98-335-01p

Mr Arnold Foudin,
USDA-APHIS
4700 River Rd.,
Unit 147
Riversdale, Md., 20737
USA

Nov.25, 1998

Dear Mr Foudin,

I hope you enjoyed your Thanksgiving break and are not now suffering the effects of over-indulgence, as I did last month during our Thanksgiving holiday.

After receiving your letter of July 31, 1998 with an opinion on transgenic flax "CDC Triffid" for crushing in the US, I have been informed that farmers in the US are interested in growing this variety, so I request now that you consider the enclosed petition for non-regulated status under 7 CFR 340, relating to CDC Triffid, so US farmers will be able to grow this cultivar next spring.

While I prepared the submission based on the guide contained in your website, much of the information has been taken from the earlier submissions to FDA and the various Canadian authorities (Agriculture and AgriFood Canada, Health Canada). I hope the information and data are clear and do not appear disjointed. There is no confidential business information in this petition.

If you have any questions or concerns, please feel free to contact me.

Sincerely,

Alan McHughen
Professor and Senior Research Scientist

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12/1/98

98-335-01p

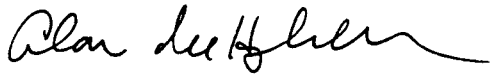
USDA Petition for determination of nonregulated status under 7 CFR 340.6 Nov. 25, 1998

Enclosed is a petition for determination on the regulatory status of *Linum usitatissimum* L. cv. "CDC Triffid", modified to provide enhanced tolerance to sulfonylurea herbicide residues in soil. Based on the data and information contained in this enclosed petition and appendices, I believe the cultivar does not present a plant pest risk and is not a threat or otherwise deleterious to human health or the environment. The enclosed petition contains no confidential business information.

CDC Triffid was scrutinized by Agriculture and Agri-Food Canada and cleared for variety registration, unrestricted environmental release in Canada, and animal feed use in May, 1996 (See Government of Canada Decision Document 98-24, Appendix 1. It is also available on the web at http://www.cfia-acia.agr.ca/english/plant/pbo/dd9824e.html). It has been growing throughout Canada ever since. Afterwards, we sought a voluntary review from Health Canada. Although dietary exposure is very small in humans, we wanted to assure consumers that consumption of CDC Triffid was no different from consuming seed of other flax cultivars. Health Canada gave human food approval in February, 1998. Similarly, we sought voluntary review by FDA in the US for human and animal feed. That "consultation" was completed in May, 1998, followed by approval from USDA for crushing in the United States, in July, 1998. There seems to be some interest in growing CDC Triffid from farmers in the flax growing area of the US, particularly in North Dakota, hence this petition.

I know of no unfavorable information regarding the subject of this petition.

I certify that to the best of my knowledge and belief, this petition contains all data, information and views relevant to the matter, whether favorable or unfavorable to the position of the undersigned, which is subject matter of this petition.



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Contents

Abbreviations:
ALS acetolactate synthase (aka Acetohydroxyacid synthase, AHAS)
NPT-II Neomycin phosphotransferase, type 2
NOS nopaline synthetase
SU sulfonylurea

1. Rationale

Sulfonylurea herbicides are popular with cereal farmers because they give good weed control in low doses. However, in some soil types (especially those with low moisture, high pH and low temperature), some classes of SU can take a long time to break down, thus requiring the farmer either to continuous crop to cereals or else to summerfallow while the SU residue deteriorates. Neither of these practices is environmentally or agronomically sustainable. In order to provide farmers with an environmentally and agronomically sustainable broadleaf crop to include in their rotations, the natural ability of flax to tolerate SU herbicides was enhanced to extend to the root tissue (Ordinary flax is tolerant to SU application, but the roots are sensitive to residual SU in the soil). With the registration of CDC Triffid, farmers can continue to use SU on their cereal crops, knowing they can rotate in subsequent years with a broadleaf crop and thus avoid non-sustainable practices.

2. Biology of *Linum*

Linum usitatissimum L. is commonly known as flax, flaxseed or linseed. The common names are often used interchangeably in North America (including in this document). It is the only member of the genus grown as a crop, although some *Linum* species have limited ornamental use.

Linum usitatissimum L. grows throughout the temperate regions of the world, either for oil (linseed) or for fibre (flax) or both. Nowhere (except perhaps Canada) is it a major crop. Although uncertain, it is widely accepted that linseed flax originated in the area east of the Mediterranean towards India because of the diverse forms found in the region (Zeven and Zhukovsky, 1975). Oilseed flax (linseed) was initially grown in southwest Asia, while the fibre type flax was developed around the Mediterranean. Oilseed types developed for agronomic cultivation were selected from among plant types with indehiscent or semi-dehiscent bolls, to minimize seed loss through shattering (Lay and Dybing, 1989). Further information is available in "The Biology of *Linum usitatissimum* L. (Flax)", Regulatory Directive Dir94-10, published by the Government of

Canada, appendix 1. It is also available on the web at http://www.cfia-acia.agr.ca/english/plant/pbo/dir9410e.html

Reproduction: Linseed flax is almost entirely self-pollinating. Pollen is relatively heavy and "sticky", reducing the incidence of wind-assisted cross-pollination to near nil. Linseed has little attraction for bees and other insects (Beard and Comstock, 1980), so insect derived cross-pollination is also very limited. Due to the flower structure and development, and because the pollen is viable for only a few hours, there is very little outcrossing by any means in the environment. Linseed is technically compatible (when facilitated by human intervention) with some other species of the *Linum* genus. None of these compatible species is important, either as weeds or as crops. In one study, over 8,000 observations were made on the variety "Bison" and natural outcrossing was nil (Appendix 1; Government of Canada Regulatory Directive Dir94-10; 1994). Within-cultivar cross-pollination is also low. An early study shows linseed flax plants sown 30cm apart (in which case the floral inflorescence would be in physical contact at anthesis due to the "spread"), produced less than 2% hybrid progeny seed (Henry and Tu; 1928). Linseed produces seed as its exclusive means of reproduction in the environment (it can be micropropagated *in vitro* in a laboratory). Linseed does not produce tubers, rhizomes or other vegetative propagules. The seeds are produced in closed capsules called bolls. In most cultivated genotypes, the bolls remain closed and so do not disperse the seeds even at maturity. Each boll can enclose up to ten seeds. In typical agronomic production, only seven or eight seeds usually develop in each boll.

Weediness: *Linum* is a primary colonizer, growing in a "disturbed land" habitat, as it is a poor competitor with other plants. In the wild (ie. unmanaged ecosystems), *Linum* plants are quickly displaced because of their poor competitive ability (Appendix 1: The Biology of *Linum usitatissimum* L. (Flax); Government of Canada Regulatory Directive Dir94-10; 1994).

The non-transformed parent cultivar: The parental genotype is cv. "Norlin", a major commercial flax cultivar in western Canada over the past several years. It is also grown commercially in the US.

3. Description of transformation system

An *Agrobacterium tumefaciens* strain carrying a disabled Ti plasmid vector was used to deliver the novel DNA to a Norlin hypocotyl segment *in vitro*. *Agrobacterium tumefaciens* strain C58 was the parental bacterium, containing a disabled Ti plasmid pGV3850, (Zambryski et al., 1983). Into this Ti plasmid was inserted a cointegrating vector plasmid, pGH6, consisting of a 5.8KB fragment of a sulfonylurea-resistant *Arabidopsis thaliana* Acetolactate Synthase (*als*) gene, Neomycin phosphotransferase-II (*npt*-II) and a nopaline synthase (*nos*) gene (Haughn et al, 1988).

After inoculation with this diasarmed Agrobacterium, a shoot was subsequently induced to develop from a transformed cell on the hypocotyl. The shoot was isolated and placed in a rooting medium to develop roots. After roots developed, the plantlet was transferred to soil and grown to maturity, with flowers self-pollinated. Seeds were collected from the regenerant plant. CDC Triffid was developed as an inbred from this initial transformant. Details of the transformation procedure are contained in McHughen (1989).

4. T-DNA genes and regulatory sequences

Table 1.

A list of fragments in the T-DNA and their source:

Fragment	Donor Source	Size (Kb)	Function
LB-Hind III fragment 10	*A. tumefaciens* C58	6.4	left border
Ampicillin resistance	*E. coli* pBR322	0.8	prok. selection
Origin of replication	*E. coli* pBR322	3.3	prok. origin
ALS fragment	*Arabidopsis thaliana*	5.8	herb. resistance
pNOS-NPT-II-NOS	*E. coli* Tn 5;	1.5	plant selection
Spectinomycin resistance	*E. coli*	2.5	prok. selection
NOS and RB	*A. tumefaciens* C58	2.9	plant marker
NOS-RB, Hind III fragment 23	*A. tumefaciens* C58	3.2	plant marker, Right border

Each fragment has the function as following:

1. LB fragment provides a left border, or limit to the amount of DNA transferred from the Agrobacterium to the plant cell. The LB region is a specific DNA base sequence that serves as a stop signal to the transfer process.
2. Ampicillin resistance fragment provides a means to identify and select bacterial colonies containing the desired plasmid. It is not expressed in the GM linseed
3. Origin of replication: allows the plasmid to replicate, so each bacterial daughter cell will have a copy of the plasmid.
4. *als* fragment: this is the critical feature; it provides the plant with the ability to grow in the presence of a soil contaminant deleterious to ordinary linseed plants. Linseed flax is naturally tolerant of sulfonylureas (SUs), but this tolerance is weak in the root tissues. When a farmer's land is contaminated by sulfonylurea residues, as is the case is some parts of western Canada, the farmer must practice non-sustainable rotations, such as continuous monoculture to cereals or summerfallowing, as we have no other broadleaf crops capable of surviving in contaminant SU residue. This gene fragment extends flax's natural ability to withstand SU to the root tissues, thus enabling farmers to include a broadleaf crop (ie. CDC Triffid) in the rotation until the contaminant SU residues degrade naturally (which can take up to several years). Without this flax, farmers with the SU residue problem are forced into environmentally and agronomically non-sustainable practices. With CDC Triffid, those farmers can adopt an environmentally and agronomically sustainable rotation.
5. *npt*-II fragment: allows selection of plant cells successfully transformed with the T-DNA, as it permits the plant cells to grow in the presence of a selection agent (kanamycin) which would inhibit growth of ordinary flax cells.
6. Spectinomycin resistance: this fragment provides a second means to identify and select bacterial colonies with the desired plasmid. It is not expressed in CDC Triffid.
7. *nos* and RB: this fragment results in production of nopaline by the successfully transformed plant cell. Nopaline is easily detected using a simple test. However, it is obsolete now, as it is weak and many ordinary plan species (eg. Soybean) produce nopaline.
8. *nos* and RB, Hind III fragment: this provides another copy of the *nos* gene (see above) and also the right border DNA sequence, which marks the beginning point of transferred DNA.

These fragments are described more fully in Zambryski et al., (1983) or Haughn et al, (1988). Note: the *npt*-II gene is regulated by *A. tumefaciens nos* promoter and terminator. Other genes are regulated by their "native" regulatory sequences. None of these fragments or their products has been reported to be toxic or otherwise harmful.

Three plant active genes have been introduced into CDC Triffid. Two genes provide traits novel to the species, the third gene provides a modification of an existing trait. The two novel genes (and their respective traits) are the markers, *nos* and *npt*-II.

The nopaline synthase (NOS; EC 1.5.1.19) (DNA sequence: Depicker *et al.*, 1982) gene comes from the Hind III fragment 23 of pTIC58, (along with the Right Border (RB)). It was used as a scorable marker in early plant transformation work because its activity is easily scored on the basis of a simple electrophoresis assay, which detects minute quantities of the product, nopaline. Unfortunately, nopaline was discovered to be produced naturally by many plants, for example soybean and cotton (Christou et al., 1986) so it could not be used as a reliable indicator of transformation due to the possibility of "false positives" from endogenous nopaline present in experimental plant tissues. While nopaline is associated with wild type, nopaline-type *Agrobacterium tumefaciens*, it is not relevant to the pathogenic functions of *Agrobacterium*.

Neomycin phosphotransferase type II (NPT-II, aka APH(3')II; EC 2.7.1.95), originally isolated from *E. coli* transposon Tn5, is a commonly used selectable marker in plant transformation work (DNA Sequence: Beck et al., 1982). The gene product renders plant cells resistant to aminoglycosidic antibiotics such as kanamycin, so putatively transformed plant cells expressing the gene will be able to survive in a selection medium containing the appropriate aminoglycoside. Indeed, kanamycin was used as the primary selection agent in the identification of the original cells that eventually gave rise to CDC Triffid.

The third active gene, a modified Acetolactate synthase (ALS, AHAS; EC 4.1.3.18) gene from *Arabidopsis thaliana*, (DNA sequence: Haughn et al., 1988) is homologous to endogenous linseed ALS. The gene product is an intermediate enzyme in the biosynthetic pathway for the amino acids leucine, valine and isoleucine. The difference between the endogenous ALS and the introduced ALS is that the endogenous ALS from linseed is sensitive to sulfonylurea type herbicides. In farming practice, conventional flax (like Norlin) cannot productively grow in soil containing residue of sulfonylurea herbicide, a common weed control chemical for cereal crops. Some commercial formulations of sulfonylurea herbicides can persist in soil for months or years, precluding the practice of sustainable rotations involving "broadleaf" (dicot) crops; farmers must either summerfallow or continuous crop to cereals. Having this gene introduced into flax gives such farmers an agronomically and environmentally sustainable rotational option (McHughen and Holm, 1995).

The *als* gene used here is modified from the original *Arabidopsis als* by the substitution of a single base change, resulting in a single amino acid change in the protein. This modification is described more fully in Haughn et al., 1988.

In addition to these active genes, the T-DNA contains fragments of prokaryotic genes. These are the prokaryotic ampicillin resistance gene (used for prokaryotic selection) and the spectinomycin resistance gene (also used for prokaryotic selection). These genes are under prokaryotic regulation and therefore are not expressed in the eukaryotic flax. They are destroyed during the processing of the linseed.

None of the fragments is known to be harmful to humans, other animals or the environment.

The T-DNA as inserted into CDC Triffid is described in Figure 1 (Appendix 2)

5. Genetic analysis

The genetic elements of the T-DNA has been characterized using several methods:

Southern and restriction analyses: Southern analyses conducted on 8th generation self-pollinated seed of CDC Triffid indicate that it carries two unlinked loci of inserts. Figure 1 (Appendix 2) maps the T-DNA used to develop CDC Triffid. The probe used was a segment of the *nos* gene (fig 1).

Restriction analyses were conducted on genomic DNA of the parent linseed cultivar Norlin as well as on CDC Triffid. NCO 1, Hind III and Dra I were the enzymes used (approximate cutting sites shown on Fig 1). NCO 1 cuts within the probe sequence, while Hind III cuts between the two *nos* genes in the T-DNA but not in the probe itself. Dra I cuts the T-DNA but not within or between the probe sequences.
In the Southern blot (Figure 2; Appendix 3), the NCO digest shows five bands, the smallest one with the heavier intensity due to it being the internal fragment (composed of two probe-homologous sequences/haploid genome), the other four bands represent the different size fragments 5' to each locus of insertion. The map predicts the distance between the two NOS sequences to be approx. 3Kb; the most intense band in the NCO I lane is about 3Kb. No bands appear in the lane using DNA from the parental cultivar Norlin (left lane), showing that *nos* or *nos*-homologous sequences are not present in the parental genome.

The Hind III digest for CDC Triffid shows three bands. The smallest, most intense band represents the internal fragment. The two lighter bands represent the (predominantly) plant DNA fragment 3' to each of the two loci of insertion. The internal band, approx. 9Kb, is consistent with the distance on the T-DNA map (Fig. 1).

In the Ti plasmid DNA of the *Agrobacterium* vector used to develop CDC Triffid, Hind III generates a 3.2 Kb fragment consisting of the Right Border (RB) and the proximal *nos* sequence, along with a portion of non-T-DNA 3' of the RB. The presence of such a fragment here would indicate that the DNA transferred from the bacterium was not limited to the sequence bounded by the right border. Note here (Fig. 2) that the fragment is missing from the Hind III digest of CDC Triffid (but larger fragments are present)

indicating that the T-DNA actually transferred into CDC Triffid was limited to the RB and did not extend into the non-T-DNA region of the Ti plasmid.

The Hind III digest for Norlin (left lane) shows no bands, again indicating that the parental cultivar Norlin lacks *nos* or *nos*-homologous sequences.

The Dra I digest, in the rightmost pair of lanes (Fig. 2) shows two bands for CDC Triffid, indicating two different loci of T-DNA insertion. Again, the Dra I digest and probe of the Norlin lane was clear, establishing that Norlin lacks *nos* and *nos* homologous sequences.

The fidelity of the Left Border (LB) region is less certain than that of the RB, as it has not been directly mapped in CDC Triffid. It has been established that the LB is typically less precise than the RB, with the actual terminus as much as 70 to 100 bases from the expected position (Zambryski et al., 1982). Therefore, it is possible that the actual left border in CDC Triffid carries some DNA from the Ti plasmid 5' to the LB. Is the presence of 70 to 100 or so DNA bases 3' of the LB likely to present a problem? The LB terminus is in the Hind III fragment 10 of Ti plasmid C58, about 6.4Kb. The actual LB sequence is about halfway into this fragment. There are no ORFs on this fragment 3' to the LB (that is, into the T-DNA; see Fig. 1), so the closest ORF in the T-DNA is the prokaryotic amp-R gene from the adjoining pBR322 fragment. This ORF is oriented to read away from the LB, so "read-through" would run into the T-DNA, not away from it in the direction of the LB and beyond.

The C58 Ti plasmid was fully characterised in the late 1970s- early 1980s. DePicker et al. (1980) provided a complete restriction map (including the Hind III fragment containing the LB), while Holsters et al. (1980) provided a functional map. The closest genetic elements mapped 5' to the LB on the Ti plasmid are the *vir* genes. More recently, Hirooka and Kado (1986) showed the *vir* locus to be about 6Kb away from the LB. Searches of the literature and of genetic databases failed to find any genetic elements on the Ti plasmid upstream of the LB not already reported. With about 6Kb between the LB and the closest upstream ORF on the Ti plasmid, and the "infidelity" of the LB being on the order of 70 to 100 bases, it is extremely unlikely that transfer and expression of any additional active elements occurred inadvertently in CDC Triffid.

Expression of *npt*-II: Western: A commercial ELISA assay (Manufacturer: 5-Prime-3-Prime, Inc.) was used on seed of CDC Triffid to quantify NPT-II present. The NPT-II protein content, in picograms NPT-II protein/mg of total protein of seven seed samples was: 217 pg/mg; 165pg/mg; 613pg/mg; 379pg/mg; 950pg/mg; 381pg/mg; and 319pg/mg, for a mean quantity of NPT-II enzyme of 432ng of NPT-II protein per gram of total protein. Note: this assay was conducted on fresh seed, as processing (crushing) of linseed denatures NPT-II.

For comparison, assays conducted on fresh leaf tissue show NPT-II enzyme content to be less than 0.001% of total protein, with the greatest single reading from twenty samples

being 7900ng/g, or 0.00079% of total protein. These data are in accordance with values reported for other transgenic plants and species carrying the same *npt*-II gene.

PCR NPT-II: DNA primers were designed to amplify the NPT-II sequences in CDC Triffid. In a recent assay using 8th generation homozygous plant material, all eight samples generated a band of the appropriate expected size for *npt*-II DNA, while none of the six parental Norlin plants produced any band with the primer. See appendix 4, figure 3.

Expression of Nopaline Synthetase: Nopaline synthase expression is determined by the presence of nopaline. Nopaline is assayed using a simple, yet highly sensitive, paper chromatographic assay first described by Otten and Schilperoort (1978). The following data show nopaline content in various tissues from seedlings and pre-flowering plants of CDC Triffid.

In seedlings, Root tissue contained an average of 0.29 ug/mg root tissue;
Stem tissue contained an average of 0.16 ug/mg stem tissue, and
Leaf tissue contained an average of 0.64 ug/mg leaf tissue

In pre-flowering plants,
Root tissue contained an average of 0.16 ug/mg root tissue;
Stem tissue contained an average of 0.07 ug/mg stem tissue, and
Leaf tissue contained an average of 0.49 ug/mg leaf tissue

Kanamycin resistance assay: Expression of the *npt*-II gene can be characterised using any of several different assays. Seed germination on media containing 500mg/l kanamycin provides an easy visual assay, as those seedlings expressing the NPT-II grow normally, while those not expressing NPT-II show inhibited root growth, with elongation arrested by about 1cm of growth. Seeds from 8th generation plants of CDC Triffid, as well as seeds from the parental cultivar Norlin, were plated on medium with 500mg/l kanamycin. All 19 Norlin seedlings were affected, while none of the 168 CDC Triffid seedlings were affected in this medium.

Another assay to characterize expression of NPT-II is a leaf recallusing test. In this, a piece of leaf tissue is placed on a tissue culture medium known to induce callus growth from leaf pieces of the parental Norlin cultivar. When 200mg/l of kanamycin is added to the medium prior to inoculation, Norlin leaf pieces fail to develop callus. In one assay, 24 Norlin leaf pieces were inoculated on the kanamycin-containing medium, none of these grew callus. However, when 168 leaf pieces from CDC Triffid were inoculated onto the same medium, all leaf pieces developed callus tissue. Identical results were obtained when the original inoculum was stem or root tissue.

Sulfonylurea resistance assay: An assay similar to that for the NPT-II expression was developed to characterize the activity of the introduced *als* gene. The parental cultivar

Norlin suffers dramatic root growth suppression in the presence of sulfonylurea herbicides. The introduced *als* gene provides an enzyme with reduced sensitivity to this class of herbicide. Chlorsulfuron (a type of sulfonylurea) was added to the germination medium at 100nM concentration. All 21 seedlings of the parental cultivar Norlin were severely affected by the chlorsulfuron, while none of the 163 seedlings of CDC Triffid was affected

Another assay to characterize expression of the introduced *als* gene is a leaf recallusing test. In this, a piece of leaf tissue is placed on a tissue culture medium known to induce callus growth from leaf pieces of the parental Norlin cultivar. When 100nM chlorsulfuron is added to the medium prior to inoculation, Norlin leaf pieces fail to develop callus. In one assay, 24 Norlin leaf pieces were inoculated on the chlorsulfuron-containing medium, none of these grew any callus. However, when 168 leaf pieces from CDC Triffid were inoculated onto the same medium, all leaf pieces developed callus tissue. Identical results were obtained when the original inoculum was stem or root tissue.

Expression of ALS: ALS is expressed throughout the plant. Note: ALS is an endogenous enzyme in linseed. The ALS enzyme activity assay cannot distinguish between the endogenous enzyme and the product of the introduced *als* gene. Therefore, the total ALS expression in CDC Triffid should be predicted to be somewhat higher than the parental plant (ie. In CDC Triffid, the activity of the endogenous ALS plus the activity of the introduced ALS, vs. the activity of the endogenous ALS alone for the parental plant).

The ALS assay used was described by Devine et al. (1991) and is based on enzyme activity as measured by production of acetolactate. In the parental Norlin control, ALS activity was 56.3 nmol/mg/hr (mean of two samples). CDC Triffid enzyme activity was 88.85 nmol/mg/hr (mean of two samples). As predicted, CDC Triffid does have higher ALS activity. However, this greater activity does not appear to adversely affect growth (as determined by agronomic parameters) or metabolism (amino acid profile is comparable to the parental line). It should be noted that, although the ALS *enzyme* activity differs, there are no significant differences between CDC Triffid and the parental line for the three relevant amino acids (leucine, isoleucine and valine). In fact, there are no significant differences in content of any amino acid between CDC Triffid and parental Norlin.

Mendelian inheritance

The initial transgenic regenerant was allowed to set self-pollinated seed. A sample of 25 seeds was plated on media containing chlorsulfuron (a sulfonylurea) and allowed to germinate. Progeny expressing the modified ALS gene are capable of growing normally on this medium, while wild-type seedlings show severely stunted root growth. In these seedling growth experiments, 24 seedlings grew normally, while one had severely stunted root growth. If a single insertion of T-DNA were present, the expected result would have about 75% of the progeny grow normally in the presence of the SU, while the remaining 25% would show stunted root growth, a ratio of 3:1. If there were two loci of insertion, the expected result would be a 15:1 resistant to sensitive ratio. Chi-square analysis

supported a segregation ratio of 15:1, indicating two independent chromosomal inserts. This result verifies the Southern analyses, conducted on homozygous material at 8th generation (see above sections).

5. Agronomic performance

This GM flax has been grown in field trials since 1989. Agronomic performance has been studied and published in McHughen and Rowland, 1991; McHughen and Holm, 1991 McHughen and Holm, 1995, and McHughen et al., 1997. Briefly, all agronomic parameters measured, including seed yield, maturity, height, seed weight oil content, protein, etc. were all similar (ie. not significantly different from) the parental Norlin. For example, in two years of national field trials in Canada, in three different soil and environmental zones, totaling 29 different station years, the overall seed yield averaged 2050kg/ha for Norlin, and 2000kg/ha for CDC Triffid. In no individual trial or group of trials was the seed yield substantially different between the parent Norlin and CDC Triffid. (Note: in these publications, CDC Triffid was referred to by its experimental number, 12115, but its national field testing system number, FP967, or by its registered name, CDC Triffid. These designations all refer to the same genotype).

The only anti-nutritional component of linseed flax, cyanogenic glycoside, was present in both Norlin and CDC Triffid in similar quantities. CDC Triffid, on the basis of meritorious agronomic performance in Canadian national competitive field trials, was recommended for variety registration by the competent authority (PRRCG) in February, 1994. The only time differences are noted between Norlin and CDC Triffid is in the presence of SU herbicide, when Norlin is adversely affected, and CDC Triffid shows no impact from the herbicide. Data from field trials are presented in appendix 5.

6. Environmental consequences

(a) mode or modes and/or rate of reproduction

None of the introduced genes was intended or expected to have any effect on modes or rate of reproduction. The recipient plant and its parent reproduce by way of seed. The rate of reproduction (measured by seed production) is similar. CDC Triffid has been grown in confined and unconfined field trials in Canada since 1989; unusual modes or rates of reproduction have NOT been reported at any time. The rate of reproduction, as measured by seed yield, is comparable to the parental cultivar. In two years of national field trials in Canada, in three different soil and environmental zones, totaling 29 different station years, the overall seed yield averaged 2050kg/ha for Norlin, and 2000kg/ha for FP967 (CDC Triffid). In no individual trial or group of trials was the seed yield substantially different between the parent Norlin and CDC Triffid.

(b) dissemination

None of the introduced genes was intended or expected to have any effect on dissemination. The recipient plant and its parent both produce seed in bolls. CDC Triffid has not changed in this aspect, and the production of seeds (ie. seed yield) is similar. See also discussion of seed persistence and invasiveness under (c) survivability below. None

of the introduced genes was intended or expected to have any effect on seed dissemination

(c) survivability

None of the introduced genes was intended or expected to have any effect on survivability. Our studies show survivability of seed of the recipient CDC Triffid and the parent Norlin is similar.

The parental plant is a poor biological competitor with little persistence or invasiveness (Government of Canada Regulatory Directive Dir94-10; 1994). The introduced genes have no expected influence over persistence or invasiveness. Therefore it is unlikely that CDC Triffid will pose any significant risk of increased persistence or invasiveness.

9. Adverse consequences

None of the introduced genes was intended or expected to alter the likelihood of transfer of genetic material from CDC Triffid to other organisms. The only known mechanism of gene transfer from linseed to other plants (including other linseed plants) is via pollen. Cross-pollination is a rare event in linseed; in one study, over 8,000 observations were made on the variety "Bison" and natural outcrossing was nil (Government of Canada Regulatory Directive Dir94-10; 1994). In the study of Henry and Tu (1928), linseed plants sown 30cm apart (such that their inflorescences were touching) had less than 2% hybridization. Subsequent studies have confirmed this low rate of pollen transfer. In 1988 and 1989, studies conducted by the University of Saskatchewan using transgenic linseed verified these results. In these recent studies, homozygous NPT-II containing flax plants were sown between rows of the parent, non-transgenic cultivar. The rows were spaced 30 cm apart, such that, at flowering, the inflorescences were in physical contact with those from the neighboring rows (one on each side). Ten rows of wild-type linseed plants were sown at 30cm intervals in either side, such that the most distant plants were (30cm*10 rows=) 3 meters away from the transgenic linseed plants. Seeds were harvested and analyzed for NPT-II in putative heterozygotic seeds. Of over 600 seeds sampled from wild-type plants, only 2 seeds contained the NPT-II gene indicating hybridization from transgenic pollen; both of these hybrid seeds were from wild-type linseed plants growing 30cm from a transgenic plant. Therefore, gene transfer from CDC Triffid is minimal, similar to that of the parental cultivar Norlin. Furthermore, none of the introduced genes is intended or expected to provide any selective advantage or disadvantage to any sexually compatible plant species in the natural environment.

There are three active genes introduced into CDC Triffid. One, *npt*-II, confers resistance to aminoglycosidic antibiotics such as kanamycin. This type of chemical is not normally found in the wild, so no advantage will be afforded any opportunistic recipient organism. The second active gene is the modified *als*, which confers resistance to the sulfonylurea herbicides. These chemicals are not normally found in the environment, other than in managed agronomic settings (ie. a farm). If an opportunistic organism were to receive the modified *als*, it might provide an advantage in the managed environment in the presence of sulfonylurea herbicide if it did not already have resistance to these herbicides. However, many plants already have natural resistance to these herbicides.

Regardless, it is recommended farm practice to "rotate" herbicides in order to deal with novel herbicide resistant plants, regardless of how the novel trait was acquired. In any case, the *als* gene will not provide a selective advantage or disadvantage in the natural environment.

The third gene, *nos*, regulates production of nopaline, which gives no selective advantage or disadvantage to either CDC Triffid or any sexually compatible species.

CDC Triffid has been grown under confined conditions in Canada from 1989 to 1995, and under unconfined conditions since 1996. The results of the site monitoring program and farmers' commentaries indicate there is no negative environmental impact from the release and cultivation of these plants.

Flax is not closely related to other species, either crops, weeds or other native plants. It does not have major interactions with pollinators as it is largely self-pollinating (although some insects do occasionally visit the flowers, it is rare (Beard and Comstock, 1980)). Interactions with other organisms are minimal. There are few pathogens of commercial cultivars of linseed; *Melampsora lini* and *Fusarium oxysporum* f. sp. *lini* can cause damage to some cultivars in some years. Similarly, there are few insects interested in linseed; flax bollworm and aphids can cause damage in some years in some locations, but these interactions are not considered significant. None of the introduced genes are known to or were intended to alter any interactions with target organisms. CDC Triffid and its parent cultivar (Norlin) continue to have similar relationships with major pathogens (*Melampsori lini* and Fusarium *oxysporum* f. sp. *lini*.), the primary organisms involved in interactions with flax.

Natural toxins: cyanogenic glycosides

The only potentially harmful characteristic, living or dead, in this species (including CDC Triffid as well as its parent) is the presence of small amounts of cyanogenic glycosides. Linseed naturally produces cyanogenic glycosides. The concentration is highly variable, depending on particular year of crop growth, location of growth and cultivar; environment seems to play a substantial role in final cyanogenic glycoside content (Bhatty, 1993; Oomah et al., 1992). A typical range for Canadian cultivars (including Norlin, the parent of CDC Triffid) is 6.0 to 9.2 mg/g of seed (Bhatty, 1993; Oomah et al., 1992). Our analyses comparing cyanogenic glycoside content of CDC Triffid with its parent cultivar Norlin shows no significant difference between the two, with a CDC Triffid reading of 7.2 (±0.24)mg/g of seed and a reading of 7.0 (±0.15)mg/g of seed for Norlin. This difference is not statistically significant.

References

Beard, B.H. and V.E. Comstock. 1980. Flax. *In:* Hybridization of Crop Plants. W. Fehr and H. Hadley (eds.) American Society of Agronomy, Madison, Wisc. USA.

Beck, E., Ludwig, G., Auerswald, E.A., Reiss,B. and H. Schaller. 1982. Nucleotide sequence and exact localization of the neomycin phosphotransferase gene from Tn5. Gene 19, 327-336

Bhatty, R.S. 1993. Further compositional analyses of flax: mucilage, trypsin inhibitors and hydrocyanic acid. JAOCS 70:899-904.

Christou, P., S. Platt and M. Anderson.1986. Opine synthesis in wild -type plant tissue. Plant Physiol. 82:218-221.

DePicker, A., M. De Wilde, G. De Vos, R. De Vos, M. Van Montagu and J. Schell. 1980. Molecular cloning of overlapping segments of the nopaline Ti-plasmid pTiC58 as a means to restriction endonuclease mapping. Plasmid 3:193-211.

Depicker, A., Stachel, S., Dhaese, P., Zambryski, P. and H.M Goodman. 1982. Nopaline synthase: transcript mapping and DNA sequence. J. Mol. Appl. Genet. 1 (6), 561-573

Devine, M.D., M.A.S. Marles and L. Hall. 1991. Inhibition of acetolactate synthase in susceptible and resistant biotypes of *Stellaria media.* Pesticide Science 31:273-280.

Government of Canada Regulatory Directive Dir94-10. 1994. The Biology of *Linum usitatissimum* L. (Flax). Web site: http://www.cfia-acia.agr.ca/english/plant/pbo/dir9410e.html

Haughn , G., J. Smith, B. Mazur and C. Somerville. 1988. Transformation with a mutant *Arabidopsis* acetolactate synthase gene renders tobacco resistant to sulfonylurea herbicides. Mol. Gen. Genet. 204:430-434.

Henry, A.W. and C. Tu. 1928. Natural crossing in flax. J. Amer. Soc. Agron. 20:1183-1192.

Hirooka, T. and C. I. Kado. 1986. Location of the right boundary of the virulence region on *Agrobacterium tumefaciens* plasmid pTiC58 and a host-specifying gene next to the boundary. J. Bacteriology 168: 237-243.

Holsters, M., B. Silva, F. Van Vliet, C. Genetello, M. De Block, P. Dhaese, A. Depicker, D. Inze, G. Engler, R. Villarroel, M. Van Montagu and J. Schell. 1980. The functional organization of the nopaline *A. tumefaciens* plasmid pTiC58. Plasmid 3:212-230.

Lay C. and C. Dybing, 1989. Linseed. *In*: Oil Crops of the World. G. Robbelen, R.K. Downey and A. Ashri, (eds.) McGraw-Hill, NY.

McHughen, A. 1989. *Agrobacterium* mediated transfer of chlorsulfuron resistance to commercial flax cultivars. Plant Cell Reports 8:445-449.

McHughen, A. and G.G. Rowland. 1991. The effect of T-DNA on the agronomic performance of transgenic flax plants. Euphytica 55: 269-275.

McHughen, A. and F.A. Holm. 1991. Herbicide resistant transgenic flax field test: agronomic perfomance in normal and sulfonylurea –containing soils. Euphytica 55: 49-56.

McHughen A. and F. A. Holm. 1995. Transgenic flax with environmentally and agronomically sustainable attributes. Transgenic Research 4: 3-11.

McHughen, A.G.G. Rowland, F.A. Holm, R.S. Bhatty, and E.O. Kenaschuk. 1997. CDC Triffid transgenic flax. Can. J. Plant Science 77:641-643. (see appendix 5)

Oomah , D. G. Mazza and E.O. Kenaschuk. 1992. Cyanogenic compounds in flax. J. Agric. Food Chem. 40: 1346-1349.

Otten, L.A.B.M. and R.A. Schilperoort. 1978. A rapid micro scale method for the detection of lysopine and nopaline dehydrogenase activities. Biochimica-et-Biophysica-Acta. 527: 497-500.

Zambryski, P., A. Depicker, K. Kruger and H. Goodman. 1982. Tumor induction by Agrobacterium tumefaciens: Analysis of the boundaries of T-DNA. J. Molecular and Applied Genetics 1: 361-370.

Zambryski, P. H. Joos, C. Gentello, J. Leemans, M. Van Montagu and J. Schell. 1983. Ti plasmid vector for the introduction of DNA into plant cells without alteration of their normal regeneration capacity. EMBO J. 2: 2143-2150.

Zeven, A.C. and P.M. Zhukovsky. 1975. Dictionary of Cultivated Plants and their Centres of Diversity. Centre for Agricultural Publishing and Documentation, Wageningen.

Appendix 1.

- Government of Canada Regulatory Directive Dir 94-10; The Biology *of Linum usitatissimum* L. (Flax). Web site: http://www.cfia-acia.agr.ca/english/plant/pbo/dir9410e.html

- Government of Canada: Plant Biotechnology Office, Decision Document 98-24, Determination of the Safety of the Crop Development Centre's 'CDC Triffid" a flax (*Linum usitatissimum* L.) variety tolerant to soil residues of triasulfuron and metsulfuron methyl. Web site: http://www.cfia-acia.agr.ca/english/plant/pbo/dd9824e.html

- Government of Canada: Variety Registration Certificate for FP967, "CDC Triffid"

- Government of Canada: Letter from Health Canada, permitting human food use of CDC Triffid linseed in Canada.

- Government of United States: Letter from FDA, United States, in completion of consultation on the use of CDC Triffid for human food and animal feed in the United States.

- Government of United States: Letter from USDA, permitting CDC Triffid linseed into the United States for crushing purposes.

Appendix 2.

Figure 1 consists of map of novel DNA in CDC Triffid, with major restriction sites, sized fragments and expressed genes marked. Also indicated is the portion of the *nos* gene used as a probe for the Southern blot (appendix 3; figure 2).

Appendix 3.

Figure 2 consists of the Southern blot comparing CDC Triffid with parental Norlin genomic DNA after restriction with three enzymes and probing with a *nos* fragment probe from the T-DNA.

Appendix 4.

Figure 3 shows the result of a *npt*-II PCR reaction in CDC Triffid and Norlin control, indicating the presence of *npt*-II DNA in all CDC Triffid samples (as well as in the positive bacterial control), but neither of the Norlin control samples.

Appendix 5.

- Request for support for cultivar registration, summarizing agronomic and other relevant data comparing CDC Triffid to the parental cultivar 'Norlin'.

- Proximate analyses of CDC Triffid and parental Norlin.

- Agronomic performance of CDC Triffid, contained in McHughen et al., Canad. J. Plant Science 77:641-643.1997.

Interdepartmental Executive Committee on Pest Management
Le Comité interministériel exécutif sur la lutte antiparasitaire

Regulatory Directive **Dir94-10**

The Biology of *Linum usitatissimum L.* (Flax)

A companion document to the Assessment Criteria for Determining Environmental Safety of Plants with Novel Traits

This document replaces Regulatory Proposal 94-03

(publié aussi en français) **December 16, 1994**

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Part A - General Information

A1.0 Background

Since 1988, Agriculture and Agri-Food Canada has been regulating the field testing in Canada of agricultural and horticultural crop plants with novel traits (PNT's). "Plants with novel traits" are defined as a plant variety/genotype possessing characteristics that demonstrate neither familiarity nor substantial equivalence to those present in a distinct, stable population of a cultivated species of seed in Canada and that have been intentionally selected, created or introduced into a population of that species through a specific genetic change. "Familiarity" is defined as the knowledge of the characteristics of a plant species and experience with the use of that plant species in Canada. "Substantial equivalence" is defined as the equivalence of a novel trait within a particular plant species, in terms of its specific use and safety to the environment and human health, to those in that same species, that are in use and generally considered as safe in Canada, based on valid scientific rationale.

The PNT's can either be derived from recombinant DNA technologies, or from traditional plant breeding. Regulated field testing is necessary when the PNT's have traits of concern, i.e., the traits themselves, their presence in a particular plant species or their use are: 1) considered unfamiliar when compared with products already in the market; 2) not considered substantially equivalent to similar, familiar plant types of the same species already in use, and regarded as safe.

Before PNT's may be authorized for unconfined release, they must be assessed for environmental safety. Regulatory guidelines entitled *Assessment Criteria for Determining Environmental Safety of Plants with Novel Traits* have been developed to define criteria and information requirements that must be considered in the environmental assessment of PNT's to ensure environmental safety, in the absence of confinement conditions.

A2.0 Scope

The present document is a companion document to the regulatory guidelines *Assessment Criteria for Determining Environmental Safety of Plants with Novel Traits*. It is intended to provide background information on the biology of *Linum usitatissimum* L., its centres of origin, its related species, and the potential for gene introgression from *Linum usitatissimum* into relatives, and details of the life forms with which it interacts.

Such species-specific information will serve as a guide for addressing some information requirements of Part D of the regulatory guidelines. Specifically, it will be used to determine whether there are significantly different/altered interactions with other life forms, resulting from the PNT's novel gene products, which could potentially cause the PNT to become a weed of agriculture, become invasive of natural habitats, or be otherwise harmful to the environment.

The conclusions drawn in this document about the biology of *Linum usitatissimum* only relate to plants of this species with no novel traits. Novel traits of concern might confer new characteristics to the plant, that could impact on the environment pursuant to their unconfined release.

Part B - The Biology of *Linum usitatissimum*

B1.0 General Description, Cultivation and Use as a Crop Plant

Linum usitatissimum L. is a species of the family Linaceae (Flax family). It is an erect, herbaceous annual which branches corymbosely above the main stem (Fernald, 1950). Two types of *L. usitatissimum* are cultivated: the **linseed type**, grown for oil extracted from the seed, is a relatively short plant which produces many secondary branches compared to the **flax type**, grown for the fibre extracted from the stem, which is taller and is less branched (Gill, 1987). *L. usitatissimum* has a short tap root with fibrous branches which may extend 90 - 120 cm in light soils. Leaves are simple, sessile, linear-lanceolate with entire margins, and are borne on stems and branches. The inflorescence is a loose terminal raceme or cyme. Flowers are borne on long erect pedicels, are hermaphrodite, hypogynous and are composed of five sepals, five petals (blue), five stamens, and a compound pistil of five carpels each separated by a false septum. The fruit is a capsule, composed of 5 carpels and may contain up to 10 seeds. The seed is oval, lenticular, 4-6 mm long with a smooth, shiny surface, brown to light-brown in colour. Seeds contain 35-45% oil and 20-25% protein (Gill, 1987; Fernald, 1950).

Canada is a major producing country along with Argentina, India, the USA and Russia; most Canadian flaxseed is exported as linseed. Traditionally, the oil pressed from the seed (linseed oil) has been used for a variety of industrial purposes and the oil-free meal could be fed to livestock (boiling with water is advised to counteract the effect of the cyanogenetic glycoside linamarin). There is no commercial production of fibre flax in Canada. Recently, plant breeders have been successful in developing a low linolenic-acid edible oil flax for human consumption. In addition to usage of seed for industrial purposes, whole flaxseed is used extensively in baked goods in Europe (Daun, 1993).

Flax is grown primarily in the three prairie provinces of western Canada, specifically in southern Manitoba, Saskatchewan and Alberta. It grows best on heavy loam soils that retain moisture well. Because of its limited root system, flax does not grow well on sandy, moisture-limited soils. Flax is moderately tolerant to salinity provided that soil nutrients are present at adequate levels and that moisture is not limiting at germination. Good weed control is essential as flax is a poor competitor.

Flax may be grown in rotation with cereals or corn but not following potatoes or sugar beets (because of problems with root diseases) or following a previous flax crop. A three-year period is recommended between flax crops to avoid fusarium wilt. Flax may grow poorly after canola or mustard; control of volunteers may minimize the detrimental effects.

Shallow tillage prior to seeding is recommended; this provides a firm seedbed and reduces the number of weed seeds brought to the surface. No or minimum till practices are also beneficial because of better soil organic matter content, moisture retention, and reduced crusting problems that can impair seedling emergence (Daun, 1993).

Seeding is usually done when soil temperatures are warm (mid-May on the prairies), at a rate of 30 to 40 kg/ha and no deeper than 2.5 to 4 cm. If the seed coat has been damaged at harvest, soil-borne fungi may infect the seed; therefore, seed treatment with a fungicide will increase seedling emergence and vigour. Flax does not require as much fertilizer as cereals but will benefit if nitrogen or phosphorus is limiting.

Most varieties of flax are resistant to wilt (*Fusarium oxysporum* f.sp.*lini*) and rust (*Melampspora lini*). Rhizoctonia root rot may be a problem under certain conditions. Weeds must be controlled in the flax crop to minimize losses due to competition.

Flax is usually swathed when 74% of the seed bolls (capsules) have matured and turned brown and, when dry, it can be threshed with a combine. If both plant and seeds are sufficiently dry, straight combining can be done. The combine must be adjusted carefully when threshing flax to prevent seed coat damage and loss of seeds to the ground (Daun, 1993).

B2.0 The Centres of Origin of the Species

The origins of *L. usitatissimum* L. (n = 15), one of the oldest of cultivated plants is uncertain (Lay and Dybing, 1989). Remains of flax, possibly *L. angustifolium* Huds. (n = 15), have been found associated with archaeological remains of early civilizations. The most likely progenitor is *L. angustifolium* but other species such as *L. bienne* Mill. may have contributed some germplasm (Lay and Dybing, 1989). It is generally accepted that, because of the very diverse forms of flax found in an area east of the Mediterranean Sea towards India, flax originated in this area (Zeven and Zhukovsky, 1975). Seed-type flax grown for expressible oil was grown in southwestern Asia, while the fibre types were developed primarily in the Mediterranean. Lay and Dybing (1989) suggest that selection for annual plants with indehiscent or partially dehiscent seed-bearing capsules has resulted in genotypes suitable for modern agriculture.

B3.0 The Reproductive Biology of *L. usitatissimum*

Cultivated flax is an annual reproducing by means of seed. Because of its flower structure and because its "sticky pollen" is rarely transferred by insects (Beard and Comstock, 1980), flax is a highly self-pollinated species. The pollen is viable for only a few hours, from the time of anther dehiscence until about the time the petals dehisce - between 4 and 7 hours (Lay and Dybing, 1989; Dillman, 1938). As the flower opens, the anthers come together and form a cap over the stigma. Dillman (1938) in studying natural crossing in flax reported the range of natural crossing from 0-5%, there being variation among genotypes. In over 8,000 observations in the flax variety "Bison" no natural crossing was observed.

B4.0 Cultivated *L. usitatissimum* as a Volunteer Weed

As with all crops cultivated and harvested at the field scale, some seed may escape harvest and remain in the soil until the following season when it germinates either before or following the seeding of the succeeding crop. In some instances, the volunteers may give considerable competition to the seeded crop and warrant chemical and/or mechanical control. The problem of volunteer plants in succeeding crops is common to most field crop species. Much depends on the management practices used in the production of the crop, e.g., whether the plants have disbursed seed at the time of harvest, the setting of the harvesting equipment, and speed of the harvesting operation which will determine whether more or less seed is lost by the harvester.

B5.0 Summary of Ecology of *L. usitatissimum* and its Progenitors

L. usitatissimum and its progenitors are plants of "disturbed land" habitats. In un-managed ecosystems these species may be considered as "primary colonizers", i.e., plant species that are the first to take advantage of disturbed land where they would compete against plants of similar types for space. Unless the habitats are disturbed on a regular basis, such as on cliff edges, open sites where soil is light or sandy and windblown, river edges and the edges of pathways made by animals, populations of these types of plants will become displaced by intermediaries and finally by plants that will form climax ecologies such as perennial grasses on prairies and tree species and perennial shrubs in forests.

In crop production systems, poor management practices or other circumstances may result in large numbers of seed of *L. usitatissimum* not being harvested and thus finding their way back to the soil. These seed may cause volunteer "weed" problems in succeeding crops, especially if they occur at high density.

Part C - *Linum usitatissimum*: Related Species

C1.0 Inter Species/Genus Hybridization

Important in considering the potential environmental impact following the unconfined release of genetically modified *L. usitatissimum* is an understanding of the possible development of hybrids through interspecific and intergeneric crosses between the crop and related species. Development of hybrids could result in the introgression of the novel traits into these related species and resulting in:

- the related species becoming more weedy
- the introduction of a novel trait with potential for ecosystem disruption into the related species.

The genus *Linum*, to which *L. usitatissimum* belongs, contains more than 100 species differing in chromosome number from 2n = 16, 18, 30, 36 and 60 (Seetharam, 1972). Among nine *Linum* species with chromosome 2n = 30, Gill and Yermanos (1967a) reported the following successful hybridization events with *L. usitatissimum* as one of the parents:

L. usitatissimum x *L. angustifolium*
L. usitatissimum x *L. africanum*
L. corymbiferum x *L. usitatissimum*
L. usitatissimum x *L. decumbens*
L. nervosum x *L. usitatissimum*
L. pallescens x *L. usitatissimum*

Seetharam (1972) reported the following successful crosses among Linum species with 2n = 30:

L. angustifolium x *L. usitatissimum*
L. hirsutum x *L. usitatissimum*
L. floccosum x *L. usitatissimum*
L. tenue x *L. usitatissimum*
L. africanum x *L. usitatissimum*
L. pallescens x *L. usitatissimum*

and, Bari and Godward (1970) the following:

L. africanum x *L. usitatissimum*
L. pallescens x *L. usitatissimum*

Seetharam (1972) attempted crosses between different species having different chromosome numbers, but without any success. Gill and Yermanos (1976b) reported similar results.

C2.0 Potential for Introgression of Genes from *L. usitatissimum* into Relatives

None of the species listed in section C1.0 above are reported in the Canadian flora. The commonest wild *Linum* species reported is *L. lewisii* Pursh, a perennial with a chromosome number 2n = 18. Moss (1983) reports that *L. rigidum* Pursh, a large, yellow-flowered annual, is present on open slopes and grasslands of southern Alberta; it has also been reported on dry open soil in Manitoba (Fernald, 1950). Budd (1987) reports that *L. rigidum* is common locally on sandhills and on very light sandy soils. It is not generally common, but plentiful where found. Scoggan (1978) reports a similar distribution for *L. rigidum*. There are no reports of hybridization between *L. usitatissimum* and *L. rigidum*. There appear to be no relatives in Canada with which *L. usitatissimum* can outcross to form hybrids.

C3.0 Occurrence of *L. usitatissimum* in Canada

Other than in cultivation, *L. usitatissimum* is reported found as an escape in waste places and along roadsides.

Part D - Potential Interactions of *L. usitatissimum* with Other Life Forms

Table 1 is intended to be used to guide applicants in their considerations of potential impacts of the release of the PNT on non-target organisms.

The intention is not to require comparison data between the PNT and its *L. usitatissimum* counterpart(s) for all interactions. Depending on the novel traits, applicants might decide to submit data for only some of the interactions. Sound scientific rationale will be required to justify the decision that data would be useless or irrelevant for the remaining interactions. For example, the applicant might choose not to provide data on the potential for gene transfer from the PNT to related species if it can be clearly shown that the novel trait will not affect reproductive characteristics of *L. usitatissimum*, either directly or indirectly.

Some of the life forms are listed as categories (i.e., pollinators, mychorrhizal fungi, animal browsers, birds, soil microbes, and soil insects). When, because of the novel traits, a concern is perceived for these specific categories, applicants will be required to provide detailed information on interactions with indicator species in each category.

Where the impact of the PNT on another life form (target or non-target organism) is significant, secondary effects may need to be considered.

Table 1. Potential Interactions of *L. usitatissimum* with other life forms during its life cycle.

"X" indicates the type of interaction between the listed organisms and *L. usitatissimum* (information requirements may be waived if valid scientific rationale is provided).

	Interaction with *L. usitatissimum*			
Other life forms	**Pathogen**	**Symbiont or Beneficial Organism**	**Consumer**	**Gene transfer**
Melampsora lini	X			
Fusarium oxysporum f.sp. *lini*	X			
Septoria linicola	X			
Rhizoctonia solani	X			
Polyspora lini	X			
Colletotrichum lini	X			
Seedling blight (specify)	X			
Other diseases (specify)	X			
Aster yellows mycoplasma	X			
Flax bollworm			X	
Aphid			X	
Beneficial insects		X		
Mychorrhizal fungi		X		
Birds			X	
Animal browsers			X	
Soil microbes		X	X	
Earthworms			X	
Soil insects			X	
Other *L. usitatissimum*				X
Others				

Bibliography

Bari, G. and M. B. E. Godward. 1970. Interspecific crosses in Linum, Euphytica 19: 443 -446.

Beard, B.H. and V.E. Comstock. 1980. Flax. In Hybridization of Crop Plants. W.R. Fehr and H.H. Hadley (eds.), American Society of Agronomy - Crop Science Society of America, Madison, WI.

Budd, A. C. 1987. Budd's Flora of the Canadian Prairie Provinces, Agriculture Canada.

Daun, J.K. 1993. Flaxseed. p. 853 to 860 In: Grains and Oilseeds, 4th ed. Vol. 2, Canadian International Grains Institute, Winnipeg, MB

Dillman, A. C. 1938. Natural Crossing in Flax, J. Am. Soc. Agron. 30: 279 - 286.

Fernald, M.L. 1950. Gray's Manual of Botany. Eighth edition (Corrected Printing, R.C. Rollins, 1970). D. Van Nostrand Company, New York, NY. 1632 p.

Gill, K.S. 1987. Linseed. Publications and Information Division, Indian Council of Agricultural Research, New Delhi. 386 p.

Gill, K. S. and D. M. Yermanos. 1967a. Cytogenetic studies on the Genus Linum I. Hybrids among taxa with 15 as the haploid chromosome number. Crop Sci. 7: 623 - 627.

Gill, K. S. and D. M. Yermanos. 1967b. Cytogenetic studies on the Genus Linum II. Hybrids among taxa with 9 as the haploid chromosome number. Crop Sci. 7: 627 - 631.

Lay, C. L. and C. D. Dybing. 1989. Linseed. pp. 416 - 430 In: Oil Crops of the World edited by G. Röbbelen, R. K. Downey and A. Ashri. McGraw-Hill, New York.

Moss, E. H. 1983. Flora of Alberta (2nd. Ed Revised by J. G. Packer), University of Toronto Press.

Scoggan, H. J. 1978. The Flora of Canada, Part 3, National Museum of Natural Sciences, Ottawa, Canada.

Seetharam, A. 1972. Interspecific Hybridization in Linum. Euphytica 21: 489 - 495.

Zeven, A. C. and P. M. Zhukovsky. 1975. Dictionary of Cultivated Plants and their Centres of Diversity. Centre for Agricultural Publishing and Documentation, Wageningen.

Plant Biotechnology Office
Plant Health and Production Division

Decision Document 98-24: Determination of the Safety of the Crop Development Centre's 'CDC Triffid', a Flax (Linum usitatissimum L.) Variety Tolerant to Soil Residues of Triasulfuron and Metsulfuron-methyl

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This Decision Document has been prepared to explain the regulatory decision reached under the guidelines Dir94-08: Assessment Criteria for Determining Environmental Safety of Plants with Novel Traits and its companion document Dir94-10: The Biology of Linum usitatissimum L. (Flax), and the guidelines Dir95-03: Guidelines for the Assessment of Livestock Feed from Plants with Novel Traits.

The Canadian Food Inspection Agency (CFIA), specifically the Plant Biotechnology Office and the Feed Section of the Plant Products Division, with input from the Plant Health Risk Assessment Unit, CFIA, has evaluated information submitted by the Crop Development Centre regarding CDC Triffid. This plant was transformed with genes conferring tolerance to soil residues of the herbicides triasulfuron and metsulfuron- methyl, and resistance to the antibiotic kanamycin and production of nopaline as selectable markers. The CFIA has determined that this plant with novel traits (PNT) should pose no concerns with respect to environmental safety, the safety of livestock consuming feed derived from the PNT, and is considered substantially equivalent to flax by-products currently approved as livestock feed.

Unconfined release into the environment and livestock feed use of CDC Triffid is therefore authorized. Also, any other L. usitatissimum lines and intra-specific hybrids resulting from the same transformation event, and all their descendants, may be released provided that: no inter-specific crosses are performed; the intended use of the plants is the same; and that it is known following thorough characterization that such plants do not display any additional novel traits and are substantially equivalent to currently grown flax in terms of their potential environmental impact and livestock feed safety.

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I. Brief Identification of the Plants with Novel Traits (PNT's)

Designation(s) of the PNT:	CDC Triffid (experimental designation, FP967)
Applicant:	Crop Development Centre of the University of Saskatchewan
Plant Species:	Linum usitatissimum L. (flax, linseed)
Novel Traits:	Tolerance to soil residues of triasulfuron and metsulfuron- methyl; kanamycin (antibiotic) resistance; production of nopaline.
Trait Introduction Method:	Agrobacterium-mediated transformation.
Proposed Use of PNT's:	For cultivation in soils containing residues of triasulfuron and metsulfuron-methyl in areas of Canada where flax is usually cultivated. This flax is to be grown for the extraction of

linseed oil, and for animal feed (linseed meal).

II. Background Information

The Crop Development Centre of the University of Saskatchewan (CDC) has developed a flax line tolerant to soil residues of triasulfuron and metsulfuron-methyl which may result from a previous year's application of the products at labelled rates. Soil residues of these sulfonylurea herbicides, which are registered for use in Western Canada to control broadleaf weeds in wheat (triasulfuron and metsulfuron-methyl) and barley (metsulfuron-methyl), may persist at biologically active rates for several years subsequent to their use. Rotational crop options are restricted during this period of time as commercially unacceptable injury to many crops, including flax, may occur. Only crops such as wheat, oats and barley can be grown in these soils in the season following application of these herbicides or the land must be summer-fallowed. Flax may not be grown until 22 to 34 months after the use of either metsulfuron methyl or triasulfuron. Neither of these sulfonylurea herbicides, nor any other sulfonylurea herbicide is registered for use on flax. While it has been reported that flax may be tolerant to low, postemergent rates of certain sulfonylurea herbicides, researchers have recently reported severe injury to flax (cv. Norlin) when these post emergent sprays were applied (Wall, D.A. and Kenaschuk, E.O. 1996. Flax tolerance to thifensulfuron and tribenuron. Can. J. Plant Sci. 76:899-905).

The flax described herein, designated as CDC Triffid, was developed by the CDC to be cultivated the year following the use of triasulfuron or metsulfuron-methyl to provide an alternative to both the continuous cropping of wheat and barley on these soils and to summer-fallowing during this time.

The development of CDC Triffid was based on recombinant DNA technology and Agrobacterium-mediated transformation. An altered acetolactate synthase enzyme (ALS) from Arabidopsis thaliana was integrated into the genomic DNA of flax to confer tolerance to chlorosulfuron. Two other genes were also inserted: one conferring resistance to kanamycin, the other coding for the enzyme nopaline synthase. Both of these traits were used to select successful transformants in vitro.

CDC Triffid was field tested in Canada, under confined conditions, in Saskatchewan, Manitoba and Alberta from 1989 to 1995.

The CDC has submitted data and information on the identity of CDC Triffid, a description of the modification method, information on the

stability of the insertion of the genes, activity of ALS in CDC Triffid compared to its non-modified counterpart cv. Norlin, molecular characterization of the kanamycin resistance gene, and levels of expression of the kanamycin resistance and nopaline synthase genes. The potential toxicity and the allergenicity of the novel proteins were assessed. Agronomic characteristics such as seed production, time to maturity and plant height were compared to those of the unmodified flax cv. Norlin, its closest counterpart.

The Plant Biotechnology Office of the Plant Products Division, with input from the Plant Health Risk Assessment Unit, on behalf of the Plant Protection Division, CFIA, has reviewed the information submitted by the CDC for the determination of environmental safety, based on the following assessment criteria as described in the regulatory directive Dir94-08: Assessment Criteria for Determining Environmental Safety of Plants with Novel Traits:

* potential of the PNT to become a weed of agriculture or be invasive of natural habitats
* potential for gene flow to wild relatives whose hybrid offspring may become more weedy or more invasive
* potential for the PNT to become a plant pest
* potential impact of the PNT or its gene products on non-target species, including humans
* potential impact on biodiversity

The Feed Section of the Plant Products Division, CFIA, has also reviewed the information submitted by the CDC based on the following assessment criteria for determining safety and efficacy of livestock feed, as described in the regulatory directive Dir95-03: Guidelines for the Assessment of Livestock Feed from Plants with Novel Traits:

* potential impact on livestock
* potential impact on livestock nutrition

III. Description of the Novel Traits

1. Tolerance to Soil Residues of Triasulfuron and Metsulfuron-methyl

* Sulfonylurea herbicides, such as triasulfuron and metsulfuron-methyl, target and bind to the enzyme acetolactate synthase (ALS) thereby inhibiting the biosynthesis of the branched chain amino acids valine, leucine and isoleucine and resulting in the accumulation of toxic levels of alpha-ketoglutarate.

* In addition to its native ALS gene, CDC Triffid contains an als

gene from a chlorsulfuron tolerant line of A. thaliana. This variant als gene differs from the wild type A. thaliana gene by one nucleotide and the resulting ALS enzyme differs by one amino acid from the wild type ALS enzyme. The inserted als gene is linked to its native promoter and terminator.

* Enzyme extracts from CDC Triffid exhibited a slightly higher ALS activity compared to its non-modified counterpart cv. Norlin. Whereas the statistical significance of this higher activity could not be verified, it may be expected due to the presence of at least two additional copies of the als gene in CDC Triffid.

2. Kanamycin Resistance

* Kanamycin is an aminoglycosidic antibiotic that binds to bacterial ribosomes thus disrupting normal protein synthesis and killing the bacterial cell.

* The kanamycin-resistance gene, nptII, isolated from the bacterium Escherichia coli, codes for an enzyme, neomycin phosphotransferase II (NPTII) that phosphorylates kanamycin. This prevents kanamycin from binding to ribosomes and renders the cells resistant to the antibiotic. Plant cells that have been co-cultured with A. tumefaciens are plated onto a growth medium containing kanamycin. Those plant cells that have been successfully transformed and thus contain the kanamycin-resistance gene, are able to grow on this medium.

* The nptII gene is linked to a constitutive promoter. The CDC has demonstrated, using PCR analysis, that the inserted nptII gene was unaltered when compared to the native E. coli gene. The gene product, NPTII, was detected in seeds, cotyledons and leaves of CDC Triffid.

* NPTII is ubiquitous in the environment. It degrades rapidly in vitro in simulated mammalian gastric and intestinal fluids.

3. Nopaline Synthase

* Nopaline synthase is an enzyme which catalyses the synthesis of nopaline, an opine which is formed as the result of the condensation of the amino acid arginine and alpha-ketoglutaric acid. When wild-type A. tumefaciens infects a host plant, the opine synthase gene present on the T-DNA region of the Ti plasmid of the bacterium directs infected host cells to synthesize an opine, such as nopaline. The type of opine produced is specific to the

particular strain of A. tumefaciens. Opines are metabolized as a source of carbon and nitrogen only by a bacterium possessing a Ti-plasmid and the gene specific for catabolism of the particular opine.

* The nopaline synthase gene, nos, is linked to its native promoter which is known to be constitutive with a slightly stronger expression in roots.

* Levels of nopaline were quantified at two growth stages in CDC Triffid: seedling and pre-flowering, and was detected in roots, stems and leaves. Expression in roots ranged from 0.05 to 0.63 mg of nopaline/g tissue (fresh weight) at the seedling stage, and 0.07 to 0.47 mg/g (f.w.) at pre-flowering; in stems, at 0.01 to 0.33 mg/g (f.w.) at the seedling stage, and 0.02 to 0.12 mg/g (f.w.) at pre-flowering; and leaves, at 0.05 to 2.4 mg/g (f.w.) at the seedling stage, and 0.23 to 0.72 mg/g (f.w.) at pre- flowering. Nopaline was not detected in seeds.

* A sequence homology search for nopaline synthase revealed similarity only to other opine synthases and no similarity was found to known sequenced toxins or allergens.

* This trait was introduced to permit the identification of transformed plant embryos. Nopaline was detected in successfully transformed plant embryos.

4. Other novel DNA sequences

Genes conferring resistance to the antibiotics ampicillin, carbenicillin and spectinomycin were present in an E. coli vector used as an intermediary during the cloning of the target sequences to be transferred to flax. These three antibiotic resistance genes were under the control of a bacterial promoter which is active only in bacteria and not active in plants. These genes are therefore not expected to be expressed in CDC Triffid. Portions of Hind III fragments 10 and 23 (Zambryski, P., Joos, H., Genetello, C., Leemans, J., Van Montagu, M., and Schell, J. 1983. Ti plasmid vector for the introduction of DNA into plant cells without alteration of their normal regeneration capacity. EMBO 2:2143-2150.) from the nopaline Ti plasmid pTiC58 were also incorporated.

5. Development Method

* The flax cv. Norlin was transformed using the disarmed A. tumefaciens Ti-plasmid vector pGV3850. The vector contained the

T-DNA region of an A. tumefaciens plasmid from which oncogenic regions had been deleted and replaced with the mutant als gene and the kanamycin resistance gene.

* Successful transformants were selected in vitro, on the basis of expression of kanamycin resistance and presence of nopaline. These were subsequently grown on medium containing chlorsulfuron to confirm the expression of the inserted chlorsulfuron-tolerant als gene.

6. Stability of Insertion of the Traits

Data were obtained from the literature (Mc Sheffrey, S. A., Mc Hughen, A., and Devine, M. D. 1992. Characterization of transgenic sulfonylurea-resistant flax (Linum usitatissimum). Theoretical and Applied Genetics 84:480-486.) showing that the genes inserted into CDC Triffid were integrated in at least two unlinked loci, with a possible third locus exhibiting partial linkage to one of the other two. The expression of the genes remained stable after eight generations of selfing CDC Triffid plants.

IV. Assessment Criteria for Environmental Safety

1. Potential of the PNT to Become a Weed of Agriculture or Become Invasive of Natural Habitats

The biology of flax (Linum usitatissimum L.), described in Dir94-10: The Biology of Linum usitatissimum L. (Flax), states that unmodified plants of this species are not invasive of unmanaged habitats in Canada, nor are they considered a weed.

CFIA evaluated information submitted by the CDC, on the reproductive and survival biology of CDC Triffid, and determined that growth habit, vegetative vigour, and seed yield, were substantially equivalent to its non-modified counterpart. No genes for cold tolerance or winter hibernation were inserted in CDC Triffid.

As described in Dir94-10, flax may appear in fields where the previous year's flax crop was harvested late resulting in seed pod shattering and escape of seed to the ground. As flax is a poor competitor, the presence of volunteers in a competitive succeeding crop such as cereals or canola would not be expected to cause significant yield loss. Flax volunteers can, however, cause difficulties as volunteers still green at harvest may interfere with crop harvesting and causing grain storage problems (Flax Council of Canada. 1996. Growing Flax: Production, Management and Diagnostic Guide. 3rd edition (Flax Council of Canada, 465 - 167 Lombard

Avenue, Winnipeg, Manitoba, R3B 0T6).). CDC Triffid may also volunteer in soils where it is intended to be grown (i.e. in soils containing residues of triasulfuron and metsulfuron-methyl). Since CDC Triffid does not possess any novel traits that would confer increased competitiveness, these volunteers would not be expected to differ in their seriousness as a weed compared with other non-transformed flax varieties. Some suppression of CDC Triffid volunteers, as well as volunteers of other flax varieties can be provided with the use of herbicides that contain dichlorprop or dicamba or with a recently registered herbicide containing quinclorac.

The above considerations, together with the fact that the novel traits have no intended effects on weediness or invasiveness, led the CFIA to conclude that CDC Triffid does not possess altered weed or invasiveness potential compared to currently commercialized flax.

2. Potential for Gene Flow to Wild Relatives Whose Hybrid Offspring May Become More Weedy or More Invasive

The biology of flax, as described in Dir94-10, indicates that there are no wild relatives in Canada that can freely hybridize with L. usitatissimum. The CFIA therefore concludes that gene flow from CDC Triffid to wild relatives is not possible in Canada.

Flax is predominantly a self-pollinating species, with a rate of natural outcrossing ranging from 0 to 5%, and this species possess does not possess any mechanism for wind or insect pollination. Thus gene flow to other L. usitatissimum is expected to occur at low frequency, if at all.

3. Altered Pest Potential

The intended effects of the novel traits are unrelated to plant pest potential, and flax is not a plant pest in Canada (Dir94-10). Furthermore, agronomic characteristics of CDC Triffid were shown to be within the range of values displayed by currently commercialized flax, which suggests that CDC Triffid has not been inadvertently altered. The CFIA has therefore determined that CDC Triffid does not display any altered pest potential.

4. Potential Impact on Non-Target Organisms

As discussed in Section III.3, CDC Triffid produces nopaline due to expression of the nos gene. Only A. tumefaciens strains of the nopaline sub-group and some Pseudomonas species produce the enzyme necessary for the catabolism of nopaline. Since oncogenic A. tumefaciens strains utilize this compound as a carbon and nitrogen source, the presence of

nopaline in soils that have been cultivated to CDC Triffid may provide an environment for enrichment of this soil bacterium. Many dicotyledonous plants (dicots) and some gymnosperms (e.g. conifers) are susceptible to infection by A. tumefaciens. Monocotyledonous plants (monocots), such as small grain cereals, are not susceptible to infection by this bacterium.

A residual effects trial was conducted by the CDC in 1995, where a monocot and a dicot crop were grown on soil cultivated the previous year to CDC Triffid and its non-modified counterpart cv. Norlin. Crown galls were not detected on the dicot crop in the year of cultivation and there were no observed differences in plant counts of the dicot crop in those plots previously cultivated to CDC Triffid when compared to cv. Norlin.

NOTE: The effect on dicot crops (e.g. canola, lentils) grown in soils previously cultivated to CDC Triffid flax has not been fully assessed. As with all other flax varieties, cultivation of CDC Triffid for more than one growing season should not be encouraged and rotation with monocots (e.g. cereals) should be advised. It is presently recommended that flax not be grown the year following a previous flax crop in order to prevent the buildup of root diseases (e.g. Melampsora lini), and a three-year period is recommended between flax crops to avoid fusarium wilt (Dir94-10). These recommendations should be judiciously followed, and monitoring for the presence of crown galls in subsequent dicotyledonous crops should also be encouraged.

5. Potential impact on biodiversity

CDC Triffid flax does not possess novel phenotypic characteristics which would extend its use beyond the current geographic range of flax production in Canada. Since flax does not outcross to wild relatives in Canada, there will be no transfer of novel traits to unmanaged environments.

The presence of nopaline in plant tissue may contribute to the enrichment of A. tumefaciens in soils where CDC Triffid has been cultivated. A. tumefaciens is a ubiquitous soil bacterium and crown gall disease is a common occurrence in many agro-ecosystems. Given this, enrichment of A. tumefaciens in soils planted to CDC Triffid should not have a negative impact on the biodiversity of the rhizosphere.

Cultivation of CDC Triffid will increase rotational cropping options in areas where triasulfuron and metsulfuron-methyl have been used. CDC Triffid should not be planted in consecutive growing seasons in a single rotation.

VI. Nutritional Assessment Criteria for Use as Livestock Feed

1. Nutritional Composition of PNT

Proximate analysis data for FP967 versus cv. Norlin (control) obtained from samples from three Saskatchewan plots in 1994 were evaluated. There were no differences in protein or ether extract content between the two lines. Crude fibre was significantly higher in FP967 than in Norlin, but was within the normal range for flax. For the amino acids valine, leucine, isoleucine and arginine, data were obtained from two samples of FP967 per site from eight sites, and from two samples of cv. Norlin from one site. There were no differences between cv. Norlin and FP967 in these amino acids.

2. Antinutritional Factors

The concentration of total cyanogenic compounds (total of linamarin, linustatin, neolinustatin) measured in three samples of each line, taken from one site, showed no differences between FP967 and cv. Norlin.

VII. Regulatory Decision

Based on the review of data and information submitted by the Crop Development Centre, the Plant Biotechnology Office of the Plant Products Division, CFIA, has concluded that the novel genes and their corresponding traits in CDC Triffid should not confer any characteristics that would result in intended or unintended ecological effects following unconfined release, as long as this plant is properly managed. Proper management should include discouraging the continuous cropping of CDC Triffid.

Based on the review of data submitted to the Feed Section of the Plant Products Division, CFIA, concludes that CDC Triffid with its novel traits does not in itself raise any concerns regarding livestock safety or nutritional composition. Flax seeds, flax oil, and flax meal are currently listed in Schedule IV of the Feeds Regulations and are, therefore, approved for use in livestock feeds in Canada. CDC Triffid has been determined to be substantially equivalent to traditional flax varieties, therefore, it meets the present ingredient definition of flax seeds and derivatives and is approved for use in Canada.

Unconfined release into the environment and livestock feed use of CDC Triffid is therefore authorized. Also, any other L. usitatissimum lines and intra-specific hybrids resulting from the same transformation event, and all their descendants, may be released, provided that: no inter-specific crosses are performed; the intended use is the same; and that

it is known following thorough characterization that these plants do not display any additional novel traits and, are substantially equivalent to currently grown flax, in terms of their potential environmental impact and livestock feed safety.

This bulletin is published by the Plant Products Division, Canadian Food Inspection Agency. For further information, please contact the Plant Biotechnology Office or the Feed Section of the Plant Products Division at the following address:

Canadian Food Inspection Agency
Plant Products Division
59 Camelot Drive
Nepean, Ontario K1A 0Y9

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March 2,1998

Plant Biotechnology Office
Plant Health and Production Division
Canadian Food Inspection Agency

Agriculture and Agri-Food Canada
Agriculture et Agroalimentaire Canada
Food Production and Inspection Branch
Direction générale de la production et de l'inspection des aliments

CERTIFICATE OF REGISTRATION

This is to certify that under the Canada Seeds Act

the ***oilseed flax*** *variety designated* ***CDC Triffid***

has been registered in accordance with the terms and conditions of

Sections 68 and 69 of the Seeds Regulations.

REGISTRATION DATE	*REGISTRATION NUMBER*
May 8, 1996	*4338*

Registrar
Variety Registration Office
Plant Products Division

Canada

AAFC / AAC4286 (95/09)

Health Canada
Santé Canada

Health Protection Branch
Direction générale de la protection de la santé

Tunney's Pasture
Ottawa, Ontario
K1A 0L2

February 16, 1998

Dr. Alan McHughen
Crop Development Centre
University of Saskatchewan
51 campus Drive
Saskatoon, Saskatchewan
S7N 5A8

Dear Dr. McHughen:

This will refer to the Novel Food Submission concerning sulfonylurea tolerant flax, specifically CDC Triffid Flax. Officers of the Health Protection Branch have reviewed the information that the Crop Development Centre of the University of Saskatchewan provided for assessment of the acceptability of the subject transgenic flax for sale as human food in Canada.

According to the submitted information, the procedure used in developing the subject flax, designated CDC Triffid Flax, involved the introduction of an acetolactate synthase (ALS) gene from a mutant *Arabidopsis*. In the mutant, the ALS gene has a single base pair substitution which results in a single amino acid change in the translated protein. The resulting ALS enzyme is not sensitive to the sulfonylurea herbicide. In addition to the ALS gene, several marker genes were also included for the purpose of development.

As a result of this genetic modification, the sulfonylurea tolerant CDC Triffid Flax contains the following novel constituents:

.../2

(1) the mutant ALS gene;

(2) the sulfonylurea tolerant enzyme acetolactate synthase encoded by the mutant ALS gene;

(3) the nopaline synthase gene as a selectable marker;

(4) the nopaline synthase enzyme encoded by the nopaline synthase gene;

(5) the *nptII* gene;

(6) the neomycin phosphotransferase II enzyme encoded by the *nptII* gene providing resistance to the antibiotic kanamycin;

(7) the ampicillin resistance gene; and,

(8) the spectinomycin/streptomycin gene.

The ampicillin resistance and spectinomycin/streptomycin resistance genes, though present in the transgenic flax, are under the control of bacterial promoters and are not expressed in CDC Triffid Flax.

The result of this genetic modification is the expression of the mutant ALS enzyme in CDC Triffid Flax, conferring tolerance to sulfonylurea herbicide.

Based on our evaluation of the submitted data, we have no objection to the sale of sulfonylurea tolerant CDC Triffid Flax as human food in the same usages as other flax varieties in Canada.

It should be noted that this opinion is solely with respect to the suitability for sale as human food of CDC Triffid Flax. It is the continuing responsibility of the Crop Development Centre of the University of

.../3

Saskatchewan to ensure that its products are in compliance with all applicable statutory and regulatory requirements.

Please note that we are providing our colleagues in the Canadian Food Inspection Agency (CFIA) with a copy of this letter in regard to that Agency's responsibility respecting animal feeds, environmental release and labelling issues. We are also providing our colleagues in the Pest Management Regulatory Agency (PMRA) with a copy of this letter for their information.

Yours truly,

George M. Paterson, Ph.D.
Director General
Food Directorate

c.c. Dr. A. MacKenzie, CFIA
Dr. C. Franklin, PMRA

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
Washington DC 20204

MAY 15 1998

Dr. Alan McHughen
Crop Development Center
University of Saskatchewan
51 Campus Drive
Saskatoon SK S7N 5A8
Canada

Dear Dr. McHughen:

This is in regard to your genetically modified flax variety, CDC Triffid, about which you initiated consultations with the Agency on October 2, 1997. According to the submission, the new flax variety produces acetolactate synthase (ALS protein), which is derived from *Arabidopsis*, and confers tolerance to sulfonylurea herbicides.

As part of bringing your consultation with FDA regarding this product to closure, you submitted a summary of your safety and nutritional assessment concerning CDC Triffid flax on October 27, 1997, and February 6 and 10, 1998. These communications informed FDA of the steps taken by you to ensure that the product complies with the legal and regulatory requirements that fall within FDA's jurisdiction. Based on the safety and nutritional assessments you have conducted, it is our understanding that you have concluded that flax seed derived from the new variety is not materially different in composition, safety, and other relevant parameters from flax seed currently on the market, and that the genetically modified flax does not raise issues that would require premarket review or approval by FDA. All materials relevant to this notification have been placed in a file designated BNF0050. This file will be maintained in the Office of Premarket Approval.

Based on the information you have presented to FDA, we have no further questions concerning CDC Triffid flax at this time. However, as you are aware, it is your responsibility to ensure that foods marketed by you are safe, wholesome and in compliance with all applicable legal and regulatory requirements.

Sincerely yours,

Laura M Tarantino

Alan M. Rulis, Ph.D.
Director
Office of Premarket Approval
Center for Food Safety
and Applied Nutrition

United States Department of Agriculture

Marketing and Regulatory Programs

Animal and Plant Health Inspection Service

4700 River Road
Riverdale, MD 20737

Professor Alan McHughen
Crop Development Centre
University of Saskatchewan
51 Campus Drive
Saskatoon, SK S7N 5AB
CANADA

July 31, 1998

Dear Professor McHughen:

I am writing in response to your letter of May 15, in which you requested an opinion on the regulatory authority of USDA-APHIS with respect to the transgenic flax variety 'CDC Triffid'.

Based upon the information in your letter and the intended use of this plant material for processing, we believe that this variety should pose no plant pest risk. Therefore, APHIS will not consider seed of CDC Triffid, moved from Canada to the United States for processing, as regulated articles under our regulations (7 CFR Part 340).

We have based our decision on the factors summarized below:

1. The intended use of the flax seed is processing by crushing. After processing, the remaining plant material is nonviable.

2. For years, flax seed has been shipped from Canada for processing at facilities located in the United States. APHIS is unaware of any plant pest problems that have been associated with such seed shipments or the handling of the remaining plant material after processing of the seeds.

3. APHIS takes note of the analysis and approvals of this material conducted by Agriculture and Agri-Food Canada and Health Canada.

4. APHIS also believes that standard industry practices for the shipment of the material to a processing plant are adequate and should not present any plant pest risk. There is no indication that the shipment of other flax varieties has ever resulted in a plant pest risk.

I emphasize that our opinion regarding this variety is limited to the conditions that you have described, namely, shipment of seed to processing plants in the United States. Under the circumstance of this intended use (i.e., shipment to a processing facility), APHIS would not regulate this flax variety under APHIS regulations found under 7 CFR Part 340.

However, if this plant material is shipped for other uses or purposes, it may be subject to regulation under 7 CFR Part 340. In particular, cultivation in the United States would require you to petition for a determination of non-regulatory status. Details on such a petition are given at our web site at www.aphis.usda.gov/biotech.

If you have any further questions about this matter, please feel free to contact me.

Sincerely,

Arnold Foudin

Arnold Foudin
Assistant Director
Scientific Services
Plant Protection and Quarantine

Appendix 2, appendix 3

Figure 1 maps the T-DNA in the plasmid used to develop CDC Triffid. The map shows the structural genes (ALS, NPT-II and two NOS, as well as the left border and both right border sequences and the probe DNA (thick line in NOS sequence). Restriction analyses were conducted on Norlin and CDC Triffid genomic DNA, using NCO I and Hind III (approximate sites shown on fig. 1). NCO I cuts within the probe sequence, while Hind III cuts in between the two NOS genes in the T-DNA but not in the probe DNA itself. In addition, genomic DNA from CDC Triffid and Norlin were restricted with Dra I, which cuts the T-DNA, but not in or between the probe sequences.

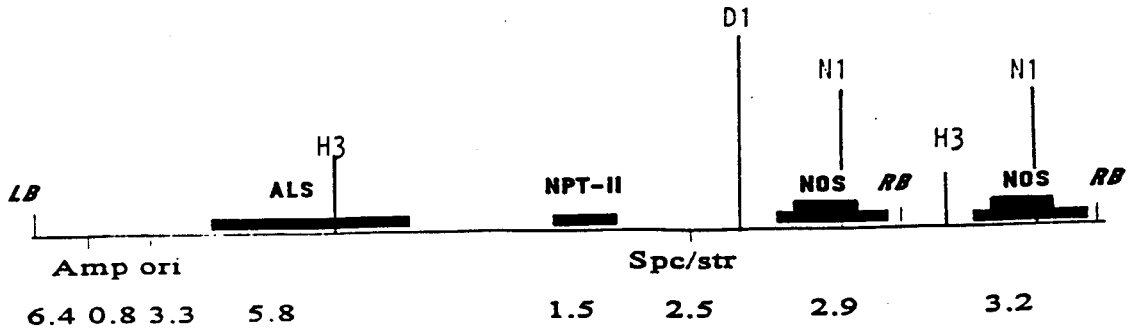
In the Southern (figure 2), the NCO I digest shows five bands, the smallest one with the heavier intensity likely due to it being the internal fragment (composed of two probe- homologous sequences/haploid genome), the other four bands likely representing the different size fragments 5' to each locus of insertion and those 3' to each locus of insertion. The map predicts the distance between the two NOS sequences to be approx 3Kb; the most intense band in the NCO I lane is about 3Kb. No bands appeared in the lane using DNA from the non-transgenic parent line Norlin (left lane).

The Hind III digest for Norlin (left lane) gives no bands. The Hind III digest for CDC Triffid gives three bands: the smallest one is most intense, probably representing the internal fragment, and two lighter bands, probably representing the (predominantly) plant DNA fragment 3' to each of the two loci of insertion. The internal band, approx. 9Kb, was predicted from the map (fig 1).

In the Agrobacteria, pGV3850 includes a 3.2kb Hind III fragment from pTiC58, a nopaline type Agrobacterium; this fragment consists of the RB and the proximal NOS sequence, along with a portion of plasmid DNA 3' of the RB. The presence of such a fragment from CDC Triffid DNA would indicate the DNA transferred from the bacterium was not limited to the sequence bounded by the Right Border. Note here that the fragment is missing from the Hind III digest, indicating the T-DNA was indeed limited to the RB, and did not extend into the non-T-DNA region of the plasmid.

The Dra I digest, probed in the rightmost pair of lanes, shows two bands for CDC Triffid, indicating two diffferent loci of insertion. Again, the Norlin lane was clear.

Taken collectively, these data show there are two insertions of T-DNA in CDC Triffid at different loci, that the transferred DNA does not include bacterial DNA outside the T-DNA, and that the T-DNA is stable over several seed generations.



Hind III = H3
NCO 1 = N1
Dra 1 = D1

Figure 1: T-DNA in CDC Triffid.

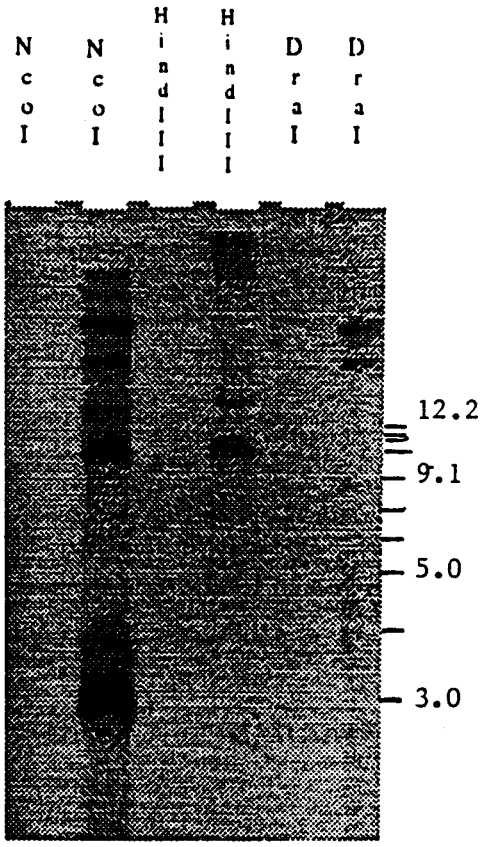
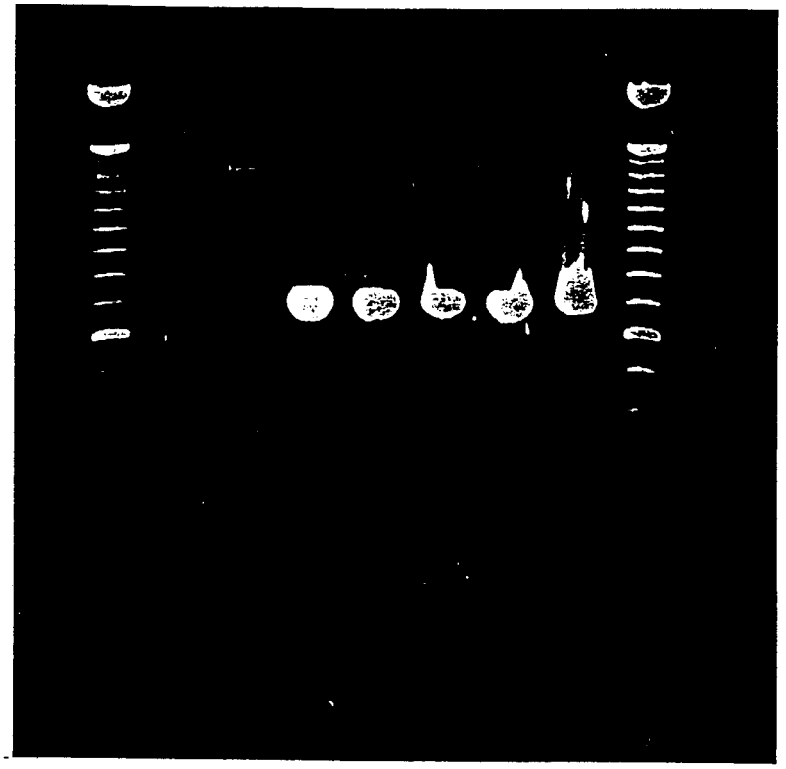


Figure 2. Southern of Norlin and CDC Triffid genomic DNA. From left: Nco I digest of Norlin; Nco I digest of CDC Triffid; Hind III digest of Norlin; Hind III digest of CDC Triffid; Dra I digest of Norlin; Dra I digest of CDC Triffid. Gibco-BRL 1kb marker on right.
(from Sigfrid, K. BSA Hons. Thesis, Characterizing the T-DNA insert in CDC Triffid flax. Univ. of Saskatchewan, 1997.

Appendix 4, figure 3



1 2 3 4 5 6 7

Figure 3. PCR reaction using genomic DNA from Norlin (lane 1 and 2) or from several different samples of DNA from FP967 (lanes 3,4,5, and 6). Lane 7 contained DNA from the inoculating bacteria. The primers, based on the *npt*-II DNA sequence, were GAGGCTATTCGGCTATGACTG and ATCGGGAGCGGCGATACCGTA, which gives a band of approx. 700 bases. All the FP967 samples contained the target sequences, while neither of the parental Norlin samples did.

Table 3. Wilt disease reactions for FP967 and check varieties

Entry	Fusarium wilt reaction		
	1992 Morden	1993 Morden	1993 Indian head
Bison	1.4	2.4	2.1
Redwing	2.4	3.8	3.0
McGregor	2.7	3.8	2.4
Norlin	2.4	3.8	2.5
Vimy	1.5	3.3	2.5
Somme	2.6	4.0	2.6
Flanders	2.9	4.3	2.5
AC Linora	1.5	2.6	2.3
FP967	2.6	3.6	2.9

Wilt reading were on a scale of 1-9 (1=healthy, most vigorous; 9= severely wilted or dead plants). Scores are means of 3 readings of 4 replications (2-row plots). In no case was there a significant difference between FP967 and Norlin.

Percentage susceptible to Rust was zero, for both Norlin and FP967. Seedlings were exposed to race 371 under controlled growthroom conditions.

Table 4a. Yield data for 1991 trial (harvested May 5, 1992), presented as the mean of four reps, in kg/ha. Means followed by the same letter do not differ significantly (p= 0.05; Duncan's Multiple Range Test).

genotype	untreated	Metsulfuron methyl 4.5g/ha	Metsulfuron methyl 9.0g/ha	Triasulfuron 8.0g/ha	Triasulfuron 25g/ha
McGregor	877.4 a-e	514.1 f-l	421.0 i-l	463.1 h-l	328.0 j-l
Norlin	787.1 a-g	567.9 e-l	507.4 f-l	391.3 jkl	464.2 h-l
FP967	868.9 a-e	1106.1 a	948.6 abc	897.6 a-d	777.6 b-h

Table 4b. Yield of 1992 trial (harvested Oct. 23, 1992), presented as the mean of four reps for each treatment, in kg/ha equivalent. Means followed by the same letter do not differ significantly (p= 0.05; Duncan's multiple range test.)

genotype	untreated	Metsulfuron methyl 4.5g/ha	Metsulfuron methyl 9.0g/ha	Triasulfuron 8.0g/ha	Triasulfuron 25g/ha
McGregor	1258 a-e	659 f-j	665 f-j	844 c-i	626 g-j
Norlin	1192 a-f	813 d-i	662 f-j	1105 a-h	558 hij
FP967	1396 abc	1535 ab	1661 a	1515 ab	1199 a-f

Table 4c. Yield of 1993 trial (harvested Oct. 8, 1993), presented as the mean of four reps for each treatment, in kg/ha equivalent. Means followed by the same letter do not differ significantly (p=0.05; Duncan's multiple range test).

		Metsulfuron methyl		Triasulfuron	
genotype	untreated	4.5g/ha	9g/ha	8g/ha	25g/ha
Norlin	2368 a-e	1943 c-g	1240 h	2333 a-e	1952 b-g
Vimy	2168 a-f	2148 a-f	1592 fgh	2611 ab	1787 eh
Somme	2257 a-e	1598 fgh	1329 gh	2673 a	1869 d-g
FP967	2228 a-f	2449 a-d	2614 ab	2448 a-d	2627 a

Table 4d. Yield as percentage of Norlin in untreated soil. Data average of three years (1991-1993), except McGregor (1991, 1992), and Somme and Vimy (1993).

		Metsulfuron methyl		Triasulfuron	
Genotype	untreated	4.5g/ha	9g/ha	8g/ha	25g/ha
Norlin	100	74	58	81	63
Vimy	92	91	67	110	75
Somme	95	67	56	113	79
McGregor	109	61	55	65	48
FP967	107	125	124	115	104

Appendix 5 ii

Supplementary data for FP967. Alan McHughen *et al.* Feb, 1994

Theoretically, there should be no critical effect of the transformation process on overall composition of FP967 compared to traditional flax. Similarly, because of the nature of the novel trait being an enhancement of natural resistance to sulfonylureas, the composition of FP967 grown in soil containing residue of the herbicide is not expected to differ significantly from FP967 grown in the absence of sulfonylurea, or indeed, from traditional flax genotypes grown in the absence of sulfonyurea.

Supplementary table 1. Data on the composition of FP967 grown in soil containing metsulfuron methyl at double recommended dose, breeder seed of FP967 (grown in the absence of sulfonylureas) and the observed range for traditional flax for each parameter. These "standards" are taken from recent publications from Canadian labs (Bhatty, R.S. JAOCS 67:79, 1990; JAOCS 70: 899,1993. Oomah et al., J Agr.Food Chem. 40: 1346, 1992). All data for FP967 were dervived from samples grown at the Kernen Research Farm, while the data for the "normal" range were from flax grown at varied locations.

Component	FP967 in 2xSU	FP967 Breeder	Normal range
Ash %	5.2	5.6	5.4-7.8
Protein %	36.3	34.5	35.0-45.6
Oil %	40.6	40.8	n.a.
Viscosity, cS	21.3	19.5	23.3-68.2
Cyanogenic Glycosides mg/g	n.a.	7.7	6.0-9.2
Major minerals (mg/g)			
P	8.5	8.8	7.3-12.6
K	11.6	11.8	9.1-15.8
S	3.7	3.6	3.8-4.2
Ca	3.9	3.8	3.9-7.4
Minor minerals (ug/g)			
Cu	16.4	12.0	17.4-24.2
Fe	131.1	115.4	157.4-274.0
Mn	59.8	36.1	47.8-71.3
B	35.7	38.1	n.a.

Appendix 5

Statistical table (t-test) for proximate analysis data of Norlin flax vs. FP967 flax conducted by Sask. Feed Testing lab. For all means, Norlin had 12 samples, while FP967 had 13. Abbreviations: s.d. standard deviation; d.f. degrees of freedom; sig? significance; n.s. not significant.

	Norlin		FP967				
%	mean	s.d.	mean	s.d.	t-value	d.f.	sig?
Moisture	4.94	0.23	4.87	0.91	0.26	14	n.s.
Crude Protein	21.1	0.84	21.29	0.84	0.53	23	n.s.
Total ash	2.88	0.24	2.87	0.22	0.1	22	n.s.
Crude fibre	7.63	0.71	8.13	0.59	1.9	22	n.s.

Appendix 2 Feb 2/94

Request for Support for Registration

Common name: Flax

Latin name: *Linum usitatissimum* L.

Experimental designation: FP 967

Proposers: A. McHughen, G.G. Rowland, F.A. Holm, E.O. Kenaschuk* and R.S. Bhatty. Crop Development Centre, University of Saskatchewan, Saskatoon, Sask., S7N 0W0. (*:Agriculture Canada Research Station, Morden, Manitoba R0J 1G0)

Origin and Breeding:

FP 967 was developed at the Crop Development Centre, University of Saskatchewan. The line originated as a single Norlin plant, into which was inserted a modified acetolactate synthase gene from *Arabidopsis*. This gene confers upon the line the ability to grow in soil containing residue of sulfonylurea herbicides. In essence, this flax line is basically the same as Norlin, but with the additional attribute of being able to be included in a rotation with cereals where sulfonylureas (eg. metsulfuron methyl, (Ally; DuPont) or triasulfuron (Amber; Ciba-Geigy) were used to control broadleaf weeds.

In addition to the *Arabidopsis* ALS gene, FP967 also carries two genes of no agronomic relevance, a neomycin phosphotransferase-2 gene and a nopaline synthase gene, for identification purposes.

Varietal characteristics:

FP967 is similar to Norlin in most respects. It is an early maturing variety with seed yield, seed weight, oil content and quality similar to the check varieties, especially Norlin (Tables 1,2). Lodging resistance is similar to Norlin, based on results from the 1992 Co-operative test and regional trials (lodging data from 1993 not available at present time). Like Norlin, FP967 is immune to Rust race 371, and is moderately resistant to Fusarium wilt (Table 3). Under conditions of sulfonylurea residue activity in the soil, FP967 significantly outperforms all other commercial flax cultivars tested (Tables 4,a-d). The presence of sulfonylurea in the soil at the concentrations tested also does not significantly affect other agronomic or quality characteristics, in that the results are similar to the results in untreated soil. That is, the presence of sulfonylurea does not significantly affect FP967 (see Tables 4,a-d, and McHughen and Holm, Euphytica, **55**: 46-56, 1991, for specific data).

Adaptation:

FP967 is well adapted across the flax growing area of western Canada. However, it will be of special interest to those producers who use long residual action sulfonylurea herbicides on their cereal crops, as FP967 will provide a broadleaf crop option in their rotation. Currently, such farmers must either summerfallow or continuous crop to cereals. FP967 will provide an alternative to those agronomically and environmentally undesirable practices.

Performance:

-strengths

The major advantage of FP967 is the ability to be incorporated into a rotation where long residual acting sulfonylurea herbicides are used to control weeds in cereal crops.

-weaknesses

While there are no significant weaknesses to FP967, producers who do not use long residual sulfonylureas could probably find other commercial flax cultivars as good as or better suited to their operation.

Seed supply

Approx. 200kg of Breeder seed of FP967 has been generated.

Table 1. Performance of FP967 in Co-operative tests.
Average agronomic and quality data, 1992 and 1993

Western Canada:

Entry	Yield '00kg/ha	Yield as % Norlin	Maturity (days)	height (cm)	seed wt mg	oil cont. %	iodine no.	protein %
Norlin	20.5	100	115.6	63.8	5.9	44.5	192	32.3
McGregor	20.6	100	120.9	65.8	5.4	45.3	192	32.4
Vimy	17.2	84	115.7	63.9	6.2	45.6	194	32.6
Somme	19.8	97	115.2	63.8	5.7	44.7	196	32.4
Flanders	21.3	104	118.2	61.6	5.4	46.7	195	32.1
AC Linora	20.6	98	117.4	63.1	5.7	48.4	195	32.7
FP967	20.0	98	114.4	63.1	5.9	44.3	191	32.3
LSD 5%	0.5		1.2	1.0	0.2	0.6	1.6	0.2
No. of tests	29		19	32	23	32	32	18

Black soil zone

Entry	Yield '00kg/ha	Yield as % Norlin	Maturity (days)	height (cm)	seed wt mg	oil cont. %	iodine no.	protein %
Norlin	20.4	100	115.1	69.9	5.8	43.7	190	33.4
McGregor	20.9	102	121.0	73.1	5.4	44.6	192	33.1
Vimy	15.1	74	114.4	69.2	5.9	44.5	192	33.6
Somme	19.2	94	115.0	69.5	5.5	43.9	195	33.0
Flanders	20.8	102	117.9	68.0	5.3	45.9	195	33.3
AC Linora	20.7	101	117.0	69.0	5.6	45.4	195	33.3
FP967	19.8	97	114.6	68.3	5.8	43.6	190	32.9
LSD 5%	0.8		1.2	1.2	0.2	0.6	1.7	0.2
No. of tests	14		12	14	14	14	14	10

Brown soil zone

Entry	Yield '00kg/ha	Yield as % Norlin	Maturity (days)	height (cm)	seed wt mg	oil cont. %	iodine no.	protein %
Norlin	20.6	100	117.6	60.9	5.8	45.1	192	30.5
McGregor	20.2	98	122.3	62.3	5.4	45.9	191	30.7
Vimy	19.0	92	118.6	61.2	6.2	46.5	194	30.2
Somme	20.3	99	116.8	61.3	5.7	45.3	196	30.5
Flanders	21.5	104	120.5	59.0	5.4	47.4	195	29.8
AC Linora	20.3	99	118.8	60.3	5.7	47.1	196	31.3
FP967	20.4	99	115	60.7	5.8	44.8	191	31.1
LSD 5%	0.3		1.3	1.0	0.2	0.7	1.5	1.5
No. of tests	14		6	14	14	14	14	6

Black and grey soil zone

Entry	Yield '00kg/ha	Yield as % Norlin	Maturity (days)	height (cm)	seed wt mg	oil cont. %	iodine no.	protein %
Norlin	20.3	100	108.5	52.4	6.4	45.4	195	32.9
McGregor	21.5	106	112.3	52.7	5.9	45.8	196	34.0
Vimy	19.6	97	113.3	54.6	7.0	46.6	197	34.5
Somme	20.0	99	108.8	52.5	6.4	45.5	199	35.0
Flanders	23.7	117	108.8	48.2	6.1	47.0	199	33.3
AC Linora	22.3	110	113.8	52.1	6.3	47.0	197	34.0
FP967	18.8	93	109.5	52.9	6.5	45.3	195	33.1
LSD 5%	3.7		1.8	1.1	0.2	0.6	0.6	1.4
No. of tests	1*		1#	4	4	4	4	2

Table 2. Performance of FP967.
Summary of Agronomic and Quality data
Late seeded co-operative test; 1992, 1993

Entry	Yield '00kg/ha	Yield as % Norlin	Maturity (days)	height (cm)	seed wt mg	oil cont. %	iodine no.	protein %
Norlin	17.4	100	109.3	70.5	5.7	44.4	194	
McGregor	16.3	94	118.6	75.7	5.2	45.2	196	
Vimy	15.6	90	111.8	71.0	6.0	45.4	195	N/A
Somme	16.0	92	109.9	71.7	5.3	44.0	199	
Flanders	17.5	101	117.6	72.2	5.1	46.4	199	
AC Linora	17.8	102	113.1	71.2	5.5	46.2	197	
FP967	17.9	103	108.2	69.6	5.7	43.7	193	
LSD 5%	0.6		2.2	1.4	0.2	0.7	1.6	
No. of tests	7		7	7	7	7	7	

CULTIVAR DESCRIPTION

CDC Triffid transgenic flax

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McHughen, A., Rowland, G. G., Holm, F. A., Bhatty, R. S. and Kenaschuk, E. O. 1997. **CDC Triffid transgenic flax.** Can. J. Plant Sci. 77: 641–643. CDC Triffid is a transgenic sulfonylurea herbicide resistant cultivar with agronomic features similar to NorLin. It is intended to provide a sustainable, broadleaf cropping option to summerfallowing or continuous cropping to cereals in soils previously treated with residual sulfonylurea herbicides. CDC Triffid flax was developed at the Crop Development Centre, University of Saskatchewan.

Key words: Flax, linseed, *Linum usitatissimum* L., cultivar description, transgenic, biotechnology, sulfonylurea, herbicide resistance, herbicide residue, germplasm

McHughen, A., Rowland, G. G., Holm, F. A., Bhatty, R. S. et Kenaschuk, E. O. 1997. **Lin transgénique CDC Triffid.** Can. J. Plant Sci. 77: 641–643. CDC Triffid est un cultivar transgénique de lin, résistant aux herbicides de la famille de la sulfonylurée. Dans les sols précédemment traités à ce type d'herbicides, il offre une option durable de culture dicotylédone comme alternative à la jachère ou à la monoculture de céréales. Il a été mis au point au centre de phytotechnie de l'Université de la Saskatchewan.

Mots clés: Lin, graine de lin, *Linum usitatissimum* L. description de cultivar, transgénique, biotechnologie, sulfonylurée, résistance aux herbicides, résidus d'herbicide, matériel génétique

Sulfonylurea herbicides are popular among cereal farmers. However, some sulfonylurea based herbicides do not readily break down in some soil types, especially those with low moisture and high pH, and can persist for several years (Saskatchewan Agriculture and Food 1996). This residual activity limits rotational options and forces farmers into such non-sustainable practices as summerfallowing or continuous cropping to cereals, as we currently have no broadleaf crop species capable of withstanding the residual effect of sulfonylureas.

To address this problem, we introduced into NorLin flax a gene conferring resistance to those registered sulfonylurea herbicides with soil residual activity (metsulfuron methyl [Ally™, DuPont] and triasulfuron [Amber™, Ciba-Geigy]). The resulting line, CDC Triffid, performed similarly to NorLin except CDC Triffid can also be grown, without detriment, in soil containing sulfonylurea residue. This line permits a sustainable broadleaf cropping rotation option for farmers who have used or wish to use soil residual sulfonylurea herbicides.

Breeding Methods and Pedigree

The gene conferring resistance is a modified Acetolactate synthase gene from *Arabidopsis*, originally cloned and described by Haughn et al. (1988). The gene was coupled to the bacterial-origin marker genes nopaline synthase and neomycin phosphotransferase II (NPT-II) in a plasmid and introduced into a disarmed *Agrobacterium tumefaciens* (McHughen 1989). Hypocotyl segments from NorLin flax were inoculated with the *Agrobacterium* in vitro, and shoots were regenerated, selected and maintained to maturity; details of the process are presented in McHughen 1989. The transgenic regenerants were selfed and progeny were assayed for expression of the Arabidopsis ALS gene. Several such lines were produced, and two lines were evaluated in the Flax Co-op test, as FP967 and FP968. FP967, displaying meritorious agronomic performance, both in the presence of the herbicides and in the absence, was proposed for registration to the PRRCG in February 1994. Because CDC Triffid is transgenic, the line also required regulatory evaluation of environmental and animal health impacts, yet those regulations were not in place at the time of the PRRCG approval. CDC Triffid awaited the ancillary regulations, was evaluated, determined to be substantially equivalent to traditional flax varieties for environmental and animal health issues, and was issued certificate no. 4338 from Agriculture and Agri-Food Canada on 8 May 1996. However, CDC Triffid cannot be marketed as a human food product unless and until it clears regulatory scrutiny by Health Canada.

Performance

CO-OP TRIALS. In the absence of sulfonylurea herbicide residue, CDC Triffid performs in a manner similar to its parent, NorLin, and comparable to other check cultivars (Table 1). The agronomic differences between NorLin and CDC Triffid are minor; none is statistically significant. In western Canada, CDC Triffid matured a day earlier than NorLin, resulting in a 2% seed yield deficit. The plant height was 0.5 cm shorter and oil content 0.2% less; other measured

Table 1. Performance of CDC Triffid in Flax Co-operative trials. Agronomic and quality data, 1992 and 1993 for Western Canada[z]

Entry	Yield (t ha^{-1})	Yield as % NorLin	Maturity (d)	Height (cm)	Seed wt (mg)	Seed oily (%)	Iodine no.	Protein (%)
CDC Triffid	2.00	98	114.4	63.1	5.9	44.3	191	32.3
NorLin	2.05	100	115.6	63.8	5.9	44.5	192	32.3
McGregor	2.06	100	120.9	65.8	5.4	45.3	192	32.4
Vimy	1.72	84	115.7	63.9	6.2	45.6	194	32.6
Somme	1.98	97	115.2	63.8	5.7	44.7	196	32.4
Flanders	2.13	104	118.2	61.6	5.4	46.7	195	32.1
AC Linora	2.06	98	117.4	63.1	5.7	48.4	195	32.7
LSD 5%	0.05		1.2	1.0	0.2	0.6	1.6	0.2
Station years	29		19	32	23	32	32	18

[z]Cultivar means at each site were used for statistical analysis without partitioning for year effects; thus each station year of data was treated as one replicate.
[y]Whole seed, dry matter basis, by nuclear magnetic resonance.

Table 2. Effect of sulfonylurea herbicide residue (at 1× and 2× or 3× recommended dose; grams of active ingredient per ha (g a.i. ha^{-1})) on seed yield (g m^{-2}) of NorLin and CDC Triffid[z]

Herbicide (years tested)	g a.i. ha^{-1}	Seed yield (g m^{-2}) NorLin	Seed yield (g m^{-2}) CDC Triffid	Seed yield of CDC Triffid as % of NorLin
Ally™ (4)	4.5 (1×)	115	187	162
	9.0 (2×)	68	190	279
Amber™ (4)	8.0 (1×)	146	188	128
	25.0 (3×)	104	177	170
Glean™ (2)	11.3 (1×)	59	110	186
	22.5 (2×)	30	119	397
Untreated (2)	0.0	99	114	115
Untreated (4)	0.0	157	166	106

[z]Summary table only; for statistical analyses see McHughen and Holm, 1995 and Holm and McHughen, 1995. All trials conducted at Saskatoon, SK, between 1991 and 1994.

parameters were also virtually identical. Again, none of the differences was statistically significant, and are in the order of those expected in comparing the performance of a population developed from a single seed with the performance of the bulk parental genotype. Like NorLin, CDC Triffid is therefore well adapted to all flax growing regions of western Canada.

HERBICIDE RESISTANCE. The relative differences between CDC Triffid and NorLin become readily evident when sown into soil containing residues of sulfonylurea herbicides. The first such trials involved chlorsulfuron (Glean™, DuPont), but this was discontinued when Glean™ was voluntarily removed from the market, partly due to problems with its long soil residual activity. It was replaced in our studies with the only other two registered sulfonylureas exhibiting residual activity, metsulfuron methyl (Ally™, DuPont) and triasulfuron (Amber™, Ciba-Geigy). In these trials, which involved incorporating the herbicides into the soil the season prior to seeding with the flax (in order to more closely mimic a commercial production situation), CDC Triffid (tested as line 12115) was not affected by the herbicide residue, in that there was no difference in performance between the herbicide treated (both at the standard and double or triple recommended doses) and the untreated check plots. However, NorLin (and other check cultivars (data not shown here)) suffered dramatically when sown into any of the herbicide treated plots. Full data on these trials are published in McHughen and Holm (1995), and Holm and McHughen (1995). A summary table, provided here (Table 2), shows the percentage increase in seed yield of CDC Triffid over NorLin in four years of trials (1991–1994) in Saskatoon, SK.

Table 3. Ancillary data on the composition of CDC Triffid grown in soil containing metsulfuron methyl at 2× recommended dose, breeder seed of CDC Triffid (grown in the absence of sulfonylureas) and the normal range for traditional flax for each parameter

Component	CDC Triffid 2× sulfonylurea	CDC Triffid breeder (no herbicide)	Normal range[z]
Ash (%)	5.2	5.6	5.4–7.8
Protein (%)[y]	36.3	34.5	35.0–45.6
Oil (%)[x]	40.6	40.8	NA
Viscosity, (cS)	21.3	19.5	23.3–68.2
Cyanogenic glycosides (mg g^{-1})	NA	7.7	6.0–9.2
Major minerals			
P (mg g^{-1})	8.5	8.8	7.3–12.6
K (mg g^{-1})	11.6	11.8	9.1–15.8
S (mg g^{-1})	3.7	3.6	3.8–4.2
Ca (mg g^{-1})	3.9	3.8	3.9–7.4
Minor minerals			
Cu (μg g^{-1})	16.4	12.0	17.4–24.2
Fe (μg g^{-1})	131.1	115.4	157.4–274.0
Mn (μg g^{-1})	59.8	36.1	47.8–71.3
B (μg g^{-1})	35.7	38.1	NA

[z]The "standards" are taken from recent publications from Canadian labs (Bhatty and Cherdkiatgumchai 1990, 1993; Oomah et al. 1992). All data for CDC Triffid were derived from single plot samples grown at the Kernen Crop Research Farm, Saskatoon, in 1993 while the data for the "normal" range were from flax grown at varied locations (see cited papers for details).
[y]Protein values based on oil-free meal analysis.
[x]Oil percentage of whole seed, by wet extraction.

Other Characteristics

Theoretically, there should be no critical effect of the transformation process on overall composition of CDC Triffid compared with traditional flax. Similarly, because of the nature of the novel trait being an enhancement of natural resistance to sulfonylureas, the composition of CDC Triffid grown in soil containing residue of the herbicide is not

Table 4. Comparison between CDC Triffid and NorLin for total seed oil fatty acids (%)[z]

Line	Palmitic *16:0*	Stearic *18:0*	Oleic *18:1*	Linoleic *18:2*	Linolenic *18:3*
NorLin	5.2	2.7	20.9	13.6	57.6
CDC Triffid	5.3	2.8	21.0	13.5	57.4
LSD[y]	0.1	0.1	0.6	0.9	1.0

[z]Composition determined by gas-liquid chromatography; data from 1993 Flax Co-op trial (17 stations).
[y]LSD from entire trial; paired field replications were bulked to provide two lab replications for each line.

expected to differ substantially from CDC Triffid grown in the absence of a sulfonylurea herbicide, or indeed, from traditional flax genotypes grown in the absence of a sulfonylurea herbicide. However, to allay concerns that the genetic transformation process was so novel and fundamentally beyond the realm of orthodox genetics as to cause such changes, a number of common parameters were measured and data presented in Table 3. As expected, there were no substantial differences.

Like NorLin, CDC Triffid is immune to Rust (*Melampsora lini*) race 371, and is moderately resistant to wilt (*Fusarium oxysporum*). It is similar to other commercial flax cultivars, especially NorLin, in almost all respects. As an industrial oilseed crop, a critical consideration is oil content (Table 1 and Table 3) and oil profile; Table 4 shows the composition of oil from CDC Triffid and NorLin to be virtually identical.

While there are no outstanding weaknesses, the only major advantage of CDC Triffid is its ability to withstand sulfonylurea herbicide residue, so it will be of especial interest to those farmers who use sulfonylurea herbicides.

Maintenance and Distribution of Pedigreed Seed

Breeder seed of CDC Triffid is maintained at the Crop Development Centre, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7K 5A8. Value Added Seeds, Inc., Lumsden, Saskatchewan, Canada S0G 3C0, has multiplication and distribution rights to CDC Triffid.

Many people were involved in the development and evaluation of CDC Triffid, and special mention and thanks must go to Robin Browne, Patti Schryer, Ellis Clayton, Gerry Stuber, Gary Farkas and Marvin Swartz. Partial funding also came from several sources, including the Crop Development Centre, ADF, NSERC and WGRF.

Bhatty, R. S. and Cherdkiatgumchai, P. 1990. Compositional analysis of laboratory-prepared and commercial samples of linseed meal and of hull isolated from flax. J. Am. Oil Chem. Soc. **67**: 79–84.

Bhatty, R. S. 1993. Further compositional analyses of flax: mucilage, trypsin inhibitors and hydrocyanic acid. J. Am. Oil Chem. Soc. **70**: 899–904.

Oomah, B. D., Mazza, G. and Kenaschuk, E. O. 1992. Cyanogenic compounds in flax. J. Agric. Food Chem. **40**: 1346–1348.

Holm, F. A. and McHughen, A. 1995. Breeding flax for herbicide resistance. *In* Proc. Western Canada Agronomy Workshop. Potash and Phosphate Institute. Red Deer, AB.

McHughen, A. 1989. *Agrobacterium* mediated transfer of chlorsulfuron resistance to commercial flax cultivars. Plant Cell Rep. **8**: 445–449.

McHughen, A. and Holm, F. A. 1995. Transgenic flax with environmentally and agronomically sustainable attributes. Transgenic Res. **4**: 3–11.

Haughn, G., Smith, J., Mazur B. and Somerville, C. 1988. Transformation with a mutant *Arabidopsis* acetolactate synthase gene renders tobacco resistant to sulfonylurea herbicides. Mol. Gen. Genet. **211**: 266–271.

Saskatchewan Agriculture and Food. 1996. Crop Protection Guide '96. Government of Saskatchewan, Regina, SK.