Theor Appl Genet (1989) 78:525–530

Microspore mutagenesis and selection: Canola plants with field tolerance to the imidazolinones

E.B. Swanson*, M.J. Herrgesell, M. Arnoldo, D.W. Sippell and R.S.C. Wong

Allelix Crop Technologies, 6850 Goreway Drive, Mississauga, Ontario, L4V1P1, Canada

Received May 22, 1989; Accepted June 27, 1989
Communicated by G. Wenzel

Summary. In vitro microspore mutagenesis and selection was used to produce five fertile double-haploid imidazolinone-tolerant canola plants. The S_2 plants of three of the mutants were resistant to at least the field-recommended levels of Assert and Pursuit. One mutant was tolerant to between five and ten times the field-recommended rates of Pursuit and Scepter. Two semi-dominant mutants, representing two unlinked genes, were combined to produce an F_1 hybrid which was superior in imidazolinone tolerance to either of the heterozygous mutants alone. Evaluation of the mutants under field conditions indicated that this hybrid and the original homozygous mutants could tolerate at least two times the field-recommended rates of Assert. The field results indicated the mutants were unaffected in seed yield, maturity, quality and disease tolerance. These genes represent a potentially valuable new herbicide resistance system for canola, which has little effect on yield, quality or maturity. The mutants could be used to provide tolerance to several imidazolinones including Scepter, Pursuit and Assert.

Key words: Microspores – Haploid – Pursuit – Assert – Tolerance

Introduction

Most oilseed rape (*Brassica napus* and *B. campestris*) seed produced in Canada is canola quality. Canola oil has less than 2.0% erucic acid, and the oil-free meal has less than 30 μm/gram of glucosinolate (Genser and Eskin 1987).

Pioneering research in Brassica microspore culture (Lichter 1982, 1985, Keller and Armstrong 1983; Choung and Beversdorf 1985) led to the development of techniques to efficiently isolate large numbers of microspore-derived embryos (Swanson et al. 1987). The first reported use of microspore mutagenesis and selection resulted in *Brassica* plants with tolerance to chlorsulfuron (Glean) (Swanson et al. 1988).

The *Brassica* microspore system is a powerful alternative to other in vitro selection techniques. Some of the advantages of the microspore selection system have been recently reviewed (Swanson 1989) and include the use of large single-cell haploid populations, low levels of somaclonal variation, efficient and uniform mutagenic applications, expression of recessive traits and a final product which breeds true for the selected trait. Perhaps the most valuable characteristic of this technique is that over 90% of the microspore-derived embryos can give rise to plants (Swanson et al. 1987).

The enzyme, acetohydroxyacid synthase (AHAS), is the first enzyme common to the biosynthesis of leucine, valine and isoleucine, and is the primary site of action for the inhibitory effects of the sulfonylureas (Ray 1984), triazole pyrimidines (Schloss et al. 1988) and the imidazolinones (Shaner et al. 1984). Mutants tolerant to the sulfonylureas have been isolated in several plants, including tobacco (Chaleff and Ray 1984), *Arabidopsis thaliana* (Haughn and Sommerville 1986), soybeans (Sebastion and Chaleff 1987), *Brassica napus* (Swanson et al. 1988) and *Datura innoxia* (Saxena and King 1988) and those tolerant to the imidazolinones have been isolated in corn (Shaner and Anderson 1985).

This paper reports on the characterization of imidazolinone-tolerant and -resistant mutants derived from in vitro mutagenesis and selection of microspores of *Brassica napus*.

* To whom correspondence should be addressed

Materials and methods

Mutant isolation and characterization

Brassica napus (cv Topas) microspores were isolated, mutagenized and cultured, essentially as outlined by Swanson et al. (1988) using 20 μM ethyl nitrosourea (ENU). The mutagenized microspores and non-mutagenized controls were cultured in microspore medium containing 40 µg/l AC 263,499 (5-ethyl-2-[4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl] nicotinic acid), tradenamed 'Pursuit'. Technical grade Pursuit was provided by American Cyanamid Company of Princeton, New Jersey. Surviving embryos were subcultured to B5 medium (Gamborg et al. 1968) with 0.45% agarose for 5 days, and then transferred to the same medium with 50 µg/l Pursuit. Plants were regenerated and transferred to soil.

Small haploid plantlets with active root growth were removed, rinsed with water and placed in a beaker with 0.2% colchicine for approximately 6 h. The colchicine-treated plants were rinsed with water and repotted. Selfed seed and reciprocally backcrossed seed of mutant plants to 'Topas' was collected from fertile sectors of the colchicine-treated plants. Several generations of selfed seed (S_2 and S_3) of the mutants were planted in the greenhouse for seed increase and crossing. Reciprocally crossed seed of the first two Pursuit mutant double-haploid plants ($P_1 \times P_2$ and $P_2 \times P_1$) were produced by hand-emasculation and crossing of S_2 plants of P_1 and P_2 in the greenhouse. Seed from these crosses was then planted and the F_1 plants selfed to produce F_2 seed for linkage and inheritance studies.

Biochemical analysis for acetohydroxyacid synthase (AHAS) from leaf extracts was performed as outlined by Singh et al. (1988) using three replicates.

Greenhouse herbicide trials

Seeds from the mutant plants and the parental cultivar (Topas) were planted in Pro Mix-C (Plant Products, Toronto, Canada), which had previously been sprayed PPI (pre-plant incorporated) with 0, 50, 100, 300, and 500 g/ha of Pursuit or a second imidazolinone, Scepter (2-[4-isopropyl-4-methyl]-5-oxo-1H-imidazol-2-yl-3-quinolinecarboxylic acid). A spray chamber (Research Instruments, Guelph, Ontario, Canada) containing a boom sprayer with a flat fan nozzle (TeeJet SS8002) was calibrated to deliver the selected herbicide applications. Ten replicates per treatment were used.

Post emergent (PE) applications of Pursuit and Scepter were applied when plants had reached the 3–5 leaf stage using the levels indicated for PPI applications. Agral 90 (0.15%) was added as a surfactant for the PE tests.

Assert (methyl 6-[4-isopropyl-4-methyl-5-oxo-2-imidazoline-2-yl]-m-toluate), another imidazolinone, was applied PE at the 3–5 leaf stage using 0, 400, 600, 800, 1000 and 1200 g/ha. Ten replicates were used per treatment.

The fatty acid composition of the P_1, P_2 mutants and the Topas parental material was determined by gas-liquid chromatography of methyl esters of the fatty acids, following the methods of Daun et al. (1983). The glucosinolate level was similarly determined using gas liquid chromatography to analyze pertrimethylsilyl desulfoglucosinolates, following the procedure of Heaney and Fenwick (1980).

To determine the level of blackleg (*Leptosphaeria maculans*) tolerance in the P_1 and P_2, pycnidiospores (10 µl at a suspension concentration of 10^7 spores/ml) were injected with a hypodermic syringe into the base of the stem of young plants. The plants were incubated in the greenhouse at 21°–24 °C, and the length of the lesions was measured at 3-week intervals. Three plants of Westar, Topas, P_1 and P_2 were tested.

Field trials

Field trials of the P_1, P_2 and the $P_1 \times P_2$ hybrid were planted May 13 in Rosebank, Manitoba to test the tolerance of the mutants to post emergent applications of Assert. The seeds were planted using a Wintersteiger (International Precision, Lincoln, Nebraska) plotspider. The plots were randomized as a split plot design with herbicide levels as main factors. The seeds were planted in standard 1×6 meter plots, using three replicates of 900 seeds per plot. When the plants had reached the 3–5 leaf stage, 0, 200, 400, 600, 800 g/ha of Assert and 100 and 300 g/ha of Pursuit were applied with 0.15% Agral 90. Data was collected on maturity by estimating the days left till full stand maturity at day 96 post planting, and the plants were subsequently combined at day 106. Seed yields were taken after direct combining and drying the seed to approximately 7.0% moisture.

A similar trial which included PE applications of Pursuit and Assert was planted in Alloa, Ontario on June 10. The late planting date was due to severe drought stress in the summer of 1988. This trial had insufficent time to fully mature but provided dramatic visual evidence of herbicide tolerance under field conditions.

Results

The first ten putative Pursuit-tolerant embryos developed from separate microspore selection experiments involving from 6,000 to over 50,000 potential embryos. All ten of these tolerant embryos produced plants and half of these plants produced selfed progeny with higher tolerance to Pursuit as whole plants (Fig. 1). The other five lines either had very low tolerance or no tolerance to Pursuit as whole plants.

The tolerant phenotypes were easily distinguishable from the parental Topas canola plants. Canola plants were killed by 20 g/ha and were seriously damaged by much lower rates. The tolerant selections withstood rates of Pursuit from 50 to 500 g/ha (Fig. 1). P_1 and PM-6 tolerated levels of Pursuit approaching two times the field-recommended rate of 50 g/ha, while P_2 tolerated rates of Pursuit up to 500 g/ha or ten times the recommended rate. While PM-3 and PM-4 were tolerant to Pursuit, the plants exhibited growth inhibition even at 20 g/ha and were very severely set back by 50 g/ha. The P_2 plants also tolerated more than five times the field-recommended rate for Scepter (results not shown). All the mutants exhibited similar tolerance levels whether the herbicide was applied PE or PPI.

For the purposes of the greenhouse spray chamber experiments, resistance was defined as plants showing no visible effects following the application of the herbicide. Highly tolerant plants were defined as plants with growth rates slightly reduced by the herbicide but with little visible damage. Low tolerant plants were defined as plants which were severely set back and exhibited various damage symptoms, including necrosis of leaves and apical meristems and prolific branching. Sensitive plants were defined as being killed by the herbicide.

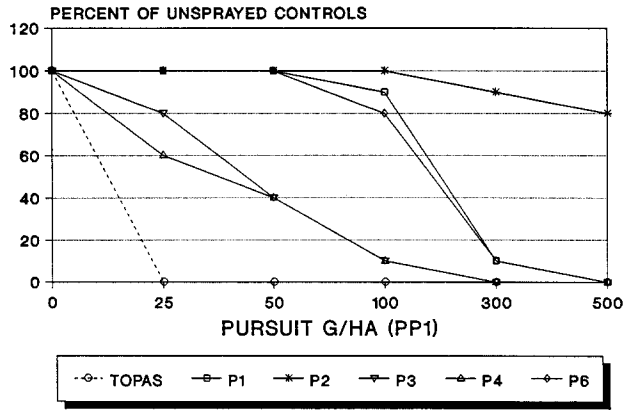

Fig. 1. The tolerance of the selfed progeny of five microspore-selected double-haploids. Seeds were germinated in soil sprayed with Pursuit (PPI). Plants were harvested after 4 weeks and the fresh weights were compared against unsprayed controls

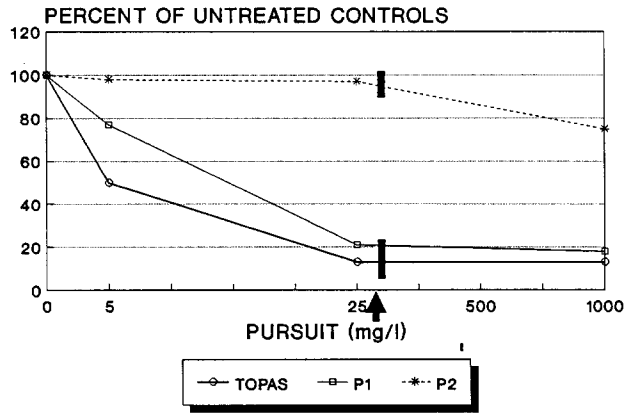

Fig. 2. The AHAS enzyme activity extracted from Topas, P_1 and P_2 (*bar* denotes change in scale for the X axis)

The tolerance of the heterozygotes ($P_2 \times$ Topas or $P_1 \times$ Topas) was intermediate compared to the tolerance between the homozygous resistant and -susceptible parents (Table 1). This data support the hypothesis that the mutants were semidominantly inherited.

The tolerance of progeny from reciprocal crosses between the P_1 and P_2 mutants and the parental Topas cultivar were similar (results not shown), which indicates that the mutations were encoded by the nucleus. The F_1 progeny of the two mutants ($P_1 \times P_2$) produced plants with a level of Pursuit tolerance consistently superior to either heterozygote alone, and this indicates that the tolerance mechanisms are complementary (additive). Similarly, we have recently selected F_2 segregants of the $P_1 \times P_2$ cross which tolerate levels of Pursuit above that tolerated by either homozygous mutant alone (data not shown), and these transgressive segregants also indicate the two genes are likely to be complementary. Similar results were obtained using Assert. The F_2 segregation

Table 1. The herbicide tolerance of P_1 and P_2 and F_1 and F_2 progeny planted in soil treated with 100 g/ha of Pursuit

Genotype	S	LT	HT	R	Total
Topas (T)	20	0	0	0	20
P_1	0	0	16	4	20
P_2	0	0	1	19	20
$P_1 \times T$	0	12	8	0	20
$P_2 \times T$	0	0	3	17	20
F_2 ($P_1 \times P_2$)	12[a]	56	67	45	180

[a] Evidence for P_1 and P_2 being separate unlinked genes is indicated by appearance of sensitive types in F_2. S = Sensitive, LT = Low Tolerance, HT = High Tolerance, R = Resistent

Table 2. The seed yield in grams/plot from standard 1 × 6 meter field plots sprayed post emergently with Assert. Seed was dried down to approximately 7% moisture before weighing

Genotype	Level of Assert applied post emergently Grams/hectare				
	0	200	400	600	800
Topas	725a	172c	120d	0	0
P_1	966b	903a	914b	937b	957ab
P_2	883ab	771b	711c	757c	902b
$P_1 \times P_2$	927b	945a	1,137a	1,144a	1,046a

[a] Numbers within a column that are followed by the same letter are not significantly different at the 5% level, according to Duncan's multiple range test

Table 3. The affect of Assert applications on the plant maturity of Topas, P_1, P_2, and the F_1 hybrid $P_1 \times P_2$.

Genotype	Days till full plant maturity Assert in grams per hectare				
	0	200	400	600	800
Topas	3[a]	12	–	–	–
P_1	1	0	0	1	2
P_2	7	4	5	6	7
$P_1 \times P_2$	3	4	4	4	4

[a] Estimated days remaining till full stand maturity at 96 days post-planting

pattern outlined in Table 1 indicates that the two genes were separate and unlinked or very far apart. This conclusion was evident by the appearance of sensitive types in the F_2 in a ratio that was not significantly different from a dihybrid cross for two separate and unlinked genes. There is little chance of misclassifying the sensitive types, as the tolerant types are clearly distinguishable even at 20 g/ha.

The P_2 mutant produced an AHAS enzyme that was greater than 500 times more tolerant to Pursuit than the wild type (Fig. 2). The AHAS enzyme from P_1 was only

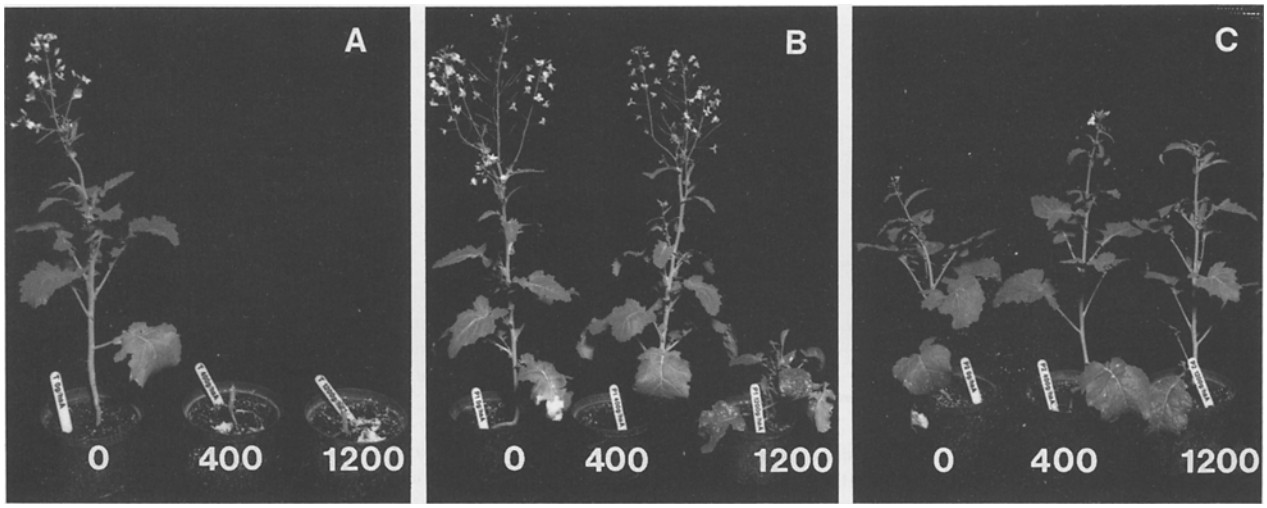

Fig. 3A–C. The relative herbicide tolerance of P_1 and P_2 to post emergent applications of 400 and 1200 g/ha of Assert. **A** = Topas, **B** = P_1, **C** = P_2

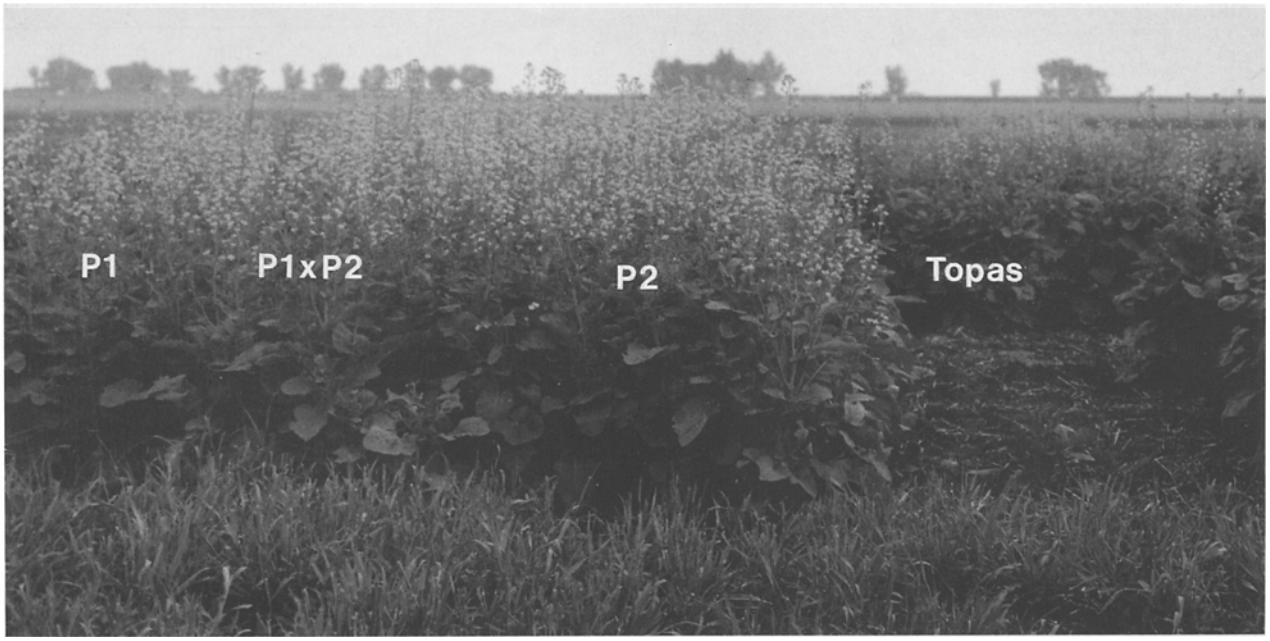

Fig. 4. The field tolerance of Topas, P_1, P_2 and $P_1 \times P_2$ hybrids after application of 800 g/ha of Assert

slightly more tolerant than the wild-type enzyme obtained from the Topas parent plant.

P_1 and P_2 were tolerant to 500 and 1200 g/ha of Assert, respectively, in greenhouse spray chamber tests (Fig. 3). P_1 was very tolerant to Assert applications up to 500–600 g/ha, but showed increasing damage symptoms above 600 g/ha to 1200 g/ha Assert, where severe damage was evident.

The field tolerance of the mutants to Assert was better than that observed in the spray chamber tests. The tolerance of these mutants in the field was visibly dramatic (Fig. 4). The P_1 and P_2 pure lines and their hybrid ($P_1 \times P_2$) withstood Assert applications up to 800 g/ha with no yield penalty (Table 2). The hybrid between the two mutants appeared to show some heterosis for seed yield at increasing herbicide applications. The application of Assert did not affect the plant maturity of the mutants (Table 3). While the P_1 mutant was slightly earlier than Topas and P_2 was slightly later, the hybrid was very similar to Topas. The mutants were the same as the Topas parent for canola quality (glucosinolates and erucic acid levels), tolerance to blackleg (*Leptosphaeria mac-*

ulans) and fatty acid profiles. Further, no unusual disease symptoms were evident in the field trials for any of the herbicide mutants tested.

Discussion

Imidazolinone-tolerant plants have been isolated in corn (Shaner and Anderson 1985). Anderson and Georgeson (1989) characterized two types of imidazolinone-tolerant corn mutants. XA17 was a mutant with cross tolerance to the sulfonylureas and QJ22 was a mutant selective only for the imidazolinones. The development of these corn mutants into commercial lines has been initiated, but it may be as long as 10 years from the time of isolation before the tolerance is backcrossed into elite inbreds (Newhouse et al. 1989). While no data was provided, preliminary field tests appear to indicate the mutations have little effect on height, flowering date or yield (Anderson and Georgeson 1989).

In vitro microspore mutagenesis and selection is a rapid method of producing mutants with added value characteristics. The isolation, regeneration, inheritance and field evaluation of the first two microspore-derived mutants of the present study was completed within one year. All the lines selected from microspore selection have bred true for the herbicide tolerance trait and none of the mutant lines evaluated in the field suffered any measurable penalty in yield, quality, disease resistance or maturity. One of the mutants (P_1) matured slightly earlier than Topas, and the other (P_2) matured slightly later. However, the Topas cultivar normally consists of plants with slightly different maturity rates (unpublished results) and the range of maturities observed in the mutants may reflect the natural variation found within the cultivar. The tolerance of the mutants to blackleg was examined in the greenhouse studies because it is a major disease problem within canola (Gabrielson 1983). Other diseases were evaluated in the field, however, the drought conditions in 1988 limited any disease problems.

The only herbicide-resistant mutant which has been produced for commercial sale was developed by transferring a spontaneous atrazine-tolerant *Brassica rapa* mutant (Maltais and Bouchard 1978) to canola. The research resulted in the release of the canola cultivar 'Triton'. However, the mutant canola plants yielded only 80% of the normal canola cultivars and had almost 2.5% less oil (Beversdorf and Hume 1984).

The first report of in vitro microspore mutagenesis and selection in plants (Swanson et al. 1988) resulted in *Brassica napus* plants with tolerance to chlorsulfuron (Glean). These mutants were tolerant to levels of chlorsulfuron which could provide resistance to soil residues of chlorsulfuron. Several other papers have appeared on the isolation of sulfonylurea-tolerant plants (Chaleff and Ray 1984; Creason and Chaleff 1988), and the transfer of a herbicide-tolerant AHAS gene from *Arabidopsis* to tobacco (Haughn et al. 1988). The site of action of the imidazolinones and the sulfonylureas on the AHAS enzyme has recently been shown to be similar in bacteria (Schloss et al. 1988).

The resistance level of P_2 is probably due to a modification in a major isozyme of AHAS, while the P_1 tolerance mechanism is less clear. It may involve a modification to an AHAS enzyme normally produced in lower amounts in *Brassica*.

All of the imidazolinone compounds tested would control the most troublesome weeds in canola (i.e. wild mustards and stinkweed). However, the application of Pursuit as a single broad-spectrum herbicide could reduce the chemical cost of weed control in canola and reduce the problems associated with controlling specific regional weed spectrums. In the present study, several imidazolinone-tolerant mutants were produced, involving at least two separate and unlinked genes conferring tolerance to the imidazolinones. Either mutant could withstand Assert and Pursuit applications well above the recommended field rates, although P_1 showed some damage in greenhouse trials. P_2 could also withstand more than five times the field-recommended rate for Scepter.

The yield advantage of the hybrid ($P_1 \times P_2$) in the field trials may be due to the natural heterosis which is evident within the cultivar Topas. Random selection of these two mutant plants from the Topas cultivar combined with hand-emasculation and crossing (ensuring close to 100% hybrid seed) could account for the observed heterosis.

It is likely that weeds tolerant to the imidazolinones will develop if the herbicides are utilized continuously, as occurred with the development of triazine-resistant weeds in corn. However, Canola is planted in a 3- or 4-year rotation with a variety of other crops. Thus, the continuous application of a particular herbicide could be avoided in canola. Imidazolinone tolerance in *Brassica napus* could have several potential advantages for the crop. It would permit tolerant *Brassica* plants to be grown in any rotation where the rotational crop uses imidazolinones and where the residues might damage a fall/winter canola crop or a rotational spring canola crop. The imidazolinone-tolerant canola lines would enable the farmer to select a herbicide with very low use rates and mammalian toxicity. Some of the canola mutants tested have tolerated levels of imidazolinone approaching ten times the field-recommended rates. Therefore, the farmer would be assured of a very wide safety margin during application.

The first two microspore-derived mutants (P_1 and P_2) were similar to the Topas parent for yield, maturity, disease resistance and oil quality. Therefore, the microspore

selection method outlined did not alter the selected plants for other critical genes.

There is little doubt that more and more commercially applicable herbicide-tolerant lines will be isolated and become powerful products for the farmer. However, unless these lines are exploited judiciously, nature will quickly produce abundant tolerant weeds and greatly reduce their usefulness. This may be especially true in cases involving the imidazolinones and the sulfonylureas, because they exhibit exceptional product effectiveness and environmental attractiveness and, thus, may tend to be over-utilized.

Acknowledgements. We are grateful to Cyanamid Canada for providing financial support for this research.

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