Weed Science, 48:688–694. 2000

Pollen flow between herbicide-resistant *Brassica napus* is the cause of multiple-resistant *B. napus* volunteers[1]

Linda Hall
Corresponding author. Agronomy Unit, Alberta Agriculture, Food, and Rural Development, 6903 116 Street, Edmonton, AB, Canada T6H 5Z2; linda.hall@agric.gov.ab.ca

Keith Topinka
Agronomy Unit, Alberta Agriculture, Food, and Rural Development, 6903 116 Street, Edmonton, AB, Canada T6H 5Z2

John Huffman
Regional Advisory Services, Alberta Agriculture, Food, and Rural Development, 1001 Provincial Building, 10320 99 Street, Grand Prairie, AB, Canada T8V 6J4

Lesley Davis
Allen Good
Department of Biological Sciences, University of Alberta, Edmonton, AB, Canada T6G 2E9

A field in which *Brassica napus* volunteers were not controlled by several applications of glyphosate was investigated in 1998. This field had been planted with glufosinate-resistant and imidazolinone-resistant *B. napus* in 1997 and was adjacent to a field that had grown glyphosate-resistant *B. napus.* Mature volunteer *B. napus* were collected on a 50- by 100-m grid in the field. Progeny from 34 volunteers were sprayed with glyphosate at 440 g ae ha^{-1}, and the survivors were sprayed with either glufosinate or imazethapyr at 400 or 50 g ai ha^{-1}, respectively. Where seed numbers permitted (14 volunteers), seedlings were also sprayed sequentially with glyphosate, glufosinate, and imazethapyr, at 440 g ae ha^{-1}, 400 g ai ha^{-1}, and 50 g ai ha^{-1}, respectively. In total, 15 volunteers had progeny that were between 66 and 82% resistant to glyphosate, consistent with the predicted 3:1 resistant : susceptible ratio. Volunteer *B. napus* plants with glyphosate-resistant seedlings were most common close to the putative pollen source; however, a plant with glyphosate-resistant progeny was collected 500 m from the adjacent field edge. Seedlings from all nine volunteers collected from the glufosinate-resistant area showed multiple resistance to glyphosate and glufosinate, whereas seedlings from 10 of 20 volunteers collected from the imidazolinone-resistant area showed resistance to imazethapyr and glyphosate. DNA extraction and restriction fragment length polymorphism (RFLP) analysis of seedlings confirmed that mature *B. napus* volunteers were hybrids resulting from pollen transfer rather than inadvertent seed movement between fields. Two seedlings from the 924 screened were resistant to all three herbicides. Progeny from these self-pollinated individuals were resistant to glyphosate and glufosinate at the predicted 3:1 resistant : susceptible ratio and resistant to imazethapyr at the predicted 15:1 resistant : susceptible ratio. Sequential crossing of three herbicide-resistant varieties is the most likely explanation for the observed multiple herbicide resistance. Integrated management techniques, including suitable crop and herbicide rotations, herbicide mixtures, and nonchemical controls should be used to reduce the incidence and negative effect of *B. napus* volunteers with multiple herbicide resistance.

Nomenclature: *Brassica napus* L., oil-seed rape; ALS, acetolactate synthase; EPSP, enolpyruvylshikimate-3-phosphate; glufosinate; glyphosate; imazethapyr.

Key words: Pollen transfer, herbicide tolerance, volunteer canola, oilseed rape.

In Canada, herbicide-resistant *Brassica napus* became commercially available in 1995, and of the 5.2 million ha of *B. napus* grown in 1998, 51% was herbicide resistant (Downey 1999). The introduction of herbicide-resistant *B. napus* has offered producers a variety of new, safe, and effective strategies for weed control. Despite rapid adoption, agronomic tribulations have been predictable and minor.

Four types of herbicide-resistant *B. napus* are commonly grown: glufosinate-, bromoxynil-, and glyphosate-resistant varieties, genetically modified by the insertion of novel genes encoding specific herbicide resistance, and a nontransgenic imidazolinone-resistant type. Glufosinate resistance is conferred by a single *bar* gene coding for phosphinothricin acetyltransferase (G), an enzyme that inactivates glufosinate ammonium (Canadian Food Inspection Agency 1995a; De Block et al. 1987; Wohlleben et al. 1988). Glufosinate-resistant 'Innovator' is presumed to be homozygous for glufosinate resistance. Glyphosate resistance is conferred by introduction of a single genetic construct containing two genes (R): one encoding for an herbicide-insensitive enolpyruvylshikimate phosphate (EPSP) synthase, the enzyme inhibited by glyphosate, and the second encoding for an enzyme that degrades glyphosate (Canadian Food Inspection Agency 1996; Barry et al. 1992; Padgette et al. 1992). The transgene segregates as a single locus and 'Quest' is known to be homozygous. Plants that contain a single copy of either glyphosate or glufosinate genes (termed hemizygous because the transgene is absent on the homologous chromosome) are herbicide resistant. These transgenes segregate similarly to a single dominant gene. Imidazolinone-resistant *B. napus* lines were selected with herbicide-insensitive acetolactate synthase (ALS) enzymes at two different and unlinked loci (Shaner et al. 1996). '45A71' is homozygous for both mutations (Canadian Food Inspection Agency 1995b) here designated I^A and I^B. Resistance is a semidominant trait; however, the relative resistance conferred differs between loci, and resistance is additive when both loci are present.

The use of herbicide-resistant *B. napus* has raised several weed management concerns. First, resistant volunteer *B. napus* may act as a weed and require different strategies to control than conventional *B. napus* (Keeler et al. 1996; Squire et al. 1997). Volunteer *B. napus* is among the 20 most common weeds in Alberta fields, occurring as a resid-

ual weed in 11.8 and 10.5% of all wheat and barley fields surveyed in Alberta in 1997, respectively (Thomas et al. 1998). Volunteer *B. napus* can persist up to 4 yr after planting (Thomas and Leeson 2000). Glyphosate-resistant *B. napus* remains uncontrolled where glyphosate alone is used for preseeding or chemical fallow weed control. Should other glyphosate-resistant crops be introduced, in-crop control of glyphosate-resistant *B. napus* will not be possible with glyphosate alone. The only current use of glufosinate in Western Canada is for weed control in glufosinate-resistant *B. napus.* Imidazolinone-resistant *B. napus* is uncontrolled in crops that receive only ALS-inhibiting herbicides for broadleaf weed control. However, all volunteer *B. napus* can be controlled by alternative herbicides, including inexpensive auxinic herbicides such as 2,4-D and MCPA or by using herbicide mixtures or nonchemical weed control strategies.

A second concern is the transfer of resistant genes to weedy relatives (Kareiva et al. 1994). *Brassica napus* is an allotetraploid for which the two progenitors are *B. campestris* L. = *B. rapa* L. (birdsrape mustard) and *B. oleracea* (Chèvre et al. 1996). Cruciferous weeds usually occur where *B. napus* is grown but species present and the potential for gene transfer varies between regions and continents. In Western Canada, cruciferous weeds include *Sinapis arvensis* = *B. kaber* (DC.) L. C. Wheeler (wild mustard), *Raphanus raphanistrum* (wild radish), and *Erucastrum gallicum* (dog mustard). Introgression into genomes of *B. kaber* appears unlikely (Lefol et al. 1996). Crosses between *B. napus* and *R. raphanistrum* and *E. gallicum* have produced F1 hybrids (Lefol et al. 1997). *Brassica napus* and *R. raphanistrum* hybrids were vigorous but mostly sterile, whereas *B. napus* and *E. gallicum* hybrids were not vigorous but produced fertile progeny that resembled *E. gallicum* when back-crossed to the *E. gallicum* parent. However to date, there have been no reports from field or greenhouse crosses of cruciferous weeds with herbicide-resistant genes conveyed from *B. napus.* (R. K. Downey, personal communication).

Finally, the transfer of herbicide resistance between varieties of *B. napus* may result in *B. napus* volunteers resistant to two or more herbicides. *Brassica napus* has interplant outcrossing rates of 21.8% (Rakow and Woods 1987). Outcrossing diminishes with distance (Stringam and Downey 1978). Pollen has moved long distances, although its viability was not determined (Timmons et al. 1996). Under field conditions, transfer from one field to another generally results in less than 1% out-crossing in the first 100 m (Downey 1999). However, assuming a 0.2% out-crossing rate in a field yielding 1,400 kg ha^{-1} with a harvest loss of 5%, Downey (1999) estimated some 35,000 out-crossed seeds (3.5 seeds m^{-2}) would remain in the recipient field. Fall or spring frost and cultivation during seedbed preparation would kill most but not all of the plants.

In spring 1997, a producer in Alberta, Canada, planted 15 ha of glufosinate-resistant *B. napus* Innovator, and the balance of the field with imidazolinone-resistant *B. napus,* 45A71 (Figure 1, Field 1). To the west, in an adjacent field (Field 2), he planted glyphosate-resistant *B. napus* Quest (Figure 1). The two fields were separated by a 22-m road allowance. Seeding and harvesting operations were separated in time, and equipment was used in other fields between operations in Fields 1 and 2. Therefore, seed movement via equipment from field to field was unlikely.

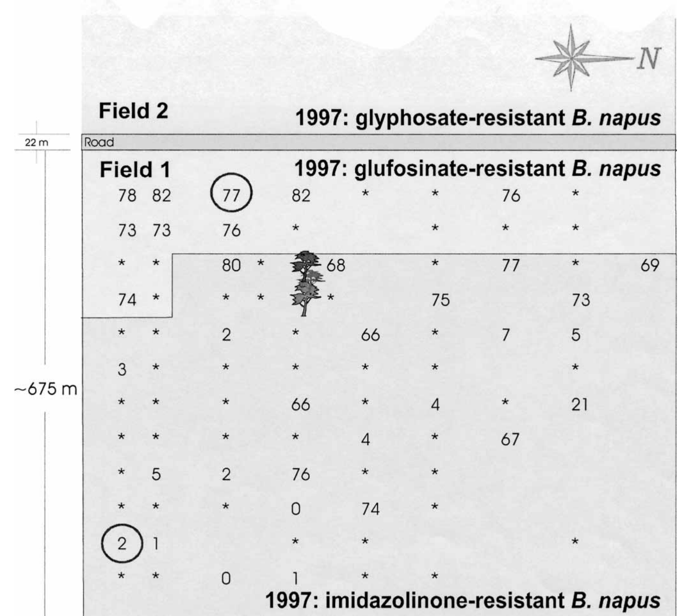

FIGURE 1. The percentage of glyphosate resistance in progeny and the position of the volunteers collected in Field 1. Locations where volunteers were collected but not tested are indicated by asterisks (*), and locations where no volunteers were present are blank. Locations of the maternal parents of triple-resistant plants A and B are circled. The relative positions of Fields 1 and 2 and a treed noncrop area are also indicated.

In 1998, the south section of Field 2 was planted with imidazolinone-resistant *B. napus* (variety 45A71). Field 1 was fallowed, with glyphosate applied for weed control at 0.9 kg ae ha^{-1} on May 13 and at 0.6 kg ae ha^{-1} on June 19. The west half of the field received a third application of glyphosate (0.4 kg ae ha^{-1}) on August 6. *Brassica napus* plants were reported in Field 1, apparently unaffected by glyphosate application.

This report documents an investigation into the cause of glyphosate-resistant volunteer *B. napus* in a field that had not previously grown a glyphosate-resistant *B. napus* variety.

Materials and Methods

Field Mapping and Sampling

Field 1 was mapped using an AIM Navigator global positioning system with Omnistar differential correction mounted on an all-terrain vehicle. A 50- by 50-m grid was established, and plants were collected on a 50- by 100-m sampling interval (Figure 1). Where grid points intersected noncrop areas, they were shifted to the nearest location where samples could be taken. In September 1998, one mature volunteer *B. napus* plant at each location was collected (where present) for subsequent greenhouse testing and genetic analysis.

Greenhouse Herbicide Resistance Testing

For each of 34 volunteers sampled, four replicates of 25 seeds were planted in a soilless peat mixture and grown in a greenhouse to determine herbicide resistance. Where seed numbers permitted (14 volunteers), four replicates of 25 seeds were also planted to test for resistance to all three

herbicides. In total, 5,670 seedlings emerged for resistance testing. Seeds of glufosinate-resistant 'Invigor 2153' *B. napus*, glyphosate-resistant Quest, and imidazolinone-resistant 45A71 were also planted for use as susceptible or resistant controls to verify herbicide performance.

At the cotyledon stage, all test seedlings were sprayed with glyphosate at 440 g ae ha^{-1} at 200 kPa in aqueous solution applied at 100 L ha^{-1} using a track sprayer.[2] Plants were categorized as susceptible or resistant 18 d after application. Controls of glyphosate-resistant Quest and glyphosate-susceptible 45A71 were also treated to confirm herbicide efficacy and levels of resistance in a greenhouse.

Seedlings that survived the glyphosate application were subsequently tested at the three-leaf stage for glufosinate resistance (400 g ai ha^{-1}) or imazethapyr resistance (50 g ai ha^{-1}). Additional seedlings were sprayed with imazethapyr (50 g ai ha^{-1}) at the three-leaf stage and glufosinate (400 g ai ha^{-1}) 3 d later. Glyphosate-resistant Quest, glufosinate-resistant Invigor 2153, and imidazolinone-resistant 45A71 were also sprayed to confirm herbicide efficacy and levels of resistance. Plants were categorized as susceptible or resistant 8 to 15 d after herbicide application.

Experiments to test herbicide resistance were designed as randomized complete blocks with four replicates. Chi-square estimates of fit to the hypothesized resistance : susceptibility ratios were performed on each replicate for each herbicide, combining all replicates, ($P < 0.05$) using SAS version 6.11.

Genetic Hypothesis and Assumptions

To determine if resistant volunteers were the result of pollen movement or inadvertent seed transfer, the following assumptions were made. Glyphosate and glufosinate transgenes and imidazolinone resistance genes all segregated independently. Quest, Innovator, and 45A71 were homozygous with respect to glyphosate, glufosinate, or imidazolinone resistance, respectively. Glyphosate and glufosinate resistance segregated as a single dominant gene, with the phenotype of the hemizygotes (Rr or Gg) equivalent to the homozygotes (RR or GG). Imidazolinone resistance segregated as two semidominant genes, I^A and I^B, and the presence of either resistant allele was sufficient to confer resistance. We assumed imidazolinone resistance conferred by heterozygous individuals could not be phenotypically distinguished from the homozygous resistant individuals (I^AI^A, I^BI^B) at a single herbicide rate, and the only susceptible allelic combination was homozygous for both susceptible genes (i^Ai^A, i^Bi^B).

If resistant gene movement by pollen were responsible for the observed glyphosate resistance, volunteers collected in September 1998 would be hybrids. They would have received resistance from the maternal parent, either glufosinate or imidazolinone resistance depending on field location, and glyphosate resistance from the pollen source. Their progeny would segregate with a 3:1, resistant : susceptible ratio for glufosinate and glyphosate and a 15:1, resistant : susceptible ratio for imidazolinone, except as perturbed by the potential 20% out-crossing during 1998.

If inadvertent seed transfer in 1997 was responsible for observed glyphosate-resistant volunteers, most (79%) of the 1998 volunteers would be homozygous for glyphosate resistance because of self-fertilization. Thus, the majority of progeny would also be homozygous for glyphosate resistance. Out-crossing in 1998 could not be distinguished because hybrid seed (Rr) also would be glyphosate resistant. A volunteer with seeds 100% resistant to glyphosate would indicate seed transfer as the cause of glyphosate-resistant volunteers.

Glufosinate-Resistant Spot Test

A nonlethal test (Kumar 1997) was conducted to determine the resistance of plants to glufosinate for subsequent DNA extraction and restriction fragment length polymorphism (RFLP) testing. A 0.045% (v/v) aqueous solution of glufosinate was applied on a 0.5-cm^2 area of one leaf on each of 40 seedlings that had survived a glyphosate application. Evaluation of glufosinate resistance was conducted 5 d after application. Treated leaves of susceptible plants were wilted or desiccated, while the rest of the plant appeared healthy and suitable for DNA extraction. Resistant plants exhibited no visible symptoms of injury. Plants were characterized as resistant or susceptible.

Plant Material, DNA Extractions, and Southern Hybridizations

Genomic DNA was extracted in duplicate from Quest, Invigor 2153, and 45A71 seedlings and from eight glyphosate-resistant seedlings using the protocol of Sharpe et al. (1995). All plants were grown in a growth chamber with a 16-h, 20/15 C day/night temperature and 65% relative humidity. Leaves of each plant were removed and either lyophilized at 0 C and stored at room temperature or immediately ground in liquid nitrogen and used for DNA extraction. Four seedlings were progeny from a single resistant volunteer (1-2 to 1-6), whereas the remaining four seedlings were two progeny each of two separate glyphosate-resistant volunteers. The DNA from these plants was digested with different restriction enzymes, including *Bam*HI and *Eco*R1,[2] and separated on agarose gels. After transfer to nylon membranes, Southern hybridizations were performed using a number of different probes as described in Sillito et al. (2000). Individual alleles were scored as present (A) or absent (0) for the specified marker loci.

Multiple-Resistant Individuals

Two plants (A and B) identified as surviving all three herbicides were allowed to self-pollinate and were maintained in the greenhouse until maturity. Seeds were collected and tested for resistance and cross-resistance patterns. From each plant, four replicates of 25 seeds were planted and sprayed with glufosinate (400 g ai ha^{-1}) at the cotyledon stage. Survivors were tested with imazethapyr (50 g ai ha^{-1}) after 7 d, and those that survived received glyphosate (440 g ai ha^{-1}) 10 d later. Four replicates of 25 seeds from each of two plants received a glyphosate application (440 g ai ha^{-1}) at the cotyledon stage, followed by glufosinate (400 g ai ha^{-1}) 11 d later, then imazethapyr (50 g ai ha^{-1}) after 6 d. Imazethapyr (50 g ai ha^{-1}) was applied to four replicates of 25 seedlings at the first-leaf stage. Plants were categorized as susceptible or resistant.

TABLE 1. Patterns of resistance and cross-resistance to glyphosate and glufosinate and allele segregation scored with three different probes following DNA digestion by restriction enzymes *Eco*R1 or *Bam*HI. Each allele is scored as present (A) or absent (0). The first four rows are scores from duplicate parental lines: R1 and R2, glyphosate-resistant Quest and G1 and G2, glufosinate-resistant Invigor 2153. The following rows are scores from eight individuals, with the first number representing the volunteer and the second individual progeny.

Plant	Glyphosate resistance	Glufosinate resistance	EC2D1 *Eco*RI	EC2C7 *Eco*RI	R89998 *Bam*H1
R1	R	S	A	A	0
R2	R	S	A	0	0
G1	S	R	0	0	A
G2	S	R	0	0	A
1–2	R	S	A	0	A
1–4	R	R	A	0	0
1–5	R	R	A	0	A
1–6	R	R	A	A	A
2–4	R	R	A	0	A
2–5	R	R	0	0	0
4–2	R	S	A	A	nd[a]
4–6	R	R	A	A	nd

[a] nd, no data.

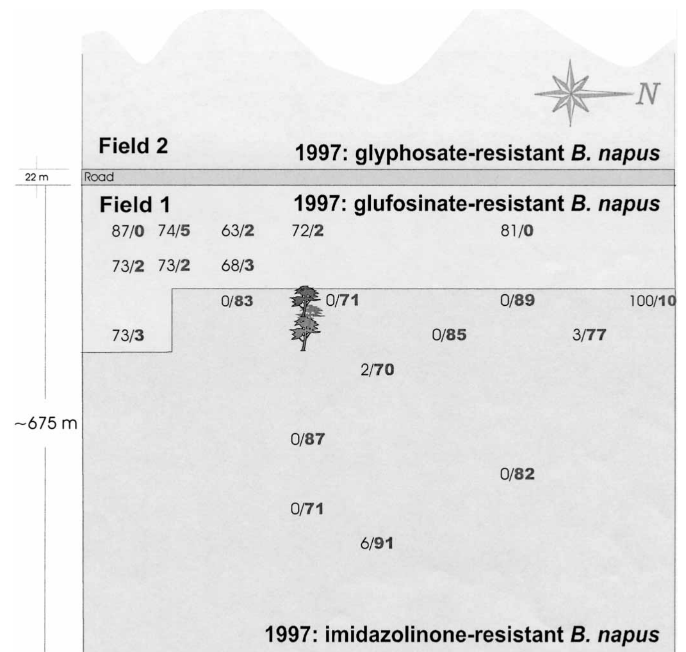

FIGURE 2. The percentage of glufosinate (left) and imazethapyr (right) resistance in progeny that survived glyphosate application and the position of the volunteer collected in Field 1.

Results and Discussion

Glyphosate Resistance

Of the 5,670 seedlings that emerged, 48% were resistant to glyphosate. Seedlings from eight volunteers were not significantly different from the 1% glyphosate resistance of the susceptible checks. Seedlings from an additional six volunteers had between 4 and 21% resistance (Figure 1). Twenty volunteers had between 66 and 82% glyphosate-resistant seeds (Figure 1). Of these twenty plants, 16 had resistance ratios which were not significantly different than predicted (3:1, resistant : susceptible). These ratios were consistent with seed set following pollen transfer from a glyphosate-resistant source in 1997, followed by segregation in 1998. Plants collected close to Field 2, the putative pollen source, were most likely to have glyphosate-resistant seed. However, this area received a third glyphosate application in 1998, which almost certainly reduced the number of susceptible volunteers. Both pollen movement and seed movement within the field at harvest may have influenced position of volunteers. However, glyphosate resistance was located far away. A mature *B. napus* volunteer 500 m from the western field edge produced seedlings with 74% glyphosate resistance.

Glyphosate-resistant controls were 92% resistant to glyphosate, consistent with reports of commercially available seed (G. Stringam, personal communication). None of the volunteers tested had resistance equivalent to glyphosate-resistant controls, indicating inadvertent seed transfer during harvesting, or seeding was not the source of glyphosate-resistant *B. napus* volunteers.

Glufosinate Resistance

Glufosinate resistance was anticipated only in the area where glufosinate-resistant *B. napus* had been seeded in 1997. Seedlings from all nine plants from this area that were glyphosate-resistant showed cross-resistance to glufosinate, and seven of the nine fit the 3:1 resistance expectations for glufosinate (Figure 2). Two samples fell outside of the expectations (63 and 87%).

In the area seeded to imidazolinone-resistant 45A71 in 1997, one of 19 volunteers produced more than 6% glufosinate-resistant progeny. Seedlings were glufosinate resistant (100%) and only 10% were imazethapyr resistant. The volunteer was located at the north edge of the field, within 25 m of the estimated Innovator/45A71 boundary. Because the cross-resistance pattern closely resembles that from the volunteers growing in the glufosinate-resistant area, it is probable that seed transfer at harvest in 1997 contributed to the position of this hybrid.

Imazethapyr Resistance

Imazethapyr resistance of seedlings that survived the glyphosate application was tested. Imazethapyr resistance was anticipated only in the area where 45A71 had been seeded in 1997. It was difficult to categorize treated seedlings into distinct resistant and susceptible classes. Ten of the 11 volunteers from the imidazolinone-resistant area showed resistance to imazethapyr, but only three of the 10 fit the 15:1, resistant : susceptible expectations (Figure 2). One was susceptible (10%, described above). Seedlings from seven plants had a lower frequency of resistance than predicted, probably because of difficulty in distinguishing between susceptible seedlings and heterozygotes with low levels of resistance. In the area seeded to glufosinate-resistant Innovator in 1997, none of the nine volunteers resistant to glyphosate had more than 5% resistance to imazethapyr.

Analysis of Resistant Hybrids Using RFLP Markers

DNA analyses were conducted on glyphosate-resistant seedlings. Plants were then nondestructively tested for glu-

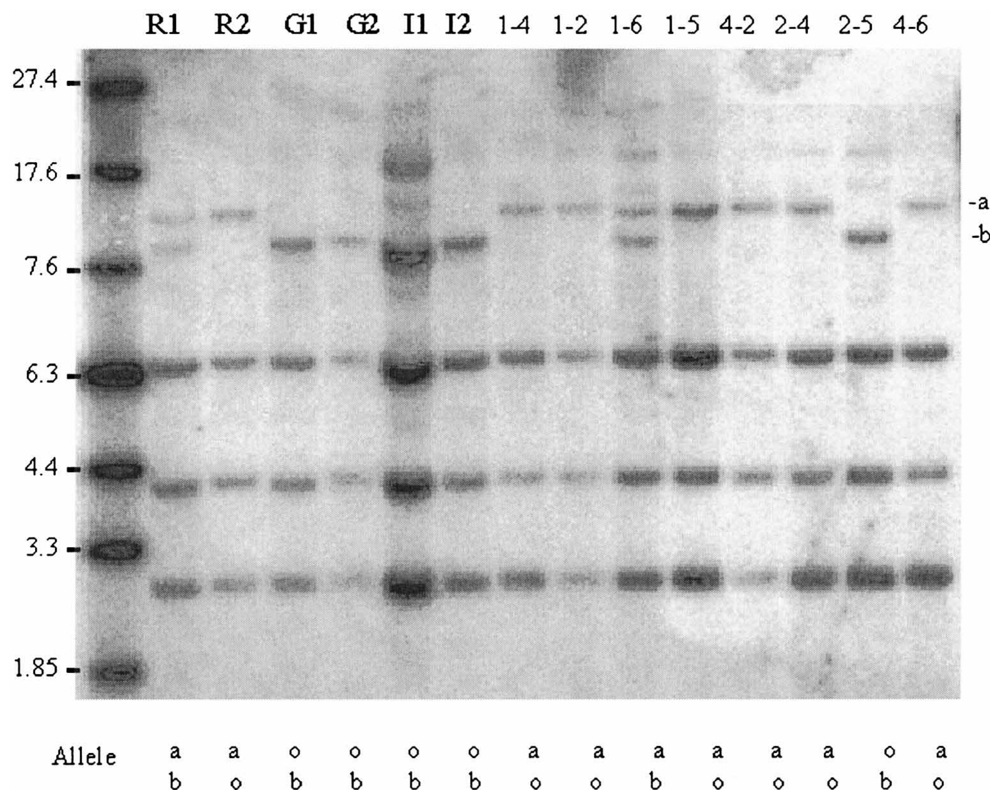

FIGURE 3. Representative RFLP pattern of duplicate glyphosate-resistant Quest (R1, R2), glufosinate-resistant Invigor 2153 (G1, G2), imidazolinone-resistant 45A71 (I1, I2), and progeny from three *Brassica napus* volunteers. The first number indicates the volunteer (maternal parent) and the second the individual seedling. Each locus is designated with a lowercase letter, as shown to the right and at the bottom. To the left are size standards in kilobase pairs (kb). This figure represents a single probe (pEC2D1) with the DNA cut with a single restriction enzyme (EcoR1).

fosinate resistance as described above. Glufosinate-resistant and -susceptible plants from specific volunteers were then analyzed using specific RFLP markers. Four progeny were tested from one resistant volunteer (1-2 to 1-6) and two progeny from each of two other resistant volunteers (2-4, 2-5 and 4-2, 4-6). Of these progeny, six of eight were hybrids based on herbicide resistance tested; that is, they were cross-resistant to glyphosate and glufosinate. Additionally, five of eight plants were hybrids based on DNA blots using two restriction enzymes and three independent probes. Plants were considered to be hybrids if they contained definitive bands from both parents (Table 1, Figure 3).

Only one plant (4-2) could not be differentiated from Quest, the glyphosate-resistant parent. Resistance testing and RFLP data alone suggest that Quest seed contamination had occurred. However, plants 4-2 and 4-6 share a common maternal parent. The maternal volunteer produced some seeds resistant to both glufosinate and glyphosate and that contained DNA from both glyphosate- and glufosinate-resistant cultivars. It would appear that Plant 4-2 was susceptible to glufosinate as a result of the parental volunteer (Plant 4) being hemizygous for the glufosinate resistance transgene, followed by segregation of this transgene in the self-pollinated plant. In RFLP analysis, when only two different alleles are scored, there is a 25% probability this allele combination could have occurred randomly.

All the lines of evidence support the conclusion that multiple-herbicide-resistant genotypes at this location arose as a result of pollen transfer between different cultivars of *B. napus*. First, there was a higher likelihood of glyphosate resistance closer to the putative glyphosate resistance pollen source (Field 2). Second, all glyphosate-resistant plants were also cross-resistant to another herbicide, and herbicide resistance ratios were generally consistent with pollen transfer in 1997 followed by segregation of resistance genes in 1998. Finally, DNA marker gene analysis of seedlings confirmed hybridization of the volunteer *B. napus*.

Glyphosate/Imazethapyr/Glufosinate Resistance

Of the 924 seedlings tested for resistance to all three herbicides, only two survived (0.2%). They originated from two volunteers, the first collected close to the western edge of Field 1, in the area planted to glufosinate-resistant *B. napus* in 1997, and the second was located in the southeast corner of the field in the area planted with imidazolinone-resistant *B. napus* (Figure 1). Initial screenings of seedlings from the first volunteer showed 77% glyphosate resistance, 63% resistance to glufosinate, and 2% resistance to imazethapyr. A single plant of 100 tested survived all three herbicides (Plant A). In contrast, only 2% of the seedlings from the second volunteer survived glyphosate. A single individual of the 100 tested survived all three herbicides (Plant B). When progeny of self-pollinated Plant A and Plant B were tested for resistance, they showed a high level of resistance to glyphosate, glufosinate, and imazethapyr. Resistance ratios conformed to the predicted 3:1 resistant : susceptible ratio for glyphosate and glufosinate resistance and generally conformed to the predicted 15:1, resistant : susceptible ratio for imazethapyr resistance (Table 2).

TABLE 2. Percentage of survivors of the progeny of the multiple-herbicide-resistant plants to each herbicide application. The sequence of herbicide applications is shown. The predicted resistant : susceptible ratio and the lack of significant difference between predicted and actual is indicated.

Plant	Herbicide sequence	Progeny survival		
		Mean	Range	Predicted resistance ratio[a]
		%		
A	Glyphosate (R)	73	65–78	3:1*
	Glufosinate (G)	77	62–91	3:1*
	Imazethapyr (I)	89	80–95	15:1*
	Glyphosate/glufosinate	79	69–92	3:1*
	Glufosinate/imazethapyr	96	88–100	15:1*
	Imazethapyr/glyphosate	nd[b]		
	Glyphosate/glufosinate/imazethapyr	91	80–100	15:1*
	Glufosinate/imazethapyr/glyphosate	73	54–86	3:1*
	Imazethapyr/glyphosate/glufosinate	nd		
Predicted genotype			R/r; (I^A/i^A I^B/i^B); G/g	
B	Glyphosate	72	52–83	3:1*
	Glufosinate	71	58–89	3:1*
	Imazethapyr	89	75–100	15:1*
	Glyphosate/glufosinate	80	61–100	3:1*
	Glufosinate/imazethapyr	98	95–100	15:1*
	Imazethapyr/glyphosate	nd		
	Glyphosate/glufosinate/imazethapyr	100	100	15:1
	Glufosinate/imazethapyr/glyphosate	68	58–89	3:1*
	Imazethapyr/glyphosate/glufosinate	nd		
Predicted genotype			R/r; (I^A/i^A I^B/i^B); G/g	

[a] Asterisk indicates observed ratio is not significantly different than the predicted ratio according to chi-square estimates at $P > 0.05$.
[b] nd, no data.

Development of multiple resistance for Plant A is consistent with glyphosate resistance transferred by pollen to the glufosinate-resistant maternal plant in 1997 (Figure 4). The progeny were glyphosate resistant and survived selection by glyphosate in chemical fallow applications. It probably received pollen from an imidazolinone-resistant *B. napus* pollen source, either from a volunteer or possibly a more distant crop source. At least one of the resulting progeny was resistant to all three herbicides (Plant A). The progeny of self-pollinated plants include susceptible and all three combinations of double resistance and some triple-resistant individuals.

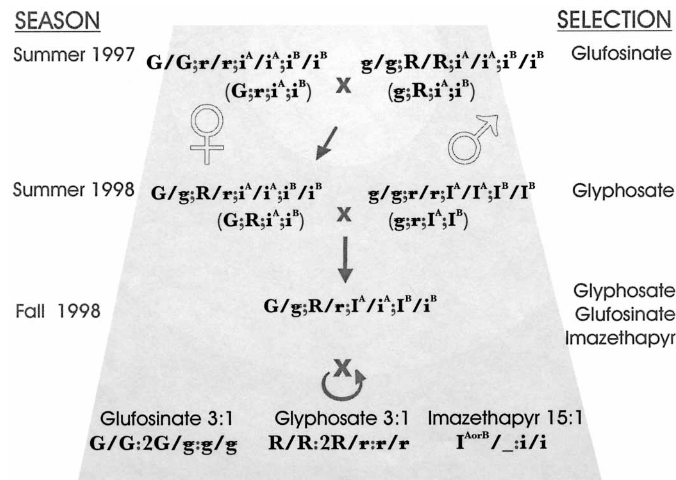

FIGURE 4. Proposed genotype of parents and gametes leading to the triple-resistant Plant A and the predicted genotype and phenotype of the progeny following self-fertilization. G, glufosinate-resistant transgene; R, glyphosate-resistance transgene, and I^A, i^A, I^B, i^B represent dominant and recessive imidazolinone resistance genes, respectively, conferring imidazolinone resistance. An r or g indicates the lack of the transgene. A G/g or R/r plant is hemizygous for glyphosate or glufosinate resistance, respectively. The herbicide applied both in field or greenhouse is indicated on the right and an indication of year and season is provided on the left.

The sequence of events resulting in Plant B probably differed. Field position (Figure 1) and resistance profile of progeny from Plant B (Table 2) suggest Plant B arose as follows. An imidazolinone-resistant maternal plant was fertilized by glufosinate-resistant pollen followed by seed set. A volunteer (arising from this event) inadvertently survived chemical fallow glyphosate applications. This volunteer probably out-crossed with a glyphosate-resistant volunteer. A few seeds collected from this mature volunteer would show resistance to glyphosate (2%), and at least one (Plant B) was resistant to all three herbicides. Although order of crossing and the resistance status of the maternal plants were probably different, progeny from self-pollinated Plants A and B were predicted to have identical resistance ratios.

This is the first report of multiple-herbicide-resistant volunteers after 3 yr of commercial production of herbicide-resistant *B. napus*. The circumstances leading to the discovery of cross-resistant volunteer *B. napus* were atypical, because the producer placed three different herbicide-resistant pollen sources in close proximity in 1997 and then selected with glyphosate alone during chemical fallow operations in 1998. However, given the > 20% out-crossing rate (Rakow and Woods 1987), the 2.6 million ha of herbicide-resistant *B. napus* grown in Western Canada (Downey 1999), the proximity of many *B. napus* fields, the 4-yr potential seed

bank life (Thomas and Leeson 2000), and the incidence of *B. napus* volunteers as residual weeds (Thomas et al. 1998), we can speculate that multiple-resistant volunteer *B. napus* is not rare and may be common. The lack of reported multiple-resistant volunteers suggests multiple-resistant *B. napus* volunteers are being controlled by chemical and nonchemical management strategies and are therefore not an agronomic concern to most producers.

The presence of a few triple-resistant mutations illustrates the rapidity and distance with which genes may transfer between varieties of an out-crossing species. Although pollen flow and seed movement with harvesting equipment may have contributed to the spread of multiple-resistant individuals, a single triple-resistant individual was located more than 550 m from the putative pollen source 17 mo after seeding. It also indicates the potential for many cross-resistance patterns as new herbicide-resistant systems are developed and herbicide-resistant *B. napus* varieties proliferate.

Although multiple herbicide-resistant volunteers are not a major weed concern as yet, the presence of herbicide-resistant genes in *B. napus* volunteers has implications for seed and product purity. Potential pollen movement is greater than the 100-m isolation distance currently regulated for seed growers (Stringam and Downey 1978; Timmons et al. 1996). Therefore, seed may contain small amounts of unrecognized herbicide-resistant seeds as a contaminant. Also, because organically grown *B. napus* is prohibited from containing genetically modified DNA, long distance movement of transgenes in pollen would prevent organic *B. napus* from being grown, except in isolated areas. Some consumers are questioning products made from genetically enhanced crops. Should growers decide to stop growing herbicide-resistant or genetically enhanced *B. napus,* volunteers have the potential to transfer herbicide resistance genes and transgenes to future *B. napus* crops for up to 4 yr.

Source of Materials

[1] Research Instruments Mfg Co. Ltd, Guelph, ON, Canada.

[2] Amersham Pharmacia Biotech, Inc., 500 Boule Morgan Blvd., Baie d'Urfe, QC, Canada H9X 3V1.

Acknowledgments

The authors thank Dr. Keith Downey, Agriculture and Agri-Food Canada, Saskatoon, SK, for advice on experiments and his careful review of the manuscript; Dr. Gary Stringam, University of Alberta, Edmonton, for editorial assistance; and Phil Thomas, Alberta Agriculture, Food, and Rural Development (AAFRD), for assistance with media and extension. Sue Jess and Lisa Raatz, AAFRD, provided technical assistance and Dr. Rong-Cai Yang, AAFRD, statistical assistance. Monsanto Canada, Ltd., Cyanamid Crop Protection, and AgrEvo Canada Ltd. supplied glyphosate, imazethapyr, and glufosinate for greenhouse experiments.

Literature Cited

Barry, G., G. Kishore, S. Padgette, et al. 1992. Inhibitors of amino acid biosynthesis: strategies for imparting glyphosate tolerance to crop plants. Pages 139–145 *in* B. J. Singh, H. E. Flores, and J. C. Shannon, eds. Current Topics in Plant Physiology, an American Society of Plant Physiologists Series; Biosynthesis and Molecular Regulation of Amino Acids in Plants. Rockville, MD: American Society of Plant Physiologists.

Canadian Food Inspection Agency. 1995a. Decision document DD95-01: Determination of Environmental Safety of AgrEvo Canada Inc.'s Glufosinate Ammonium-Tolerant Canola. http://www.cfia-acia.agr.ca/english/plaveg/pbo/old/dd9501e.shtml. Accessed March 13, 2000.

Canadian Food Inspection Agency. 1995b. Decision document DD95-03: Determination of Environmental Safety of Pioneer Hi-Bred International Inc.'s Imidazolinone-Tolerant Canola. http://www.cfiaacia.agr.ca/english/plaveg/pbo/old/dd9503e.shtml. Accessed March 13, 2000.

Canadian Food Inspection Agency. 1996. Decision document: DD96-07: Determination of Environmental Safety of Monsanto Canada Inc.'s Roundup® Herbicide-Tolerant *Brassica napus* Canola Line GT73. http://www.cfia-acia.agr.ca/english/plaveg/pbo/old/dd9502e.shtml. Accessed March 13, 2000.

Chèvre, A. M., F. Eber, A. Baranger, M. C. Kerlan, P. Barret, G. Festoc, P. Vallée, and M. Renard. 1996. Interspecific gene flow as a component of risk assessment for transgenic *Brassicas*. Acta Hortic. 407:169–179.

De Block, M., J. Botterman, M. Vandewiele, et al. 1987. Engineering herbicide resistance in plants by expression of a detoxifying enzyme. EMBO J. 6(9):2513–2518.

Downey, R. K. 1999. Risk assessment of out-crossing of transgenic *Brassica,* with focus on *B. rapa* and *B. napus. In* Proceedings of the 10th International Rapeseed Congress, Canberra, Australia.

Kareiva, P., W. Morris, and C. M. Jacobi. 1994. Studying and managing the risk of cross-fertilization between transgenic crops and wild relatives. Mol. Ecol. 3:15–21.

Keeler, K. H., C. E. Turner, and M. R. Bolick. 1996. Movement of crop transgenes into wild plants. Pages 303–330 *in* S. O. Duke, ed. Herbicide-Resistant Crops: Agricultural, Environmental, Economic, Regulatory, and Technical Aspects. Boca Raton, FL: CRC Press.

Kumar, A. 1997. Performance of Glufosinate-Tolerant Susceptible Near-Isogenic Lines of *Brassica napus* L. M.Sc. thesis. University of Saskatchewan, Saskatoon, Canada. 117 p.

Lefol, E., V. Danielou, and H. Darmency. 1996. Predicting hybridization between transgenic oilseed rape and wild mustard. Field Crops Res. 45:153–161.

Lefol, E., G. Séguin-Swartz, and R. K. Downey. 1997. Sexual hybridization in crosses of cultivated *Brassica* species with *Erucastrum gallicum* and *Raphanus raphanistrum*: potential for gene introgression. Euphytica 95: 127–139.

Padgette, S. R., D. B. Re, G. F. Barry, D. E. Eichholtz, X. Dalannay, R. L. Fuchs, G. M. Kishore, and R. T. Fraley. 1992. New weed control opportunities: development of soybeans with a Roundup Ready™ gene. Pages 53–84 *in* S. O. Duke, ed. Herbicide-Resistant Crops: Agricultural, Environmental, Economic, Regulatory, and Technical Aspects. Boca Raton, FL: CRC Press.

Rakow, G. and D. Woods. 1987. Out-crossing in rape and mustard under Saskatchewan prairie conditions. Can. J. Plant Sci. 67:147–151.

Shaner, D. L., N. F. Bascomb, and W. Smith 1996. Imidazolinone-resistant crops: selection, characterization, and management. Pages 143–157 *in* S. O. Duke, ed. Herbicide-Resistant Crops: Agricultural, Environmental, Economic, Regulatory, and Technical Aspects. Boca Raton, FL: CRC Press.

Sharpe, A. G., I.A.P. Parkin, D. J. Keith, and D. J. Lydiate. 1995. Frequent nonreciprocal translocation in the amphidiploid genome of oilseed rape (*Brassia napus*). Genome 38:1112–1121.

Sillito, D., I.A.P. Parkin, R. Mayerhofer, D. J. Lydiate, and A. G. Good. 2000. *Arabidopsis thaliana,* a source of candidate disease resistance genes for *Brassica napus.* Genome. 43:452–460.

Squire, G. R., D. Burn, and J. W. Crawford. 1997. A model for the impact of herbicide tolerance on the performance of oilseed rape as a volunteer weed. Ann. Appl. Biol. 131:315–338.

Stringam, G. R. and R. K. Downey. 1978. Effectiveness of isolation distance in turnip rape. Can. J. Plant Sci. 58:427–434.

Thomas, G., B. L. Frick, and L. M. Hall. 1998. Alberta Weed Survey of Cereal and Oilseed Crops in 1997. Saskatoon, SK: Agriculture and Agri-Food Canada Weed Survey Series Publ. 98-2. 283 p.

Thomas, G. A. and J. Y. Leeson. 2000. Persistence of volunteer wheat and canola using weed survey data. Page 94 *in* Proceeding of the 1999 Expert Committee on Weeds, Ottawa, Ontario, Canada.

Timmons, A. M., J. M. Charters, J. W. Crawford, et al. 1996. Risks from transgenic crops. Nature 380:487.

Wohlleben, W., W. Arnold, I. Broer, D. Hillemann. E. Strauch, and A. Pühler. 1988. Nucleotide sequence of the phosphinothricin *N*-acetyltransferace gene from *Streptomyces viridonchromogenes* Tü494 and its expression in *Nicotiana tabacum.* Gene 70:25–37.

Received April 4, 2000, and approved July 1, 2000.