kb signal (Figure V-1A). Roundup Ready alfalfa event J101 DNA (lanes 3 and 6) produced a single hybridization signal at approximately 2.2 kb. No unexpected bands were detected, indicating that Roundup Ready alfalfa event J101 does not contain any additional P-eFMV promoter element sequences other than that associated with the intact *HSP70-ctp2-cp4 epsps* gene.

Probe 2 for Roundup Ready Alfalfa Event J101. Results of the analysis for Roundup Ready alfalfa event J101 are shown in Figure V-5. R2336 control DNA for (lane 2) showed no detectable hybridization signals, as expected for the negative control. *Pst* I restriction fragments of the plasmid PV-MSHT4 mixed with R2336 control DNA (lanes 4 and 5) produced the expected size bands at approximately 1.2 kb and 2.2 kb (Figure V-1A). Roundup Ready alfalfa event J101 DNA (lanes 3 and 6) produced both the expected approximately 1.2 kb and 2.2 kb hybridization signals. No unexpected bands were detected, indicating that Roundup Ready alfalfa event J101 does not contain any additional *HSP70-ctp2-cp4 epsps* coding region element sequences other than that associated with the intact *HSP70-ctp2-cp4 epsps* gene cassette.

Probe 3 for Roundup Ready Alfalfa Event J101. Results of the analysis for Roundup Ready alfalfa event J101 are shown in Figure V-6. R2336 control DNA (lane 2) yielded no hybridization signals, as expected. *Pst* I restriction fragments of the plasmid PV-MSHT4 mixed with R2336 control DNA (lanes 4 and 5) produced the expected size band at approximately 1.2 kb (Figure V-1A). Roundup Ready alfalfa event J101 DNA (lanes 3 and 6) produced a band at approximately 1.2 kb. No unexpected bands were detected, indicating that Roundup Ready alfalfa event J101 does not contain any additional E9 3' polyadenylation sequence other than that associated with the intact *HSP70-ctp2-cp4 epsps* gene cassette.

Analysis for the Left Border Region in Roundup Ready Alfalfa Event J101.

The plasmid used to generate the Roundup Ready alfalfa events contains right and left border sequences that delineate the T-DNA that is transferred into the plant chromosome (Rogers and Klee, 1987; Zambryski, 1992). The most common mechanism known for insertion of DNA into the plant genome using *Agrobacterium*-based plant transformation suggests that initiation of T-DNA transfer begins at one border sequence and continues through the T-DNA to the next border sequence (Tinland, 1996). The Southern blot analyses that have been described thus far encompass the majority of the T-DNA sequence that would be transferred into the Roundup Ready alfalfa events. However, there is a small segment of the T-DNA following the E9 3' polyadenylation signal sequence up to the actual left border sequence that was not encompassed in any of the previously described analyses. Therefore, in order to demonstrate that at least a portion of this left border region is present in Roundup Ready alfalfa event J101, as would be expected, a DNA probe specific to the left border region was generated and used for Southern blot analysis. Test and control genomic DNA were digested with the restriction endonuclease *Sph* I, which

cleaves once within the T-DNA between the *ctp2* and *cp4 epsps* genetic elements, to release two border fragments (Figure V-1B). Control DNA was spiked with two gel-purified *Sph* I restriction fragments of the plasmid PV-MSHT4 to serve as hybridization controls. The Southern blot was probed with the left border region of the plasmid PV-MSHT4 (Probe 4). Observation of a signal would demonstrate the presence of at least a portion of the left border region. The results of the analysis for Roundup Ready alfalfa event J101 are shown in Figure V-7. Digested DNA from the control R2336 yielded no signal, as expected (lane 2). *Sph* I restriction fragments of the plasmid PV-MSHT4 spiked into a matrix of digested R2336 control DNA (lanes 4 and 5) produced the predicted size band of approximately 7.5 kb (Figure V-1A). The long and short runs (lanes 3 and 6) of Roundup Ready alfalfa event J101 DNA yielded a hybridization signal of approximately 13.0 kb. It is therefore concluded that Roundup Ready alfalfa event J101 minimally contains a portion of the left border region, as expected. Additionally, the size of the hybridization signal is consistent with the size of the signal obtained in the copy number analysis (Figure V-3). Based on these results, it can also be concluded that Roundup Ready alfalfa event J101 does not have any additional left border region DNA sequences other than those associated with the T-DNA insertion.

Analysis for Backbone in Roundup Ready Alfalfa Event J101. Roundup Ready alfalfa event J101 was analyzed for the presence of backbone sequences from the plasmid PV-MSHT4. Results from this analysis are presented in Figure V-8. Test and control genomic DNA were digested with the restriction endonucleases *Sal* I, *Sca* I and *Xba* I, which do not cut within the vector PV-MSHT4. A *Hin*d III + *Not* I restriction fragment, containing the backbone region of the plasmid PV-MSHT4, was mixed with control R2336 genomic DNA and digested with *Sal* I, *Sca* I and *Xba* I to serve as a positive hybridization control. The blot was probed with two overlapping probes (Probes 5 and 6) that span the backbone present in plasmid PV-MSHT4 (Figure V-1A). Negative control R2336 genomic DNA (lane 2) showed no detectable hybridization bands, as expected. Plasmid PV-MSHT4 *Hin*d III + *Not* I restriction fragment mixed with R2336 control DNA (lanes 4 and 5) produced one expected size band at approximately 5.6 kb. Roundup Ready alfalfa event J101 (lanes 3 and 6) showed no detectable hybridization bands. This result establishes that Roundup Ready alfalfa event J101 does not contain any detectable backbone sequence from the transformation vector PV-MSHT4.

Predicted Insert Map for Event J101. A predicted map of the insert was constructed on the basis of data derived from Southern Blots analyses conducted on J101. This insert map is presented in Figure V-9.

A.2.b. Results for Roundup Ready alfalfa event J163

Insert Number Analysis for Roundup Ready Alfalfa Event J163. The number of inserts in Roundup Ready alfalfa event J163 was evaluated by digesting test and control genomic DNA with the restriction endonucleases *Sal* I, *Sca* I and *Xba* I, all of which do not cleave within the plasmid PV-MSHT4. Control DNA was spiked with the two gel-purified *Sph* I restriction fragments of the vector PV-MSHT4 to serve as positive hybridization controls. The blot was probed with Probe 1 and Probe 2 (Figure V-1A). If a single insert was present within the plant genome, the restriction endonuclease and probe combination would yield a single restriction fragment containing the inserted T-DNA and adjacent plant genomic DNA. The number of restriction fragments observed would reflect the number of

inserts present that contain the elements encompassed in the probes. The results of the insert number analysis for Roundup Ready alfalfa event J163 are shown in Figure V-10. Lane 3, containing digested DNA from the control line R2336, yielded no signal, as expected. *Sph* I restriction fragments of the plasmid PV-MSHT4 spiked into a matrix of previously digested R2336 control DNA (lanes 4 and 5) produced the predicted size bands of approximately 1.5 kb and 7.5 kb (Figure V-1A). Roundup Ready alfalfa event J163 yielded a single hybridization signal of ~8.4 kb in both the long and short runs (lanes 2 and 6). These results, in conjunction with the results from the E9 3' polyadenylation signal and left border region Southern blots discussed below, establish that Roundup Ready alfalfa event J163 contains a single insert.

Copy Number Analysis for Roundup Ready Alfalfa Event J163. The number of copies of the T-DNA was assessed for Roundup Ready alfalfa event J163 by digesting test and control genomic DNA with the restriction endonuclease *Sph* I, which cleaves once within the T-DNA (Figure V-1B). The blot was probed with Probes 1 and 2 (Figure V-1A). Digested control DNA was spiked with the two gel-purified *Sph* I restriction fragments from the plasmid PV-MSHT4 (Figure V-1A) to serve as positive hybridization controls. If a single copy of the T-DNA was present at the locus of integration, the *Sph* I restriction endonuclease would yield two border fragments, each containing a portion of the inserted T-DNA along with the associated alfalfa genomic DNA flanking the insert. When probed with Probe 1 and Probe 2, the detection of two hybridization signals would indicate the presence of a single copy of the T-DNA, containing the elements encompassed on the probes, at the site of integration. The results of this analysis are shown in Figure V-11. Digested DNA from the conventional control line R2336 yielded no signal, as expected (lane 2). *Sph* I restriction fragments of the plasmid PV-MSHT4 spiked into a matrix of digested R2336 control DNA (lanes 4 and 5) produced the predicted size bands of approximately 1.5 kb and 7.5 kb. The long and short runs of Roundup Ready alfalfa event J163 digested DNA yielded bands of approximately 3.6 kb and 1.9 kb (lanes 3 and 6). Based on these results, in conjunction with the results from the E9 3' polyadenylation signal and left border region Southern blots discussed below, it is concluded that the Roundup Ready alfalfa event J163 contains a single copy of the T-DNA at the site of integration.

Cassette Intactness for Roundup Ready Alfalfa Event J163. In order to demonstrate the cassette integrity for Roundup Ready alfalfa event J163, test and control DNA were digested with the restriction endonucleases *Dra* I and *Mfe* I. The restriction endonuclease *Dra* I cuts twice within the T-DNA, once at the beginning of the P-eFMV promoter region and again at the beginning of the HSP70 leader sequence. The restriction endonuclease *Mfe* I cuts twice within the T-DNA, once at the end of the E9 3' polyadenylation sequence, and again near the left border (Figure V-1B). Together, the two restriction enzymes would be expected to release three restriction fragments of ~1.2 kb, ~2.3 kb, and ~0.4 kb from an intact *cp4 epsps* gene cassette (Figure V-1A). Control DNA was spiked with the plasmid PV-MSHT4 prior to digestion to serve as a positive hybridization control. Individual Southern blots were probed separately with the P-eFMV promoter region (Probe 1), the HSP70-*ctp2-cp4 epsps* coding region (Probe 2), and the E9 3' polyadenylation sequence (Probe 3). The following results would be expected if the *cp4 epsps* gene cassette were intact: 1) probing the *Dra* I + *Mfe* I digested Roundup Ready alfalfa genomic DNA with Probe 1 would result in an ~1.2 kb hybridization signal, 2) probing the *Dra* I + *Mfe* I

digested Roundup Ready alfalfa genomic DNA with Probe 2 would result in a single hybridization signal of ~2.3 kb, and 3) probing the *Dra* I + *Mfe* I digested Roundup Ready alfalfa genomic DNA with Probe 3 would result in an ~2.3 kb hybridization signal.

Probe 1 for Roundup Ready Alfalfa Event J163. Results of the analysis for Roundup Ready alfalfa event J163 are shown in Figure V-12. R2336 control DNA (lane 2) showed no detectable hybridization signals, as expected for the negative control. R2336 control DNA mixed with plasmid PV-MSHT4 in lanes 4 and 5 yielded the expected ~1.2 kb signal (Figure V-1A). Roundup Ready alfalfa event J163 genomic DNA digested with *Dra* I + *Mfe* I (lanes 3 and 6) produced a single band at approximately 1.3 kb. The hybridization signal in the test sample lanes appears to be slightly larger than expected. Analysis of the DNA sequence flanking the 5' end of the insert in Roundup Ready alfalfa event J163 has shown that the *Dra* I site predicted at the beginning of the insert is not present. Instead, a *Dra* I site was found in the genomic DNA sequence flanking the 5' end of the insert (data not shown). As a result, the restriction fragment in Roundup Ready alfalfa event J163 is slightly larger than that predicted based on the plasmid PV-MSHT4 map. No unexpected bands were detected, indicating that Roundup Ready alfalfa event J163 does not contain any additional enhanced P-eFMV promoter element DNA sequence other than that associated with the intact HSP70- ctp2-*cp4 epsps* gene cassette.

Probe 2 for Roundup Ready Alfalfa Event J163. Results of the analysis for Roundup Ready alfalfa event J163 are shown in Figure V-13. R2336 control DNA (lane 2) showed no detectable hybridization signals, as expected for the negative control R2336 control DNA mixed with plasmid PV-MSHT4 in lanes 4 and 5 yielded the expected ~2.3 kb signal (Figure V-1A). Roundup Ready alfalfa event J163 genomic DNA digested with *Dra* I + *Mfe* I (lanes 3 and 6) produced a single band at approximately 2.3 kb. No unexpected bands were detected, indicating that Roundup Ready alfalfa event J163 does not contain any additional *cp4 epsps* genetic element DNA sequence other than that associated with the intact HSP70- ctp2-*cp4 epsps* gene cassette.

Probe 3 for Roundup Ready Alfalfa Event J163. Results of the analysis for Roundup Ready alfalfa event J163 are shown in Figure V-14. Conventional alfalfa R2336 control DNA (lane 2) yielded faint hybridization signals at approximately 0.4 kb, 0.6 kb, and 1.1 kb. R2336 control DNA mixed with plasmid PV-MSHT4 in lanes 4 and 5 yielded the expected ~2.3 kb signal (Figure V-1A), as well as additional faint hybridization signals at ~0.4 kb ~0.6 kb, and ~1.1 kb. Roundup Ready alfalfa event J163 genomic DNA digested with *Dra* I + *Mfe* I (lanes 3 and 6) produced a band at approximately 2.3 kb as well as hybridization signals at ~0.4 kb, ~0.6 kb, and ~1.1 kb. The ~0.4 kb, ~0.6 kb, and ~1.1 kb bands observed in the positive controls (lanes 4 and 5), and in the test sample lanes (3 and 6) are also observed in the negative control (lane 2). These additional bands are therefore concluded to be the result of hybridization of Probe 3 to endogenous alfalfa DNA sequences that bear similarity to the probe sequence. No additional unexpected bands were detected, indicating that Roundup ready alfalfa event J163 does not contain any additional E9 3' genetic element DNA sequence other than that associated with the intact HSP70- ctp2-*cp4 epsps* gene cassette.

Analysis for the Left Border Region in Roundup Ready Alfalfa Event J163. In order to demonstrate that at least a portion of the left border region of the T-DNA is present in Roundup Ready alfalfa event J163, a DNA probe specific to the left border region was generated and used for Southern blot analysis. Test and control genomic DNA was digested with the restriction endonuclease *Sph* I, which cleaves once within the T-DNA between the ctp2 and cp4 epsps genetic elements to release two border, fragments (Figure V-1B). Control DNA was spiked with two gel- purified *Sph* I restriction fragments of the plasmid PV-MSHT4 to serve as hybridization controls. The Southern blot was probed with the left border region of the plasmid PV-MSHT4 (Probe 4). The results of the analysis for Roundup Ready alfalfa event J163 are shown in Figure V-15. Digested DNA from the control R2336 yielded no signal as expected (lane 2). *Sph* I restriction fragments of the plasmid PV-MSHT4 spiked into a matrix of digested R2336 control DNA (lanes 4 and 5) produced the predicted size band of approximately 7.5 kb (Figure V-1A). The long and short runs (lanes 3 and 6) of Roundup Ready alfalfa event J163 DNA yielded a hybridization signal of approximately 3.6 kb. It is therefore concluded that Roundup Ready alfalfa event J163 minimally contains a portion of the left border region as expected. Additionally, the size of the hybridization signal is consistent with the size of the signal obtained in the copy number analysis (Figure V-11). Based on these results it can also be concluded that Roundup Ready alfalfa event J163 does not have any additional left border region DNA sequences other than those associated with the T-DNA insertion.

Analysis for Backbone in Roundup Ready Alfalfa Event J163. Roundup Ready alfalfa event J163 was analyzed for the presence of backbone sequences from the plasmid PV-MSHT4. Results from this analysis are presented in Figure V-16. Test and control genomic DNA were digested with the restriction endonucleases *Sal* I, *Sca* I and *Xba* I, which do not cut within the vector PV-MSHT4. A *Hin*d III + *Not* I restriction fragment, containing the backbone region of the plasmid PV-MSHT4, was mixed with control R2336 genomic DNA and digested with *Sal* I, *Sca* I and *Xba* I to serve as a positive hybridization control. The blot was probed with two overlapping probes (Probes 5 and 6) that span the backbone present in plasmid PV-MSHT4 (Figure V-1A). Negative control R2336 genomic DNA (lane 2) showed no detectable hybridization bands as expected. Plasmid PV-MSHT4 *Hin*d III + *Not* I restriction fragment mixed with R2336 control DNA (lanes 4 and 5) produced one expected size band at approximately 5.6 kb. Roundup Ready alfalfa event J163 (lanes 3 and 6) showed no detectable hybridization bands. This result establishes that Roundup Ready alfalfa event J163 does not contain any detectable backbone sequence from the transformation vector PV-MSHT4.

Predicted insert map for event J163. A predicted map of the insert was constructed on the basis of data derived from Southern Blots analyses conducted on J163. This insert map is presented in Figure V-17.

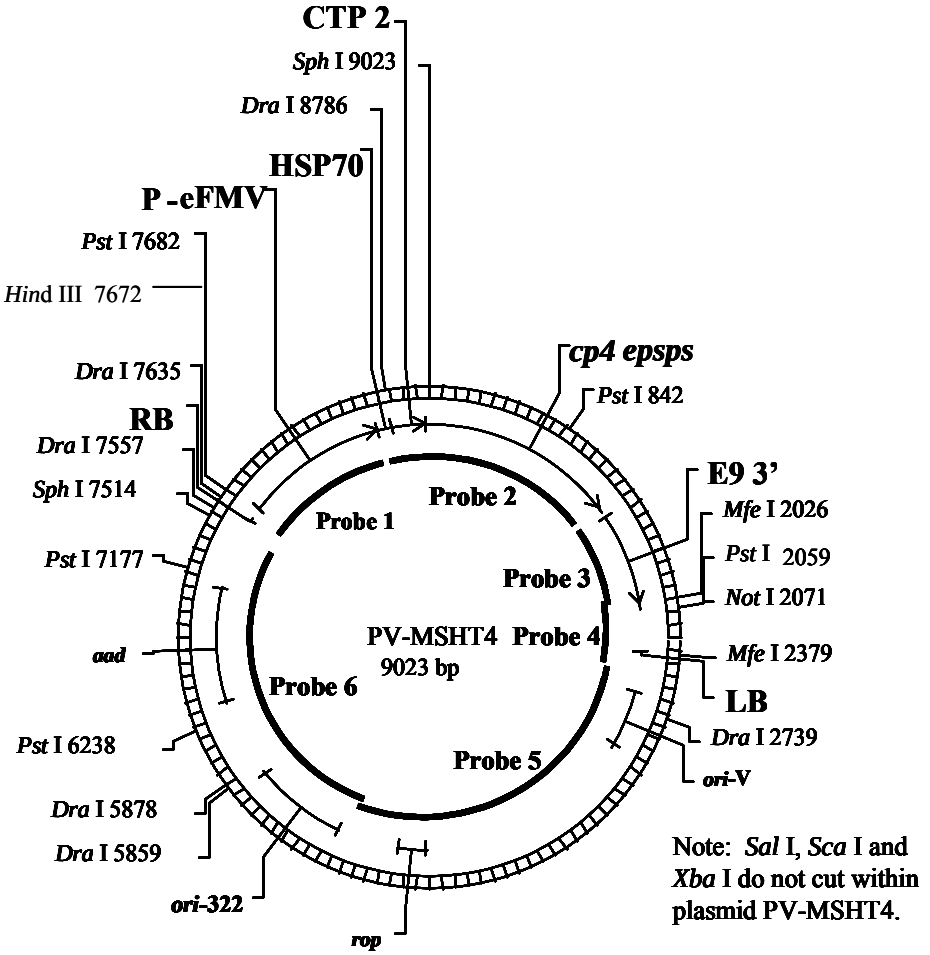
A.3. Stability of the Inserted T-DNA in Roundup Ready Alfalfa Events J101 and J163

The stability of the T-DNA insertions in Roundup Ready alfalfa events J101 and J163 was determined by analyzing the T_0 and dihomogenic Syn 1 generations. Because alfalfa can be vegetatively propagated, the original T_0 plants that were regenerated from the R2336 callus tissue have been maintained. The breeding history for Roundup Ready alfalfa is shown in Figure V-18. Tissue was obtained from the original transformants (T_0) as well as

from plants in the advanced breeding program. Genomic DNA samples from the T_0 generation of Roundup Ready alfalfa events J101 and J163 and the dihomogenic Syn 1 generation J101 x J163, as well as the R2336 control, were digested with the restriction endonuclease *Sph* I. R2336 control DNA was spiked with the plasmid PV-MSHT4 to serve as the positive hybridization control. The blot was probed with Probes 1 and 2. The results from this experiment are shown in Figure V-19. No hybridization was observed in the negative control as expected (lane 2). R2336 control DNA spiked with plasmid PV-MSHT4 (Figure V-1A) and digested with the restriction endonuclease *Sph* I produced the predicted size bands of approximately 1.5 kb and 7.5 kb (lane 3). Roundup Ready alfalfa event J101 yielded the expected band sizes of 13.0 kb and 6.5 kb (lane 4). Roundup Ready alfalfa event J163 yielded the expected size bands of 3.6 kb and 1.9 kb (lane 6). Lane 9 containing *Sph* I digested DNA from the dihomogenic J101 x J163 Syn 1 generation yielded hybridization signals of 13.0 kb, 6.5 kb, 3.6 kb, and 1.9 kb. These band sizes are consistent with those produced by the T_0 simplex events J101 (13.0 kb and 6.5 kb) and J163 (3.6 and 1.9 kb). Lanes 5, 7, 8, 10, 11, 12, and 13 contain Roundup Ready alfalfa eventsJ101 and J163, as well as other Roundup Ready alfalfa events that are not being pursued commercially. These data demonstrate that the T-DNA insertion in Roundup Ready alfalfa event J101 and the T-DNA insertion in Roundup Ready alfalfa event J163 are stable in the T_0 and the dihomogenic Syn 1 generations.

A.4. Conclusions – Molecular Characterization

Roundup Ready alfalfa events J101 and J163 were produced by Agrobacterium-mediated transformation of alfalfa cultivar R2336 with plasmid vector PV-MSHT4 containing the *cp4 epsps* gene cassette. The following conclusions can be made for Roundup Ready alfalfa events J101 and J163. Both events contain a single copy of the T-DNA at a single locus of integration. The cp4 epsps gene cassette for both events is intact. No additional elements from the transformation vector PV-MSHT4, linked or unlinked to the intact cassette in each event, were detected in either of the alfalfa plant genomes. Additionally, neither event contains any detectable plasmid backbone sequence. These data generated through Southern blot analyses support the conclusion that only the expected full length CP4 EPSPS protein should be encoded by the inserts in Roundup Ready alfalfa events J101 and J163. Stability analysis for Roundup Ready alfalfa events J101 and J163 has demonstrated that each event was observed to be stable in the T_0 and dihomogenic Syn 1 generations.



Probe	Start Position	End Position	Total Length (bp)
Probe 1 (P-eFMV)	7669	8682	1014
Probe 2 (HSP70-ctp2-*cp4 epsps*)	8692	1392	1724
Probe 3 (E9 3')	1403	2034	632
Probe 4 (left border region)	2023	2385	363
Probe 5 (backbone)	2414	4986	2573
Probe 6 (backbone)	4890	7484	2595

Figure V-1A. Plasmid Map of PV-MSHT4 with Probe and Restriction Enzyme Locations

A circular map of the plasmid vector PV-MSHT4 used in the transformation events J101 and J163 with genetic elements annotated is shown above. Restriction sites with positions relative to the size of the plasmid vector for endonucleases used in the Southern analysis are shown. Probes used in the Southern analysis are detailed in the accompanying table. The portion of the plasmid transferred to the plant genome begins near the right border, extends through the *cp4 epsps* coding region, and ends near the left border.

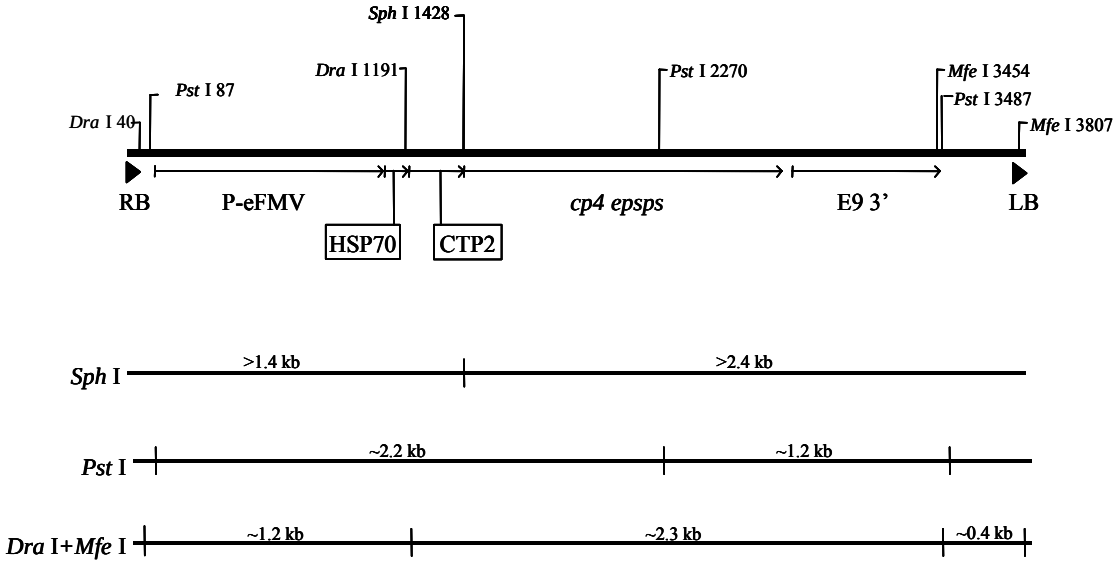


Figure V-1B. Schematic Representation of the T-DNA from the Transformation Vector PV-MSHT4 that is Present in Roundup Ready Alfalfa Events J101 and J163
A linear map of the T-DNA from the transformation vector PV-MSHT4 is shown. Genetic elements annotated within the T-DNA are represented by arrows to indicate the direction of transcription. The left and right borders (LB and RB respectively) are denoted by triangles. The predicted sizes of restriction fragments for each enzyme or enzyme combination used in Southern blot analysis are illustrated beneath the schematic. Fragment sizes were calculated based on the linear map. Roundup Ready alfalfa events J101 and J163 each contain one copy of the T-DNA at a single integration locus. Although the left and right borders are illustrated on the map of the T-DNA, they may or may not be fully intact in each transformation event. Although several restriction enzymes are shown, each one may not have been used in the analysis of both Roundup Ready alfalfa events.

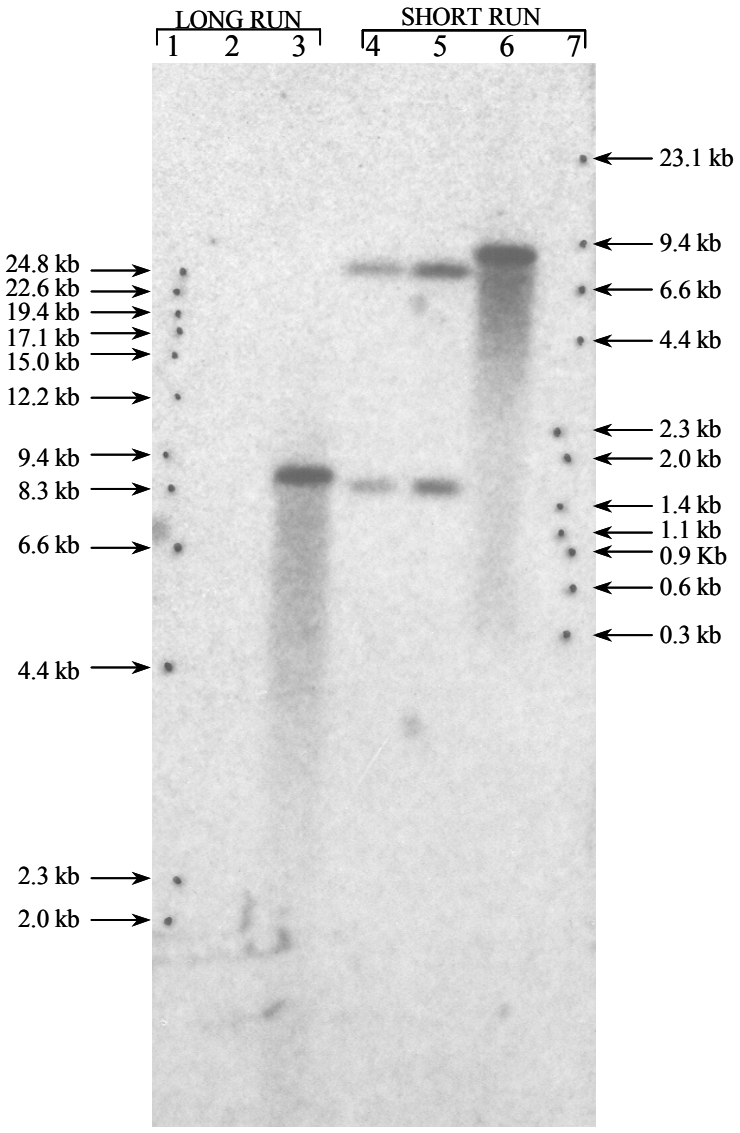


Figure V-2. Southern Blot Analysis of Roundup Ready Alfalfa Event J101: Insert Number

Ten micrograms of R2336 conventional and twenty micrograms of Roundup Ready alfalfa event J101 genomic DNA extracted from leaf tissue were digested with the restriction endonucleases *Sal* I, *Sca* I and *Xba* I. The blot was probed with the P-eFMV (Probe 1) and *cp4 epsps* (Probe 2) genetic elements. Lane designations are as follows:

Lane 1: High Molecular Weight DNA Ladder (Gibco BRL) and Marker II (Roche)
2: R2336 [10 µg]
3: Roundup Ready alfalfa event J101 [10 µg]
4: R2336 [10 µg] spiked with *Sph* I restriction fragments of PV-MSHT4 (0.5 copy)
5: R2336 [10 µg] spiked with *Sph* I restriction fragments of PV-MSHT4 (1 copy)
6: Roundup Ready alfalfa event J101 [10 µg]
7: Markers II and IX (Roche)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium bromide stained gel.

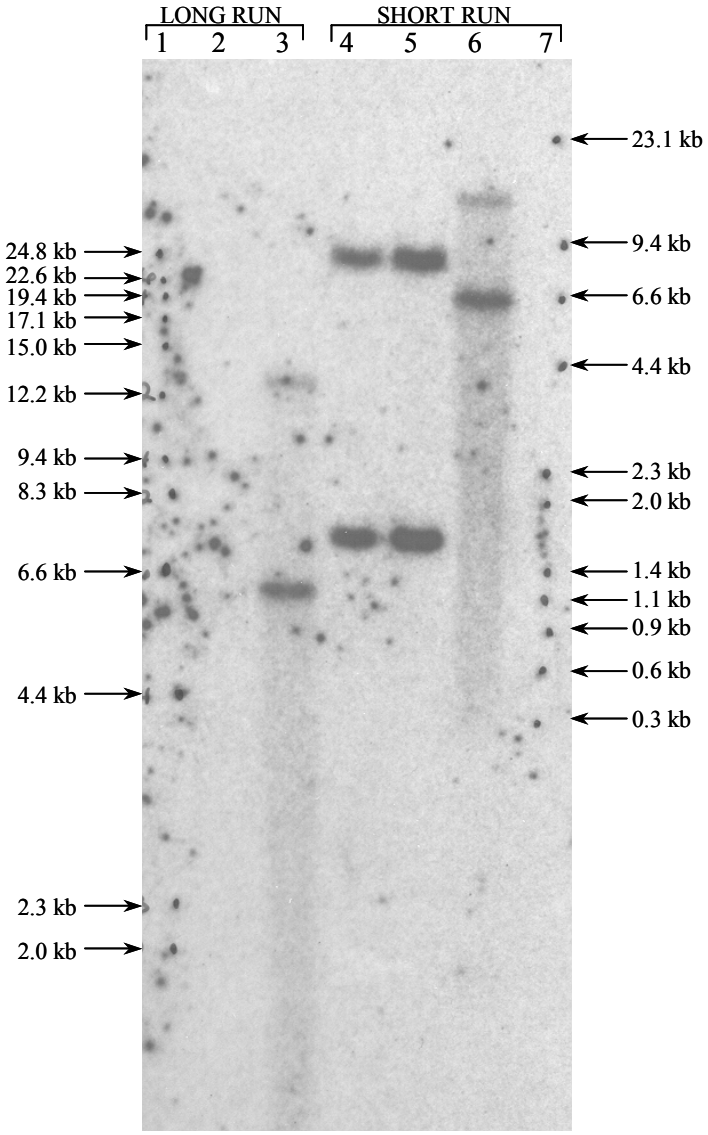


Figure V-3. Southern Blot Analysis of Roundup Ready Alfalfa Event J101: Copy Number
Ten micrograms of R2336 conventional and twenty micrograms of Roundup Ready alfalfa event J101 genomic DNA extracted from leaf tissue were digested with the restriction endonuclease *Sph* I. The blot was probed with the P-eFMV (Probe 1) and *cp4 epsps* (Probe 2) genetic elements. Lane designations are as follows:

Lane 1: High Molecular Weight DNA Ladder (Gibco BRL) and Marker II (Roche)
2: R2336 [10 μg]
3: Roundup Ready alfalfa event J101 [10 μg]
4: R2336 [10 μg] spiked with *Sph* I restriction fragments of PV-MSHT4 (0.5 copy)
5: R2336 [10 μg] spiked with *Sph* I restriction fragments of PV-MSHT4 (1 copy)
6: Roundup Ready alfalfa event J101 [10 μg]
7: Markers II and IX (Roche)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium bromide stained gel.

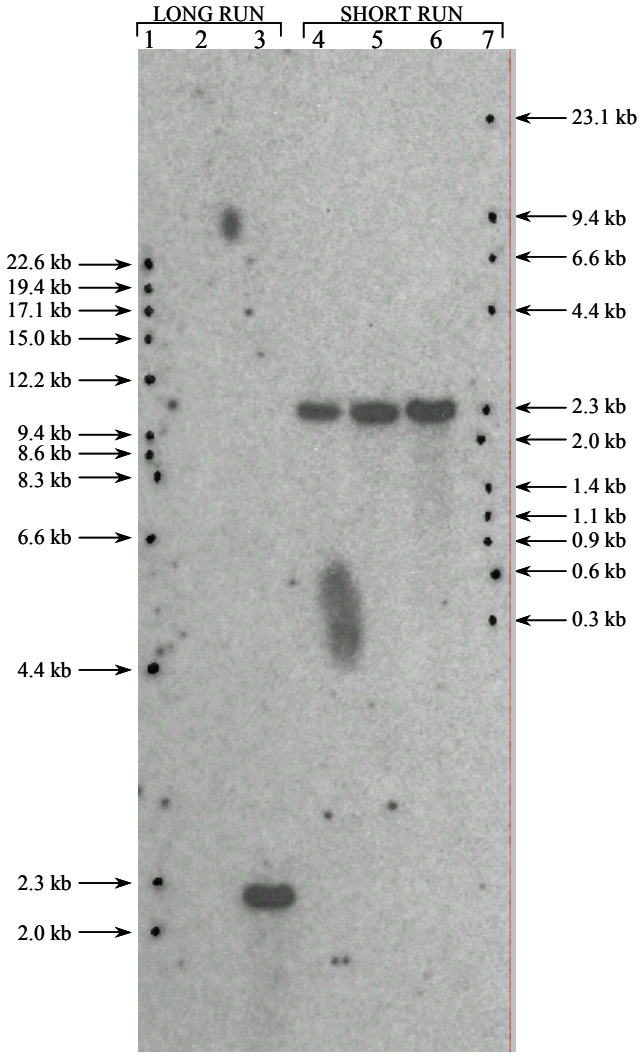


Figure V-4. Southern Blot Analysis of Roundup Ready Alfalfa Event J101: Cassette Intactness (P-eFMV)

Ten micrograms of R2336 conventional and twenty micrograms of Roundup Ready alfalfa event J101 genomic DNA extracted from leaf tissue were digested with the restriction endonuclease *Pst* I. The blot was probed with the P-eFMV (Probe 1) genetic element. Lane designations are as follows:

Lane 1: High Molecular Weight DNA Ladder (Gibco BRL) and Marker II (Roche)
2: R2336 [10 µg]
3: Roundup Ready alfalfa event J101 [10 µg]
4: R2336 [10 µg] spiked with two *Pst* I restriction fragments of PV-MSHT4 (0.5 copy)
5: R2336 [10 µg] spiked with two *Pst* I restriction fragments of PV-MSHT4 (1 copy)
6: Roundup Ready alfalfa event J101 [10 µg]
7: Markers II and IX (Roche)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium bromide stained gel.

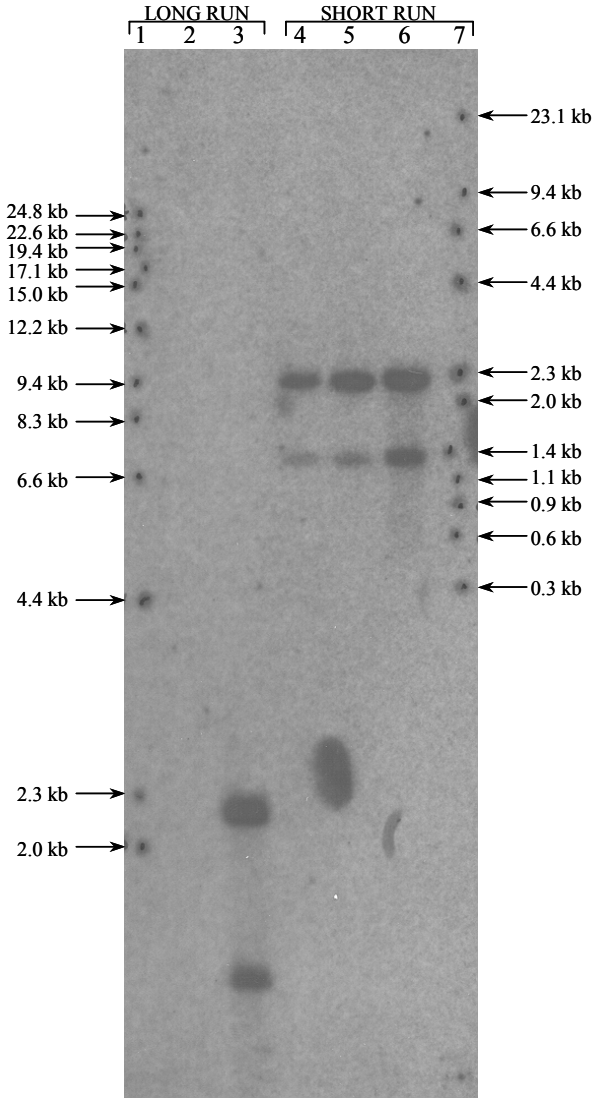


Figure V-5. Southern Blot Analysis of Roundup Ready Alfalfa Event J101: Cassette Intactness (HSP70-ctp2-*cp4 epsps*)

Ten micrograms of R2336 conventional and twenty micrograms of Roundup Ready alfalfa event J101 genomic DNA extracted from leaf tissue were digested with the restriction endonuclease *Pst* I. The blot was probed with the HSP70-ctp2-*cp4 epsps* (Probe 2) genetic elements. Lane designations are as follows:

Lane 1: High Molecular Weight DNA Ladder (Gibco BRL) and Marker II (Roche)
2: R2336 [10 µg]
3: Roundup Ready alfalfa event J101 [10 µg]
4: R2336 [10 µg] spiked with two *Pst* I restriction fragments of PV-MSHT4 (0.5 copy)
5: R2336 [10 µg] spiked with two *Pst* I restriction fragments of PV-MSHT4 (1 copy)
6: Roundpu Ready alfalfa event J101 [10 µg]
7: Markers II and IX (Roche)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium bromide stained gel

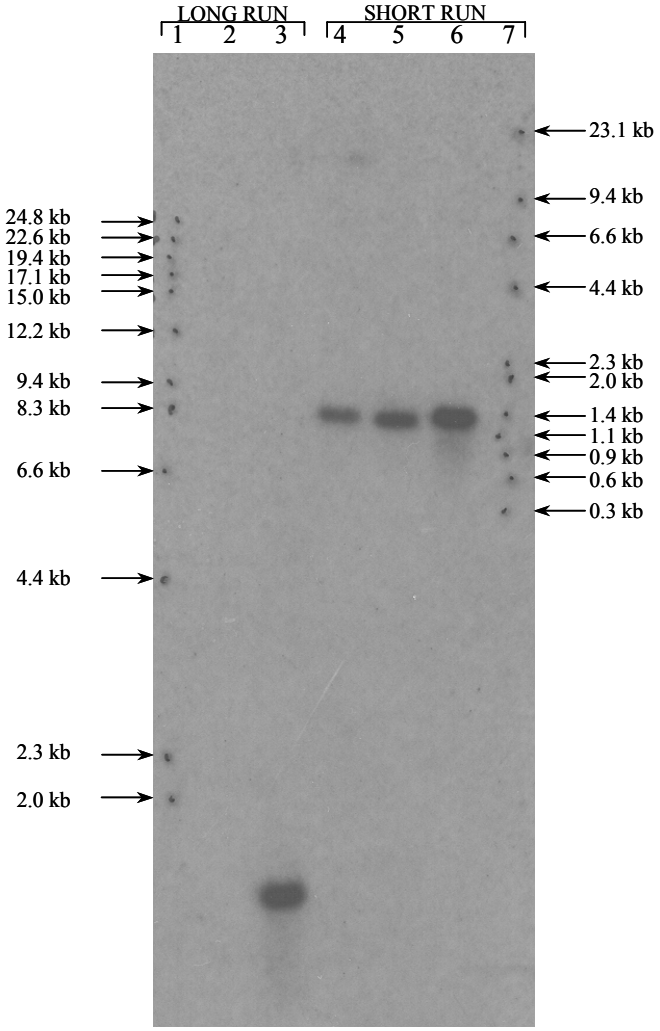


Figure V-6. Southern Blot Analysis of Roundup Ready Alfalfa Event J101: Cassette Intactness (E9 3')

Ten micrograms of R2336 conventional and twenty micrograms of Roundup Ready alfalfa event J101 genomic DNA extracted from leaf tissue were digested with the restriction endonuclease *Pst* I. The blot was probed with the E9 3' (Probe 3) genetic element. Lane designations are as follows:

Lane 1: High Molecular Weight DNA Ladder (Gibco BRL) and Marker II (Roche)
2: R2336 [10 μg]
3: Roundup Ready alfalfa event J101 [10 μg]
4: R2336 [10 μg] spiked with two *Pst* I restriction fragments of PV-MSHT4 (0.5 copy)
5: R2336 [10 μg] spiked with two *Pst* I restriction fragments of PV-MSHT4 (1 copy)
6: Roundup Ready alfalfa event J101 [10 μg]
7 : Markers II and IX (Roche)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium bromide stained gel.

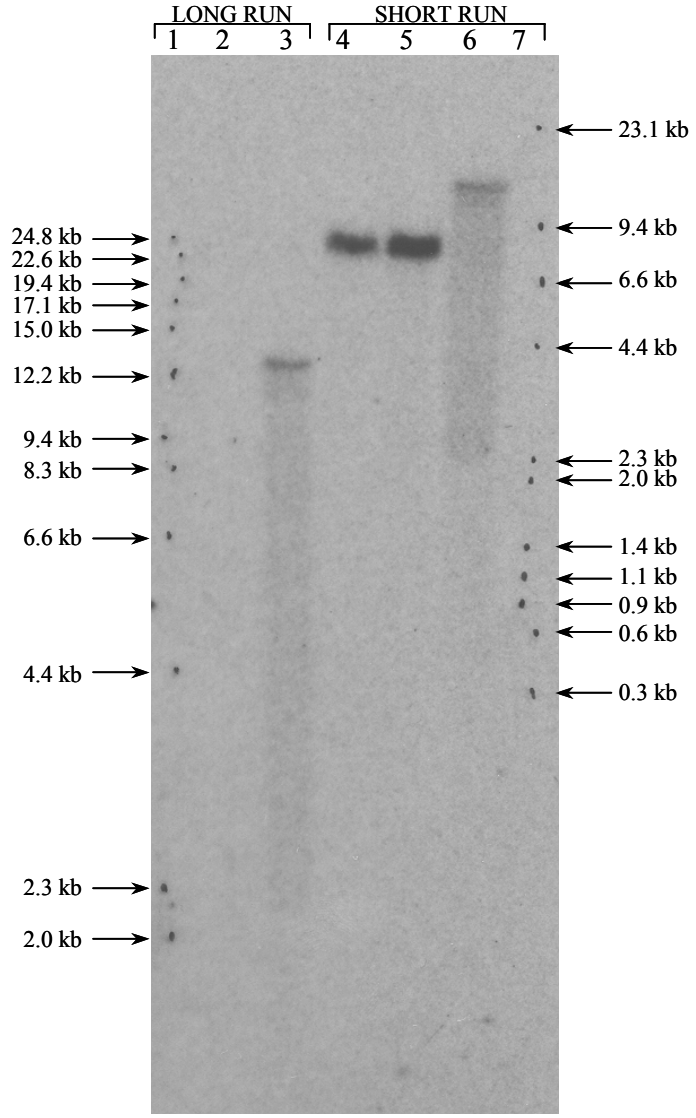


Figure V-7. Southern Blot Analysis of Roundup Ready Alfalfa Event J101: Left Border Region

Ten micrograms of R2336 conventional and twenty micrograms of Roundup Ready alfalfa event J101 genomic DNA extracted from leaf tissue were digested with the restriction endonuclease *Sph* I. The blot was probed with the left border region (Probe 4) of the plasmid PV-MSHT4 genetic elements. Lane designations are as follows:

Lane 1: High Molecular Weight DNA Ladder (Gibco BRL) and Marker II (Roche)
2: R2336 [10 µg]
3: Roundup Ready alfalfa event J101 [10 µg]
4: R2336 [10 µg] spiked with *Sph* I restriction fragments of PV-MSHT4 (0.5 copy)
5: R2336 [10 µg] spiked with *Sph* I restriction fragments of PV-MSHT4 (1 copy)
6: Roundup Ready alfalfa event J101 [10 µg]
7; Markers II and IX (Roche

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium bromide stained gel.

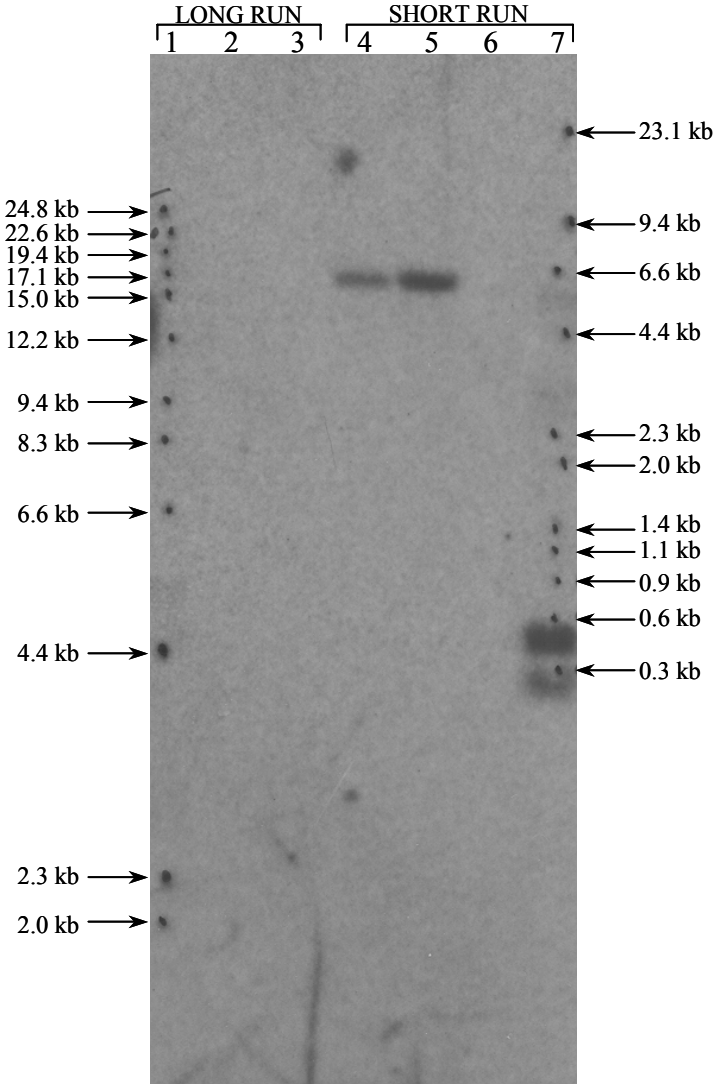


Figure V-8. Southern Blot Analysis of Roundup Ready Alfalfa Event J101: Backbone

Ten micrograms of R2336 conventional and twenty micrograms of Roundup Ready alfalfa event J101 genomic DNA extracted from leaf tissue were digested with the restriction endonucleases *Sal* I, *Sca* I and *Xba* I. The blot was probed with the backbone region of the plasmid PV-MSHT4 (Probes 5 and 6). Lane designations are as follows:

Lane 1: High Molecular Weight DNA Ladder (Gibco BRL) and Marker II (Roche)
2: R2336 [10 µg]
3: Roundup Ready alfalfa event J101 [10 µg]
4: R2336 [10 µg] spiked with *Hin*d III + *Not* I restriction fragment of PV-MSHT4 (0.5 copy)
5: R2336 [10 µg] spiked with *Hin*d III + *Not* I restriction fragment of PV-MSHT4 (1 copy)
6: Roundup Ready alfalfa event J101 [10 µg]
7: Markers II and IX (Roche)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium bromide stained gel.

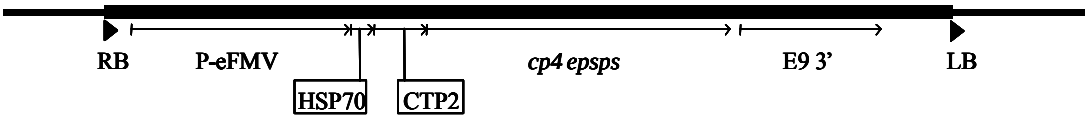


Figure V-9. Schematic Representation of the Insert in Roundup Ready Alfalfa Event J101

A schematic of the insert in Roundup Ready alfalfa J101 is shown above. The bold heavy line represents the genetic material inserted into the alfalfa genome. The lighter line to the left and right of the insert represents genomic DNA. Individual genetic elements are identified below the insert. The map was developed on the basis of Southern blot characterization data for J101.

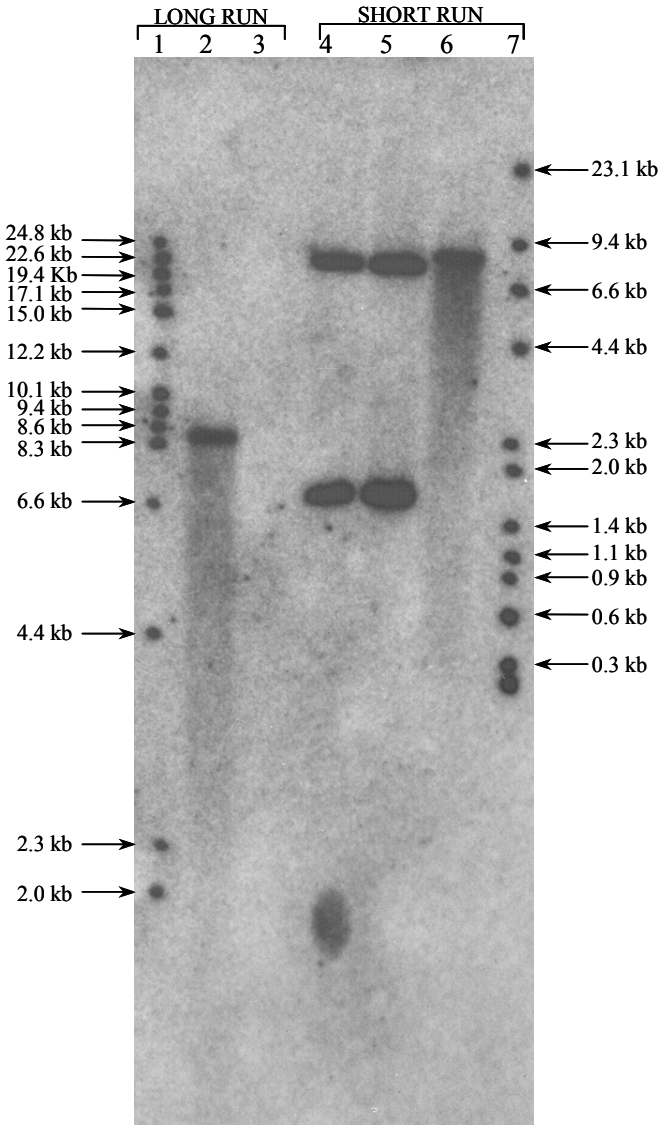


Figure V-10. Southern Blot Analysis of Roundup Ready Alfalfa Event J163: Insert Number

Ten micrograms of R2336 conventional and twenty micrograms of Roundup Ready alfalfa event J163 genomic DNA extracted from leaf tissue were digested with the restriction endonucleases *Sal* I, *Sca* I and *Xba* I. The blot was probed with the P-eFMV (Probe 1) and *cp4 epsps* (Probe 2) genetic elements. Lane designations are as follows:

Lane 1: High Molecular Weight DNA Ladder (Gibco BRL) and Marker II (Roche)
2: Roundup Ready alfalfa event J163 [10 µg]
3: R2336 [10 µg]
4: R2336 [10 µg] spiked with *Sph* I restriction fragments of PV-MSHT4 (0.5 copy)
5: R2336 [10 µg] spiked with *Sph* I restriction fragments of PV-MSHT4 (1 copy)
6: Roundup Ready alfalfa event J163 [10 µg]
7: Markers II and IX (Roche)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium bromide stained gel.

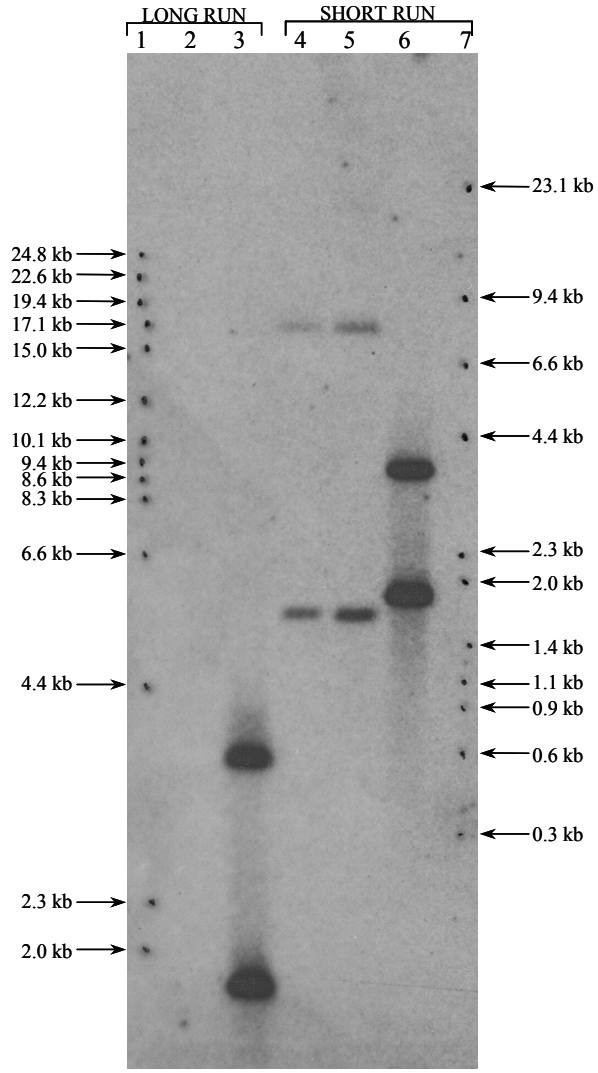


Figure V-11. Southern Blot Analysis of Roundup Ready Alfalfa Event J163: Copy Number

Ten micrograms of R2336 conventional and twenty micrograms of Roundup Ready alfalfa event J163 genomic DNA extracted from leaf tissue were digested with the restriction endonuclease *Sph* I. The blot was probed with the P-eFMV (Probe 1) and *cp4 epsps* (Probe 2) genetic elements. Lane designations are as follows:

Lane 1: High Molecular Weight DNA Ladder (Gibco BRL) and Marker II (Roche)
2: R2336 [10 µg]
3: Roundup Ready alfalfa event J163 [10 µg]
4: R2336 [10 µg] spiked with *Sph* I restriction fragments of PV-MSHT4 (0.5 copy)
5: R2336 [10 µg] spiked with *Sph* I restriction fragments of PV-MSHT4 (1 copy)
6: Roundup Ready alfalfa event J163 [10 µg]
7: Markers II and IX (Roche)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium bromide stained gel.

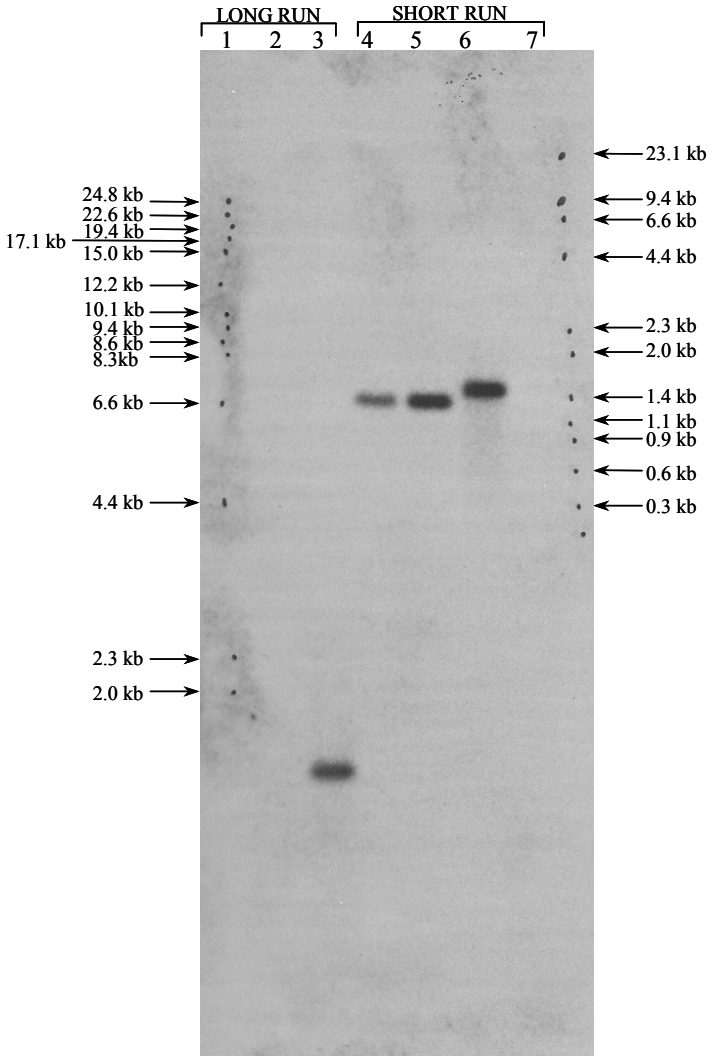


Figure V-12. Southern Blot Analysis of Roundup Ready Alfalfa Event J163: Cassette intactness (P-eFMV)

Ten micrograms of R2336 conventional and twenty micrograms of Roundup Ready alfalfa event J163 genomic DNA extracted from leaf tissue were digested with the restriction endonuclease combination *Dra* I+*Mfe* I. The blot was probed with the P-eFMV (Probe 1) genetic element. Lane designations are as follows:

Lane 1: High Molecular Weight DNA Ladder (Gibco BRL) and Marker II (Roche)
2: R2336 [10 µg]
3: Roundup Ready alfalfa event J163 [10 µg]
4: R2336 [10 µg] spiked with PV-MSHT4 (0.5 copy
5: R2336 [10 µg] spiked with PV-MSHT4 (1 copy)
6: Roundup Ready alfalfa event J163 [10 µg]
7: Markers II and IX (Roche)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium bromide stained gel.

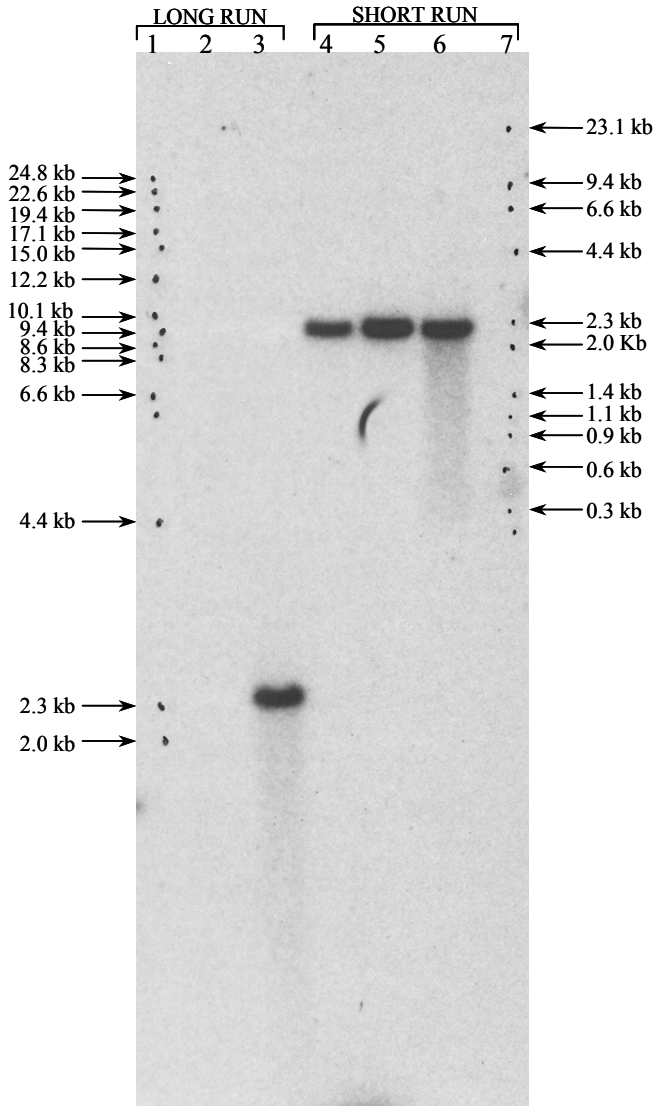


Figure V-13. Southern Blot Analysis of Roundup Ready Alfalfa Event J163: Cassette Intactness (HSP70-ctp2-*cp4 epsps*)

Ten micrograms of R2336 conventional and twenty micrograms of Roundup Ready alfalfa event J163 genomic DNA extracted from leaf tissue were digested with the restriction endonuclease combination *Dra* I+*Mfe* I. The blot was probed with the HSP70-ctp2-*cp4 epsps* (Probe 2) genetic elements. Lane designations are as follows:

Lane 1: High Molecular Weight DNA Ladder (Gibco BRL) and Marker II (Roche)
2: R2336 [10 µg]
3: Roundup Ready alfalfa J163 [10 µg]
4: R2336 [10 µg] spiked with PV-MSHT4 (0.5 copy)
5: R2336 [10 µg] spiked with PV-MSHT4 (1 copy)
6: Roundup Ready alfalfa event J163 [10 µg]
7: Markers II and IX (Roche)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium bromide stained gel.

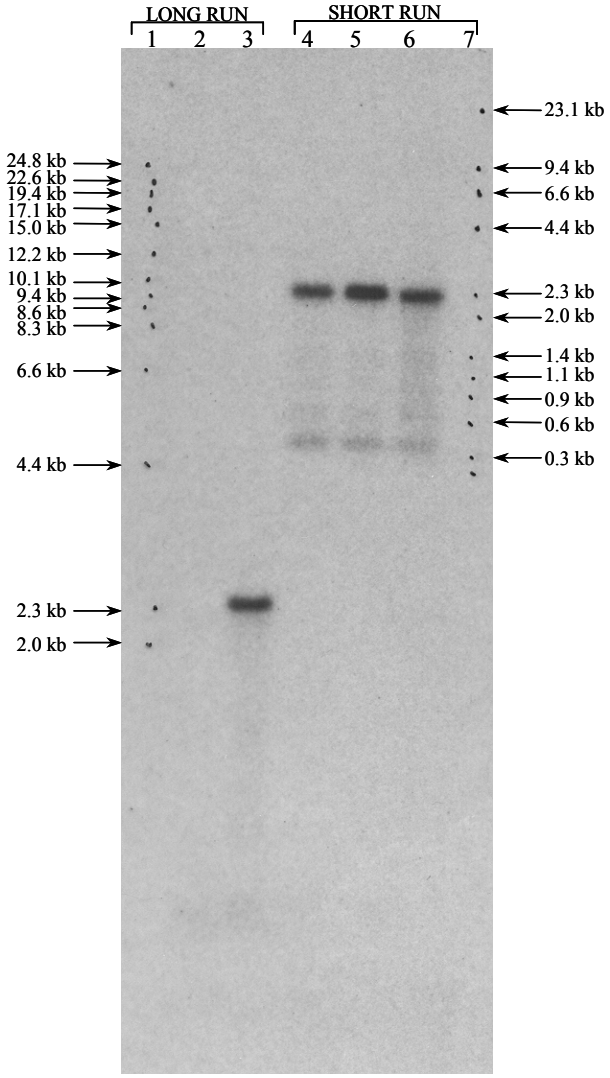


Figure V-14. Southern Blot Analysis of Roundup Ready Alfalfa Event J163: Cassette Intactness (E9 3')

Ten micrograms of R2336 conventional and twenty micrograms of Roundup Ready alfalfa event J163 genomic DNA extracted from leaf tissue were digested with the restriction endonuclease combination *Dra* I+*Mfe* I. The blot was probed with the E9 3' (Probe 3) genetic element. Lane designations are as follows:

Lane 1: High Molecular Weight DNA Ladder (Gibco BRL) and Marker II (Roche)
2: R2336 [10 µg]
3: Roundup Ready alfalfa event J163 [10 µg]
4: R2336 [10 µg] spiked with PV-MSHT4 (0.5 copy
5: R2336 [10 µg] spiked with PV-MSHT4 (1 copy)
6: Roundup Ready alfalfa event J163 [10 µg]
7: Markers II and IX (Roche)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium bromide stained gel.

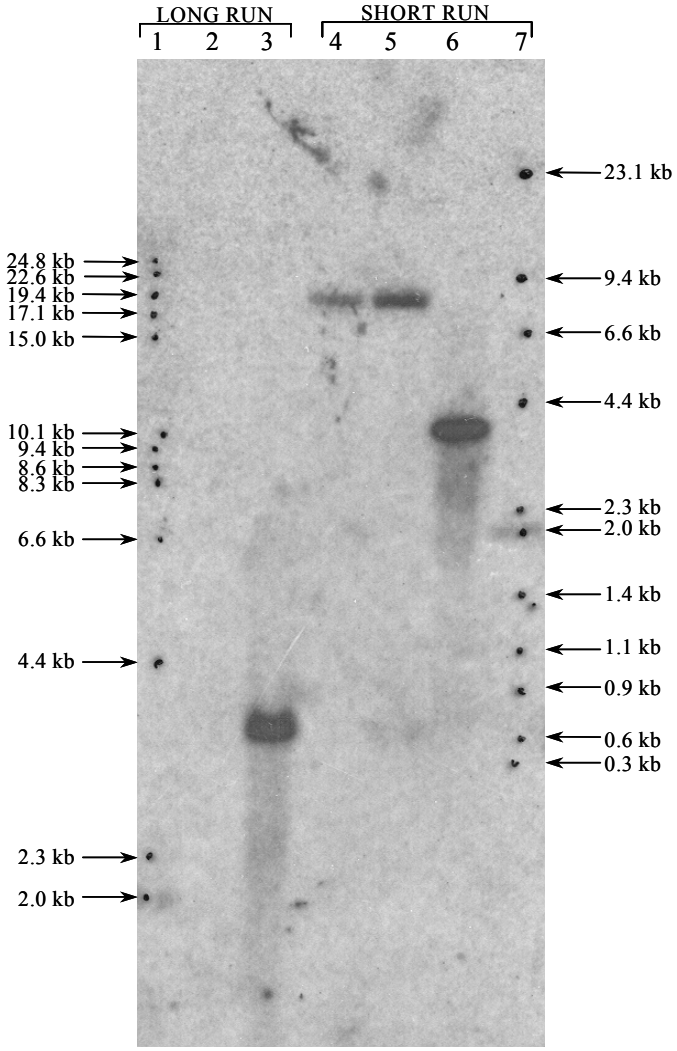


Figure V-15. Southern Blot Analysis of Roundup Ready Alfalfa Event J163: Left Border Region

Ten micrograms of R2336 conventional and twenty micrograms of Roundup Ready alfalfa event J163 genomic DNA extracted from leaf tissue were digested with the restriction endonuclease *Sph* I. The blot was probed with the left border region (Probe 4) of the plasmid PV-MSHT4 genetic elements. Lane designations are as follows:

Lane 1: High Molecular Weight DNA Ladder (Gibco BRL) and Marker II (Roche)
2: R2336 [10 µg]
3: Roundup Ready alfalfa event J163 [10 µg]
4: R2336 [10 µg] spiked with *Sph* I restriction fragments of PV-MSHT4 (0.5 copy)
5: R2336 [10 µg] spiked with *Sph* I restriction fragments of PV-MSHT4 (1 copy)
6: Roundup Ready alfalfa event J163 [10 µg]
7: Markers II and IX (Roche)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium bromide stained gel.

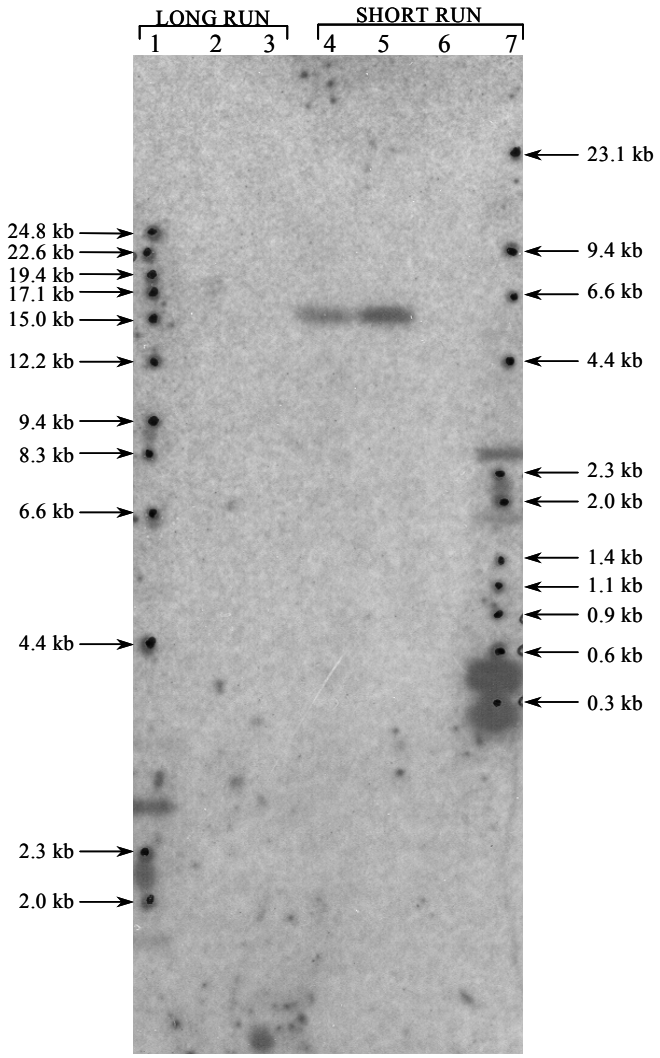


Figure V-16. Southern Blot Analysis of Roundup Ready Alfalfa Event J163: Backbone

Ten micrograms of R2336 conventional and twenty micrograms of Roundup Ready alfalfa event J163 genomic DNA extracted from leaf tissue were digested with the restriction endonucleases *Sal* I, *Sca* I and *Xba* I. The blot was probed with the backbone region of the plasmid PV-MSHT4 (Probes 5 and 6). Lane designations are as follows:

Lane 1: High Molecular Weight DNA Ladder (Gibco BRL) and Marker II (Roche)
2: R2336 [10 µg]
3: Roundup Ready alfalfa event J163 [10 µg]
4: R2336 [10 µg] spiked with *Hin*d III + *Not* I restriction fragment of PV-MSHT4 (0.5 copy)
5: R2336 [10 µg] spiked with *Hin*d III + *Not* I restriction fragment of PV-MSHT4 (1 copy)
6: Roundup Ready alfalfa event J163 [10 µg]
7: Markers II and IX (Roche)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium bromide stained gel.

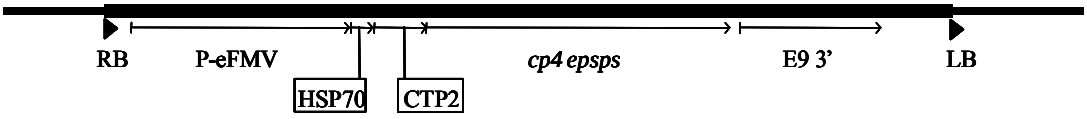
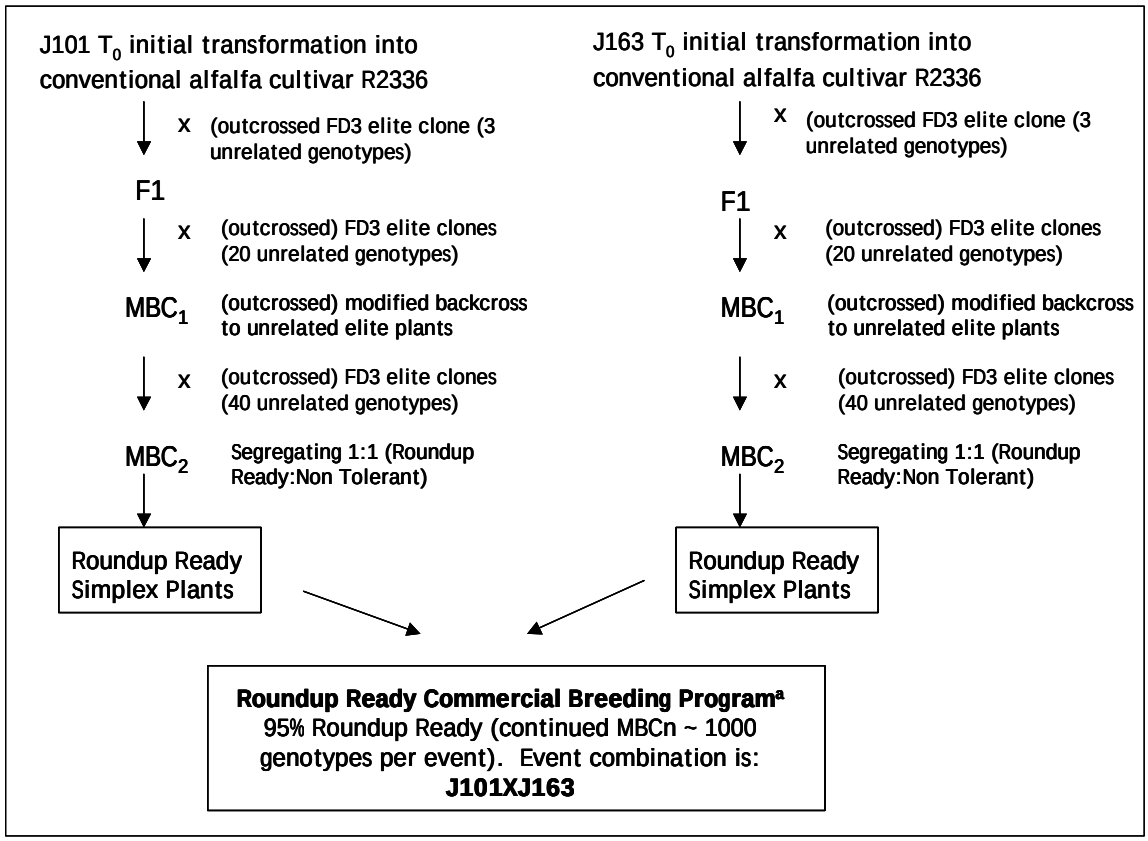


Figure V-17. Schematic Representation of the Insert in Roundup Ready Alfalfa Event J163

A schematic of the insertion in J163 is shown above. The bold heavy line represents the genetic material inserted into the alfalfa genome. The lighter line to the left and right of the insert represents genomic DNA. Individual genetic elements are identified below the insert. The map was developed on the basis of Southern blot characterization data for J163.



[a]Dihomogenenic Syn 1 generation
FD = fall dormancy
x = hybrid cross
MBC = modified backcross

Figure V-18. Roundup Ready Alfalfa Breeding History

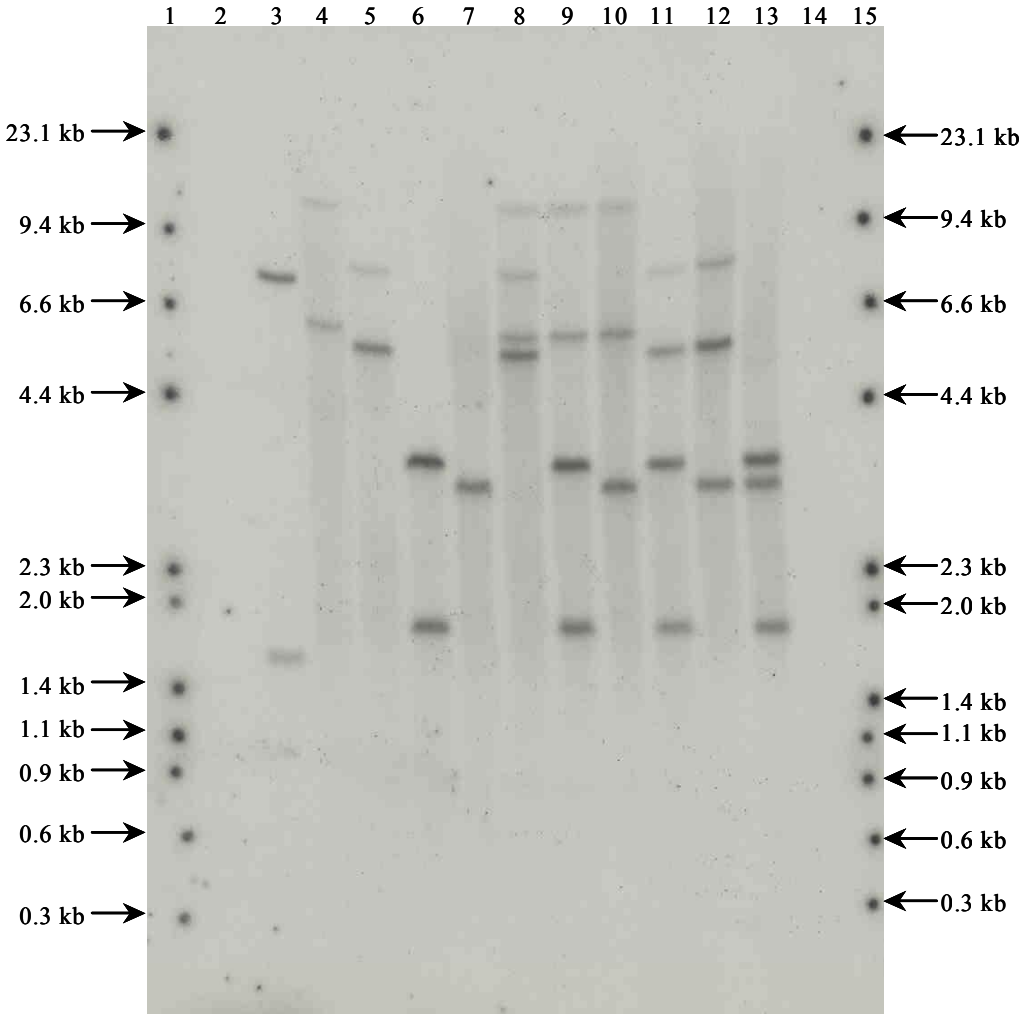


Figure V-19. Stability Analysis of Roundup Ready Alfalfa Events J101 and J163
Ten micrograms of R2336 conventional and ten micrograms of genomic DNA from the test substances were digested with the restriction endonuclease *Sph* I. The blot was probed with the P-eFMV (Probe 1) and HSP70-ctp2-*cp4 epsps* (Probe 2). Lane designations are as follows:

Lane
1: Markers II and IX (Roche)
2: R2336 [10 µg]
3: R2336 [10 µg] spiked with plasmid PV-MSHT4 (1 copy)
4: Roundup Ready alfalfa event J101 [10 µg]
5: Event dropped from development
6: Roundup Ready alfalfa event J163 [10 µg]
7: Event dropped from development
8: Line dropped development
9: J101 x J163 [10 µg]
10: Line dropped from development
11: Line dropped from development
12: Line dropped from development
13: Line dropped from development
14: Empty
15: Markers II and IX (Roche)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium bromide stained gel.

B. Segregation Data

Inheritance of the Roundup Ready insert was determined through five generations where the Roundup Ready gene was introgressed into elite alfalfa germplasm. The J101 and J163 T_0 plants were initially outcrossed to a small number of elite FD3 clones. Roundup Ready progeny from the initial F1 cross were outcrossed to a second set of unrelated elite FD3 clones to produce the modified backcross 1 generation (MBC_1). Subsequent MBC_n generations (MBC_2-MBC_4) were produced by outcrossing Roundup Ready progeny from the previous generation to elite conventional clones from a variety of fall dormancy groups (Fall Dormancy and Non-dormancy). For all of the crossing cycles described above, the Roundup Ready parent was used as the pollen donor. Segregation data for four of the five generations of populations derived from modified backcrosses made between F1 plants hemizygous for the *cp4 epsps* gene and derived from plants containing Roundup Ready alfalfa events and elite parental plants are presented in Table V-1. The inheritance of the introduced DNA in the progenies from the modified backcrosses was monitored phenotypically at the whole plant level by application of Roundup at the two- to three-leaf stage in a greenhouse.

Statistical significance for the segregation data was determined using Chi square analysis. For these analyses a Chi square value (χ^2) was determined as follows: $\chi^2 = \sum [(|o-e|-0.5)^2/e]$, where o = observed frequencies for each class, e = expected frequencies for each class and 0.5 = Yates correction factor for Chi square analysis with one degree of freedom (df) (Little and Hills, 1978). The calculated Chi square value was compared to a table of Chi square values to determine whether the observed frequencies fit the expectation for a single insert at p = 0.05 and/or p = 0.01.

Chi square analysis showed that the majority of the values were not significantly different. However, four of the 14 Chi-square values (Table V-1) indicate a significant difference between the observed and expected values with three of the four values below the predicted value and one above. Much of the variability can probably be attributed to differences in the rate of setting of self-seed observed with different alfalfa populations. As part of the Roundup Ready alfalfa breeding development program Forage Genetics has used the Roundup Ready trait to estimate the level of selfing in hand crosses of alfalfa (McCaslin and Temple, 2003). Research conducted by Forage Genetics indicates that under greenhouse conditions, when non-emasculated hand crosses are made by experienced researchers, up to 10% of the seed produced can be the result of selfing. Data presented in Table V-1 were developed using Roundup Ready alfalfa pollen donors and conventional alfalfa plants as the females. Therefore, given the predicted level of selfing, a 45% inheritance ratio would be expected. In practice, Forage Genetics has observed a 43-47% inheritance ratio for the Roundup Ready trait in the on-going forward breeding program. The remaining statistically significant observation at 60% is clearly above the predicted value of 50%, however, the sample size used for this determination was based on a very small number of observations (only 133 test samples), thus random sampling error may have contributed to the unexpectedly high inheritance ratio for this population estimate.

The remaining observation at 60% is clearly above the predicted value of 50%. However, the sample size used for this determination was based on a very small number

of observations (only 133 test samples), and thus may have contributed to sampling errors resulting in the unexpectedly high inheritance ratio.

In summary, data presented in Table V-1 show that the Roundup Ready trait was stably maintained through five generations in alfalfa. On the basis of Chi square analyses of the inheritance data, it is concluded that the Roundup Ready trait in alfalfa plants containing J101 or J163 is inherited in a one-locus Mendelian fashion. These results are also consistent with the genetic analysis described in this section, thus confirming that J101 and J163 are present at a single locus.

Table V-1. Phenotypic Segregation Data for Roundup Ready Alfalfa Events J101 and J163.

Dormancy Group	Event(s)	Generation	Number Tested	Number Tolerant	Roundup Ready % Tolerant	Roundup Ready % Expected	Chi-Square Value	Significance
ALL	J101	F1	133	80	60.00	50.00	5.083	*
ALL	J163	F1	131	65	49.60	50.00	0.000	NS
ALL	J101	MBC1	405	170	42.00	50.00	10.114	*
ALL	J163	MBC1	404	194	48.00	50.00	0.557	NS
FD	J101	MBC2	565	259	45.84	50.00	3.745	NS
FD	J163	MBC2	578	276	47.75	50.00	1.081	NS
ND	J101	MBC2	201	101	50.25	50.00	0.000	NS
ND	J163	MBC2	180	80	44.44	50.00	2.006	NS
ALL	J101	MBC2	766	360	47.00	50.00	2.644	NS
ALL	J163	MBC2	758	356	46.97	50.00	2.672	NS
FD	J101	MBC3	1523	663	43.53	50.00	25.224	*
FD	J163	MBC3	1543	689	44.65	50.00	17.731	*
FD	J101	MBC4	155	80	51.61	50.00	0.103	NS
FD	J163	MBC4	172	82	47.67	50.00	0.285	NS

* = Significantly different ($p < 0.05$)
NS = NotSignificant

C. Characterization of the CP4 EPSPS Protein Produced by Event J101

The purpose of the protein characterization was to assess the physicochemical and functional properties of the CP4 EPSPS protein isolated from Roundup Ready alfalfa event J101 and to compare it with the *E. coli*-produced CP4 EPSPS protein used in previous safety studies.

A panel of analytical tests, some utilizing the *E. coli*-produced CP4 EPSPS protein as a reference standard, was used to characterize the plant-produced CP4 EPSPS protein. The analytical tests were: (1) BCA total protein assay; (2) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and densitometry; (3) immunoblotting; (4) glycosylation analysis; (5) enzyme activity assay; (6) N-terminal sequence analysis; and (7) matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry.

The identity of the plant-produced protein was confirmed using data from immunoblot analyses, N-terminal sequence analyses and MALDI-TOF mass spectrometry. On the basis of western blot analysis, the electrophoretic mobility and immunoreactivity of the plant-produced CP4 EPSPS protein were similar to the *E. coli*-produced CP4 EPSPS reference standard. The amino acids on the N-terminus of the protein were sequenced. The amino acid sequence was consistent with the predicted sequence of amino acids translated from the *cp4 epsps* gene. MALDI-TOF mass spectral analysis of the tryptic digest of the CP4 EPSPS isolated from Roundup Ready alfalfa event J101 yielded peptide sequences consistent with the peptide sequences of the *E. coli*-produced CP4 EPSPS. Approximately 53.4% of the expected 455 amino acid sequence comprising the *in planta* CP4 EPSPS protein was identified using MALDI-TOF mass spectrometry.

The approximate molecular weight of the plant-produced CP4 EPSPS protein, estimated using densitometric analysis of a Brilliant Blue G-Colloidal stained SDS-polyacrylamide gel, was observed to be 43.6 kDa. The molecular weight of the plant-produced CP4 EPSPS, as determined by the mass average (MH+) molecular weight using MALDI-TOF mass spectrometry, was 47037.3 Da, consistent with the calculated molecular weight of CP4 EPSPS. The functional activities of the plant-produced CP4 EPSPS protein and the *E. coli*-produced CP4 EPSPS reference standard were determined using a phosphate release assay. The specific activities for the plant-produced and *E. coli*-produced CP4 EPSPS were estimated to be 5.5 U/mg total protein and 3.9 U/mg total protein, respectively.

C.1. Protein Purification

The plant-produced CP4 EPSPS protein was isolated from an alfalfa plant containing event J101. The plant was a ramet produced from the original T_0 transformant that had been maintained in tissue culture. CP4 EPSPS was extracted and purified from forage obtained from the plant using a combination of ammonium sulfate fractionation, hydrophobic interaction chromatography, anion exchange chromatography, and affinity chromatography.

C.2. N-terminal Sequence Analysis

The results of the N-terminal sequence analysis of the plant-produced CP4 EPSPS protein are summarized in Table V-2. The experimentally determined N-terminal sequence for the plant-produced CP4 EPSPS, isolated from alfalfa event J101, confirmed the expected amino acid N-terminal sequence. There were two sequences observed in the CP4 EPSPS protein isolated from alfalfa event J101. Both are consistent with the N-terminus of the CP4 EPSPS protein. One sequence starts at residue six, serine, and the other sequence starts at residue five, alanine. The observation of two N-terminal sequences for plant-produced CP4 EPSPS is not uncommon; other studies have previously reported a similar finding with plant purified CP4 EPSPS from soybean, canola and cotton (Harrison et al., 1996). The initiator methionine is normally removed in eukaryotic systems via the action of a methionine aminopeptidase (Arfin and Bradshaw, 1988). The loss of a few N-terminal amino acid residues may be because of protease action when plant cells are homogenized. Collectively, the N-terminal sequence data confirm that the correct protein has been isolated from the forage sample of alfalfa event J101 and the N-terminal sequence observed is consistent with the N-terminal sequence of *E. coli*-produced CP4 EPSPS reference standard.

C.3. MALDI-TOF MS Analysis

The average mass (MH+) of the CP4 EPSPS for alfalfa was estimated using MALDI-TOF mass spectrometry after desalting the sample. The actual mass observed for the plant-produced CP4 EPSPS was 47,037 kDa. The theoretical mass is 47,614 kDa, which was calculated using DNAStar, based on the full length of 455 amino acids (Figure V-20). If corrected for amino acids 6-455 as predicted by N-terminal sequencing, the calculated mass would be 47,104 kDa.

Table V-2. N-Terminal Amino Acid Sequence Analysis of CP4 EPSPS Isolated from Roundup Ready Alfalfa Event J101.

The predicted amino acid sequence (residues 1-20 of 455) of the plant-produced CP4 EPSPS protein was deduced from the coding region of the full-length *cp4 epsps* gene present in Roundup Ready alfalfa event J101. The observed sequences (1 and 2) were obtained from N-terminal sequencing where the CP4 EPSPS protein isolated from alfalfa event J101 was sequenced through 15 cycles. Undesignated amino acid assignments are shown as an "X" and tentative amino acid assignments are shown in parentheses using the single letter amino acid code[a].

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
PREDICTED	M	L	H	G	A	S	S	R	P	A	T	A	R	K	S	S	G	L	S	G
						|	|	|	|	|	|	|	|	|		|	|	|		
OBSERVED-1						S	S	R	P	A	T	A	R	K	X	S	G	L	X	X
					|	|	|	|	|	|		|	|	|	|	|		
OBSERVED-2					A	S	S	R	P	A	T	X	R	K	S	S	G	L	(S)	

[a] The single letter IUPAC-IUB amino acid code is **A**, alanine; **G**, glycine; **H**, histidine; **K**, lysine; **L**, leucine; **M**, methionine; **P**, proline; **R**, arginine; **S**, serine; and **T**, threonine.

The identity of the plant-produced CP4 EPSPS protein was assessed using MALDI-TOF mass spectrometry. Prior to analysis, the protein sample was chemically reduced, alkylated and proteolytically digested with trypsin. MALDI-TOF is considered an indirect means of establishing protein identity. The ability to identify a protein using this method is dependent on matching a sufficient number of observed mass fragments to expected (theoretical) mass fragments. A protein can typically be identified when 40% of the amino acids in the protein are identified by matching experimental masses for the tryptic peptide fragments to the expected masses for the fragments (Jiménez et al., 1998).

Differences of less than one dalton between the observed mass and its theoretical mass fragment were required to be designated as a match. Matches were made without consideration for potential amino acid modifications. A total of 20 observed mass fragments matched the expected tryptic digest mass fragments from the deduced amino acid sequence of the CP4 EPSPS protein (Table V-3). The identified masses were used to assemble a coverage map indicating the matched peptide sequences for the entire protein (Figure V-20). Sufficient coverage (53.4%) was obtained to confirm the identity of the plant-produced CP4 EPSPS protein.

Table V-3. Summary of MALDI-TOF Mass Spectrometry Tryptic Masses Observed for CP4 EPSPS Isolated from Roundup Ready Alfalfa Event J101.

Observed Mass (Da)					Expected		aa	
no Zip-tip[a]	Wash 1[b]	Wash 2[b]	Wash 3[b]	Wash 4[b]	Mass (Da)	Δ[c]	Position	Sequence
		568.38		568.25				
				586.42				
599.27		599.39			599.33	0.06	29-33	SISHR
		616.40			616.34	-0.06	128-132	RPMGR
643.97				644.17				
		698.53	698.58		698.46	-0.07	152-157	LPVTLR
711.38		711.53	711.55	711.61	711.45	0.07	133-138	VLNPLR
				855.25				
		863.56	863.59		863.46	-0.10	15-23	SSGLSGTVR
		872.55			872.45	-0.10	313-320	GVTVPEDR[d]
		872.55			872.52	-0.03	358-366	LSAVANGLK[d]
948.43		948.63	948.65		948.52	0.09	161-168	TPTPITYR
				1060.30				
				1066.31				
1115.46		1115.71	1115.74	1115.83	1115.57	0.11	295-305	LAGGEDVADLR
1311.52		1311.80	1311.84	1311.93				
1357.57		1357.87	1357.90	1358.00	1357.71	0.14	146-157	SEDGDRLPVTLR
1359.52			1359.86	1359.96	1359.64	0.12	34-46	SFMFGGLASGETR[d]
1359.52			1359.86	1359.96	1359.72	0.20	354-366	ESDRLSAVANGLK[d]
			1559.04	1559.17	1558.83	-0.21	47-61	ITGLLEGEDVINTGK

Table V-3. Summary of MALDI-TOF Mass Spectrometry Tryptic Masses Observed for CP4 EPSPS Isolated from Roundup Ready Alfalfa Event J101 (cont'd)

Observed Mass (Da)					Expected		aa	
no Zip-tip[a]	Wash 1[b]	Wash 2[b]	Wash 3[b]	Wash 4[b]	Mass (Da)	Δ[c]	Position	Sequence
1646.67		1647.03	1647.06	1647.18	1646.84	0.17	389-405	GLGNASGAAVATHLDHR
		1705.03	1705.06					
1746.58			1747.01					
1763.62		1764.01	1764.05		1763.81	0.19	367-382	LNGVDCDEGETSLVVR
			1946.24	1946.39	1946.05	-0.19	4-23	GASSRPATARKSSGLSGTVR
	1994.18		1994.25	1994.38	1993.97	-0.21	206-224	MLQGFGANLTVETDADGVR
2134.92	2135.33		2135.47	2135.60				
2182.92	2183.34		2183.47	2183.59	2183.17	0.25	275-294	TGLILTLQEMGADIEVINPR
			2319.65					
			2367.66	2367.79	2367.33	-0.33	178-200	SAVLLAGLNTPGITTVIEPIMTR
				3202.16				
			3244.93	3245.05	3244.52	-0.41	73-104	EGDTWIIDGVGNGGLLAPEAPLDF GNAATGCR

[a] Sample, 0.3 µL, was analyzed directly prior to desalting.
[b] The Zip-tip was washed with 0.1% (v/v) trifluoroacetic acid containing acetonitrile at varying concentrations [0, 20, 50 and 90% (v/v)] acetonitrile.
[c] A difference of less than one Dalton between the observed and expected mass was considered a match.
[d] Two expected fragments having nearly identical masses were matched to one observed mass.

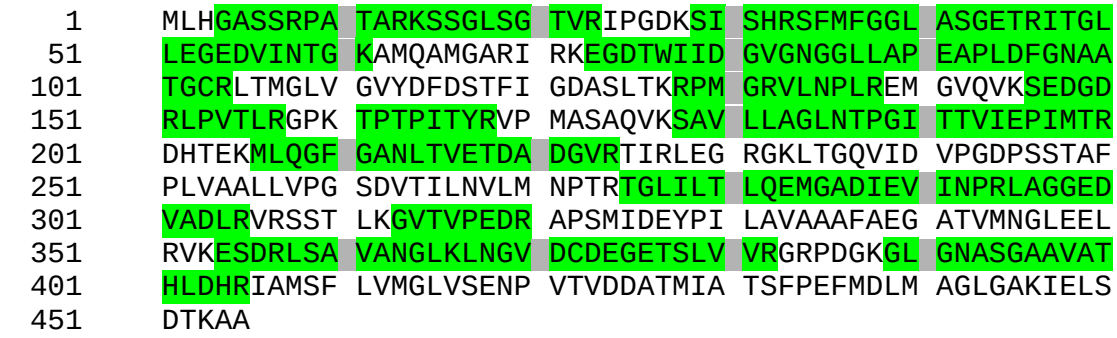
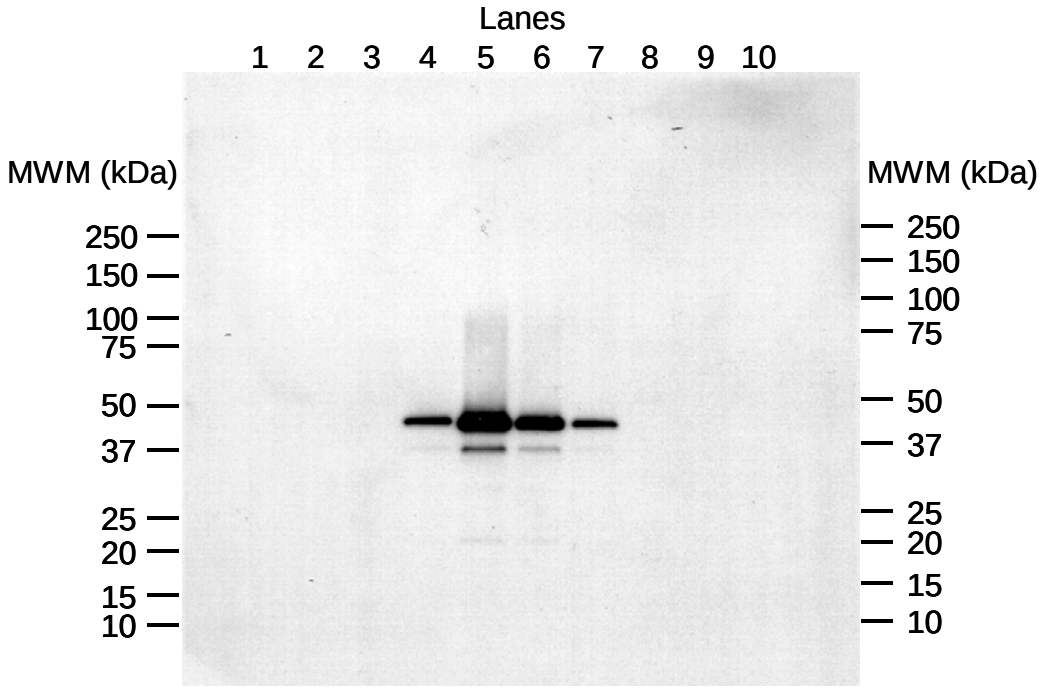


Figure V-20. MALDI-TOF Coverage Map of CP4 EPSPS Isolated from Roundup Ready Alfalfa Event J101.

The amino acid sequence of the plant-produced CP4 EPSPS protein was deduced from the coding region of the full-length *cp4 epsps* gene present in Roundup Ready alfalfa event J101. Shaded regions correspond to identified peptides. Approximately 53.4% (243 of 455 amino acids) of the expected protein sequence was identified.

C.4. Immunoblot Analysis – Immunoreactivity

Immunoblot analysis was performed using goat anti-CP4 EPSPS serum, which was produced using the *E. coli*-produced CP4 EPSPS protein as the antigen. The test substance was loaded at 8.4, 5.0 and 1.7 ng total protein per lane compared to 2.0 ng CP4 EPSPS per lane for the reference standard. As expected, the immunoreactive signal increased with increased levels of the plant-produced CP4 EPSPS protein (Figure V-21). Furthermore, the western blot analysis showed that the CP4 EPSPS protein isolated from alfalfa event J101 elicited comparable immunoreactivity and equivalent electrophoretic mobility to the *E. coli*-produced CP4 EPSPS reference standard. Also visible are two lower molecular weight immunoreactive bands; in lanes 4-7 a band is visible at approximately 37 kDa and in lane 5 and 6, a band is visible at 20 kDa. Both of the lower immunoreactive bands were likely formed by proteolytic degradation of CP4 EPSPS protein during the protein extraction process. The observed similarity in protein mobility and the immunoreactivity of both plant- and *E. coli* –produced CP4 EPSPS proteins confirms that plant-produced CP4 EPSPS protein is equivalent to *E. coli*-produced CP4 EPSPS reference standard.



Lane	Sample	Amount (µg)
1	Blank	—
2	Blank	—
3	Pre Stained MW Markers (Bio-Rad, Cat #: 161-0374)	—
4	*E. coli*-produced CP4 EPSPS reference standard	2.0
5	Plant-produced CP4 EPSPS from alfalfa event J101	8.4
6	Plant-produced CP4 EPSPS from alfalfa event J101	5.0
7	Plant-produced CP4 EPSPS from alfalfa event J101	1.7
8	Prestained MW markers (Bio-Rad, Cat #: 161-0374)	—
9	Blank	—
10	Blank	—

Figure V-21. Immunoblot Analysis of CP4 EPSPS Isolated from Roundup Ready Alfalfa Event J101.

Samples of plant-produced CP4 EPSPS and *E. coli*-produced CP4 EPSPS reference standard were separated by 4→20% SDS-PAGE, electrotransferred to a PVDF membrane and detected using CP4 EPSPS polyclonal antisera, followed by development using the ECL system (45 sec exposure shown). Amount refers to total protein loaded per lane, except for CP4 EPSPS reference standard, where amount refers to CP4 EPSPS protein. Approximate molecular weights (kDa) correspond to the markers loaded in Lanes 3 and 8.

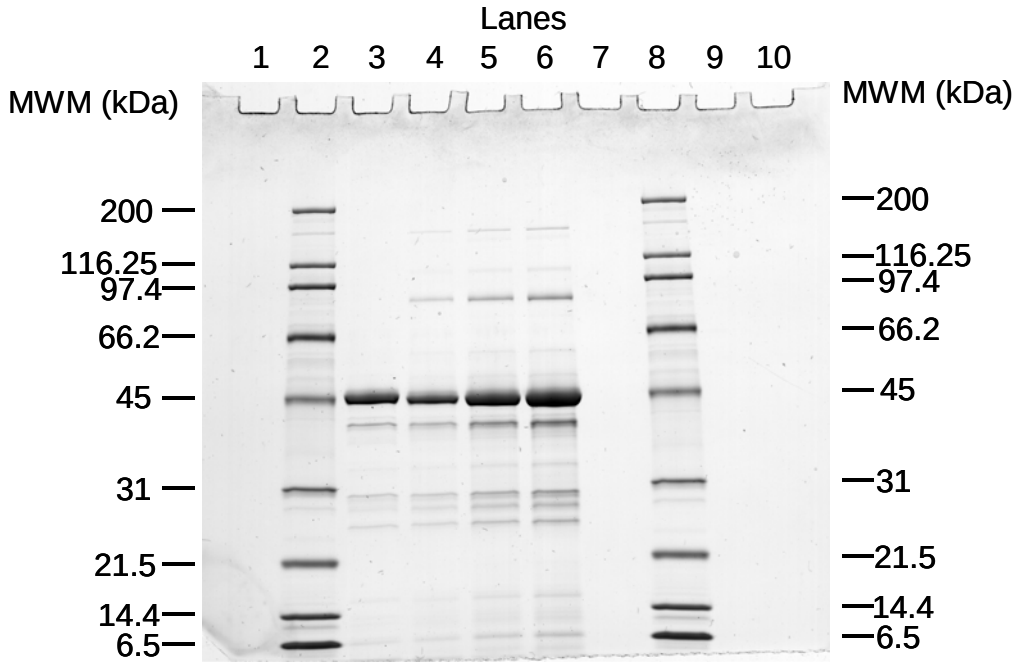
C.5. Molecular Weight and Purity Determination

The plant-produced CP4 EPSPS protein was separated using SDS-PAGE and stained with Brilliant Blue G-Colloidal stain (Figure V-22). Purity and molecular weight were estimated using densitometric analysis and are summarized in Table V-4. The purity values were averaged from loads of 1.7, 3.4, and 5.0 μg total protein per lane (Lanes 4-6, Figure V-22). The predominant protein band in the plant-purified sample had an average molecular weight of approximately 43.6 kDa. Because this protein migrated to an identical molecular weight as that of the *E. coli* reference standard analyzed concurrently, this protein was assumed to be the plant-produced CP4 EPSPS. The average purity was estimated to be 65.2%.

The molecular weight of the plant-produced CP4 EPSPS protein was further confirmed using MALDI-TOF mass spectrometry. The average mass (MH+), determined from three separate spectral acquisitions, was 47,037 kDa, compared to a calculated value of 47,614 kDa. While the observed value was very close to the calculated value, this difference (576.45 Da) is consistent with the absence of 4-5 residues from the N-terminus.

Table V-4. Protein Molecular Weight and Purity Estimation of CP4 EPSPS Isolated from Roundup Ready Alfalfa Event J101.
Relative percent quantities of each visible band were derived from densitometric analysis of the SDS polyacrylamide gel shown in Figure V-22, Lanes 4-6 (test substance). The test substance molecular weights were calculated from the molecular weight markers (Figure V-22, Lanes 2 and 8) using the manufacturer's supplied molecular weight values. The average molecular weight and purity of the plant-produced CP4 EPSPS protein was assessed to be ~43.6 kDa and 65.2 % (shown in bold values), respectively.

1.7 μg Load (Figure V-22, Lane 4)		3.4 μg Load (Figure V-22, Lane 5)		5.0 μg Load (Figure V-22, Lane 6)	
Mol. Wt. (kDa)	**Relative Qty (%)**	**Mol. Wt. (kDa)**	**Relative Qty (%)**	**Mol. Wt. (kDa)**	**Relative Qty (%)**
158.04	0.8	157.75	1.1	157.45	1.2
—	—	108.17	0.4	107.76	0.6
86.77	4.4	86.31	5.8	85.63	6.7
44.15	**71.9**	**43.44**	**65.2**	**43.08**	**58.6**
40.23	10.0	40.11	11.5	40.04	11.9
33.57	0.7	33.78	0.8	33.71	1.1
29.80	4.2	29.86	4.4	29.84	6.1
27.92	2.5	27.98	3.6	27.95	5.6
25.80	2.9	25.85	3.0	25.82	4.1
16.04	0.5	16.19	1.1	16.19	1.1
—	—	12.10	0.6	11.97	0.7
7.15	2.1	7.40	2.5	7.39	2.4



Lane	Sample	Amount (µg)
1	Blank	—
2	MW Markers (Bio-Rad, Cat #: 161-0317)	0.5 µg/band
3	*E. coli*-produced CP4 EPSPS reference standard	2.0
4	Plant-produced CP4 EPSPS from alfalfa event J101	1.7
5	Plant-produced CP4 EPSPS from alfalfa event J101	3.4
6	Plant-produced CP4 EPSPS from alfalfa event J101	5.0
7	Blank	—
8	MW Markers (Bio-Rad, Cat #: 161-0317)	0.5 µg/band
9	Blank	—
10	Blank	—

Figure V-22. SDS-PAGE Purity and Molecular Weight Analysis of CP4 EPSPS Isolated from Roundup Ready Alfalfa Event J101.

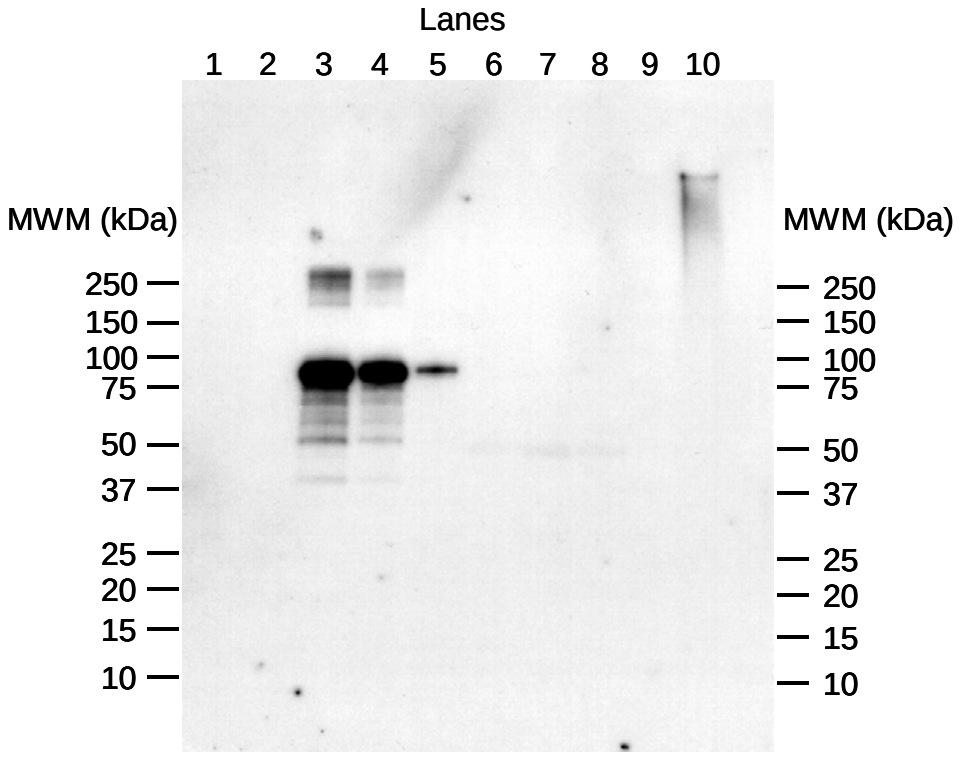
Samples of the plant-produced CP4 EPSPS and *E. coli*-produced CP4 EPSPS reference protein were loaded as indicated on a 4→20% polyacrylamide gel. Amount refers to total protein loaded per lane, except for CP4 EPSPS reference standard, where amount refers to CP4 EPSPS protein. Approximate molecular weights (kDa) correspond to the markers loaded in Lanes 2 and 8. Following electrophoresis, the Brilliant Blue G-Colloidal stained gel was analyzed densitometrically (see Table V-4).

C.6. Functional Activity

The specific activity of the plant-produced CP4 EPSPS protein was estimated using a phosphate release assay. The estimated specific activity of the plant-produced CP4 EPSPS was 5.5 U/mg total protein. The specific activity of the *E. coli*-produced CP4 EPSPS reference standard protein, which was analyzed concurrently, was 3.9 U/mg total protein. These values were within the range of specific activities of CP4 EPSPS protein reported in other studies (Harrison et al., 1996). The enzyme assay demonstrated the plant-produced CP4 EPSPS was as active as *E. coli*-produced CP4 EPSPS protein and thus plant-produced protein is functionally equivalent to the *E. coli*-produced protein with respect to S-3-P dependent CP4 EPSPS enzyme mediated release of the phosphate group from PEP.

C.7. Glycosylation Analysis

Many eukaryotic proteins are posttranslationally modified with carbohydrate moieties (Rademacher et al., 1988). These carbohydrate moieties may be complex, branched polysaccharide structures or simple monosaccharides. To test whether posttranslational glycosylation of the plant-produced CP4 EPSPS protein occurred, the plant-produced CP4 EPSPS protein was analyzed for covalently bound carbohydrate using the ECL glycoprotein detection system kit of Amersham Pharmacia Biotech, Inc. (Piscataway, NJ). The *E. coli*-produced CP4 EPSPS reference standard protein (negative control) and the transferrin protein (positive control) were also analyzed concurrently. The results are presented in Figure V-23. A total of three film exposures (1 min, 2 min, and 4 min) were produced. The exposure shown in Figure V-23 was produced from a scan of the 2 min film exposure. The positive control (transferrin) was clearly detected from the highest concentration of 1.0 μg/lane to the lowest concentration of 0.10 μg/lane, in a concentration dependent manner. However, at the longest film exposure-time of 4 min (data not shown), a barely discernable band, close to the expected position for the CP4 EPSPS protein, was observed for the test substance and for the *E. coli*-produced CP4 EPSPS protein (considered as the negative control). Glycosylation of proteins is a posttranslational process, which occurs exclusively in eukaryotic organisms and is not observed in prokaryotic organisms such as *E. coli*. Therefore, the very faint band observed for the test and reference CP4 EPSPS proteins on the 4 min exposure is likely attributable to a weak nonspecific interaction between the detection reagent (Streptavidin-HRP conjugate) and protein mass bound to the blot. Additionally, N-linked glycosylation would result in an apparent increase in the molecular weight of the plant-produced CP4 EPSPS protein. However, this was not observed for the average mass determination of the plant-produced CP4 EPSPS protein using MALDI-TOF mass spectrometry. The glycosylation analysis demonstrated that there is no detectable glycosylation of the CP4 EPSPS protein produced in alfalfa and thus the plant-produced protein is equivalent to the *E. coli*-produced CP4 EPSPS reference standard with respect to glycosylation.



Lane	Sample	Amount (µg)
1	Blank	—
2	Pre Stained MW Markers (Bio-Rad, Cat #: 161-0374)	—
3	Transferrin (Positive control)	1.0
4	Transferrin (Positive control)	0.50
5	Transferrin (Positive control)	0.10
6	*E. coli*-produced CP4 EPSPS reference standard	1.0
7	Plant-produced CP4 EPSPS from alfalfa event J101	1.7
8	Plant-produced CP4 EPSPS from alfalfa event J101	0.83
9	Pre Stained MW Markers (Bio-Rad, Cat #: 161-0374)	—
10	Blank	—

Figure V-23. Glycosylation Analysis of CP4 EPSPS Isolated from Roundup Ready Alfalfa Event J101.
Samples of plant-produced CP4 EPSPS, *E. coli*-produced CP4 EPSPS reference standard (negative control) and transferrin (positive control) were separated by SDS-PAGE (4→20%) and electrotransferred to PVDF membrane. If present, the protein-bound carbohydrate moiety is labeled with biotin, and detected with streptavidin-horseradish peroxidase and enhanced chemiluminescence (2 min exposure shown). Amount refers to total protein loaded per lane, except for CP4 EPSPS reference standard, where amount refers to CP4 EPSPS protein. Approximate molecular weights (kDa) correspond to the markers loaded in Lanes 2 and 9.

C.8. Conclusions for Event J101 CP4 EPSPS Protein Characterization

The physicochemical and functional properties of the plant-produced CP4 EPSPS protein isolated from Roundup Ready alfalfa event J101 were assessed. A battery of analytical tests was used to characterize the physicochemical and functional properties of the plant-produced CP4 EPSPS protein. The *E. coli*-produced CP4 EPSPS protein was included in selected analyses as a reference standard. The analytical tests included (1) BCA total protein assay, (2) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and densitometry, (3) immunoblotting, (4) glycosylation analysis, (5) enzymatic assay, (6) N-terminal sequence analysis, and (7) MALDI-TOF mass spectrometry.

The results of N-terminal sequence analysis of the plant-produced CP4 EPSPS protein were consistent with the expected CP4 EPSPS protein N-terminal sequence. Immunoblot and MALDI-TOF mass spectrometric analyses also provided data to confirm the identity of the plant-produced CP4 EPSPS protein. A phosphate release assay confirmed that the CP4 EPSPS protein isolated from alfalfa event J101 was functionally active.

SDS-PAGE, immunoblot analysis, glycosylation analysis and a functional assay were performed to evaluate the equivalence of the plant-produced CP4 EPSPS protein to the *E. coli*-produced CP4 EPSPS reference standard protein. The plant-produced CP4 EPSPS protein was considered to be equivalent to the *E. coli*-produced CP4 EPSPS reference standard protein based on comparable electrophoretic mobility, enzyme activity, immunoreactivity and absence of detectable glycosylation. Moreover, N-terminal sequence data and MALDI-TOF data clearly establish the identity of plant-produced CP4 EPSPS.

Collectively, these data establish the physicochemical and functional properties of the CP4 EPSPS protein isolated from alfalfa event J101 and establish its chemical and functional equivalence to the *E. coli*-produced CP4 EPSPS protein used in previous safety studies.

D. Characterization of the CP4 EPSPS Protein Produced by Event J163

The purpose, material and methods used for the characterization of the CP4 EPSPS protein produced by alfalfa event J163 were the same as previously described for event J101, except that the CP4 EPSPS protein was isolated from an alfalfa plant containing event J163. (Background information on the transformation vector and the CP4 EPSPS protein has been described in Section IV above).

D.1. N-terminal Sequence Analysis

The results of the N-terminal sequence analysis of the plant-produced CP4 EPSPS protein are summarized in Table V-5. The experimentally determined N-terminal sequence for the plant-produced CP4 EPSPS isolated from alfalfa event J163 confirmed the expected amino acid sequence. There were two sequences observed in the CP4 EPSPS protein isolated from alfalfa event J163. Both are consistent with the N-terminus of the CP4 EPSPS protein. One sequence starts at residue six, serine, and the other sequence starts at residue five, alanine. The observation of two N-terminal sequences for plant-produced CP4 EPSPS is not uncommon; other studies have previously reported similar

observations with plant-purified CP4 EPSPS from soybean, canola and cotton (Harrison et al., 1996). The initiator methionine is normally removed in eukaryotic systems via the action of a methionine aminopeptidase (Arfin and Bradshaw, 1988). The loss of a few N-terminal amino acid residues may be because of protease action when plant cells are homogenized. Collectively, the N-terminal sequence data confirm that the correct protein has been isolated from the forage tissue of alfalfa event J163 and the N-terminal sequence observed is consistent with the N-terminal sequence of the *E. coli*-produced CP4 EPSPS reference standard.

Table V-5. N-terminal Amino Acid Sequence Analysis of CP4 EPSPS Isolated from Roundup Ready Alfalfa Event J163.
The predicted amino acid sequence (residues 1-20 of 455) of the plant-produced CP4 EPSPS protein was deduced from the coding region of the full-length *cp4 epsps* gene present in Roundup Ready alfalfa event J163. The observed sequences (1 and 2) were obtained from N-terminal sequencing where the CP4 EPSPS protein isolated from alfalfa event J163 was sequenced through 15 cycles. Undesignated amino acid assignments are shown as an "X" and amino acid assignments are shown using the single letter amino acid code[a].

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
PREDICTED	M	L	H	G	A	S	S	R	P	A	T	A	R	K	S	S	G	L	S	G
OBSERVED-1						S	S	R	P	A	T	A	R	K	S	S	G	L	X	X
OBSERVED-2					A	S	X	R	P	A	T	X	R	K	X	S	G	L	X	

[a] The single letter IUPAC-IUB amino acid code is **A**, alanine; **G**, glycine; **H**, histidine; **K**, lysine; **L**, leucine; **M**, methionine; **P**, proline; **R**, arginine; **S**, serine; and **T**, threonine

D.2. MALDI-TOF MS Analysis

The average mass (MH+) of the test substance was estimated using MALDI-TOF mass spectrometry after desalting the sample. The actual mass observed for the plant-produced CP4 EPSPS was 47,032 kDa. The theoretical mass is 47,614 kDa, which was calculated using DNAStar, based on the full length of 455 amino acids (Figure V-24). If corrected for amino acids 6-455 as predicted by N-terminal sequencing, the calculated mass would be 47,104 kDa.

The identity of the plant-produced CP4 EPSPS protein was assessed using MALDI-TOF mass spectrometry. Prior to analysis, the protein sample was chemically reduced, alkylated and proteolytically digested with trypsin. MALDI-TOF is considered an indirect means of establishing protein identity. The ability to identify a protein using this method is dependent on matching a sufficient number of observed mass fragments to expected (theoretical) mass fragments. A protein can typically be identified when 40% of

the amino acids in the protein are identified by matching experimental masses for the tryptic peptide fragments to the expected masses for the fragments (Jiménez *et al*., 1998).

Differences of less than one dalton between the observed mass and its theoretical mass fragment were required to be designated as a match. Matches were made without consideration for potential amino acid modifications. A total of 21 observed mass fragments matched the expected tryptic digest mass fragments from the deduced amino acid sequence of the CP4 EPSPS protein (Table V-6). The identified masses were used to assemble a coverage map indicating the matched peptide sequences for the entire protein (Figure V-24). Sufficient coverage (54.7%) was obtained to confirm the identity of the plant-produced CP4 EPSPS protein.

Table V-6. Summary of MALDI-TOF Mass Spectrometry Tryptic Masses Observed for CP4 EPSPS Isolated from Roundup Ready Alfalfa Event J163.

Observed Mass (Da)					Expected		aa	
no Zip-tip[a]	Wash 1[b]	Wash 2[b]	Wash 3[b]	Wash 4[b]	Mass (Da)	Δ[c]	Position	Sequence
	568.34	568.37						
				586.43				
	599.33	599.38	599.44		599.33	0.00	29-33	SISHR
629.27	629.34				629.29	0.02	201-205	DHTEK[d]
629.27	629.34				629.34	0.07	383-388	GRPDGK[d]
640.52								
				644.18				
	650.06							
668.55								
		698.52	698.59		698.46	-0.06	152-157	LPVTLR
711.38	711.47	711.51	711.59	711.61	711.45	0.07	133-138	VLNPLR
	739.41							
	787.42	787.47						
		863.54	863.63		863.46	-0.08	15-23	SSGLSGTVR
	872.49	872.54	872.63		872.45	-0.04	313-320	GVTVPEDR[d]
	872.49	872.54	872.63		872.52	0.03	358-366	LSAVANGLK[d]
948.42	948.55	948.61	948.71	948.74	948.52	0.10	161-168	TPTPITYR
1115.48	1115.61	1115.69	1115.79	1115.82	1115.57	0.09	295-305	LAGGEDVADLR
	1311.69	1311.78	1311.91	1311.94				
	1357.76	1357.85	1357.98	1358.01	1357.71	-0.05	146-157	SEDGDRLPVTLR
	1359.72		1359.93	1359.95	1359.64	-0.08	34-46	SFMFGGLASGETR[d]

Table V-6. Summary of MALDI-TOF Mass Spectrometry Tryptic Masses Observed for CP4 EPSPS Isolated from Roundup Ready Alfalfa Event J163, (continued).

Observed Mass (Da)					Expected		aa	
no Zip-tip[a]	Wash 1[b]	Wash 2[b]	Wash 3[b]	Wash 4[b]	Mass (Da)	Δ[c]	Position	Sequence
	1359.72		1359.93	1359.95	1359.72	0.00	354-366	ESDRLSAVANGLK[d]
	1558.88		1559.13	1559.17	1558.83	-0.05	47-61	ITGLLEGEDVINTGK
	1646.88	1647.00	1647.15	1647.20	1646.84	-0.04	389-405	GLGNASGAAVATHLDHR
	1746.82		1747.12	1747.15				
	1763.86	1763.98	1764.14	1764.17	1763.81	-0.05	367-382	LNGVDCDEGETSLVVR
	1946.03		1946.34		1946.05	0.02	4-23	GASSRPATARKSSGLSGTVR
	1994.03		1994.34	1994.40	1993.97	-0.06	206-224	MLQGFGANLTVETDADGVR
	2183.22			2183.61	2183.17	-0.05	275-294	TGLILTLQEMGADIEVINPR
	2319.39		2319.75					
	2367.38		2367.77	2367.82	2367.33	-0.05	178-200	SAVLLAGLNTPGITTVIEPIMTR
	3244.56		3245.17	3245.13	3244.52	-0.04	73-104	EGDTWIIDGVGNGGLLAPEAPL DFGNAATGCR

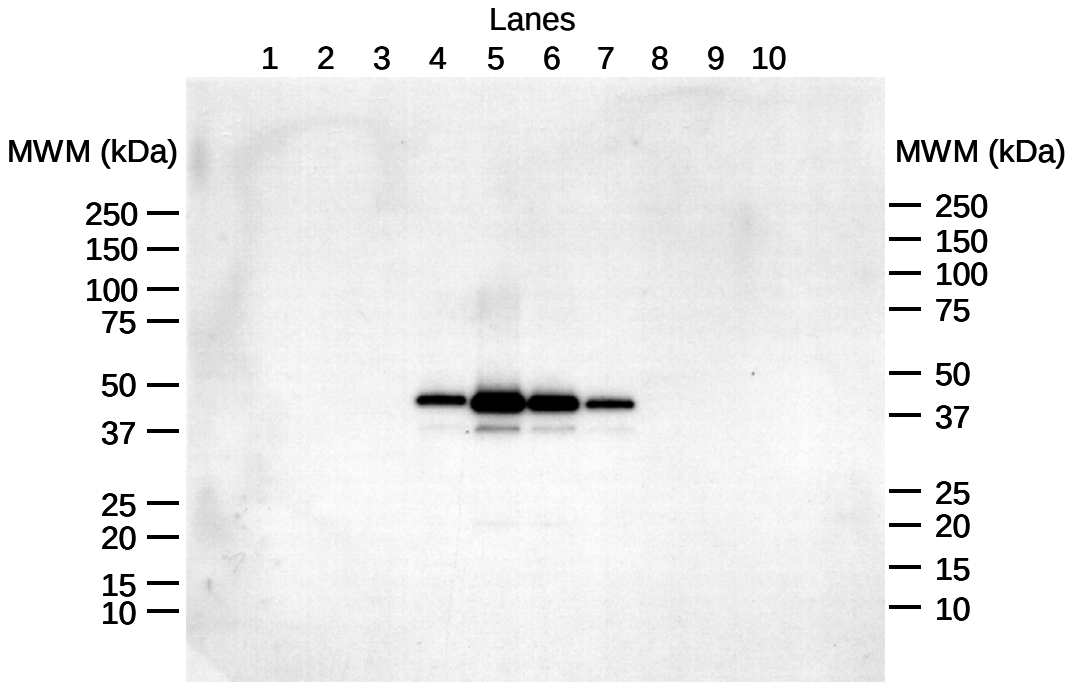
[a] Sample, 0.3 µL, was analyzed directly prior to desalting.
[b] The Zip-tip was washed with 0.1% (v/v) trifluoroacetic acid containing acetonitrile at varying concentrations [0, 20, 50 and 90% (v/v) acetonitrile]
[c] A difference of less than one Dalton between the observed and expected mass was considered a match.
[d] Two expected fragments having nearly identical masses were matched to one observed mass.

```
  1   MLHGASSRPA TARKSSGLSG TVRIPGDKSI SHRSFMFGGL ASGETRITGL
 51   LEGEDVINTG KAMQAMGARI RKEGDTWIID GVGNGGLLAP EAPLDFGNAA
101   TGCRLTMGLV GVYDFDSTFI GDASLTKRPM GRVLNPLREM GVQVKSEDGD
151   RLPVTLRGPK TPTPITYRVP MASAQVKSAV LLAGLNTPGI TTVIEPIMTR
201   DHTEKMLQGF GANLTVETDA DGVRTIRLEG RGKLTGQVID VPGDPSSTAF
251   PLVAALLVPG SDVTILNVLM NPTRTGLILT LQEMGADIEV INPRLAGGED
301   VADLRVRSST LKGVTVPEDR APSMIDEYPI LAVAAAFAEG ATVMNGLEEL
351   RVKESDRLSA VANGLKLNGV DCDEGETSLV VRGRPDGKGL GNASGAAVAT
401   HLDHRIAMSF LVMGLVSENP VTVDDATMIA TSFPEFMDLM AGLGAKIELS
451   DTKAA
```

Figure V-24. MALDI-TOF Coverage Map of CP4 EPSPS Isolated from Roundup Ready Alfalfa Event J163.
The amino acid sequence of the plant-produced CP4 EPSPS protein was deduced from the coding region of the full-length *cp4 epsps* gene present in Roundup Ready alfalfa event J163. Shaded regions correspond to identified peptides. Approximately 54.7% (249 of 455 amino acids) of the expected protein sequence was identified.

D.3. Immunoblot Analysis – Immunoreactivity

Immunoblot analysis was performed using goat anti-CP4 EPSPS serum, which was produced using the *E. coli*-produced CP4 EPSPS protein as the antigen. The alfalfa-derived CP4 EPSPS protein was loaded at 7.3, 4.4 and 1.5 ng total protein per lane compared to 2.0 ng CP4 EPSPS protein per lane for the reference standard. As expected, the immunoreactive signal increased with increased levels of the plant-produced CP4 EPSPS protein (Figure V-25). Furthermore, the western blot analysis showed that the CP4 EPSPS protein isolated from alfalfa event J163 elicited comparable immunoreactivity and equivalent electrophoretic mobility to the *E. coli*-produced CP4 EPSPS reference standard. Also visible are two lower molecular weight immunoreactive bands; in lanes 4-7 a band is visible at approximately 37 kDa and in lane 5 and 6, a band is visible at 20 kDa. Both of the lower immunoreactive bands were likely formed by proteolytic degradation of CP4 EPSPS protein during the protein extraction process. The observed similarity in protein mobility and the immunoreactivity of both plant- and *E. coli* –produced CP4 EPSPS proteins further demonstrates that the plant-produced CP4 EPSPS protein is equivalent to the *E. coli*-produced CP4 EPSPS reference standard.



Lane	Sample	Amount (µg)
1	Blank	—
2	Blank	—
3	Pre Stained MW Markers (Bio-Rad, Cat #: 161-0374)	—
4	*E. coli*-produced CP4 EPSPS reference standard	2.0
5	Plant-produced CP4 EPSPS from alfalfa event J163	7.3
6	Plant-produced CP4 EPSPS from alfalfa event J163	4.4
7	Plant-produced CP4 EPSPS from alfalfa event J163	1.5
8	Prestained MW markers (Bio-Rad, Cat #: 161-0374)	—
9	Blank	—
10	Blank	—

Figure V-25. Immunoblot Analysis of CP4 EPSPS Isolated from Roundup Ready Alfalfa Event J163.
Samples of plant-produced CP4 EPSPS and *E. coli*-produced CP4 EPSPS reference standard were separated by 4→20% SDS-PAGE, electrotransferred to a PVDF membrane and detected using CP4 EPSPS polyclonal antisera followed by development using the ECL system (45 sec exposure shown). Amount refers to total protein loaded per lane, except for CP4 EPSPS reference standard, where amount refers to CP4 EPSPS protein. Approximate molecular weights (kDa) correspond to the markers loaded in Lanes 3 and 8.

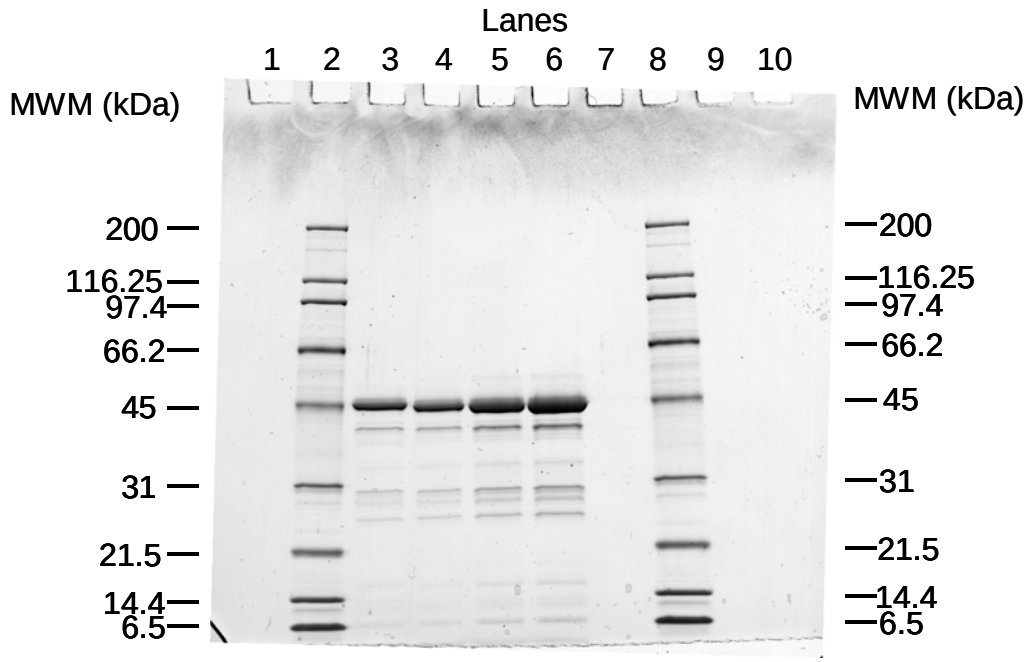
D.4. Molecular Weight and Purity Determination

The plant-produced CP4 EPSPS protein was separated using SDS-PAGE and stained with Brilliant Blue G-Colloidal stain (Figure V-26). Purity and molecular weight were estimated using densitometric analysis and are summarized in Table V-7. The purity values were averaged from loads of 1.5, 2.9, and 4.4 μg total protein per lane (Lanes 4-6, Figure V-25). The predominant band in the plant-purified sample had an average molecular weight of approximately 43.3 kDa. Because this protein migrated with an identical molecular weight as that of the *E. coli* reference standard, this protein was assumed to be the plant-produced CP4 EPSPS. The average purity was estimated to be 72.2%.

The molecular weight of the plant-produced CP4 EPSPS protein was further confirmed using MALDI-TOF mass spectrometry. The average mass (MH+), determined from three separate spectral acquisitions, was 47,032 kDa, compared to a calculated value of 47,614 kDa. While the observed value was very close to the calculated value, this difference (582.2 daltons) is consistent with the absence of five residues from the N-terminus based on N-terminal sequence analysis.

Table V-7. Protein Molecular Weight and Purity Estimation of CP4 EPSPS Isolated from Roundup Ready Alfalfa Event J163.
Relative percent quantities of each visible band were derived from densitometric analysis of the SDS polyacrylamide gel shown in Figure V-26, Lanes 4-6 (test substance). The test substance molecular weights were calculated from the molecular weight markers (Figure V-26, Lanes 2 and 8) using the manufacturer's supplied molecular weight values. The average molecular weight and purity of the plant-produced CP4 EPSPS protein were assessed to be ~43.3 kDa and 72.2 % (shown in bold values), respectively.

1.5 μg Load (Figure V-26, Lane 4)		2.9 μg Load (Figure V-26, Lane 5)		4.4 μg Load (Figure V-26, Lane 6)	
Mol. Wt. (kDa)	**Relative Qty (%)**	**Mol. Wt. (kDa)**	**Relative Qty (%)**	**Mol. Wt. (kDa)**	**Relative Qty (%)**
43.72	**77.1**	**43.15**	**70.5**	**42.91**	**69.0**
40.03	9.2	39.98	10.5	40.05	11.6
—	—	33.76	1.3	33.85	1.4
29.79	5.0	29.88	6.7	29.91	6.7
27.96	2.6	28.10	3.0	28.13	3.2
25.75	3.1	25.87	3.8	25.91	4.2
15.96	0.8	16.00	1.1	16.05	1.1
6.92	2.1	6.94	2.9	7.03	2.8



Lane	Sample	Amount (µg)
1	Blank	—
2	MW Markers (Bio-Rad, Cat #: 161-0317)	0.5 µg/band
3	*E. coli*-produced CP4 EPSPS reference standard	2.0
4	Plant-produced CP4 EPSPS from alfalfa event J163	1.5
5	Plant-produced CP4 EPSPS from alfalfa event J163	2.9
6	Plant-produced CP4 EPSPS from alfalfa event J163	4.4
7	Blank	—
8	MW Markers (Bio-Rad, Cat #: 161-0317)	0.5 µg/band
9	Blank	—
10	Blank	—

Figure V-26. SDS-PAGE Purity and Molecular Weight Analysis of CP4 EPSPS Isolated from Roundup Ready Alfalfa Event J163.

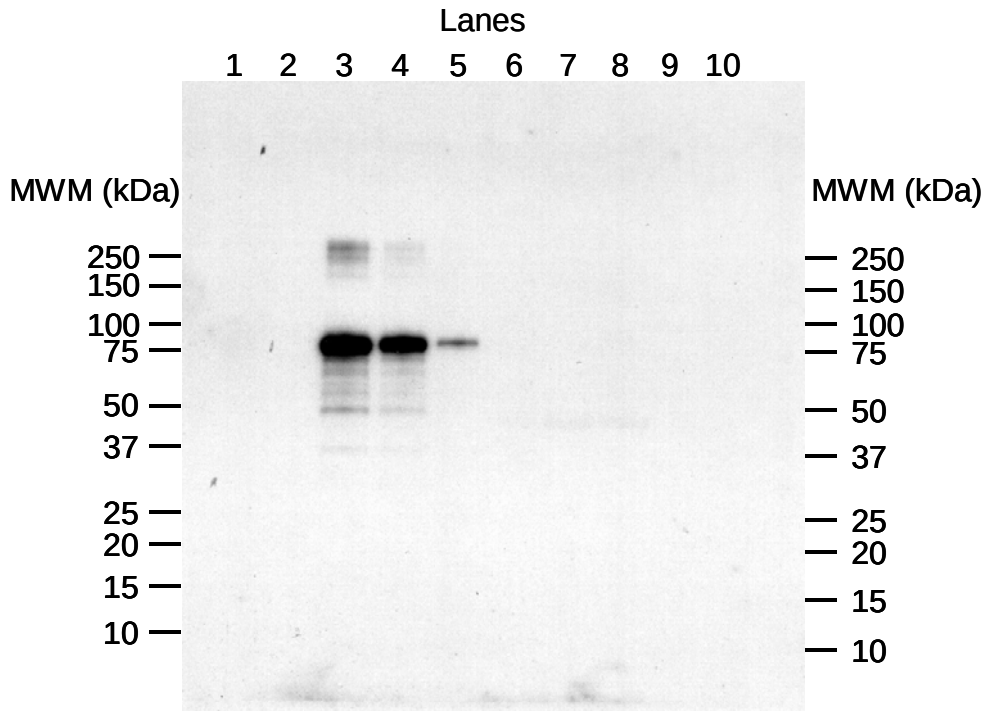
Samples of the plant-produced CP4 EPSPS and *E. coli*-produced CP4 EPSPS reference protein were loaded as indicated on a 4→20% polyacrylamide gel. Amount refers to total protein loaded per lane, except for CP4 EPSPS reference standard, where amount refers to CP4 EPSPS protein. Approximate molecular weights (kDa) correspond to the markers loaded in Lanes 2 and 8. Following electrophoresis, the Brilliant Blue G-Colloidal stained gel was analyzed densitometrically (see Table V-7).

D.5. Functional Activity

The specific activity of the plant-produced CP4 EPSPS protein was estimated using a phosphate release assay. The estimated specific activity of the plant-produced CP4 EPSPS was 7.3 U/mg total protein. The specific activity of the *E. coli*-produced CP4 EPSPS reference standard protein, which was analyzed concurrently, was 4.7 U/mg total protein. These values were within the range of specific activities of CP4 EPSPS protein reported in other studies (Harrison et al., 1996). The enzyme assay demonstrated the plant-produced CP4 EPSPS protein was as active as *E. coli*-produced CP4 EPSPS protein and thus the plant-produced protein is functionally equivalent to the *E. coli*-produced protein with respect to CP4 EPSPS enzyme mediated release of the phosphate group from PEP.

D.6. Glycosylation Analysis

Many eukaryotic proteins are post-translationally modified with carbohydrate moieties (Rademacher et al., 1988). These carbohydrate moieties may be complex, branched polysaccharide structures or simple monosaccharides. To test whether potential post-translational glycosylation of the plant-produced CP4 EPSPS protein occurred, the isolated plant-produced CP4 EPSPS protein was analyzed for covalently bound carbohydrate using the ECL glycoprotein detection system kit of Amersham Pharmacia Biotech, Inc. (Piscataway, NJ). The *E. coli*-produced CP4 EPSPS reference standard protein (negative control) and the transferrin protein (positive control) were also analyzed concurrently. The results are presented in Figure V-27. A total of three film exposures (1 min, 2 min, and 4 min) were produced. The exposure shown in Figure V-27 was produced from a scan of the 2 min film exposure. The positive control (transferrin) was clearly detected from the highest concentration of 1.0 μg/lane to the lowest concentration of 0.10 μg/lane, in a concentration dependent manner. However, at the longest film exposure-time of 4 min (data not shown), a barely discernable band, close to the expected position for the CP4 EPSPS protein, was observed for the test substance and for the *E. coli*-produced CP4 EPSPS protein (considered as the negative control). Glycosylation of proteins is a post-translational process, which occurs exclusively in eukaryotic organisms and is not observed in prokaryotic organisms such as *E.* coli. Therefore, the very faint band observed for the test and reference CP4 EPSPS proteins on the 4 min exposure is attributable to a very weak nonspecific interaction between the detection reagent (Streptavidin-HRP conjugate) and protein mass bound to the blot. Additionally, N-linked glycosylation would result in an apparent increase in the molecular weight of the plant-produced CP4 EPSPS protein. However, this was not observed for the average mass determination of the plant-produced CP4 EPSPS protein using MALDI-TOF mass spectrometry. The glycosylation analysis demonstrated that there is no detectable glycosylation of the CP4 EPSPS protein produced in alfalfa and thus the plant-produced protein is equivalent to the *E. coli*-produced CP4 EPSPS reference standard with respect to glycosylation.



Lane	Sample	Amount (µg)
1	Blank	—
2	Pre Stained MW Markers (Bio-Rad, Cat #: 161-0374)	—
3	Transferrin (Positive control)	1.0
4	Transferrin (Positive control)	0.50
5	Transferrin (Positive control)	0.10
6	*E. coli*-produced CP4 EPSPS reference standard	1.0
7	Plant-produced CP4 EPSPS from alfalfa event J163	1.5
8	Plant-produced CP4 EPSPS from alfalfa event J163	0.73
9	Pre Stained MW Markers (Bio-Rad, Cat #: 161-0374)	—
10	Blank	—

Figure V-27. Glycosylation Analysis of CP4 EPSPS Isolated from Roundup Ready Alfalfa Event J163.

Samples of plant-produced CP4 EPSPS, *E. coli*-produced CP4 EPSPS reference standard (negative control) and transferrin (positive control) were separated by SDS-PAGE (4→20%) and electrotransferred to PVDF membrane. If present, the protein-bound carbohydrate moiety is labeled with biotin, and detected with streptavidin-horseradish peroxidase and enhanced chemiluminescence (2 min exposure shown). Amount refers to total protein loaded per lane, except for CP4 EPSPS reference standard, where amount refers to CP4 EPSPS protein. Approximate molecular weights (kDa) correspond to the markers loaded in Lanes 2 and 9.

D.7. Conclusions for Event J163 CP4 EPSPS Protein Characterization

The physicochemical and functional properties of the plant-produced CP4 EPSPS protein isolated from Roundup Ready alfalfa event J163 were assessed. A battery of analytical tests was used to characterize the physicochemical and functional properties of the plant-produced CP4 EPSPS protein. The *E. coli*-produced CP4 EPSPS protein was included in selected analyses as a reference standard. The analytical tests included (1) BCA total protein assay, (2) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and densitometry, (3) immunoblotting, (4) glycosylation analysis, (5) enzymatic assay, (6) N-terminal sequence analysis, and (7) MALDI-TOF mass spectrometry.

The results of N-terminal sequence analysis of the plant-produced CP4 EPSPS protein were consistent with the expected CP4 EPSPS protein N-terminal sequence. Immunoblot and MALDI-TOF mass spectrometric analyses also provided data to confirm the identity of the plant-produced CP4 EPSPS protein. A phosphate release assay confirmed that the CP4 EPSPS protein isolated from alfalfa event J163 was functionally active.

SDS-PAGE (molecular weight), immunoblot analysis, glycosylation analysis and a functional assay were performed to evaluate the equivalence of the plant-produced CP4 EPSPS protein to the *E. coli*-produced CP4 EPSPS reference standard protein. The plant-produced CP4 EPSPS protein was considered to be equivalent to the *E. coli*-produced CP4 EPSPS reference standard protein based on comparable electrophoretic mobility, enzyme activity, immunoreactivity and absence of detectable glycosylation. Moreover, N-terminal sequence and MALDI-TOF data clearly establish the identity of plant-produced CP4 EPSPS.

Collectively, these data establish the physicochemical and functional properties of the CP4 EPSPS protein isolated from alfalfa event J163 and establish its chemical and functional equivalence to the *E. coli*-produced CP4 EPSPS protein used in previous safety studies.

E. CP4 EPSPS Levels in Roundup Ready Alfalfa

The level of the CP4 EPSPS protein in Roundup Ready alfalfa forage was estimated using a validated Enzyme Linked ImmunoSorbent Assay (ELISA). The extraction of CP4 EPSPS from forage tissue as well as the ELISA method, and sample analyses are described below.

E.1. Sample Preparation for ELISA Analysis.

Alfalfa forage was harvested from the field and stored frozen at -20ºC. Tissues were ground to a powder using a Waring blender. Dry ice was added to the blender to keep the tissue frozen during the grinding process. CP4 EPSPS was extracted from frozen forage tissue using a Tris-borate (TBA) buffer at an optimized tissue-to-buffer volume ratio of 1:50. TBA buffer consists of 100 mM Tris base, 100 mM $Na_2B_4O_7 \cdot 10H_2O$, 5 mM $MgCl_2$, 0.05% (v/v) Tween-20 at pH 7.8, and 0.2% (w/v) L-ascorbic acid. Tissues were disrupted in a linear shaker (Harbil Mixer) for approximately 3.5 min. The insoluble plant debris was then separated from the liquid extract using a serum filter. The clarified supernatants were aliquoted into pre-labeled tubes and stored in a -80°C freezer.

E.2. ELISA Method.

The assay method was a double antibody sandwich ELISA wherein mouse monoclonal anitbody specific to the CP4 EPSPS protein was used as the capture antibody followed by detection of bound CP4 EPSPS with goat polyclonal anti-CP4 EPSPS antibody. A detailed description of the antibody reagents and method is provided below.

E.2.a. Assay reagents

CP4 EPSPS Protein Standard. The CP4 EPSPS protein standard, lot # 5192245, was produced by fermentation of *Escherichia coli* that was transformed with a plasmid containing the *cp4 epsps* coding sequence from *Agrobacterium sp*. strain CP4. Following purification, the CP4 EPSPS protein standard was dissolved in a buffer containing 50% (v/v) glycerol, 50 mM Tris-HCl, 50 mM KCl, and 2 mM DTT. The protein standard was stored in aliquots at approximately -20°C. The total protein concentration of the purified standard was determined to be 3.96 mg/ml. The purity of this lot was determined to be >85% by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Coomassie staining, and subsequent band analysis by densitometry. The standard concentration of 3.96 mg/ml was utilized to generate the ELISA data and was not corrected for purity.

Anti-CP4 EPSPS Antibodies. Antibodies specific to the CP4 EPSPS protein were generated in mice and goats using the CP4 EPSPS standard (described above) as antigen. Specificity of goat or mouse antisera was demonstrated during development of the ELISA wherein goat or mouse antibodies were selected on the basis of high specific binding to the CP4 EPSPS standard and absence of non-specific binding to endogenous alfalfa proteins. Antiserum generation and specificity testing were similar to that described by Rogan et al. (1992). Mouse monoclonal antibody clone 39B6 (IgG2a isotype, kappa light chain; lot # 6199732), specific for the CP4 EPSPS protein, was purified from mouse ascites fluid using Protein-A Sepharose affinity chromatography. The concentration of the purified IgG2a was determined to be 3.2 mg/ml by spectrophotometric methods. Production of the 39B6 monoclonal antibody was performed by TSD Bioservices, Inc. (Newark, DE). The purified antibody was stored in a buffer (pH 7.2) containing 0.02 M $Na_2HPO_4 \cdot 7H_2O$, 0.15 M NaCl, and 15 ppm ProClin 300 (Sigma, St. Louis, MO). Goat polyclonal anti-CP4 EPSPS antibody (Harlan Bioproducts for Science, Indianapolis, IN) conjugated to horseradish peroxidase (HRP) was used as the ELISA detection antibody. The polyclonal anti-CP4 EPSPS antibody was purified from goat sera using Protein-G affinity chromatography (TechServ Associates, St. Louis, MO). The antibody conjugated to HRP was stored in a solution containing 0.02 M KH_2PO_4, 0.15 M NaCl and 0.01% thimerosal, pH 7.3. The conjugated antibody was diluted 1:100 in a stabilizing buffer (Stabilzyme, Surmodics, Eden Prairie, MN), assigned lot # 6558639, aliquoted, and stored at approximately 4°C.

ELISA Procedure. Mouse anti-CP4 EPSPS monoclonal antibody was diluted in antibody coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) and immobilized onto 96-well microtiter plates at 1.0 μg/ml followed by incubation in a 4°C refrigerator for $\geq$ 8 h. Plates were washed in 1X phosphate buffered saline (10 mM

$Na_2HPO_4 \cdot 7H_2O$, 1 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl at pH 7.4) with 0.05% (v/v) Tween-20 (1X PBST) prior to performing the assay. Samples and standards diluted in TBA were added to the microtiter plate at 100 µl per well and allowed to incubate for 1 h in a 37° C incubator. After the sample incubation period, the plates were again washed with 1X PBST and the detection antibody, goat anti-CP4 EPSPS conjugated to HRP, was further diluted 1:250 in stabilizing buffer, added at 100 µl per well, and incubated for 1 h at 37°C. Following this incubation, plates were washed as before and developed by adding 100 µl per well of the enzyme substrate 3,3',5,5' tetramethylbenzidine (TMB, Kirkegaard and Perry Laboratories, Gaithersburg, MD) for 10 min at room temperature. The enzymatic reaction was terminated by the addition of 100 µl per well of 3 M H_3PO_4. The CP4 EPSPS protein absorbance readings were determined at a wavelength of 450 nm with a simultaneous reference reading at 650 nm that was subtracted from the 450 nm reading. Absorbance readings were determined using a Molecular Devices SpectraMax Plus microplate reader (Molecular Devices, Sunnyvale, CA) and data reduction analyses were performed using Molecular Devices SOFTmax PRO version 2.4.1. Quantitation of CP4 EPSPS protein levels was accomplished by interpolation from a seven-point standard curve that ranged in concentration from 0.313 to 20.0 ng/ml.

Positive and Negative Quality Controls. Positive quality control (QC) samples (lot # 6844589-B) and negative QC samples (lot # 6844589-C) were used during the ELISA development and validation. The positive and negative QC samples were comprised of extracts prepared from alfalfa leaf tissue that was provided by Forage Genetics International. The average amount of CP4 EPSPS protein detected by ELISA in the quality control extracts was determined in twenty-five independent assays. The range of CP4 EPSPS protein in the quality controls was determined as the mean $\pm$ 3 standard deviations. The range for the positive QC sample, lot # 6844589-B, was 0.6 – 3.3 ng/ml and the negative QC sample was always less than the assay LOQ (0.313 ng/ml).

E.3. Estimation of CP4 EPSPS in Alfalfa Forage Samples

To estimate the levels of CP4 EPSPS protein, alfalfa varieties containing one of each of the events, and the paired combination of both events, were planted at six field sites in the spring of 2001. Sites selected represented geographies where alfalfa is typically grown in the United States. A randomized, four-replicate complete block experimental design was used at all field sites. Samples were obtained from each replicate for ELISA analysis. Because alfalfa is a perennial plant that can be harvested multiple times over the length of the growing season, the CP4 EPSPS protein level was determined at two different times during the growing season and from two different years of forage growth (2001 and 2002). Forage was harvested at all sites when plants were at the early to late bud stage.

The level of the CP4 EPSPS protein in forage collected from the alfalfa varieties are presented in Table V-8. The mean levels of the CP4 EPSPS protein across two seasons and from multiple cuttings were 257 and 270 µg/g on a tissue fresh weight (tfw) basis for alfalfa plants that contained event J101 and J163, respectively. While there was greater variation in the levels of the CP4 EPSPS enzyme in forage obtained from plants that contained two transformation events, combining the two inserts to make the Syn 1

population did not cause an additive effect on the level of the CP4 EPSPS protein produced by alfalfa plants. The mean level of the CP4 EPSPS protein in the synthetic alfalfa population, produced using events J101 and J163, across two seasons and from multiple cuttings was 252 μg/g tfw. The level of the CP4 EPSPS protein was estimated in forage samples obtained from two seasons (2001 and 2002) at six sites and from multiple cuttings in each season at three of the sites. As expected, the level of the CP4 EPSPS protein varied at each sampling time point. The majority of the samples obtained from the second growing season had lower levels of CP4 EPSPS. These differences were not biologically meaningful and are likely attributable to assay variation, differences associated with stage of tissue growth and environmental factors. Observations from the field trials from which the forage samples were obtained confirmed that the tolerance to Roundup agricultural herbicides was excellent and consistent across the two events and the combined event.

Table V-8. CP4 EPSPS Levels in Roundup Ready Alfalfa Events J101, J163 and the Paired Event Combination.

		Levels of CP4 EPSPS Protein in Forage[a].					
		Events and Year of Forage Sampling					
		2001			2002		
Site	Cut Number	J101	J163	J101XJ163	J101	J163	J101XJ163
Wisconsin	1	300	330	260	200	140	150
Iowa	1	300	380	290	210	150	180
New York	1	270	290	280	220	180	140
Illinois	1	260	320	290	270	310	200
Illinois	2	230	330	270	280	290	230
Washington	1	220	270	330	160	140	120
Washington	2	340	290	360	220	240	310
California	1	270	320	390	240	220	120
California	2	290	320	340	340	340	280
	Mean	276	317	312	238	223	192
	Range Low	220	270	260	160	140	120
	Range High	340	380	390	340	340	310

[a]Concentration is given in micrograms/gram tissue fresh weight.

F. Biochemical Properties of EPSPS Proteins and Homology to EPSPSs Derived from a Variety of Plant and Microbial Sources

The EPSPS protein has a well-characterized catalytic function in plants, bacteria and fungi, it catalyses a non-rate limiting step in the shikimate pathway involved in aromatic amino acid biosynthesis in plants. The enzymatic activity of EPSPS's from a variety of glyphosate-tolerant, and sensitive plant and microbial sources has been extensively characterized. It has been established that just like native EPSPS's; CP4 EPSPS is highly specific for its natural substrates, shikimate-3-phosphate and phosphoenolpyruvate

(Gruys and Sikorski, 1999). This characterization included an examination of the three-dimensional folding patterns and active site homology. The shikimate pathway is not present in mammals, which contributes to the selective toxicity of glyphosate.

The CP4 EPSPS protein produced by Roundup Ready alfalfa is comparable in many ways to the family of EPSPS enzymes commonly found in a wide variety of food sources. The *cp4 epsps* gene has been completely sequenced and encodes a 47.6 kDa protein consisting of a single polypeptide of 455 amino acids (Padgette et al., 1996). The CP4 EPSPS protein shows significant homology to EPSPS's naturally present in all crops and in fungal and microbial food sources such as Baker's yeast (*Saccharomyces cerevisiae*) and *Bacillus subtilis* (Mountain, 1989) which have a history of safe human consumption, as shown in Table V-9. This similarity of the CP4 EPSPS protein to EPSPS's in a variety of foods supports the lack of health concerns and extensive human and animal consumption of the family of EPSPS proteins.

A search of GenBank (National Center for Biotechnology Information) for alfalfa EPSPS sequences has come up blank indicating that as of December 2002 the alfalfa EPSPS has not been cloned and sequenced. The quantity of alfalfa DNA sequence submitted to GenBank is very limited and there have been no large-scale genome alfalfa sequencing efforts. However, a relatively close relative of alfalfa, the annual cool season Medic, *Medicago truncatula*, has been adopted as the model legume for genomics research. The goal of this research is to provide a comparative analysis of molecular, genetic and biological information across legumes. Currently, the EST database contains over 170,000 sequences and their computed assembly as 26,000 unigenes from around 30 libraries (Lamblin et al., 2003). Very high conservation at the both individual gene sequence and gene structure exists between the two species. A search of the *Medicago truncatula* databases in late December 2002 also failed to identify a candidate EPSPS. Based on the extensive *Medicago truncatula* sequence data available, it would be expected that the alfalfa EPSPS enzyme would be similar to other EPSPS's, particularly those from other legumes such as soybean.

Crops containing the Roundup Ready trait have been commercialized since 1996. Since that time, there have been no reports of unintended health effects on bees or other insects foraging on crops that contain the Roundup Ready trait. The mechanism of action for the CP4 EPSPS protein is well known (Padgette et al., 1996). The CP4 EPSPS protein has no known effect on insects or any other animal species.

Table V-9. Comparison of Deduced Amino Acid Sequences of CP4 EPSPS to other EPSPSs

	Soybean (*G. max*)	Maize (*Z. mays*)	Petunia (*P. hybrida*)	*E. coli*	*B. subtilis*	*S. cerevisiae*
CP4 EPSPS						
% sequence identity	26	24	23	26	41	30
% sequence similarity	51	49	50	52	59	54

VI. Phenotypic Evaluation

This section provides a comprehensive evaluation of Roundup Ready alfalfa including phenotypic characteristics, field-testing information, USDA-APHIS field reports, and crop compositional assessments that were used to reach a determination that Roundup Ready alfalfa events J101 and J163 are no more likely to pose a plant pest risk than conventional alfalfa.

In evaluating the phenotypic characteristics of event J101 and event J163, data were collected that address specific characteristics suggested by USDA-APHIS. These phenotypic characteristics have been grouped into five general categories: 1) dormancy, germination and emergence; 2) vegetative growth; 3) reproductive growth; 4) disease, insect, and abiotic stressor interactions; and 5) symbiotic organisms. An overview of the phenotypic characteristics is presented in Table VI-1.

The phenotypic evaluation is based on both laboratory experiments and replicated, multi-site field trials conducted over five years (1999-2003) by agronomists and scientists who are familiar with the production and evaluation of alfalfa. In each of these assessments, event J101 and event J163, are compared to an appropriate alfalfa control.

Roundup Ready alfalfa varieties will be commercialized using a combination of two different *cp4 epsps* insertion events combined through a conventional breeding process. A Forage Genetics International (FGI) proprietary conventional breeding method (patent pending)[1] has been developed for rapid introgression of a gene into alfalfa varieties. In the FGI breeding process, one copy of the *cp4 epsps* gene is required at each of two different, independently segregating loci. The two independent gene loci are products of two separate *cp4 epsps* single copy insertion events. The population of alfalfa plants in commercial Roundup Ready varieties will consist of individual plants with the *cp4 epsps* gene insert copy number ranging from zero to eight, contributed by either of the two transformation events. A description of the distribution of the *cp4 epsps* gene copy number in a Roundup Ready alfalfa population is presented in Appendix 1. Plants with seven to eight copies are extremely rare, the majority of plants will contain two copies of the *cp4 epsps* gene. The variation in copy number is due to the genetics associated with alfalfa breeding and because varieties are comprised of a heterogeneous population of individuals.

While the genetics of the commercial paired event population will be more complicated than most annual crops, the primary objective of these phenotypic studies was to compare phenotypic parameters of Roundup Ready alfalfa plants containing either event J101 or event J163 with these same parameters in control and reference alfalfa plants. Data derived from single event plants and appropriate control or references served as the foundation upon which the risk assessment was based. However, data were also developed for synthetic generations of plants containing both events and are presented in

[1] FGI has filed a U. S. patent application (US-2002-0042928-A1) relating to a novel method of conventional breeding alfalfa with high transgene trait transmission in the commercial product: "Methods for Maximizing Expression of Transgenic Traits in Autopolyploid Plants."

this section as confirmatory information to support the conclusions generated from single event populations.

Table VI-1. Phenotypic Characteristics Measured for Roundup Ready Alfalfa Events J101 and J163

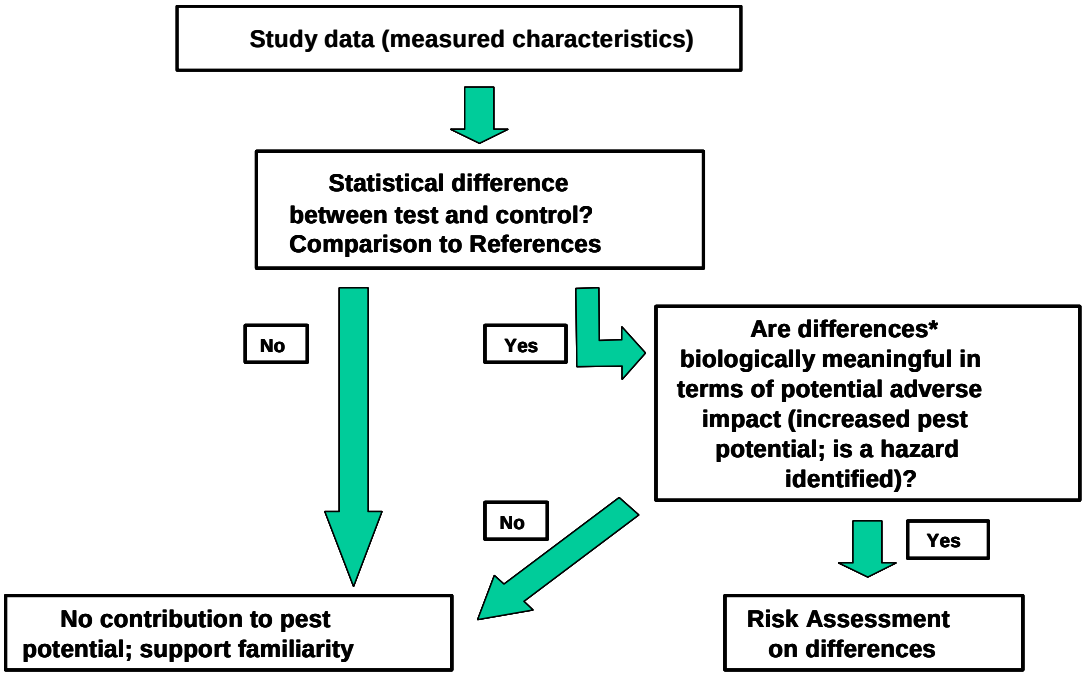
General Assessment Characteristic	Specific Characteristic Assessed	Characteristic Measured	Data Table Location	Figure Location
Dormancy / Germination / Emergence	Seed dormancy	Dormancy, Germination	VI-2-4 VI-5, VI-6	NA[1]
	Seedling emergence	Emerged plants, Seedling vigor	VI-10-13	NA
Vegetative Growth	Overwintering capacity	Spring vigor Spring stand	VI-10-13, VI-17, VI-22	NA
	Vegetative biomass	Forage yield	VI-10-13, VI-22	NA
	Vegetative Regrowth	Regrowth after cutting	VI-10-12	NA
	Habit (basic morphology)	Crop growth stage Fall growth habit	VI-10-13 VI-14, VI-24	NA
Reproductive Growth	Habit (basic morphology)	Flower morphology Flower color	VI-27	VI-10-15
	Fertility or infertility & pollen viability	Pollen load/flower Pollen viability Self fertility Pollen morphology Pollen germination	VI-26-29	VI-17, VI-19-21
	Seed production	Seed yield Seed morphology Seed weight Seed number / flower number	VI-30-32	VI-18
Disease, Insect & Abiotic Stressors	Species and frequency	Differential susceptibility to pests or abiotic stressors	VI-16, VI-18-20	NA
Symbiotic Organisms	Status of symbiotic relationship	Nodule mass/morphology Yield, Nitrogen, Amino acids	VI-34-35, VI-39	VI-22-23

[1]Not applicable. No figure was produced to support this characteristic.

A. Phenotypic Characteristics

Phenotypic characteristic information is used in assessing plant pest risk by evaluating for biologically meaningful differences between the biotechnology derived crop and the unmodified crop in terms of pest potential. A tiered approach is used to assess whether a difference is, or is not, biologically meaningful. As such, evaluation of phenotypic characteristics is designed according to the biology of the crop using replicated plots at multiple locations with appropriate controls and references. When no statistically significant differences are detected between the genetically modified crop and its nonmodified control, a conclusion of no difference in pest potential can be made. If a statistically significant and biologically meaningful difference in a characteristic were detected, its effect on pest potential can then be assessed, as shown in the schematic diagram below.

Schematic diagram of data interpretation methods:



*Consider direction & magnitude of change and interaction of differences

Statistically significant changes in one characteristic are considered in terms of the direction of the change (contributing to or detracting from weed potential), its magnitude (outside the range of the unmodified organism), and within the context of other observed changes. Interpretation of detected differences in an ecological risk assessment should

focus on those differences that are biologically meaningful. Differences detected in a characteristic must be considered alone and in the context of: 1) differences detected in other measured characteristics; 2) contributions to enhanced pest potential of the crop itself; and 3) potential effects of trait transfer to a wild or weedy species. For example, a significant difference in a growth characteristic may not be biologically meaningful in terms of weed potential if it is not outside the range typical for alfalfa or if a change in another parameter is in the direction toward less weed potential. A careful assessment must be used to distinguish between meaningful changes toward increased pest potential and differences associated with natural plant variation or random experimental error. A finding of no meaningful difference can be concluded only after an evaluation of all the data collected on the characteristics measured.

B. Description of Test, Control and Reference Substances Used for Phenotypic Studies

To understand the terminology used to describe test, control and reference populations used for the studies presented in this section, a brief review of alfalfa breeding is needed. A more thorough discussion of breeding terminology, the breeding of conventional alfalfa and the breeding of Roundup Ready alfalfa, plus key terms used throughout this section, is presented in Appendix 1.

A typical alfalfa variety may have 10 to 200 parent plants that were initially crossed in isolation to form the breeder generation seed (Figure VI-1). The breeder seed of commercial alfalfa varieties is produced by the random intercrossing (open pollination) of all parent plants. An alfalfa variety is maintained through multiple seed generations beyond breeder seed via the open pollination of their progeny in isolation from other alfalfa varieties or pollen sources. Plant varieties bred in this way are called synthetic varieties (Rumbaugh et al., 1988).

Individual plants within a synthetic variety are genotypically and phenotypically heterogeneous, i.e., no two individuals within the variety are exactly alike. Synthetic alfalfa varieties are closed populations that segregate, within a defined range, for most morphological traits and naturally occurring genetic markers. Because alfalfa varieties are segregating heterogeneous populations, alfalfa varieties are routinely described in terms appropriate to populations (mean or percent trait expression). For example, alfalfa variety registration agencies require that the pest resistance of a variety be described as the mean percentage of plants that express the segregating trait when the population is tested under standardized conditions.

A typical commercial seed increase process is illustrated in Figure VI-1. Commercial seed of alfalfa varieties is commonly produced according to the following sequence: 1) a set of superior alfalfa plants (usually 10-200 genotypes, also known as Syn 0 parents) are identified by an alfalfa breeder for use as parent plants for a new variety, and the Syn 0 parents are randomly intercrossed to produce the first synthetic generation of seed (Syn 1 seed); 2) Syn 2 generation seed is produced from a random, isolated intercross of Syn 1 plants; and 3) Syn 3 seed is produced from a random, isolated intercross of Syn 2 plants. Breeder, Foundation and Certified seed classes are defined at the discretion of the plant breeder during the variety registration process and are typically Syn 1, Syn 2 and Syn 3 generations, respectively. Most commercial seed varieties sold to alfalfa forage

producers are Certified Seed, although noncertified seed (seed produced without official oversight) is also sold.

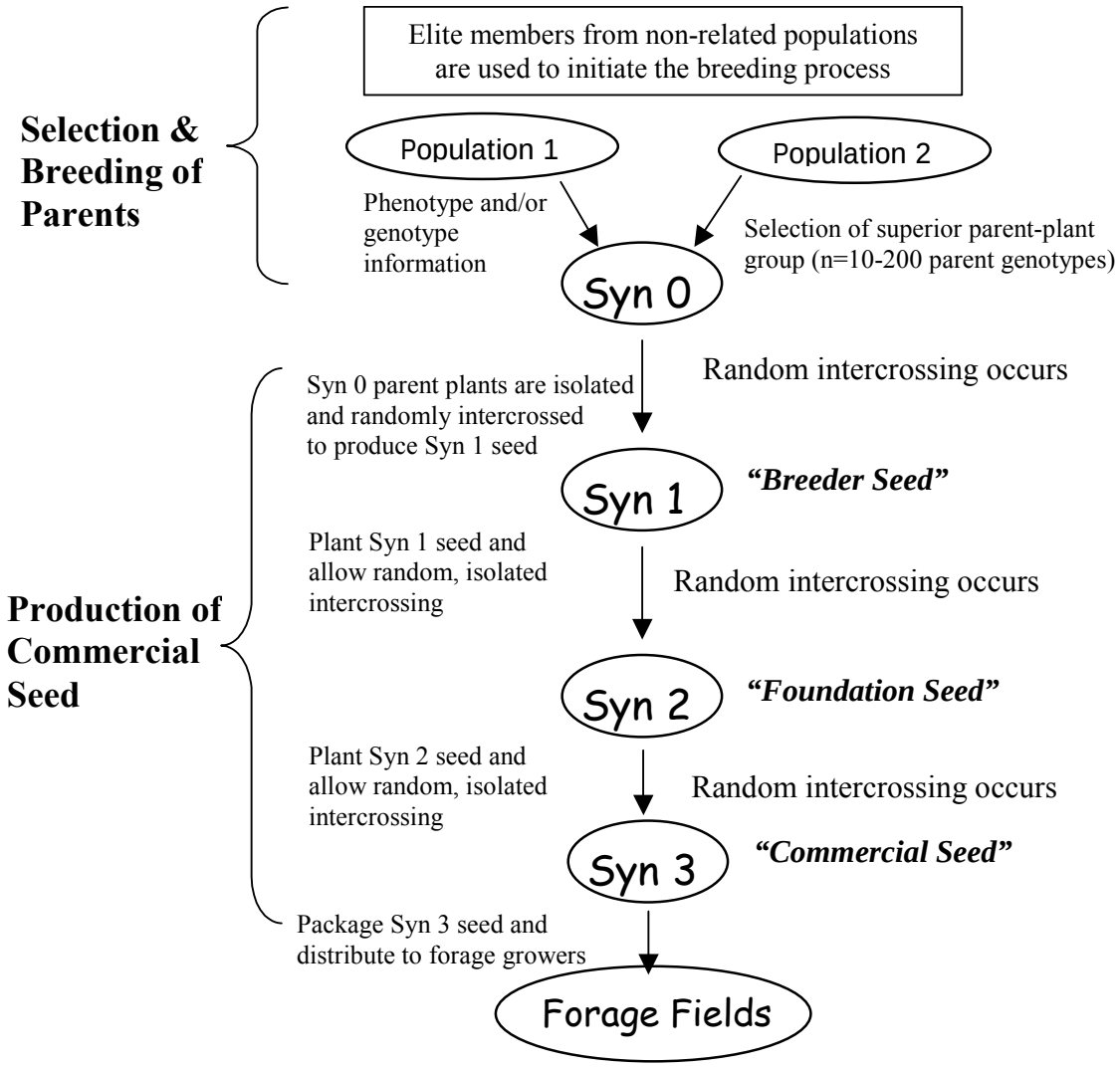


Figure VI-1. Commercial Alfalfa Synthetic Variety Breeding Schematic.

B.1. Development and Characterization of Near-Isogenic Control Populations

Alfalfa is an outcrossing autotetraploid. This creates a unique challenge for the development of appropriate negative controls for use in safety assessment studies where a near-isogenic line would be needed as a comparator. Unlike most transgenic crop species

commercialized to date, it is not possible to self-pollinate a single genotype of alfalfa to generate seed of adequate vigor for these studies. The limited amount of self-progeny seed (S1) that could be produced would suffer from inbreeding depression (Rumbaugh et al., 1988) and would segregate for most traits. S1 progeny would be unlikely to generate second generation seed successfully and would produce highly inbred seedlings with very poor vigor. Clearly, such material would be unacceptable for field studies performed to evaluate the impact of the trait or transformation process on alfalfa plants containing event J101 and event J163.

A closely related (near-isogenic) population of control plants was therefore used in the majority of the studies performed to assess phenotypic characteristics. The control population developed for these studies was bred to synchronize the background genetics of the control and test populations (populations containing event J101 or J163). The control population was developed from null (also known as nulliplex or negative) segregants obtained from the Roundup Ready alfalfa breeding program. Derivation of the null control population was as follows. A generalized progeny map describing the breeding process used to produce the control population is presented in Figure VI-8. Ancestors of the control plant starting material were subjected to, and resulted from, the genetic modification process. During the process of ensuing natural breeding (hand outcrossing) and directed selection for the *cp4 epsps* gene, a representative, *cp4 epeps* gene-segregating seed source was developed and characterized as follows. From this common source MBC2 generation, a nonselectively sampled subset of control alfalfa plants was genetically identified to be null segregating siblings (i.e., lacking the *cp4 epsps* gene) and used as the control plant starting material ancestors. A second nonselectively sampled subset was identified as positive segregating siblings (i.e., *cp4 epsps* present) and used as the test plant starting material ancestors. Segregation of a single-copy, dominant gene is the normal, predicted outcome in segregating seed families prior to fixing of the trait (i.e., allele saturation achieved through natural, intensive plant breeding). Using Southern blot analysis, it was determined that the null siblings did not contain the *cp4 epsps* gene nor any elements of the plasmid used during the plant transformation process. CP4 EPSPS-specific ELISA assay of the null segregants also did not detect the protein product of the *cp4 epsps* gene. The null siblings were then used as parents to produce a control seed population for the phenotypic studies presented in this section. Control plants had common background genetics representative to the test plants but did not express the CP4 EPSPS protein. The control plants also provided a background matrix for use in subsequent analytical evaluations of alfalfa tissues collected from field-grown plants. Other than intentional selection for the presence/absence of a *cp4 epsps* event, there were no other selection criteria imposed during the near-isopopulation (or test population) development process.

B.2. Development and Characterization of Null Segregant Progenitors

The following section describes in detail the development and characterization of the alfalfa progenitors that were used to produce null segregant control alfalfa populations for the studies described in this petition. MBC2 seedlings from transformations using plasmid PV-MSHT4 to develop alfalfa transformation events J101 and J163 from the fall dormancy background four (FD4) were nondestructively screened for the expression of

the CP4 EPSPS protein using a lateral flow immunoassay technique. The negative segregants and seedlings containing the *cp4 epsps* gene segregated approximately 1:1 as expected because of the modified backcrossing breeding strategy being employed. The negative MBC2 segregants were subsequently screened for the presence of the *cp4 epsps* gene by PCR. The PCR analysis was used to identify: 1) any plants that contained the *cp4 epsps* gene but the gene had been silenced, and 2) any samples where the CP4 EPSPS protein assay had failed. All plants identified as negative for CP4 EPSPS protein also tested negative for the *cp4 epsps* nucleotide sequence as determined by PCR.

The negative segregants were subjected to Southern blot analysis to verify the absence of any plasmid-derived DNA elements. The Southern blot analysis was conducted at Montana State University using standard operating procedures supplied by Monsanto. The genomic DNA from the negative segregants and suitable negative and positive controls was hybridized to probes for the eFMV promoter region, the *cp4 epsps* gene and two elements of the vector backbone. One element was located on either side of the T-DNA border sequences outside of the right and left borders. The combination of these four probes represents greater than 95% of the sequence from PV-MSHT4.

A total of 53 putative negative segregant plants were subjected to Southern blot analysis. No hybridization signal was observed with any of the negative segregant plants probed with the eFMV promoter and *cp4 epsps* probes or the vector backbone probes. Samples 14-27 trace to T_0 event J101, samples 42-53 trace to T_0 event J163. Figures VI-2 to VI-7 show the results of the Southern blot analysis. Figures VI-2, VI-4 and VI-6 show the analysis of the 53 plants using the combined eFMV promoter and *cp4 epsps* coding region probes. The genomic DNA was digested simultaneously with restriction enzymes *Sal1*, *Sca1* and *Xba1*. DNA sequence analysis had previously confirmed that none of these restriction enzymes cleave within the T-DNA region and that each of the events generates an event-specific fingerprint based on the position of flanking sites for the three enzymes (see Section V of this petition). On each blot, a lane containing PV-MSH4 plasmid (9023 bp) linearized with *BamHI* provided a positive control for hybridization. In addition, the representative T_0 event for the samples on a given blot was also included. All three blots were exposed for approximately four days. Following removal of the first probe by stripping the filter according to the manufacturer's instructions, a process that was confirmed by reexposure of the blots to x-ray film, the blots were rehybridized using a combined vector backbone probe (see figure legend for details). Figures VI-3, VI-5 and VI-7 show the results of hybridization with the two vector backbone probes. As expected, only the R2336 sample spiked with PV-MSHT4 plasmid showed any hybridization signal for vector backbone sequences. This also confirmed results derived from previous analyses that indicated that the events J101 and J163 were free from vector backbone sequences

It was concluded on the basis of Southern blot analyses that the 53 plants analyzed did not contain any detectable DNA sequences that could be traced to the transformation vector PV-MSHT4. As such, they were considered to be negative segregants suitable for seed production to provide control materials for subsequent product characterization and ecological safety assessment studies.

B.3. Creation of MBC3 Negative Control Seed Lots

The fall-dormant MBC2 negative segregant plants from the analysis described above were randomly inter-pollinated by hand to form approximately 2000 seed (the Syn 0 generation). A 90-plant subsample of the Syn 0 generation was grown in the greenhouse and inter-mated by hand to produce approximately 10 grams (approximately 4000 seed) of the MBC2-derived negative control Syn 1 generation during fall 2000. Although this amount of seed was adequate to meet the spring 2001 planting needs, it was insufficient for additional field trial control needs. Therefore additional seed increases were done under screen enclosure cages in 2001 and 2002 as described below.

2001: After the 10 g Syn1 generation greenhouse seed was harvested from the 90 Syn0 plants (described above), the plant stems were clipped back and plants were maintained and allowed to grow vegetatively in the greenhouse until April 24, 2001, when they were transplanted to the field. The 90 plants were space-transplanted into a single 20 x 20 ft plot with nine rows of ten plants each with 24 inch spacing between rows and 20 inch spacing within rows at the FGI Western Research Station in Idaho. When the plants reached the early bud stage, the entire plot was covered with a 24 ft x 36 ft X 6 ft (width x length x center height) pollinator-proof screen enclosure or cage; the screen fabric was mounted over a half-hoop type frame system. The lower edges of the screen material were buried under the soil to prevent pollinators from escaping. Research personnel had access to the cage interior using a full height, zippered opening located on one end. At 10% bloom, alfalfa leafcutting bees were placed into the cage as emerging pupae and sheltered in predrilled bee boards hung above the canopy within the cage. The bee stocking rate was gradually increased as bloom became more abundant and was maintained at a maximum stocking rate of approximately 2.75 lbs loose pupae cells/cage (i.e., ~2 gallons by volume per acre—the recommended stocking rate for full bloom alfalfa seed fields in Idaho). The cage was sprinkler-irrigated, insect pests were managed with labeled insecticides, and weeds were controlled by hand-hoeing. Plants were pollinated for eight weeks and seed was allowed to fully mature before the plot was sprayed with chemical desiccant in mid-August. Seedpods were harvested seven days later using small hand tools, air-dried (approximately 110 ^{o}F) and belt-threshed on research-scale equipment to remove non-seed debris, and the screen cage was removed from the plot. The seed produced from the 2001 screen cage was a second increase of the control population Syn1 generation.

After seed harvest, the stems of all plants were clipped back to three inch height and allowed to overwinter until 2002. The Syn1 J101 and J163 test materials that were grown under screen cages were produced according to the same method and were grown under separate screen enclosures adjacent to the control seed increase cage.

2002: The overwintered plants were maintained through the winter dormancy period, survived and produced a third increase of Syn1 generation seed in 2002. In 2002, the plots were managed the same as in 2001 with respect to cage enclosures, pollinators, seed harvest, and general plot management. Plots were chemically desiccated on August 23, 2002, and harvested eight days later.

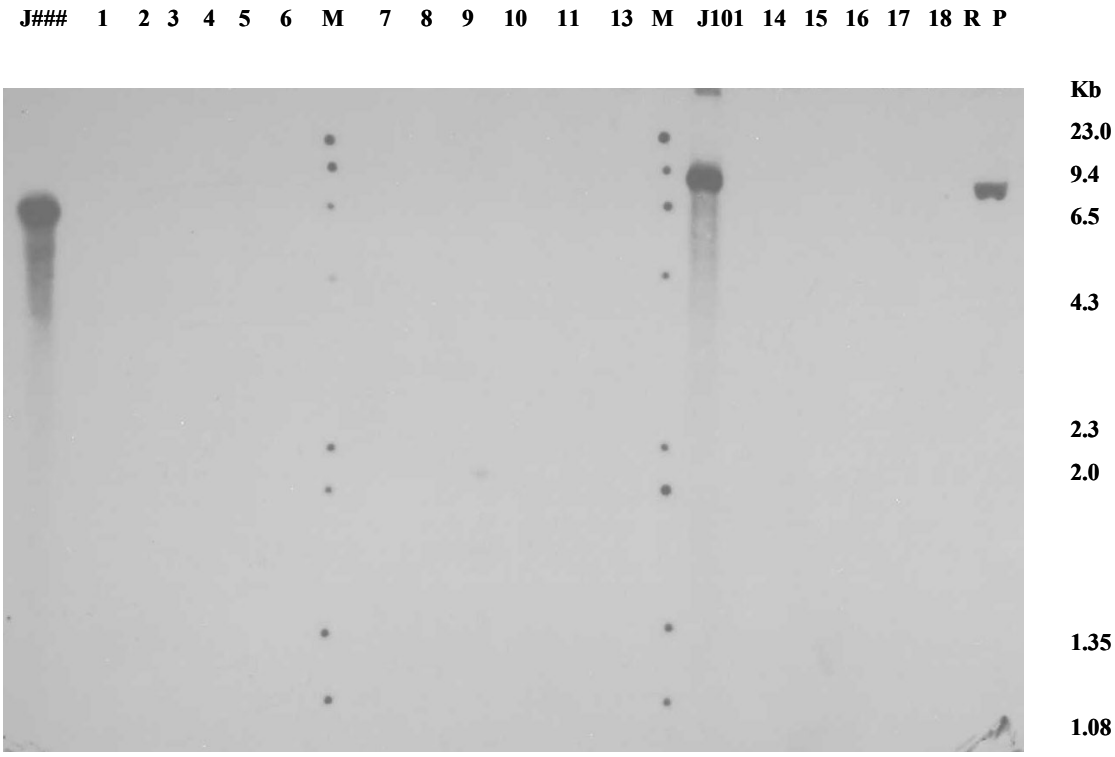


Figure VI-2. Evaluation of Negative Segregant Plants in MBC2 Population by Southern Blot Analysis, Series One.

Fifteen micrograms of genomic DNA per lane was digested with *Sal 1*, *Sca1* and *Xba1*, run on a 1% agarose gel and transferred to Hybond-N+ (Amersham) according to the manufacturers instructions. The blot was hybridized with a probe consisting of probes 1 and 2, encompassing the eFMV promoter region (Probe 1) and HSP70-*cp4 epsps* synthetic region (Probe 2) of plasmid PV-MSHT4. Both fragments were amplified by PCR from the plasmid using standard procedures. Samples numbered 1-18 inclusive were putative negative segregants from the MBC2 population. Lanes J### (an event that is not the subject of this petition) and J101 contained DNA isolated from the respective T_0 event plants. Lane R represented 15 ug of negative control DNA (R2336). Lane P contained 15 ug of R2336 DNA spiked with 30 pg PV-MSHT4 plasmid DNA linearized by restriction with *BamHI*. The positions of *HindIII* cut λ DNA standards are indicated to the left of the blot and also by dots in two lanes within the blot (Lane M).

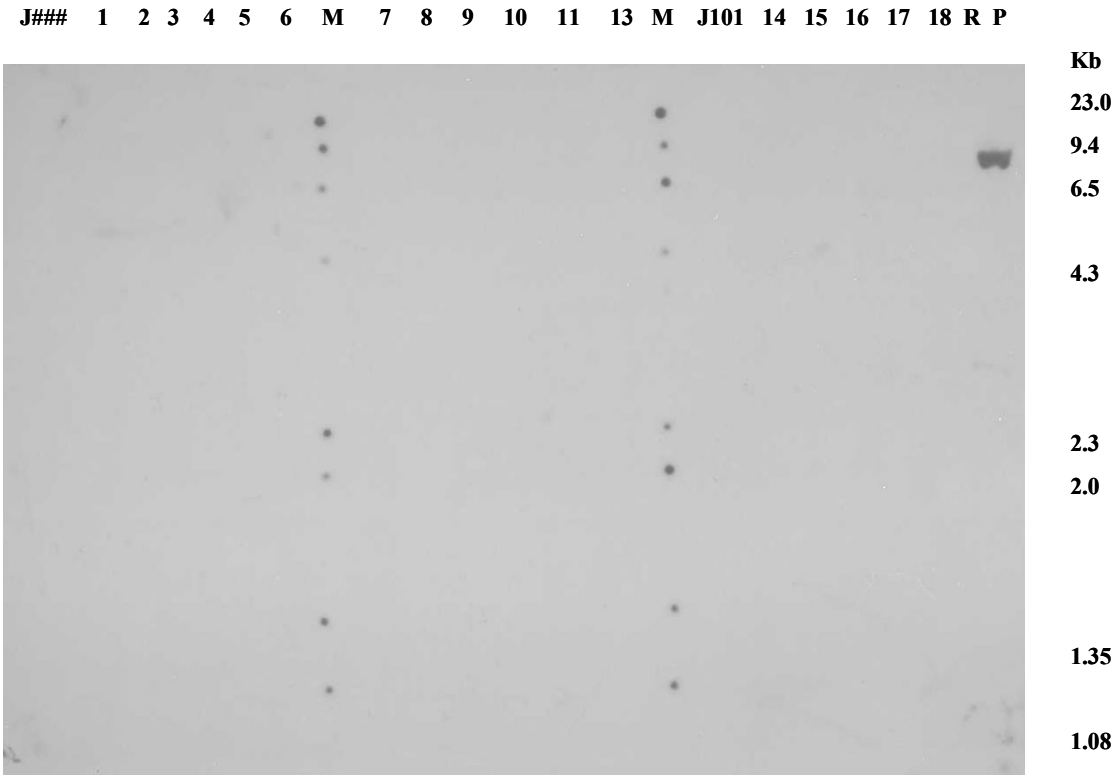


Figure VI-3. Evaluation of Negative Segregant Plants in MBC2 Population by Southern Blot Analysis, Series Two.

Fifteen micrograms of genomic DNA per lane was digested with *Sal 1*, *Sca1* and *Xba1*, run on a 1% agarose gel and transferred to Hybond-N+ (Amersham) according to the manufacturers instructions. The blot from Figure VI-2 was stripped and rehybridized with a vector backbone probe consisting of probes 5 and 6, encompassing the *Ori*-V region (Probe 5) and the *Ori*-322 and spectinomycin antibiotic resistance-coding region (Probe 6) of plasmid PV-MSHT4. Both fragments were amplified by PCR from the plasmid using standard procedures. Samples numbered 1-18 inclusive were putative negative segregants from the MBC2 population. Lanes J### (an event that is not subject of this petition) and J101 contained DNA isolated from the respective T_0 event plants. Lane R represented 15 ug of negative control DNA (R2336). Lane P contained 15 ug of R2336 DNA spiked with 30 pg PV-MSHT4 plasmid DNA linearized by restriction with *BamHI*. The positions of *HindIII* cut λ DNA standards are indicated to the left of the blot and also by dots in two lanes within the blot (Lane M).

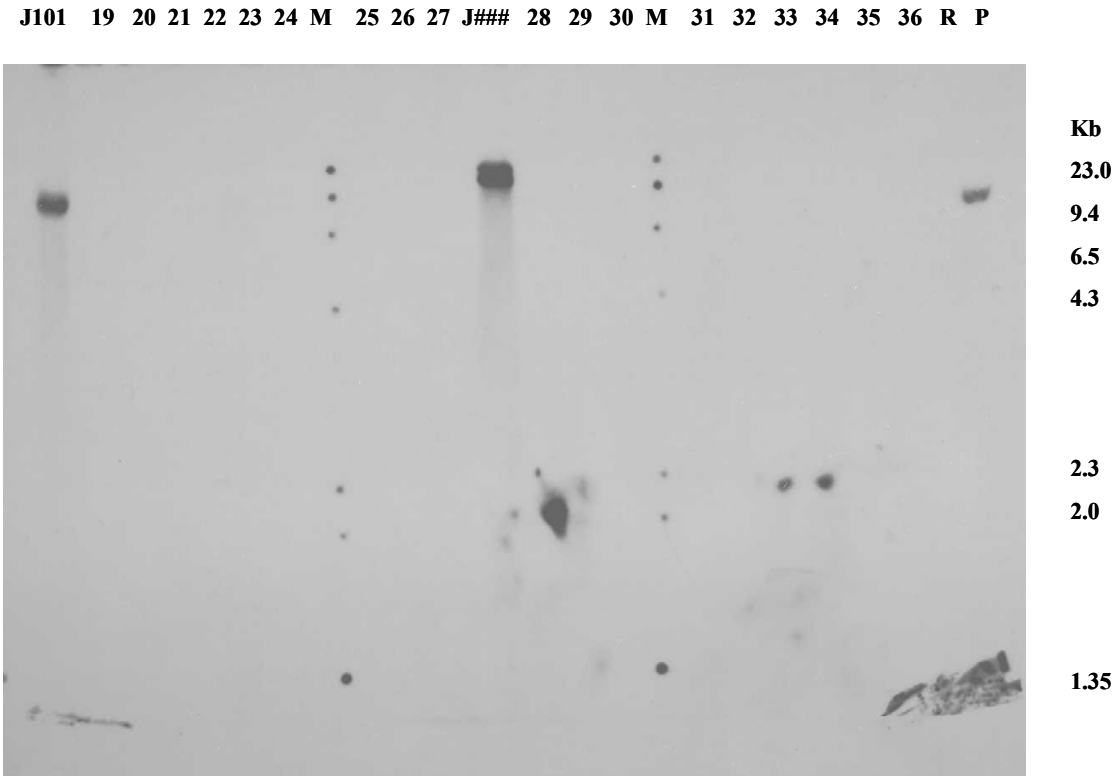


Figure VI-4. Evaluation of Negative Segregant Plants in MBC2 Population by Southern Blot Analysis, Series Three.

Fifteen micrograms of genomic DNA per lane was digested with *Sal 1*, *Sca1* and *Xba1*, run on a 1% agarose gel and transferred to Hybond-N+ (Amersham) according to the manufacturers instructions. The blot was hybridized with a probe consisting of probes 1 and 2, encompassing the eFMV promoter region (Probe 1) and HSP70-CP4 EPSPS syn region (Probe 2) of plasmid PV-MSHT4. Both fragments were amplified by PCR from the plasmid using standard procedures. Samples numbered 19-36 inclusive were putative negative segregants from the MBC2 population. Lanes J101 and J### (an event that is not subject of this petition) contained DNA isolated from the respective T_0 event plants. Lane R represented 15 ug of negative control DNA (R2336). Lane P contained 15 ug of R2336 DNA spiked with 30 pg PV-MSHT4 plasmid DNA linearized by restriction with *BamHI*. The positions of *HindIII* cut λ DNA standards are indicated to the left of the blot and also by dots in two lanes within the blot (lane M).

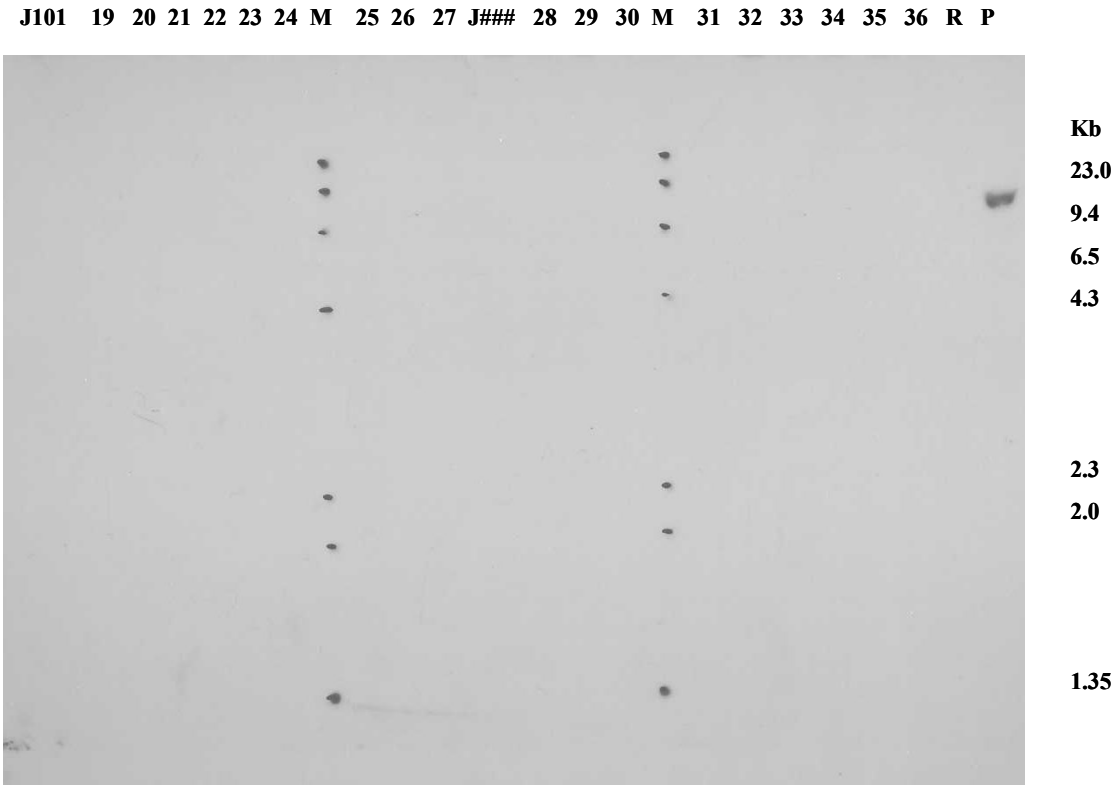


Figure VI-5. Evaluation of Negative Segregant Plants in MBC2 Population by Southern Blot Analysis, Series Four.

Fifteen micrograms of genomic DNA per lane was digested with *Sal 1*, *Sca1* and *Xba1*, run on a 1% agarose gel and transferred to Hybond-N+ (Amersham) according to the manufacturers instructions. The blot from figure VI-4 was stripped and rehybridized with a vector backbone probe consisting of probes 5 and 6, encompassing the *Ori*-V region (Probe 5) and the *Ori*-322 and spectinomycin antibiotic resistance-coding region (Probe 6) of plasmid PV-MSHT4. Both fragments were amplified by PCR from the plasmid using standard procedures. Samples numbered 19-36 inclusive were putative negative segregants from the MBC2 population. Lanes J101 and J### (an event that is not subject of this petition) contained DNA isolated from the respective T_0 event plants. Lane R represented 15 ug of negative control DNA (R2336). Lane P contained 15 ug of R2336 DNA spiked with 30 pg PV-MSHT4 plasmid DNA linearized by restriction with *BamHI*. The positions of *HindIII* cut λ DNA standards are indicated to the left of the blot and also by dots in two lanes within the blot (Lane M).

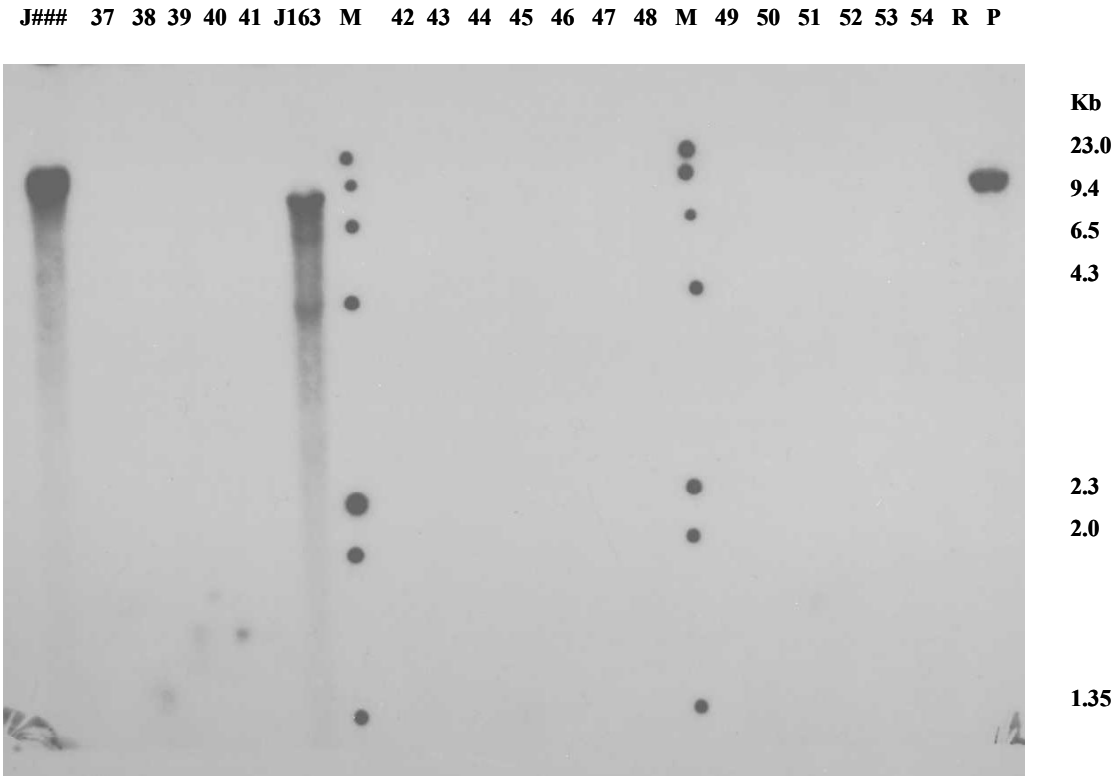


Figure VI-6. Evaluation of Negative Segregant Plants in MBC2 Population by Southern Blot Analysis, Series Five.

Fifteen micrograms of genomic DNA per lane was digested with *Sal 1*, *Sca1* and *Xba1*, run on a 1% agarose gel and transferred to Hybond-N+ (Amersham) according to the manufacturers instructions. The blot was hybridized with a probe consisting of probes 1 and 2, encompassing the eFMV promoter region (Probe 1) and HSP70-CP4 EPSPS syn region (Probe 2) of plasmid PV-MSHT4. Both fragments were amplified by PCR from the plasmid using standard procedures. Samples numbered 37-53 inclusive were putative negative segregants from the MBC2 population. Lanes J### (an event that is not subject of this petition) and J163 contained DNA isolated from the respective T_0 event plants. Lane R represented 15 ug of negative control DNA (R2336). Lane P contained 15 ug of R2336 DNA spiked with 30 pg PV-MSHT4 plasmid DNA linearized by restriction with *BamHI*. The positions of *HindIII* cut λ DNA standards are indicated to the left of the blot and also by dots in two lanes within the blot (Lane M).

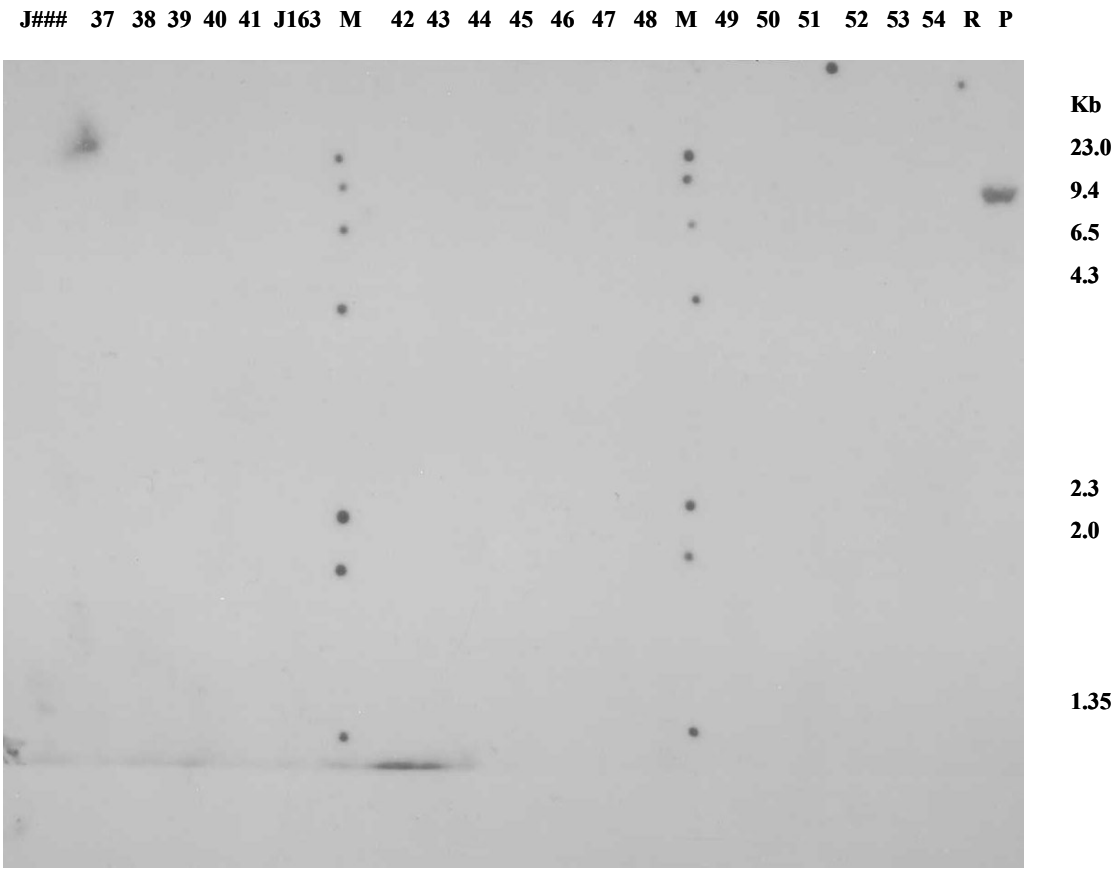


Figure VI-7. Evaluation of Negative Segregant Plants in MBC2 Population by Southern Blot Analysis, Series Six.

Fifteen micrograms of genomic DNA per lane was digested with *Sal 1*, *Sca1* and *Xba1*, run on a 1% agarose gel and transferred to Hybond-N+ (Amersham) according to the manufactures instructions. The blot from figure VI-6 was stripped and rehybridized with a vector backbone probe consisting of probes 5 and 6, encompassing the *Ori*-V region (Probe 5) and the *Ori*-322 and spectinomycin antibiotic resistance-coding region (Probe 6) of plasmid PV-MSHT4. Both fragments were amplified by PCR from the plasmid using standard procedures. Samples numbered 37-53 inclusive were putative negative segregants from the MBC2 population. Lanes J163 and J### (an event that is not subject of this petition) contained DNA isolated from the respective T_0 event plants. Lane R represented 15 ug of negative control DNA (R2336). Lane P contained 15 ug of R2336 DNA spiked with 30 pg PV-MSHT4 plasmid DNA linearized by restriction with *BamHI*. The positions of *HindIII* cut λ DNA standards are indicated to the left of the blot and also by dots in two lanes within the blot (Lane M).

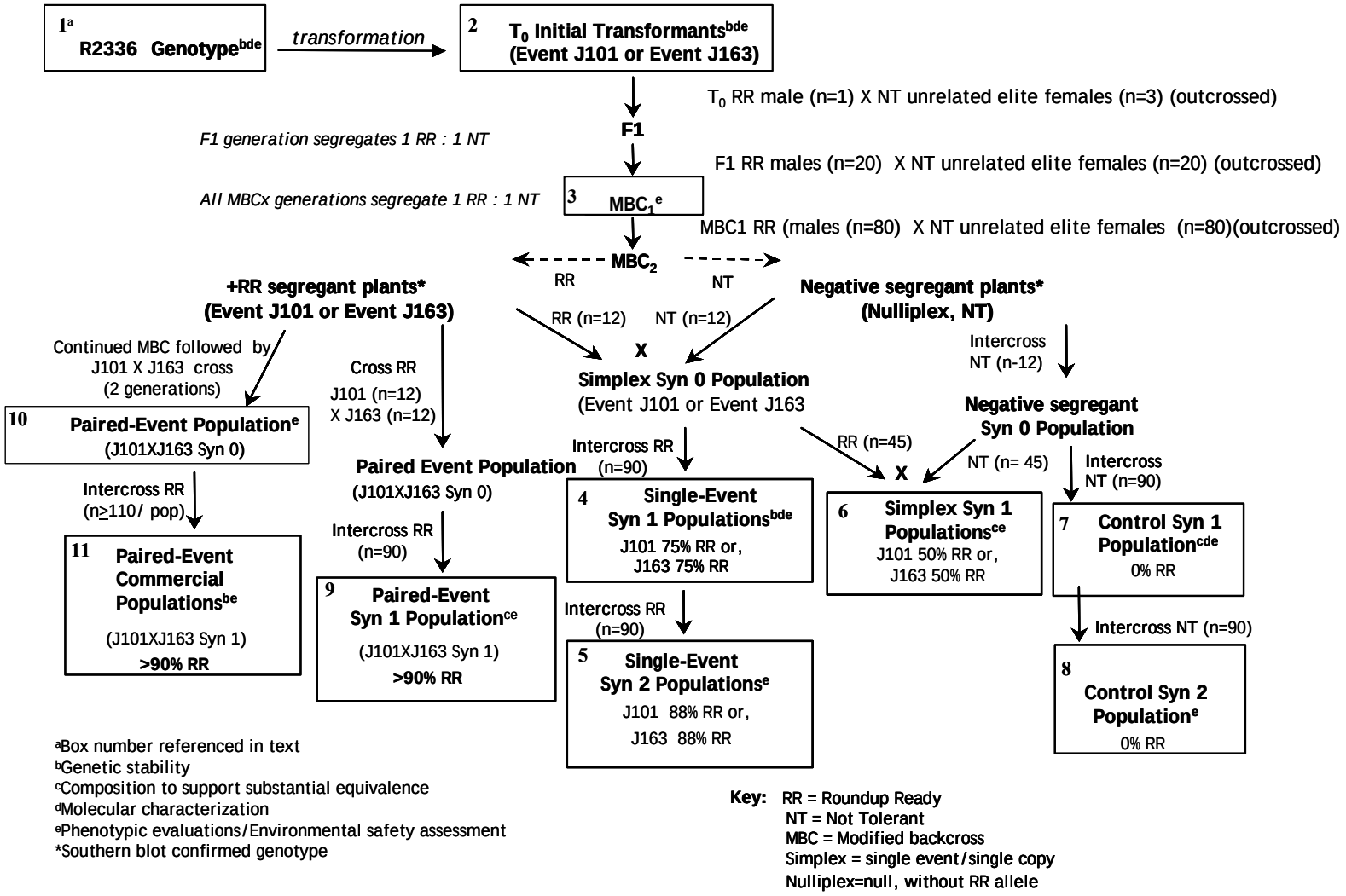


Figure VI-8. Roundup Ready Alfalfa Progeny Map

B.4. Development of Roundup Ready Test Event Progenitors

In addition to the development of the negative control population, positive-segregating siblings of the null plants were identified from the same J101 or J163 MBC2 populations and seed was increased in parallel with the control material as described in Section B.3., as outlined in Figure VI-8 or as described specifically within study discussions herein. The presence of the single intended *cp4 epsps* event was verified using Southern blot analysis and confirmed by event-specific PCR and the expression of CP4 EPSPS protein. The J101 and J163 siblings to the MBC2 negative segregants were used as parents to produce separate near-isogenic test seed populations (Figure VI-8) for the phenotypic studies presented in this section.

A generalized progeny map describing the development and source of test substances used for the product characterization and ecological safety assessment is presented in Figure VI-8.

C. Dormancy and Germination Evaluations

Seed dormancy is an important characteristic that is often associated with plants that are weeds (Anderson, 1996). Dormancy mechanisms, including hard seed, vary with species and tend to include complex processes. For most crops, the number of hard seed is negligible or nonexistent. However, when alfalfa seed is produced, a portion of the seed is hard; that is, the seeds do not absorb water after a prescribed period of time because of an impermeable seed coat (AOSA, 2002). The percentage of hard seed in alfalfa can range from 0 to 100% of a particular seed lot, depending on genetic factors, environmental conditions during and after seed maturation, and harvesting methods (Bass et al., 1988).

In the commercial alfalfa seed industry, standardized germination assays are used to measure the germination potential and dormancy of alfalfa seed lots (AOSA, 2002). Seed characteristics measured in the standardized assay include percentages of normal germinated, abnormal germinated, hard (dormant) ungerminated, and dead seed. Normal alfalfa seeds that readily imbibe water through the seed coat layer will immediately germinate within a broad range of temperatures (5-35^{o}C), with 19-25^{o}C being optimal (McElgunn, 1973; Townsend and McGinnies, 1972; Stone et al., 1979; Ungar, 1967). Hard (dormant) seed is defined as seed that does not imbibe water or germinate within the first seven days of the assay at 20^{o}C. A change in the percentage of hard seed within a seed lot is typically measured as an inverse effect on other germination components (e.g., as percentage hard seed decreases, there usually will be a corresponding increase in percentage normal germinated seed).

Three separate experiments were conducted to evaluate seed dormancy and germination characteristics for Roundup Ready alfalfa events J101 and J163. The first two experiments (experiments one and two) evaluated seed harvested in 2001 and 2002 from identical plots, and seed germination was evaluated using eight different temperature regimes. A third experiment was conducted to evaluate the percentage of hard seed of potential Roundup Ready alfalfa commercial variety seed lots containing alfalfa events J101 and J163.

Results from seed harvested in 2001 indicated an increased percentage of hard seed produced by Roundup Ready alfalfa events compared to the control. Germination and dormancy results from seed harvested in 2002 did not show a difference in percentage of

hard seed between Roundup Ready and control alfalfa. A combined, overall statistical analyses of data from both seasons indicated a trend towards increased percentage hard seed in Roundup Ready alfalfa relative to the control. However, the results of the combined analysis were largely weighted by the magnitude of the differences observed in the 2001 experiment. The results from the third experiment confirmed that there were no statistical differences for any of the seed germination characteristics (including hard seed) of seed produced by Roundup Ready alfalfa plants compared to seed produced by conventional alfalfa plants. Collectively, it was concluded that no biologically meaningful changes in seed dormancy were associated with seed produced by Roundup Ready alfalfa plants containing event J101 or event J163. Experimental methodology and results for these three experiments are presented in detail in Sections C.1, C.2 and C.3 below. Overall discussion and conclusions for the three seed dormancy experiments are presented in Section C.4.

C.1. Dormancy and Germination; Seed Harvested in 2001

Starting seed materials were produced by Forage Genetics International in Canyon County, Idaho, during summer 2001. All seed used was unscarified. Seed materials included the two single-event test populations, J101 and J163; one paired-event test population, J101XJ163 (confirmatory); one control (null) population, and a set of conventional reference variety populations. Seed materials to be tested were produced from plants grown using either pollinator-proof screen cages or distance to isolate the plants and minimize cross-pollination. The single-event, control, and four reference variety populations were grown in close proximity at one location under separate screen isolation cages so that all materials could be grown at a uniform single location, albeit under artificial cage micro environments. Confirmatory data developed for the paired-event population represents the commercial product concept for Roundup Ready alfalfa, so the paired-event and four reference reference variety populations were grown in physically isolated, distant field plots (without screen cages) representing typical commercial seed production practices.

C.1.a. Test, control, and reference materials

The test materials were single-event J101 or J163, and paired-event J101XJ163, Syn 1 Roundup Ready alfalfa populations (Figure VI-8: single-event test materials, Box 4, ~75% trait purity; paired-event test material, Box 11, ~95% trait purity). The control material was a Syn 1 alfalfa population developed for use as an appropriate null control (Figure VI-8, Box 7). The control and reference materials provided background values for dormancy and germination characteristics against which the single-event test materials were compared. The reference materials were commercially-destined, FGI experimental, conventional alfalfa variety populations (syn 2 generation). Three of the four reference varieties were common between the screen caged and the spatially isolated (no-cage) seed production environments (FGI-3S11, FGI-4S33 and FGI-4S41), however, the fourth experimental variety in each set was not the same (due to stock seed quantity limitations). Therefore, two different, but highly similar experimental, FGI reference varieties, 5S45 and 5S52, were used because both were deemed by the breeder to be appropriate comparitors for the test materials and both had very similar breeding pedigrees. The reference materials

provided control background values for dormancy and germination characteristics against which the paired-event test material was compared.

C.1.b. Methods

Seed was harvested and germination characteristics of the harvested seed were tested at eight different temperature regimes in growth chambers. Each of the eight growth chambers was maintained dark under one of the following temperature regimes: constant target temperature of approximately 5°, 10°, 20°, 30°, or 40°C, or alternating target temperature of approximately 5/20°, 10/20° or 20/30°C. The alternating temperature regimes were maintained for 16 hours at the lower temperature and eight hours at the higher temperature. Each temperature regime was considered an experimental block. Standardized germination tests for commercial alfalfa seed lots are conducted at a single, constant temperature of 20°C, which is most suitable for alfalfa seed germination (AOSA, 2002). The AOSA methods further specify that the temperature should not exceed 20°C. In this experiment, a range of temperature regimes was used to more thoroughly evaluate the effect of temperature on seed germination characteristics, particularly seed dormancy (hard seed).

Rolled germination towels containing exactly 100 seeds of each test, control, or reference material were prepared according to AOSA standards (AOSA 2002), procedures described in the testing laboratory's procedures manual, and appropriate standard operating procedures (SOPs). Eight germination towels (eight replications) were prepared for each test, control, and reference seed material for placement in each of the eight temperature regimes.

Each rolled germination towel was checked periodically for normal germinated, abnormal germinated, hard (dormant) ungerminated, and dead (degenerated) seed as defined by AOSA standards (AOSA, 2002). Hard ungerminated seeds were considered viable. Observations were made five, seven, and 12 days after initiation.

C.1.c. Statistical analysis

Because alfalfa varieties are produced and maintained as heterogeneous populations, each single-event and paired-event test material evaluated contained null segregant individuals. The population of alfalfa plants in each single-event Roundup Ready alfalfa test population consisted of individual plants with the *cp4 epsps* gene insert copy number ranging from 0 to 2. It was not technically possible to selectively remove the nulls from the starting seed materials prior to conducting the experiment. Consequently, for each comparative assessment between test and control populations, the values of each measured characteristic were inherently more similar than if each population were 100% trait-positive or trait-negative because null individuals occurred in both the test and control populations. The predicted proportion of null individuals in each single-event and paired-event test materials were 25 and 5 %, respectively (Figure VI-8, Box 4 and Box 11). The actual proportions of null individuals for J101, J163 and J101XJ163 were 23, 21 and 7%, respectively (greenhouse trait purity assay, data not shown). Because 77 to 93% of the individuals in each test population possessed the test trait, significant differences in the measured characteristics would have been detectable.

Variance analysis was conducted according to a split-plot design with temperature regime as the main-plot factor and alfalfa population type (test, control, or reference) as the subplot factor for each characteristic measured, but without a block effect because only one germination chamber was used for each temperature regime.

Data were analyzed using Statistical Analysis Software (SAS) Release 6.12 to compare test, control, and reference materials for cumulative values of the percentage of hard, normal germinated, abnormal germinated, and dead seed among the three observations (5, 7, and 12 days after initiation). Comparisons were made between each test population and the control and between each test population and the mean of the reference variety populations. As stated previously, temperature regimes above and below 20°C were included in the experimental design to evaluate the effect of temperature on germination characteristics. Because of the potential effect of temperature on germination characteristics, comparisons were not made across temperature regimes for an event. Within a temperature regime, a P-value less than 0.05 indicated a statistically significant difference between the mean of a test material population and the mean of the control population (or the mean of the four reference variety populations) at the 5% level.

C.1.d. Results and discussion

Mean seed germination results for the two single-event test populations (J101 and J163), the control population and the mean of four reference variety populations, all of which were grown using screened isolation cages, are presented in Table VI-2. This table presents the percentage of hard-ungerminated seed, normal germinated seed, abnormal germinated seed, and dead seed for each temperature regime tested. J101 and J163 had higher percentages of hard seed compared to the control population and to the mean of the reference variety populations at all eight-temperature regimes. Mean values for these two measured characteristics were also outside the observed range of values for the reference variety populations. Because increased percentages of hard seed for J101 and J163 may indicate an increased pest potential from dormancy, additional experiments were conducted to determine whether seed hardness was consistent across growing seasons and in commercial seed lots. Data from these additional experiments are presented in Sections C.2 and C.3 below.

As noted above, differences in the percentage of abnormal germinated and the percentage of dead seed were detected between one or both of the single-event test populations when compared to the control or mean of the reference variety populations at all eight temperature regimes. However, the percentages of abnormal germinated and dead seed for all test, control, and reference variety populations were relatively low (< 5%) overall, with the exception of percentage dead seed at 40°C. The 40°C temperature was extremely warm for alfalfa seed germination and therefore it was not unexpected that larger percentages of abnormal germinated and dead seed were observed. No trends for percentage abnormal germinated or dead seed were indicated from the data; therefore, differences detected among test materials for these two measured characteristics were likely due to experimental error.

Comparisons of the paired-event test population (J101XJ163) with the mean of four reference variety populations grown using spatial isolation (and without screen cages) are presented in Table VI-3. As stated previously, the starting seed materials were produced

from plants grown with or without screen isolation cages. Because of a significant interaction detected for the cage effect, data from seed materials produced with cages (single-event materials and associated references) had to be analyzed separately from data from seed materials produced without cages (paired-event materials and associated references).

J101XJ163 had a higher percentage of hard seed with a concurrently lower percentage of normal germinated seed compared to the mean of the reference variety populations at all eight temperature regimes (Table VI-3). All mean values for these two measured characteristics were also outside the observed range of values for the reference variety populations, with the exception of percentage normal germinated seed at the 40°C temperature regime.

Differences in the percentage of abnormal germinated and the percentage of dead seed were detected between J101XJ163 and the mean of the reference variety populations at approximately half of the temperature regimes (Table VI-3). However, the percentages of abnormal germinated and dead seed for J101XJ163 and the reference populations were relatively low (typically < 7%) at all temperature regimes except 40°C. No trends for the percentage of abnormal germinated or dead seed were indicated from the data; therefore, differences detected for these two measured characteristics at each temperature regime were likely because of random experimental error.

The data from seed harvested in 2001 indicate that the J101, J163, and J101XJ163 seed materials had an increased percentage of hard seed, a decreased percentage of normal germinated seed, and similar percentages of abnormal germinated and dead seed when compared to their respective control population and reference variety populations. The increased percentage of hard seed in the test materials warranted further analysis to determine whether the differences detected were because of environmental effects on the percentage of hard seed during seed production, small differences in harvesting or handling activities, or, if they were the result of unexpected changes in dormancy resulting from the genetic modification process. Germination and dormancy data for seed harvested in 2002 and 2003 are presented in Sections C.2 and C.3, respectively.

Table VI-2. Mean Percent Hard, Normal Germinated, Abnormal Germinated, and Dead Seed at Eight Temperature Regimes for Single-Event, Control, and Reference Variety Alfalfa Populations Produced Under Screen Cages in 2001.

Temperature Regime (°C)	Identification Code	Mean[1]			
		Hard (%)	Normal (%)	Abnormal (%)	Dead (%)
5	J101	57.6*†	38.5*†	0.4	3.5*
	J163	70.6*†	25.3*†	1.1*†	3.0*
	Control	43.1	56.5	0.0	0.4
	Reference Mean[2]	37.0	60.2	0.1	2.7
	Reference Range[3]	20-55	42-78	0-1	0-9
10	J101	60.8*†	38.8*†	0.0*†	0.5
	J163	68.3*†	31.0*†	0.0*†	0.8*
	Control	39.4	59.9	0.8	0.0
	Reference Mean[2]	29.9	67.9	1.2	1.0
	Reference Range[3]	17-47	53-80	0-3	0-6
20	J101	55.6*†	40.5*†	0.5*	3.4*†
	J163	64.0*†	30.8*†	0.6*	4.6*†
	Control	36.3	63.1	0.0	0.6
	Reference Mean[2]	29.2	69.1	0.5	1.3
	Reference Range[3]	17-43	54-82	0-3	0-5
30	J101	58.9*†	36.9*†	1.4*	2.9*†
	J163	65.8*†	28.8*†	1.3	4.3*†
	Control	34.8	64.1	0.3	0.9
	Reference Mean[2]	27.0	70.7	0.9	1.4
	Reference Range[3]	15-43	55-80	0-3	0-6

* Indicates a statistically significant difference between a single-event population (J101 or J163) and the control population at $P \leq 0.05$.

† Indicates a statistically significant difference between a single-event population (J101 or J163) and the mean of reference variety populations at $P \leq 0.05$.

[1]Mean percentage hard ungerminated, normal germinated, abnormal germinated, and dead seed. Seed were produced under screened isolation cages and harvested in 2001 as described in the text.

[2]Reference Mean = mean of combined data for all four reference variety populations, FGI-3S11, FGI-4S33, FGI-4S41, and FGI-5S45.

[3]Reference Range = minimum and maximum values of combined data for all four reference variety populations FGI-3S11, FGI-4S33, FGI-4S41, and FGI-5S45.

Table VI-2 (continued). Mean Percent Hard, Normal Germinated, Abnormal Germinated, and Dead Seed at Eight Temperature Regimes for Single-Event, Control, and Reference Variety Alfalfa Populations Produced Under Screen Cages in 2001.

Temperature Regime (° C)[4]	Identification Code	Mean[1]			
		Hard (%)	Normal (%)	Abnormal (%)	Dead (%)
40	J101	56.3*†	29.6*†	4.6*	9.6
	J163	62.6*†	18.6*†	1.3†	17.6*†
	Control	33.1	52.6	1.6	12.6
	Reference Mean[2]	22.1	61.9	3.9	12.0
	Reference Range[3]	12-38	49-76	0-13	3-20
5/20	J101	59.1*†	37.5*†	0.0	3.4†
	J163	68.0*†	27.4*†	1.5*†	3.1†
	Control	38.1	59.9	0.4	1.6
	Reference Mean[2]	30.0	68.2	0.5	1.3
	Reference Range[3]	20-44	54-77	0-5	0-4
10/20	J101	55.8*†	42.6*†	0.6	1.0
	J163	67.3*†	29.3*†	1.0*	2.5†
	Control	38.0	60.8	0.0	1.3
	Reference Mean[2]	29.7	68.9	0.2	1.2
	Reference Range[3]	15-44	56-83	0-1	0-5
20/30	J101	58.4*†	38.9*†	1.1	1.6
	J163	69.6*†	26.5*†	2.1*†	1.8†
	Control	38.3	59.8	0.8	1.3
	Reference Mean[2]	27.5	71.2	0.7	0.6
	Reference Range[3]	18-39	59-81	0-3	0-2

* Indicates a statistically significant difference between a single-event population (J101 or J163) and the control population at $P \leq 0.05$.

† Indicates a statistically significant difference between a single-event population (J101 or J163) and the mean of reference variety populations at $P \leq 0.05$.

[1]Mean percentage hard ungerminated, normal germinated, abnormal germinated, and dead seed. Seed were produced under screened isolation cages and harvested in 2001 as described in the text.

[2]Reference Mean = mean of combined data for all four reference variety populations, FGI-3S11, FGI-4S33, FGI-4S41, and FGI-5S45.

[3]Reference Range = minimum and maximum values of combined data for all four reference variety populations, FGI-3S11, FGI-4S33, FGI-4S41, and FGI-5S45.

[4]Alternating temperature regimes (5/20, 10/20, and 20/30) were maintained for 16 h at the lower temperature and 8 h at the higher temperature.

Table VI-3. Confirmatory Mean Percentage of Hard, Normal Germinated, Abnormal Germinated, and Dead Seed at Eight Temperature Regimes for Paired-Event and Reference-Variety Alfalfa Populations Produced Without Screen Cages in 2001.

Temperature Regime (°C)[4]	Identification Code	Mean[1]			
		Hard (%)	Normal (%)	Abnormal (%)	Dead (%)
5	J101XJ163	56.3*	41.3*	0.0	2.5
	Reference Mean[2]	21.7	75.1	0.4	2.9
	Reference Range[3]	15-32	65-84	0-2	0-8
10	J101XJ163	50.5*	42.6*	2.5	4.4*
	Reference Mean[2]	17.3	79.3	1.4	1.9
	Reference Range[3]	5-28	71-93	0-5	0-8
20	J101XJ163	49.4*	40.9*	4.0*	5.8*
	Reference Mean[2]	15.7	78.8	2.2	3.3
	Reference Range[3]	1-28	66-89	0-9	0-11
30	J101XJ163	49.9*	40.1*	1.6	8.4*
	Reference Mean[2]	14.1	80.5	1.8	3.6
	Reference Range[3]	6-23	69-90	0-6	0-10
40	J101XJ163	42.8*	42.4*	2.1*	12.8*
	Reference Mean[2]	13.5	64.2	14.3	8.0
	Reference Range[3]	5-24	42-87	2-25	1-21
5/20	J101XJ163	57.4*	38.4*	1.9*	2.3
	Reference Mean[2]	17.9	78.4	0.4	3.3
	Reference Range[3]	5-32	63-95	0-3	0-9
10/20	J101XJ163	51.1*	44.1*	1.3	3.4
	Reference Mean[2]	17.0	78.5	1.8	2.7
	Reference Range[3]	8-41	54-89	0-7	0-7
20/30	J101XJ163	49.4*	39.8*	4.4*	6.5*
	Reference Mean[2]	11.5	83.0	2.2	3.4
	Reference Range[3]	6-17	75-94	0-5	0-10

* Indicates a statistically significant difference between the paired-event (J101-J163) and the mean of reference variety populations at $P \leq 0.05$.

[1]Mean percentage hard ungerminated, normal germinated, abnormal germinated, and dead seed. Seed were produced in the field and harvested in 2001 as described in the text.

[2]Reference Mean = mean of combined data for all four reference variety populations, FGI-3S11, FGI-4S33, FGI-4S41, and FGI-5S52.

[3]Reference Range = minimum and maximum values of combined data for all four reference variety populations, FGI-3S11, FGI-4S33, FGI-4S41, and FGI-5S52.

[4]Alternating temperature regimes (5/20°C, 10/20°C, and 20/30°C) were maintained for 16 hours at the lower temperature and 8 h at the higher temperature.

C.2. Seed Dormancy and Germination; Seed Harvested in 2002

Because of the unexpected results observed for seed harvested in 2001, a second experiment was conducted to provide additional germination data on Roundup Ready alfalfa seed populations containing test events J101 and J163 compared to the control alfalfa seed population and four conventional reference variety alfalfa seed populations.

C.2.a. Test, control and reference materials and methods

Starting seed materials were produced by FGI in Canyon County, Idaho, during summer 2002. Seed materials included the two single-event test populations, J101 and J163 (See Figure VI-8, Box 4; 75% trait purity); one control (null) population (Figure VI-8, Box 7); and four reference variety populations, FGI-3S11, FGI-4S33, FGI-4S41, and FGI-5S45. Starting seed material was harvested from the same caged plants used to generate starting seed materials for seed harvested in 2001, and described in Section C.1. The parental plants were maintained during 2001 and 2002 and fresh seed was harvested from them each year. All seed were unscarified. Confirmatory data for the paired-event population were not generated because the plots without cages had been destroyed after the 2001 seed harvest.

Experimental methods and statistical analysis were as described previously for seed harvested in 2001, with the exception of the addition of a germination characteristic category for firm ungerminated seed. Firm ungerminated seeds were those that had imbibed water (swollen) but had not germinated by day 12. The AOSA standards do not specify a firm ungerminated category for alfalfa seed (AOSA, 2002). In the 2001 experiment, all firm ungerminated seed at day 12 were included in the total of normal germinated seed. In the 2002 experiment, all firm ungerminated seed at day 12 were identified separately.

C.2.b. Results and discussion

Roundup Ready alfalfa event J101:

Germination and dormancy results from seed harvested in 2002 showed that J101 had lower percentages of hard seed compared to the control population at all eight temperature regimes with concurrent increases in percentages of normal germinated seed in all but the 40°C temperature regime (Table VI-4). J101 had lower percentages of hard seed compared to the mean of the reference varieties at the 5, 10, 20, 30, 5/20, and 10/20°C temperature regimes, with increases in percentages of normal seed in those same temperature regimes as well as the 20/30° C temperature regime. The percentage of hard seed for J101 was within the observed range of values for the reference variety populations in all but the 20 and 5/20°C temperature regimes. Percentage normal germinated seed for J101 was within the observed range of values for the reference variety populations at the 5, 10, 40 and 10/20°C temperature regimes.

The percentage of abnormal germinated and the percentage of dead seed were low (<5.5%), with the exception of the percentage of dead seed at 40°C. Few differences were identified between J101 and the control population or the mean of the reference varieties. In the 5° and 40°C temperature regimes, firm ungerminated seeds remained on the final day of the experiment. As stated above, the AOSA standards do not specify a firm

ungerminated category for alfalfa seed (AOSA, 2002). This seed characteristic appeared to be the result of temperatures above and below the optimal range for alfalfa seed germination (e.g., 5^{o} and 40^{o}C). In this experiment, all firm ungerminated seed at day 12 were identified separately to more accurately describe the observed temperature effect. The percentage of firm ungerminated seed for J101 did not differ from the control population in either temperature regime. The percentage of firm ungerminated seed was lower for J101 in the 5° C temperature regime when compared to the mean of the reference variety populations.

Across the eight temperature regimes, J101 had a decreased percentage of hard seed, increased percentage of normal germinated seed, and similar percentages of abnormal germinated, dead, and firm ungerminated seed when compared to the control population or to the mean of the reference varieties. A decreased percentage of hard seed for J101 would not contribute to increased pest potential from dormancy compared to the control.

Roundup Ready alfalfa event J163:

J163 had lower percentages of hard seed compared to the control population at all eight temperature regimes, with concurrent increases in the percentages of normal germinated seed in all but the 5°, 40° and 20/30°C temperature regimes (Table VI-4). J163 had lower percentage of hard seed compared to the mean of the reference varieties in all temperature regimes except 20/30°C, with concurrent increases in percentages of normal germinated seed in all temperature regimes except 40°C. Percentages of hard and normal germinated seed for J163 were within the observed range of values for the reference variety populations in all eight temperature regimes.

The percentage of abnormal germinated and the percentage of dead seed were low (< 10%), with the exception of percentage dead seed at 40°C. Few differences were identified between J163 and the control population or the mean of the reference varieties. In the 5° and 40°C temperature regimes, firm ungerminated seed remained on the final day of the experiment. The percentage of firm ungerminated seed for J163 was not different from the control population in either temperature regime. The percentage of firm ungerminated seed was higher for J163 in the 40°C temperature regime when compared to the mean of the reference variety populations.

Across the eight temperature regimes, J163 had a decreased percentage of hard seed, an increased percentage of normal germinated seed, and similar percentages of abnormal germinated and firm ungerminated seed compared to the control population or to the mean of the reference variety populations. Across temperature regimes, a small increase was detected in percentage of dead seed for J163 compared to the control population, although no differences were detected when compared to the mean of the reference variety populations. A decreased percentage of hard seed for J163 would not contribute to increased pest potential from dormancy.

Data from this second germination experiment indicate that the J101 and J163 seed materials had reduced percentage of hard seed, increased percentage of normal germinated seed and similar percentages of abnormal germinated, dead, and firm ungerminated seed when compared to the control population and reference variety populations.

Table VI-4. Mean Percent Hard, Normal, Dead and Firm Ungerminated Seed at Eight Temperatures Regimes for Single-Event, Control, and Reference Variety Alfalfa Populations Harvested in 2002 (Grown Under Screen Cage).

Temperature Regime (°C)	Identification Code	Mean[1]				
		Hard (%)	Normal (%)	Abnormal (%)	Dead (%)	Firm Ungerminated (%)
5	J101	50.5*†	39.8*†	3.4	3.0†	3.4†
	J163	48.6*†	32.6†	5.5†	6.3*	7.0
	Control	60.3	27.3	3.9	3.0	5.5
	Reference Mean[2]	56.9	27.4	3.2	5.8	6.6
	Reference Range[3]	43-72	11-40	0-11	0-14	0-15
10	J101	44.6*†	47.8*†	3.5	4.1†	0.0
	J163	46.0*†	42.5*†	3.8	7.8*	0.0
	Control	58.0	35.5	2.4	4.1	0.0
	Reference Mean[2]	53.2	36.5	2.9	7.4	0.0
	Reference Range[3]	37-66	23-51	0-10	0-16	0.0
20	J101	41.8*†	48.0*†	5.4*	4.9	0.0
	J163	47.3*†	42.0*†	3.3	7.5*	0.0
	Control	59.0	34.6	2.5	3.8	0.0
	Reference Mean[2]	54.7	34.4	4.3	6.6	0.0
	Reference Range[3]	45-65	25-42	0-9	0-16	0.0
30	J101	44.9*†	46.8*†	3.8	4.6†	0.0
	J163	45.4*†	42.8*†	3.9	8.0	0.0
	Control	57.6	33.4	4.0	5.0	0.0
	Reference Mean[2]	53.9	33.5	4.1	8.5	0.0
	Reference Range[3]	41-70	22-46	0-11	0-19	0.0

* Indicates a statistically significant difference between a single-event population (J101 or J163) and the control population at $P \leq 0.05$.

† Indicates a statistically significant difference between a single-event population (J101 or J163) and the mean of reference variety populations at $P \leq 0.05$.

[1]Mean percentage hard ungerminated, normal germinated, abnormal germinated, dead and firm ungerminated seed. Seed were produced under screened isolation cages and harvested in 2002 as described in the text.

[2]Reference Mean = mean of combined data for all four reference variety populations, FGI-3S11, FGI-4S33, FGI-4S41, and FGI-5S45 (n = 32).

[3]Reference Range = minimum and maximum values of combined data for all four reference variety populations, FGI-3S11, FGI-4S33, FGI-4S41, and FGI-5S45 (n = 32).

Table VI-4 (continued). Mean Percent Hard, Normal, Dead and Firm Ungerminated Seed at Eight Temperatures Regimes for Single-Event, Control, and Reference Variety Alfalfa Populations Harvested in 2002 (Grown Under Screen Cage).

Temperature Regime (°C)[4]	Identification Code	Mean[1]				
		Hard (%)	Normal (%)	Abnormal (%)	Dead (%)	Firm Ungerminated (%)
40	J101	47.6*	16.5	4.6	11.9	19.4
	J163	45.8*	12.3	3.9	13.8	24.4†
	Control	56.4	10.6	3.6	8.6	20.6
	Reference Mean[2]	51.6	14.4	4.1	13.6	16.3
	Reference Range[3]	34-65	4-30	0-12	1-36	2-31
5/20	J101	45.8*	46.3*†	2.9	5.1	0.0
	J163	47.9*	42.9*†	2.8	6.5	0.0
	Control	59.0	33.3	3.4	4.4	0.0
	Reference Mean[2]	55.2	35.1	3.3	6.4	0.0
	Reference Range[3]	46-70	26-44	0-10	1-17	0-0
10/20	J101	45.3*	46.4*†	4.9	3.5†	0.0
	J163	44.0*	44.0*†	4.6	7.6*	0.0
	Control	55.8	37.3	2.6	4.4	0.0
	Reference Mean[2]	55.0	35.1	3.4	6.5	0.0
	Reference Range[3]	42-68	24-48	0-8	1-14	0.0
20/30	J101	45.8*	45.4*†	4.4	5.3†	0.0
	J163	45.1*	39.5†	5.5*	9.9*	0.0
	Control	54.4	37.8	2.6	5.1	0.0
	Reference Mean[2]	49.8	35.2	5.6	9.5	0.0
	Reference Range[3]	33-63	28-44	0-18	3-27	0.0

* Indicates a statistically significant difference between a single-event population (J101 or J163) and the control population at $P \leq 0.05$.

† Indicates a statistically significant difference between a single-event population (J101 or J163) and the mean of reference variety populations at $P \leq 0.05$.

[1]Mean percentage hard ungerminated, normal germinated, abnormal germinated, dead and firm ungerminated seed. Seed were harvested in 2002 as described in the text.

[2]Reference Mean = mean of combined data for all four reference variety populations, FGI-3S11, FGI-4S33, FGI-4S41, and FGI-5S45 (n = 32).

[3]Reference Range = minimum and maximum values of combined data for all four reference variety populations, FGI-3S11, FGI-4S33, FGI-4S41, and FGI-5S45 (n = 32).

[4]Alternating temperature regimes (5/20, 10/20, and 20/30) were maintained for 16 h at the lower temperature and 8 h at the higher temperature.

C.2.c. Combined statistical analysis of 2001 and 2002 seed dormancy data

Seed dormancy data derived from the 2001 and 2002 experiments were subjected to a combined statistical analysis. Combined data from the 2001 and 2002 experiments were analyzed using Statistical Analysis Software (SAS) Release 6.12 to compare test, control, and reference materials for cumulative values of the percentage of normal germinated, abnormal germinated, hard, firm ungerminated, and dead seed among the three observations (five, seven, and 12 days after initiation). Within a temperature regime, a p-value less than 0.05 indicated a statistically significant difference at the five percent level of significance between the mean of a test material population and the mean of the control population or the mean of the four reference variety populations. Because of the potential effect of temperature regime on germination characteristics, statistical comparisons were not made across temperature regimes for an event.

C.2.d. Results and discussion

The results from the combined data analysis are presented in Table VI-5. J101 had higher percentages of hard seed compared to the control population at five of the eight temperature regimes (10°, 30°, 40°, 5/20°, and 20/30°C). These differences detected in percentage hard seed were concurrent with lower percentages of normal germinated seed compared to the control. However, mean values for these two measured characteristics were within the observed range of values for the reference variety populations. Slight increases in percentage abnormal germinated seed were detected for J101 compared to the control at the 20°, 40°, and 10/20°C temperature regimes. Slight increases in percentage dead seed were detected for J101 compared to the control at the 5° and 20°C temperature regimes. All firm ungerminated seed data were from the 2002 experiment and this seed germination characteristic was only observed in the 5° and 40°C temperature regimes. No differences were detected in percentage firm ungerminated seed between J101 and the control.

J163 had higher percentages of hard seed and concurrently lower percentages of normal germinated seed compared to the control population at all eight temperature regimes (Table VI-5). However, mean values for these two measured characteristics were within the observed range of values for the reference variety populations. Slight increases in percentage abnormal germinated seed were detected for J163 compared to the control at the 5°, 10/20°, and 20/30°C temperature regimes. Slight increases in percentage dead seed were detected for J163 compared to the control at all eight temperature regimes. All firm ungerminated seed data were from the 2002 experiment and this seed germination characteristic was only observed in the 5° and 40°C temperature regimes. No differences were detected in percentage firm ungerminated seed between J163 and the control.

As noted above, differences in the percentage of abnormal germinated and the percentage of dead seed were detected between one or both of the single-event test populations when compared to the control or mean of the reference variety populations at all eight temperature regimes. However, the percentages of abnormal germinated and dead seed for all test, control, and reference variety populations were relatively low (< 6%) overall, with the exception of percentage dead seed at 40°C. The 40°C temperature was extremely warm

for alfalfa seed germination and therefore it was not unexpected that larger percentages of abnormal germinated and dead seed were observed at that temperature. No trends for percentage abnormal germinated seed were indicated from the data; therefore, differences detected among test materials for this measured characteristics were likely due to random experimental error. A trend toward slightly increased percentage dead seed was observed for J163 compared to the control; however, it is unlikely that this small change is biologically meaningful. Further, increased percentage dead seed for the test material would not contribute to increased weediness potential.

The combined data analysis from seed harvested in 2001 and 2002 indicate that the J101 and J163 seed materials had an increased percentage of hard seed, a decreased percentage of normal germinated seed, and had similar percentages of abnormal germinated and dead seed when compared to their respective control populations (Table VI-5). It is important to consider that the results of this analysis summarized data from seed lots collected from identical plants during two different growing seasons. Combining the data from these two experiments represented an average percentage for each germination characteristic. However, the results of the combined analysis were largely weighted by the magnitude of the differences observed in the 2001 experiment. Opposite trends were observed for percentages of hard and normal germinated seed for both J101 and J163 compared to the control between the 2001 and 2002 experiments (Tables VI-2 and VI-4, respectively). These opposite trends between the two experiments indicated that the increased percentage hard seed observed in the 2001 experiment appears not to be the result of unexpected changes in seed dormancy resulting from the genetic modification process, but rather it was because of environmental effects during seed production or other subtle differences in seed harvesting methods. As discussed in Section C.3, data from seed harvested in 2003 have confirmed this conclusion.

Table VI-5. Mean Percentage Hard, Normal, Abnormal, Dead and Firm Ungerminated Seed at Eight Temperature Regimes for Single-Event, Control, and Reference Variety Alfalfa Populations for Pooled Data Across 2001 and 2002 Dormancy Studies (Screen Cage Only).

Temp. Regime (°C)	Identification Code	Mean[1]				
		Hard (%)	Normal (%)	Abnorm. (%)	Dead (%)	Firm ungerminated (%)
5	J101	54.1	39.1	1.9	3.3*	1.7
	J163	59.6*	28.9*	3.3*	4.6*	3.5
	Control	51.7	41.9	1.9	1.7	2.8
	Reference Mean[2]	47.0	43.8	1.6	4.3	3.3
	Reference Range[3]	20-72	11-78	0-11	0-14	0-15
10	J101	52.7*	43.3*	1.8	2.3	0.0
	J163	57.1*	36.8*	1.9	4.3*	0.0
	Control	48.7	47.7	1.6	2.1	0.0
	Reference Mean[2]	41.7	52.0	2.0	4.3	0.0
	Reference Range[3]	17-66	23-80	0-10	0-16	0-0
20	J101	48.7	44.3*	2.9*	4.1*	0.0
	J163	55.6*	36.4*	1.9	6.1*	0.0
	Control	47.6	48.9	1.3	2.2	0.0
	Reference Mean[2]	42.1	51.5	2.4	4.0	0.0
	Reference Range[3]	17-65	25-82	0-9	0-16	0-0
30	J101	51.9*	41.8*	2.6	3.8	0.0
	J163	55.6*	35.8*	2.6	6.1*	0.0
	Control	46.2	48.8	2.1	2.9	0.0
	Reference Mean[2]	40.7	51.8	2.5	5.0	0.0
	Reference Range[3]	15-70	22-80	0-11	0-19	0-0

* Indicates a statistically significant difference between a single-event population (J101 or J163) and the control population at $P \leq 0.05$.

[1]Mean percentage hard ungerminated, normal germinated, abnormal germinated, dead, and firm ungerminated seed.

[2]Reference Mean = mean of combined data for all four reference variety populations, FGI-3S11, FGI-4S33, FGI-4S41, and FGI-5S45 (n = 64).

[3]Reference Range = minimum and maximum values observed among all four reference variety populations, FGI-3S11, FGI-4S33, FGI-4S41, and FGI-5S45 (n = 64).

[4]Alternating temperature regimes (5/20, 10/20, and 20/30) were maintained for 16 h at the lower temperature and 8 h at the higher temperature.

Table VI-5 (continued). Mean Percentage Hard, Normal, Abnormal, Dead and Firm Ungerminated Seed at Eight Temperature Regimes for Single-Event, Control, and Reference Variety Alfalfa Populations for Pooled Data Across 2001 and 2002 Dormancy Studies (Screen Cage Only).

Temp. Regime (°C)[4]	Identification Code	Mean[1]				
		Hard (%)	Normal (%)	Abnorm. (%)	Dead (%)	Firm ungerminated (%)
40	J101	51.7*	22.6*	4.6*	10.8	10.3
	J163	53.6*	15.2*	2.7	15.5*	13.0
	Control	44.8	31.6	2.6	10.6	10.3
	Reference Mean[2]	37.6	37.0	4.0	12.9	8.6
	Reference Range[3]	12-65	4-76	0-13	1-36	0-31
5/20	J101	52.4*	41.9*	1.4	4.3	0.0
	J163	57.9*	35.1*	2.1	4.8*	0.0
	Control	48.6	46.6	1.9	3.0	0.0
	Reference Mean[2]	43.0	51.2	2.0	3.9	0.0
	Reference Range[3]	20-70	26-77	0-10	0-17	0-0
10/20	J101	50.5	44.5*	2.8*	2.3	0.0
	J163	55.6*	36.6*	2.8*	5.1*	0.0
	Control	46.9	49.0	1.3	2.8	0.0
	Reference Mean[2]	41.7	52.8	1.7	3.7	0.0
	Reference Range[3]	15-68	24-83	0-8	0-14	0-0
20/30	J101	52.1*	42.1*	2.8	3.4	0.0
	J163	57.4*	33.0*	3.8*	5.8*	0.0
	Control	46.3	48.8	1.7	3.2	0.0
	Reference Mean[2]	38.7	53.2	3.1	5.1	0.0
	Reference Range[3]	18-63	28-81	0-18	0-27	0-0

* Indicates a statistically significant difference between a single-event population (J101 or J163) and the control population at $P \leq 0.05$.

[1]Mean percentage hard ungerminated, normal germinated, abnormal germinated, dead, and firm ungerminated seed.

[2]Reference Mean = mean of combined data for all four reference variety populations, FGI-3S11, FGI-4S33, FGI-4S41, and FGI-5S45 (n = 64).

[3]Reference Range = minimum and maximum values observed among all four reference variety populations, FGI-3S11, FGI-4S33, FGI-4S41, and FGI-5S45 (n = 64).

[4]Alternating temperature regimes (5/20, 10/20, and 20/30) were maintained for 16 h at the lower temperature and 8 h at the higher temperature.

C.3. Seed Dormancy and Germination; Commercial Track Seed Lots

Seed germination tests are routinely performed for alfalfa seed lots during the variety breeding process. Because of the high variability in percentage hard seed in alfalfa observed in the previous two studies, seed hardness data from non-scarified commercial paired-event J101XJ163 Roundup Ready alfalfa seed lots was collected. These data were developed to confirm conclusions derived from the previous two studies where single-event seed were compared to the null segregant seed, and to determine whether the trend observed for increased percentage hard seed in the 2001 study was also observed in commercial seed lots.

C.3.a. Test, control, and reference materials

Starting seed materials included ten paired-event test populations (J101XJ163) and ten conventionally bred control populations. A genetically broad-based germplasm population was used as the common parental source population from which both the test and control materials were bred (the source population was an FGI elite fall-dormant, winterhardy, hay-type germplasm). Test materials were the seed of ten Roundup Ready alfalfa populations (Syn 1 generation experimental varieties). A progeny map showing the breeding of the test material is presented in Figure VI-8 (test populations are in Box 11, 95% trait purity). In all test populations the test events J101 and J163 were both present as each was bred according to the FGI Roundup Ready two-event product development plan. Control materials were the seed of ten conventional synthetic breeding alfalfa populations (Syn 1 generation experimental varieties). The control materials shared a common genetic ancestry (pedigree) with the test populations.

Test and control seed from each of the ten test and control populations were produced in 2003 at two similarly managed spatially isolated field sites in Idaho. Test and control seed were produced in two spatially isolated seed production fields (approximately 3 acres each). Field sites were typical for alfalfa seed production. Plants were established using space-planted transplants (18 inches between plants within rows, 28 inches between rows, approximately 2,300 transplants in each of the ten plots per field). The ten individual plots within a field were separated from all others in the field by approximately 25 feet of fallow ground surrounding the plot on all sides.

C.3.b Methods

Seeds were tested using standardized germination assays at a single temperature of 20°C (AOSA 2002). Four replicates of 100 to 104 seed were evaluated in each germination towel for each test and control population. Data were recorded for the number of normal germinated, abnormal germinated, hard (dormant) ungerminated, and dead (degenerated) seed as defined by AOSA standards (AOSA 2002). The percentage of seed observed in each germination characteristic category was calculated based on the number of seeds tested per germination towel.

C.3.c. Statistical comparison of means

The mean values for the measured characteristics were compared for the 20 individual test and control populations using analysis of variance (randomized complete block with four replications and 20 subpopulations). Next, the overall test material mean and overall control material mean were calculated by averaging all subpopulation data for the ten test and ten control subpopulations, respectively (i.e., each overall mean was based on n=40 observations). A second analysis of variance was conducted to compare the overall means for each characteristic. Differences between means were tested using the least significant difference (LSD) at significance level of 0.05 ($p \leq 0.05$). Statistical calculations were made using Statistix for Windows software, version 2.2 (Analytical Software, Tallahassee, Florida, USA).

C.3.d. Results and discussion

Seed germination and dormancy data are summarized in Table VI-6. The overall mean percentage hard seed (dormant seed) for the test and control materials were 30.5 and 30.8 percent, respectively. Overall mean percent normal seed were 68.8 and 68.5 percent for the test and reference seed, respectively. The overall percentages for abnormal and dead seed were identical for the test and references seed (abnormal = 0.3; dead = 0.4). The overall range of values for each of the categories was also highly comparable; the ten populations within each of the test subpopulations expressed variation comparable and similar in magnitude to the variation demonstrated for the control subpopulations. Therefore, in this regard, the test material was also similar to the control material. Statistical analysis of the means showed that there were no statistically significant differences detected between the overall test and overall control material means for percentages of hard seed, normal germinated, abnormal germinated, dead or total viable seed. On the basis of results from this experiment, it is concluded that the germination characteristics of seed produced by Roundup Ready alfalfa are highly comparable to those of seed produced by conventional alfalfa varieties.

Table VI-6. Mean Percentage Hard Ungerminated, Normal Germinated, Abnormal Germinated, and Dead Seed for Ten Test (J101XJ163) and Ten Control Alfalfa Populations.

Test Seed[1]						Control Seed[1]					
Population	Mean[2]					Population	Mean[2]				
	Hard (%)	Normal (%)	Abnormal (%)	Dead (%)	Viable (%)		Hard (%)	Normal (%)	Abnormal (%)	Dead (%)	Viable (%)
RR03-1	36.0	63.0	0.5	0.5	99.5	CC03-11	23.3	75.8	0.5	0.5	99.5
RR03-2	22.8	76.5	0.8	0.0	100.0	CC03-12	30.3	68.8	0.8	0.3	99.8
RR03-3	39.3	60.3	0.0	0.5	99.5	CC03-13	37.8	61.8	0.3	0.3	99.8
RR03-4	29.3	70.0	0.3	0.5	99.5	CC03-14	24.3	75.0	0.0	0.8	99.3
RR03-5	38.5	60.5	0.5	0.5	99.5	CC03-15	19.5	80.3	0.3	0.0	100.0
RR03-6	24.3	75.8	0.0	0.0	100.0	CC03-16	31.5	67.8	0.5	0.3	99.8
RR03-7	25.5	74.0	0.5	0.0	100.0	CC03-17	35.0	64.5	0.3	0.3	99.8
RR03-8	31.5	68.0	0.3	0.3	99.8	CC03-18	41.0	58.0	0.3	0.8	99.3
RR03-9	26.3	73.3	0.0	0.5	99.5	CC03-19	35.0	64.8	0.0	0.3	99.8
RR03-10	32.0	67.0	0.3	0.8	99.3	CC03-20	30.5	68.8	0.5	0.3	99.8
Overall Mean[3]	**30.5**	**68.8**	**0.3**	**0.4**	**99.7**	-	**30.8**	**68.5**	**0.3**	**0.4**	**99.7**
SD	5.9	6.1	0.6	0.7	0.7	-	6.70	6.75	0.62	0.62	0.62
Range[4]	23-39	60 –77	0.0 –0.8	0.0 –0.8	99 –100	-	19 –41	58 –80	0 –0.8	0 –0.8	99 –100
						95% CI[5]	28.66 –32.94	66.37 –70.68	0.01 – 0.05	0.02 – 0.05	99.45 – 99.85

[1]Test and control seeds were produced in Ada and Canyon counties, Idaho, respectively, during 2003 under USDA Notification Number 03-346-15n.
[2]Individual subpopulation (variety lot) means based on 4 replicates of approximately 100 seed each.
[3]Overall test and control material means (in boldface) within a variable type were not statistically significantly different at $P \geq 0.05$ using analysis of variance. Overall test population and control population means were based on the pooled means over the ten populations, two locations in each group (n=40 observations).
[4]Range = minimum and maximum values of combined data for ten individual populations.
[5]95% Confidence Interval for overall control population means.

C.4. Overall Discussion and Conclusion; Seed Dormancy

Seed was harvested from Roundup Ready alfalfa plants in three consecutive growing seasons and the germination and dormancy of these seeds were evaluated. Results from seed harvested in 2001 showed that seed harvested from Roundup Ready alfalfa plants containing events J101 and J163 had a higher percentage of hard seed in comparison to seed produced by control and reference alfalfa populations. Because of the high variability in seed dormancy and germination that has been historically associated with alfalfa seed, additional data were needed to determine whether the increase in dormancy was because of the Roundup Ready trait or other conditions during seed production and harvest. Seed harvested in 2002 from the same alfalfa plants containing event J101 or J163 did not show an increase in percentage hard seed compared to control and reference variety populations. This result was further confirmed when percent hard seed was evaluated in Roundup Ready alfalfa commercial track seed lots harvested in 2003 where no statistically significant differences in percent hard seed was observed. Collectively, the results of these germination experiments confirm that the percentage of hard seed in alfalfa populations is inherently highly variable and sensitive to environmental conditions under which the plants are grown and other conditions associated with the harvest of the seed. The differences observed in the hardness of seed harvested were not reproducible in two subsequent seasons, and therefore are not considered to be due to the Roundup Ready trait.

In considering the seed dormancy results from these three experiments, some discussion of seed hardness in conventional alfalfa is warranted. In natural populations, seed dormancy is an adaptation to extreme climatic conditions. The percentage of hard seed in alfalfa is influenced by edaphic and climatic conditions during and after seed maturation, by genetics and by harvesting techniques. An example of the variation observed in seed hardness in alfalfa because of environmental conditions was described by Bass et al. (1988), where seed harvested from a single variety of alfalfa grown in the same field near Wapato, WA was observed to vary from 24 to 69 percent hard seed over two consecutive growing seasons.

Data from FGI on commercial alfalfa displays considerable variation in seed hardness in lots harvested from production areas throughout North America. Seed dormancy data from 77 FGI commercial seed lots harvested from 1999 to 2001 are presented in Table VI-7. Seed harvested from alfalfa plants grown in the warm production areas of California and Arizona was less hard (<20%) than seed grown in the cooler climates in the Pacific Northwest (20-60%) or in western Canada (50-80%). Overall, seed hardness of the Roundup Ready alfalfa material evaluated in the three experiments described above was well within the range considered normal for mechanically harvested alfalfa seed in the Pacific Northwestern region where it was grown.

On the basis of the understanding of the factors that are important in determining seed hardness, there are several possible explanations for the overall variation in seed hardness and trend towards increased hardness in Roundup Ready alfalfa seed produced in 2001. In 2001, plants were grown under slightly different growing conditions, and harvested seed was processed using different equipment. Soil variations and climatic conditions are known to have a very strong

influence on the percentage of hard seed produced in alfalfa. It is possible that these conflicting study results were because of uncontrolled differences in the separate growing environments (plots) under which the starting seeds were produced. An array of small differences in environmental conditions under which starting material was produced (e.g., soil type, soil alkalinity, soil moisture content, irrigation water and soil salinity, cumulative growing degrees, or relative humidity) may have occurred, impacted seed maturation rate, and thereby affected the percentage of hard seed in each of the study years.

In alfalfa seed, the thickened outer wall of the palisade cells creates a moisture impermeable barrier (Bass et al., 1988). A minute break in this barrier causes the seed to imbibe water and initiate germination. Mechanical harvesting of seed may decrease the hardness of seed, as generally less that 60% of mechanically-harvested seed are hard, whereas hand-harvested seed lots may be 100% hard (Bass et al., 1988). In these experiments, separate seed thrashing equipment was used for Roundup Ready alfalfa seed than for control and reference seed. FGI had specifically purchased new seed thrashing equipment to be used separately from equipment used for thrashing conventional seed. This was done to assure absolute containment of the regulated Roundup Ready alfalfa seed in compliance with USDA regulations. The equipment used was of a design that allowed for more thorough cleaning between lots relative to the older equipment used to thresh the control/reference materials. Therefore, in 2001, the test and control/reference seed lots may have been inadvertently threshed in a relatively more or less abrasive fashion, which could have resulted in the differences in measured hard seed percentage for the seed lots for the Roundup Ready alfalfa lots. Because all Roundup Ready seed were processed using this new equipment, it could account for the differences observed. By the time this equipment was used in 2002 and 2003, it was broken in and would thus not contribute to differences.

In conclusion, the increased percentage of hard seed from alfalfa plants containing events J101 and J163 observed in the first experiment may have been because of environmental and seed harvesting conditions during 2001, and is not likely due to the genetic modification process. The trend towards increased percentage hard seed was not observed in two subsequent experiments or during extensive breeding and field-testing of Roundup Ready alfalfa since 1998. Regardless of differences observed in relative hard seed percentages, no increased pest potential for the J101 and J163 test materials would be expected from this seed dormancy characteristic because high hard seed percentage is very common and historically the norm for traditional alfalfa seed. Even though alfalfa produces seed with highly variable seed hardness, this has not impacted its weediness characteristics because alfalfa is not listed as a weed in North America even though it has been grown for forage since the late 1800s and is widely known to produce typically greater than 60% hard seed in some northern regions. Seed dormancy is one characteristic that may be associated with plants that are weeds (Anderson 1996). However, all plant species that exhibit high seed dormancy are not *a priori* weedy or invasive species. The potential for seed dormancy is but one of several characteristics that may increase the likelihood that a plant may exhibit weedy properties.

Table VI-7. Percentage Hard Seed and Total Germination of 77 Commercially Grown Alfalfa Reference Variety Seed Lots Produced in the U.S. or Canada During 1999-2001.

	State or Province of Seed Production[1]										
	ID	MT	NV	OR	UT	WA	WY	AB	TX	AZ	CA
Hard seed (%)											
Mean	**25.8**	**29.0**	**24.7**	**19.0**	**21.6**	**14.3**	**34.7**	**66.2**	**13.6**	**5.0**	**4.6**
± SD	10.9	15.9	12.8	5.4	12.0	12.1	13.6	8.5	10.2	na	3.2
Minimum	12.0	13.0	6.0	12.0	11.0	5.0	14.0	56.0	2.0	5.0	0.0
Maximum	43.0	55.0	48.0	24.0	46.0	38.0	55.0	76.0	28.0	5.0	9.0
Total germination (%)											
Mean	**93.1**	**95.0**	**94.7**	**93.0**	**95.6**	**94.0**	**95.1**	**97.0**	**92.6**	**86.0**	**93.4**
± SD	1.5	2.6	1.6	2.7	1.7	1.7	2.0	1.6	1.5	na	2.1
Minimum	90.0	91.0	92.0	91.0	93.0	92.0	92.0	95.0	91.0	86.0	90.0
Maximum	96.0	99.0	97.0	98.0	98.0	97.0	99.0	99.0	94.0	86.0	97.0
No. of samples	11	7	10	6	7	7	11	5	5	1	7

[1] State and province abbreviations, respectively: Idaho, Montana, Nevada, Oregon, Utah, Washington, Wyoming, Alberta (Canada), Texas, Arizona and California

D. Phenotypic Comparative Studies

Comparative information on plant growth and development characteristics is useful in assessing whether the genetic modification process changed the plant's weed potential. The purpose of these trials was to assess whether the genetic modification altered the plant growth or development characteristics of Roundup Ready alfalfa events J101 or J163 compared to the control, such that weed potential was altered.

Phenotypic data were collected from three separate field trials. For the first study, detailed quantitative information was collected from alfalfa plants established in seeded plots during the fall of 2001. Field trials were conducted at four locations. Information collected from this study includes data taken from three years, the establishment year and first and second forage production seasons. Plants were not treated with any Roundup agricultural herbicide. For the second study, detailed phenotypic information was obtained from plants treated with Roundup agricultural herbicide. Information from this six-location field trial also spans two growing

seasons. Trials were established in 2001 from transplants that were reared in a greenhouse. The third study includes phenotypic information collected over four growing seasons, including observations taken during the productive phase and into stand decline. Plots were not treated with Roundup agricultural herbicides. The field trial was established at a single location in 2000 from transplants that were treated with Roundup agricultural herbicide and reared in a greenhouse. These three studies are discussed in detail below.

D.1. 2001-2003 Phenotypic Studies; Phenotypic Study Number One

During Fall 2001 and continuing through Fall 2003, Monsanto conducted field tests of J101, J163, and J101XJ163 confirmatory alfalfa populations at four locations to evaluate phenotypic equivalency. Field trials were established at contract research facilities in California (CA); Iowa (IA); Illinois (IL); and Wisconsin (WI). These four locations provided a range of environmental and agronomic conditions representative of major alfalfa growing regions where commercial production of Roundup Ready alfalfa is expected. Agronomic practices used to prepare and maintain each field site were characteristic of each respective region. Field trials were conducted under USDA Notification Numbers 01-164-03n and 01-163-02n.

D.1.a. Materials and methods

Starting seed materials used for this study were the same as described in Section C.1. At each field site, study materials were established in a randomized complete block design with four replications (except for the Wisconsin site, which contained only three replications). Each plot consisted of one row, planted on 30-inch centers, 15 ft in length (16 ft at the Wisconsin site). Alleys between replications (perpendicular to row direction) were 3-10 ft wide. To minimize any edge effect, a one-row border of a conventional alfalfa variety was planted on both sides of the field plot area parallel to row direction. These border rows were the same length and row spacing as the single-row plots.

Starting seed materials for this study were scarified and inoculated with 170 g *Sinorhizobium meliloti* inoculum per 25 kg of seed. At each site, the plots were maintained according to the standard alfalfa production practices in the respective regions.

It was acknowledged that null individuals within test material populations were susceptible to Roundup herbicide and thus could have been selectively removed with a postemergence glyphosate application. However, to ensure that all plots (test, control, and reference) were managed uniformly, glyphosate was not applied to any of the plots. Consequently, for each comparative assessment between test and control populations, the values of each measured characteristic were inherently more similar than if each population were 100% trait-positive or trait-negative because null individuals occurred in both the test and control populations. While this resulted in a slightly reduced ability to detect statistically significant differences, Roundup Ready alfalfa populations sold commercially will be composed of segregating individuals.

Data for phenotypic characteristics were collected in the establishment year (2001) and in the first and second forage production years (2002-2003).

Descriptions for each phenotypic characteristic evaluated in this study are as follows:

Seedling emergence. Two to three weeks after seeding in the fall of 2001, emerged seedlings were counted in three randomly selected 1-ft segments of each plot.

Seedling vigor. Three to four weeks after seeding in the fall of 2001, seedling vigor for each plot was rated on a 1-10 scale, where 1 = dead and 10 = excellent vigor and growth of entire plot.

Spring vigor. After regrowth was initiated in the spring of 2002 and 2003 and plants reached a height of 4-6 inches, spring vigor for each plot was rated on a 1-10 scale, where 1 = dead and 10 = excellent vigor and growth of entire plot.

Spring stand. After regrowth was initiated in the spring of 2002 and 2003 and plants reached a height of 4-6 inches, spring recovery for each plot was visually assessed as the percentage of plot surviving winter, where 100% stand equaled a uniform, full stand (without gaps) along the entire single-row plot.

Forage yield. At each field site, plots were harvested for forage throughout the 2002 and 2003 forage production seasons timed according to local agronomic practices (approximately 4-6 week intervals). At each cutting, all plants were cut 2-3 inches above the soil surface and laid on the plot, and total fresh weight (lbs. per plot) was recorded within 48 hours. Forage harvested from the study area was devitalized either by burning, composting, or tilling it into a designated disposal site. During both the 2002 and 2003 forage production seasons, six cuttings were taken at CA, five at IL, and four at IA and WI.

Crop growth stage at cutting. At the second and third cuttings at each field site in 2002 and 2003 (only the third cutting at the WI site in 2003), crop growth stage was determined using the Mean Stage by Count (MSC) method (Kalu and Fick, 1981). Approximately 35-45 random alfalfa plant stems from each plot were collected after clipping but before devitalization. The stems were separated by growth stage as defined in Table VI-8. The MSC was calculated for each plot using the following formula: MSC = [Σ(stage number * number of stems in stage)]/total number of stems evaluated.

Regrowth after cutting. Ten to 15 days after each cutting in 2002 and 2003, regrowth was rated for each plot on a 1-10 scale, where 1 = dead and 10 = excellent vigor and growth of entire plot. The number of regrowth ratings at each field site in 2002 and 2003 corresponded to the number of cuttings.

Fall plant height. Three to four weeks after the final cutting in 2002 and 2003, plant height (inches) was taken for three randomly selected plants per plot. Heights were measured from the soil surface to the top of the plant. Fall plant height was observed for an evaluation of fall dormancy, where taller plant height may indicate lower fall dormancy. Plant heights were not measured in the fall of the planting year (2001).

Fall growth habit. Three to four weeks after the final cutting in 2002 and 2003, plant populations per plot were categorically scored for fall growth habit as upright, prostrate, or a mixture of both types.

Biotic and abiotic stressors. Each field site was periodically rated for the presence of various biotic (e.g., insects, diseases, weeds) and abiotic stressors. Test, control and reference alfalfa population responses to the stressors were also specifically noted.

D.1.b. Statistical analysis

Variance analysis for each field site (CA, IA, IL, and WI) was conducted according to a randomized complete block design with four replications (the WI site had three replications) using Statistical Analysis Software (SAS Version 8.2, SAS Institute, Inc. 1999-2001). Characteristics analyzed were seedling emergence, seedling vigor, spring vigor, spring stand, forage yield for individual cuttings, total yearly (cumulative) forage yield, crop growth stage (at cuttings 2 and 3), regrowth ratings after each cutting, fall plant height, and fall growth habit. Because the starting seed for this study were produced from plants grown with and without cages, data from the single-event control and reference populations (established from starting seed produced with cages as described in Section C.1) were analyzed separately from data from the paired-event control and references populations (established from starting seed produced without cages), due to a "cage effect" observed for some of the phenotypic parameters evaluated.

The statistical comparisons were made within each field site and year for all measured characteristics and across all field sites and years for all measured characteristics, except individual forage yield cuttings and regrowth after cuttings. It was not statistically appropriate to pool data for these two characteristics across sites and years because of the different number of forage cuttings between sites. The level of statistical significance was predetermined to be at the 5% level of significance ($p \leq 0.05$).

D.1.c. Results and discussion

Results will be discussed separately for the J101, J163 and J101XJ163 Roundup Ready alfalfa populations. J101 compared to the control and reference populations will be addressed first, followed by J163 and then J101XJ163. Dates for data collection at each location are presented in Table VI-9.

Phenotypic comparison of J101 to the control and references; 2002. In the 2002 by-site analysis for J101 compared to the control, a total of 70 comparisons were made. The phenotypic characteristics of J101, control and reference alfalfa populations are presented in Table VI-10. There were no differences detected between J101 and the control at any of the sites for seedling emergence, spring vigor, spring stand, crop growth stage (cutting 3), forage yield (cuttings 1, 2, 4, 5, 6, and total), regrowth after cutting (cuttings 2, 3, 4, 5, and 6), and fall plant height. No differences were detected between J101 and the control for any of the measured characteristics at the CA and IA sites. At the IL and WI locations, a total of four differences were detected between J101 and the control for four of the measured characteristics. At the IL site, the crop growth stage rating (cutting 2) was higher for J101 compared to the control (4.3 vs. 3.7). At the WI site, seedling vigor was lower (6.7 vs. 8.0); forage yield (cutting 3) was higher (6.5 vs. 5.7 t/a); and regrowth after cutting (cutting 1) was lower (9.0 vs. 10.0) for J101 compared to the control. The differences described above were randomly distributed among the measured characteristics and field sites, and the mean value

of each characteristic was within the range of values observed for the reference populations at each field site (with the exception of reduced seedling vigor at the WI site).

Trends toward decreased seedling vigor and regrowth after cutting would not contribute to increased plant weed potential, while increased crop growth stage rating (i.e., less time to reach reproductive maturity) and forage yield could indicate an increase in weed potential if the trait were transferred to feral alfalfa or a wild relative. The increased crop growth stage rating (cutting 2) was only observed at one site and the magnitude of this difference is likely not biologically meaningful with respect to weed potential as crop growth stage ratings 3 and 4 are both related to flower bud stages (Table VI-8). Increased forage yield was only observed for one cutting interval at the WI site and was not observed for total yearly forage yield or for any cuttings at the other three sites.

Phenotypic comparison of J101 to the control and references; 2003. In the 2003 by-site analysis, a total of 59 comparisons were made between J101 and the control. The phenotypic characteristics of J101, control and reference alfalfa populations are presented in Table VI-10. There were no differences detected between J101 and the control at any of the sites for spring vigor, spring stand, forage yield (cuttings 1, 2, 3, 5, and 6), regrowth after cutting (cuttings 1, 2, 3, 4, 5, and 6), and fall plant height. No differences were detected between J101 and the control for any of the measured characteristics at the IA site. At the CA, IL and WI locations, a total of five differences were detected between J101 and the control for four of the measured characteristics. At the CA and WI sites, the crop growth stage rating (cutting 3) was higher for J101 compared to the control (5.4 vs. 4.9 and 4.4 vs. 4.1, respectively). At the IL site, crop growth stage rating (cutting 2) was higher for J101 compared to the control (3.8 vs. 3.4). Also at the WI site, forage yields (cutting 4 and total) were higher for J101 compare to the control (5.1 vs. 4.7 t/a and 16.8 vs. 15.2 t/a, respectively). The differences described above were randomly distributed among the measured characteristics and field sites, and the mean value of each characteristic was within the range of values observed for the reference populations at each field site.

Trends toward an increased crop growth stage rating and forage yield could indicate an increase in weed potential if the trait were transferred to feral alfalfa or a wild relative. Increased crop growth stage rating (cutting 2) was only observed at one site and the magnitude of this difference is likely not biologically meaningful with respect to weed potential as both J101 and the control were within the early flower bud stage (Table VI-8). Increased forage yield was only observed for one cutting interval at the WI site and was not observed for any cuttings at the other three sites. Increased total yearly forage yield was only observed at the WI site.

Phenotypic comparison of J101 to the control and references; Combined analysis. No consistent trends for changes were detected when data were pooled across sites and years (Table VI-11). Where significant differences were noted in phenotypic parameters in the single year analyses, [e.g., 2002: changes in seedling vigor, crop growth stage (cutting 2), or total yearly forage; 2003: crop growth stage (cutting 2) or total yearly forage yield] differences were no longer significant. One difference was detected between J101 and the

control. Crop growth stage rating (cutting 3) was higher for J101 compared to the control (4.3 vs. 4.1) (Table VI-11). In the by-site by-year analysis, the crop growth stage rating (cutting 3) was higher for J101 compared to the control at two sites in 2003 and was not different at the other two sites in 2003 or at any of the four sites in 2002. The differences detected at the two sites in 2003 contributed largely to the difference detected across sites and years. The magnitude of the difference detected in the pooled analysis is likely not biologically meaningful with respect to weed potential because crop growth stage was assessed qualitatively on a 0-6 categorical scale and both J101 and the control were within the late flower bud stage (Table VI-8). Thus, the difference is likely an artifact of the assessment method.

Conclusions for phenotypic evaluation of J101. The results from the by-site by-year and across-site across-year analyses for J101 support the conclusion that the plant phenotype was not unintentionally altered by genetic modification. Each of the differences detected is unlikely to be of biological significance with respect to its impact on weed potential of the crop itself or transference of the trait to feral alfalfa. The low-magnitude of differences detected in the measured characteristics, which resulted in values within the range common for alfalfa, are unlikely to affect the weed potential of alfalfa.

Phenotypic comparison of J163 to control and references. In the 2002 by-site analysis, a total of 70 comparisons were made between J163 and the control. The phenotypic characteristics of J163, control and reference alfalfa populations are presented in Table VI-10. There were no differences detected between J163 and the control at any of the sites for seedling emergence, seedling vigor, spring vigor, spring stand, forage yield (cuttings 1, 2, 5, 6, and total), regrowth after cutting (cuttings 2, 3, 4, and 6), and fall plant height. No differences were detected between J163 and the control for any of the measured characteristic at the CA and IA sites. At the IL and WI locations, a total of six differences were detected between J163 and the control for six of the measured characteristics. At the IL site, both crop growth stage ratings were higher for J163 compared to the control [cutting 2 (4.4 vs. 3.7); cutting 3 (4.8 vs. 4.1)]. Also at the IL site, forage yield (cutting 4) was lower (1.1 vs. 1.3 t/a) and regrowth after cutting (cutting 5) was lower (8.3 vs. 9.0) for J163 compared to the control. At the WI site, forage yield (cutting 3) was higher (6.6 vs. 5.7 t/a) and regrowth after cutting (cutting 1) was lower (9.0 vs. 10.0) for J163 compared to the control. These differences were randomly distributed among the measured characteristics and field sites, and the mean value of each characteristic was within the range of values observed for the reference populations at each field site.

Trends toward decreased forage yield and regrowth after cutting would not contribute to increased weed potential, while increased crop growth stage rating and forage yield could indicate an increase in weed potential if the trait were transferred to feral alfalfa. Increased crop growth stage ratings (cutting 2 and 3) were only observed at one site and the magnitude of these differences is likely not biologically meaningful with respect to weed potential as growth stage ratings 3 and 4 are both related to flower bud stages (Table VI-8). Increased

forage yield was only observed for one cutting interval at the WI site and was not observed for total yearly forage yield or for any cuttings at the other three sites.

Phenotypic comparison of J163 to the control and references; 2003. In the 2003 by-site analysis, a total of 59 comparisons were made between J163 and the control. The phenotypic characteristics of J101, control and reference alfalfa populations are presentd in Table VI-10. There were no differences detected between J163 and the control at any of the sites for spring vigor, spring stand, forage yield (cuttings 1, 2, 4, and 5), regrowth after cutting (cuttings 3, 4, 5, and 6), and fall plant height. A total of eight differences were detected between J163 and the control for seven of the measured characteristics. At the CA site, forage yield (cutting 6) was lower for J163 compared to the control (4.7 vs. 5.8 t/a). The crop growth stage rating (cutting 2) was higher for J163 compared to the control at the IA (4.0 vs. 3.5) and IL sites (3.8 vs. 3.4). At the WI site, crop growth stage rating (cutting 3) was higher for J163 compared to the control (4.6 vs. 4.1). Also at the IL site, regrowth after cutting (cutting 2) was lower for J163 compared to the control (8.0 vs. 8.5). At the WI site, forage yields (cutting 3 and total) were higher (5.4 vs. 4.6 t/a and 16.7 vs. 15.2 t/a, respectively) and regrowth after cutting (cutting 1) was higher (8.3 vs. 7.3) for J163 compared to the control. These differences were randomly distributed among the measured characteristics and field sites, and the mean value of each characteristic was within the range of values observed for the reference populations at each field site [with the exception of forage yield (cutting 6) at the CA site].

A trend toward decreased forage yield and regrowth after cutting would not contribute to increased weed potential, while increased crop growth stage rating, forage yield, and regrowth after cutting could indicate an increase in weed potential if the trait were transferred to feral alfalfa. Increased crop growth stage rating was observed at two sites for cutting 2 and at one site for cutting 3. The magnitude of these differences is likely not biologically meaningful with respect to weed potential as growth stage ratings 3 and 4 are both related to flower bud stages (Table VI-8). Increased forage yield was observed for only one cutting interval at the WI site and was not observed for any cuttings at the other three sites. Increased total yearly forage yield was only observed at the WI site.

Phenotypic comparison of J163 to the control and references; Combined analysis. No trends were observed for changes in any measured characteristic when the data were pooled across sites and years (Table VI-11). Thus, the detected differences in the by-site by-year analysis of crop growth stage and yield are unlikely to be biologically meaningful in terms of plant weed potential of the crop itself or if the trait were transferred to feral alfalfa.

Regrowth after cutting data were not pooled across sites and years; however, increased regrowth after cutting was observed for only one cutting interval at the WI site in 2003 and was not observed for any cuttings at the other three sites in 2003 or for any cuttings at any of the four sites in 2002. Thus, the detected differences in the by-site by-year analysis of crop growth stage, yield, and regrowth after cutting are unlikely to be biologically meaningful in terms of plant weed potential of the crop itself or if the trait were transferred to feral alfalfa.

Conclusions for phenotypic evaluation of J163. The results from the by-site by-year and across-site across-year analyses for J163 support the conclusion that the plant phenotype was

not unintentionally altered by the trait or genetic modification process. Each of the differences detected in the by-site by-year analysis is unlikely to be of biological significance with respect to its impact on weed potential of the crop itself or transference of the trait to feral alfalfa. The low-magnitude differences detected in the measured characteristics, which resulted in values within the range common for alfalfa, are unlikely to affect the weed potential of alfalfa.

Phenotypic Comparison of J101X J163 to References; *Cage effect.* As mentioned previously, a significant cage condition by alfalfa population interaction was detected for the three reference populations (FGI-3S11, FGI-4S33, and FGI-4S41) for which starting seed were produced from plants grown with and without cages. Because the starting seed for the control population was produced under cage and the starting seed for the paired-event population was produced without a cage, the paired-event population could not be compared to the control population.

Phenotypic comparison of J101XJ163 to references; 2002. In the 2002 by-site analysis, a total of 70 comparisons were made between J101XJ163 and the mean of the reference populations. The phenotypic characteristics of J101XJ163 and reference alfalfa populations are presented in Table VI-12. There were no differences detected between J101XJ163 and the mean of the references at any of the sites for seedling emergence, seedling vigor, spring stand, crop growth stage (cutting 3), forage yield (cuttings 1, 3, 4, 5, 6, and total), regrowth after cutting (cuttings 1, 2, 3, 5, and 6), and fall plant height. No differences were detected between J101XJ163 and the mean of the references for any of the measured characteristics at the IA site. At the CA, IL and WI sites, a total of four differences were detected between J101XJ163 and the mean of the references for four of the measured characteristics. At the CA site, the crop growth stage rating (cutting 2) and forage yield (cutting 2) were higher ([4.6 vs. 3.9] and [2.0 vs. 1.5 t/a], respectively). At the IL site, regrowth after cutting (cutting 4) was lower for J101XJ163 compared to the mean of the references (7.8 vs. 8.2). At the WI site, spring vigor was higher for J101XJ163 compared to the mean of the references (8.7 vs. 8.0). The differences were randomly distributed among the measured characteristics and field sites, and the mean value of each characteristic was within the range of values observed for the reference populations at each field site.

A trend toward decreased regrowth after cutting would not contribute to increased weed potential, while increased crop growth stage rating, forage yield, and spring vigor could indicate an increase in weed potential if the trait were transferred to feral alfalfa. An increased crop growth stage rating (cutting 2) was only observed at one site and the magnitude of this difference is likely not biologically meaningful with respect to weed potential as growth stage ratings 3 and 4 are both related to flower bud stages (Table VI-8). Increased forage yield was only observed for one cutting interval at the CA site and was not observed for total yearly forage yield or for any cuttings at the other three sites. Increased spring vigor was only observed at one site.

Phenotypic comparison of J101XJ163 to references; 2003. In the 2003 by-site analysis, a total of 59 comparisons were made between J101XJ163 and the mean of the reference

populations. The phenotypic characteristics of J101XJ163 and reference alfalfa populations are presented in Table VI-12. There were no differences detected between J101XJ163 and the mean of the references at any of the sites for spring vigor, spring stand, crop growth stage (cuttings 2 and 3), forage yield (cuttings 3, 5, 6, and total), regrowth after cutting (cuttings 2, 3, and 6), and fall plant height. Furthermore, no differences were detected between J101XJ163 and the mean of the references for any of the measured characteristics at the IA site. At the CA, IL and WI locations, a total of six differences were detected between J101XJ163 and the mean of the references among six of the measured characteristics. Forage yield was lower for J101XJ163 compared to the mean of the references for cutting 2 (8.1 vs. 9.9 t/a) at the CA site and for cutting 1 (5.9 vs. 6.7 t/a) and cutting 4 (1.7 vs. 1.9 t/a) at the IL site. Also at the CA site, regrowth after cutting (cutting 5) was higher for J101XJ163 compared to the mean of the references (8.8 vs. 7.9). Regrowth after cutting was lower for J101XJ163 compared to the mean of the references for cutting 1 (7.0 vs. 7.9) and cutting 4 (7.0 vs. 8.0) at the WI site. The differences were randomly distributed among the measured characteristics and field sites, and the mean value of each characteristic was within the range of values observed for the reference populations at each field site.

A trend toward decreased forage yield and regrowth after cutting would not contribute to increased weed potential, while increased regrowth after cutting could indicate an increase in weed potential if the trait were transferred to feral alfalfa. Regrowth after cutting data were not pooled across sites and years (see Statistical Analysis section); however, increased regrowth after cutting was observed for only one cutting interval at the CA site in 2003 and was not observed for any cuttings at the other three sites in 2003 or for any cuttings at any of the four sites in 2002. Thus, it is unlikely that the detected differences in the by-site by-year analysis of regrowth after cutting are biologically meaningful in terms of plant weed potential of the crop itself or if the trait were transferred to feral alfalfa.

Phenotypic comparison of J101XJ163 to references; Combined analysis. No consistent trends for increased crop growth stage rating, forage yield, or spring vigor were detected when data were pooled across sites and years (Table VI-13). Thus, the differences detected in the by-site by-year analysis of these characteristics are unlikely to be biologically meaningful in terms of plant weed potential of the crop itself or if the trait were transferred to feral alfalfa. In the pooled analysis, two differences were detected between J101XJ163 and the mean of the references (Table VI-13). First, forage yield (total) was lower for J101XJ163 compared to the mean of the references (16.9 vs. 18.0 t/a); however, an interaction detected between alfalfa population, site, and year suggests a consistent trend across sites and years did not occur. In the by-site by-year analysis, there were no differences between J101XJ163 and the mean of the references for forage yield (total) at any of the sites in 2002 or 2003. Forage yield from three cutting intervals was lower for J101XJ163 compared to the mean of the references among two sites in 2003 and may have contributed to this difference. A trend toward decreased total yearly forage yield would not contribute to increased weed potential. Second, fall plant height was lower for J101XJ163 compared to the mean of the references (8.1 vs 8.6 in.). The magnitude of a 0.5-inch difference in plant height is likely not biologically meaningful with respect to weed potential. In the by-site by-year analysis, there were no

differences between J101XJ163 and the mean of the references for fall plant height at any of the sites in 2002 or 2003. Furthermore, a trend toward slightly decreased fall plant height would not contribute to increased weed potential.

Conclusions for phenotypic evaluation of J101XJ163. The results from the by-site by-year and across-site across-year analyses for J101XJ163 support the conclusion that the plant phenotype was not unintentionally altered by genetic modification. It is unlikely that each of the differences detected is of biological significance with respect to its impact on weed potential of the crop itself or transference of the trait to feral alfalfa. The low-magnitude differences detected in the measured characteristics, which resulted in values within the range common for alfalfa, are unlikely to affect the weed potential of alfalfa.

D.1.d. Fall growth habit

Plant breeders use fall growth habit measurements as one estimate of fall dormancy. Fall dormancy is a natural physiological response mechanism to terminate fall growth of alfalfa plants growing in more northern latitudes. For example, upright fall plant growth is associated with lower fall dormancy. In this study, after the final cutting of the 2002 and 2003 seasons, plant populations per plot were categorically scored for fall growth habit as upright, prostrate, or a mixture of both types. Data are presented as the percentage of replications of each test, control, or reference population across sites and years categorized as each fall growth habit type (Tables VI-14 and VI-15). Each test, control, and reference population demonstrated a range in growth habit among sites and years and populations tended to be more upright or a mixture of both types. No differences were detected between J101 or J163 and the control for any of the fall growth habit types. Furthermore, this was also confirmed by the paired-event population J101XJ163, where no differences were detected for any of the fall growth habit types when compared to the mean of the references. These results support the conclusion that the plant phenotype of J101, J163, and J101XJ163 was not unintentionally altered by genetic modification.

D.1.e. Biotic and abiotic stressor observations

Each field site was periodically rated for various biotic (e.g., insects, diseases, weeds) and abiotic stressors. Specific stressors were not necessarily common to all field sites and, therefore, varied between sites. Approximately every four to six weeks throughout each growing season, the principal investigator qualitatively assessed the level at which commonly occurring stressors were present in the study area. The response of the plants to the stressor was evaluated with specific emphasis on whether there were observable differences between test, control and reference populations. Observations were made a total of 17, 15, 16, and 14 times throughout the 2001, 2002, and 2003 growing seasons at the CA, IA, IL, and WI sites, respectively (Table VI-16). Out of a total of 118 abiotic, 185 disease, 214 insect, 112 weed, and 34 other stressor observations, no differences among test, control, and reference plots were noted. These data could not be subjected to statistical analyses; however, these results support the conclusion that the ecological interactions of J101 or J163 were not unintentionally altered by genetic modification. This was confirmed by the observations of the paired-event population J101XJ163.

D.1.f. Conclusions; 2001-2003 phenotypic study

This study assessed whether the presence of the *cp4 epsps* coding sequence or the presence of the CP4 EPSPS protein altered the phenotypic characteristics and/or ecological interactions of J101 or J163 Roundup Ready alfalfa compared to the control. The characteristics measured provide crop biology data useful in assessing equivalence and familiarity in the context of ecological risk assessment. All phenotypic characteristic data for which there were no detected differences between J101 or J163 alfalfa and the control support a conclusion of phenotypic equivalence as it relates to familiarity and a lack of increased weed potential. Detected differences were evaluated alone, in consideration of other observed differences, and for trends across sites and years. Each detected difference was considered with respect to its impact on increased weed potential of the crop itself and if the trait were transferred to feral alfalfa. The results from this study indicate that the Roundup Ready trait in J101, J163, or J101XJ163 alfalfa is unlikely to confer a selective advantage to alfalfa that would result in increased weed potential and confirm the conclusion that the Roundup Ready trait has no effect on altering the measured phenotypic characteristics of alfalfa.

Table VI-8. Definition of Morphological Stages of Development of Individual Alfalfa Stems used to Calculate Crop Growth Stage.

Growth Stage Number	Growth Stage Name	Growth Stage Definition[1]
0	Early vegetative	Stem length ≤ 6 inches; no buds, flowers or seed pods
1	Mid vegetative	Stem length 6 to 12 inches; no buds, flowers or seed pods
2	Late vegetative	Stem length ≥ 12 inches; no buds, flowers or seed pods
3	Early flower bud	1 to 2 nodes with flower buds; no flowers or seed pods
4	Late flower bud	≥ 3 nodes with flower buds; no flowers or seed pods
5	Early flower	One node with one open flower (standard open); no seed pods
6	Late flower	≥ 2 nodes with open flowers; no seed pods

[1] Crop growth stage data were collected at the second and third cuttings at each field site. Crop growth stage was determined using the Mean Stage by Count (MSC) method (Kalu and Fick 1981). Approximately 35-45 random stems from each plot were collected after clipping and separated by growth stage. The MSC was calculated for each plot using the following formula: MSC = [Σ(stage number * number of stems in stage)]/total number of stems evaluated.

Table VI-9. Dates of Data Collection Events During 2001-2003 at CA, IA, IL, and WI.

	Observation Date							
	CA		IA		IL		WI	
Characteristic	2002	2003	2002	2003	2002	2003	2002	2003
Emergence/ vigor								
Seedling emergence[1]	11/09/2001	N/A	09/24/2001	N/A	09/18/2001	N/A	09/06/2001	N/A
Seedling vigor[1]	11/09/2001	N/A	09/24/2001	N/A	09/24/2001	N/A	09/21/2001	N/A
Spring vigor	03/07/2002	4/21/2003	04/22/2002	04/18/2003	04/15/2002	04/11/2003	04/29/2002	04/28/2003
Spring stand	03/07/2002	4/21/2003	04/22/2002	04/18/2003	04/15/2002	04/11/2003	04/29/2002	04/28/2003
Crop growth stage								
Cutting #2	05/29-30/2002	5/28/2003	06/19/2002	07/01/2003	06/14/2002	06/19/2003	07/10/2002	N/A
Cutting #3	07/11-12/2002	6/30/2003	07/17/2002	07/30/2003	07/16/2002	07/18/2003	08/20/2002	07/30/2003
Forage Yield								
Cutting #1	04/30/2002	4/21-22/2003	05/17/2002	06/02/2003	05/15/2002	05/12/2003	06/10/2002	05/30/2003
Cutting #2	05/29/2002	5/28/2003	06/19/2002	07/01/2003	06/13/2002	06/18/2003	07/10/2002	06/30/2003
Cutting #3	07/11/2002	6/30/2003	07/17/2002	07/30/2003	07/15/2002	07/17/2003	08/20/2002	08/01/2003
Cutting #4	08/15/2002	8/06/2003	08/31/2002	09/13/2003	08/12/2002	08/18/2003	09/16/2002	09/02/2003
Cutting #5	09/16/2002	9/10/2003	N/A	N/A	09/09/2002	09/18/2003	N/A	N/A
Cutting #6	10/31/2002	10/17/2003	N/A	N/A	N/A	N/A	N/A	N/A
Regrowth after cutting								
Cutting #1	05/08/2002	5/02/2003	06/01/2002	06/16/2003	05/30/2002	05/24/2003	06/20/2002	06/11/2003
Cutting #2	06/18/2002	6/06/2003	07/01/2002	07/10/2003	06/26/2002	07/01/2003	07/22/2002	07/13/2003
Cutting #3	07/23-24/2002	7/14/2003	08/01/2002	08/11/2003	07/29/2002	07/31/2003	08/30/2002	08/11/2003
Cutting #4	08/29/2002	8/18/2003	09/10/2002	09/24/2003	08/26/2002	08/29/2003	09/30/2002	09/11/2003
Cutting #5	09/30/2002	9/22/2003	N/A	N/A	09/24/2002	10/02/2003	N/A	N/A
Cutting #6	11/12/2002	10/31/2003	N/A	N/A	N/A	N/A	N/A	N/A
Fall plant height/ growth habit	11/25/2002	11/14/2003	09/28/2002	10/07/2003	10/01/2002	10/10/2003	10/22/2002	09/22/2003

[1] Seedling emergence and seedling vigor data were only collected in 2001 (the year the plots were seeded).
N/A = not applicable.
Field trials conducted under USDA notification numbers:01-163-02n and 01-164-03n.

Table VI-10. The Phenotypic Characteristics of J101 and J163 Compared to the Control During 2001-2003.

Phenotypic Characteristics for California, 2001-2003; USDA Number 01-163-02n								
	Alfalfa Population							
	J101		J163		Control		Ref. Min. – Max.[1]	
Characteristic	2002	2003	2002	2003	2002	2003	2002	2003
Emergence and vigor								
Seedling emergence[2]	6.3	N/A	6.0	N/A	6.9	N/A	3.7-8.0	N/A
Seedling vigor[3, 4]	8.0	N/A	8.3	N/A	8.3	N/A	7.0-9.0	N/A
Spring vigor[4]	7.0	8.5	7.0	8.5	6.5	8.8	5.0-10.0	7.0-10.0
Spring stand (%)	87.5	92.5	87.5	92.5	80.0	97.5	70.0-90.0	70.0-100.0
Crop growth stage[5]								
Cutting #2	4.1	3.8	4.0	3.6	3.9	4.1	2.7-4.9	3.6-4.8
Cutting #3	5.0	5.4*	4.4	4.9	4.7	4.9	4.1-5.2	4.7-5.6
Forage Yield (t/a)[6]								
Cutting #1	2.5	7.3	2.3	7.8	2.1	7.3	1.1-6.0	5.5-11.3
Cutting #2	1.8	8.7	1.5	8.9	1.5	10.5	1.1-2.6	7.0-12.5
Cutting #3	2.1	8.1	1.9	7.6	1.9	9.4	1.1-3.2	4.9-11.4
Cutting #4	2.0	4.6	2.1	4.3	2.5	4.4	1.6-3.4	3.3-6.0
Cutting #5	2.8	4.8	2.5	4.3	2.8	5.3	2.0-3.9	3.4-6.6
Cutting #6	3.9	5.0	3.5	4.7*	4.2	5.8	1.7-5.1	5.2-7.7
Total	15.1	38.5	13.7	37.5	14.9	42.6	9.4-23.1	30.8-54.5
Regrowth after cutting[4]								
Cutting #1	7.5	8.5	7.8	8.3	7.5	8.0	6.0-9.0	7.0-9.0
Cutting #2	7.8	8.8	7.8	8.3	7.5	8.3	6.0-9.0	7.0-9.0
Cutting #3	7.8	8.3	7.3	8.0	8.3	8.5	7.0-9.0	8.0-9.0
Cutting #4	8.0	7.5	8.0	7.8	8.0	7.5	7.0-9.0	6.0-9.0
Cutting #5	7.8	8.3	8.0	8.0	8.5	8.3	7.0-9.0	7.0-9.0
Cutting #6	8.3	8.5	8.0	8.3	8.3	8.0	6.0-9.0	7.0-9.0
Fall plant height[7]	3.6	10.4	3.2	9.3	4.1	10.4	2.9-6.8	8.4-12.6

***** Indicates a significant difference between a single-event population (J101 or J163) and the control population at $p \leq 0.05$.

N/A = not applicable.

[1] Reference Min. – Max. = minimum and maximum observed values from among the four reference populations from which starting seed were produced *with* screen cages (n = 16).

[2] Number of emerged seedling counted in three randomly selected 1-ft segments of each plot. Seedling emergence data were only collected in 2001 (the year the plots were seeded).

[3] Seedling vigor data were only collected in 2001.

[4] Rated for each plot on a 1-10 scale, where 1 = dead and 10 = excellent vigor and growth of entire plot.

[5] Crop growth stage calculated using the Mean Stage by Count method (Table VI-8).

[6] Forage yield measured as fresh weight and reported in tons/acre.

[7] Mean height in inches of three randomly selected plants per plot measured from the soil surface to the top of the plant.

Table VI-10 (continued). The Phenotypic Characteristics of J101 and J163 Compared to the Control During 2001-2003.

Phenotypic Characteristics for Iowa, 2001-2003; USDA Number 01-163-02n								
	Alfalfa Population							
	J101		J163		Control		Ref. Min. – Max.[1]	
Characteristic	2002	2003	2002	2003	2002	2003	2002	2003
Emergence and vigor								
Seedling emergence[2]	10.9	N/A	9.8	N/A	7.5	N/A	1.7-13.7	N/A
Seedling vigor[3, 4]	7.5	N/A	7.5	N/A	7.5	N/A	6.0-9.0	N/A
Spring vigor[4]	8.3	9.0	7.8	9.3	7.3	9.5	4.0-10.0	7.0-10.0
Spring stand (%)	92.5	87.5	85.0	87.5	85.0	92.5	40.0-100.0	60.0-100.0
Crop growth stage[5]								
Cutting #2	3.7	3.8	3.6	4.0*	3.4	3.5	2.4-4.2	3.3-4.4
Cutting #3	2.9	3.4	2.6	3.5	2.8	3.1	1.1-3.5	3.1-3.8
Forage Yield (t/a)[6]								
Cutting #1	6.4	7.0	5.2	7.1	5.1	7.2	0.9-8.1	5.0-8.8
Cutting #2	3.4	2.2	2.8	2.6	2.6	2.5	1.2-4.4	1.6-3.3
Cutting #3	1.2	1.6	1.2	1.6	1.0	1.7	0.3-2.3	1.1-2.9
Cutting #4	2.0	2.3	1.8	2.3	2.0	2.3	0.9-2.8	1.7-3.6
Total	13.0	13.1	11.0	13.5	10.8	13.7	3.8-15.8	11.3-16.3
Regrowth after cutting[4]								
Cutting #1	9.0	7.8	6.8	7.5	7.0	7.8	3.0-10.0	6.0-9.0
Cutting #2	8.3	8.8	8.0	8.5	7.3	8.5	5.0-9.0	7.0-10.0
Cutting #3	8.0	8.3	7.8	8.3	7.0	8.0	5.0-9.0	5.0-10.0
Cutting #4	8.8	7.8	8.0	7.5	7.5	7.0	4.0-10.0	6.0-9.0
Fall plant height (in.)[7]	10.3	5.8	9.3	5.3	9.0	5.5	7.7-13.7	3.7-8.3

***** Indicates a significant difference between a single-event population (J101 or J163) and the control population at $p \leq 0.05$.
N/A = not applicable.
[1]Reference Min. – Max. = minimum and maximum observed values from among the four reference populations from which starting seed were produced *with* screen cages (n = 16).
[2] Number of emerged seedling counted in three randomly selected 1-ft segments of each plot. Seedling emergence data were only collected in 2001 (the year the plots were seeded).
[3] Seedling vigor data were only collected in 2001.
[4] Rated for each plot on a 1-10 scale, where 1 = dead and 10 = excellent vigor and growth of entire plot.
[5] Crop growth stage calculated using the Mean Stage by Count method (Table VI-8).
[6] Forage yield measured as fresh weight and reported in tons/acre.
[7] Mean of three randomly selected plants per plot measured from the soil surface to the top of the plant.

Table VI-10 (continued). The Phenotypic Characteristics of J101 and J163 Compared to the Control During 2001-2003.

Phenotypic Characteristics for Illinois, 2001-2003; USDA Number 01-163-02n								
	Alfalfa Population							
	J101		J163		Control		Reference Min. – Max.[1]	
Characteristic	2002	2003	2002	2003	2002	2003	2002	2003
Emergence and vigor								
Seedling emergence[2]	12.7	N/A	14.8	N/A	10.1	N/A	6.7-15.0	N/A
Seedling vigor[3, 4]	6.8	N/A	6.5	N/A	4.8	N/A	4.0-8.0	N/A
Spring vigor[4]	7.0	9.0	8.3	9.0	7.3	8.8	6.0-9.0	8.0-9.0
Spring stand (%)	82.5	90.0	85.0	90.0	90.0	90.0	70.0-90.0	80.0-90.0
Crop growth stage[5]								
Cutting #2	4.3*	3.8*	4.4*	3.8*	3.7	3.4	3.7-5.0	3.3-4.3
Cutting #3	4.2	4.9	4.8*	4.9	4.1	4.9	4.0-5.3	3.9-5.5
Forage Yield (t/a)[6]								
Cutting #1	1.3	7.3	1.2	6.6	1.3	6.9	0.4-1.8	5.3-7.9
Cutting #2	1.6	5.8	1.5	5.6	1.4	5.5	0.8-2.0	3.7-6.7
Cutting #3	0.8	3.0	0.7	2.6	0.7	2.9	0.4-1.1	1.9-3.3
Cutting #4	1.2	1.9	1.1*	1.7	1.3	1.8	0.8-1.5	1.2-2.2
Cutting #5	1.3	2.4	1.1	2.4	1.3	2.6	1.0-1.6	1.8-2.9
Total	6.1	20.3	5.6	18.7	6.0	19.7	3.6-7.5	13.9-22.4
Regrowth after cutting[4]								
Cutting #1	7.3	8.0	7.5	8.0	7.3	8.0	6.0-8.0	8.0-8.0
Cutting #2	7.0	8.5	7.3	8.0*	7.3	8.5	6.0-8.0	8.0-9.0
Cutting #3	7.8	7.8	7.8	7.5	8.0	8.0	8.0-8.0	7.0-8.0
Cutting #4	7.8	7.5	7.8	7.3	7.5	7.8	7.0-9.0	7.0-8.0
Cutting #5	9.0	8.0	8.3*	7.8	9.0	8.0	8.0-9.0	8.0-8.0
Fall plant height (in.)[7]	10.6	6.3	9.8	5.9	10.3	6.3	9.3-12.0	5.3-8.0

* Indicates a significant difference between a single-event population (J101 or J163) and the control population at $p \leq 0.05$.
N/A = not applicable.
[1] Reference Min. – Max. = minimum and maximum observed values from among the four reference populations from which starting seed were produced *with* screen cages (n = 15).
[2] Number of emerged seedling counted in three randomly selected 1-ft segments of each plot. Seedling emergence data were only collected in 2001 (the year the plots were seeded).
[3] Seedling vigor data were only collected in 2001.
[4] Rated for each plot on a 1-10 scale, where 1 = dead and 10 = excellent vigor and growth of entire plot.
[5] Crop growth stage calculated using the Mean Stage by Count method (Table VI-8).
[6] Forage yield measured as fresh weight and reported in tons/acre.
[7] Mean of three randomly selected plants per plot measured from the soil surface to the top of the plant.

Table VI-10 (continued). The Phenotypic Characteristics of J101 and J163 Compared to the Control During 2001-2003.

Phenotypic Characteristics for Wisconsin, 2001-2003; USDA Number 01-164-03n								
	Alfalfa Population							
	J101		J163		Control		Reference Min. – Max.[1]	
Characteristic	2002	2003	2002	2003	2002	2003	2002	2003
Emergence and vigor								
Seedling emergence[2]	25.8	N/A	26.2	N/A	29.4	N/A	19.7-30.7	N/A
Seedling vigor[3, 4]	6.7*	N/A	7.0	N/A	8.0	N/A	7.0-8.0	N/A
Spring vigor[4]	8.3	9.3	8.7	9.3	9.0	9.0	8.0-9.0	9.0-10.0
Spring stand (%)	96.7	96.7	96.7	96.7	100.0	100.0	90.0-100.0	90.0-100.0
Crop growth stage[5]								
Cutting #2	3.9	N/A	3.9	N/A	3.9	N/A	3.8-3.9	N/A
Cutting #3	4.1	4.4*	4.1	4.6*	4.1	4.1	4.0-4.3	4.1-4.7
Forage Yield (t/a)[6]								
Cutting #1	5.9	6.5	5.7	6.4	5.8	6.0	5.3-6.9	5.5-6.4
Cutting #2	5.1	N/A	4.5	N/A	4.4	N/A	4.3-5.6	N/A
Cutting #3	6.5*	5.1	6.6*	5.4*	5.7	4.6	5.8-7.0	4.5-5.7
Cutting #4	5.5	5.1*	6.0	4.9	5.8	4.7	5.4-6.2	4.8-5.6
Total	21.2	16.8*	22.7	16.7*	21.7	15.2	21.5-24.8	15.1-17.6
Regrowth after cutting[4]								
Cutting #1	9.0*	8.0	9.0*	8.3*	10.0	7.3	8.0-10.0	7.0-9.0
Cutting #2	9.7	N/A	9.3	N/A	9.3	N/A	8.0-10.0	N/A
Cutting #3	9.0	8.7	8.7	8.3	9.7	8.0	8.0-10.0	7.0-9.0
Cutting #4	8.7	8.0	9.0	7.3	9.3	8.0	8.0-10.0	7.0-9.0
Fall plant height (in.)[7]	4.4	12.6	5.1	13.8	5.0	12.3	4.5-6.8	11.7-15.7

* Indicates a significant difference between a single-event population (J101 or J163) and the control population at $p \leq 0.05$.
N/A = not applicable.
[1] Reference Min. – Max. = minimum and maximum observed values from among the four reference populations from which starting seed were produced *with* screen cages (n = 12).
[2] Number of emerged seedling counted in three randomly selected 1-ft segments of each plot. Seedling emergence data were only collected in 2001 (the year the plots were seeded).
[3] Seedling vigor data were only collected in 2001.
[4] Rated for each plot on a 1-10 scale, where 1 = dead and 10 = excellent vigor and growth of entire plot.
[5] Crop growth stage calculated using the Mean Stage by Count method (Table VI-8).
[6] Forage yield measured as fresh weight and reported in tons/acre.
[7] Mean of three randomly selected plants per plot measured from the soil surface to the top of the plant.

Table VI-11. The Phenotypic Characteristics of J101 and J163 Compared to the Control Pooled Across Sites and Years.

Phenotypic Characteristics for CA, IA, IL and WI, 2001-2003; USDA Numbers 01-163-02n and 01-164-03n				
	Alfalfa Population			
Characteristic	J101	J163	Control	Reference Min. – Max.[1]
Emergence and vigor				
Seedling emergence[2]	13.1	13.4	12.4	1.7-30.7
Seedling vigor[3, 4]	7.3	7.3	7.1	4.0-9.0
Spring vigor[4]	8.3	8.4	8.2	4.0-10.0
Spring stand (%)	90.3	89.7	91.3	40.0-100.0
Crop growth stage[5]				
Cutting #2	3.9	3.9	3.7	2.4-5.0
Cutting #3	4.3*	4.2	4.1	1.1-5.6
Total Forage Yield (t/a)[6]	17.9	17.3	18.0	3.6-54.5
Fall plant height (in.)[7]	8.0	7.6	7.8	2.9-15.7

***** Indicates a significant difference between a single-event population (J101 or J163) and the control population at $p \leq 0.05$.

[1] Reference Min. – Max. = minimum and maximum observed values from among the four reference populations from which starting seed were produced *with* screen cages (n = 118).

[2] Number of emerged seedling counted in three randomly selected 1-ft segments of each plot. Seedling emergence data were only collected in 2001 (the year the plots were seeded) and, therefore, are only pooled across sites.

[3] Seedling vigor data were only collected in 2001 and, therefore, are only pooled across sites.

[4] Rated for each plot on a 1-10 scale, where 1 = dead and 10 = excellent vigor and growth of entire plot.

[5] Crop growth stage calculated using the Mean Stage by Count method (Table VI-8). Crop growth stage (cutting 2) does not include data from the WI site from 2003.

[6] Forage yield measured as fresh weight and reported in tons/acre.

[7] Mean of three randomly selected plants per plot measured from the soil surface to the top of the plant.

Table VI-12. The Phenotypic Characteristics of J101XJ163 Compared to the Mean of the References During 2001-2003.

Phenotypic Characteristics for California, 2001-2003; USDA Number 01-163-02n						
	Alfalfa Population					
	J101XJ163		Reference[1]		Reference Min. – Max.[2]	
Characteristic	2002	2003	2002	2003	2002	2003
Emergence and vigor						
Seedling emergence[3]	5.9	N/A	6.0	N/A	5.0-8.7	N/A
Seedling vigor[4, 5]	8.0	N/A	8.0	N/A	7.0-9.0	N/A
Spring vigor[5]	7.5	8.3	7.2	8.6	5.0-9.0	8.0-9.0
Spring stand (%)	85.0	97.5	83.1	89.7	70.0-90.0	70.0-100.0
Crop growth stage[6]						
Cutting #2	4.6*	3.9	3.9	3.9	3.1-4.8	3.2-4.6
Cutting #3	4.7	5.3	4.8	5.2	4.3-5.0	4.5-5.5
Forage Yield (t/a)[7]						
Cutting #1	3.2	7.3	2.7	8.2	1.2-5.3	5.5-10.7
Cutting #2	2.0*	8.1*	1.5	9.9	0.8-2.8	7.0-12.9
Cutting #3	2.3	6.6	1.9	8.4	1.1-3.5	4.4-12.3
Cutting #4	2.6	3.7	2.5	4.2	1.3-4.1	1.9-5.7
Cutting #5	2.6	4.1	3.0	4.5	1.9-4.3	2.5-6.0
Cutting #6	3.3	4.8	3.8	5.5	2.1-5.8	2.9-7.6
Total	15.9	34.6	15.4	40.7	9.0-24.9	24.2-50.9
Regrowth after cutting[5]						
Cutting #1	7.8	8.0	7.8	8.1	6.0-9.0	6.0-9.0
Cutting #2	8.0	8.3	8.1	8.3	6.0-9.0	7.0-9.0
Cutting #3	8.3	8.5	8.1	8.2	7.0-9.0	7.0-9.0
Cutting #4	8.0	8.0	8.0	7.4	7.0-9.0	6.0-9.0
Cutting #5	8.0	8.8*	8.2	7.9	7.0-9.0	7.0-9.0
Cutting #6	7.5	8.3	8.0	8.1	7.0-9.0	7.0-9.0
Fall plant height (in.)[8]	4.0	10.4	4.5	11.0	2.8-6.9	7.5-18.5

* Indicates a significant difference between the paired-event population (J101XJ163) and the mean of the reference populations at $p \leq 0.05$.

N/A = not applicable.

[1] Reference = mean of combined data from among the four reference populations from which starting seed were produced *without* screen cages.

[2] Reference Min. – Max. = minimum and maximum observed values from among the four reference populations (n = 16).

[3] Number of emerged seedling counted in three randomly selected 1-ft segments of each plot. Seedling emergence data were only collected in 2001 (the year the plots were seeded).

[4] Seedling vigor data were only collected in 2001.

[5] Rated for each plot on a 1-10 scale, where 1 = dead and 10 = excellent vigor and growth of entire plot.

[6] Crop growth stage calculated using the Mean Stage by Count method (Table VI-8).

[7] Forage yield measured as fresh weight and reported in tons/acre.

[8] Mean of three randomly selected plants per plot measured from the soil surface to the top of the plant.

Table VI-12 (continued). The Phenotypic Characteristics of J101XJ163 Compared to the Mean of the References During 2001-2003.

Phenotypic Characteristics for Iowa, 2001-2003; USDA Number 01-163-02n						
	Alfalfa Population					
	J101XJ163		Reference[1]		Reference Min. – Max.[2]	
Characteristic	2002	2003	2002	2003	2002	2003
Emergence and vigor						
Seedling emergence[3]	6.8	N/A	8.1	N/A	1.7-15.0	N/A
Seedling vigor[4, 5]	6.8	N/A	7.8	N/A	6.0-9.0	N/A
Spring vigor[5]	7.0	8.8	7.6	9.1	5.0-10.0	5.0-10.0
Spring stand (%)	82.5	90.0	85.0	88.8	40.0-100.0	60.0-100.0
Crop growth stage[6]						
Cutting #2	3.7	3.5	3.8	3.6	3.2-4.3	3.0-4.2
Cutting #3	2.6	3.5	2.8	3.5	1.9-3.7	3.1-3.9
Forage Yield (t/a)[7]						
Cutting #1	4.5	6.8	5.3	7.1	0.9-8.1	5.5-8.6
Cutting #2	2.5	2.4	2.8	2.3	1.2-4.1	0.9-3.2
Cutting #3	1.2	1.7	1.2	1.6	0.3-2.0	0.9-2.7
Cutting #4	2.2	2.5	2.0	2.5	0.8-2.8	2.0-3.3
Total	10.4	13.4	11.3	13.5	4.5-15.9	10.3-15.5
Regrowth after cutting[5]						
Cutting #1	6.5	8.0	7.5	7.6	3.0-10.0	6.0-9.0
Cutting #2	7.5	8.8	7.2	8.9	4.0-9.0	8.0-10.0
Cutting #3	7.0	8.5	7.1	7.9	4.0-9.0	6.0-10.0
Cutting #4	7.5	7.3	7.5	7.4	4.0-10.0	6.0-9.0
Fall plant height (in.)[8]	9.5	5.8	10.3	6.1	8.0-12.3	3.7-8.3

***** Indicates a significant difference between the paired-event population (J101XJ163) and the mean of the reference populations at $p \leq 0.05$ (none detected).
N/A = not applicable.
[1] Reference = mean of combined data from among the four reference populations from which starting seed were produced *without* screen cages.
[2] Reference Min. – Max. = minimum and maximum observed values from among the four reference populations (n = 16).
[3] Number of emerged seedling counted in three randomly selected 1-ft segments of each plot. Seedling emergence data were only collected in 2001 (the year the plots were seeded).
[4] Seedling vigor data were only collected in 2001.
[5] Rated for each plot on a 1-10 scale, where 1 = dead and 10 = excellent vigor and growth of entire plot.
[6] Crop growth stage calculated using the Mean Stage by Count method (Table VI-8).
[7] Forage yield measured as fresh weight and reported in tons/acre.
[8] Mean of three randomly selected plants per plot measured from the soil surface to the top of the plant.

Table VI-12 (continued). The Phenotypic Characteristics of J101XJ163 Compared to the Mean of the References During 2001-2003.

Phenotypic Characteristics for Illinois, 2001-2003; USDA Number 01-163-02n						
	Alfalfa Population					
	J101XJ163		Reference[1]		Reference Min. – Max.[2]	
Characteristic	2002	2003	2002	2003	2002	2003
Emergence and vigor						
Seedling emergence[3]	13.3	N/A	13.5	N/A	8.7-21.3	N/A
Seedling vigor[4, 5]	5.8	N/A	6.1	N/A	4.0-9.0	N/A
Spring vigor[5]	7.5	8.5	7.3	9.0	5.0-9.0	9.0-9.0
Spring stand (%)	85.0	87.5	84.0	90.0	80.0-90.0	90.0-90.0
Crop growth stage[6]						
Cutting #2	4.2	3.7	4.3	3.7	3.8-5.5	3.3-4.2
Cutting #3	4.6	5.3	4.4	5.1	3.6-5.0	4.1-5.7
Forage Yield (t/a)[7]						
Cutting #1	1.2	5.9*	1.3	6.7	0.7-2.1	5.6-8.0
Cutting #2	1.5	5.7	1.5	5.3	0.7-2.0	3.6-6.7
Cutting #3	0.8	2.7	0.8	2.7	0.4-1.1	1.6-3.9
Cutting #4	1.2	1.7*	1.3	1.9	0.7-1.6	1.2-2.3
Cutting #5	1.2	2.2	1.3	2.5	0.9-1.5	1.7-2.8
Total	5.9	18.2	6.1	19.1	4.4-8.0	14.1-22.9
Regrowth after cutting[5]						
Cutting #1	7.5	8.0	7.3	7.9	5.0-9.0	7.0-8.0
Cutting #2	7.5	8.3	7.4	8.5	6.0-8.0	8.0-9.0
Cutting #3	8.0	8.0	8.1	8.1	7.0-9.0	8.0-9.0
Cutting #4	7.8*	7.8	8.2	7.9	7.0-9.0	7.0-9.0
Cutting #5	9.0	8.0	8.9	8.0	8.0-9.0	8.0-8.0
Fall plant height (in.)[8]	10.7	6.7	10.8	7.1	9.0-12.3	5.7-7.7

* Indicates a significant difference between the paired-event population (J101XJ163) and the mean of the reference populations at $p \leq 0.05$.
N/A = not applicable.
[1] Reference = mean of combined data from among the four reference populations from which starting seed were produced *without* screen cages.
[2] Reference Min. – Max. = minimum and maximum observed values from among the four reference populations (n = 15).
[3] Number of emerged seedling counted in three randomly selected 1-ft segments of each plot. Seedling emergence data were only collected in 2001 (the year the plots were seeded).
[4] Seedling vigor data were only collected in 2001.
[5] Rated for each plot on a 1-10 scale, where 1 = dead and 10 = excellent vigor and growth of entire plot.
[6] Crop growth stage calculated using the Mean Stage by Count method (Table VI-8).
[7] Forage yield measured as fresh weight and reported in tons/acre.
[8] Mean of three randomly selected plants per plot measured from the soil surface to the top of the plant.

Table VI-12 (continued). The Phenotypic Characteristics of J101 × J163 Compared to the Mean of the References During 2001-2003 at WI.

Phenotypic Characteristics for Wisconsin, 2001-2003; USDA Number 01-164-03n						
	Alfalfa Population					
	J101XJ163		Reference[1]		Reference Min. – Max.[2]	
Characteristic	2002	2003	2002	2003	2002	2003
Emergence and vigor						
Seedling emergence[3]	27.9	N/A	29.1	N/A	19.7-40.3	N/A
Seedling vigor[4, 5]	7.0	N/A	7.3	N/A	7.0-8.0	N/A
Spring vigor[5]	8.7*	9.3	8.0	9.1	7.0-9.0	8.0-10.0
Spring stand (%)	96.7	100.0	95.0	93.3	90.0-100.0	80.0-100.0
Crop growth stage[6]						
Cutting #2	3.9	N/A	3.9	N/A	3.8-4.0	N/A
Cutting #3	4.1	4.1	4.1	4.3	4.1-4.2	4.0-4.7
Forage Yield (t/a)[7]						
Cutting #1	5.8	6.2	5.7	5.8	5.1-6.9	5.0-6.4
Cutting #2	4.5	N/A	4.4	N/A	3.7-5.9	N/A
Cutting #3	6.1	4.9	6.2	5.2	5.5-7.1	4.7-6.0
Cutting #4	5.5	4.7	5.7	5.1	5.0-6.3	4.3-5.9
Total	21.9	15.8	22.0	16.1	19.3-24.8	14.4-18.4
Regrowth after cutting[5]						
Cutting #1	8.0	7.0*	8.8	7.9	8.0-10.0	7.0-9.0
Cutting #2	8.3	N/A	9.3	N/A	7.0-10.0	N/A
Cutting #3	9.3	7.3	9.1	8.3	8.0-10.0	8.0-10.0
Cutting #4	8.7	7.0*	9.3	8.0	8.0-10.0	7.0-9.0
Fall plant height (in.)[8]	5.3	12.6	5.4	13.8	4.5-6.7	12.3-15.0

* Indicates a significant difference between the paired-event population (J101XJ163) and the mean of the reference populations at $p \leq 0.05$.
N/A = not applicable.
[1] Reference = mean of combined data from among the four reference populations from which starting seed were produced *without* screen cages.
[2] Reference Min. – Max. = minimum and maximum observed values from among the four reference populations (n = 12).
[3] Number of emerged seedling counted in three randomly selected 1-ft segments of each plot. Seedling emergence data were only collected in 2001 (the year the plots were seeded).
[4] Seedling vigor data were only collected in 2001.
[5] Rated for each plot on a 1-10 scale, where 1 = dead and 10 = excellent vigor and growth of entire plot.
[6] Crop growth stage calculated using the Mean Stage by Count method (Table VI-8).
[7] Forage yield measured as fresh weight and reported in tons/acre.
[8] Mean of three randomly selected plants per plot measured from the soil surface to the top of the plant.

Table VI-13. The Phenotypic Characteristics of J101XJ163 Compared to the Mean of the References Pooled Across Sites and Years.

Phenotypic Characteristics for CA, IA, IL and WI, 2001-2003; USDA Numbers 01-163-02n and 01-164-03n			
	Alfalfa Population		
Characteristic	J101XJ163	Reference[1]	Reference Min. – Max.[2]
Emergence and vigor			
Seedling emergence[3]	12.5	13.2	1.7-40.3
Seedling vigor[4, 5]	6.9	7.3	4.0-9.0
Spring vigor[5]	8.1	8.2	5.0-10.0
Spring stand (%)	90.0	88.3	40.0-100.0
Crop growth stage[6]			
Cutting #2	4.0	3.9	3.0-5.5
Cutting #3	4.3	4.3	1.9-5.7
Total Forage Yield (t/a)[7]	16.9*†	18.0	4.4-50.9
Fall plant height (in.)[8]	8.1*	8.6	2.8-18.5

***** Indicates a significant difference between the paired-event population (J101XJ163) and the mean of the reference populations at $p \leq 0.05$.

† Indicates a significant interaction between alfalfa population, site, and year at $p \leq 0.05$.

[1] Reference = mean of combined data from among the four reference populations from which starting seed were produced *without* screen cages.

[2] Reference Min. – Max. = minimum and maximum observed values from among the four reference populations (n = 118).

[3] Number of emerged seedling counted in three randomly selected 1-ft segments of each plot. Seedling emergence data were only collected in 2001 (the year the plots were seeded) and, therefore, are only pooled across sites.

[4] Seedling vigor data were only collected in 2001 and, therefore, are only pooled across sites.

[5] Rated for each plot on a 1-10 scale, where 1 = dead and 10 = excellent vigor and growth of entire plot.

[6] Crop growth stage calculated using the Mean Stage by Count method (Table VI-8). Crop growth stage (cutting 2) does not include data from the WI site from 2003.

[7] Forage yield measured as fresh weight and reported in tons/acre.

[8] Mean of three randomly selected plants per plot measured from the soil surface to the top of the plant

Table VI-14. Fall Growth Habit of J101 and J163 Compared to the Control Pooled Across Sites and Years.

Alfalfa Population								
J101			J163			Control		
U[1]	P[1]	M[1]	U	P	M	U	P	M
63.3%	6.7%	30.0%	50.0%	3.3%	46.7%	60.0%	0.0%	40.0%

* Indicates a significant difference between a single-event population (J101 or J163) and the control population at $p \leq 0.05$ (none detected).

[1] Plant populations per plot were categorically scored for fall growth habit as upright (U), prostrate (P), or a mixture (M) of both types. Data are presented as the percentage of replications of each test or control population across sites and years categorized as each fall growth habit type.

Field trials conducted under USDA notification numbers: 01-163-02n and 01-164-03n.

Table VI-15. Fall Growth Habit of J101XJ163 Compared to the Mean of the References Pooled Across Sites and Years.

Alfalfa Population					
J101XJ163			Reference[2]		
U[1]	P[1]	M[1]	U	P	M
56.7%	3.3%	40.0%	58.5%	4.2%	37.3%

* Indicates a significant difference between the paired-event population (J101XJ163) and the mean of the reference populations at $p \leq 0.05$ (none detected).

[1] Plant populations per plot were categorically scored for fall growth habit as upright (U), prostrate (P), or a mixture (M) of both types. Data are presented as the percentage of replications of each test or reference population across sites and years categorized as each fall growth habit type.

[2] Reference = mean of combined data from among the four reference populations from which starting seed were produced *without* screen cages.

Field trials conducted under USDA notification numbers: 01-163-02n and 01-164-03n.

Table VI-16. Observations for Biotic and Abiotic Stressors Among Alfalfa Test, Control, and Reference Plots.

Biotic and Abiotic Stressors for California, 2001-2003; USDA Number 01-163-02n								
	Observation dates[1, 2]							
Stressor	**04/04/02**	**05/01/02**	**05/29/02**	**06/26/02**	**07/24/02**	**08/21/02**	**09/19/02**	**10/21/02**
Disease								
Bacterial wilt	none	none	none	none	none	none	none	none
Insect								
Alfalfa caterpillar	none	none	none	none	none	none	none	none
Alfalfa weevil	none	none	none	none	none	none	none	none
Beet armyworm	none	none	none	none	none	none	none	none
Cucumber beetle	none	none	none	none	none	none	none	none
Lygus	none	none	none	**slight**	none	none	none	none
Pea aphid	none	none	none	none	none	none	none	none
Weed								
Common groundsel	**slight**	none	none	none	none	none	none	none
Fiddleneck	**moderate**	**moderate**	none	none	none	none	none	none
London rocket	none	**moderate**	none	none	none	none	none	none
Purslane	none	none	none	none	none	none	none	none
Shepherd's purse	none	**moderate**	none	none	none	none	none	none
Other								
Gopher	none	**slight**	none	none	none	**moderate**	**moderate**	**slight**
Rabbit	none	none	none	none	none	none	none	none

[1]If a stressor was noted, it was rated as slight, moderate, or severe.
[2]No differences between test, control or reference alfalfa population responses were noted to any stressor on any observation date.

Table VI-16 (Continued). Observations for Biotic and Abiotic Stressors Among Alfalfa Test, Control, and Reference Plots.

Biotic and Abiotic Stressors for California, 2001-2003; USDA Number 01-163-02n									
	Observation dates[1, 2]								
Stressor	**02/27/03**	**03/21/03**	**04/22/03**	**05/28/03**	**06/30/03**	**07/30/03**	**08/29/03**	**09/29/03**	**10/28/03**
Insect									
Armyworm	none	**moderate**	**moderate**	none	**slight**	**slight**	none	none	none
Leafhoppers	none	none	none	none	**moderate**	none	none	none	none
Loopers	none	none	**moderate**	none	**slight**	none	**slight**	none	none
Lygus	none	none	none	none	**moderate/ severe**	none	**slight**	none	none
Weed									
Annual sowthistle	**slight**	**slight**	none	none	none	**moderate**	**slight**	**slight**	**slight**
Chickweed	**slight**	**slight**	none	none	none	none	none	none	none
Fiddleneck	none	none	none	none	none	**slight**	none	none	none
Hairy fleabane	none	**slight**	none	none	none	none	none	none	none
London rocket	none	none	**slight**	none	none	none	none	none	none
Milkweed	none	none	none	none	none	none	none	none	none
Shepherd's purse	**slight**	none	none	none	none	none	none	none	none
Witchgrass	none	none	none	**moderate**	none	**slight**	none	none	none
Other									
Gopher	none	none	**moderate**	**slight**	**moderate/ severe**	**slight**	none	none	none
Rabbit	none	none	none	none	none	none	none	none	none

[1]If a stressor was noted, it was rated as slight, moderate, or severe.

[2] No differences between test, control or reference alfalfa population responses were noted to any stressor on any observation date.

Table VI-16 (Continued). Observations for Biotic and Abiotic Stressors Among Alfalfa Test, Control, and Reference Plots.

Biotic and Abiotic Stressors for Iowa, 2001-2003; USDA Number 01-163-02n								
	Observation dates[1, 2]							
Stressor	**09/28/01**	**10/29/01**	**04/11/02**	**05/13/02**	**06/04/02**	**07/16/02**	**08/15/02**	**09/10/02**
Abiotic								
Drought	**moderate**	none	none	none	none	none	**moderate**	**moderate**
Heat	none	none	none	none	none	**moderate**	none	**moderate**
Disease								
Anthracnose	none	none	none	none	none	none	none	none
Common leaf spot	none	none	none	none	none	none	none	none
Crown rot	none	none	none	none	none	none	none	none
Insect								
Alfalfa weevil	none	none	none	none	none	none	none	none
Grasshopper	none	none	none	none	none	none	**slight**	**slight**
Leafhopper	none	none	none	none	none	**slight**	**slight**	none
Spittle bug	none	none	none	none	none	none	none	none

[1] If a stressor was noted, it was rated as slight, moderate, or severe.

[2] No differences between test, control or reference alfalfa population responses were noted to any stressor on any observation date.

Table VI-16 (Continued). Observations for Biotic and Abiotic Stressors Among Alfalfa Test, Control, and Reference Plots.

Biotic and Abiotic Stressors for Iowa, 2001-2003; USDA Number 01-163-02n							
	Observation dates[1, 2]						
Stressor	**04/01/03**	**05/01/03**	**06/05/03**	**07/06/03**	**08/13/03**	**09/05/03**	**10/13/03**
Abiotic							
Drought	none	none	none	none	none	**moderate**	none
Disease							
Anthracnose	none	none	none	none	none	none	none
Common leaf spot	none	none	none	none	none	none	none
Crown rot	none	none	none	none	none	none	none
Insects							
Alfalfa weevil	none	none	none	none	none	none	none
Grasshopper	none	none	none	none	none	**moderate**	**slight**
Potato leafhopper	none	none	none	none	none	none	none
Spittle bug	none	none	none	none	none	none	none

[1]If a stressor was noted, it was rated as slight, moderate, or severe.

[2] No differences between test, control or reference alfalfa population responses were noted to any stressor on any observation date.

.

Table VI-16 (continued). Observations for Biotic and Abiotic Stressors Among Alfalfa Test, Control, and Reference Plots.

Biotic and Abiotic Stressors for Illinois, 2001-2003; USDA Number 01-163-02n								
	Observation dates[1, 2]							
Stressor	**10/03/01**	**04/12/02**	**05/09/02**	**06/08/02**	**07/09/02**	**08/07/02**	**09/07/02**	**10/07/02**
Abiotic								
Drought	none	none	none	none	none	none	none	none
Heat	none	none	none	none	none	none	none	none
Disease								
Anthracnose	none	none	none	none	none	none	none	none
Black stem	none	none	none	none	none	none	none	none
Common leaf spot	none	none	none	**moderate**	none	none	none	none
Insect								
Alfalfa weevil	none	none	**slight**	none	none	none	none	none
Blister beetle	none	none	none	none	none	none	none	none
Leafhopper	none	none	none	none	none	none	**slight**	none

[1]If a stressor was noted, it was rated as slight, moderate, or severe.
[2]No differences between test, control or reference alfalfa population responses were noted to any stressor on any observation date.

Table VI-16 (Continued). Observations for Biotic and Abiotic Stressors Among Alfalfa Test, Control, and Reference Plots.

Biotic and Abiotic Stressors for Illinois, 2001-2003; USDA Number 01-163-02n								
	Observation dates[1, 2]							
Stressor	**04/09/03**	**05/05/03**	**06/04/03**	**07/16/03**	**08/15/03**	**09/12/03**	**10/13/03**	**11/03/03**
Abiotic								
Drought	none	none	none	none	none	none	none	none
Heat	none	none	none	none	none	none	none	none
Disease								
Anthracnose	none	none	none	none	none	none	none	none
Black stem	none	none	none	none	none	none	none	none
Common leaf spot	none	none	none	none	none	none	none	none
Insect								
Alfalfa weevil	none	**slight**	none	none	none	none	none	none
Blister beetle	none	none	none	none	none	none	none	none
Leafhopper	none	none	none	none	none	none	none	none
Plant bugs	none	none	**slight**	none	none	none	none	none

[1]If a stressor was noted, it was rated as slight, moderate, or severe.

[2]No differences between test, control or reference alfalfa population responses were noted to any stressor on any observation date.

Table VI-16 (continued). Observations for Biotic and Abiotic Stressors Among Alfalfa Test, Control, and Reference Plots.

Biotic and Abiotic Stressors for Wisconsin, 2001-2003; USDA Number 01-164-03n							
	Observation dates[1, 2]						
Stressor	**09/21/01**	**10/13/01**	**04/29/02**	**06/09/02**	**07/10/02**	**08/20/02**	**09/30/02**
Abiotic							
Cold	none	moderate	none	none	none	none	none
Drought	**moderate**	none	none	none	moderate	none	none
Flooding	none	none	none	none	none	none	none
Frost	none	**moderate**	none	none	none	none	**slight**
Heat	none	none	none	none	**moderate**	none	none
Disease							
Common leaf spot	none	none	none	none	none	none	none
Leptosphaerulina leaf spot	none	none	none	none	**slight**	**slight**	none
Sclerotinia crown and stem rot	none	none	none	none	none	none	none
Spring black stem	none	none	**slight**	**slight**	none	none	**slight**
Stemphyllium leaf spot	none	none	none	none	none	**slight**	none
Insect							
Potato leafhopper	none	none	none	none	**slight**	**slight**	none

[1]If a stressor was noted, it was rated as slight, moderate, or severe.

[2]No differences between test, control or reference alfalfa population responses were noted to any stressor on any observation date.

.

Table VI-16 (Continued). Observations for Biotic and Abiotic Stressors Among Alfalfa Test, Control, and Reference Plots.

Biotic and Abiotic Stressors for Wisconsin 2001-2003; USDA Number 01-164-03n							
	Observation dates[1, 2]						
Stressor	**04/23/03**	**05/08/2003**	**06/05/03**	**07/02/03**	**08/05/03**	**08/15/03**	**09/29/03**
Abiotic							
Cold	none	slight	slight	none	none	none	none
Drought	none	none	none	slight	slight	none	none
Heat	none	none	none	none	none	none	none
Wet soils	slight	slight	none	none	none	none	none
Disease							
Anthracnose	none	none	none	none	none	none	none
Crown rot complex	none	none	none	none	none	none	none
Leptosphaerulina leaf spot	none	none	none	none	none	none	none
Spring black stem	none	none	none	none	none	none	none
Stemphyllium leaf spot	none	none	none	none	none	none	none
Summer black stem	none	none	none	none	none	none	none
Verticillium wilt	none	none	none	none	none	none	none
Insect							
Potato leafhopper	none	none	none	none	slight	slight	none

[1]If a stressor was noted, it was rated as slight, moderate, or severe.

[2]No differences between test, control or reference alfalfa population responses were noted to any stressor on any observation date.

D.2 Phenotypic Study Number Two

Alfalfa field trials were established in 2001 at six locations that represent a range of typical environmental and agronomic conditions for commercial alfalfa production. The primary purpose of this field study was to generate test, control and reference substances for use in subsequent product characterization studies. Phenotypic observations were also taken throughout the course of the field trials. These observations were used to document the condition of the plants from which forage samples were obtained. These observations are included in this section as supplemental data to support the conclusions made from information presented in Section D.1.

Field sites were located in the states of California, Iowa, Illinois, New York, Washington, and Wisconsin. The following data were taken on all plots during 2001 and 2002: general growth and vigor, disease incidence, and insect incidence. Growth and vigor were rated for each plot using a scale from one to five, where one = very weak and five = excellent. In 2001, plots were also rated on a numerical scale for insect and disease susceptibility. Insect and disease susceptibility was rated on a qualitative basis in 2002. Using analysis of variance, no significant differences were detected in the combined overall (over season, years and sites) growth/vigor ratings in Roundup Ready versus control alfalfa, nor for insect and disease susceptibility (over season and site).

This study was conducted under USDA Notification Number 01-029-12n.

D.2.a. Test, control and reference materials

The starting test material consisted of Roundup Ready alfalfa populations containing event J101, J163 and the paired event J101XJ163. The single-event plants were from a Syn 1 generation bred to specifically contain a single copy of the *cp4 epsps* gene from either event J101 or event J163 (Figure VI-8, Box 6; with null segregants removed). The paired event was a Syn 1 generation produced through conventional breeding (Figure VI-8, Box 9; with null segregants removed). The control plant material was a Syn 1 population derived from null segregant progenitors and did not contain the *cp4 epsps* gene (Figure VI-8, Box 7). The four reference alfalfa varieties grown at each site were commercially available varieties adapted to each region.

All test, control and reference plants were phenotypically and genotypically screened prior to planting to confirm that the test material contained the event(s) and that the control plants did not contain either of the two events. Unlike the previous study described in Section D.1, there were no null segregating plants in the test populations transplanted into the fields, i.e., all test plants had the Roundup Ready trait.

D.2.b. Methods

Starting test, control and reference transplants were planted in a randomized complete block design with four replications. Each plot was planted with 49 alfalfa transplants. Each replicated block was separated by at least a 15-foot alley, and each plot within each replication was separated by at least a 10-foot buffer.

Plots were maintained as weed free as possible by hand-weeding or application of herbicides registered for use in alfalfa. All plots containing plants with the Roundup Ready events were treated with two quarts of Roundup Ultra herbicide per acre a few

weeks after transplanting. Plots were treated again up to three times (each application was 2 qt product/A) throughout the growing season. (Note: 2.0 qt/acre of Roundup Ultra herbicide is equivalent to 2.0 lb/acre of glyphosate in the form of its isopropylamine salt, or 1.5 lb/acre of glyphosate acid equivalent). In the 2002 growing season, plots that contained the Roundup Ready alfalfa plants were treated with up to 6 qt/A in separate 2 qt/A applications. Treatments were designed to be within proposed labeled rates for the application of a Roundup agricultural herbicide to Roundup Ready alfalfa.

D.2.c. Results and discussion

The growth and vigor ratings of the test, control and reference varieties are presented in Table VI-17. In general, the growth and vigor of the test, control and reference varieties were good to excellent at all of the locations across the two seasons. The exceptions noted were at the Iowa location and for the end of the season observations at the New York location. Early adverse weather conditions at Iowa (cold and moist conditions) were partly responsible for early, reduced vigor and growth scores. Table VI-18 presents the means derived from insect and disease scores for test control and reference populations. Insect and disease ratings were assigned numerical scores in 2001, and data were recorded as stressors in 2002. Stressor data for the 2002 season are reported in Table VI-19. While several stressors were noted to be present at all six locations, no differences were noted between the test, control and treated plots. For the 2001 season (Table VI-18), the following insects or diseases were noted. Black mold was present at the California site at the 12/17/01 observation date. Potato leafhoppers were observed at the Illinois site in plots on 5/23/01 and 6/25/01. Leaf spot was observed in the plots on 11/24/01. Potato leafhoppers were observed at the Iowa location on 7/31/01. At the Wisconsin location, potato leafhoppers were observed in all of the plots on 6/27/01 and 8/7/01. While numerical differences were noted between the test, control, and reference lines, there were no consistent trends across all of the locations that could be attributed to the trait or transformation event regarding the susceptibility to stressors that frequent alfalfa production fields.

Analysis of variance testing was conducted across sites, years, and observations for growth and vigor, insect damage, and disease damage using Statistical Analysis Software (SAS Version 8.2, SAS Institute, Inc. 1999-2001). The three Roundup Ready alfalfa lines (J101, J163, and J101XJ163) were separately compared to the control, while the minimum and maximum observed values were determined for the references. There were no significant differences detected at the 5% level of significance ($p > 0.05$) between J101, J163, or J101XJ163 and the control for any of the measured characteristics (Table VI-20).

In conclusion, growth/vigor, plant-insect and plant-disease interactions across two growing seasons were not changed between the test and control or reference populations. These results support a conclusion of no altered past potential for Roundup Ready alfalfa plants containing either event J101 or J163.

Table VI-17. Alfalfa Growth and Vigor Scores for the Single-Event Populations J101 and J163 and the Paired-Event Population J101XJ163 During 2001 and 2002.

		Observation		Alfalfa Population[1, 2]					
Site	Year	No.	Date	J101	J163	J101 X J163	Control	Ref mean[3]	Ref range[4]
CA	2001	1	05/11/01	4.0	4.0	4.0	3.8	3.9	3.8 - 4.0
		2	07/18/01	4.5	5.0	5.0	4.8	4.8	4.5 - 5.0
		3	08/14/01	4.8	4.8	4.3	4.3	4.4	4.0 - 4.8
		4	09/25/01	4.3	4.5	4.3	4.1	4.3	4.0 - 4.5
		5	12/17/01	4.8	4.3	4.0	3.8	4.1	4.0 - 4.3
	2002	1	03/27/02	4.0	4.0	4.0	4.0	4.1	4.0 - 4.3
		2	04/10/02	4.0	4.0	4.3	3.8	4.4	4.0 - 4.8
		3	05/21/02	4.8	4.0	5.0	4.3	4.3	4.0 - 4.5
		4	06/18/02	4.0	4.8	4.0	4.3	4.2	4.0 - 4.3
		5	07/19/02	4.3	4.3	4.5	4.8	4.3	4.0 - 5.0
IA	2001	1	05/15/01	3.5	3.5	3.5	3.0	2.7	2.0 - 3.0
		2	06/11/01	4.0	4.0	2.8	4.0	3.5	3.3 - 3.8
		3	07/31/01	4.5	4.5	4.5	5.0	4.8	4.8 - 5.0
		4	09/06/01	4.8	5.0	4.6	4.8	5.0	4.9 - 5.0
		5	11/12/01	4.0	4.5	3.5	3.0	3.3	2.5 - 4.8
	2002	1	04/22/02	4.3	4.5	3.5	3.8	3.3	3.0 - 3.5
		2	07/01/02	3.8	4.8	3.8	4.5	4.0	3.3 - 4.8
		3	08/01/02	3.5	5.0	3.8	4.0	3.4	3.0 - 3.8
IL	2001	1	05/08/01	4.6	4.9	4.6	4.8	4.8	4.8 - 4.9
		2	05/23/01	4.5	4.8	4.9	4.5	4.7	4.5 - 4.8
		3	06/25/01	4.3	4.5	4.8	4.1	4.4	4.4 - 4.5
		4	07/25/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
		5	11/24/01	4.8	5.0	5.0	4.5	4.6	4.5 - 4.8
	2002	1	04/25/02	4.8	4.3	4.3	4.5	4.4	4.2 - 4.9
		2	06/19/02	4.8	4.8	4.4	4.8	4.3	4.1 - 4.7
		3	07/20/02	4.4	4.0	4.2	4.0	4.3	4.1 - 4.4

[1]Mean characteristic values for test, control, and reference variety alfalfa populations.
[2]Observation score: M = dead plot; 1 = very weak, thin growth; 5 = excellent vigor and growth.
[3]Ref mean = mean of combined data for all four reference variety populations. CA: WL325HQ, 5454, Sommerset, and Cimmarron VR; IA: WL325HQ, 5454, Alfagraze, and LegenDairy YPQ; IL: WL325HQ, 5454, Ranger, and Magnum IV.
[4]Ref range = minimum and maximum values among all four reference variety populations.
Data developed under USDA Notification Number: 01-029-12n.

Table VI-17 (continued). Alfalfa Growth and Vigor Scores for the Single-Event Populations J101 and J163 and the Paired-Event Population J101XJ163 during 2001 and 2002.

		Observation		Alfalfa Population[1, 2]					
Site	Year	No.	Date	J101	J163	J101 X J163	Cont.	Ref mean[3]	Ref range[4]
NY	2001	1	05/16/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
		2	07/05/01	4.0	4.0	4.0	4.0	4.0	4.0 - 4.0
		3	09/22/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
		4	11/01/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
	2002	1	05/03/02	5.0	4.3	2.8	4.0	4.0	3.5 – 4.5
		2	08/13/02	5.0	5.0	4.0	4.8	4.8	4.5 - 5.0
		3	10/10/02	5.0	5.0	4.5	4.5	4.8	4.3 - 5.0
		4	10/29/02	2.0	2.0	2.0	2.0	2.0	2.0 - 2.0
WA	2001	1	05/10/01	4.3	4.3	4.0	4.3	3.9	3.8 - 4.0
		2	06/15/01	4.0	4.0	4.0	3.9	4.0	4.0 - 4.0
		3	07/06/01	4.8	4.8	5.0	5.0	4.8	4.5 - 5.0
		4	08/10/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
		5	10/29/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
	2002	1	04/23/02	5.0	5.0	4.8	4.8	4.5	3.8 - 5.0
		2	06/28/02	5.0	5.0	4.5	4.8	4.6	4.5 - 5.0
WI	2001	1	05/14/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
		2	06/27/01	4.0	4.0	3.5	4.0	3.9	3.8 - 4.0
		3	08/07/01	4.0	4.0	4.0	4.0	4.0	4.0 - 4.0
		4	10/03/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
		5	11/29/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
	2002	1	04/24/02	4.8	4.8	4.5	4.8	4.6	4.5 - 4.8
		2	05/29/02	5.0	5.0	4.8	4.8	4.8	4.8 - 5.0
		3	07/09/02	4.0	4.0	3.8	3.5	3.9	3.8 - 4.0

[1]Mean characteristic values for test, control, and reference variety alfalfa populations.
[2]Observation score: M = dead plot; 1 = very weak, thin growth; 5 = excellent vigor and growth.
[3]Ref mean = mean of combined data for all four reference variety populations. NY: WL325HQ, 5454, Oneida VR, and Macon; WA: WL325HQ, 5454, WL252HQ, and Vernema; WI: WL325HQ, 5454, Vernal, and Innovator + Z.
[4]Ref range = minimum and maximum values among all four reference variety populations.
Data developed under USDA Notification Number: 01-029-12n.

Table VI-18. Alfalfa Insect and Disease Observations for the Single-Event Populations J101 and J163 and Paired-Event Population J101XJ163 During 2001.

	Observation			Alfalfa Population[1, 2]					
Site	Charac-teristic	No.	Date	J101	J163	J101 X J163	Null	Ref mean[3]	Ref range[4]
CA	Insect	1	05/11/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
		2	07/18/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
		3	08/14/01	4.5	5.0	4.8	5.0	4.8	4.5 - 5.0
		4	09/25/01	4.8	5.0	5.0	5.0	5.0	5.0 - 5.0
		5	12/17/01	4.8	4.5	4.3	4.5	4.8	4.5 - 5.0
	Disease	1	05/11/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
		2	07/18/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
		3	08/14/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
		4	09/25/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
		5	12/17/01	4.8	4.5	4.3	4.3	4.3	3.8 - 4.8
IA	Insect	1	05/15/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
		2	06/11/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
		3	07/31/01	4.0	4.0	3.8	4.8	4.6	4.3 - 5.0
		4	09/06/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
		5	11/12/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
	Disease	1	05/15/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
		2	06/11/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
		3	07/31/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
		4	09/06/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
		5	11/12/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
IL	Insect	1	05/08/01	5.0	5.0	5.00	5.0	5.0	5.0 - 5.0
		2	05/23/01	3.0	3.3	3.00	3.0	3.0	3.0 - 3.0
		3	06/25/01	1.0	1.0	1.00	1.0	1.0	1.0 - 1.0
		4	07/25/01	5.0	5.0	5.00	5.0	5.0	5.0 - 5.0
		5	11/24/01	5.0	5.0	5.00	5.0	5.0	5.0 - 5.0
	Disease	1	05/08/01	5.0	5.0	5.00	5.0	5.0	5.0 - 5.0
		2	05/23/01	5.0	5.0	5.00	5.0	5.0	5.0 - 5.0
		3	06/25/01	5.0	5.0	5.00	5.0	4.9	4.8 - 5.0
		4	07/25/01	5.0	5.0	5.00	5.0	5.0	5.0 - 5.0
		5	11/24/01	4.0	4.3	4.50	4.3	4.3	4.3 - 4.5

[1]Mean characteristic values for test, control, and reference variety alfalfa populations.
[2]Observation score: M = dead plot; 1 = very weak, thin growth; 5 = excellent vigor and growth.
[3]Ref mean = mean of combined data for all four reference variety populations: CA: WL325HQ, 5454, Sommerset, and Cimmarron VR; IA: WL325HQ, 5454, Alfagraze, and LegenDairy YPQ; IL: WL325HQ, 5454, Ranger, and Magnum IV.
[4]Ref range = minimum and maximum values among all four reference variety populations.
Data developed under USDA Notification Number: 01-029-12n.

Table VI-18 (continued). Alfalfa Insect and Disease Observations for Single-Event Populations J101 and J163 and Paired-Event Population J101XJ163 during 2001.

	Observation			Alfalfa Population[1, 2]					
Site	Charact-eristic	No.	Date	J101	J163	J101 X J163	Null	Ref mean[3]	Ref range[4]
NY	Insect	1	05/16/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
		2	07/05/01	1.0	1.0	1.0	1.0	1.0	1.0 - 1.0
		3	09/22/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
		4	11/01/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
	Disease	1	05/16/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
		2	07/05/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
		3	09/22/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
		4	11/01/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
WA	Insect	1	05/10/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
		2	06/15/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
		3	07/06/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
		4	08/10/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
		5	10/29/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
	Disease	1	05/10/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
		2	06/15/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
		3	07/06/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
		4	08/10/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
		5	10/29/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
WI	Insect	1	05/14/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
		2	06/2701	2.0	2.0	2.0	2.0	2.0	2.0 - 2.0
		3	08/07/01	4.0	4.0	4.0	4.0	3.9	3.5 - 4.0
		4	10/03/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
		5	11/29/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
	Disease	1	05/14/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
		2	06/27/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
		3	08/07/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
		4	10/03/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0
		5	11/29/01	5.0	5.0	5.0	5.0	5.0	5.0 - 5.0

[1]Mean characteristic values for test, control, and reference variety alfalfa populations.
[2]Observation score: M = dead plot; 1 = very weak, thin growth; 5 = excellent vigor and growth.
[3]Ref mean = mean of combined data for all four reference variety populations. NY: WL325HQ, 5454, Oneida VR, and Macon; WA: WL325HQ, 5454, WL252HQ, and Vernema; WI: WL325HQ, 5454, Vernal, and Innovator + Z.
[4]Ref range = minimum and maximum values among all four reference variety populations.
Data developed under USDA Notification Number: 01-029-12n.

Table VI-19. Alfalfa Biotic and Abiotic Stressor Observations for the Single-Event Populations J101 and J163 and the Paired-Event Population J101XJ163 during 2002.

	Observation				
Site	**No.**	**Date**	**Stressor or symptom**	**Level of stressor[1]**	**Plot differences**
CA	1	01/24/02	None	-	-
	2	02/21/02	None	-	-
	3	03/15/02	Gophers	Moderate	No
	4	04/18/02	Gophers	Slight	No
	5	04/18/02	Rabbits	Slight	No
	6	04/18/02	Beet armyworm	Slight	No
	7	05/16/02	Lygus bug	Moderate	No
	8	06/14/02	Gophers	Moderate	No
	9	07/18/02	Gophers	Severe	No
	10	07/18/02	Rabbits	Moderate	No
	11	08/16/02	Gophers	Moderate	No
	12	08/16/02	Rabbits	Slight	No
IA	1	04/11/02	None	-	-
	2	05/13/02	None	-	-
	3	06/01/02	Spittle bug	Slight	No
	4	07/01/02	Heat	Moderate	No
	5	08/07/02	Heat	Moderate	No
	6	08/07/02	Leafhopper	Slight	No
IL	1	04/12/02	None	-	-
	2	05/09/02	Alfalfa Weevil	Slight	No
	3	05/15/02	Excess Water	Moderate	No
	4	05/20/02	Leaf Spot	Moderate	No
	5	05/20/02	Water Stress	Severe	No
	6	05/20/02	Potato Leafhopper	Slight	No
	7	05/30/02	Potato Leafhopper	Slight	No
	8	05/30/02	Wet Feet (flooding)	Severe	No
	9	06/13/02	Potato Leafhopper	Severe	No
	10	07/09/02	None	-	-
	11	08/07/02	None	-	-
	12	08/17/02	Heat	Moderate	No
	13	08/16/02	Drought	Severe	No

[1]Level of stressor: slight, moderate or severe.
Data developed under USDA Notification Number: 01-029-12n.

Table VI-19 (continued). Alfalfa Biotic and Abiotic Stressor Observations for the Single-Event Populations J101 and J163 and the Paired-Event Population J101XJ163 during 2002.

	Observation				
Site	**No.**	**Date**	**Stressor or symptom**	**Level of stressor[1]**	**Plot differences**
NY	1	05/30/02	Cold	Moderate	No
	2	05/30/02	Weevil	Moderate	No
	3	06/24/02	Potato Leafhopper	Moderate	No
	4	06/24/02	Weeds	Slight	No
	5	06/24/02	Grasses	Slight	No
	6	07/17/02	Potato Leafhopper	Moderate	No
	7	08/01/02	Potato Leafhopper	Slight	No
	8	08/26/02	Potato Leafhopper	Slight	No
WA	1	06/11/02	Leaf Spot	Slight	No
	2	06/11/02	Leafhopper	Slight	No
	3	07/02/02	Leaf Spot	Slight	No
	4	07/02/02	Heat	Slight	No
	5	07/02/02	Leafhopper	Slight	No
	6	07/23/02	Heat	Slight	No
	7	07/23/02	Leafhopper	Slight	No
WI	1	January	None	-	-
	2	February	None	-	-
	3	March	None	-	-
	4	04/24/02	Potato Leafhopper	None	No
	5	04/24/02	Drought	None	No
	6	05/29/02	Potato Leafhopper	None	No
	7	05/29/02	Drought	None	No
	8	06/21/02	Potato Leafhopper	Moderate	No
	9	06/21/02	Drought	None	-
	10	07/08/02	Potato Leafhopper	Moderate	No
	11	07/08/02	Drought	Moderate	No

[1]Level of stressor: slight, moderate or severe.

Data developed under USDA Notification Number: 01-029-12n.

Table VI-20. Growth/Vigor and Insect and Disease Damage of J101, J163, and J101XJ163 Compared to the Control Pooled Across Sites, Years, and Observations.

	Alfalfa Population[1]				
Characteristic	J101	J163	J101 x J163	Control	Ref. Min. – Max.[2]
Growth and Vigor[3]	4.5	4.5	4.3	4.3	3.0 – 5.0
Insect Damage[4]	4.4	4.4	4.4	4.5	3.8 – 5.0
Disease Damage[4]	5.0	5.0	5.0	5.0	4.6 – 5.0

[1]No significant differences were detected between J101, J163, or J101XJ163 and the control at $P \leq 0.05$ for any of the measured characteristics.

[2]Ref. Min. – Max. = minimum and maximum observed values from among all reference populations (n = 192 for Growth and Vigor; n = 96 for Insect Damage and Disease Damage).

[3]Growth and Vigor was evaluated periodically in 2001 and 2002 using the following rating scale: M (dead plot), 1 (weak plot, thin growth) to 5 (excellent vigor/growth).

[4]Insect Damage and Disease Damage were evaluated periodically in 2001 using the following rating scale: M (dead plot), 1 (76-100% plants with symptoms) to 5 (0-5% plants with symptoms).

Data developed under USDA Notification Number: 01-029-12n.

D.3. Phenotypic Study Number Three

The following phenotypic information was collected by Forage Genetics International during field trials performed for event selection purposes. This information is provided because it includes phenotypic data over the useful life of an alfalfa stand and provides additional supporting information to confirm the conclusions derived from previously-described research in Sections D.1 and D.2 for newly established and first and second forage production years.

Several phenotypic characteristics of test alfalfa populations containing Roundup Ready event J101 or Roundup Ready event J163 were compared to those of a near-isogenic control population and a conventional alfalfa reference population. A 40-month field experiment was conducted by Forage Genetics International, West Salem, Wisconsin, from 1999 through 2002. Vegetative vigor, forage yield, survival, pest tolerance, environmental stress tolerance, and fall dormancy reaction were assessed in a randomized, complete block design field study for four growing years and included three winter dormancy periods. The vegetative vigor, forage yield, survival, pest tolerance, environmental stress tolerance, and fall dormancy reaction of the single-event, J101 or J163 Roundup Ready alfalfa test populations were similar to that of the near-isogenic control alfalfa population during the four growing seasons.

This study was conducted under USDA Notification Numbers 99-047-03n, 00-063-18n, 01-010-09n and 02-007-08n.

D.3.a. Test, control and reference populations

The test populations were single-event, simplex MBC1 populations containing either event J101 or event J163 (Figure VI-8, positive Roundup Ready segregants from Box 3 with null segregants removed). The populations were not homozygous and therefore segregated for the Roundup tolerance trait. So that all test plants in the study contained the event of interest, the nontolerant, null segregants were removed from each test population by application of Roundup UltraMAX herbicide (3 lb a.e./A rate, applied with a hand-sprayer 16 days after planting). Tolerant plants were transplanted to the field. Therefore, unlike the previous study described in Section D.1, and similar to the study in Section D.2., there were no null segregating plants in the test populations transplanted into the fields, i.e., all test plants were Roundup Ready.

The control was the null segregant (nulliplex) MBC1 population selected specifically to serve as a near-isogenic control population for the event test lines (Figure VI-8, null segregants from Box 3). The control population was the MBC1 null segregant population identified using a nondestructive assay prior to Roundup herbicide application to the remaining MBC1 test seedlings.

The reference population was a fall-dormant, commercial alfalfa variety (DK134).

D.3.b. Field procedure and experimental design

Alfalfa plants were hand-transplanted to the field at the same location in a randomized complete block design with four replicates and eight plants per replicate. Each experimental unit was a nine-foot-long, space-transplanted row (five plants with 15 inches between plants within rows and 30 inches between rows). Weeds were controlled throughout the experiment's duration with mechanical cultivation, and the plots were managed according to locally recommended practices for optimum alfalfa forage production (e.g., fertility, insect pest control, harvest schedule, etc.). Pests were monitored and insecticides were applied three times during mid-summer to control potato leafhoppers (*Emposca fabae*).

Forage yield (fresh forage weight, g/plant). The field was hand-harvested 12 times and the fresh weight per plot was recorded at seven dates during the study duration.

Vigor (1-10 score). Vigor was subjectively scored ten times during the test duration so that data on growth and development would be collected at a variety of growth stages, plant age and under a range of seasonal environments. Vigor was scored from 1 (dead) to 10 (highest vigor).

Survival (%). Plant survival was measured on 14 dates throughout the test duration.

Recovery after cutting, spring recovery, fall growth, disease and insect susceptibility and general appearance. These characteristics were subjectively scored (1= poorest to 10=best vigor or appearance) or continuously monitored throughout the study period.

D.3.c. Results and discussion

The Roundup Ready alfalfa event J101 population was not different from the control population for mean forage yield and survival at all dates (Tables VI-21 and VI-22). The J101 population was not different from the control population for plant vigor score at all

dates except at one of the ten scoring dates where it was greater than the control, 07/09/99 (Table VI-23). The overall mean values for the J101 population forage yield, vigor and survival were within the range of the control population. There was no overall trend toward increased vegetative yield, vigor or survival of the J101 population when compared to the control population throughout the four growing seasons. Fall dormancy reaction was similar between J101 and the control population (Table VI-24).

The Roundup Ready afalfa event J163 population was not different from the control population for mean forage yield, vigor score and survival at all dates (Tables VI-21, VI-22 and VI-23). The overall mean values for the J163 population forage yield, vigor and survival were within the range of the control population. There was no overall trend toward increased vegetative yield, vigor or survival of the J163 population when compared to the control population throughout the four growing seasons. Fall dormancy reaction was similar between J163 and the control population (Table VI-24).

The Roundup Ready alfalfa event J101 population was not different from the reference population on four of seven harvest dates. However, on three dates, the forage yield of J101 was significantly greater than the reference population mean (Table VI-21). Overall, the J101 population had significantly greater forage yield than the reference population. The J101 population vigor mean was not different from the mean of the reference population for all comparison dates except two, where the J101 population had significantly greater vigor than the reference mean (Table VI-23). The overall vigor mean for J101, however, was similar when compared to the reference population mean. Mean survival between the J101 and the reference populations was similar throughout the study (Table VI-22) and fall dormancy reaction was also similar (Table VI-24).

The Roundup Ready alfalfa event J163 population was not different from the reference population on six of seven harvest dates. However, on one date, the forage yield of J163 was significantly greater than the reference population mean (Table VI-21). Overall, the J163 population had similar forage yield when compared to the reference population. The J163 population vigor mean was not different from the mean of the reference population for all comparison dates except two, where the J163 population had significantly greater vigor than the reference mean (Table VI-23). The overall vigor mean for J163 was also significantly greater when compared to the reference population mean, indicating that the J163 population tended to be more vigorous overall than the reference population. Mean survival between the J163 and the reference populations was similar throughout the study (Table VI-22) and fall dormancy reaction was also similar (Table VI-24).

In addition to the characteristics discussed above, plants were observed for their reaction to endemic diseases, insect pests and environmental stressors. All plants appeared normal and typical in all aspects of their growth, development and reaction to endemic diseases, insect pests and environmental stressors. Potato leafhoppers were the only pest species that were actively controlled, and there was little or no visible damage to the plants when the entire plot area was uniformly sprayed with insecticide. In each of the four years during the month of June, potato leafhoppers migrated into the region and populated the research plots and all other alfalfa plots growing on the research farm. Therefore, all research plots were treated. There were no differences between populations or plants for reaction to potato leafhopper, i.e., all plants were uniformly

susceptible as anticipated (the base germplasm was susceptible). The other endemic pests that were observed during routine monitoring were present at very low levels and did not differentially affect populations. The endemic pests included: spring blackstem, summer blackstem, Leptospherulina leafspot, bacterial wilt, Verticillium wilt, aphids, plant bugs and thrips. Additionally, there were no observed differences for reaction to heat, drought, wind, saturated soil (because of frequent rains) or frost.

In general, the reference population tended to be somewhat less vigorous than the control or test populations. The four alfalfa synthetic populations compared in the study are similar in base germplasm (fall-dormant, hay-type background germplasm) but they had been bred from different parent genotypes (i.e., they are different synthetic varieties; the test and control populations were related by MBC1 source population in contrast to the reference population that was bred independently). Therefore, the relatively modest difference between the reference population means and the control and test population means likely were because of differences in population pedigree background genetics and related to the fact that the test and control MBC1 populations were forward-bred (a type of modified backcross breeding for population improvement) during their development. Therefore, the modest increase in vigor evident for the control and test populations versus the reference population, may have been a result of population background genetics, rather than an effect of the Roundup Ready alfalfa events.

Table VI-21. Forage Yield Measured as Fresh Weight Grams per Plant of Roundup Ready Event J101, Event J163, Control and Reference Populations at West Salem, Wisconsin, 1999-2002.

Harvest Date	Forage Yield in g/plant[1] (range)			
	Event J101[2]	Event J163[2]	Control	Reference
07/26/99	311[†] ± 65	272 ± 68	245 ± 75	202 ± 43
	(249-392)	(172-315)	(154-337)	(149-254)
09/03/99	317[†] ± 20	316[†] ± 42	334 ± 7	285 ± 5
	(292-338)	(270-372)	(325-343)	(279-291)
05/23/00	494 ± 62	542 ± 33	528 ± 19	472 ± 115
	(403-545)	(505-585)	(505-551)	(331-613)
05/22/01	355[†] ± 20	334 ± 85	346 ± 9	226 ± 2
	(334-378)	(238-444)	(335-357)	(204-248)
06/28/01	307 ± 49	277 ± 53	307± 31	270 ± 7
	(255-3690	(223-347)	(268-345)	(261-279)
08/30/01	177 ± 41	149 ± 45	159 ± 12	180 ± 21
	(127-2160	(101-2040	(144-1730	(155-206)
06/07/02	337 ± 41	339 ± 24	374 ± 19	352 ± 28
	(300-394)	(306-363)	(350-397)	(318-386)
Overall	2298[†] ± 172	2230 ± 166	2265 ± 51	1989 ± 120
	(2062-2432)	(2044-2438)	(2202-2328)	(1840-2134)

[1] Mean of four replications, ± standard deviation and range.
[2] Means of test population were not different from control population means.
[†] Mean of test population was significantly different than the mean for the reference population ($P<0.05$).
Data were developed under USDA Notification Numbers: 99-047-03n, 00-063-18n, 01-010-09n and 02-007-08n.

Table VI-22. Mean Percent Survival of Roundup Ready Event J101, Event J163, Control and Reference Populations at West Salem, Wisconsin, 1999-2002.

Survival Count Date	Survival % [1] (range)			
	Event J101[2]	Event J163[2]	Control	Reference
05/27/99	100	100	100	100
06/07/99	100	100	100	100
06/15/99	100	100	100	100
07/09/99	100	100	100	100
07/26/99	100	100	100	100
08/03/99	100	100	100	100
08/12/99	100	100	100	100
04/06/00	100	100	100	100
05/23/00	100	100	100	100
07/21/00	100	100	100	100
08/31/00	100	100	100	100
04/27/01	100	100	94 $\pm$ 5 (88-100)	100
05/08/02	66 $\pm$ 6[3] (63-75)	66 $\pm$ 6 (63-75)	75 $\pm$ 10 (63-88)	75 $\pm$ 10 (63-88)
06/07/02	59 $\pm$ 16 (38-75)	56 $\pm$ 16 (38-75)	63 $\pm$ 10 (50-70)	69 $\pm$ 15 (50-88)

[1] Mean of four replications, $\pm$ standard deviation and range.
[2] Means of test population were not different from control or reference population means.
[3]Numbers after $\pm$ are standard deviation.
Data were developed under USDA Notification Numbers: 99-047-03n, 00-063-18n, 01-010-09n and 02-007-08n.

Table VI-23. Mean Plant Vigor of Roundup Ready Event J101, Event J163, Control and Reference Populations at West Salem, Wisconsin, 1999-2002.

Vigor Score Date	Vigor Score [1] (range)			
	Event J101	Event J163	Control	Reference
05/27/99	6.75	7.00	7.50	7.00
06/07/99	7.25	7.75†	6.98	6.75
06/15/99	6.93†	7.23†	7.18	6.30
07/09/99	7.70*	7.50	6.78	6.00
08/03/99	6.56	6.70	6.70	6.40
08/12/99	6.85	6.95	6.90	6.70
07/21/00	7.33	7.48	7.20	7.10
08/31/00	7.78	7.40	7.30	7.28
04/27/01	5.40†	5.35	5.03	4.28
05/8/02	3.25	3.25	4.00	3.50
Overall	6.58 ± 0.20[2] (6.37-6.77)	6.66† ± 0.22 (6.41-6.89)	6.56 ± 0.04 (6.51-6.60)	6.13 ± 0.09 (6.06-6.24)

[1] Mean of four replications, ± standard deviation and range. 1=dead, 10=best vigor.

[2]Numbers after ± are standard deviation.

* Mean of test population was significantly different from the mean for the control population ($P<0.05$).

† Mean of test population was significantly different than the mean for the reference population ($P<0.05$).

Data were developed under USDA Notification Numbers: 99-047-03n, 00-063-18n, 01-010-09n and 02-007-08n.

Table VI-24. Mean Fall Dormancy Score of Roundup Ready Event J101, Event J163, Control and Reference Populations at West Salem, Wisconsin, 1999.

	Fall Dormancy Score [1]			
	Event J101[2]	Event J163[2]	Control	Reference
Mean ± standard deviation	3.1 ± 0.3	3.3 ± 0.1	3.1 ± 0.0	3.3 ± 0.1
Range	2.8-3.4	3.2-3.3	3.1-3.1	3.2-3.4

[1] Mean of four replications, ± standard deviation and range.
[2] Means of test population were not different from control or reference population means.
Data were developed under USDA Notification Numbers 99-047-03n, 00-063-18n, 01-010-09n and 02-007-08n.

In conclusion, the vegetative vigor, forage yield, survival, pest tolerance, environmental stress tolerance, and fall dormancy reaction of the single event J101 or J163 Roundup Ready test populations were not different from that of the near-isogenic control alfalfa population during the four growing seasons. When compared to the reference population, the J101 and J163 test populations tended to have somewhat greater yield and vigor. These differences may have resulted from genetic pedigree differences between the test and reference populations because the populations were not directly related. Over the four-season duration of the study, there were no trends toward sustained or biologically meaningful differences in terms of pest potential between the J101 and J163 populations and the control population.

D.4. Overall Conclusion: Plant Phenotypic Comparisons

Three separate phenotypic studies were conducted to assess familiarity and to evaluate the pest potential of alfalfa populations containing event J101 or J163. Data developed from these studies represent a broad range of environmental conditions and agronomic practices that Roundup Ready alfalfa would likely encounter. In the first study, information was presented from trials that were designed based on discussions with APHIS and conducted to specifically assess the pest potential of alfalfa populations containing the Roundup Ready alfalfa events. The second and third studies include phenotypic information collected during the normal course of product development. During the normal course of plant variety development and event selection, plant breeders typically collect a considerable amount of information regarding the phenotype of plants containing a transformation event. These data include both quantitative and qualitative data regarding the plants agronomic performance and susceptibility to insects and diseases. This information serves as the basis for elimination and advancement of events during the development of a crop containing a genetically modified trait. Qualitative data were taken for the test, control and reference plants grown at each site. While these data were not subjected to statistical analysis, they represent observations that are typically recorded by plant breeders, plant pathologists and agronomists to

evaluate the qualities of a plant variety. These types of observations are extremely useful and are commonly used to make variety selections. Collectively, the characteristics measured in these three studies provide crop biology data useful in assessing risk, including equivalence, as it relates to pest potential, and familiarity. The phenotypic data presented do not indicate any enhanced weed potential for the Roundup Ready alfalfa single-event populations J101 and J163, or from the confirmatory data on the paired-event population J101XJ163, compared to the control and reference variety populations.

E. Reproductive Characteristics

E.1. Flower Morphology and Phenotypic Characteristics

Flower morphology, color, pollen germination, pollen load per flower, self-fertility and seed morphology of alfalfa plants with the Roundup Ready alfalfa events J101, J163 and J101XJ163 were compared to the nontransformed parental control line, R2336.

The viability and morphology of pollen was evaluated in two separate experiments. In the first experiment, pollen was harvested from T_0 J101, J163 or control alfalfa plants and the morphology and viability of pollen compared. In this experiment, pollen was harvested from single test or control plants, which precluded statistical comparisons of viability data. In the second experiment, pollen was harvested from multiple plants and the viability of pollen from J101, J163, J101XJ163 and control was subjected to statistical analysis.

E.1.a. Materials and methods

The R2336 isoline control was a single FGI proprietary alfalfa genotype (clone) (Figure VI-8, Box 1). The two T_0 test events, J101 and J163 (T_0 initial transformants) were derived from R2336 tissue *via Agrobacterium* transformation and regeneration from culture during 1997-98 at Montana State University (Figure VI-8, Box 2). The R2336 control, J101 and J163 plants (i.e., one genotype of each) were shipped from Montana State University to Forage Genetics International, West Salem, Wisconsin, and planted in greenhouse pots. FGI made clones of each of the three genotypes by rooting two vegetative stem cuttings (ramets) from each of the original T_0 mother plants in December 1998.

Each of the ramets was verified for identity using event-specific PCR analysis at FGI. The plants had been maintained on the same greenhouse bench at 20-25 C with $\geq$18 hr photoperiod to encourage reproductive structure development. All plants of the three genotypes (clones) were grown alongside one another and the plants were clipped and fertilized bimonthly to maintain good plant health and vigor. Flowers used for these comparisons were from four-year-old plants.

Fully developed, freshly opened flowers were measured to document their morphology, size and color. A diagram of a typical alfalfa flower is presented in Figure VI-9. Table VI-25 lists the characteristics observed, the parameter measured and the number of observations compared for each genotype. Whole racemes, individual flowers on a raceme (also known as florets), and dissected flower parts were measured and representative stems, racemes, flowers, and flower parts were photographed. Because no seed was produced by selfing the J101 T_0, J163 T_0 or R2336 plants, seed morphology was observed using other materials. Single event J101, J163 and appropriate null control

seed were produced as the product of a seed yield study conducted in the greenhouse at FGI, West Salem, Wisconsin in 2002 (see Section F.1. for seed production details; Figure VI-8, test materials- Box 5, 88% trait purity and, control material- Box 8). Paired-event J101XJ163 and appropriate control material seeds were grown in the field during 2003 (see Section C.3.a. for seed production details; Figure VI-8, test materials- Box 11). Most of the measurements of the characteristics are self-explanatory, i.e., counts or length. The remaining measurements are described in more detail below:

Pollen load/flower. The relative amount of pollen per 100 individual flowers was subjectively scored. Flowers were tripped by gentle squeezing between the thumb and index finger, causing the keel petals to split open. The relative size of the pollen mass deposited on the standard petal was scored as follows: 1=none; 2=scant; 3=moderate; and 4=abundant.

Pollen load/flower. Anthers (with pollen load intact) were carefully excised from untripped, newly opened flowers. The anthers (and the closely associated stamen and pistil tissues) of three flowers were placed into 1 ml of distilled water in a test tube. Pollen was released from the anthers by vigorous agitation using a vortex mixer. Ten replicates (three flowers per replicate) were used. Pollen grains/ml were immediately determined using a blood cell counting chamber. Mean pollen load/flower was calculated by dividing the total count/ml by three for each replicate.

Pollen germination. Pollen samples suspended in water were spotted onto sterile pollen germination media containing 15% sucrose, 0.4 mM $CaCl_2$, 0.4 mM H_3BO_4 and 1% agar in petri dishes (Carpenter et al., 1992). Petri dishes were incubated at 25^{o}C for 75 to 90 minutes and monitored for pollen germination. Pollen germination was counted under a microscope using suitable levels of magnification (approximately 30X). For each genotype, ten replicate fields of view were counted with approximately 30-80 pollen grains/field/spot. Representative photographs were taken using available magnification.

Self-fertility. Flowers were self-pollinated in two ways and the number of resulting normal seeds were counted. In one measurement, the undisturbed flowers were allowed to self-trip. In the second measurement, the flowers were manually tripped by gentle mechanical pressure, i.e., the raceme was gently rolled between the thumb and index finger, causing the flower to trip and self-pollinate. Ten freshly opened racemes per genotype per method were labeled and monitored.

Seed morphology. Seed were harvested from Roundup Ready alfalfa plants (single and paired event) and from conventional alfalfa varieties. Photographs of the seed were taken so that visual comparisons could be made.

E.1.b. Results and discussion

The mean, standard deviation (SD) and range of observed values for each of the quantitative characteristics are presented in Table VI-26 for the control, event J101 and event J163. Two-sample T-tests did not detect any differences between flowers obtained from plants containing event J101 versus the control or flowers obtained from plants containing event J163 versus the control for any of the characteristics measured ($P \geq 0.05$). Therefore, it was concluded that Roundup Ready alfalfa containing either event J101 or

event J163 was not different from the control alfalfa for any quantitative reproductive morphological characteristics assessed.

The observed values for each of the qualitative flower characters assessed are presented in Table VI-27. All racemes, flowers, staminal columns and seed had typical morphology. Because none of the qualitative measurements within a characteristic varied among experimental units, no statistical analysis was performed.

Representative comparative photographs for the qualitative flower characteristics are presented in Figures VI-10 to VI-16 and described below. All flowers were classified as variegated (Class 2) dark purple (Subclass 1) (Barnes, 1972) (Figures VI-10 and VI-11). Flower colors for all genotypes progressively changed with age, as is typical for alfalfa flowers (Figure VI-12) (Barnes, 1972). Nascent, unopened flowers were very bright pink-purple. After opening, flowers changed to dark purple variegated (Class 2.1), and, as they aged, the flowers gradually faded to light purple with green and yellow pigments becoming more apparent. A description of flower color change with aging has been published by Barnes (1972).

Flower morphology was typical in all aspects for all flowers. All flower buds developed normally and progressively from raceme base to distal tip (Figures VI-12). All flowers opened normally, with keel petals closed around the sexual column, i.e., the blooms remain closed until mechanically tripped or auto-tripped (left photos in Figures VI-13 and VI-14). Flowers were then intentionally manually or mechanically tripped to split the keel petal suture, causing the release of the sexual column for photographs, self-pollination and measurement (right photos in Figures VI-13 and VI-14). Raceme development and attachment were typical for all genotypes (Figure VI-15).

Comparative photographs of test and control sexual columns are presented in Figure VI-16, where the sexual columns were dissected away from the corolla and calyx. All flowers demonstrated typical staminal morphology, wherein nine stamens are fused to form a tube around the style and one stamen develops free and unfused to the others (Teuber and Brick, 1988). All sexual columns were firm and arched (Figure VI-16).

The percent pollen germination did not differ between test and control plants, with approximately 50% of the pollen germinating within the first 90 minutes *in vitro* at 25ºC. Pollen tube emergence began within the first 15 minutes for all samples; no additional germination was noted at incubation times >60 minutes. Single pollen tubes emerged from all pollen grains; all tubes grew directly away from the pollen grain and most were slightly curved (Figure VI-17). There were no differences observed in pollen tube development or growth between control and test events.

As expected, no seed was produced from self fertilization; however, comparative photographs of seed produced by J101, J163 and control alfalfa populations are presented in Figure VI-18, panel A. Seed size and shape is normally variable within a seed lot (Teuber and Brick, 1988). Seed size and color varied slightly within each test or control lot, but there were no atypical morphology evident or differences between test and control seed lots. Seed size (mg/seed) for Roundup Ready alfalfa and controls are reported in Section VI, Subsection F of this petition. A photograph of seed produced by the paired-event population and conventional alfalfa is presented in Figure VI-18, panel

B. No morphological differences were observed between seed produced by the paired event population in comparison to that produced by conventional alfalfa varieties.

The isogenic control plant, R2336, is a male-fertile but self-incompatible (self-infertile) genotype (i.e., viable pollen is produced, but it will not self-fertilize). Self-incompatible genotypes are extremely common in conventional alfalfa, and self-compatible types are relatively rare (Viands et al., 1988). There were no pods or seeds that developed on any of the racemes for control J101 or J163 plants. Therefore, self-fertility—or more specifically, the lack of self-fertility—was not changed by the presence of either of the test events.

In summary, the data from the numerous comparisons made in this assessment indicate that Roundup Ready alfalfa events J101 and J163 do not differ from control alfalfa with regard to any floral characteristic and support a conclusion of no altered pest potential for alfalfa plants containing event J101 or event J163 compared to the nontransformed variety.

Table VI-25. Characters Observed or Measured and Number of Flowers Used for J101, J163, and Control Alfalfa Plants.

Character	Measurement	No. of observations per genotype
Flower color	Color classification[1]	60 intact racemes
No. of flowers/raceme	Count/rachis	60 intact racemes
Attachment of rachis to stem	Count normal vs. abnormal	60 intact racemes
Attachment of flowers to rachis	Count normal vs. abnormal	60 intact racemes
Flower ripening pattern/raceme	Count normal vs. abnormal	60 intact racemes
Standard petal	Length (mm)	100 individual flowers
Keel petals	Length (mm)	100 individual flowers
Calyx tube	Diameter (mm)	100 individual flowers
Sexual column	Length (mm)	100 individual flowers
Pollen load (subjective)	Subjective score 1 to 4 [2]	100 individual flowers
Pollen load (quantitative)	Pollen grains/flower	10 replicates of 3 flowers/rep
Pollen germination	*In vitro* germination (%)	10 replicates of 3 flowers/rep
Gross flower morphology	Count normal vs. abnormal	100 individual flowers
Self-fertility, auto-tripped [3]	Count of seeds produced	10 racemes (ca. 20 flowers/raceme)
Self-fertility, tripped [4]	Count of seeds produced	10 racemes (ca. 20 flowers/raceme)

[1] Color classification per Barnes, 1972.
[2] Subjective score: 1=no pollen to 4= abundant pollen.
[3] Flowers were not tripped by the researchers.
[4] Flowers were tripped by the researchers by rolling the raceme between thumb and index finger.

Table VI-26. Flower and Pollen Measurements of Roundup Ready Alfalfa Plants and Control.

Character [1]	Control	Event J101	Event J163
Number of flowers / raceme:			
Mean ± SD [2]	20.15±5.24	20.30±5.48	20.62±5.40
Range	13 - 34	11 - 33	12 - 31
Standard petal (mm length):			
Mean± SD	11.49±0.61	11.50±0.56	11.62±0.60
Range	10 - 13	10 - 13	10 - 13
Keel petals (mm length):			
Mean± SD	7.82±0.50	7.79±0.48	7.83±0.51
Range	7 - 9	7 - 9	7 - 9
Calyx tube (mm diameter):			
Mean± SD	1.875±0.22	1.925±0.18	1.925±0.18
Range	1.5 – 2.0	1.5 – 2.0	1.5 – 2.0
Sexual column (mm length):			
Mean± SD	7.80±0.55	7.75±0.54	7.81±0.54
Range	7 - 9	7 - 9	7 - 10
Pollen load, quantitative (grains/flower):			
Mean± SD	2519 ± 480	2500 ± 352	2519 ± 411
Range	1852 – 3148	2037 – 3148	2037 – 3148
Pollen germination (%):			
Mean± SD	51.91 ± 5.65	50.93 ± 4.19	50.05 ± 4.22
Range	41.86 – 57.63	42.00 – 57.45	43.14– 56.86

Quantitative assessment of flowers collected from control J101 and J163 alfalfa plants. Means within a character were not statistically different for any of the control vs. J101 or control vs. J163 comparisons ($P \geq 0.05$).

[1] Data are based on the number of observations given for the character in Table VI-25.
[2] Standard deviation (SD) of the mean.

Table VI-27. Qualitative Asessment of Flower, Pollen, Seed, and Fertility Measurements of Roundup Ready Alfalfa Events J101 and J163 Versus Control.

Character [1]	Control	Event J101	Event J163
Flower color (class) [2]	Class 2.1: variegated dark purple	Class 2.1: variegated dark purple	Class 2.1: variegated dark purple
Flower general morphology	normal	normal	normal
Rachis attachment to stem	normal	normal	normal
Attachment of flowers to rachis	normal	normal	normal
Flower ripening pattern/raceme	normal	normal	normal
Pollen load/flower, subjective[3]	Class 4: abundant	Class 4: abundant	Class 4: abundant
Self-fertility, auto-tripped[4]	100% self-infertile	100% self-infertile	100% self-infertile
Self-fertility, mechanically tripped [5]	100% self-infertile	100% self-infertile	100% self-infertile
Seed morphology	normal	normal	normal

Qualitative measurements within a character did not vary among experimental units, so standard deviations and statistics were not calculated.

[1] Data are based on the number of observations given for the character in Table 1.

[2] Color classification per Barnes, 1972.

[3] Subjective score: 1=no pollen to 4= abundant pollen.

[4] Flowers were not tripped by the researchers.

[5] Flowers were tripped by the researchers by rolling the raceme between thumb and index finger.

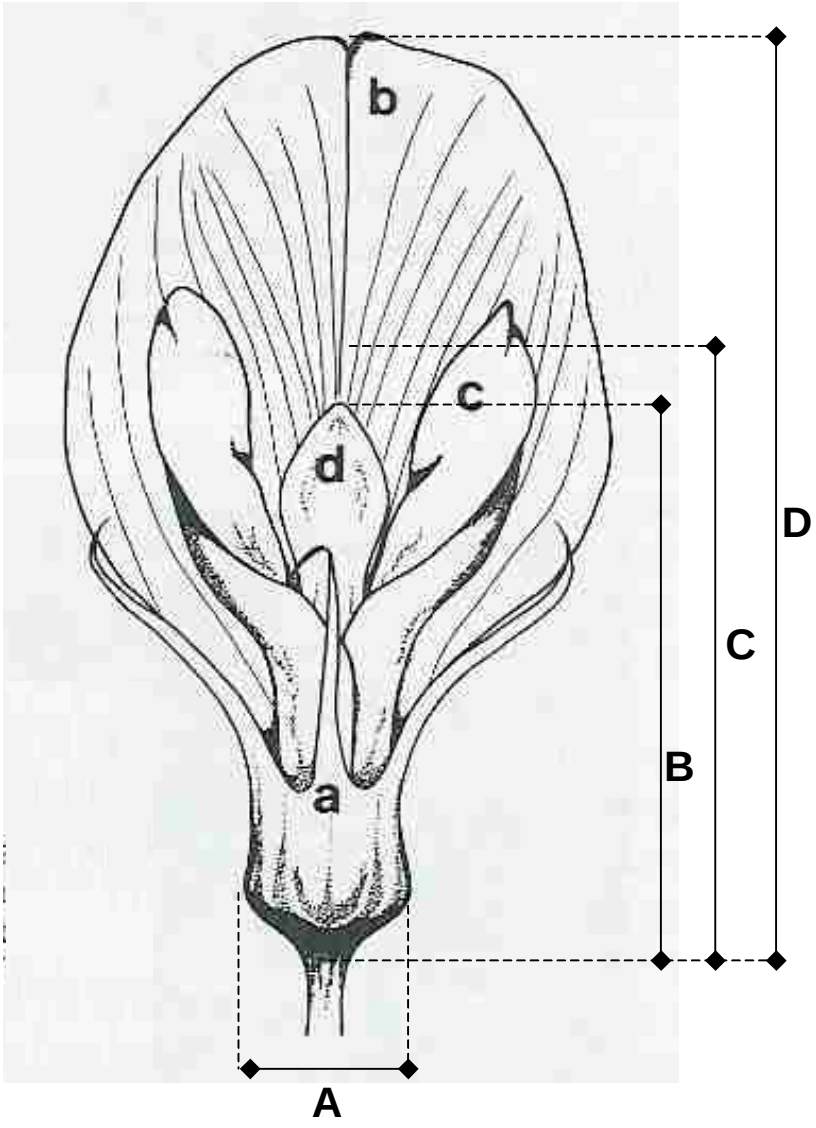
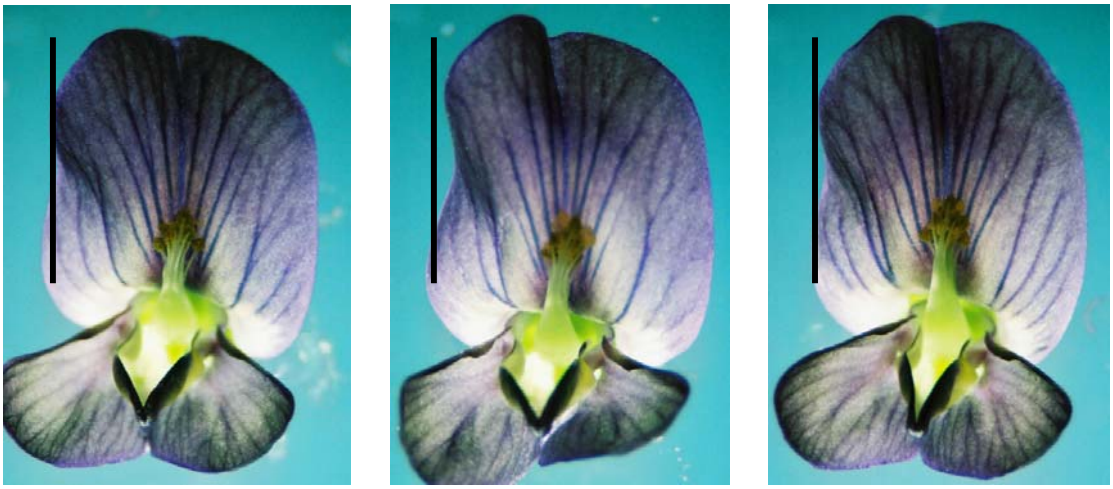


Figure VI-9. Diagram of an Untripped (Unopened) Alfalfa Flower.

a-sepals, **b**-standard petal, **c**-wing petals, and **d**-fused keel petals which conceal the sexual column. Intact flowers were tripped to reveal the sexual column and measurements were recorded for: **A**-diameter of calyx, **B**- length of keel petals, **C**-length of sexual column after release from keel petals (sexual column is not shown in the diagram) and, **D**-length of standard petal.

The figure was produced by Dr. Larry Teuber of the University of California-Davis.



Event J101 Control Event J163

Figure VI-10. Whole-Flower Front View of Event J101, Control and Event J163.

Flowers were mechanically tripped by the researcher to release the staminal column, anthers and pollen mass. Bar = 5 mm.

Event J101 **Control** **Event J163**

Figure VI-11. Whole-Flower Side-View of J101, Control and J163.

Flowers were mechanically tripped by the researcher to reveal the staminal column, anthers and pollen mass.

Event J101 **Control** **Event J163**

Figure VI-12. Whole Raceme Comparison.

Alfalfa flowers matured and changed color in a normal progression from basal to distal tip within each rachis. Flower color gradually faded as flowers aged (youngest to oldest racemes from top to bottom, respectively, within a photograph).

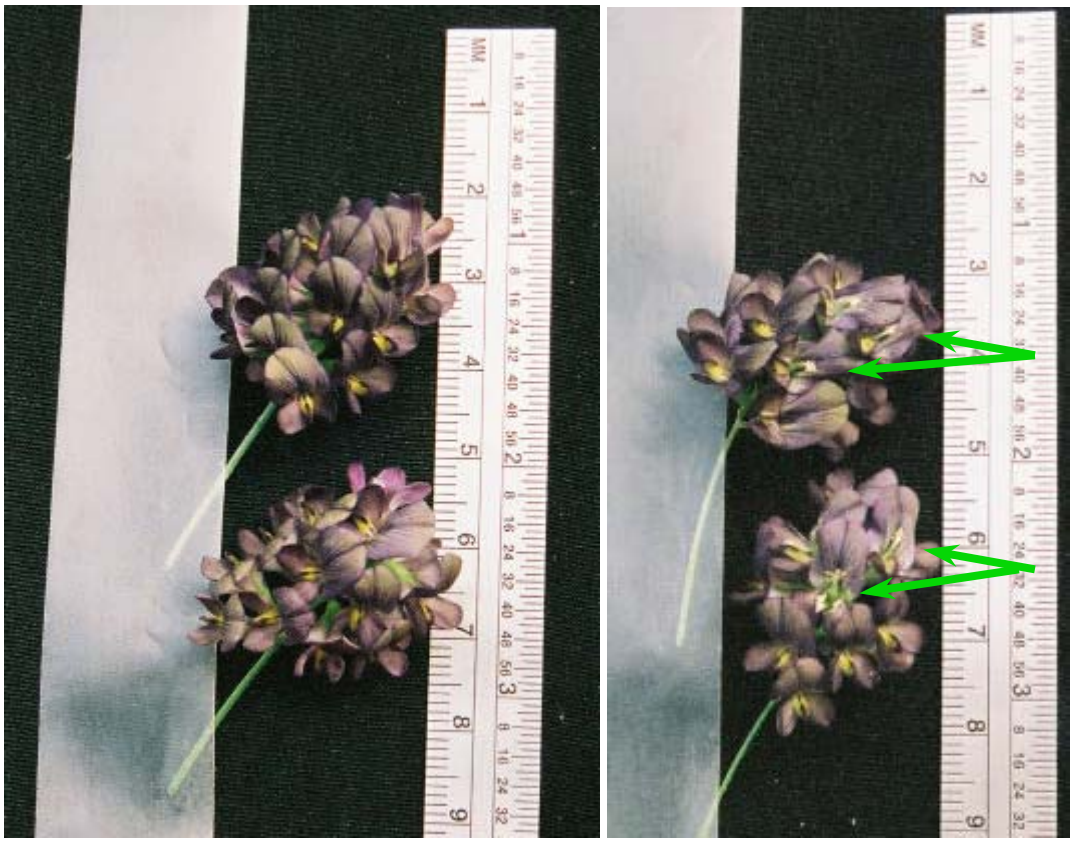


Figure VI-13. Event J101 and Control Whole Racemes.

Control (upper raceme) and Event J101 (lower raceme). In the left photograph, no flowers are tripped, whereas, in the right photograph, two flowers (indicated with arrows) in each raceme were mechanically tripped to reveal the sexual column.

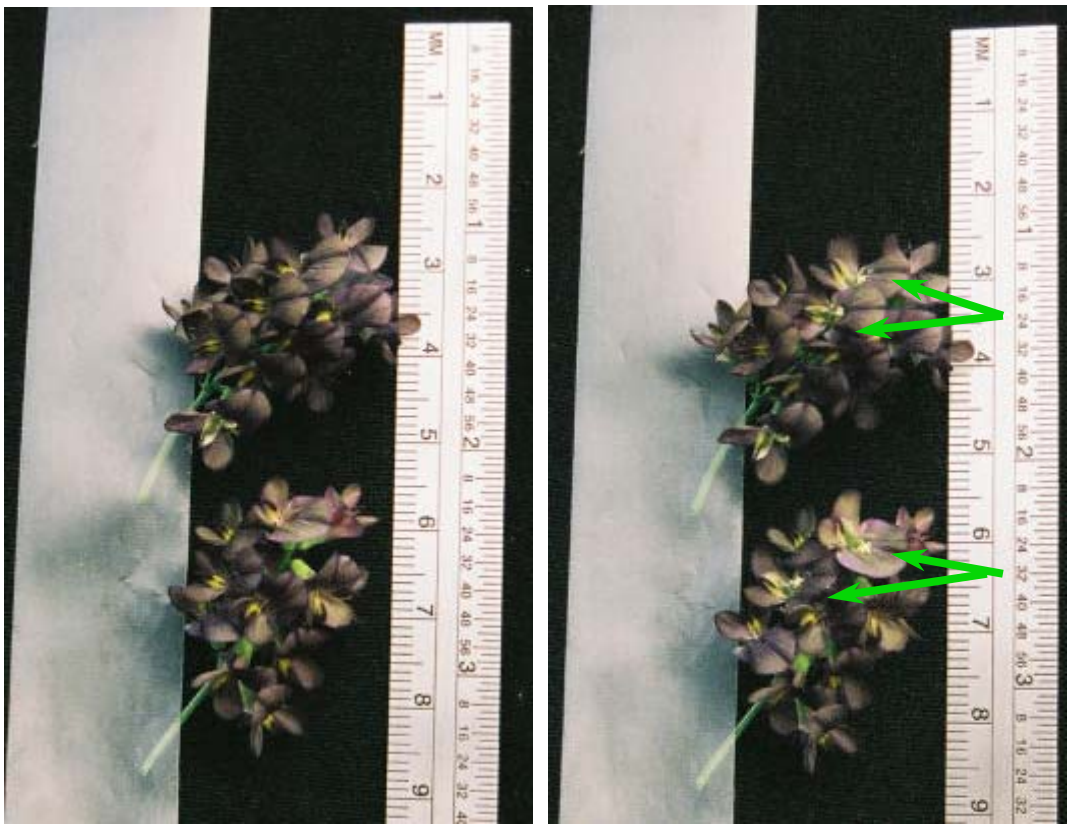


Figure VI-14. Event J163 and Control Whole Racemes.

Control (upper raceme) and Event J163 (lower raceme). In the left photograph, no flowers are tripped, whereas, in the right photograph, two flowers (indicated with arrows) in each raceme were mechanically tripped to reveal the sexual column.

Event J101 vs. Control **Event J163 vs. Control**

Figure VI-15. Raceme Attachment
Attachment of racemes for Test (left stem in each image) and Control (right stem in each image).

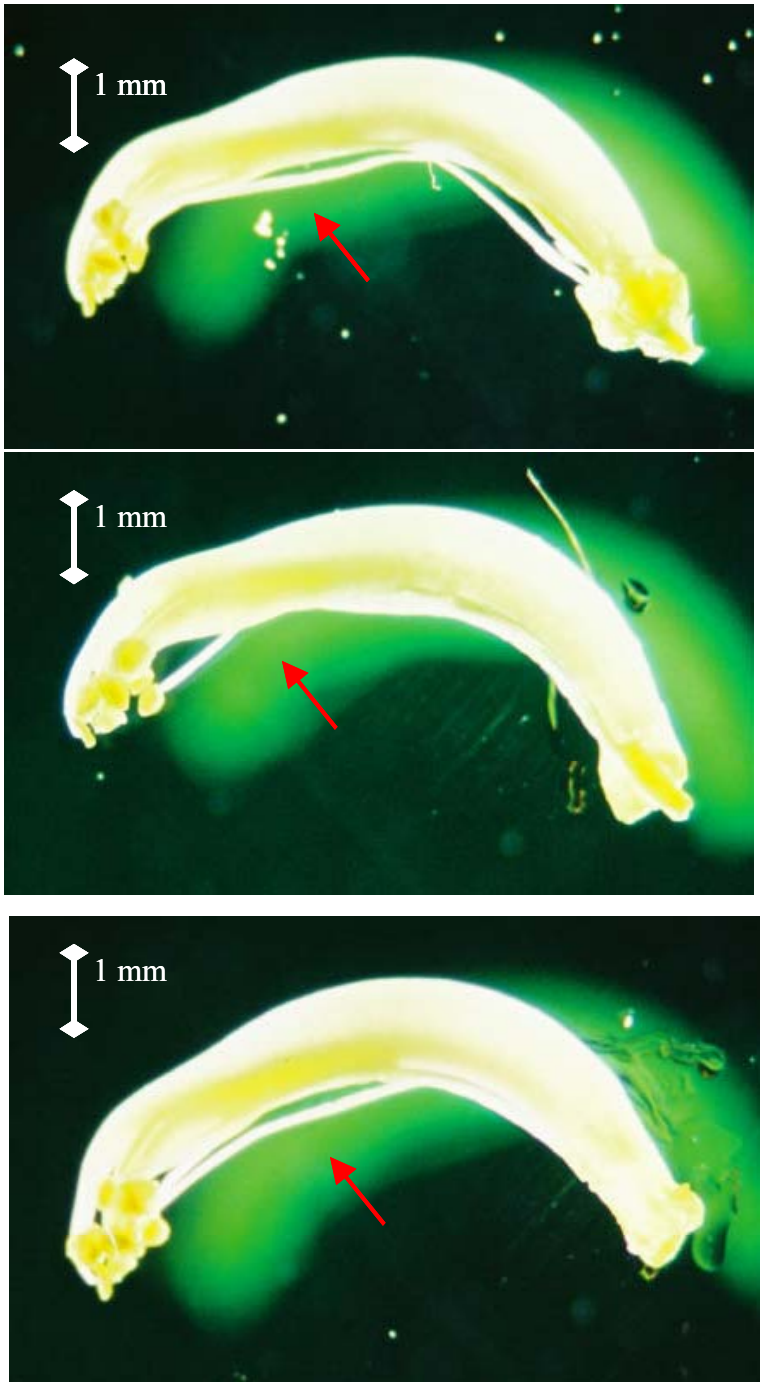
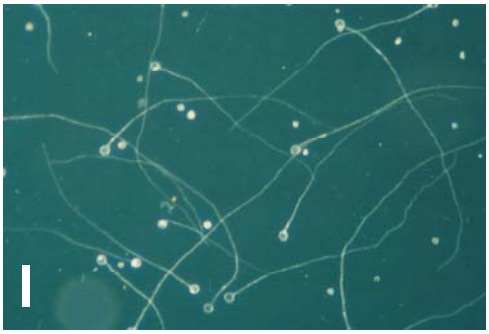


Figure VI-16. Excised Sexual Column of Event J101, Control and Event J163 Flowers.
Bar = 1 mm.

Event J101:



Control:

Event J163:

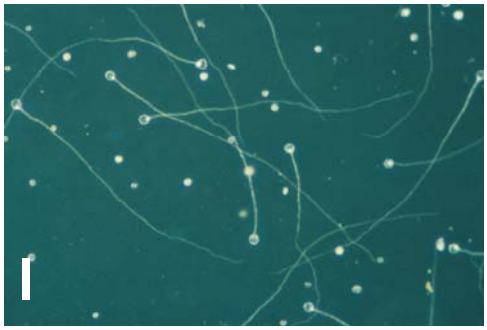


Figure VI-17. *In vitro* Pollen Germination of Control, J101 and J163 after 90 Minutes on Germination Medium at 25ºC.

Bar = ~ 250 µm

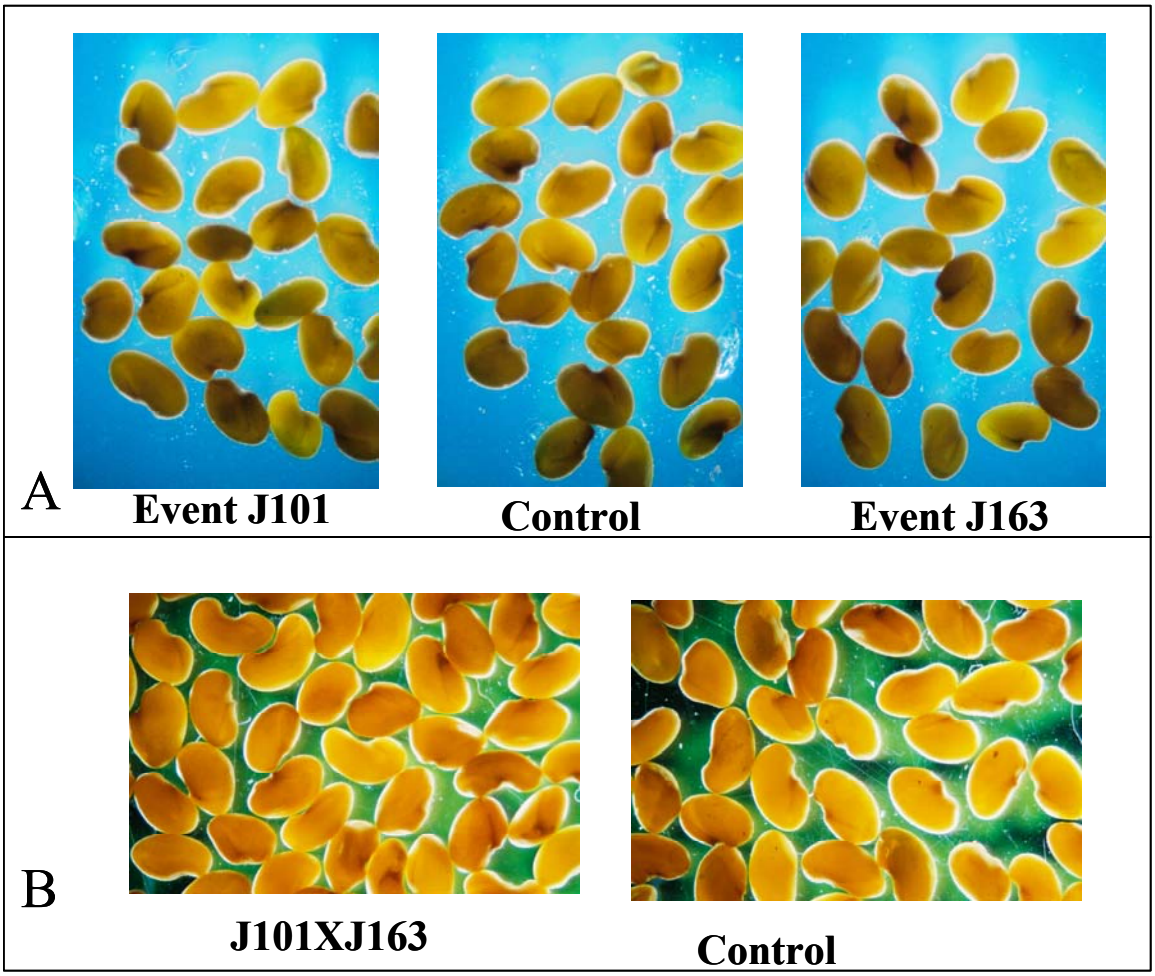


Figure VI-18. Seed of Control, J101, J163 and J101XJ163

Panel A shows the whole seed profile of J101, control and J163 seed. Panel B shows the whole seed profile of J101XJ163 and conventional alfalfa seed. Seed were photographed at 13X magnification.

E.2. Pollen Morphology / Viability

In this assessment, the morphology and viability of pollen from Roundup Ready alfalfa were compared to pollen from control alfalfa. Pollen viability and morphology were evaluated in two separate experiments. In the first experiment, pollen was obtained from a single T_0 ramet of an alfalfa plant containing event J101 or J163 and a single ramet of the nontransformed parental control alfalfa genotype (R2336). Multiple pollen grains were evaluated, and numerical comparisons of means and ranges were used to determine whether there was any change in the

morphology or viability of pollen produced by Roundup Ready alfalfa plants; however, these data were not appropriate for statistical analysis because pollen was not obtained from multiple plants. Therefore, a second experiment was conducted where the design allowed for statistical analyses to be performed. In this experiment, pollen was harvested from multiple ramets from the same T_0 vegetatively-propagated plants (clones) containing event J101, J163 or R2336 control and from non-isogenic paired event alfalfa populations. Viability data were subjected to statistical analyses. Methods and results summaries for both experiments are presented below.

E.2.a. Materials and methods; Experiment one

All plants were grown in a growth chamber. Test or control pollen was obtained from a single Roundup Ready alfalfa plant that was a vegetative propagule (ramet) from one of the T_0 initial transformants [Figure VI-8, Box 2 (test); Box 1 (control)]. Three racemes from each of the three source plants were collected. The individually selected racemes contained a minimum of 8-12 healthy, untripped florets in bloom. The three racemes were placed in a Petri dish containing water-moistened filter paper.

All open, untripped florets were removed from each of the three racemes per genotype and grouped together in a pile (i.e., each pile consisted of florets from a single genotype). Ten florets were non-selectively chosen from each pile for evaluation. Pollen from each floret was identified as a subsample.

Each of three glass microscope slides consisted of ten pollen subsamples from a single genotype. Ten water-repellent circles were drawn on each slide using a hydrophobic slide-marking pen. The circles sequestered the pollen and stain solution in defined locations on the slides and prevented cross-contamination of individual subsamples. A dissection microscope was used to dissect the florets and extract the pollen onto the slides. The petals were removed from the florets to expose the anthers. A single floret was pressed into the center of each water-repellant circle to trip the floret and release the pollen onto the slide. The pollen in each circle (ranging from approximately 300 to over 1000 grains) was evaluated as a single subsample. Twenty microliters of a 1:10 diluted stain solution (Alexander, 1969) were placed over the pollen in each circle, and the pollen was allowed to soak in the stain overnight at 4^{o}C before microscopy.

Three of the ten pollen subsamples from each genotype were then randomly selected for measurement. The subsamples were viewed under 200x magnification. Pollen visible under magnification was determined to be either viable or nonviable based on staining color (Alexander, 1969), in which viable pollen grains, which maintain a round shape in the hydrated state are stained red, while dead pollen grains are stained light blue and vary in the degree of dehydration based on collapse of the pollen wall. Ten representative, viable pollen grains per subsample were selected. Pollen diameter was measured on only viable pollen grains, using a reference scale within the microscope eyepiece. Pollen diameters were measured along the x- and y-axes, and the values were averaged to estimate pollen diameter. Digital photographs of three pollen subsamples were taken.

E.2.b. Results; Experiment one

The diameter of viable pollen grains from the test events and control is presented in Table VI-28. Pollen derived from Roundup Ready alfalfa event J101 had a mean pollen grain diameter of 32.1 μm and a range of 32.0-35.0 μm. Pollen derived from Roundup Ready alfalfa event J163 had a mean pollen grain diameter of 32.3 μm and a range of 32.0-35.0 μm. Pollen from the R2336 control had a mean pollen grain diameter of 32.1 μm and a range of 32.0-34.0 μm. Photographs of the three subsamples per genotype used in this assessment are reproduced in Figures VI-19, VI-20, and VI-21. The photographs provide a visual representation of the data and do not necessarily capture all of the pollen within the subsamples. No gross differences in pollen diameter or overall pollen morphology were observed in these samples.

Data on the viability of pollen collected from the control and test events are presented in Table VI-28. Pollen derived from Roundup Ready alfalfa event J101 had a mean pollen viability of 69% and a range of 57-78%. Pollen derived from Roundup Ready alfalfa event J163 had a mean pollen viability of 57% and a range of 50-70%. Pollen derived from the R2336 control had a mean pollen viability of 58% and a range of 50-64%. No gross differences in pollen viability were observed.

E.2.c. Materials and methods; Experiment two

All plants were grown in a greenhouse. For each of the Roundup Ready test events J101 or J163, pollen was obtained from three Roundup Ready alfalfa plants that were genotypically identical vegetative propagules (ramets belonging to the same clone) from either J101 or J163 T_0 initial transformants (clone mother-plants) (Figure VI-8, Box 2) and the control material was likewise from three vegatative propagules of the non-transformed parental genotype R2336 (clone mother-plant) (Figure VI-8, Box 1). Ramets were maintained in pots (one plant per pot with three pots per clone). In addition, and separately within the same greenhouse, J101XJ163 and conventional control plants were grown from seed planted in flats (approximately 190 to 200 plants/flat). The seeded test and control populations were FGI experimental Syn 1 varieties and were derived from a common germplasm source. The flats containing the J101XJ163 population were treated with glyphosate to remove null segregants (approximately 5%) prior to sampling. Pollen harvest and staining were essentially identical to that described for experiment one. For the vegetatively propagated plants, six individual flowers were collected from each of the three pots within each of the three clones in the study. For the seeded flats, six individual flowers were non-selectively collected from a separate plant within each of three flats grown for the test or control. Each of the five means in the study was based on 18 pollen samples (i.e., three replicates of six flowers per material).

There were two statistical analyses of variance completed; both were conducted using analysis of variance for a randomized complete block design with three replications and six observations per replicate as follows. One anaysis compared the vegetatively propagated isogenic plants (J101 versus control, and J163 versus control), and a separate statistical analysis compared the confirmatory J101XJ163 population to a conventional control population grown from seed. Separate analyses were done because different control groups were used. Both statistical analyses were conducted using Statixtix® for Windows (Version 2.2) analytical software (Analytical Software, Tallahassee, FL).

E.2.d. Results; Experiment two

The mean and range of percentage of viable pollen, the observed range of values and the standard deviation of the mean are presented for the controls, J101, J163 and J101XJ163 in Table VI-29. The sample T-test P-value statistic for each comparison is also given. There were no differences ($P \geq 0.05$) between test and control materials for percentage of viable pollen in either comparison group. Mean pollen viability was, 63.7, 65.3, 61.8 percent for J101, J163 and control clones, respectively (Table VI-29). The J101XJ163 and conventional control populations had 64.6 and 64.9 percent viable pollen, respectively (Table VI-29).

E.2.e. Overall conclusion/Discussion

Pollen viability was evaluated in two separate experiments. In the first experiment, the pollen from ten flowers per plant was evaluated, but only one plant of each test or control line was used. The diameter, morphology and viability of pollen obtained from Roundup Ready plants was comparable to that obtained from control alfalfa. These data were not subjected to statistical analysis because there was no replication of test and control plants. Pollen viability results from the first experiment demonstrated a high degree of within-plant variability (sub sample means varied by ±14 to 21%) but, in general, the data indicated that mean pollen viability for J101 or J163 was similar to or slightly greater than the control mean. In the second experiment, plants were replicated, the number of samples evaluated was greater and pollen viability between test and control was statistically analyzed. On the basis of these data, it is concluded that the mean diameter of the pollen and the percentage viable pollen produced by J101 or J163 Roundup Ready test plants was not different from the isogenic control (R2336) mean. Data from the J101XJ163 population and the conventional control population also confirmed that there is no effect of the J101 or J163 events on pollen viability in alfalfa.

Results from these experiments indicate that Roundup Ready alfalfa J101 and J163 have similar size and percentage viable pollen to control alfalfa. Further, the confirmatory data on the paired event J101XJ163 population had similar percentage viable pollen compared to pollen from a control population. There were no statistically significant differences in the size or viability of pollen produced by Roundup Ready alfalfa compared to control alfalfa. On the basis of this information, no increased pest potential for alfalfa populations containing events J101 and J163 would be expected.

Table VI-28. Mean and Range for Pollen Diameter and Pollen Viability of Roundup Ready Events J101 (T_0), J163 (T_0) and Control (Experiment One).

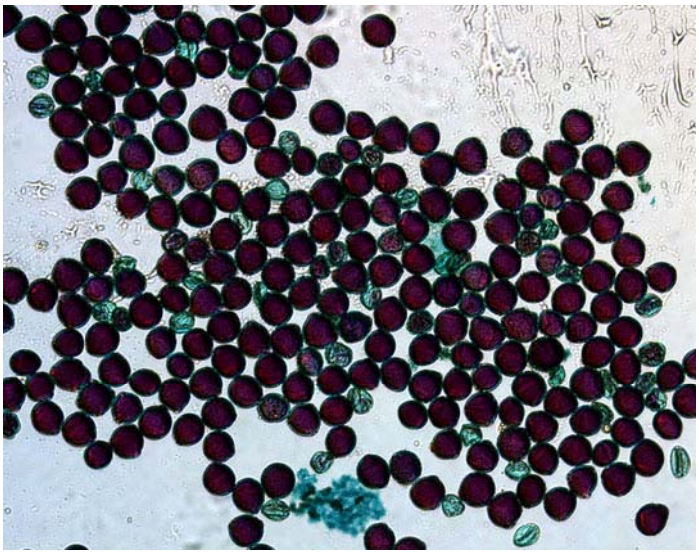
	Pollen Diameter[1]			Viable Pollen[2]		
Test or Control Pollen[3]	**Mean[4]**	**Min.**	**Max.**	**Mean[5]**	**Min.**	**Max.**
	μM			Percent		
J101	32.1	32.0	35.0	69	57	78
J163	32.3	32.0	35.0	57	50	70
Control (R2336)	32.1	32.0	34.0	58	50	64

[1] Pollen diameter was evaluated only for viable pollen grains. The diameter of each pollen grain was defined as the average length of the x- and y-axes.
[2] Viable pollen grains were stained red; nonviable pollen grains were stained light blue.
[3] J101 and J163 contain the *cp4 epsps* coding sequence; R2336 does not contain the *cp4 epsps* coding sequence.
[4]Mean and range of values based on test or control pollen collected from thirty pollen grains (n=30).
[5]Mean and range of values based on test or control pollen collected from approximately 750 pollen grains. (n=750)

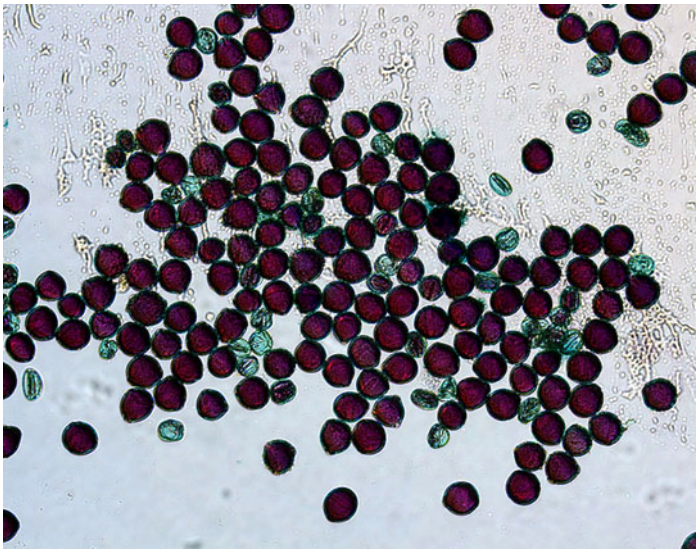
Table VI-29. Percentage Viable Pollen of Roundup Ready Events J101 (T_0), J163 (T_0), Control (R2336), and Confirmatory J101XJ163 Populations of and Control (Experiment Two).

	Viable Pollen[1]		
Test or Control Pollen	**Mean ± SD**	**Min.**	**Max.**
J101	63.7 ± 7.9[2]	52	89
J163	65.3 ± 11.4	51	87
Control (R2336)	61.8 ± 7.3	47	75
J101XJ163	64.6 ± 5.6[3]	56	74
Control (conventional)	64.9 ± 8.3	47	76

[1]Viable pollen grains were stained red; nonviable pollen grains were stained light blue.
[2]Mean, standard deviation (S.D.) and range of values are based on 3 replicates of 6 samples per clone (n=18 observations). Means were nonsignificantly (n.s.) different [P-value=0.4067 for clones; LSD (0.05)=5.1%]. Statistical comparisons were made between J101 and the control (R2336) and between J163 and control (R2336).
[3]Mean, standard deviation (S.D.) and range of values are based on 3 replicates of 6 samples per population (n=18 observations). Means were nonsignificantly (n.s.) different [P-value=0.8717 for clones; LSD (0.05)=4.9%]. Statistical comparisons were made between J101XJ163 and the control (experimental Syn 1 generation populations for test and conventional control materials).



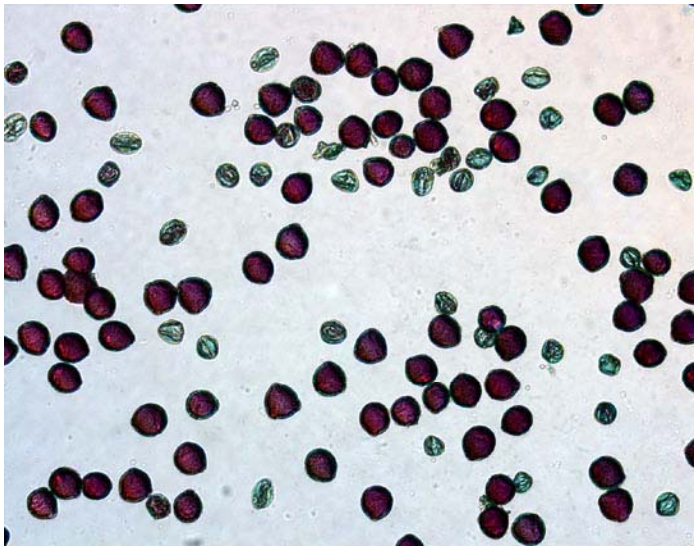
Subsample #1



Subsample #2

Figure VI-19. Overall Morphology of Three Subsamples of Alfalfa Pollen from the Single Source Plant Containing Event J101 under 200X Magnification.

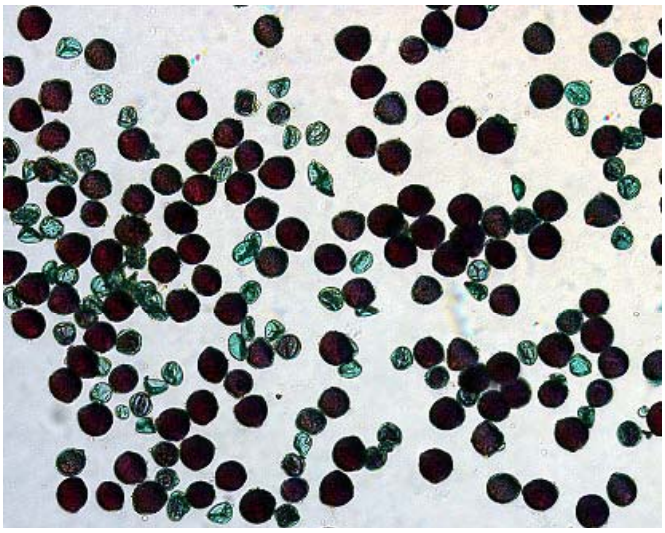
Note: The larger, dark pollen grains are viable; the smaller, shriveled, lighter pollen grains are nonviable.



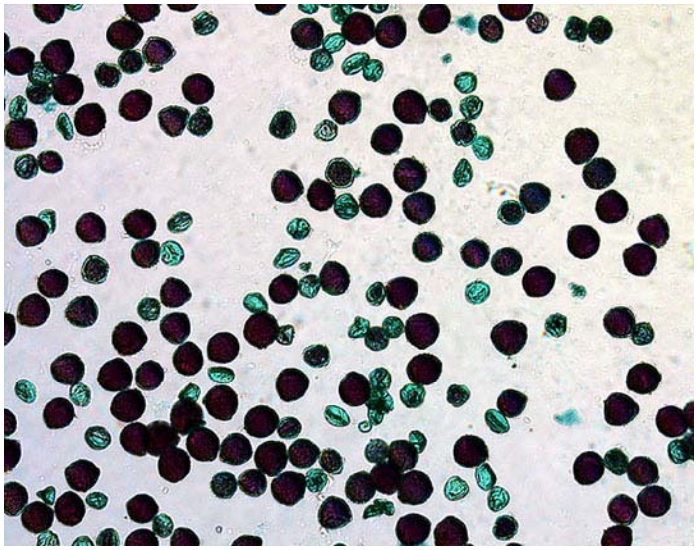
Subsample #3

Figure VI-19 (continued). Overall Morphology of Three Subsamples of Alfalfa Pollen from the Single Source Plant Containing Event J101 under 200X Magnification.

Note: The larger, dark pollen grains are viable; the smaller, shriveled, lighter pollen grains are nonviable.



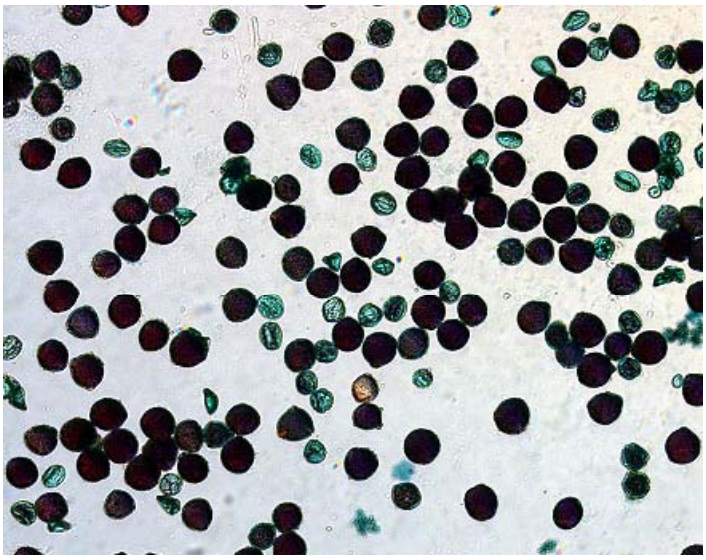
Subsample #1



Subsample #2

Figure VI-20. Overall Morphology of Three Subsamples of Alfalfa Pollen from the Single Source Plant Containing Event J163 under 200X Magnification.

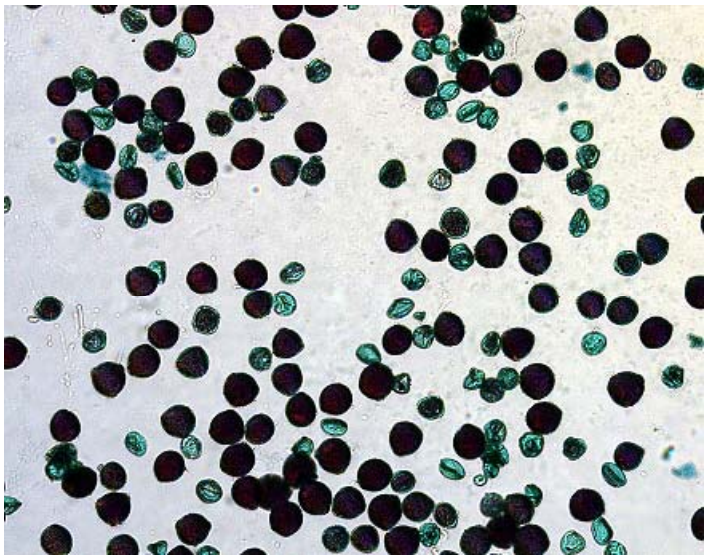
Note: The larger, dark pollen grains are viable; the smaller, shriveled, lighter pollen grains are nonviable.



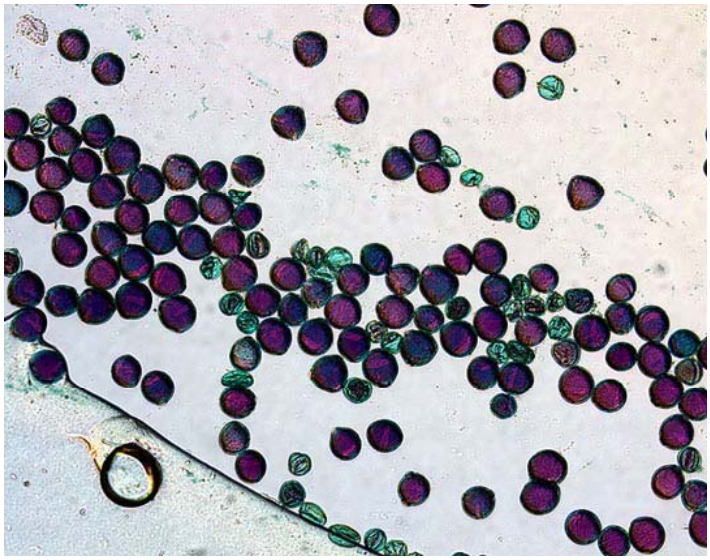
Subsample #3

Figure VI-20 (continued). Overall Morphology of Three Subsamples of Alfalfa Pollen from the Single Source Plant Containing Event J163 under 200X Magnification.

Note: The larger, dark pollen grains are viable; the smaller, shriveled, lighter pollen grains are nonviable.



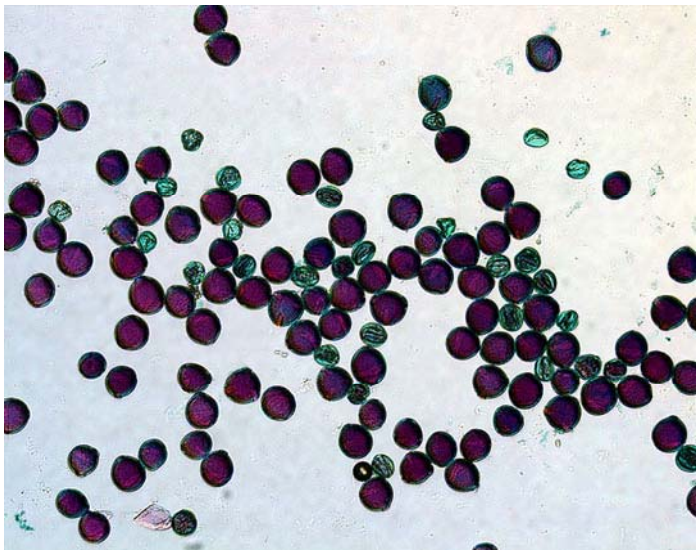
Subsample #1



Subsample #2

Figure VI-21. Overall Morphology of Three Subsamples of Alfalfa Pollen from the Single Control Plant under 200X Magnification.

Note: The larger, dark pollen grains are viable; the smaller, shriveled, lighter pollen grains are nonviable.



Subsample #3

Figure VI-21 (continued). Overall Morphology of Three Subsamples of Alfalfa Pollen from the Single Control Plant under 200X Magnification.

Note: The larger, dark pollen grains are viable; the smaller, shriveled, lighter pollen grains are nonviable.

F. Seed Yield

High fecundity may be associated with increased weediness potential. A significant change in seed yield for alfalfa plants containing the Roundup Ready events could indicate a change in the weediness potential of alfalfa through an change in reproductive potential. Therefore, seed yield of the alfalfa populations containing event J101 or J163 was evaluated.

Seed yield was compared between alfalfa populations containing events J101 or J163 and control or reference alfalfa populations. Seed yield was assessed under both greenhouse and field conditions. The greenhouse represented an environment where both pollinator and environmental variables could be more highly controlled than in a field situation. Sections F.1, F.2 and F.3 below present seed yield data derived from greenhouse and field experiments conducted with alfalfa plants containing events J101, J163 and control or reference alfalfa populations. In addition, confirmatory data are presented from populations containing both events.

F.1. Greenhouse Seed Yield

The seed yield characteristics of test alfalfa populations containing Roundup Ready alfalfa events J101 or J163 were compared to those of control and reference alfalfa populations. All plants were grown in a randomized complete block design in which hand-pollination was used to produce seed. Seed yield (g) and seed weight (mg/seed)

were measured and the mean number of seed per flower pollinated was calculated. Forage Genetics International, West Salem, Wisconsin, conducted this experiment.

F.1.a. Test, control and reference populations

The test materials were single-event, synthetic generation 1 (Syn 1) populations containing either event J101 or event J163 (Figure VI-8, Box 4, null segregants removed). The base populations were not homozygous and therefore segregated for the glyphosate tolerance trait. So that all test plants in the study contained the event of interest, the nontolerant, null segregants were removed from each test population by application of Roundup UltraMAX herbicide (3 lb a.e./A rate, applied with a hand-sprayer 16 days after planting). Tolerant plants were transplanted to greenhouse pots and hand-pollinated. There were no remaining null segregating plants in the tested populations, i.e., all test plants were Roundup Ready.

The control was the null segregant (nulliplex) Syn 1 population (Figure VI-8, Box 7).

The reference populations, RV207 (FGI-4S33) and RV308 (FGI-4S41), were two FGI conventional, Syn 1 experimental alfalfa populations. The reference populations (conventional experimental varieties) were chosen from representative conventional alfalfa varieties.

F.1.b. Methods

Alfalfa seeds were planted in the greenhouse at FGI, West Salem, Wisconsin, and grown under a 16- to18-hour photoperiod at 70-76^{o}F. Each of the test, control and reference populations was replicated six times in a randomized complete block (RCB) experiment, with ten 6-inch pots/replicate and three plants/pot (i.e., 30 plants per experimental unit). The 30 plants within an experimental unit were randomly intercrossed (cross-pollinated) by hand. The hand-pollination method attempted to mimic the random, cross-pollination activities of a bee, as described below.

Alfalfa flowers must be mechanically opened or tripped to affect pollination (Rincker et al., 1988). Each alfalfa flower is perfect (pollen and ovules are within the same flower); however, most alfalfa plants are genetically self-incompatible and require cross-pollination with pollen from another genotype to form seed. In nature, cross-pollination is facilitated by certain species of pollinating bees. In the greenhouse, tripping may be accomplished by hand using any small, stiff instrument (such as a toothpick) to mimic the action of bees. In this study, hand-pollinations were accomplished using handmade, disposable tools called pollen boats. Pollen boats were made from fine grit emery paper, ¾ x 2 inch strips, cut to a point on one end. The pointed end of the pollen boat was deeply inserted along the axis of the standard petal, which caused the flower to trip and pollinate. In the same motion, the anthers deposited a mass of pollen on the tip of the tool. The pollen-laden tool was reused for all pollinations within the experimental unit that took place on that day (approximately a two-hour period of time), thereby cross-pollinating the flowers with fresh pollen from other members of the same 30-plant experimental unit. The number of flowers pollinated per day was recorded and was dependent upon the number of fresh, open flowers available in the group (approximately 200 to 350 per day). Each group was pollinated at three- or four-day intervals until a minimum cumulative total of 1000 pollinations were completed.

Seed was harvested when the pods and seeds had fully ripened. Seed from all plants in an experimental unit was bulked, air dried for two days at 110^{o}F, hand-threshed to remove pod material, and weighed. Seed yield was compared by adjusting total seed yield (grams) by the exact total number of flowers pollinated per replicate X 1000, thereby calculating the seed (g) produced per 1000 pollinations. Mean seed weight (mg/seed) for each experimental unit was measured by counting the number of seeds in a small seed sample (0.5-1.0 gram). The number of seed produced per flower was calculated by dividing the total number of seeds produced by the number of pollinations for the experimental unit.

F.1.c. Results and discussion

Individual comparisons of the two single-event test populations, J101 and J163, to the control population and to the mean of reference variety populations are presented in Table VI-30. All plants within the study bloomed abundantly and, after hand cross-pollination, produced normal, tightly-coiled pods and normal seeds.

Mean seed yield, mean seed weight and the mean number of seeds produced per flower pollinated were not different ($P \geq 0.05$) between the Roundup Ready alfalfa populations containing event J101 and J163 and the control and reference populations. These data indicate that plants containing Roundup Ready alfalfa events J101 and J163 have similar seed yield potential, seed weight and seed production per flower when compared to the control and/or reference plants. On the basis of these data, it was concluded that Roundup Ready alfalfa events J101 and J163 did not affect seed yield characteristics.

Table VI-30. Greenhouse Study Seed Characteristic Data for Roundup Ready Events J101 and J163, Control and Reference Populations.

	Seed yield (g/1000 pollinations)*†		Seed weight (mg/seed) *†		Seed per flower*†	
Population	**Mean** [1]	**Range**	**Mean** [1]	**Range**	**Mean** [1]	**Range**
J101	**11.0** ± 2.8	6.3 – 14.0	**2.45** ± 0.12	2.32 – 2.64	**4.50** ± 1.12	2.57 – 5.71
J163	**8.9** ± 1.3	7.4 – 10.2	**2.49** ± 0.08	2.41 – 2.60	**3.59** ± 0.51	2.96 – 4.10
Control	**11.4** ± 2.8	7.3 – 14.7	**2.51** ± 0.12	2.30 – 2.61	**4.54** ± 1.12	2.91 – 5.86
Reference	**11.2** ± 2.1	8.1 – 15.1	**2.38** ± 0.19	2.13 – 2.84	**4.70** ± 0.92	3.37 – 6.38

*† There were no statistically significant differences between either single-event population (J101 or J163) and the control population or the reference population ($P \geq 0.05$).
[1]Mean seed yield (g) per 1000 pollinations ± standard deviation of the mean with n=6 replications, except for the reference mean, where data was combined for the two reference populations (n=12).

F.2. Seed Yield from Field-Grown Alfalfa Plants; Commercial Breeding Populations; Trial One

The seed yield characteristics of test alfalfa populations containing Roundup Ready alfalfa event J101 or J163 were compared to those of control and reference alfalfa populations. All plants were grown in a randomized complete block design field study in which plants were pollinated using leafcutter bees to produce the seed. This experiment was conducted by Forage Genetics International in Idaho, during 1999 under USDA Notification Number 99-047-03n.

F.2.a. Test, control and reference populations

The test materials were single-event, simplex MBC1 populations containing either event J101 or event J163 (Figure VI-8, positive Roundup Ready segregants from Box 3). The populations were not homozygous and therefore segregated for the Roundup herbicide tolerance trait. So that all test plants in the study contained the event of interest, the nontolerant, null segregants were removed from each test population by application of Roundup UltraMAX herbicide (3 lb a.e./A rate, applied with a hand-sprayer 16 days after planting). Tolerant plants were transplanted to the field. There were no remaining null segregating plants in the tested populations, i.e., all test plants were Roundup Ready.

The control was the null segregant MBC1 population (Figure VI-8, null segregants from Box 3).

The reference population was comprised of two conventional commercial alfalfa varieties (DK134 and Sutter) that represented a range of traditional alfalfa varieties.

F.2.b. Methods

Alfalfa plants were hand-transplanted to the field at Forage Genetics International's Research Facility in Idaho, in a randomized complete block design experiment with six replicates and five plants/replicate. Each experimental unit was an eight-foot-long, space-transplanted row (five plants with 24 inches between plants within rows and 30 inches between rows). Weeds were controlled throughout the experiment's duration with mechanical cultivation and the plots were managed according to locally recommended practices for alfalfa seed production (e.g., flood irrigation, fertility, insect pest control, pollinator management, desiccant application, etc.). The field was spatially isolated from all other alfalfa, and allowed to produce seed. At the 10% bloom stage, newly emerging leafcutter bee pupae were placed in a commercial-style domicile in which the bees were sheltered in predrilled nesting boards. The bee stocking rate was gradually increased as bloom became more abundant and was maintained at a maximum stocking rate of approximately two gallons of loose cell pupae per acre—the recommended stocking rate for full bloom alfalfa seed fields in the area. Plants were pollinated for six weeks, sprayed with chemical desiccant, harvested seven days later using hand equipment, air-dried (approximately 110^{o}F), threshed on research-scale equipment, and weighed. Mean seed yield (g) per plant was calculated for each experimental unit.

F.2.c. Results and discussion

Individual comparisons of the two single-event test populations J101 or J163 to the control population and to the mean of reference population are presented in Table VI-31. There were no statistically significant differences ($P \geq 0.05$). All plants bloomed and, after pollination, produced normal, tightly-coiled pods and normal seeds.

Mean seed yield per plant was not different between Roundup Ready alfalfa events J101 and J163, and the control, and reference populations. These data indicate that alfalfa plants that contain the Roundup Ready alfalfa events J101 or J163 have similar seed yield potential when compared to the control and the reference plants.

Table VI-31. Seed Yield of Roundup Ready Event J101, Event J163, Control, and Reference Populations Grown in the Field in Idaho, in 1999.

Population	Seed Yield (g/plant) Mean [1, 2] ± SD	Range (g/plant)
J101	20.40 ± 3.61	13 - 23
J163	16.27 ± 6.78	8 - 26
Control	18.73 ± 8.69	3 - 28
Reference	18.43 ± 6.62	5 - 29

[1] There were no statistically significant differences between either single-event population (J101 or J163) compared to the control population or compared to the reference population ($P \geq 0.05$).

[2] Mean seed yield ± standard deviation (SD) of the mean with n=6 replications, except for the reference, where four reference populations were pooled, n=24.

Data developed under USDA Notification Number: 99-047-03n.

F.3. Confirmatory Seed Yield from Field-Grown Alfalfa Plants: Commercial Breeding Populations; Trial Two

The seed yield of a commercially destined, paired-event Roundup Ready alfalfa (J101XJ163) test population was compared to that of three conventional control alfalfa populations. Seed production plots were grown by Forage Genetics International in Idaho, during 2001 and plants were pollinated by leafcutter bees (*Megachile rotundata* F.). These data were developed using seed production practices commonly used by alfalfa breeders during variety development and are provided as confirmatory information. This trial was conducted under USDA Notification Number 01-058-10n.

F.3.a. Test and control/reference materials

The test material was a paired-event (J101XJ163) breeding population (Figure VI-8, Box 10, null segregants removed). Specifically, the test plants on which the seed yields were measured, were simultaneously being used to produce Syn 1 Roundup Ready alfalfa breeder generation seed (i.e., the seed yield was measured on the Syn 0 parent plants of commercial variety). The test population set was comprised of four similar subpopulations: all four were derived from hay-type, fall-dormant base germplasm and all plants contained copies of both Roundup Ready alfalfa events J101 and J163, i.e., plants were dihomogenous (Samac and Temple, in press). Each of the four test subpopulations was an experimental Roundup Ready alfalfa variety that was under development for potential commercial use, i.e., four Syn 0 subpopulations were pollinated to produce four Syn 1 (breeder generation) seed lots analogous to the control subpopulations described in the next paragraph.

There were three control population sets (or groups) used in this study: Set 1) hay-type, non dormant germplasm group with 15 unique Syn 0 subpopulations (Location 1); Set 2) hay-type, fall-dormant germplasm group with 15 unique Syn 0 subpopulations within Set 2 (Location 2); and, Set 3) hay-type fall-dormant germplasm group with 63 unique Syn 0

subpopulations (Location 3). Each of the 93 control subpopulations was a different experimental conventional variety that was under development as a potential commercial variety, i.e., FGI produced 93 Syn 0 conventional subpopulations divided across three seed increase locations where they were pollinated to produce 93 individual Syn 1 (breeder generation) seed lots. The control populations also served as the reference populations for this study.

F.3.b. Results and discussion

Seed yield data are presented in Table VI-32. No statistical comparisons were made. Mean seed yield per plant was numerically very similar between the test population (28.25 g/plant) and two of the three control populations (27.13 and 28.27 g/plant). Control population Set 3 had lower seed production per plant compared to the test and the other two control populations. Control Set 3 may have had lower seed yield than the other populations because of location-related factors such as uncontrolled environmental variation (soil moisture, soil type), variation in pest insect levels, or differences in pollinator density or activity. Seed yield of alfalfa is highly influenced by environment, cultural management and pollinator activity (Rincker et al., 1988). For all subpopulations, general growth observations indicated that plants appeared phenotypically true to type regardless of the presence or absence of the test events. The mean amount of seed produced per plant for the Roundup Ready alfalfa test population was within the range of values observed for each of the three control populations and was near the midpoint of the value range for two of the three control populations.

It was concluded that the Roundup Ready events J101 and J163, when combined by using traditional breeding methods, did not alter the seed yield potential of alfalfa. These data support the findings of related previously described field and greenhouse studies in which the seed yield single-event of Roundup Ready alfalfa populations was not different from control and reference alfalfa populations.

Table VI-32. Confirmatory Seed Yield of the Roundup Ready Paired-Event Test Population (J101XJ163) and Three Conventional Control Populations Grown with Spatial Isolation at Four Locations in Idaho, in 2001.

Population	Seed Yield (g/plant) Mean[1] ± SD	Range (g/plant)
Control Set 1 (Location 1)	27.13 ± 5.99	19 - 35
Control Set 2 (Location 2)	28.27 ± 6.32	16 - 37
Control Set 3 (Location 3)	16.51 ± 4.85	5 - 30
Test Set: J101XJ163 (Location 4)[2]	28.25 ± 2.75	25 - 31

[1]Mean and standard deviation (SD) for n=4 test subpopulations, and n=15, 15 or 63 control subpopulations for sets 1, 2, 3, respectively.
[2]Location four conducted under USDA Notification Number: 01-058-10n.

G. Field Testing and USDA-APHIS Reports

The agronomic evaluation of events J101 and J163 and other alfalfa plants with the Roundup Ready trait also included observational information on disease/pest susceptibility and phenotypic assessments from product evaluation field trials conducted over six growing seasons. These observations provide confirmatory information to the quantitative agronomic characterization data provided in Sections A through G of this Part VI.

The confirmatory information includes a qualitative assessment between events J101 and J163 and the control line or commercial alfalfa varieties observed in U.S. field trials. These field trials were established for several product evaluation purposes, including yield testing, efficacy evaluation (weed control), genotype evaluation, glyphosate-tolerance testing, volunteer control, glyphosate residue trials, etc. The field designs for these trials varied, with some field trials being replicated at multiple sites, while other trials were nonreplicated single sites. Similarly, the comparator for events J101 and J163 in these trials varied according to the purpose of the trial. These field trials were conducted under USDA-APHIS notifications between 1998 and 2003, as presented in Table VI-33.

The broad geographic distribution of the Roundup Ready alfalfa test sites in the U.S. has exposed the test, control and reference materials to a wide range of naturally occurring diseases and disease complexes. The major diseases of economic importance in the U.S. are those alfalfa pathogens that impact the foliar, crown, root, vascular and seedling health of alfalfa plants. The majority of alfalfa diseases are caused by fungi. However, nematodes, bacteria, viruses and other microbes also incite economic losses in alfalfa production (Leath et al., 1988). The major economic diseases that occurred in the test locations included, but were not limited to: seedling damping-off (e.g., fungal genera

such as *Pythium, Phytophthora, Aphanomyces*); foliar diseases (e.g., fungal genera such as *Leptosphaerulina, Colletotrichum, Peronospora, Phoma, Stemphylium, Cercospora*, and stem nematodes like *Ditylenchus*); and root rots, vascular wilts and crown diseases (e.g., fungal genera such as *Phytophthora, Verticillium, Fusarium, Phoma*, and bacterial wilt caused by *Clavibacter*).

The major insect pest species that are economically important in alfalfa vary widely among regions in the U.S. The broad geographic distribution of the Roundup Ready alfalfa test sites in the U.S. has exposed the test, control and reference materials to a wide range of naturally occurring insect pests. The major economic insects included, but were not limited to: potato leafhoppers (*Empoasca fabae*), aphids [pea (*Acyrthosiphon pisum*), blue (*A. kondoi*) and spotted alfalfa aphids (*Therioaphis maculata*)], alfalfa weevil (*Hypera postica*), lygus bugs (*Lygus* species), other plant bug species (family *Miridae*) and alfalfa caterpillars (various lepidopteran species). The results of the disease and pest susceptibility observations were provided in the final reports submitted to USDA-APHIS at the conclusion of the notification period for each field trial listed in Table VI-33. The results from these observations consistently showed no meaningful differences in the disease and insect susceptibility between events J101 and J163 or synthetic populations developed using both events and the conventional control lines or commercial reference varieties. While occasional differences were noted at some field sites, there were no concurrent trends of differences across field sites or years, which indicates the few observed differences were likely due to random experimental variation.

Similarly, the results of the agronomic observations provided in the final reports submitted to USDA-APHIS showed no meaningful differences in the agronomic characteristics assessed between events J101, J163 or synthetic populations developed using both events and the conventional control line or commercial reference varieties. While occasional differences were noted at some field sites, there were no concurrent trends of differences across field sites or years, which indicate that the few observed differences were likely due to random experimental variation.

These observational data corroborate the conclusion of no enhanced pest or weediness potential of Roundup Ready alfalfa events J101 or J163 compared to conventional alfalfa, as discussed in Subsections A through G of this Section VI.

Table VI-33. USDA Notifications Relevant to the Field-Testing of Events J101 and J163.

Year	USDA Notification Number	Approved Release Sites (by state) Covered by Notification
1998 Field Trial	98-093-08n	IA, ID, IN, WI
1999 Field Trials	99-047-03n	IA, ID, IN, WI
2000 Field Trials	00-040-10n	WA
	00-053-07n	ID
	00-053-14n	IA, WI
	00-053-17n	IA, ID, IN, WI
	00-055-03n	CA, WA
	00-063-18n	IA, ID, IN, WI
	00-069-04n	WI
	00-139-01n	IA, ID, IN, WI
	00-139-02n	CA
	00-171-02n	IN, PA
	00-182-04n	CA, IA, IL, NY, WA
	00-207-01n	UT
	00-243-06n	CA
	00-272-04n	CA
2001 Field Trials	00-009-08n	ID
	01-009-04n	IA, WI
	01-009-05n	WI
	01-010-09n	IA, IN, WI
	01-011-03n	MN, ND, NE, VA, WI
	01-016-33n	ID, IN, WI
	01-017-08n	PA
	01-017-09n	ID , WA
	01-053-08n	CA,WA
	01-058-09n	NY, PA
	01-058-10n	ID
	01-080-05n	IA, MO, NE, NY, OH, PA, WI
	01-092-07n	CO, ID, OR, UT
	01-092-08n	CA
	01-107-01n	ID

Table VI-33 (continued). USDA Notifications Relevant to the Field Testing of Events J101 and J163.

Year	USDA Notification Number	Approved Release Sites (by state) Covered by Notification
2001 Field Trials (cont'd)	01-108-09n	CA
	01-010-09n	IA, IN, WI
	01-011-03n	MN, ND, NE, VA, WI
	01-156-01n	AZ
	01-159-01n	MI
	01-163-01n	UT
	01-163-02n	CA, IA, IL, NY, WA
	01-164-07n	CO
	01-205-04n	CA
	01-205-05n	CA
	01-211-06n	SD
	01-164-01n	WA
	01-164-02n	ID
	01-164-04n	IA
2002 Field Trials	02-004-12n	WI
	02-004-13n	WI
	02-004-14n	ID
	02-004-17n	PA
	02-010-08n	IN
	02-010-09n	ID
	02-010-11n	IA
	02-011-01n	MT
	02-011-02n	OK
	02-028-30n	NE
	02-044-11n	ID
	02-046-16n	TX
	02-046-19n	TX
	02-046-22n	CA
	02-051-11n	CA
	02-051-24n	MN
	02-051-27n	CT, MA
	02-052-06n	IA, MN
	02-056-08n	TX

Table VI-33 (continued). USDA Notifications Relevant to the Field Testing of Events J101 and J163.

Year	USDA Notification Number	Approved Release Sites (by state) Covered by Notification
2002 Field Trials (cont'd)	02-056-12n	ID
	02-060-08n	IA
	02-060-09n	ID
	02-077-14n	TX
	02-077-22n	WA
	02-078-04n	TX
	02-093-09n	NM
	02-099-01n	CO, ID, UT, OR
	02-170-02n	CA
	02-205-02n	OR
	02-206-01n	KS
	02-212-04n	KS
Trials in Progress	01-029-12n*	CA, IA, IL, NY, WA, WI
	01-164-03n*	WI
	01-164-05n*	IN
	01-164-06n*	PA
	01-205-06n*	CA
	01-211-08n*	CA
	01-211-09n*	NY
	01-219-02n*	ID
	01-236-03n*	GA
	01-243-10n*	CA
	01-275-02n*	TX
	02-004-15n*	ID, IN, WI
	02-007-08n*	WI
	02-010-10n*	ID
	02-028-29n*	MT
	02-044-10n*	WY, PA
	02-044-15n*	MI, PA
	02-046-24n*	CA
	02-046-25n*	CA
	02-046-26n*	CA
	02-051-17n*	WI
	02-051-20n	NY, PA

*Interim Field Test Report has been filed with APHIS.

Table VI-33 (continued). USDA Notifications Relevant to the Field Testing of Events J101 and J163.

Year	USDA Notification Number	Approved Release Sites (by state) Covered by Notification
Trials in progress	02-051-21n*	OH
	02-051-23n*	IL
	02-051-26n*	KY, MI
	02-053-04n*	IN
	02-084-19n*	NE, WY
	02-105-04n	SD
	02-193-02n	IA, MO, NE
	02-212-05n*	OK
	02-214-09n	GA
	02-220-16n	TN
	02-247-07n	CA
	02-346-12n	IN
	02-346-14n	ID
	02-346-15n	ID
	02-346-16n	IA
	02-346-17n	MT
	02-346-18n	OK
	02-352-01n	IA, MN
	02-352-02n	CA
	03-021-15n	CO
	03-021-17n	MT
	03-021-18n	OR
	03-034-30n	ID
	03-043-09n	TX
	03-043-10n	IA
	03-052-19n	TX
	03-052-21n	OK
	03-062-03n	NY
	03-062-04n	WV
	03-098-02n	CA
	03-098-03n	MO
	03-098-04n	MN
	03-098-06n	WI
	03-121-05n	PA
	03-121-06n	KY
	03-184-03n	OK

*Interim Field Test Report has been filed with APHIS.

Table VI-33 (continued). USDA Notifications Relevant to the Field Testing of Events J101 and J163.

Year	USDA Notification Number	Approved Release Sites (by state) Covered by Notification
Trials in Progress	03-184-04n	NE
	03-184-05n	MT
	03-184-06n	CO
	03-191-01n	IA
	03-191-02n	PA
	03-191-04n	WI
	03-191-03n	ID
	03-191-05n	IN
	03-191-06n	WA
	03-202-10n	WI
	03-218-01n	CA
	03-247-01n	CA
	03-247-02n	CA
	03-021-19n	ND
	03-247-04n	CA
	03-021-21n	ND
	03-022-03n	ND
	03-021-22n	ND
	03-021-23n	ND
	03-304-03n	ID
	03-304-04n	ID
	03-304-05n	ID
	03-310-02n	MS
	03-314-03n	AL
	03-022-04n	ND
	03-318-04n	IL
	03-318-05n	IL
	03-324-01n	ID
	03-324-02n	ID
	03-325-01n	CO
	03-328-02n	TX
	03-345-01n	OR
	03-345-03n	WA
	03-350-01n	NE
	04-005-01n	CA
	04-013-02n	CA, IA, IL, WI

Table VI-33 (continued). USDA Notifications Relevant to the Field Testing of Events J101 and J163.

Year	USDA Notification Number	Approved Release Sites (by state) Covered by Notification
Trials in progress	04-036-02n	MT, WY
	04-030-10n	KY, TN
	04-030-14n	CO
	04-007-01n	CA, IA, IL, NY, WA, WI

H. Crop Compositional Assessment

The composition of forage produced by Roundup Ready alfalfa plants containing either event J101, J163, or the paired events J101XJ163 was measured and compared to the composition of control and conventional alfalfa forage. Forage was harvested from plants grown in the field trials described in Section VI-D.2. The experimental design and herbicide treatment have been previously described. This study was conducted under USDA Notification Number 01-029-12n. A brief description of the methods used for forage production follows. The alfalfa varieties were grown at five replicated field sites across the alfalfa-producing regions of the U.S. during the 2001 field season. Field sites were located in the states of California, Illinois, New York, Washington and Wisconsin. Five of the field sites from this trial were used to generate forage for compositional analyses. Plots were established using plants that were reared in a greenhouse and transplanted to the field. A randomized complete block design with four replicates per treatment was used at each location. Roundup Ready alfalfa plants were simplex (single copy, single event) or a synthetic population of Roundup Ready alfalfa plants (Syn 1) that contained a combination of events J101 and J163, generated through conventional breeding (J101XJ163). Plots containing Roundup Ready alfalfa lines were treated with a Roundup agricultural herbicide at expected commercial treatment rates. At each of the five field sites, four commercially available alfalfa varieties were also grown (12 unique commercial varieties in total).

Forage samples were collected from all plots and analyzed for nutritional components. Compositional analyses of the forage samples included proximates (protein, fat, ash and moisture), acid detergent fiber (ADF), neutral detergent fiber (NDF), lignin, amino acids, and minerals (calcium, copper, iron, magnesium, manganese, phosphorous, potassium, sodium and zinc), as well as carbohydrates by calculation. In all, 35 different components were analyzed to assess the composition of Roundup Ready alfalfa.

Statistical analyses of the compositional data were conducted using a mixed model analysis of variance from a combination of all five trials. Statistical evaluation of the composition data involved comparison of the forage from the alfalfa test lines to the nontransgenic control. Statistically significant differences were determined at the 5% level of significance ($P \leq 0.05$). Using the data for each component obtained from the 12 unique commercial varieties, a 99% tolerance interval was calculated to contain, with 95% confidence, 99% of the values contained in the population of commercial alfalfa

varieties. For those comparisons in which the test was statistically different from the control, the test range was compared to the 99% tolerance interval in order to determine if the test range were within the interval and therefore considered to be part of the population of the commercial alfalfa varieties.

A summary of the results and conclusions derived from analyses conducted on forage derived from the Roundup Ready alfalfa plants containing event J101, J163, or the confirmatory synthetic population, J101XJ163, is presented below.

Table VI-34 presents the combined site summary of the results derived from the analyses of forage collected from alfalfa populations containing event J101, J163 or the confirmatory data derived from alfalfa populations containing both events, and from the control and reference varieties. A discussion of results will be limited to those that were statistically different. Table VI-35 presents a summary of the statistically significant differences observed between J101, J163, J110XJ163 (confirmatory) and the control line. Results from the analyses conducted on forage samples derived from alfalfa plants containing event J101 indicated that there were three statistically significant differences observed between the test and nontransgenic control: cystine, glutamic acid, and tyrosine. Results from the analyses conducted on forage samples derived from alfalfa plants containing event J163 indicated that there were seven statistically significant differences observed between the test and nontransgenic control: cystine, histidine, lysine, tyrosine, acid detergent fiber, lignin, and neutral detergent fiber. Confirmatory results from the analyses conducted on forage samples derived from alfalfa populations containing the J101XJ163 paired event population indicated that there were eleven statistically significant differences observed between the test and nontransgenic control: cystine, isoleucine, phenylalanine, proline, tyrosine, neutral detergent fiber, calcium, iron, ash, carbohydrates, and moisture. For the means of the analytes that were statistically significantly different from the control, the values were within the 99% tolerance interval developed from the conventional alfalfa varieties grown at the same locations. Literature ranges for the analytes measured are presented in Table VI-36.

While individual site data are not presented in this summary, the range of iron was unusually high, and primarily because of the iron levels at one location (New York). Because iron is a particularly problematic mineral to measure because of soil contamination (discussed below), additional samples of alfalfa forage were harvested from the same New York field plots in 2002 and analyzed for ash (indicator of soil contamination), iron and moisture content. Data for these second-year analyses are presented in Tables VI-37 and VI-38. The results from these analyses showed that the ash and iron content returned to levels consistent with those observed for conventional alfalfa forage (Table VI-36). It was therefore hypothesized that the high levels of iron and ash observed in 2001 were because of surface contamination by soil.

The following information supports this hypothesis. According to Kabata-Pendias and Pendias (1992), plants with iron concentrations above 1000 ppm usually show some phenotypic effect such as injured leaves, a bronzing of the leaves or necrotic spots. At the New York site, there were no adverse iron toxicity effects observed, suggesting that the iron was not absorbed by the plants. Secondly, iron is an extremely abundant mineral in the soil and accurate measurement of iron in plants has been noted to be problematic because of soil contamination and the iron level actually in the plant can often be masked

by introduced iron from the soil (Jones, 1972; Pais and Jones, 1997; Bickoff et al., 1972). It was therefore concluded that because the plants showed no phenotypic effects associated with iron toxicity and because the iron levels in the samples obtained from the New York site in 2002 decreased, the high iron levels observed in samples derived from 2001 were not indigenous to the plant and were likely because of soil surface contamination. Further, the high iron levels were not observed at the other locations in the trial.

High ash levels are an indirect indicator of soil contamination. Ash represents the remaining components of the sample after incineration, including other soil minerals and silica not measured in these analyses. Hence, the ash component would also be an indicator of the level of soil in a sample. Samples were taken at the New York location using standard forage harvesting practices and were not rinsed to remove residual soil that may have stuck to the surface of the plants. Because the plots were separated by bare ground at this location, it is reasonable to assume that some soil dusted the plants, resulting in iron contamination.

In summary, compositional data were generated and statistical analyses performed on Roundup Ready alfalfa containing events J101 and J163. As expected, statistically significant differences were observed for the concentration of some of the analytes in comparison to the control. Where values were different, the mean was within the 99% tolerance interval developed for the analyte using conventional alfalfa reference varieties. Hence, it is unlikely that these differences are biologically meaningful. These data are consistent with the conclusion that forage produced by alfalfa plants containing event J101 or J163 is comparable to forage produced by control or conventional alfalfa varieties. These compositional data support the conclusions derived from other phenotypic studies presented in this section where no biologically meaningful changes were associated with alfalfa populations containing event J101 or event J163.

Table VI-34. Composition of Forage Derived from Alfalfa Population Containing Event J101, J163, J101XJ163 vs. Control and Reference Varieties.

			Difference (Test minus Control)			
Amino Acid (% Total AA[1])	Line	Mean ± S.E.[2] (Range)	Mean ± S.E. (Range)	95% C.I. (Lower, Upper)	P-Value	(Com. Ref.[3]) [99% T. I.[4]]
Alanine	Control	6.19 ± 0.097				(5.93 - 6.93)
		(6.01 - 6.56)				[5.55, 6.80]
	J101	6.19 ± 0.097	0.0015 ± 0.063	-0.13, 0.13	0.981	
		(5.99 - 6.69)	(-0.22 - 0.36)			
	J163	6.27 ± 0.097	0.084 ± 0.063	-0.044, 0.21	0.190	
		(5.96 - 6.93)	(-0.19 - 0.75)			
	J101X163	6.20 ± 0.097	0.011 ± 0.063	-0.12, 0.14	0.866	
		(6.00 - 6.79)	(-0.20 - 0.61)			
Arginine	Control	5.64 ± 0.063				(5.40 - 5.90)
		(5.40 - 6.23)				[4.98, 6.21]
	J101	5.60 ± 0.063	-0.049 ± 0.057	-0.17, 0.068	0.399	
		(5.34 - 5.84)	(-0.64 - 0.25)			
	J163	5.58 ± 0.063	-0.060 ± 0.057	-0.18, 0.056	0.299	
		(5.32 - 5.82)	(-0.51 - 0.27)			
	J101X163	5.56 ± 0.063	-0.088 ± 0.058	-0.21, 0.029	0.137	
		(5.10 - 5.99)	(-0.75 - 0.44)			

[1]AA – Amino acid.
[2]S.E. – Standard error of the mean.
[3]Com. Ref. – Commercial references. Data in parenthesis are the range of values derived from commercial reference varieties.
[4] T.I. – Tolerance interval. With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were se to zero.
Data developed under USDA Notification Number 01-029-12n.

Table VI-34 (continued). Composition of Forage Derived from Alfalfa Population Containing Event J101, J163, J101XJ163 vs. Control and Reference Varieties.

			Difference (Test minus Control)			
Amino Acid (% Total AA[1])	Line	Mean ± S.E.[2] (Range)	Mean ± S.E. (Range)	95% C.I. (Lower, Upper)	P-Value	(Com. Ref.[3]) [99% T. I.[4]]
Aspartic Acid	Control	12.86 ± 0.37				(11.83 - 15.40)
		(10.95 - 16.22)				[9.75, 16.61]
	J101	13.28 ± 0.37	0.42 ± 0.25	-0.090, 0.93	0.103	
		(12.02 - 17.22)	(-1.49 - 3.13)			
	J163	13.34 ± 0.37	0.48 ± 0.25	-0.023, 0.99	0.060	
		(11.63 - 15.62)	(-1.67 - 2.27)			
	J101X163	13.16 ± 0.37	0.31 ± 0.25	-0.21, 0.82	0.234	
		(12.05 - 14.34)	(-1.22 - 2.40)			
Cystine	Control	1.41 ± 0.057				(1.23 - 1.76)
		(1.17 - 1.59)				[1.01, 1.96]
	101	1.56 ± 0.057	0.15 ± 0.042	0.065, 0.23	<0.001	
		(1.36 - 1.86)	(-0.16 - 0.64)			
	J163	1.56 ± 0.057	0.15 ± 0.042	0.062, 0.23	<0.001	
		(1.35 - 1.90)	(-0.15 - 0.69)			
	J101 X 163	1.57 ± 0.057	0.16 ± 0.042	0.070, 0.24	<0.001	
		(1.41 - 1.84)	(-0.091 - 0.63)			

[1]AA – Amino acid.
[2]S.E. – Standard error of the mean.
[3]Com. Ref. – Commercial references. Data in parenthesis are the range of values derived from commercial reference varieties.
[4] T.I. – Tolerance interval. With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.
Data developed under USDA Notification Number 01-029-12n.

Table VI-34 (continued). Composition of Forage Derived from Alfalfa Population Containing Event J101, J163, J101XJ163 vs. Control and Reference Varieties.

			Difference (Test minus Control)			
Amino Acid (% Total AA[1])	Line	Mean ± S.E.[2] (Range)	Mean ± S.E. (Range)	95% C.I. (Lower, Upper)	P-Value	(Com. Ref.[3]) [99% T. I.[4]]
Glutamic Acid	Control	11.10 ± 0.077				(10.75 - 11.62)
		(10.85 - 11.79)				[10.28, 11.77]
	J101	10.95 ± 0.077	-0.15 ± 0.069	-0.29,-0.015	0.031	
		(10.64 - 11.34)	(-0.77 - 0.30)			
	J163	11.02 ± 0.077	-0.075 ± 0.069	-0.21, 0.065	0.285	
		(10.64 - 11.42)	(-0.53 - 0.35)			
	J101X163	11.03 ± 0.077	-0.069 ± 0.069	-0.21, 0.072	0.327	
		(10.70 - 11.33)	(-0.89 - 0.38)			
Glycine	Control	5.56 ± 0.044				(5.35 - 5.64)
		(5.39 - 5.97)				[5.11, 5.84]
	J101	5.52 ± 0.044	-0.034 ± 0.039	-0.11, 0.044	0.381	
		(5.37 - 5.77)	(-0.43 - 0.14)			
	J163	5.54 ± 0.044	-0.023 ± 0.039	-0.10, 0.056	0.562	
		(5.35 - 5.79)	(-0.30 - 0.20)			
	J101X163	5.61 ± 0.044	0.051 ± 0.039	-0.028, 0.13	0.195	
		(5.46 - 6.23)	(-0.36 - 0.62)			

[1]AA – Amino acid.
[2]S.E. – Standard error of the mean.
[3]Com. Ref. – Commercial references. Data in parenthesis are the range of values derived from commercial reference varieties.
[4] T.I. – Tolerance interval. With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.
Data developed under USDA Notification Number 01-029-12n.

Table VI-34 (continued). Composition of Forage Derived from Alfalfa Population Containing Event J101, J163, J101XJ163 vs. Control and Reference Varieties.

			Difference (Test minus Control)			
Amino Acid (% Total AA[1])	Line	Mean ± S.E.[2] (Range)	Mean ± S.E. (Range)	95% C.I. (Lower, Upper)	P-Value	(Com. Ref.[3]) [99% T. I.[4]]
Histidine	Control	2.76 ± 0.044				(2.43 - 2.96)
		(2.57 - 3.01)				[2.25, 3.22]
	J101	2.74 ± 0.044	-0.028 ± 0.032	-0.094, 0.038	0.391	
		(2.43 - 2.91)	(-0.42 - 0.14)			
	J163	2.67 ± 0.044	-0.098 ± 0.032	-0.16, -0.032	0.004	
		(2.44 - 2.85)	(-0.56 - 0.088)			
	J101X163	2.70 ± 0.045	-0.064 ± 0.033	-0.13, 0.0017	0.055	
		(2.44 - 2.88)	(-0.39 - 0.15)			
Isoleucine	Control	4.94 ± 0.052				(4.60 - 5.20)
		(4.65 - 5.31)				[4.25, 5.58]
	J101	4.93 ± 0.052	-0.010 ± 0.037	-0.083, 0.062	0.784	
		(4.48 - 5.17)	(-0.52 - 0.34)			
	J163	4.91 ± 0.052	-0.029 ± 0.037	-0.10, 0.044	0.434	
		(4.69 - 5.29)	(-0.56 - 0.47)			
	J101X163	4.86 ± 0.052	-0.083 ± 0.037	-0.16, -0.0093	0.027	
		(4.64 - 5.14)	(-0.60 - 0.20)			

[1]AA – Amino acid.
[2]S.E. – Standard error of the mean.
[3]Com. Ref. – Commercial references. Data in parenthesis are the range of values derived from commercial reference varieties.
[4] T.I. – Tolerance interval. With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.
Data developed under USDA Notification Number 01-029-12n.

Table VI-34 (continued). Composition of Forage Derived from Alfalfa Population Containing Event J101, J163, J101XJ163 vs. Control and Reference Varieties.

			Difference (Test minus Control)			
Amino Acid (% Total AA[1])	Line	Mean ± S.E.[2] (Range)	Mean ± S.E. (Range)	95% C.I. (Lower, Upper)	P-Value	(Com. Ref.[3]) [99% T. I.[4]]
Leucine	Control	8.66 ± 0.059				(8.36 - 8.90)
		(8.32 - 9.12)				[8.08, 9.07]
	J101	8.60 ± 0.059	-0.056 ± 0.057	-0.17, 0.059	0.327	
		(8.08 - 8.87)	(-0.48 - 0.26)			
	J163	8.59 ± 0.059	-0.072 ± 0.057	-0.19, 0.044	0.214	
		(8.25 - 8.97)	(-0.61 - 0.25)			
	J101X163	8.55 ± 0.060	-0.11 ± 0.057	-0.23, .0020	0.053	
		(8.24 - 8.88)	(-0.59 - 0.27)			
Lysine	Control	7.05 ± 0.098				(6.27 - 7.48)
		(6.62 - 7.34)				[6.26, 7.85]
	J101	7.07 ± 0.098	0.026 ± 0.060	-0.093, 0.14	0.669	
		(6.43 - 7.53)	(-0.77 - 0.45)			
	J163	6.89 ± 0.098	-0.16 ± 0.060	-0.28, -0.039	0.009	
		(6.50 - 7.37)	(-0.76 - 0.28)			
	J101X163	6.94 ± 0.098	-0.11 ± 0.061	-0.23, 0.013	0.079	
		(6.55 - 7.39)	(-0.70 - 0.31)			

[1]AA – Amino acid.
[2]S.E. – Standard error of the mean.
[3]Com. Ref. – Commercial references. Data in parenthesis are the range of values derived from commercial reference varieties.
[4] T.I. – Tolerance interval. With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.
Data developed under USDA Notification Number 01-029-12n.

Table VI-34 (continued). Composition of Forage Derived from Alfalfa Population Containing Event J101, J163, J101XJ163 vs. Control and Reference Varieties.

			Difference (Test minus Control)			
Amino Acid (% Total AA[1])	Line	Mean ± S.E.[2] (Range)	Mean ± S.E. (Range)	95% CI (Lower, Upper)	P-Value	(Com. Ref.[3]) [99% T. I.[4]]
Methionine	Control	1.89 ± 0.031				(1.67 - 2.10)
		(1.57 - 2.16)				[1.56, 2.30]
	J101	1.88 ± 0.031	-0.015 ± 0.040	-0.096, 0.065	0.701	
		(1.64 - 2.17)	(-0.37 - 0.27)			
	J163	1.91 ± 0.031	0.017 ± 0.040	-0.064, 0.098	0.672	
		(1.64 - 2.16)	(-0.29 - 0.36)			
	J101X163	1.90 ± 0.031	0.011 ± 0.040	-0.070, 0.093	0.778	
		(1.71 - 2.21)	(-0.32 - 0.31)			
Phenylalanine	Control	5.67 ± 0.065				(5.40 - 6.16)
		(5.32 - 6.47)				[4.64, 6.61]
	J101	5.61 ± 0.065	-0.062 ± 0.049	-0.16, 0.039	0.220	
		(5.20 - 6.23)	(-0.73 - 0.48)			
	J163	5.57 ± 0.065	-0.096 ± 0.049	-0.20, 0.0044	0.060	
		(5.33 - 5.99)	(-0.88 - 0.24)			
	J101X163	5.54 ± 0.066	-0.12 ± 0.050	-0.23, -0.023	0.017	
		(5.39 - 6.06)	(-0.92 - 0.31)			

[1]AA – Amino acid.
[2]S.E. – Standard error of the mean.
[3]Com. Ref. – Commercial references. Data in parenthesis are the range of values derived from commercial reference varieties.
[4] T.I. – Tolerance interval. With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.
Data developed under USDA Notification Number 01-029-12n.

Table VI-34 (continued). Composition of Forage Derived from Alfalfa Population Containing Event J101, J163, J101XJ163 vs. Control and Reference Varieties.

			Difference (Test minus Control)			
Amino Acid (% Total AA[1])	Line	Mean ± S.E.[2] (Range)	Mean ± S.E. (Range)	95% C.I. (Lower, Upper)	P-Value	(Com. R.[3]) [99% T. I.[4]]
Proline	Control	5.28 ± 0.11				(4.86 - 5.73)
		(4.32 - 5.97)				[4.57, 6.06]
	J101	5.29 ± 0.11	0.011 ± 0.079	-0.15, 0.17	0.889	
		(4.93 - 5.82)	(-0.46 - 1.24)			
	J163	5.37 ± 0.11	0.090 ± 0.079	-0.071, 0.25	0.264	
		(4.75 - 5.91)	(-0.22 - 1.27)			
	J101X163	5.49 ± 0.11	0.21 ± 0.080	0.048, 0.37	0.012	
		(5.06 - 6.16)	(-0.51 - 0.97)			
Serine	Control	5.36 ± 0.11				(4.92 - 5.91)
		(4.87 - 5.73)				[4.31, 6.57]
	J101	5.41 ± 0.11	0.051 ± 0.073	-0.096, 0.20	0.485	
		(4.93 - 5.97)	(-0.63 - 0.70)			
	J163	5.32 ± 0.11	-0.041 ± 0.073	-0.19, 0.11	0.578	
		(4.78 - 5.80)	(-0.79 - 0.54)			
	J101X163	5.45 ± 0.11	0.086 ± 0.073	-0.063, 0.23	0.248	
		(5.05 - 5.92)	(-0.37 - 0.72)			

[1]AA – Amino acid.
[2]S.E. – Standard error of the mean.
[3]Com. Ref. – Commercial references. Data in parenthesis are the range of values derived from commercial reference varieties.
[4] T.I. – Tolerance interval. With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.
Data developed under USDA Notification Number 01-029-12n.

Table VI-34 (continued). Composition of Forage Derived from Alfalfa Population Containing Event J101, J163, J101XJ163 vs. Control and Reference Varieties.

			Difference (Test minus Control)			
Amino Acid (% Total AA[1])	Line	Mean ± S.E.[2] (Range)	Mean ± S.E. (Range)	95% C.I. (Lower, Upper)	P-Value	(Com. Ref.[3]) [99% T. I.[4]]
Threonine	Control	4.57 ± 0.067				(4.10 - 4.85)
		(4.07 - 4.79)				[3.63, 5.48]
	J101	4.54 ± 0.067	-0.029 ± 0.051	-0.13, 0.074	0.575	
		(4.23 - 4.84)	(-0.37 - 0.27)			
	J163	4.60 ± 0.067	0.035 ± 0.051	-0.068, 0.14	0.497	
		(4.36 - 4.81)	(-0.38 - 0.31)			
	J101X163	4.59 ± 0.067	0.023 ± 0.051	-0.081, 0.13	0.661	
		(4.13 - 4.88)	(-0.30 - 0.30)			
Tryptophan	Control	1.22 ± 0.056				(0.86 - 1.38)
		(0.81 - 1.48)				[0.62, 1.84]
	J101	1.15 ± 0.056	-0.073 ± 0.044	-0.16, 0.016	0.104	
		(0.73 - 1.42)	(-0.49 - 0.36)			
	J163	1.15 ± 0.056	-0.075 ± 0.044	-0.16, 0.013	0.093	
		(0.78 - 1.48)	(-0.38 - 0.38)			
	J101X163	1.19 ± 0.057	-0.036 ± 0.044	-0.12, 0.054	0.424	
		(0.86 - 1.45)	(-0.36 - 0.40)			

[1]AA – Amino acid.
[2]S.E. – Standard error of the mean.
[3]Com. Ref. – Commercial references. Data in parenthesis are the range of values derived from commercial reference varieties.
[4] T.I. – Tolerance interval. With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.
Data developed under USDA Notification Number 01-029-12n.

Table VI-34 (continued). Composition of Forage Derived from Alfalfa Population Containing Event J101, J163, J101XJ163 vs. Control and Reference Varieties.

			Difference (Test minus Control)			
Amino Acid (% Total AA[1])	Line	Mean ± S.E.[2] (Range)	Mean ± S.E. (Range)	95% C.I. (Lower, Upper)	P-Value	(Com. Ref.[3]) [99% T. I.[4]]
Tyrosine	Control	3.83 ± 0.045				(3.30 - 3.94)
		(3.46 - 4.51)				[3.33, 4.07]
	J101	3.68 ± 0.045	-0.15 ± 0.052	-0.25 , -0.044	0.005	
		(3.23 - 3.94)	(-0.79 - 0.41)			
	J163	3.69 ± 0.045	-0.14 ± 0.052	-0.24, -0.036	0.008	
		(3.19 - 3.86)	(-0.80 - 0.15)			
	J101X163	3.69 ± 0.046	-0.14 ± 0.053	-0.25, -0.037	0.007	
		(3.18 - 3.89)	(-1.14 - 0.36)			
Valine	Control	6.01 ± 0.051				(5.69 - 6.26)
		(5.58 - 6.41)				[5.36, 6.63]
	J101	6.01 ± 0.051	-0.00012 ± 0.052	-0.10, 0.10	0.998	
		(5.60 - 6.24)	(-0.44 - 0.56)			
	J163	6.01 ± 0.051	0.0071 ± 0.052	-0.096, 0.11	0.892	
		(5.74 - 6.35)	(-0.37 - 0.70)			
	J101X163	6.00 ± 0.052	-0.010 ± 0.053	-0.11, 0.094	0.842	
		(5.82 - 6.27)	(-0.59 - 0.44)			

[1]AA – Amino acid.
[2]S.E. – Standard error of the mean.
[3]Com. Ref. – Commercial references. Data in parenthesis are the range of values derived from commercial reference varieties.
[4] T.I. – Tolerance interval. With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.
Data developed under USDA Notification Number 01-029-12n.

Table VI-34 (continued). Composition of Forage Derived from Alfalfa Population Containing Event J101, J163, J101XJ163 vs. Control and Reference Varieties.

			Difference (Test minus Control)			
Analyte (% DW[1])	Line	Mean ± S.E.[2] (Range)	Mean ± S.E. (Range)	95% C.I. (Lower, Upper)	P-Value	(Com. Ref.[3]) [99% T. I.[4]]
Acid Detergent Fiber	Control	25.79 ± 1.61				(23.12 - 33.39)
		(18.81 - 33.47)				[15.76,40.19]
	J101	26.83 ± 1.61	1.04 ± 0.92	-0.78, 2.86	0.259	
		(21.65 - 32.38)	(-5.04 - 5.77)			
	J163	28.31 ± 1.61	2.52 ± 0.92	0.70, 4.35	0.006	
		(20.00 - 39.67)	(-5.54 - 12.86)			
	J101X163	27.01 ± 1.62	1.22 ± 0.94	-0.62, 3.07	0.192	
		(22.09 - 33.91)	(-5.13 - 5.75)			
Lignin	Control	5.07 ± 0.56				(3.86 - 9.65)
		(1.64 - 8.10)				[0,12.92]
	J101	5.78 ± 0.56	0.71 ± 0.39	-0.063, 1.48	0.071	
		(3.86 - 9.11)	(-1.70 - 4.12)			
	J163	6.01 ± 0.56	0.94 ± 0.39	0.17, 1.71	0.017	
		(3.94 - 8.13)	(-1.43 - 5.51)			
	J101XJ163	5.31 ± 0.56	0.24 ± 0.40	-0.54, 1.03	0.543	
		(3.48 - 8.16)	(-2.00 - 2.06)			

[1]Percent dry weight.
[2]S.E. – Standard error of the mean.
[3]Com. Ref. – Commercial references. Data in parenthesis are the range of values derived from commercial reference varieties.
[4]T.I. – Tolerance interval. With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.
Data developed under USDA Notification Number 01-029-12n.

Table VI-34 (continued). Composition of Forage Derived from Alfalfa Population Containing Event J101, J163, J101XJ163 vs. Control and Reference Varieties.

			Difference (Test minus Null)			
Analyte (% DW[1])	Line	Mean ± S.E.[2] (Range)	Mean ± S.E. (Range)	95% C.I. (Lower, Upper)	P-Value	(Com. Ref.[3]) [99% T. I.[4]]
Neutral Detergent Fiber	Null	28.09 ± 1.37				(26.53 - 35.72)
		(22.25 - 32.07)				[20.01, 41.80]
	J101	29.49 ± 1.37	1.40 ± 1.02	-0.68, 3.47	0.181	
		(25.22 - 34.05)	(-3.68 - 5.79)			
	J163	30.94 ± 1.37	2.85 ± 1.02	0.77, 4.92	0.008	
		(24.49 - 43.57)	(-4.07 - 14.78)			
	J101X163	30.64 ± 1.38	2.54 ± 1.03	0.45, 4.64	0.018	
Calcium	Null	1.12 ± 0.070				(0.90 - 1.53)
		(0.88 - 1.44)				[0.48, 1.89]
	J101	1.14 ± 0.070	0.022 ± 0.044	-0.067, 0.11	0.623	
		(0.94 - 1.51)	(-0.29 - 0.28)			
	J163	1.12 ± 0.070	0.0049 ± 0.044	-0.084, 0.094	0.911	
		(0.91 - 1.58)	(-0.20 - 0.40)			
	J101X163	1.01 ± 0.070	-0.10 ± 0.044	-0.19, -0.015	0.023	
		(0.81 - 1.38)	(-0.40 - 0.22)			

[1]Percent dry weight..
[2]S.E. – Standard error of the mean.
[3]Com. Ref. – Commercial references. Data in parenthesis are the range of values derived from commercial reference varieties.
[4] T.I. – Tolerance interval. With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.
Data developed under USDA Notification Number 01-029-12n.

Table VI-34 (continued). Composition of Forage Derived from Alfalfa Population Containing Event J101, J163, J101XJ163 vs. Control and Reference Varieties.

			Difference (Test minus Control)			
Analyte (mg/kg DW[1])	Line	Mean ± S.E.[2] (Range)	Mean ± S.E. (Range)	95% C.I. (Lower, Upper)	P-Value	(Com. Ref.[3]) [99% T. I.[4]]
Copper	Control	9.41 ± 0.68				(5.29 - 10.18)
	J101	(6.76 - 17.10)			0.451	[3.12, 12.64]
		(6.32 - 11.72)	(-9.20 - 4.65)			
	J163	9.15 ± 0.68	-0.25 ± 0.59	-1.45, 0.95	0.672	
		(6.66 - 19.49)	(-7.39 - 10.49)			
	J101X163	8.24 ± 0.68	-1.17 ± 0.59	-2.37, 0.039	0.057	
		(6.42 - 12.28)	(-9.22 - 3.85)			
Iron	Control	410.19 ± 230.60				(235.53 - 1538.46)
		(184.32 - 764.23)				[0, 892.57]
	J101	563.39 ± 230.60	153.20 ± 115.24	-80.90, 387.30	0.192	
		(240.21 - 1553.40)	(-123.45 - 876.41)			
	J163	614.37 ± 230.60	204.18 ± 115.24	-29.91, 438.28	0.085	
		(218.23 - 1882.35)	(-259.76 - 1230.18)			
	J101 X 163	730.93 ± 230.85	320.74 ± 115.74	85.75, 555.73	0.008	
		(199.10 - 2196.43)	(-176.38 - 1530.12)			

[1]DW – Dry Weight.
[2]S.E. – Standard error of the mean.
[3]Com. Ref. – Commercial references. Data in parenthesis are the range of values derived from commercial reference varieties.
[4] T.I. – Tolerance interval. With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.
Data developed under USDA Notification Number 01-029-12n.

Table VI-34 (continued). Composition of Forage Derived from Alfalfa Population Containing Event J101, J163, J101XJ163 vs. Control and Reference Varieties.

			Difference (Test minus Control)			
Analyte (mg/kg DW[1])	Line	Mean ± S.E.[2] (Range)	Mean ± S.E. (Range)	95% C.I. (Lower, Upper)	P-Value	(Com. Ref.[3]) [99% T. I.[4]]
Magnesium (% DW)	Control	0.26 ± 0.051				(0.11 - 0.45)
		(0.11 - 0.54)				[0, 0.68]
	J101	0.27 ± 0.051	0.012 ± 0.015	-0.019, 0.042	0.447	
		(0.12 - 0.60)	(-0.073 - 0.17)			
	J163	0.27 ± 0.051	0.011 ± 0.015	-0.020, 0.042	0.471	
		(0.12 - 0.52)	(-0.045 - 0.15)			
	J101X163	0.24 ± 0.051	-0.019 ± 0.015	-0.050, 0.012	0.230	
		(0.10 - 0.38)	(-0.16 - 0.062)			
Manganese (mg/kg DW)	Control	54.04 ± 8.57				(34.60 - 109.50)
		(32.97 - 81.01)				[0, 120.37]
	J101	56.72 ± 8.57	2.68 ± 4.29	-6.03, 11.39	0.535	
		(35.20 - 95.45)	(-19.59 - 47.89)			
	J163	62.36 ± 8.57	8.32 ± 4.29	-0.38, 17.03	0.060	
		(30.29 - 117.23)	(-18.90 - 53.03)			
	J101X163	61.83 ± 8.60	7.80 ± 4.34	-1.01, 16.60	0.080	
		(35.90 - 112.95)	(-8.69 - 32.46)			

[1]DW – Dry Weight.
[2]S.E. – Standard error of the mean.
[3]Com. Ref. – Commercial references. Data in parenthesis are the range of values derived from commercial reference varieties.
[4] T.I. – Tolerance interval. With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.
Data developed under USDA Notification Number 01-029-12n.

Table VI-34 (continued). Composition of Forage Derived from Alfalfa Population Containing Event J101, J163, J101XJ163 vs. Control and Reference Varieties.

			Difference (Test minus Control)			
Analyte (% DW[1])	Line	Mean ± S.E.[2] (Range)	Mean ± S.E. (Range)	95% C.I. (Lower, Upper)	P-Value	(Com. Ref.[3]) [99% T. I.[4]]
Phosphorus	Control	0.33 ± 0.027				(0.22 - 0.45)
		(0.25 - 0.45)				[0.095, 0.54]
	J101	0.34 ± 0.027	0.0057 ± 0.0075	-0.0096, 0.021	0.456	
		(0.22 - 0.48)	(-0.082 - 0.14)			
	J163	0.33 ± 0.027	0.0016 ± 0.0075	-0.014, 0.017	0.832	
		(0.24 - 0.49)	(-0.090 - 0.077)			
	J101X163	0.32 ± 0.027	-0.012 ± 0.0076	-0.027, 0.0035	0.124	
		(0.22 - 0.42)	(-0.088 - 0.12)			
Potassium	Control	3.08 ± 0.41				(1.39 - 4.31)
		(1.57 - 4.30)				[0.38, 5.75]
	J101	3.07 ± 0.41	-0.011 ± 0.10	-0.22, 0.19	0.914	
		(1.48 - 4.61)	(-0.74 - 1.14)			
	J163	3.01 ± 0.41	-0.074 ± 0.10	-0.28, 0.13	0.468	
		(1.18 - 4.41)	(-0.50 - 0.53)			
	J101X163	2.96 ± 0.41	-0.12 ± 0.10	-0.33, 0.083	0.233	
		(0.85 - 4.32)	(-1.37 - 1.08)			

[1]DW – Dry Weight.
[2]S.E. – Standard error of the mean.
[3]Com. Ref. – Commercial references. Data in parenthesis are the range of values derived from commercial reference varieties.
[4] T.I. – Tolerance interval. With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.
Data developed under USDA Notification Number 01-029-12n.

Table VI-34 (continued). Composition of Forage Derived from Alfalfa Population Containing Event J101, J163, J101XJ163 vs. Control and Reference Varieties.

			Difference (Test minus Control)			
Analyte (% or mg/kg DW[1])	Line	Mean ± S.E.[2] (Range)	Mean ± S.E. (Range)	95% C.I. (Lower, Upper)	P-Value	(Com. Ref.[3]) [99% T. I.[4]]
Sodium (% DW)	Control	0.079 ± 0.041				(0.017 - 0.21)
		(0.018 - 0.23)				[0, 0.31]
	J101	0.087 ± 0.041	0.0085 ± 0.015	-0.022, 0.039	0.573	
		(0.018 - 0.25)	(-0.053 - 0.11)			
	J163	0.092 ± 0.041	0.013 ± 0.015	-0.017, 0.043	0.388	
		(0.017 - 0.24)	(-0.019 - 0.071)			
	J101X163	0.10 ± 0.041	0.025 ± 0.015	-0.0060, 0.055	0.112	
		(0.017 - 0.38)	(-0.025 - 0.15)			
Zinc (mg/kg DW)	Control	29.58 ± 2.93				(18.09 - 35.98)
		(16.70 - 46.15)				[5.05, 50.21]
	J101	30.86 ± 2.93	1.28 ± 1.12	-0.99, 3.56	0.259	
		(18.28 - 44.76)	(-10.27 - 11.32)			
	J163	29.25 ± 2.93	-0.33 ± 1.12	-2.60, 1.95	0.771	
		(16.45 - 40.36)	(-17.06 - 9.51)			
	J101X163	28.61 ± 2.94	-0.98 ± 1.14	-3.28, 1.33	0.395	
		(17.01 - 37.28)	(-11.19 - 10.66)			

[1]DW – Dry Weight.
[2]S.E. – Standard error of the mean.
[3]Com. Ref. – Commercial references. Data in parenthesis are the range of values derived from commercial reference varieties.
[4] T.I. – Tolerance interval. With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.
Data developed under USDA Notification Number 01-029-12n.

Table VI-34 (continued). Composition of Forage Derived from Alfalfa Population Containing Event J101, J163, J101XJ163 vs. Control and Reference Varieties.

			Difference (Test minus Control)			
Analyte (% DW[1])	Line	Mean ± S.E.[2] (Range)	Mean ± S.E. (Range)	95% C.I. (Lower, Upper)	P-Value	(Com. Ref.[3]) [99% T. I.[4]]
Ash	Control	11.31 ± 2.46				(8.58 - 15.25)
		(8.44 - 15.04)				[5.59, 16.40]
	J101	13.48 ± 2.46	2.18 ± 1.21	-0.29, 4.64	0.081	
		(8.55 - 28.59)	(-1.53 - 13.55)			
	J163	13.23 ± 2.46	1.92 ± 1.21	-0.55, 4.38	0.123	
		(8.87 - 26.13)	(-1.29 - 11.09)			
	J101X163	14.41 ± 2.46	3.10 ± 1.22	0.63, 5.58	0.015	
		(8.26 - 32.50)	(-1.09 - 18.12)			
Carbohydrates	Control	65.08 ± 3.01				(58.03 - 74.38)
		(55.44 - 73.53)				[46.29, 85.59]
	J101	63.32 ± 3.01	-1.76 ± 0.93	-3.64, 0.12	0.065	
		(50.30 - 73.64)	(-9.89 - 9.32)			
	J163	63.29 ± 3.01	-1.78 ± 0.93	-3.67, 0.097	0.062	
		(51.37 - 73.39)	(-8.82 - 4.77)			
	J101X163	63.10 ± 3.01	-1.98 ± 0.93	-3.88, -0.085	0.041	
		(48.03 - 74.71)	(-11.57 - 7.00)			

[1]DW – Dry Weight.
[2]S.E. – Standard error of the mean.
[3]Com. Ref. – Commercial references. Data in parenthesis are the range of values derived from commercial reference varieties.
[4] T.I. – Tolerance interval. With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.
Data developed under USDA Notification Number 01-029-12n.

Table VI-34 (continued). Composition of Forage Derived from Alfalfa Population Containing Event J101, J163, J101XJ163 vs. Control and Reference Varieties.

			Difference (Test minus Control)			
Analyte (% FW[1] or % DW[2])	Line	Mean ± S.E.[3] (Range)	Mean ± S.E. (Range)	95% C.I. (Lower, Upper)	P-Value	(Com. R.ef[4]) [99% T. I.[5]]
Moisture (% FW)	Control	76.77 ± 1.64				(70.90 - 82.10)
		(70.70 - 84.20)				[62.91, 88.67]
	J101	77.11 ± 1.64	0.34 ± 0.48	-0.65, 1.32	0.492	
		(71.10 - 82.40)	(-4.60 - 5.70)			
	J163	77.01 ± 1.64	0.24 ± 0.48	-0.75, 1.22	0.629	
		(71.00 - 83.30)	(-3.30 - 4.50)			
	J101X163	75.78 ± 1.64	-0.99 ± 0.49	-1.98, -0.0023	0.049	
		(70.70 - 83.10)	(-7.80 - 4.70)			
Protein (% DW)	Control	21.35 ± 1.24				(15.29 - 25.81)
		(16.02 - 28.20)				[7.98, 33.81]
	J101	21.01 ± 1.24	-0.35 ± 0.52	-1.40, 0.70	0.505	
		(15.44 - 24.89)	(-5.99 - 5.85)			
	J163	21.21 ± 1.24	-0.15 ± 0.52	-1.20, 0.91	0.779	
		(15.80 - 26.32)	(-3.46 - 5.57)			
	J101X163	20.49 ± 1.24	-0.87 ± 0.52	-1.93, 0.19	0.105	
		(15.53 - 27.11)	(-5.93 - 8.85)			

[1]FW – Fresh Weight.
[2]DW- Dry Weight.
[3]S.E. – Standard error of the mean.
[4]Com. Ref. – Commercial references. Data in parenthesis are the range of values derived from commercial reference varieties.
[5] T.I. – Tolerance interval. With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.
Data developed under USDA Notification Number 01-029-12n.

Table VI-34 (continued). Composition of Forage Derived from Alfalfa Population Containing Event J101, J163, J101XJ163 vs. Control and Reference Varieties.

			Difference (Test minus Control)			
Analyte (% DW[1])	Line	Mean ± S.E.[2] (Range)	Mean ± S.E. (Range)	95% C.I. (Lower, Upper)	P-Value	(Com. Ref.[3]) [99% T. I.[4]]
Total Fat	Control	2.26 ± 0.17				(1.33 – 3.15)
		(1.45 – 3.58)				[0, 4.61]
	J101	2.19 ± 0.17	-0.065 ± 0.16	-0.39, 0.26	0.685	
		(1.27 – 4.01)	(-1.80 – 0.88)			
	J163	2.27 ± 0.17	0.014 ± 0.16	-0.31, 0.34	0.932	
		(1.21 – 3.68)	(-1.67 – 0.78)			
	J101X163	2.12 ± 0.17	-0.14 ± 0.16	-0.47, 0.18	0.387	
		(1.50 – 3.13)	(-1.24 – 1.37)			

[1]DW – Dry weight.
[2]S.E. – Standard error of the mean.
[3]Com. Ref. – Commercial references. Data in parenthesis are the range of values derived from commercial reference varieties.
[4] T.I. – Tolerance interval. With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.
Data developed under USDA Notification Number 01-029-12n.

Table VI-35. Summary of Statistically Significant Differences Comparing Test Line J101, J163 and J101XJ163 to the Control

Site/Component (Units)[1]	Mean Test Event	Control Mean	Mean Diff. (% of Control)	Signif-icance (P-value)	Test Event Range	99%Tolerance Interval[2]
Test Event J101						
Cystine (% total AA)	1.56	1.41	10.61	<0.001	1.36-1.86	1.01, 1.96
Glutamic acid (% total AA)	10.95	11.10	-1.39	0.031	10.64-11.34	10.28, 11.77
Tyrosine (% total AA)	3.68	3.83	-3.82	0.005	3.23-3.94	3.33, 4.07
Test Event J163						
Cystine (% total AA)	1.56	1.41	10.40	0.001	1.35-1.90	1.01, 1.96
Histidine (% total AA)	2.67	2.76	-3.55	0.004	2.44-2.85	2.25, 3.22
Lysine (% total AA)	6.89	7.05	-2.24	0.009	6.50-7.37	6.26, 7.85
Tyrosine (% total AA)	3.69	3.83	-3.62	0.008	3.19-3.86	3.33, 4.07
Acid detergent fiber (% dw)	28.31	25.79	9.79	0.006	20.00-39.67	15.76, 40.19
Lignin (% dw)	6.01	5.07	18.54	0.017	3.94-8.13	0, 12.92
Neutral detergent fiber (% dw)	30.94	28.09	10.13	0.008	24.49-43.57	20.01, 41.80
Test Events J101XJ163						
Cystine (% total AA)	1.57	1.41	11.01	<0.001	1.41-1.84	1.01, 1.96
Isoleucine (% total AA)	4.86	4.94	-1.67	0.027	4.64-5.14	4.25, 5.58
Phenylalanine (% total AA)	5.54	5.67	-2.19	0.017	5.39-6.06	4.64, 6.61
Proline (% total AA)	5.49	5.28	3.97	0.012	5.06-6.16	4.57, 6.06
Tyrosine (% total AA)	3.69	3.83	-3.70	0.007	3.18-3.89	3.33, 4.07
Neutral detergent fiber (% dw)	30.64	28.09	9.05	0.018	21.87-39.73	20.01, 41.80
Calcium (% dw)	1.01	1.12	-9.35	0.023	0.81-1.38	0.48, 1.89
Iron (mg/kg dw)	730.93	410.19	78.19	0.008	199.10-2196.43	0, 892.57
Ash (% dw)	14.41	11.31	27.46	0.015	8.26-32.50	5.59, 16.40
Carbohydrates (% dw)	63.10	65.08	-3.04	0.041	48.03-74.71	46.29, 85.59
Moisture (% fw)	75.78	76.77	-1.29	0.049	70.70-83.10	62.91, 88.67

[1]dw=dry weight; fw=fresh weight; AA=amino acids; [2]99% tolerance interval is the statistical population assessed from the reference substances analyzed with the test and control. Data developed under USDA Notification Number 01-029-12n.

Table VI-36. Literature Ranges for Proximates, Fiber, Amino Acids and Minerals in Alfalfa Forage.

Component[a]	Literature Range	Component[a]	Literature Range
Amino Acids (% dw)		**Proximates**	
Alanine	0.93-1.21[d]	Ash (% dw)	9.5[b]; 5.8-7.5[d]
Arginine	0.81[b]; 0.86-1.08[d]	Carbohydrates (% dw)	-
Aspartic acid	1.97-2.15[d]	Fat, total (% dw)	-
Cystine	0.34[b]	Moisture (% fw)	76-77[b]
Glutamic acid	1.88-2.40[d]	Protein (% dw)	17-27[b]; 17-21.5[d]
Glycine	0.75[b]; 0.82-1.10[d]		
Histidine	0.38[b]; 0.48-0.60[d]	**Fiber (% dw)**	
Isoleucine	0.67[b]; 0.77-0.95[d]	Acid detergent fiber	13-37[b]
Leucine	1.19[b]; 1.35-1.62[d]	Lignin	7[b]; 4.5-7.6[c]
Lysine	0.90[b]; 1.06-1.16[d]	Neutral detergent fiber	40-47[b]; 31.2-44.1[c]
Methionine	0.21[b]; 0.28-0.37[d]		
Phenylalanine	0.78[b]; 0.87-1.08[d]	**Minerals**	
Proline	0.65-1.26[d]	Calcium (% dw)	1.96[b]; 1.39-2.30[e]
Serine	0.72[b]; 0.76-0.95[d]	Copper (mg/kg dw)	10[b]; 3-4[d]; 12-52[e]
Threonine	0.66[b]; 0.78-1.11[d]	Iron (mg/kg dw)	286[b]; 66-78[d]; 204-489[e]
Tryptophan	-	Magnesium (% dw)	0.27[b]; 0.35-0.49[d]; 0.21-0.30[e]
Tyrosine	0.53[b]; 0.66-0.83[d]	Manganese (mg/kg dw)	43[b]; 48-60[d]; 39-46[e]
Valine	0.88[b]; 0.91-1.18[d]	Phosphorous (% dw)	0.30[b]; 0.24-0.34[d]; 0.24-0.42[e]
		Potassium (% dw)	2.09[b]; 1.34-2.35[d]; 1.48-2.78[e]
		Sodium (% dw)	0.19[b]; 0.0024-0.19[e]
		Zinc (mg/kg dw)	18[b]; 30-65[d]

[a]fw=fresh weight; dw=dry weight
[b]Proximate, fiber and mineral values were measured in fresh alfalfa and amino acid values were measured in alfalfa hay, sun-cured (National Research Council, 1982).
[c]Fiber values were measured in dried alfalfa forage (Julier et al., 2000).
[d]Proximate, mineral and amino acid values were measured in freeze-dried alfalfa forage at the first flower stage (Smith, 1969).
[e]Mineral values were measured in dried alfalfa tissue (Townsend et al., 1998).
Conversions: % dw x 10 = g/kg dw; % dw x 10 x fraction of sample that is dry matter = mg/g fw; g/kg dw x 10^3 = mg/kg d

Table VI-37. Iron Content of 2001 Roundup Ready Alfalfa Forage Samples (2nd Cutting) and 2002 Roundup Ready Alfalfa Forage Samples (1st cutting) from the New York Field Site.

				Moisture (% fw)		Ash (% dw)		Iron (ppm dw)	
Line	**Substance**	**Plot #**	**Rep #**	**2001**	**2002**	**2001**	**2002**	**2001**	**2002**
J101	Test	114	1	81.5	83.9	24.54	11.24	1249	319
J101	Test	203	2	81.1	78.9	14.39	7.44	778	212
J101	Test	305	3	79.4	75.7	23.59	7.94	1553	209
J101	Test	406	4	79.4	74.7	28.59	9.41	1408	458
Average				80.4	78.3	22.78	9.01	1247	300
J163	Test	102	1	82.6	81.9	14.43	8.40	661	225
J163	Test	208	2	77.9	79.3	24.30	10.92	1882	422
J163	Test	309	3	78.7	77.8	18.17	8.33	1146	309
J163	Test	404	4	76.2	76.6	26.13	10.64	1878	453
Average				78.9	78.9	20.76	9.57	1392	352
J101XJ163	Test	106	1	78.5	79.1	26.93	9.04	2158	255
J101XJ163	Test	301	2	74.8	74.2	32.50	6.82	1952	370
J101XJ163	Test	410	3	77.6	78.1	30.09	8.45	2196	263
Average				77.0	77.1	29.84	8.10	2102	296

Data developed under USDA Notification Number 01-029-12n.

Table VI-38. Iron Content of 2001 Control and Reference Alfalfa Forage Samples (2nd Cutting) and 2002 Control and Reference Alfalfa Forage Samples (1st Cutting) from the New York Field Site

				Moisture (% fw)		Ash (% dw)		Iron (ppm dw)	
Line	Substance	Plot No.	Rep No.	2001	2002	2001	2002	2001	2002
Null	Control	103	1	79.3	81.1	13.53	9.15	628	198
Null	Control	207	2	79.3	80.4	13.62	8.37	652	202
Null	Control	315	3	77.4	79.6	14.38	9.17	677	273
Null	Control	405	4	75.4	75.4	15.04	9.23	764	467
Average				77.9	79.1	14.14	8.98	680	285
5454	Reference	107	1	78.3	79.9	11.11	9.85	401	269
Macon	Reference	104	1	78.1	83.0	12.56	10.59	383	183
Oneida VR	Reference	108	1	77.3	78.9	12.16	8.48	529	170
WL325HQ	Reference	115	1	77.9	82.5	15.25	14.40	1538	674
Average				77.9	81.1	12.77	10.83	713	324

Data developed under USDA Notification Number 01-029-12n.

I. Symbiotic Organisms

Members of the bacterial family *Rhizobiaceae* are known to form a highly complex and specific symbiotic relationship with leguminous plants. The *Rhizobium*-legume symbiosis results in the formation of root nodules, providing an environment in which differentiated bacteria called bacteroids are capable of reducing or fixing atmospheric nitrogen. The product of nitrogen fixation, ammonia, can then be utilized by the plant. High rates of nitrogen fixation from symbiosis are typically observed in alfalfa (Vance et al., 1988), and the interaction between *Sinorhizobium meliloti* (formerly *Rhizobium meliloti*) and alfalfa has been the subject of scientific investigation (Ferguson et al., 2002).

Historically, several parameters have been evaluated to assess potential biological or environmental effects on nitrogen fixation by *Rhizobium*-legume interactions. These include nodule formation (mass and/or number), root and shoot dry mass, total protein, total nitrogen, and forage yield. At a more specific level, nitrogen-containing components such as amino acids or ureides, and nodule leghemoglobin (the oxygen-carrying protein that plays a critical role in the symbiosis) have been measured, and methods for the analysis of acetylene reduction and ^{15}N isotope dilution have also been utilized to assess nitrogen fixation capacity (Goos et al., 2002; King et al., 2001; and Vance et al., 1988). The above parameters are appropriate for evaluating nitrogen-fixing symbionts in alfalfa. However, the major nitrogen-containing compounds found in alfalfa xylem sap as a product of nitrogen fixation by symbionts are asparagine and aspartate, rather than ureides (Vance et al., 1988).

The symbiotic relationship between *Sinorhizobium meliloti* and Roundup Ready alfalfa was assessed using several of the typical parameters discussed above. Nodule formation (mass and/or nodule number and phenotype) was evaluated on Roundup Ready field- and greenhouse-grown plants. For this evaluation, nodules on Roundup Ready plants were compared to nodules formed on control plants grown under the same conditions. In addition to nodule formation, total protein, forage yield, amino acids (asparagine and aspartate) were measured in Roundup Ready and control alfalfa. Forage yield, total protein and amino acid data were presented and discussed previously in Sections D and G. Their relevance to symbiosis in Roundup Ready alfalfa will be further discussed below. Materials, methods and results from nodule formation experiments are also summarized below.

I.1. Nodule Formation

Nodule formation was evaluated quantitatively by counting the number of nodules per plant in greenhouse-grown seedlings of Roundup Ready and control alfalfa and through a qualitative assessment of the phenotype of nodules formed on field-grown mature Roundup Ready and control alfalfa plants.

I.1.a. Nodule formation on greenhouse grown seedlings

The test substances for this experiment were J101 and J163 alfalfa populations (Figure VI-8, Box 4, approximately 75% trait purity) as well as the J101XJ163 (confirmatory) paired event synthetic population (Figure VI-8, Box 11, approximately 95% trait purity). The control substance was a similar null control synthetic population (Figure VI-8, Box 7). Just prior to planting, the seeds were inoculated using a peat-based commercial alfalfa seed

inoculant product containing *Sinorhizobium meliloti*. Seedlings were grown hydroponically within a coarse vermiculite support media and fertilized bi-weekly with a liquid fertilizer mixture that contained the required macro- and micronutrients but did not contain nitrogen (which would have inhibited nodulation). The experiment was conducted as a randomized complete block with four replications of 100 seeds per population. Sixteen days after planting, 15 seedlings per population per replicate were nonselectively chosen for evaluation. The number of nodules per seedling and the whole seedling fresh weight was measured for each seedling. It was not possible to weigh the nodules, as they were small and tightly associated with root tissue.

Analysis of variance was conducted to compare mean nodules per seedling and whole seedling weight between test and control plants. Differences between means were tested using the least significant difference (LSD) at a significance level of 0.05 ($P \leq 0.05$). Statistical calculations were made using Statistix for Windows software, version 2.2 (Analytical Software, Tallahassee, Florida, USA).

Mean nodules per seedling and the overall range of nodules per seedling, as well as the mean seedling fresh weight and range for test and control plants are presented in Table VI-39. The mean number of nodules and mean plant weight were not statistically different ($P > 0.05$) for all Roundup Ready test materials when compared to control materials (Table VI-39). The color, size, shape, distribution and appearance of the nodules on all plants were similar between the test and control materials (see Figure VI-22).

I.1.b. Phenotype of nodules on mature alfalfa plants

A phenotypic comparison was conducted to assess the gross morphology of the nodules attached to roots of mature field-grown test and control plants growing in an FGI breeding nursery. Plants at the nursery were evaluated when they were approximately one year old. The J101 and J163 single event Roundup Ready alfalfa populations used for this comparison were MBC six populations (Figure VI-8, grandparents to Box 10 with null segregants removed). Paired event plants were plants shown in Figure VI-8, Box 11 (null segregants removed). Nodulation was assessed on J101 and J163 as well as J101XJ163 test populations and compared to nodules on a similar control synthetic population (all test and control plants were derived from a common pedigree). All plants (except for controls) were treated with a Roundup agricultural herbicide in the previous season. For this assessment, roots of four plants of each of the four populations were exhumed and examined for the relative abundance, size and color of the nodules. Because of the large size of each root mass and the inability to recover intact smaller diameter roots with nodules from soil, it was not feasible to obtain an accurate nodule count per plant. Nodules were gathered from all plants and collectively bulked according to the source material and shipped to the laboratory for examination. A representative photograph of nodule formation on a mature J101XJ163 plant is presented in Figure VI-23, panel a. Photographs of nodules associated with J101, J163 and control plants are presented in Figure VI-23, panel b. The color, size, shape, distribution and appearance of the nodules were similar between the test and control materials.

I.2. Forage Yield, Total Protein and Amino Acids

Results from evaluating phenotypic characteristics, including crop compositional data, indicate that there are no biologically meaningful differences between Roundup Ready alfalfa and control or conventional alfalfa populations (see Section VI). In regard to nitrogen-fixing symbionts in Roundup Ready alfalfa plants, results from the analysis of forage for total protein, the amino acids asparagine and aspartate, and forage yield were used to assess the *Sinorhizobium* symbiotic relationship for J101 or J163 alfalfa populations, control and reference varieties.

The composition of forage produced by Roundup Ready alfalfa plants was measured and compared to the composition of control and conventional alfalfa forage. Forage was harvested from plants grown in the field trials described in Section D.2. As indicators of nitrogen fixation by *Sinorhizobium* symbionts of alfalfa, no statistically significant differences in total protein or the amino acids asparagine and aspartate (Table VI-34, note the analysis for aspartate represents a pooled value for asparagine and aspartate) were observed between Roundup Ready alfalfa plants and the control and reference varieties. It must be noted that total protein was derived from empirical measurement of total nitrogen as described by Bradstreet (1965) and Kalthoff and Sandell (1948). Thus, total protein also reflects nitrogen levels in the plant.

Forage yield of alfalfa plants was evaluated in two separate field trials in which plots were not treated with Roundup agricultural herbicides. Further descriptions of the field trials can be found in Section D. The first study was conducted at four locations using alfalfa populations containing J101, J163, and J101XJ163 and control and reference varieties (See Section D.1). Starting seed materials for the study were inoculated with 170 g *Sinorhizobium meliloti* inoculum per 25 kg of seed. With the exception of the Iowa location, plots were not fertilized with nitrogen. Forage yield was measured at each cutting and as total fresh weight in tons/acre (Tables VI-10 through VI-13). While some statistically significant differences were observed for an individual cutting or cumulative forage yield between Roundup Ready alfalfa populations compared to the control populations, the mean value for forage yield was within the range of values observed for the reference varieties at each site. Forage yield was also evaluated in a second field trial (See Section D.3). The field was hand-harvested 12 times and the fresh weight per plot was recorded at seven dates during the study duration (Table VI-21). The mean forage yield at each harvest date and overall yield for Roundup ready alfalfa populations was not different from the control population and not less than that for the reference population.

I.3. Conclusions, Symbiotic Organisms

On the basis of nodule formation data and phenotypic information reported, in particular known indicators of nitrogen fixation by *Sinorhizobium* symbionts in alfalfa such as total protein, asparagine and aspartate, and forage yield, no biologically meaningful differences were detected between Roundup Ready alfalfa J101, J163, or J101XJ163 populations and control or reference populations. The results support the conclusion that there are no meaningful differences in symbiont interactions with J101 or J163 alfalfa populations compared to the control or reference alfalfa populations.

Table VI-39. Number of Nodules and Seedling Weight of Roundup Ready J101, J163 and J101XJ163 and Control Syn1 Populations.

Population	Number of nodules per seedling		Whole seedling weight, fresh weight (g)	
	Mean ±SD[1]	Range	Mean ±SD[1]	Range
J101	5.3±1.8	2 - 10	8.5±1.7	6 - 14
J163	4.9±1.6	2 - 11	8.0±1.6	5 - 13
J101XJ163	5.8 ±1.7	2 - 10	8.3 ±1.6	5 - 14
Control	5.2 ±1.7	2 - 10	8.2 ±2.0	4 - 13
P-value	0.0575 (ns)	-	0.4779 (ns)	-
LSD (0.05)	0.6	-	0.6	-

[1]Mean, standard deviation (S.D.) and range of values are based on four replicates of 15 plants per replicate (n=60 observations). Means were nonsignificantly (n.s.) different ($p \leq 0.05$).

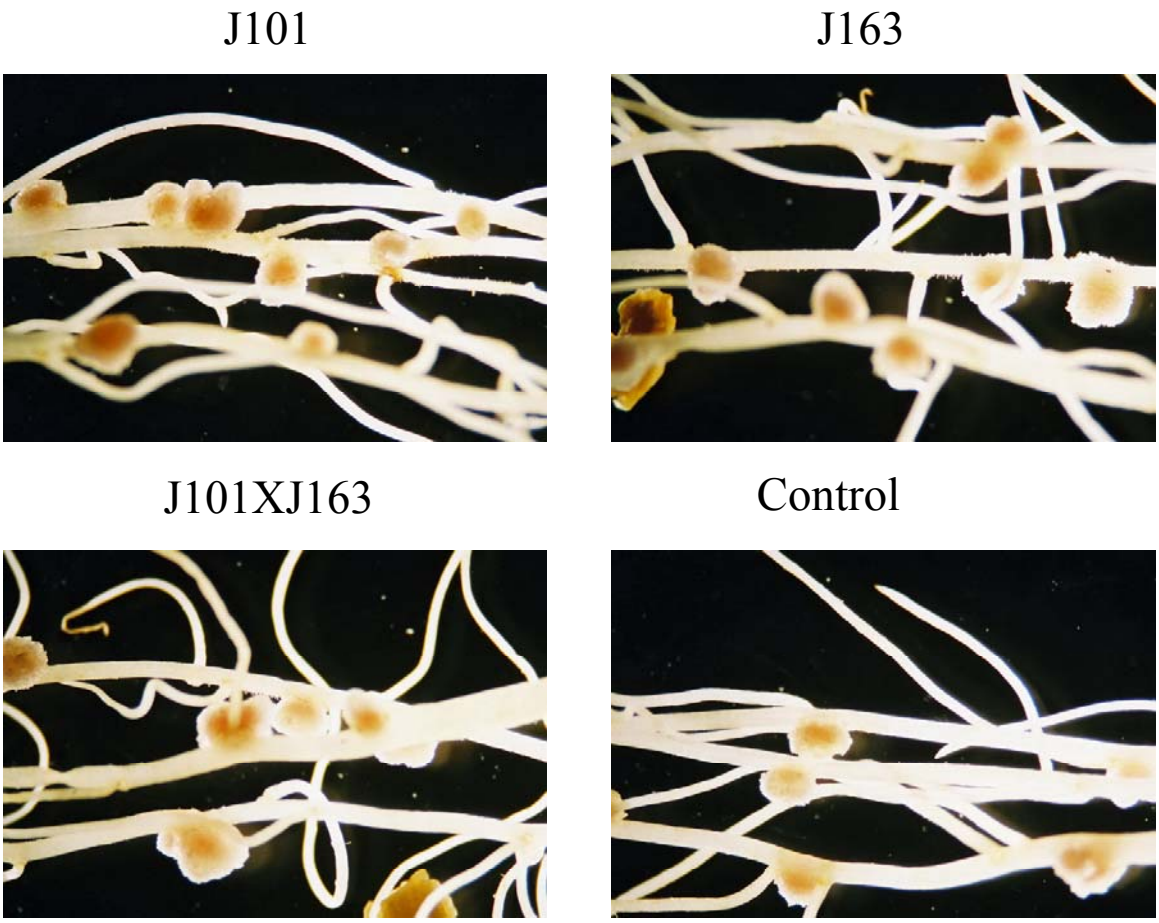


Figure VI-22. Nodule Formation on Roundup Ready and Control Alfalfa Seedling Roots.

Representative photographs were taken of roots of Roundup Ready or control alfalfa populations. Roots with nodules shown above were photographed at 13X magnification.

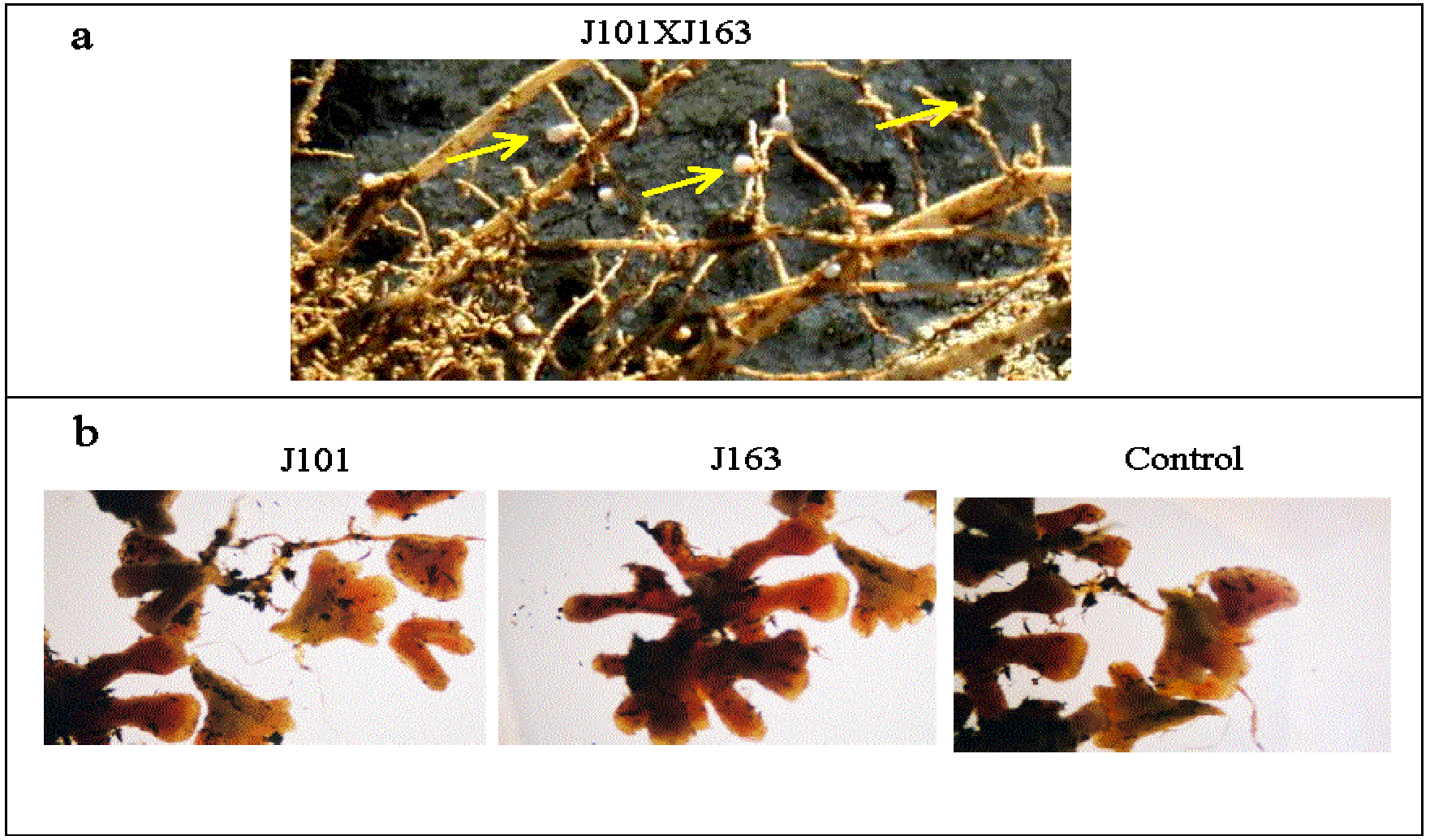


Figure VI-23. Nodule Phenotype on Mature Roundup Ready and Control Alfalfa Roots.

Panel a shows nodule formation (yellow arrows) on roots of a mature Roundup Ready (J101XJ163) plant. Panel b shows phenotype of nodules associated with the roots of J101, J163 and control alfalfa plants. Nodules were photographed at 13X magnification. Plants were harvested from a field in California under USDA permit number 01-205-05n planted without addition of *Sinorhizobium* inoculum as previous experience indicated that there was adequate native Sino*rhizobium* populations in the soil because of long-term use of alfalfa in crop rotations.

J. Conclusions of Phenotypic Evaluation

Monsanto and Forage Genetics International have performed a thorough characterization of Roundup Ready alfalfa populations containing events J101 and J163. Information was presented to assess whether the trait, the transformation process, or ensuing tissue culture process produced alfalfa events that would impact the plant pest characteristics of alfalfa differently than those observed for the control or conventional alfalfa varieties. Additional phenotypic, agronomic and compositional information also were provided to assess familiarity. The characterizations conducted took into consideration the biology of alfalfa and provided information relevant to the assessment of plant pest risk and familiarity. For alfalfa, characteristics that may impact plant pest risk include enhanced growth, vigor or stand longevity; changes in susceptibility to plant pests and diseases; increases in seed yield; and increases in seed dormancy. The overall conclusions from this extensive characterization were that there are no biologically meaningful differences between alfalfa populations that contain event J101 or J163 and the nontransformed alfalfa control or alfalfa reference variety populations. Crop compositional data and other phenotypic and agronomic data provided also lead to the conclusion that alfalfa populations containing event J101 or J163 were not different from the nontransformed control or conventional alfalfa populations. On the basis of these data, it is concluded that there is no increased pest potential of Roundup Ready alfalfa populations containing either of the two events, and that other than the intentional change caused by the trait, the phenotype of alfalfa has not been changed as a result of the trait or transformation process.

VII. Agronomic Practices and Environmental Consequences

This section will review alfalfa's importance as a crop; current agronomic practices for the establishment, management, and termination of alfalfa seed and forage stands on a regional basis; current weed-control options compared to anticipated weed control practices for Roundup Ready alfalfa; and the likely economic and biological impacts resulting from adoption of Roundup Ready alfalfa. In addition, agronomic practices for management of Roundup Ready or volunteer alfalfa are addressed.

A. Importance of Alfalfa

Alfalfa grown for forage is a major crop in the U.S. More than 20 million acres have been planted annually since 1950. Acreage peaked in the mid-1960s at approximately 30 million acres and more recently has been relatively constant at approximately 25 million acres (Table VII-1). Alfalfa acreage in the North Central and Northeastern regions has decreased since the 1960s, but it has increased slightly in the West and Southwest. Acreage in the Southern region has remained more or less constant during this period. Average production in 1950 was 2.2 ton/A. Yields increased to 3 ton/A in the mid-1970s and plateaued around 3.2 to 3.5 ton/A in the 1990s, resulting in an average production of 81.5 million tons with a value of nearly $62 billion (average price per ton was $88.50 in the 1990s). Thus, alfalfa is and will continue to be an important crop in the U.S.

Table VII-1. Changes in Alfalfa Acreage for Four Regions of the U.S. by Decade from 1950 to 2000.

Region[1]	1951-60	1961-1970	1971-80	1981-1990	1991-2000
N. Central	16,770[2]	18,240	17,175	16,720	14,690
West & SW	5,655	6,305	6,655	6,620	6,985
N. Eastern	1,850	2,300	2,135	2,100	1,650
Southern	1,575	1,455	1,445	1,535	1,405
Total	25,845	28,300	27,410	26,975	24,730

[1] N. Central region: ND, SD, NE, KS, MO, KY, MN, OH, MI, IN, IL, IA, WI
West & SW region: WA, OR, ID, MT, NM, AZ, CA, NV, UT, CO, WY
N. Eastern region: ME, MA, VT, NH, NJ, CT, DE, NY, PA, RI, MD
Southern region: AR, TX, OK, LA, MS, AL, FL, SC, NC, GA, TN, VA, WV

[2]Data are presented in thousands of acres and averaged over the decade (USDA, 2002).

B. Current Agronomic Practices for Alfalfa Forage and Seed Production

B.1. Overview

Alfalfa (*Medicago sativa* L.) is a widely adapted perennial species grown for forage in most regions of the United States. The distribution of alfalfa grown as forage across the U.S. is presented in Figure VII-1. Fields grown for alfalfa hay are harvested multiple times per growing season, with the number of cuttings per year dependent upon the length of the growing season. The production of good quality hay demands that the field be harvested at

or before prolific bloom development (i.e., ~10% open flowers), which precludes the production of seed. Therefore, with few exceptions in certain western geographies, an alfalfa field produces either hay or seed, but not both products in a single growing season.

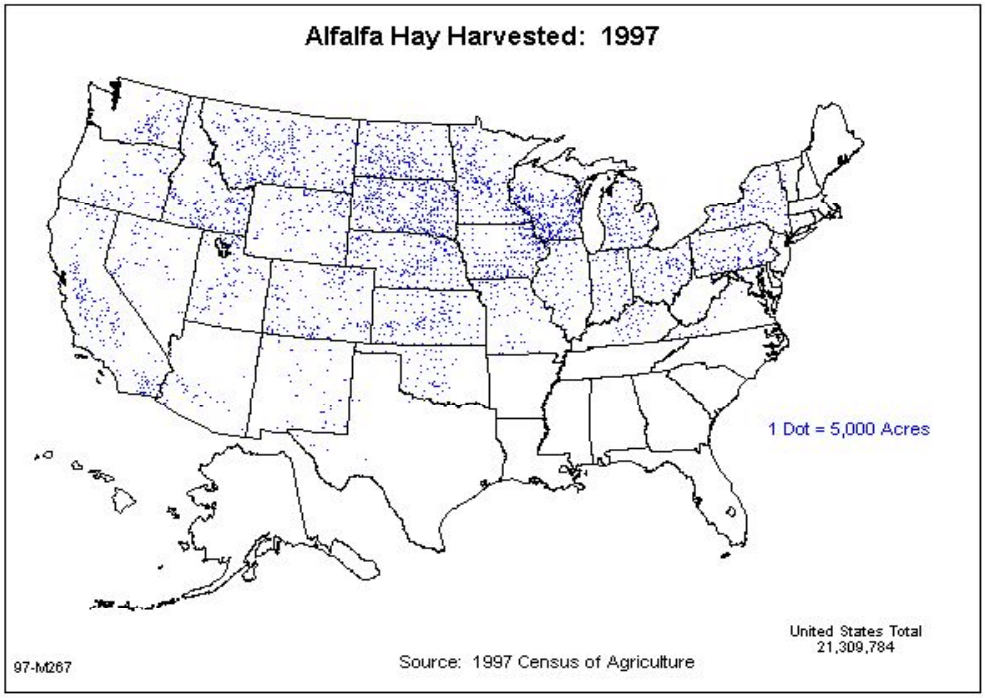


Figure VII-1. Alfalfa Hay (Forage) Acres Harvested in the United States, 1997

In contrast to the broad geographic distribution for forage production, most commercial alfalfa seed production is highly concentrated in irrigated regions of the West (Figure VII-2). The U.S. alfalfa seed crop value, yield, and number of acres planted varies from year to year. The National Agricultural Statistics Service does not report alfalfa seed production statistics but, during the five-year period from 1988 through 1992, alfalfa seed was produced on 172,000 to 227,000 acres nationally. The average yield of clean seed was 432 lb/A and the seed was valued at $1.08/lb (approximately $90M) (Hower et al., 1999). Because of overproduction of seed in the past five years, there has been a trend in recent years toward relatively fewer acres being grown for seed production. Nearly all alfalfa seed is used for the establishment of hay fields, with a minor amount used as field stock seed or for sprouting purposes. Alfalfa seed is not consumed as a grain and therefore not used directly as a food or feed product.

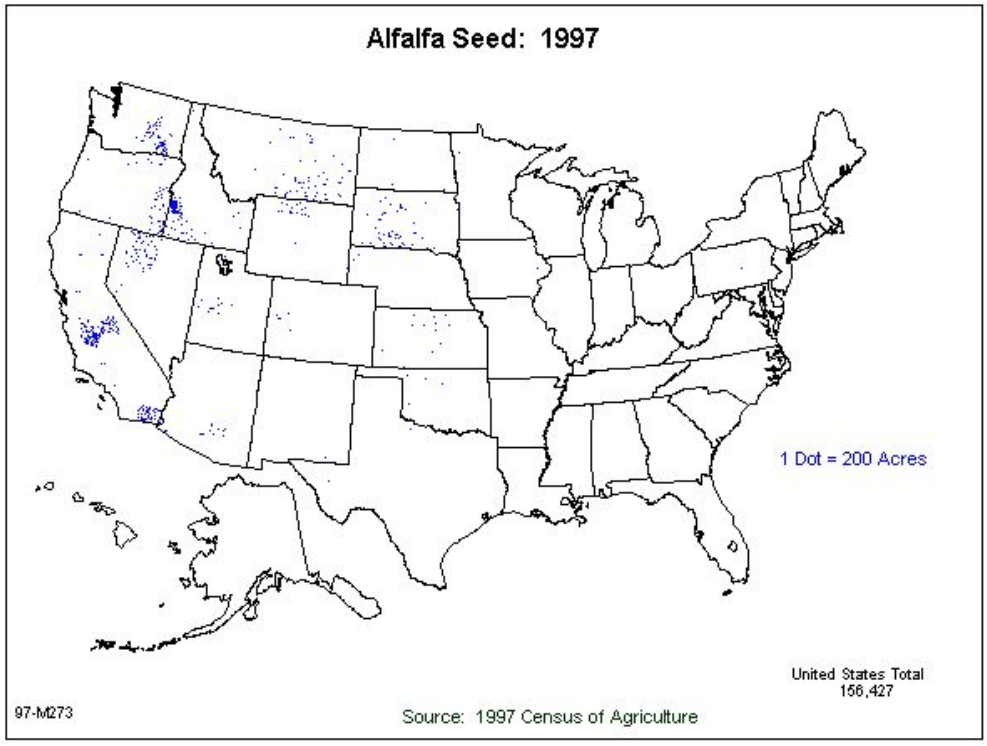


Figure VII-2. Alfalfa Seed Acres Harvested in the United States, 1997

Agronomic practices for alfalfa forage and seed production differ significantly across the nation, as discussed in Section B.8. below. Regardless of geography, the key objective of most alfalfa forage growers is agronomic management of alfalfa as an important source of direct income (e.g., cash hay or seed product sales), indirect income (e.g., livestock products), and beneficial rotational crop. Alfalfa improves soil tilth and, because it is a legume that biologically fixes nitrogen, benefits the rotational crop with the nitrogen that remains in the soil after alfalfa stand take-out (termination). Alfalfa is also a preferred crop on cultivated slopes because it enhances soil conservation by reducing soil run-off potential and the average number of soil tillage operations per year.

B.2. Establishment of Alfalfa for Forage Production

Alfalfa grown for forage production is commonly seeded either alone (called direct, clear, or solo seeding) or it may be sown with a companion crop such as oat (*Avena sativa* L.)—a practice also known as establishment with a nurse or cover crop. If solo-seeded, herbicides are usually used. A key reason for using a cereal companion crop is that the companion crop establishes very quickly, thereby displacing most weeds and protecting the field from soil erosion. The companion crop is maintained with the alfalfa for a temporary period. Stands established by solo or companion crop seeding are nearly always managed for pure-stand alfalfa forage production. In contrast, alfalfa may be only one component established in a mixed-species forage production setting where the species mixture is intentionally maintained for the duration of the stand (e.g., pasture or grazing lands).

Depending upon geography, alfalfa may be established successfully in the spring or later in the growing season (i.e., late summer/fall). In the U.S., 69% of alfalfa acres are spring-seeded and the remaining 31% are planted during late summer and early fall (Hower et al., 1999). According to the 1988-1992 USDA National Agricultural Pesticide Impact Assessment Program (NAPAIP) Survey for alfalfa (Hower et al., 1999), companion-crop seeding is practiced on 43% of spring seeded acres and only 5% of late-summer or fall seedings in the U.S. Because alfalfa is a perennial crop with stands lasting approximately five years on average, only about 18.4% (4.3 M acres) of the 24 M acres devoted to alfalfa forage production in the U.S. are newly seeded each year (Hower et al., 1999; USDA, 2002). In the upper Midwest, there has been a trend toward an increase in solo-seeding, as demonstrated by the fact that oat acreage in this region has declined dramatically from 45 M acres in 1950 to only 4.5 M in 2000, while alfalfa acreage has declined only slightly during this same period (Table VII-1). Therefore it can be inferred that fewer alfalfa acres are being established with a companion crop of oats.

B.3. Establishment of Alfalfa for Seed Production

Commercial seed production in alfalfa occurs almost exclusively in the Pacific Northwest and western states where late-summer seed ripening may occur without damage from rain, heavy dew or high humidity. Approximately 18% of the seed production stands are reseeded each year (Hower et al., 1999). Less than 200,000 acres are managed for alfalfa seed production in the U.S. and average stand-life is approximately 5 years (Hower et al., 1999). Alfalfa grown for the dedicated purpose of commercial seed production is always established alone without a companion crop and 100% of seed acres are established using herbicides (Hower et al., 1999). Fall establishment predominates (58%) because first year seed yields in fall-established stand are greater than for spring seedings. Unlike alfalfa grown for forage, alfalfa seed fields are planted at a low seeding rate (<2 lb. seed/acre) in widely spaced rows. Weeds may be a significant problem between and within the rows of alfalfa. Weed control during establishment is critical because weeds compete with the alfalfa plants, produce abundant seed, and directly reduce the seed yield of the stand. Although all seed growers establish alfalfa using herbicides, shallow tillage is also practiced to improve weed control. After stand establishment, growers may mechanically thin stands by disking because fields with fewer and larger alfalfa plants tend to have higher per acre seed yield, therefore, weeds may be especially problematic where alfalfa plant density is lower. The majority of the dedicated alfalfa seed production fields (i.e., not including catch-crop fields) are established according to the terms outlined in a commercial seed grower contract (e.g., specifications for variety, seed generation, length of stand allowed, and restrictions or prohibitions on certain weed species allowed in the delivered seed product).

B.4. Alfalfa Forage Production

Alfalfa hay may be harvested as haylage (ensiled forage) and dry hay bales, fed without storage as wilted greenchop, or directly grazed by livestock. The predominant method of hay harvest and resulting value per harvested ton of forage varies according to geography, forage quality and abundance. Forage is typically harvested multiple times throughout the season from the same plot. The number of harvests varies from two to 11 per year depending upon the variety and geography where it is grown. When grown for forage production, alfalfa may be grown in pure stands or mixed with various other forage species (e.g., cool season grass

mixtures with or without other legumes such as forage peas, birdsfoot trefoil [*Lotus corniculatus* L.] or clover [*Trifolium* L. spp.]). The use of mixed stands is widespread in the eastern and southern regions of the U.S., where pure-stand alfalfa production is challenged by climate and/or soil-type. In general, species mixtures compete with alfalfa plants for available nutrients, light, space and moisture and thereby limit the hay's market value and alfalfa yield (i.e., percent alfalfa composition or tonnage). Therefore, an increasing number of forage fields are solo (direct) seeded to exploit the benefits of pure-stand alfalfa. Herbicides are usually used during direct-stand establishment.

B.5. Alfalfa Seed Production

Commercial alfalfa seed production for all variety types occurs almost exclusively in irrigated regions of the western United States (Figure VII-2). Alfalfa is a legume in which seeds form inside a tightly curled seedpod after pollination. During the four- to six-week seed maturation period, it is important that the seedpod or curl does not become moist. Moisture during seed ripening can cause the pods to split open and result in yield loss. The ripening seed may sprout inside the pod and/or the harvested seed may have poor germination quality. Therefore, alfalfa seed is primarily produced in the Pacific Northwest and Desert Southwest where moisture can be controlled through irrigation management and summer temperatures are favorable for pollinator activity and rapid seed ripening. In general, seed of non-dormant varieties is produced in the Desert Southwest and dormant variety seed is produced in the Pacific Northwest. Seed yield is highly variable within and among regions, years, and fields and is highly influenced by management, pollinators, pests and environment (Rincker et al., 1988). Seed is typically harvested once per year.

Weeds in seed production fields are controlled by cultivation, preplant, preemergent and postemergent herbicides. Patches of alfalfa fields that contain uncontrolled, prohibited or parasitic weed species such as dodder (*Cuscuta* L. spp.), may be burned to remove the weed or left unharvested. Seed growers are paid for the clean (pure) alfalfa seed they produce and must pay the seed conditioner for cleaning the seed. Therefore, alfalfa seed growers carefully monitor and manage their production fields for weeds.

Seed companies contract the majority of commercial alfalfa seed production. The stand-life of a certified seed field is usually two to four years and is usually predetermined in the contract terms; non-certified seed fields are generally maintained several years longer. Seed producers mechanically thin stands and/or plant alfalfa sparsely (<2 lb/A), in rows or on beds, and introduce large quantities of cultured bees to pollinate the field. Depending upon the relative market prices for forage versus common or variety not stated (VNS) alfalfa seed, a minor proportion of the U.S. alfalfa seed crop is produced without contract as a catch-crop, where solid-seeded hay fields are allowed to set and ripen seed instead of being cut for forage. This practice occurs sporadically in the Plains, Pacific Northwest and Desert Southwest but is absent in the humid Midwest and East regions. The yield and species purity of catch-crop seed are significantly inferior to and of lower value than seed produced under optimum seed crop growing conditions.

B.6. Alfalfa Stand Take-Out Practices

Alfalfa stand take-out (termination) of forage production fields is most often accomplished using conventional or reduced tillage in the fall either with or without the use of herbicides.

Less frequently, stands will be spring-terminated and again the use of herbicides is variable. The use of herbicide(s) for take-out depends on cost, the efficacy of tillage, herbicide sensitivity of the next rotational crop and available options to manage volunteer or surviving alfalfa plants in the rotational crop. For termination of seed production fields, multiple, shallow tillage operations are used, followed by irrigation to induce germination of dropped seed and the decomposition of the killed plants. Herbicides in combination with shallow tillage may be used to kill the seed parent plants and recently germinated seedlings. Generally, in a terminated forage production field, volunteer alfalfa arises from viable alfalfa crowns. In former seed production fields, volunteers may be either from surviving or newly germinated plants. Because the terminated alfalfa stand contributes nitrogen to the soil, non-legumes such as corn (*Zea mays* L.), small grains, cotton (*Gossypium hirsutum* L.), safflower (*Carthamus tinctorius* L.), or vegetable crops frequently follow alfalfa in the rotation. Immediate reseeding of alfalfa is not recommended because of the potential for autotoxicity; decomposing alfalfa plants release water-soluble compounds into the soil that impair germination, vigor, and yield of alfalfa (Tesar and Marble, 1988). The autotoxic effect is correlated to the amount of decomposing alfalfa residue present. The effect subsides after thorough decomposition and appears to be of limited significance when very sparse or young stands precede the new alfalfa seeding.

B.7. Cultivar Breeding and Geographic Adaptation

Alfalfa varieties are bred for adaptation to one or more of the major growing regions where climate, soil type and photoperiod affect crop growth and agronomic management regime (Figure VII-3). There are nine basic germplasm pools that have been used by plant breeders to produce the > 200 registered alfalfa varieties available today (Barnes et al., 1977). Alfalfa producers may choose among numerous regionally adapted varieties to best match their objectives for forage yield and quality, persistence, and resistance to insects, nematodes and diseases, etc. Alfalfa breeders may test new alfalfa strains for adaptation and performance in several regions. During the variety registration and review process by the USDA Plant Variety Protection Office and/or National Alfalfa and Miscellaneous Legume Variety Review Board, alfalfa breeders rate applicant varieties on approximately 15 key agronomic, phenotypic, and pest resistance attributes.

Alfalfa varieties may be divided into three broad groups according to their response to day length: fall-dormant (winterhardy and photoperiod sensitive), non-dormant (winter-active and photoperiod insensitive) and semi-dormant (intermediate photoperiod sensitivity and with limited winterhardiness). The dormancy reaction may be used to predict a variety's general region of adaptation and is measured according to a standard protocol whereby Class 1 is very dormant and Class 11 is extremely non-dormant (Teuber et al., 1998). Class 1-3 varieties are grown where the winters are severe and growing season is short in contrast to Class 9-11 varieties that grow year-round in the irrigated desert areas of the Southwest. Forage production is greatest in non-dormant regions (3.7 T dry matter/A/yr) and less where the growing season is shorter and fall-dormant varieties are used (2.7 T dry matter/A/yr) (Hower et al., 1999). As a consequence of yield potential and crop value differences among regions and seasons, relative grower inputs such as expenditures for seed and weed management also vary widely among regions. Alfalfa forage producers choose alfalfa varieties based on their priorities for yield potential, forage quality potential, pest resistances as well as seed brand, price and other personal preferences.

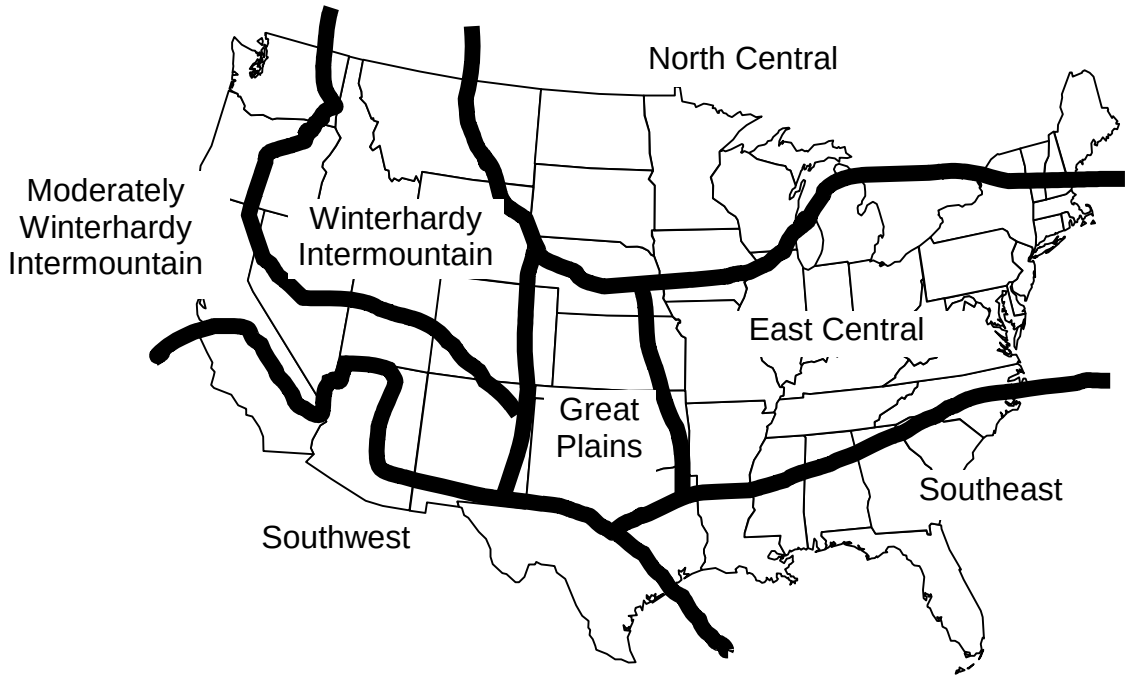


Figure VII-3. Areas of Alfalfa Adaptation in the United States.
Regions recognized by the USDA-Plant Variety Protection Office and National Alfalfa and Miscellaneous Legume Variety Review Board.

B.8. Regional Agronomic Management of Alfalfa Grown for Forage (Hay) and Seed

Although alfalfa is grown across the United States, there are regional differences in production and management. The following sections briefly discuss regional agronomic practices for production of seed and/or hay.

B.8.a. Southwest Region

Alfalfa is a highly valued crop in the Southwest Region where both forage and seed production occur separately.

Seed production is concentrated in a few key valleys of the region where a combination of a long growing season, high temperature, moderate to low altitude, infrequent late season rains and availability of irrigation result in exceptionally good conditions for alfalfa seed production. Seed growers in these valleys can produce high yields (>1000 lb/A) with a low percentage of hard seed (5-15%) (Rincker et al., 1988). Twenty-five to 30 percent of the U.S. alfalfa seed crop is produced in the Imperial Valley and San Joaquin Valleys of California.

Forage production areas are more widely distributed in the Southwest region, but tend to be concentrated near the primary forage consumers—California's dairy industry and alfalfa processing facilities. Most of the high quality forage in the region is sold by hay producers or brokers to dairies and hay processors. Hay cubing, bale compressing, meal and pelleting

companies process alfalfa hay for domestic and export markets. Primary export markets are Japan and other Pacific Rim countries. Many dairies, brokers or processors contract with growers and specify a minimum forage quality. The price of the alfalfa hay is based on forage quality (lab test), appearance (color and leafiness), odor, species purity (lack of weeds), market demand, and abundance of hay within each forage quality class. Alfalfa hay that contains poisonous or prohibited weed forage or seeds will have significantly reduced marketability and value.

In the Imperial Valley, non-dormant alfalfa varieties continue to produce growth nearly year-round and are harvested eight to eleven times per year. Semi-dormant varieties are well adapted to the middle to northern San Joaquin and Sacramento Valleys and are cut six to eight times per year. Although somewhat lower yielding, late-fall-dormant varieties may be grown instead because they produce a higher forage quality feedstuff. Alfalfa varieties are generally selected for high forage yield (ton/A) but forage quality potential is a higher priority for some hay growers. Often, a single forage producer in California will grow several varieties belonging to different dormancy classes so that field cutting schedules may be managed to optimally balance yield, quality and date-to-market.

About two-thirds of alfalfa fields are planted in the fall (September and October) with the balance planted in the early spring (February to mid-March). Stand establishment is made difficult by the many species of weeds that germinate during the cool, wet fall and winter and early spring seasons (Hower et al., 1999). Establishment later in the growing season is impaired by high temperature. Average stand-life for hay fields is between five and six years.

B.8.b. North Central Region (Northern Corn and Dairy Belt)

Alfalfa is not grown for commercial seed production in this region. In the North Central region, winterhardy, fall-dormant varieties (fall-dormant classes 2 to 4) are harvested primarily as haylage and dry hay and fed to dairy cattle. Less commonly, alfalfa growers may graze, greenchop or sell dry hay for dairy, beef or horse feeding. This region grows more than half of the total acres of alfalfa forage in the U.S. (>12 million A/yr). Deep, fertile soils and moderate precipitation in the region make alfalfa a highly productive and economically important crop.

Forage is typically harvested two to five times per year with more harvests (cuttings) in the southern areas that have a longer growing season and adequate moisture. High forage yield (dry matter tonnage), winterhardiness, stand persistence, disease resistance, and feed value (forage quality) are grower priorities in this region. Average hay yield is approximately 2.71 T/A (Hower et al., 1999). Stand-life is typically three to five years—less where the interval between cuttings is short (<34 days) and more where the interval is longer (>40 days). Alfalfa is valued for its contribution to dairy rations, as a deep-rooted, nitrogen-fixing rotational crop for corn and grain rotations, and as a crop well suited to sloped, errodable hillsides where the soil-erosion potential makes growth of row crops problematic.

Weed control is critical to successful alfalfa stand establishment. Seeding typically occurs in the spring (76%), when moisture and weed competition are high (Hower et al., 1999). In the spring, direct or clear seeding (seeding only alfalfa) is practiced on 55% of the new acres, and 55% of the acres are treated with herbicides before or after seeding. The remaining 45% of the spring-planted acres are seeded with a companion crop such as oats to help control

weeds and/or stabilize the soil until the alfalfa is well established. Late-summer seeding accounts for 24% of the alfalfa acres established in this region. Late-summer seeded acres have less potential weed competition, but are more likely to be significantly injured by an early killing frost or lack of rainfall than are spring seedings. Companion crop seeding is practiced on only 5% of the late-summer seedings. Of the 95% of late-summer seeded acres, 85% are treated with herbicides and 51% of these are treated with paraquat (Hower et al., 1999).

Dairy cattle consume most of the forage produced in this region. Milk production is improved by feeding high quality, highly digestible feedstuffs, and a large proportion of the dairy diet may be alfalfa. Consequently, alfalfa forage quality is recognized as economically important in this region. Most hay producers in the region state that forage of high quality is difficult to harvest because of the presence of weeds and relatively frequent precipitation during harvest activities. Weeds such as foxtail (*Setaria* L. spp.), quackgrass (*Elytrigia repens* L.), Canada thistle (*Cirsium arvense* L.), pigweed (*Amaranthus* L.), lambsquarters (*Chenopodium album* L.), mustard (*Brassica* spp.) and volunteer grains compete aggressively with alfalfa and reduce forage quality and palatability.

B.8.c. East Central Region

Alfalfa management and priorities in the East Central Region are most similar to the North Central Region. Farmland in the East Central Region is generally hillier and thus the potential for soil erosion after establishment following conventional-tillage of alfalfa can be severe. Alfalfa is more commonly grown in mixtures with other forage legumes and grasses, although some pure-stand alfalfa is grown. Alfalfa pure-stands and alfalfa mixtures may be pastured and grazed. Alfalfa grown for pure stands is most commonly established with a companion crop such as oats or barley (*Hordeum vulgare* L.) to reduce weed competition and soil erosion potential. In reduced, minimum, or no-till alfalfa establishment, weed pressure can limit stand establishment. Precipitation is greater during the spring and summer in this region, which leads to increased weed pressure during alfalfa stand establishment.

Alfalfa fields in this region are harvested three or four times per year. Alfalfa variety selection is based primarily on yield potential and varieties grown in this region are predominately in the fall dormancy 4 category. Insect and disease pests are more economically important [e.g., potato leafhopper, alfalfa weevil, spring black stem, Sclerotinia (*Sclerotinia trifolium*) crown and stem rot]. Forage yield and quality are generally lower than in other regions because of challenges with weeds, pests, shallow soil depths and weather. Humidity, precipitation and topography make dry hay and haylage harvest operations more protracted and difficult to achieve.

B.8.d. Southeast Region

Soils in the Southeast Region tend to be highly weathered and acidic. Alfalfa is not productive or successful where low pH impairs the nitrogen-fixing symbiotic bacteria (*Sinorhizobium meliloti*) and makes metals such as aluminum highly available. Alfalfa is sensitive to aluminum as germination and growth are significantly reduced. Soil amendments to increase soil pH are not readily available in this region and may not be used because of cost. Thus, alfalfa is not a widely grown forage crop in the Southeast. Alfalfa is most often grown as one component in forage species mixture and mixed stands are

commonly grazed rather than harvested and stored. Relative to forage grass species, alfalfa mixtures may have improved mid-summer forage production and higher protein levels throughout the season. Alfalfa varieties selected for use in the Southeast range from late-fall-dormant to semi-dormant types (fall-dormancy classes 4 to 7).

B.8.e. Moderately Winterhardy, Winterhardy Intermountain and Great Plains Regions

These regions include areas of the Pacific Northwest and Great Plains where alfalfa is grown for both forage and seed production.

Seed production is concentrated in several irrigated areas within the region such as the Treasure Valley of Idaho, the Columbia River Basin of Washington, and isolated valleys throughout the Pacific Northwest (Figure VII-2). Catch-crop seed production occurs sporadically in this region.

Winters along the western slope of the Rocky Mountains are relatively moderate, so higher yielding, less fall-dormant varieties are planted. The alfalfas grown in this region are predominantly moderately fall-dormant varieties (fall-dormancy class 4-5), although in some areas, semi-dormant varieties are also used (fall-dormancy 6-7). The winterhardy Intermountain Region has a shorter growing season and more severe winters so more fall-dormant varieties with good winterhardiness are grown. Fall-dormancy group 3-5 varieties with good winter hardiness are planted in the Great Plains Region where alfalfa fields may be without consistent snow cover for protection from the wind and low temperatures during the winter.

Grower priorities for alfalfa varieties are forage yield, forage quality and resistance to pests. Important in these regions are several yield and stand-limiting pests, such as alfalfa stem nematode, root-knot nematode, pea (*Pisum sativa* L.) spotted alfalfa aphid, and Verticillium wilt. Alfalfa is an important rotational crop in the region. Customary stand-lives for seed and forage production acres are generally two to four and three to five years, respectively.

Alfalfa is primarily direct-seeded without a companion crop in the fall, although spring establishment is also used. In the spring, companion seeding is practiced only on sloping or wind-swept land. When seeded alone, alfalfa is usually established using preplant incorporated herbicides with use of postemergent herbicides to control weeds in the established stand. Many species of weeds germinate throughout the growing season, causing alfalfa yield and forage quality losses. Weed-control challenges for seed and forage production are similar to those in the Southwest Region. Forage is harvested as dry hay and haylage for feeding to dairy and beef cattle; dryland areas may be grazed. Forage is harvested two to six times per year depending upon water availability, variety grown, and length of the growing season.

C. Impact of Weeds in Alfalfa Forage and Seed Production

An important component of alfalfa production in U.S. agriculture is the impact of weeds in alfalfa forage. Weeds affect alfalfa forage production both directly and indirectly. They consistently reduce the yield of the harvested legume but not the total harvested biomass. However, the primary negative impact of weeds in alfalfa is the loss in forage quality. Weeds can significantly reduce forage quality depending upon the weed species present, their stage of development, protein and energy levels, and digestibility. Grass weeds like quackgrass often seriously reduce the forage quality of harvested hay or silage, especially

when fed to dairy cows (Dutt et al., 1979). Dandelion (*Taraxacum*), white cockle (*Silene alba*) and curly dock (*Rumex crispus* L.) are examples of weeds that can increase the drying time of forage because some are wetter at the time of cutting than alfalfa (Doll, 1994). This is of importance when making dry hay in regions where rainfall after cutting, and before harvesting can decrease hay quality, especially if the hay is nearly ready to harvest when rain occurs. Some weeds may increase the drying time by a full day. This impact is minimal if the forage is harvested as silage but can be of great significance when the forage is harvested as dry hay. Weeds can also reduce forage palatability and lower the nitrogen credits available to rotational crops. These effects are summarized by Doll (1986).

Many different weeds can infest alfalfa fields. In a nation-wide survey (Hower et al., 1999), 81 species in spring seeded, 93 species in fall seeded and 98 species in established alfalfa were reported (Table VII-2). The most common annuals were foxtail, pigweeds, lambsquarter, shepherd's purse (*Capsella bursa-pastoris* L.), pennycress (*Thlaspi arvense* L.) and downy bromegrass (*Bromus inermis* L.). The perennial weeds included quackgrass, common dandelion and curly dock. The only biennial noted was musk thistle (*Carduus nutans* L.); however, in the Midwest, burdock, another biennial weed, is appearing with increasing frequency in established alfalfa. Hower et al. (1999) also lists the important weeds in alfalfa seed production systems. Most of these species are controlled by 0.75 lb a.e./A of glyphosate, especially when applied in the fall. However, yellow nutsedge (*Cyperus esculentus* L.), curly dock, common dandelion and orchardgrass (*Dachtylis golmerata* L.) are not readily controlled at this rate early in the growing season, and higher rates must be used.

Table VII-2. The Number of Weed Species Classified by Their Life Cycle for Three Alfalfa Forage Production Environments in the U.S.[1]

Weed Life Cycle	Alfalfa environment		
	Spring Seeded	Fall Seeded	Established
Summer Annual	31	30	31
Winter Annual	21	36	32
Perennial	29	27	35
Total Number	81	93	98

[1]Data summarized from Hower et al. (1999).

Weed management in alfalfa is complicated by the large number of weeds that are of economic importance in the crop, the differences in management required for spring-seedings, fall-seedings, and established stands, and environmental and economic variables that exist between growing regions. Cultural and mechanical weed-management practices are used extensively in alfalfa, but are reported to be substantially less effective than herbicides. Thus, herbicide use is critical to alfalfa management in many areas.

Herbicide use is proportionately greater in the West than in other regions of the U.S. (Hower et al., 1999). This may be due in part to the West's more arid environment in which weed competition has a greater economic impact. In many areas of the North Central and

Northeast regions of the U.S., alfalfa hay is produced and used on the farm. Income from alfalfa hay is generated indirectly through livestock enterprises rather than directly from hay, so there is less recognition of benefits from improved forage quality associated with weed management. In the West, hay is more often grown and sold as a cash crop. The price of hay increases if weeds are controlled (Hower et al., 1999). Gianessi et al. (2002) estimate that the value of alfalfa hay, in California alone, is reduced $21 M/year because of weeds in harvested hay. According to an estimate by the 1988-1992 National Agricultural Pesticide Impact Assessment Program Survey (Hower et al., 1999), the economic benefit of all herbicides used on alfalfa seed and hay acres is $336 M. This benefit was calculated by comparing current integrated weed management strategies and use rates versus estimated production costs and crop value if only cultural methods were used without any herbicides.

The value of all forage is based on the production of milk, meat or animal health and welfare. Dairy scientists and forage agronomists have developed a set of equations known as Milk2000 to give producers the ability to estimate milk production outcome per ton of forage fed based on the quality factors of the forage being fed (Shaver et al., 2001). If, for example, the neutral detergent fiber (NDF) as a percentage of dry matter improves (decreases) from 45 to 42% because of better weed control, milk production would increase by 600 lb/A based on forage production yields of five tons dry matter/A. Such an improvement in forage quality is well within the realm of possibility with the removal of low quality weeds from a well-managed field.

Many of the same impacts of weeds on forage production also apply to seed production (e.g., yield and quality reduction because of weed competition and seed contamination). Alfalfa seed fields are carefully managed to control weeds. Hower et al. (1999) lists the important weeds in alfalfa seed production systems. According to Hower et al. (1999), 100% of commercial seed production fields are treated with herbicides. Hence, weed control is an important component for production of a high-quality seed crop. The primary impact of weeds during seed production is the reduction of seed yields and contamination of alfalfa seed with weed seeds which can increase the seed lot cleaning cost and reduce the seed price. In addition, the presence of regulated weeds may prohibit sale of the seed.

D. Agronomic and Economic Impact of Roundup Ready Alfalfa

Roundup Ready alfalfa technology will offer alfalfa forage and seed producers a new tool to control weeds before, during, and after alfalfa stand establishment. There are many benefits of reducing weeds in alfalfa forage production because weeds negatively impact stand density and vigor, forage quality, market value, palatability and field drying time. Weed seed species and weed quantity in forage or seed products may also negatively affect species purity, marketability, and value of the products. The introduction of Roundup Ready alfalfa will have many benefits over current weed control practices. Use of Roundup Ready technology will simplify and improve weed management in stand establishment; enhance the flexibility of weed control in established stands; improve forage quality by reducing the weed content of harvested forage; provide growers with a more efficient weed control system; and will allow the use of a herbicide that is a more environmentally acceptable alternative to some of the herbicides currently used for weed management in alfalfa.

Alfalfa is one of the first perennial plant species to be developed for herbicide tolerance. The agronomic and economic dynamics of this technology in a perennial crop have some

similarities and differences to its use in annual cropping systems such as Roundup Ready soybean (*Glycine max* L.) and corn. For Roundup Ready annual crops, Roundup agricultural herbicides may be applied preplant and/or at planting, followed by one or more in-crop applications; multiple applications are especially common in minimum-till or no-till established fields. Similarly, in a perennial Roundup Ready crop such as alfalfa, some fields may be treated one or more times per year with a Roundup agricultural herbicide to control weeds. Unlike the annual crops, however, the perennial Roundup Ready plants will be treated across multiple growing seasons.

The following sections will review the herbicides currently used during seedling establishment, in established alfalfa forage and seed production fields, followed by the potential changes in these weed-control practices that may accompany the introduction of Roundup Ready alfalfa varieties during each of these phases. In addition, the economic impact associated with the adoption of Roundup Ready alfalfa will also be discussed. Dr. Jerry Doll of the University of Wisconsin contributed the following sections (Sections D.1. – D.7). Dr. Doll is a Weed Scientist with extensive knowledge of weed-control issues confronting alfalfa forage growers.

D.1. Current Herbicide Options for Seedling Establishment of Alfalfa

Most solo-seeded alfalfa in the U.S. is established with herbicides, with 52% and 66% of spring and fall planted acres, respectively, treated with herbicides (Hower et al., 1999). In total, about 1.49 M acres of alfalfa are established annually using herbicides (Hower et al., 1999). The use of preplant incorporated herbicides such as EPTC (s-ethyl dipropylthiocarbamate), benefin and trifluralin has declined in light of the development of effective postemergence herbicide options such as sethoxydim, clethodim, imazethapyr and imazamox to compliment or replace the older postemergence options of 2,4-DB and bromoxynil.

A review of the characteristics and limitations of each commonly used alfalfa herbicide compared to glyphosate herbicides that would be used in Roundup Ready alfalfa is presented below.

- EPTC is a shoot inhibitor that provides excellent annual grass control and also controls important broadleaf weeds such as common lambsquarters and pigweed. It is the only alfalfa herbicide that suppresses yellow nutsedge. It is weak on weeds in the mustard family and eastern black nightshade (*Solanum ptycanthum*). EPTC requires immediate mechanical incorporation into the soil or must be applied via chemigation to avoid losing the active ingredient through vaporization. This product has a slight to moderate risk of stunting alfalfa in the first few weeks after application.
- Benefin and trifluralin are root-inhibiting dinitroanaline herbicides that control annual grasses and many annual broadleaves. Ragweed (*Ambrosia* L.), nightshades, most mustards and velvetleaf (*Abutilon theophrasti*) are not controlled. The risk of crop injury is minimal. If not mixed into the soil surface within 12 hours of application, some of the active ingredient could be lost to photodecomposition.
- Bromoxynil is a postemergence product that controls many annual broadleaves and reduces the competition of giant and green foxtail. It is particularly effective on common

lambsquarters and mustards but is weak on pigweed species. Crop injury is highly correlated with air temperature at the time of application (treatment cannot be made in the upper Midwest if the high temperature will exceed 70º F the day of application and for the next three days). This restriction, in conjunction with the labeled requirement to not treat alfalfa until plants have four trifoliate leaves, limits the number of days suitable for bromoxynil treatment in states east of the Mississippi. Most states west of the Mississippi can use an 80º F cutoff temperature.

- 2,4-DB [4-(2,4-dichlorophenoxy) butyric acid)] is seldom used in new seeding for annual broadleaf control because it has a 60-day harvest interval and is relatively expensive. This is the only product with a serious conflict in harvest interval in newly seeded alfalfa. 2,4-DB is deficient in controlling several common broadleaf weeds in forages; examples include nightshades, smartweed (*Polygonum pensylvanicum* L.), and most weeds in the mustard family.
- Imazethapyr and imazamox are imidazolinone herbicides that control many annual broadleaves and several annual grasses. These products have similar activity with the exception that imazamox is more effective on common lambsquarters and foxtails than imazethapyr and has less soil persistence. They are the only postemergence herbicides that have residual activity and control weeds that would otherwise appear after the application. Numerous ALS (acetolactate synthase) resistant weed biotypes have appeared in grain crop production systems.
- Sethoxydim and clethodim are graminicides that kill only grasses (all annuals and several perennials). Thus, they fit fields with a predominance of grasses or they need to be tank-mixed with a broadleaf product. Both are used to kill oats grown as a temporary cover crop and after alfalfa establishment when yellow foxtail, barnyardgrass (*Echinochloa crus-galli* L.) and large or smooth crabgrass (*Digitaria* L.) appear in mid- or late-summer.

Reports of herbicide use in alfalfa include paraquat and glyphosate as burndown herbicides in no-till systems. The introduction of Roundup Ready alfalfa may well increase the adoption of no-till alfalfa establishment as in soybean (Fawcett and Towery, 2002) because a wide range of both annual and perennial weed species can be controlled both before and after seeding. Paraquat is a restricted-use pesticide because of its relatively high acute toxicity (LD_{50} approximately 90 mg/kg).

D.2. Impact of Roundup Ready Alfalfa on Seedling Establishment Practices

Forage agronomists long have known that the initial establishment condition of alfalfa is a strong predictor of stand productivity and longevity. Thus, the ability to establish an alfalfa stand free of most biotic stresses will have benefits both initially and in later production years. Vigorous, dense pure-stand alfalfa establishment will be facilitated by the improved weed control of the Roundup Ready alfalfa technology, because the competitive stress from weeds and/or a companion crop will be greatly reduced compared to current practices. In the seeding year, weeds are often a serious competitor with alfalfa, especially in direct-seeded systems. Alfalfa planted in the spring grows slow, relative to the rapid grown of most weeds or companion small grains, and, therefore, alfalfa has a competitive disadvantage during the seedling establishment period. Even though alfalfa tolerates early season competition (as evidenced by its survival with a companion seeding), weeds will rarely provide the same

level of feed value or palatability as pure alfalfa. If the alfalfa stand density or vigor is compromised by weed or companion-crop competition during establishment, other stressors such as drought, insect pests, disease, wet soil conditions, etc., may further weaken and thin the stand. Stressed, thin alfalfa stands will allow weeds to reappear more quickly than if the stand is dense and the plants regrow vigorously.

The following key aspects summarize the potential changes that may occur to weed management in alfalfa establishment after Roundup Ready alfalfa is available to the alfalfa industry.

Improved spring establishment. Spring is the typical time that forage alfalfa is seeded in most of the U.S., and 76% of acres are spring-seeded in the Northeast and North Central Regions (Hower et al., 1999). The use of Roundup Ready alfalfa varieties could replace the use of trifluralin, EPTC, imazethapyr, imazamox, sethoxydim, clethodim and bromoxynil herbicides that are currently used on this acreage. Some producers, particularly in the Northeast, that currently avoid no-till/reduced-till and spring plantings primarily because of weed pressure potential, may return to the spring establishment option with Roundup Ready alfalfa varieties. Roundup Ready technology would be compatible with companion-crop methods where soil erosion is of concern. While late-season establishment offers some advantages, it also has the risks of insufficient moisture, which can result in poor, late, or uneven germination, and damage from early frost, etc.

Improved late-season establishment. The availability of Roundup Ready alfalfa varieties also will provide producers with excellent weed control during late-summer and fall establishment. In the northern and northeastern regions, late summer seeding is gaining in popularity while in the southern and western regions most alfalfa (64%) is currently established in the fall (Hower et al., 1999). In the North Central and Northeast regions, herbicides are seldom needed for late summer seedings because most summer annual weeds are well past their peak germination period. Unless weed pressure from summer annuals is moderate to heavy, no herbicide is needed because frost will kill this class of weeds. For those growers who seed alfalfa following winter wheat (*Triticum aestivum* L.) harvest, volunteer wheat is often the most serious weed present. Current options of sethoxydim and clethodim may not give complete control because wheat is not easily killed by their mode of action and proper application timing is essential. The use of a Roundup agricultural herbicide will improve the level of wheat control and provide a wider application window than current herbicide alternatives.

Harvest interval will not limit weed management decisions. The preharvest interval is only a serious concern for 2,4-DB because it has a 60-day preharvest interval in new seedings and 30 days in established alfalfa. The preharvest interval of 30 days for imazethapyr is not normally a concern. Preharvest intervals of 20 days or less are not a concern, because herbicides must be applied at least 21 days before harvest to achieve the expected weed control or suppression. Because Roundup agricultural herbicides can be used in conventional alfalfa varieties for stand renovation treatment with only a 36-hour preharvest interval, it is assumed that a relatively short preharvest interval also will be labeled for Roundup Ready alfalfa varieties.

Perennial weed control will improve. Perennial weeds often are controlled prior to seeding alfalfa primarily because herbicides available for use after seeding have little if any activity

on perennial species. The notable exceptions would be sethoxydim and clethodim, which effectively suppress several perennial grasses such as quackgrass and wirestem muhly (*Muhlenbergia frondosa*). The availability of glyphosate for in-crop use will allow producers to control most perennial species effectively in all years of the stand, including the seeding year.

Application timing will be more flexible. Currently, postemergence products like bromoxynil have serious limitations for use when very warm temperatures may occur during and after application, while 2,4-DB, bromoxynil and the imidazolinones require that weeds be relatively small when treated. Roundup agricultural herbicides can be applied under any growing season temperature regime and will control taller weeds better than most other postemergent herbicide alternatives.

The spectrum of weeds controlled will increase. No herbicide product currently available controls the full range of weeds found in most alfalfa fields. Roundup agricultural herbicides should control nearly all weeds present at the time of application, even problem grassy weeds such as foxtails, barnyardgrass, annual bluegrass (*Poa* L. spp.), crabgrass, as well as annual broadleaf weeds such as kochia (*Kochia scoparia* L.) and dandelion. Roundup agricultural herbicides are well recognized for their broad spectrum weed control. In a recent review of how well 16 different herbicides controlled 48 different annual and perennial weed species found in California alfalfa production fields, glyphosate provided full control of 42 species, partial control of four species, did not control one species, and no data existed for the remaining one species (University of California, 2001). The other 15 herbicides described in the review provided full control of only 11 to 24 species each. The susceptibility of weeds to the 16 herbicides is presented in Tables VII-3, VII-4 and VII-5 for spring/summer; perennial/ biennial/nutsedge, and winter annual weeds, respectively.

Table VII-3. Susceptibility of Annual Spring / Summer Weeds to Herbicide Control.

	Herbicide															
Annual Weeds	**benefin**	**bromoxynil**	**diuron**	**EPTC**	**paraquat**	**pronamide**	**metribuzin**	**sethoxydim**	**trifluralin**	**hexazinone**	**glyphosate**	**2,4-DB**	**imazethapyr —high rate**	**clethodim**	**norflurazon**	**pelargonic acid**
barnyardgrass (*Echinochloa crys-galli* L.)	C	N	P	C	P	C	C	C	C	P	C	N	C	C	C	C
cupgrass, prarie (*Erichloa contracta*)	C	N	P	P	P	C	-	C	C	P	C	N	N	C	C	-
dodders (*Cuscuta* L. spp.)	N	N	N	N	P	P	N	N	C	N	C	N	P	N	-	-
foxtail, green (*Setaria vividis* L.)	C	N	C	C	-	C	P	C	C	C	P	N	C	C	C	-
foxtail, yellow (*Setaria glauca* L.)	C	N	P	C	N	P	P	C	C	C	C	N	C	C	C	-
goosefoot, nettleleaf (*Chenopodium murale* L.)	P	C	C	C	C	C	C	N	C	C	C	C	O	N	-	C
Goosegrass (*Eleusine indica* L.)	P	N	C	C	P	C	P	-	C	-	C	N	-	C	C	-
Junglerice (*Echinocloa colona* L.)	C	N	C	C	P	C	P	C	C	C	C	N	P	C	C	C
knotweed, prostrate (*Polygonum aviculare* L.)	C	P	C	P	P	C	N	N	C	C	C	P	C	N	-	C
lambsquarters, common (*Chenopodium album* L.)	C	C	C	C	N	C	P	N	P	C	C	C	P	N	-	-

C = Control; N = No control; P = Partial control; - = No information; Source: University of California Pest Management Guidelines, Alfalfa: Susceptibility of Weeds to Herbicide Control, 2001

Table VII-3 (continued). Susceptibility of Annual Spring / Summer Weeds to Herbicide Control.

	Herbicide															
Annual Weeds	**benefin**	**bromoxynil**	**diuron**	**EPTC**	**paraquat**	**pronamide**	**metribuzin**	**sethoxydim**	**trifluralin**	**hexazinone**	**glyphosate**	**2,4-DB**	**imazethapyr - high rate**	**clethodim**	**norflurazon**	**pelargonic acid**
nightshades (*Solanum* L. spp.)	N	C	C	C	P	C	N	N	N	C	C	C	C	N	C	-
pigweeds (*Amaranthus* L. spp.)	C	P	C	C	P	C	C	N	C	C	C	C	C	N	C	-
stinkgrass (*Eragrostis cilianensis*)	C	N	C	C	P	C	P	C	C	P	C	N	N	-	C	-
thistle, Russian (*Salsola kali* L.)	P	C	N	P	P	P	P	N	P	P	C	P	P	N	-	C
Witchgrass (*panicum capilarre* L.)	C	N	N	C	P	C	C	C	C	P	C	N	N	C	C	C

C = Control; N = No control; P = Partial control; - = No information

Source: University of California Pest Management Guidelines, Alfalfa: Susceptibility of Weeds to Herbicide Control, 2001

Table VII-4. Susceptibility of Perennial, Biennial, and Nutsedge Spring / Summer Weeds to Herbicide Control.

	Herbicide															
Perennial, Biennial, and Nutsedge	**benefin**	**bromoxynil**	**diuron**	**EPTC**	**paraquat**	**pronamide**	**metribuzin**	**sethoxydim**	**trifluralin**	**hexazinone**	**glyphosate**	**2,4-DB**	**imazethapyr - high rate**	**clethodim**	**norflurazon**	**pelargonic acid**
barley, foxtail (*Hordeum jabatum* L.)	P	N	P	P	P	C	C	C	P	P	C	N	N	C	C	-
Bermudagrass (*Cynadon dactylon* L.)	P	N	N	P	N	N	N	P	N	N	C	N	N	P	P	-
bindweed, field (*Convulvulus arvensis* L.)	N	N	-	N	N	-	-	N	P	-	P	-	N	N	N	N
Dandelion (*Taraxacum*)	N	N	-	-	P	N	P	N	N	P	C	C*	N	N	-	-
johnsongrass (seedling) (*Sorgum halepense* L.)	P	N	C	C	-	C	N	C	-	C	C	-	C	C	C	-
nutsedge, yellow (*Cyperus esculentus* L.)	N	N	N	P	N	N	P	N	N	N	P	N	P	N	P	N
plaintain, buckhorn (*Plantago lanceolata* L.)	N	N	N	-	P	-	-	N	N	N	P	C*	N	N	P	-
Quackgrass (*Elytrigia repens* L.)	-	N	P	P	N	C	-	L	N	P	C	N	P	-	-	N

C = control; P = partial control; N = no control; - = no information; L = controlled according to label

* Control only in seedling stage.

Source: University of California Pest Management Guidelines, Alfalfa: Susceptibility of Weeds to Herbicide Control. 2001

Table VII-5. Susceptibility of Winter Annual Weeds to Herbicide Control.

	Herbicides															
Annual Weeds	**benefin**	**bromoxynil**	**diuron**	**EPTC**	**paraquat**	**pronamide**	**metribuzin**	**sethoxydim**	**trifluralin**	**hexazinone**	**glyphosate**	**2,4-DB**	**imazethapyr - high rate**	**clethodim**	**norflurazon**	**pelargonic acid**
barley, hare (*Hordeum leporinum*)	P	N	P	P	C	C	C	P	C	P	C	N	N	C	C	-
bluegrass, annual (*Poa annua* L.)	C	N	C	C	P	C	P	N	C	P	C	N	P	C	C	C
bluegrass, bulbous (*Poa bulbosa* L.)	-	N	N	-	C	C	C	N	P	P	C	N	N	N	-	-
brome, downy (*Bromus tectorum* L.)	P	N	P	C	C	C	C	C	C	C	C	N	N	C	C	-
canarygrasses (*Philaris* L., spp.)	C	N	C	C	P	C	C	C	C	P	C	N	P	P	C	-
cereal, volunteer	P	N	C	C	P	C	P	P	N	P	C	N	N	C	C	-
chickweed, common (*Sellaria media* L.)	C	N	C	C	P	P	C	N	P	P	C	N	C	N	C	C
Fiddlenecks (*Amsinckia* Lem. Spp.)	C	C	C	P	P	C	C	N	C	C	C	N	P	N	P	C
filarees (*Erodium* L. spp.)	N	P	C	N	N	N	C	N	N	C	C	P	C	N	N	-
flixweed (*Descurainia sophia* L.)	N	C	C	N	C	N	C	N	N	C	C	C	C	N	N	-

C = control; P = partial control; N = no control; - = no information.
Source: University of California Pest Management Guidelines, Alfalfa: Susceptibility of Weeds to Herbicide Control. 2001

Table VII-5 (continued). Susceptibility of Winter Annual Weeds to Herbicide Control.

	Herbicides															
Annual Weeds	**benefin**	**bromoxynil**	**diuron**	**EPTC**	**paraquat**	**pronamide**	**metribuzin**	**sethoxydim**	**trifluralin**	**hexazinone**	**glyphosate**	**2,4-DB**	**imazethapyr - high rate**	**clethodim**	**norflurazon**	**pelargonic acid**
groundsel, common (*Senecio vulgaris* L.)	N	P	N	P	P	N	P	N	N	C	C	N	P	N	N	C
lettuce, prickly (*Latuca serriola* L.)	N	P	P	C	P	N	C	N	N	C	C	C	N	N	N	-
mallow, little (cheeseweed) (*Malva parviflora* L.)	N	P	P	N	N	N	C	N	N	P	C	N	C	N	P	P
miner's lettuce (*Claytonia perfoliata*)	P	P	C	N	C	P	-	N	C	C	C	N	C	N	P	-
Mustards (Brassicaceae family)	N	C	P	N	P	P	C	N	N	C	C	C	C	N	C	-
nettle, burning (*Urtica urens* L.)	C	P	C	P	P	C	C	N	C	C	N	P	C	N	N	-
oat, wild (*Avena fatua* L.)	N	N	N	C	P	C	N	C	N	P	C	N	P	C	C	-
Pepperweeds (*Lepidium* L. spp.)	-	C	C	-	C	N	C	N	N	P	-	C	C	N	N	-
radish, wild (*Raphanus raphinistrum* L.)	N	C	C	N	P	P	C	N	N	C	C	N	C	N	C	-

C = control; P = partial control; N = no control; - = no information.
Source: University of California Pest Management Guidelines, Alfalfa: Susceptibility of Weeds to Herbicide Control. 2001

Table VII-5 (continued). Susceptibility of Winter Annual Weeds to Herbicide Control.

	Herbicides															
Annual Weeds	**benefin**	**bromoxynil**	**diuron**	**EPTC**	**paraquat**	**pronamide**	**metribuzin**	**sethoxydim**	**trifluralin**	**hexazinone**	**glyphosate**	**2,4-DB**	**imazethapyr - high rate**	**clethodim**	**norflurazon**	**pelargonic acid**
rocket, London (*Sisymbrium irio* L.)	N	C	C	P	C	C	C	N	N	C	C	P	C	N	C	-
ryegrass, Italian (*Lolium multiflorum*)	C	N	P	C	C	C	C	C	C	C	C	N	N	C	C	-
ryegrasses (*Lolium* L. spp.)	C	N	P	C	C	C	P	C	C	P	C	N	N	C	C	-
shepherd's purse (*Capsela bursa-pastoris* L)	N	C	C	P	P	C	C	N	N	C	C	P	C	N	C	-
sowthistles (*Sanchus* L. spp)	N	C	P	C	N	P	N	N	C	C	C	C	N	N	P	-
starthistle, yellow (*Centaurea solstitialis* L.)	P	P	C	C	P	N	-	N	N	C	C	C	N	N	N	-

C = control; P = partial control; N = no control; - = no information.
Source: University of California Pest Management Guidelines, Alfalfa: Susceptibility of Weeds to Herbicide Control. 2001.

Crop injury will be minimal. All currently used herbicides that control broadleaf weeds in newly seeded alfalfa have the potential to injure the crop. Roundup agricultural herbicides used over the top of Roundup Ready alfalfa will significantly minimize this risk.

No-till alfalfa establishment will be enhanced. No-till alfalfa establishment is appropriate in any field with the potential for soil erosion caused by wind or water. The no-till seed drills available today make this a sound alfalfa seeding method. Alfalfa acreage established with no-till methods is currently very low because of the difficulty in controlling weeds before and after planting. Roundup Ready soybean varieties have increased the number of acres established using no-till seeding methods (Fawcett and Towery, 2002) and the same likely will happen for alfalfa establishment when Roundup Ready alfalfa varieties are available. This will allow producers to have better weed control in no-till and reduced-till plantings with or without using a companion crop.

Faster and more efficient take-out of small grain companion crop. While the overall acreage planted in oats is declining, the use of oats as a temporary (early season only) companion crop for alfalfa is growing in popularity in the upper Midwest. If well-cleaned seed oats are used, this establishment system may be relatively weed-free and the current use of sethoxydim or clethodim to kill the oats is effective. However, an advantage of using the Roundup Ready technology in conjunction with companion-crop establishment is that the broadleaf weeds would also be controlled if glyphosate were used in place of clethodim or sethoxydim. In a recent field study, oats used as a temporary companion crop were controlled several days faster with Roundup agricultural herbicide than with ACCase inhibitors, thereby ending the oat competition sooner and benefiting the growth of the young alfalfa plants (Doll, 2002; Doll et al., 2002)).

D.3. Current Herbicide Options in Established Alfalfa

On a national level, relatively few acres of established alfalfa are currently treated with herbicides (Table VII-6). Approximately 5.1 M acres (26%) of established forage alfalfa in the U.S. were treated with a herbicide during the 1988-1992 NAPAIP survey period (Hower et al., 1999). The primary reasons for the limited use of herbicides on established alfalfa are: 1) most herbicide treatments are relatively expensive; 2) several of the herbicides can only be applied to dormant or nearly dormant alfalfa during the early spring to avoid crop injury, which is not convenient; 3) some weeds have few efficacious herbicide options (curly dock and broadleaf plantain, for example); and 4) the logical and common practice is to terminate thin or weedy alfalfa stands and rotate to another crop for one or two seasons.

Table VII-6. Estimated Herbicide Use in the U.S. in Alfalfa.

		Percent Acres Treated		Total lb a.i. used (1000s)	
Herbicide	lb a.i./A applied[1]	New/ Established plantings[2]	New and Established plantings (combined)[3]	New/ Established plantings[2]	New and Established plantings (combined)[3]
benefin	1.2	<1/na[4]	<1	128/na	119
bromoxynil	0.40	<1/na	<1	18/na	37
clethodim	0.126	nm[4]	<1	nm	4
diruron	1.4	na/<1	<1	na/286	271
EPTC	3.0	<1/na	1	493/na	695
glyphosate	0.85	1/<1	<1	293/154	175
hexazinone	0.60	na/4	2	na/671	316
imazethapyr	0.055	<1/1	2	12/20	28
metribuzin	0.56	na/5	3	na/593	319
norflurazon	1.3	nm	<1	nm	43
paraquat	0.50	<1/4	3	80/366	355
pronamide	1.2	na/<1	<1	na/125	24
sethoxydim	0.23	3/3	3	30/150	132
terbacil	0.50	na/1	<1	na/195	47
trifluralin	1.6	<1/2	3	32/375	950
2,4-DB	1.0	1/<1	2	222/177	389
Total	--	29%		4,420	3,904

[1] Application rates from Gianessi (1997)
[2] Data from Hower et al. (1999) for 1990 new versus established plantings; alfalfa acreage reported as 23,000,000 acres.
[3] Data from Gianessi (1997) for mid 1990s for new and established plantings combined; alfalfa acreage reported as 21,300,000.
[4] na = not applied in this phase; nm = not marketed at that time

A review of the characteristics of the herbicides used on established alfalfa and their limitations is presented below.

- Sethoxydim and clethodim provide excellent annual grass control, reasonable suppression of several perennial grasses (e.g., quackgrass), have a wide window of application, and have no risk of crop injury. Glyphosate will not improve the weed control options of annual grasses in established alfalfa, but will be advantageous for perennial grass species—especially for bluegrass, orchardgrass, bromegrass, and timothy (*Phleum pratense* L.) control. Because the graminicides do not control any broadleaf weeds, another herbicide(s) must be applied with potentially different timing to control broadleaf species.
- Metribuzin is a photosystem II inhibitor that must be applied to dormant alfalfa or impregnated onto dry fertilizer and applied to dry foliage and before plants are four

inches tall in early spring to avoid crop injury. There are no mid-season opportunities to apply metribuzin. Metribuzin controls nearly all winter and summer annual weeds and effectively suppresses several perennial weeds, including common dandelion. Quackgrass and other perennial grasses are often suppressed through the first cutting. Most crops can be planted the year after a spring application of metribuzin was made to established alfalfa.

- Another photosystem II inhibitor, hexazinone, has similar uses and the weeds controlled are very similar to those described for metribuzin. Hexazinone allows growers more flexibility to apply it as a spray in the spring because it can be applied to alfalfa with up to two inches of new growth. Only field corn can be planted the year after a hexazinone application.
- Diuron is used in established alfalfa in western states. It is another photosystem II inhibitor with considerable persistence in the soil. It is particularly effective on winter annual weeds in fall seeded or established stands. Rotation to other crops is not recommended for two years after a diuron application.
- Terbacil is a very persistent photosystem II inhibitor that kills several important perennial weeds in alfalfa. Terbacil is rarely used in the Midwest and Northeast because of its persistence, it cannot be used in California, and it has a surface and groundwater advisory statement on the label.
- Norfurazon is another photosystem II inhibitor that can be used in established stands of alfalfa in many southern and western states. It is less persistent than terbacil.
- Pronamide is a shoot inhibitor that controls several annual grass and winter annual weeds and can suppress quackgrass in established stands. The price limits pronamide use west of the Mississippi and a 120-day interval between application and harvest east of the Mississippi curtails nearly all use of this product except in the West.
- Pelargonic acid is a relatively new, non-selective, broadspectrum, burn-down, contact herbicide that is considered by some to be a non-traditional herbicide with low toxicity and low environmental persistence. Therefore, its use may be allowed on certain "pesticide-free" or specialty fields. Pelargonic acid controls small seedling weeds but only suppresses established weeds. It works better above 70ºF and therefore, even as a burn-down, it is not well suited for use during alfalfa's dormant period. Relative to other herbicides that may be used immediately after harvest, it is more expensive, caustic to equipment and less effective. Pelargonic acid is not widely used.

D.4. Impact of Roundup Ready Alfalfa on Established Stands

Several of the advantages described for stand establishment (Section D.2.) will also apply to established alfalfa stands. These include improved flexibility of herbicide application, a broader spectrum of weeds controlled, and the improved environmental profile of Roundup agricultural herbicides. There are several additional advantages of the Roundup Ready weed control system that apply to established stands, and these are discussed below.

Herbicide treatment as needed. After Roundup Ready alfalfa is established, the frequency and timing of herbicide use can be tailored to the integrated pest management

principle of treat as needed (within labeled use rates). Unlike the restrictive timing options available for most herbicides applied in established alfalfa, Roundup Ready technology will allow producers to treat and target weed problems as they arise during any point in the growing season. Most other herbicides can only be applied when the crop is dormant, which means weeds cannot be controlled when first observed but only at the end of the season or very early in the next year.

In the midwest and northeast regions, a single fall application of a Roundup agricultural herbicide will keep fields free of most common perennial and winter annual weeds for the following growing year (Doll, 2003). If winter annuals like shepherd's purse, chickweed (*Sellaria media* L.) or pennycress appear, then a fall treatment of glyphosate would effectively control these weeds. Such species are very common in the South and West and most late-summer and fall-seeded alfalfa in these regions is routinely treated with herbicides. Such applications normally will not be needed the first year of full alfalfa production, but in the second and subsequent years, fall applications of Roundup herbicide likely will be a common practice. In the South and West where the growing season is long, herbicide applications may be needed the first year after establishment; in subsequent years, two applications of Roundup herbicide—one during the growing season and a second during the fall— likely will be needed to keep established stands free of weeds through and beyond the long harvest period. When perennial weeds are present, 0.75 lb a.e. glyphosate per acre will generally provide control of most species. Lower rates may be satisfactory for many summer and winter annual weeds because vigorous alfalfa crop competition and mechanical harvest will follow the herbicide application(s).

Improved mixed stand and pasture management options. Those growers planting forage grasses with alfalfa likely will not use Roundup Ready alfalfa in their systems. However, after establishment, some producers may wish to remove a weak or undesired grass stand and reestablish a different or more vigorous grass mix with their alfalfa. As dairy-based grazing systems continue expanding, some growers may wish to have this flexibility. For these forage producers, long stand life is highly desired, and Roundup Ready alfalfa will offer advantages over conventional varieties and may be adopted from both a weed management and forage grass management perspective.

In the southern U.S., alfalfa has the potential to be used in livestock grazing systems. Varieties that tolerate grazing now exist (Smith and Bouton, 1993), and incorporating the glyphosate tolerance trait into them would offer several interesting opportunities. The most obvious opportunity would be to control undesirable warm season grasses such as bermudagrass (*Cynadon dactylon* L.), which goes dormant in the summer, and perennial weeds like horsenettle (*Solanum carolinense* L.), which is not grazed because of spines on the stems and leaves. Use of Roundup Ready alfalfa also would allow producers to rejuvenate pastures or switch between forage grasses within an existing alfalfa planting. For example, producers could kill fescue (*Festuca* L. spp.) using Roundup herbicide and establish orchardgrass (*Dachtylis golmerata* L.) without needing to replant the alfalfa pasture component if Roundup Ready alfalfa were planted (Bouton, 2002).

A novel approach to perennial weed management in pastures is foreseen with the advent of Roundup Ready alfalfa. Weeds like Canada thistle, horsenettle, goldenrod (*Solidago*

canadensis L.), groundcherry (*Physalis* L. spp.), common milkweed (*Asclepias syriaca* L.) and other hard-to-kill perennial herbaceous species present formidable weed management challenges in pastures. Livestock producers may find that they can gradually eradicate these infestations by planting Roundup Ready alfalfa and applying a Roundup agricultural herbicide judiciously over a two- to four-year period. During this period, the pasture can be grazed or the alfalfa harvested as forage. The Roundup Ready alfalfa could be inter-seeded with forage grasses or other legumes as desired or as the alfalfa stand begins to decline.

Weed-free hay. Some regions of the U.S. produce significant quantities of alfalfa hay or processed hay products. Both producers and buyers put a high value on weed-free forage, and a national program has been introduced for certification that the hay is free of weeds (Schoenig, 2002). In the ten states that have implemented this program, buyers are assured that certified hay is free of 54 noxious weeds, i.e., all of the species on the legally declared noxious weed lists in those states. Buyers can purchase alfalfa hay without the concern of importing new weeds onto private farms or federally managed lands. The price of hay is based on the actual feed value of the hay being purchased. To the extent that Roundup Ready alfalfa varieties will ensure the control of noxious, prohibited, or regulated weeds and enhance feed value, these varieties will be viewed as advantageous and adopted by many cash hay producers who use these programs.

There are two other areas where weed-free hay is a priority. One is for producers who market hay to horse or companion animal owners. In this case, it is not a question of feed value *per se* but rather the aesthetics and/or the assurance that the purchased hay is free of weeds. Weedy hay may not even have a sale value in some of these markets. Additionally, in an effort to protect ecosystems and native plant communities on many federal lands, the U.S. Forest System has required that all hay, straw or mulch brought in for livestock feeding or other purposes on these lands must be certified as noxious weed-free (USDA Forest Service, 2002; U.S. Forest Service, 1995). According to one U.S. Secretary of the Interior (Pimental, 2003), ranchers spend about $5 billion each year to control invasive nonindigenous weeds in pastures and rangelands. Nevertheless, these weeds continue to spread into pastures, fields and native areas and, thus the demand for weed-free hay (and weed-free seed) products is likely to increase in the future.

Control of dodder. There is recent evidence that Roundup Ready alfalfa technology may be the first crop-safe method of controlling dodder (*Cuscuta* spp.), a parasitic weed on alfalfa. Research conducted by Reisen et al. (2002), showed that Roundup agricultural herbicides control dodder after the parasite attaches to the host alfalfa plant. Seeds of dodder are prohibited or restricted in alfalfa hay and seed for most markets.

D.5. Weed Control in Seed Production and Impact of Roundup Ready Alfalfa

Approximately 18.3% of alfalfa seed acres (≤37,000) are newly established each year, with about half of the total acres established in each fall and spring. Hower et al. (1999) reported that 100% of seed acres are established using herbicides and greater than 98% of established acres are treated. Benefin, 2,4-DB, bromoxynil, sethoxydim and EPTC were used on the greatest number of spring- and fall-seeded acres. In established stands, EPTC, diuron, triflualin, and hexazinone were used in greatest quantity. Use of a

Roundup agricultural herbicide in Roundup Ready alfalfa would offer growers improved flexibility in application timing and a new mode of action compared to currently used herbicides. The review of weed spectra controlled, mode of action, rotational considerations, and environmental effects of currently used products, discussed above, also pertains directly to alfalfa grown for seed. In addition, dodder is an important alfalfa weed seed contaminant that is currently very difficult or impossible to control after it attaches to the alfalfa host, and due to its similar seed size and shape, it is also difficult and costly to remove from commercial seedlots.

D.6. Impact of Roundup Ready Alfalfa on Herbicide Use in Alfalfa

While numerous herbicides are available for use in alfalfa, the acreage of established alfalfa treated with herbicides is relatively low, except in the west. Between 23 to 29% of the total U.S. alfalfa acreage is treated with herbicides (Table VII-6). Using the average of these values (26%) and the average use rate of 0.72 lb ai/acre (the average from the Hower and Gianessi surveys of 4.2 M total lb active ingredient used over 5.8 M acres), the amount of active ingredient used per acre may not change significantly with the introduction of Roundup Ready alfalfa. This assumes that the typical use rate of 0.75 lb ae/A of glyphosate in Roundup Ready soybean and corn also will be used in Roundup Ready alfalfa. However, the rate per acre could increase if producers make more than one application per year, especially in the southern and western regions. Gianessi et al. (2002) estimate that in California the use of glyphosate in herbicide-tolerant alfalfa varieties could average 1.5 lb ai/A/ yr, a 0.2 lb/A/yr increase over the current average herbicide use rate in that state. However, as described above, to achieve weed control comparable to glyphosate using currently available herbicide alternatives (trifluralin, EPTC, and imazethapyr) would require 4.7 lb ai/A/yr., a substantial increase over the amount estimated by Gianessi et al. (2002). The treat-as-needed approach, and the flexibility and effectiveness of the Roundup Ready weed control system may not result in an overall increase in herbicide use. Regardless, the current herbicides used (some of which are persistent in the environment and/or carry environmental or worker safety warnings), would be replaced by glyphosate, a generally more environmentally acceptable alternative.

D.7. Economics of Roundup Ready Alfalfa

Alfalfa forage growers in the West likely would see a significant economic and environmental benefit from annual or semi-annual Roundup herbicide applications to Roundup Ready alfalfa because forage quality is a concern and the impact of weed competition may be considerable during most of the year. It is estimated that two applications of glyphosate at 0.75 lb a.i./A would provide effective season-long control of troublesome weeds in California alfalfa fields. It is estimated that the Roundup herbicide cost would be $15/A and that a seed premium of $5/A would be charged. As mentioned previously, glyphosate is well recognized for the broad spectrum of weeds it controls. In fact, if a grower were to use a season-long herbicide control program that provides the same spectrum and performance as glyphosate , it would require the use of trifluralin, EPTC, and imazethapyr. The level of performance provided by these three herbicides would be full control of 38 species, partial control of eight species, and no control of two

species. Based on information provided in Table VII-6, this weed control program would require 4.7 lb. a.i./A/yr. at a cost of $45/A, for an annual increase of 3.2 M pounds of herbicide active ingredient at a cost of $25 M. When compared to the anticipated product concept for the Roundup Ready alfalfa weed control program, herbicide use would increase by 3.2 lb. a.i./A and cost would increase by $25/A.

As noted in an earlier section, even modest improvements in relative feed value translate into significant economic gains. For instance, if the NDF decreases from 45 to 42% in the forage harvested from a million acres (5%) of the current alfalfa acreage, and if the alfalfa yields five tons/A, this NDF change would increase milk production by 600 lb/A. Even at today's low milk prices near $10/cwt, the added value would be $60/A and $60 M for the area assumed in the example.

The impact of Roundup Ready alfalfa in established alfalfa will vary with the length of the rotation. Producers in the Midwest and East who keep stands for more than three years after establishment are the most likely to benefit from improved weed control. Few producers harvesting alfalfa for only two years after establishment before rotating to corn will find a need to use any herbicide in the established phase of production. An assessment of rotations that included one to four years of established alfalfa in rotation with one to three years of corn (a total of six rotations) found that the four-year rotation (seeding year, two alfalfa harvest years and one year of corn) was the most profitable (Frank, 1994). In these tight rotations, it is unlikely that weeds will reach levels of concern in only two full seasons of alfalfa.

An important consideration is whether weed pressure determines the endpoint of alfalfa in the crop rotation. In short rotations it is unlikely, but for those growers who prefer to keep alfalfa for four or more years, weeds probably are a significant consideration, albeit an indirect one. Forage agronomists and weed scientists generally accept that weeds indicate a declining forage stand and a point will be reached at which even if weeds are controlled, the stand will be below the desired alfalfa population levels of four to five plants or 55 stems/sq. foot.

The value of longer stand life should also be considered. In general, forage agronomists do not recommend extending the life of the stand beyond three years (seeding year plus two production years) if yields decline by 0.5 to 1.0 ton/acre per year (Frank, 1994). However, if control of weeds resulted in a relatively constant yield, the stand could be maintained rather than reseeding. This would allow amortization of the high cost of establishment over more years, reducing the production cost per acre over the life of the stand. For example, Hendrickson (2002) reported that the input costs to establish an acre of alfalfa in Wisconsin are $172; the input costs in subsequent years are $88/acre. Thus, it is more economical to maintain an existing stand than reseed if yields do not decline as the stand ages.

E. Gene Flow

Gene flow is a natural process in the reproduction of many plants. It can occur between plants that are sexually compatible through a number of mechanisms or by movement of viable seeds, which can grow within new populations and possibly exchange genes with the new population. There are no sexually compatible wild relatives of alfalfa in North America; however, natural gene flow between cultivated and feral alfalfa commonly occurs. This Section reviews the biology of alfalfa pollination, relevant information regarding gene flow under commercial seed and forage production, and the results of recently completed studies to measure gene flow in alfalfa. Since alfalfa is a plant that can become established outside of cultivation, the abundance of feral populations found within six states is documented within this section. While the data collected indicates that there are no biologically meaningful risks associated with gene flow from Roundup Ready alfalfa varieties, the consequences and management of gene flow will be addressed in Section F under the stewardship of Roundup Ready alfalfa.

E.1. Center of Origin/Potential for Gene Transfer to Wild Relatives

As discussed in Section II-E, cultivated and closely related species of alfalfa originated in Asia Minor, Transcaucasia, Turkmenistan and Iran. Presently, particularly in Europe, Asia, the Middle East, and North Africa, native populations of various members in the *M. sativa* complex, as well as other perennial *Medicago* species, exist to which cultivated alfalfa would hybridize (Sinskaya, 1961; Lesins and Lesins, 1979; Ivanov, 1988). Alfalfa does not naturally hybridize with any related wild relatives in North America.

Cultivated alfalfa, *M. sativa* ssp. *sativa*, is a tetrasomic tetraploid ($2n = 4x = 32$), characterized by purple flowers and coiled pods (Quiros and Bauchan, 1988). Subspecies *falcata* occurs both as tetraploid and diploid ($2n = 2x = 16$) accessions and has yellow flowers and straight to sickle-shaped pods. Purple-flowered *M. sativa* ssp. *coerulea* is a diploid form of *M. sativa* ssp. *sativa*. Gene flow between species that differ in ploidy level is possible through the production of unreduced ($2n$) gametes (McCoy and Bingham, 1988). The production of occasional $2n$ gametes in some *M. sativa* plants occurs (Pfeiffer and Binham, 1983). All other members of the *M. sativa* complex readily cross-pollinate with cultivated alfalfa; ssp. x *varia* is actually the hybrid of ssp. *sativa* and *falcata*.

The *M. sativa* complex has been successfully hybridized with 12 other perennial *Medicago* species and, additionally, a hybrid between *M. sativa* and *M. arborea* was produced *via* protoplast fusion (Nenz et al., 1996). Interspecific hybrids are very unlikely to occur in nature as most hybrid embryos abort during embryogenesis (except crosses with closely related *M. prostrata* and *M. glomerata* already discussed in Section II-E). Many of the literature reports of successful interspecific hybridization were accomplished only by using artificial embryo culture techniques and/or trispecies bridging (McCoy and Smith, 1986; McCoy and Bingham, 1988).

Of the *Medicago* species reported to hybridize with *Medicaco sativa* (discussed in Section II-E) only *Medicago lupulina* grows wild in North America. *Medicago lupulina* (black medic) is described by Turkington and Cavers (1979) as an annual, biennial, or

short-lived perennial; it is a native of Europe and Western Asia that has become naturalized in the U.S. and Canada. Black medic grows very rapidly in lawns and waste areas where frequent cutting cycles may be used and its seeds often contaminate forage legume seeds such as white clover, red clover and alfalfa. Due to its aggressive behavior, black medic is considered a weed in the U.S. and Canada (Turkington and Cavers, 1979). Since *Medicago lupulina* and *Medicago sativa* grow in close proximity to each other in North America, some discussion of their potential for cross hybridization is warranted.

While there are reports of successful hybridization between *Medicago sativa* and *Medicago lupulina* (Southworth, 1928; Fryer, 1930; Shrock, 1943) it is generally agreed that these putative hybrids were due to faulty breeding techniques resulting in self-hybridization and were not truly hybrids. According to Lesins and Gillies (1972), *M. sativa* and *M. lupulina* do not hybridize and the earlier reports were incorrect. Lesins and Giles stated that the few, unconfirmed hybrids reported earlier were more likely to have been weak, distorted *M. sativa* selfed inbreds mistaken as interspecific hybrids. Fridriksson and Bolton (1963) measured embryo development in stigmas of *M. sativa* after pollination with several highly incompatible *Medicago* species. including *M. arborea*, *M. blancheana*, *M. lupulina*, *M. marina*, *M. platycarpos*, *M. rigidula*, *M. ruthenica* and *M. scutellata*. While none of the crosses produced mature embryos, all crosses except for *M. lupulina* resulted in the initiation of the early stages of embryonic growth. Specifically, crosses with *M. lupulina* showed no evidence of fertilization. The lack of genetic compatibility between *M. sativa* and *M. lupulina* is also supported by restriction fragment length polymorphism (RFLP) analysis. Valizadeh et al. (1996) analyzed chloroplast DNA polymorphisms among nine *Medicago* ssp. representing four subgenera. Of the species analyzed, it was found that *M. sativa* and *M. lupulina* were the most genetically distant from each other. These statements and findings refute much earlier claims of hybridization between *M. sativa* and *M. lupulina* (Southworth, 1928, Fryer, 1930 and Schrock, 1943).

Contemporary experts in *Medicago* genetics, taxonomy and breeding also agree that *Medicago sativa* does not naturally hybridize with *Medicago lupulina* based on their professional experience. Three *Medicago* experts were specifically asked to address the potential for cross hybridization between *Medicago sativa* and *Medicago lupulina*. Their responses to these questions are presented in Appendix 4. On the basis of expert testimony provided by these experts, it was concluded that natural hybridizaton between perennial and annual medics (specifically *Medicago sativa* and *Medicago lupulina*) is extremely unlikely and has never been achieved after numerous attempts over many years by experts. On the basis of the biology of alfalfa and practical breeding experience by skilled alfalfa breeders it can be concluded that natural hybridization with other wild relatives of *Medicago sativa* in North America is extremely improbable.

There are considerable biological barriers between annual and perennial medics that make the likelihood of their cross hybridization extremely low. *Medicago sativa,* a perennial, requires insects for pollination. According to Bauchan (2004, Appendix 4) all annual *Medicago* species are self-pollinating. According to Lesins and Gillies (1972), perennial *Medicago* species (e.g., *Medicago sativa*) do not naturally hybridize with any of the annual *Medicago* species; in this report they stated that, "No annuals hybridize

with perennials." Lesins and Lesins (1979), Fridriksson and Bolton (1963) and Bauchan (Appendix 4) have tested the natural and artificial hybridization of a number of *Medicago* annuals with *M. sativa*. In their studies, early embryos and/or pod formation were occasionally observed but viable hybrid seeds were not produced. To date, even artificial embryo rescue-assisted hybridizations have occurred only for *M. scutellata* (Sangduen, et al. 1982) and *M. rugosa* (Piccirilli and Arcioni, 1992) and in both cases the hybrid was sterile and eventually died without producing offspring. Further, ploidy and karyotype differences between alfalfa and the annual medic species present major barriers to gene flow. Alfalfa is a tetraploid with 32 chromosomes whereas, *M. scutellata* and *M. rugosa* are tetraploid but have 30 chromosomes, while all of the other annual *Medicago* species are diploids with either 16 or 14 chromosomes (McCoy and Bingham, 1988; Quiros and Bauchan, 1988).

E.2. Gene Flow During Commercial Seed Production

Alfalfa requires insect pollinators for cross-pollination. Alfalfa is exclusively pollinated by bees and a relatively small number of bee species can effectively pollinate alfalfa flowers. Predominant species that are important for alfalfa seed production include leafcutter bees (*Megachile rotunda*), honeybees (*Apis mellifera*) and alkali bees (*Nomia melanderi*). In alfalfa, the leafcutter bee is the preferred pollinator in many regions of the U.S. because it is efficient. However, the leafcutter bee is not used extensively in warmer regions of the U.S. (e.g., California and Arizona) because it does not tolerate heat very well. In situations where alfalfa seed is being produced for commercial purposes, bees are purposely stocked into the field.

To reduce the amount of bee-mediated gene movement between varieties during seed production, alfalfa varietal purity is achieved by maintaining adequate isolation distances between alfalfa seed production fields. Current seed production isolation standards are 274 meters and 50 meters for foundation and certified seed classes, respectively.

Forage Genetics International and Monsanto have conducted studies to measure the effectiveness of current isolation standards. A brief summary of the results and conclusions drawn from these studies follows. A more detailed summary of these results and a review of other recently completed alfalfa gene flow studies are presented in Appendix 5. Studies were conducted using the Roundup Ready trait as a marker to measure gene flow in moderate to small sized plots. This research examined the movement of the *cp4 epsps* gene from Roundup Ready alfalfa to conventional alfalfa pollen trap plots under seed production conditions and provides data that can be used to assess the potential for gene flow between commercial-scale seed production fields (McCaslin et al., 2001; Fitzpatrick et al., 2002). Leafcutter bees were introduced as pollinators in all of the studies to ensure pollination. Combined information from three years of research showed that the upper bound (99.9% confidence) of gene flow at 274 meters was approximately 0.3 percent and at 152 meters was approximately 1.7 percent. In two of the years, 2000 and 2002, gene flow was not detected at 610 meters or at distances > 825 meters, respectively.

Information obtained from these studies confirms that spatial isolation remains an effective means to maintain the purity of both conventional and Roundup Ready alfalfa

varieties. This information will be provided to alfalfa seed producers and members of the alfalfa seed industry community (e.g., State Crop Improvement Agents) so that they can determine appropriate isolation distances needed to meet the various varietal purity requirements of different seed markets.

E.3. Gene Flow During Forage Production

Pollen dispersal, also called pollen flow, is one of the ways genes can move between plants. However, not all pollen dispersal results in gene flow. Gene flow is defined as the *successful* transfer of genetic material. Because pollen-mediated gene flow only can occur between individual plants of the same or sexually compatible species, gene flow from pollen occurs only when pollen (the male gamete) is deposited on the stigma of a plant, fertilizes the ovule (female gamete) of that plant, and viable seed is produced.

Alfalfa is cultivated for its animal feeding value, and the nutritive value of alfalfa decreases after flowering. Alfalfa managed for forage production is generally cut on a calendar schedule with multiple harvests within a growing year (two to eleven cuttings per year depending upon geographic region). The harvest interval is dependent upon weather conditions and optimally coincides with the early flower to 10% bloom growth stages. For many regions, this interval is 28-35 days in length during the growing season, an interval inadequate to initiate full bloom or ripen seed. Alfalfa requires four or more weeks of adequate temperature and photoperiod to grow and form floral buds and an additional four to six weeks to form mature seed on pollinated blooms. Forage harvest periodically removes the entire plant canopy where blooms or seed might form. Growth of the canopy must be reinitiated from vegetative crown buds (as occurs in the spring) or from the elongation of lower stem axillary buds. Therefore, alfalfa managed as forage will have little contribution to pollen-mediated gene flow under production conditions because there will be few if any open flowers in the standing canopy or mature seeds in the harvested forage.

As noted above, gene flow from Roundup Ready alfalfa grown for forage to conventional alfalfa hay production fields will be predictably far less than that which would be expected to occur when alfalfa is grown for seed. However, some gene flow could occur during the early flowering stage or from mismanaged forage production fields to scattered feral alfalfa populations. Studies have been performed by St. Amand et al. (2000) to examine gene movement from alfalfa forage fields. A review of the experimental design and general conclusions from this study are found in Appendix 5. As expected, the results from this study showed that some gene flow does occur from forage production fields to feral alfalfa populations.

E.4. Assessment of Gene Flow to Feral Alfalfa

Jenczewski et al. (1999) and St. Amand et al. (2000) have shown that gene flow occurs naturally between cultivated and wild alfalfa populations using both isozyme markers and the analysis of quantitative traits. These studies show that cross-pollination between cultivated alfalfa and feral *M. sativa* occurs, particularly in regions with abundant native or naturalized populations. While it is known that alfalfa populations escape cultivation, much of the available information is anecdotal. To better steward the longevity and

efficacy of Roundup Ready alfalfa, a two-year biogeographic survey of feral alfalfa was conducted. The information obtained documents the presence and relative abundance of feral alfalfa in six states, thereby enabling an estimate of the extent to which gene flow from cultivated alfalfa occurs. The states surveyed were California, Idaho, Pennsylvania, South Dakota, Washington and Wisconsin. Information from this survey defines the extent of naturalized alfalfa populations that survive outside of cultivation. The results from this survey are summarized below.

E.5. Six-State Survey of Feral Alfalfa Populations

A biogeographic survey was performed to document and characterize feral alfalfa populations within six major alfalfa production states of the U.S.

Eleven counties in Idaho and ten counties in both Pennsylvania and Wisconsin were surveyed for feral and cultivated alfalfa populations in 2001. Ten counties in both California and Washington and six counties in South Dakota were surveyed for feral alfalfa populations in 2002. The counties surveyed were selected based on the largest acreage of alfalfa forage or seed production. The survey methods incorporated three strategies to ensure adequate identification and description of existing feral alfalfa populations within each state. The first strategy involved preselecting a set of survey sites prior to initiating the survey (without knowledge of terrain or vegetation) to avoid sampling bias. The second strategy involved selecting a second set of satellite survey sites by scouting for feral alfalfa populations within a prescribed distance from each preseleted survey site (satellite sites). Satellite sites were selected at or near the three-mile distance, whether or not feral alfalfa was present. Finally, the third strategy involved recording the frequency of feral alfalfa populations observed along the entire travel route. Within each county, 20 survey sites were selected along the predetermined travel route. Selected survey sites consisted of ten preselected sites and ten corresponding satellite sites in each county. In Washington, the survey was limited to ten preselected survey sites in each county selected along the travel route. Among the six states, data were collected from 1040 individual survey sites. Data collected at each preselected and satellite survey site included the exact geographic location, the occurrence of feral and cultivated alfalfa populations, coverage area of observed feral populations, and the proximate distance between observed feral and cultivated populations. In addition to survey site data, average distance between all feral populations observed along the entire travel route within each county was calculated for all states surveyed, with the exception of Wisconsin, where these data were not collected. The results of this survey are discussed by state.

Idaho

In Idaho, feral alfalfa occurred within and/or near 17 and 41% of the preselected and satellite sites, respectively, averaged across the 11 counties surveyed. For all states surveyed, the satellite sites were expected to have a higher occurrence of feral alfalfa compared to corresponding preselected sites because the researchers were intentionally scouting for feral alfalfa when selecting satellite sites. Cultivated alfalfa occurred within and/or near 44 and 43% of the preselected and satellite sites, respectively. A similar occurrence rate for cultivated alfalfa between the two survey site types was expected

because there was an equal probability of cultivated alfalfa occurrence per site, regardless of survey site selection method. Feral and cultivated populations occurred within 2000 m of each other in approximately three sites per county, and in approximately half of these sites the average distance between feral and cultivated populations was less than 20 m. Among all counties surveyed in Idaho, average mean coverage area was 1.7 % per site (in sites where feral alfalfa occurred) and 0.5% per all 20 sites per county. Feral alfalfa did not occur more frequently in the counties located in the intensive alfalfa seed-production region of Idaho compared to the other counties located in regions primarily devoted to forage production. The average distance between feral populations observed along the entire travel route in Idaho was greater than 12 miles.

Pennsylvania

In Pennsylvania, feral alfalfa occurred in 57% of the satellite sites. Feral and cultivated populations occurred together in 10 and 21% of the preselected and satellite sites, respectively. Among all counties surveyed, average mean coverage area was 1.2 % per site (in sites where feral alfalfa occurred) and 0.5% per all 20 sites per county. The average distance between feral populations observed along the travel routes within each county in Pennsylvania was approximately one population every six to twelve miles. Within Centre and Franklin Counties, feral populations were observed approximately every one to three miles.

Wisconsin

In Wisconsin, feral alfalfa occurred in 47% of the satellite sites and was present in 70-80% of the satellite survey sites for Dane, Grant, Shawano, and Vernon Counties. Feral and cultivated populations occurred together in 9 and 31% of the preselected and satellite sites, respectively, and, in nearly three-fourths of these sites, the average distance between feral and cultivated populations was less than 20 m. Average mean coverage area was 1.7% per site (in sites where feral alfalfa occurred) and 0.5% per all 20 sites per county in Wisconsin. Feral alfalfa frequency ratings were not recorded in Wisconsin; thus the approximate distance between feral alfalfa populations could not be calculated.

California

In California, feral alfalfa occurred within and/or near 27 and 67% of the preseleted and satellite sites, respectively, averaged across the ten counties surveyed. Cultivated alfalfa occurred within and/or near 38 and 52% of the preseleted and satellite sites, respectively. Among all counties surveyed in California, average mean coverage area was 4.2 % per site (in sites where feral alfalfa occurred) and 1.7% per all 20 sites. Feral and cultivated populations occurred within 2000 m of each other in approximately six sites per county and in approximately half of these sites the average distance between feral and cultivated populations was less than 20 m. Feral alfalfa did not occur more frequently in the counties located in the intensive alfalfa seed production regions of California compared to the other counties located in regions primarily devoted to forage production. The average distance between feral populations observed along the entire travel route in California was approximately three to six miles.

South Dakota

In South Dakota, feral alfalfa occurred in 63 and 82% of the preseleted and satellite survey sites, respectively. Feral and cultivated populations occurred together in 40% of the satellite sites. Among all counties surveyed, average mean coverage area was 5.4 % per site (in sites where feral alfalfa occurred) and 3.5% per all 20 sites. The average distance between feral populations observed along the travel routes within each county in South Dakota was approximately one population every three to six miles. Within Hand, Harding, and Tripp Counties, feral populations were observed approximately every one to three miles.

Washington

In Washington, feral alfalfa occurred in 10% of the preseleted sites and feral and cultivated populations occurred together in 2% of preseleted sites. Data were not collected from satellite survey sites in eight out of the ten counties surveyed; therefore, satellite survey site results are not presented. The average distance between feral populations observed along the entire travel route was >12 miles. Feral alfalfa did not occur more frequently in the counties located in the intensive alfalfa seed production regions of Washington compared to the other counties located in regions primarily devoted to forage production.

In conclusion, we have documented that alfalfa survives to a small extent outside of cultivation. This was anticipated because alfalfa is a highly adapted plant that is cultivated broadly across the U.S. However, the abundance of feral alfalfa has not been previously described. The results from this survey likely are representative of other states in the U.S. where alfalfa is produced for seed or forage. It is reasonable to assume that feral alfalfa populations also exist to a minor extent in other locations where alfalfa is produced. The occurence of feral alfalfa near seed-production sites was less than that observed where forage was produced; this may be because of the importance that seed producers place on maintaining isolation during seed production to assure genetic purity of alfalfa varieties.

It must be noted that while alfalfa does survive outside of cultivation, these scattered feral populations are not recognized as noxious or invasive weed species (USDA-APHIS, 2000; USDA-APHIS, 2002; USDA-NRCS, 2003; USDA-ARS, 2003). Furthermore, information discussed below in Section F.5 describes the role that glyphosate plays where control of alfalfa may be needed. Briefly, glyphosate has a very limited role in the control of feral alfalfa because glyphosate is not the only herbicide used, nor is it the herbicide of choice for control of alfalfa.

E.6. Transfer of Genetic Information to Species with which Alfalfa Cannot Interbreed

We are aware of no reports of the transfer of genetic material from alfalfa to species with which alfalfa cannot interbreed (i.e., horizontal gene transfer).

E.7. Conclusion – Gene Flow

Gene flow is a naturally occurring process mediated by bees in alfalfa. Information presented in this Section showed that the *cp4 epsps* gene behaves like any gene in alfalfa

and can be transferred between different alfalfa varieties. Monsanto and Forage Genetics International have documented the occurrence of feral populations in six states, and the results from this survey show that both feral and cultivated populations occur in close proximity to one another. It is logical to assume that with the introduction of Roundup Ready alfalfa technology, gene flow will occur between cultivated and feral alfalfa populations. The consequence of gene flow to feral populations has been partially addressed in Section VI of this document where it was shown that there are no biologically meaningful risks associated with gene flow from Roundup Ready alfalfa varieties. Section F below will further address potential consequences of gene flow to feral alfalfa populations and during commercial seed and forage production.

F. Stewardship of Roundup Ready Alfalfa

The stewardship of crops improved through the use of biotechnology has long been recognized by Monsanto as a key component to the successful introduction, production, and long-term use of this technology in agricultural production systems. Components of the stewardship program for Roundup Ready alfalfa varieties will include: 1) providing users of Roundup Ready alfalfa with appropriate crop rotation practices, thereby enabling a smooth transition in and out of Roundup Ready alfalfa; 2) providing vegetation control personnel (e.g., highway department personnel) with control options for feral alfalfa; 3) using grower agreements to prevent unauthorized seed production; 4) providing the alfalfa seed production industry with gene flow information and analytical tools that will be used in the production of conventional and Roundup Ready alfalfa varieties; and 5) providing Monsanto field personnel with ongoing training to address anticipated and unforeseen issues that may arise because of the introduction of the technology. The following sections address the elements of Monsanto's stewardship program for Roundup Ready alfalfa varieties, which will be in place prior to product launch.

F.1. Crop Rotation Systems for Roundup Ready Alfalfa Varieties

Alfalfa has long been recognized as an excellent component in a crop rotation cycle for its ability to fix nitrogen and improve soil structure (Entz et al., 2002). Rotation with wheat, oats, barley, potato (*Solanum tuberosum* L.), sugar beet (*Beta vulgaris* L.), and corn are expected to continue as currently practiced because nonglyphosate herbicides are available to manage alfalfa volunteers in each crop. Alfalfa rotation with soybean will remain uncommon because of the lack of nitrogen benefit from alternating between consecutive plantings of legumes. Although the success of rotation with cotton will depend largely on mechanical control of volunteer alfalfa, the number of acres is expected to be low. State production rankings (USDA/ERS, 2002) indicate that only California appeared in the Top 10 list for both cotton and alfalfa production. Market research (Marketing Horizons, Inc., 1996) indicated that only 10% of alfalfa acres in California were rotated to cotton. Overall, by following Monsanto's recommendations, it is expected that the introduction of Roundup Ready alfalfa will have negligible impact on crop rotation practices.

As part of ongoing product stewardship, Monsanto plans to maintain its cropping systems research program. Research is currently underway with academic researchers at universities such as Cornell University, Pennsylvania State University, the University of

Wisconsin, the University of Arizona, Texas A&M University, and the University of California. Specific areas of research include the impact of Roundup Ready alfalfa on direct seeding systems, crop rotations, weed spectrum shifts, and overall grower profitability.

F.2. Stand Termination

Unlike annual crops where harvest generally coincides with the end of the plant's lifecycle, alfalfa is harvested multiple times throughout the life of the stand, and the stand is generally only terminated when yield and/or quality diminish. Alfalfa stand take-out is achieved through the use of tillage, herbicides, or a combination of both. To minimize the inconvenience of alfalfa volunteers in subsequent rotation crops, Monsanto recognizes the importance of establishing and communicating successful stand termination recommendations. Such recommendations will be an integral part of the practices presented in technology use guides as well as to growers during training for use of Roundup Ready alfalfa technology.

A survey of herbicide labels contained within the Crop Data Management System's (CDMS) Ag Product Label Service (APLS, 2002) database indicated that 2,4-D, chlorpyralid (Stinger®), dicamba - dimethylamin salt (Banvel®), dicamba - diglycolamine salt (Clarity®), diflufenzopyr + dichloro-o-anisic acid (Distinct®), glufosinate (Liberty®), glyphosate (Roundup), and primsulfuron-methyl (Beacon®) were labeled for control of alfalfa. Independent research has demonstrated that dicamba, 2,4-D, tank mixtures of dicamba and 2,4-D, and clopyralid were often more effective than glyphosate for terminating alfalfa stands (Endres, 1999; Mayerle, 2002; Manitoba Agriculture and Food, 2002).

Monsanto also is conducting research to validate the performance of existing alternative (nonglyphosate) herbicide products for stand take-out. Results to date demonstrate that tillage is highly effective for stand take-out (Table VII-7). At the timepoint that coincided with eight days after stand take-out herbicide treatment, five days before tillage, and 21 days before irrigation, there were virtually no differences observed between the tilled and untilled treatments. For example, Roundup Ready alfalfa control by 2,4-D amine at 1 lb ai/A in tilled and non-tilled treatments was 51% and 55 %, respectively. However 28 days later, at a point that coincides with 36 days after stand take-out herbicide treatment, 22 days after tillage (tilled treatment only), and seven days after irrigation, the same 2,4-D amine treatment provided 100% and 18% control of Roundup Ready alfalfa in tilled and non-tilled treatments, respectively. These results were consistent with those reported by Boerboom (2002). Also, stand take-out can be made more effective when followed by frost (Rawlinson and Martin, 1999; Manitoba Agriculture and Food, 2002) or extreme heat (McCloskey, 2001). Furthermore, the

® Stinger is a registered trademark of Dow Agro Sciences LLC
® Banvel is a registered trademark of Micro Flo Company LLC
® Clarity is a registered trademark of BASF Ag Products
® Distinct is a registered trademark of BASF AG Products
® Liberty is a registered trademark of Bayer Crop Sciences
® Beacon is a registered trademark of Syngenta Crop Protection, Inc.

effectiveness of stand take-out herbicides was equivalent in comparisons of Roundup Ready alfalfa and conventional alfalfa (Table VII-8). The percent alfalfa control for each observation point was identical within an observation timt point for each treatment when Roundup Ready alfalfa and conventional alfalfa were compared.

F.3. Management of Volunteer Roundup Ready Alfalfa in Conventional Crops

With the introduction of this technology, some occurrence of Roundup Ready alfalfa volunteers is anticipated in subsequent rotation crops. Since the introduction of herbicide tolerant crops in 1996, volunteers of these crops have been successfully managed using established control practices, such as alternative herbicides and tillage. When combined with effective stand take-out recommendations, a successful management strategy for volunteer Roundup Ready alfalfa is expected to utilize the same or similar control practices.

Depending on the rotational crop chosen to follow alfalfa, growers currently use a combination of tillage and herbicide treatments, both prior to planting and after crop emergence, for volunteer alfalfa control. A survey of herbicide labels contained within the Crop Data Management System's (CDMS) Ag Product Label Service (APLS, 2002) database and the Crop Protection Reference (C&P Press, 2002) indicates that 2,4-D, dicamba - dimethylamin salt (Banvel), dicamba - diglycolamine salt (Clarity), chlorpyralid (Stinger), rimsulfuron (Matrix®), primisulfuron-methyl (Beacon) and diflufenzopyr + dichloro-o-anisic acid (Distinct) are registered for in-crop use to remove volunteer alfalfa in 35 crops. The effectiveness was described as control in 54 occurrences and suppression in eight occurrences. All crops, except potato, tomato (*Solanum lycopersicum* L.), and popcorn, had at least one herbicide available where the effectiveness was described as control (Table VII-9). Monsanto has conducted trials to determine the effectiveness of some of the recommended treatments in conventional tillage and no-till production systems. In conventional tillage systems (Table VII-8), wheat was planted after Roundup Ready alfalfa. At two months after planting wheat, 100% of the Roundup Ready alfalfa was controlled by clopyralid, clopyralid + 2,4-D, or 2,4-D + dicamba. In the no-till production system (Table VII-10), no-till corn was planted one week after spraying 2,4-D LV ester or paraquat for Roundup Ready alfalfa stand take-out. A broad cross sample of volunteer alfalfa treatments was applied to the corn three weeks later. Volunteer alfalfa control at six weeks after treatment ranged from 25 to 95%, and was most efficacious when using 2,4-D as the stand take-out treatment.

The seven herbicides labeled for control of volunteer alfalfa in rotation crops are also labeled for control of feral alfalfa in 15 non-crop settings, such as roadsides, fencerows, and ditch banks. The effectiveness was described as control in 27 occurrences and suppression in 12 occurrences. All 15 non-crop settings had at least one herbicide available where the effectiveness was described as control (Table VII-11).

Four of the herbicides (2,4-D, dicamba - dimethylamine salt, dicamba - diglycolamine salt, and chlorpyralid) labeled for the control of volunteer alfalfa in rotation crops are also labeled for control of feral alfalfa in 22 forestry, turf or municipal settings, such as

® Matrix is a registered trademark of E.I. Dupont de Nemours & Co, Inc.

Douglas fir (*Pseudotsuga* spp.), turfgrass, or golf courses. The effectiveness was described as control in 25 occurrences and suppression in four occurrences. All settings, except bermudagrass, bluegrass, and fescue had at least one herbicide available where effectiveness was described as control (Table VII-12).

Table VII-7. Stand Take-Out of Roundup Ready Alfalfa: Tilled vs. Non-Tilled.

Days After:		Tilled				Non-Tilled			
Treatment		8	14	22	36	8	14	22	36
Tillage		-5	1	8	22	N/A	N/A	N/A	N/A
Irrigation		-21	-15	-7	7	-21	-15	-7	7
Stand Take-out Treatment	**Rate (lb ai/A)**	**Percent control of Roundup Ready Alfalfa**							
Untreated	0	0	100	100	100	0	0	0	15
2,4-D amine	1	51	100	100	100	55	50	66	18
2,4-D amine	1.5	66	100	100	100	68	62	81	44
Clarity (dicamba - diglycolamine salt)	2	72	100	100	100	71	72	85	89
2,4-D amine + Clarity	1 + 0.5	69	100	100	100	72	70	86	89
2,4-D amine + Clarity	1 + 1	71	100	100	100	76	74	89	94
Crossbow™ (triclopyr + 2,4-D)	3	80	100	100	100	81	90	97	94
Confront (clopyralid + triclopyr)	1	74	100	100	100	76	76	91	92
Stinger + 2,4-D amine	0.29 + 1.5	74	100	100	100	76	80	90	92
Millinium Ultra (dicamba + clopyralid + 2,4-D)	2.8	79	100	100	100	79	75	90	92
Rely® (glufosinate)	1.25	79	100	100	98	84	25	19	35

Experiment: 2002143051.
Conducted by the University of California Cooperative Extension Service.
Conducted under USDA Notification Number: 02-046-26n.

™ Crossbow is a registered trademark of Dow Agro Sciences LLC
® Rely is a registered trademark of Bayer Crop Sciences

Table VII-8. Alfalfa Stand Take-out and Percent of Volunteer Alfalfa Control in Conventional Tilled Wheat

		Paraquat + Diuron (0.75 lb ai/A)		Clopyralid (0.25 lb ai/A)		Clopyralid + 2,4-D (0.6 lb ai/A)		2,4-D + Dicamba – Diglycolamine Salt 2,4-D + Clarity (3 + 0.5 lb ai/A)	
Activity	**Date**	**RR[a]**	**non RR**	**RR**	**non RR**	**RR**	**non RR**	**RR**	**non RR**
Stand take-out treatment applied to alfalfa	10/16/2001								
Efficacy evaluation of stand take-out	10/22/2001	80.0	80.0	38.8	38.8	37.5	37.5	60.0	60.0
Efficacy evaluation of stand take-out	10/30/2001	80.0	80.0	50.0	50.0	60.0	60.0	70.0	70.0
Efficacy evaluation of stand take-out	11/19/2001	85.0	85.0	60.0	60.0	70.0	70.0	80.0	80.0
Tillage (sweep)	12/01/2001								
Tillage (disc)	02/15/2002								
Seedbed preparation (cultivator + packer)	03/14/2002								
Wheat planted	03/19/2002								
Efficacy evaluation	04/08/2002	47.5	47.5	100.0	100.0	99.8	99.8	99.8	99.8
Volunteer alfalfa management treatment applied[b]	04/24/2002								
Efficacy evaluation of volunteer management	05/16/2002	70.0	70.0	100.0	100.0	100.0	100.0	100.0	100.0

[a]Roundup Ready
[b](2,4-D + dicamba - diglycolamine salt)
Conducted under USDA Notification Number: 01-053-08n.

Table VII-9. Herbicides Registered for In-Crop Use to Control Volunteer Alfalfa.

Crop	2,4-D	dicamba – dimethylamin salt	dicamba - diglycolamine salt	chlorpyralid	rimsulfuron	primsulfuron-methyl	diflufenzopyr + dichloro-o-anisic acid
Asparagus (*Asparagus officinalis* L.)			S	C			
Barley, Fall (*Hordeum vulgare* L.)	C			C			
Barley, Spring (*Hordeum vulgare* L.)	C			C			
Barley,Winter (*Hordeum vulgare* L.)	C			C			
Table Beets (for seed) (*Beta vulgaris* L.)				C			
Broccoli (for seed) (*Brassica oleracea* var *botrytis* L.)				C			
Cabbage (for seed) (*Brassica oleracea* L.)				C			
Cauliflower (for seed)				C			
Corn, Field (*Zea mays* L.)	C	S	S	C		S	S
Corn, Pop (*Zea mays* L.)		S					
Corn, Sweet (*Zea mays* L.)	C						
Crop Stubble	C						
CRP Areas[1]	C	C	C				
Fallow Ground	C	C	C				
Meadowfoam (for seed) (*Limnanthes* spp)				C			
Millets (*Pennisetum glaucum* L.)	C						
Oats, Fall (*Avena sativa* L.)	C			C			
Oat, Spring (*Avena sativa* L.)	C			C			
Oat, Winter (*Avena sativa* L.)				C			
Pastures	C	C	C	C			
Peppermint (*Mentha* X *piperita* L.)				C			

C = Control; S = Suppress

[1]CRP=Conservation Reserve Program or similar set-aside conservation government spposored programs.

Table VII-9 (continued). Herbicides Registered for In Crop Use to Control Volunteer Alfalfa.

Crop	2,4-D	dicamba – dimethylamin salt	dicamba - diglycolamine salt	chlorpyralid	rimsulfuron	primsulfuron-methyl	diflufenzopyr + dichloro-o-anisic acid
Potatoes (*Solanum tuberosum* L.)					S		
Rye (*Secale cereale* L.)	C	C					
Sorghum, Grain (*Sorghum bicolor* L.)	C	C					
Sorghum, Milo (*Sorghum bicolor* L.)	C	C					
Spearmint (*Mentha spicata* L.)				C			
Sudan Grass (*Sorghum bicolor* L.)		C					
Sugar Beets				C			
Sugar Cane (*Saccharum officinarum* L.)	C	C	C				
Swiss Chard (for seed)				C			
Tomato (*Solanum lycopersicum* L.)					S		
Wheat, Durum (*Triticum durum* L.)	C			C			
Wheat, Fall (*Triticum aestivum* L.)	C			C			
Wheat, Spring (*Triticum aestivum* L.)	C			C			
Wheat, Winter (*Triticum aestivum* L.)	C			C			

C = Control; S = Suppress

Table VII-10. Volunteer Alfalfa Control in No-Till Corn.

Alfalfa Stand Take-out Treatment[1]	Rate (lb ai/A)	Volunteer Alfalfa Treatment	Rate (lb ai/A)	Volunteer Alfalfa Control (Percent)
Applied 05/07/2001		**Applied 06/06 or 06/08/2001**		**07/26/2001**
2,4-D LV ester	1	dicamba	0.206	25
2,4-D LV ester	1	primisulfuron	0.0356	95
2,4-D LV ester	1	diflufenzopyr + dicamba	0.262	75
2,4-D LV ester	1	diflufenzopyr	0.175	75
2,4-D LV ester	1	dicamba + atrazine	1.4	75
2,4-D LV ester	1	clopyralid	0.187	95
2,4-D LV ester	1	flumetsulam + clopyralid	0.214	95
2,4-D LV ester	1	dicamba - diglycolamine salt	0.25	95
2,4-D LV ester	1	2,4-D LV ester	0.25	95
2,4-D LV ester	1	halosulfuron + dicamba	0.0313 + 0.125	95
2,4-D LV ester	1	glufosinate + atrazine	1.34	85
2,4-D LV ester	1	glufosinate	0.46	95
paraquat	1.13	dicamba + diflufenzopyr	0.206	25
paraquat	1.13	primisulfuron	0.0356	25
paraquat	1.13	diflufenzopyr	0.262	25
paraquat	1.13	diflufenzopyr	0.175	25
paraquat	1.13	dicamba	1.4	50
paraquat	1.13	clopyralid	0.187	85
paraquat	1.13	flumetsulam+clopyralid	0.214	50
paraquat	1.13	dicamba	0.25	50
paraquat	1.13	2,4-D LV ester	0.25	75
paraquat	1.13	halosulfuron + dicamba	0.0313 + 0.125	85
paraquat	1.13	atrizine + glufosinate	1.34	85

[1]Stand was planted on 5/14/2001.

Table VII-11. Herbicides Registered for Control of Feral Alfalfa in Non Crop Settings.

Area	2,4-D	dicamba - dimethylamin salt	chlorpyralid	triasulfuron	dicamba - diglycolamine salt	diflufenzopyr + dichloro-o-anisic acid
Air Fields	C					
Canals	C					
Cemeteries	C					
Ditch Banks	C			S		
Ditches, Irrigation	C					
Drain Ditches	C	C		S		
Fence Rows	C	C	C			S
Highways	C					
Non Crops Areas	C	C	C	S	C	S
Pipelines		C				S
Railroads	C	C		S		S
Rangeland	C	C	C	S	C	
Rights-Of-Way	C	C		S		S
Roadsides	C	C		S		
Wasteland		C				

C = Control
S = Suppress

Table VII-12. Herbicides Registered for Control of Feral Alfalfa in Forestry, Turf and Municipal Settings.

Crop	2,4-D	dicamba - dimethylamin salt	dicamba – diglycolamine salt	chlorpyralid
Balsam Fir (*Abies balsamea* L.)				C
Bermudagrass (*Cynadon dactylon* L.)		S		
Blue Spruce (*Picea pungens*)				C
Bluegrass (*Poa* L. spp.)		S		
Cottonwood (*Populus deltoides*)				C
Douglas Fir (*Pseudotsuga* spp.)	C			C
Eucalyptus (*Eucalyptus* spp.)				C
Fescue (*Festuca* L. spp.)		S		
Fraser Fir (*Abies fraseri*)	C			C
Golf Courses	C			
Grand Fir (*Abies grandis*)				C
Grasses	C	S		C
Parks	C			
Loblolly Pine (*Pinus tieda* L.)				C
Slash Pine (*Pinus elliottii*)				C
Lodgepole Pine (*Pinus contorta*)				C
Longleaf Pine (*Pinus palustris*)				C
Poplars (*Populus* L. spp.)				C
Turfgrass	C	C	C	
Noble Fir (*Abies procera*)	C			C
Poderosa Pine (*Pinus ponderosa*)				C
White Pine (*Pinus* L. spp.)				C

C = Control
S = Suppress

F.4. Impact of Roundup Ready Alfalfa on Current Crop Rotation Practices

Current crop rotation options or patterns in the U.S. where alfalfa is grown are expected to remain the same upon the introduction of Roundup Ready alfalfa. Alfalfa-to-alfalfa rotations are uncommon because of potential autotoxicity and the inefficient use of residual soil nitrogen credits, i.e., the preferred rotation is to a non-leguminous crop. Each year, 18% of existing alfalfa stands are terminated and rotated to a different crop (Hower et al., 1999). Alfalfa is commonly used as rotational crop only in those states where forage-consuming livestock operations are widespread. Only four states (Wisconsin, California, Idaho and Pennsylvania) practice a hay/pasture to major crop rotation on greater than 5% of their acres (Padgitt et al., 2000). In the other 24 states where data indicated use of a hay/pasture to major crop rotation, the practice was limited to $\leq$5% of their major crop acreage. Therefore, the primary use of alfalfa in rotational crop systems is overwhelmingly represented by the states of Wisconsin, California, Idaho and Pennsylvania where the practice occurs on 31, 15, 10 and 10% of major crop acres, respectively (Padgitt et al., 2000). In 2002, approximately 28% of the newly-seeded U.S. alfalfa acreage was planted within these four states (940,000 of 3.3 million acres) (USDA-NASS, 2003).

It is expected that Roundup Ready alfalfa technology will be adopted almost exclusively by producers who desire pure-stand alfalfa, rather than by mixed-species forage producers. Crop report data are available for forage produced as hay and pasture crops (Padgitt et al., 2000) or alfalfa and alfalfa mixtures (USDA, 2003); however, data on the use of pure stand alfalfa as a rotational crop are lacking. Using USDA 2003 Crop Report data, it is possible to estimate the annual acreage that is potentially rotated with alfalfa and alfalfa mixtures and what percentage of that acreage would be to a Roundup Ready crop (Table VII-13). The calculations assume that all newly seeded alfalfa acres are rotated from a crop other than alfalfa and that a similar percentage of alfalfa rotation is used for all of the major rotational crops within a state. From these calculations, and assuming a 50% adoption rate for Roundup Ready alfalfa, the number of acres annually rotated with Roundup Ready alfalfa is estimated as 142,300 acres nationwide (Table VII-13). For the U.S., approximately 300 million acres were harvested in 2002 for all crops and, of this total, 144 million acres were considered to likely be non-leguminous major crop rotational partners with alfalfa (Table VII-13). Given these assumptions, on an annual basis less than 0.05% of the total crop acres or less than 0.1% of common rotational major crop acreage is expected to be rotated from Roundup Ready alfalfa to another Roundup Ready crop.

In anticipation of commercialization, Monsanto has developed Roundup Ready alfalfa stand termination (Section VII-F.2), crop rotation (Section VII-F.1) and volunteer management strategies (Section VII-F.3). In all regions of the country, alfalfa is most frequently rotated to a non-legume crop, with corn or a small grain most commonly following alfalfa. Regionally important crops such as potato, cotton or sugar beet may also be used in rotation with alfalfa. Rotations with corn, wheat, oats, barley, potato and sugar beets are expected to continue as currently practiced because nonglyphosate herbicides with different modes of action are available to manage weeds and volunteer alfalfa in each of these rotational crops. Corn is the only one of these crops where there

is significant adoption of existing Roundup Ready varieties; approximately 7% of the U.S. corn acres planted in 2002 were Roundup Ready (USDA-NASS, 2003). Soybeans are not commonly rotated with alfalfa because both crops are nitrogen-fixing legumes; therefore, rotations involving soybeans (including Roundup Ready soybean varieties) are not anticipated. Cotton is currently rotated with alfalfa in California on approximately 10% of the alfalfa acreage. Nationwide, herbicide-tolerant cotton varieties, including Roundup Ready cotton varieties, represent 58% of the total cotton acres (USDA, 2001). Success of the alfalfa to cotton rotation will remain largely dependent upon diligent alfalfa stand take-out followed by mechanical control of alfalfa volunteers, as described in Section F.2.

Rotations from Roundup Ready crops with limited adoption (canola, sugarbeets) or under development (wheat, bentgrass) to Roundup Ready alfalfa are considered to be very manageable. Volunteer Roundup Ready wheat and volunteer Roundup Ready bentgrass will be easily controlled in Roundup Ready alfalfa with the use of the graminicides clethodim or sethoxydim (Section D.1.). Volunteer Roundup Ready canola and volunteer Roundup Ready sugarbeet will be easily controlled in Roundup Ready alfalfa with the use of imazethapyr and repeated alfalfa harvest clipping. Imazethapyr is recognized for its outstanding control of weeds in the Brassicaceae/*Cruciferae* (mustard) and *Chenopodiaceae* (goosefoot and sugarbeet) families, as well as the 40-month plant back rotation restriction to sugarbeets and canola following an imazethapyr application.

F.5. Volunteer Management Recommendations

Crop rotations can be divided into two main groups for alfalfa, grass crops and broadleaf crops. More herbicide alternatives exist for management of volunteer alfalfa in grass crops. The general recommendations for controlling volunteer Roundup Ready alfalfa will likely be:

- *Diligent stand take-out.* Use appropriate commercially available herbicide treatments (Stand Termination, Section F.2) alone for reduced tillage systems or in combination with tillage to terminate the alfalfa stand. When possible, time the application to precede a pending freeze or extreme heat.
- *Establish a clean start.* If necessary, utilize tillage and/or additional herbicide application(s) after alfalfa stand termination, and before the planting of the subsequent rotation crop.
- *Plan for success.* Rotate to crops with known mechanical or herbicidal methods for managing volunteer alfalfa.
    - It is acknowledged that rotations to select broadleaf crops are not advisable if the grower is not willing to implement the recommended stand termination practices.
    - In the event that no known mechanical or herbicidal methods exist to manage volunteer alfalfa, it is suggested that a crop with established volunteer alfalfa management practices be introduced into the rotation.
- *Timely execution*. Execute in-crop mechanical or herbicidal treatments (Table VII-9) for managing alfalfa volunteers in a timely manner.

Monsanto, in collaboration with the academic community, has developed firsthand knowledge about successful stand take-out and volunteer management practices for Roundup Ready alfalfa. The recommendations will be communicated in series of training and technical documents (Technology Use Guides, Regional Technical Bulletins, and Fact Sheets, Section F.8 as per current Monsanto practice). In the event that a grower is unsuccessful in taking out Roundup Ready alfalfa, a local Monsanto field scientist will assist in developing a recommendation tailored to address the specific circumstances. The recommendation would likely include a combination of cultural control practices, mechanical control practices and/or use of the herbicide products listed in Tables VII-11 and VII-12.

Table VII-13. Annual Roundup Ready Alfalfa Use Estimates for Major Crop Rotations

	Column Number									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
State	Alfalfa total acres seeded[1] (1000's)	Major crops that follow alfalfa in rotation	Total harvested acres[1] (1000's)	Per crop proportion of state total[2]	Major crop acres rotated with alfalfa[3] (1000's)	Major crop acres rotated with RR alfalfa[4] (1000's)	National use rate for the RR rotational crop option[1]	Acres where RR crop may follow RR alfalfa[5] (1000's)	Annual use of all alfalfa in major crop rotations[6]	Estimated annual use of RR alfalfa in major crop rotations[7]
WI		corn	3630	0.87	434	217	7%	15.0		
		wheat	177	0.04	21	11				
		potato	83	0.02	10	5				
		other small grains[8]	290	0.07	35	17				
State Total	**500**		**4180**	**1**	**500**	**250**		**15.0**	**12.0%**	**0.36%**
CA		corn	540	0.30	48	24	7%	1.7		
		wheat	390	0.22	35	18				
		potato	45	0.02	3	2				
		cotton-upland	477	0.26	42	21	58%	12.2		
		cotton-pima	209	0.12	19	9				
		other small grains	102	0.06	9	4				
		sugarbeet	49	0.02	4	2				
State Total	**160**		**1812**	**1.00**	**160**	**80**		**13.9**	**8.7%**	**0.77%**

Table VII-13 (continued). Annual Roundup Ready Alfalfa Use Estimates for Major Crop Rotations

	Column number									
	1	2	3	4	5	6	7	8	9	10
State	Alfalfa total acres seeded[1] (1000's)	Major crops that follow alfalfa in rotation	Total harvested acres[1] (1000's)	Per crop proportion of state total[2]	Major crop acres rotated with alfalfa[3] (1000's)	Major crop acres rotated with RR alfalfa[4] (1000's)	National use rate for the RR rotational crop option[1]	Acres where RR crop may follow RR alfalfa[5] (1000's)	Annual use of all alfalfa in major crop rotations[6]	Estimated annual use of RR alfalfa in major crop rotations[7]
ID		corn	185	0.07	12	6	7%	0.4		
		wheat	1200	0.44	75	38				
		potato	373	0.14	23	12				
		other small grains	735	0.27	46	23				
		sugarbeet	210	0.08	13	7				
State Total	**170**		**2703**	**1.00**	**170**	**85**		**0.4**	**6.3%**	**0.01%**
PA		corn	1430	0.79	87	43	7%	3.0		
		wheat	185	0.10	11	6				
		potato	14	0.01	1	<1				
		other small grains	175	0.10	11	5				
State Total	**110**		**1804**	**1.00**	**110**	**55**		**3.0**	**6.1%**	**0.17%**

Table VII-13 (continued). Annual Roundup Ready Alfalfa Use Estimates for Major Crop Rotations

State	Column Number 1: Alfalfa total acres seeded[1] (1000's)	2: Major crops that follow alfalfa in rotation	3: Total harvested acres[1] (1000's)	4: Per crop proportion of state total[2]	5: Major crop acres rotated with alfalfa[3] (1000's)	6: Major crop acres rotated with RR alfalfa[4] (1000's)	7: National use rate for the RR rotational crop option[1]	8: Acres where RR crop may follow RR alfalfa[5] (1000's)	9: Annual use of all alfalfa in major crop rotations[6]	10: Estimated annual use of RR alfalfa in major crop rotations[7]
State Totals	**940**		**10499**		**940**	**735**		**32.3**	**9.0%**	**0.31%**
U.S. Overall		corn	76,803	0.534	1762	881	7%	61.7		
		wheat	45,817	0.318	1049	524				
		potato	1277	0.009	30	15				
		cotton-upland	12,171	0.085	280	140	58%	81.2		
		cotton-pima	242	0.002	7	4				
		other small grains	6,233	0.043	142	71				
		sugarbeet	1,361	0.009	30	15				
U.S. Total	**3300**		**143,904**	**1.00**	**3300**	**1650**		**142.9**	**2.3%**	**<0.10%**

[1]Acres seeded or harvested by state or biotech crop planting percentage in 2002 (USDA-NASS 2003).
[2]Crop acres (column 3: by crop) divided by total acres for major crops (column 3: subtotal) in the state or U.S.
[3]Calculated acres of alfalfa (pure stands) established annually in rotation with major rotational crop (column 1 x column 4).
[4]Calculated by multiplying total acres of alfalfa (column 5) by estimated 50% adoption rate for Roundup Ready alfalfa.
[5]Calculated by multiplying estimated acres rotated to Roundup Ready alfalfa annually (column 6) by nationwide adoption rate for Roundup Ready (RR) rotational crop option (column 7).
[6]Calculated by dividing annual acres rotated with alfalfa (column 5) by total crop acres for the rotational crops (column 3).
[7]Calculated by dividing annual estimated acres rotated with Roundup Ready (RR) alfalfa (column 8) by total crop acres for rotational crops (column 3).
[8]Small grain crops includes acreage for oat, barley.

F.6. Control Options for Feral Alfalfa

It is expected that after Roundup Ready alfalfa is commercialized, the trait will eventually migrate into naturalized populations from cultivated alfalfa. In order to assess the potential for the trait to become a concern in naturalized (i.e., feral) alfalfa, information was gathered to determine whether the current naturalized populations are controlled with herbicides, and, if so, whether Roundup agricultural herbicides are used to control naturalized alfalfa. To address these questions, information is presented below describing the role herbicides (including Roundup agricultural herbicides) have in control of feral alfalfa.

The potential environmental consequences on feral alfalfa because of introduction of Roundup Ready alfalfa varieties, was evaluated by obtaining information from weed control experts in eight states including Arizona, California, Idaho, Pennsylvania, Oregon, South Dakota, Washington and Wisconsin. The objective of the survey was to determine whether feral alfalfa is a plant species targeted for control and, if controlled, what herbicides were used. In addition, respondents were asked to comment on what control measures would be used should feral alfalfa populations be suspected of containing individuals with the Roundup Ready trait. Responses were received from 13 experts. The respones are included in Appendix 3.

These weed control experts were asked to answer the following three questions:

1) To the best of your knowledge, is feral alfalfa a weed species that is controlled on roadsides or other unmanaged areas in your state?
2) If alfalfa is a species that is controlled, what are the herbicides currently used for control?
3) If you suspected that the Roundup Ready trait was possibly present in feral alfalfa populations, what herbicides would you use to control feral alfalfa?

A brief summary and conclusions drawn from these responses follows. In general, alfalfa is not a species specifically targeted for weed control in unmanaged areas or on roadsides. In fact, it was noted that alfalfa may be considered a desirable species along some roadsides in South Dakota and Wisconsin and is encouraged to grow. However, it was noted that where weeds are controlled (e.g., where bare ground is desired, for example along roadsides or irrigation canals) alfalfa could be one of the plant species controlled. In the majority of situations, respondents noted that glyphosate was not the herbicide of choice to control feral alfalfa. It was specifically noted that glyphosate is not extremely effective on alfalfa and respondents listed several other herbicides that are typically used because they provide better control. While Roundup agricultural herbicides were listed as one of the herbicides that could be used, it was not the only herbicide used. Feral alfalfa that may contain the Roundup Ready trait would therefore not be expected to be impacted from the selective pressure of glyphosate.

The majority of the respondents offered numerous control options that are available should one suspect that feral alfalfa may contain the Roundup Ready trait. As a result of these responses, it was generally concluded that there would be no environmental impact if feral alfalfa contained the Roundup Ready trait. There were two exceptions. In one

situation, glyphosate was mentioned as the only herbicide used to control weeds where bare ground is desired. In certain regions in California, glyphosate is the only herbicide labelled for control of weeds on banks of irrigation ditches with running water. It is reasoned that feral alfalfa with the Roundup Ready trait would not become a management problem because alfalfa is not known to be an invasive species, and the vast majority of alfalfa is grown as forage that is harvested prior to extensive bloom or seed formation. Therefore, few if any seed would be available to be introduced into irrigation canals. Introduction of the seed likely would be accidental because of misapplication during field establishment. In the event that feral alfalfa with the Roundup Ready trait were to appear in irrigation canals, mechanical weed removal or spot burning would be the best control options. Finally, irrigation canals have extended water-free periods during the off-season; thus, there would be a window of opportunity for the application of non-glyphosate herbicides for weed control.

Related to the issue of feral alfalfa management, one respondent mentioned that alfalfa might appear as a volunteer plant on land recently rotated from alfalfa to newly established fruit trees. While this is not truly a feral situation, but rather inadequate stand termination, Roundup herbicide was mentioned as the herbicide to manage volunteer alfalfa in this type of rotation. To prevent the creation of a problematic situation, the alfalfa control emphasis will change to aggressive alfalfa stand termination practices, prior to the planting of fruit trees, as opposed to waiting to control the alfalfa after it appears in newly planted fruit trees. Specifically, growers rely primarily on tillage to remove alfalfa in areas destined for orchard planting. Following the strategy outlined in Section F.5, Volunteer Management Recommendations, growers will be advised to augment the current practice by applying a suitable stand take-out herbicide before tillage. For the period between stand take-out and tree planting, the grower will be advised to keep the area clean with additional tillage and/or a suitable stand take-out herbicide.

F.7. Management of Gene Flow During Alfalfa Seed and Forage Production

It is expected that the current isolation distances for seed production will be adequate for production of the majority of Roundup Ready and conventional alfalfa varieties. However, information from the gene flow studies will be shared with the alfalfa seed production industry so that seed producers can meet the specific varietal purity standards dictated by various markets.

With the introduction of Roundup Ready alfalfa varieties, seed producers will need to implement seed quality programs similar to the seed quality programs already used for the production of other crops improved through the use of biotechnology. The introduction of the Roundup Ready trait will allow for a more accurate assessment of the genetic purity of alfalfa varieties. Monsanto is committed to working with alfalfa seed producers by providing the alfalfa seed production industry with the tools needed to detect the *cp4 epsps* gene and/or CP4 EPSPS protein. Use of these tools and appropriate seed-production strategies will allow the alfalfa industry to produce seed to meet market specifications.

Forage producers who purchase Roundup Ready alfalfa will sign a grower agreement that strictly prohibits the production of seed, that is, the use of the seed is solely for forage production. Only seed producers that are trained, licensed, and contracted to produce Roundup Ready alfalfa seed will be authorized to do so.

F.8. Technical Support

With the introduction of Roundup Ready alfalfa technology, Monsanto will provide growers with technical training to address the agronomic management of Roundup Ready alfalfa varieties. A broad overview on how to use the technology and stewardship obligations will be provided in a Technology Use Guide specifically developed for management of Roundup Ready alfalfa varieties. It will provide general guidelines, such as strategies for stand take-out and volunteer management, and be distributed nationally to every grower licensed to use Monsanto technology. Unique information needs that are production area specific will be addressed in Regional Technical Bulletins and distributed primarily within the production area for which the Regional Technical Bulletin is created. Examples of unique information needs include specific product and practice recommendations for stand take-out and volunteer management in anticipated crop rotations with Roundup Ready alfalfa. Issue-specific information needs, such management of a weed known to be resistant to glyphosate, will be addressed in a Fact Sheet and distributed on an as-needed basis. Monsanto currently uses Technology Use Guides, Regional Technical Bulletins, and Fact Sheets to support its commercialized products.

Monsanto has a vast network of field scientists located across the U.S., representing all agricultural and industrial/turf/ornamental markets. This network is responsible for providing technical support to users of Monsanto products and technology. In the event that a Roundup agricultural herbicide fails to control weeds in alfalfa, Monsanto will launch a thorough investigation to determine the cause. If feral alfalfa containing the Roundup Ready trait were to create a management challenge in any of these markets, local Monsanto field scientists will assist in developing practices tailored to address the specific circumstances. Those practices would likely include a combination of cultural control practices, mechanical control practices and/or use of the herbicide products listed in Tables VII-11 and VII-12.

F.9. Conclusions

Roundup Ready technology represents the first biotechnology-derived trait introduced in alfalfa. Biotechnology-derived improvements hold great promise for this crop and other traits under development. For these traits to be successful in alfalfa, the alfalfa industry will need to follow practices that have allowed for successful introduction of biotechnology-derived traits in other crops like soybean and corn. Monsanto and Forage Genetics International intend to provide information and guidance to the alfalfa industry to facilitate the introduction and long-term use of this product. These guidelines discussed above highlight the basic components of the stewardship program for Roundup Ready alfalfa varieties. These components include:

- Viable stand rotation practices

- Control options for volunteer and feral alfalfa plants
- Management of Roundup Ready technology through grower agreements
- Analytical detection methods for use by the alfalfa industry
- Ongoing technical support

Implementation of this program will help ensure successful introduction and use of the Roundup Ready weed control system in alfalfa.

G. Weed Resistance

The risk of weeds developing resistance and the potential impact of resistance on the usefulness of a herbicide vary greatly across different modes of action and are dependent on a combination of different factors. Monsanto considers product stewardship to be a fundamental component of customer service and business practices and invests considerably in research to understand the proper uses and stewardship of the glyphosate molecule. This research includes an evaluation of some of the factors that can contribute to the development of weed resistance.

The introduction of Roundup Ready alfalfa varieties with the potential for repeated glyphosate applications to growing alfalfa will likely raise the question of how this may affect the appearance of glyphosate-resistant weed biotypes and whether the adoption of Roundup Ready alfalfa will encourage this situation. The risk factors for developing herbicide resistance are considerably different in forages because weeds rarely produce seed and fields are repeatedly mown during the growing season. Annual weeds are generally only present in the seeding year and perhaps again as the stand declines in the third or later years. Individual weeds that might have a gene for tolerance or resistance to any herbicide used in alfalfa usually will be killed mechanically before viable seed is produced when alfalfa is harvested. This is a case of built-in integrated control (cultural and chemical), which is often lacking in row crops, orchards and cereal crops, where the four cases of confirmaed tolerance/resistance to glyphosate herbicides have occurred to date.

Weeds that could potentially shift to resistant biotypes in alfalfa fields are those that produce seed, even in well-managed fields. These include common dandelion, shepherd's purse, downy bromegrass, crabgrasses, broadleaf plantain and chickweeds. Weed scientists consider the risk of weeds developing resistance to glyphosate as low. With good stewardship, careful crop monitoring, and integrated management practices, resistance can be greatly delayed or prevented well into the future. Further information regarding development of glyphosate-resistant weeds is presented in Appendix 2.

Section VIII. Adverse Consequences of Introduction

Monsanto and Forage Genetics International know of no unfavorable results or observations associated with Roundup Ready alfalfa events J101 or J163 that would result in adverse consequences of introduction. The substantial benefits to alfalfa seed and forage producers and the environmental benefits of Roundup agricultural herbicides over current herbicides used for weed control in alfalfa have been described in this petition. Alfalfa plants containing events J101 or J163 show no unintentional phenotypic or agronomic differences when compared to conventional alfalfa. Forage produced by alfalfa plants containing these events was shown to be substantially equivalent to forage produced by conventional alfalfa varieties. The only difference between alfalfa plants containing events J101 or J163 is the expression of the CP4 EPSPS protein and resulting tolerance to Roundup agricultural herbicides. The *cp4 epsps* gene and CP4 EPSPS protein produced by these events have been well characterized. On the basis of information presented in this petition, it is concluded that the trait and the transformation process did not confer plant pest characteristics on alfalfa plants containing these events. Therefore, Monsanto and Forage Genetics International request a determination from APHIS that Roundup Ready alfalfa events J101 and J163 and any progenies derived from breeding of these events into other alfalfa varieties no longer be considered regulated articles under 7 CFR Part 340.

References

Acharya, S.N., Stout, D.G., Brook, B., Thompson, D. 1999. Cultivar and storage effects on germination and hard seed content of alfalfa. Can. J. Plant Sci. 79:201-208.

Alexander, M.P. 1969. Differential staining of aborted and nonaborted pollen. Stain Technology 44:117-122.

Anderson, W.P. 1996. P. 27-37. *In* Weed Science Principles and Applications, 3rd ed. West Publishing Company, St. Paul, Minnesota.

AOSA, Association of Official Seed Analysts. 1998. Rules for Testing Seeds. AOSA, Lincoln, Nebraska. 122 pp.

AOSA, Association of Official Seed Analysts. 2002. Rules for Testing Seeds. AOSA, Lincoln, Nebraska. 123 pp.

APLS, Ag Product Label Service. June 2002. [http://www.cdms.net/webapls.asp] Accessed 04/06/2004.

Arfin, S.M., and Bradshaw, R.A. 1988. Cotranslational processing and protein turnover in eukaryotic cells. Biochemistry 27:7979-7984.

Austin, S., Bingham, T., Mathews, D.E., Shahan, M.N., Will, J., Burgess, R.R. 1995. Production and field performance of transgenic alfalfa (*Medicago sativa* L.) expressing alpha-amylase and manganese-dependent lignin peroxidase. Euphytica 85:381-393.

Barker, R.F., Idler, K.B., Thompson, D.V. and Kemp, J.D. 1983. Nucleotide sequence of the T-DNA region from the *Agrobacterium tumefaciens* octopine Ti plasmid pTi15955. Plant Mol. Biol. 2:335-350.

Barnes, D. K. 1972. A system for visually classifying alfalfa flower color. Agriculture Handbook No. 424. U.S. Department of Agriculture. Note: Publication is out of print, copies available at [http://www.naaic.org/Resources/colorguide/flowercolor.html] Accessed 4/06/2004.

Barnes, D.K., Bingham, E.T., Murphy, R.P., Hunt, O.J., Beard, D.F., Skrdla, W.H., and Teuber, L.R. 1977. Alfalfa Germplasm in the United States: Genetic Vulnerability, Use, Improvement and Maintenance. USDA Tech. Bull. 1571. U.S. Government Printing Office, Washington, D.C.

Barnes, D.K., and Sheaffer, C.C. 1995. Alfalfa. P. 205-216. *In* Barnes, R.F., Miller, D.A., and Nelson, C.J. (ed.) Forages. Iowa State Univ., Ames, Iowa.

Barry, G., Kishore, G., Padgette, S., Taylor, M., Kolacz, K., Weldon, M., Re, D., Eichholtz, D., Fincher, K., and Hallas, L. 1992. Inhibitors of amino acid biosynthesis: strategies for imparting glyphosate tolerance to crop plants. P. 139-145. *In* Singh, *et al.,* (eds.) Biosynthesis and Molecular Regulation of Amino Acids in Plants. American Society of Plant Physiologists.

Bass, L.N., Gunn, C.R., Hesterman, O.B., and Roos, E.E. 1988. Seed physiology, seedling performance, and seed sprouting. P. 961-979. *In* Hanson, A.A., Barnes, D.K., and Hill Jr., R.R. (ed.) Alfalfa and Alfalfa Improvement. ASA-CSSA-SSSA, Madison, Wisconsin. 1084 pp.

Bickoff, E.M., Kohler, G.O., and Smith, D. 1972. Chemical composition of herbage. P. 247-282. *In* Hanson, C.H. (ed.) Alfalfa Science and Technology. ASA-CSSA-SSSA, Madison, Wisconsin. 812 pp.

Boerboom, C. 2002. Answers to Three Common Phone Calls: How Can I Control Alfalfa Postemergence in Soybeans? Wisconsin Crop Manager. [http://ipcm.wisc.edu/wcm/pdfs/2002/02-7weeds4.html]. Accessed 04/06/2004.

Bouton, J.H. 2002. Professor of Agronomy. University of Georgia. Personal communication.

Bradstreet, R.B. 1965. The Kjeldahl Method for Organic Nitrogen. Academic Press, New York, New York.

Burnette, B.L., Taylor, M.L., Lakemeyer, L.L., Smith, C.E., Bailey, M.R., and Nida, D.L. 1993. Characterization of 5-*enol*-pyruvyl-shikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4 (CP4 EPSPS) produced by glyphosate-tolerant cotton and assessment of equivalence relative to CP4 EPSPS produced by *E. coli*. (Monsanto Company, St. Louis, Missouri) Study 93-01-30-02, Technical Report MSL-13166.

C&P Press. 2002. Crop Protection Reference. [http://www.greenbook.net] Accessed 4/06/04.

Carpenter, J. L., Ploense, S. E., Snustad, D. P., and Silflow, C. D. 1992. Preferential expression of an α–tubulin gene of 'anadensis' in pollen. The Plant Cell 4:557-571.

Corruzzi, G., Broglie, R., Edwards, C., and Chua, N-H. 1984. Tissue-specific and light-regulated expression of a pea nuclear gene encoding the small subunit of ribulose-1,5-bisphosphate carboxylase. EMBO J. 3:1671-1679.

Della-Cioppa, G., Bauer, C.S., Klein, B.K., Shah, D.M., Fraley, R.T., and Kishore, G.M. 1986. Translocation of the precursor of 5-enolpyruvylshikimate-3-phosphate synthase into chloroplasts of higher plants *in vitro*. Proc. Natl. Acad. Sci. USA 83:6873-6877.

Depicker, A., Stachel. S., Dhaese, P., Zambryski, P., and Goodman, H.M. 1982. Nopaline synthase: Transcript mapping and DNA sequence. J. Molec. Appl. Genet. 1:561-573.

Doll, J.D. 1986. Do weeds affect forage quality? Proc. 16th National Alfalfa Improvement Symposium. Ft. Wayne, Indiana. pp. 161-170.

Doll, J.D. 1994. Protein, moisture content and feed value of forage weeds. *In* Proc. North Central Weed Science Society 49:94.

Doll, J. D. 2002. Weed Management in Roundup Ready alfalfa. *In* Weed Control Results Vol. 31, pages C-8 to C-10.

Doll, J.D. 2003. Future of Roundup Ready Alfalfa. *In* Proc. Wis. Fertilizer, AgLime and Pest Management Conf. 42:245-251.

Doll, J.D., Fitzpatrick, S., and Undersander, D.J. 2002. Glyphosate resistant alfalfa. *In* Proc. North Central Weed Sci. Soc. Abstr. No. 200. St. Louis, MO.

Dutt, T.E., Harvey, R.G., Fawcett, R.S., Jorgensen, N.A., Larsen, H.J., and Schlough, D.A. 1979. Forage quality and animal performance as influenced by quackgrass control in alfalfa with pronamide. Weed Sci. 27:127-132.

Endres, G. 1999. Control of Established Alfalfa, Fargo, 1993. North Dakota State University Extension Service Pro Crop Database. [http://www.ag.ndsu.nodak.edu/aginfo/procrop/leg/killal04.htm]. Accessed 04/06/2004.

Entz, M.H., Ominski, P.D., Mohr, R.,. Schoofs, A., Forster, D., Bullied, W.J., Shirtliffe, S., and Bamford, K.C.. 2002. Forages Improve Efficiency of Prairie Cropping Systems. University of Manitoba Department of Plant Science Extension Bulletin. [http://www.umanitoba.ca/faculties/afs/plant_science/extension/agrif95.html] Accessed 04/06/04.

Fawcett, R. and Towery, D. 2002. Conservation Tillage and Plant Biotechnology: How New Technologies Can Improve the Environment By Reducing the Need to Plow. Conservation Technology Information Center. West Lafayette, IN

Ferguson, G.P., Roop, R.M., and Walker, G.C. 2002. Deficiency of a *Sinorhizobium meliloti bac*A mutant in alfalfa symbiosis correlates with alteration of the cell envelope. J. Bacteriol. 184:5625-5632.

Fitzpatrick, S., Reisen, P. and McCaslin, M. 2002. Alfalfa pollen-mediated gene flow studies, 2000-2001. *In* Report of the 38th North Amer. Alfalfa Improvement Conference. Sacramento, California. [http://www.naaic.org/Meetings/National/2002meeting/2002Abstracts/Fitzpatrick.pdf]. Accessed 04/06/2004.

Fitzpatrick, S., Reisen, P. and McCaslin, M. 2002. Alfalfa pollen-mediated gene flow studies, 2002. Forage Genetics International. [Pending posting at, http://www.foragegenetics.com/News.htm]. Copies available upon request until posted.

Fling, M E., Kopf, J., and Richards, C. 1985. Nucleotide sequence of the transposon Tn7 gene encoding as aminsglycoside-modifying enzyme, 3'(9)-*O*-nucleotidyltransferase. Nuc. Acids Res. 13:7095-7106.

Frank, G. 1994. Cost of production in rotations of alfalfa and corn silage. Proc. Wis. Forage Council Annual Meeting. Available at: [http://cdp.wisc.edu/jenny/crop/rotation.pdf]. Accessed 04/06/2004.

Fridriksson, S., and Bolton, J.L. 1963. Development of the embryo of *Medicago sativa* L. after normal fertilization and after pollination by other species of *Medicago*. Can. J. Bot. 41:23-33.

Fyrer, J.R. 1930. Cytological studies in *Medicago, Melilotis and Trigonella*. Can. J. Res. 3:3-50.

Gianessi, L. 1997. National Pesticide Use Database. National Center for Food and Agricultural Policy, Washington, D.C. Available at: [http://www.ncfap.org/database/default.htm]. Accessed 04/06/2004.

Gianessi, L.P., Silvers, C.S., Sankula, S. Carpenter, J.E. 2002. Plant biotechnology: Current and potential impact for improving pest management in U.S. agriculture an analysis of 40 case studies. [http://www.ncfap.org] Accessed 04/06/2004.

Giza, P.E. and Huang, R.C. 1989. A self-inducing runaway-replication plasmid expression system utilizing the Rop protein. Gene 78:73-84.

Goos, R.J., Johnson, B., Reinert, M., Helms, T., Henson, R., and Martin, L. 2002. Effect of Roundup on nitrogen fixation by Roundup-Ready soybeans. *In* Proc. of the Great Plains Soil Fertility Conference. Denver, Colorado.

Gruys, K.J., and Sikorski, J.A. 1999. Inhibitors of tryptophan, phenylalanine and tyrosine biosynthesis as herbicides. P. 357-384 *In* Singh, B. (ed.) Plant Amino Acids: Biochemistry and Biotechnology. Marcel Dekker, Inc., New York, New York.

Hanson, A.A., Barnes, D.K., and Hill, Jr., R.R. (eds.). 1988. Alfalfa and Alfalfa Improvement, Agronomy Monograph no. 29. ASA-CSSA-SSSA, Madison, Wisconsin. 1084 pp.

Harrison, L., Bailey, M., Naylor, M., Ream, J., Hammond, B., Nida, D., Burnette, B., Nickson, T., Mitsky, T., Taylor, M., Fuchs, R. and Padgette, S. 1996. The expressed protein in glyphosate-tolerant soybean, 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4, is rapidly digested *in vitro* and is not toxic to acutely gavaged mice. J. Nutr. 126:728-740.

Hendrickson, S. 2002. Economics of alfalfa persistence. P. 5. *In* Manitowoc Co. Extension Newsletter, Country Corner, Nov., 2002 (cites Univ. of Wisconsin agronomy advice on this topic from May, 1999).

Hower, A.A., Harper, J.K., and Harvey, R.G. 1999. The Importance of Pesticides and Other Pest Management Practices in U.S. Alfalfa Production. U.S.D.A. National Agricultural Pesticide Impact Assessment Program. NAPIAP Document No. 2-CA-99. 221 pp.

Ivanov, A.I. 1988. Alfalfa. Amerind Publishing Co., New Delhi.

Jenczewski, E., Prosperi, J.-M. and Ronfort, J. 1999. Evidence for gene flow between wild and cultivated *Medicago sativa* (Leguminosae) based on allozyme markers and quantitative traits. Amer. J. Bot. 86:677-687.

Jiménez, C. R., Huang, L., Qui, Y., and Burlingame, A. L. 1998. In-gel digestion of proteins for MALDI-MS fingerprint mapping. Current Protocols in Protein Science Supplement 14:16.4.1-16.4.5.

Jones, Jr., J.B. 1972. Plant tissue analysis for micronutrients. P. 325-334. *In* Mortvedt, J.J., Giordano, P.M., and Lindsay, W.L. (eds.) Micronutrients in Agriculture. Soil Science Society of America, Madison, Wisconsin.

Julier, B., Huyghe, C., and Ecalle, C. 2000. Within- and among-cultivar genetic variation in alfalfa: Forage quality, morphology, and yield. Crop Sci. 40:365-369.

Kabata-Pendias, A., and Pendias, H. 1992. Elements of group VIII. P. 271-276. *In* Trace Elements in Soils and Plants. CRC Press, Boca Raton, Florida.

Kalthoff, I.M., and Sandell, E.B. 1948. Quantitative Inorganic Analysis. MacMillan, New York, New York.

Kaul, B.A., and Fick, G.W. 1981. Quantifying morphological development of alfalfa for studies of herbage quality. Crop Sci. 21:267-271.

King, C.A, Purcel, L.C., and Vories, E.D. 2001. Plant growth and nitrogenase activity of glyphosate-tolerant soybean in response to foliar glyphosate applications. Agronomy J. 93:179-186.

Kishore, G., and Shah, D. 1988. Amino acid biosynthesis inhibitors as herbicides. Ann. Rev. Biochem. 57:627-663.

Klee, H.J., Muskopf, Y.M., and Gasser, C.S. 1987. Cloning of an *Arabidopsis* gene encoding 5-enolpyruvylshikimate-3-phosphate synthase: Sequence analysis and manipulation to obtain glyphosate-tolerant plants. Mol. Gen. Genet. 210:437-442.

Lamblin, A-F J., Crow, J. A., Johnson, J. E., Silverstein, K.A., Kunau, T. M., Kilian, A., Benz, D., Stromvik, M., Endre, G., VandenBosch, K. A., Cook, D. R., Young, N. D., and Retzel, E. F. 2003. MtDB: A database for personalized data mining of the model legume *Medicago truncatula* transcriptome. Nucleic Acids Res. 31:196-201.

Leath, K.T., Erwin, D.C., and Griffin, G.D. 1988. Diseases and nematodes. P. 621-670. *In* Hanson, A.A., Barnes, D.K., and Hill Jr., R.R. (eds.) Alfalfa and Alfalfa Improvement. ASA-CSSA-SSSA, Madison, Wisconsin. 1084 pp.

Lesins, K.A., and Lesins, I. 1979. Genus *Medicago* (Leguminosae): A taxogenetic study. Dr. W. Junk Publishers, Kluwer, Dordrecht, The Netherlands.

Lesins, K.A., and Gillies, C.B. 1972. Taxonomy and cytogenetics of *Medicago*. P. 53-86. *In* Hanson, C.H. (ed.) Alfalfa Science and Technology. ASA-CSSA-SSSA, Madison, Wisconsin. 812 pp.

Little, T.M., and Hills, J.F. 1978. Analysis of counts. *In* Agricultural Experimentation, Design and Analysis. John Wiley and Sons, Inc., New York, New York.

Manitoba Agriculture and Food. 2002. Getting Out of Alfalfa with Herbicides. University of Manitoba Faculty of Agriculture and Food Sciences. [http://www.gov.mb.ca/agriculture/crops/forages/bjb00s12.html]. Accessed 04/06/2004.

Marketing Horizons, Inc. 1996. Roundup Ready Alfalfa Market Opportunity Study. St. Louis, Missouri.

Mayerle, G. 2002. Eradicating Alfalfa For Direct Seeding. The Newsletter of the Saskatchewan Soil Conservation Association, Issue 33. [http://ssca.usask.ca/Issue33/Garry2.htm]. Accessed on 04/063/2004.

McCaslin, M., Fitzpatrick, S., and Reisen, P. 2001. Summary of 2000 alfalfa pollen flow experiment. [http://foragegenetics.com/News.htm]. Accessed on 04/06/2004.

McCaslin, M. and Temple, S. 2003. Use of the Roundup Ready trait to estimate selfing in hand crosses of alfalfa. Central Alfalfa Improvement Conference, July 21-25, 2003 (virtual meeting). http://www.naaic.org/Meetings/Central2003/CAIC2003VirtualMeeting.htm#GMO). Accessed 04/06/2004.

McCloskey, W.B. 2001. Associate Specialist, Weed Sciences. University of Arizona. Personal Communication.

McCoy, T.J. and Bingham, E.T. 1988. Cytology and cytogenetics of alfalfa. P. 737-776. *In* Hanson, A.A., Barnes, D.K., and Hill Jr., R.R. (eds.) Alfalfa and Alfalfa Improvement. ASA-CSSA-SSSA, Madison, Wisconsin. 1084 pp.

McCoy, T.J., and Smith, LY. 1986. Interspecific hybridization of perennial *Medicago* species using ovule-embryo culture. Theor. Appl. Genet. 71:772-783.

McElgunn, J.D. 1973. Germination response of forage legumes to constant and alternating temperatures. Can. J. Plant Sci. 53:797-800.

McKenzie, J.S., Paquin, R., and Duke, S.H. 1988. Cold and heat tolerance. P. 259-302. *In* Hanson, A.A., Barnes, D.K., and Hill Jr., R.R. (eds.) Alfalfa and Alfalfa Improvement. ASA-CSSA-SSSA, Madison, Wisconsin. 1084 pp.

Michaud, R., Lehman, W.F., and Rumbaugh, M.D. 1988. World distribution and historical development. P. 25-91. *In* Hanson, A.A., Barnes, D.K., and Hill Jr., R.R. (eds.) Alfalfa and Alfalfa Improvement. ASA-CSSA-SSSA, Madison, Wisconsin. 1084 pp.

Mountain, A. 1989. Gene expression systems for *Bacillus subtilis*. P. 73-114. *In* Harwood, C.R. (ed.) *Bacillus*. Plenum Press, New York.

NASS, National Agricultural Statistics Service. 2002. United States Department of Agriculture: Track Records-U.S. Crop Production. [http://jan.mannlib.cornell.edu/data-sets/crops/96120/track02b.htm#hay]. Accessed 04/06/2004.

National Research Council. 1982. United States-Canadian tables of feed composition. P. 8-146. *In* Nutritional Data for United States and Canadian Feeds 3rd revision. National Academy Press, Washington, D.C.

Nenz, E., Pupilli, F., Damiani, F., and Arcioni, S. 1996. Somatic hybrid plants between the forage legumes *Medicago sativa* L. and *Medicago arborea* L. Theor. Appl. Genet. 93:183-189.

OECD, Organisation for Economic Co-operation and Development. 1999. Consensus Document on General Information Concerning the Genes and Their Enzymes that Confer Tolerance to Glyphosate Herbicide. OECD Environmental Health and Safety Publications, Paris.

Padgette, S.R., Re, D.B., Barry, G.F., Eichhlotz, D.E., Delannay, X., Fuchs, R.L., Kishore, G.M. and Fraley, R.T. 1996. New weed control opportunities: Development of soybeans with a Roundup Ready™ gene. *In* Duke, O.S. (ed.) Herbicide-Resistant Crops: Agricultural, Environmental, Economic, Regulatory, and Technical Aspects. CRC Press, New York.

Padgitt, M., Newton, D., Penn, R., and Sandretto, C. 2000. General Crop Management Practices. P. 53-64. *In* Production Practices for Major Crops in U.S. Agriculture, 1990-97. USDA-Economic Research Service Statistical Bulletin No. 969. 114 pp. [http://www.ers.usda.gov/publications/sb969/sb969e.pdf]. Accessed 04/06/2004.

Pais, I., and Jones Jr., J.B. 1997. Trace elements. P. 112-114. *In* The Handbook of Trace Elements. CRC Press, Boca Raton, Florida.

Pfeiffer, T.W., and Bingham, E.T. 1983. Abnormal meiosis in alfalfa, *Medicago sativa*: Cytology of 2*n* egg and 4*n* pollen formation. Can. J. Genet. Cytol. 25:107-112.

Pimental, D., Lach, L., Zuniga, R., and Morrison, D. Environmental and economic costs associated with non-indigenous species in the United States. [http://www.news.cornell.edu/releases/Jan99/species_costs.html] Accessed on 04/06/2004.

Piccirilli, M., and Arcioni, S. 1992. New interspecific hybrids in the genus *Medicago* through *in vitro* culture of fertilized ovules. P. 325-330. *In* Ottaviano, E., Mulcahy, D.L., Sari Gorla, M. and Bergamini Mulcahy, G. (eds.) Angiosperm Pollen and Ovules. Springer-Verlag New York, Inc.

Quiros, C.F., and Bauchan, G.R. 1988. The genus *Medicago* and the origin of the *Medicago sativa* complex. P. 93-124. *In* Hanson, A.A., Barnes, D.K., and Hill Jr., R.R. (eds.) Alfalfa and Alfalfa Improvement. ASA-CSSA-SSSA, Madison, Wisconsin. 1084 pp.

Rademacher, T. W., Parekh, R. B., and Dwek, R. A. 1988. Glycobiology. *Annu Rev Biochem* 57:785-838.

Rawlinson, J., A. Martin. 1999. Fall's Best for Killing Alfalfa Stands. University of Nebraska Institute of Agriculture and Natural Resources Cooperative Extension Crop Watch News Service. [http://cropwatch.unl.edu/archives/1999/crop99-22.htm – fall] Accessed 04/06/2004.

Reisen, P., McCaslin, M., and Johannsen, N. 2002. Dodder control in Roundup Ready alfalfa. *In* Report of the 38th North American Alfalfa Improvement Conference. July 27-30, 2002. Sacramento, California. [http://www.naaic.org/Meetings/National/2002meeting/2002Abstracts/Reisen.pdf] Accessed 04/06/2004.

Richins, R., Scholthof, H., and Shepard, R. 1987. Sequence of figwort mosaic virus DNA (Caulimovirus Group). Nucleic Acids Res. 15:8451-8466.

Rincker, C.M., Marble, V.L., Brown, D.E., and Johansen, C.A. 1988. Seed production practices. P. 985-1021. *In* Hanson, A.A., Barnes, D.K., and Hill Jr., R.R. (eds.) Alfalfa and Alfalfa Improvement. ASA-CSSA-SSSA, Madison, Wisconsin. 1084 pp.

Rochester, D.E., Winer, J.A., and Shah, D.M. 1986. The structure and expression of maize genes encoding the major heat shock protein, hsp 70. The EMBO J. 5:451-458.

Rogan, G.J., Ream, J. E., Berberich, S.A., and Fuchs, R.L. 1992. Development and validation of an enzyme-linked immunosorbent assay for neomycin phosphotransferase II in genetically modified cotton tissue extracts. J. Agric. Food Chem. 40:1453-1458.

Rogers, S. G., and Klee, H. 1987. Pathways of plant genetic manipulation employing *Agrobacterium*. P. 179-203. *In* Hohn, T. and Schell, J. (eds.) Plant Gene Research, Plant DNA Infectious Agents, Vol. IV. Springer-Verlag Wien Publisher, New York, New York.

Rumbaugh, M.D., Caddel, J.L., and Rowe, D.E. 1988. Breeding and quantitative genetics. P. 777-808. *In* Hanson, A.A., Barnes, D.K., and Hill Jr., R.R. (eds.) Alfalfa and Alfalfa Improvement. ASA-CSSA-SSSA, Madison, Wisconsin. 1084 pp.

Samac, D.A. and Temple, S.J. (In press.) Development and Utilization of Transformation in *Medicago* species. *In* Liang, G.H. and Skinner, D.Z. (eds.) Genetically Modified Crops: Their Development, Uses, and Risks. Haworth Press, Inc.

Sangduen, N., Sorensen, E.L., and Liang, G.H. 1982. A perennial x annual *Medicago* cross. Can. J. Genet. Cytol. 24:361-365.

Schoenig, S. 2002. Certified weed free forage: An emerging program for western states. *In* Proc. Western Alfalfa and Forage Conference, December 11-13, 2002, Sparks, Nevada. [http://alfalfa.ucdavis.edu/SUBPAGES/2002Symposium/pdf/Schoenig%20-%20Accomplishments%20Since%20January%201996.pdf]. Accessed, 04/06/2004.

Schulz, A., Krüper, A., and Amrhein, N. 1985. Differential sensitivity of bacterial 5-enolpyruvylshikimate-3-phosphate synthases to the herbicide glyphosate. FEMS Microbiology Letters 28:297-301.

Shaver, R., Undersander, D., Schwab, E., Hoffman, P., Lauer, J., Combs, D., Coors, J. 2001. Milk2000: Combining yield and quality into a single term. [http://www.uwex.edu/ces/forage/pubs/milk2000.htm]. Accessed 04/06/2004.

Schrock, O. 1943. Beobachtungen an einem Bastard zwischen Luzerne (*Medicago media*) und Gelbklee (*Medicago lupulina*) and seiner Nachkommenschaft. Der Zuchter 15:4-10.

Sinskaya, E.N. (ed.). 1961. Flora of cultivated plants of the USSR. XII. Part 1. Medic, sweetclover, fenugreek. Jerusalem, Israel Program for Scientific Translation.

Skinner, K., Smith, L., and Rice, L. 2000. Using noxious weed lists to prioritize targets for developing weed management strategies. Weed Sci. 48:640-644.

Small, E. and Jomphe, M. 1989. A synopsis of the genus *Medicago* (Leguminosae). Can. J. Bot. 67:3260-3294.

Smith, S.R., Jr., and Bouton, J.H. 1993. Selection within alfalfa cultivars for persistence under continuous stocking. Crop Sci. 33:1321-1328.

Smith, D. 1969. Influence of temperature on the yield and chemical composition of Vernal alfalfa at first flower. Agronomy J. 61:470-472.

Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503-517.

Southworth, W. 1928. Influences which tend to effect seed production in alfalfa and an attempt to raise a high seed producing strain by hybridization. Sci. Agric. 9:1-29.

St. Amand, P.C., Skinner, D.Z., and Peaden, R.N. 2000. Risk of alfalfa transgene dissemination and scale-dependent effects. Theor. Appl. Genet. 101:107-114.

Stalker, D. M., Thomas, C. M., and Helinski, D. R. 1981. Nucleotide sequence of the region of the origin of replication of the broad host range plasmid RK2. Mol. Gen. Genetics 181:8-12.

Steinrucken, H. and Amrhein, N. 1980. The herbicide glyphosate is a potent inhibitor of 5-enolpyruvylshikimic acid-3-phosphate synthase. Biochem. Biophys. Res. Commun. 94:1207-1212.

Stone, J.E., Marx, D.B., and Dobrenz, A.K. 1979. Interaction of sodium chloride and temperature on germination of two alfalfa cultivars. Agronomy J. 71:425-427.

Sutcliffe, J.G. 1978. Complete nucleotide sequence of the *Escherichia coli* plasmid pBR322. Symposia on Quantitative Biology 43:77-103.

Tesar, M.B. and Marble, V.L. 1988. Alfalfa establishment. P. 303-332. *In* Hanson, A.A., Barnes, D.K., and Hill Jr., R.R. (eds.) Alfalfa and Alfalfa Improvement. ASA-CSSA-SSSA, Madison, Wisconsin. 1084 pp.

Teuber, L.R., Taggard, K.L., Gibbs, L.K., Orloff, S.E., Mueller, S.C., Frate, C.A., Putnam, D.H., and Volenec, J.J. 1998. Check cultivars, locations, and management of fall dormancy evaluation. P. 25. *In* Proc. 36th North American Alfalfa Improvement Conference, Bozeman, Montana. [http://www.naaic.org/Publications/1998Proc/abstracts/Teuber.html]. Accessed 04/06/2004.

Teuber, L.R. and. Brick, M.A. 1988. Morphology and anatomy. P. 125-162. *In* Hanson, A.A., Barnes, D.K., and Hill Jr., R.R. (eds.) Alfalfa and Alfalfa Improvement. ASA-CSSA-SSSA, Madison, Wisconsin. 1084 pp.

Tinland, B. 1996. The integration of T-DNA into plant genomes. Trends in Plant Sci. 1:178-184.

Townsend, M.S., Henning, J.A., Smith, D.W., Ray, I.M., and Currier, C.G. 1998. Differential concentration of ten minerals among four ancestral alfalfa germplasms. Crop Sci. 39:574-578.

Townsend, C.E., and McGinnies, W.J. 1972. Temperature requirements for seed germination of several forage legumes. Agronomy J. 73:329-332.

Turkington, R. and Cavers, P.B. 1979. The biology of Canadian weeds. 33. *Medicago lupulina* L. Can. J. Plant Sci. 59:99-110.

Ungar, I.A. 1967. Influence of salinity and temperature on seed germination. Ohio J. Sci. 67:120-123.

University of California Pest Management Guidelines. 2001. Alfalfa: Susceptibility of Weeds to Herbicide Control, 2001. [http://www.ipm.ucdavis.edu/PMG/r1700411.html#WINTER]. Accessed 04/06/2004.

U.S. Forest Service. 1995. 2081-Management of noxious weeds. Forest Service Manual. Amendment No. 2000-95-5 (Nov. 29, 1995). [http://www.fs.fed.us/dirindexhome/fsm/2000/2080.txt] Accessed 04/06/2004.

USDA. 1998. Field crops final estimates, 1992-97. Stat. Bull. 947a. USDA-NASS, Washington, DC.

USDA-APHIS. 2000. Federal noxious weed list (09/08/2000 update). [http://www.aphis.usda.gov/ppq/permits/fnwsbycat-e.PDF] Accessed 04.06/2004.

USDA. 2001. Acreage (June 28. 2002). [http://usda.mannlib.cornell.edu/reports/nassr/field/pcp-bba/acrg0602.pdf] Accessed 04/06/2004.

USDA. 2003. [http://usda.mannlib.cornell.edu/reports/nassr/field/pcp-bba/acrg0602.pdf] Accessed 04/06/2004.

USDA-APHIS, Animal and Plant Health Inspection Service. 2002. National Plant Board State Regulated Noxious Weeds. [http://www.aphis.usda.gov/npb/statenw.html]. Accessed 04/06/2004.

USDA-ARS, Agricultural Research Service. 2003. Invaders Database System. [http://invader.dbs.umt.edu/Noxious_Weeds/noxlist.asp] Accessed March 23, 2003).

USDA-ERS, Economic Research Service. 2002. Economic Research Service/USDA [http://www.ers.usda.gov/publications/sb969/sb969e.pdf]. Accessed 04/06/2004.

USDA Forest Service. 2002. U.S. Forest service requires the use of noxious weed-free hay (Aug. 5, 2002). [http://www.fs.fed.us/r2/psicc/sanc/weed.shtml] Accessed 4/21/03.

USDA-NASS, National Agricultural Statistics Service. 2002. Washington D.C. [www.nass.usda.gov:81/ipedb/]. Accessed 04/06/2004.

USDA-NASS, National Agricultural Statistics Service. 2003. Acreage, June 2002. Agricultural Statistics Board. [http://www.ers.usda.gov] Accessed April, 2003.

USDA-NRCS. 2003. USDA-National Resource Conservation Service. 2003 Plants database. [http://plants.usda.gov/cgi_bin/topics.cgi]. Accessed 04/06/2004.

Valizadeh, M., Kang, K.K., Kanno, A., and Kameya, T. 1996. Breeding Science 46:7-10.

Vance, C.P., Heichel, G.H., and Phillips, D.A. 1988. Nodulation and symbiotic dinitrogen fixation. P. 229-257. *In* Hanson, A.A., Barnes, D.K., and Hill Jr., R.R. (eds.) Alfalfa and Alfalfa Improvement. ASA-CSSA-SSSA, Madison, Wisconsin. 1084 pp.

Viands, D.R., P. Sun, and D.K. Barnes. 1988. Pollination control: Mechanical and sterility. P. 931-960. *In* Hanson, A.A., Barnes, D.K., and Hill Jr., R.R. (eds.) Alfalfa and Alfalfa Improvement. ASA-CSSA-SSSA, Madison, Wisconsin. 1084 pp.

Walker, K.A. and Sato, S.J. 1981. Morphogenesis in callus tissue of *Medicago sativa*: the role of ammonium ion in somatic embryogenesis. Plant Cell, Tissue and Organ Culture 1:109-121.

Zambryski, P. 1992. Chronicles from the *Agrobacterium*-plant cell DNA transfer story. Annu. Rev. Plant Physiol. Plant Mol. Biol. 43:465-490.

APPENDIX 1

Roundup Ready Alfalfa Product Development

Mark McCaslin and Sharie Fitzpatrick

Forage Genetics International

Introduction and Statement of Purpose

Forage Genetics International (FGI) and Monsanto are jointly developing Roundup Ready alfalfa technology. In 1997 Montana State University scientists began conducting *Agrobacterium*-mediated transformation of an elite FGI alfalfa clone using Monsanto vectors (McCaslin and Fitzpatrick, 2000). Alfalfa has been genetically modified to contain the *cp4 epsps* gene. Production of the CP4 EPSPS enzyme in alfalfa plants confers resistance to glyphosate, the active ingredient in Roundup agricultural herbicides (Padgette et al., 1996). Commercial introduction of Roundup Ready alfalfa is expected to provide significant benefits to farmers by lowering the input cost for weed control. Roundup agricultural herbicides are very effective herbicides and have a favorable environmental profile; their use is expected to provide environmental benefits over current chemical herbicides used to control weeds in alfalfa. Roundup Ready alfalfa has been evaluated in controlled field tests in the United States since 1998.

After it is approved for environmental release and feed/food uses, the trait will be available to alfalfa producers in a broad range of genetic backgrounds adapted to all major markets where alfalfa is grown. The purpose of this document is to provide background information on the genetics and commercial breeding of alfalfa and to describe Roundup Ready alfalfa varieties and their development process. Technical terms used in this document are defined in the Appendix.

Introduction to the Genetics and Commercial Breeding of Alfalfa

Genetics and Pollination Characteristics of the Species

Alfalfa (*Medicago sativa* L.) is a perennial, autotetraploid plant with eight sets of chromosomes (x=8). In alfalfa there are four copies of each chromosome (2n=4x=32); gametes contain a pair of each chromosome (n=2x=16).

Alfalfa requires insect pollinators for cross-pollination. Alfalfa is exclusively pollinated by bees; a relatively small number of bee species effectively pollinate alfalfa flowers. Predominant species that are important for alfalfa seed production include leafcutter bees (*Megachile rotunda*), honeybees (*Apis mellifera*) and alkali bees (*Nomia melanderi*).

Most alfalfa plants exhibit various forms of genetic self-incompatibility or self-sterility and will not successfully self-pollinate (Viands et al., 1988). Alfalfa is adversely affected by inbreeding, i.e., self-fertilized plants commonly demonstrate a dramatic reduction in forage and seed yield potential (Rumbaugh et al., 1988). Inbreeding depression may be because of the loss of heterosis and/or accumulation and unmasking of deleterious recessive alleles that occur as a result of self-pollination and/or pollination among close relatives. Therefore, commercial alfalfa breeding programs are structured to avoid significant inbreeding and the resulting negative effects of inbreeding depression (Rumbaugh et al., 1988).

Alfalfa does not naturally hybridize with any other plant species found in North America (Quiros and Bauchan, 1988). No perennial *Medicago* species are present naturally in the Americas, Australia, New Zealand, or South Africa. The only exception in North

America is *M. lupulina*, a widely naturalized plant existing in both annual and perennial forms (Lesins and Lesins, 1979; Turkington and Cavers, 1979). Alfalfa-*lupulina* hybrids do not occur (Lesins and Gillies, 1972), therefore, no risk for alfalfa interspecific hybridization exists in North America, however natural cross-pollination to the scattered, feral populations of *M. sativa* would be possible.

Commercial Alfalfa Plant Breeding and Variety Development Overview

Alfalfa is one of the most important forage crops in the United States. Varieties are primarily bred for forage yield (vegetative production), forage quality, longevity and adaptation to a broad geographic area. A typical alfalfa variety may have ten to 200 parent plants that were initially crossed in isolation to form the breeder generation seed (Figure 1). The breeder seed of commercial alfalfa varieties is produced by the random intercrossing (open pollination) of all parent plants. An alfalfa variety is maintained through multiple seed generations beyond breeder seed via the open pollination of their progeny in isolation from other alfalfa varieties or pollen sources. Plant varieties bred in this way are called synthetic varieties (Rumbaugh et al., 1988).

Individual plants within a synthetic variety are genotypically and phenotypically heterogeneous, i.e., no two individuals within the variety are exactly alike. Synthetic alfalfa varieties are closed populations that segregate, within a defined range, for most morphological traits and naturally occurring genetic markers. Because alfalfa varieties are segregating heterogeneous populations, alfalfa varieties are routinely described in terms appropriate to populations (mean or % trait expression). For example, alfalfa variety registration agencies require that the pest resistance of a variety be described as the mean percent of plants that express the segregating trait when the population is tested under standardized conditions.

Commercial Seed Increase of Alfalfa Varieties

A typical commercial seed increase process is illustrated in Figure 1. Commercial seed of alfalfa varieties is commonly produced according to the following sequence: (1) A set of superior alfalfa plants (usually 10-200 genotypes, also known as Syn 0 parents) are identified by an alfalfa breeder for use as parent plants for a new variety. The Syn 0 parents are randomly intercrossed to produce the first synthetic generation of seed (Syn 1 seed); (2) Syn 2 generation seed is produced from a random, isolated intercross of Syn 1 plants; and, (3) Syn 3 seed is produced from a random, isolated intercross of Syn 2 plants. Breeder, Foundation and Certified seed classes are defined at the discretion of the plant breeder during the variety registration process and are typically Syn 1, Syn 2 and Syn 3 generations, respectively. Most commercial seed varieties sold to alfalfa forage producers are Certified Seed, although noncertified seed (seed produced without official oversight) is also sold.

Commercial Variety Testing and Registration

Alfalfa breeders typically use Syn 1 and Syn 2 generation seed to establish variety testing and evaluation experiments. The U.S. alfalfa variety registration and review process is based on data from tests initiated with Syn 1 generation seed although other Syn generation test data is also recognized.

Roundup Ready Alfalfa: Product Concept and Product Development Strategies

The Roundup Ready Alfalfa Variety Product Concept

Commercially available Roundup Ready alfalfa varieties will have three requirements:

(1) high Roundup Ready trait purity: $\geq$ 90% of plants in the variety must be tolerant to Roundup agricultural herbicides.

(2) each Roundup Ready alfalfa variety will be broadly adapted within the region(s) of its intended use; and,

(3) forage yield potential must meet or exceed FGI-Monsanto standards.

Achieving the Product Concept: Conventional Breeding Strategies

High trait purity is a requirement for Roundup Ready alfalfa varieties. To increase trait purity, conventional breeding methods may be used to increase or concentrate the number of copies of a gene at the genetic locus. Gene complement terminology in diploid and tetraploid species is illustrated in Figures 2 and 3.

While alfalfa is a tetraploid, breeding processes used for a diploid species will be reviewed to provide examples of strategies used to achieve high trait purity. In a diploid species for the transgenic locus A, it is relatively easy, with selfing and selection, to develop lines that are homozygous (AA) at the transgenic locus from a hemizygous (Ax) plant (e.g., original T_0 transgenic or a backcrossed breeding line). Diploid homozygous (AA) lines can be used as parents in the production of F_1 hybrids or as breeding lines for the development of self-pollinated varieties. In both of these diploid breeding systems, 100% of the plants in the resulting varieties will have the desired transgenic dominant phenotype with Ax or AA genotypes (Figure 2).

Because alfalfa is an autotetraploid and does not self, an alternate strategy is needed to achieve high trait purity and minimize inbreeding depression. Theoretically, high trait purity in an autotetraploid may be achieved by repeated phenotypic selection (i.e., spaying with a Roundup agricultural herbicide to select plants that carry the *cp4 epsps* gene) within a closed population, thereby saturating a single locus (A) and the trait will be fixed, i.e., will not segregate. Very high trait purity (>95%) in a tetraploid synthetic variety requires that the parent plants are nearly homozygous at the single transgenic locus (all parents must be AAAA [quadriplex] or AAAx [triplex] so that the trait will segregate very little in the subsequent generations). Selection of parental plants would require multiple cycles of phenotypic selection coupled with one or more generations of progeny testing to identify triplex and/or quadriplex individuals in a closed population.

Although high trait purity could be accomplished using the single-event breeding strategy, the method has several significant disadvantages and risks, which make it impractical for breeding commercial alfalfa varieties, including the following.

- Inbreeding and inbreeding depression (poor vigor and low seed yield) would be expressed in the selected triplex and quadriplex plants and in the varieties that they would produce. Multiple generations of recurrent selection in a closed population often results in the intercrossing of individuals that are closely related to one another, thus inbreeding depression is likely.

- Relatively few potential parents would be identified because the triplex and quadriplex genotypes occur in low frequency compared to simplex and duplex genotypes. Further confounding this problem would be that the initial number of plants available for the progeny testing process may be limited by very poor seed production on inbred plants and/or from crosses between closely related individuals.
- Genetic drift is another likely, undesirable outcome associated with multiple cycles of selection within closed populations. The agronomic characteristics of small breeding subpopulations often differ from the original source population because of chance or random sampling errors that may be repeated through multiple generations.
- The protracted process of identifying a relatively small number of parent plants would take an alfalfa breeding team several years to accomplish. Each generation of crossing, progeny testing or reselection would take approximately six to twelve months and multiple generations of selection, combined with progeny testing, would be required.

The amount of time, resources and labor required for vigorous population development would significantly limit the type and number of germplasm backgrounds used for breeding and would result in few Roundup Ready alfalfa varieties being commercialized. Thus, the single-event conventional breeding strategy discussed above would be difficult to use to achieve the Roundup Ready alfalfa product concept objectives of multiple varieties with very high trait purity, high yield and broad adaptation.

Overview and Key Features of the FGI Method

Because of significant product performance risk factors associated with the previously-described single-event breeding methods, FGI has developed an alternative conventional breeding strategy that achieves the product concept trait purity objectives without the product performance risks and associated resource requirements discussed earlier. An example of the method is shown in Figure 4 and is described below.

High trait purity without the aforementioned challenges has been accomplished using a FGI-proprietary conventional breeding method (patent pending)[1]. The FGI method (the two-event breeding strategy) identifies nonrelated plants that contain one or more copies of the same Roundup Ready gene (*cp4 epsps*) at each of two independent loci for use as Syn 0 parents. These two-event genotypes are called dihomogenic plants (Samac and Temple, in press). The intercrossing of dihomogenic plants results in populations with $\geq$90% trait purity in the commercial generation seed product. The FGI method is very similar to the traditional breeding process that has been used commercially to combine or stack two different transgenic traits in diploids, e.g., herbicide tolerance combined with insect resistance in maize. The difference is that the two transgenic events to be combined code for the same protein rather than two different proteins.

[1]FGI has filed a U. S. patent application (US-2002-0042928-A1) relating to a novel conventional method of breeding alfalfa with high transgene trait transmission in the commercial product: "Methods for Maximizing Expression of Transgenic Traits in Autopolyploid Plants."

In the FGI two-event breeding strategy, one copy of the *cp4 epsps* gene is required at each of two different, independently segregating loci, in contrast to the single-event breeding method which requires multiple copies of *cp4 epsps* at a single locus. The two independent gene loci are products of two separate *cp4 epsps* gene insertion events (e.g., *cp4 epsps* at locus A and *cp4 epsps* at locus B). The FGI breeding method identifies plants with one or more copies of the gene at each of two loci. Plants containing Event A and Event B can be rapidly identified using an event-specific polymerase chain reaction (ES-PCR) laboratory technique (Figure 5). The two independent events are subsequently combined via traditional F_1 crossing between two nonrelated plants that each contain one of the independent events (Figure 4).

Selection and Breeding of Roundup Ready Alfalfa Parent Plants

Alfalfa breeders have traditionally used a multiple year, field-based selection program to identify elite plants with high forage yield potential, excellent longevity and high forage quality. In the Roundup Ready alfalfa breeding program, numerous field selected, elite conventional (nontolerant) plants will be crossed to Roundup Ready alfalfa breeding lines containing one of the events. Seed will be harvested from the only the conventional plants and the filial population will segregate 1 (Axxx):1 (xxxx) for the Roundup Ready alfalfa phenotype. An application of Roundup agricultural herbicide will be used to eliminate nulliplex plants that do not contain a copy of the *cp4 epsps* gene. This selection is based on phenotype alone, and is called phenotypic selection. Multiple cycles of phenotypic selection and intercrossing of desired plants is termed phenotypic recurrent selection (PRS). The notation PRS*x* denotes *x* cycles of phenotypic selection. Multiple PRS cycles can be used to increase the gene frequency within numerous, broad-based Roundup Ready alfalfa breeding lines that contain a single event. Subsequently, alfalfa breeders may use field selection to identify Roundup Ready alfalfa plants with superior agronomic characteristics for use as experimental Roundup Ready breeding-line parents.

Next, elite Roundup Ready alfalfa plants containing a single event (A) will be crossed with nonrelated, elite Roundup Ready alfalfa plants containing a second, independent event (B). The progeny from this cross will segregate approximately as follows: ¼ will be nulliplex and susceptible to glyphosate; ¼ will carry only the gene at the A locus and are tolerant to glyphosate; ¼ will carry only the gene at the B locus and are tolerant to glyphosate, and ¼ will carry the gene at both the A and B loci (dihomogenic) and are tolerant to glyphosate. Dihomogenic segregants are identified using event-specific PCR markers (Figure 5). This type of selection is based on the genotype of the selected plant (i.e., the dihomogenic genotype) and is called genotypic selection. The process of repeating cycles of genotypic selection is called genotypic recurrent selection (GRS). A combination of single or multiple cycles of PRS and GRS is used to generate Syn 0 plants that, when intercrossed, produce a Syn 1 population that contains two events, has very high Roundup Ready trait purity, and expresses little to no inbreeding depression or genetic drift.

A detailed example of the FGI two-event breeding strategy is depicted in Figure 4. Population A is hemizygous simplex (Axxx) for Roundup Ready Event A. One cycle of phenotypic selection for glyphosate tolerance is used to increase gene frequency in the

population (Pop A PRS_1). Similarly, Population B is hemizygous simplex (Byyy) for Roundup Ready Event B. One cycle of phenotypic selection for glyphosate tolerance is used to increase gene frequency in the population (Pop B PRS_1). Individuals from Pop A PRS_1 are crossed to individuals from Pop B PRS_1. The progeny resulting from the A x B cross are sprayed with a Roundup agricultural herbicide to eliminate the null segregants. The tolerant plants are genotypically analyzed with Event-A and Event-B specific PCR assays to identify the segregants that contain at least one copy of Event A *and* at least one copy of Event B (i.e., the AB dihomogenic genotypes). The dihomogenic plants (Pop AB PRS_1) are grouped into Syn 0 parent plants sets (each set contains approximately 10-200 plants). The Syn 0 parent plants are intercrossed to form a PRS_1GRS_1 Syn 1 Roundup Ready alfalfa population, which serves as breeder seed for an experimental Roundup Ready alfalfa variety. The subsequent synthetic generations will segregate for the *cp4 epsps* genotype (Table 1 and Figures 6 and 7).

Similar Syn 1 populations have been derived by two consecutive cycles of genotypic recurrent selection (i.e., GRS_2). Compared to PRS_1GRS_1, the GRS_2 selection regime results in a small increase in trait purity in the Syn 1, 2 and 3 generations. Commercial Roundup Ready alfalfa varieties are being developed using these strategies as the selection schemes result in Roundup Ready varieties with high trait purity (Samac and Temple, in press).

The currently available, Roundup Ready event-specific PCR technique does not distinguish among genotypes with various gene copies of the *cp4 epsps* gene at a single locus of the Roundup Ready gene {e.g., simplex [Axxx], duplex [AAxx], triplex [AAAx] and/or quadriplex [AAAA] cannot be differentiated). All these genotypes are qualitatively identified by the technique as A---, where the genetic condition at that locus on the other three copies of the same chromosome is unknown. This limitation is not critical for the FGI breeding method because the commercial product will meet the trait purity standard threshold. The full genotype at the locus could be estimated based on breeding line pedigrees and Mendelian segregation predictions, or accurately deduced using progeny tests, but is not necessary for product development.

The FGI breeding method successfully and reliably produces high trait purity in the Syn 1, Syn 2 and Syn 3 generations; however, Syn 0 parent plants must have at least a single copy of the *cp4 epsps* gene at *both* of the two loci. Additional gene copy of either Event A or Event B on the second, third and/or fourth copies of the same chromosomes in the Syn 0 parent plants will slightly enhance trait purity in subsequent synthetic generations. The same Roundup Ready phenotype is accomplished whether one or more than one copy of the *cp4 epsps* gene is in the plant.

Production of Roundup Ready Synthetic Generation Alfalfa Seed

The application of this breeding strategy for the production of Roundup Ready alfalfa will be accomplished as follows: genotypically selected, dihomogenic Syn 0 parent stock plants will be intercrossed to produce the Syn 1 seed generation needed for experimental line testing and variety advancement to commercial production. The Roundup Ready Syn 1 and Roundup Ready Syn 2 alfalfa seed generations will be planted in successive seed increase field plots, and the fields will be sprayed with Roundup agricultural

herbicide before bloom to control weeds and remove segregating plants that did not inherit a copy of the *cp4 epsps* gene. Mendelian segregation ratios have been used to predict the trait purity in the Syn 1, Syn 2 and Syn 3 generations as 95.3%, 94.5%[2] and 95.3%, respectively, for the PRS_1GRS_1 selection strategy, and 97.0%, 97.3% and 97.5% for the Syn 1, Syn 2 and Syn 3 generations in the GRS_2 selection strategy, respectively.

Genetic Makeup of Individual Plants in a Variety

Theoretically, the number of *cp4 epsps* copies contributed from Event A, Event B or a combination of Event A and Event B individual plants within a Roundup Ready alfalfa population could range from zero to eight; in total, 25 different genetic classes are possible (Table 1). The expected genotypic frequencies of the three synthetic generations resulting from the FGI breeding method for Roundup Ready alfalfa can be calculated. For example, Figure 6 details the predicted frequency of Roundup Ready gene classes for the Syn 1, Syn 2 and Syn 3 generations using the PRS_1GRS_1 breeding strategy (Figure 4). Using this information, the mean number of *cp4 epsps* copies/plant can be estimated for each population. The mean number of gene copies/plant in the Syn 1, Syn 2 and Syn 3 generations are 2.28, 2.39 and 2.51, respectively. The mean increases slightly as the number of synthetic cycles increases. This is because of the phenotypic selection for glyphosate tolerance of the generation's parent plants prior to each intercross (i.e., the progressive elimination of recessive, null, x and y alleles via elimination of the tolerant plants from the population prior to bloom in the Syn 2 and Syn 3 seed production fields). If similar high trait purity were fixed using the single-event breeding method (see page 3), the number of *cp4 epsps* copies in an autotetraploid would be close to three to four because of the need for near saturation at the locus. Therefore, the FGI two-event breeding method will result in a lower number of *cp4 epsps* copies in Roundup Ready alfalfa varieties (Figure 7) than a single event breeding strategy.

Trait Stability Throughout the Breeding Process

Trait stability data were evaluated through analysis of inheritance data through eight generations. Segregation data were analyzed from single and paired event populations to determine whether the trait segregated in the expected Mendelian pattern for a single gene. Data were analyzed leading up to the Syn 1 dihomogenic population.

Segregation data for single events

Inheritance of the Roundup Ready insert was determined through modified backcross (MBCn) generations where the Roundup Ready gene was introgressed into elite commercial alfalfa germplasm. The J101 and J163 T_0 plants were initially out-crossed to a small number of elite non-Roundup Ready fall-dormant clones. Roundup Ready progeny from the initial F1 cross were crossed to a second set of unrelated elite fall-dormant clones to produce the modified backcross 1 generation (MBC_1). Subsequent

[2] The small decline in Syn 2 trait purity is predicted (using autotetraploid Punnet Square calculations) because of the normal, unmasking of null segregants when the unfixed, *cp4 epsps* genes at two loci randomly and independently segregate and resort.

MBC_n generations (MBC_2-MBC_4) were produced by forward-crossing Roundup Ready progeny from the previous generation to elite conventional clones that represented a range of fall dormancy groups [fall-dormant (FD) and non-dormant (ND)]. For all the crossing cycles described above, the Roundup Ready parents were used as the pollen parents and non-Roundup Ready plants were used as seed (female) parents. Segregation data for five generations of plants hemizygous for the *cp4 epsps* gene (event J101 or J163) are presented in Table 2. The inheritance of the introduced DNA in the progenies from the MBC generations was monitored phenotypically at the whole plant level by application of Roundup herbicide at the two to three leaf stage in a greenhouse.

Statistical significance for the segregation data was determined using Chi square analysis. For these analyses a Chi square value (χ^2) was determined as follows: $\chi^2 = \sum [(|o-e|-0.5)^2/e]$, where o = observed frequencies for each class, e = expected frequencies for each class and 0.5 = Yates correction factor for Chi square analysis with one degree of freedom (df) (Little and Hills, 1978). The calculated Chi square value was compared to a table of Chi square values to determine whether the observed frequencies fit the expectation for a single insert at p = 0.05.

On the basis of data presented in Table 2, it is concluded that the T-DNA integrated into the alfalfa genome at a single locus in both events J101 and J163, thus supporting a one-locus Mendelian inheritance model for the gene. Data presented in Table 2 shows that the insert was stably maintained and normally inherited through five generations.

Chi square analysis showed that the majority of the values were not significantly different. However, four of the 14 Chi-square values (Table 2) indicate a significant difference between the observed and expected values with three of the four values below the predicted value and one above. Much of the variability can probably be attributed to differences in the rate of setting of self-seed observed with different alfalfa populations. As part of the Roundup Ready alfalfa breeding development program Forage Genetics has used the Roundup Ready trait to estimate the level of selfing in hand crosses of alfalfa (McCaslin and Temple, 2003). Research conducted by Forage Genetics indicates that under greenhouse conditions, when non-emasculated hand crosses are made by experienced researchers, up to 10% of the seed produced can be the result of selfing. Data presented in Table 2 were developed using Roundup Ready alfalfa pollen donors and conventional alfalfa plants as the females. Therefore, given the predicted level of selfing, a 45% inheritance ratio would be expected. In practice, Forage Genetics has observed a 43-47% inheritance ratio for the Roundup Ready trait in the on-going forward breeding program (see Table 2). The remaining statistically significant observation at 60% is clearly above the predicted value of 50%, however, the sample size used for this determination was based on a very small number of observations (only 133 test samples), thus random sampling error may have contributed to the unexpectedly high inheritance ratio for this population estimate.

In summary, data presented in Table 2 show that the Roundup Ready trait was stably maintained through five generations in alfalfa. On the basis of Chi square analyses of the inheritance data, it is concluded that the Roundup Ready trait in alfalfa plants containing J101 or J163 is each inherited in a one-locus Mendelian fashion.

Segregation data for dihomogenic populations

Dihomogenic Syn 1 populations were produced as described in Figure 4. One cycle of phenotypic recurrent selection (PRS) was used to create two separate PRS1 populations: one containing only event J101 and one containing only event J163. Next, F1 crosses were made between random plants of these two populations, each plant in the cross thus contained one or two copies of either Roundup Ready alfalfa transgenic event J101 or J163 (i.e. F1 cross J101 x J163). An F1 seed population was produced by bulking the seed from the individual crosses. Event-specific PCR markers were used to identify dihomogenic plants (i.e. F1 plants containing both independent events) in the segregating progeny of this F1 cross. The dihomogenic F1 plants were randomly intercrossed to produce Syn1 seed. Three thousand six hundred and sixty one Syn1 seeds were established in the greenhouse and evaluated phenotypically at the whole plant level by application of Roundup herbicide at the two to three leaf stage. Event-specific PCR was used to establish the genotype of the Roundup tolerant Syn1 plants (Table 3).

Statistical significance for the segregation data was determined using Chi square analysis. For these analyses a Chi square value (χ^2) was determined as follows: $\chi^2 = \sum [(|o-e|-0.5)^2/e]$, where o = observed frequencies for each class, e = expected frequencies for each class (Little and Hills, 1978). The calculated Chi square value was compared to a table of Chi square values to determine whether the observed frequencies fit the expectation for a two independent inserts at $p = 0.05$. The expected frequency was calculated using a model for Mendelian inheritance of two independent loci.

On the basis of data presented in Table 3, it is concluded that the population behaves as predicted for normal Mendelian inheritance of two independent loci, confirming the J101 and J163 transgenic events are not genetically linked (i.e., they are not on the same chromosome). Data in Table 3 support that J101 and J163 are inherited normally through at least eight generations as the syn 1 progeny evaluated were three sexual generations beyond the MBC4 (fifth generation) discussed above.

These inheritance data confirm that the genetic inserts associated with the Roundup Ready alfalfa transgenic events J101 and J163, are genetically stable over multiple sexual generations and remain stable when combined in dihomogenic populations.

Conclusion

The FGI breeding method for Roundup Ready alfalfa varieties outlined in this document has been used to produce Roundup Ready alfalfa populations with trait purity at the predicted levels. Observed values of the Roundup Ready trait meet expected values through the eight generations of crossing described herein. The genotypic and phenotypic data gathered by FGI and Monsanto verify that the *cp4 epsps* genes are genetically stable and phenotypically effective throughout the breeding process.

Literature Cited

Lesins, K. and Gillies, C.B. 1972. Taxonomy and cytogenetics of Medicago. Pages 53-86. In: C.H. Hanson (ed.) Alfalfa Science and Technology. Agronomy Monograph no. 15, ASA-CSSA-SSSA, Madison, WI. 812 pp.

Lesins, K.A. and Lesins, I. 1979. Genus Medicago (Leguminosae): A taxogenetic study. Kluwer, Dordrecht, The Netherlands.

Little and Hills. 1978. Analysis of counts. In Agricultural Experimentation, Design and Analysis. John Wiley and Sons, Inc., NY.

McCaslin, M. and Fitzpatrick, S. 2000. Roundup Ready alfalfa (abstract). In: Volume 9 of the American Forage and Grassland Council Proceedings and the 37th Report of the North American Alfalfa Improvement Conference Proceedings. Phillips, M. (ed.). pp. 396-400.

McCaslin, M. and Temple, S. 2003. Use of the Roundup Ready Trait to Estimate Selfing in Hand Crosses of Alfalfa. Presentation 2003 CAIC Meeting. http://www.naaic.org/Meetings/Central2003/CAIC2003VirtualMeeting.htm#GMO).

Padgette, S., Re, D., Barry, G., Eichholtz, D., Dellanay, X., Fuchs, R., Kishore, G., Fraley, R. 1996. New weed control opportunities: Development of soybean with a Roundup Ready™ gene. Pages 53-84. In: Herbicide Resistant Crops. Duke, S. (ed.) CRC Press.

Quiros, C.F. and Bauchan, G.B. 1988. The genus Medicago and the origin of the Medicago sativa complex. In A.A. Hanson, D.K. Barnes and R.R. Hill, Jr. (ed.) Alfalfa and alfalfa improvement. ASA-CSSA-SSSA, Madison, WI. pp. 93-124.

Rumbaugh, M.D., Caddel, J.L., Rowe, D.E. 1988. Breeding and quantitative genetics. In: Hanson, A.A. (ed.) Alfalfa and Alfalfa Improvement. Agronomy 29: 777-805.

Samac, D. A. and Temple, S. J. (In press.) Development and Utilization of Transformation in Medicago species. In: Genetically Modified Crops: Their Development, Uses, and Risks. Liang, G. H. and Skinner, D. Z. (ed.). Haworth Press, Inc.

Turkington, R. and Cavers, P.B. 1979. The biology of Canadian weeds. Medicago lupulina L. Can. J. Plant Sci. 59:99-110.

Viands, D. R., Sun, P. and Barnes, D. K. 1988. Pollination control: mechanical and sterility. In: Hanson, A.A. (ed.) Alfalfa and Alfalfa Improvement. Agronomy 29: 931-960.

Figure 1. Commercial alfalfa synthetic variety breeding schematic.

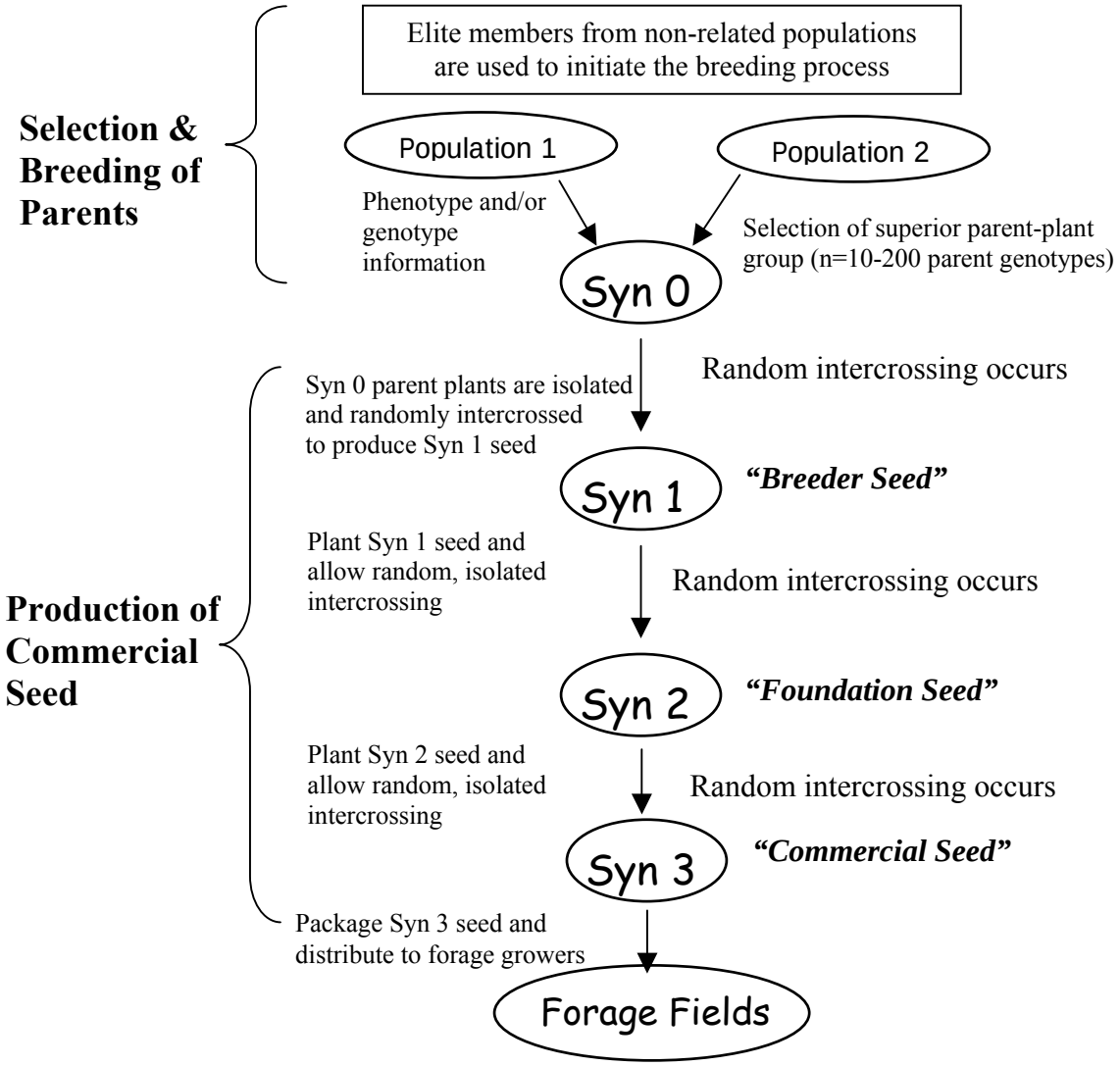
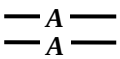


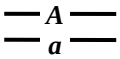
Figure 2. Comparison of terminology for nontransgenic (native) and transgenic genotypes at a single locus for diploids.

Diploid plant species have a pair of two similar or homologous chromosomes in vegetative cells (2n=2x); gametes are haploid (n=x). Maize and soybeans are examples of diploid species. Frequency of genotypes may not be equal and depends upon the pedigree of the population.

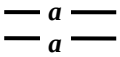
1) Hypothetical native gene with dominant (***A***) and recessive (***a***) alleles on homologous chromosome pairs:



Homozygous duplex for dominant allele (AA)

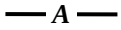


Heterozygous simplex for dominant allele (Aa)

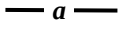


Nulliplex for dominant allele (aa)

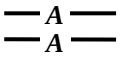
Gametes possible- ratio depends upon source population (haploid, n=x):



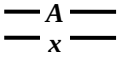
or



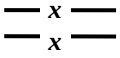
2) Hypothetical dominant transgene (***A***) and null condition (***x***) on homologous chromosome pairs:



Homozygous duplex for dominant transgene (AA)



Hemizygous simplex for dominant transgene (Ax)
(e.g., initial transformant T_0 genotype)



Nulliplex, without transgene (xx)

Gametes possible- ratio depends upon source population (haploid, n=x):

or

Figure 3. Terminology for transgenic genotypes at a single locus in tetraploids.

Tetraploid plant species have a set of four similar or homologous chromosomes (4x) in vegetative cells (2n); gametes are diploid (n=2x). Alfalfa is an example of an autotetraploid species in which the four chromosome copies randomly segregate in pairs during meiosis. Frequency of genotypes may not be equal and depends upon the pedigree of the population.

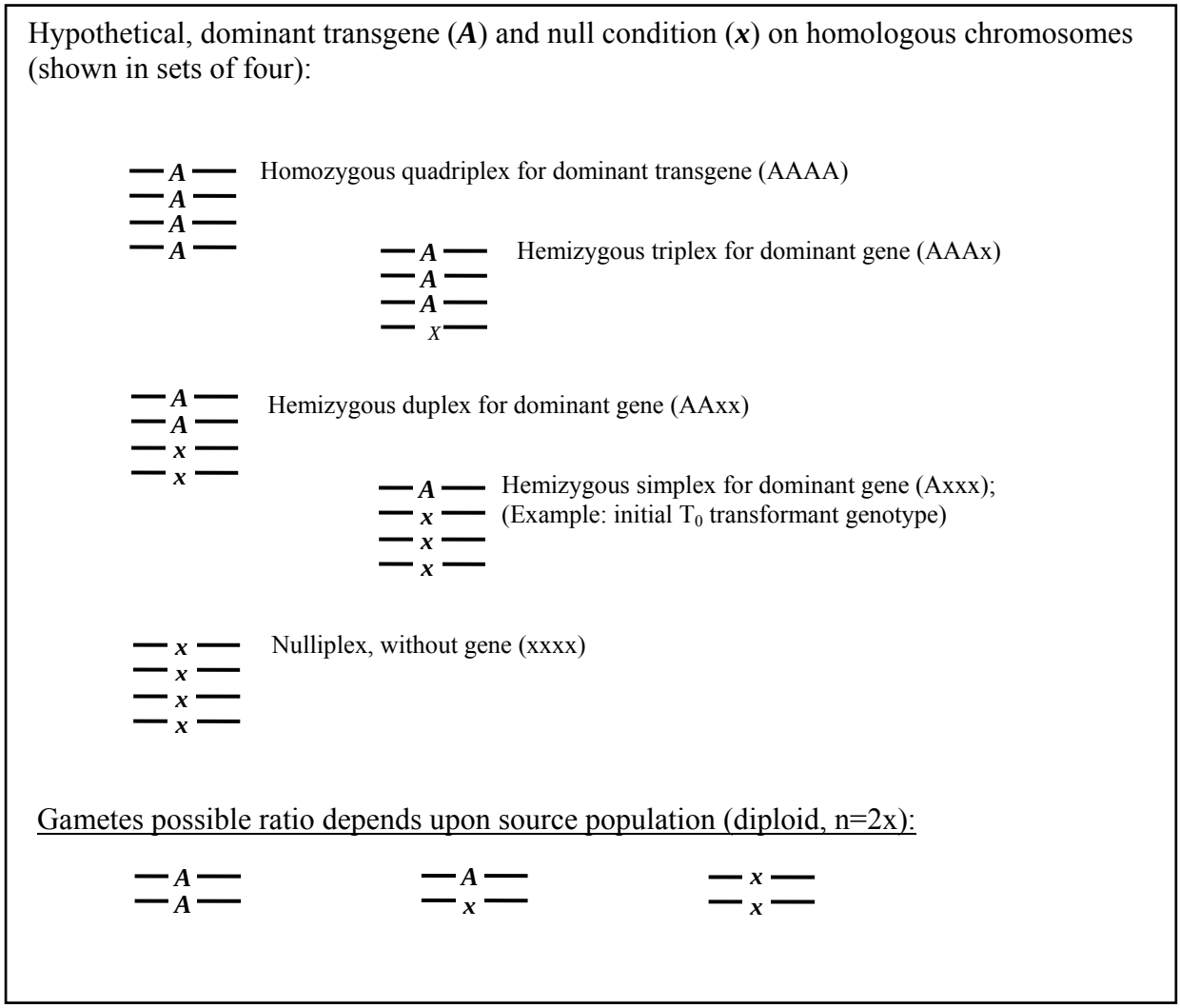


Figure 4. FGI Roundup Ready alfalfa synthetic variety breeding schematic.

Example is for varieties developed using the PRS1GRS1 selection method.

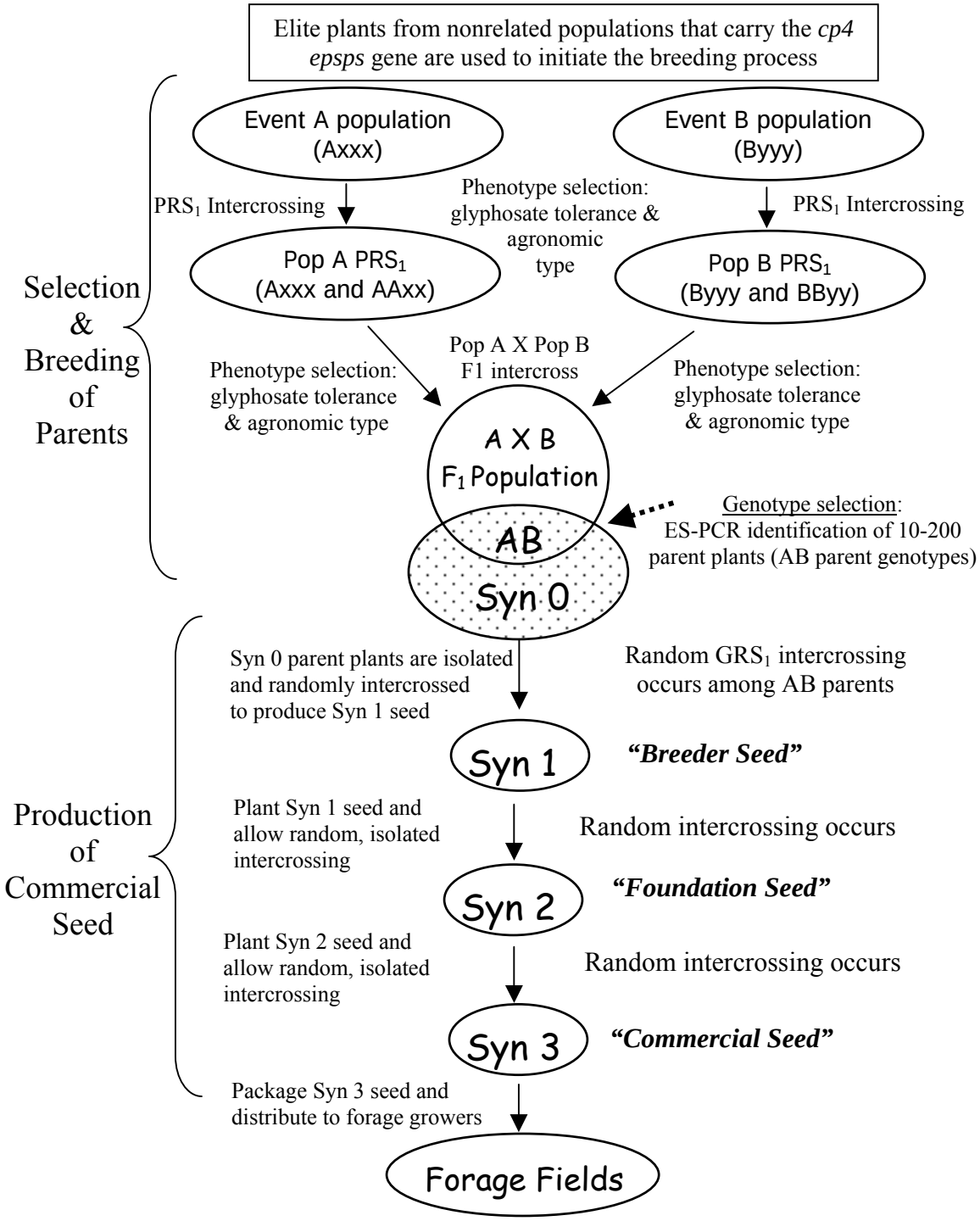


Figure 5. Illustration of dihomogenic plant selection technique.
The event specific-polymerase chain reaction (ES-PCR) laboratory technique is used to identify dihomogenic (AB) plants from a segregating population of superior Roundup Ready alfalfa candidate plants. The candidate plants are progeny from various Event A x Event B crosses that are not closely related by pedigree. All plants tested in the example except the negative control (Neg. Control) have the Roundup Ready phenotype. Only the AB plants such as plants 1, 5 and 9 are selected for use as Roundup Ready variety parent stock plants because they have two independently segregating copies of *cp4 epsps*. Starting with AB parents, high Roundup Ready trait purity (>90%) will be maintained through all successive synthetic generations.

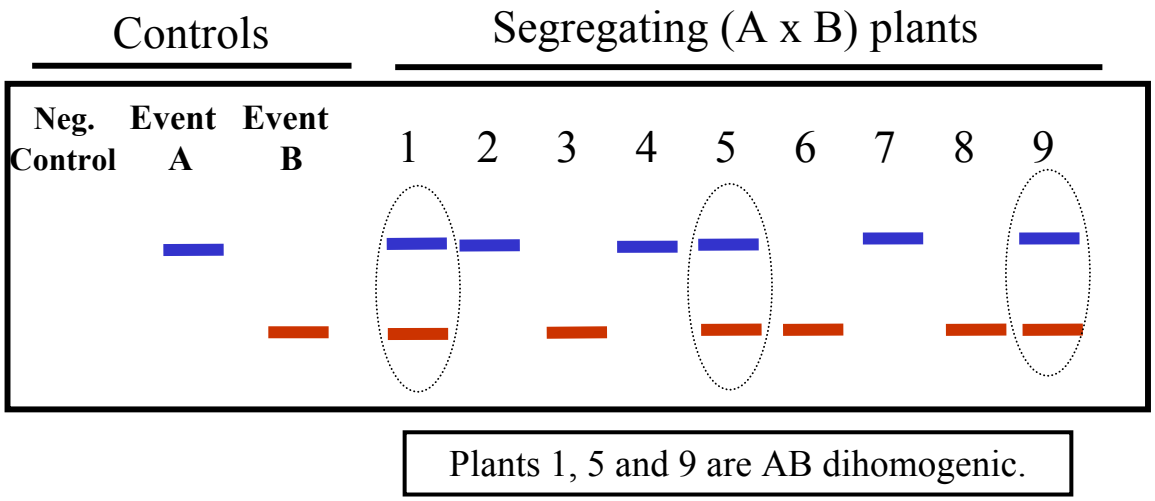


Figure 6. Changes in genotypic frequency with advancing synthetic generations. The model presented assumes parents of the Syn 1 were selected using one cycle each of phenotypic and genotypic recurrent selection (PRS_1GRS_1). Reciprocal genotypes for the each of the two events (A and B) are summed into a single bar, e.g., 1 copy of A and 3 copies of B (1,3) and 3 copies of A and 1 copy of B (3,1) occur in 1:1 ratio and are summed and shown in bar 3, 1. Genotypic frequencies do not shift significantly through successive synthetic generations (see Table 1 for Syn3 data).

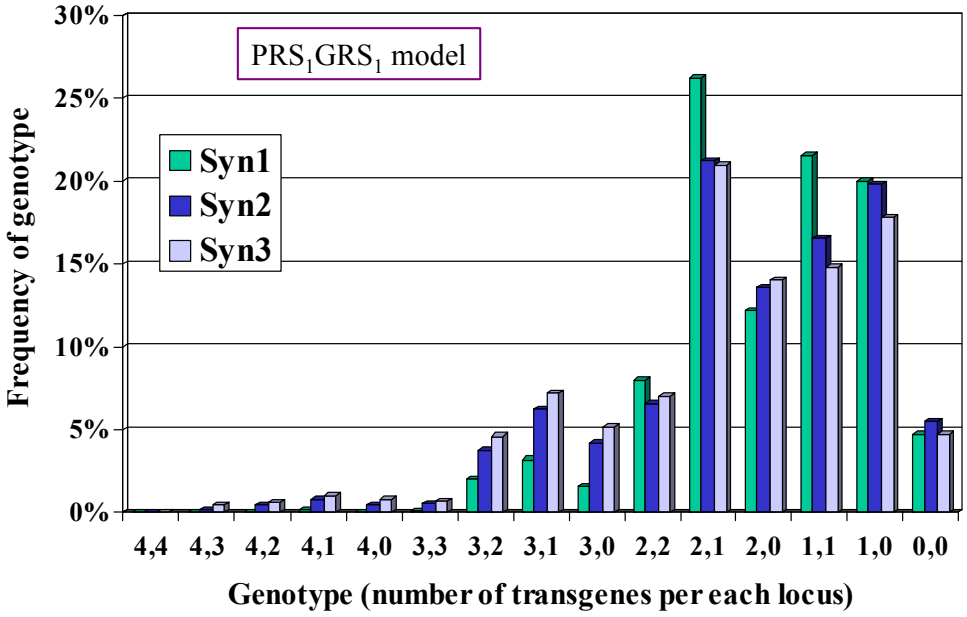


Figure 7. Percent of population and number of *cp4 epsps* copies per plant in a Roundup Ready alfalfa variety developed using the FGI breeding method. Example is for segregation in PRS_1 GRS_1 selected varieties (Syn 3 generation, percent in class after glyphosate application). Mean for Syn 3 population is 2.51 *cp4 epsps* copies/plant and 92.73% of the population has four or fewer copies (see Table 1 for data). In contrast, nearly 100% of plants would have four copies (AAAA) using the single-event breeding strategy to develop Roundup Ready alfalfa varieties. Therefore, the FGI two-event breeding method results in fewer gene copies in a population than a single-event breeding strategy of similar trait purity.

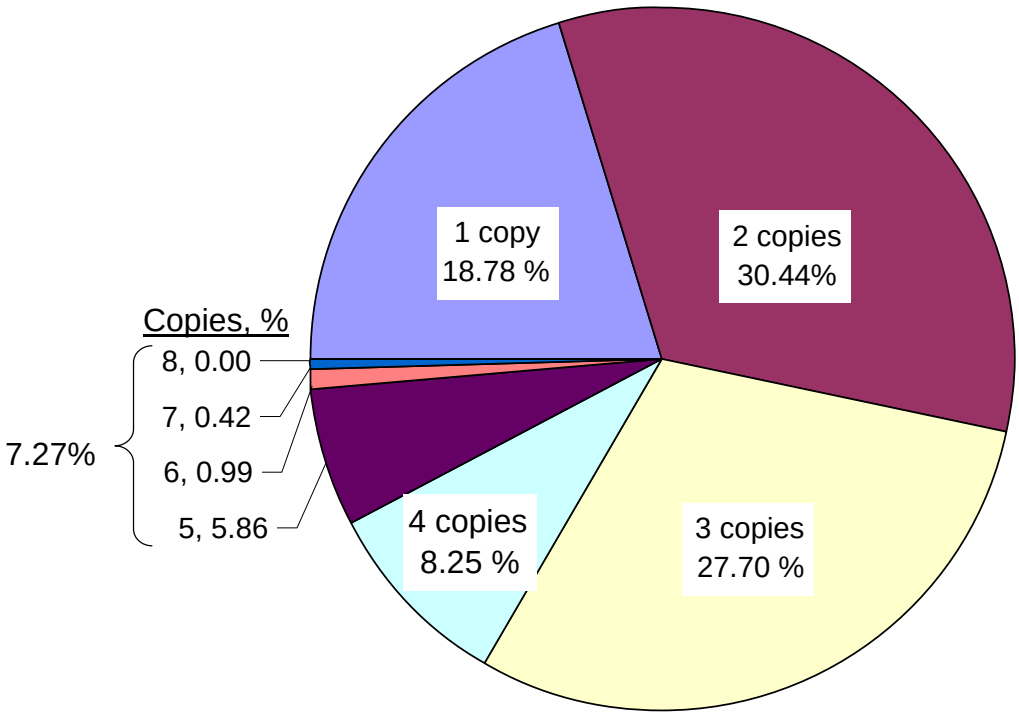


Table 1. Expected frequency of *cp4 epsps* genotype classes in Roundup Ready alfalfa varieties developed using the FGI two-event breeding method.
Genotype is given, followed by the total number of *cp4 epsps* copies per plant (number in parentheses). Example is for segregation in PRS_1 GRS_1 selected varieties (Syn 3 generation): percent in class before glyphosate application to the population and percent in class after glyphosate application, i.e., adjusted for loss of null nontolerant class [post application % in brackets]. Alfalfa synthetic varieties typically segregate for most or all traits and genotypic markers.

	Locus B genetic states (Event B)				
Locus A genetic states (Event A)	BBBB	BBBy	BByy	Byyy	yyyy
AAAA	BBBB AAAA (8)[1] 0.00%[2] [0.00%][3]	BBBy AAAA (7) 0.20% [0.21%]	BByy AAAA (6) 0.30% [0.31%]	Byyy AAAA (5) 0.50% [0.52%]	yyyy AAAA (4) 0.40% [0.42%]
AAAx	BBBB AAAx (7) 0.20% [0.21%]	BBBy AAAx (6) 0.35% [0.37%]	BByy AAAx (5) 2.30% [2.41%]	Byyy AAAx (4) 3.60% [3.78%]	yyyy AAAx (3) 2.70% [2.83%]
AAxx	BBBB AAxx (6) 0.30% [0.31%]	BBBy AAxx (5) 2.30% [2.41%]	BByy AAxx (4) 7.05% [7.41%]	Byyy AAxx (3) 10.50% [11.02%]	yyyy AAxx (2) 7.10% [7.45%]
Ayyy	BBBB Axxx (5) 0.50% [0.52%]	BBBy Axxx (4) 3.60% [3.78%]	BByy Axxx (3) 10.50% [11.02%]	Byyy Axxx (2) 14.80% [15.54%]	yyyy Axxx (1) 8.95% [9.39%]
xxxx	BBBB xxxx (4) 0.40% [0.42%]	BBBy xxxx (3) 2.70% [2.83%]	BByy xxxx (2) 7.10% [7.45%]	Byyy xxxx (1) 8.95% [9.39%]	yyyy[4] xxxx (0) 4.70% [0.00%]

[1]Number of total copies of the *cp4 epsps* gene in parenthesis
[2]Percent of population in class prior to glyphosate application
[3]Percent of population in class after glyphosate application; % adjusted for removal of nontolerant null plants.
[4]Plants with this genetic class do not inherit a copy of the *cp4 epsps* gene and will not survive treatment with glyphosate. It is predicted that approximately 5% of the plants within a variety will have this genetic state (Samac and Temple, in press).

Table 2. Phenotypic Segregation Data for Roundup Ready Alfalfa Events J101 and J163.

Dormancy Group[1]	Event(s)	Generation [2]	Number Tested	Number Roundup Tolerant	Roundup Ready % Tolerant	Roundup Ready % Expected	Chi-Square Value (χ^2)	Significance of χ^2
ALL	J101	F1	133	80	60.00	50.00	5.083	*
ALL	J163	F1	131	65	49.60	50.00	0.000	NS
ALL	J101	MBC1	405	170	42.00	50.00	10.114	*
ALL	J163	MBC1	404	194	48.00	50.00	0.557	NS
FD	J101	MBC2	565	259	45.84	50.00	3.745	NS
FD	J163	MBC2	578	276	47.75	50.00	1.081	NS
ND	J101	MBC2	201	101	50.25	50.00	0.000	NS
ND	J163	MBC2	180	80	44.44	50.00	2.006	NS
ALL	J101	MBC2	766	360	47.00	50.00	2.644	NS
ALL	J163	MBC2	758	356	46.97	50.00	2.672	NS
FD	J101	MBC3	1523	663	43.53	50.00	25.224	*
FD	J163	MBC3	1543	689	44.65	50.00	17.731	*
FD	J101	MBC4	155	80	51.61	50.00	0.103	NS
FD	J163	MBC4	172	82	47.67	50.00	0.285	NS

* = Significantly different ($p < 0.05$); NS = Not Significant

[1] Fall dormancy reaction of base germplasm into which the events were forward crossed (FD=fall dormant, ND=non dormant and ALL= FD and ND combined overall mean for the generation.

[2] Generations of sexual crossing beyond initial transformant (T0), where, F1 is the first generation and the modified backcross 4 (MBC4) is the fifth generation.

Table 3. Genotypic Segregation Data for a Dihomogenic (J101XJ163) Roundup Ready Alfalfa Syn1 Population[a].

Genotype[b]	Actual	Predicted	Chi Square Value (χ^2)	Significance of χ^2
Null	170	170	0.00	NS[c]
J101 only	659	632	1.17	NS
J163 only	641	632	0.13	NS
J101+J163	2191	2227	0.60	NS
Total	3661			

[a]Actual segregation data for a Syn 1 population resulting from the PRS1GRS1 two-event breeding strategy. Eight generations of crossing have occurred beyond the T_0 (three generations followed the MBC4 generation described in Table 2). Refer to Figure 4 for schematic representation of the breeding method.

[b]Null progeny identified in phenotypic assay, Roundup Ready progeny genotype determined by event-specific PCR

[c]NS = Not Significant ($p<0.05$)

Appendix Terminology and Examples of Use

Autotetraploid: An organism with four basic sets (x) of chromosomes (i.e., 4x). The four chromosomes of a set are assumed to segregate randomly in meiosis producing n=2x gametes.

Backcross (BC): A cross of a hybrid to either one of its parents

Breeder seed/Breeder generation: Seed produced by a variety's sponsor and used to produce foundation seed. For alfalfa varieties, breeder seed is typically Syn 1 generation. The breeder of the variety must define the Syn generation(s) allowed as breeder seed when the alfalfa variety is registered.

Certified Seed: Seed of a registered, recognized variety that has been produced under conditions meeting the minimum requirements of the certifying agency. Examples of requirements to produce Certified Class Seed: seed field must meet field isolation criteria; field cropping history requirement; and, seed stock must meet requirements.

Commercial generation seed: Seed produced by a variety's sponsor from foundation generation plants and sold to the end users. For alfalfa, commercial variety seed is typically certified and of the Syn 3 generation. The breeder of the variety must define the Syn generation(s) allowed as commercial seed when the alfalfa variety is registered.

Dihomogenic: Plants that carry at least one copy of each of two independent, transgenic events. Dihomogenic plants are produced from a cross between parents with independent transgenic events. These parents trace to T_0 plants or progeny that are simplex for the gene, or plants/populations derived there from. Example: Dihomogenic plants with a single allele at each of the two loci are classified as 1,1-dihomogenic (i.e., AxxxByyy); plants duplex for the gene at one locus and triplex at another are 2, 3- dihomogenic (AAxxBBBy), etc.

Diploid: An organism with two chromosomes of each kind (x). Gametes produced in meiosis are normally haploid, where n=x and G=2n.

Event: A unique integration of the gene sequence into the host plant's genomic DNA. The initial transformant is designated as the T_0 plant.

Foundation seed/Foundation generation: Seed produced by a variety's sponsor and may be used to produce Certified class seed. For alfalfa varieties, Foundation Seed is typically Syn 2 generation. The breeder of the variety must define the Syn generation(s) allowed as Foundation Seed when the alfalfa variety is registered.

Genetic drift: The unpredictable changes in gene and genotypic frequencies that may occur in small populations because of random processes.

Genotypic recurrent selection (GRS): A method of breeding designed to concentrate desired genes through the method of repeated selection that is based on the genotype of each generation's parent plants.

Genotypic selection: Selection of an individual based solely on the heritable elements (genes) it contains.

Germplasm backgrounds: The various subpopulations within a species that, as a group, demonstrate distinct, inherited physical attributes. Examples: alfalfa has various genetic subgroups that respond differentially to photoperiod (fall-dormant vs. non dormant backgrounds) or have unique crown attributes (creeping-rooted types that are well suited to extreme winters or pasture grazing systems).

Hemizygous: The genetic condition that occurs at a locus when a dominant gene (A) is integrated into DNA of a chromosome (Ax in a diploid or, Axxx in a tetraploid) and no recessive allele (a) exists at that genetic position in nontransformed plants. The genetic position in the nontransformed members of the chromosome pair or set is represented by x.

Inbreeding depression: The dramatic reduction in plant vigor observed in inbred plants of certain species. Inbreeding depression may result from the unmasking of deleterious recessive alleles when closely related plants are intercrossed or self-pollination occurs.

Locus (Loci): The position occupied by a gene in a chromosome.

Modified backcross (MBC): a cross of a hybrid plant to a plant from either one of its parent's source populations. MBC crossing schemes may be used to avoid the interbreeding of close relatives (i.e., avoid inbreeding depression in progeny) and to forward-breed hybrid plants to new elite parents for population improvement. For example, an F1 cross is made between a Plant 1 (a Roundup Ready plant, pollen source, male) and an unrelated plant, Plant 2 (elite conventional genotype, seed-parent, female). Next, the MBC generation 1 (MBC1) cross is made between an F1 hybrid plant, Plant 3 (pollen parent) and Plant 4 (a plant that is phenotypically or genotypically similar to, but not identical to Plant 2). In this way, vigorous, non-inbred, MBC1 seed/progeny are produced in alfalfa without inbreeding effects or the interbreeding closely-related plants.

MBCx: a specified modified backcross generation (x) to which a trait (gene) has been bred, where x is the number of generations past the F1 hybrid generation.**Allele:** One of a pair or series of forms of a gene which are alternative in inheritance because they are situated at the same locus in homologous chromosomes.

Open pollination: Random pollination among plants within a closed (isolated) population of plants. In alfalfa, open pollination is accomplished by insect pollinators.

Outcross: A cross to a plant of a different genotype within the same species. In alfalfa, outcrossing may be accomplished by hand or insect pollination.

Phenotypic selection: Selection of an individual based solely on physical attributes or morphological traits.

Phenotypic recurrent selection (PRS): A method of breeding which concentrates the genes through repeated selection for the desired phenotype (i.e., physical appearance is used to select parent plants for the next generation of seed). Example: in the Roundup Ready alfalfa breeding method, seedlings are sprayed with a Roundup agricultural herbicide. Plants are identified by physical appearance as tolerant or nontolerant. Tolerant survivors are used as Roundup Ready progenitors of a Roundup Ready alfalfa synthetic variety or line. In the segregating, synthetic generations, the same Roundup Ready phenotype is achieved whether the plants have only one, or greater than one, segregating Roundup Ready genes in the plant.

Progeny testing: A cross of the unknown genotype (e.g., AAxx) with a known tester plant genotype, (e.g., xxxx). The genotype of the unknown parent is deduced by the observed segregation ratio of the progeny resulting from the cross.

Recurrent selection: The use of repeated or sequential parent plant selection methods to produce gene frequency changes in subsequent generations.

Roundup Ready event-specific polymerase chain reaction (ES-PCR): Laboratory technique that can amplify specific DNA regions that trace to unique gene insertion events. Using ES-PCR reactions, where one PCR primer anneals to the gene and the other to a unique alfalfa DNA sequence adjacent to the gene insertion position, DNA tracing to each T_0 Roundup Ready event can be readily distinguished from other DNA from all other events.

Roundup Ready trait purity: See Trait purity.

Synthetic variety/ Synthetic variety: A variety (or cultivar) produced by crossing *inter se* a set of selected genotypes with subsequent maintenance of the variety by open pollination (natural hybridization) in an isolated crossing block. The successive generations of the variety produced by open pollination are termed Syn 1, Syn 2, …and Syn X. Syn 0 plants are the set of Syn 1 progenitor genotypes.

Synthetic Generation (Syn): See Synthetic variety.

Trait purity: The percent of plants in a population that express a desired phenotype for a genetic trait. Roundup Ready trait purity is the percent of plants in a population that ar tolerant to glyphosate as conferred by the presence of one or more copies of the *cp*4 *epsps* gene.

Transgenic locus: The chromosomal location into which DNA has been integrated.

APPENDIX 2

Appearance of Glyphosate-Resistant Weeds

Appearance of Glyphosate-Resistant Weeds

Monsanto considers product stewardship to be a fundamental component of customer service and business practices. The potential for weeds to become resistant to glyphosate is important to Monsanto because resistance can adversely impact the utility and life cycle of our products if it is not managed properly. The risk of weeds developing resistance and the potential impact on the usefulness of a herbicide vary greatly across different herbicidal modes of action and are dependent on a combination of factors. As leaders in the development and stewardship of glyphosate products for almost 30 years, Monsanto has invested considerably in research to understand the proper uses and stewardship of the glyphosate molecule. This research includes an evaluation of some of the factors that can contribute to the development of weed resistance.

A. The Herbicide Glyphosate

Glyphosate (N-phosphonomethyl-glycine) (CAS Registry #: 1071-83-6), the active ingredient in the Roundup family of nonselective, foliar-applied, postemergent agricultural herbicides, is among the world's most widely used herbicidal active ingredients. Glyphosate is highly effective against the majority of annual and perennial grasses and broad-leaved weeds. Glyphosate kills plant cells by inhibition of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), an enzyme involved in the shikimic acid pathway for aromatic amino acid biosynthesis in plants and microorganisms (Franz et al., 1997). This aromatic amino acid pathway is not present in mammalian metabolic systems (Cole, 1985). This mode of action contributes to the selective toxicity of glyphosate toward plants and to the low risk to human health from the use of glyphosate according to label directions. A comprehensive human safety evaluation and risk assessment concluded that glyphosate has low toxicity to mammals, is not a carcinogen, does not adversely affect reproduction and development, and does not bioaccumulate in mammals (Williams et al., 2000). Glyphosate has favorable environmental characteristics, including a low potential to move to through soil to reach ground water and that it is degraded over time by soil microbes. Because it binds tightly to soil, glyphosate's bioavailability is reduced immediately after use, which is why glyphosate has no residual soil activity. An ecotoxicological risk assessment concluded that the use of glyphosate does not pose an unreasonable risk of adverse effects to nontarget species, such as birds and fish, when used according to label directions (Giesy et al., 2000).

B. Characteristics Related to Resistance

Today, some 171 herbicide-resistant species and 284 biotypes within those species have been identified (Heap, 2004). Most of these are resistant to the triazine family of herbicides (Holt and Le Baron, 1990; Le Baron, 1991; Shaner, 1995). Resistance usually has developed because of the long residual activity of these herbicides with the capability to control weeds over a long period and the selection pressure exerted by the repeated use of herbicides with a single target site and a specific mode of action. Using these criteria, and based on current use data, glyphosate is considered to be a herbicide with a low risk

for weed resistance (Benbrook, 1991). Nonetheless, a question has been raised as to whether the introduction of crops tolerant to a specific herbicide, such as glyphosate, may lead to the occurrence of weeds resistant to that particular herbicide.

It is important to recognize that weed resistance is a herbicide-related issue, not a crop-related issue. The use of a specific herbicide with a herbicide-tolerant crop is no different than the use of a selective herbicide in a conventional crop from a weed resistance standpoint. While the incidence of weed resistance is often associated with repeated applications of a herbicide product, its development depends very much on the specific herbicide chemistry in question as well as the plant's ability to inactivate it. Some herbicide products are much more prone to develop herbicide resistance than others. Glyphosate has been used extensively for three decades with very few cases of resistance development, particularly in relation to many other herbicides. This is largely because of many unique properties of glyphosate that make the development of resistance unlikely (Heering et al., 2004), including highly specific target sites in the plant, limited metabolism in plants, and a lack of soil residual activity. A summary of those factors is provided below.

B.1. Target site specificity

Target site alteration is a common resistance mechanism among many herbicide classes, such as acetolactate synthase (ALS) inhibitors and triazines, but is less likely for glyphosate.

A herbicide's mode of action is classified by the interference of a critical metabolic process in the plant by binding to a target protein and disrupting the required function. The specificity of this interaction is critical for the opportunity to develop target site-mediated resistance. Because the herbicide comes into contact with discreet amino acids during protein binding, changing one of these contact point amino acids can interrupt this binding.

The specificity of inhibitor binding is dependent on the number and type of the amino acids serving as contact points and can be measured indirectly by counting the number of unique compounds that can bind to the same site. On one extreme, the only herbicide compound known to bind to EPSPS is glyphosate, demonstrating that the binding is highly specific. Single amino acid substitutions near the active site making glyphosate binding slightly weaker have been observed; however, these enzymes are also less fit. Similarly, high specificity is observed for glutamine synthetase, binding three compounds including phosphinothricin in the active site (Crespo et al., 1999). Paraquat and diquat are the only two herbicides inhibiting photosystem I. No target site mutations have been reported to be responsible for resistance in these systems (Powles and Holtum, 1994).

On the other extreme are target enzymes that are efficiently inhibited by a wide array of compounds, e.g., ALS is inhibited by 53 separate herbicide compounds and acetyl CoA carboxylase (ACCase) is inhibited by 21 separate herbicide compounds that bind both within and outside the active site (HRAC, 2002; Tranel and Wright, 2002). These cases demonstrate that numerous noncritical amino acids are involved outside of the active site, offering a relatively large range of permissible mutations. In these two cases, a single

amino acid change can result in virtual immunity to these classes of herbicides and has directly led to the preponderance of resistant weed species for these mode of actions (MOAs), 79 and 30, respectively.

Glyphosate competes for the binding site of the second substrate, phosphoenolpyruvate (PEP), in the active site of EPSPS and is a transition state inhibitor of the reaction (Steinrücken and Amrhein, 1984). This was recently verified by x-ray crystal structure (Schonbrunn et al., 2001). As a transition state inhibitor, glyphosate binds only to the key catalytic residues in the active site. Catalytic residues are critical for function and cannot be changed without a lethal or serious fitness penalty. Furthermore, very few selective changes can occur near the active site of the enzyme to alter the competitiveness of glyphosate without interfering with normal catalytic function. Therefore, target site resistance is highly unlikely for glyphosate. This was further illustrated in that laboratory selection for glyphosate resistance using whole plant or cell/tissue culture techniques were unsuccessful (Jander et al., 2003; Widholm et al., 2001; OECD, 1999).

B.2. Metabolism in plants

Metabolism of the herbicide active moiety is often a principal mechanism for the development of herbicide resistance. The lack of glyphosate metabolism or significantly slow glyphosate metabolism has been reported in multiple plant species and reviewed in various publications (Duke, 1988; Coupland, 1985). Therefore, this mechanism is unlikely to confer resistance to glyphosate in plants.

B.3. Soil residual activity

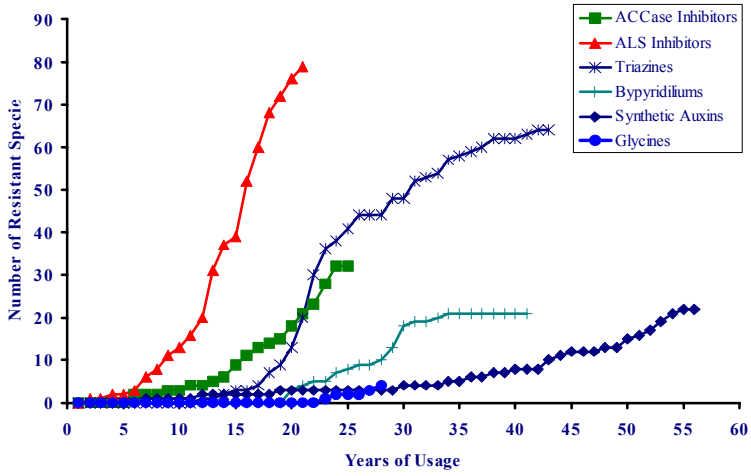
Herbicides with soil residual activity dissipate over time in the soil, resulting in a sublethal exposure and low dose selection pressure over a period of time. Glyphosate adsorption to soils occurs rapidly, usually within one hour (Franz et al., 1997). Soil-bound glyphosate is therefore unavailable to plant roots, so the impact of sublethal doses over time is eliminated. The fact that glyphosate is only active foliarly allows for the use of a high dose weed management strategy.

The graph in Figure 1 illustrates the occurence of weed resistance over time to various herbicide families. The different slopes observed are largely because of the factors described above, which relate to chemistry and function, in addition to levels of exposure in the field. Glyphosate is a member of the glycine family of herbicides, which has experienced very limited cases of resistance despite almost three decades of use. On the other hand, numerous weed species have developed resistance to the ALS inhibitors and triazine families even after they were available for only a relatively short period of time (Heap, 2004).

It is also important to recognize that each herbicide targets a large number of weeds, so the development of resistance in certain species does not mean the herbicide is no longer useful to the grower for control of other species. For example, resistance of certain weeds to imidazolinone and sulfonylurea chemistries developed within three to five years of their introduction into cropping systems. Nevertheless, Pursuit (imidazolinone) herbicide maintained a 60% share of the U.S. soybean herbicide market despite the presence of a large number of resistant weeds because it was used in combination with

other herbicides that controlled the resistant species. How weed resistance impacts the use of a particular herbicide varies greatly depending on the herbicide chemistry, the biology of the weed, the availability of other control practices and the diligence with which it is managed.

Figure 1. Number of Herbicide Resistant Weed Species Found Over Time[1]



[1]Heap, 2004 (www.weedscience.com)

C. Weeds Resistant to Glyphosate

Weed resistance is generally defined as the naturally occurring inheritable ability of some weed biotypes within a given weed population to survive a herbicide treatment that should, under normal use conditions, effectively control that weed population. Thus, a resistant weed must demonstrate two criteria: 1) the ability to survive application rates of a herbicide product that once were effective in controlling it; and 2) the ability to pass the resistance trait to seeds. Procedures to confirm resistance generally require both field and greenhouse analyses, particularly if the level of resistance is relatively low as is generally seen for cases of glyphosate resistance.

Herbicide tolerance differs from resistance in that the species is not controlled but has the inherent ability to survive applications of the herbicide from the beginning. In other words, the species does not develop tolerance through selection but is naturally tolerant.

As part of our current product stewardship and customer service policy, Monsanto investigates cases of unsatisfactory weed control to determine the cause, as described in the performance evaluation program outlined in section E of this Appendix. Weed control failures following application of Roundup agricultural herbicides are most often the result of management and/or environmental issues and are very rarely the result of

herbicide resistance. The procedures included in Monsanto's performance evaluation program provide early detection of potential resistance, field and greenhouse protocols to investigate suspected cases and mitigation procedures to respond to confirmed cases of glyphosate resistance.

To date, biotypes of only four weed species resistant to glyphosate have been identified. In all cases, Monsanto worked with local scientists to identify alternative control options that have been effective in managing the resistant biotype.

C.1. *Lolium rigidum*

In 1996 in Australia, it was reported that a biotype of annual ryegrass (*Lolium rigidum*) was surviving application of recommended rates of glyphosate (Pratley et al., 1996). Collaboration was established with Charles Sturt University to develop an agronomic understanding of the biotype and investigate the mechanism of resistance. Where the biotype has been found, it has occurred within isolated patches within a field and does not appear to be widespread. The resistant biotype is easily controlled within conservation and conventional tillage systems with other herbicides, tillage or seed removal.

A large body of biochemical and molecular biology experiments between Australian ryegrass biotypes resistant and susceptible to glyphosate indicate that the observed resistance is because of a combination of factors. The mechanism of resistance appears to be multi-genic and caused by a complex inheritance pattern, which is unlikely to occur across a wide range of other species. The mechanism is yet to be fully defined despite significant research effort; however, reduced cellular transport of glyphosate has been proposed (Lorraine-Colwill et al., 2003).

The resistant annual ryegrass biotype has also been observed in orchard systems of California and South Africa. Similar to the Australian locations, these fields are small and isolated. Monsanto established collaborations with local scientists to identify alternative control mechanisms, and the use of other herbicides, tillage, mowing and seed removal have been very effective in controlling the ryegrass.

Ryegrass species can become weed problems in spring and fall seeded alfalfa (Hower et al., 1999). In the event that glyphosate resistant annual ryegrass is found in Roundup Ready alfalfa stands, a variety of post emergence, preemergence and post/preemergence alternative herbicides currently used in alfalfa exist for its control. Products include diuron, hexazinone, sethoxydim, and trifluralin. Further, seed of annual ryegrass is unlikely to mature in properly managed alfalfa fields.

C.2. *Lolium multiflorum*

A population of Italian ryegrass (*Lolium multiflorum*) was reported to survive labeled rates of glyphosate by a scientist conducting greenhouse and field trials in Chile. A second population was also found in Brazil. Monsanto conducted field and greenhouse trials to confirm the resistance and worked with the researcher in Chile to identify alternative control options. The resistant biotypes have been found on a few farms and are easily controlled through tank mixes with other herbicides and cultural practices.

Italian ryegrass is noted as a weed in spring and fall seeded alfalfa (Hower et al., 1999). In the event that glyphosate-resistant Italian ryegrass is found in Roundup Ready alfalfa stands, preemergence and postemergence alternative herbicides currently used in alfalfa exist for its control. Products include clethodim, pronamide, and norflurazon.

C.3. *Eleusine indica*

A population of *Eleusine indica (*goosegrass*)* was reported to survive labeled rates of glyphosate in some orchard systems in Malaysia. Monsanto entered into collaborations with the Universiti Kebangsaan Malaysia (National University of Malaysia) and identified alternative control options to effectively manage the resistant biotype. Extensive molecular investigations determined that some of the resistant goosegrass plants have a modified EPSPS that is 2-4 times less sensitive to glyphosate than more sensitive biotypes (Baerson et al., 2002). Partial sequencing of the EPSPS synthase gene in the R biotype of resistant goosegrass confirmed that a mutation has occurred, where there has been a substitution of proline with serine or threonine at amino acid 106 (Ng et al., 2003). This mutation may account for these resistant goosegrass plants that are less sensitive to glyphosate. However, some individuals did not exhibit the enzyme modification, suggesting that different mechanisms may be at play or resistance may be because of a combination of factors.

The resistant biotypes are easily controlled through application timing (applying glyphosate during the early growth stages), use of other herbicides, tillage and other cultural control practices.

Goosegrass is a warm season annual grass that has a low, creeping growth habit. Goosegrass is noted as a weed in alfalfa grown in the Northeast U.S. (Hower et al., 1999). In the event that glyphosate resistant goosegrass is found in Roundup Ready alfalfa stands, a variety preemergence and postemergence alternative herbicides currently used in alfalfa exist for its control. Products include clethodim, EPTC, sethoxydim, pronamide, and norflurazon.

C.4. *Conyza canadensis*

Laboratory and field investigations confirmed the presence of a glyphosate-resistant biotype of marestail (Conyza canadensis) in certain states of the eastern and southern U.S. (VanGessel, 2001). The mechanism of resistance in the marestail biotype is currently under investigation. Findings thus far have been presented at regional and national weed science meetings and submitted for publication (Feng et al., 2004 in press)

Investigations thus far indicate that this biotype has a heritable resistance ranging up to approximately 6-8X field herbicide application rates. Current data indicates that the heritance is dominant and transmitted by a singular nuclear gene. Resistance is not due to over-expression of EPSPS, glyphosate metabolism or reduction in glyphosate retention or uptake. Resistance is also not due to target site mutation, as the three isozymes of EPSPS identified in marestail were identical in sensitive and resistance lines. Our results demonstrate a strong correlation between impaired glyphosate translocation and resistance. Tissues from both S and R biotypes showed elevated levels of shikimate, suggesting that EPSPS remained sensitive to glyphosate. Analysis of tissue shikimate levels relative to those of glyphosate demonstrated a reduced efficiency of EPSPS inhibition in the R biotypes. Our results are consistent with an exclusion mechanismfor glyphosate resistance. Our current working hypothesis is that marestail resistance results from an alteration of glyphosate distribution that impairs its phloem loading and plastidic import.

The resistant marestail biotype has been observed in conventional and Roundup Ready cotton and soybean fields. As in other cases, Monsanto responded to weed control inquiries and alternative weed control options were provided. One of the most effective ways to minimize the resistant biotype is by planting a cover crop, such as wheat, that can compete with marestail and limit its fall and winter germination. In addition, growers are advised to use a tank-mix of glyphosate with Clarity for cotton and glyphosate with 2,4-D or Amplify for soybeans in their burn down treatment. If marestail is present in-crop, then growers are advised to use MSCA plus diuron for cotton and glyphosate plus Amplify for soybeans.

As part of Monsanto's stewardship program, EPA approval of a supplemental label was obtained for use in counties where the resistant biotype has been confirmed. Growers in those counties are instructed to use the alternative control options, regardless of whether or not they had trouble controlling marestail on their farm the previous season, as a means to minimize spread of the resistant biotype. It has been recommended to growers in surrounding areas where the resistant biotype has not been confirmed that they use the alternative control options if mare's-tail has been a difficult weed for them to control. This stewardship program has proven effective in controlling the glyphosate-resistant biotype and minimizing its spread beyond the south and eastern regions of the U.S.

With regard to alfalfa, marestail is not listed as a weed in alfalfa (Hower et al., 1999). However, in the event that glyphosate resistant marestail is found in Roundup Ready alfalfa stands, metribuzin, a preemergence alternative herbicide currently used in alfalfa, is available for its control.

C.5. Other Species

In addition to the species described above, two weed species hairy fleabane (*Conyza bonariensis*) and buckhorn plantain (*Plantago lanceolata*), are reported (however not confirmed by Monsanto) to be resistant to glyphosate (Heap, 2004). Both resistant species are limited to South Africa. Non-glyphosate herbicides are available for selective control of these two species in alfalfa and provide suppression under most conditions.

Fleabane and plantain species are listed as weeds in U.S. alfalfa fields (Hower et al., 1999). In the event that glyphosate resistance in hairy fleabane and/or buckhorn plantain is confirmed, and these species are found in Roundup Ready alfalfa stands, the first course of action will be to recommend those herbicides that provide suppression. If either species reaches a level of infestation that has a negative economic impact on alfalfa yield and/or quality, it will be recommended that the alfalfa stand be terminated with a tank mixture of dicamba and 2,4-D. This alfalfa stand take-out herbicide tank mixture is also effective for the control of hairy fleabane and buckhorn plantain.

Other scenarios considered involve indirect impacts of other Roundup Ready crops and their accompanying weed control systems on Roundup Ready alfalfa. The first scenario involves the selection of glyphosate resistant weeds through use of a different Roundup Ready weed control system. In this hypothetical scenario, a major weed in one system that is a minor weed in the other becomes a problem weed in both systems through increased selection of resistant plants. For example, annual bluegrass (*Poa annua*) is noted as a major weed for creeping bentgrass and a minor weed for alfalfa. The second hypothetical scenario involves gene flow from a Roundup Ready crop to a sexually compatible wild relative that is a weed in alfalfa. Plant species that are weeds in alfalfa with a crop relative that has the Roundup Ready trait includes wild mustards (*Sinapis arvensis*) and wild radish (*Raphanus raphanistrum*); both species are wild relatives of canola (*Brassica, napus*). Both of these scenarios are unlikely because: (1) rotations with creeping bentgrass and alfalfa are very uncommon, thus there is practically no opportunity for this to occur, and (2) cross-hybridization of canola with wild mustard is only possible through embryo rescue and ovule culture, and cross hybridization with wild radish does not result in the stable integration of genetic material from canola; hence, natural gene flow under field conditions is extremely unlikely to both species (Eastham and Sweet, 2002; USDA, 2004). Regardless, should these or other unforeseen scenarios occur, these cases would be treated no differently than cases of actual glyphosate resistant weeds as described above. Through appropriate stewardship, including identification of the problem weed and herbicide resistance mechanism and the use of a broad cross-section of pre- and postemergence grass and broadleaf herbicides currently available in alfalfa, control of most resistant species will be accomplished.

In summary, Monsanto has effective product stewardship and customer service practices established to directly work with the grower communities and provide appropriate control measures for glyphosate-resistant weeds. Monsanto has collaborated with academic institutions to study these glyphosate-resistant biotypes, and findings have been communicated to the scientific community through publications in peer-reviewed scientific journals and scientific meetings.

D. Weed Management Strategies for Glyphosate

A key element of good weed management is using the correct rate of glyphosate at the appropriate window of application for the weed species and size present. Appropriate herbicide doses result in higher weed mortality and lower frequency of resistance genes in the surviving population (Matthews, 1994). However, low herbicide rates may allow both heterozygous and homozygous resistant individuals to survive (Maxwell and Mortimer, 1994), further contributing to the build up of resistant alleles in a population.

Because resistance is dependent upon the accumulation of relatively weak genes, which appears to be the case for one or more of the four weed species that have developed resistance to glyphosate, using a lethal dose of herbicide is critical.

Results that support these strategies are beginning to emerge from field research studies at several universities (Roush et al., 1990). Various weed management programs have been evaluated since 1998 to determine how they impact weed population dynamics. Studies were initiated in Colorado, Kansas, Nebraska, Wyoming (Wilson and Stahlman, 2003), and Wisconsin (Stoltenburg, 2002) to evaluate the continuous use of Roundup Ready technology with exclusive use of glyphosate or inclusion of herbicides with other modes of action and rotation away from Roundup Ready technology. These treatment regimes were compared to a conventional herbicide program for each crop evaluated. General observations after five years are: (1) Use of a continuous Roundup Ready cropping system with either glyphosate alone at labeled rates or incorporation of herbicides with other modes of action resulted in excellent weed control with no weed shifts or resistance reported; (2) use of glyphosate at below labeled rates resulted in a weed shift to common lambsquarters at two locations (Nebraska and Wyoming); and (3) in Wisconsin, ALS-resistant giant ragweed was selected for in the broad-spectrum residual herbicide regime implemented in the conventional corn cropping system. The continuous glyphosate system (using labeled rates) resulted in no significant weed shifts.

By using glyphosate at the recommended lethal dose, the buildup of weeds with greater inherent tolerance or any potential resistance alleles has been avoided over the duration of these studies. These results indicate that continuous Roundup Ready systems used over five years did not create weed shifts or resistant weeds when the correct rate of glyphosate was applied and good weed management was practiced.

E. Glyphosate Stewardship Program

Commercial experience, field trials and laboratory research demonstrate that one of the most important stewardship practices is achieving maximum control of the weeds. This can be accomplished by using the correct rate of glyphosate at the appropriate window of application for the weed species and size present, and using other tools or practices as necessary.

As the recognized leader in the development and commercialization of glyphosate, Monsanto is committed to the proper use and long-term effectiveness of glyphosate through a four-part stewardship program: developing appropriate weed control recommendations; conducting research to refine and update recommendations; educating growers on the importance of good weed management practices; and responding to repeated weed control inquiries through a performance evaluation program.

E.1. Development of local weed management recommendations to ensure maximum practical control is achieved

Weed control recommendations in product labels and informational materials are based on local needs to promote the use of the management tool(s) that are most appropriate technically and economically for each region. Furthermore, growers are instructed to

apply the same principles when making weed control decisions for their own farm operation. Multiple agronomic factors, including weed spectrum and population size, application rate and timing, herbicide resistance status (where applicable) and an assessment of past and current farming practices used in the region or on the specific operation are considered to ensure appropriate recommendations for the use of glyphosate to provide effective weed control. Carefully developing and regularly updating the use recommendations for glyphosate are fundamental to Monsanto's stewardship program.

Weed spectrum: Weed spectrum refers to all of the weed species present in a grower's field and the surrounding areas that may impact those fields. The spectrum may vary across regions, farm operations, and even among fields within a farm operation depending on environmental conditions and other factors. Weed control programs should be tailored on a case-by-case basis by identifying the target weeds present, considering the efficacy of glyphosate and other weed management tools against those particular weeds, and assessing if any are unlikely to be controlled sufficiently with glyphosate alone, i.e., the weeds are not included on the product label, are difficult to control based on agronomic and/or environmental conditions, or have documented resistance to glyphosate. Specific formulations, rates, application parameters, and additional control tools are recommended as necessary to optimize control of all weeds in that system.

Species that are tolerant to glyphosate, such as *Equisetum arvensis* (Field horseweed), are occasionally described as resistant. This characterization is technically inappropriate because glyphosate is not commercially effective on those weeds and they generally are not listed as controlled on Roundup product labels. Other species, such as *Convolvulus arvensis* (Field bindweed) that are listed on the label may be partially tolerant or difficult-to-control with glyphosate alone. In these cases, additional herbicides are usually recommended to be tank-mixed with glyphosate. Still other species, such as *Abutilon theophrasti* (Velvetleaf), may be listed as controlled by glyphosate on the label but a tank-mix recommendation for additional herbicide may be used in the field because of sensitive environmental or herbicide application conditions in certain counties or seasons.

Application Rate: Application rate is integral to the correct use of glyphosate and critical to obtain effective weed control. Significant research has been conducted to identify the appropriate rate of glyphosate required for a particular weed at various growth stages in various agronomic and environmental conditions. These rates are included in rate tables provided in product labels and other materials. In addition, Monsanto recommends that growers use the rate necessary to target the most difficult to control weed in the field to minimize weed escapes. When using tank mixes, growers should consider the potential impacts on glyphosate efficacy through antagonism or below-recommended rates and make adjustments accordingly.

Application Timing: Application timing is based on the growth stage of weeds, the size/biomass of weeds and the agronomic and environmental conditions at the time of application. Delaying the application of glyphosate and allowing weeds to grow too large before applying the recommended rate of glyphosate will result in poor efficacy.

Applying glyphosate at a time when weeds are under agronomic stress (e.g., insect/ disease pressure) or environmental stress (e.g., moisture/drought/cold condition) can also result in poor weed control.

Compensating for a delayed application through subsequent applications may not be effective, because the first application may inhibit weed growth and impair the efficacy of the second application because weeds may not be in an active growth process.

Correct application timing is dependent on the combined management of the weed spectrum, the size and layout of the farm operation and the feasibility to make timely applications of all weeds in the fields with the labor and equipment available. Monsanto recommends an application timeline that targets susceptible growth stages of all weeds, and, where applicable, includes recommendations for inclusion of additional control tools as necessary to optimize control of all weeds on that farm.

Finally, it is important to assess the current agronomic practices used in a particular region or farm operation to integrate the glyphosate recommendations into the grower's preferred management system. Variables such as tillage methods, crop rotations, other herbicide programs, other agronomic practices and the resistance status of the weeds to herbicides other than glyphosate can impact the spectrum of weeds present and the tools available to the grower.

Weed management recommendations communicated to growers also incorporate other components of the glyphosate stewardship program including use of high-quality seed, employing sanitary practices such as cleaning equipment between fields, and scouting fields and reporting instances of unsatisfactory weed control for follow up investigation.

E.2. Research

A fundamental component of Monsanto's leadership in glyphosate stewardship is research on the recommended use of glyphosate and factors impacting its effectiveness. In addition to extensive analyses conducted to determine the correct application rate of glyphosate prior to product registration, ongoing agronomic evaluations are conducted at the local level to refine weed management recommendations for specific weed species in specific locations.

Weed efficacy trials are part of ongoing efforts by Monsanto to tailor recommendations to fit local conditions and grower needs. Application rate and timing, additional control tools and other factors are included in these analyses. As a result of weed efficacy trials, changes are made to specific weed control recommendations in product labeling, and modifications to local recommendations are communicated to growers through informational sheets and other methods.

E.3. Education and communication efforts

Another key element of effective product stewardship and appropriate product use is education to ensure that growers understand and implement effective weed management plans and recommendations. Monsanto communicates weed management recommendations through multiple channels and materials to multiple audiences.

All Monsanto technical and sales field representatives are required to take a weed management training course to understand the glyphosate stewardship program and the importance of proper product use. The training program is supported by ongoing weed management updates that highlight seasonal conditions and recommendations.

All of Monsanto's weed management and product use recommendations are based on, and are consistent with, federally approved product labeling. Monsanto weed management recommendations and the importance of sound agronomic practices are communicated to growers, dealers and retailers, academic extension agents and crop consultants through multiple tools:

- Technology training programs: Highlighting weed management principles, weed management plans and practical management guidelines.
- Technology use guide: Includes summary tables outlining appropriate product use rate and timing for different weed species and sizes.
- Grower meetings: Conducted prior to planting to emphasize the importance of following local application recommendations.
- Marketing programs: Designed to reinforce and encourage the continued adoption and use of weed management recommendations by the grower (e.g., recommended rate and timing of application, and additional weed control tools when applicable).
- Informational Sheets: Provided to growers and dealers/retailers to highlight local recommendations for specific weeds.

As with most stewardship efforts, education is key to help growers and other stakeholders understand the importance of proper product use and encourage those practices in the field.

E.4. Performance inquiry evaluation and weed resistance management plan

To support and enhance Monsanto's weed management principles and recommendations, Monsanto has implemented a performance evaluation program based on grower performance inquiries and field trial observations. The goal of the program is to adapt, modify and improve Monsanto's weed control recommendations, with a focus on:

- Particular weeds and growing conditions;
- Providing product support to customers who are not satisfied with their level of weed control; and
- Identifying and investigating potential cases of glyphosate resistance early so that mitigation strategies can be implemented.

The grower generally reports instances of unsatisfactory weed control following a glyphosate application to either Monsanto or the retailer. Monsanto investigates these inquiries immediately, because it is important to maintain the customer's satisfaction and is part of the stewardship committment.

The vast majority of inquiries are because of application error or environmental conditions at the time of herbicide application. A system is in place to investigate a repeated performance inquiry for a specific weed within a specific field that occurs within the same growing season. The investigation considers the various factors that could account for ineffective weed control such as (but not limited to):

- Application rate and timing;
- Plant size and growth stage;
- Application equipment set up and calibration; and
- Environmental and agronomic conditions at time of application.

In all cases, the first priority is to provide control options to the grower so that satisfactory weed control is achieved for that growing season. The majority of repeated product performance inquiries are because of improper application or environmental/ agronomic conditions and, when properly addressed, are not repeated. However, if unsatisfactory weed control occurs again in that field and does not appear to be because of application or growing condition factors, then steps are taken to determine whether resistance is the cause, as outlined in the Monsanto Weed Resistance Management Plan.

The Monsanto Weed Resistance Management Plan consists of three elements:

- Identification process for potential cases of glyphosate resistance;
- Initiation of steps to respond to cases of suspected resistance; and
- Development and communication of guidelines to incorporate resistance mitigation into weed management recommendations.

Identification of potential cases of glyphosate resistance is accomplished through evaluation of product performance inquiries and local field trials. These efforts provide an early indication of ineffective weed control that may indicate potential resistance.

If the follow up investigation clearly indicates that the observation is because of application error or agronomic/environmental conditions, then appropriate control options are recommended to the grower for that season and the grower receives increased education on the importance of proper product use. The vast majority of weed control inquiries fall into this category.

If repeated lack of control is observed and does not appear to be because of application error or environmental conditions, then a field investigation is conducted by Monsanto to analyze control of the weed more thoroughly.

Weeds must be actively growing in order for glyphosate to be effective. Application error or environmental conditions that result in insufficient glyphosate to kill the weed often stunt its growth such that subsequent applications by the grower are ineffective. Monsanto's field investigations at this stage remove that artifact by ensuring that the

weeds tested are in an active growth phase. The vast majority of field investigations do not repeat the insufficient control reported by the grower. If the field investigation confirms that agronomic factors accounted for the observation, then the grower receives further education on proper application recommendations.

In addition, the internal network of Monsanto technical managers and sales representatives in the surrounding area are notified to highlight any problematic environmental conditions or application practices that may be common in that area. Critical information regarding location, weed species, weed size, rate used and the potential reason for lack of control is documented, and the information is reviewed annually by the appropriate technical manager to identify any trends or learnings that need to be incorporated into the weed management recommendations.

If the reported observation is repeated in the field investigation, then a detailed performance inquiry is conducted and greenhouse trials are initiated. If greenhouse trials do not repeat the observation and the weed is clearly controlled at label rates, then a thorough follow-up visit is conducted with the grower to review the application recommendations and conditions of his operation that may be impacting weed control. Monsanto's internal network of agronomic managers is notified of the results to raise awareness of performance inquiries on that particular weed the following season. If the greenhouse efficacy trials do indicate insufficient control at label rates, then detailed studies are conducted to determine if the weed is resistant.

Resistance is confirmed if the following two criteria are fulfilled either through greenhouse and field data or experience with similar cases:

- The suspect plant is demonstrated to tolerate labeled rates of glyphosate that previously were effective in controlling it, and
- The suspect plant is capable of passing that ability to offspring (i.e., the trait is heritable).

Additional field trials generally are initiated simultaneously as these investigations are conducted to identify the most effective and efficient alternative control options for that weed in various growing conditions. The research may be conducted internally by Monsanto as well as through collaboration with external researchers.

If resistance is confirmed, then the scientific and grower communities are notified as appropriate and a weed resistance mitigation plan is implemented. The mitigation plan is designed to manage the resistant biotype through effective and economical weed management recommendations implemented by the grower. The scope and level of intensity of the mitigation plan will vary depending on a combination of the following factors:

- Biology and field characteristics of the weed species (seed shed, seed dormancy, etc.);
- Importance of the weed species in the agricultural system;

- Resistance status of the weed species to other herbicides with alternate modes of action; and
- Availability of alternative control options.

These factors are analyzed in combination with economic and practical management considerations to develop a tailored mitigation strategy that is technically appropriate for the particular weed and incorporates practical management strategies that can be implemented by the grower.

After development, the mitigation plan is communicated to the grower community through Federally approved supplemental labeling, informational fact sheets, retailer training programs, agriculture media or other means, as appropriate.

The final step of the Weed Resistance Management Plan may include extensive genetic, biochemical or physiological analyses of confirmed cases of glyphosate resistance in order to elucidate the mechanism of resistance. Findings of this research are communicated to the scientific community through scientific meetings and publications, and information pertinent to field applications is incorporated into weed management recommendations.

F. Summary

Development of weed resistance is a complex process that is very difficult to accurately predict, and no single agronomic practice will mitigate resistance for all herbicides or all weeds. As a result, weed resistance must be managed on a case-by-case basis and management programs need to be tailored to the particular herbicide and grower needs. Using good weed management principles built upon achieving high levels of control through proper application rate, choice of cultural practices and appropriate companion weed control tools will allow glyphosate to continue to be used effectively.

The key principles for effective stewardship of glyphosate use, including use in Roundup Ready crops, are: 1) basing recommendations on local needs and using the tools necessary to optimize weed control; 2) proper rate and timing of herbicide application; and 3) responding rapidly to instances of unsatisfactory weed control.

G. References

Baerson, S.R., D.J. Rodriguez, M. Tran, Y. Feng, N. Biest, and G.M. Dill. 2002. Glyphosate-resistant goosegrass. Identification of a mutation in the target enzyme 5-enolpyruvylshikimate-3-phosphate synthase. Plant Physiology 129:1265-1275.

Benbrook, C. 1991. Racing against the clock. agrichemical age, pp. 32-33.

Cole, D.J. 1985. *Mode of action of glyphosate – a literature analysis*. *In* The Herbicide Glyphosate. E. Grossbard and A. Atkinson, Ed. Butterworth's & Co. Boston, 1985. pp. 48-75.

Coupland, D. 1985. *In* The Herbicide Glyphosate; Grossbard, E: Atkinson, D. (Eds.), Butterworths, London, pp. 25-34.

Crespo, J.L., M.G. Guereo, and F.J. Florencio. 1999. Mutational analysis of Asp51 of *Anabaena azollae* glutamine synthetase. D51E mutation confers resistance to the active site inhibitors L-methionine-DL-sulfoximine and phosphinothricin. European Journal of Biochemistry 266:1202-1209.

Duke, S.O. 1998. *In* Herbicides: Chemistry, Degradation and Mode of Action; Kearney, P.C. & Kaufman, D.D. (Eds.), Marcel Decker Inc., New York, 1988, 3: 1-70.

Eastham, K and Sweet, J. 2002. Genetically modified organisms (GMO's): The significance of gene flow through pollen transfer. Environmental issue report No. 28. European Environment Agency, Copenhagen, 77 pp.

Feng, P.C.C., M. Tran, T. Chiu, R.D. Sammons, G.R. Heck, and C.A. CaJacob. 2004. Investigations into Glyphosate Resistance Horseweed (Conyza Canadensis): Retention, Uptake, Translocation. Weed Science (*in press)*.

Franz, J.E., M.K. Mao, and J.A. Sikorski. 1997. *In* Glyphosate: A Unique Global Herbicide. ACS Monograph 189. 1997 American Chemical Society, Washington, D.C.

Giesy JP, Dobson S, Solomon KR. 2000. Ecotoxicological risk assessment for Roundup herbicide. Reviews of Environmental Contamination and Toxicology 167: 35-120.

Heap, I. 2004. www.weedscience.com

Heering, D.C., G.A. Elmore, N.L. DiNicola, and R.D. Sammons. 2004. Sustainability and stewardship of glyphosate and Roundup Ready® crops. Weed Technology (*submitted*).

Herbicide Resistance Action Committee. 2002. Classification of herbicides according to mode of action**.** http://www.plantprotection.org/HRAC/.

Holt, J.S. and H.M. Le Baron. 1990. Significance and distribution of herbicide resistance. Weed Technology 4:141-149.

Hower, A.A., J.K. Harper and R.G. Harvey. 1999. The importance of pesticides and other pest management practices in U.S. alfalfa production. National Agricultural Pesticide Impact Assessment Program (NAPIAP). Pub. No. 2-CA-99. 221 pp.58-91

Jander, G., S.R. Baerson, J.A. Hudak, K.A. Gonzalez, K.J. Gruys, and R.L. Last. 2003. Ethylmethanesulfonate saturation mutagenesis in Arabidopsis to determine frequency of herbicide resistance. Plant Physiology 131:139-146.

Le Baron, H. M. 1991. Herbicide resistant weeds continue to spread. Resistant Pest Management Newsletter 3:36-37.

Lorraine-Colwill, D. F., S.B. Powles, T.R. Hawkes, P.H. Hollinshead, S.A.J.. Warner. and C. Preston. 2003. Investigations into the mechanism of glyphosate resistance in *Lolium rigidum*. Pest. Biochem. Physiol. 74:62-72.

Matthews, J.M. 1994. Management of herbicide resistant populations. *In* Herbicide Resistance in Plants, Biology and Biochemistry; S.B. Powles and J.A.M. Holtum (eds.). Lewis, Ann Arbor. pp. 317-335.

Maxwell, B. D. and A.M. Mortimer. 1994. Selection for herbicide resistance. *In* Herbicide Resistance in Plants, Biology and Biochemistry; S.B. Powles and J.A.M. Holtum (eds.). Lewis, Ann Arbor. pp. 1-26.

Ng, C.H., R. Wickneswari, S. Salmijah, Y.T. Teng and B.S. Ismail. 2003. Gene polymorphisms in glyphosate-resistant and –susceptible biotypes of *Eleusine indica* from Malaysia. Weed Research 43:108-115.

OECD. 1999. Consensus Document on General Information Concerning the Genes and Their Enzymes that Confer Tolerance to Glyphosate Herbicide. OECD Environmental Health and Safety Publications, Paris, ENV/JM/MONO(99)9. http://www.oecd.org/ehs/

Pratley, J., P. Baines, P. Eberbach, M. Incerti, and J. Broster. 1996. Glyphosate resistance in annual ryegrass. Proc. Eleventh Ann. Conf. Grassld. Soc. NSW, pp. 122.

Powles, S.B., and J.A.M. Holtum. 1994. *In* Herbicide Resistance in Plants, Biology and Biochemistry. Lewis, Ann Arbor. pp. 353.

Roush, M.L., S.R. Radosevich, and M.B. Maxwell. 1990. Future outlook for herbicide-resistance research. Weed Technology 4:208-214.

Sammons, R.D., G.R. Heck, M. Tran, P. Ratliff, P.C.C. Feng, and C.A. CaJacob. 2004. Investigations into Glyphosate Resistant Horseweed (Conyza canadensis): Genetic and Phenotypic Characterization. Weed Science (*submitted*).

Schonbrunn, E., S. Eschenburg, W.A. Shuttleworth, J.V. Schloss, N. Amrhein, J.N.S. Evans, and W. Kabsch. 2001. Interaction of the herbicide glyphosate with its target enzyme EPSP synthase in atomic detail. Proc. Natl. Acad. Sci. U.S.A. 98:1376-1380.

Shaner, D.L. 1995. Herbicide Resistance: Where are we? How did we get here? Where are we going? Weed Technology 9:850-856.

Steinrücken, H.C., and N. Amrhein. 1980. The herbicide glyphosate is a potent inhibitor of 5-enolpyruvylshikimic acid-3-phosphate synthase. Biochem. Biophys. Res. Commun. 94 (4):1207-1212.

Stoltenberg, D. E. 2002. Weed Management and Agronomic risks associated with glyphosate-resistant corn and soybean cropping systems. Proceedings of the 2002 Wisconsin Fertilizer, Aglime and Pest Management Conf. 41:200-208.

Trannel, P. and T.R. Wright. 2002. Resistance of weeds to ALS-inhibiting herbicides: what have we learned? Weed Science 50:700-712.

USDA. 2004. Response to Monsanto petition 98-216-01 for determination od nonregulated status for glyphosate-tolerant canola line RT73. Environmental assessment and finding of no significant impact. Accessed, March 16, 2004. http://www.aphis.usda.gov/brs/aphisdocs2/98_21601p_com.pdf

VanGessel, M.J. 2001. Glyphosate resistant horseweed from Delaware. Weed Science 49:703-705.

Widholm, J.M., A.R. Chinnala, J.H. Ryu, H.S. Song, T. Eggett, and J.E. Brotherton. 2001. Glyphosate selection of gene amplification in suspension cultures of 3 plant species. Physiol. Plant. 112:540-545.

Williams GM, Kroes R, Munro IC. 2000. Safety evaluation and risk assessment of the herbicide Roundup and its active ingredient, glyphosate, for humans. Reg Toxicol Pharmacol 31(2):117-165.

Wilson, R.G. and P. Stahlman. 2003. Maintain the rate. pp. 12-13, Nebraska Farmer, June edition.

APPENDIX 3

Letters from Weed Control Experts

UNIVERSITY of CALIFORNIA
Agriculture & Natural Resources
COOPERATIVE EXTENSION • MADERA COUNTY
328 Madera Avenue • Madera, CA 93637-5465
TEL (559) 675-7879 FAX (559) 675-0639

January 14, 2003

Glen Rogan
Monsanto Regulatory Affairs (BB3N)
700 Chesterfield Parkway
Chesterfield, MO 63017

Dear Dr. Rogan:

Feral or volunteer alfalfa plants on roadsides and other unmanaged areas is not a problem of any concern. More of a concern are volunteer alfalfa plants as weed problems in a subsequent cotton or corn crop. The following are responses to your questions.

1 Roadside weed control is most often accomplished with the use of glyphosate by both county and state agencies. But, as stated above, feral alfalfa plants are normally a small percentage of roadside weed problems.

2. Again, glyphosate is the main herbicide used, but it or any other herbicide is not specifically targeted to alfalfa.

3. The control of feral alfalfa would best be accomplished with a phenoxy herbicide. In a study I conducted in 2002, Roundup Ready alfalfa was controlled between 92 and 94% by several phenoxy or hormone type herbicide formulations. This creates a problem in the San Joaquin Valley of California, where the use of phenoxy herbicides are prohibited between March and October of each year. The reason for this CDFA regulation is prevention of injury to the many broadleaf crops, such as cotton and grapes, by either drift of the fuming action of many of the phenoxy herbicides.

If you have other questions, please do not hesitate to contact me.

Sincerely,

Ron Vargas

Ron Vargas
Farm Advisor

RV/nj

University of California, U.S. Department of Agriculture and Madera County Cooperating

UNIVERSITY of CALIFORNIA
Agriculture & Natural Resources
COOPERATIVE EXTENSION • SAN JOAQUIN COUNTY
420 South Wilson Way, Stockton CA 95205-6299 Telephone: (209) 468-2085
Fax: (209) 462-5181 Web: http://cesanjoaquin.ucdavis.edu

January 13, 2003

Glen Rogan
Monsanto Regulatory Affairs (BB3N)
700 Chesterfield Parkway
Chesterfield, MO 63017

Dear Mr Rogan,

This letter is in response to your letter requesting my comments on the following questions.

To the best of your knowledge, is feral alfalfa a weed species that is controlled on roadsides or other unmanaged areas in your state?

To the best of my knowledge, feral alfalfa occurs occasionally but is not a major problem along roadsides or unmanaged areas. I am not aware to what extent it is a problem Statewide.

If alfalfa is a species that is controlled, what are the herbicides currently used for control?

Agriculturally, Glyphosate is the primary herbicide used for ditch banks, levees, field edges and roadsides to control alfalfa. A tank mix combining oxyfluorfen and/or 24-D is common where conditions and weed species meet local restrictions. The California State Transportations system and San Joaquin county weed abatement programs use glyphosate alone or may include soil residual herbicide for roadside vegetation management.

If you suspected that the Roundup Ready trait was possibly present in feral alfalfa populations, what herbicides would you use to control feral alfalfa?

That is one of the questions now being researched in production alfalfa! Roundup is the primary herbicide for chemical alfalfa removal prior to discing or plowing. Currently, trials are under way evaluating removal of established alfalfa plants with alternate herbicides in an agricultural setting. The Phenoxy herbicides, specifically 2,4D and dicamba look promising and can be a stopgap to volunteer glyphosate plants. However, Phenoxy type herbicides have many restrictions in California. In non-cropped areas, triclopyr would be effective on feral alfalfa if registration were approved.

Sincerely,

Mick Canevari
Farm Advisor

College of Agriculture and Life Sciences
Department of Plant Sciences
Forbes Building #36
Room 303

THE UNIVERSITY OF
ARIZONA
TUCSON ARIZONA

P.O. Box 210036
Tucson, AZ 85721-0036
(520) 621-1977
FAX: (520) 621-7186

January 20, 2003

Glen Rogan
Monsanto Regulatory Affairs (BB3N)
700 Chesterfield Parkway
Chesterfield, MO 63017

Dear Mr. Rogan:

In view of the fact that Forage Genetics International (FGI) and Monsanto Company are jointly developing Roundup Ready alfalfa technology for weed control in alfalfa, I would like to address a couple of issues regarding feral alfalfa populations in the state of Arizona.

Feral alfalfa is not a weed species that is controlled on roadsides or in other unmanaged areas in Arizona. The occurrence of feral alfalfa populations in Arizona is rare due to the arid environment in desert agricultural areas and the necessity of irrigation for any type of crop production in the state. Thus, I have never seen feral alfalfa along roadsides, canals, ditch-banks and other rights-of-ways where it would be subject to control by herbicides or by glyphosate specifically. I have also traveled extensively in wildlands, Indian reservations, National Parks and lands administered by the Bureau of Land Management in Arizona and I have not seen feral alfalfa in these lands. Most of these areas are also quite arid making the survival of escaped crop species outside of agricultural areas problematic. Thus, since its occurrence is rare or nonexistent, feral alfalfa is not a species controlled using herbicides in the unmanaged areas of Arizona.

Glyphosate herbicides are used by some growers to terminate or kill alfalfa fields in preparation for rotating to another crop or to plant alfalfa again. In anticipation of the introduction of Roundup Ready alfalfa and the necessity of chemically terminating Roundup Ready alfalfa fields, I conducted extensive research on the efficacy of various herbicides in controlling alfalfa when used alone or in combination with tillage. The best and most economical treatment is a mixture of 2,4-D and dicamba in a 2:1 ratio at a total rate of 1 to 1.5 lb a.i./acre followed by a mechanical weed control operation that disturbs the soil (e.g., disking or tillage). If tillage is not used, a higher rate of herbicide and/or two sequential herbicide applications may be required for complete control. In non-crop rights-of-ways, preemergence herbicides (e.g., diuron, imazapyr and others) could be combined with postemergence herbicide treatments to prevent the reoccurrence or regrowth of feral alfalfa were it to appear in these rights-of-ways.

Sincerely,

William B. McCloskey, Ph.D.
Associate Specialist, Weed Science

School of Renewable Natural Resources
College of Agriculture and Life Sciences
School of Family and Consumer Sciences

COOPERATIVE EXTENSION
WASHINGTON STATE UNIVERSITY
Prosser Irrigated Agriculture Research and Extension Center

December 20, 2002

Glen Rogan
Monsanto Regulatory Affairs (BB3N)
700 Chesterfield Parkway
Chesterfield, MO 63017

Dear Mr. Rogan:

In regards to your 3 questions regarding feral alfalfa.

1 To the best of your knowledge, is feral alfalfa a weed species that is controlled on roadsides or other unmanaged areas in your state?

Feral alfalfa is sometimes controlled on roadsides and other unmanaged areas. Whether it is controlled or not on roadsides depends on where it is growing. If growing on the shoulder where vegetation is not wanted, the alfalfa will be controlled. If growing in the area where vegetation is wanted, it is usually not controlled.

2. If alfalfa is a species that is controlled, what are the herbicides currently used for control?

Glyphosate is not usually the herbicide of choice on roadsides to control alfalfa as it does not control the plant very well. On the bareground areas several of the soil residual products will control alfalfa as will clopyralid, triclopyr and dicamba. However, on irrigation ditchbanks glyphosate is often used to try to control the plant since the only other herbicide available is 2,4-D and that is not that effective.

3. If you suspected that the Roundup Ready trait was possibly present in feral alfalfa populations, what herbicides would you use to control feral alfalfa?

On the bareground areas several of the soil residual products could be used to control alfalfa as wouldl clopyralid, triclopyr and dicamba. Irrigation ditchbanks there is no other alternative at this time. Repeated applications of 2,4-D would suppress, however we are only allowed to make one application per year.

Another issue of feral alfalfa as a weed is in managed areas, precisely orchards and vineyards. The best treatment we have, for what it is worth, is glyphosate. There are no other herbicides presently labeled in orchards and vineyards that will effectively control

24106 N. Bunn Road, Prosser, WA 99350-8694
509-786-2226 • Fax: 509-786-9370 • TDD: 1-800-833-6388

Cooperating agencies: Washington State University, U.S. Department of Agriculture, and Washington counties. Cooperative Extension programs and employment are available to all without discrimination. Evidence of noncompliance may be reported through your local Cooperative Extension office.

alfalfa without the application being followed with deep tillage. Deep tillage is not an option in vineyards and orchards.

A common practice to kill alfalfa stands out in preparation to going to another crop is to use glyphosate followed by deep tillage 10 days later. However, there is an option to glyphosate which is as effective and is less ccostly. That option is to apply 2,4-D and plow 10 days later, however, growers are using glyphosate and plowing as the preferred method.

If you have further questions, please let me know.

Sincerely,

Robert Parker
Extension Weed Scientist

South Dakota State University
SDSU

College of Agriculture and Biological Sciences

Plant Science Department

Box 2140C, NPB 247
SDSU
Brookings, SD 57007-2141
Phone: 605-688-4020
FAX: 605-688-4452

31 December 2002

Mr. Glen Rogan
Monsanto Regulatory Affairs (BB3N)
700 Chesterfield Parkway
Chesterfield, MO 63017

Dear Mr. Rogan:

Following are responses to three questions sent to us by Dr. Sharie Fitzpatrick at Forage Genetics International.

Q1. To the best of your knowledge, is feral alfalfa a weed species that is controlled on roadsides or other unmanaged areas in your state?
A1. No, feral alfalfa is not a weed species in roadsides. In fact, alfalfa is planted with other species along most roads (including interstate highways) in South Dakota.
Q2. If alfalfa is a species that is controlled, what are the herbicides or cultural management practices that are currently used for its control?
A2. Again, alfalfa is a desired component of most roadside mixtures. Therefore, it will not typically be controlled with herbicides or with other practices.
Q3. If you suspected that the Roundup Ready trait was possibly present in feral alfalfa populations, what herbicides or management practices would you use to control feral alfalfa?
A3. Most managers would probably use a herbicide such as 2,4-D, Dicamba, or Tordon to attempt to eliminate Roundup resistant feral alfalfa. One or more tillage operations may also be used if a new seeding is to be done.

It is important to note that many miles of roadsides are harvested each year. Producers who successfully bid a segment of road or highway are allowed to cut in the roadside after a specific date, usually after 14 July. This date is set to allow for optimum wildlife nesting habitat, specifically pheasants. Essentially all of the feral alfalfa will have flowered by this date allowing significant outcrossing to occur. There are quite a number of organic producers in South Dakota. The presence of Roundup resistant alfalfa close to their operations (either on roadsides or in adjacent fields) may threaten their organic status.

Please contact us if you need further clarification about feral alfalfa in South Dakota.

Sincerely,

Dr. Vance Owens
Forage Research Agronomist

Dr. Sharon Clay
Weed Scientist

cc: Dr. Sharie Fitzpatrick

PLANT SCIENCE is a multidisciplinary department offering teaching, research, extension, and service in Agronomy (Crops, Soils, and Water), Entomology, Plant Pathology, and Weed Science.

COOPERATIVE EXTENSION
Washington State University
Department of Crop and Soil Sciences

P.O. Box 646420
Pullman, WA 99164-6420
509-335-2915 FAX 509-335-1758
TDD 1-800-833-6388

December 23, 2002

Glen Rogan
Monsanto Regulatory Affairs (BB3N)
700 Chesterfield Parkway
Chesterfield, MO 63017

Dear Glen:

Sheldon Blank asked me to respond to three specific questions regarding control of feral alfalfa and any potential control problems due to glyphosate-resistant feral alfalfa. I am pleased to respond to these questions.

1) To the best of your knowledge, is feral alfalfa a weed species that is controlled on roadsides or other unmanaged areas in your state?
My best estimate is that feral alfalfa is controlled in less than 1% of the area where it occurs in the state. Furthermore, in most areas where it is controlled, the goal is total vegetation management (bare ground) and feral alfalfa is not the dominant species. Examples of those areas include roadsides, industrial areas, parking lots, etc.

2) If alfalfa is a species that is controlled, what are the herbicides currently used for control?
Where it is controlled, I would guess that weed control is achieved through a combination of products including; Roundup, long residual sulfonylureas, fluroxypyr, picloram, bromacil, phenoxies, and possibly other materials in combination with one or more of those previously listed.

3) If you suspected that the Roundup Ready trait was possibly present in feral alfalfa populations, what herbicides would you use to control feral alfalfa?
Sulfonylureas, fluroxypyr, quinclorac, picloram, bromacil, phenoxies, and any number of additional herbicides depending upon the site where control is necessary.

In addition to my answering these questions, I would like to add that I do not see a problem controlling glyphosate-resistant feral alfalfa in the state of Washington. While glyphosate would not provide control, many other economical control options are available.

Please let me know if you have any questions.

Sincerely,

Joseph P. Yenish
Extension Weed Scientist
Washington State University

Cooperating agencies: Washington State University and the U.S. Department of Agriculture, and Washington counties. Cooperative Extension programs and employment are available to all without discrimination. Evidence of noncompliance may be reported through your local Cooperative Extension office.

ROGAN, GLENNON J [AG/1005]

From: Sharie Fitzpatrick [sfitzpatrick@foragegenetics.com]
Sent: Friday, January 24, 2003 3:48 PM
To: Simko, Ben
Subject: RE: Request for information on feral alfalfa

Ben,
Thank you for your time in addressing these questions. I have forwarded this on to Glen Rogan at Monsanto.
Regards,
Sharie

At 10:02 AM 1/24/2003 -0800, you wrote:
>Greetings Sharie:
>
>I am responding to your questions regarding feral alfalfa populations and
>herbicidal control. My responses are limited to my knowledge and
>experience with the local semi arid environments of the Malheur County and
>E Oregon. Other potential contacts would be Dr. Corey Ransom at our OSU
>Malheur Experiment Station 541 889 2174 and our statewide weed extension
>specialist Dr. Jed Colquhoun 541 737 8868 (OSU campus)
>
>1) To the best of your knowledge, is feral alfalfa a weed species that is
>controlled on roadsides or other unmanaged areas in your state? I spoke
>with our county roadside public applicator, Greg Hollopeter. He has
>covered many miles of county right of way in performing his duties and
>right of way spraying. His opinion is that the incidence of road side
>feral alfalfa is very low. His spray program includes use of 2,4D
>herbicide or mixtures of 2,4D plus dicamba. No additional cultivation is
>done on roadside right of ways. He doesn't consider feral alfalfa as a
>prevalent weed problem in the disturbed roadside environment at this time.
>2) If alfalfa is a species that is controlled, what are the herbicides or
>cultural management practices that are currently used for its control? In
>cropping systems of Malheur county alfalfa is control with both
>cultivation and herbicides use in process of producing onions, sugar
>beets, potatoes and small grains. Alfalfa seed growers take out old
>established stands to rotate out of a variety using a combination of 2,4D
>or 2,4D&dicamba along with aggressive cultivation operations, (disc, plow,
>or crowners). In a roadside or right of way situation products containing
>2,4D, dicamba, or maybe chlopyrilid would be used.
>3) If you suspected that the Roundup Ready trait was possibly present in
>feral alfalfa populations, what herbicides or management practices would
>you use to control feral alfalfa? Again in a roadside or right of way
>situation products containing 2,4D, dicamba, or maybe chlopyrilid would be
>used. Mechanical or cultivation control as a practice is not common.
>
>
>Some additional comments and suggestions.
>1) By contacting the departments of agriculture in the PNW you might get
>lists of county weed control officials who could also give input on the
>question of feral alfalfa as a noxious weed issue and control practices.
>
>2) Would FGI or Monsanto be interested in providing grants to PNW branch
>experiment stations to screen for herbicide efficacy against feral alfalfa
>if indeed via pollen drift feral RR alfalfa becomes a public right of way
>noxious weed problem. Dr. Ransom would be a possible cooperator for this
>type of research.
>
>
>Because I am running up against your deadline would you forward this
>e-mail to your Monsanto contact.

>
>Best regards,
>
>Ben
>
>Ben Simko
>Crops Extension Agent
>Malheur County Extension Office
>Oregon State University
>710 SW 5th Ave
>Ontario, OR 97914
>541.881.1417 FAX: 541.889.8840
>ben.simko@orst.edu
>
>
> -----Original Message-----
>From: Sharie Fitzpatrick [mailto:sfitzpatrick@foragegenetics.com]
>Sent: Friday, December 20, 2002 2:19 PM
>To: Simko, Ben
>Subject: Request for information on feral alfalfa
>
> << File: Request for feral alfalfa control information.doc >> << File:
> ATT397576.txt >> Dear Dr. Simko:
>
>As you know, FGI and Monsanto are preparing a petition to the USDA to
>request deregulation of Roundup Ready alfalfa. In 2002, there was a
>biogeographic survey of feral alfalfa distribution conducted in Washington
>and Idaho and several other states. We'd like to ask for your help in
>gathering an additional piece of information for the petition. FGI and
>Monsanto would like to ask your opinion and response to three questions
>regarding feral alfalfa populations in Oregon and the Pacific Northwest
>(see attached). Glen Rogan of Monsanto will be collecting the responses
>(letters) in January for inclusion with the upcoming Roundup Ready petition
>to the USDA.
>
>We would greatly appreciate your participation in this process. If you feel
>that we should contact an additional person in the region (e.g., an
>extension weed scientist), please drop me a note to let me know who else
>would be an appropriate source of this information.
>
>Please contact me if you have any questions.
>
>Happy Holidays!
>
>Regards,
>
>Sharie

Glenn E. Shewmaker, Ph.D.
Extension Forage Specialist
P.O. Box 1827
Twin Falls, ID 83303-1827

208-736-3600
Fax: 208-736-0843
Email: gshew@uidaho.edu

University of Idaho
College of Agricultural & Life Sciences

January 27, 2003

Mr. Glen Rogan
Monsanto Regulatory Affairs (BB3N)
700 Chesterfield Parkway
Chesterfield, MO 63017

Dear Mr. Rogan:

I am extension forage specialist with the University of Idaho. It is my opinion that:

1) Feral alfalfa as a weed species is not targeted for control on roadsides or other unmanaged areas in our state.
2) Although alfalfa is a species that is not targeted for control, 2,4-D would commonly be used to control other broadleaf weeds.
3) I would use 2,4-D or a variety of other herbicides which are available to control alfalfa if I suspected that the Roundup Ready trait was possibly present in feral alfalfa populations.

Sincerely,

Glenn E. Shewmaker

Glenn E. Shewmaker
Extension Forage Specialist

UW Extension

Department of Agronomy

University of Wisconsin–Extension
1575 Linden Drive
Madison, WI 53706
608-262-1390 / 1391
608-262-5217 (fax)
800-947-3529 (TTY)

December 26, 2002

Glen Rogan
Monsanto Regulatory Affairs (BB3N)
700 Chesterfield Parkway
Chesterfield MO 63017

Dear Dr Rogan:

I have been asked to comment on the presence and importance of feral alfalfa in Wisconsin. The questions asked and my responses are as follows:

1. To the best of your knowledge, is feral alfalfa a weed species that is controlled on roadsides or other unmanaged areas in your state?

I am not aware of any concerns of feral alfalfa in roadsides or similar areas. I have trained right –of – way personnel in our Applicator Training Program for 25 years and no one has ever commented on or asked for help in controlling alfalfa. In contrast, efforts are sometimes made to establish legumes along roadsides. In years past, birdsfoot trefoil was used and now it seems that clovers are more common. Thus, legumes along roadsides are viewed as desired species, with the occasional exception of sweet clover which can invade adjacent non-disturbed sites.

2. If alfalfa is a species that is controlled, what are the herbicides or cultural management practices that are currently used for its control?

No control is practiced. A further comment would be that if present in roadsides, alfalfa populations are low, further reducing the likelihood that it would be considered undesirable.

3. If you suspected that the Roundup Ready trait was possibly present in feral alfalfa populations, what herbicides or management practices would you use to control feral alfalfa?

The clear choices would be herbicides with a growth regulator mode of action for two reasons.

UW-Extension provides equal opportunities in employment and programming including Title IX and ADA.

1. They are selective which leaves all grass species. No one wants to eliminate all growing plants along roadsides and glyphosate would do so.

2. They are more effective. Glyphosate is fair at best in killing alfalfa in the spring and summer. Growth regulators like decamba, clopyrolid and 2, 4-D) are more effective.

Sincerely yours,

Jerry Doll

Jerry Doll
Extension Weed Scientist

University of Wisconsin-Madison

College of Agricultural and Life Sciences
Department of Agronomy
1575 Linden Drive
Madison, Wisconsin 53706-1597
Phone: 608-262-1390/1391
FAX: 608-262-5217

Improving Agriculture Through Crop Biotechnology, Genetics and Production Research

December 30, 2002

Glen Rogan
Monsanto Regulatory Affairs (BB3N)
700 Chesterfield Parkway
Chesterfield, MO 63017

Dear Sir:

Regarding your questions:

1) To the best of your knowledge, is feral alfalfa a weed species that is controlled on roadsides or other unmanaged areas in your state? No it is not specifically controlled on roadsides, except incidental to mowing which is usually sufficient to prevent seed set.
2) If alfalfa is a species that is controlled, what are the herbicides or cultural management practices that are currently used for its control? N/A
3) If you suspected that the Roundup Ready trait was possibly present in feral alfalfa populations, what herbicides or management practices would you use to control feral alfalfa? I would use 2,4-D or Banvel.

Sincerely,

Dr. Dan Undersander
Professor of Agronomy

cc Sharie Fitzpatrick

• *Providing equal opportunities in employment and programming.*

PENNSTATE

Crop and Soil Sciences
Cooperative Extension

(814) 865-2543
Fax: (814) 863-7043

The Pennsylvania State University
116 Agricultural Sciences and Industries Building
University Park, PA 16802-3504

December 20, 2002

Glen Rogan
Monsanto Regulatory Affairs (BB3N)
700 Chesterfield Parkway
Chesterfield, MO 63017

Dear Glen:

Below are the responses to your questions.

To the best of your knowledge, is feral alfalfa a weed species that is controlled on roadsides or other unmanaged areas in your state?

No

If alfalfa is a species that is controlled, what are the herbicides or cultural management practices that are currently used for its control?

Not Applicable

If you suspected that the Roundup Ready trait was possibly present in feral alfalfa populations, what herbicides or management practices would you use to control feral alfalfa?

Probably a spot treatment with Banvel and 2,4-D

Sincerely,

M. H. Hall

Marvin H. Hall
Professor of Forage Management

APPENDIX 4

Expert Testimony

Hybridization of *Medicago sativa* with *Medicago lupulina*

could be produced at all, which based on my first point seems very unlikely, then for hybridization to be a success under natural conditions, the following conditions would need to be met. Alfalfa pollen would need to be applied to the stigmas of black medic florets before the medic pollen was shed. Without a tripping mechanism like alfalfa, placing pollen on the stigma within the very small florets of black medic would be difficult. We do not know if stigmas of medic are receptive at this time. If alfalfa pollen were deposited simultaneously with or after black medic pollen shed, hybridization would be very unlikely, given the competition with self pollen. Or medic pollen would need to be transferred to alfalfa. Given that little pollen is produced by black medic and no tripping mechanism to effectively place the pollen on the pollinator, hybridization seems unlikely in the face of substantial amounts of alfalfa pollen that will also be present. In either case, a pollinator will need to transfer the pollen, but no data is present about whether any alfalfa pollinators visit black medic flowers. In casual observation, I have not noticed pollinators on black medic.

Third, black medic is quite distant from alfalfa evolutionarily. Its morphology has several major character differences with other *Medicago* species; for example, it is the only species in the genus with single seeded pods and a compact, clover-like inflorescence. Thus, hybridization between black medic and alfalfa would be unlikely based on evolutionary grounds, which supports the lack of conclusive evidence of artificial hybrids.

In summary, the reports of successful hybrids between black medic and alfalfa in the literature appear to be unreliable, and given the distinctive morphology and evolutionary distance between the species, hybridization would appear to be unlikely. I conclude that the possibility of natural crossing between the species to be essentially zero.

Please contact me if you have further questions.

Best regards,

E. Charles Brummer
Associate Professor

MONTANA STATE UNIVERSITY BOZEMAN

Office of the Vice President for Research, Creativity & Technology Transfer

P.O. Box 172460
Bozeman, MT 59717-2460
Fax (406) 994-2893
E-mail research@montana.edu

Research and Creative Activities	(406) 994-2891
Grants and Contracts	(406) 994-2381
Pre-Award Services	(406) 994-6240
Technology Transfer	(406) 994-7868

January 30, 2004

To: Glen Rogan
Monsanto Company
800 N. Lindbergh Blvd
Creve Coeur, MO 63167

From: Thomas J. McCoy, Ph.D.
Vice President for Research, Creativity
and Technology Transfer

Re: *Medicago Interspecific Hybridization*

You have put together an excellent document regarding *Medicago interspecific hybridization*. This is a through document and also completely accurate.

I agree with the statements made in this document and will also add some comments of my own.

As a result of 25 years of research with *Medicago* species I am totally convinced there is absolutely no way alfalfa will naturally hybridize with any of the annual *Medicago* species. Given the insurmountable sexual barrier between the perennial and annual *Medicago* species I have often stated that the annual *Medicago* species should be placed in a separate genus. I am also totally convinced that *M. sativa* will never hybridize in nature with *M. lupulina* and agree with Lesins' statement that the earlier reports of Southworh (1928) and Schrock (1943) are incorrect. I am certain that if molecular markers would have been able to be utilized they would have demonstrated these plants were self-pollinated and not hybrids.

During a six-year period (1980 – 1986) while I was employed as a Research Geneticist with USDA/ARS my group conducted massive experiments aimed at hybridizing alfalfa with other *Medicago* species. The results of this work have been summarized in your Table II-1. We made literally tens of thousands attempted hybridizatios for most of the species combinations we tried, including *Medicago lupulina*. Also given that we found that some species will only hybridize successfully if unequal ploidy levels were used, e.g. *M. sativa* x *M. papillosa* and *M. sativa* x *M. dzhwawakehtica* we even produced (using colchicines doubling) some *tetraploid M. lupulina*. Regardless of ploidy level combination *M. sativa 2x* and *4x* with *M. lupulina 2x* and *M. sativa 2x* and *4x* with *M. lupulina 4x* we were unsuccessful in even achieving successful fertilization.

The one possible route to a *M. sativa* x *M. lupulina* hybrid would be through somatic hybridization using protoplast fusion; however, this could hardly be considered as something possible in nature. Furthermore based on the results with somatic hybrids of other distantly related species such a somatic hybrid would likely be weak and sterile.

APPENDIX 5

Gene Flow in Alfalfa

Gene Flow in Alfalfa

Introduction

One area of interest regarding the application of biotechnology to the improvement of crops is the potential for movement of a gene into populations of wild relatives or to neighboring cultivated or feral plants via pollen movement. Alfalfa does not naturally hybridize with any other species in North America. Cultivated and feral (noncultivated) populations of alfalfa may inter-pollinate if requisite conditions and appropriate pollinator species are present and active. The purpose of this section is to provide background information on the biology and agronomics of alfalfa and its pollination characteristics, and summarize current knowledge regarding gene flow via pollen movement in alfalfa.

Alfalfa (*Medicago sativa* L.): Biology, Genetics and Pollination, Seed and Forage Management Practices

Alfalfa is a perennial forage crop species that is predominantly cross-pollinated and dependent upon bees for pollination. Unlike vegetable or grain crops, alfalfa forage fields are harvested at regular intervals throughout the growing season, which precludes prolific flowering and seed set (Sheaffer et al., 1988). Hence, some of the issues regarding uncontrolled proliferation of a trait will not impact alfalfa managed for forage or hay production because pollen is available to pollinators for a very limited duration and/or little to no ripened seed will be produced. Self-pollination can occur in alfalfa, but the occurrence is relatively low because of the interactions of self-incompatibility or self-sterility systems with severe inbreeding depression (Viands et al., 1988). Further, in a forage field, if viable seeds are produced and dropped to the soil, the resultant germlings are unlikely to be successful in self-perpetuation because of intense interplant competition and effects of autotoxicity (Tesar and Marble, 1988).

Alfalfa is an exclusively insect-pollinated crop that, unlike other insect-pollinated crops, is pollinated by a small number of insect species, namely, certain bee species. Alfalfa flowers have an explosive tripping mechanism that may be triggered by bees visiting the flower to collect nectar or pollen. After it is tripped, the stigma of the flower becomes lodged into the groove of the standard petal of the flower. Tripped flowers cannot be fertilized again. Because of the nonreversible tripping mechanism within the alfalfa flower, each alfalfa bloom may be pollinated only a single time, by a single pollinating insect. Flowers do not shed pollen to the wind. After pollination, alfalfa seed requires four to six weeks of adequate growing conditions to ripen. Rainfall during the ripening time will cause decreased seed yield and poor seed quality (e.g., reductions in seedling vigor and reduced percent germination because of fungal pathogen infection of the seed, or seed will sprout prematurely and die while it is still in the pod). Commercial production of the alfalfa seed crop, therefore, is largely confined to the western regions of the United States where late season rain is unlikely and irrigation is used.

In most regions of the world, alfalfa is cultivated for its animal feeding value and is grazed or mechanically harvested as haylage, greenchop, forage or hay. Hay may be further processed into dehydrated bales, pressed cubes or blended into feed concentrates.

As an animal feedstuff, alfalfa's optimum economic value occurs just as the vegetative growth period is transitioning into the flowering phase. Flowers and developing embryos, although they may be transiently present in a field managed as forage, are removed along with the vegetative growth before pollination or seed ripening. Alfalfa managed for forage production is cut on a calendar schedule with multiple harvests within a growing year (two to eleven cuttings per year depending upon the geographic region). The harvest interval is dependent upon weather conditions and optimally coincides with the early flower to 10% bloom growth stage. For many regions, this interval is 28-35 days in length during the growing season, an interval inadequate to initiate full bloom or ripen seed. Alfalfa requires four or more weeks of adequate temperature and photoperiod to grow and form floral buds and an additional four to six weeks to form mature seed on pollinated flowers. Forage harvest, by definition, periodically removes the entire plant canopy where blooms or seed might form. Growth of the canopy must be reinitiated from vegetative crown buds (as occurs in the Spring) or from the elongation of lower stem axillary buds.

Gene and Pollen Flow in Alfalfa

Discussion of gene flow should begin by distinguishing between gene flow and pollen flow per se. In alfalfa, pollen flow may occur any time a pollinating insect carries pollen away from source plants. Events subsequent to this determine whether pollen-mediated gene flow will occur. Gene flow cannot occur without the simultaneous occurrence of *all* of the following conditions: 1) presence of source blooms, 2) active and appropriate insect pollinators, 3) receptive blooms outside the cultivated area and within the flight radius of the insect and, 4) pollinated blooms must be allowed four to six weeks to ripen seed. Only ripened, mature seed that develops has any long-term impact on the unintended flow of genes in alfalfa. While true embryos are found in developing alfalfa pods approximately 120 hours after fertilization (Bass et al., 1988), it takes a minimum of four weeks for embryos to ripen mature seed under optimal seed production conditions. Because alfalfa managed for forage production is typically cut on a 28- to 35-day cycle, few if any mature seeds will be produced. Properly managed forage production fields will have few blooms because the plots will be harvested according to optimal forage management practices (i.e., forage harvests are made at the early flower to 10% bloom stage). Hence, gene flow to commercial alfalfa seed stock fields or to small, feral populations is of central concern when discussing gene flow.

Two separate groups of recent studies have been conducted which examine the incidence of pollen-mediated gene flow in alfalfa. One group of studies examined the movement of the *cp4 epsps* gene from Roundup Ready alfalfa to conventional alfalfa under commercial seed production conditions and used introduced leafcutter (*Megachile rotandata*) bees as pollinators during the 2000, 2001 and 2002 growing seasons (McCaslin et al., 2001; Fitzpatrick et al., 2002). The studies were conducted with the purpose of gaining a more thorough understanding of alfalfa pollen flow dynamics in commercial alfalfa seed production settings so that alfalfa seed producers may set reasonable and informed varietal isolation standards for the production of high quality conventional and biotech alfalfa seed products.

A second group of studies, conducted by St. Amand et al. (2000), studied the movement of a native alfalfa marker genes from alfalfa fields to simulated and natural feral plants (see summary and discussion, below). Taken together, the studies provide data that may be used to estimate the potential for pollen-mediated gene flow to uncontrolled, feral alfalfa populations and between commercial-scale seed or forage production fields. Key conclusions from these studies, which are discussed in detail below, are:

- Spatial isolation is an effective means to mitigate pollen-mediated gene flow in alfalfa to levels observed for other crops.
- Mitigation of pollen-mediated gene flow in alfalfa is possible if adequate spatial isolation is used in combination with observance of other recommended agronomic and pollinator management practices. The presence of a border crop is not required to mitigate pollen-mediated gene flow if adequate spatial isolation is used.
- Borders (alfalfa or non-alfalfa) may be a useful supplemental method to further reduce pollen flow between small or inadequately spatially isolated fields, however, additional data is needed to clarify the effectiveness of borders in bee-pollinated crops.
- Long-range (0.5-0.6 mile) gene flow is possible from commercial-scale seed and hay fields to cultivated or feral alfalfa that is allowed to ripen mature seed.

In most alfalfa seed-growing areas, naturally occurring populations of those species of bees capable of tripping and producing cross-pollination are either nonexistent or in such low number that commercial production of seed alfalfa would be impossible without the deliberate production, introduction, and management of certain bee species to provide adequate pollination (Arnett, 2002). The three species of bees used for this purpose include the honey bee (*Apis mellifera*), the alkali bee (*Nomia melanderi*), and the alfalfa leafcutting bee (*Megachile rotundata*). Although maximum foraging radius for each of the three species is dependent on the abundance of nectar and pollen resources, leafcutter bees are considered to have the shortest routine foraging distance (<1/4 mile) followed by the honey bee (ca. 1 mile) and alkali bee (<3 miles). A general recent review of principles of bee pollination may be found in *Crop Pollination by Bees* (Delaplane and Mayer, 2000).

Review and discussion of findings: Pollen-mediated gene flow using *cp4 epsps* as a marker gene

A group of four studies conducted over a three-year period by Forage Genetics International (McCaslin, et al. 2001, Fitzpatrick et al., 2002) measured pollen-mediated gene flow between genetically marked gene source fields (Roundup Ready alfalfa) and nonmarked pollen trap seed production plots (conventional alfalfa).

Three studies were conducted in an Idaho irrigated alfalfa seed production area under agronomic management typical for the Pacific Northwest Region where leafcutter bees are the introduced pollinator. Location, isolation distances, plot size, the number of replicate plots, cardinal direction of the trap relative to the source, and inter-plot land cover are given in Table 1. In 2000, all but one of the plots was separated from the source plot by fallow, simulating a worst-case inter-plot management where bees would

have no physical or visual barriers to movement. In contrast, all other trap plots during 2000-2002 were spatially isolated by land planted to a variety of crops and/or terrain typical for the region (e.g., winter wheat, onions, sweet corn and roadways, etc.). All alfalfa plots (0.03 to 2 acres each) were stocked with leafcutter bees and were managed and pollinated according to the recommended practices for commercial seed production. In addition to the high density of intentionally introduced leafcutter bees (ca. 2 gallons of loose cell pupae/A), a low number of honeybees were observed pollinating alfalfa flowers in the plots. A greenhouse seedling assay was used to measure gene flow between source and trap plots wherein, a subsample of between 4,200 to 30,000 seed from each trap plot replicate were assayed for the presence or absence of Roundup Ready trait. The upper bound of true gene flow was calculated using the method of Remund et al. (2001). Specifically, the sum of the Roundup Ready seed and the total number of seed tested at that distance per location per year were compared, the 99.9% confidence interval upper limit was calculated and used to estimate the upper bound of true gene flow for that isolation distance.

Results from these studies demonstrated that pollen-mediated gene flow diminished with increasing distance from the source (Table 2 and Figure 1). Gene flow among the fallow-surrounded, worst-case management plots was 1.39%, 0.32% and 0.07% at 500, 1000 and 1500 ft, respectively. No gene flow (0.0000%) was detected at 2000 ft in 2000, however, one of 30,000 seeds tested (0.032%) carried the trait when isolation was ½ mile (2640 ft) in 2002. Gene flow was not detected at ¾ or 1 mile isolation distances. Observed gene flow (Y_{obs}) is described by the equation, $Y_{obs}= (1x10^{10}) (X^{-3.6262})$, $R^2=0.9391$; and, the upper bound of the 99.9% confidence interval for gene flow, Y_{CI} is calculated as, $Y_{CI}= (4x10^{6})(X^{-2.3673})$, $R^2=0.9728$.

Findings from these replicated studies demonstrate that alfalfa seed production fields may be effectively insolated from undesired pollen flow using spatial isolation and adherence to currently recommended pollinator management practices. Currently, 165 ft (50 m) and 600 ft (183 m) isolation are required for production of certified and foundation class alfalfa seed, respectively and foundation fields less than 2 acres in size are required to have 900 ft (274 m) isolation (Brown et al., 1986). As all trap plots were <2 acres in size, at 900 ft isolation the 99.9% confidence interval upper bound for gene flow (Y_{CI}) and mean observed gene flow may be estimated as approximately 0.4 and 0.2%, respectively. These values would indicate that current foundation seed field isolation standards would be sufficient to produce foundation class seed with >99% varietal purity.

It is interesting to note that the amount of pollen-mediated gene flow in alfalfa (an insect-pollinated crop), as observed by Forage Genetics 2000-2002, approximates that observed for corn (a wind-pollinated crop). For instance, in a study conducted by Haskell and Dow (1951), outcrossing in corn was 2.33, 0.48 and 0.20% for plants located 125, 300 and 500 m from source plants, respectively.

Also in 2002, a fourth Forage Genetics field study was conducted in which, pollen-mediated gene flow from a worst-case forage production source to nearby alfalfa seed production trap plots was measured. The plots were grown near Touchet, Washington, and separated by fallow or mowed grass alleys. During June and July (i.e., the peak pollinator activity period for the region), the forage production gene source plot was

intentionally allowed to produce copious and sustained bloom (50% bloom stage when harvested), thereby simulating a very poorly managed, low-forage quality hay field. Nearby, unmarked seed production trap plots (conventional alfalfa) were pollinated with introduced leafcutter bees. Alfalfa gene flow was measured with 150 and 300 ft spatial isolation distances (2 replicates each) from the Roundup Ready marked forage source plot (Table 2). These distances were selected so as to bracket the certified class seed field isolation distance of 165 ft.

Observed means of gene flow with 150 ft (46 m) or 300 ft (92 m) isolation were 0.21 and 0.23%, respectively and, the upper bounds of true gene flow (99.9% confidence) were 0.30 and 0.32%, respectively (Table 2). These values are less than or similar to values observed for seed plot to seed plot flow with 500 to 1000 ft isolation (Table 2). The data indicate that poorly managed hay production fields located near to alfalfa seed production fields, have far less potential for gene flow than similarly located seed production fields (Figure 1).

Review and discussion of related literature

Pollen traps have been shown to be effective for herbicide-tolerant canola (Staniland et al., 2000). In alfalfa, pollen-mediated gene flow field studies were conducted by St. Amand et al. (2000) where they utilized two different native marker techniques with three study objectives, specifically the measurement of pollen-mediated gene flow: 1) within seed fields, 2) from fields to roadside alfalfa and, 3) among feral alfalfa plants.

In the first study, a naturally occurring variant of the alfalfa glutamine synthase (GS) gene was used as a marker to monitor within seed field gene flow from marked source plants to surrounding plants not containing the variant gene. Minor gene flow (0.2%) was detected 4 m or less from the 1 m^2 (nonreplicated) source plot and zero gene flow was detected $\geq$6 m from the source plants. The authors discuss that these results would indicate that pollen traps or borders might be effective to mitigate alfalfa pollen flow. It should be noted that in this study, the area covered by the pollen trap plants from which +GS seed was harvested (i.e., the nonmarked alfalfa border plants located $\leq$ 6 m from the edge of the 1 m^2 gene source plot) was 134 times greater than the area occupied by the single block of gene source plants—a ratio that would be unmanageable for commercial alfalfa seed or forage production. Same-species border crops would be problematic for regulated and/or commercial alfalfa seed fields where genetic purity of the target seed product is of central concern, i.e., the use of a nontransgenic synthetic alfalfa variety to surround a Roundup Ready seed field would reduce trait purity, reduce varietal purity and likely preclude varietal certification because spatial isolation standards would not be met. Non-alfalfa borders may have incompatible agronomic managment and/or irrigation requirements when grown in the same field with alfalfa seed production which would impact the crop's potential efficacy as a pollen trap. Additionally, because alfalfa is not a preferred source of pollen for pollen-collecting bees (Arnett, 2002), the non-alfalfa border might be more attractive to bees than the alfalfa. This could reduce pollinator activity on the alfalfa and/or unintentionally attract more rouge or scout bees from distant colonies than the non-bordered alfalfa alone would attract and inadvertently result in longer-distance pollen-mediated gene flow. Another challenge would be managing species purity during seed harvest and cleaning. If seed from the border species mixed

into the alfalfa seed product, seed cleaning costs would increase or, if the border crop seeds were very difficult to segregate from the alfalfa seed, even a slight decrease in species purity would significantly impair the alfalfa seed's value or marketability. For example, the presence in a lot of alfalfa seed of other small-seeded legume seeds, such as birdsfoot trefoil or red clover, negatively impacts species purity. In significant quantities, it will prevent alfalfa seed certification and decrease the alfalfa seed lot's value.

In the second and third study components, St. Amand et al. (2000) used two marker systems, the GS marker and a RAPD marker, to measure gene flow to unclipped ramets of a single, marker-free, genetic clone planted as twelve–1 m^2 pollen traps along a roadside at each of four locations. The pollen traps, intended to simulate feral alfalfa, were distributed along a single vector at each location at increasing intervals from each of the pollen sources (i.e., 0, 20, 40, 60, 80, 100, 200, 300, 400, 500, 750 and 1000 m distance). Source fields were managed either for seed or for hay production and were considered either commercial-scale (ca. 0.13 ha or 1.74 ha, for seed or hay, respectively) or research-scale (2 m^2 for both seed and hay). The RAPD or GS markers were used as the pollen-tracking system for the commercial- or research-scale plots, respectively. Plots were located in the states of Washington and Kansas. The small and large seed-production plots in Washington were stocked with leafcutter bees, while all other plots relied on natural populations of native bees for pollination.

Data indicated that pollen flow from research plots was minimal, with 1% to 2% flow at 0 to 100 m and no flow was detected at distances greater than 200 m. These data would support the conclusion that 200 m (640 ft) isolation zone (without border crop) would mitigate pollen outflow from small plots.

Gene flow from the four large fields to the 1 m^2 pollen traps was notably higher, with 25% to 35% outcrossing measured at 1000 m. Data are presented for percent outcrossing, but the gross number of seed produced on each trap or trap plant (the sample size) was not reported. In other words, if few seeds were produced a very low number of outcrosses would equal 25% (e.g., 10/40), whereas, if normal seed set occurred, numerous outcrosses and multiple bee visits would have been necessary for 25% outcrossing (e.g., 100/400). The number of seed produced on the trap plants would be important for direct comparison of the clone of artificial feral plants to naturally occurring feral plants. Plants within and among traps were of the same genotype (genetic clone), which was likely, self-incompatible to some degree. Therefore, the plants within a trap were unlikely to form seed with any plant *except* one whose pollen was carried from the source field. Therefore, interpretation of this data is not straightforward. While use of the clonal-trap is an appropriate technique to measure the maximum distance of pollen flow, it may result in artificially high estimates for gene flow to natural (non-clonal) feral alfalfa plants. It may be assumed that the naturally occurring outcrossing mechanism prevalent in cultivated alfalfa is also prevalent in feral alfalfa populations. Therefore, the potential for self-incompatibility among the trap clone ramets (nearest neighbors) may have acted to select for pollen carried from nongenetically related individuals. Thus, the use of the clones may have artificially skewed the outcrossing frequency toward long-distance pollen sources relative to what may have been found if the trap plants had been cross-compatible with each other (nearer sources) as is found in

nature. In a diverse, heterogeneous, natural, feral population, there would be no bias for or against any source of non-self pollen and proportionately more progeny would trace to pollen carried shorter distances.

Other factors that may have enhanced flow to the trap plots include the relative attractiveness of the plants with respect to other plants along the roadsides and the close proximity of the traps. First, the close proximity and unidirectional, regular placement of the twelve trap plots may have influenced bee movement by forming a highly attractive, pollinator-conduit or bridge from one trap to the next. It is important to note that the relatively high frequency of outcrossing reported at 1000 m took place at the distal end of the regularly spaced, unidirectional trap plots. Although the findings may be relevant to high-density feral populations, it would be inappropriate to apply the findings to long-distance flow with 1000 m of true isolation from the nearest alfalfa pollen source (e.g., between 1000 m isolated seed fields). Secondly, the trap plants were not clipped or managed in any way that would have made the twelve 1 m^2 areas less attractive to bees and this would simulate feral alfalfa only if it were growing in unmanaged roadsides or wild areas.

The key results from these studies show that gene flow is extremely minor from small-scale research plots and that long-distance gene flow from commercial-scale field sources is possible.

References Cited

Arnett, W.H. 2002. Alfalfa pollinators. Report prepared by Dr. Harold Arnett, Professor of Entomology (Emeritus), University of Nevada, for Forage Genetics International. (Copies available on request.)

Bass, L.N., C.R. Gunn, O.B. Hesterman, and E.E. Roos. 1988. Seed physiology, seedling performance, and seed sprouting. *In* A.A. Hanson, D.K. Barnes and R.R. Hill, Jr. (ed.) Alfalfa and alfalfa improvement. ASA-CSSA-SSSA, Madison, WI.

Brown, D. E., Grandstaff, E. L., Hanna, M. R., Hanson, A. A., Marble, V. L., Moutray, J. B. 1986. Committee Report on Alfalfa Field Isolation. Page 123 *In*: Report of the Thirtieth North American Alfalfa Improvement Conference. July 27-31, 1986.

Delaplane, K. S. and Mayer, D. F. 2000. Crop Pollination by Bees. CABI Publishing Company. 344 pp.

Fitzpatrick, S., Reisen, P. and McCaslin, M. 2002. Alfalfa pollen-mediated gene flow studies, 2000-2001. Page 23 In: Report of the 38th North American Alfalfa Improvement Conference. Sacramento, California.

Haskell, G. and Dow. P. 1951. Studies with sweet corn. V. Seed setting with distance from pollen source. Empire Journal of Experimental Agriculture (London) 19: 45-50.

McCaslin, M., Fitzpatrick, S., Reisen, P. 2001. Summary of 2000 alfalfa pollen flow experiment. 8 pages. [http://foragegenetics.com/Gene%20Flow%20in%20alf%202000.pdf] Accessed on 4/19/03

Remund, K. M., Dixon, D. A., Wright, D. L., and Holden, L. R. 2001. Statistical considerations in seed purity testing for transgenic traits. Seed Science Research 11: 101-119.

St. Amand, P.C., D.Z. Skinner and R.N. Peaden. 2000. Risk of alfalfa transgene dissemination and scale-dependent effects. *Theor. Appl. Genet*. 101: 107-114.

Sheaffer, C.C., Lacefield, G.D. and Marble, V.L. 1988. Cutting schedules and stands. Pages 411-437 *In*: Alfalfa and Alfalfa Improvement- Agronomy Monograph no. 29. Hanson, A. A. editor. ASA-CSSA-SSSA, Madison, WI. 1084 pp.

Staniland, B.K., McVetty, P.B.E., Friesen, L.F., Yarrow, S., Freyssinet, G., Freyssinet, M. 2000. Effectiveness of border areas in confining the spread of transgenic *Brassica napus* pollen. *Can. J. Plant Sci.* 80: 521-526.

Tesar, M. B. and Marble, V. L. 1988. Alfalfa establishment. Pages 303-332. *In*: Alfalfa and Alfalfa Improvement- Agronomy Monograph no. 29. Hanson, A. A. editor. ASA-CSSA-SSSA, Madison, WI . 1084 pp.

Viands, D.R., Sun, P. and Barnes, D.K. 1988. Pollination control: mechanical and sterility. p. 931-960. *In*: Alfalfa and Alfalfa Improvement- Agronomy Monograph no. 29. Hanson, A. A. editor. ASA-CSSA-SSSA, Madison, WI. 1084 pp.

Table 1. Summary of pollen-mediated gene flow study design for experiments conducted by Forage Genetics International using *cp4-epsps* as the marker gene in the pollen source plot. Location, isolation distance between trap and source, number of replicates per distance, plot size, trap plot cardinal direction from source and inter-plot land cover are given. USDA Notification Numbers: 00-053-07n (2000); 01-009-08n (2001); 02-020-09n (2002).

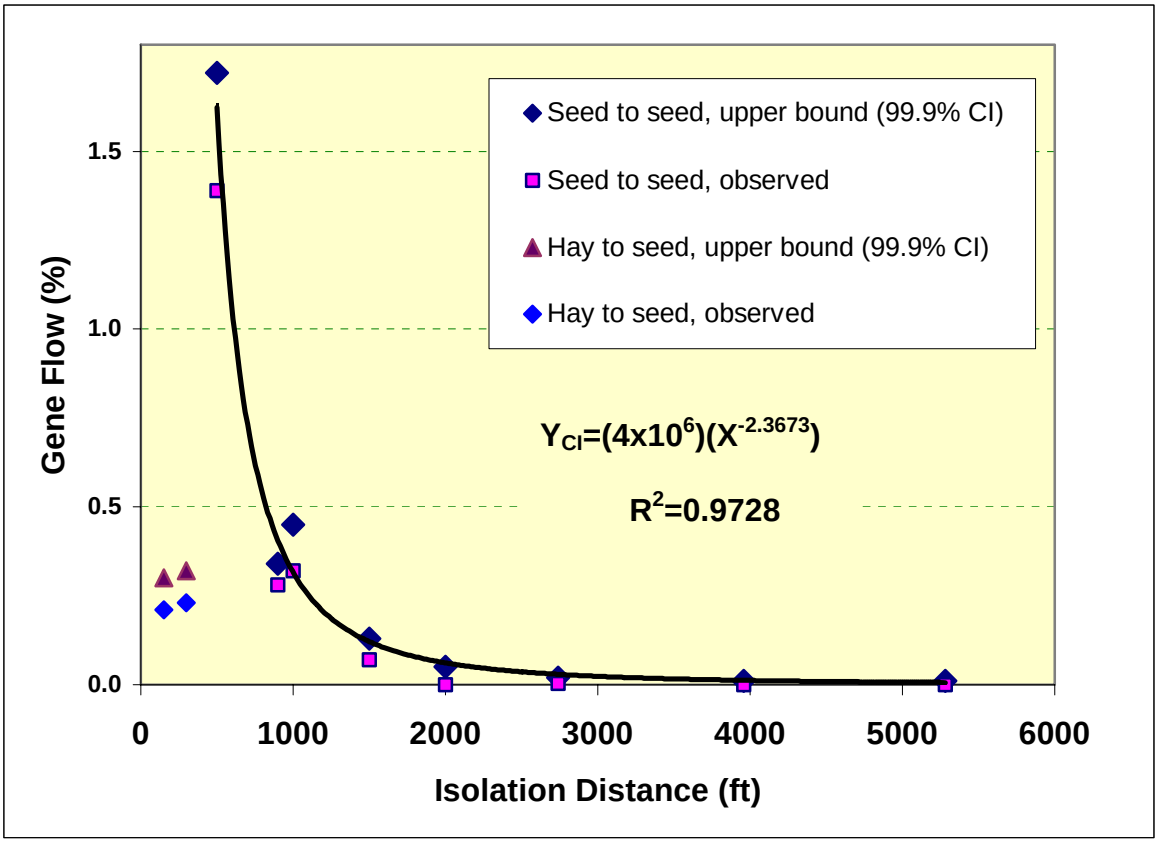
Year, gene source management type and location:	2000 Study	2001 Study	2002 Studies	
	Seed field Idaho	**Seed field** Idaho	**Seed field** Idaho	**Forage field** Washington
ISOLATION DISTANCE				
0 ft	Source (1 A)	Source (1.6 A)	Source (1 A)	Source (0.1 A)
150 ft	-	-	-	2 reps (0.01 A) [c] Rep 1: S. Rep 2:N.
300 ft	-	-	-	2 reps (0.01 A) [c] Rep 1: E. Rep 2: W.
500 ft	4 reps (0.03 A) [c] 4 reps: N.	-	-	-
900 ft	-	2 reps (0.7 – 1.6 A) [a] Rep 1: N. Rep 2: N.E.	-	-
1000 ft	4 reps (0.03 A) [c] 4 reps: N.	-	-	-
1500 ft	4 reps (0.03 A) [c] 4 reps: N.	2 reps (1.6 A) [a] Rep 1: W. Rep 2: N.W.	2 reps (1 A) [a, b] Rep 1: S.W. Rep 2: S.E.	-
2000 ft	1 rep (2 A) [a] N.W.	-	-	-
2640 ft (1/2 mi)	-	-	2 reps (1 A) [a, b] Rep 1: S.W. Rep 2: S.E.	-
3960 ft (3/4 mi)	-	-	2 reps (1 A) [a, b] Rep 1: S.W. Rep 2: S.E.	-
5280 ft (1 mi)	-	-	2 reps (1 A) [a, b] Rep 1: N.W. Rep 2: S.E.	-

Inter-plot land cover:
[a] Various crop species typical for the area (e.g., onions, corn, wheat, etc.)
[b] Roadway
[c] Fallow

Table 2. Summary of pollen-mediated gene flow data for experiments conducted by Forage Genetics International using *cp4-epsps* as the marker gene in the pollen source plot. Gene flow was measured by planting trap plot seed in a greenhouse glyphosate tolerance assay and applying Roundup agricultural herbicide to detect the presence of the source plot pollen marker gene (i.e., the Roundup Ready phenotype). Data is presented for mean observed gene flow (%) and the estimated upper bound of true gene flow (% in parentheses) based on the total number of seed assayed per distance per year (i.e., the 99.9% confidence interval upper limit) following the method of Remund et al. (2001). USDA Notification Numbers: 00-053-07n (2000); 01-009-08n (2001); 02-020-09n (2002).

Year, gene source management type:	2000 Study	2001 Study	2002 Studies		Mean Across 1-3 Years
	Seed field	Seed field	Seed field	Forage field	
Isolation distance					
150 ft	-	-	-	0.21% (0.30%)	**0.21% (0.30%)**
300 ft	-	-	-	0.23% (0.32%)	**0.23% (0.32%)**
500 ft	1.39% (1.72%)	-	-	-	**1.39% (1.72%)**
900 ft	-	0.28% (0.34%)	-	-	**0.28% (0.34%)**
1000 ft	0.32% (0.45%)	-	-	-	**0.32% (0.45%)**
1500 ft	0.07% (0.17%)	0.13% (0.17%)	0.032% (0.06%)	-	**0.08% (0.13%)**
2000 ft	0.00% (0.05%)	-	-	-	**0.00% (0.05%)**
2640 ft (1/2 mi)	-	-	0.003% (0.02%)	-	**0.003% (0.02%)**
3960 ft (3/4 mi)	-	-	0.0000% (0.01%)	-	**0.0000% (0.01%)**
5280 ft (1 mi)	-	-	0.0000% (0.01%)	-	**0.0000% (0.01%)**
Mean no. seed tested per trap:	14, 750	41,250	60,000	32,400	

FIGURE 1. Three-year summary of alfalfa gene flow for seed production using leafcutter bees with 150 ft to 1 mile isolation distance from gene source plot. Values given are the observed and upper bound of true gene flow (99.9% confidence) based on all data collected during 2000, 2001 and 2002 field studies. The equation for, Y_{CI} = upper bound of 99% confidence interval at X (ft) isolation distance was calculated using seed production source plots only. USDA Notification Numbers: 00-053-07n (2000); 01-009-08n (2001); 02-020-09n (2002).



Addendum 1

Petition for Determination of Nonregulated Status: Roundup Ready® Alfalfa (*Medicago sativa* L.) Events J101 and J163

USDA Petition Number: 04-110-01p

Monsanto Number: 04-AL-116U

OECD Unique Identifiers:
Event J101 - MON-ØØ1Ø1-8
Event J163 - MON-ØØ163-7

PART ONE:

Request from USDA: Please submit information on the likelihood that RR alfalfa seed will be used for sprouting and then consumed by humans.

Response: Information on the safety of Roundup Ready alfalfa has been submitted to the U.S. Food and Drug Administration (FDA BNF# 0084) and is currently being evaluated. FDA has previously assessed the safety of the CP4 EPSPS protein that is present in other Roundup Ready crops. In addition, EPA has granted an exemption from the requirement for a tolerance for the CP4 EPSPS protein [FR 61(150): (40338)]. Given the safety of the introduced protein and the substantial equivalence of harvested material, we have concluded that Roundup Ready alfalfa is as safe and nutritious as conventional alfalfa for food and feed uses. This would include the potential use of Roundup Ready alfalfa seed for production of sprouts. However, the likelihood that the trait would be intentionally used for the production of sprouts is low for the reasons given below.

All Roundup Ready alfalfa seed will be produced under production contracts and field management practices that will preclude the legal sale of the seed for food use. Under the terms of the production contract, the seed will be produced exclusively for field planting stock purposes with all seed production (for the foreseeable future) occurring exclusively in the United States. Only seed producers that are trained, licensed and contracted to produce Roundup Ready alfalfa seed will be authorized to do so. As described in Section F. of USDA petition number 04-110-01P, forage producers who purchase Roundup Ready alfalfa seed will be required to sign a grower agreement that expressly prohibits the production of seed. Roundup Ready alfalfa seed will be commercially available as registered alfalfa varieties that will be clearly identified as a Roundup Ready variety on each seed bag, seed tag and on the purchase agreement (limited use license). Alfalfa seeds for planting purposes are commonly coated with seed treatments as a means to enhance stand establishment (Brick, 2002) or to uniquely identify certain proprietary varieties. All Roundup Ready alfalfa seed will be pre-treated with a colored coating that will contain bacterial (*Sinorhizobium meliloti*) inoculant to promote nodulation and/or a fungicide such as metalaxyl for control of seedling damping off fungi. Because treated seeds and seed produced for planting purposes should not be used for sprouting purposes (DeWaal, 1998; Oregon State University, 2004), the coating applied to Roundup Ready alfalfa seed along with packaging in labeled seed bags will uniquely identify seed that contains the trait, and preclude its use as a starting material for the production of alfalfa sprouts.

The vast majority of alfalfa seed that is produced in the U.S. is for planting purposes with a relatively small amount (estimated to be approximately 7%) used for the production of sprouts (Bass et al., 1988). A considerable amount of alfalfa seed is also imported from outside of the U.S. specifically for sprout production (FDA, 1999). Alfalfa sprouts are the most common form of green sprouts available to consumers. In 1998 the total U. S. market for all types of sprouts was valued at approximately $25 million with over 400 growers producing 300,000 tons of sprouts annually (Kurtweil, 1999). Outbreaks of food-borne illness associated with sprouts containing the microbial pathogens *Salmonella* spp, *Escherichia coli* O157:H7 or

Listeria monocytogenes have resulted in greater scrutiny of seed production practices in recent years and heightened the awareness of seed sources used for sprouting (FDA, 1999; CFIA, 2001). Seed have been identified as the primary source of these microbial contaminants (Puohiniemi et al., 1991; CDC, 1997; Mahon et al., 1997). Therefore, the sprouting industry endorses the use of certified sprouting seed to avoid these outbreaks (International Specialty Supply, 2004). Criteria evaluated for certification include seed production practices such as field history, pesticide/herbicide use and origin of seed. While it is not known how widely certified seed are used by the industry, according to the U.S. FDA, approximately 83 % of sprout manufacturers surveyed reported using traceable seed sources, thus confirming that the industry is knowledgeable of the source of seeds used for sprout production (FDA, 1999).

Roundup Ready alfalfa will be marketed only when a consultation with FDA has been completed allowing for food and feed use of the product. It is not the intention of Monsanto or FGI to introduce Roundup Ready alfalfa for sprout seed production. It is unlikely that sprout seed producers and sprout producers will be impacted by the introduction of Roundup Ready alfalfa because multiple measures will be implemented by both companies to avoid this use. These measures include, grower agreements and seed production contracts specifically limiting use of the crop for forage and seed for planting purposes, respectively, and colored seed inoculants/treatment and seed packaging clearly identifying the material as Roundup Ready alfalfa seed. Sprout producers and sprout seed producers purchasing seed will be able to avoid Roundup Ready alfalfa seed because of the colorant, seed bag label and mandatory grower agreement. Sprout seed producers who plant conventional alfalfa seed will be able to avoid the trait through appropriate isolation of their seed production fields from other seed production fields. Furthermore, systems are in place in the spouting industry to help maintain the identity and suitability of sprouting seed, including seed certification. In combination, these systems will effectively limit the presence of Roundup Ready alfalfa in seed for sprouting purposes.

References, Part One

Bass, L.N., Gunn, C.R., Hesterman, O.B., and Roos, E.E. 1988. Seed physiology, seedling performance, and seed sprouting. P. 961-979. *In* Hanson, A.A., Barnes, D.K., and Hill Jr., R.R. (ed.) Alfalfa and Alfalfa Improvement. ASA-CSSA-SSSA, Madison, Wisconsin. 1084 pp.

Brick, M.A. 2002. Soil; Legume Seed Inoculants. Colorado State Cooperative Extension Crop Series No. 305. http://www.ext.colostate.edu/pubs/crops/00305.pdf [accessed 9/2004].

Centers for Disease Control and Prevention. 1997. Outbreaks of *Escherichia coli* O157:H7 associated with eating alfalfa sprouts - Michigan and Virginia, June-July 1997. Morbid. Mortal. Weekly Rep. 46: 741-744.

CFIA. 2001. Canadian Food Inspection Agency. Draft code of practice for hygienic production of sprouted seeds. http://www.inspection.gc.ca/english/plaveg/fresh/sprointe.shtml [accessed 9/2004].

DeWaal, C.S. 1998. Remarks of Caroline Smith DeWaal Director of Food Safety FDA's Public Meeting to Review at the Current Science Relating to Sprouts and Needed Control Measures. http://www.cfsan.fda.gov/~lrd/fr991027.html [accessed 9/2004]

FDA. 1999. Microbiological Safety Evaluations and Recommendations on Sprouted Seeds. U.S. Food and Drug Administration. National Advisory Committee on Microbiological Criteria for Food. http://vm.cfsan.fda.gov/~mow/sprouts2.html [accessed 9/2004]

International Specialty Supply, web site. http://www.sproutnet.com/seed_certificate.htm [accessed, 8/2004]

Kurtweil P. 1999. Questions keep sprouting about sprouts. FDA Consumer News; 33: 18-22. http://www.fda.gov/fdac/features/1999/199_sprt.html [accessed 9/2004]

Mahon, B.E., A. Ponka, W. Hall, K. Komatsu, L. Beuchat, S. Shiflett, A. Siitonen, G. Cage, M. Lambert, P. Hayes, N. Bean, P. Griffin, and L. Slutsker. 1997. An international outbreak of *Salmonella* infections caused by alfalfa sprouts grown from contaminated seed. J. Infect. Dis. 175:876-882.

Oregon State University. 2004. Commercial Vegetable Production Guides; Sprout Production. http://oregonstate.edu/Dept/NWREC/beansprt.html [accessed 9/2004]

Puohiniemi, R., T. Heiskanen, and A. Siitonen. 1997. Molecular epidemiology of two international sprout-borne *Salmonella* outbreaks. J. Clin. Microbiol. 35:2487-2491.

PART TWO:

Request from USDA: P 324 - Valizadeh, M., Kang, K.K., Kanno, A., and Kameya, T. 1996. Breeding Science 46:7-10. Please provide title and correct journal name, if incorrect.

Response: The correct citation is listed below.

Valizadeh, M., K-K. Kang, A. Kanno, and T. Kameya. 1996. Analysis of genetic distance among nine *Medicago* species by using DNA polymorphisms. Breeding Sci. 46(1): 7-10.

PART THREE:

Request from USDA: P 384 – William Curran letter: Please provide the results of 2003 trials mentioned in the letter.

Response: The results of the trial mentioned by Dr. Curran are summarized on the following four pages.

Controlling Roundup Ready Alfalfa in No-till Roundup Ready Corn

Jennifer Ralston
Technical Development Manager
Monsanto Company

Introduction

Alfalfa is not considered to be a serious weed problem in corn as there are several effective methods (tillage and herbicides) for alfalfa stand removal prior to planting corn. Several herbicides are also labeled for control of alfalfa in corn.

The use of Roundup agricultural herbicides applied pre-harvest to alfalfa prior to rotating to corn in the spring is becoming more common in the northeastern and midwestern U.S. This allows a final crop to be harvested as hay or silage shortly after the herbicide application while suppressing the alfalfa stand, and controlling weedy grasses and broadleaves that may be present. When Roundup Ready alfalfa is introduced, effective programs to control remaining alfalfa plants in the subsequent conventional or Roundup Ready corn crop will need to be identified for growers that choose this rotation. The objective of this study was to evaluate herbicides in no-till corn for control of Roundup Ready alfalfa.

Materials & Methods

The study was conducted in Centre County, Pennsylvania in the spring and summer of 2004 under USDA Notification Number 02-051-20n. The Roundup Ready alfalfa was established in the spring of 2002 and was going into its third year of production. Each plot was 300 square feet, measuring 10 feet wide by 30 feet long. The alfalfa was cut for the final time on May 18, 2004. Roundup Ready corn was subsequently planted into the alfalfa field using a no-till planter on May 24, 2004. Several different herbicide products that are labeled for use in corn were applied at two different timings in-crop for control of alfalfa (Table 1). Ammonium sulfate was added to the spray solution for all treatments that included Roundup Weather MAX herbicide (treatments 1-9). Treatments one through three were applied one day after planting (DAP) on May 25, 2004 and treatments four through nine were applied to corn at the five-leaf stage on June 2, 2004. Corn injury was evaluated as percent phytotoxicity on June 15, 2004, which was 21 days after the first application timing and 13 days after the second application timing. Alfalfa control was rated as percent control compared to the check plots (treatment 1) at 7, 21 and 34 days after the first application timing. Clopyralid (Stinger) was applied after the June 28 evaluation to treatments 1 – 3 and as a spot treatment throughout the trial when necessary to ensure complete control of the alfalfa. Each herbicide treatment was replicated three times and data presented represent the mean of the three replications. Data were analyzed using the Student-Newman-Keuls means separation procedure, $P \leq .05$.

Table 1. Herbicide Treatments

Treat-ment #	Herbicide	Active ingredients	Rate	Timing
1	Harness XTRA (check plot)	acetochlor + atrazine	4.2 lbs ai/ac[1]	1 DAP[2]
2	Harness XTRA 2,4-D LVE Banvel	acetochlor + atrazine 2,4-D ester dicamba	4.2 lbs ai/ac 0.5 lbs ai/ac 0.5 lbs ai/ac	1 DAP
3	Harness XTRA 2,4-D LVE Banvel	acetochlor + atrazine 2,4-D ester dicamba	4.2 lbs ai/ac 0.25 lbs ai/ac 0.25 lbs ai/ac	1 DAP
4	Roundup WeatherMAX Degree XTRA 2,4-D LVE	Glyphosate acetochlor + atrazine 2,4-D ester	0.75 lb ae/ac 3.03 lb ai/ac 0.5 lb ai/ac 2.0 lb/ac	9 DAP
5	Roundup WeatherMAX Degree XTRA Banvel	Glyphosate acetochlor + atrazine dicamba	0.75 lb ae/ac 3.03 lb ai/ac 0.5 lb ai/ac 2.0 lb/ac	9 DAP
6	Roundup WeatherMAX Degree XTRA 2,4-D LVE Banvel	Glyphosate acetochlor + atrazine 2,4-D ester dicamba	0.75 lb ae/ac 3.03 lb ai/ac 0.25 lb ai/ac 0.25 lb ai/ac 2.0 lb/ac	9 DAP
7	Roundup WeatherMAX Degree XTRA Hornet	Glyphosate acetochlor + atrazine clopyralid + flumetsulam	0.75 lb ae/ac 3.03 lb ai/ac 0.196 lb ai/ac 2.0 lb/ac	9 DAP
8	Roundup WeatherMAX Degree Yukon	Glyphosate acetochlor halosulfuron + dicamba	0.75 lb ae/ac 1.9 lb ai/ac 0.169 lb ai/ac 2.0 lb/ac	9 DAP
9	Roundup WeatherMAX Degree Marksman	Glyphosate acetochlor + atrazine atrazine + dicamba	0.75 lb ae/ac 1.9 lb ai/ac 1.4 lb ai/ac 2.0 lb/ac	9 DAP

[1]ai – active ingredient, ae – acid equivalent
[2]DAP – days after planting.

Results & Discussion

All evaluated treatments showed complete safety to the corn crop. There was no evidence of crop injury in any of the treated plots (Table 2).

Alfalfa control ratings by treatment are listed in Table 2. The early postemergence application of Harness XTRA alone (treatment 1) served as the control plot. Harness XTRA has been shown to be safe to corn but has little effect on alfalfa plants. However, Harness XRTRA effectively controls many grass and small-seeded broadleaf weeds that would also be present making it possible, in comparing across treatments, to better evaluate alfalfa control provided by the other herbicides used.

As expected, by four weeks after treatment, there was no evidence of alfalfa control with treatment 1.

Of the nine treatments in this study, six of them provided excellent alfalfa control with 91% or greater control at the final rating on June 28, 2004. The percent control values for the six most effective treatments were not significantly different from one another.

These data demonstrated that early postemergence applications of corn herbicides (Harness XTRA, Degree, and Degree XTRA) applied in tank mixtures with other broadleaf corn herbicides (Banvel, 2,4-D, Marksman and Hornet) effectively controlled Roundup Ready alfalfa in a Roundup Ready corn crop. In addition, no injury to the corn crop was observed for any of the herbicide treatments. The results also showed that the higher labeled rates of 2,4-D and Banvel (0.5 vs 0.25 lbs ai/ac) were necessary for improved alfalfa control as demonstrated by the significant difference in efficacy between treatments two and three. In-crop treatments with Yukon provided less than acceptable alfalfa control.

Growers who plan to rotate from Roundup Ready alfalfa to Roundup Ready corn in the spring would be able to select from at least three different active ingredients (2,4-D, dicamba, and clopyralid) that are currently labeled for use in corn to effectively remove Roundup Ready alfalfa and other weeds that were not controlled by previous control procedures.

Table 2. Herbicide injury to Roundup Ready corn and control of Roundup Ready alfalfa in a rotation with Roundup Ready corn under several herbicide treatment regimes.

Treat-ment #	Herbicide	Corn Injury % phyto June 15, 2004	Alfalfa % control June 1, 2004	Alfalfa % control June 15, 2004	Alfalfa % control June 28, 2004
1	Harness XTRA (check plot)	0 a[1]	36 b	2 d	0 c
2	Harness XTRA / 2,4-D LVE / Banvel	0 a	97 a	98 a	93 a
3	Harness XTRA / 2,4-D LVE / Banvel	0 a	96 a	67 bc	67 b
4	Roundup WeatherMAX / Degree XTRA 2,4-D LVE	0 a	0 c	88 ab	95 a
5	Roundup WeatherMAX / Degree XTRA Banvel	0 a	0 c	75 abc	91 a
6	Roundup WeatherMAX /Degree XTRA 2,4-D LVE / Banvel	0 a	0 c	82 ab	93 a
7	Roundup WeatherMAX /Degree XTRA Hornet	0 a	0 c	91 ab	98 a
8	Roundup WeatherMAX/ Degree Yukon	0 a	0 c	57 c	53 b
9	Roundup WeatherMAX /Degree Marksman	0 a	0 c	79 ab	94 a
	LSD ($P \leq .05$)	0.0	4.0	16.2	16.3

[1]Means within each column followed by different letters are significantly different from one another ($P \leq .05$, Student-Newman-Keuls).
Study conducted under USDA Notification Number 02-051-20n.