Report on Project Entitled

Isolation Effectiveness in Canola Pedigree Seed Production

R.K. Downey and H. Beckie

Agriculture and Agri-Food Canada
Saskatoon Research Centre
SASKATOON, SK

Executive Summary

Seventy samples of Certified canola seed from 14 different herbicide susceptible, open-pollinated canola varieties, produced in 2000, were supplied by growers and/or seed companies. The number of samples supplied per variety varied between one and 37. Only 37 growers returned completed questionnaires providing detailed isolation information. At least 2000 seeds from each of the 70 samples were screened for the presence of Roundup and/or Liberty tolerant genes using selective herbicide germination tests. Two of the 14 varieties greatly exceeded the 0.25% maximum for the presence of other varieties permitted by the Association of Official Seed Certifying Agencies (AOSCA). One of these varieties had a combined Roundup plus Liberty contamination of 0.81% while the other contained 0.69% contamination. A third variety marginally exceeded the allowed maximum at 0.28%. The contamination found in these varieties almost certainly occurred in the breeding nurseries during variety development rather than from outcrossing during pedigree seed production.

Five varieties provided examples that Certified seed true to variety can be produced under the present CSGA regulations, provided the Breeder and Foundation seed lots are free of contamination. When the three varieties that exceeded the AOSCA genetic purity standard are excluded, only three of the remaining 54 samples exceeded the 0.25% genetic purity limit. Of these, one sample (D_2, 0.35%) was harvested from a field isolated by 500m, one sample (G_1, 0.37%) by 1207m and a third (E_1, 0.57%) by 100m from a field sown to an unknown canola variety. The data suggest that the contamination in samples E_1 and D_2 arose from an admixture during the seeding, harvesting or cleaning operations while in sample G_1, the Foundation seed is the most likely source.

The test results indicate that the present CSGA isolation regulations for canola are adequate to produce Certified seed lots that will meet or exceed genetic purity standards of AOSCA, provided the Breeder and Foundation seed lots meet or exceed the mandatory genetic purity level of 99.95%. **It is recommended** that no changes be made in the present canola isolation standards. However, **it is recommended** that the CSGA require all Breeder and Foundation seed lots be tested for the presence of foreign herbicide tolerant genes to ensure genetic purity standards are met. (Note: fast and accurate test methods are lacking for the imidazolinone and bromoxynil tolerant genes). It should be realized that even when the genetic purity standards are met, the sowing of a herbicide susceptible variety will almost certainly result in a significant population of herbicide tolerant plants within that field.

Unsolicited samples from five different Roundup tolerant varieties were received. The percent of susceptible seedlings in these samples varied from 1.8 to 6.8%, with an average of 3.7%. Thus, all were above the genetic purity standard, unless the official variety description defined presence and amount of susceptible seed. **It is recommended** that the varietal descriptions of herbicide tolerant varieties be reviewed and modified as required.

Plant breeders, seed companies and institutions have been advised through oral presentations to test and take the necessary steps to ensure their Breeder and Foundation seed meets the current genetic purity standards of AOSCA and CSGA. However, **it is recommended** that the CSGA officially bring these standards to the attention of the seed industry and institute mandatory testing where feasible.

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AAFC Research Centre Saskatoon SK

Objective

The objective of this project was to assess isolation distance effectiveness in maintaining the purity of pedigree open pollinated canola varieties as it relates to the presence of inserted herbicide tolerant (HT) genes that impart tolerance to glyphosate (Roundup) and/or glufosinate (Liberty).

Seed Samples Sources

A list of growers, producing Certified seed of herbicide susceptible, open pollinated canola varieties, was provided by the Canadian Seed Growers Association (CSGA) on a confidential basis. The list contained the names of some 122 growers. All *B. napus* growers on the list were sent the attached questionnaire.

Only 16 growers responded, providing a seed sample of their Certified 2000 production and a completed questionnaire. Five of the 16 growers sent in seed samples from a Roundup tolerant variety. To overcome the short fall in number of samples, arrangements were made with two seed companies whereby seed samples from contract production of their varieties were made available and a combined effort by researchers and the companies was made to encourage completion and submission of the related questionnaires. As a consequence we obtained a total of 70 seed samples from 14 different herbicide susceptible (HS) open pollinated varieties, plus five seed samples from five different Roundup Ready varieties. Unfortunately a large percentage of the 70 HS samples represented only a few varieties (Table 1). All seed samples received had been grown under conditions that met or exceeded the CSGA regulations for the production of Certified canola seed.

Methods

The original plan was to draw 40 subsamples of 50 seeds each from each submitted seed sample, grind the seed in buffered aqueous solution, let stand for 2 minutes and insert an immunoassay test strip to detect the presence of one or more seeds containing a Roundup Ready or a Liberty gene. However, a more discriminating test for the presence of the Roundup gene, developed by the CFIA seed lab became available. In this test 50 seeds are placed in covered germination boxes each of which contained two germination blotters moistened with 21 ml of a 400 ppm solution of Roundup. The seed is then incubated in the boxes for 6-7 days at 25-15_C day/night temperature with an 8 hour day. Herbicide tolerant seedlings were readily identified by their long tapering roots, elongated hypocotyls and the presence of secondary roots.

A similar test for the detection of seeds and seedlings carrying the Liberty gene was published about the same time as the Roundup test became available, [Pfeilsetter, E., Matzk, A., Feldmann, S.D. and Schiemann, J. 2000. Rapid efficient screening of phosphinothricin tolerant oilseed rape (*Brassica napus*) with a novel germination test. Euphytica 113: 119-124.] However, the published protocol called for the germination of rape or canola seed, using an 18 hour day and a day/night temperature of 20/18°C, on filter paper soaked with a 0.005% phosphinothriein solution. The germination trays were watered every 4-5 days with 250ml of a 0.005% herbicide solution. Seedlings were rated for tolerance to glufosinate after 10 days. This procedure in our hands did not provide the desired level of discrimination between glufosinate tolerant and susceptible seedlings. Thus the procedure was modified by using the same protocol as for the Roundup assay except that the double blotters were moistened with 21ml. of a 0.06% solution of Liberty. These testing methods allowed the classification of each individual seed as opposed to the testing of a bulk subsample of 50 seeds.

Twenty germination boxes per sample, each with 50 seeds, were incubated and assessed. This procedure was repeated several days later so that in all 2000 seeds per sample were subjected to germination testing on Roundup and Liberty containing media. Seedlings were classified as tolerant, susceptible or dead and the percent tolerant seedlings calculated by dividing the number of tolerant seedlings by the total number of live germinated seedlings, multiplied by 100.

To verify the accuracy of the herbicide tolerant classification of the germination seedlings, two independent measures were employed. First, in blind experiments, one researcher placed and recorded the location of Roundup or Liberty tolerant seeds in among seeds from herbicide susceptible varieties in the germination boxes. After 6 to 7 days incubation, a second researcher, without knowing the number or location of the herbicide tolerant seeds, correctly located and classified the herbicide tolerant seedlings. Second, all seedlings classified as tolerant to Liberty herbicide were analyzed for the presence of the Liberty gene using the "Trait LL Leaf and Seed Test Kit" developed by Strategic Diagnostics Inc. and marketed in Canada by Biovet. Seedlings classified as Liberty tolerant were macerated with a glass rod in Eppendorff vials filled with a 4:1 solution of distilled water: Trait Sample Buffer. A trait "LL Test Strip" was inserted into each vial and read after 10 minutes exposure when the control line at the top of the Test Strip was clearly visible. All seedlings classified as Liberty tolerant gave a positive Test Strip reaction. Seedlings classified as Roundup resistant were similar tested using the "Trait RUR Bulk Soybean Test Kit" developed and supplied by the same companies as the Liberty test kits. Again, all seedlings classified as being Roundup tolerant that were subjected to the immunoassay test strip analysis gave a positive response. Since Roundup tolerant seedlings proved to be easily recognized and all seedlings tested gave positive results, test strip verification of seedlings classified as Roundup tolerant was discontinued after the first 1000 seeds sample from each Certified seed lot. To further verify the accuracy of the herbicide classifications, the variety Westar was used as a herbicide susceptible check variety in all tests and the Roundup tolerant variety LG3345 and the open-pollinated Liberty

tolerant variety SW Flare were used as checks in the Roundup and Liberty testing, respectively.

Table 1. Number of herbicide susceptible Certified seed samples received and analyzed and number of completed questionnaires returned per variety plus number of samples of Roundup tolerant varieties and questionnaires received.

Variety code	Herbicide classification	No. samples analyzed	No. of returned questionnaires
A	HS	37	7
B	HS	10	9
C	HS	5	1
D	HS	5	4
E	HS	3	3
F	HS	2	0
G	HS	1	1
H	HS	1	1
I	HS	1	1
J	HS	1	1
K	HS	1	1
L	HS	1	1
M	HS	1	1
N	HS	1	1
O	RT[1]	1	1
P	RT	1	1
Q	RT	1	1
R	RT	1	1
S	RT	1	1

[1]HS = Herbicide susceptible, RT = Roundup tolerant

The size of sample required to accurately assess the level of contamination is an important aspect of this study as well as an important factor for the seed trade in that a

high level of confidence is necessary to ensure the test conducted by the seller and the buyer will result in the same or nearly the same value. It was calculated that the sample size required to give a 99% chance that the buyer would obtain the same or a lower percentage of the herbicide tolerant gene, for various contamination limits, would be as follows:

Contamination level %	Sample size required
0.05	8000
0.2	2200
0.5	600

The genetic and crop standards for canola seed as laid down by the Association of Official Seed Certifying Agencies (AOSCA) state that the maximum presence of other varieties permitted is 0.05% for Foundation seed and 0.25% for Certified seed. (AOSCA. 1999. Genetic and Crop Standards. p. 2-37). Thus, the 2000 seeds analyzed for each Certified seed lot in this project is larger than that required to determine if a seed lot would meet the Certified standard for the presence of other varieties, at the 99% confidence level.

Results

It is evident from the data that some varieties almost certainly had an unacceptable percentage of the Roundup and/or Liberty genes in their Foundation seed (Table 2). Varieties B and N are clear examples where contamination from both the Roundup and Liberty genes probably occurred in the breeding nurseries during the development of these varieties. Variety C was probably similarly affected with the presence of the Liberty gene being the main contamination source. On the other hand, varieties A, F, J, K and L provide examples that Certified seed true to variety can be produced provided the Foundation and Breeder seed lots are pure.

If varieties B, C and N are excluded only 3 of the remaining 54 samples exceeded the 0.25% standard for the presence of other varieties when the total of both the Roundup and Liberty genes are considered, namely one sample (D_2) from variety D (0.35%), one (E_1) from variety E (0.57%) and one from variety G (0.37%) (see Appendix I). In examining the isolation distances reported by growers of these seed lots, the sample D_2 was isolated by 500m, the E_1 sample by 100m from field sown to an unknown canola variety and the G_1 sample was isolated by 1207m. Since only one sample was obtained of variety G, it is not possible to determine if the level of contamination resulted from outcrossing in the Certified production field or from an impure Foundation lot. However, given the isolation distance, the Foundation seed is the most likely source.

Table 2. Average level of the presence of the Roundup and Liberty genes in Certified seed lots of 14 different non-herbicide tolerant canola varieties produced in Canada in 2000.

Variety	No. of	% Roundup gene		% Liberty gene		% total cont.	
code	samples	Ave.	Range	Ave.	Range	Ave.	Range
A	37	0.021	0.00-0.15	0.01	0.00-0.10	0.03	0.00-0.15
B	10	0.40	0.20-0.61	0.29	0.10-0.67	0.69	0.31-1.02
C	5	0.04	0.00-0.10	0.24	0.10-0.36	0.28	0.15-0.46
D	5	0.08	0.00-0.30	0.04	0.00-0.05	0.12	0.05-0.35
E	3	0.24	0.05-0.57	0.00		0.24	0.05-0.57
F	2	0.05	0.00-0.10	0.02	0.00-0.05	0.07	0.00-0.15
G	1	0.37		0		0.37	
H	1	0.06		0.05		0.11	
I	1	0.21		0		0.21	
J	1	0		0		0	
K	1	0.05		0		0.05	
L	1	0		0		0	
M	1	0.10		0		0.10	
N	1	0.35		0.46		0.81	

The Sample of D_2 that contained 0.35% foreign genes is not easily explained since the Certified production field was isolated by 500m and three other samples tested from the same variety each contained only 0.05% and a fourth 0.10% contamination. It is assumed, but not known at this time, that all five samples of variety D originated from the same Foundation seed lot. However, the level of contamination is higher than one might expect from either sampling error or pollen movement. Thus, the most likely explanation is admixture either in the seeding, harvesting or seed cleaning operations. Sample E_1 also exceeded the 0.25% contamination limit with a value of 0.57%. The other two samples tested from this variety contained only 0.05 and 0.10% of the Roundup gene (Appendix I). If all three fields were sown with the same Foundation seed lot, the 0.57% value is too large to be considered a sampling error. The E_2 Certified seed producing field was isolated by 100m from a canola field sown with an unknown variety. However, even if the adjacent field was a Roundup tolerant variety the level of contamination is still far higher than would be expected from pollen

movement given the data gathered from outcrossing studies between adjacent fields with different herbicide tolerances. Thus, the major source of contamination in this sample is likely to be a small amount of mechanical mixing during the seeding, harvesting or cleaning operations.

One sample (B_{11a}) from variety B, which is not included in the above summary of results, had an unusually high level of contamination of both the Roundup and Liberty genes (Table 3). The sample was produced by a very experienced grower and was isolated from the closest canola field (sown to a Roundup tolerant variety) by 792m. Since at least four of the growers reported growing their non-herbicide tolerant Certified canola fields within 100 to 120m of a Roundup tolerant field and only had contamination levels between 0.00 and 0.10%, the contamination level of 7.20% in this sample with 792m isolation had to have resulted from an admixture or an impure Foundation lot. To be certain that there had not been an error in the sampling of this seed lot, a second sample was provided by the grower. This second sample, B_{11b} was obtained from what was left in the bottom of the empty bin in which the seed lot had been stored. Although the total level of contamination was lower at 2.78% it was far too high to have resulted from pollen flow. The fact that the contamination included a significant amount of both Roundup and Liberty genes indicates a problem with the Foundation seed lot. It should be noted that the same grower produced a Certified seed lot of variety D under an identical isolation distance with the presence of only 0.05% of the Roundup gene.

Table 3. Presence of Roundup and Liberty genes in two samples of the outlier Certified seed lot from variety B.

Sample	Code	% Roundup	% Liberty	Total R + L
Original	B_{11a}	6.84	0.36	7.2
Second	B_{11b}	4.69	0.58	5.27

Although it was not part of the project, the proportion of susceptible seeds present in five Certified samples of Roundup tolerant varieties, submitted by growers, was determined (Table 4).

Table 4. Average percent of Roundup susceptible seeds in Certified seed of Roundup Ready varieties submitted by growers from their 2000 crop.

Variety code	No. of samples	Average % susceptible seed
O	1	1.8
P	1	3.42
Q	1	3.28
R	1	3.44

These data suggest that Roundup tolerant varieties presently on the market are unlikely to meet the genetic purity standard of 99.75% unless the variety description characterizes the presence of such susceptible seed as a varietal characteristic. The presence of these susceptible seeds would not affect the agronomic performance of these varieties since such susceptible seedlings would be eliminated in the early seedling stage when the commercial field was sprayed with Roundup. The presence of susceptible seedlings in these varieties probably arises from the dominant genetic characteristic of the Roundup herbicide tolerant trait and the resulting difficulty of maintaining all plants within such a variety homozygous for the Roundup tolerant trait. In hind sight, we should have also tested these Roundup tolerant seed lots for the presence of the Liberty gene.

Discussion

The results indicated that canola breeders and seed companies must use much greater care in making sure that Breeder and Foundation seed of their varieties are as free of foreign genes as possible. Until late in 2000, the technology to rapidly and economically test for the presence and amount of Roundup and Liberty tolerant genes was not available. However, germination herbicide tests described in this report are now available and the industry and regulators now have a fast and economical means to detect the presence and percentage of foreign genes. Also, the availability of immunoassay test sticks and kits to identify plants carrying a Roundup or Liberty gene also makes it possible to eliminate such plants during Breeder seed production. Thus, it is expected that future Breeder and Foundation seed lots will be free of foreign genes or at least meet the required 99.95% genetic purity. It is recommended that the CSGA make it mandatory to have all Foundation seed lots tested for the presence of foreign herbicide tolerant traits to ensure all Foundation lots meet the required standard of purity.

The results from testing variety A demonstrate that if care is taken to produce clean Breeder seed and seed growers are aware of potential problems with outcrossing, it is quite possible to produce genetically pure Certified seed. It is also clear that varieties B, G and N, and probably C, should be withdrawn or Breeder and Foundation seed sources be cleaned up.

It is concluded that although the presently required 100m isolation is insufficient to prevent zero outcrossing in pedigree seed fields, such an isolation distance is more than adequate to produce Certified seed of the required 99.75% genetic purity. However, it must be remembered that the seed lots on which this survey is based have been grown on fields which would not have previously grown a herbicide-tolerant variety, since the first production of such varieties did not occur until 1995. Thus, in the future, volunteer herbicide-tolerant canola may add to the level of contamination in non-herbicide tolerant pedigree seed.

Although 99.75% genetic purity appears to be a stringent standard and has resulted in a highly marketable product for many years, the fact that the presence of individual genes can now be readily and precisely identified and quantified, greatly tightens the specifications. Further, customers who purchase herbicide susceptible varieties in many cases have expected 100% genetic purity only to find that when they use Roundup alone to clean up weeds and volunteers the following year a significant amount of Roundup tolerant plants survive. Canola growers, agronomists and seedsmen need to understand that because canola seed is small (1000 seeds=4 grams) and seeding rates are normally high, about 2.75kg/ac or 688,000 seeds/ac, the number of herbicide tolerant seeds sown per acre, even at an acceptable 0.05% level of contamination, would result in 344 herbicide tolerant seeds being planted per acre. Not all such seeds would produce plants but certainly there would be enough to provide a large population of herbicide tolerant volunteers over the entire field in the spring of the following year. Thus, it is not surprising that such situations have occurred and been reported in the press by surprised and sometimes angry producers. However, it is important to keep in mind that the only herbicide tolerant form that has created concern to date is Roundup tolerance. This is so even though Liberty tolerant plants are also present, but in a lower proportion in many fields. The presence of Liberty tolerant plants goes unnoticed only because, unlike Roundup, Liberty is only used as a herbicide for Liberty tolerant varieties. Many producers have failed to recognize or accept that Roundup is now a selective herbicide and is no longer a broad spectrum herbicide that will give complete vegetative control in spring burnoff and chem fallow operations.

In summary, the present isolation distance of 100m provides adequate but not complete protection from foreign pollen. Provided that the Breeder seed has 100% genetic purity, or nearly so, the Foundation and Certified respective standards of 99.95 and 99.75% should be easily met. However, unless canola pedigree seed growers take extra care to control canola volunteers in the years between canola pedigree production, such volunteers could raise the presence of foreign genes to unacceptable levels. The large number of canola seeds normally planted per acre plus the high probability that a small percentage of herbicide tolerant seeds will be present in most Certified seed lots has and will continue to result in significant herbicide tolerant plant populations in most commercial canola fields.

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Appendix I. Number of seedlings rated for susceptibility and tolerance to the herbicides Roundup and Liberty and the percent herbicide tolerance present in each Certified seed sample produced in the year 2000.

	Variety	Roundup			Liberty			R + L
Can No.	Code	No. Sus.	No. Tol.	% Tol.	No. Sus.	No. Tol.	% Tol.	Total Tol. %
17	A_1	1984	2	0.1	1984	0	0	0.1
18	A_2	1992	0	0	1988	1	0.05	0.05
19	A_3	1997	0	0	1991	0	0	0
20	A_4	1990	0	0	1991	0	0	0
21	A_5	1992	0	0	1986	0	0	0
22	A_6	1869	0	0	1887	0	0	0
23	A_7	1969	0	0	1972	0	0	0
24	A_8	1993	0	0	1989	0	0	0
25	A_9	1985	1	0.05	1979	0	0	0.05
26	A_{10}	1975	0	0	1972	0	0	0
27	A_{11}	1992	0	0	1994	0	0	0
28	A_{12}	1990	0	0	1993	0	0	0
29	A_{13}	1995	0	0	1990	0	0	0
30	A_{14}	1986	0	0	1977	0	0	0
32	A_{15}	1970	1	0.05	1955	0	0	0.05
34	A_{16}	1983	0	0	1987	0	0	0
35	A_{17}	1944	3	0.15	1947	0	0	0.15
36	A_{18}	1994	0	0	1991	0	0	0
37	A_{19}	1994	0	0	1984	0	0	0
39	A_{20}	1988	0	0	1983	0	0	0
41	A_{21}	1917	0	0	1939	0	0	0
42	A_{22}	1923	0	0	1928	1	0.05	0.05
43	A_{23}	1988	0	0	1986	0	0	0

	Variety	Roundup			Liberty			R + L
Can No.	Code	No. Sus.	No. Tol.	% Tol.	No. Sus.	No. Tol.	% Tol.	Total Tol. %
45	A_{24}	1936	0	0	1929	2	0.1	0.1
46	A_{25}	1976	0	0	1950	0	0	0
47	A_{26}	1985	3	0.15	1981	0	0	0.15
48	A_{27}	1988	3	0.15	1974	0	0	0.15
50	A_{28}	1995	0	0	1988	0	0	0
51	A_{29}	1967	0	0	1963	0	0	0
52	A_{30}	1987	1	0.05	1980	0	0	0.05
54	A_{31}	1980	0	0	1979	0	0	0
56	A_{32}	1972	0	0	1983	0	0	0
57	A_{33}	1981	2	0.1	1988	1	0.05	0.15
58	A_{34}	1995	0	0	1996	0	0	0
59	A_{35}	1997	0	0	1993	0	0	0
60	A_{36}	1997	0	0	1996	0	0	0
61	A_{37}	1989	0	0	1987	0	0	0
69	B_1	1987	8	0.4	1975	4	0.2	0.6
71	B_2	1904	4	0.21	1913	2	0.1	0.31
72	B_3	1934	8	0.41	1891	4	0.22	0.63
73	B_4	1976	7	0.35	1970	6	0.3	0.65
74	B_5	1930	5	0.26	1929	5	0.26	0.52
75	B_6	1976	4	0.2	1959	5	0.26	0.46
76	B_7	1959	11	0.56	1952	9	0.46	1.02
78	B_8	1948	12	0.61	1929	1	0.05	0.65
79	B_9	1965	12	0.61	1950	6	0.41	1.02
8	B_{10}	2931	10	0.34	1936	13	0.67	1.01
	B_{11a}	1836	135	6.84	1956	7	0.36	7.2
70	B_{11b}	1833	37	1.98	1855	15	0.8	2.78

	Variety	Roundup			Liberty			R + L
Can No.	Code	No. Sus.	No. Tol.	% Tol.	No. Sus.	No. Tol.	% Tol.	Total Tol. %
33	C_1	1930	1	0.05	1941	2	0.1	0.15
31	C_2	1976	2	0.1	1967	7	0.36	0.46
40	C_3	1997	0	0	1991	6	0.3	0.3
53	C_4	1980	0	0	1967	3	0.15	0.15
11	C_5	1993	1	0.05	1985	6	0.3	0.35
63	D_1	1962	1	0.05	1965	0	0	0.05
64	D_2	1985	6	0.3	1987	1	0.05	0.35
65	D_3	1981	0	0	1978	1	0.05	0.05
67	D_4	2000	0	0	1992	1	0.05	0.05
68	D_5	1986	1	0.05	1977	1	0.05	0.1
3	E_1	2956	17	0.57	1971	0	0	0.57
6	E_2	1948	1	0.05	1965	0	0	0.05
15	E_3	1922	2	0.1	1632	0	0	0.1
44	F_1	1982	2	0.1	1980	1	0.05	0.15
55	F_2	1976	0	0	1981	0	0	0
1	G_1	2959	11	0.37	1969	0	0	0.37
14	H_1	1742	1	0.06	1921	1	0.05	0.11
5	I_1	1872	4	0.21	1890	0	0	0.21
7	J_1	1955	0	0	1904	0	0	0
9	K_1	1944	1	0.05	1946	0	0	0.05
10	L_1	1987	0	0	1985	0	0	0
13	M_1	1960	2	0.1	1977	0	0	0.1
80	N_1	1981	7	0.35	1970	9	0.46	0.81