95-256-01p

DuPont Agricultural Products
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DUPONT

DuPont Agricultural Products

September 11, 1995

Mr. Michael A. Lidsky, J.D., LL.M
Deputy Director, Biotechnology
Coordination and Technical Assistance,
BBEP, APHIS, USDA
4700 River Road
Riverdale, MD 20737

Dear Mr. Lidsky:

PETITION FOR DETERMINATION OF NONREGULATED STATUS:
SULFONYLUREA TOLERANT COTTON LINE 19-51A

DuPont Agricultural Products submits this petition under 7-CFR 340.6 to request that sulfonylurea tolerant cotton line 19-51a and all progenies derived from crosses between line 19-51a and traditional cotton varieties no longer be considered a regulated article under regulations 7-CFR part 340.

The petition does not contain any trade secret or confidential business information.

Sincerely,

Edward W. Raleigh

Edward W. Raleigh
Manager
Biotechnology Regulatory Affairs

EWR/lkb
Enclosure

95-256-01p

TITLE

PETITION FOR THE DETERMINATION OF NONREGULATED STATUS

SULFONYLUREA TOLERANT COTTON LINE 19-51A

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

Edward W Raleigh

Sept. 11, 1995

Edward W. Raleigh
Manager, Biotechnology Regulatory Affairs

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Contains no confidential business information

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 relevant data and information known to the petitioner which are unfavorable to the petition.

Edward W. Raleigh
Manager, Biotechnology Regulatory Affairs

Sept 11, 1995
Date

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I. INTRODUCTION

DuPont Agricultural Products is submitting to the Animal and Plant Health Inspection Service (APHIS) a Petition for a Determination of Nonregulated Status for cotton with an inserted gene which encodes an acetolactate synthase (ALS) enzyme which is tolerant to sulfonylurea herbicides and provides enhanced tolerance to Staple® herbicide. This petition requests that APHIS determine that line 19-51a and any cotton lines derived from crosses between line 19-51a and other non-transgenic cotton varieties no longer be considered a regulated article under 7-CFR part 340.

Sulfonylurea herbicides and Staple® herbicide control weeds by inhibiting ALS, the enzyme that catalyzes the first common step in the biosynthesis of the essential amino acids isoleucine, leucine, and valine. Crop selectivity is based on the ability of crop plants to metabolize the herbicides to inactive products. A second mechanism for selectivity, that of altering ALS sensitivity to inhibition by sulfonylurea herbicides, was utilized to produce the cotton line 19-51a which is the subject of this exemption petition.

DuPont has tested different lines of cotton containing an ALS gene expressing a tolerant form of the ALS enzyme, with different promoters, terminators and with or without an antibiotic resistance gene. In the line we wish to have exempted, 19-51a, there is no antibiotic resistance gene since the herbicide tolerance conferred by the inserted ALS gene was the basis of selection, and the ALS gene, promoter and terminator are all derived from plants.

ALS enzymes are found in all plants, and also have been isolated from fungi and bacteria, with some of the ALS from bacteria being naturally resistant to sulfonylurea herbicides. Mammals do not contain ALS which helps explain the low mammalian toxicity of the sulfonylurea and Staple® herbicides. The amino acid sequences of a variety of ALS enzymes, including soybeans, sugarbeet, corn, canola, *Arabidopsis*, and tobacco, have been determined and they are highly conserved throughout the entire length of the mature protein. Humans and mammals consuming plant food would be exposed to a wide variety of very similar ALS enzymes; consumption of foods derived from bacteria or fungi would expand the variability of consumed ALS enzymes.

The ALS gene in cotton line 19-51a is a chimeric gene derived from two different tobacco ALS genes that both encode herbicide sensitive versions of ALS. Two resistance mutations were introduced into one of the ALS genes by in vitro site-directed mutagenesis. A DNA fragment containing the resistance mutations was moved into the second ALS gene by using a common restriction enzyme fragment. The gene introduced into cotton line 19-51a, designated chimeric S4-HrA, encodes a resistant form of ALS with resistance attributable to two amino acid changes in the protein sequence.

The chimeric S4-HrA gene was transformed into *Agrobacterium tumefaciens* strain LBA4404, a "disarmed" strain of *Agrobacterium* in which the genes responsible for induction of tumors in plants have been deleted from the Ti plasmid. The T-DNA introduced into cotton cultivar Coker 312, other than the ALS gene, contains no other intact prokaryotic or eukaryotic coding sequences.

Polymerase chain reaction (PCR) analyses confirmed that the chimeric S4-HrA gene was stably integrated into the cotton genome and transmitted through normal sexual reproduction. Southern blot analyses indicated that two copies of the gene, in tandem repeat, had been introduced at one locus and that DNA beyond the left and right borders had not been introduced into the cotton. Thus the only enzyme expressed by DNA inserted into cotton line 19-51a is the resistant ALS enzyme, which confers tolerance to sulfonylurea herbicides and enhances the safety of Staple® herbicide.

The use of cotton plants derived from line 19-51a would enable the farmer to utilize certain sulfonylurea herbicides at a small fraction of an ounce of active ingredient per acre and would increase the safety in use of Staple® herbicide at one to two ounces of active ingredient (a.i) per acre, especially under more adverse growing conditions. DuPont sulfonylurea herbicides, used alone or in combination with Staple® could be used to provide over-the-top broadleaf weed control, a void in current cotton weed control programs. These herbicides could eventually eliminate or reduce the number of soil applications and present the greatest opportunity to reduce the total amount of herbicide used yearly in U. S. cotton production. The post emergence activity of these herbicides would allow growers to treat weeds only when needed, which fits in well with conservation (reduced) tillage programs.

Cotton line 19-51a was tested under APHIS field release permits at two sites in two states in 1991 (91-025-02), at six sites in three states in 1992 (91-358-01), at six sites

in three locations in 1993 (93-053-01), under APHIS notification in the winter 1993/1994 in Puerto Rico (93-250-03N) and in 1994 under APHIS notification at approximately 19 locations in nine states (94-021-09N, 94-069-06N, 94-090-08N, 94-095-12N, 94-103-02N, 94-104-01N, 94-109-02N and 94-090-09N. In 1995, cotton line 19-51a has been or is currently being tested at approximately 50 sites in 14 states under APHIS notifications 95-026-01N, 95-066-07N, 95-066-08N, 95-088-01N, 95-060-04N, 95-066-09N and 95-066-10N. The data from these trials, mapping of cotton plant development, results from laboratory experiments, and literature references demonstrate that line 19-51a is not a plant pest, does not demonstrate any weediness potential greater than that seen in non-transgenic cotton and does not have any selective advantage over non-transgenic cotton except in those instances where the cotton is treated with a herbicide which is active towards ALS. Compositional analyses confirm that levels of important constituents of cotton seed from line 19-51a, including key antinutritional factors, are within the normal range for cotton. Cotton line 19-51a differs from other cotton varieties only in its resistance to sulfonylurea herbicides and Staple® herbicide.

Employing transformation to achieve this end offered two advantages over mutational breeding. First, the tolerance phenotype encoded by the tobacco S4-HrA gene had been well characterized in transgenic tobacco and tomato prior to cotton transformation. The spectrum of tolerances to a broad range of ALS inhibitors had been studied in these transformants, and the heritability and stability of the phenotype had been demonstrated repeatedly. Second, the influence of chromosomal position on gene expression causes each transformant to express the tolerance phenotype at a slightly different level. This offers the opportunity to select for the optimal level of tolerance for a given crop/herbicide combination. In contrast, nothing can be known about the products of mutational breeding before their selection, and little control can be exercised over the level or stability of trait expression. The greater levels of understanding and control possible with transformation offered clear advantages in this project.

II. BIOLOGY OF COTTON

A. *Origin and General Characteristics of Cotton*

All cotton species are of the genus, *Gossypium*. All members of this genus produce spinnable fibers (lint) on the seedcoat. The genus *Gossypium* is diverse and has 39 species (Freyxell, 1979). Four species of *Gossypium* are used in commercial cotton; two (*Gossypium arboreum* and *Gossypium herbaceum*) are diploids (n = 13) of Middle East or Old World origin and two (*Gossypium barbadense* and *Gossypium hirsutum*) are tetraploids (n = 26).

In the Americas, it appears that *Gossypium barbadense* evolved as a wild species in South America and was in fact domesticated in Peru (Lee, 1984). *Gossypium hirsutum* occurs in the wild in Central America, northern South America and in the West Indies. No form of *Gossypium hirsutum* has a history of growing wild as a perennial plant in what is today the continental United States .

Basically, cotton is a perennial of tropical and semi-tropical origins. Through natural crossing and by selection, agronomically acceptable cultivars evolved and were developed that could be grown as annual commercial crops in temperate zones (Niles and Feaster, 1984).

Although commercial cotton (*G. hirsutum; G. barbadense*) has the potential to cross pollinate, it is commonly self-pollinating. In the U.S., cross pollination may occur when the concentration of honey bees (*Apis mellifera*) and bumble bees (*Bombus spp.*) is high and insecticide use is absent (McGregor 1976). Even under these conditions conducive to crossings, pollination occurs over relatively short distances with 660 feet established as the buffer required for certified seed production by 7-CFR 201.

B. *Cotton Production*

Essentially, cotton production in the United States lies in a tier of 15 states stretching from Virginia to California. Over 95% of the cotton grown in the United States is Upland (*Gossypium hirsutum*) with Pima (*G. barbadense*) comprising less than

5% of the remaining acres. The value of cotton in the United States in 1990 was $5.1 billion for lint and $739 million for seed (Niles and Feaster, 1984).

The distribution of the cotton acres from 1974 to 1980 is described in the Table I.

Table I
Average annual harvested production of cotton in the USA, 1974 through 1980†

State	Hectares (1000s)	Bales (1000s)
Alabama	156.4	335.7
Arizona	202.1	1080.0
Arkansas	324.9	727.7
California	532.4	2617.0
Florida	2.4	6.4
Georgia	81.3	171.0
Louisiana	207.1	534.0
Mississippi	520.7	1342.3
Missouri	93.6	192.7
New Mexico	49.1	117.0
North Carolina	29.1	63.3
Oklahoma	194.5	312.0
South Carolina	58.6	133.3
Tennessee	128.7	231.3
Texas	2263.2	3765.1
Other States	1.5	2.8
Totals	**4845.4**	**11631.6**

† Taken from Niles and Feaster, 1984.

Since cotton originated in the tropics, the plant becomes inactive at temperatures below 15° C and is easily killed by frost (Waddle, 1984). Climate generally sets the geographic limits for cotton production areas.

Cotton is grown in areas giving 200 or more frost-free days. This area lies south of 36° N latitude except for a small area below the junction of the Ohio and Mississippi rivers, a small area in central Oklahoma, and the northern portion of the San Joaquin Valley in California where cotton is grown close to 37° N. There is a direct relationship between potential yield and the number of growing days (Waddle, 1984).

Gossypium species in the world occur in arid parts of the tropics and subtropics (Fryxell, 1979). In US commercial production, an acceptable cotton crop requires at least 20 inches of water during the growing season. Cotton is usually irrigated when annual rainfall is below 16 inches. Supplemental irrigation is used as needed in the 16 to 35 inch rainfall zone. Most of the cotton produced in higher rainfall areas is not irrigated. Cotton is adapted for production on a wide range of soil types as long as fertility is adequate and the soil's physical condition allows adequate drainage and root penetration (Waddle, 1984).

The two components of cotton production which are sold in commerce are lint and seed. The primary component is lint which is used in textile and clothing production worldwide. Approximately 40% of seed cotton by weight is lint (Niles and Feaster, 1984). From cotton seed both processed and unprocessed components are used. The processed and refined component of cotton seed oil is consumed by humans. Unprocessed cotton seed, meal and hulls are primarily used for livestock feed (Waddle, 1984).

C. *Consideration of Risk Assessment and Weediness Potential in Cotton*

The USDA/APHIS Environmental Assessment dated February, 1994 with the companion response to Calgene's Petition to exempt their transgenic cotton from APHIS oversight (P93-186-01) include detailed information on the biology of cotton and the potential for cotton to become a weed or to outcross with other cotton or with wild relatives. The Environmental Assessment notes that "*Gossypium hirsutum* does not show

any appreciable weedy characteristics. The genus also seems to be devoid of any such characteristics; although some New World cottons show tendencies to "weediness" (Fryxell, 1979; Haselwood et al., 1983), the genus shows no particular weedy aggressive tendencies. The standard texts and list of weeds give no indication that cotton is clearly regarded as a weed anywhere (Holm et al., 1979; Muenscher, 1980; Reed, 1970; Weed Science Society of America, (1989).

The same Environmental Assessment further notes that "None of the relatives of cotton found in the United States (*G. barbadense, G. thurberi* and *G. tomentosum*) shows any definite weed tendencies." The compatible species are *G. hirsutum, G. barbadense and G. tomentosum*, with some uncertainty about the possibility for pollination of the latter wild relative.

The Calgene exemption petition P93-196-01 reports on outcrossing studies which confirm the low potential for their transgenic cotton to cross pollinate with neighboring cotton plants, as is the case with non-transgenic cotton. Cotton is a self-pollinating crop, the pollen is heavy and not readily dispersed by the wind and any cross pollination would be via insects, especially bees. The Calgene studies indicated a rapid decline in the frequency of outcrossing as the distance from the pollen source is increased, with approximately 0.007 % outcrossing at 5-6 meters.

The SU-tolerant ALS enzyme expressed in cotton line 19-51a essentially only differs from the multitude of ALS enzymes in other plants in its tolerance to sulfonylurea (and Staple®) herbicides. The genetic material introduced into cotton to produce cotton line 19-51a only encodes the tolerant ALS enzyme, and such an ALS enzyme could be bred into cotton plants by traditional plant techniques such as mutational breeding. Thus, line 19-51a is essentially equivalent to cotton lines that could be produced by classical plant breeding techniques. The *Agrobacterium tumefaciens* used to produce line 19-51a was disarmed, and the laboratory, growth chamber, and greenhouse testing, plus extensive field testing over the past four years have confirmed the absence of any plant pest characteristics.

Based on the above, there is no reason to believe that cotton line 19-51a would ever: 1) exhibit any increased weediness relative to the non-transgenic varieties; 2) lead to an increase in weediness in any plant with which it could successfully interbreed; 3) have any significant impact on any beneficial organisms, any threatened or endangered

species, or any other non-target organism; 4) cause any damage to processed agricultural commodities. Any possible, albeit low-level, outcrossing between cotton line 19-51a and other wild or cultivated cotton or cotton relatives would not be expected to otherwise impact such plants any differently from outcrossing from cotton produced by other plant breeding methods.

III. THE TRANSFORMATION AND VECTOR SYSTEM

A. *Transformation Procedure*

A binary vector (Bevan 1984) based *Agrobacterium tumefaciens* plant transformation system was used to transfer the chimeric S4-HrA gene to cotton. In this system the *Agrobacterium* is 'disarmed' or nonpathogenic since the genes responsible for tumor induction have been deleted from the Ti plasmid. The binary vector system involves two plasmids that function in *trans* to introduce the DNA into plants. The first plasmid, the disarmed Ti plasmid, encodes the *vir* genes necessary for DNA transfer to the plant but lacks the T-DNA region. The second plasmid contains the genes to be transferred to the plant between the left and right borders of the T-DNA. Genes on the first plasmid are not transferred to the transgenic plant.

The plasmid, pMH26 (Figure 1), used for cotton transformation was constructed by cloning the sulfonylurea tolerant gene into the binary plasmid pZH1 (Figure 2) which is based on vectors described by Leemans and Deblaere (1988). pZH1 contains the origin of replication and the ampicillin resistance (β-lactamase) gene from pBR322 (Sutcliffe 1979) allowing maintenance and replication of the plasmid in *E. coli*, the replication (rep) and stability (sta) regions of the *Pseudomonas aeruginosa* plasmid pVS1 (Itoh *et al.* 1984) allowing replication in *Agrobacterium*, T-DNA borders described in van den Elzen *et al.* (1985), and a portion of the polylinker from pUC19 (Yanisch-Perron *et al.* 1985). The sulfonylurea tolerance gene was inserted into the pUC19 polylinker between the T-DNA borders of pZH1 as a 4.5 kB Sal I to Pst I fragment to give pMH26. The DNA contained between the left and right borders of pMH26 is described in Table II.

The chimeric S4-HrA gene was transformed into *Agrobacterium tumefaciens* strain LBA4404 (Ooms *et al.* 1981, Ooms *et al.* 1982, Hoekema *et al.* 1983) by bacterial conjugation. LBA4404 is a 'disarmed' strain of *Agrobacterium* in which the genes responsible for induction of tumors in plants have been deleted from the Ti plasmid. Since neither the Ti plasmid nor the binary vector contain any of the genes responsible for crown gall tumorigenesis, these strains cannot cause crown gall disease.

Figure 1
Map of plasmid pMH26
RB = T-DNA right border, LB = T-DNA left border

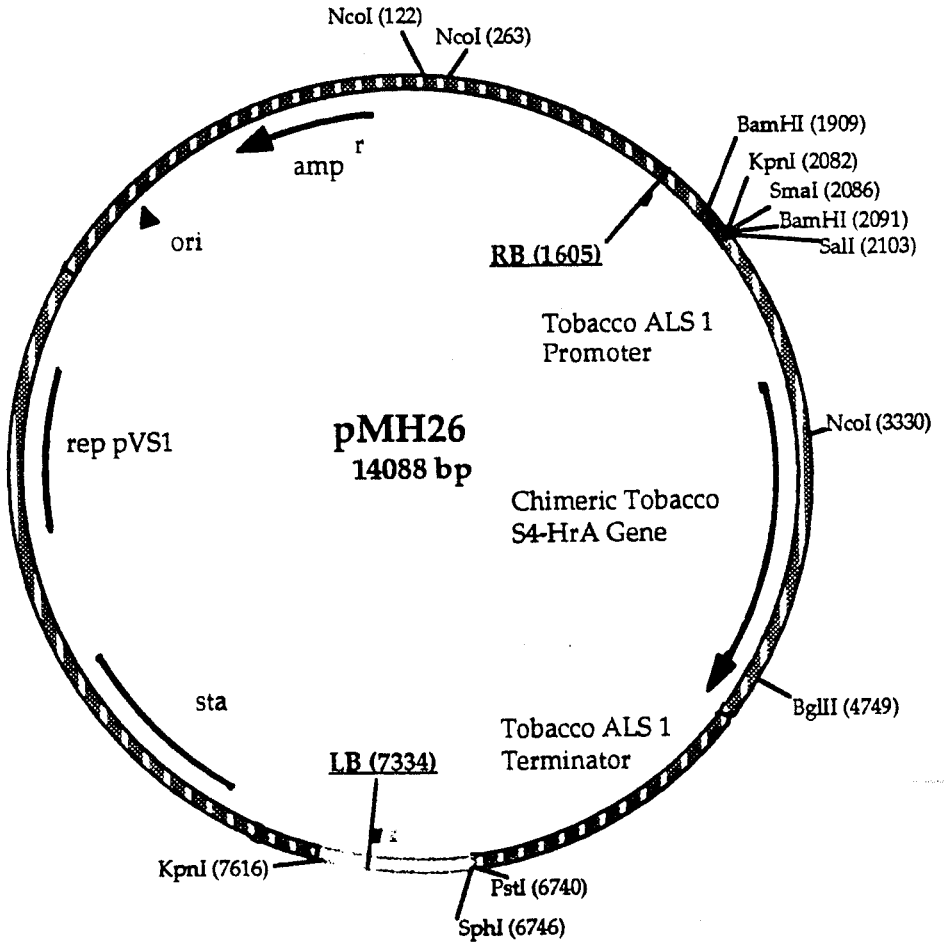
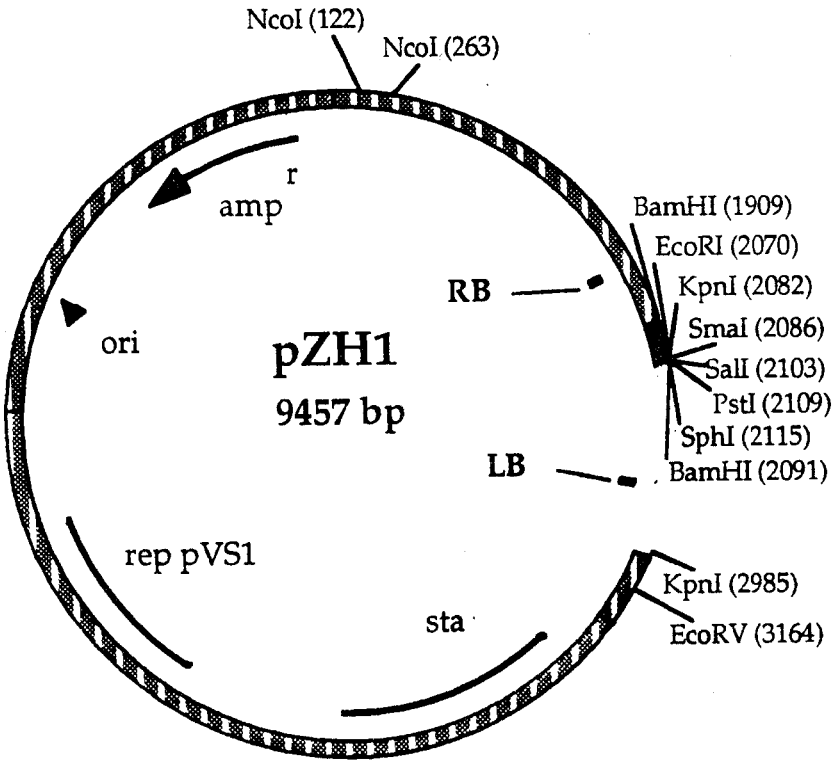


Figure 2
Map of Binary Vector pZH1



The T-DNA on plasmid pMH26 was introduced into cotton cultivar Coker 312 by inoculation of seedling hypocotyl segments with the *Agrobacterium* strain described in the previous section. After two days of co-cultivation, the hypocotyl pieces were placed on medium containing 25 ppb chlorsulfuron to select for transformed callus growth. Tolerant calli were transferred sequentially to embryo induction and maturation media to produce plantlets. Plantlets were transplanted to soil and grown to maturity in a greenhouse. One sulfonylurea tolerant transgenic line, 19-51a, containing the integrated T-DNA from pMH26 was selected for further development.

Table II

Description of the DNA contained between the left and right borders on plasmid pMH26

The base pair locations refer to the numbers relative to those on the map of pMH26 as shown in Figure 1.

Genetic Element	Base Pair Location	Description of Gene Function
RB	1605 - 1628	Right border 24 bp direct repeat from the Ti plasmid, pTiACH5 of *Agrobacterium tumefaciens* for transfer of the T-DNA (van den Elzen *et al.* 1985).
OCS Enhancer	1629 - 1909	Octopine Synthase Enhancer from *Agrobacterium tumefaciens* Ti plasmid, pTiACH5. The upstream region of the octopine synthase promoter which enhances gene expression from downstream promoters (Ellis *et al.* 1987).
pUC19 Polylinker	1910 - 2103	A portion of the pUC19 polylinker (Yanisch-Perron *et al.* 1985). It contains a segment of the coding region of the *E. coli* LacZα' gene but does not contain any of the signals required for expression of this gene.
P-ALS	2104 -2980	The tobacco ALS 1 promoter (Mazur *et al.* 1987, Lee *et al.* 1988, Keeler *et al. 1993*).
Chimeric S4-HrA	2981 - 4975	Coding region of the chimeric tobacco ALS gene (Hartnett *et al.* 1990). ALS 1 coding sequences extend from bp 2981 to bp 3330 and from bp 4749 to bp 4975. ALS 2 coding sequence extend from bp 3331 to bp 4748.
ALS Terminator	4976 - 6739	The tobacco ALS 1 terminator (unpublished sequence).
pUC19 Polylinker	6740 - 6755	A portion of the pUC19 polylinker (Yanisch-Perron *et al.* 1985). It contains a segment of the coding region of the *E. coli* LacZα' gene but does not contain any of the signals required for expression of this gene.
Ti Plasmid DNA	6756 - 7307	A segment of DNA from the octopine Ti-plasmid, pTiA6 (van den Elzen *et al.* 1985). The DNA was isolated from a region upstream of T-DNA gene 5. The sequence does not contain any of the identified promoter signals for gene 5 nor any portion of the coding region of gene 5 (Leemans *et al.* 1982).
LB	7308 - 7332	Left border 24 bp direct repeat for transfer of the T-DNA.

B. *The Sulfonylurea Tolerance Gene*

The sulfonylureas are a group of compounds which inhibit acetolactate synthase (ALS), the enzyme that catalyzes the first common step in the biosynthesis of the essential amino acids isoleucine, leucine, and valine (LaRossa and Schloss 1984, LaRossa and Falco 1984). These compounds inhibit plant growth by inactivating an enzyme in an essential amino acid biosynthetic pathway. Essential amino acids are not produced by mammals and they therefore lack the target enzyme, contributing to the low mammalian toxicity of the sulfonylureas. The chimeric S4-HrA gene expresses an herbicide tolerant ALS (Chaleff and Mauvais 1984, Mazur *et al.* 1987) which allows the cotton plant to produce the essential amino acids in the presence of the sulfonylureas. The development of sulfonylurea tolerant plants has been documented previously (Mazur *et al.* 1989, Haughn *et al.* 1988, Lee *et al.* 1988).

ALS genes have been isolated from bacteria, fungi and plants (Friden *et al.* 1985, Falco *et al.* 1985, Mazur *et al.* 1987) and thus the enzyme is present in food derived from these sources. The deduced amino acid sequence from three ALS isozymes in *E. coli* and *Salmonella typhimurium* has shown that these isozymes have three highly conserved regions between four non-conserved regions. Plant enzymes are also highly conserved within these domains but the highly conserved regions between plant enzymes extends through the entire length of the mature protein. Table III indicates the level of amino acid sequence similarity and identity between a wide variety of plant ALS enzymes compared to the chimeric S4-HrA enzyme. Although the majority of the ALS enzymes found in nature are sensitive to inhibition by sulfonylureas there are examples of naturally tolerant ALS enzymes, such as the ALS isozyme I of *E. coli* (LaRossa and Smulski 1984).

Table III

Percent similarity and percent identity between the Chimeric S4-HrA and other plant ALS enzymes

Percent identity represents the number of amino acid residues that are identical between the compared sequences divided by the total number of amino acid residues compared. Percent similarity is generally a higher number than percent identity since the similarity calculation takes into account replacement of amino acids with similar amino acids in both charge and structure. The numbers would indicate a higher level of identity if the chloroplast transit peptides were not included in these calculations.

ALS ORIGIN	PERCENT SIMILARITY	PERCENT IDENTITY
Arabidopsis	87	79
Brassica 1	85	73
Brassica 2	89	82
Corn 1	83	71
Corn 2	82	70
Soybean 1	86	76
Soybean 2	88	80
Sugarbeet	88	83
Tobacco 1	99	98
Tobacco 2	99	99

The chimeric S4-HrA gene introduced into cotton was derived from tobacco (*Nicotiana tabacum* cv. Xanthi) variants using plant cell culture techniques. Callus generated from a haploid tobacco plant was exposed to concentrations of chlorsulfuron or sulfometuron methyl (two different sulfonylurea herbicides) which completely inhibited cell growth. Several tolerant cell lines were selected and plants were regenerated from each. These plants displayed up to a 100-fold increase in tolerance to foliar application of chlorsulfuron. Genetic analysis indicated that tolerance in these lines was due to dominant nuclear mutations at either of two unlinked chromosomal loci (Chaleff and Ray 1984).

Callus lines derived from plants homozygous for one of the mutations were further selected on a concentration of sulfometuron methyl which completely inhibited the growth of the variant line. One line tolerant to this higher herbicide concentration was

identified and plants were regenerated. Genetic analysis revealed that the increased tolerance was due to a second mutation linked to the first. Plants homozygous for both mutations displayed at least a 1000-fold increase in tolerance to foliar applications of chlorsulfuron when compared with non-variant parental genotype (Chaleff *et al.* 1987).

Biochemical and genetic studies in tobacco established that the primary site of action of sulfonylureas in higher plants is ALS and that tolerance in the mutants described above was due to the production of an insensitive form of the ALS enzyme (Chaleff and Mauvais 1984). The ALS genes from the single and double mutant tobacco lines were isolated by constructing bacteriophage lambda genomic libraries with DNA from the individual variant lines and screening the libraries with a tobacco ALS gene which had been previously isolated from a wild type tobacco DNA library (Mazur *et al.* 1987). Phage clones containing DNA fragment which hybridized to the probe were identified and the location of the ALS genes restriction enzyme mapped. The clones fell into two classes which correlated with the two loci, hereafter referred to as ALS 1 and ALS 2, defined by the genetic analyses described above. DNA sequence analysis of the genes encoding sulfonylurea-sensitive and insensitive forms of ALS from each locus identified the mutation sites conferring sulfonylurea tolerance (Lee *et al.* 1988).

The chimeric S4-HrA gene was constructed by combining fragments of DNA from the ALS 1 and ALS 2 genes (Hartnett *et al.* 1990). Mutations analogous to those in the double mutant tobacco line were introduced into the sensitive version of ALS 2 by site-directed mutagenesis and then a restriction fragment containing the two introduced mutations was subcloned into the sensitive version of the ALS 1 gene. The two mutations introduced into the ALS 2 gene result in a proline to alanine substitution at amino acid position 191 and a tryptophan to leucine substitution at amino acid position 568. The chimeric S4-HrA gene, under the control of the ALS 1 gene promoter and 3' terminator, was cloned into pZH1 to create pMH26 as described above. Figure 3 shows the complete nucleotide sequence of the DNA contained between the T-DNA left and right borders in pMH26.

Figure 3
Sequence of the T-DNA region found in plasmid pMH26

The amino acid sequence is deduced from the chimeric S4-HrA gene sequence. Bold underlines indicate the 24 bp repeats of the T-DNA right and left borders. Restriction enzyme sites used for Southern blot analyses and the locations of the S4 and HrA mutations are noted. (Refer to Table II for additional descriptions of the T-DNA.)

RB (1605)

1605 AATTACAACGGTATATATCCTGCCAGTCAGCATCATCACACCAAAAGTTAGGCCCGAATAGTTTGA

1671 AATTAGAAAGCTCGCAATTGAGGTCTACAGGCCAAATTCGCTCTTAGCCGTACAATATTACTCACC
1737 GGTGCGATGCCCCCCATCGTAGGTGAAGGTGGAAATTAATGATCCATCTTGAGACCACAGGCCCAC
1803 AACAGCTACCAGTTTCCTCAAGGGTCCACCAAAAACGTAAGCGCTTACGTACATGGTCGATAAGAA

BamHI (1909)

1869 AAGGCAATTTGTAGATGTTAACATCCAACGTCGCTTTCAGGGATCCATTCGCCATTCAGGCTGCGC
1935 AACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGT
2001 GCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCC

SmaI (2086)
KpnI (2082) BamHI (2091) SalI (2103)

2067 AGTGAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACTCTAGTGTACAAGATTGGGATGTG
2133 AAGGCTCAAGGATGTGAATTGATACTCTCATCAGGGGGAGTTAATACGTGTTGTACTCTTTTTTCC
2199 TTACAAGATTTTGACCCACTGGGTTTTTCTTGCAAGGTTTTTAACGAGGCAACCAAAAGGCGTATT
2265 TCTAAACATGTGTACTTTTTTTCCTTCACTAGGATTTTTTTCCTATATGATTTTTTCCTAATAAGG
2331 TTTTAACGAGGCACATTATCTATGGACATCCAAGGGGGAGTGTTATAAGAAAAATCAAATTATGGT
2397 GGATGTCTACTCTTCCTCCATGATCTTCTCAAATGCTTAATGACATATTCAATGACATATTTCTAT
2463 GCTTAATGACATATTTTTCTTCACTTTTCATGCCTATATAAAGGCCTTGTAATAGATAGAAAAATA
2529 CAAATAATTGAAGAAGAAATAAAAATCTCTTATCTCTATATTTCTTAGCTTGTTTTTTTTTTGTTC
2595 TATATTGTTACTTTGAGCTATATTTCATAACAGCATTCACATTCTTTTTCCATAGTCTTTTTTCCC
2661 TTTTATATTTTAATTTACTGAAGTAACAAATACTTCCACTTCTTTCTTCTTCCCACCCTCCTAAAT
2727 ATATCCAACATCTCATTTTTCTTTTCCCCAATTCTCAGACATTTTAATCTTTCTTTTCTATTTATT
2793 TTCTTCATATTTTGATCTCTCTTCCATTTGTTCTCATCCATTTTCGCTATTCACGTGAATTCAATC
2859 AAGTAGGACCCTTTCAGTTTCGTGGCGCTCTCGTCTTCTCAGCTTAATATAAAACCAACCACACAC
2925 CATCTACATTGCCCTTTCCTTTCAGTTTCGTCTCTCACTGCTCTCATTCAACAATAATGGCGGCGG
1▶MetAlaAlaA

2991 CTGCGGCGGCTCCATCTCCCTCTTTCTCCAAAACCCTATCGTCCTCCTCCTCCAAATCCTCCACCC
4▶laAlaAlaAlaProSer ProSer PheSer LysThr LeuSer Ser Ser Ser Ser LysSer Ser Thr L

3057 TCCTCCCTAGATCCACCTTCCCTTTCCCCCACCACCCCCACAAAACCACCCCACCACCCCTCCACC
26▶euLeuProArgSer Thr PheProPheProHisHisProHisLysThr Thr ProProProLeuHisL

3123 TCACCCCCACCCACATTCACAGCCAACGCCGTCGTTTCACCATCTCCAATGTCATTTCCACTACCC
48▶euThr ProThr HisIleHisSer GlnArgArgArgPheThr IleSerAsnValIleSer Thr Thr G

3189 AAAAAGTTTCCGAGACCCAAAAAGCCGAAACTTTCGTTTCCCGTTTTGCCCCTGACGAACCCAGAA
70▶InLysVal Ser GluThr GlnLysAlaGluThr PheVal Ser ArgPheAlaProAspGluProArgL

3255 AGGGTTCCGACGTTCTCGTGGAGGCCCTCGAAAGAGAAGGGGTTACGGACGTTTTTGCGTACCCAG
92▶ysGlySerAspVal LeuVal GluAlaLeuGluArgGluGlyVal ThrAspVal PheAlaTyrProG

NcoI (3330)

3321 GCGGCGCTTCCATGGAGATTCACCAAGCTTTGACCCGTTCAAGCATCATCCGCAACGTGCTGCCAC
114▶lyGlyAlaSerMetGluIleHisGlnAlaLeuThrArgSer Ser IleIleArgAsnVal LeuProA

3387 GTCACGAGCAGGGCGGTGTCTTCGCCGCTGAGGGTTACGCACGCGCCACCGGATTTCCCGGCGTTT
136▶rgHisGluGlnGlyGlyVal PheAlaAlaGluGlyTyrAlaArgAlaThr GlyPheProGlyVal C

3453 GCATTGCCACCTCTGGCCCCGGCGCCACCAATCTCGTCAGCGGCCTCGCTGACGCGCTACTGGATA
158▶ysIleAlaThr Ser GlyProGlyAlaThrAsnLeuVal Ser GlyLeuAlaAspAlaLeuLeuAspS

S4 Pro --> Ala (3551)

3519 GCGTCCCCATTGTTGCTATAACAGGTCAAGTGGCACGTAGGATGATAGGTACTGATGCTTTTCAGG
180▶erValProIleValAlaIleThr GlyGlnValAlaArgArgMetIleGlyThrAspAlaPheGlnG

```
3585 AAACTCCTATTGTTGAGGTAACTAGATCGATTACCAAGCATAATTATCTCGTTATGGACGTAGAGG
 202▶ luThr Pro Ile Val Glu Val Thr Arg Ser Ile Thr Lys His Asn Tyr Leu Val Met Asp Val Glu A

3651 ATATTCCTAGGGTTGTACGTGAAGCTTTTTTCCTCGCGAGATCGGGCCGGCCTGGCCCTATTTTGA
 224▶ sp Ile Pro Arg Val Val Arg Glu Ala Phe Phe Leu Ala Arg Ser Gly Arg Pro Gly Pro Ile Leu I

3717 TTGATGTACCTAAGGATATTCAGCAACAATTGGTGATACCTGACTGGGATCAGCCAATGAGGTTAC
 246▶ Ie Asp Val Pro Lys Asp Ile Gln Gln Gln Leu Val Ile Pro Asp Trp Asp Gln Pro Met Arg Leu P

3783 CTGGTTACATGTCTAGGTTGCCTAAATTGCCCAATGAGATGCTTTTAGAACAAATTGTTAGGCTTA
 268▶ r o Gly Tyr Met Ser Arg Leu Pro Lys Leu Pro Asn Glu Met Leu Leu Glu Gln I Ie Val Arg Leu I

3849 TTTCTGAGTCAAAGAAGCCTGTTTTGTATGTGGGGGGTGGGTGTTCGCAATCGAGTGAGGACTTGA
 290▶ Ie Ser Glu Ser Lys Lys Pro Val Leu Tyr Val Gly Gly Gly Cys Ser Gln Ser Ser Glu Asp Leu A

3915 GACGATTCGTGGAGCTCACGGGTATCCCCGTGGCAAGTACTTTGATGGGTCTTGGAGCTTTTCCAA
 312▶ r g Arg Phe Val Glu Leu Thr Gly Ile Pro Val Ala Ser Thr Leu Met Gly Leu Gly Ala Phe Pro T

3981 CTGGGGATGAGCTTTCCCTTTCAATGTTGGGTATGCATGGTACTGTTTATGCTAATTATGCTGTGG
 334▶ hr Gly Asp Glu Leu Ser Leu Ser Met Leu Gly Met His Gly Thr Val Tyr Ala Asn Tyr Ala Val A

4047 ACAGTAGTGATTTGTTGCTCGCATTTGGGGTGAGGTTTGATGATAGAGTTACTGGAAAGTTAGAAG
 356▶ sp Ser Ser Asp Leu Leu Leu Ala Phe Gly Val Arg Phe Asp Asp Arg Val Thr Gly Lys Leu Glu A

4113 CTTTTGCTAGCCGAGCAAAAATTGTTCACATTGATATTGATTCAGCTGAGATTGGAAAGAACAAGC
 378▶ la Phe Ala Ser Arg Ala Leu Ile Val His Ile Asp Ile Asp Ser Ala Glu Ile Gly Lys Asn Lys G

4179 AGCCTCATGTTTCCATTTGTGCAGATATCAAGTTGGCGTTACAGGGTTTGAATTCGATACTGGAGA
 400▶ In Pro His Val Ser Ile Cys Ala Asp Ile Leu Lys Leu Ala Leu Gln Gly Leu Asn Ser Ile Leu Glu S

4245 GTAAGGAAGGTAAACTGAAGTTGGATTTTTCTGCTTGGAGGCAGGAGTTGACGGAGCAGAAAGTGA
 422▶ er Lys Glu Gly Lys Leu Lys Leu Asp Phe Ser Ala Trp Arg Gln Glu Leu Thr Glu Gln Lys Val L

4311 AGCACCCATTGAACTTTAAAACTTTTGGTGATGCAATTCCTCCGCAATATGCTATCCAGGTTCTAG
 444▶ ys His Pro Leu Asn Phe Lys Thr Phe Gly Asp Ala Ile Pro Pro Gln Tyr Ala Ile Gln Val Leu A

4377 ATGAGTTAACTAATGGGAATGCTATTATAAGTACTGGTGTGGGGCAACACCAGATGTGGGCTGCTC
 466▶ sp Glu Leu Thr Asn Gly Asn Ala Ile Ile Ser Thr Gly Val Gly Gln His Gln Met Trp Ala Ala G

4443 AATACTATAAGTACAGAAAGCCACGCCAATGGTTGACATCTGGTGGATTAGGAGCAATGGGATTTG
 488▶ In Tyr Tyr Lys Tyr Arg Lys Pro Arg Gln Trp Leu Thr Ser Gly Gly Leu Gly Ala Met Gly Phe G

4509 GTTTGCCCGCTGCTATTGGTGCGGCTGTTGGAAGACCGGATGAAGTTGTGGTTGACATTGATGGTG
 510▶ ly Leu Pro Ala Ala Ile Gly Ala Ala Ala Val Gly Arg Pro Asp Glu Val Val Val Asp Ile Asp Gly A

4575 ATGGCAGTTTCATCATGAATGTGCAGGAGCTTGCAACAATTAAGGTGGAGAATCTCCCAGTTAAGA
 532▶ sp Gly Ser Phe Ile Met Asn Val Gln Glu Leu Ala Thr Ile Lys Val Glu Asn Leu Pro Val Lys I

                                                    HrA Trp --> Leu (4682)
4641 TTATGTTACTGAATAATCAACACTTGGGAATGGTGGTTCAATTGGAGGATCGGTTCTATAAGGCTA
 554▶ Ie Met Leu Leu Asn Asn Gln His Leu Gly Met Val Val Gln Leu Glu Asp Arg Phe Tyr Lys Ala A

                                                    BglII (4749)
4707 ACAGAGCACACACATACCTGGGGAATCCTTCTAATGAGGCGGAGATCTTTCCTAATATGTTGAAAT
 576▶ sn Arg Ala His Thr Tyr Leu Gly Asn Pro Ser Asn Glu Ala Glu Ile Phe Pro Asn Met Leu Lys P

4773 TTGCAGAGGCTTGTGGCGTACCTGCTGCGAGAGTGACACACAGGGATGATCTTAGAGCGGCTATTC
 598▶ he Ala Glu Ala Cys Gly Val Pro Ala Ala Arg Val Thr His Arg Asp Asp Leu Arg Ala Ala Ile G

4839 AAAAGATGTTAGACACTCCTGGGCCATACTTGTTGGATGTGATTGTACCTCATCAGGAACATGTTC
 620▶ In Lys Met Leu Asp Thr Pro Gly Pro Tyr Leu Leu Asp Val Ile Val Pro His Gln Glu His Val L

4905 TACCTATGATTCCCAGTGGCGGGGCTTTCAAAGATGTGATCACAGAGGGTGACGGGAGAAGTTCCT
 642▶ eu Pro Met Ile Pro Ser Gly Gly Ala Phe Lys Asp Val Ile Thr Glu Gly Asp Gly Arg Ser Ser T

4971 ATTGACTTTGAGGTGCTACAGAGCTAGTTCTAGGCCTTGTATTATCTAAAATAAACTTCTATTAAA
 664▶ y r• • •

5037 CCAAAAATGTTATGTCTATTAGTTTGTTATTAGTTTTTCCGTGGCTTTGCTCATTGTCAGTGTTGT

5103 ACTATTAAGTAGTTGATATTATGTTTGCTTTAAGTTTTGCATCATCTCGCTTTGGTTTTGAATGTG

5169 AAGGATTTCAGCAATGTTTCATTCTCTATTCGCAACATCCAGTCGGTATCCGGAGCTCTATGTAGT

5235 ATGTCTGGAGAGATTAATTTCTAGTGGAGTAGTTTAGTGCGATAAAGTTAGCTTGTTCCACATTTT

5301 TATTTTCGTAACCTGGGTCAGATTAACTTCCTCTTTAGGTTGGAATGCAATCCCTATTTGGGCTTT
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5367 CTCTTAATTTCATTATTGAAATTGTTGGCTTTTAATCTGAGCAAGTTGATTTGCAGCTTTCTCTCT
5433 TGAGTCCTAGCGAGCAATACGTTATCTCTGTCTCCTATTTCTTAGTGGATAATCTTATGATGGAAA
5499 TCTGTGGAGATAGGAAACTGTTGACTGCTCATTCTCTCTCTTTGTGAGGCGTCTGACAGTTACGCT
5565 TTCAATCTATAGCAGTTCGATCAGACTTTCTTTGCTTATAACAATACTAGATACAGTTGGACCGTG
5631 TAGCACGGGCCCAACAATTTGTGTAACGTTATCTTTAATTCAAAATATCTTTCATGATTTTTGTAA
5697 TTTATTAAGAAAATCAGTTAATAACATAAATGACTAAGTTAGAGTAGCATCTTTGTACCATTTTGC
5763 CCATTGCTTTTTATTTTCTTGTTTCAATGTTATTTGACAATATAGATGTATTTTCCTGCATCTATA
5829 GCATTAGCAGTCTTGTTAATATGAGCTAGTATATTGGTTTTTATGGCAATACTTCAATATCTTATT
5895 AGTTACTGCACCATGAAAAGAAATAAAGTTCTCTAATAGAAAATAAGAAACTGTGTTACAGCTCAA
5961 ATATTCTCATTAGATTGATACTTACTTCAGGAAAGAAAATATTGTACACAACTCCTACATTTTGTT
6027 ATATTCTGGGAAAACTTCAACAATATGAAGTCCAACAAAAATCATTCTATCATCAAGAATAAAGGG
6093 AAGTCGCATTGGAAAAACAGCAGCATCAACTTGTCAATTCTATTCTAGAGCTCTCTATTGGCAGAA
6159 TCATGTGGTCCAGGTTCTCTCTATCTGCACAATCTTTTGGTTCAGGTTTTTCTTGCCGATAATTTT
6225 CTGTAATTCAACTATAACTATCCTGGATGGTAGTGTGTAAGCACAGTGGATGTCATGGTCAGAATA
6291 GAACAACTGCTGCGGATTTTTTGCATCAAATGTGAGTTCTTTTTTGGACGTCTACTTTTGTTTCCT
6357 ATAGCTTTCCTTGTTTGCTTCCCCATCTCCCTTTTCAGCTCAAGGAAACGGTGAAAAGATGGTTGT
6423 GGTAGAACTCACATTTGATGTAGACATCCACCATAATTTGATTTTTCTTATAACACTCCCCCTTGG
6489 ATGTCCATAGATAATGTGCCTCGTTAAAACCTTCTTAGGAAAAAATCATATAGGAAAAAAATCCTA
6555 GTGAAGGAAAAAAAGTACACATGTTTAGAAATACGCCTTTTGGTTGCCTCGTTAAAAACCTTGCAA
6621 GAAAAACCCAGTGGGTCAAAATCTTGTAAGGAAAAAAGAGTACAACACGTATTAACTCCCCCTGAT

SphI (6746)

PstI (6740)

6687 GAGAGTATCAATTCACATCCTTGAGCCTTCACATCCCAATCTTGTACACTAGACTGCAGGCATGCA
6753 AGCTCGAGTGGTGGATTTATCACAAATGGGACCCGCCGCCGACAGAGGTGTGATGTTAGGCCAGG
6819 ACTTTGAAAATTTGCGCAACTATCGTATAGTGGCCGACAAATTGACGCCGAGTTGACAGACTGCCT
6885 AGCATTTGAGTGAATTATGTGAGGTAATGGGCTACACTGAATTGGTAGCTCAAACTGTCAGTATTT
6951 ATGTATATGAGTGTATATTTTCGCATAATCTCAGACCAATCTGAAGATGAAATGGGTATCTGGGAA
7017 TGGCGAAATCAAGGCATCGATCGTGAAGTTTCTCATCTAAGCCCCCATTTGGACGTGAATGTAGAC
7083 ACGTCGAAATAAAGATTTCCGAATTAGAATAATTTGTTTATTGCTTTCGCCTATAAATACGACGGA
7149 TCGTAATTTGTCGTTTTATCAAAATGTACTTTCATTTTATAATAACGCTGCGGACATCTACATTTT
7215 TGAATTGAAAAAAAAATTGGTAATTACTCTTTCTTTTTCTCCATATTGACCATCATACTCATTGCTG

LB (7333)

7281 ATCCATGTAGATTTCCCGGACATGAAGCCATTTACAATTGAATATATCCTGCC

C. *Genes Contained on pMH26 which are not Transferred to Cotton Line 19-51a*

The mechanism of T-DNA transfer to plant cells by *Agrobacterium* transformation has been studied extensively (for reviews see Binns and Thomashow 1988, Zambryski 1992). Reports have indicated that integration of the T-DNA into the plant is not always precise and regions outside of the T-DNA on the vector can integrate into the plant genome (Holsters *et al.* 1983). For this reason a description of the genes outside of the T-DNA borders in pMH26 is given below. Cotton line 19-51a does not contain plasmid DNA beyond the borders as shown by Southern blot analysis (Southern 1975) described in Section IV.

Plasmid sequences from *E. coli* include the ampicillin resistance gene, the origin of replication (*ori* ColE1) and a fragment of the tetracycline resistance gene (the fragment includes the tetracycline resistance gene promoter and the 5' end of the coding sequence but does not express an intact protein) from pBR322 (Sutcliffe 1979). The ampicillin resistance gene is necessary for selection and maintenance of the plasmid in *E. coli* and *ori* is necessary for the replication of the plasmid in *E. coli*. *E. coli* is not a plant pest and the DNA sequences are not present in cotton line 19-51a.

Additional *Agrobacterium* DNA sequences from the Ti plasmids, pTiAch5 and pTiA6, are present outside of the 24bp repeats of the right and left borders, respectively, in pMH26. A region of Ti plasmid DNA known as overdrive (Peralta *et al.* 1986) that stimulates T-DNA transfer in an orientation-independent manner is located just outside of the right border. The remainder of the Ti plasmid DNA in pMH26 is not involved in T-DNA transfer or tumorigenesis.

A second origin of replication in pMH26 is derived from the pVS1 plasmid of *Pseudomonas aeruginosa* (Itoh *et al.* 1984) and permits plasmid replication in *Agrobacterium tumefaciens*. Two regions of pVS1 are necessary for replication in *Agrobacterium*; the *Pseudomonas* origin of replication, *ori* pVS1, and the *sta* gene which stabilizes plasmid replication.

Agrobacterium and *Pseudomonas* are regulated articles since they are considered plant pests. The DNA sequences discussed above from these organisms are not

integrated into the cotton genome of 19-51a, hence they do not confer any plant pest characteristics on cotton.

IV. GENETIC, MOLECULAR, AND BIOCHEMICAL ANALYSIS OF LINE 19-51A

A. *Mendelian Inheritance of Herbicide Tolerance in Cotton Line 19-51a*

Cotton line 19-51a was an initial R_0 plant selected on 25 ppb chlorsulfuron as described previously. Progeny derived from self-pollination of plant 19-51a were tested for inheritance and expression of the introduced ALS gene by two methods: 1) callus formation from seedling cotyledons on tissue culture medium containing chlorsulfuron and 2) seed germination in soil supplemented with chlorsulfuron. In the callus assay, 28 of 35 seedlings formed callus on medium containing 50 ppb chlorsulfuron; untransformed control seedlings failed to form callus on this medium. In the soil germination test, 18 of 25 progeny plants grew vigorously in soil saturated with 100 ppb chlorsulfuron; untransformed control seedlings failed to develop beyond the cotyledonary stage in this treatment. These experiments demonstrate that sulfonylurea tolerance segregates in transformed cotton line 19-51a as a dominant Mendelian trait. The ratio of tolerant to sensitive progeny was statistically indistinguishable from 3:1 in each assay, suggesting that the gene has integrated into a single chromosomal locus.

R_1 seedlings which survived the soil germination test described above were either homozygous or heterozygous for the tolerance gene. All were grown to maturity and self-pollinated. R_2 seeds derived from these self-pollinations were again screened by germination in soil saturated with 100 ppb chlorsulfuron in order to identify homozygous lines. If tolerance were expressed at a single locus, one-third of the R_2 lines would be expected to be homozygous. Seven of 18 R_2 lines were homozygous in this test, confirming that the trait is expressed at a single locus.

The genetic stability of expression of the ALS gene in line 19-51a can be examined by looking at segregation of tolerance in elite lines derived through repeated rounds of backcrossing. An R_2 plant homozygous for the resistance gene was used to make F_1 crosses with elite lines DP51, DP5415, and DP5690. Tolerant progeny were backcrossed five times to the elite parents, and then selfed to produce BC_5F_2 plants. These plants were treated with a sulfonylurea herbicide in the field in 1994 to eliminate sensitive segregants; tolerant plants self-pollinated to produce BC_5F_3 lines.

150 BC_5F_3 lines for each elite variety were planted in the field in 1995 and sprayed with a sulfonylurea herbicide to identify homozygous lines. Again, since sensitive segregants were removed during the previous generation, one-third of the lines should be homozygous for the tolerance gene if it has continued to segregate as a single locus through ten generations of self-pollinations and crosses. Results are summarized in the following table, confirming the genetic stability of expression of the ALS gene in 19-51a and its derivatives.

Table IV
Segregation of sulfonylurea tolerance in line 19-51a and elite derivatives

Line	Generation	Number Lines Tested	Expected Homozygous	Observed Homozygous
19-51a	R2	18	6	7
DP51-SU	BC_5F_3	150	50	36
DP5415-SU	BC_5F_3	150	50	41
DP5690-SU	BC_5F_3	150	50	52

B. Molecular Analysis of Sulfonylurea Tolerant Cotton

1. PCR analysis on R2 progeny

DNA was purified from leaf tissue of eight randomly selected homozygous R_2 progeny of plant 19-51a by the method of Hall *et al.* (1978). The DNA was analyzed by PCR assay to confirm that the chimeric S4-HrA gene was stably integrated into the cotton genome and transmitted through normal sexual reproduction. Two different sets of primers were used to determine the presence of the transgene. The first set of primers produced a 642 bp fragment internal to the ALS 2 coding region of the chimeric S4-HrA gene and the second set of primers produced a 494 bp fragment extending from the promoter of ALS 1 into the beginning of the ALS 2 fragment. All of the eight transgenics produced a band of the expected sizes for both sets of primers (Figures 4 and 5). A faint 642 bp band present in the wild type cotton DNA sample (Coker 312, negative control sample) for the first set of primers was probably due to contamination of the sample or due to amplification of the cotton ALS gene since these primers are located

in the highly homologous region of plant ALS genes. These data confirmed that the gene was stably integrated into the genome of transformant 19-51a and was transmitted through at least two generations of self-pollination.

Figure 4
PCR on R2 Progeny of Cotton Line 19-51a; Chimeric S4-Hra coding sequence primers

Cotton genomic DNA from leaves of eight R2 homozygous progeny of line 19-51a was analyzed using PCR to confirm the presence of the chimeric S4-HrA gene. The positive control is pMH26 plasmid DNA and the negative control is DNA prepared from the wild type cotton line Coker 312. The primer set was designed to produce a 642 base pair (bp) fragment from the coding sequence of the chimeric S4-HrA gene beginning from the first resistance mutation. The 5' primer was 5'-CAGGTCAAGTGGCACGTAGGATG-3' which extends from bp 3540 to 3562 of the T-DNA sequence shown in Figure 3 and the 3' primer was 5'-GGCTGCTTGTTCTTTCCAATCT-3' which extends from bp 4182 to 4161 of the T-DNA sequence shown in Figure 3.

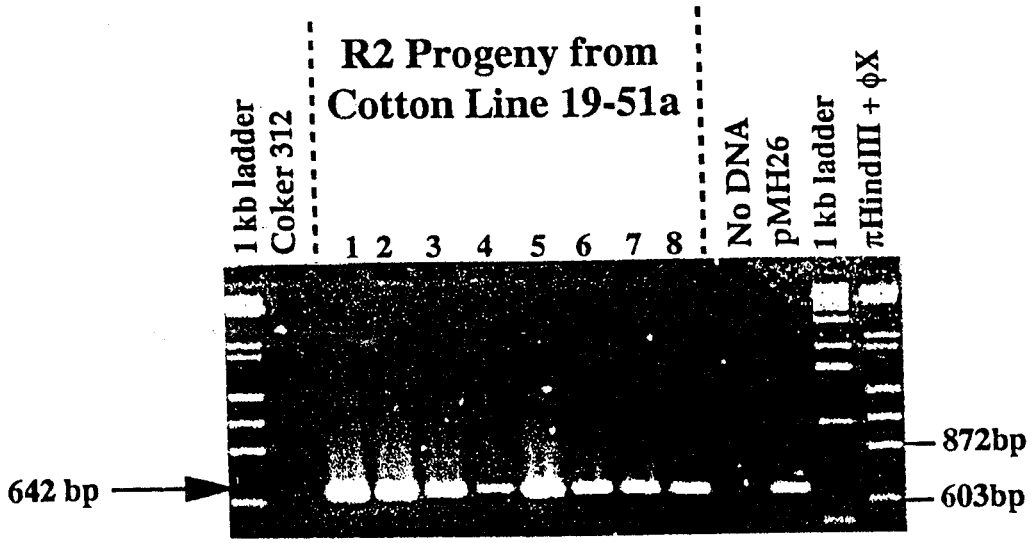
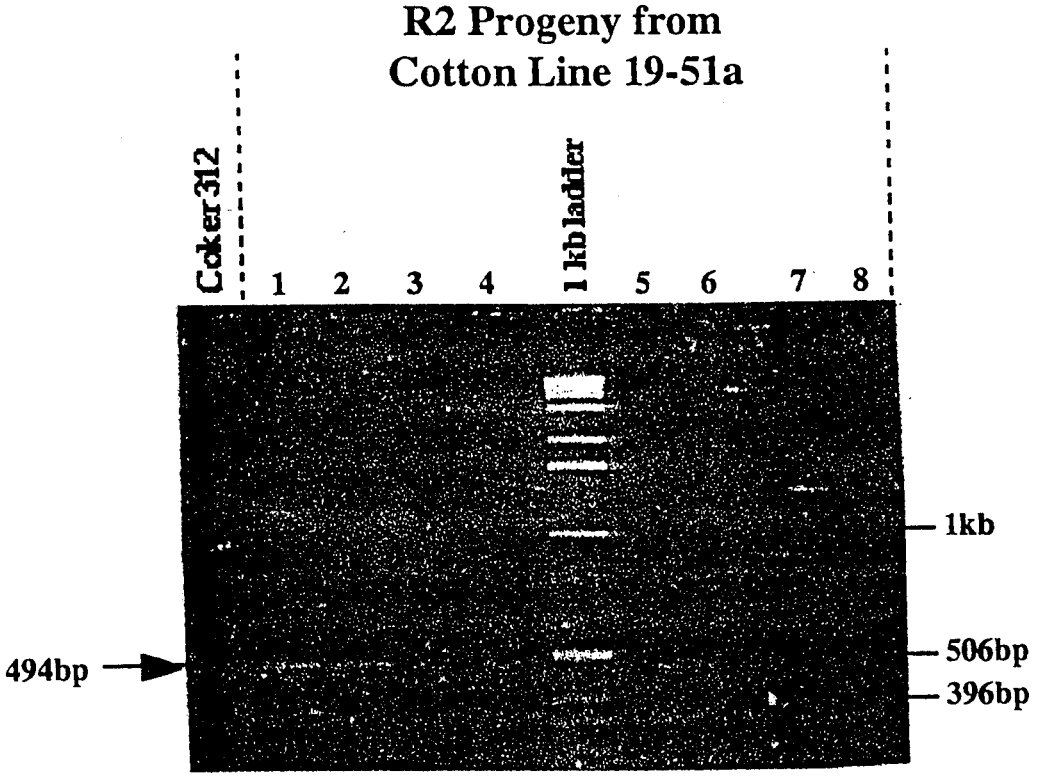


Figure 5

PCR on R2 Progeny of Cotton Line 19-51a; ALS 1 promoter to ALS 2 fragment sequence primers

Cotton genomic DNA from leaves of eight R2 homozygous progeny of line 19-51a was analyzed using PCR to confirm the presence of the chimeric S4-HrA gene. The negative control is DNA prepared from the wild type cotton line Coker 312. The primer set was designed to produce a 494 base pair (bp) fragment extending from the ALS 1 promoter to just beyond the Nco I site where ALS 1 and ALS 2 are joined. The 5' primer was 5'-CAAGTAGGACCCTTTCAGTTTCG-3' which extends from bp 2858 to 2880 of the T-DNA sequence shown in Figure 3 and the 3' primer was 5'-CAAAGCTTGGTGAATCTCCATGG-3' which extends from bp 3352 to 3330 of the T-DNA sequence shown in Figure 3.



2. Southern blot analysis of cotton line 19-51a

Southern blot analysis (Southern 1975) was carried out on genomic DNA from cotton line 19-51a to characterize the inserted T-DNA more thoroughly. The Southern blots were designed to address four main issues concerning the inserted T-DNA: 1) the number of loci into which the chimeric S4-HrA gene was inserted, 2) the number of copies of the chimeric S4-HrA gene inserted into the cotton genome, 3) the organization of the T-DNA insert and 4) to determine if the insert extends beyond the T-DNA borders.

a. Hybridization probes used for Southern blot analysis

Two different hybridization probes were used to analyze the DNA insertion event in cotton line 19-51a. A DNA fragment encompassing the entire length of the chimeric S4-HrA gene was isolated by the restriction enzymes Bam HI to Pst I from plasmid pMH26 (Figure 1) and was used as a probe to address the first three issues listed above. The second probe was a mixture of two different DNA fragments, one located just outside of the T-DNA right border and the other just outside of the left border. Both of these DNA fragments were produced by PCR from plasmid pMH26 and subsequent gel isolation and purification. Their sequence and location on pMH26 are indicated in Figure 6. Figure 7 is a schematic representation of the probes relative to the T-DNA insert and it also indicates the location of the restriction enzymes used in the Southern blot analysis described below.

DNA was isolated from Coker 312 and from two different F1 lines both derived from the original cotton line 19-51a using etiolated cotyledons. Cotton seeds from the F1 lines were planted in soil and allowed to germinate in the dark, ten to twenty cotyledons were harvested after ten days and combined to prepare DNA by the method described by Hall *et al.* (1978). Several different restriction enzymes were used to digest DNA from the F1 lines and Coker 312, the DNA subjected to agarose gel electrophoresis, blotted onto nylon membranes and hybridized to a digoxigenin labeled chimeric S4-HrA probe. Visualization of hybridized probe was carried out by reacting the membrane with anti-digoxigenin alkaline phosphatase conjugate followed by chemiluminescent detection with Lumi-Phos™ 530 as described in Boehringer Mannheim's "The Genius™ System User's Guide for Filter Hybridization".

Figure 6

Southern blot probes outside of T-DNA borders

The two probes produced by PCR result in an external RB probe of 545 bp in length and an external LB probe of 520 bp in length and are indicated by the thin solid lines below the DNA sequence. The bp numbers correspond to the bp numbers in plasmid pMH26 as shown in Figure 1. The thick solid lines are the 24 bp repeats of the right and left border from the T-DNA, which are not included in the probe. Only a small portion of the DNA sequences between the left and right border are shown in this figure; refer to Figure 3 for the complete sequence between the borders.

1059 GGCTTGAGCAACAGCTGCCGTGGAACTGGACATCCAAGACACTGAGTGCTCAGGCGGCCTGACC

1123 TGCGGCCTTCACCGGATACTTACCCCATTATCGCAGATTGCGATGAAGCATCAGCGTCATTCAG

1187 CAATCTTGCCAAAGTATGCAGGCTCGCGAGAATCGACGTGCGAAACCGGCTGGTTGCGCCAAAG

1251 ATCCGCTTGCGGAGCGGTCGAACATTCATGCTGGGACTTCAAGAGGTCGAGTAGAGGAAGAACC

1315 GGAAAGGTTGCACCGGAAAATATGCGTTCCTTTGGAGAGCGCCTCATGGACGTGAACAAATCGC

1379 CCGGACCAAGGATGCCACGGATACAAAAGCTCGCGAAGCTCGGTCCCGTGGGTGTTCTGTCGTC

1443 TCGTTGTACAACGAAATCCATTCCCATTCCGCGCTCAAGATGGCTTCCCCTCGGCAGTTCATCA

1507 GGGCTAAATCAATCTAGCCGACTTGTCCGGTGAAATGGGCTGCACTCCAACAGAAACAATCAAA

RB (1605)

1571 CAAACATACACAGCGACTTATTCACACGAGCTCAAATTACAACGGTATATATCCTGCCAGTCAG

1635 CATCATCACACCAAAAGTTAGGCCC

LB (7334)

7283 CCATGTAGATTTCCCGGACATGAAGCCATTTACAATTGAATATATCCTGCCGCCGCTGCCGCTTT

7348 GCACCCGGTGGAGCTTGCATGTTGGTTTCTACGCAGAACTGAGCCGGTTAGGCAGATAATTTCCA

7413 TTGAGAACTGAGCCATGTGCACCTTCCCCCCAACACGGTGAGCGACGGGGCAACGGAGTGATCCA

7478 CATGGGACTTTTAAACATCATCCGTCGGATGGCGTTGCGAGAGAAGCAGTCGATCCGTGAGATCA

7543 GCCGACGCACCGGGCAGGCGCGCAACACGATCGCAAAGTATTTGAACGCAGGTACAATCGAGCCG

7608 ACGTTCACGGTACCGGAACGACCAAGCAAGCTAGCTTTAATGCGGTAGTTTATCACAGTTAAATT

7673 GCTAACGCAGTCAGGCACCGTGTATGAAATCTAACAATGCGCTCATCGTCATCCTCGGCACCGTC

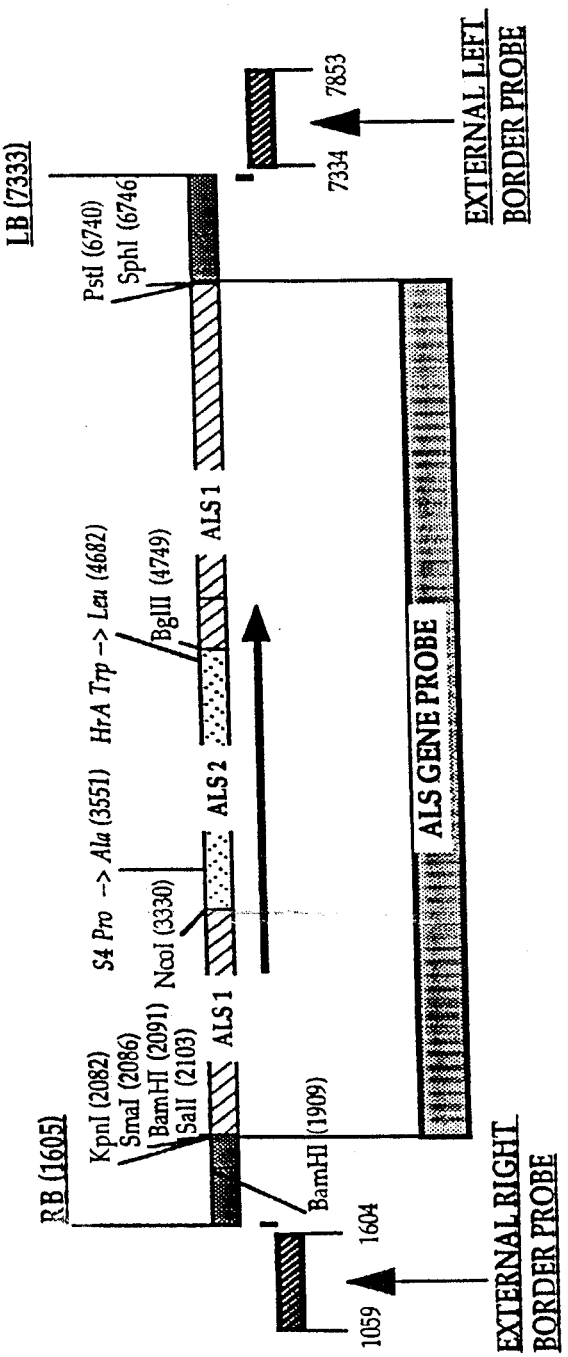
7738 ACCCTGGATGCTGTAGGCATAGGCTTGGTTATGCCGGTACTGCCGGGCCTCTTGCGGGATATCGT

7803 CCATTCCGACAGCATCGCCAGTCACTATGGCGTGCTGCTAGCGCTATATGCG

Figure 7

Schematic representation of the T-DNA region found in plasmid pMH26

The hybridization probes used for Southern blot analysis are indicated below the map of the T-DNA. The base pair numbers refer to the locations on plasmid pMH26 as seen in Figure 1.



b. Southern blot analysis with the Chimeric S4-HrA Probe to determine number of genetic loci, number of copies and organization of the T-DNA insert

The number of border junction fragments (DNA fragments extending from the T-DNA into the plant genome) present on a Southern blot is used to determine the number of insertion loci (Jorgenson *et al.* 1987). For each copy of the T-DNA inserted there is one right border junction fragment and one left border junction fragment which can be detected on a Southern with the correct restriction enzyme digests of the plant DNA. Simple insertions of single T-DNA regions result in equal numbers of right and left border junction fragments which also equals the number of copies of the T-DNA inserted into the genome. If multiple insertions of the T-DNA occur at one site then the number of right and left border junction fragments are no longer equal. Hybridization signals of varying intensity for different DNA fragments due to more than one copy of the probe target within one fragment, are also a strong indication of multiple insertions at one locus. The orientation of the tandem inserts can be determined since the size of fragments between T-DNA inserts can be calculated based on the known sequence and restriction enzyme sites in the T-DNA. With a combination of the correct choice of restriction enzymes and probe, the T-DNA insert in line cotton 19-51a was determined to contain two copies of the T-DNA arranged as an inverted repeat at one locus (Figure 8). Tandem or inverted repeat T-DNA insertions are not unusual and have been previously described in the literature (Jorgenson *et al.* 1987).

Table V describes the hybridizing fragment sizes expected from a single T-DNA insert and an inverted repeat T-DNA insert and compares them to the hybridizing fragment sizes seen on Southern blot analysis of cotton line 19-51a (Figures 9). The hybridizing restriction enzyme fragments correlated with the sizes expected for an inverted repeat of the T-DNA. Digests for right border fragments (Sph I and Pst I) resulted in two bands suggesting the presence of two copies of the inserted T-DNA either in one or two loci. However, digests for left border fragments (Bam HI, Kpn I, and Sma I) each produced only one strongly hybridizing band of approximately the same size and equal to the fragment size expected for an inverted repeat. Additional digests with enzymes internal to the chimeric S4-HrA gene (Nco I, Bgl II and Nco + Bgl II) resulted in fragments sizes equal to an inverted repeat arrangement of the T-DNA within the

resolution of the technique (Table V and Figure 8). These internal digests also produced bands with strong hybridization signals and confirmed the number of T-DNA to plant border junctions. One locus as determined by Southern blot analysis confirms the Mendelian inheritance pattern as discussed in Section IVA.

Figure 8
Schematic diagram of inverted repeat structure of the T-DNA from pMH26 in Cotton Line 19-51a.

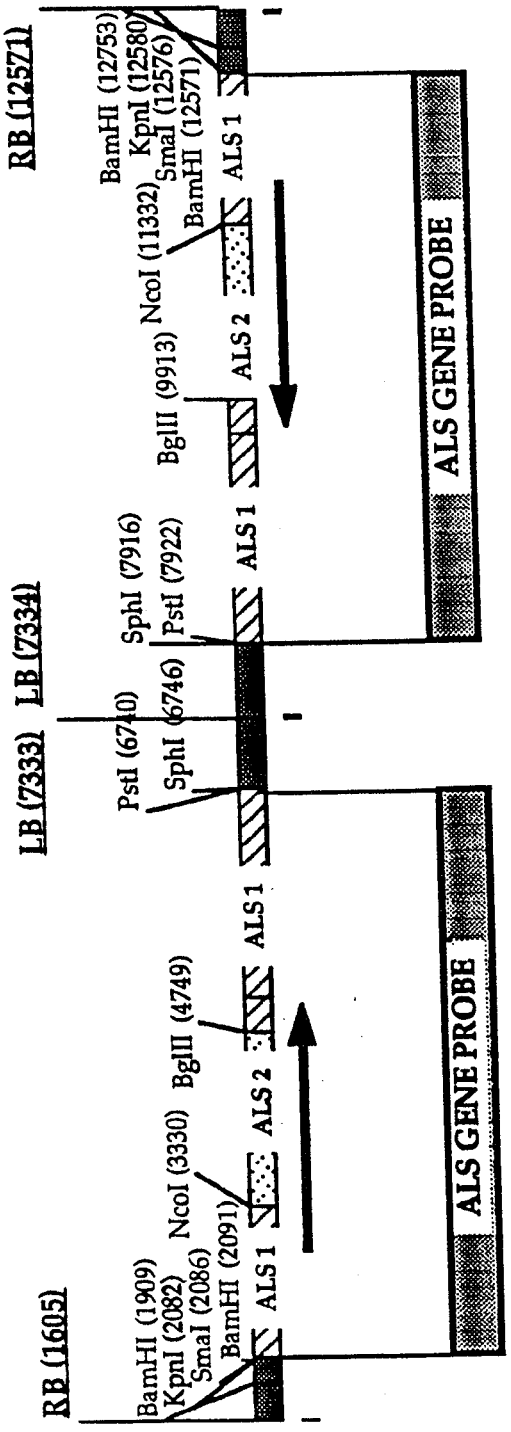


Figure 9

Southern blot analysis of Cotton Line 19-51a: Chimeric S4-HrA probe

Cotton genomic DNA (20 µg) prepared from etiolated cotyledons from Coker 312 and from two different F1 lines (F1-1 and F1-2) derived from cotton line 19-51a was digested with the enzymes indicated above the lanes and subjected to Southern blot analysis using the chimeric S4-HrA probe. The marker lane contains 1 kb ladder from BRL. Abbreviations: Bam HI (B), Bgl II (Bg), Kpn I (K), Nco I (N), Pst I (P), Sma I (Sm). Sph I (Sp), Uncut (U). The various digests are divided between blots A, B and C.

A)

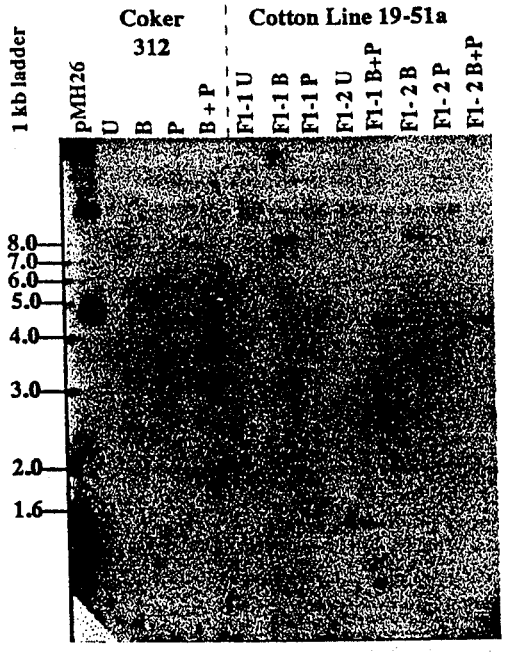
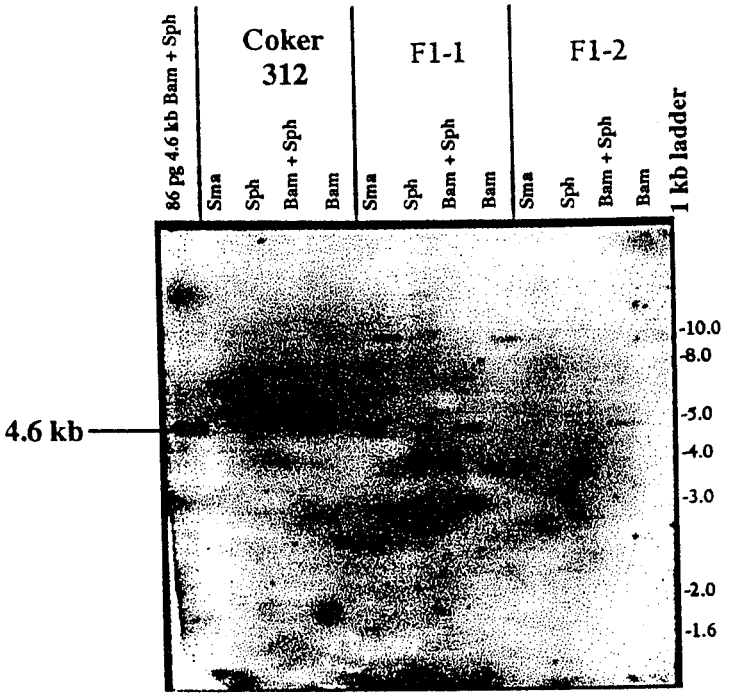


Figure 9 (Continued)

B)



C)

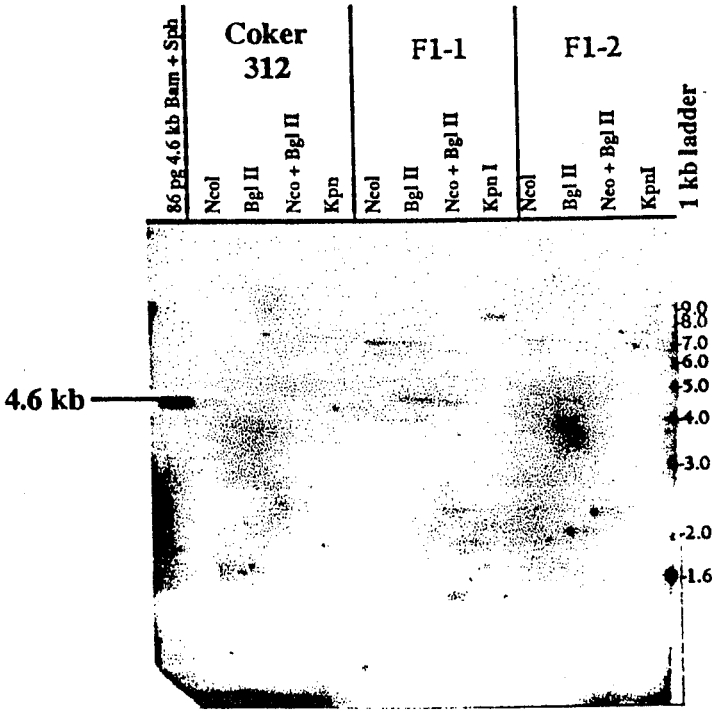


Table V

Southern blot analysis on Cotton Line 19-51a

The first column lists the restriction enzymes used on cotton DNA from Coker 312 and two F1 lines derived from cotton line 19-51a for Southern blot analysis. The Southerns were probed with the ALS gene probe. Column two indicates the expected fragment sizes that would be seen on a Southern of a single T-DNA insertion event and column three indicates the expected sizes for an inverted repeat of the T-DNA. Column four lists the actual fragment sizes visualized on the Southerns. Italicized numbers indicate that the fragment should be larger than the indicated number (i.e. a border fragment). Underlined numbers indicate a strong hybridization signal relative to the other bands on the Southern.

RESTRICTION ENZYMES	SINGLE INSERT	INVERTED REPEAT	COTTON LINE 19-51A
Bam HI	*> 5242 bp*	10480 bp	10200 bp
Pst I	*> 5140 bp*	*> 5140 bp* *> 5136 bp*	> 12000 bp > 12000 bp
Bam HI + Pst I	4653 bp	4653 bp 4645 bp	4600 bp
Sma I	*> 5245 bp*	10490 bp	10500 bp
Sph	*> 5146 bp*	*> 5146 bp* *> 5142 bp*	> 12000 bp 11000 bp
Bam HI + Sph I	4659 bp	4659 bp 4651 bp	4500 bp
Nco I	*> 4003 bp* *> 1726 bp*	8002 bp *> 1730 bp* *> 1726 bp*	7900 bp 6900 bp 2200 bp
Bgl II	*> 3145 bp* *> 2584 bp*	5164 bp *> 3149 bp* *> 3145 bp*	7600 bp 4600 bp 3900 bp
Nco I + Bgl II	*> 2584 bp* *> 1726 bp* 1419 bp	5164 bp *> 1730 bp* *> 1726 bp* 1419 bp 1419 bp	5900 bp 4600 bp 2200 bp 1400 bp
Kpn I	*> 5247 bp*	10498 bp	10000 bp

c. *Southern blot analysis with the External Border Probes for DNA outside of the T-DNA borders*

T-DNA inserts are not precise DNA segments since the T-DNA to plant junctions can occur within 10-40 bp of the right border and within 30-2000 bp of the left border (Jorgensen *et al.* 1987 and Holsters *et al.* 1983). The variability of the DNA around the borders can be either a truncation of the T-DNA or an extension of the T-DNA beyond the borders. In order to confirm that the integrated T-DNA did not contain DNA sequences external to the border the external border probes as described in Section IV.B.2a were used as probes on DNA from the two F1 lines derived from cotton line 19-51a.

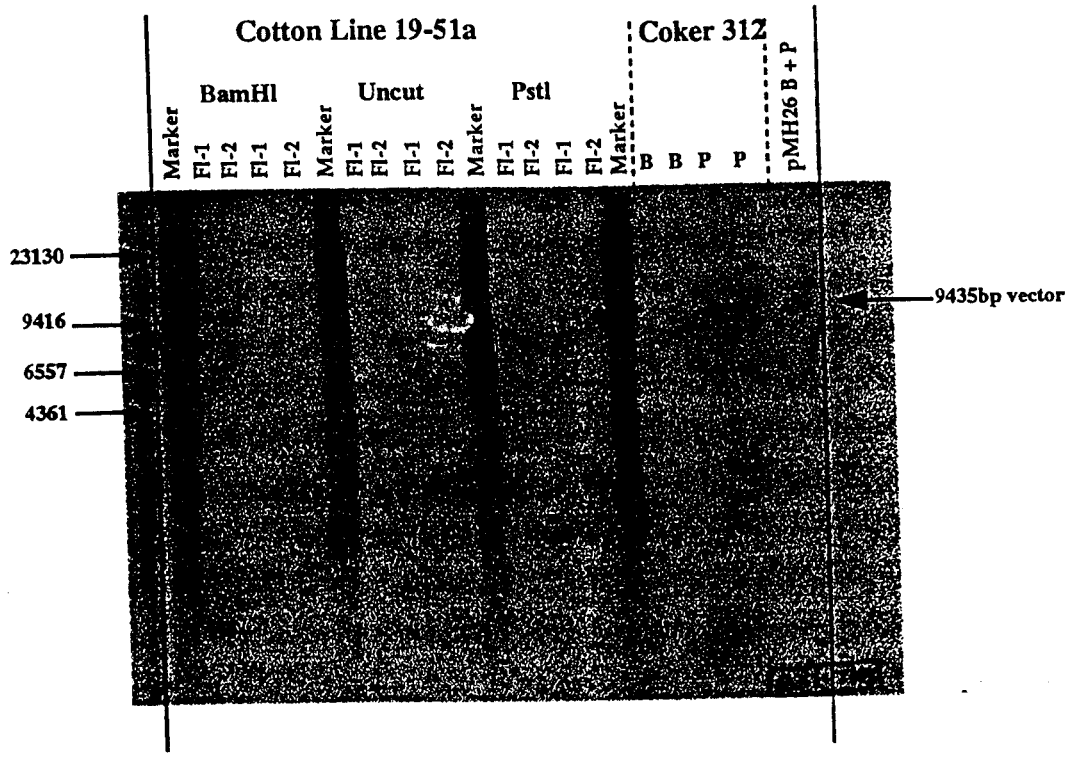
Southern blot analysis with the two external border probes (Section IV.B.2.a and in Figure 6) indicated that no DNA beyond the T-DNA borders integrated into cotton line 19-51a (Figure 10). DNA was prepared as described above and digested with either Bam HI or Pst I and hybridized to the two ^{32}P-labeled external border probes mixed together in the same hybridization reaction. No hybridization was detectable in any of the cotton DNA lanes; the probe did hybridize to the expected 10.0 kb band in the control plasmid DNA lane. The same blot produced the expected hybridization band pattern when hybridized to ^{32}P-labeled chimeric S4-HrA probe. These results indicated that only the T-DNA region of pMH26 integrated into cotton line 19-51a.

C. *Stability of the T-DNA Insert*

The combined data from PCR and Southern analyses as discussed above confirm that the T-DNA was stably integrated into the genome. PCR results confirmed the presence of the chimeric S4-HrA gene in the cotton genome following two generations of self-pollinations indicating that the gene was stably integrated into the cotton genome and transmitted through normal sexual reproduction. Southern blot analysis confirmed the presence of the T-DNA following several breeding cycles and determined the structure of the T-DNA insert as an inverted repeat at a single locus in the cotton genome. The identical Southern blot hybridization band pattern in two different F1 lines derived from cotton line 19-51a further confirmed the stability of the insert.

Figure 10
Southern blot analysis of Cotton Line 19-51a: External border probes

Cotton genomic DNA (20 µg) prepared from etiolated cotyledons from Coker 312 and two different F1 lines (F1-1 and F1-2) derived from cotton line 19-51a was digested with the enzymes indicated above the lanes and subjected to Southern blot analysis using the two external border probes. The positive control was 150 pg of pMH26 digested with the indicated enzymes. The marker lane contain DNA/Hind III from BRL. Abbreviations: Bam HI (B) and Pst I (P).



D. *Biochemical Analysis of Cotton Line 19-51a: ALS Enzyme Assays*

Partially purified extracts prepared from leaves of transgenic cotton plants were assayed *in vitro* for ALS activity using the method described by Chaleff and Mauvais (1984). Four BC_3F_6 transgenic plants were assayed: two DP51-SU and two DP5690-SU. These plants contain a mixture of tolerant ALS encoded by the introduced tobacco gene and sensitive ALS encoded by endogenous cotton genes. Four non-transgenic plants were also assayed: two DP51 and two DP5690. These plants contain only sensitive ALS encoded by endogenous cotton genes. Four reactions were run for each extract: two with no herbicide and two containing 100 ppb of the sulfonylurea herbicide chlorsulfuron. Results for duplicate assays were averaged.

In this assay, the reaction product of ALS, acetolactate, is converted to acetoin by an acid quench. Acetoin is then converted, by reaction with creatine and α-napthol, to a chromogen which absorbs at 530 nm. In the following table, ALS specific activities are reported as Δabs530 per mg protein per minute. In addition, for each plant, ALS activity in the presence of chlorsulfuron is expressed as a percentage of activity in the absence of chlorsulfuron. The concentration of chlorsulfuron included in the assay is sufficient to completely inhibit the sensitive cotton ALS; the tolerant ALS encoded by the introduced S4-HrA tobacco gene is not inhibited at this concentration. A small amount of acetoin is produced through another pathway, accounting for a background chromogen production (5-6% of total) in chlorsulfuron-inhibited extracts.

Table VI
Specific activity of ALS in transgenic and non-transgenic cotton pants

Specific activity is reported as Δabs530 per mg protein per minute; each value is the average of two reactions. Each extract was assayed in the presence and absence of a concentration of chlorsulfuron sufficient to completely inhibit sensitive ALS. The designation SU indicates a transgenic plant.

Plant	Specific Activity No Chlorsulfuron	Specific Activity 100 ppb Chlorsulfuron	% Uninhibited Activity
DP51 A	0.65	0.04	6
DP51 B	0.65	0.03	5
DP51-SU A	0.71	0.39	55
DP51-SU B	0.40	0.21	54
DP5690 A	0.38	0.02	6
DP5690 B	0.55	0.03	5
DP5690-SU A	0.76	0.39	52
DP5690-SU B	0.86	0.50	58

The increase in ALS specific activity caused by the introduction of the S4-HrA tobacco gene can be estimated using two different methods. First, the specific activities of extracts from non-transgenic plants can be compared with those from transgenic plants, using the results from reactions without chlorsulfuron. The average specific activity of the transgenic extracts, 0.68, is slightly higher than that of the non-transgenic extracts, 0.56. However, this difference is not statistically significant. The table reveals that specific activity varies from plant to plant, whether transgenic or non-transgenic. Thousands of ALS activity assays performed on many different species during the past ten years at DuPont have repeatedly demonstrated this kind of plant-to-plant variation.

A more reliable method for estimating the increase in specific activity caused by introduction of the S4-HrA tobacco ALS gene involves comparison of specific activities measured in the presence and absence of chlorsulfuron at a concentration which completely inhibits sensitive cotton ALS, but does not inhibit the tolerant tobacco ALS. Since this method compares activities separately for each plant extract, the effect of plant-to-plant variation is eliminated. In the assays lacking chlorsulfuron, specific activity is a

mixture of tolerant ALS activity encoded by the introduced tobacco gene and sensitive ALS activity encoded by endogenous cotton genes. In the assays containing chlorsulfuron, only the tobacco ALS activity is measured.

The table demonstrates that 100 ppb chlorsulfuron virtually eliminates ALS activity in non-transgenic extracts. As noted above, the small amount of residual chromogen production in these assays is due to a non-ALS activity which produces acetoin. In the extracts prepared from transgenic plants, 100 ppb chlorsulfuron consistently inhibits about 50% of the total ALS activity, regardless of the actual activity level in a given plant. This suggests that roughly one-half of the ALS activity in the transgenic plants is encoded by endogenous genes, and one-half by the introduced gene. Assuming that the introduced gene does not increase or decrease the endogenous ALS activity, these data imply that ALS specific activity is increased no more than two-fold in cotton plants derived from transgenic line 19-51a.

V. SEED COMPOSITIONAL ANALYSES OF LINE 19-51A

Cottonseed is processed into oil, meal, hulls, and linters which are used in a large variety of applications. The most important applications involve the use of meal as a fiber and protein source in animal feed, particularly for ruminants such as cattle, and the use of refined oil in the manufacture of many human food products (Cherry and Leffler 1984).

Compositional analyses were performed on acid-delinted cottonseed samples of transgenic line 19-51a and non-transgenic Coker 312 collected from a replicated yield trial in Greenville, MS. Samples were collected from plots which were not treated with sulfonylurea herbicide, since such treatment would have seriously injured the Coker 312 control plants. Compositional values reported in the tables below are the means of five replicates for each cultivar. Any differences significant at the $p = 0.05$ level are noted.

A. *Protein and Oil Content of Seeds from Line 19-51a and Coker 312*

Several studies have found that cottonseed protein and oil contents vary significantly across cultivars, environments, and year (Lawhon *et al.* 1976, Turner *et al.* 1976, Cherry *et al.* 1978, Kohel 1980, Cherry 1983, Kohel *et al.* 1985). Kohel *et al.* surveyed the protein content of 747 accessions from the Stoneville Germplasm Collection, which represented Upland cotton cultivars, breeding lines, and genetic stocks adapted to the U.S. Cotton Belt. Protein content was found to range from 16 to 32% in this collection. Kohel examined twenty cultivars from the same collection (ten high oil and ten low oil) at two locations over four years, and found that oil content ranged from 17 to 31%.

Protein and oil content of the 19-51a and Coker 312 seed samples collected at Greenville are summarized in the table below. Protein content was determined using AOAC Official Methods 955.04 and 954.01, and oil content was determined using AOCS Official Method Ba 3-38 (Appendix 1). Contents are expressed as percentages of delinted seed weight.

Differences between the transgenic and non-transgenic samples are not significant at the p = 0.05 level. Protein contents for both lines fall within the range determined by Kohel *et al.*, and oil contents fall within the range determined by Kohel.

Table VII
Protein and oil content of line 19-51a and Coker 312

Line	Protein Content (% seed weight)	Oil Content (% seed weight)
19-51a	27.70	20.75
Coker 312	27.74	20.47

B. *Amino Acid Composition of Meal from Line 19-51a and Coker 312*

Cottonseed meal is an important ingredient in various animal feed formulations. Formulators pay particular attention to the amino acid composition of feed additives in order to assure the proper balance of essential amino acids for each stage of animal development. The cellular target of the sulfonylurea herbicides, acetolactate synthase (ALS), is a key regulatory enzyme in the biosynthesis of three essential amino acids: leucine, isoleucine, and valine. Since herbicide tolerance in line 19-51a is mediated by the expression of an altered form of ALS, the amino acid composition of its seed meal was examined to ensure that no changes in composition occurred as the result of unintended modification of the regulatory properties of ALS.

Amino acid composition of the 19-51a and Coker 312 seed samples collected at Greenville are summarized in the table below. Amino acid hydrolysates were prepared from de-fatted meal samples, separated by HPLC, and quantified by the ninhydrin reaction (see procedure in Appendix 2). Compositions are expressed as percentages of total amino acids.

Differences between the transgenic and non-transgenic samples are not significant at the p = 0.05 level for all amino acids except glutamate and aspartate. The absolute differences for these two amino acids are quite small: glutamate is higher (21.0 vs. 20.8%) and aspartate lower (9.8 vs. 10.0%) in the transgenic line. No significant differences were observed for any of the essential amino acids. The table also includes

amino acid ranges determined for sixteen cotton cultivars by Lawhon *et al.* (1977). All values for both 19-51a and Coker 312 fall within these ranges except aspartate, which is slightly higher than the literature value in both lines.

Table VIII
Amino acid composition of meal from Line 19-51a and Coker 312 (as % of total amino acids)

Amino Acid	19-51a	Coker 312	Lawhon *et al.*
Cysteine	1.72	1.74	----
Aspartate	9.84	10.02	8.6 - 9.5
Methionine	1.62	1.63	1.2 - 1.8
Threonine	3.15	3.16	2.8 - 3.2
Serine	4.27	4.19	3.9 - 4.4
Glutamate	21.01	20.82	19.9 - 22.4
Glycine	4.34	4.40	3.7 - 4.6
Alanine	3.94	3.98	3.6 - 4.2
Valine	4.68	4.70	4.1 - 4.8
Isoleucine	3.23	3.24	2.8 - 3.4
Leucine	5.89	5.84	5.3 - 6.1
Tyrosine	2.76	2.72	1.6 - 3.6
Phenyalanine	5.71	5.63	5.0 - 6.2
Lysine	4.50	4.53	4.2 - 4.6
Histidine	2.93	2.89	2.6 - 2.9
Arginine	12.44	12.51	10.9 - 13.2

C. *Fatty Acid Composition of Oil from Line 19-51a and Coker 312*

Cottonseed oil is used in a large number of human food applications, particularly for commercial baking and frying, and as a component of bottled cooking and salad oils. It is also used in the manufacture of mayonnaise and margarine.

Fatty acid composition of oil extracted from the 19-51a and Coker 312 seed samples collected at Greenville are summarized in the table below. Fatty acid methyl

esters were prepared from hexane-extracted oil, and separated and quantified by gas chromatography (see procedure in Appendix 3). Compositions are expressed as percentages of total fatty acids.

Differences between the transgenic and non-transgenic samples are significant at the $p = 0.05$ level for myristic, linoleic and linolenic acids. However, absolute differences are very small, and all values for both lines fall within the ranges adopted by the FAO/WHO Codex Alimentarius Committee on Fats and Oils in 1993.

Table IX
Fatty acid composition of oil from Line 19-51a and Coker 312 (as % of total fatty acids)

Fatty Acid	19-51a	Coker 312	Codex Standard
Myristic	0.65	0.71	0.5 - 2.5
Palmitic	22.50	23.04	17 - 29
Palmitoleic	0.76	0.75	0.5 - 1.5
Stearic	2.16	2.23	1.0 - 4.0
Oleic	15.33	15.86	13 - 44
Linoleic	57.99	56.70	33 - 58
Arachidic	0.37	0.39	< 0.5
Linolenic	0.24	0.32	0.1 - 2.1

D. Toxicant Levels in Seed from Line 19-51a and Coker 312

Gossypol

Gossypol is a phenolic compound found in glands in various parts of the cotton plant, including the seed (Adams *et al.* 1960, Abou-Donia 1976, 1989). Cottonseed has been reported to contain from 0.4 to 1.7% gossypol, with variation between cultivars and growth environments. Gossypol has a wide range of toxicological effects in animals, especially non-ruminants. These include direct effects on the biochemistry and

physiology of the animal; they also include indirect effects caused by reactions between gossypol and proteins in the animal diet, which lower the availability of key amino acids.

Free and total gossypol contents of the 19-51a and Coker 312 seed samples collected at Greenville are summarized in the table below. Free gossypol content was determined using AOCS Official Method Ba 8-78, and total gossypol was determined using AOCS Official Method Ba 7-58 (Appendix 4). Contents are expressed as percentages of delinted seed weight.

Both free and total gossypol are significantly higher in the transgenic line, at the $p = 0.05$ level. However, total gossypol values for both lines fall in the middle of the 0.4-1.7% range reported in the literature.

Table X
Gossypol content of seeds from Line 19-51a and Coker 312 (as % of seed weight)

Line	Free Gossypol	Total Gossypol
19-51a	0.852	0.901
Coker 312	0.789	0.832

Cyclopropene Fatty Acids

Cottonseed oil contains several cyclopropene fatty acids; the most abundant are malvalic and sterculic acids. Animal feeding studies have shown that these fatty acids inhibit the conversion of stearic to oleic acid, changing the ratio of saturated to monounsaturated fatty acids in eggs, milk, and animal body fat (Johnson et al. 1967). Most of the cyclopropene fatty acids are removed during the deodorization step of oil refining (Mattson 1973).

Cyclopropene fatty acid content of oil extracted from the 19-51a and Coker 312 seed samples collected at Greenville are summarized in the table below. Hexane-extracted oil was subjected to the Halphen reaction (AOAC Method 974.19) (Appendix 5), in which cyclopropene fatty acids react with sulfur in carbon disulfide to produce a chromogen which absorbs at 547 nm. Results are expressed as abs547 per

100 mg of oil. The difference between the values for 19-51a and Coker 312 are not significant at the p = 0.05 level.

Table XI

Cyclopropene fatty acid levels in oils from Line 19-51a and Coker 312

Oil Source	Abs547/100 mg Oil
19-51a	0.586
Coker 312	0.592
Refined Cottonseed Oil (House of Tsang Wok Oil)	0.224
Refined Corn Oil (Mazola)	0.000

VI. FIELD PERFORMANCE OF LINE 19-51A AND ITS DERIVATIVES

A. *Herbicide Tolerance of Parental and Sulfonylurea Tolerant Cotton*

From 1991 to 1994, thirty-five field trials were conducted by DuPont or its cooperators in Arkansas, Delaware, Georgia, Louisiana, Mississippi, North Carolina, Puerto Rico, South Carolina, Tennessee, and Texas. In 1995 approximately 50 field trials were or are currently being conducted at approximately 50 sites in 14 states (see Section XII for field test reports, including reports for completed 1995 tests). In these trials, various sulfonylurea and other ALS-inhibiting herbicides were applied to line 19-51a or its elite derivatives, as well as to non-transgenic parental lines.

The following table summarizes the response to one of these herbicides: DPX-M6316, a sulfonylurea which is highly injurious to cotton. Data are presented from 1992 and 1994. In 1992, the R5 generation of the original 19-51a transformant was compared with its parental line, Coker 312. In 1994, three lines created by backcrossing the ALS gene into elite varieties DP51, DP5415, and DP5690 were compared with the recurrent parents.

Tolerance is expressed using a visual injury rating system in which 0 = No Injury and 100 = Dead Plant. Ratings were taken 11-17 days after herbicide treatment. Injury ratings lower than 15 are considered to be acceptable at this interval after post-emergence herbicide treatment in cotton. Most trials included four to six replicate plots for each treatment.

Table XII
Sulfonylurea herbicide tolerance in Line 19-51a and its elite derivatives

Plots were treated with M6316 at 0.125-oz.-a.i./ac post-emeregence at first to third true leaf stage of cotton.

DPX M6316	0.125 OZ A.I./AC	POST-EMERGENCE	
Generation	Number of Trials	Average Injury Non-Transgenic	Average Injury Transgenic
R_5	3	89.0 (s.d. = 15.5)	5.0 (s.d. = 1.4)
BC_3F_5	9	87.3 (s.d. = 6.9)	7.9 (s.d. = 5.5)

These data indicate that the level of tolerance has remained stable through eight generations of backcrossing and self-pollination in three different elite genetic backgrounds.

B. *Seed Germination, Disease Responses, and Insect Responses of Parental and Sulfonylurea Tolerant Cotton*

In addition to evaluation for tolerance to herbicides, plants in the thirty-five trials were examined by the various investigators for differences in germination, disease symptoms, and insect responses. They were asked to note any observable responses that differed from typical cotton responses, particularly when comparing the untreated sulfonylurea-tolerant to parental lines.

Early injury ratings made in the 7-21 day period after planting or treatment take into account comparative reductions in plant populations, stunting, leaf malformation, visual symptoms of chlorosis, necrosis of stem and leaf parts, and insect feeding. In none of the trials were any differences seen in these parameters apart from herbicide tolerance. Where stand counts were taken, no differences were seen between transgenic and non-transgenic varieties.

Later ratings take into account differences in insect populations and feeding. Specific insect counts are taken if visual differences are noted but none were needed. Again, in none of the tests were any differences noted.

C. *Seed Survival and Overwintering Comparisons of Parental and Sulfonylurea-Tolerant lines*

Seed of 19-51a and Coker 312 were planted in the Fall of 1993 at typical cotton harvest timing in Greenville, MS and McAllen, TX. (Appendix 6). The land was treated according to normal cotton practices for the areas where the tests were conducted. Greenville represents the heart of the Mississippi Delta cotton region. McAllen represents the southernmost region of cotton production in the U.S., with the mildest winter and earliest planting dates for cotton.

In neither trial did any of the sulfonylurea tolerant or parental cotton seed survive to the following season. Tests were maintained until beyond the latest planting time for cotton in the Spring of 1994 in both of the geographic areas. In the Greenville trial, when seed were examined after a short time in the soil (2 months or less), they were found to be necrotic and decaying. This was true of both parental and sulfonylurea-tolerant lines.

D. *Yield, Fiber Quality and Seed Index of Parental and Sulfonylurea Tolerant Cotton*

In 1994, the Delta and Pineland Company conducted trials in Mississippi, South Carolina, and Arizona to compare the yield and fiber characteristics of three elite sulfonylurea tolerant cotton lines with their non-transgenic parental lines. The transgenic lines tested were NuCOTN 64, NuCOTN 66, and NuCOTN 68 which were the BC_3F_5 derivatives of crosses between line 19-51a and elite lines DP51, DP5415, and DP5690, respectively. The tests were not treated with sulfonylurea herbicides.

A report summarizing the trials, written by several Delta and Pineland Company scientists, is included in this section. The authors found "... no significant differences in lint yield and fiber strength between each of the cultivars and their corresponding ALS tolerant strains. Where significant differences were found, they generally were positive

improvements over the original cultivar. The traits in which improvements were noted include earliness, plant vigor, increased seed size, micronaire, and fiber length."

One set of observations in the D&PL report is particularly notable. Mapping of node and boll development suggests that all three transgenic lines mature earlier than the corresponding non-transgenic lines, without any decrease in lint yield. This earlier maturation can have several potential benefits:

- First, it could allow the cotton to be harvested earlier in the growing season. Early harvest lessens the risks of crop loss or lower grade cotton which can be the consequences of late season weather storms.

- Second, a shorter growing season decreases the amount of irrigation and number of pesticide applications necessary to bring the crop to harvest.

FIELD COMPARISONS OF ALS HERBICIDE TOLERANT STRAINS AND CORRESPONDING NON-TOLERANT STRAINS FROM WHICHTHEY WERE DERIVED

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Abstract

After breeding three backcross generations on three distinct cotton cultivars to incorporate tolerance to several of DuPont's Acetolactate Synthase (ALS) herbicide compounds in these cultivars, it was necessary to field test the new ALS tolerant strains in order to determine the effectiveness of the breeding. Fields tests in South Carolina, Mississippi and Arizona were conducted under normal cultural practices for each of the locations. No ALS compounds were applied to any of the plots. The three cultivars, DP 5415, DP 5690 and DP 51 and their corresponding ALS tolerant strains were compared for fiber and agronomic traits. There were very few differences between each of the cultivars and their corresponding ALS tolerant strain. There were no significant differences in lint yield and fiber strength between the non-tolerant cultivars and their corresponding ALS tolerant strains. Where significant differences were observed, they generally were positive improvements over the original cultivar. The traits in which improvements were noted include earliness, plant vigor, increased seed size, micronaire and fiber length. In the future, the ALS tolerant strain of DP 5415 will be known as NuCOTN 66, the ALS tolerant strain of DP 5690 will be known as NuCOTN 68 and the ALS tolerant strain of DP 51 will be known as NuCOTN 64.

Introduction

The introduction of novel genes into the genetic matter of cotton, both Gossypium hirsutum and Gossypium barbadense, is somewhat complex. This is due to the fact that when using Agrobacterium tumefaciens as a vector for the foreign gene being introduced into cotton, the gene is first introduced into the genetic matter of a single plant cell and then it is necessary to regenerate an entire plant from the single cell (Umbeck et al. 1987). It is at the regeneration stage that the process becomes complex with cotton. Only a very few specific cotton cultivars regenerate relatively easily (Trollinder and Xhixian 1989). Therefore, most modern and superior cotton cultivars cannot be transformed directly.

Coker 312 is one of the cultivars of which an entire plant can be generated from a single cell. It is probably the cotton cultivar most commonly used for the direct introduction of novel transgenic genes. However, it is an older cultivar and therefore is

not satisfactory as a cultivar to be grown by modern cotton producers. It is also a hairy leafed cultivar which is not acceptable to many farmers as their grades for their lint may suffer if they do not grow smoothleaf cultivars. For these reasons, transformed Coker 312 has been used as a donor parent in backcrossing schemes to introduce the transgenic gene into modern, high yielding, agronomically superior, smoothleaf cultivars which also carry superior fiber characteristics.

The insertion of a novel gene into the genetic material of cotton is a random event. Depending on which chromosome and where on a particular chromosome the random insertion occurs, there may be deleterious traits found in the transgenic plants and their offspring.

A backcrossing program was initiated to develop cultivars tolerant to several of DuPont's Acetolactate Synthase (ALS) herbicide compounds but which otherwise carried the agronomic and fiber characteristics of the modern cultivars, DP 5415, DP 5690 and DP 51. Because of the possible problems which can be related to the introduction of novel genes into cotton germplasm and the backcrossing schemes employed to refine transgenic germplasm into commercially desirable cultivars, it was necessary to field test the ALS herbicide tolerant cotton strains resulting from the backcrossing program to determine whether the new strains were at least equal to their respective recurrent parents for both agronomic and fiber characteristics when no ALS herbicide was applied. The objective of this study was to determine if breeding to the BC_3 generation was sufficient to develop the transgenic germplasm to this level of refinement and to confirm that there were no heritable deleterious effects of the original gene insertion into the Coker 312.

Materials and Methods

Three recurrent parent cultivars, DP 5415, DP 5690 and DP 51, and their respective transgenic backcross strains were tested in field tests in three distinct cotton growing areas of the United States. The locations were Hartsville, SC, Scott, MS and Casa Grande, AZ. These were replicated small plot tests. Data were collected on fiber and agronomic characteristics. The agronomic data collected included those of plant mapping. None of the plots were sprayed with an ALS herbicide compound. Cultural practices for each location were those generally used in that geographic area.

Results and Discussion

It became apparent during the backcrossing program and early field selection process that there were no obvious deleterious effects caused by this particular insertion of the ALS tolerant gene into the cotton genome. The same donor parent of Coker 312 was used in the backcrossing programs to create each of the three transgenic strains tested

in this study. Therefore, each of the three new transgenic populations involved the identical gene and the same insertion.In some cases, there were significant differences for various characteristic between the three distinct cultivars, DP 5415, DP 5690 and DP 51, or between the three locations. This is to be expected as these three original cultivars do vary in some characteristics. We also expected differences between locations. However, our primary reason for conducting this study was to compare the differences between each of the three recurrent parents, DP 5415, DP 5690 and DP 51 and their corresponding ALS tolerant backcross strain. Only those data which are pertinent to this question will be addressed here.

On comparing agronomic (Table 1) and fiber (Table 2) data analyzed across the three locations it is apparent that breeding to the BC_3 generation has been successful in developing strains which carry the novel gene for ALS herbicide tolerance and which are very close to the respective recurrent parents in both agronomic and fiber traits.

Each of the three ALS tolerant strains is smoothleaf as selection was directed toward this trait.

There were no significant differences in lint yield between each of the recurrent parents and the corresponding ALS tolerant strain.

There were no significant differences between DP 5690 and DP 51 and their respective ALS tolerant strains for lint percent. However, there was a significant difference between the DP 5415 and it's corresponding ALS tolerant strain. Obviously, this lower lint percent did not have an adverse effect on yield. This lower lint percent is correlated to the higher seed index for the ALS tolerant DP 5415 strain. The significantly higher seed index for the ALS tolerant DP 5415 is a positive result of the backcrossing as DP 5415 has a rather small seed. There were no significant differences in seed indexes between DP 5690 and DP 51 and their corresponding ALS tolerant strains.

There were no significant differences for final plant height between DP 5690 and DP 51 and their respective ALS tolerant strains. The ALS tolerant strain of DP 5415 was significantly shorter than DP 5415 and this is in agreement with data to be presented later indicating the ALS tolerant strain is slightly earlier.

There were no significant differences between DP 5415 and DP 5690 and their corresponding ALS tolerant strains for vegetative nodes to first fruiting branch. However, there were significantly fewer vegetative nodes to first fruiting branch on the ALS tolerant DP 51 than on DP 51 and this may indicate that the ALS tolerant DP 51 is slightly earlier.

There were no significant differences in number of fruiting branches between DP 5415, DP 5690 and DP 51 and their corresponding ALS tolerant strains.

There were no significant differences in height to node ratio between DP 5415 and DP 5690 and their corresponding tolerant strains. However, there was a significant increase in height to node ratio for the ALS tolerant DP 51 over DP 51 and this may indicate that the ALS tolerant DP 51 is slightly more vigorous than DP 51.

There was an increase in the percent of total bolls at position one on the ALS tolerant DP 5415 and this again may be an indication that it is earlier than DP 5415. There were no significant differences between DP 5690 and DP 51 and their corresponding ALS tolerant strains.

There were no significant differences in percent retention at the bottom five fruiting positions and in percent retention in the 95% zone between DP 5415, DP 5690 and DP 51 and their corresponding ALS tolerant strains.

There were significantly fewer nodes in the 95% zone for the ALS tolerant strains of DP 5415 and DP 5690 than with the non-tolerant corresponding cultivars. Fewer nodes indicate the plants are cutting out earlier and again this indicates that the ALS tolerant strains of DP 5415 and DP 5690 are earlier than DP 5415 and DP 5690, respectively. There was no significant difference in number of nodes for the 95% zone between DP 51 and the ALS tolerant strain of DP 51.

When observing the distribution of harvestable bolls there are fewer on the right side of the curve for the ALS tolerant strain of DP 5415 than there are for the non-tolerant DP 5415 indicating again that the ALS tolerant strain is earlier (Figure 1). At first glance, it appears that the opposite is true for the ALS tolerant strain of DP 5690 as there are more harvestable bolls on the right side of the curve for the ALS tolerant strain than for DP 5690 (Figure 2). However, because the transgenic version had more bolls on nodes 14 to 19, it accumulated 95% of all harvestable bolls at a lower node than did the non-tolerant DP 5690. The distribution of bolls is very similar for the ALS tolerant strain of DP 51 and DP 51 (Figure 3).

Fiber characteristics, with a few generally positive exceptions, were very similar between the three cultivars and their corresponding ALS tolerant strains.

Micronaire was significantly lower for the ALS tolerant DP 51 strain than for DP 51. There were no significant differences in micronaire between DP 5415 and DP 5690 and their corresponding ALS tolerant strains.

Fiber was significantly longer for the ALS tolerant strains of DP 5415 and DP 5690 than for their corresponding non-tolerant cultivars. There was no significant difference in length between DP 51 and its corresponding ALS tolerant strain.

There was no significant difference in fiber length uniformity between DP 5415 and the ALS tolerant strain. The ALS tolerant strain of DP 5690 had a higher percent of length

uniformity than DP 5690. The ALS tolerant strain of DP 51 had a lower percent of length uniformity than DP 51.There were no significant differences in fiber strength between DP 5415, DP 5690 and DP 51 and their corresponding ALS tolerant strains.

Fiber elongation for the ALS tolerant strain of DP 5415 was one tenth of a percent less than that of DP 5415. There were no significant differences between the fiber elongation of DP 5690 and DP 51 and their corresponding ALS tolerant strains.

Conclusions

It is apparent that the backcrossing programs to incorporate tolerance to several of the DuPont ALS herbicides into the cultivars of DP 5415, DP 5690 and DP 51 have been successful. When there have been variances in characteristics from the original cultivars most of them have been positive differences for the new ALS tolerant strains. The ALS tolerant strains of DP 5415, DP 5690 and DP 51 may be slightly earlier than their original corresponding cultivars. The ALS tolerant strain of DP 51 may be more vigorous than DP 51. The seeds of the ALS tolerant DP 5415 are larger than those of DP 5415. Micronaire of the ALS tolerant strain of DP 51 is lower than that of DP 51 and fibers of the ALS tolerant strains of DP 5415 and DP 5690 are longer than those of the corresponding non-tolerant cultivars.

The ALS tolerant strain of DP 5415 will be known as NuCOTN 66 when released as a cultivar. The ALS tolerant strain of DP 5690 will be known as NuCOTN 68 when released as a cultivar. The ALS tolerant strain of DP 51 will be known as NuCOTN 64 when released as a cultivar.

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Table 1. Agronomic Data Averaged Over Trials in South Carolina, Mississippi and Arizona.

Cultivar or Strain	Lint Yield	Lint %	Seed Index	Ht (in)	Veg Nodes to 1st FB	# FB	HNR	%Total Bolls Pos. 1	% Ret Bot 5 Zone	%Ret 95% Zone	Nodes 95% Zone
DP 5415	1088	40.5	8.32	42.3	5.8	15.5	1.93	46.5	52.5	49.7	19.0
DP 5415 ALS	1032	39.7	8.92	40.3	5.8	15.3	1.92	49.7	50.2	48.6	18.2
DP 5690	1126	39.7	9.52	46.5	5.8	17.2	2.03	50.2	48.7	48.1	19.9
DP 5690 ALS	1074	39.9	9.76	45.4	5.9	19.4	2.04	51.3	54.1	50.3	19.1
DP 51	1181	39.0	9.80	41.0	5.5	16.4	1.88	53.9	71.2	61.9	17.5
DP 51 ALS	1191	38.9	9.84	40.0	5.2	16.0	1.96	56.4	65.6	60.3	17.4
LSD .05	73	0.5	0.46	2.8	0.3	2.8	0.04	2.7	5.7	3.2	0.7
CV	23.6	4.7	3.7	19.8	19.1	21.7	16.5	23.1	31.2	7.9	9.6

Table 2. Fiber Data Averaged Over Trials in South Carolina, Mississippi and Arizona

	Mic.	Length (inches)	Length Unif (%)	Strength (g/tex)	Elong. (%)
DP 5415	4.49	1.156	83.3	28.9	8.3
DP 5415 ALS	4.59	1.173	83.4	29.5	8.2
DP 5690	4.47	1.145	82.9	30.2	7.7
DP 5690 ALS	4.41	1.167	83.5	30.6	7.7
DP 51	4.50	1.147	83.1	26.6	7.9
DP 51 ALS	4.33	1.152	82.7	27.0	7.9
LSD .05	0.14	0.009	0.4	0.7	0.1
CV	9.6	2.6	1.6	7.0	8.8

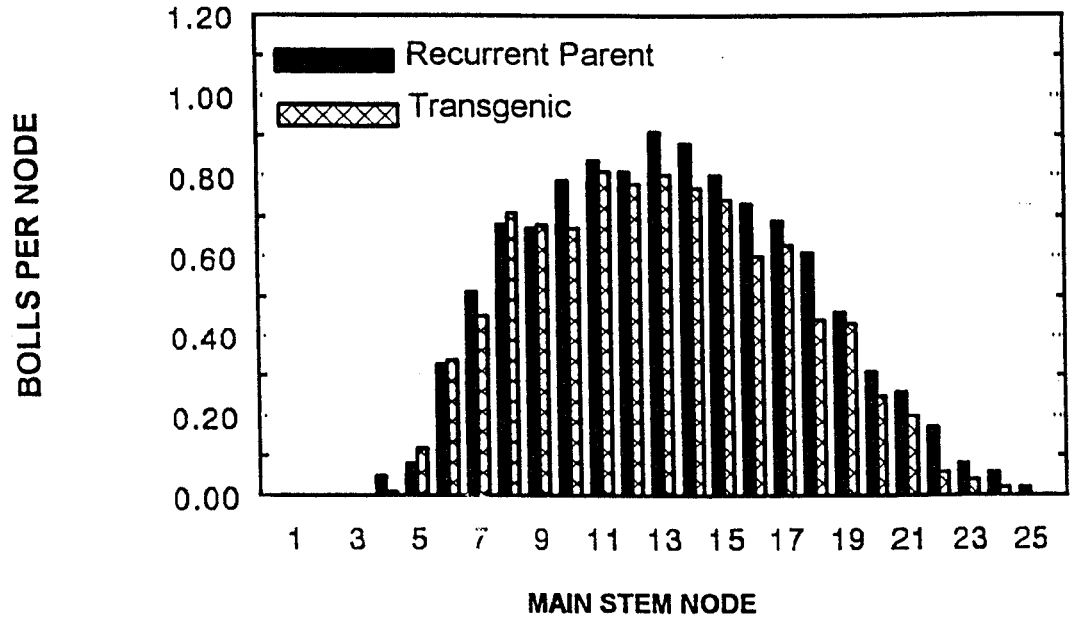

Figure 1. Distribution of harvestable bolls for ALS tolerant DP 5415 averaged over trials in South Carolina, Mississippi and Arizona.

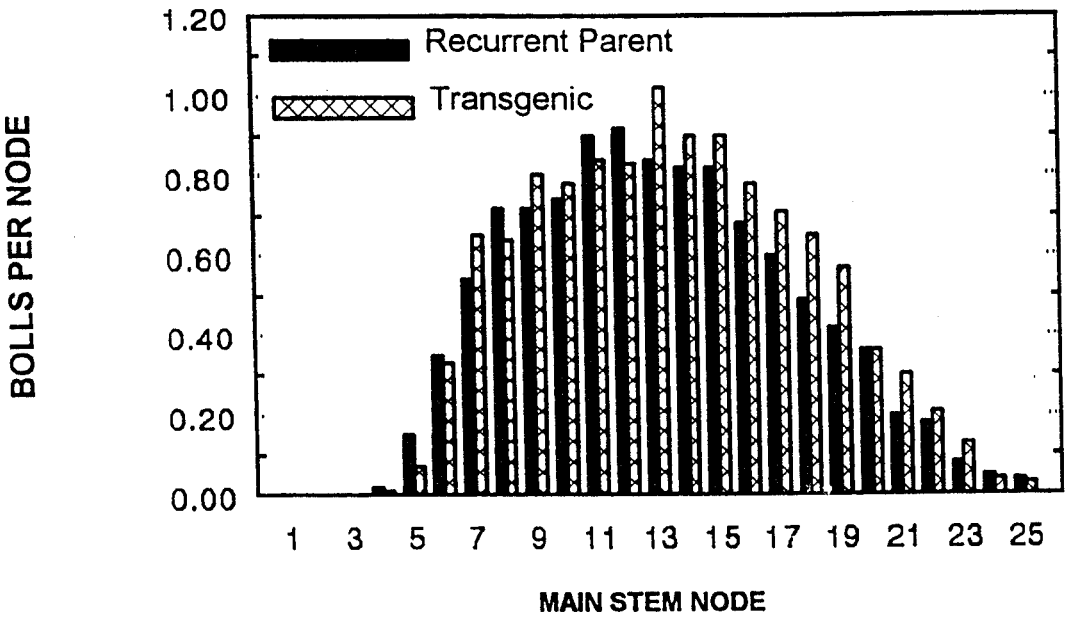

Figure 2. Distribution of harvestable bolls for ALS tolerant DP 5690 averaged over trials in South Carolina, Mississippi Arizona.

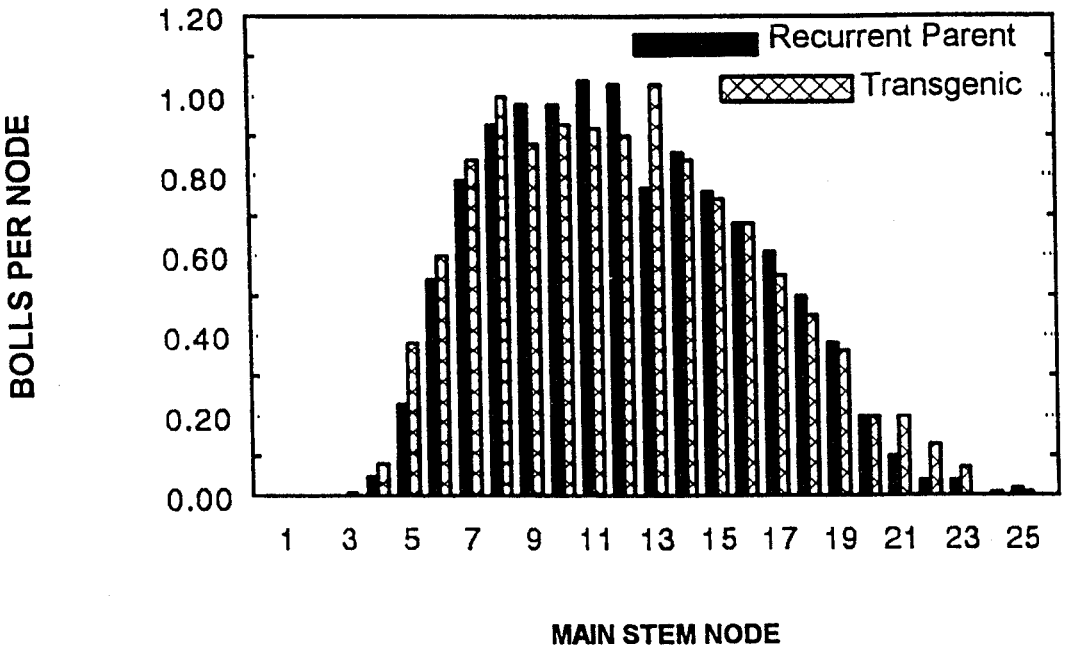

Figure 3. Distribution of harvestable bolls for ALS tolerant DP 51 averaged over trials in South Carolina, Mississippi and Arizona.

VII. ENVIRONMENTAL CONSEQUENCES OF THE INTRODUCTION OF COTTON LINE 19-51A

A. *Gene Product*

As reviewed in section II, use of the transgenic cotton plant would have no effect on the weediness of cotton or related species. The only difference between line 19-51a and its non-transgenic counterpart is the tolerance to sulfonylurea herbicides. This trait is conferred by the introduction of a gene which encodes an ALS enzyme that is tolerant to sulfonylurea herbicides. The tolerant ALS enzyme represents a single example of an ALS enzyme, one of the myriad of ALS enzymes found in plants and bacteria throughout nature. Indeed some ALS enzymes found in bacteria are naturally tolerant to sulfonylurea herbicides so this trait is not unique to the enzyme expressed by the gene introduced into line 19-51a. The effect of any sulfonylurea tolerant ALS that possibly could be released into the soil from cotton debris would be expected to be no different from the effect of any other ALS enzyme that could come from any plants, or from bacteria in the soil.

B. *ALS Gene*

As pointed out in the USDA Environmental Assessment of a field test which included cotton line 19-51a (Permit number 91-025-02, dated April 29, 1991), "Horizontal movement of the introduced genes is not known to be possible. The vector acts by delivering the gene to the cotton genome where it is stably inserted into the cotton chromosomal DNA. The vector does not survive in the transformed plants. No mechanism that can transfer an inserted gene from a chromosome of a transformed plant to a chromosome of another organism has been shown to exist in nature." Due the fact that ALS genes, including those that express a sulfonylurea tolerant ALS enzyme, are already present in soil bacteria, even if there were horizontal gene transfer, it would be of no concern.

C. *Cotton Line 19-51A*

Section II discussed the weediness and outcrossing potential of cotton; the following discussions will center on the environmental consequences from the use of herbicides on cotton line 19-51a.

1. Current Weed Control Practices

Weed control programs can vary greatly from one cotton growing region to another across the U.S. The number of herbicide applications can range from three treatments in the southeast, Arizona and California, to five to six in the mid-south and east Texas.

Table XIII

Examples of regional herbicide programs used in conventional tillage systems*

Conservation tillage production systems could include an early preplant/ burndown herbicide followed by a pre-grass control product versus applying Treflan ppi. All postemergent treatments could remain basically the same as in conventional tillage system.

Region	Product	Rate (Lb. AI/A)	*Timing
Southeast	Treflan	0.75	PPI
	Cotoran + Zorial	1.0 + 1.0	PRE
	Cotoran + MSMA	1.5 + 2.0	Early Post-Direct
	Bladex® + MSMA	0.75 + 2.0	Late Post/Layby
Mid-South	Treflan	1.0	PPI
	Cotoran + Zorial	1.2 + 0.75	PRE
	Cotoran + MSMA	0.8 + 2.0	Early Post-Direct
	Bladex® + MSMA	0.8 + 2.0	Mid-Post
	Bladex®	1.0	Layby
Arizona	Treflan + Caparol	0.75 + 1.4	PPI
	Karmex®	0.4	Mid-Post
	Bladex®	1.2	Layby
California	Treflan + Caparol	0.75 + 1.4	PPI
	Caparol	1.6	Layby
East Texas	Treflan	0.6	PPI
	Caparol	0.8	PRE
	Cotoran + MSMA	0.8 + 2.0	Mid-Post
	Bladex® + MSMA	0.8 + 2.0	Layby

Timings:
PPI = Preplant incorporated
PRE = Preemergence to crop and weeds
Early Post-Direct = 3"-5" cotton
Mid-Post = 6"-9" cotton
Late Post = 10"-12" cotton
Layby = Just prior to crop canopy closure

2. Herbicide Use Reduction

The introduction of sulfonylurea-tolerant cotton plants could significantly alter the total amount (Lb./A) and number of herbicide treatments in a particular weed control program in cotton. Staple® herbicide, a new family of low use rate chemistry (pyrimidinyl carboxy) being developed by DuPont, will be one ALS inhibitor herbicide that fills an historical void in cotton weed control programs -- over-the-top broadleaf weed control. Several DuPont sulfonylurea (SU) herbicides used alone or in combination with Staple® could broaden the number of low use rate products available for over-the-top treatments.

Cotton farmers, in several surveys, have declared a need for an effective broadleaf herbicide that doesn't injure their crop (Patterson and Marks, 1986; Weaver, 1986; Sanders, 1986). Many would also prefer to apply herbicides by over-the-top applications. Most soil applied herbicides in use today can adversely affect cotton plants, especially on soils that are low in organic matter (Guy, 1994). The effect could be delayed maturity and/or yield loss and also increased insecticide usage and grower costs. The increased crop safety from SU-tolerant cotton plants and the effective broadleaf weed control from Staple® and SU herbicides, like M6316, fill the "most wanted" needs of these cotton growers. The aforementioned herbicides could eventually eliminate or reduce the number of soil applied applications each year and significantly reduce the total amount or load of herbicides being applied in cotton.

For example, when comparing labeled maximum seasonal application rates of various herbicides applied on all of Mississippi's 1.2 million acres of cotton, DuPont's Staple® and M6316 herbicides, in conjunction with SU-tolerant cotton plants, offer the greatest opportunity to lower the total herbicide load in U.S. cotton production.

Table XIV

Maximum seasonal application rates for selected cotton herbicides on all Mississippi cotton acres

		Total Lb. A.I.	
Product	**Lb. AI/A/Season**	**Broadcast**	**33% Band**
Caparol 4L	6.2	7,440,000	2,445,200
Cotoran 4L	5.0	6,000,000	2,000,000
Bladex® 90DF	6.0	7,200,000	2,400,000
Buctril® 4EC	1.5	1,800,000	**1,800,000
Karmex® 80DF	2.2	2,640,000	871,200
MSMA 6SC	4.0	4,800,000	1,600,000
Zorial 80DF	2.0	2,400,000	1,200,000
*Staple® 80SP	0.125	144,000	48,000
*Staple®+ M6316	0.07	84,000	28,000

*Not yet registered
** Buctril® label allows using single maximum rate concentrated in the banded area.

Crop safety, efficacy and reduced pesticide load are all possible when SU-tolerant cotton plants are the foundation for the over-the-top herbicides Staple® and M6316.

At the present time, the focus on M6316 as a premix partner with Staple® has to do with M6316's efficacy and short half-life or soil residual. Basically, any rotational crop can be planted 60 days after an application of M6316.

Staple® has an excellent biological fit for the weed spectrum found in the arid cotton growing region of West Texas. Under these climatic conditions Staple® tends to biodegrade relatively slowly. The normal 2x safety factor in use rate to rotational crops does not always apply under these conditions when grain sorghum or corn are the crops to be planted following Staple® applications in cotton. Current data from this area

suggests that neither of these crops may be planted in the season following an application of Staple®.

It is thought that a mixture of a reduced rate of Staple® plus M6316 could alleviate this rotational concern. Additional field recrop and efficacy studies have been initiated to confirm.

3. Environmental Risk

The low use rate chemistry of Staple® and M6316 will allow a reduction in the total pounds of herbicide active ingredient used in cotton weed control programs. Staple® use rates will range from 1 - 1.5 ounces active ingredient per acre with 1.0 ounce active being the rate used in most situations. M6316 use rates will range from 0.062 - 0.125 ounces active ingredient per acre. The rates of postemergent, over-the-top herbicides will not be affected by soil type or soil organic matter.

Both of these herbicides control weeds by blocking the ALS enzyme that is necessary for the production of several essential amino acids. This enzyme is only found in plants and not in animals. Having this particular mode of activity offers excellent safety to mammals, fish and birds.

Staple® and M6316 are non-volatile which will greatly reduce the probability of off-target movement that has been documented with the herbicide Command®. In SU-tolerant cotton, Staple® and Staple® + M6316 combinations will control several pigweed species, pitted morningglory, Pennsylvania smartweed, cocklebur and redweed that Command is used for without the off-target movement concerns.

4. Expanded IPM/Conservation Tillage

Staple® and M6316 postemergence activity on broadleaf weeds and safety to SU-tolerant cotton would allow growers to target their herbicide applications on an as needed basis. Not all weeds emerge at the same time in a given field so a grower may choose to spot spray with Staple® or Staple® + M6316 while cultivating.

Cotton growers, in general, employ crop consultants to scout fields for insecticide recommendations. Many of these insect consultants are also expanding their business to include herbicide recommendations. As these consultants perform early season insect scouting, they could diagnose weed control needs as well. This would allow a more targeted field by field approach for the early postemergence treatment of weed escapes from preplant incorporated and preemergence applications.

Weeds also harbor insects and nematodes. Elimination of these host plants reduces the need for treatment of these pests.

For cotton producers in the U.S., tillage has been one of the most important production tools available. The average number of tillage operations varies from state to state, but generally involves 8 to 12 tillage operations. At least half of these are specifically for the control of weeds (Bryson, Keeley, 1992). In one survey, the two most frequently cited disadvantages for reduced-tillage in cotton production are the increased cost for weed control and weed control difficulties (Bryson, Keeley, 1992).

Worsham (1977) stated that successful weed control holds the key to the success or failure of reduced tillage. SU-tolerant cotton and the ready availability of low rate, effective herbicides like Staple® and M6316 will provide considerable impetus for increased use of conservation (reduced) tillage cotton production. Soil residues of the previous crop (stubble and surface residues) remaining on the soil as a mulch should reduce wind and water erosion in cotton as in other crops.

5. Storage and Transportation Safety

Staple® and M6316 are dry formulations that will be packaged in water soluble film (WSF) bags that allow safer handling and economical storage. These WSF bags will be contained in a resealable barrier bag. The barrier bags are stored in cardboard cartons for shipment. This form of packaging allows minimal exposure to the product itself. The barrier bags and cardboard cartons can be disposed of in the normal manner for regular trash since there is no contact of these materials with the herbicide.

This unique form of packaging provides minimal exposure to the product throughout the spray operation - handling the WSF bags, mixing and loading. It provides less handling exposure and no chemical splash back when adding the product bags to the

spray tank. This packaging concept could be viewed as a "closed system" approach to mixer/loader exposure.

Staple® and M6316 do not freeze, therefore storage costs are less than that of liquid products. These low use rate products will require less storage space for distributors, dealers and farmers. This could provide a cost benefit, especially for dealers and distributors, which, hopefully, can be passed along to the farmer. Reduced need for space along with lower weight materials versus liquid formulations could translate into reduced shipping costs as well.

SU-tolerant cotton will play a lead role in allowing the expanded usage of these low rate, safe and effective herbicides in cotton production.

6. Persistence

Staple®, under non-sterile aerobic soil conditions, degrades with a calculated half-life of approximately 60 days. Carbon dioxide was the principal degradation product under these conditions. Minor metabolites were detected, but did not accumulate to any significant degree.

The photodegradation of Staple® in a silt loam soil degraded with a mean calculated half-life of 43 days. In a buffered aqueous solution at pH 5, 7 and 9, the photolysis rate of Staple® was rapid with average half-lives of 11, 13 and 15 days, respectively. Photolytic degradation may also play a significant role in the dissipation of Staple® in the environment.

At a test site in Rochelle, IL, M6316 applied to a Hanagan silt loam soil (pH 5.4) has a calculated half-life of 6 days. In a Newark, DE Keyport silt loam (pH 5.2) test, the soil half-life was 2 days for M6316.

The soil photolysis half-life of M6316 was calculated to be 18 days. In a buffered aqueous solution at pH 5, 7 and 9, the photolysis rate for M6316 was an average of 5 days irrespective of pH.

Besides being rapidly degraded in the environment, the low environmental impact of Staple® and M6316 is due, in part, to the fact that these herbicides selectively inhibit

acetolactate synthase (ALS), a key enzyme found in plants but not in animals. Also, Staple® and M6316 are practically non-toxic to avian and insect species.

The introduction of SU-tolerant cotton allows these environmentally compatible herbicides to become an intricate part of a cotton grower's weed control program.

7. Resistant Weed Management

One benefit of sulfonylurea herbicides is that they focus on a single enzyme system, allowing very low use rates. However, this mode of action also increases the chance that weed biotypes with resistant enzymes may occur. The following factors may influence the spread of resistant biotypes:

- The ratio of susceptible biotypes to resistant biotypes that naturally occur in a given weed population.
- Exclusive, repeated use of the same herbicide, or herbicides with the same mode of action, on the same crop in a given field.
- Use of long residual herbicides that control susceptible biotypes effectively and thus give resistant biotypes the chance to spread rapidly.

To manage resistant weeds once they have appeared, re-treat the problem area in a timely manner with a broadleaf herbicide that has a different mode of action. To delay or prevent the occurrence of resistant weeds, use the following guidelines:

- Rotate crops and choose herbicides with different modes of action.
- Use sulfonylurea herbicides in tank mixes and/or sequential treatments with other herbicides that have different modes of action.
- Choose herbicides with short residuals whenever possible and use those with long residuals sparingly so resistant weed biotypes do not have a chance to flourish.
- Balance the weed management program by using tillage, where practical, in conjunction with herbicides.
- Do not allow weed escapes to go to seed.

Staple® and M6316 will be positioned as only a part of a cotton farmer's total weed control program. These herbicides, used alone or in combination, will be applied

primarily at the early postemergent timing (cotyledon - 4 leaf cotton) of application. This will allow any weed escapes to be controlled by herbicides with different modes of action such as Bladex®, Karmex® or MSMA. These particular herbicides are already being used in just such a way today and are readily available to cotton farmers

Used in a program approach, Staple® and M6316 should provide many years of safe and effective broadleaf weed control for U.S. cotton farmers.

Table XV

Examples of typical full season weed control programs incorporating Staple® and M6316 herbicides

Product	*Timing
Treflan	PPI
Cotoran	PRE
Staple® + M6316	Early Post-Direct
Bladex® or Bladex® + MSMA	Layby
Treflan	PPI
Cotoran + Zorial	PRE
Staple® + M6316	EP
Bladex® + MSMA	Mid-Post
Bladex®	Layby
Prowl + Cotoran	PRE
Staple® + M6316	EP
Bladex® + MSMA	MP
Bladex® or Karmex®	Layby
Treflan or Prowl	PPI or PRE
Staple® + M6316	EP
Bladex® + MSMA	MP
Bladex® or Karmex®	Layby
Treflan or Prowl	PPI or PRE
Staple® + M6316	EP
Staple® + M6316	MP (as needed)
Bladex® or Bladex® + MSMA	Layby/Spot treat

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4. Animal Feed

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North Carolina Department of Agriculture

965.16 Sampling of Animal Feed

Procedure

Use slotted single or double tube, or slotted tube and rod, all with pointed ends.

Take ≥500 g sample, 1 kg preferred, as follows: Lay bag horizontally and remove core diagonally from end to end. Det. number of cores as follows: From lots of 1–10 bags, sample all bags; from lot of ≥11, sample 10 bags. Take 1 core from each bag sampled, except that for lots of 1–4 bags take enough diagonal cores from each bag to total ≥5 cores. For bulk feeds draw ≥10 cores from different regions; in sampling small containers (≤10 lb) 1 package is enough. Reduce composite sample to amt required, preferably by riffling, or by mixing thoroly on clean oil-cloth or paper and quartering. Place sample in air-tight container.

A sample from less than these numbers of bags may be declared an official sample if guarantor agrees. For samples that cannot be representatively taken with probe described, use other sampling means.

950.02 Animal Feed

Preparation of Sample

Final Action

Grind sample to pass sieve with circular openings 1 mm (1/25") diam. and mix thoroly. If sample cannot be ground, reduce to as fine condition as possible. Do not grind molasses feeds.

Refs.: JAOAC 33, 424(1950); 41, 223(1958); 48, 658(1965); 51, 467(1968).

934.01 Moisture in Animal Feed

Drying in Vacuo at 95–100°

Final Action

Dry amt sample contg ca 2 g dry material to const wt at 95–100° under pressure ≤100 mm Hg (ca 5 hr). For feeds with high molasses content, use temp. ≤70° and pressure ≤50 mm Hg. Use covered Al dish ≥50 mm diam. and ≤40 mm deep. Report loss in wt as moisture.

Ref.: JAOAC 17, 68(1934); 51, 467(1968); 60, 322(1977).

925.04 Moisture in Animal Feed

By Distillation with Toluene

Final Action

A. Apparatus

Connect 250 mL flask of Pyrex or other resistant glass by means of Bidwell-Sterling moisture receiver to 500 mm Liebig condenser. Calibrate receiver, 5 mL capacity, by distg known amts H_2O into graduated column, and estg column of H_2O to 0.01 mL. Clean tube and condenser with chromic acid cleaning mixt., rinse thoroly with H_2O, then alcohol, and dry in oven to prevent undue amt H_2O from adhering to inner surfaces during detn.

B. Determination

If sample is likely to bump, add dry sand to cover bottom of flask. Add enough toluene to cover sample completely (ca 75 mL). Weigh and introduce enough sample into toluene to give 2–5 mL H_2O and connect app. Fill receiving tube with toluene, pouring it thru top of condenser. Bring to boil and distil slowly, ca 2 drops/sec, until most of the H_2O passes over; then increase rate of distn to ca 4 drops/sec.

When all H_2O is apparently over, wash down condenser by pouring toluene in at top, continuing distn short time to see whether any more H_2O distils over; if it does, repeat washing-down process. If any H_2O remains in condenser, remove by brushing down with tube brush attached to Cu wire and satd with toluene, washing down condenser at same time. (Entire process is usually completed within 1 hr.) Let receiving tube come to room temp. If any drops adhere to sides of tube, force them down, using Cu wire with end wrapped with rubber band. Read vol. H_2O and calc. to %.

Refs.: JAOAC 8, 295(1925); 9, 30(1926).

920.36* Moisture in Animal Feed

Drying without Heat over Sulfuric Acid

Final Action
Surplus 1974

See 7.006–7.007, 12th ed.

930.15 Moisture in Animal Feed

Drying at 135°

Final Action

(Not to be used when fat detn is to be made on same sample)

Regulate air oven to 135±2°. Using low, covered Al dishes, 934.01, weigh ca 2 g sample into each dish and shake until contents are evenly distributed. With covers removed, place dishes and covers in oven as quickly as possible and dry samples 2 hr. Place covers on dishes and transfer to desiccator to cool. Weigh, and calc. loss in wt as H_2O.

Refs.: JAOAC 13, 173(1930); 14, 152(1931); 17, 178(1934); 18, 80(1935).

953.07 Moisture in Animal Feed in Highly Acid Milk By-products

Final Action

Add ca 2 g ZnO, freshly ignited or oven dried, to flat-bottom dish ≥5 cm diam. and weigh. Add ca 1 g sample and

weigh quickly. Add ca 5 mL H_2O and distribute sample evenly on bottom of dish. Heat on steam bath, exposing max. surface of dish bottom to live steam until apparently dry. Heat at 98–100° in air oven 3 hr or to const wt. Cool in desiccator; then weigh quickly. Det. wt residue. Dil. with twice its vol. CO_2-free H_2O. Add 2 mL phthln, and titr. with 0.1N NaOH to first persistent pink. Calc. as % lactic acid by wt. (1 mL 0.1N NaOH = 0.0090 g lactic acid.). To compensate for H_2O formed when acid is neutzd by ZnO, add 0.1 g to residue wt for each g acid (as lactic) in weighed sample. Report % residue (corrected) as total solids.

Refs.: JAOAC 36, 213(1953); 37, 253(1954).

942.05 Ash of Animal Feed

Final Action

Weigh 2 g sample into porcelain crucible and place in temp. controlled furnace preheated to 600°. Hold at this temp. 2 hr. Transfer crucible directly to desiccator, cool, and weigh immediately, reporting % ash to first decimal place.

Refs.: JAOAC 25, 857(1942); 26, 220(1943).

935.11* Protein in Animal Feed

Qualitative Tests

Final Action
Surplus 1965

A. Biuret Test

See 22.012–22.013, 10th ed.

B. Millon Test

See 22.014–22.015, 10th ed.

C. Glyoxylic Acid Test (Hopkins-Cole)

See 22.016–22.017, 10th ed.

D. Adamkiewicz Test

See 22.018, 10th ed.

E. Xanthoproteic Test

See 22.019, 10th ed.

954.01 Protein (Crude) in Animal Feed

Kjeldahl Method

Final Action

Det. N as in 955.04. Multiply result by 6.25, or in case of wheat grains by 5.70.

Refs.: JAOAC 37, 241(1954); 38, 56(1955).

988.05 Protein (Crude) in Animal Feed

$CuSO_4/TiO_2$ Mixed Catalyst Kjeldahl Method

First Action 1988

(*Caution:* See safety notes on sulfuric acid and sodium hydroxide.)

A. Principle

Sample is digested in H_2SO_4, using $CuSO_4/TiO_2$ as catalysts, converting N to NH_3 which is distd and titrd.

B. Reagents

(a) *Sodium hydroxide soln.*—Dissolve ca 450 g NaOH pellets or flakes (low N) in H_2O, cool, and dil. to 1 L; or use soln with sp. gr. ≥1.36.

(b) *Boiling stones.*—Alundum, 8–14 mesh (No. 1590-D18; Thomas Scientific Co.).

(c) *Methyl red indicator.*—Dissolve 1 g Me red (Na salt) in 100 mL MeOH.

(d) *Hydrochloric or sulfuric acid std soln.*—0.5N. Prep. as in 936.15 or 890.01.

(e) *Sodium hydroxide std soln.*—0.1N. Prep. as in 936.16.

After stdzg both acid and base by methods suggested in (d) and (e), also check one against the other. In addn, check entire method by analyzing NIST Std Ref. material No. 194, $NH_4H_2PO_4$, certified 12.15% N, and a high purity lysine·HCl.

C. Apparatus

(a) *Digestion.*—Kjeldahl flasks with capacity of 500–800 mL.

(b) *Distillation.*—Digestion flask (e.g., Corning Glass No. 2020) connected to distn trap by rubber stopper. Distn trap is connected to condenser with low-S tubing. Outlet of condenser tube should be <4 mm diam.

D. Determination

Weigh 0.250–1.000 g sample into digestion flask. Add 16.7 g K_2SO_4, 0.01 g anhyd. $CuSO_4$, 0.6 g TiO_2, 0.3 g pumice, 0.5–1.0 g Alundum granules, and 20 mL H_2SO_4. (Add addnl 1.0 mL H_2SO_4 for each 0.1 g fat or 0.2 g other org. matter if sample wt is >1 g.)

Include at least 1 sample of high purity lysine·HCl in each day's run as check of correctness of digestion parameters. If recovery is not complete, make appropriate adjustments.

To digest sample, first adjust heat to bring 250 mL H_2O at 25° to rolling boil in 5 min. Add a few boiling chips to prevent superheating. Then heat samples at this 5-min boil rate until dense white fumes clear bulb of flask (ca 10 min), swirl gently, and continue heating addnl 40 min. (Note: Reagent proportions, heat input, and digestion time are critical factors—do not change.) Cool, cautiously add about 250 mL H_2O, and cool to room temp. (Note: Add H_2O as soon as possible to reduce amt of caking. If excessive bumping occurs during distn, increase diln H_2O from 250 mL to ca 300 mL.)

Prep. titrn beaker by adding appropriate vol. of acid std soln to amt of H_2O such that condenser tip will be sufficiently immersed to trap all NH_3 evolved. Add 3–4 drops of indicator soln (c).

Add addnl 0.5–1.0 g Alundum granules to cooled digestion flask. Optionally, 2–3 drops of tributyl citrate may also be added to reduce foaming. Slowly down side of flask, add sufficient NaOH soln (a) such that mixt. will be strongly alk. Immediately connect flask to distn app., mix completely, and distill at ca 7.5-min boil rate until ≥150 mL distillate is collected in titrn beaker.

Titr. excess std acid in distillate with NaOH std soln (e). Correct for blank detn on reagents. Calc. % nitrogen:

$$\%N = \{[(N_{acid})(mL_{acid}) - (mL_{blk})(N_{NaOH}) - (mL_{NaOH})(N_{NaOH})] \times 1400.67\}/mg\ sample$$

where mL_{NaOH} = mL std base needed to titr. sample; mL_{acid} = mL std acid used for that sample; mL_{blk} = mL std base needed to titr. 1 mL std acid minus mL std base needed to titr. reagent blank carried thru method and distd into 1 mL std acid; N_{acid} = normality of std acid; N_{NaOH} = normality of std base. Calc. % crude protein, defined as 6.25 × % nitrogen, or 5.7 × % nitrogen for wheat grains.

Ref.: JAOAC 70, 907(1987).

A.O.C.S. Official Method Ba 3-38

Revised April 1954
Reapproved 1969

Oil

Definition: This method determines the substances extracted by petroleum ether under the conditions of the test.

Scope: Applicable to cottonseed meats, and cake and meal from cottonseed, soybeans, peanuts, and flaxseed.

Apparatus:

1. Butt type extraction apparatus, assembled exactly as indicated in A.O.C.S. method Aa 4-38 (89). See Apparatus section in method Aa 4-38 (89) and Figure 1 below.

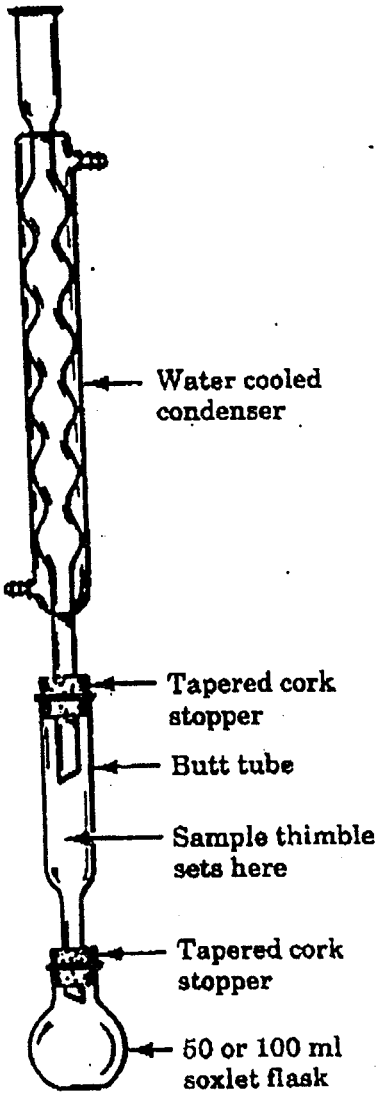


Figure 1. Butt-type extraction apparatus.

2. Filter paper S & S No. 597, Whatman No. 2, Reeve-Angel No. 211, or equivalent, 150 mm diameter.
3. Absorbent cotton, free of petroleum ether extractables.
4. Porcelain mortar and pestle. The mortar must be at least 4 inches id at the top. The pestle handle must be large enough to afford a firm hand grip. The inner surface of the mortar is kept rough by occasionally grinding with sand.
5. Sieve, U.S. No. 20.
6. Sieve, U.S. No. 30.
7. Laboratory mill suitable for grinding the samples to a maximum particle size of U.S. No. 20 sieve, except for linseed meal for which a No. 30 sieve should be used.

Reagents:

1. Petroleum ether, A.O.C.S. Specification H 2-41 (see Notes, Caution).

Preparation of Sample:

1. Grind the 100 g portion from A.O.C.S Official Method Ba 2a-38, Preparation of Sample section, through the laboratory mill to a uniform fineness, about 20 mesh (about 30 mesh for linseed meal). Immediately return to an air-tight container. Oil, ground-moisture and ammonia are determined on this portion.

Procedure:

1. Meal and Ground Cake or Pellets -

(a) Weigh 5 g of the ground sample into a filter paper and enclose in a second filter paper, folded in such a fashion as to prevent escape of the meal (see Figure 2). The second paper is left open at the top like a thimble. A piece of absorbent cotton may be placed in the top of the thimble to distribute the solvent as it drops on the sample.

(b) Place wrapped sample in the Butt extraction tube and assemble the apparatus as shown in Figure 1. Put about 25 mL of petroleum ether into the tared extraction flask before attaching to the tube.

(c) Heat on a water bath or electric hot plate at such a rate that the solvent will drop from the condenser on the center of the thimble at the rate of at least 150 drops per minute.

(d) Keep the volume of solvent fairly constant by adding enough to make up for any that may be

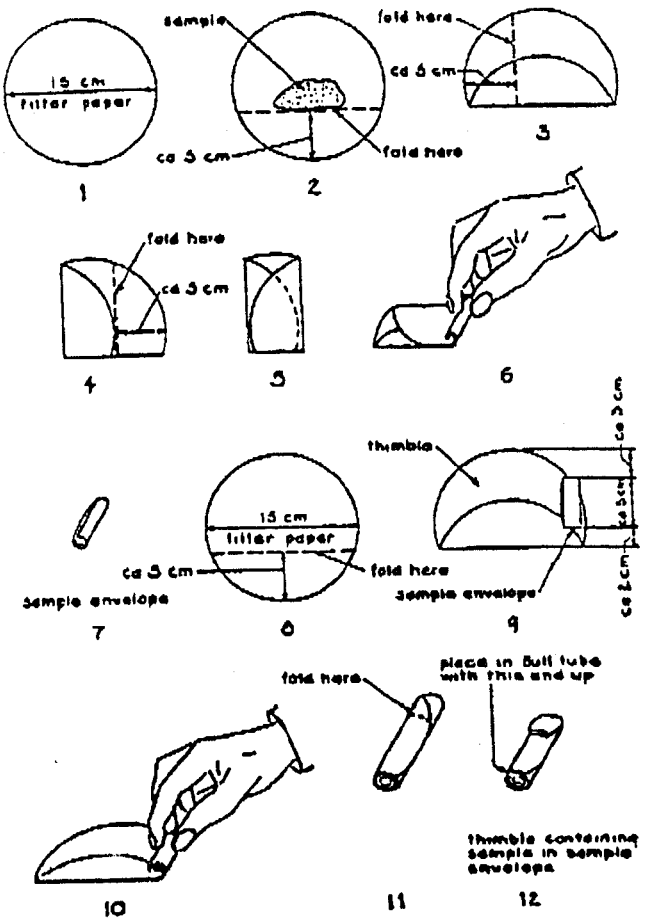


Figure 2. Folding filter paper for oil extraction.

lost due to evaporation. Continue extraction for 3 hrs.

(e) Cool and disconnect the extraction flask. Evaporate the petroleum ether on a steam or water bath until no odor of solvent remains. A gentle stream of clean, dry nitrogen may be used to facilitate removal of the solvent. Cool to room temperature, carefully remove any moisture or dirt from the outside of the flask and weigh. Repeat heating until constant weight is obtained.

(f) Determine the moisture in the ground sample as directed in A.O.C.S. Official Method Ba 2a-38.

2. Cottonseed Meats -

(a) Weigh accurately about 2 g of the ground sample and proceed as directed in Procedure, 1, (a) above, continuing the extraction for 2 hrs only.

(b) Remove the thimble from the Butt tube, allow the solvent to evaporate from the filter paper and sample at room temperature. Then carefully transfer the sample to the mortar so that there will be no loss. Grind the sample in the mortar with the pestle for at least 1 minute or with at least 100 vigorous strokes. Use no abrasive.

(c) Return the reground sample to the same filter paper and continue the extraction as before for 2 additional hrs. From here on, proceed as directed in Procedure 1, (d), (e) and (f).

Calculations:

1. Oil, $\% = \dfrac{\text{Weight of oil} \times 100}{\text{Weight of sample}}$

2. The percentage oil may be calculated to any desired moisture basis with the following formula

$$\text{Oil, desired moisture basis, \%} = \frac{\text{F (100 − \% moisture desired)}}{\text{(100 − \% moisture in sample analyzed)}}$$

Where —

F = % oil in sample analyzed.

Notes:

Caution

Petroleum ether is extremely flammable. Avoid static electricity. The explosive limits in air are 1 to 6%. A fume hood should be used at all times when using petroleum ether.

Appendix 2

1. Weigh out a sample which will contain 20 mg of protein into a glass weigh boat. Transfer the sample to a 25 x 150 mm glass test tube, record the weight (100 mg of cottonseed meal will give the approximate 20 mg of protein required). At this time it is also necessary to weigh out a moisture sample. Weigh between 1 and 2 grams into a previously weighed aluminum dish. This sample is to be dried at 130°C for 2 hours, desiccated, then re-weighed for moisture determination. Moisture is calculated as follows:

   % moisture content = (before oven wt-after oven wt)/(before oven wt-tare wt) *100

2. Prepare Performic Acid solution. Allow to stand at room temperature in the fume hood for 1 hour, then place in the freezer for at least 30 minutes to cool to approximately 0°C.

   for 18 tubes mix - 54 mls of 88% Formic Acid
   6 mls of 30% Hydrogen Peroxide

3. 30 Minutes prior to the addition of Performic Acid place the sample tubes in an ice bath and put the ice bath in the refrigerator.

4. After 30 minutes add 3 mls of the cold Performic Acid to each tube. Put a cap on and place the tube back in the ice bath in the refrigerator.

5. After the allotted time period prepare a solution of Sodium Metabisulfite, cool for 30 minutes and then add 1.5 mls to each tube. Allow to stand for 1.5 to 2.0 hours with the caps off.

   for 18 tubes mix - 9.6 g of Sodium Metabisulfite
   30 mls of distilled water

6. After the time period add 100 microliters of Phenol to each tube.

7. Add 4.5 mls of 12 N trace metal grade HCl. At this time turn on the digiblock, 145°C.

8. De-gas each sample by placing the vacuum line stopper in the test tube. After most of the bubbling has stopped, place the tube in the sonicator for 30 seconds. (The sonicator should be filled with distilled water with a small amount of detergent added to the water. It should be allowed to sonicate for 10 minutes prior to usage to remove any gas bubbles. The vacuum line should be fitted with a liquid nitrogen trap when in use.) After removing the vacuum line stopper from the tube immediately cap the tube tightly.

9. Place the samples in the digiblock and check the temperature. Remember the correct setting is 145°C for 4 hours. After 30 minutes check the caps and tighten as necessary.

10. After the proper time has elapsed remove the tubes from the digiblock, put them into a test tube rack and cool in a sink partly filled with ice water for 1 hour.

11. Remove the samples from the sink and in the hood remove the caps. There will be a slight pressure release so it would be best to wear gloves at this time.

12. Add 9 mls of a 6 N Sodium Hydroxide solution at this time.

In a 1 liter flask place 240 g of NaOH pellets and approximately 800 mls of distilled water. Stir slowly, the reaction is exothermic so the flask will become quite hot. Allow the solution to cool and then bring to volume.

13. Put the caps back on and place the test tubes back in the sink for about 15 minutes.

14. After cooling remove from the sink and add 10 mls of a 0.05 mg/ml solution of L-alpha-n-butyric acid (LABA), the internal standard, to each tube.

    Put 1 gram of LABA into a 250 ml volumetric flask, add 250 mls of Citrate Buffer (this gives a 4mg/ml solution). Take 6.25 mls of this LABA solution and dilute to 500 mls in a volumetric flask with citrate buffer (this will give the 0.05 mg/ml solution required).

15. Transfer each test tube to a marked 50 mls volumetric flask and bring to volume with distilled water. Cap and place on a rotator for 15 minutes.

16. Transfer 0.5 mls of each sample to a test tube and add 4.5 mls of Citrate Buffer. Filter through a 3 ml disposable syringe (must be disposed of by crushing the barrel of the syringe with a hammer) with a 0.2 micrometer nylon acrodisc filter (Gelman Science) into a HPLC autosampler vial. Seal and store in the freezer for later use.

17. Amino acid hydrolysate samples are separated and quantified on a Beckman System Gold HPLC, via cation exchange, using a 3 mm x 25 cm Sperogel column n the sodium phase, with elution buffers and program as supplied by Beckman, and detection via ninhydrin reaction with visible detection at 570 nm.

18. Hydrolyzed and diluted samples in autosampler vials are loaded into the autosampler trays. Each set of twelve samples is preceded by two test samples to evaluate instrument stability, and then one injection each of two standards to establish a two-point standard curve. Standards are prepared previously to mimic the ratio of amino acids common in grain samples, and use L-alpha-amino-n-butyric acid as internal standard.

Appendix 3

FATTY ACID SAMPLE PREPARATION

1. Scoop approximately 15 to 30 mg of cotton seed into a 13 x 100 mm test tube.
2. Add 1 ml of Hexane to each tube using the 1 ml jet pipet.
3. Add 1 ml of 1% Sodium Methoxide solution to each tube.
4. Cap the tubes, then place them on a rotator for 15 minutes.
5. After 15 minutes, uncap the tubes and add 1 mls of 1% Acetic Acid to each tube.
6. Recap and shake briefly, then centrifuge at 2500 RPM for 5 minutes.
7. Transfer the top layer (hexane layer) into the GC autosampler vials, cap, and seal. Store in the freezer until the samples are ready for analysis.

ANALYSIS OF FATTY ACID METHYL ESTERS BY GAS CHROMATOGRAPHY

Fatty acid methyl esters are separated and quantified by injecting one microliter onto a Supelco SPM-2330 fused silica capillary column, 0.32 mm x 15 m, with 0.2 micron film thickness, in a dual column Hewlett Packard 5890 Gas Chromatograph with FID detection. Percent fatty acids are reported as percent of total on a peak area basis.

A.O.C.S. Official Method Ba 8-78

Replaces Ba 8-58
Official 1963
Updated 1967
Reapproved 1969

Total Gossypol

Definition: Total Gossypol defines gossypol and gossypol derivatives, both free and bound, in cottonseed products which are capable of reacting with 3-amino-1-propanol in dimethylformamide solution to form a diaminopropanol complex, which then reacts with aniline to form dianilinogossypol under the conditions of the method. Gossypol, gossypol analogs, and gossypol derivatives having an available aldehyde moiety fare measured by the method (see Notes, 6).

Scope: Applicable to decorticated, glanded and glandless cottonseed, cottonseed flour, cooked cottonseed meats, cottonseed press cake, cottonseed meal, crude cottonseed oil, and cottonseed soapstock (see Notes, 1).

Apparatus:

1. Disk mill, Bauer Brothers No. 148, or equivalent, with plates separated to just break the seed.
2. Cutting mill, Wiley, with 1 mm and 2 mm screens.
3. Water bath, for operation at 95-100 C, with clamps for supporting 50 and 25 mL Volumetric flasks. Metal washers can be placed over the neck of flasks for support.
4. Spectrophotometer, for operation at 440 nm 1 cm light-path cells. A colorimeter can be used if equipped with a filter with maximum transmission in range of 440-460 nm (see Notes, 2).
5. Volumetric flasks, 25, 50, and 100 mL.
6. Pipets, volumetric, class A, 1, 2, 4, 5 and 10 mL.
7. Filter paper, medium retention, 11 cm diameter (Whatman No. 2, S & S 597, or equivalent); creped paper for oil filtration (Eaton and Dikeman 617, Reeve-Angel 230, S & S 478, or equivalent).

Reagents:

1. Solvents, reagent grade (see Notes, Caution) — isopropyl alcohol (2-propanol), n-hexane (boiling range 68-69 C), dimethylformamide (N,N-dimethylformamide), 3-amino- 1-propanol (propanolamine), free of color, glacial acetic acid and aniline. The aniline should be redistilled over zinc dust using water cooled condenser. Discard first and last 10% of distillate, and store in brown bottle in refrigerator when not in use. Redistill when reagent blank (Procedure, step 8) absorbance exceeds 0.022.
2. Isopropyl alcohol-hexane mixture, 60 volumes of isopropyl alcohol, and 40 volumes of n-hexane.
3. Complexing reagent, prepared by pipetting 2 mL of 3-amino-1-propanol and 10 mL glacial acetic acid into a 100 mL volumetric flask, cooling to room temperature, and diluting to volume with dimethylformamide. Prepare reagent weekly, and store in refrigerator when not in use.
4. Gossypol, or gossypol acetic acid, primary standard quality. Gossypol and gossypol acetic acid standards are available from — Atomergic Chemetals Corp., 100 Fairchild Ave., Plainview, NY 11803; Sigma Chemicals, P.O. Box 14508 St. Louis, MO 63178; Chemical Dynamics Corp., 3001 Hadley Rd., South Plainfield, NJ 07080; Aldrich Chemical Co., P.O. Box 355, Milwaukee, WI 53201. Determine purity as in AOCS Ba 7-58 (Reagents, 4). Absorptivity of 39.1-39.9 for gossypol and 35.1-35.8 for gossypol acetic acid indicate satisfactory purity in range of 98-100%. Gossypol acetic acid contains 89.62% gossypol by weight, or 0.8962 mg gossypol per mg of gossypol acetic acid.
5. Standard gossypol solution, prepared by weighing 25 mg of primary standard gossypol, or 27.9 mg of gossypol acetic acid into a 50 mL volumetric flask. Dissolve in and make up to volume with the complexing reagent (Reagents, 3). Solution is stable up to 1-week if stored in refrigerator. If exact amounts of either gossypol or gossypol acetic acid were weighed as indicated, either solution contains 0.50 mg gossypol per mL. Multiply mg gossypol acetic acid by 0.8962 to obtain mg of gossypol.

Preparation of Sample:

1. Cottonseed —
   De-hull about 50 g sample as in AOCS method Aa 2-38, using Bauer mill with plates separated so that seed are just broken. Screen on 4-6 mesh sieve to remove meats from hulls and lint. Grind meats through 2 mm screen in a Wiley mill, avoiding overheating meats and expressing oil.
2. Cooked meats —
   Reduce moisture to 5% or below by air equilibration, or by blowing air over meats. Grind through 2 mm screen in a Wiley mill.
3. Press cake and meal —
   Prepare sample as in AOCS method Ba 1-38 and grind 50 g through 1 mm screen in a Wiley mill.
4. Crude oil —
   Heat sample to 50 C, mix well, and filter through paper suitable for oil filtration (Apparatus, 7). Analyze filtered sample within 1 day, or store at

Total Gossypol

-20 C (0 F) if analysis is delayed. Gossypol in oil undergoes changes if stored at room temperature and above.

5. Soapstock —
Heat to 50 C, mix well before with-drawing analytical sample. Store under same conditions as for crude oil.

Sample Size:

Sample weight and aliquot used for aniline reaction depend on expected total gossypol content. Ideally, the analytical sample should contain from 0.5-5.0 mg of gossypol, and the aliquot for the aniline reaction about 0.1 mg gossypol. Table 1 below is a guide, and will provide mg of gossypol for aniline reaction within the range used for calibration.

Procedure:

1. Weigh analytical sample (see Sample Size and Table 1) on analytical balance accurate to ± 0.001 g and transfer to a 50 mL volumetric flask. A small piece of aluminum foil or weighing paper is useful for weighing and transferring sample to flask. Add 10 mL complexing reagent (Reagents, 3) by pipet, washing down sample material adhering to neck of flask. A rapid delivery pipet may be used (see Notes, 5).
2. Prepare reagent blank containing 10 mL of complexing reagent in a 50 mL volumetric flask.
3. Heat sample and blank in water bath (95-100 C) for 30 min, cool to room temperature, dilute to volume with isopropyl alcohol-hexane mixture (Reagents, 2) and mix well.
4. Filter sample extract through 11 cm medium retention paper (Apparatus, 7) into a 50 mL glass stoppered Erlenmeyer flask, discarding first 5 mL of filtrate.
5. Pipet duplicate aliquots of sample extract (see Sample Size and Table 1) into 25 mL volumetric flasks. Pipet duplicate blank aliquots of same volume as sample aliquot into 25 mL volumetric flasks.
6. Dilute one set of sample and blank aliquots to volume with the isopropyl alcohol-hexane mixture, and reserve as reference solutions for absorbance measurement.
7. Add 2 mL aniline, by pipet, to the other set of sample and reagent blank aliquots, heat in a water bath (95-100 C) for 30 min. Cool to room temperature, dilute to volume with the isopropyl alcohol-hexane mixture, and mix well. Allow to stand for 1 hr at room temperature after dilution (see Notes, 3).
8. With a spectrophotometer (Apparatus, 4) at 440 nm, or a suitable spectrophotometer, determine absorbance of reagent blank reacted with aniline (Procedure, 7), using blank aliquot without aniline (Procedure, 6) as reference solution.
9. Determine absorbance of sample aliquot reacted with aniline (Procedure, 7), using diluted sample aliquot without aniline (Procedure, 6) as reference solution. Subtract absorbance of reagent blank (Procedure, 8) from that of sample aliquot reacted with aniline to obtain corrected absorbance (see Notes 2, 3). 10. From corrected absorbance of sample aliquot (Procedure, 9), determine mg of gossypol in sample aliquot by multiplying absorbance by either the mean calibration factor or reference to a calibration graph (Calibration, step 7).

Calibration (see Notes, 4):

1. Pipet duplicate 1, 2, 4, 6, 8 and 10 mL aliquots of the standard gossypol solution (Reagents, 5) into 50 mL volumetric flasks. To each standard add sufficient volume of complexing reagent (Reagents, 3) to make volume 10 mL. Use 10 mL of complexing reagent as a blank.
2. Heat flasks in a water bath (95-100 C) for 30 min, cool to room temperature, dilute to volume with the isopropyl alcohol-hexane solution and mix well.

Table 1. Sample size for gossypol analysis.

Expected total gossypol %	Expected total gossypol ppm	Sample weight g	Aliquot mL
0.002-0.01	20-100	5.0	10
0.01-0.05	100-500	5.0	5
0.05-0.10	500-1,000	5.0	2
0.10-0.20	1,000-2,000	2.0	2
0.20-0.40	2,000-4,000	1.0	2
0.40-0.60	4,000-6,000	0.75	2
0.60-1.0	6,000-10,000	0.50	2
1.0-2.0		0.20	2
2.0-4.0		0.20	1

3. Pipet duplicate 2 mL aliquots of each standard and the reagent blank into 25 mL volumetric flasks.
4. Dilute one set of standard aliquots and the reagent blank to volume with the isopropyl alcohol-hexane solution and reserve as reference solutions for absorbance measurements.
5. Add 2 mL aniline to the other set of standard aliquots and the blank, heat in water bath (95-100 C) for 30 min, cool to room temperature, dilute to volume with the isopropyl alcohol-hexane solution and mix well. Allow to stand for 1 hr at room temperature before determining absorbance.
6. Determine Absorbance of reagent blank as in Procedure, 8 and of the standard aliquots as in Procedure, 9. Subtract absorbance of reagent blank from absorbance of each standard to obtain corrected absorbance for use in following step.
7. Calculate a calibration factor by dividing mg of gossypol in standards by corrected absorbance of each standard to obtain calibration factors.

   Factor, F, = mg gossypol in standard/corrected absorbance

   Calculate the average of the factors determined for each of the standards, and use this average Factor in Procedure, step 10, to calculate mg gossypol in sample aliquots.
8. In event a colorimeter is used, rather than a spectrophotometer, the Factors in determined in the Calibration, step 7, may vary with gossypol concentration. If this occurs, it is necessary to plot mg gossypol in each standard against the appropriate corrected absorbance as determined in Calibration, step 6, and use the plot to determine mg of gossypol in sample aliquots in Procedure, step 10.

Calculations:

1. Calculate % total gossypol in sample as follows

$$\text{Total gossypol, \%} = \frac{5 \times G}{W \times V}$$

Where -

G = mg gossypol in sample aliquot (Procedure, 10).

W = weight of sample in grams.

V = volume of sample aliquot used for analysis (Procedure, 9).

2. If gossypol concentration is desifed in parts per million

(ppm) use the following equation -

$$\text{Total Gossypol, ppm} = \frac{5 \times G}{W \times V} \times 10{,}000$$

Precision:

Collaborative study established the following precision estimates for the method. As the % gossypol decreases, precision (coefficient of variation, CV) becomes poorer. Precision in the 0.50-0.10% range was estimated to be 3.5-7.0% and for the 0.06-0.01% range the precision was estimated to be 10.0-17.0%.

Table 2. Precision values for gossypol determination.

Total Gossypol, %	Std. Dev.	CV, %
0.970	0.0102	1.1
1.200	0.0146	1.2
1.376	0.0117	0.9
0.208	0.0062	3.0

Notes:

Caution

Isopropyl alcohol and n-hexane are flammable solvents. They should not be used near an open flame. The use of a properly operating fume hood is recommended when using these solvents. The TLV for hexane is 50 ppm in air. OSHA recommends that exposure not exceed 350 ng/M^3 for a time weighted average. Hexane vapor causes lung irritation and produces neurotoxic effects.

Aniline is an allergin and is toxic if absorbed through the skin. The TLV in air is 2 ppm. Protective clothing and a properly operating fume hood should be used when using aniline.

Dimethylformamide is a strong irritant to skin and tissue. It is toxic by skin absorption. It is a moderate fire risk. The TLV is 10 ppm in air.

3-amino-1-propanol (propanolamine) is a tissue irritant. Avoid breathing vapors and contact with the skin.

Glacial acetic acid is moderately toxic by ingestion and inhalation. It is a strong irritant to skin and tissue. The TLV in air 10 ppm.

Numbered Notes

1. This method may not be applicable to feeds containing whole, unprocessed cottonseed. Components in the feed interfere with this method and may give false positive results. At the time of the revision of this method, the A.O.C.S. Technical Committee had alternate methodology under review, but no satisfactory method has yet been found. For feed samples containing whole, untreated cottonseed, the analyst may want to try alternate published HPLC methods.
2. Absorption maximum of the dianilinogossypol reaction product is at 440 nm, but may vary with

the wavelength accuracy of the instrument. All absorbance measurements should be made at the actual maximum for the spectrophotometer used.

3. After the aniline reaction (Procedure, 7) extracts of samples, and standards, exhibit slight increase in absorbance up to 1 hr, and are stable for 1-5 hrs. For purposes such as plant control, absorbance measurements can be made immediately after aniline reaction and dilution, provided that the calibration factors (Calibration, steps 5 to 7) are determined under the same conditions. A variation of about 10 min in determining absorbance will result in about 2% relative error in total gossypol.
4. Once a given spectrophotometer is calibrated, calibration factors are valid for long-time use, and need not be repeated except for occasional check of instrument performance. Matched cells for the absorbance measurements should be used, or suitable absorbance corrections applied to unmatched cells, using the isopropyl alcohol-hexane mixture for cell comparison.
5. Extracts of sample treated with the complexing reagent (Procedure, steps 1 to 4) are exceptionally stable, and if necessary, can be stored under refrigeration for several days before conducting the aniline spectrophotometric reaction.
6. See JAOCS 35: 93-97 (1958) for details and verification of the method.

GLEAN FC	✓ HARMONY	HARMONY EXTRA [2]	
chlorsulfuron	thifensulfuron methyl	tribenuron methyl + thifensulfuron methyl	**Common Name**
DPX-W4189	DPX-M6316	DPX-R9674	**Code Name**
2-Chloro-N-[[(4-methoxy-6-methyl-1,3,5,-triazin-2-yl) amino]carbonyl]-benzenesulfonamide	Methyl 3-[[[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)-amino]carbonyl] amino]sulfonyl]-2-thiophenecarboxylate		**Chemical Name**
64902-72-3	79277-27-3		**CAS Registry No.**
$C_{12}H_{12}ClN_5O_4S$	$C_{12}H_{13}N_5O_6S_2$		**Molecular Formula**
357.78	387.40		**Molecular Weight**
Crystalline Solid	Solid	Dry Flowable	**Physical State**
White	White	Light Beige	**Color**
None	None	Slightly Pungent	**Odor**
Caution	Caution	Caution	**Signal Word**
IV	IV	IV	**Toxicity Class**
174-178 °C	186 °C		**Melting Point**
			Aqueous Solubility at 25 °C
g/L	g/L		**pH**
0.587	0.223		**5**
31.8	2.24		**7**
–	8.83		**9**
$2.3x10^{-11}$ mm Hg	$1.3x10^{-10}$ mm Hg		**Vapor Pressure at 25 °C**
$3.4x10^{-16}$ atm-m^3/mol	$4.3x10^{-15}$ atm-m^3/mol		**Henry's Law Constant at 25 °C**

Continued on next page

GLEAN FC	HARMONY	HARMONY EXTRA [2]	
			Octanol/Water Partition Coefficient at 25 °C
K_{ow}	K_{ow}		
2.13	1.60		**pH 5**
0.10	0.02		**7**
0.04	0.01		**9**
3.6 pK_a	4 pK_a		**Dissociation Constant**
0.69	1.49		**Specific Gravity at 25 °C**
0.66 g/mL	0.59 g/mL	1.56 g/mL	**Bulk Density**
Nonflammable Noncorrosive	Nonflammable Noncorrosive	Nonflammable Noncorrosive	**Safety Attributes**
			Hydrolysis Half-life at 45 °C
hr	hr		
5.7	8.3		**pH 4**
42	39		**5**
1235	250		**7**
303	10		**9**
°C g/L	mg/L at 25 °C		**Solubility in:**
22 57	11.9		***Acetone***
- -	7.3		***Acetonitrile***
- -	-		***Carbon tetrachloride***
- -	0.9		***Ethanol***
- -	2.6		***Ethyl acetate***
22 <0.01	<0.1		***Hexane***
22 14	2.6		***Methanol***
22 102	27.5		***Methylene chloride***
22 3	-		***Toluene***
25 100-125 ppm	-		***Water (distilled, deionized)***
- -	-		***Water (unbuffered)***
- -	- 24		***Water (pH 4)***
25 0.3	260		***Water (pH 5)***
- -	2400		***Water (pH 6)***
25 27.9	-		***Water (pH 7)***
- -	0.2		***Xylene***
Stable when stored in the original closed container	Stable when stored in normal conditions and at normal temperatures	Stable when stored in normal conditions and at normal temperatures	**Storage Stability**

DU PONT AGRICULTURAL PRODUCTS
Product Physical, Chemical, and Environmental Data Sheet

Product Identification

Common Name	Thifensulfuron Methyl (DPX-M63136)

Environmental Properties (continued)

					Data Type	Reference
Photolysis Half-life	t1/2	pH	% OM	T (°C)		
- Soil	17.6 days	5.4	4.3	25	E	AMR-505-86
- Water	*4.9 days	5	X	25	E	AMR-511-86
	*5.3 days	7		25		AMR-511-86
	*5.4 days	9		25		AMR-511-86
- Air		X	X			
Hydrolysis Half-life	at pH 5	6 days (0.5 ppm); 4 days (5 ppm)			E	AMR-224-84
	at pH 7	>30 days (0.5 ppm); >30 days (5 ppm)			E	AMR-224-84
	at pH 9	>30 days (0.5 ppm); >30 days (5 ppm)			E	AMR-224-84
Aerobic Aquatic Half-life	t1/2	pH	T (°C)	Location		
Anaerobic Aquatic Half-life	t1/2	pH	T (°C)	Location		
	2 1/2 - 3 weeks	4.9	25	Fayetteville, N.C.	E	AMR-540-86
	2 1/2 - 3 weeks	7.5	25	Rochelle, Ill.	E	AMR-540-86
	2 1/2 - 3 weeks	6.5	25	Landenberg, PA	E	AMR-540-86

*Corrected for hydrolysis component

DU PONT AGRICULTURAL PRODUCTS
Product Physical, Chemical, and Environmental Data Sheet

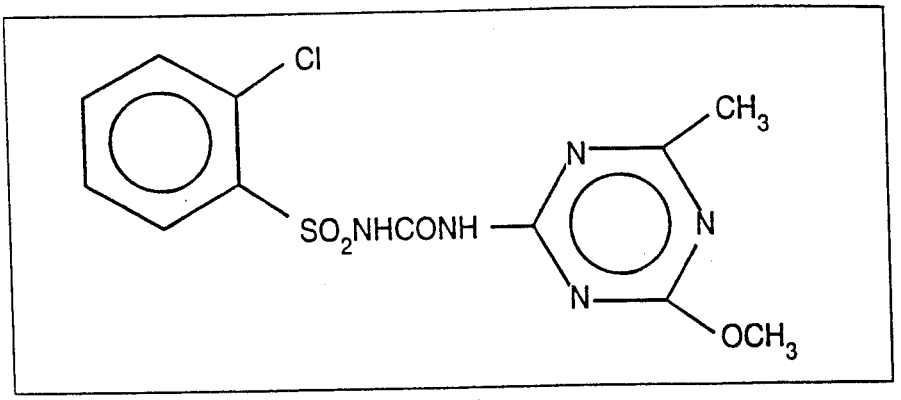
Environmental Properties (continued)

Soil Adsorption Coefficient (K_d) and Soil Partition Coefficient (K_{oc}) @25°C	K_d	K_{oc}	pH	% OM	Soil Type	Location	Data Type	Reference
	0.08	19.2	6.6	1.1	Woodstown sandy loam	Dover, Del.	E	AMR-286-84
	0.19	19.5	6.5	2.1	Cecil sandy loam	Raleigh, N.C.	E	AMR-286-84
	1.38	62.9	5.4	4.3	Flanagam silt loam	Rochelle, Ill.	E	AMR-286-84
	1.25	31.5	5.2	7.5	Keyport silt loam	Newark, Del.	E	AMR-286-84

Aerobic Soil Half-life	t1/2	pH	% OM	T (°C)	Soil Type	Location	Data Type	Reference
	6 days	5.4	4.3	25	Flanagan silt loam	Rochelle, Ill.	E	AMR-236-84; revision 1
	2 days	5.2	7.5	25	Keyport silt loam	Newark, Del.	E	AMR-236-84; revision 1

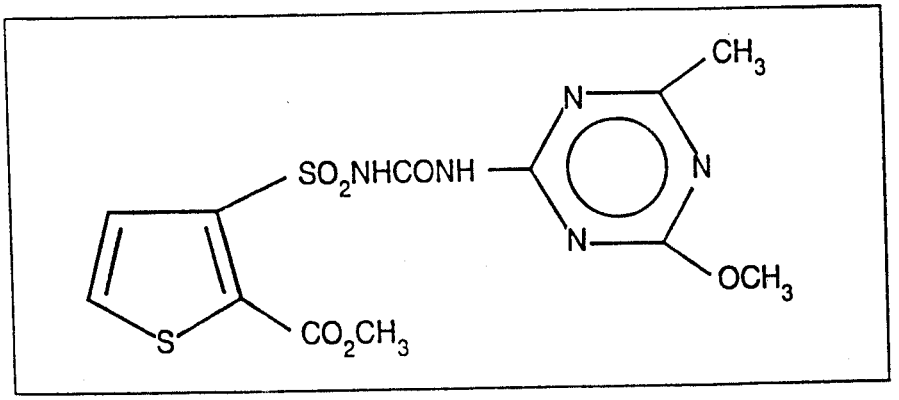
Structural Formulas (continued)

GLEAN FC



chlorsulfuron

✓ HARMONY



thifensulfuron methyl

FINESSE

The active ingredients in FINESSE are metsulfuron methyl and chlorsulfuron. See the figures under ALLY and GLEAN FC for the structural formulas.

HARMONY EXTRA

The active ingredients in HARMONY EXTRA are tribenuron methyl and thifensulfuron methyl. See the figures under EXPRESS and HARMONY for the structural formulas.

HARMONY Toxicology

ACUTE TOXICOLOGY [1, 2, 3]

Acute Oral
- LD_{50} Rat ..... >5,000 mg/kg (virtually nontoxic)

Acute Dermal
- LD_{50} Rabbit ..... >2,000 mg/kg (slightly to moderately toxic)

Acute Inhalation
- LC_{50} Rat ..... >7.9 mg/L^3 (very low toxicity)

Eye Contact
- Rabbit ..... moderate irritant

Skin Contact
- Rabbit ..... not an irritant
- Guinea pig ..... not a sensitizer

AVIAN [4]

Mallard duck
- LD_{50} ..... >2,510 mg/kg (virtually nontoxic)
- LC_{50} ..... >5,620 ppm (virtually nontoxic)

Bobwhite quail
- LD_{50} ..... 481 mg/kg (moderately toxic)
- LC_{50} ..... >5,620 ppm (virtually nontoxic)

AQUATIC [4]

Bluegill sunfish
- LC_{50} ..... >100 ppm (virtually nontoxic)

Rainbow trout
- LC_{50} ..... >100 ppm (virtually nontoxic)

Daphnia magna
- EC_{50} ..... 470 ppm (virtually nontoxic)

NONTARGET INSECT [4]

Honeybee
- LD_{50} ..... >12.5 µg/bee (virtually nontoxic)

MUTAGENICITY AND GENETIC TOXICITY STUDIES [4]

Thifensulfuron methyl, the active ingredient in HARMONY, has tested negative for genetic damage in these tests: Ames bacterial assay, *in vitro* Chinese hamster ovary assay, *in vitro* unscheduled DNA synthesis, *in vitro* cytogenetic assay, and mouse micronucleus assay.

CHRONIC AND SUBCHRONIC TOXICITY STUDIES [4]

In toxicology studies, thifensulfuron methyl showed no effect on rat reproduction or lactation at any dose up to 2,500 ppm. There were no gross or microscopic effects on the offspring at any dose tested. Developmental toxicity studies showed no birth defects in rats or rabbits at doses up to 800 mg/kg and 650 mg/kg, respectively. No tumorous effects were observed in eighteen-month mouse-feeding studies or two-year rat-feeding studies. At 2,500 ppm, lower body weights and weight gains were observed in rats. At the 500 and 2,500 ppm doses, slightly lowered serum sodium concentrations were observed in rats. This lowering was not biologically significant.

CARCINOGENICITY [4]

No component of HARMONY is listed as a carcinogen by the International Agency for Research on Cancer (IARC), the National Toxicology Program (NTP), the Occupational Safety and Health Administration (OSHA), or the American Conference of Governmental Industrial Hygienists (ACGIH).

1 LD_{50} is the dose of chemical per kilogram of body weight that is lethal to 50% of the animals tested.
2 LC_{50} is the concentration of material in air or water that is lethal to 50% of the animals tested.
3 EC_{50} is the concentration of material in water that produces an effect on 50% of the species tested.
4 Tests were conducted with the active ingredient only.

Performance Characteristics (continued)

ALLY is recommended for use on land dedicated to the production of wheat (including durum) and barley with rotation options available for other crops, including oats, proso millet, dryland grain sorghum, dryland corn, soybeans, flax, sunflower, safflower, alfalfa hay, and dry beans. In areas that have a short growing season, prolonged periods of low soil temperature, and low annual rainfall, ALLY can remain in the soil for 34 months or more and can injure certain crops rotated to previously treated land. For information on regional soil residual activity, crop rotation recommendations, and field or LRB℠ bioassay, see the product specimen label.

EXPRESS and HARMONY EXTRA are recommended for postemergence treatment of wheat (including durum), barley, and oats. (Registration of HARMONY EXTRA for use on oats is pending and expected in 1992.) EXPRESS and HARMONY EXTRA have very little soil activity. They are rapidly broken down by chemical hydrolysis and by microbes under aerobic soil conditions. Any rotational crop can be planted 60 days after an EXPRESS or HARMONY EXTRA application.

FINESSE and GLEAN FC are recommended for use on land that has a soil pH no higher than 7.9 and that is dedicated to the long-term production of cereal grains. The soil residual activity of FINESSE and GLEAN FC can injure crops other than wheat, barley, oats, rye, or triticale for fourteen months to four years after application. Low soil pH, high soil temperature, and moist soil conditions all enhance breakdown of FINESSE and GLEAN FC. To ensure crop rotation flexibility, do not use FINESSE or GLEAN FC on all wheat or barley acreage. For information on regional soil residual activity, crop rotation recommendations, and field or LRB℠ bioassay, see the product specimen label.

HARMONY is recommended for postemergence treatment of wheat (including durum) and barley. HARMONY is rapidly broken down by microbes under aerobic soil conditions. Any rotational crop can be planted 60 days after the application of HARMONY.

Personal Safety Information

SAFETY PRECAUTIONS

Observe the following guidelines when using Du Pont sulfonylurea cereal herbicides:

- Use only with adequate ventilation and avoid breathing vapors, mist, or dust.
- Avoid herbicide contact with eyes, skin, or clothing.
- Wash thoroughly after handling herbicide material.
- Wash clothing after herbicide use.
- Keep herbicide away from heat, sparks, and flames.
- Do not consume food, drink, or tobacco in areas that may be contaminated by herbicide material.

PERSONAL PROTECTIVE EQUIPMENT

During normal use and handling of sulfonylurea herbicides, no personal protective equipment is required. During abnormally high or continuous herbicide exposure, use an approved pesticide respirator.

During industrial herbicide handling and formulation, wear safety glasses, coverall chemical-splash goggles, and a face shield to protect face and eyes from splashed or sprayed herbicide material.

FIRST AID

Inhalation: Du Pont sulfonylurea herbicide compounds are not likely to be hazardous by inhalation. If overexposure does occur, remove the victim to fresh air. Provide oxygen if breathing is difficult, and if the victim is not breathing, give artificial respiration. Get medical attention.

Skin contact: Flush with water after excessive contact.

Eye contact: Immediately flush eyes with plenty of water for at least 15 minutes while holding eyelids open. Get medical attention.

Ingestion: No specific intervention is necessary. Du Pont sulfonylurea compounds are not likely to be hazardous by ingestion. Consult a physician if necessary.

MEDICAL EMERGENCIES

For medical emergencies involving sulfonylurea herbicides or related formulations of any other Du Pont agrichemical product, call toll free:

1-800-441-3637

This is a 24-hour Du Pont product information and emergency response number in Wilmington, Delaware. A caller using this number should state, "This is a medical emergency." He or she will be placed in contact with the Du Pont physician on duty and should be prepared to state the name of the Du Pont product involved and the attending physician's name, address, and phone number.

Material Safety Information

HEALTH, SAFETY, AND HANDLING INFORMATION

Individuals handling Du Pont sulfonylurea herbicides are responsible for storage and application safety. Observe the precautions outlined in the following sections when working with sulfonylurea herbicides. Material Safety Data Sheets (MSDS) are available for information on material safety and handling. For more information on how to use these herbicides safely, see the product labels.

HAZARDOUS REACTIVITY

Instability: Stable under normal temperature and storage conditions
Incompatibility: None reasonably foreseeable
Polymerization: Will not occur

FIRE AND EXPLOSION INFORMATION

Du Pont sulfonylurea herbicides are not a fire or explosion hazard, but, like most organic powders or crystals, may form explosive mixtures in air under severe dusting conditions. The following extinguishing media may be used: water spray, foam, dry chemical, or CO_2.

FIREFIGHTING INSTRUCTIONS

Evacuate personnel to a safe area and wear self-contained breathing apparatus during firefighting. Use water spray to control the fire.

If the area is heavily exposed to fire and conditions permit, let the fire burn itself out. Water may increase the contamination hazard.

SPILL, LEAK, OR RELEASE

Shovel or sweep up spilled dry material. For spilled liquid mixtures, dike the spill and prevent the liquid from entering sewers, waterways, or low areas. Use appropriate personal protective equipment during cleanup procedures. Review the Fire and Explosion Hazards and Safety Precautions sections of the MSDS before proceeding with a cleanup.

If a significant spill occurs, call CHEMTREC:

1-800-424-9300

WASTE DISPOSAL

Treat, store, transport, and dispose of sulfonylurea herbicide wastes according to applicable federal, state/provincial, and local regulations. Dispose of wastes either on site or at an approved waste disposal facility. Do not flush herbicide waste to surface water or sanitary sewer systems, and do not contaminate water, food, or feed by improper storage, disposal, or equipment cleanout.

1995 DUPONT TRANSGENIC COTTON TRIAL

APHIS Reference Number:	95-026-01n, TEST 1
Trait:	Sulfonylurea herbicide tolerance
Researcher's Name:	ROBERT E. SEAY
Researcher's Address:	HWY.454 GREENVILLE,MS
Researcher's Phone:	(601) 378-3699
Cooperator's Name:	SAME AS ABOVE
Cooperator's Address:	
ɔoperator's Phone:	
Trial Location:	DUPONT FIELD STATION, HWY.454 GREENVILLE, MS
Original Transgenic Line:	19-51A
Lines in Trial:	NUCOTN 68 (TRANSGENIC) DP5690
Number of Herbicide Treatments:	22
Number of Reps per Treatment:	3
Date Planted:	12/MAY/95
Date of Emergence:	19/MAY/95 FOR BOTH
Estimated Percent Germination:	85% FOR BOTH
Date of First Bloom:	01/JUL/95 FOR BOTH LINES IN THE UNTREATED
Insect Scouting:	SCOUTING WAS DONE ON 5/26,6/9,6/23 AND 7/1. NO DIFFERENCES WERE NOTED BETWEEN LINES. A MODERATE BUILD UP OF APHIDS WAS NOTED ON 6/9 EVALUATION. SCOUTING WAS DONE BY R.E.SEAY
Disease Scouting:	SCOUTING WAS DONE ON THE SAME DATES AS ABOVE AND NO DISEASE WAS NOTED IN EITHER LINE. SCOUTED BY R.E.SEAY

Date of Trial Termination:	01/JUL/95
Method of Termination:	BUSH HOG AND DOUBLE DISK
Disposition of Seed Cotton:	TERMINATED AT FIRST BLOOM

1995 DUPONT TRANSGENIC COTTON TRIAL

APHIS Reference Number:	95-021-01N, TEST 2
Trait:	Sulfonylurea herbicide tolerance
Researcher's Name:	ROBERT E. SEAY
Researcher's Address:	HWY.454, GREENVILLE,MS.
Researcher's Phone:	(601) 378-3699
Cooperator's Name:	SAME AS ABOVE
Cooperator's Address:	
Cooperator's Phone:	
Trial Location:	DUPONT FIELD STATION, HWY.454 GREENVILLE,MS.
Original Transgenic Line:	19-51A
Lines in TriaL:	NUCOTN 68 (TRANSGENIC) DP5690
Number of Herbicide Treatments:	12
Number of Reps per Treatment:	3
Date Planted:	12/MAY/95
Date of Emergence:	19/MAY/95 FOR BOTH LINES
Estimated Percent Germination:	85% FOR BOTH LINES
Date of First Bloom:	01/JUL/95 FOR BOTH LINES IN THE UNTREATED
Insect Scouting:	SCOUTING WAS DONE ON 5/26,6/9,6/23 AND 7/1. NO DIFFERENCES WERE NOTED BETWEEN LINES. A MODERATE BUILD UP OF APHIDS WAS NOTED ON 6/9 OBSERVATION. SCOUTING WAS PERPORMED BY R.E.SEAY.
ɔease Scouting:	SCOUTING DATES WERE THE SAME AS ABOVE WITH NO DISEASES NOTED IN EITHER LINE AT ANY DATE. SCOUTING DONE BY R.E.SEAY
Date of Trial Termination:	01/JUL/95

APHIS PERMIT No:94-069-06N
TEST No:CEZ94012
INVESTIGATOR:Pitts
CITY:Idalou
COUNTY:Lubbock
STATE:Texas
PROJECT No:USA-94-343
COTTON: Transgenic line 19-51a (DP5690SU)

STAGE	DATE
preemergence	
3-5 leaf stage	24UN94
3-5leaf stage	27JUN94
squaring	06JUL94
squaring	22JUL94

RESULTS WITH STAPLE, STAPLE +M6316

Treatment	Appl. Rate (oz ai/A)	Eval. Date	% Injury DP5690SU	% Injury Parent	Stand counts/10' of row DP5690SU	Stand counts/10' of row Parent
1. staple	.25	6-27-94			43.3	47.3
		7-06-94	0	4.3		
		7-22-94	0	1.7		
2. staple	.5	6-27-94			46.3	49.0
		7-06-94	0	3.3		
		7-22-94	0	0		
3. staple	1	6-27-94			45.3	53.0
		7-06-94	2.7	11.7		
		7-22-94	0	0		
4. m6316+staple	.031+.25	6-27-94			37.7	49.7
		7-06-94	8.3	63.3		
		7-22-94	0	41.7		
5. m6316+staple	.063+.5	6-27-94			41.7	45.7
		7-06-94	6	66.7		
		7-22-94	1.7	53.		
6. m6316+staple	.125+1	6-27-94			41.3	59.0
		7-06-94	7.7	76.7		
		7-22-94	0	51.7		
7. staple+m6316	.046+.25	6-27-94			42.7	49.0
		7-06-94	9.3	71.7		
		7-22-94	2.3	51.7		
8. m6316+staple	.093+.5	6-27-94			37.0	50.3
		7-06-94	7.7	76.7		
		7-22-94	0	71.7		
9. m6316+staple	.187+1	6-27-94			40.3	49.0
		7-06-94	9.3	76.7		
		7-22-94	77.3			
10. m6316+staple	.063+.25	6-27--94			40.7	43.7
		7-06-94	3.3	71.7		
		7-22-94	0	70.0		

Treatment	Appl. Rate (oz ai/A)	Eval. Date	% Injury DP5415SU	% Injury Parent	Stand counts/10' of row DP5690SU	Stand counts/10' of row Parent
11. m6316+staple	.125+.5	6-27-94			48.3	52.3
		7-06-94	6.7	70.0		
		7-22-94	0	73.3		
12. m6316+staple	.25+1	6-27-94			39.0	52.3
		7-06-94	15.0	75.0		
		7-22-94	0	85.0		
13. m6316	.031	6-27-94			46.3	50.7
		7-06-94	12.7	67.7		
		7-22-94	0	48.3		
14. m6316	.063	6-27-94			43.7	52.0
		7-06-94	5.0	70.0		
		7-22-94	0	65.0		
15. m6316	.125	6-27-94			39.7	49.0
		7-06-94	11.7	73.3		
		7-22-94	0	81.7		
16. m6316	.25	6-27-94			44.7	49.0
		7-06-94	9.3	77.7		
		7-22-94	0	86.0		
17. untreated		6-27-94			40.0	48.7
		7-06-94	0	0		
		7-22-94	0	0		

COMMENTS: TG cotton Dpl-5690TG and the parental variety DPL-5690 were planted in split plot design and treated postemergence with various ratios of staple + DPX-M6316. Low weed populations prevented collection of weed control data. In stand counts , no significant difference was seen in stand plant between DPL-5690TG and DPL-5690 varieties. In addition, no differences were seen in plant vigor between transgenic and conventional cotton. At 12 DAT, crop injury from Staple, DPX-M6316 and mixtures of Staple plus DPX-M6316 averaged 0-15% in transgenic cotton DPL-5690TG. However, all treatments caused unacceptable injury in conventional DPL-5690, except Staple 0.25-1.0 oz ai/ A. There was no significant difference seen in plant stand or vigor between TG cotton DPL-5690TG and parent DPL-5690. DPX-M6316 and mixtures of Staple plus DPX-M6316 caused unacceptable injury in conventional cotton but not in trangenic cotton.

1994 DUPONT TRANSGENIC COTTON TRIAL

APHIS Reference Number: 94-095-06N

Trait: Sulfonylurea herbicide tolerance

Researcher's Name: E. A. Drummond

Researcher's Address: Scott, Mississippi

Researcher's Phone: 601-742-3351

Trial Location: Scott, Mississippi

Original Transgenic Line: 19-51A

Lines in Trial (Names): DP5690-SU

Date Planted: April 26, 1994

Trial Size: 50 acres

Quantity Seed Planted: 13 lbs.

Date of Emergence: May 8, 1994

Estimated Percent Germination: 75%

Date of first Picking: October 29, 1994 (rain delayed harvest)

Date of Trial Termination: November 20, 1994

Method of Termination: Bush hog, plow

Disposition of Seed Cotton: fuzzy seed in bulk storage

Amount of fiber: 835.6 lb./ac

Disposition of fiber: going to commerce

1994 DUPONT TRANSGENIC COTTON TRIAL

APHIS Reference Number: 94-095-06N

Trait: Sulfonylurea herbicide tolerance

Researcher's Name: E. A. Drummond

Researcher's Address: Scott, Mississippi

Researcher's Phone: 601-742-3351

Trial Location: Scott, Mississippi

Original Transgenic Line: 19-51A

Lines in Trial (Names): DP51-SU

Date Planted: May 2, 1994

Trial Size: 30 acres

Quantity Seed Planted: 13 lbs.

Date of Emergence: May 13, 1994

Estimated Percent Germination: 75%

Date of first Picking: October 1, 1994

Date of Trial Termination: November 1, 1994

Method of Termination: Bush hog, plow

Amount of cleaned seed: 420 - 50 lb. sx

Disposition of Seed: delinted, cleaned and bagged, cleanouts dumped back on field

Amount of fiber: 734.6 lb./ac

Disposition of fiber: going to commerce

1994 DUPONT TRANSGENIC COTTON TRIAL

APHIS Reference Number: 94-095-06N

Trait: Sulfonylurea herbicide tolerance

Researcher's Name: E. A. Drummond

Researcher's Address: Scott, Mississippi

Researcher's Phone: 601-742-3351

Trial Location: Scott, Mississippi

Original Transgenic Line: 19-51A

Lines in Trial (Names): DP5415-SU

Date Planted: April 25, 1994

Trial Size: 50 acres

Quantity Seed Planted: 13 lbs.

Date of Emergence: May 7, 1994

Estimated Percent Germination: 75%

Date of first Picking: September 30, 1994

Date of Trial Termination: November 1, 1994

Method of Termination: Bush hog, plow

Amount of cleaned seed: 849 - 50 lb. sx

Disposition of Seed: delinted, cleaned and bagged, cleanouts dumped back on field

Amount of fiber: 899.8 lb./ac

Disposition of fiber: going to commerce

THIS PERMIT No:94-090-08N
TEST No:FGA94110
INVESTIGATOR: SEAY
CITY:Greenville
COUNTY:Washington
STATE:Mississippi
PROJECT No:USA-94-341
COTTON: Transgenic line 19-51a (DP51SU)

STAGE	DATE
preemergence	
3-5 leaf stage	10JUN94
3-5 leaf stage	17JUN94
3-5 leaf stage	24JUN94
squaring	04JUL94
1st bloom	08JUL94

RESULTS WITH STAPLE, STAPLE +M6316

Treatment	Appl. Rate (oz ai/A)	Eval. Date	% Injury DP51SU	% Injury Parent	Stand counts/10' of row DP51SU	Stand counts/10' of row Parent
1. m6316	.063	6-24-94	15.0	90.0		
		7-04-94	6.7	90.0	35.3	32.0
		7-08-94				
2. m6316	.125	6-24-94	11.7	90.0		
		7-04-94	13.3	91.7	34.0	330.7
		7-08-94				
3. m6316	.25	6-24-94	30.0	90.0		
		7-04-94	23.3	91.7	32.0	34.0
		7-08-94				
4. staple	1	6-24-94	0	0		
		7-04-94	0	0	35.3	353
		7-08-94				
5. staple	2	6-24-94	0	0		
		7-04-94	0	0	34.0	313
		7-08-94				
6. untreated		6-24-94	0	0		
		7-04-94	0	0	28.0	307
		7-08-94				

COMMENTS: Both DP51 non transgenic cotton were planted May 18, 1994. Plot size was a split design composed of two rows transgenic and two rows non-transgenic cotton with one row of each being treated, plot length 35 feet. Application was made June 19, 1994 to 3 leaf cotton. Evaluations were made 7, 14, 24 and 28 days after treatment. For the parent material cotton all treatments showed significant injury at all rates with the exception of Staple both 1 and 2 oz which indicated no injury at the 14 and 24 DAT rating. Observations made at each evaluation date indicated no differences in insect or disease pressure in SU resistant or parental cotton.

APHIS PERMIT No:94-090-08N
TEST No:FGA94111
INVESTIGATOR:Seay
CITY:Greenville
COUNTY:Washington
STATE:Mississippi
PROJECT No:USA-94-342
COTTON: Transgenic line 19-51a (DP51SU)

STAGE	DATE
preemergence	20May94
3-5 leaf stage	10JUN94
3-5 leaf stage	17JUN94
squaring	24JUN94
squaring	04JUL94
1st bloom	08JUL94
1st open bolls	20SEP94
60% open bolls	01NOV94

RESULTS WITH STAPLE, STAPLE +M6316

Treatment	Appl. Rate (oz ai/A)	Eval. Date	% Injury		Stand counts/10' of row		LB/AC Yield	
			DP51SU	Parent	DP51SU	Parent	DP51SU	Parent
1. staple (pre)	1	6-17-94	8.8	10.0				
		6-24-94	0	0				
		7-04-94	0	0				
		7-08-94			37.5	38.5		
		11-01-94					2730.6	2986.4
2. staple (pre)	2	6-17-94	11.3	11.3				
		6-24-94	0	0				
		7-04-94	0	0				
		7-08-94			33.3	34.5		
		11-01-94					2632.2	2853.6
3. staple (post)	1	6-17-94	6.3	13.8				
		6-24-94	0	0				
		7-04-94	0	0				
		7-08-94	34.5	33.5				
		11-01-91					2853.6	2902.8
4. staple (post)	2	6-17-94	8.8	18.8				
		6-24-94	2.5	2.5				
		7-04-94	2.5	2.5				
		7-08-94			34.0	30.5		
		11-01-94					2804.4	2435.4
5. untreated		6-17-94	0	0				
		7-04-94	0	0				
		11-01-94					2558.4	2706.0

COMMENTS: All plots should be planted in a split design with TG cotton and normal cotton varieties side by side. Requirements specified by APHIS (Notification, buffer areas, and crop destruction where applicable) should be followed. Plots should be maintained as weed free as possible. Both DP51 Transgenic and DP51 were planted May 18, 1994. Plot size was a split design composed of two rows of transgenic (north side) and two rows of non- transgenic cotton with both rows being treated. Plot length

/as 35 feet and by 6.3 wide. Applications were made 5/20 for preemergence and 5/10 for postemergence applications. Evaluations were made at 28, 35, and 45 days after pre-application and 7, 14, and 24 days after postemergence applications. Yields were taken November 1, 1004 and poundage was adjusted for plant stand. Staple pre and post on either cotton did not adversely affect yield compared to the non-treated control. No differences were noted between the transgenic and non-transgenic cotton in insect or disease populations.

APHIS PERMIT No:94-090-08N
TEST No:FGA94112
INVESTIGATOR:SEAY
CITY:Greenville
COUNTY:Washington
STATE:Mississippi
PROJECT No:USA-94-343
COTTON: Transgenic line 19-51a (DP51SU)

STAGE	DATE
preemergence	
3-5 leaf stage	10JUN94
3-5 leaf stage	17JUN94
3-5 leaf stage	24JUN94
squaring	4JUL94
1st bloom	07JUL94

RESULTS WITH STAPLE, STAPLE +M6316

Treatment	Appl. Rate (oz ai/A)	Eval. Date	% Injury DP51SU	% Injury Parent	Stand counts/100' of row DP51SU	Stand counts/100' of row Parent
1. staple	.25	6-17-94	0	0		
		6-24-94	0	0		
		7-04-94	0	0		
		7-07-94			243.3	326.7
2. staple	.5	6-17-94	3.3	6.7		
		6-24-94	0	0		
		7-04-94	0	0		
		7-07-94			280.0	233.3
3. staple	1	6-17-94	0	6.7		
		6-24-94	0	0		
		7-04-94	0	0		
		7-07-94			300.0	296.7
4. m6316+staple	.031+.25	6-17-94	3.3	50.0		
		6-24-94	5.0	86.7		
		7-04-94	0	73.3		
		7-07-94			323.3	333.3
5. m6316+staple	.063+.5	6-17-94	8.3	60.0		
		6-24-94	5.0	90.0		
		7-04-94	0	73.3		
		7-07-94			253.3	283.3
6. m6316+staple	.125+1	6-17-94	8.3	70.0		
		6-24-94	6.7	90.0		
		7-04-94	0	91.7		
		7-07-94	256.7	326.7		

Treatment	Appl. Rate (oz ai/A)	Eval. Date	% Injury DP51SU	% Injury Parent	Stand counts/100' of row DP51SU	Stand counts/100' of row Parent
7. m6316+staple	.046+.25	6-17-94	5.0	56.7		
		6-24-94	5.0	86.		
		7-04-94	3.3	83.3		
		7-07-94			320.0	303.3
8.m6316+staple	.093+.5	6-17-94	6.7	66.7		
		6-24-94	5.0	90.0		
		7-04-94	0	90.0		
		7-07-94			266.7	313.3
9. m6316+staple	.187+1	6-17-94	10.0	63.3		
		6-24-94	6.7	90.0		
		7-04-94	0	91.7		
		7-07-94			316.7	280.0
10. m6316+staple	.063+.25	6-17-94	3.3	63.3		
		6-24-94	5.0	86.7		
		7-04-94	0	90.0		
		7-07-94			306.7	286.7
11. m6316+staple	.125+.5	6-17-94	10.0	66.7		
		6-24-94	5.0	90.0		
		7-04-94	3.3	91.7		
		7-07-94			303.3	300
12. m6316+staple	.25+1	6-17-94	5.0	60.0		
		6-24-94	3.3	88.3		
		7-04-94	0	83.3		
		7-07-94			320.0	346.7
13. m6316	.031	6-17-94	3.3	50.0		
		6-27-94	6.7	80.0		
		7-04-94	0	71.7		
		7-07-94			303.3	306.7
14. m6316	.063	6-17-94	5.0	66.7		
		6-24-94	5.0	86.7		
		7-04-94	0	90.0		
		7-07-94			290.0	306.7
15. m6316	.125	6-17-94	10.0	63.3		
		6-24-94	11.7	90.0		
		7-04-94	10.0	91.7		
		7-07-94			280.0	310.0
16.m6316	.25	6-17-94	5.0	50.0		
		6-24-94	1.7	86.7		
		7-04-94	10.0	80.0		
		7-07-94			296.7	290.0
17.untreated		6-17-94	0	0		
		6-24-94	0	0		
		7-04-94	0	0		
		7-07-94			340.0	283.3

COMMENTS: DPL51 transgenic and DPL51 non-tansgenic cotton were planted May 18, 1994. The plots were in a split plot design composed of 2 rows each of transgenic and non-transgenic variety cotton. The 2 center rows, one row of each variety, were treated. Plot length was 35 feet replicated 3 times. Post- emergence application was made June 10, 1994 to 3 leaf cotton. Evaluations were made 7, 14, 24 and 27 days after treatment. No treatment caused significant injury to transgenic cotton but the most injurious treatments were M6316 at .125 and .25 oz ai alone. All treatments with the exception of Staple alone caused significant crop injury to DPL51 parent cotton. All injury ratings with M6316 alone or in combination with Staple exhibited greater than 50% injury. At each evaluation date, observations were made to determine differences in Disease or Insect populations, no differences were noted between the Transgenic and Non-Transgenic plots.

APHIS PERMIT No:94-090-08N
TEST No:FGA94113
INVESTIGATOR:SEAY
CITY:Greenville
COUNTY:Washington
STATE:Mississippi
PROJECT No:USA-94-344
COTTON: Transgenic line 19-51a (DP51SU)

STAGE	DATE
preemergence	20MAY94
3-5 leaf stage	10JUN94
3-5 leaf stage	17JUN94
3-5 leaf stage	25JUN94
squaring	04JUL94
1st bloom	08JUL94
60% bloom	31OCT94

RESULTS WITH STAPLE, STAPLE +M6316

Treatment	Appl. Rate (oz ai/A)	Eval. Date	% Injury DP51SU	% Injury Parent	Stand counts/10' of row DP51SU	Stand counts/10' of row Parent	LB/AC Yield DP51SU	LB/AC Yield Parent
1. staple (pre)	1	6-17-94	2.5	2.5				
		6-25-94	0	0				
		7-04-94	0	0				
		7-08-94			26.5	34.0		
		10-31-94					2506.7	2605.0
2. staple (pre)	2	6-17-94	0	2.5				
		6-25-94	0	0				
		7-04-94	0	0				
		7-08-94			30.5	30.5		
		10-31-94					2752.4	2703.3
3. staple (post)	1	6-17-94	0	7.5				
		6-25-94	0	0				
		7-04-94	0	0				
		7-08-94			33.5	30.5		
		10-31-94					2654.1	2801.6
4. staple (post)	2	6-17-94	3.8	10.0				
		6-25-94	0	0				
		7-04-94	0	0				
		7-08-94			29.0	30.5		
		10-31-94					2678.7	277.0
5. m6316	.25	6-17-94	15.0	55.0				
		6-25-94	16.3	87.5				
		7-04-94	15.0	90.0				
		7-08-94			30.5	30.0		
		10-31-94					2703.3	196.6
6. untreated		6-17-94	0	0				
		6-25-94	0	0				
		7-04-94	0	0				
		7-08-94			32.0	32.5		
		10-31-94					255.8	2703.3

APHIS PERMIT No:94-090-08N
TEST No:FGA94113
INVESTIGATOR:SEAY
CITY:Greenville
COUNTY:Washington
STATE:Mississippi
PROJECT No:USA-94-344
COTTON: Transgenic line 19-51a (DP5415SU)

STAGE	DATE
preemergence	20MAY94
3-5 leaf stage	10JUN94
3-5 leaf stage	17JUN94
3-5 leaf stage	25JUN94
squaring	04JUL94
1st bloom	08JUL94
60% bloom	31OCT94

RESULTS WITH STAPLE, STAPLE +M6316

Treatment	Appl. Rate (oz ai/A)	Eval. Date	% Injury DP5415SU	% Injury Parent	Stand counts/10' of row DP5415SU	Stand counts/10' of row Parent	LB/AC Yield DP5415SU	LB/AC Yield Parent
1. staple (pre)	1	6-17-94	0	2.5				
		6-25-94	0	0				
		7-04-94	0	0				
		7-08-94			35.0	35.0		
		10-31-94					1769.4	1818.6
2. staple (pre)	2	6-17-94	2.5	7.5				
		6-25-94	0	0				
		7-04-94	0	0				
		7-08-94			33.0	35.0		
		10-31-94					1744.8	1548.2
3. staple (post)	1	6-17-94	0	7.5				
		6-25-94	0	0				
		7-04-94	0	0				
		7-08-94			33.5	38.0		
		10-31-94					1769.4	1867.7
4. staple (post)	2	6-17-94	5.0	10.0				
		6-25-94	0	0				
		7-04-94	0	0				
		7-08-94			40.0	36.5		
		10-31-94					1695.7	1548.2
5. m6316	.25	6-17-94	15.0	60.0				
		6-25-94	10.0	95.0				
		7-04-94	15.0	90.0				
		7-08-94			33.5	35.0		
		10-31-94					2039.7	196.6
6. untreated		6-17-94	2.5	0				
		6-25-94	0	0				
		7-04-94	0	0				
		7-08-94			34.0	32.5		
		10-31-94					1597.4	1769.4

THIS PERMIT No:94-090-08N
TEST No:FGA94113
INVESTIGATOR:SEAY
CITY:Greenville
COUNTY:Washington
STATE:Mississippi
PROJECT No:USA-94-344
COTTON: Transgenic line 19-51a (DP5690SU)

STAGE	DATE
preemergence	20MAY94
3-5 leaf stage	10JUN94
3-5 leaf stage	17JUN94
3-5 leaf stage	25JUN94
squaring	04JUL94
1st bloom	08JUL94
60% bloom	31OCT94

RESULTS WITH STAPLE, STAPLE +M6316

Treatment	Appl. Rate (oz ai/A)	Eval. Date	% Injury DP5690SU	% Injury Parent	Stand counts/10' of row DP5690SU	Stand counts/10' of row Parent	LB/AC Yield DP5415SU	LB/AC Yield Parent
1. staple (pre)	1	6-17-94	0	2.5				
		6-25-94	0	0				
		7-04-94	0	0				
		7-08-94			35.0	38.0		
		10-31-94					1916.9	1966.0
2. staple (pre)	2	6-17-94	0	0				
		6-25-94	0	0				
		7-04-94	0	0				
		7-08-94			30.0	27.0		
		10-31-94					1572.8	1695.7
3. staple (post)	1	6-17-94	0	7.5				
		6-25-94	0	0				
		7-04-94	0	0				
		7-08-94			32.5	35.5		
		10-31-94					1720.3	2088.9
4. staple (post)	2	6-17-94	5.0	10.0				
		6-25-94	0	0				
		7-04-94	0	0				
		7-08-94			38.5	33.0		
		10-31-94					1794.0	1449.9
5. m6316	.25	6-17-94	17.5	65.0				
		6-25-94	10.0	95.0				
		7-04-94	15.0	90.0				
		7-08-94			33.5	28.5		
		10-31-94					2187.2	196.6
6. untreated		6-17-94	0	0				
		6-25-94	0	0				
		7-04-94	0	0				
		7-08-94	0	0	36.0	33.5		
		10-31-94					1695.7	1523.7

COMMENTS: Three DP varieties, 51, 5415, and 5690 were planted on May 18, 1994. Each of these three varieties contained both transgenic and parent cotton. Two rows of transgenic and two rows of parent cotton from each of the three varieties were planted side by side. The plot length was 35 feet and the width was 6.3 feet per plot. Both rows of transgenic and parent cotton were treated. Pre- emergence applications were made 5-20-94 and the post-emergence applications were made 6-10-94. Crop tolerance evaluations were made 28, 35 and 45 days after pre-emergence application, and 7, 14, 24 days after post-emergence applications. No crop injury was noted 36-45 DAT after the pre-treatment or 15-24 DAT after the post-treatment of any rate of Staple to either the transgenic or parent material from any of the three varieties. M6316 indicated severe injury to the non-transgenic cotton in all varieties. The transgenic cotton averaged 15% injury from the m6316 treatment. No differences in injury could be noted between the varieties whether transgenic or non-transgenic. In general, the parent material yielded slightly higher than the transgenic material in the control plots. The only material that caused a significant yield reduction was m6316 at .25 ozai/A on the non-transgenic material. It should be noted that harvest was delayed approximately 21 days due to wet whether. No differences were noted at any time in insect or disease pressure between the three varieties- transgenic or non-transgenic.

APHIS PERMIT No:94-090-08N
TEST No:SWH94007
INVESTIGATOR:Edmund
CITY:England
COUNTY:Lonoke
STATE:Arkansas
PROJECT No:USA-94-341
COTTON: Transgenic line 19-51a (DP51SU)

STAGE	DATE
preemergence	
2nd true leaf	02JUN94
3-5 leaf stage	07JUN94
3-5 leaf stage	15JUN94
squaring	01JUL94

RESULTS WITH STAPLE, STAPLE +M6316

Treatment	Appl. Rate (oz ai/A)	Eval. Date	% Injury DP51SU	% Injury Parent
1. m6316	.063	6-07-94	8.3	48.3
		6-15-94	0	86.7
		7-01-94	0	81.7
2. m6316	.125	6-07-94	11.7	55.0
		6-15-94	8.3	90.0
		7-01-94	0	96.3
3. m6316	.25	6-07-94	21.7	58.3
		6-15-94	18.3	93.3
		7-01-94	6.7	99.0
4. staple	1	6-07-94	0	5.0
		6-15-94	0	0
		7-01-94	0	0
5.staple	2	6-07-94	0	11.7
		6-15-94	0	8.3
		7-01-94	0	6.7
6. untreated		6-07-94	0	0
		6-15-94	0	0
		7-01-94	0	0

COMMENTS: DP51 (transgenic and parent) cotton was planted in a split plot design on 5-21-94. Dual at .125 PT/AC was oversprayed for grass control. All treatments were postemergence and were sprayed on 6-2-94 with a CO2 plot sprayer in 17 GPA of water at 32 PSI using 11002 flat fan nozzles. At the time of application, the cotton was 2 leaf. Staple applications 1 and 2 ozai/A resulted in 0 and 7% crop injury at 29 DAT. Staple applications of 1 and 2 ozai/A resulted in 0% crop injury to the transgenic cotton. Transgenic vs parent growth comparison: due to variation in planting depth between rows and problems with the temik application, stand counts were not taken. Overall, no differences in growth, fruiting or susceptibility to disease or insects was observed between the transgenic and parent varieties.

APHIS PERMIT No:94-090-08N
TEST No:SWH94008
INVESTIGATOR:Edmund
CITY:England
COUNTY:Lonoke
STATE:Arkansas
PROJECT No:USA-94-342
COTTON: Transgenic line 19-51a (DP51SU)

STAGE	DATE
preemergence	12MAY94
cotyledon	26MAY94
2nd true leaf	02JUN94
3-5 leaf stage	07JUN94
3-5 leaf stage	15JUN94
squaring	01JUL94

RESULTS WITH STAPLE, STAPLE +M6316

Treatment	Appl. Rate (oz ai/A)	Eval. Date	% Injury DP51SU	% Injury Parent
1. staple (pre)	1	5-26-94	0	0
		6-07-94	0	5.0
		6-15-94	0	3.3
		7-01-94	0	0
2. staple (pre)	2	5-26-94	0	3.3
		6-07-94	0	10.0
		6-15-94	0	3.3
		7-01-94	0	0
3. staple (post)	1	6-07-94	0	15.0
		6-15-94	0	3.3
		7-01-94	0	0
4. staple (post)	2	6-07-94	0	16.7
		6-15-94	0	8.3
		7-01-94	0	6.7
5. untreated		5-26-94	0	0
		6-07-94	0	0
		6-15-94	0	0
		7-01-94	0	0

COMMENTS:At preemergence, Staple applications of 1 and 2 oz ai/A resulted in 0% crop injury. At postemergence, Staple applications of 1 and 2 ozai/A resulted in 0% crop injury. Transgenic VS parent growth comparison: due to variation in planting depth between rows and problems with the temik application, stand counts were not taken. Overall, no differences in vigor, growth, fruiting, or susceptibility to disease or insects was observed between the transgenic and parent varieties.

APHIS PERMIT No:94-090-08N
TEST No:SWG94108
INVESTIGATOR:Mitchell
CITY:Vicksburg
COUNTY:Warren
STATE:Mississippi
PROJECT No:USA-94-341
COTTON: Transgenic line 19-51a (DP51SU)

STAGE	DATE
preemergence	05JUL94
squaring	11JUL94
squaring	18JUL94
squaring	01AUG94

RESULTS WITH STAPLE, STAPLE +M6316

Treatment	Appl. Rate (oz ai/A)	Eval. Date	% Injury DP51SU	% Injury Parent
1. m6316	.063	7-11-94	11.7	36.7
		7-18-94	0	48.3
		8-01-94	0	66.7
2. m6316	.125	7-11-94	11.7	38.3
		7-18-94	0	45.0
		8-01-94	0	80.0
3. m6316	.25	7-11-94	15.0	40.0
		7-18-94	3.3	48.3
		8-01-94	0	60.0
4. staple	1	7-11-94	0	10.0
		7-18-94	1.7	1.7
		8-01-94	0	6.7
5. staple	2	7-11-94	3.3	18.3
		7-18-94	1.7	1.7
		8-01-94	0	6.7
6. untreated		7-11-94	0	0
		7-18-94	0	0
		8-01-94	0	0

COMMENTS: All plots were planted in a split plot design with TG cotton and normal cotton varieties side by side. Requirements by APHIS (notification buffer areas and crop destruction where applicable) should be followed. All plant vigor, germination and disease/insect characteristics were the same for the transgenic cotton and the parent. The transgenic cotton showed increased cotton safety to both m6316 and Staple when compared to the parent.

APHIS PERMIT No:94-090-08N, 94-109-02N
TEST No:SOG94006
INVESTIGATOR:Williams
CITY:Lexington
COUNTY:Madison
STATE:Tennessee
PROJECT No:USA-94-342
COTTON: Transgenic line 19-51a (DP51SU)

STAGE	DATE
preemergence	26MAY94
2nd true leaf	08JUN94

RESULTS WITH STAPLE, STAPLE +M6316

Treatment	Appl. Rate (oz ai/A)	Eval. Date	% Injury DP51SU	% Injury Parent
1. staple (pre)	1	7-08-94	0	0
		7-16-94	0	3.3
		7-28-94	0	0
2. staple (pre)	2	7-08-94	0	0
		7-16-94	6.7	13.3
		7-28-94	0	0
3. staple (post)	1	7-16-94	0	0
		7-28-94	0	0
4. staple (post)	2	7-16-94	0	0
		7-28-94	0	0
5. untreated		7-08-94	0	0
		7-16-94	0	0
		7-28-94	0	0

COMMENTS: Staple treatments resulted in little to no injury either on the transgenic cotton or its parent. No differences were seen in vigor, germination, or pest effects between transgenic cotton and its parent.

THIS PERMIT No:94-090-08N
TEST No:SWK94107
INVESTIGATOR:E. Castner
CITY:St. Joseph
COUNTY:Tensas
STATE:Louisiana
PROJECT No:USA-94-342
COTTON: Transgenic line 19-51a (DP5415SU)

STAGE	DATE
preemergence	20MAY94
3-5 leaf stage	13JUN94

RESULTS WITH STAPLE, STAPLE +M6316

Treatment	Appl. Rate (oz ai/A)	Eval. Date	% Injury DP5415SU	% Injury Parent	Stand counts/25' of row DP415SU	Stand counts/25' of row Parent
1. staple (pre)	1	6-15-94	0	0	99.0	86.3
		6-30-94	0	0		
		7-14-94	0	0		
		8-04-94	0	0		
2. staple (pre)	2	6-15-94	0	1.7	87.3	79.7
		6-30-94	0	1.7		
		7-14-94	0	0		
		8-04-94	0	0		
3. staple (post)	1	6-15-94			96.7	86.7
		6-30-94	0	0		
		7-14-94	0	0		
		8-04-94	0	1.7		
4.staple (post)	2	6-15-94			93.3	84.7
		6-30-94	0	0		
		7-14-94	0	0		
		8-04-94	0	1.7		
5. untreated		6-15-94	0	0	91.3	84.3
		6-30-94	0	0		
		7-14-94	0	0		
		8-04-94	0	0		

COMMENTS:Extremely heavy rainfall occurred approximately 1 1/2 hours after application. 4 row plots, 2 left rows-Dp5415 transgenic/2 right rows DP5415. 12 border rows on each side of test. 50 feet borders on front and back of tests. Stand counts were made on June 15. All four rows of each plot were counted. No differences were noted between transgenic cotton and the parent. Pre- and Post applications of Staple resulted in minimal injury (2%) to both transgenic and commercial DP5415 across all rates and rating dates. No difference was observed in plant development or fruiting (flowering).

PHIS PERMIT No:94-090-8N
TEST No:SWK94108
INVESTIGATOR:E. Castner
CITY:St. Joseph
COUNTY:Tensas
STATE:Louisiana
PROJECT No:USA-94-343
COTTON: Transgenic line 19-51a (DP5415SU)

STAGE	DATE
preemergence	
3-5 leaf stage	13JUN94

RESULTS WITH STAPLE, STAPLE +M6316

Treatment	Appl. Rate (oz ai/A)	Eval. Date	% Injury DP5415SU	% Injury Parent
1. staple	.25	6-30-94	0	0
		7-14-94	0	0
		8-04-94	0	0
2. staple	.5	6-30-94	0	0
		7-14-94	0	0
		8-04-94	0	5.0
3. staple	1	6-30-94	0	0
		7-14-94	0	0
		8-04-94	0	0
4. m6316+staple	.031+.25	6-30-94	0	33.3
		7-14-94	0	15.0
		8-04-94	0	16.7
5. m6316+staple	.063+.5	6-30-94	0	73.3
		7-14-94	0	53.3
		8-04-94	0	36.7
6. m6316+staple	.125+1	6-30-94	0	81.7
		7-14-94	0	71.7
		8-04-94	0	36.7
7. m6316+staple	.046+.25	6-30-94	0	66.7
		7-14-94	0	35.0
		8-04-94	0	20.0
8. m6316+staple	.093+.5	6-30-94	0	83.3
		7-14-94	0	68.3
		8-04-94	0	45.0
9. m6316+staple	.187+1	6-30-94	0	90.0
		7-14-94	0	83.3
		8-04-94	0	66.7
10. m6316+staple	.063+.25	6-30-94	0	78.3
		7-14-94	0	63.3
		8-04-94	0	43.3

Treatment	Appl. Rate (oz ai/A)	Eval. Date	% Injury DP5415SU	% Injury Parent
11. m6316+staple	.125+.5	6-30-94	0	86.7
		7-14-94	0	73.3
		8-04-94	0	53.3
12. m6316+staple	.25+1	6-30-94	0	90.0
		7-14-94	0	83.3
		8-04-94	0	53.3
13. m6316	.031	6-30-94	0	73.3
		7-14-94	0	46.7
		8-04-94	0	31.7
14. m6316	.063	6-30-94	0	85.0
		7-14-94	0	75.0
		8-04-94	0	60.0
15. m6316	.125	6-30-94	0	90.0
		7-14-94	0	81.7
		8-04-94	0	68.3
16. m6316	.25	6-30-94	0	90.0
		7-14-94	0	90.0
		8-04-94	0	85.0
17. untreated		6-30-94	0	0
		7-14-94	0	0
		8-04-94	0	3.3

COMMENTS: DP5415 and DP5451 transgenic cotton was planted on May 20, 1994 at the Northeast Research Station in St. Joseph, LA. Plots were 4 rows wide and 25 feet long. The left 2 rows of each plot were DP5415 transgenic and the right 2 rows were DP5415 commercial variety. Herbicide applications were made to the 2 center rows of each plot leaving an untreated check row of each variety in each plot. Postemergence applications were made on June 13, 1994. Crop injury ratings were made on June 30, July 14 and August 4 which corresponds to the 17, 31 and 52 days after treatment. All 4 rows of each plot were counted. Staple alone across all the rates and rating dates resulted in minimal injury (<5%).. No transgenic cotton injury was observed in any treatment No visual effect is noted on plant growth and development or fruiting when comparing the cotton varieties. No differences were seen in disease or insect effects between the transgenic cotton and the parental cotton.

APHIS PERMIT No:94-090-08N
TEST No:SWG94215
INVESTIGATOR:Snipes
CITY:Stoneville
COUNTY:Washington
STATE:Mississippi
PROJECT No:USA-94-343
COTTON: Transgenic line 19-51a (DP51SU)

STAGE	DATE
preemergence	
squaring	20JUN94
squaring	27JUN94

RESULTS WITH STAPLE, STAPLE +M6316

Treatment	Appl. Rate (oz ai/A)	Eval. Date	% Injury DP51SU	% Injury Parent
1. staple	.25	6-27-94	0	0
2. staple	.5	6-27-94	0	0
3. staple	1	6-27-94	0	3.0
4. m6316+staple	.031+.25	6-27-94	0	34.0
5. m6316+staple	.063+.5	6-27-94	0	41.0
6. m6316+staple	.125+1	6-27-94	0	34.0
7. m6316+staple	.046+.25	6-27-94	0	31.0
8. m6316+staple	.093+.5	6-27-94	0	44.0
9. m6316+staple	.187+1	6-27-94	0	46.0
10. m6316+staple	.063+.25	6-27-94	0	39.0
11. m6316+staple	.125+.5	6-27-94	0	38.0
12. m6316+staple	.25+1	6-27-94	0	39.0
13 m6316	.031	6-27-94	0	31.0
14. m6316	.063	6-27-94	0	29.0
15. m6316	.125	6-27-94	0	36.0
16. m6316	.25	6-27-94	0	39.0
17. untreated		6-27-94	0	0

COMMENTS: DP51SU showed no injury from any treatment evaluated. Neither the DP51SU or its parent, DP51, showed any differences in morphology, germination or in reaction to pest. DP51 was injured severely by any mixture with m6316 or m6316 alone.

APHIS PERMIT No:94-103-02N
TEST No:SOH94015
INVESTIGATOR:Hammes
CITY:Montezuma
COUNTY:
STATE:Georgia
PROJECT No:USA-94-342
COTTON: Transgenic line 19-51a (DP5415SU)

STAGE	DATE
preemergence	27MAY94
2nd true leaf	17JUN94
3-5 leaf stage	28JUN94

RESULTS WITH STAPLE, STAPLE +M6316

Treatment	Appl. Rate (oz ai/A)	Eval. Date	% Injury DP5415SU	% Injury Parent
1. staple (pre)	1	6-17-94	5.0	16.7
		6-28-94	0	6.7
2. staple (pre)	2	6-17-94	13.3	25.0
		6-28-94	0	16.7
3. staple (post)	1	6-28-94	0	0
4. staple (post)	2	6-28-94	0	11.7
5. untreated		6-17-94	10.0	10.0
		6-28-94	0	0

COMMENTS:

APHIS PERMIT No:94-021-09N
TEST No:RGV94002
INVESTIGATOR:Goldsberry
CITY:Donna
COUNTY:Hidalgo
STATE:Texas
PROJECT No:USA-94-342
COTTON: Transgenic line 19-51a (DP51SU)

STAGE	DATE
preemergence	02JUN94
cotyledon	09JUN94
2nd true leaf	16JUN94
3-5 leaf stage	21JUN94

RESULTS WITH STAPLE, STAPLE +M6316

Treatment	Appl. Rate (oz ai/A)	Eval. Date	% Injury DP51SU	% Injury Parent	Stand counts DP51SU	Stand counts Parent
1. staple	1	6-09-94			72.5	68.3
		6-16-94	0	0		
		6-27-94	0	0		
2. staple	2	6-09-94			67.5	66.5
		6-16-94	0	0		
		6-27-94	0	0		
3. staple	1	6-09-94			69.5	69.5
		6-16-94	0	0		
		6-27-94	0	0		
4. staple	2	6-09-94			71.5	66.8
		6-16-94	0	0		
		6-27-94	0	0		
5. staple	1				73.0	63.5
staple	1	6-09-94				
		6-16-94	0	0		
		6-27-94	0	0		
6. untreated		6-09-94			64.0	71.3
		6-16-94	0	0		
		6-27-94	0	0		

APHIS PERMIT No:94-021-09N
TEST No:RGV94002
INVESTIGATOR:Goldsberry
CITY:Donna
COUNTY:Hidalgo
STATE:Texas
PROJECT No:USA-94-342
COTTON: Transgenic line 19-51a (DP5690SU)

STAGE	DATE
preemergence	02JUN94
cotyledon	09JUN94
2nd true leaf	16JUN94
3-5 leaf stage	21JUN94

RESULTS WITH STAPLE, STAPLE +M6316

Treatment	Appl. Rate (oz ai/A)	Eval. Date	% Injury DP5690SU	% Injury Parent	Stand counts DP5690SU	Stand counts Parent
1. staple	1	6-09-94			93.0	88.0
		6-16-94	0	0		
		6-27-94	0	0		
2. staple	2	6-09-94			92.8	83.3
		6-16-94	0	0		
		6-27-94	0	0		
3. staple	1	6-09-94			83.8	89.3
		6-16-94	0	0		
		6-27-94	0	0		
4. staple	2	6-09-94			83.8	84.5
		6-16-94	0	0		
		6-27-94	0	0		
5. staple	1					
staple	1	6-09-94			92.8	88.3
		6-16-94	0	0		
		6-27-94	0	0		
6. untreated		6-09-94			83.0	89.0
		6-16-94	0	0		
		6-27-94	0	0		

APHIS PERMIT No:94-021-09N
TEST No:RGV94002
INVESTIGATOR:Goldsberry
CITY:Donna
COUNTY:Hidalgo
STATE:Texas
PROJECT No:USA-94-342
COTTON: Transgenic line 19-51a (DP5415SU)

STAGE	DATE
preemergence	02JUN94
cotyledon	09JUN94
2nd true leaf	16JUN94
3-5 leaf stage	21JUN94

RESULTS WITH STAPLE, STAPLE +M6316

Treatment	Appl. Rate (oz ai/A)	Eval. Date	% Injury DP5415SU	% Injury Parent	Stand counts DP5415SU	Stand counts Parent
1. staple	1	6-09-94			93.5	82.3
		6-16-94	0	0		
		6-27-94	0	0		
2. staple	2	6-09-94			88.8	97.0
		6-16-94	0	0		
		6-27-94	0	0		
3. staple	1	6-09-94			83.8	84.3
		6-16-94	0	0		
		6-27-94	0	0		
4. staple	2	6-09-94			92.0	88.8
		6-16-94	0	0		
		6-27-94	0	0		
5. staple	1					
staple	1	6-09-94			95.3	91.8
		6-16-94	0	0		
		6-27-94	0	0		
6. untreated		6-09-94			88.3	87.3
		6-16-94	0	0		
		6-27-94	0	0		

COMMENTS: No differences were seen in stands, susceptibility to insects or disease, and vigor among any varieties and their parental lines. Staple caused no apparent injury to any line of transgenic cotton or its parent.

DuPont Agricultural Products
Walker's Mill, Barley Mill Plaza
P. O. Box 80038
Wilmington, DE 19880-0038

DuPont Agricultural Products

APHIS Permit No. 93-053-01
Test No. SWH93212
Investigator: Frans
Affiliation: Univ. of AR
Cooperator: Cotton Exp. Sta.
Test Type: Crop Herbicide

City: Marianna
County: Lee
State: Arkansas
Project No: USA-93-320
Cotton: Transgenic Line 19-51a

Soil Texture: Silt Loam
Organic Matter: 1.1%
Soil PH: 6.5

Date	Stage
21 May 93	preemergence
09 Jun 93	2nd true leaf
16 Jun 93	3-5 leaf stage
25 Jun 93	squaring
07 Jul 93	fruiting

Evaluation Dates

A = 16 Jun 93
B = 25 Jun 93
C = 07 Jul 93

Results with "Staple" herbicide and DPX-M6316 herbicide treatments:

Appl. Date	Chemical	Appl. Rate	Active Ingredient	Appl. Method	Eval. Date	Days After First Appl.	% Injury	CM Height
21May93	Staple	2 OZAI/AC	80% SP	Preemerg	A	26	1.3	13.2
					B	35	.0	
					C	47	.0	
21May93	Staple	4 OZAI/AC	80% SP	Preemerg	A	26	7.5	12.8
					B	35	.0	
					C	47	2.5	
09Jun93	Staple	2 OZAI/AC	80% SP	Postembr	A	7	5.0	13.7
	Surfactant	.25 %V/V	1 lb/gal L		B	16	.0	
					C	28	1.3	
09Jun93	Staple	4 OZAI/AC	80% SP	Postembr	A	7	1.3	13.8
	Surfactant	.25 %V/V	1 lb/gal L		B	16	1.3	
					C	28	2.5	

Appl. Date	Chemical	Appl. Rate	Active Ingredient	Appl. Method	Eval. Date	Days After First Appl.	% Injury	CM Height
09Jun93	DPX-M6316	.5 OZAI/AC	25% DF	Postembr	A	7	6.3	13.4
	Surfactant	.25 %V/V	1 lb/gal L		B	16	15.0	
					C	28	7.5	
09Jun93	DPX-M6316	1 OZAI/AC	25% DF	Postembr	A	7	11.3	13.7
	Surfactant	.25 %V/V	1 lb/gal L		B	16	25.0	
					C	28	13.8	
	Untreated				A	7	3.8	13.4
					B	16	.0	
					C	28	.0	

COTTON - TRANSGENIC COTTON CROP PHYTO EVALUATION

Dr. Bob Frans, University of Arkansas
SWH-93-212, Project #: USA-93-320

- Raw data can be obtained from Dr. Bob Frans, Data File - SUTCMAR. (not in his annual report)
- Treflan at 0.75 LBAI/AC was applied over the entire test area.
- Plots were maintained weed free.
- The early post treatments were applied 6-9 to 1-2 leaf cotton.
- Plant mapping data (node development) is available if needed. Data showed no adverse effects on plant development via node counts from any of the treatments. This data was recorded up through the development of the 1st fruiting node.
- The test design was a RCB without use of split plot design. Therefore, the transgenic cotton was not directly compared to the parent material in this study. As a general observation, no difference in disease or insect pressure was observed when comparing to various non-transgenic varieties used in surrounding test plots in the same field.

DuPont Agricultural Products
Walker's Mill, Barley Mill Plaza
P. O. Box 80038
Wilmington, DE 19880-0038

DuPont Agricultural Products

APHIS Permit No. 93-053-01
Test No: SWG93210
Investigator: WHM
Affiliation: DuPont
Cooperator: Snipes
Test Type: Crop Herbicide

City: Stoneville
County: Washington
State: Mississippi
Project No: USA-93-320
Cotton: Transgenic Line 19-51a

Soil Texture: Silt Loam
Organic Matter: 1.0%
Soil PH: 6.2

Date	Stage	Evaluation Dates
04 Jun 93	preemergence	A = 06 Jul 93
28 Jun 93	3-5 leaf stage	B = 21 Jul 93

ults with "Staple" herbicide, DPX-M6316 herbicide and Cotoran/Zorial and herbicide treatments:

Appl. Date	Chemical	Appl. Rate	Active Ingredient	Appl. Method	Eval. Date	Days After First Appl.	% Injury
04Jun93	Staple	2 OZAI/AC	85% DF	Preembr	A	32	.0
					B	47	.0
04Jun93	Staple	4 OZAI/AC	85% DF	Preembr	A	32	.0
					B	47	.0
28Jun93	Staple	2 OZAI/AC	85% DF	Postembr	A	8	.0
	Surfactant	.25 %V/V	90% L		B	23	.0
28Jun93	Staple	4 OZAI/AC	85% DF	Postembr	A	8	.0
	Surfactant	.25 %V/V	90% L		B	23	.0
28Jun93	DPX-M6316	.5 OZAI/AC	25% DF	Postembr	A	8	18.0
	Surfactant	.25 %V/V	90% L		B	23	16.0
28Jun93	DPX-M6316	1 OZAI/AC	25% DF	Postembr	A	8	25.0
	Surfactant	.25 %V/V	90% L		B	23	43.0

Appl. Date	Chemical	Appl. Rate	Active Ingredient	Appl. Method	Eval. Date	Days After First Appl.	% Injury
04Jun93	Cotoran	24 OZAI/AC	4 lb/gal EC	Preembr	A	32	24.0
	Zorial	24 OZAI/AC	80% DF		B	47	3.0
	Untreated				A	32	.0
					B	47	.0

Under the conditions of this study there were no agronomic, pathological or entomological differences seen between transgenic or non-transgenic cotton. There were also no differences seen in stand count.

The only differences observable where those expressed as herbicide tolerance.

Walt

DuPont Agricultural Products
Walker's Mill, Barley Mill Plaza
P.O. Box 80038
Wilmington, DE 19880-0038

DuPont Agricultural Products

APHIS Permit No. 93-053-01
Test No: FGA93117
Investigator: R.E. Seay
Affiliation: DuPont
Station: Greenville
Test Type: Crop Herbicide

City: Greenville
County: Washington
State: Mississippi
Project No: USA-93-320
Cotton: Transgenic Line 19-51a

Soil Texture: Silt Loam
Organic Matter: .7%
Soil PH: 6.5

Date	Stage
24 May 93	preemergence
06 Jun 93	cotyledon
10 Jun 93	2nd true leaf
14 Jun 93	3-5 leaf stage
25 Jun 93	3-5 leaf stage
06 Oct 93	60% open bolls

Evaluation Dates

A = 06 Jun 93
B = 14 Jun 93
C = 25 Jun 93
D = 06 Oct 93

Results with "Staple" herbicide and DPX-M6316 herbicide treatments:

Appl. Date	Chemical	Appl. Rate	Active Ingredient	Appl. Method	Eval. Date	Days After First Appl.	% Injury	Lb/AC Yield
24May93	Staple	2 OZAI/AC	85% WP	Preembr	A	13	.0	
	WSF Solut.	.38 OZPR/AC	9.75% L		B	21	.0	
					C	32	2.5	
					D	135		1336.5
24May93	Staple	4 OZAI/AC	85% WP	Preembr	A	13	.0	
	WSF Solut.	.76 OZPR/AC	9.75 %L		B	21	2.5	
					C	32	5.0	
					D	135		1287.0
10Jun93	Staple	2 OZAI/AC	85% WP	Postembr	B	4	2.5	
	Surfactant	.25 %V/V	98% L		C	15	3.8	
	WSF Solut.	.38 OZPR/AC	9.75% L		D	118		1485.0
10Jun93	Staple	4 OZAI/AC	85% WP	Postembr	B	4	.0	
	Surfactant	.25 %V/V	98% L		C	15	7.5	
	WSF Solut.	.76 OZPR/AC	9.75% L		D	118		1336.6

Appl. Date	Chemical	Appl. Rate	Active Ingredient	Appl. Method	Eval. Date	Days After First Appl.	% Injury	Lb/AC Yield
10Jun93	DPX-M6316	.5 OZAI/AC	25% DF	Postembr	B	4	2.5	
	Surfactant	.25 %V/V	98% L		C	15	6.3	
					D	118		1311.8
10Jun93	DPX-M6316	1 OZAI/AC	25% DF	Postembr	B	4	6.3	
	Surfactant	.25 %V/V	98% L		C	15	11.3	
					D	118		1410.8
	Untreated				A	13	.0	
					B	21	.0	
					C	32	.0	
					D	135		1361.3

FIELD STATION PROJECT REPORT

OBJECTIVE:

The objectives of this study were to evaluate the crop response of various SU's when applied pre and post to transgenic cotton line 19-51A.

MATERIAL AND METHODS: (Staple and M6316 treatments):

Transgenic cotton line 19-51A was planted May 24, 1993. Plots were 2 row (6.3 ft.) x 35 ft. x 4 replicates. Treatments included Staple at 2 and 4 OZAI/AC pre and post: M6316 at 0.5 and 1 OZAI/AC pot.

Visual injury data was collected throughout this study and yield data was determined using a commercial cotton picker on October 6, 1993.

RESULTS: (Staple and M6316 treatments):

All treatments either yielded higher than the control or no more than 5% less than the control.

Plant mapping was done on this study, but no conclusions could be drawn from this mapping.

No observable differences were noted in insect pressure or incidence of disease between resistant plants or parental cotton plants. Disease and insect populations were at normal levels for commercial cotton.

DuPont Agricultural Products
Walker's Mill, Barley Mill Plaza
P O. Box 80038
Wilmington, DE 19880-0038

DuPont Agricultural Products

APHIS Permit No. 93-053-01
Test No: WEL93052
Investigator: Bierman
Affiliation: DuPont
Station: Rio Grande
Cooperator: RGVS
Test Type: Crop Herbicide

City: Donna
County: Hildago
State: Texas
Project: No: USA-93-320
Cotton: Transgenic Line 19-51a

Soil Texture: Sandy Clay Loam
Organic Matter: 1.0%
Soil PH: 7.8

Date	Stage
29 Mar 93	preemergence
04 May 93	3-5 leaf stage
07 May 93	3-5 leaf stage
11 May 93	squaring
18 May 93	squaring
24 May 93	squaring
01 Jun 93	1st bloom
09 Jul 93	fruiting
23 Aug 93	60% open bolls

Evaluation Dates

A = 07 May 93
B = 11 May 93
C = 18 May 93
D = 24 May 93
E = 01 Jun 93
F = 09 Jul 93
G = 23 Aug 93

Results with "Staple" herbicide and DPX-M6316 herbicide treatments:

Appl. Date	Chemical	Appl. Rate	Active Ingredient	Appl. Method	Eval. Date	Days After First Appl.	% Injury	Lb/AC Yield
29Mar93	Staple	2 OZAI/AC	80% WP	Preembr	A	39	.0	
	WSF Solut.	1.5 %V/V	9.75% WSG		B	43	.0	
					C	50	.0	
					D	56	.0	
					E	64	.0	
					F	102		
					G	147		1189.7
29Mar93	Staple	4 OZAI/AC	80% SP	Preembr	A	39	.0	
	WSF Solut.	1.5 %V/V	9.75% WSF		B	43	.0	
					C	50	.0	
					D	56	.0	
					E	64	.0	
					F	102	.0	
					G	147		1257.8

Appl. Date	Chemical	Appl. Rate	Active Ingredient	Appl. Method	Eval. Date	Days After First Appl.	% Injury	Lb/AC Yield
04May93	Ortho X-77	.25 %V/V	90% SL	Postembr	A	3	.0	
	Staple	2 OZAI/AC	80% WP		B	7	.0	
	WSF Solut.	1.5 %V/V	9.75% WSF		C	14	.0	
					D	20	1.3	
					E	28	.0	
					F	66	.0	
					G	111		1015.5
04May93	Ortho X-77	.25 %V/V	90% SL	Postembr	A	3	.0	
	Staple	4 OZAI/AC	80% WP		B	7	.0	
	WSF Solut.	1.5 %V/V	9.75% WSF		C	14	.0	
					D	20	2.5	
					E	28	.0	
					F	66	.0	
					G	111		999.2
29Mar93	Untreated				A	39	.0	
					B	43	.0	
					C	50	.0	
					D	56	.0	
					E	64	.0	
					F	102	.0	
					G	147		1045.4

TRANSGENIC COTTON TRIAL BY RGV STATION - DONNA, TX 1993

OBJECTIVES:

Evaluate several SU herbicides and some competitive herbicides for tolerance to transgenic cotton at the RGV Station, Donna, TX.

METHODS:

Pre and Mid post applications were made.

Weed control ratings were made using Coker 312 parent non-TG variety in an adjacent companion trial.

RESULTS: For Staple (PE-350) treatments:

There was no damage from PE-350 applied post-emergent. There was no damage early from any pre-em treatment throughout the trial period.

YIELDS:

Yield measurement was made by a two-row commercial picker which picked the entire two row plots which were 60 ft. long. The plots were separated by a single border row. Yield results directly reflected the apparent phyto from those POST treatments which showed damage in that the yields were less. The remaining treatments showed no significant injury or yield reduction.

The Coker 312 non-transgenic variety (test WEL-93-050) was planted adjacent to this test to evaluate weed control. There were no observable differences between the two varieties in response to insect damage nor were there any observable differences due to diseases. Cotton root rot is a common disease in this area and it was not seen here.

Generally, the two cotton varieties grew with no agronomic abnormalities noticed; that is the TG variety looked and grew the same as the non-TG variety.

Others involved in these trials were Dennis Goldsberry, Station Manager and Cliffton Brister also at the Station. TDA and USDA officials observed critical phases of the trial.

R. H. Bierman

DuPont Agricultural Products
Walker's Mill, Barley Mill Plaza
P. O. Box 80038
Wilmington, DE 19880-0038

DuPont Agricultural Products

APHIS Permit No. 93-053-01
Test No: CEZ93208
Investigator: Abernathy
Affiliation: TX AG Exp
Coooperator: Abernathy
Test Type: Crop Herbicide

City: Lubbock
County: Lubbock
State: Texas
Project No: USA-93-320
Cotton: Transgenic Line 19-51a

Soil Texture:	Loam
Organic Matter:	1.3%
Soil PH:	7.7

Date	Stage
19 Apr 93	presow/preplant
22 May 93	at planting
07 Jun 93	cotyledon
14 Jun 93	2nd true leaf
22 Jun 93	3-5 leaf stage
29 Jun 93	squaring
19 Jul 93	squaring
10 Aug 93	squaring

Evaluation Dates

A = 07 Jun 93
B = 22 Jun 93
C = 29 Jun 93
D = 19 Jul 93
E = 10 Aug 93

Results with "Staple" herbicide, DPX-M6316 ("Pinnacle") herbicide and Treflan herbicide treatments:

Appl. Date	Chemical	Appl. Rate	Active Ingredient	Appl. Method	Eval. Date	Days After First Appl.	% Injury
22May93	Staple	1 OZAI/AC	85% SP	Preemerg	A	16	.0
					B	31	.0
					C	38	.0
					D	58	.0
					E	80	.0
22May93	Staple	2 OZAI/AC	85% SP	Preemerg	A	16	.0
					B	31	.0
					C	38	.0
					D	58	.0
					E	80	.0

Appl. Date	Chemical	Appl. Rate	Active Ingredient	Appl. Method	Eval. Date	Days After First Appl.	% Injury
14Jun93	Staple	1 OZAI/AC	85% SP	Postembr	B	8	.0
	Surfactant	.25 %V/V	90% SC		C	15	3.0
					D	35	3.0
					E	57	.0
14Jun93	Staple	2 OZAI/AC	85% SP	Postembr	B	8	.0
	Surfactant	.25 %V/V	90% SC		C	15	5.0
					D	35	.0
					E	57	.0
14Jun93	Pinnacle (6316)	.5 OZAI/AC	25% DF	Postembr	B	8	.0
	Surfactant	.25 %V/V	90% SC		C	15	15.0
					D	35	.0
					E	57	.0
14Jun93	Pinnacle	1 OZAI/AC	25% DF	Postembr	B	8	3.0
	Surfactant	.25 %V/V	90% SC		C	15	27.0
					D	35	10.0
					E	57	.0
19Apr93	Treflan	12 OZAI/AC	4 lb/gal EC	PPI	A	49	.0
					B	64	.0
					C	71	.0
					D	91	.0
					E	113	.0
	Untreated				A	49	.0
					B	64	.0
					C	71	.0
					D	91	.0
					E	113	.0

OBJECTIVES: To evaluate the crop safety of pre and post applications of various SU herbicides to transgenic and conventional.

Results from Staple and DPX-M6316 (Pinnacle) treatments:

Test was conducted by Dr. Wayne Keeling, Texas A&M Experimental Station, Lubbock, TX. Test consisted of 3 reps. in RCB design. Data reported are treatment means.

Preemergence - no crop injury was seen in conventional or transgenic (TG) cotton to preemergence applications of Staple 1.0-2.0 OZAI/A.

Postemergence - Post applications of Staple at 1.0 and 2.0 OZAI/A caused 3%-20% injury to conventional cotton, and 3-5% injury to TG cotton. Both conventional and TG cotton quickly outgrew all visual injury symptoms. Post applications of pinnacle 0.5-1.0 OZAI caused 80-90% injury to conventional cotton. Pinnacle 0.5 and 1.0 OZAI/A caused 15-27% injury to TG cotton. However, 20 days later injury from pinnacle 0.5 to 1.0 had declined to 0% and 10%, respectively.

DISCUSSION: No crop injury was seen in TG cotton to pre-applications of Staple. Post applications of Staple 1.0-2.0 OZAI caused only 3-5% injury to TG cotton. Pinnacle at 0.5 to 1.0 OZAI caused 15-27% injury to TG cotton, but within 20 days had declined to 0% and 10%, respectively.

RECOMMENDATIONS: Continue to evaluate the crop safety of all ALS inhibitors to transgenic cotton.

Jerry Pitts

1993 SULFONYLUREA NURSERY

SITE LOCATION:

Delta and Pine Land Company Research Farm, Scott, MS in Field # 22.

NURSERY SIZE:

13 Acres

DATE PLANTED:

May 20, 1993

CULTURAL PRACTICES:

Normal cultural practices for nontransgenic cotton. The plot was not sprayed with sulfonylurea.

SEED SOURCE:

Plants containing sulfonylurea tolerance genes grown in Dominican Republic, Winter 1992-93.

NATURE OF MATERIAL:

Backcross material in following genotypes:

DP 5415	DP 5816	DPX 8516-6110
DP 5690	DELTAPINE 51	

DATE HARVESTED:

Material was harvested during October, 1993.

BORDERING OF NURSERY:

Nursery was bordered on each side with 24 border rows of nontransgenic cotton. The nursery was bordered on front on back with 80 feet of nontransgenic cotton.

DESTRUCTION OF MATERIAL:

All of nursery was harvested including border rows. Seed not saved for further research was returned to field and burned. Field was disked and plant material worked into the soil.

OBSERVATIONS ON TRANSGENIC MATERIAL:

1. No abnormal or weedy types were observed.

2. Transgenic lines were equal to the nontransgenic recurrent parents for yield, plant height, earliness, disease resistance, storm resistance and fiber characteristics.

SULFONYLUREA

PEDF2	PEDF3	PEDF4	PEDF5	FIBERCODE	ROW	TIER	PLOT #	SDWT	LINTWT	LINT%	LPDEV	MIC	MICDEV	LEN	LENDEV
DP 51, 5415, 5690				SU	STDS	n = 178		1.125	0.7014	3.469		0.3		0.036	
DP 51, 5415, 5690					STDS	n = 27		1.013	0.5253	1.339		0.3		0.02	
DP 51, 5415, 5690				SU	AVG	n = 178		4.131	2.3768	36.37		4.5		1.106	
DP 51, 5415, 5690					AVG	n = 27		4.052	2.3852	37.16		4.5		1.096	

: URDEV	T1	T1DEV	EL	: ELDEV
E-3				
.7		1.08		0.379
		1		0.347
.5		26.4		9.111
.4		25.3		9.189

DELTA AND PINE LAND COMPANY
SCOTT, MISSISSIPPI

1993 SULFONYLUREA NURSERY

Additional Information

The 1993 nursery was grown under APHIS permit number 93-053-01R of 91-358-01. USDA Field Contact person was Donald Smith (601/965-4304). Mr. Smith was contacted before planting and visited the nursery during the growing season. Contact was also made with the office of Jack Coley (601/325-3390) who was Mississippi State USDA Representative.

Sulfonylurea tolerant lines were compared with recurrent parent lines for seed yield, lint yield, lint percentage, micronaire, fiber length, fiber uniformity, fiber strength and fiber elongation:

Sulfonylurea Lines

Seed Yield	Lint Yield	Lint %	Micronaire	Length	Fiber Unif.	Fiber Strength	Fiber Elong.
4.1	2.4	36.4	4.5	1.10	83.5	26.4	9.1

Nontransgenic Checks

Seed Yield	Lint Yield	Lint %	Micronaire	Length	Fiber Unif.	Fiber Strength	Fiber Elong.
4.1	2.4	37.2	4.5	1.10	83.4	25.3	9.2

There were no statistical differences between the transgenic and nontransgenic lines.

The field was observed for volunteers after crop had been disked into soil. No volunteers were observed.

Keith R. Jones
Midsouth Cotton Breeder

DuPont Agricultural Products
Walker's Mill, Barley Mill Plaza
P. O. Box 80038
Wilmington, DE 19880-0038

DuPont Agricultural Products

APHIS Permit No. 93-053-01
Test No. SWH93212
Investigator: Frans
Affiliation: Univ. of AR
Cooperator: Cotton Exp. Sta.
Test Type: Crop Herbicide

City: Marianna
County: Lee
State: Arkansas
Project No: USA-93-320
Cotton: Transgenic Line 19-51a

Soil Texture: Silt Loam
Organic Matter: 1.1%
Soil PH: 6.5

Date	Stage
21 May 93	preemergence
09 Jun 93	2nd true leaf
16 Jun 93	3-5 leaf stage
25 Jun 93	squaring
07 Jul 93	fruiting

Evaluation Dates

A = 16 Jun 93
B = 25 Jun 93
C = 07 Jul 93

Results with "Staple" herbicide and DPX-M6316 herbicide treatments:

Appl. Date	Chemical	Appl. Rate	Active Ingredient	Appl. Method	Eval. Date	Days After First Appl.	% Injury	CM Height
21May93	Staple	2 OZAI/AC	80% SP	Preemerg	A	26	1.3	13.2
					B	35	.0	
					C	47	.0	
21May93	Staple	4 OZAI/AC	80% SP	Preemerg	A	26	7.5	12.8
					B	35	.0	
					C	47	2.5	
09Jun93	Staple	2 OZAI/AC	80% SP	Postembr	A	7	5.0	13.7
	Surfactant	.25 %V/V	1 lb/gal L		B	16	.0	
					C	28	1.3	
09Jun93	Staple	4 OZAI/AC	80% SP	Postembr	A	7	1.3	13.8
	Surfactant	.25 %V/V	1 lb/gal L		B	16	1.3	
					C	28	2.5	

1992 rDNA COTTON FIELD STUDIES
APHIS PERMIT NUMBER 91-358-01

Field studies with the third self-pollinated generation of rDNA cotton line #25-2 and the fourth self-pollinated generation of transformed line #19-51A were continued for the second year at the DuPont Field Research Station near Greenville, Mississippi, and were extended to five other locations, namely, Stoneville, Mississippi; Marianna, Arkansas; Lubbock, Texas; Donna, Texas and Scott, Mississippi. Tests at the latter location were carried out only with lines derived from #19-51A. In both of these lines, the only introduced gene is a chimeric ALS gene which encodes an acetolactate synthase enzyme which is tolerant to the sulfonylurea herbicides. This report will summarize tests carried out at all locations except Scott, MS.

At all five locations, tests were carried out to evaluate the levels of tolerance to sulfonylurea herbicides and to determine whether the introduced ALS genes have any impact on overall agronomic performance. Field studies at the DuPont Field Research Station in Greenville, Mississippi and at the DuPont Rio Grande Valley Field Station near Donna, Texas, involved flowering of the cotton plants and subsequent harvesting of cotton for experimental purposes and for additional greenhouse and field trials. In contrast, at Stoneville, Marianna, and Lubbock, the rDNA cotton plants were not allowed to flower, but were destroyed after tests had been carried out to evaluate tolerance to sulfonylurea herbicides.

The test results indicate that, apart from the tolerance to the sulfonylurea herbicides, the rDNA cotton plants were similar to the cotton plants from which they were derived, e.g., emergence and growth was similar to that of the non-rDNA Coker standard, and the plants produced normal fruiting structures. This is consistent with results from the 1991 field tests with the same cotton plants, carried out at the Greenville Field Research Station and at the DuPont Stine Research Farm near Newark, Delaware (Maryland side of farm). Field studies in 1993 will continue to evaluate not only the tolerance of the rDNA plants to sulfonylurea herbicides, but also the other characteristics of the plants, since in any breeding program aimed at the introduction of a new, desired trait, attention must also be directed towards assuring that favorable traits found in commercial lines of the plant are maintained.

AG-301 REV 10/91

DUPONT

AGRICULTURAL PRODUCTS
Walker's Mill, Barley Mill Plaza
Wilmington, Delaware 19880-0038

Transgenic Cotton Project

1992 Summary Report

This is the final report of the trangenic cotton study conducted at the Greenville Field Station during the Cropping season 1992. The following is a summary of key events.
Shipping permit 92-022-01M
Field trial permit 91-358-01

Two lines of transgenic cotton were evaluated during the 1992 season.

* 3/19 Received the 19-51A line seed from Wilm. approx. 130#.
* During April the cotton was transported to D&PL Co. in Scott, Ms (approx. 20 miles) for delinting at this facility. Jay Ellis witnessed the operation to insure no seed were lost during the delinting phase.
* 5/7 Planted the study with line 19-51A.
* 5/14 received the 25-2 line seed from Wilm. ..approx. 5#.
* 5/22 transported 25-2 seed to D&PL for delinting
* 5/27 Planted 25-2 line study
* Harvested to determine yield effects and collect subsamples for fiber quality analysis on Oct 7,1992
* Field site was destroyed via clipping stalks and discing on Oct 9 and Oct 10. All cotton not retained for fiber analysis was piled and burned at the test site.

Border rows (20-24 rows) were planted on all sides of the transgenic cotton. All border row cotton was destroyed at harvest as well.
The test area has been constantly monitored for any plants that emerge after termination of the study. No unusual plants were noted during the duration of the study. Transgenic plants grew normal and produced normal fruiting structures.

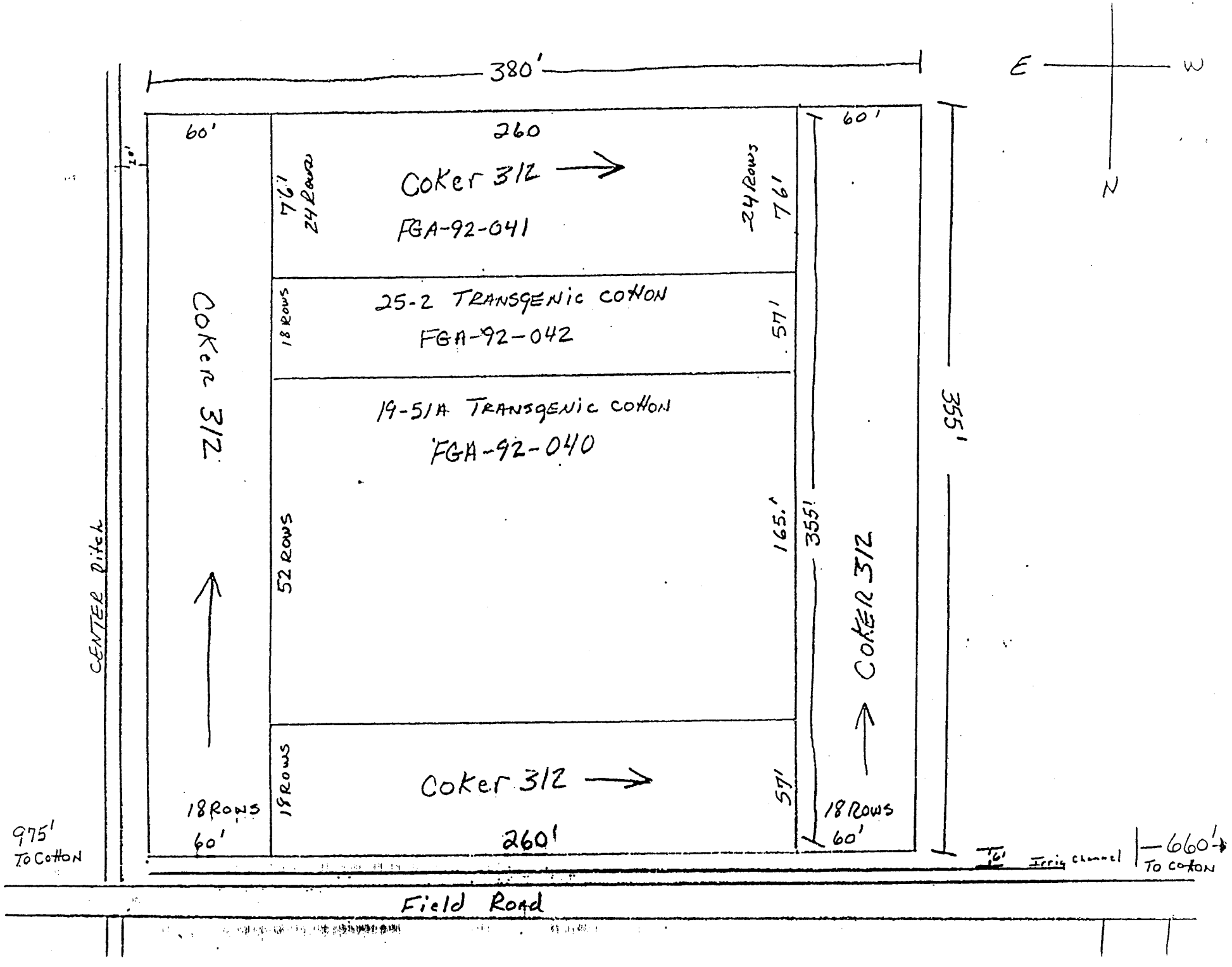
Key inspections and notifications

4/27Site inspection by Dan Fieselman
5/1 Notified Dan Fieselman of intentions to plant within the next few days
August visit to view the test site by Miriam Allred, USDA, Jackson, Ms
10/13 Site visit by Miriam Allred to verify destruction of the test site

Miriam Allred indicated acceptable plot destruction

These tests were very successful in determination of various SU tolerances and effects on crop yield.

Scotty Crowder
Field Station Manager
upont Agproducts
reenville, Ms


380'
E
W
N
60'
260
Coker 312
FGA-92-041
76'
24 Rows
24 Rows
76'
60'
25-2 TRANSGENIC COTTON
FGA-92-042
18 Rows
57'
19-51A TRANSGENIC COTTON
FGA-92-040
52 Rows
165'
355'
355'
Coker 312
Coker 312
18 Rows
57'
Coker 312
260'
18 Rows
60'
18 Rows
60'
CENTER Ditch
975'
To Cotton
660'
To Cotton
Irrig channel
Field Road

18327 Theiss Mail Rd.
Spring, TX 77379
September 17, 1992

To Whom it May Concern:

As a Manager of the DuPont Rio Grande Valley Field Station near Donna, Texas, I offer the status report of the Trans-genic cotton project completed there.

Important Dates:

Planted: April 16, 1992
Seed received about 1 week prior to this date.

Efficacy ratings:
May 19, 1992
June 24, 1992
July 16, 1992
August 19, 1992 (Yield harvest)

Observation Personnel at Planting 5/19/92:
Mr. Otis Mullins, USDA, Harlingen, Tx.
Mr. Carlos Rivas, Texas Department of Ag., Pharr, Tx.
Observation Personnel at yield harvest 8/19/92:
Same as above.
Observation Personnel at seedcotton burning 8/19/92:
Same as above.
Observatin Personnel at stalk destruction, land discing 8/19/92:
Same as above.
Other Observatins: Also, there were some casual observations throughout t growing season and to date by Mr. Rivas and Mr. Mullins.

Present status of plot area: The ground is completely turned under. Any sprouting seeds, should they occur, are soon cultivated/disced under or destroyed. The site is monitored by Dennis Goldsberry, the Station Site Manager and Cliffton Brister, Assistant Manager.
Stalk Destruction: The stalks were shredded immediately after picking and the ground was disced immediately after shredding the same day.
Implement Cleaning: All planting equipment and harvesting equipment was cleaned prior to and hand cleaned after use. It was inspected by myself, D. Goldsberry. Mr. Mullins, and Mr. Rivas.
Seed disposal: All seed produced was burned immediately after harvest. Then the area was disced under immediately for further destruction.
General Comments:

There were no abnormal or unexpectedly wierd looking cotton plants.

There were no strange herbicide responses; only the very high rate of Classic caused injury. Cotton growth was very much like the Coker 314 standard planted adjacent.

Future Activities Regarding Site: Inspections made approximately monthly for sprouting seeds by Station Personnel, R. H. Bierman and Dennis Goldsberry or Cliffton Brister, and TDA and USDA personnel at their discretion

Efficacy Report: This report has been forwarded to Ed Raleigh and Scott Crowder of DuPont.

# # #

1992 rDNA COTTON FIELD STUDIES
APHIS PERMIT NUMBER 91-358-01
LUBBOCK, TX; MARIANNA, AR; STONEVILLE, MS

Field studies at the three above locations were all terminated prior to flowering, after tests had been carried out to evaluate the tolerance of the rDNA cotton plants to sulfonylurea herbicides.

Texas Agricultural Experiment Station
Lubbock, Texas

The rDNA cotton plants were planted on June 16, 1992 and the planting was monitored by Mr. Cary Reynolds of USDA APHIS and Mr. Bill Rogers of the Texas Dept. of Agriculture. In addition both visited the test site on July 15. The cotton plants emerged and grew off normally with postemergence treatments applied on July 2. Four evaluations were made to determine effects on cotton stands, crop injury and weed control. The study was destroyed by shredding and discing on August 1, prior to cotton flowering.

Cotton Branch Experiment Station, University of Arkansas
Marianna, Arkansas

Planting of the rDNA cotton seeds was accomplished on May 19, 1992. Present for the planting was Mr. James Beville of USDA APHIS. No unusual plants or plant responses were noted. The study was terminated on July 24, 1992 by discing. Mr. Beville visited the site after the test was destroyed. No germination of additional cotton plants was noted; however, some re-growth from roots was noted, so the test area was re-disced on September 11. Subsequently the test area was monitored for re-growth.

Mississippi Agricultural and Forestry Experiment Station
Delta Branch Station
Stoneville, MS

The rDNA cotton seeds were planted on May 8, 1992 and herbicide treatments began on May 11. Dan Fieselman of USDA APHIS visited the test site on July 7 and Miriam Allred of USDA APHIS visited the test site on August 4. No unusual plant characteristics or responses were noticed. The study was destroyed on July 7, 1992, with no subsequent germination noted.

DELTA AND PINE LAND COMPANY
SCOTT, MISSISSIPPI

1992 SULFONYLUREA NURSERY

SITE LOCATION:

Delta and Pine Land Company Research Farm, Scott, MS in Field # 11.

NURSERY SIZE:

1 Acre

DATE PLANTED:

May 25, 1993

CULTURAL PRACTICES:

Normal cultural practices for nontransgenic cotton with the exception that the plot was sprayed with sulfonylurea.

SEED SOURCE:

F_2 and BC_1 populations from greenhouse at Scott.

NATURE OF MATERIAL:

Backcross material in following genotypes:

Pima S_6	DP 6166	DES 119	MD 51
Pima S_7	DP 20	DP 50	

DATE HARVESTED:

Material was not harvested.

BORDERING OF NURSERY:

Nursery was bordered on each side with 24 border rows of nontransgenic cotton. The nursery was bordered on front on back with 80 feet of nontransgenic cotton.

DESTRUCTION OF MATERIAL:

Unharvested field was cut with stalk cutter. Field was disked and plant material worked into the soil.

OBSERVATIONS ON TRANSGENIC MATERIAL:

No abnormal or weedy types were observed. Segregation for sulfonylurea tolerance was observed.

DELTA AND PINE LAND COMPANY
SCOTT, MISSISSIPPI

1992 SULFONYLUREA NURSERY

Additional Information

Bordering of nursery consumed most of the 1 acre test site. The actual acreage of transgenic cotton was less than 1/2 acre. Local PPQ Inspector contacted was Bobby Moore (601/846-7449). Contact was also made with USDA field man Donald Smith (601/965-4304).

No differences between plants containing resistant gene and nonresistant plants was observed for number of bolls, seed size and maturity.

After destruction of crop and disking of residue into the soil, no volunteer plants were observed. The 1992 nursery test site was fallowed in 1993 and no volunteers were observed during the 1993 crop year.

Keith R. Jones
Midsouth Cotton Breeder

AG-303 REV. 10/89

(-10U

DUPONT

AGRICULTURAL PRODUCTS
Walker's Mill, Barley Mill Plaza
Wilmington, Delaware 19880-0038

Transgenic Cotton Project
1991

This is a final report of the transgenic cotton trial conducted at the Greenville Field Research Station during the calendar year 1991.
Shipping permit # ..91-025-06
Field study permit#..91-025-02

* Cotton seed was received from Dupont Wilmington on 04/30/91.

* Field trial was planted on 5/15 and 5/16 1991.
* Remaining seed were shipped back to Dupont Labs for storage on 5/23
* Harvest date was 10/1/91.
* Field trial destroyed on 10/4(transgenic plants) and 10/10(non-transgenic plants). Plots were destroyed by using a clipper to mow the cotton stalks, followed by double discing.

No unusual plants were noted among any of the transgenic cotton plants that emerged in the study area. Twenty-four guard rows of non-transgenic cotton were planted on all sides of the transgenic cotton(Map attached). These border rows were so installed to assist in preventing movement of pollen to neighboring cotton. All border rows were also destroyed after the study was completed.

Any cotton yielded using the commercial cotton picker was piled and burned at the test site. Some representative samples were retained for fiber analysis in the lab.

Key inspections and notifications:

5/2 Conversation to Dan Fiselman and Ed Dyess on intentions to plant within one week.
5/13 Notification of intent to plant within days
5/16 Notification of planting
7/30 Ed Dyess(Ms Dept of Ag.) Visit to site
8/14 phone call from Dan to arrange a personal visit
9/13 Ed Dyess and Tom Harris visited Plot
10/10Miriam Allred visited plot to witness plot destruction

Miriam Allred indicated acceptable plot destruction

The test area was monitored several months to verify no plants emerged in the previously planted area.

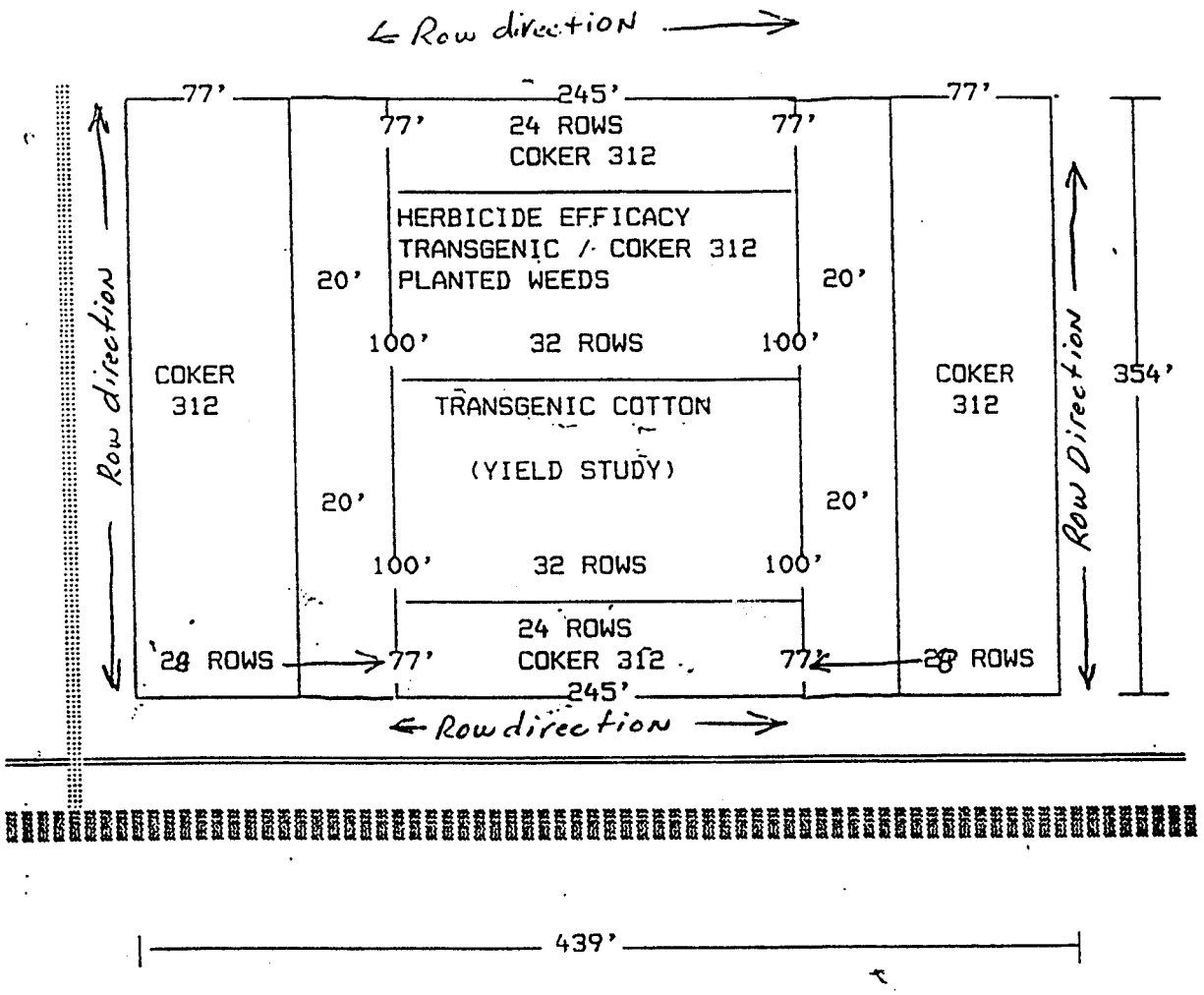
The test was very successful in determination of various SU tolerance.

Scotty Crowder
Field Station manager
Dupont Agproducts
Greenville, MS

To: Dan Fiscelman phone # 601-868-7172

From: Scotty Crowder — Dupont

Field Permit # 91-025-02



FIELD ROAD

DITCH

IRRIGATION CHANNEL

OUTLINE OF PLOTS & TESTS

THE ALLEY WAYS ARE 20 FEET BETWEEN TESTS.

THE DITCH IS 20 FEET FROM TESTS.

THERE IS 6 FEET BETWEEN IRRIGATION CHANNEL AND TESTS.

THE FIELD ROAD IS 20 FEET FROM IRRIGATION CHANNEL.

S
E W
N

Treatment	Appl. Rate (oz ai/A)	Eval. Date	% Injury DP5690SU	% Injury Parent
11. m6316+staple	.125+.5	7-13-94	51.7	98.0
		7-20-94	31.7	99.0
		8-03-94	0	99.7
12. m6316+staple	.25+1	7-13-94	46.7	74.7
		7-20-94	21.7	90.0
		8-03-94	0	90.0
13. m6316	.031	7-13-94	27.7	80.0
		7-20-94	10.0	93.7
		8-03-94	0	95.0
14. m6316	.063	7-13-94	41.7	75.0
		7-20-94	20.0	83.3
		8-03-94	0	78.3
15. m6316	.125	7-13-94	28.3	73.3
		7-20-94	8.3	86.7
		8-03-94	0	95.0
16. m6316	.25	7-13-94	46.7	96.0
		7-20-94	23.3	98.0
		8-03-94	0	100.0
17. untreated		7-13-94	33.3	33.3
		7-20-94	11.7	11.7
		8-03-94	0	0

COMMENTS: Delta Pineland 5690 varieties were used. 2 rows of each were planted per plot. At appropriate time to spray early post, the field received a lot of rain and a portion of it stood in water for a period of time. Thus treatments were put on late, cotton was 10 inches tall with 6-7 leaves in first 40 rows and 5 inches tall with 4-5 leaves in remainder of the plot. Nitrogen was applied to the test on July 11, 1994 and injury ratings on July 13 reflected the injury from the nitrogen application. Initial plant stand as seen in the check are essentially the same for both parent and TG cotton. Emergence date was June 13, 1994, 7 DAP. With TG cotton, the untreated plot showed 33% injury 1 WAT, 11.7 2 WAT again due mostly to water/nitrogen application. Only a few applications exceeded this amount of injury...5oz Staple by 12%, .25oz Staple +.063 M6316(by 30%),..5 Staple+.125 M6316 (20%), 10oz Staple+ .25oz M6316 (13%), .063M6316 (8%) and .25oz M6316 (12%). All injury was gone by 4 WAT. With the parent cotton, the untreated plot showed 33% injury 1 WAT, all treatments exceeded this amount of injury. Staple alone gave about 20% addition injury which mostly disappeared by 4 WAT. M6316 plots were highly injured (76% up by 4 WAT) with mush cotton killed. TG cotton appears relatively safe to M6316 applications (this test is hindered by excess water at normal application time thus injury picture and weed control ratings were probably affected; higher (injury) and lower control due to tall weeds.) No differences were seen as to diseases or insects at any rating dates between TG and parental cotton.

A.O.C.S. Official Method Ba 7-58
Revised 1960
Updated 1967
Reapproved 1969

Free Gossypol

Definition: The term free gossypol defines gossypol and gossypol derivatives in cottonseed products which are soluble in aqueous acetone under the conditions of the method.

Scope: Application to cottonseed, cottonseed meats, cottonseed slab and sized cake, and cottonseed meals of normal commercial production. Application to chemically treated meals should be verified before use (see Notes, 1).

Apparatus:

1. Mechanical shaker equipped to hold 250 mL Erlenmeyer flasks, and to provide vigorous agitation, Burrell "Wrist Action" shaker, or equivalent.
2. Spectrophotometer isolating a band at 40 nm and equipped with cells of 1 cm light path is preferred. Alternately a photoelectric colorimeter equipped with a filter having maximum transmittance between 440-460 nm may be used (see Notes, 2).
3. Grinding mill, Bauer Brothers, No. 148 laboratory mill with No. 6912 plate, 36,700 rpm.
4. Grinding mill, Wiley, with 1 mm screen.
5. Solid glass beads, about 6 mm in diameter.
6. Erlenmeyer flasks, 250 mL capacity, fitted with leak proof glass or polyethylene stoppers (Kimble No. 28160, size 27, or equivalent).
7. Pipets, volumetric, class A.
8. Filter paper, medium retention, 11 cm diameter circles (S & S No. 597, Whatman No. 2, or equivalent).
9. Volumetric flasks, 25, 200, and 250 mL, class A.
10. Water bath for operation at 100 C, equipped with clamps for supporting 25 mL volumetric flasks. Alternatively, rust proof metal washers slipped over the necks of the flasks may be used for stability. Operation of the bath in a well ventilated hood is recommended.

Reagents:

1. Solvents (see Notes, Caution) -

(a) Aqueous acetone, prepared by mixing 700 mL reagent grade acetone and 300 mL of distilled water.

(b) Aqueous isopropyl alcohol (2-propanol), prepared by mixing 800 mL isopropyl alcohol and 200 mL of distilled water.

(c) Aniline, prepared by distilling reagent grade aniline over a small amount of zinc dust, using an efficient water cooled condenser, and discarding the first and last 10 percent of the distillate. Store in a glass stoppered brown bottle in refrigerator. Redistill when the reagent blank (Procedure, paragraph 11) exceeds 0.022 absorbance (95% transmittance).

2. Thiourea solution, prepared by dissolve 10 g of reagent grade thiourea in distilled water and diluting to 100 mL.
3. Hydrochloric acid, 1.2 N, prepared by diluting 106 mL of concentrated hydrochloric acid (35-37% HCl) (see Notes, Caution) to 1 liter with distilled water.
4. Gossypol, primary standard quality gossypol, or gossypol acetic acid (89.61% gossypol by weight), should be used for calibration. Gossypol and gossypol acetic acid standards are available from: Atomergic Chemetals Corp., 100 Fairchild Ave., Plainview, NY 11802; Sigma Chemicals, P.O. Box 14508, St. Louis, MO 63178; Chemical Dynamics Corp., 3001 Hadley Rd., South Plainfield, NJ 07080; Aldrich Chemical Co., P.O. Box 355, Milwaukee, WI 53201. For determination of purity, accurately weigh 2 mg of gossypol or gossypol acetic acid, using a semi-micro or micro balance, into a 100 mL volumetric flask. Add about 40 mL of spectral grade cyclohexane, and warm on a steam bath, with swirling, to dissolve the compound. Cool to room temperature, and dilute to volume with cyclohexane. Using a calibrated spectrophotometer such as a Beckman Model DU, DK, DK 2-A, B; Cary Model 14 or 15; or equivalent, and matched 1.000 cm standard, or far ultraviolet, silica cells, determine the absorbance of the gossypol solution against the cyclohexane solvent at 358 nm. Calculate the absorptivity as follows

$$a = A/(c)(l)$$

Where —

a = absorptivity

A = Absorbance

c = concentration, in g/l

l = light path, 1.000 cm

The absorptivity of highest purity primary standard gossypol should be 39.9 ± 0.2, and that of highest purity primary standard gossypol acetic acid 35.8 ± 0.2. Absorptivity values in the range of 39.1-39.9 for gossypol, and 35.1-35.8 for gossypol acetic acid, (98-100% purity), denote primary standards satisfactory for calibration.

Free Gossypol

5. Standard gossypol solution, prepared by accurately weighing 25 mg of primary standard gossypol, or 27.9 mg of primary standard gossypol acetic acid, and transferring quantitatively to a 250 mL volumetric flask, using 100 mL of reagent grade acetone. Add 1.0 mL glacial acetic acid, 75 mL of distilled water, dilute to volume with acetone, and mix well. Pipet 50 mL of the above solution into a 200 mL volumetric flask, add 100 mL acetone, 45 mL distilled water and dilute to volume with acetone, and mix well. The latter standard gossypol solution contains 0.025 mg of gossypol per mL if exactly 25 mg of gossypol, or 27.9 mg of gossypol acetic acid were weighed. It is stable for 24 hrs when protected from the light.

Preparation of Sample:

1. Cottonseed —
   De-hull about 50 grams of sample, prepared as directed in A.O.C.S. Official Method Aa 2-38, using a Bauer mill with the plates separated so that the seed are just broken. Remove the meats from the hulls and lint by screening on a 4-6 mesh screen. Grind the meats in a Wiley mill to pass a 2 mm screen. Do not pre-heat cottonseed, and avoid heating during grinding.
2. Slab and sized cake and meals —
   Prepare sample as directed in A.O.C.S. Official Method Ba 1-38. Grind about 50 grams in a Wiley mill to pass a 1 mm screen.

Sample Size:

The sample weight and aliquot for analysis will depend on the free gossypol content of the cottonseed material. The Table 1 below is intended as a guide. Although most accurate values are obtained when the sample weight is 1 gram or less, it is necessary to increase the sample size for very low free gossypol meals.

Procedure:

1. Transfer the accurately weighed sample (See D) to a 250 mL. Erlenmeyer flask, and cover the bottom of the flask with glass beads.
2. Add 50 mL. of aqueous acetone (see Sample Size and Table 1) by pipet, stopper the flask with a leak proof glass or polyethylene stopper (Apparatus, 6), and shake vigorously on a mechanical shaker for 1 hour.
3. Filter through dry filter paper of medium retention (Apparatus, 8), discarding the first 5 mL of filtrate, and collecting filtrate in a small flask. Place a watch glass over the funnel to reduce evaporation during filtration.
4. Pipet appropriate duplicate aliquots of the filtrate (see Sample Size and Table 1) into 25 mL volumetric flasks.
5. To one sample aliquot, designated as solution "A", add 2 drops (0.10 mL) of 10% aqueous thiourea (Reagents, 2), 1 drop (0.05 mL) of 1.2 N HCl (Reagents, 3), and dilute to volume with aqueous isopropyl alcohol (Reagents, 1 (b)).
6. To the other sample aliquot, designated as solution "B", add 2 drops of 10% aqueous thiourea (Reagents, 2), 1 drop of 1.2 N HCl (Reagents, 3), and 2 mL of redistilled aniline (Reagents, 1, (c)). A rapid delivery pipet may be used for dispensing aniline.
7. Prepare a reagent blank containing a volume of aqueous acetone solution (Reagents, 1 (a)) equal to that of the sample aliquot, and add 2 drops of 10% aqueous thiourea (do not add any 1.2 N HCl), and 2 mL of aniline.
8. Heat the sample aliquot "B" (paragraph 6 above) and the reagent blank (paragraph 7 above) in a boiling water bath (100 C) for 30 minutes.
9. Remove the solutions from the bath, add about 10 mL of aqueous isopropyl alcohol to effect homogenous solution, and cool to room temperature in an appropriate water bath. Dilute to volume with aqueous isopropyl alcohol.

Table 1. Sample size for gossypol analysis.

Type of Sample	Expected free gossypol content	Sample weight	Aliquot size
	%	grams	ml
Meats	0.5-1.5	0.25	2
Meal	0.2-0.4	0.50	2
Meal	0.1-0.2	1.00	2
Meal	0.05-0.10	1.00	5
Meal	0.02-0.05	1.00	10
Meal	0.01-0.02	2.00	10
Meal	Below 0.01	5.00	10

10. Determine the absorbance of sample aliquot A (paragraph 5 above) at 440 nm, using aqueous isopropyl alcohol to set the instrument at 0 absorbance (100% transmittance).
11. With the instrument set at 0 absorbance (100% transmittance) with aqueous isopropyl alcohol, determine the absorbance of the reagent blank (paragraphs 7 and 8 above), taking care that a clean cuvette is used for the blank. If the reagent blank exceeds 0.022 absorbance units (below 95% transmittance), the analysis must be repeated using freshly distilled aniline.
12. Determine the absorbance of sample aliquot B (paragraphs 6 and 8 above) at 440 nm, using the reagent blank (paragraphs 7 and 8 above) to set the instrument at 0 absorbance (100% transmittance).
13. Calculate the corrected absorbance of the sample aliquot as follows —

    Corrected absorbance = (absorbance of B − absorbance of A)

Note — If the readings on solutions A and B above were taken in terms of transmittance, convert to absorbance, using the following equation —

Absorbance = (2 − logarithm transmittance)

14. From the corrected absorbance of the sample aliquot determined in paragraph 13 above, determine the mg of gossypol in the sample aliquot by reference to a calibration graph (see Calibration, paragraphs 8 and 9 below), or by use of the calibration factor (see Calibration, paragraphs 10 and 11 below).

Calibration:

1. Pipet duplicate 1, 2, 3, 4, 5, 7, 8, and 10 mL aliquots of the standard gossypol solution (0.025 mg/mL) (Reagents, 4) into 25 mL volumetric flasks.
2. To one set of aliquots, designated as "C", add 2 drops of 10% aqueous thiourea, 1 drop of 1.2 N HCl, and dilute to volume with aqueous isopropyl alcohol solution.
3. Determine the absorbance as outlined in (Procedure, paragraph 10).
4. To the other set of standard gossypol aliquots, designated as "D", add 2 drops of 10% aqueous thiourea, 2 drops of 1.2 N HCl, and 2 mL of redistilled aniline. Prepare a reagent blank containing 10 mL of aqueous acetone, 2 drops of 10% aqueous thiourea and 2 mL. of aniline (do not add any 1.2 N HCl to the reagent blank).
5. Heat the standards and the reagent blank in a boiling water bath (100 C) for 30 min, remove, cool to room temperature and dilute to volume with aqueous isopropyl alcohol solution.
6. Determine the absorbance as directed in Procedure, paragraphs 11 and 12.
7. Calculate the corrected absorbance for each standard gossypol aliquot as follows

   Corrected absorbance = (absorbance of C − absorbance of D)
8. Plot the corrected absorbance from paragraph 7 above for each gossypol standard against the corresponding milligrams of gossypol in the 25 mL volumes, on regular coordinate paper, to obtain the calibration graph.
9. If the calibration graph is non-linear (which may occur with some photoelectric colorimeters), it is necessary to refer to the calibration graph to determine the mg of gossypol in the sample aliquots in Procedure, paragraph 14.
10. If the calibration graph is linear (which should occur with most spectrophotometers), it is convenient to use a factor for calculating the mg of gossypol in sample aliquots in Procedure, paragraph 15. To obtain the factor, divide the mg of gossypol in each gossypol standard by the corresponding absorbance as follows —

$$\text{Factor} = \frac{\text{mg gossypol in 25 mL volume}}{\text{(Corrected absorbance*)}}$$

*As determined in Calibration, paragraph 7. Average the factors for all the gossypol standards.

11. The mg of gossypol in the sample aliquots (Procedure, paragraph 14) is then found by multiplying the corrected absorbance of the sample aliquot (Procedure, paragraph 13) by the calibration factor (Calibration, paragraph 10) —

    mg gossypol = corrected absorbance × factor

Calculations:

Calculate the free gossypol content as follows —

$$\text{Free gossypol, \%} = \frac{5(G)}{(W)(V)}$$

Where —

G = mg gossypol in the sample aliquot (Procedure, paragraph 14).

W = Sample weight, in grams.

V = Volume of sample aliquot used (Procedure, paragraph 4).

Notes:

Caution

Acetone, isopropyl alcohol and cyclohexane are flammable solvents. They should not be used near an open flame. The use of a properly operating fume hood is recommended when using these solvents.

Aniline is an allergin and is toxic if absorbed through the skin. The TLV in air is 2 ppm. Protective clothing and a properly operating fume hood should be used when using aniline.

Numbered Notes

1. This method may not be applicable to feeds containing whole, unprocessed cottonseed. Components in the feed interfere with this method and may give false positive results. At the time of the revision of this method, the A.O.C.S. Technical Committee had alternate methodology under review, but no satisfactory method had yet been found. For feed samples containing whole, untreated cottonseed, the analyst may want to try alternate published HPLC methods.
2. The absorption maxima of the gossypol-aniline reaction product should be at 440 nm. However, depending on the wavelength accuracy of the spectrophotometer and the band isolated, the maxima may be in the range of 440-450 nm. All absorbance measurements should be taken at the actual maxima for the spectrophotometer used.

APPENDIX 5

OILS AND FATS

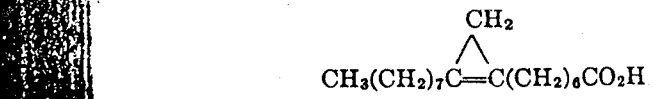
A Simplified Halphen Procedure for Cyclopropene Fatty Acids

By EUGENE C. COLEMAN[1] and DAVID FIRESTONE (Division of Chemistry and Physics, Food and Drug Administration, Washington, D.C. 20204)

A simplified Halphen procedure was developed for the quantitative determination of cyclopropene fatty acids in fats and oils. Butanol, dimethyl sulfoxide (DMSO), and mixtures of butanol and DMSO were used as reaction media in a closed system. Higher sample absorbances were produced in butanol than in mixtures of butanol and DMSO. Butanol was superior to the other solvent systems for both quantitative and qualitative analyses. The lower limit of sensitivity for the tube-butanol system for quantitative and qualitative analyses was 18 and 15 μg cyclopropene fatty acids/g oil, respectively.

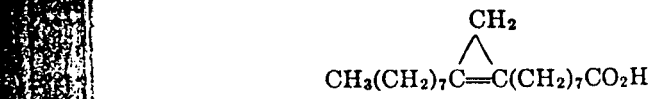
The Halphen test (1) has been used for a considerable period of time to detect the presence of cottonseed oil in other oils. Originally it was not known that the pink-to-red color produced in the Halphen test was due to the presence of 0.04–2% cyclopropenoid fatty acids (2). After it was noted that other seed oils of the order *Malvales* also produced a positive Halphen response, the suggestion was made that the cyclopropene ring was the reactive species and the test was then considered specific for that chemical moiety (3, 4).

The cyclopropenoid fatty acids (CPA) that have been identified in oils are malvalic and sterculic acids:



$$CH_3(CH_2)_7C{=}C(CH_2)_6CO_2H$$

malvalic acid



$$CH_3(CH_2)_7C{=}C(CH_2)_7CO_2H$$

sterculic acid

These fatty acids were shown to be associated with undesirable biological effects after ingestion by experimental animals (2, 5–9); some of the effects reported were growth inhibition, delay of sexual maturity, altered fatty acid distribution, fat accumulation, and cocarcinogenicity.

Although other methods have been developed for estimating the level of CPA in fats and oils, none possesses the apparent specificity or sensitivity of the Halphen test (10). The test is deficient, however, in that it is not always quantitative and reproducible. As a result, investigators have continued to search for means to improve the method and, in addition, to increase its sensitivity (11, 12).

A consideration of some of the factors that influence reactions (temperature, light, quality and quantity of reagents, and reaction medium) suggested that the reaction medium might be the cause of some of the problems associated with the test. Therefore, based on all reported reaction media (11–13), it was assumed that the product(s) formed by the interaction of CPA and sulfur-carbon disulfide did so primarily via an ionic pathway. Thus, a solvent more polar than butanol would be expected to enhance the reaction rate, thereby making the reaction go to completion or shifting its direction to favor one pathway or fewer pathways than would otherwise be favored. In this laboratory, dimethyl sulfoxide (DMSO) was investigated as a medium for the Halphen test. Reactions were carried out in closed tubes because, in the presence of DMSO, the reaction product(s) were foul-smelling and presumably toxic. The results of this investigation are reported here.

Experimental

Analytical samples were composed of cottonseed oil, cottonseed oil methyl esters, crude cottonseed oil methyl esters, or dilutions of these oils in 20% olive oil in peanut oil or in 20% olive oil methyl esters in peanut oil methyl esters. Methyl esters were prepared by the method of Brown (14). All undiluted analytical samples were analyzed for CPA by hydrobromic acid titration (15). CPA have been reported to interact with acetic acid during the hydrobromic acid titration (14, 16, 17). It was judged

[1] Present address: Office of the Assistant Director for Management, Bureau of Foods, Food and Drug Administration, Washington, D.C. 20204.

that 15–17% of the CPA interacts with acetic acid and therefore a correction factor could be used. However, because of the low CPA levels utilized in our studies, correction factors were not considered significant and were not used.

The closed system consisted of 20 × 150 mm screw-cap tubes (Pyrex, No. 9825, or equivalent) fitted with phenolic caps and Teflon liners. The size tube selected increased the cell path from 1 to 2 cm and the sample concentration used was twice that recommended in the Halphen test of Bailey *et al.* (12), giving an expected increase in sensitivity of 4.

Experiments were performed with a reaction volume of 25 ml at 110, 76, and 59°C for 2 to 24 hr. All reaction media contained 20% of 1% sulfur in carbon disulfide. In addition, the media consisted of 80% DMSO, 80% butanol, 40% DMSO-40% butanol, or 26% DMSO-54% butanol. For example, the 26% DMSO medium was prepared by mixing 5 ml 1% sulfur in carbon disulfide with 6.5 ml DMSO and 13.5 ml butanol. Triglycerides were not completely soluble in 80% DMSO and only methyl esters were analyzed at this level.

In the recommended procedure, about 200 mg oil was accurately weighed into a 5 ml volumetric flask and diluted to volume with butanol, DMSO (spectral grade, Fisher), or butanol-DMSO and transferred to the screw-cap tube. The volumetric flask was rinsed with 5 ml appropriate solvent and the rinse was added to the tube. An additional 10 ml solvent was added to the tube by pipet, followed by 5 ml 1% sulfur (precipitated, Fisher) in carbon disulfide (reagent grade). The tubes were tightly sealed with liners and caps to prevent solvent loss and were placed in a beaker containing propylene glycol. The propylene glycol was maintained at 110°C in an oil bath in the hood. The reaction was allowed to proceed 2.5 hr in the presence of light, after which time the tubes were removed and placed in a beaker containing tap water. The water was exchanged until the tubes reached room temperature; the tubes were then wiped dry and clean with a soft paper towel.

Absorbance measurements were made at the absorbance maximum of each solvent system against an oil blank. The amount of CPA was determined from a standard curve prepared from a hydrobromic acid standardized (15) reference oil (crude cottonseed oil or other CPA-containing oil) and various dilutions of the reference oil in CPA-free vegetable oil. If the reaction medium consisted of 40% or more DMSO and if the reaction was carried out in an open or closed system in the presence or absence of light, the maximum occurred at 510 nm. However, if the medium contained less than 40% DMSO and if the reaction was carried out in a closed system in the presence of light, the maximum occurred at 547 nm (Fig. 1). The shifts from 495 nm, the wavelength at which the maximum absorbance is ordinarily seen in the procedure of Bailey *et al.* (12), to 510 and 547 nm are probably associated with the variable formation of "Halphen" pigments. Bailey *et al.* separated orange and purple pigments absorbing at 490 and 520 nm, respectively. The pigment absorbing at 490 nm was further fractionated to yield compounds with molecular weights of 320, 520, and 780, and the absorbance of the pigment having a maximum at 520 nm was increased when the reaction was allowed to proceed in the presence of light (12). In our work we found that if the reaction tube containing butanol or less than 40% DMSO was covered with aluminum foil, the absorption maximum occurred at 495 nm, but this absorption was less than that obtained when the tube was exposed to light and the absorption was measured at the 547 nm maximum. The spectrophotometer used was a Bausch and Lomb Spectronic 20 (or equivalent) that was modified to hold a 20 mm tube; measurements were compared to those obtained with a Cary 14 spectrophotometer and were in close agreement. The 0.75″ tube adapter for the Spectronic 20 required a slight enlargement. Sandpaper wrapped around a conical figure was suitable for that purpose.

Results and Discussion

Samples that were treated for up to 6 hr at 76 and 59°C produced no significant color in any of the solvent systems, and the data are not shown. However, for a treatment of 2 hr at 110°C, more than a 2-fold increase in absorbance at 510 nm was observed with cottonseed oil methyl esters in 80% DMSO, compared to the absorbance at 547 nm of the same sample in 80% butanol (Table 1). In 26 and 40% DMSO, the absorbances of methyl esters and triglycerides were lower than the absorbances in 80% butanol. On the other hand, methyl esters and triglycerides in 40% DMSO produced absorbances that were nearly equal. In 26% DMSO, the absorbance of triglycerides was higher than that of methyl esters. This observation was thought to be related to a solvent effect because in 80% butanol and 26% DMSO the absorbances of triglycerides were higher than those of methyl esters. In 26 and 40% DMSO, net absorbances of methyl esters were slightly lower after 4 hr; the reason for the decrease was not clear.

Since peanut and olive oil methyl esters produced significant absorbances in 80% DMSO at 110°C (sometimes with a faint peach color), several additional ester samples were treated 2.5

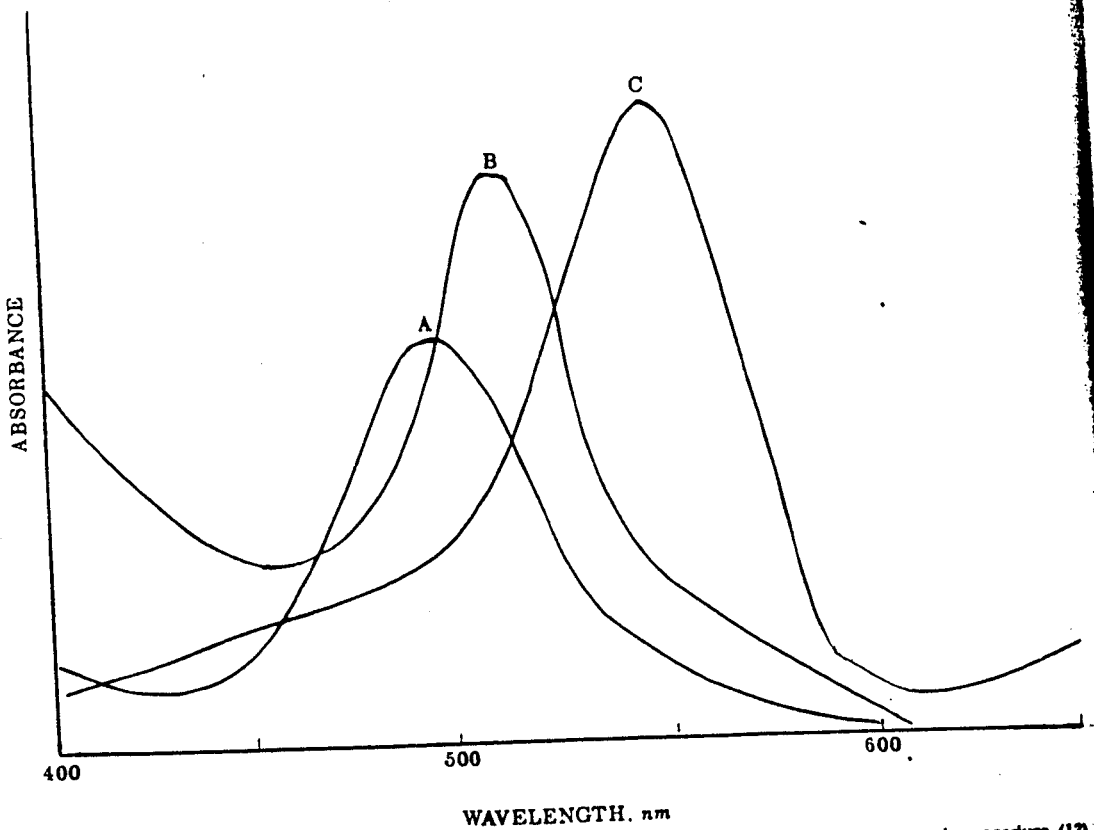
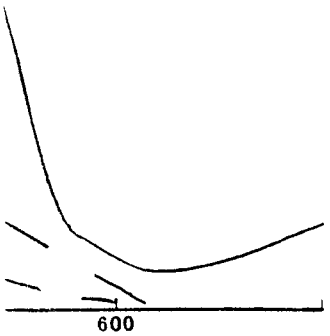


FIG. 1—Absorbance curves for Halphen reaction products: A, product obtained from Bailey *et al.* procedure (12) (reaction in butanol solvent or in 80% butanol in closed tube in absence of light); B, product obtained in 40% or more DMSO in butanol in open or closed tube in presence or absence of light; C, product obtained in 80% butanol in closed tube in presence of light.

hr at 110 and 96°C to determine if the absorbances could be reduced (Table 2). In addition, a comparison was made, in terms of absorbance, between the test performed exactly as described by Bailey *et al.* (12) and the tube system. The results indicated that the use of 80% DMSO, at 110°C, was prohibitive because of high blank absorbances. The blank oil absorbance in 80% DMSO at 96°C was acceptable, but occasionally some color was formed which destroyed the specificity of the test. Moreover, higher absorbances were obtained with the closed system regardless of the reaction medium. Absorbances obtained with the sytem of Bailey *et al.* were significantly lower (except for crude cottonseed oil methyl esters), even after multiplying by 4 to account for the shorter cell path and lower concentration. Because the actual absorbance with a prescribed method is the important factor and because the tube system at 110°C for 2.5 hr produced higher absorbances for the same quantity of material than the system of Bailey *et al.*, the tube system therefore seems to be the better procedure. Since the preparation of this report, a paper on the use of a closed system for the Halphen test has been published (18).

As mentioned earlier, no solubility problems were encountered with 40% DMSO, and triglycerides and methyl esters responded about equally in that medium; the response, however, was lower than that obtained in butanol. In order to determine (*a*) if the response of methyl esters and triglycerides in 40% DMSO was indeed equal and (*b*) if the response of triglycerides was consistently higher in 80% butanol, a series of experiments were performed with 40% DMSO and with 80% butanol at 110°C for 2.5 hr (Table 3). The absorptivities (absorbance/mg CPA) of methyl



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esters were higher than those of triglycerides in both solvent systems. On the other hand, absorptivities of both methyl esters and triglycerides in the butanol solvent were greater than those of the respective methyl esters and triglycerides in the 40% DMSO-butanol solvent. In general, the absorptivity of both methyl esters and triglycerides decreased as the concentration of CPA increased and the rate of decrease was greater in 40% DMSO-butanol than in 80% butanol.

Also of interest in this work was the question of sensitivity of the tube procedure and the 2 solvent systems for cyclopropenes. Table 4 lists cumulative data for both solvent systems. For quantitative purposes, it was desirable to work at an absorbance level that would not introduce more than a 10% relative error in the measurement. With an assumed absolute error in a transmittance measurement (ΔT) of ± 0.005, the relative error in a concentration measurement is about 10% at an absorbance of 0.022 (19). Therefore, sample sizes were chosen such that the CPA concentration would produce absorbances greater than 0.022. Above 1 g, the background of the blank oil became significant. On the other hand, for qualitative purposes, it seemed convenient to use the common criterion that a sample absorbance should be twice the background of a blank. Using these criteria, we estimated that the lower limit of sensitivity for quantitative analysis was 18 μg CPA/g oil and that, for qualitative analysis, 15 μg CPA/g oil could be reliably detected. (In a recent collaborative study of the method (20), samples were standardized by hydrobromic acid titration. The accuracy of the analyses was expressed in terms of recovery, which ranged from 79 to 111% for a CPA concentration range of 0.019 to 0.190%.) Blanks heated 2.5 hr in 40% DMSO at 110°C absorbed too strongly and could not be used for either quantitative or qualitative analysis at the sensitivity levels mentioned above; in addition, a faint peach color was occasionally formed in the oil blank and destroyed the specificity of the test.

Table 1. Halphen analysis of vegetable oils and their methyl esters in different solvent systems at 110°C

Sample[a]	Av. CPA, mg[b]	DMSO, %[c]	Av. Absorbance,[d] hr 2	4	6
			510 nm		
POE	0.00	80	0.090	0.126	0.156
CSOE	0.43	80	1.0	1.0	1.0
PO	0.00	40	0.047	0.063	0.064
POE	0.00	40	0.034	0.048	0.052
CSO	0.61	40	0.401	0.407	0.412
CSOE	0.45	40	0.354	0.366	0.368
			547 nm		
PO	0.00	26	0.015	0.022	0.025
POE	0.00	26	0.010	0.012	0.016
CSO	0.60	26	0.598	0.586	0.592
CSOE	0.46	26	0.422	0.406	0.411
PO	0.00	0	0.005	0.012	0.014
POE	0.00	0	0.000	0.006	0.008
CSO	0.58	0	0.700	0.785	0.790
CSOE	0.44	0	0.416	0.471	0.480

[a] Sample weights, approximately 200 mg. POE = peanut and olive oil methyl esters, CSOE = cottonseed oil methyl esters, PO = peanut and olive oils, CSO = cottonseed oil.
[b] CPA = cyclopropene fatty acids calculated from HBr analysis of 100% CSOE and CSO.
[c] 80% DMSO = 20 ml DMSO and 5 ml 1% S in CS_2; 40% DMSO = 10 ml DMSO, 10 ml butanol, and 5 ml 1% S in CS_2; 26% DMSO = 6.5 ml DMSO, 13.5 ml butanol, and 5 ml 1% S in CS_2; 0% DMSO = 20 ml butanol and 5 ml 1% S in CS_2.
[d] Average of 2 analyses.

Table 2. Halphen test of oils in 80% butanol and in 80% DMSO[a]

Sample[b]	Av. CPA, mg[c]	Av. Absorbance[d] Bailey *et al.* Procedure	Tubes (2.5 hr) 110°C Butanol	110°C DMSO	96°C Butanol	96°C DMSO
POE	0.00	0.000	0.008	0.090	0.004	0.010
CSOE (50%)	0.21	0.035	0.272	0.382	0.104	0.314
CSOE	0.42	0.077	0.445	0.713	0.256	0.548
CCSOE	0.60	0.205	0.693	1.3	0.401	1.2

[a] 80% Butanol = 20 ml butanol and 5 ml 1% S in CS_2; 80% DMSO = 20 ml DMSO and 5 ml 1% S in CS_2.
[b] POE = peanut and olive oil methyl esters, CSOE = cottonseed oil methyl esters, CCSOE = crude cottonseed oil methyl esters.
[c] CPA = cyclopropene fatty acids calculated from HBr analysis of 100% CSOE and CCSOE.
[d] Average analyses; wavelengths, nm: Bailey *et al.* (12) 495, butanol 547, and DMSO 510.

Table 3. Halphen analysis of vegetable oils, using 80% butanol or 40% DMSO as solvent (tubes, 2.5 hr at 110°C)[a]

Sample[b]	Butanol			DMSO-Butanol		
	Av. CPA, mg[c]	Absorbance, 547 nm[d]	Av. Dev.	Av. CPA, mg[c]	Absorbance, 510 nm[d]	Av. Dev.
POE	0.000	0.007	0.002	0.000	0.026	0.007
CCSOE (5%)	0.075	0.152 (2.03)	0.016	0.073	0.119 (1.63)	0.013
(25%)	0.353	0.542 (1.54)	0.046	0.342	0.447 (1.31)	0.014
CSOE (5%)	0.026	0.040 (1.54)	0.001	0.025	0.045 (1.80)	0.006
(10%)	0.043	0.055 (1.28)	0.007	0.043	0.067 (1.56)	0.005
(20%)	0.093	0.119 (1.28)	0.010	0.090	0.095 (1.06)	0.013
(50%)	0.228	0.264 (1.16)	0.005	0.219	0.197 (0.90)	0.004
(100%)	0.428	0.464 (1.08)	0.010	0.414	0.347 (0.88)	0.016
PO	0.000	0.007	0.003	0.000	0.032	0.004
CSO (50%)	0.245	0.250 (1.02)	0.020	0.230	0.163 (0.71)	0.014
(100%)	0.527	0.522 (0.99)	0.033	0.492	0.283 (0.58)	0.062

[a] Values are averages of 3 analyses. 80% Butanol = 20 ml butanol and 5 ml 1% S in CS_2; 40% DMSO = 10 ml DMSO, 10 ml butanol, and 5 ml 1% S in CS_2.
[b] POE = peanut and olive oil methyl esters, CCSOE = crude cottonseed oil methyl esters, CSOE = cottonseed oil methyl esters, PO = peanut and olive oil, CSO = cottonseed oil. Per cent sample in diluent oil given in parentheses.
[c] CPA = cyclopropene fatty acids calculated from HBr analysis of 100% CCSOE, CSOE, and CSO.
[d] Numbers in parentheses are normalized values, expressed as absorbance/mg CPA.

Table 4. Sensitivity of Halphen test, using 80% butanol or 40% DMSO as solvent (tubes, 2.5 hr at 110°C)[a]

Sample[b]	Butanol			DMSO-Butanol		
	Av. Sample Wt, g	Av. CPA, mg[c]	Av. Absorbance, 547 nm	Av. Sample Wt, g	Av. CPA, mg[c]	Av. Absorbance, 510 nm
POE	0.2058	0.000	0.007	0.2055	0.000	0.026
CSOE	0.2039	0.428	0.464	0.1972	0.414	0.349
CSOE (1:1)	0.2159	0.228	0.264	0.2076	0.219	0.197
(1:10)	0.2001	0.043	0.055	0.2039	0.043	0.067
(1:20)	0.4170	0.045	0.081	0.3964	0.041	0.070
POE	0.5312	0.000	0.009	0.5295	0.000	0.058
CSOE (1:30)	0.6359	0.045	0.078	0.6156	0.044	0.093
POE	1.0459	0.000	0.016	1.0233	0.000	0.126
CSOE (1:42)	0.8027	0.041	0.087	0.8287	0.040	0.125

[a] See Table 3, footnote a.
[b] See Table 3, footnote b, but values in parentheses refer to dilution ratios.
[c] CPA = cyclopropene fatty acids calculated on basis of HBr titration of 100% CSOE.

The behavior of other oils and methyl esters in this procedure is not precisely known. The oils and mixtures used in this work were typical vegetable oils, and other vegetable oils would be expected to behave similarly. Animal fat has not been analyzed by this procedure, but there are no known reasons that would suggest behavior different from that ordinarily seen (12).

Although minor components may exert some influence on the formation of the Halphen color, no specific attempts were made to ascertain the extent of their influence by the deliberate addition of known substances. In our work, we have not observed changes in color formation that could be attributed to the inadvertent presence of such components. Moreover, the presence of natural pigments in refined vegetable oils has not influenced the formation of Halphen color bodies.

Received June 20, 1972.

as evidenced by our experience in the analysis of alumina-treated and non-treated oils. In previous work with highly colored fats such as liver lipids, using the procedure of Bailey *et al.* (12), the background was considerably higher, but the use of a comparable blank permitted absorbance measurements without difficulty. In addition, later experiments showed that the amount of sample used in the recommended method can be varied up to 1 g without significantly affecting the absorbance.

Finally, results of analyses of samples containing CPA showed that 80% butanol was the preferred solvent system. Because of the increased concentration and longer cell path, actual absorbances were higher in the closed system than in the apparatus described by Bailey *et al.* (12) and this resulted in increased sensitivity. Other advantages of the closed system are the elimination of the need for special glassware and the transfer of solutions for absorbance measurements, and the stability of the color formed. The color formed was stable up to 4 hr after the reaction period; after this time a slight enhancement or a diminution of color was observed, each without any noticeable directional consistency. Furthermore, the presence or absence of air did not appear to affect the formation of color bodies as evidenced by identical spectra.

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93-05-01

APPENDIX 6

TRANSGENIC COTTON OVERWINTERING STUDY
FGA-93-130 - GREENVILLE, MS
INVESTIGATOR: ERICK SEAY

Objective:

The objective of this study is to determine if a seed source of transgenic cotton (1951-A):

- Can overwinter and germinate the following spring.
- Germinate during the cooler/colder winter months.
- Differs in any way from the parental cotton.

Materials and Methods:

Transgenic cotton seed (DP5690 SU) and a back-cross parent were planted on 10-27-93 at the DuPont Field Station in Greenville, MS. This is typical timing for the scattering of any possible seed from harvest of cotton. Fields were maintained in a manner typical for soil during winter months.

Plots of each variety were replicated 4 times, 31.5 sq. ft. in size, and contained 30 seeds each. The seed were acid de-linted and treated identically.

Evaluations:

Evaluation dates were:

11-14-93
12-15-93
2-10-94
4-13-94
6-3-94

The test was terminated on 6-3-94. The area was disked and any vegetation (weeds) destroyed.

Conclusions:

- No cotton seed germination was observed by either variety at any date.
- On 12-15-93, cotton seed of both varieties examined and found to appear in a rotted condition from the typical cool, damp conditions of the fall.

It appears that the transgenic cotton does not differ from the parent in regard to overwintering. Neither appear to have the potential to survive the fall or winter months.

93-05-01

TRANSGENIC COTTON OVERWINTERING STUDY
RHB-93-001 - DONNA, TX
INVESTIGATOR: R. H. BIERMAN

Objective:

To observe the germination and freeze responses of fall planted cotton comparing transgenic (1951-A) to its parental line.

Material and Methods:

On 10-25-93, two side by side 20 x 40' blocks of cotton were established. One block contained transgenic cotton (1951-A); the other Coker 312 (the parent).

The two blocks were marked into 4 replications in each block. No other treatments were made and cotton was left to grow or not, as is typical for harvested fields at this time of year.

At planting, temperatures of soil were warm, but, as is normal, cooler temperatures arrived in November and December.

Observations:

Observations were made on the following dates:

11-10-93	11-17-93	11-28-93
12-10-93	3-15-94	

Results and Conclusions:

On 11-10-93, stands counts were made on the germinating cotton seed by counting the number of plants per sq. ft. in the four replications. Cotton was in the cotyledon to very early first leaf stage. There were no differences in the transgenic and parent cotton (see below).

PLANTS/SQUARE FOOT

	Normal Cotton				TG Cotton			
Rep	I	II	III	IV	I	II	III	IV
	58	26	16	26	19	28	33	45
Avg.	31.50				31.25			

By 11-17-93, both cotton varieties were between the first and second leaf stage of cotton. Only normal variation was seen between the transgenic and non-transgenic cotton in terms of germination.

LEAF GROWTH STAGE

	Normal Cotton				TG Cotton			
Rep	I	II	III	IV	I	II	III	IV
	1.0	1.0	1.5	1.5	1.5	1.5	1.5	2.0
Avg.	1.25				1.65			

On 11-28-93, a freezing temperature of 30.9° F was recorded and all cotton that had emerged was killed. This is typical for a frost to occur at this time of year.

On 12-10-93, no regrowth of cotton had occurred or further germination. No differences between transgenic and the parent was observed.

On 3-15-94, plots were again observed for any further germination. None had occurred for either variety. At this time, the test was terminated since this represents atypical planting date for cotton in this area.

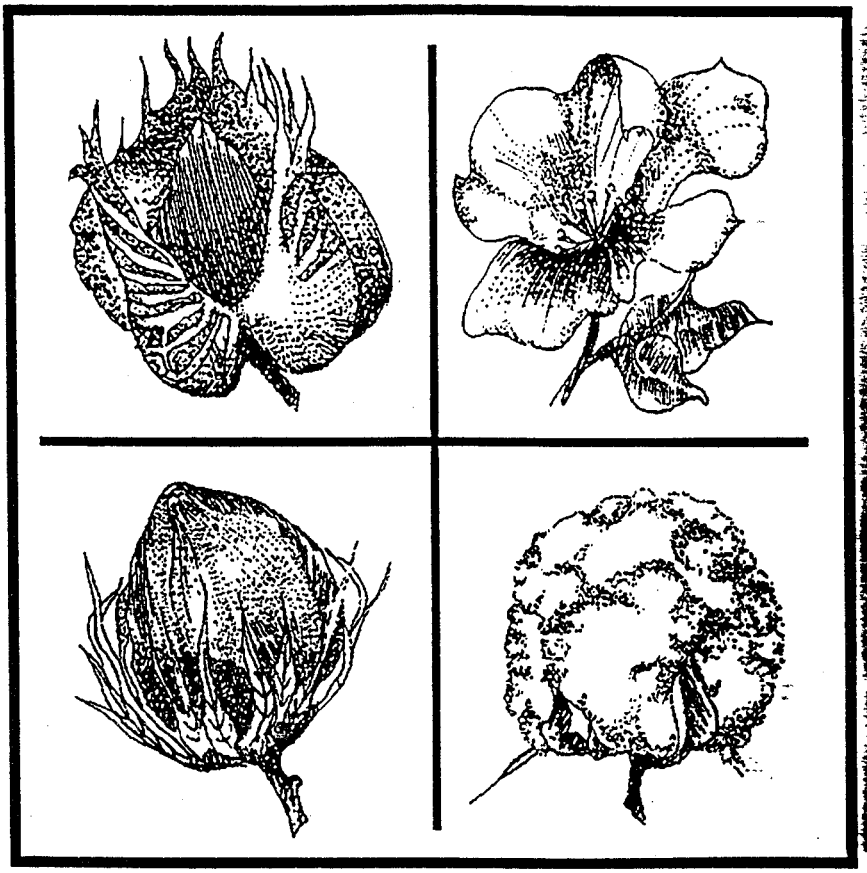
Based on these conditions and results of this trial, there was no difference in germination or cold hardiness between varieties.

In addition, Donna, TX in the Rio Grande Valley represents the Southern-most area of commercial cotton production in the U.S. Weather conditions in this trial were typical for those found year to year. Consequently, it is highly unlikely that any cotton, including 1951-A, has the potential to overwinter.

DUPONT®

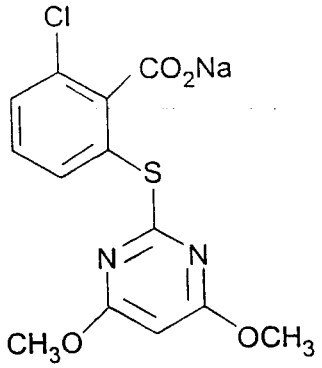
Staple®

experimental herbicide for cotton



Staple is a highly active, low-use-rate herbicide with postemergence activity for use in cotton weed control programs. The United States Environmental Protection Agency has granted DuPont an Experimental Use Permit for limited use of Staple. Staple is not registered for commercial sales. Staple applied over the top of cotton has exhibited excellent safety to cotton in university and DuPont field studies. Plant mapping and yield trials indicate no yield reductions or maturity delays from applications of Staple to cotton.

Structure of Active Ingredient DPX-PE350



CAS NO. 123343-16-8
Sodium 2-chloro-6-(4,6-dimethoxypyrimidin-2-ylthio) benzoate

The patent on pyrithiobac sodium is held by Kumiai Chemical Industry Co., Ltd.

Chemical and Physical Properties

Physical State	solid
Melting Point (decomp)	233.8–234.2°C
Water Solubility	728 g/L (20°C)
Hydrolysis	stable at pH 5, pH 7 and pH 9 at 25°C for 30 days
pH (1% wt/wt in water)	4.9
Density	1.6 g/cc

Formulation

Staple is formulated as an 85 percent water-soluble powder contained in a water-soluble pouch.

Mode of Action

Staple herbicide inhibits cell division and growth by inhibiting the plant enzyme acetolactate synthase (ALS). Cessation of growth is first observed in meristematic tissue of sensitive plants. Chlorosis, necrosis, and death of sensitive plants follow initial interruption of plant growth.

Fate in Soil

Staple degrades relatively slowly in soil, primarily by microbially mediated degradation, with an estimated half-life of approximately 60 days in laboratory studies.

Crop Rotation

Field studies to date indicate that the following crops may be planted following the use of Staple:

Crop	Interval (months)
Cotton	anytime
Spring/Winter wheat	4
Soybeans	10
Peanuts	10
Rice	10
Sorghum, grain	†
Corn, field	†

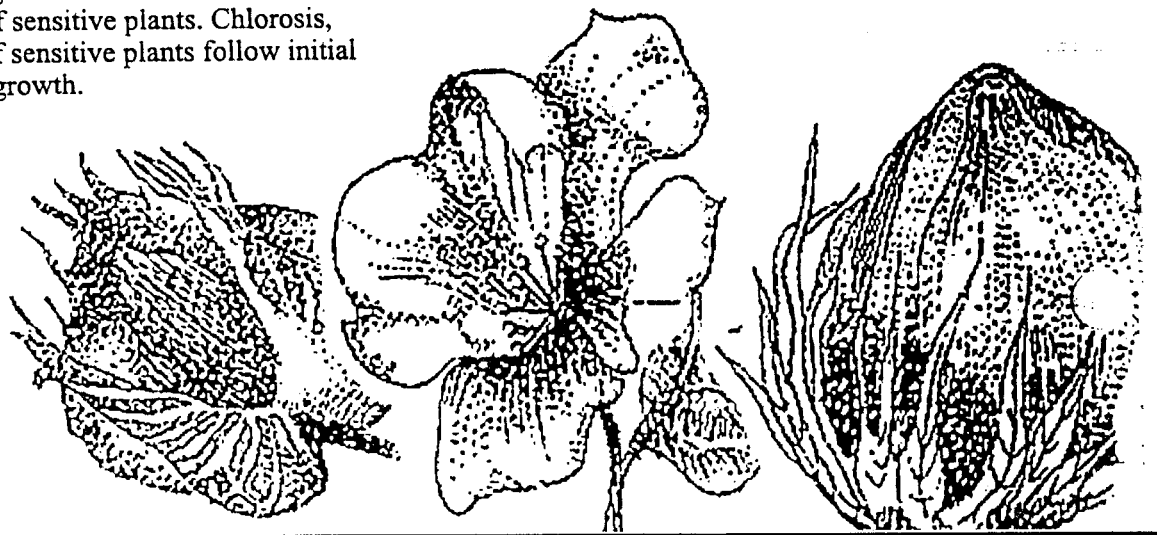
†Do not rotate to corn or grain sorghum in the season following a Staple application.

Selectivity

Selectivity is based upon the differential rate of metabolism of the active ingredients to inactive metabolites in cotton as opposed to sensitive species. Cotton metabolizes Staple quickly; susceptible weeds do not. Under certain conditions a slight temporary yellowing or chlorosis of the cotton foliage has been observed.

Adjuvants

When applying postemergence to weeds, always add a nonionic surfactant or crop oil concentrate to the spray mixture. For additional information on adjuvants, refer to the DuPont Agricultural bulletin, "Approved Adjuvants for Use with DuPont Row Crop and Cereal Herbicides."



Application Timing

Staple herbicide is most effective when applied postemergence to young, actively growing weeds. In general, weeds should be less than 4 inches (10.16 cm) in height or diameter at treatment.

Spectrum/Efficacy

Postemergence applications of Staple herbicide have provided effective control of the following weeds:

Amaranth, Palmer	*Amaranthus palmeri*
Anoda, Spurred	*Anoda cristata*
Cocklebur, Common	*Xanthium strumarium*
Coffee senna	*Cassia occidentalis*
Devils claw	*Proboscidea louisianica*
Groundcherry, Wright	*Physalis wrightii*
Jimsonweed	*Datura stramonium*
Morningglory, Entireleaf	*Ipomoea hederacea var. integriuscula*
Morningglory, Ivyleaf	*Ipomoea hederacea*
Morningglory, Pitted	*Ipomoea iacunosa*
Morningglory, Scarlet	*Ipomoea coccinea*
Morningglory, Sharppod	*Ipomoea trichocarpa*
Morningglory, Threelobe	*Ipomoea triloba*
Morningglory, Woolly	*Ipomoea hirsutula*
Nightshade, Black	*Solanum nigrum*
Nightshade, Hairy	*Solanum sarrachoides*
Pigweed, Redroot	*Amaranthus retroflexus*
Pigweed, Smooth	*Amaranthus hybridus*
Pigweed, Spiny	*Amaranthus spinosus*
Pigweed, Tumble	*Amaranthus albus*
Poinsettia, Wild	*Euphorbia heterophylla*
Sage, Lanceleaf	*Salvia reflexa*
Sesbania, Hemp	*Sesbania exaltata*
Sida, Prickly	*Sida spinosa*
Smartweed, Pennsylvania	*Polygonum pensylvanicum*
Velvetleaf	*Abutilon theophrasti*

Toxicology Data

Testing	Studies: PE350 Technical	Studies: PE350 Formulation 85 SP
Acute Oral		
LD50 Rat	3200 mg/kg	4000 mg/kg
Acute Dermal		
LD50 Rabbit	>2000 mg/kg	>2000 mg/kg
Dermal Irritation Rabbit	Non-irritant	Mild irritant
Dermal Sensitizer Guinea Pig	Not sensitizer	Not sensitizer
Acute Eye		
Eye Irritation Rabbit	Irritant, positive irritant effects cleared by 14 days	Irritant, positive irritant effects cleared by 14 days
Acute Inhalation		
LC50 Rat (4-hr. exposure)	>6.9 mg/L	>5.6 mg/L

Mutagenicity	
Ames Mutagenicity	Negative
Chinese Hamster Ovary/HGPRT	Negative
Unscheduled DNA Synthesis	Negative
In vitro chromosome aberration Human Lymphocytes	Positive
In vivo mouse micronucleus	Negative

PE350 Technical	
90-Day	
Rat	No observable effect level 10 ppm male, 500 ppm female
Mouse	No observable effect level 500 ppm
Dog	No observable effect level 5,000 ppm
Developmental/ Reproduction	
Rat Teratogenicity	No observable adverse effect level 200 mg/kg/day for maternal, 600 mg/kg/day for fetal effects
Rabbit Teratogenicity	No observable adverse effect level 300 mg/kg/day for both maternal and fetal effects
2-Generation Rat Reproduction	No observable effect level 1500 ppm

(continued)

Toxicology Data (continued)

PE350 Technical	
Aquatic	
Bluegill Sunfish 96-hour LC50	>930 mg/L
Rainbow Trout 96-hour LC50	>1000 mg/L
Daphnia magna 48-hour EC50	>1100 mg/L
Sheepshead Minnow 96-hour LC50	>145 mg/L
Mysid Shrimp 96-hour LC50	>140 mg/L
Oyster Shell Deposition 96-hour EC50	>130 mg/L
Catfish 96-hour LC50	>970 mg/L
Crawfish 96-hour LC50	>910 mg/L
Avian/Honey Bee	
Oral LD50 Bobwhite Quail	1599 mg/kg
Dietary 5-Day LC50 Bobwhite Quail	>5620 ppm
Dietary 5-Day LC50 Mallard Duck	>5620 ppm
Contact LD50 Honey Bee	>25 µg/bee

Safety and Handling

Precaution Statement

WARNING! CAUSES EYE IRRITATION. Avoid contact with eyes, skin, or clothing. Avoid breathing dust or spray mist.

User Recommendations

USERS SHOULD: Wash hands before eating, drinking, chewing gum, using tobacco, or using the toilet.

Personal Protective Equipment (PPE)

Applicators and handlers must wear: long-sleeved shirt and long pants, shoes plus socks, and protective eyewear.

Discard clothing or other adsorbent materials that have been drenched or heavily contaminated with this product's concentrate—do not reuse them. Follow manufacturer's instructions for cleaning/maintaining PPE. If no such instructions for washables exist, use detergent and hot water. Keep and wash PPE separately from other laundry.

Statement of Practical Treatment

In case of contact with eyes, immediately flush with plenty of water. In case of skin contact, wash area with plenty of soap and water. Seek medical attention if irritation persists.

Product Information: **800-574-GROW (574-4769)**

Transportation Emergency: **800-424-9300**

Medical Emergency Phone: **800-441-3637**

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