FOOD DERIVED FROM BROMOXYNIL-TOLERANT CANOLA LINE WESTAR-OXY-235

A Safety Assessment

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SUMMARY AND CONCLUSIONS

Food from bromoxynil-tolerant canola line Westar-Oxy-235 has been evaluated for its safety for human consumption. A number of criteria were used in this assessment including: a characterisation of the genes, their origin and function; the changes at the DNA, protein and whole food levels; stability of the introduced genes in the canola genome; compositional analyses; evaluation of intended and unintended changes; and the potential allergenicity and toxicity of the newly expressed proteins.

History of use

Canola is a genetic variation of rapeseed developed by plant breeders specifically for its nutritional qualities, particularly its low levels of saturated fat and naturally occurring toxins. Oil is the only product of the canola plant that is being assessed for human consumption. Canola oil is routinely used in food and has a moderately long history of safe use.

Nature of the genetic modification

Bromoxynil-tolerant canola line Westar-Oxy-235 was generated by the transfer of the *oxy* gene from the soil bacterium *Klebsiella ozaenae*, using the *Agrobacterium*-mediated transformation system. The *oxy* gene codes for the enzyme nitrilase, which converts the herbicide bromoxynil (3,5-dibromo-4-hydrobenzonitrile) into its non-phytotoxic metabolite 3,5-dibromo-4-hydroxybenzoic acid (DBHA). No other genes were transferred and the transformed canola was shown to be phenotypically and genotypically stable by segregation and mapping studies.

The modification did not involve the transfer of any antibiotic resistance genes.

Characterisation of novel protein

The new protein, nitrilase, is an enzyme specific for oxynil herbicides. It was found to be easily detectable in leaf extracts from the modified plant, but was only present at very low levels in seeds. No detectable protein was found in refined oil.

The potential toxicity and allergenicity of nitrilase was considered in the assessment. Proteins from the same family as nitrilase are ubiquitous throughout the animal and plant kingdoms, and are consumed by both animals and humans. Nitrilase itself does not have any significant similarity to known protein toxins or allergens and is rapidly digested in conditions that mimic human digestion. The absence of toxicity of nitrilase has been confirmed through acute toxicity testing in mice.

Nitrilase, also cannot be detected in refined canola oil, therefore exposure to the protein, through consumption of refined oil from bromoxynil-tolerant canola, would be zero. There is thus no evidence to indicate that there is any potential for nitrilase to be either toxic or allergenic to humans.

The potential toxicity of DBHA, the by-product of bromoxynil detoxification by nitrilase, was also considered. The evidence indicates that DBHA shows no potential to be toxic to humans at the predicted exposure levels.

Comparative analyses

Detailed compositional analyses did not reveal any consistent differences in key constituents (nutrients, anti-nutrients and toxicants) between modified canola plants and control plants, or the oils produced from them. Treatment with bromoxynil also did not affect the levels of any of the key constituents measured. The results confirmed that the levels of key constituents in bromoxynil-tolerant canola are no different to those of non-modified canola varieties.

Nutritional impact

An animal feeding study confirmed that there is no difference between bromoxynil-tolerant and control varieties of canola in their ability to support typical growth and well being.

Conclusion

No potential public health and safety concerns have been identified in the assessment of canola line Westar-Oxy-235. On the basis of the data submitted with the present application, and other available information, oil derived from bromoxynil-tolerant canola line Westar-Oxy-235 is considered to be as safe and nutritious as refined oil derived from conventional canola varieties.

INTRODUCTION

A safety assessment has been conducted on food derived from canola, which has been genetically modified to be tolerant to the oxynil family of herbicides comprising bromoxynil and ioxynil. The genetically modified canola is marketed as Navigator™ canola.

The oxynil family of herbicides act by inhibiting electron transport in photosystem II in plants. Inhibition of electron transport causes super oxide production resulting in the destruction of cell membranes and an inhibition of chlorophyll formation, leading to plant death (Comai and Stalker 1986). Tolerance to either bromoxynil (3,5-dibromo-4-hydorxybenzonitrile) or ioxynil (3,5-di-iodo-4-hydroxybenzonitrile) is achieved through expression in the plant of a bacterial nitrilase enzyme that hydrolyses the herbicide to an inactive, non-phytotoxic compound. The nitrilase is derived from the bacterium *Klebsiella pneumoniae* subspecies *ozaenae* and is responsible for rapidly degrading bromoxynil in soil. The nitrilase enables the bacterium to utilise bromoxynil as a sole source of nitrogen (McBride *et al* 1986).

Bromoxynil is particularly effective on broadleaf weeds common in canola fields. The rationale for engineering canola to be bromoxynil-tolerant is to enable bromoxynil-containing herbicides to be used for the post-emergence control of broadleaf weeds in canola crops without crop injury. The modified canola was developed for commercialisation in Canada, where it is grown for both domestic use and for export. Although the current level of trade of canola and its commodities between Canada and New Zealand and Australia is relatively small, some imported processed foods may contain genetically modified canola oil.

Canola seeds are processed into two major products, oil and meal with the oil being the only human food product being considered in this assessment. Canola meal is used principally as an animal feed. Canola oil is a premium quality oil and is used in a variety of manufactured food products including salad and cooking oil, margarine, shortening, mayonnaise, sandwich spreads, creamers and coffee whiteners. It can thus be imported as an ingredient of many processed foods.

HISTORY OF USE

Donor organism

Klebsiella ozaenae is a member of the *Enterobacteriaceae*, a group of facultative gram-negative bacteria. The European Federation of Biotechnologies has classified *K. ozaenae* as a Class 2 microorganism. This class contains microorganisms that could potentially cause disease in humans, however no known pathogenicity exists for the subspecies *ozaenae*. Bacteria of the *Klebsiella* class are widely distributed in nature, occurring naturally in the soil, water and in grain and are normal inhabitants of the intestinal tract (Krieg and Holt 1984).

Host organism

The plant species *Brassica napus* L. oleifera Metzg is more commonly known as oilseed rape, rape or rapeseed, with some cultivars referred to as canola. Rapeseed breeding began soon after the crop was introduced during the 1940s. Early rapeseed varieties were very high in the natural toxicants, erucic acid and glucosinolates, which made them unsuitable for consumption by either humans or animals. In the 1970s intensive breeding programs produced high quality varieties that were significantly lower in both erucic acid and glucosinolates. These varieties, largely *Brassica napus,* were called canola, the term denoting that these varieties contain an erucic acid level below 2% of total fatty acids and less than 30 micromoles of total glucosinolates. World production of oilseed rape in 1996-1997, was the third most important of oilseed crops behind soybean and cottonseed, but above peanut, sunflower and palm.

Presently, oilseed rape is grown primarily for its seeds, which yield about 40% oil and a high protein animal feed. Demand for canola has risen sharply, particularly the oil, which is used in margarine and other oil-based products. Canola oil-based products are routinely used in food and are considered to have a history of safe use.

DESCRIPTION OF THE GENETIC MODIFICATION

Methods used in the genetic modification

Canola (*Brassica napus* L. oleifera Metzg.) line Westar was transformed with plasmid pRPA-BL-150a using the method of *Agrobacterium tumefaciens*-mediated transformation. A disarmed (i.e. non phytopathogenic) strain of *Agrobacterium tumifaciens*, EHA 101 was used (Hood *et al* 1986). The *Agrobacterium*-mediated transformation system is well understood, and is widely used in plant biotechnology (Zambryski 1992).

Regeneration of transformed plants was done in the presence of bromoxynil as the sole selective agent. The transformation resulted in the selection of a single transformation event – Westar-Oxy-235 – which was subsequently used in sexual crosses with elite canola lines to generate the Navigator™ canola varieties used in commercial production.

Function and regulation of the novel genes

The transformation of canola with plasmid pRPA-BL-150a resulted in the transfer of a single gene expression cassette. The genetic elements contained within the gene expression cassette are described in Table 1 below and their organisation is depicted in Figure 1.

Table 1: Description of the gene expression cassette contained within pRPA-BL-150a

Genetic element	Source	Function
35S promoter	The cauliflower mosaic virus (CaMV) 35S promoter region (Gardner *et al* 1981).	A promoter for high-level constitutive (occurring in all parts of the plant and at all stages of development) gene expression in plant tissues.
Enhancer	The non-translated leader of a RuBisCO small subunit gene derived from maize (Lebrun *et al* 1987).	The non-translated leader sequence helps to stabilise mRNA and improve translation.
oxy	Gene isolated from *Klebsiella pneumoniae* subspecies *ozaenae* encoding the enzyme nitrilase (Stalker *et al* 1988).	Inactivates the herbicide bromoxynil and confers bromoxynil tolerance when expressed in plants.
NOS 3'	The 3' non-translated region of the nopaline synthase gene isolated from *Agrobacterium tumefaciens* plasmid pTi37 (Bevan *et al* 1983).	Contains signals for termination of transcription and directs polyadenylation.

The oxy gene

The *oxy* gene was isolated from the soil bacterium *Klebsiella pneumoniae* subsp. *ozaenae* and encodes an enzyme that metabolises the herbicide bromoxynil (Stalker and McBride 1987). The 1150 base pair *oxy* gene has been fully sequenced and its encoded enzyme, nitrilase, has been fully characterised (Stalker *et al* 1988). When transferred into plants, the gene, through its encoded protein, confers tolerance to the oxynil family of herbicides including bromoxynil and ioxynil. The mechanism of tolerance involves the detoxification of the herbicide by the nitrilase enzyme. This degradation effectively inactivates the herbicide and enables the normally bromoxynil-sensitive plant to survive and grow when treated with applications of the herbicide.

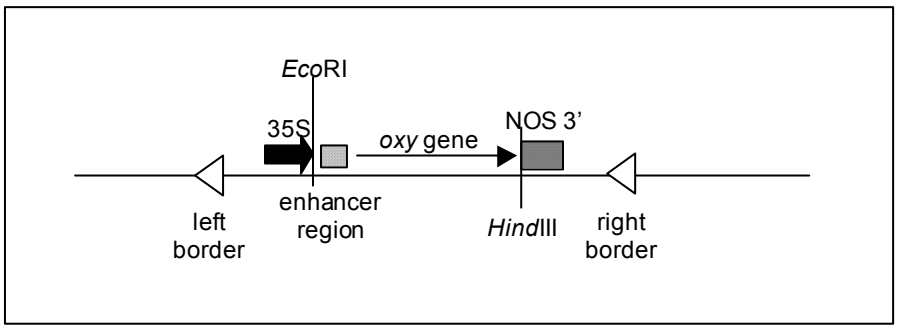
Other genetic elements

The plasmid pRPA-BL-150a is a double border binary plant transformation vector which contains well-characterised DNA segments required for the selection and replication of the plasmid in bacteria as well as the right and left borders delineating the region of DNA (T-DNA) which is transferred into the plant genomic DNA (Table 2). This is the region into which the gene expression cassette is inserted. DNA residing outside the T-DNA region does not normally get transferred into plant genomic DNA (Zambryski 1992). All DNA cloning and vector construction was carried out using the host bacterium *Escherichia coli* DH5α, a derivative of the common laboratory *E. coli* K-12 strain.

Table 2: Description of other genetic elements contained within pRPA-BL-150a

Genetic element	Source	Function
Left border	A DNA fragment of the pTiA6 plasmid containing the 24 bp nopaline-type T-DNA left border region from *A. tumefaciens* (Barker *et al* 1983).	Terminates the transfer of the T-DNA from *A. tumefaciens* to the plant genome.
Right border	A DNA fragment from the pTiA6 plasmid containing the 24 bp nopaline-type T-DNA right border region from *A. tumefaciens*. (Barker *et al* 1983).	The right border region is used to initiate T-DNA transfer from *A. tumefaciens* to the plant genome.
Genta	Gentamicin resistance gene from plasmid pH1J1 (Hirsch and Beringer 1984).	Confers resistance to the antibiotic gentamicin. Used as a marker to select transformed bacteria from non-transformed bacteria during the DNA cloning and recombination steps undertaken in the laboratory prior to transformation of the plant cells.
ori-322	Origin of replication from *E. coli* plasmid pBR322 (Bolivar *et al* 1977).	Allows for autonomous replication of plasmids in *E. coli.*

Figure 1: Diagram of the T-DNA region transferred to Westar-Oxy-235.



Characterisation of the genes in the plant

Selection of plant lines

After the transformation of Westar with pRPA-BL-150a, regenerated plantlets were taken out of tissue culture and transferred to soil. The transformed plants were then assayed for herbicide tolerance, as well as other agronomic characteristics, in order to select the best transformation event. Line Westar-Oxy-235 was subsequently selected and used for all further studies, as well as for sexual crosses with elite lines.

Characterisation of inserted T-DNA

Southern blotting (Southern 1975) was used to characterise the inserted T-DNA in terms of insert number (number of integration events), insert integrity (gene size), and sequences outside the T-DNA borders (including the gentamicin resistance gene and the plasmid origin of replication).

Genomic DNA was isolated from leaf tissue of the non-transformed parental line, Westar and from the T_3 generation of the transformed canola line, Westar-Oxy-235. To determine the insert number of the T-DNA, genomic DNA was digested with either *EcoR*1 or *Hind*III, which reside at the 5' and 3' ends of the *oxy* gene, respectively (see diagram above). The number of hybridising bands detected will represent the number of copies of the *oxy* gene present in the plant genome, and hence serves as an indicator of the number of T-DNA insertions. With either restriction digestion, only a single hybridising band was detected, indicating that only a single copy of the *oxy* gene is present in Westar-Oxy-235. No hybridising bands were detected in genomic DNA isolated from the non-transformed control. Double digestion of the genomic DNA with both *EcoR*1 and *Hind*III resulted in a single hybridising band corresponding to the size of the coding region of the *oxy* gene (1150 bp). This indicates that the entire coding region has been transferred.

To determine if any sequences from outside the T-DNA borders had been transferred to the plant genome, genomic DNA from both Westar-Oxy-235 and the parental control were probed with a DNA fragment corresponding to the *ori-322* region of pBR322. No hybridising bands were detected, indicating that the bacterial origin of replication had not been transferred.

PCR analysis was used to determine if the gentamicin resistance gene had been transferred during the transformation process. DNA extracted from leaf tissue harvested from Westar-Oxy-235 and the parental control line was used in the analysis. Plasmid DNA, containing the gentamicin resistance gene, was used as the reference substance and positive control for the analysis. No gentamicin-specific DNA fragment could be amplified from DNA extracted from Westar-Oxy-235, indicating that the gentamicin resistance gene had not been transferred.

Conclusion

A single copy of T-DNA, containing the *oxy* gene, has been integrated at a single site in Westar-Oxy-235. No rearrangements of the T-DNA were apparent and no sequences residing outside the T-DNA region, including the gentamicin resistance gene, were transferred during the transformation.

Stability of the genetic changes

The genetic stability (i.e., inheritance) and segregation of the bromoxynil-tolerant trait was monitored using data obtained from herbicide-sprayed plants and Southern blotting.

Progeny derived from the original transformation event, Westar-Oxy-235, were sprayed with oxynil herbicides at the T_2 and T_3 generations. By spraying seedlings with the herbicide and determining the Mendelian segregation ratios of the bromoxynil tolerant trait it is possible to determine the total number of functional (bromoxynil-tolerant) loci that have been integrated into an individual transformed plant. Ideally, a single genetic locus (i.e., a single insertion site) is preferred because, while not essential for the performance of the canola or the *oxy* gene, it simplifies the breeding of the trait into other elite commercial cultivars.

The segregation analysis done with the early generations derived from the original transformation event indicated the bromoxynil-tolerance trait is stably inherited by subsequent generations and that it segregates in a manner consistent with a single genetic locus.

Beyond the T_3 generation, lines homozygous for the bromoxynil-tolerant trait were selected. These lines no longer display segregation of the trait and oxynil spray screening is instead used to maintain and monitor seed purity. The maintenance of the tolerance trait over subsequent homozygous generations is thus a good measure of genetic stability. The bromoxynil-tolerant trait was found to be stably maintained over several generations produced from self-pollination, as well as in different genetic backgrounds produced through backcrossing with elite canola varieties. During the backcrossing program, the *oxy* gene was introgressed into a winter elite variety of canola called Samourai, producing Samourai-Oxy-235. Southern blotting was done on genomic DNA isolated from Samourai-Oxy-235 and compared to Westar-Oxy-235. The hybridisation patterns obtained were indistinguishable, confirming that the *oxy* gene is stably maintained in different genetic backgrounds.

Conclusion

Stability of the bromoxynil-tolerant trait was studied by backcrossing of plants containing transformation event Westar-Oxy-235 with elite canola varieties and by self-crossing followed by propagation. The bromoxynil-tolerant trait was found to segregate in a manner consistent with a single genetic locus and was also found to be stably inherited from one generation to the next. Additionally, Southern blotting demonstrated that the *oxy* gene was stably maintained in a different genetic background.

Antibiotic resistance genes

Antibiotic resistance genes can be present in some transgenic plants as a result of their use as marker genes to select transformed cells. It is generally accepted that there are no safety concerns with regard to the presence in the food of antibiotic resistance gene DNA *per se* (WHO 1993).

There have been concerns expressed, however, that there could be horizontal gene transfer of antibiotic resistance genes from ingested food to microorganisms present in the human digestive tract and that this could compromise the therapeutic use of antibiotics.

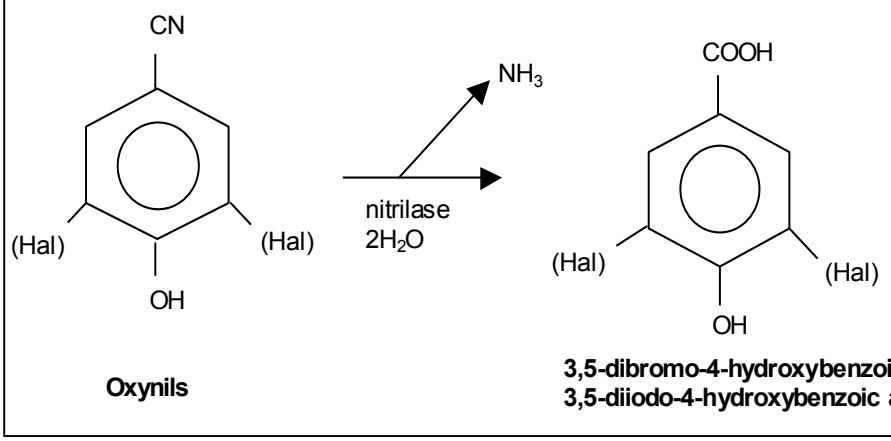
As no antibiotic resistance gene was transferred to Westar-Oxy-235 during the transformation process, this issue was not considered further in the assessment.

CHARACTERISATION OF NOVEL PROTEIN

Biochemical function and phenotypic effects

The *oxy* gene was isolated from *Klebsiella pneumoniae* subspecies *ozaenae* (McBride *et al* 1986, Stalker and McBride 1987, Stalker *et al* 1988) and encodes a 37 kDa nitrilase (EC. 3.5.5.6). This enzyme hydrolyses the oxynil herbicides into non-phytotoxic compounds: 3.5-dibromo-4-hydroxybenzoic acid or 3,5-diiodo-4-hydroxybenzoic acid and ammonia (Figure 2).

Figure 2: Oxynil degradation by the *oxy*-encoded nitrilase. (Hal is either Br or I)



Purified nitrilase has optimal activity at pH 9.2 and at a temperature of 35˚C. The pH optimum remains relatively constant at different substrate concentrations. Nitrilase activity declines to 15% at pH 7.0 and also in temperatures of 10 and 55˚C. The *oxy*-encoded nitrilase is highly specific for its substrates, exhibiting a K_m of 0.31nM and a V_{max} of 15µmole of NH_3 released/min/mg protein for bromoxynil.

Protein expression analyses

Expression levels of the *oxy*-encoded nitrilase were determined by immuno-blotting techniques using a rabbit polyclonal antibody specific to nitrilase. Analyses were done on leaf and seed tissue extracts as well as processed fractions (oil and meal) from homozygous Westar-Oxy-235 and the non-transformed parental control. A positive nitrilase signal on the immunoblot consists of a single band at 37 kDa. The protein level was quantified by comparing the intensity of the signal in the protein extracts with known amounts of purified nitrilase. The detection limit for the assay was 20 ppb nitrilase. Nitrilase was not detected in any of the protein extracts from the non-transformed parental control line. The results are summarised in Table 3.

Table 3: Nitrilase expression levels in tissue from Westar-Oxy-235

	Nitrilase expression levels		
Sample	**ng/mg total protein**	**% tissue**	**Parts per million (ppm)**
Leaf	1000	0.002	20
Seed	<10	<0.0003	<3
Meal	5	0.0002	2
Refined Oil	Not detected		

The results show that the levels of nitrilase are highest in the leaf tissue, with only relatively low amounts of nitrilase able to be detected in the seeds. In refined oil, which is the only human food product derived from canola being assessed in this application, nitrilase could not be detected (detection limit of 20 ppb).

Potential toxicity of novel protein

The protein expression data demonstrates that Westar-Oxy-235 expresses a single novel protein – nitrilase. This section of the report will therefore assess the potential toxicity of nitrilase based on the following:

- the potential for human exposure to nitrilase;
- its amino acid sequence similarity to known toxins;
- an acute oral toxicity study in mice;
- prior history of human ingestion of similar enzymes; and
- potential toxicity of bromoxynil metabolites.

Potential for human exposure to nitrilase

Refined canola oil from Westar-Oxy-235 was analysed for the presence of nitrilase, which could not be detected down to a detection limit of 20 ppb. Therefore, it is highly unlikely that humans ingesting refined oil derived from bromoxynil-tolerant canola would be exposed to any appreciable amounts of nitrilase.

Similarity to known protein toxins

Studies evaluated:

Astwood, J.D. (1997). *Klebsiella ozaenae* nitrilase (BXN) has no significant sequence similarity to known allergens or toxins. Monsanto Study Report No. MSL-15120 – submitted with Application A379 – Bromoxynil tolerant cotton.

A database of protein toxin amino acid sequences was assembled from the public domain genetic databases, which included GenPept ver. 92 (a protein database extracted from GenBank and EMBL), PIR ver. 45, and SwissProt ver. 31. Amino acid sequences were retrieved from the databases using the STRINGSEARCH program supplied with the GCG sequence analysis package version 7 (Devereux *et al* 1984). Using the DATASET program, the sequences of toxins were combined into a single database called TOXIN3.

The keyword "toxin" identified and retrieved 2662 amino acid sequences from the public domain genetic databases – this comprised the TOXIN3 database. There were no toxins in the TOXIN3 database that showed significant similarity to nitrilase.

Acute oral toxicity

Studies evaluated:
Dange, M. (1996) Nitrilase: sub-acute oral toxicity study in the mouse. Rhône-Poulenc Study SA 96267 – submitted with Application A379 – Bromoxynil tolerant cotton.

To obtain sufficient quantities of nitrilase for toxicity testing, the enzyme was expressed in *Escherichia coli* BL21 and subsequently purified as an inclusion body pellet.

An acute oral toxicity study was planned to be performed using doses up to 2000mg/kg body weight, using a suspension of nitrilase at 200mg/ml. However, the consistency of the suspension once prepared did not allow the total dose to be administered at one time. Therefore, the suspension was administered over four consecutive days at 500mg/kg body weight/day.

Four consecutive oral doses (500mg/kg body weight) of nitrilase (Batch No. JHJ0001) were administered to groups of OF1 mice (5/sex) at a dose volume of 20ml/kg. The purified nitrilase was suspended in 0.25% methylcellulose in distilled water.

All animals were checked daily for clinical signs over a period of 15 days, and their body weight recorded weekly. At termination of the study period, all animals were killed and subject to necropsy. The necropsy included the macroscopic examination of abdominal and thoracic cavities, major organs and tissues.

No clinical signs were observed during the study and there were no unscheduled deaths. The body weight gain of the animals was unaffected by the treatment and no gross findings were recorded at necropsy. The LD_{50} was designated as >500 mg/kg body weight.

History of ingestion

Nitrilase enzymes, similar to that encoded by the *oxy* gene from *Klebsiella pneumonia,* have been found in a number of plant and microbial species. Although substrates and pathways differ, it appears as though nitrilases share common functions such as hydrolysis of nitriles to carboxylic acids. Plant nitrilases can also confer resistance to some of the nitrile containing herbicides. Nitrilases have been found in a number of

important food crops such as wheat, cabbage, barley, and bananas (Buckland *et al* 1973, Thimann and Mahadevan 1964), therefore, humans have a history of exposure to similar types of proteins with no apparent ill effects ever being documented.

Potential toxicity of bromoxynil metabolites

Bromoxynil-tolerant canola plants inactivate bromoxynil by hydrolysing it to 3,5-dibromo-4-hydroxybenzoic acid (DBHA), a carboxylic acid. As this metabolite is a by-product resulting from the activity of an introduced enzyme it is important that a consideration of its safety be included in any evaluation of bromoxynil-tolerant canola. Two issues are relevant. Firstly, the actual toxicity of DBHA, and secondly, the residue levels of DBHA likely to be present in food derived from bromoxynil-tolerant canola varieties.

In relation to toxicity, the US Environment Protection Agency (EPA), in its evaluation of bromoxynil, also evaluated the toxicity of the DBHA metabolite and concluded "there was no concern that DBHA would exhibit significant toxicity over that of the parent bromoxynil" and that bromoxynil "poses negligible risk to human health at expected exposure levels" (US EPA 1998). Bromoxynil and DBHA are extremely similar in structure, varying only in that bromoxynil has a cyano (-CN) group that has been converted to a carboxyl (-COOH) group in the DBHA metabolite. Conversion to a carboxyl group is generally considered to decrease the toxicity of a molecule (US EPA 1998). The conversion to the carboxyl group should cause the DBHA to be more polar and therefore more soluble in water and less in fats. This increased water solubility, combined with the decreased fat solubility means that DBHA should be eliminated faster from the organism than its parent compound, bromoxynil. It is likely that these characteristics would also limit the amount of DBHA residue likely to be present in canola oil.

The Pest Management Regulatory Authority of Canada has recently agreed (February 2000) to the registration of bromoxynil for use on bromoxynil-tolerant canola varieties. To support this registration, a number of field trials were conducted on bromoxynil-tolerant canola between 1996 and 1997. The field trials monitored maximum residue levels of bromoxynil, as well as DBHA. The maximum residues of bromoxynil and DBHA in canola seeds, collected 71-119 days after the last application of bromoxynil, were less than 0.05 ppm each, that is, below the limit of quantitation for the method used. A further study, done with the processed fractions (oil and meal) of the seed, found no detectable residues, even with application rates 10 times that of the commercial rate, and therefore no concentration of residues with processing. Overall, the residues expected to be present in refined canola oil are effectively zero.

Conclusion

The evidence from the sub-acute toxicity study in mice does not indicate that there is any potential for nitrilase from *Klebsiella pneumoniae* subsp. *ozaenae* to be toxic to humans. Furthermore, humans are extremely unlikely to be exposed to this enzyme through the consumption of refined oil from bromoxynil-tolerant canola as the refined oil has been shown to be devoid of any detectable protein. The metabolite of

bromoxynil, DBHA, also does not show any potential to be toxic to humans at the predicted exposure levels.

Potential allergenicity of novel proteins

The concerns regarding potential allergenicity of novel proteins are two fold. Firstly, there are concerns that the ability to express new or different proteins in food will result in the transfer of allergens from one food to another, thereby causing some individuals to develop allergic reactions to food they have not previously been allergic to. Secondly, there are concerns that the transfer of novel proteins to food will lead to the development of new allergies in certain individuals. The former is more easily addressed than the latter because if an allergen is already known it is possible, using human sera or human skin tests, to test if it has been transferred. There are no reliable tests or animal models, however, which enable the prediction of the allergenic potential of novel proteins.

Instead, potential allergenicity can only be indicated by examination of a number of characteristics of the novel protein, such as whether it is derived from a known allergenic source, its physical/chemical characteristics (resistance to acid and protease degradation, amino acid sequence similarity with known allergens) and whether it is likely to be present in large amounts in the food as consumed and therefore have potential for allergic sensitisation.

Notwithstanding the lack of any detectable nitrilase in refined oil, the allergenic potential of nitrilase has been assessed according to the following:

- potential for human exposure to nitrilase;
- similarity to known allergens; and
- digestibility in simulated mammalian digestion fluids

Potential for human exposure

Refined oil from bromoxynil-tolerant canola has been found to contain no detectible nitrilase therefore humans would be extremely unlikely to be exposed to nitrilase through consumption of the oil.

Similarity to known allergens

Studies evaluated:

Astwood, J.D. (1997). *Klebsiella ozaenae* nitrilase (BXN) has no significant sequence similarity to known allergens and toxins. Monsanto Study Report No. MSL-15120 – submitted with Application A379 – Bromoxynil tolerant cotton.

A search for amino acid sequence similarity with known allergens and gliadins is a useful first approximation of potential allergenicity and potential association with coeliac disease (Fuchs and Astwood 1996, Metcalf *et al* 1996). Many protein allergens

have been characterised and their amino acid sequences are known, and importantly, their IgE binding epitopes have been mapped (Elsayad and Apold 1983, Elsayad *et al* 1991, Zhang *et al* 1992). The binding epitopes are generally between 8 and 12 amino acids in length.

To undertake the amino acid sequence comparison between nitrilase and known protein allergens and gliadins, a database of allergen and gliadin sequences was assembled from the standard public domain databases containing protein sequences (GenPept ver. 86.0, PIR ver. 41, SwissProt ver. 30). In addition, DNA sequences were retrieved from GenBank/EMBL ver. 86 as some allergen sequence entries do not appear in the protein sequence databases. The amino acid sequences of the allergens retrieved from the GenBank/EMBL database were either obtained from the GenEMBL flat files or were obtained by translation of the open reading frames in the DNA sequences. Therefore the assembled database consisted of two parts: (1) a dataset of protein sequences and (2) a supplemental database of protein sequences initially retrieved as DNA sequences. Duplicates were deleted from the assembled database and irrelevant sequences were identified by examining complete flat files or by reference to the scientific literature. The resulting database of 219 allergens and gliadins has been published in the scientific literature (Astwood *et al* 1996).

The allergen and gliadin database was then searched for sequences similar to nitrilase. A significant sequence similarity was defined as a sequence identity of greater than seven contiguous amino acids. No significant similarity between nitrilase and any of the known allergens or gliadins was identified.

Digestibility

Studies evaluated:

Aasen, E., *et al* (1997). Assessment of the digestibility of purified BXN nitrilase protein *in vitro* using mammalian digestive fate models. Monsanto Study Report No. MSL-15148 – submitted with Application A379 – Bromoxynil tolerant cotton

If proteins are to be allergenic they must be stable to the peptic and tryptic digestion and acid conditions of the digestive system if they are to pass through the intestinal mucosa to elicit an allergenic response.

The digestibility of nitrilase was determined experimentally using *in vitro* mammalian digestion models. *In vitro* studies with simulated digestion solutions have been used as models for animal digestion for a number of years and have had wide application.

To obtain sufficient quantities of purified nitrilase for testing, the enzyme was expressed in *Escherichia coli* from a cloned *Kelbsiella ozaenae* DNA fragment and purified to homogeneity (Stalker *et al* 1988). The coding region used to express nitrilase in *E. coli* was therefore identical to that transferred into Westar-Oxy-235. The molecular mass of nitrilase is approximately 37 kDa, however, the active form of the enzyme is as a dimer composed of two identical 37 kDa subunits.

Nitrilase was added to simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) and incubated at 37ºC over a series of time points. The time points for SGF were 0 sec, 15 secs, 30 secs, 1 min, 5 mins, 10 mins, 30 mins, 1 hour and for SIF the time points were 0 sec, 1 min, 5 mins, 15 mins, 30 mins, 1 hour, 2 hours, 4 hours, 8 hours and 24 hours.

Analysis of nitrilase after incubation in SGF showed that the protein is degraded to below the limit of detection within 15 seconds. Nitrilase was found to be stable in an inactive test system over the time period tested confirming that the degradation of nitrilase in the active test system is due to proteolytic activity, not to any molecular instability of nitrilase.

In SIF, nitrilase was degraded within 5 minutes of exposure. Once again, nitrilase was shown to be stable in an inactive SIF system.

The results of these studies demonstrate that nitrilase is rapidly degraded in conditions that mimic mammalian digestion, greatly minimising any potential for intact nitrilase to be absorbed by the intestinal mucosa.

Conclusion

Humans are highly unlikely to be exposed to nitrilase through the consumption of refined oil from bromoxynil-tolerant canola. Moreover, nitrilase does not possess any of the characteristics of known allergens. Therefore nitrilase has very limited potential to become a food allergen.

COMPARATIVE ANALYSES

Key nutrients

There are concerns that genetic modification will affect the overall nutritional composition of a food, or cause unintended changes that could adversely affect the safety of the product. Therefore a safety assessment of food produced from transgenic plants must include analysis of the composition of the food, based on a comparison with other commercial varieties of the crop. Generally, comparisons are made not only with the parental line but also with other non-transformed lines. If the parameter for the transformed line is within the normal range for non-transformed lines, this is considered acceptable (Hammond and Fuchs 1998).

Compositional analyses of key constituents were done on the seed, and its derived meal and oil fractions, harvested from field trials with Westar-Oxy-235 canola and its non-transformed parental control grown at various sites in Canada and France in 1992 – 1995. Data was also obtained from field trials in France in 1994 – 1995 with French canola elite lines that had been crossed with Westar-Oxy-235. Data was obtained for both bromoxynil-sprayed and unsprayed plants.

Proximate analysis

Proximate analysis was done on seed from Westar-Oxy-235, Tanto-Oxy (a hybrid between Westar-Oxy-235 and Tanto, a spring elite line used in France), and the respective parental control lines grown in field trials in Canada and France during 1995. All the bromoxynil-tolerant canola lines were treated with bromoxynil at 330 g/ha. The combined results are summarised in Table 4.

Table 4: Proximate analysis of seed harvested from Westar-Oxy-235 canola lines and control canola grown in Canada and France in 1995

Analysis	Parental control Lines Mean ± SE (n=6)	Westar-Oxy-235 canola lines Mean ± SE (n=6)
Dry matter (%)	92.85 ± 1.44	92.85 ± 1.76
Mineral content (% dry weight)	7.33 ± 1.66	8.18 ± 1.89
Nitrogen (2 reps)	4.35 ± 0.32	4.33 ± 0.20
Protein in seed (% D.W.)	27.03 ± 2.07	26.89 ± 1.26
Protein in meal (% D.W.)	45.91 ± 3.76	44.65 ± 2.84
Fat/oil (% D.W.)	41.10 ± 0.50	39.53 ± 1.76
Soluble sugars (% D.W.)	3.25 ± 0.95	2.90 ± 0.51
Total carbohydrates (% D.W.)	24.54 ± 3.76	25.40 ± 3.13
Gross energy (seed, Kcal/kg)	6491 ± 60	6494 ± 109
Gross energy (meal, Kcal/kg)	4894 ± 131	4802 ± 137

No significant differences were evident between the bromoxynil-tolerant canola lines and their parental controls in any of the major constituents.

Fatty acid analysis

New varieties of canola oil are analysed to ensure they meet certain specifications – this includes the fatty acid content. Canola oil has considerable natural variation in fatty acid composition and thus some variation in the composition of commercial canola oil is acceptable. The individual fatty acids measured were palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1 cis), linoleic acid (C18:2), and linolenic acid (C18:3), arachidic acid (C20:0), eicosenoic acid (C20:1), and behenic acid (C22:0). Values for erucic acid (C22:1) are presented in Table 8. The results are summarised in Tables 5 and 6.

Table 5: Fatty acid content (%total fatty acids) of seeds from Westar-Oxy-235 canola and control canola grown in Canada in 1992 – 1994. (see Table 7 for Codex ranges)

Fatty acid	Lines				
	Control (Unsprayed) (n =21)	Oxy-235 (Unsprayed) (n=5)	Oxy-235 (120g/ha) (n=5)	Oxy-235 (240g/ha) (n=5)	Oxy-235 (480g/ha) (n=5)
Palmitic acid	4.5 (3.9-5.9)[1]	4.4 (3.6-5.3)	4.2 (3.5-4.7)	4.1 (3.5-4.7)	4.1 (3.8-4.4)
Stearic acid	1.7 (1.2-2.5)	1.6 (1.3-1.9)	1.5 (1.4-1.6)	1.5 (1.3-1.7)	1.5 (1.3-1.7)
Oleic acid	61.9 (59.8-64.7)	63.8 (63.1-64.6)	64.0 (63.0-65.3)	63.7 (62.3-65.7)	64.4. (63.2-66.0)
Linoleic acid	19.5 (17.7-21.0)	18.4 (17.5-19.3)	17.9 (16.8-19.0)	18.3 (16.5-19.7)	17.8 (17.1-19.0)
Linolenic acid	9.0 (6.7-10.5)	9.1 (7.6-10.3)	9.6 (8.6-10.1)	9.4 (8.5-10.3)	9.5 (8.5-10.5)
Arachidic acid	0.7 (0.5-0.8)	0.7 (0.6-0.7)	0.6 (0.6-0.7)	0.6 (0.6-0.7)	0.6 (0.6-0.7)
Eicosenoic acid	1.5 (1.2-2.4)	1.4 (1.2-1.6)	1.5 (1.3-1.6)	1.4 (1.3-1.6)	1.4 (1.3-1.6)
Behenic acid	0.4 (0.3-0.5)	0.4	0.4	0.4 (0.3-0.4)	0.4 (0.3-0.4)

[1] mean value, range in parentheses

Table 6: Fatty acid content (% total fatty acids) of seeds from French elite lines crossed with Westar-Oxy-235 grown in France in 1994 – 1995

Fatty acid	CODEX Ranges	Line	
		French elite lines (Unsprayed) (n=8)	Westar-Oxy-235 X French elite lines (480g/ha) (n=8)
Palmitic acid	2.5-7.0	5.7 (5.1-6.6)[1]	5.5 (4.8-6.2)
Palmitoleic acid	0.0-0.6	0.15 (0.1-0.2)	0.15 (0.0-0.2)
Stearic acid	0.8-3.0	1.55 (1.2-2.0)	1.5 (1.2-1.9)
Oleic acid	51.0-70.0	61.4 (58.1-65.3)	60.5 (50.6-64.4)
Linoleic acid	15.0-30.0	20.9 (19.0-23.0)	21.1 (19.0-26.0)
Linolenic acid	5.0-14.0	8.4 (7.6-9.5)	8.65 (7.3-11.8)
Arachidic acid	0.2-1.2	0.5 (0.2-0.8)	0.6 (0.5-0.8)
Eicosenoic acid	0.1-4.3	1.2 (0.8-1.9)	1.2 (0.9-1.7)
Behenic acid	0.0-0.6	0.3 (0.0-0.5)	0.3 (0.2-0.5)

[1] mean value, range in parentheses

All the fatty acids measured were within the ranges specified by Codex (Table 6) for canola quality oilseed rape (Codex Alinorm 99/7 Appendix II 3.1), and no major differences between modified and control crops were identified. Treatment with bromoxynil had no significant effect on the fatty acid content.

Sterol and tocopherol analysis

The levels of sterols and tocopherols were measured in seed from control and Westar-Oxy-235 canola grown in France and Canada in 1995 as well as from an elite line (Samourai), also grown in France in 1995. The bromoxynil-tolerant canola lines were all treated with bromoxynil. The results are summarised in Table 7.

Table 7: Total sterol and tocopherol levels in bromoxynil tolerant canola and control canola grown in field trials in Canada and France in 1994 - 1995

Trial and crop		Bromoxynil treatment	Total Sterols (mg/100g oil)	Total Tocopherols (µg/g oil)
Canada (1995)	Control	-	777.3	876.0
(n=2)	Westar-Oxy-235	-	753.8	785.0
	Westar-Oxy-235	330 g/ha	760.8	808.5
France (1995)	Control	-	828.2	955.0
(n=2)	Westar-Oxy-235	-	851.2	1015.0
	Westar-Oxy-235	450 g/ha	840.2	1007.0
France (1995)	Control (Samourai)	-	922.3	739.88
(n=8)	Samourai-Oxy-235	450 g/ha	943.1	716.13
		Literature values:		424.0 – 1054.0

No major differences were evident in total sterol and tocopherol content between control and bromoxynil tolerant canola. Treatment with bromoxynil had no significant effect on either the total sterol or total tocopherol content.

Unsaponifiable matter

The Codex specification for unsaponifiable matter states that the level must be not higher than 1.5%. This component was measured for oils produced from the 1995 French and Canadian trial crops. All were below the specified level, and no significant differences were seen between control and modified crops.

Conclusion

Analysis of the compositional data of the canola seed and processed fractions indicates that there were no significant differences in the levels of key nutrients between Westar-Oxy-235 line and control lines. This was true for both untreated plants and those treated with bromoxynil.

Key toxicants

Canola contains two naturally occurring toxic compounds – erucic acid and the glucosinolates. High levels of erucic acid, a long-chain fatty acid, are considered to have cardiopathic potential based on laboratory studies with rats and the glucosinolates

have been found to possess goitrogenic properties. Because of this, canola must meet specific standards on the levels of erucic acid and glucosinolates – these are less than 2% erucic acid in the oil and less than 30μmoles of total glucosinolates in the meal.

The levels of erucic acid and the glucosinolates were measured in the oil and meal derived from seed samples taken from field trials with Westar-Oxy-235 canola and the non-transformed parental control grown at various sites in Canada and France in 1992-1995. Data was also obtained from field trials in France in 1994 – 1995 with French canola elite lines that had been crossed with Westar-Oxy-235. Data was obtained for both bromoxynil-sprayed and unsprayed plants. These data are presented in Tables 8 and 9 below.

Erucic acid

Table 8: Erucic acid content[1] of oil from Westar-Oxy-235 and elite crosses

Line	Bromoxynil treatment	Erucic acid level (% total fatty acids)
Control (n=21)	unsprayed	0.04 (0 – 2.2)
Westar-Oxy-235 (n=8)	unsprayed	0.01 (0 – 0.1)
Westar-Oxy-235 (n=5)	120 g/ha	0.0
Westar-Oxy-235 (n=5)	240 g/ha	0.0
Westar-Oxy-235 (n=5)	480 g/ha	0.0
French elite lines (n=8)	unsprayed	0.08 (0 – 0.5)
Westar-Oxy-235 x French elite lines (n=8)	480 g/ha	0.0
Literature range	-	0.0 – 2.0

[1] mean values with range in parentheses

Mean values of erucic acid in oil from Westar-Oxy-235 and elite lines expressing the bromoxynil tolerant trait were found to all be well below the limit specified for canola and comparable to that found in oil from the parental control lines. The application of bromoxynil to the plants did not result in any changes to the levels of erucic acid.

Glucosinolates

The glucosinolates are converted to more toxic compounds upon hydrolysis by myrosinase, an enzyme localised within the cells of Brassica seeds. When the seed is crushed, the enzyme acts upon the glucosinolate to produce isothiocyanates, thiocyanates and possibly nitriles depending on temperature and moisture conditions. However, during processing, a cooking step inactivates myrosinase, leaving glucosinolates intact. Some destruction and reduction of glucosinolates may occur in further processing steps. Nonetheless, breeders are encouraged to work towards the elimination of glucosinolates in canola.

There are over 100 known structural types of glucosinolates, nine of which have been monitored in canola because of the known potential toxicity of their metabolites. A group called the alkyl glucosinolates are monitored particularly closely – the sum of four of them must be less than a total of 30µmoles/g seed for the seed to be classified as canola quality – this is an industry standard agreed by various canola associations worldwide. Of similar concentration but of less concern are the indol glucosinolates, two of which are monitored. Two types from a third group of glucosinolates, the thioalkyl glucosinolates are measured but are typically present in very low concentrations.

Table 9: Glucosinolate content[1] of canola meal from Westar-Oxy-235 and elite crosses

Line	Bromoxynil treatment	Glucosinolates (µmol/g seed)		
		Alkyl	**Indol**	**Total**
Control (n=21)	unsprayed	10.27 (5.88 – 20.47)	5.21 (1.57 – 8.04)	15.48 (11.58 – 25.05)
Westar-Oxy-235 (n=5)	unsprayed	8.12 (5.76 – 10.99)	6.51 (5.04 – 8.4)	14.63 (13.45 – 16.39)
Westar-Oxy-235 (n=5)	120 g/ha	8.79 (7.06 – 11.29)	6.15 (4.53 – 7.25)	14.94 (11.82 – 17.59)
Westar-Oxy-235 (n=5)	240 g/ha	9.19 (7.06 – 13.61)	6.29 (4.92 – 8.28)	15.48 (12.29 – 19.55)
Westar-Oxy-235 (n=5)	480 g/ha	8.22 (5.17 – 13.17)	5.73 (4.28 – 6.72)	13.95 (11.59 – 19.83)
French elite lines (n=8)	unsprayed	8.46 (7.1 – 11.5)	3.33 (2.8 – 4.1)	11.75 (10.2 – 15.4)
Westar-Oxy-235 x elite lines (n=8)	480 g/ha	8.59 (4.3 – 15.7)	3.44 (2.8 – 4.4)	12.03 (7.2 – 20.1)
Literature range		7.28 – 14.4	1.82 – 11.4	6.70 – 18.50

[1] mean values with range in parentheses

The levels of total glucosinolates in the bromoxynil-tolerant canola lines were found to be well below the 30µmole maximum limit for oil-free meal and were also comparable to the levels found in the corresponding parental control lines. The application of bromoxynil to the plants did not result in any changes to the levels of glucosinolates in the meal.

Some differences were apparent in the levels of the different classes of glucosinolates. In particular, the bromoxynil-tolerant canola lines exhibit a pattern of slightly higher levels of the indol glucosinolates, compared to the parental control. This contrasts to the slightly reduced levels of the alkyl glucosinolates, compared to the parental control. Overall, this balances out to very little difference in the levels of total glucosinolates, which as stated above, are well below the industry standard of 30µmoles. As the indol glucosinolates are much less of a concern than the alkyl glucosinolates, the slightly

increased levels are not considered to pose a hazard, particularly as the meal is not intended for human consumption.

NUTRITIONAL IMPACT

In assessing the safety of food produced using gene technology, a key factor is the need to establish that the food is nutritionally adequate and will support typical growth and well being. In most cases, this can be achieved through an understanding of the genetic modification and its consequences, together with an extensive compositional analysis of the food. Where, on the basis of available data, there is still concern or doubt in this regard, carefully designed feeding studies in animals may provide further re-assurance that the food is nutritionally adequate. Such studies may be considered necessary where the compositional analysis indicates significant differences in a number of important components or nutrients, or where there is concern that the bioavailability of key nutrients may be compromised by the nature of the genetic changes to the food.

Animal feeding studies

In the case of oil derived from bromoxynil-tolerant canola there is adequate compositional data to demonstrate the nutritional adequacy of the oil. However, a feeding study on canola meal was evaluated as additional supporting information.

Feeding study in rats

A 28-day feeding study was carried out in rats to compare the effects of ingestion of canola cake made from both non-transgenic (Westar and Tanto) and transgenic (untreated and that treated with bromoxynil) canola. The study was performed in accordance with the OECD Principles of Good Laboratory Practice (OECD, 1982):

From days 1 to 28, each type of cake was administered *ad libitum* at a 10% concentration to groups of five male and five female rats. Clinical signs were recorded at least once a day throughout the study. Additional detailed physical examination was performed weekly. Body weights were measured on days –1, 1, 8, 15, and 22 and at final sacrifice. The weight of food supplied to each animal and that remaining at the end of the food consumption period was recorded for each week throughout the treatment period. From these records, the mean weekly consumption was calculated for each rat. Food spillage was also noted. For clinical pathology studies, blood samples were collected before necropsy. At necropsy macroscopic examination of the external surfaces, all orifices and all major body cavities, organs and tissues was carried out. Any significant macroscopic findings were recorded and the tissues (adrenal gland, heart, kidney, liver and spleen) samples taken.

Results

There were no mortalities and no clinical signs of toxicity in any of the groups. Neither the mean body weight, mean daily intake, haematology nor clinical chemistry was affected by the type of canola administered. Likewise, no differences were seen in the macroscopic observations, or the microscopic examination of the organs sampled.

At a level of 10% inclusion of canola cake in feed, therefore, there was no difference between the control and the transgenic canola in their ability to support growth and well being of rats.

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