

Pollen-mediated gene flow from transgenic safflower (*Carthamus tinctorius* L.) intended for plant molecular farming to conventional safflower

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Field experiments were conducted in Chile and western Canada to measure short-distance (0 to 100 m) outcrossing from transgenic safflower (*Carthamus tinctorius* L.) intended for plant molecular farming to non-transgenic commodity safflower of the same variety. The transgenic safflower used as the pollen source was transformed with a construct for seed-specific expression of a high-value protein and constitutive expression of a gene conferring resistance to the broad-spectrum herbicide glufosinate. Progeny of non-transgenic plants grown in plots adjacent to the transgenic pollen source were screened for glufosinate resistance to measure outcrossing frequency. Outcrossing frequency differed among locations: values closest to the transgenic pollen source (0 to 3 m) ranged from 0.48 to 1.67% and rapidly declined to between 0.0024 to 0.03% at distances of 50 to 100 m. At each location, outcrossing frequency was spatially heterogeneous, indicating insects or wind moved pollen asymmetrically. A power analysis assuming a binomial distribution and a range of alpha values (type 1 error) was conducted to estimate an upper and lower confidence interval for the probable transgenic seed frequency in each sample. This facilitated interpretation when large numbers of seeds were screened from the outcrossing experiments and no transgenic seeds were found. This study should aid regulators and the plant molecular farming industry in developing confinement strategies to mitigate pollen mediated gene flow from transgenic to non-transgenic safflower.

Keywords: outcrossing / safflower / plant molecular farming / gene-flow patterns / binomial distribution / power analysis / vicinism

INTRODUCTION

Safflower is grown for its seed oil throughout the Mediterranean, Europe and in the Americas, including the US and Chile. In the Canadian prairies, safflower is a minor crop grown for a small niche bird feed market with production limited to 320 to 810 ha annually (Muendel et al., 2004). Recently, safflower (*Carthamus tinctorius* L.; cv. 'Centennial') was transformed for plant molecular farming (PMF) using a construct encoding a seed-targeted high-value protein and constitutive expressed *phosphinothricin acetyltransferase (pat)* to confer resistance to the broad-spectrum herbicide glufosinate (L-phosphinothricin). One concern with this new technology

is pollen-mediated gene flow from transgenic to commodity safflower. The consequences of adventitious presence in commodity crops can be serious, including halting the development of a PMF crop. For example, pollen movement from a small experiment of transgenic corn expressing a gene for a pig vaccine intended for PMF being developed by ProdiGene Inc. resulted in cross-fertilization of a field of corn intended for food/feed in Iowa. The neighboring fields of 63 ha. of corn growing near the site were destroyed by USA government regulators (Ellstrand, 2003).

Crop species and varieties have different rates of outcrossing that are controlled genetically and influenced by the environment (for example see Beckie et al., 2003; Eastham and Sweet, 2002; Fritz and Lukaszewski,

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1989; Hanson et al., 2005). Outcrossing for both wind-pollinated and insect-pollinated species decreases rapidly with distance from the pollen source with the nearest neighbors receiving most of the pollen (Levin and Kerster, 1974). Various factors affect outcrossing among plant populations: pollinator effects including pollinator species and distance to other pollen sources; spatial and abiotic factors including distance to compatible crops, humidity, wind direction and velocity, geographic and vegetative barriers; crop species effects including the number and diversity of plant species attractive to pollinators in the area, ploidy level of the populations, shape, size and density of pollen donor and receptor plant populations, floral synchrony, floral and inflorescence position on the plant, pollen longevity, and cross-compatibility. Many of these factors interact, making predictions difficult without empirical measurements (Hoyle et al., 2007; Luna et al., 2001; Messeguer, 2003; Rognli et al., 2000). While complete confinement of pollen and seed is not possible for any crop species to date (Levin and Kerster, 1974), management that incorporates spatial, temporal or vegetative barriers could minimize pollen-mediated gene flow between crops.

Safflower genotype, floral morphology and development influence the rate of self-pollination. Claassen (1950) reported a wide range of self-pollination rates for different safflower varieties (genotypes), between 9.3 and 81.5%. Bees have been observed combing pollen from the stigmatic hairs of safflower in the early morning, which facilitates pollen reaching the stigma and encourages plant-to-plant movement of pollen (Howard et al., 1915). However, pollen delivered by an insect must outcompete the floret's own pollen to result in outcrossing.

Studies conducted in India using orange or red corolla (dominant trait) safflower types as the pollen donor and white (recessive trait) types as the pollen recipient demonstrated that safflower outcrossing decreases rapidly over relatively short distances (1 to 50 m) (Deokar and Patil, 1976; Howard et al., 1915; Kadam and Patankar, 1942). Safflower varieties examined in these studies differed greatly in outcrossing. In close proximity, cross-pollination ranged from 10 to 27% in high outcrossing varieties (Howard et al., 1915; Kadam and Patankar, 1942) and 0.08 to 1.29% for low outcrossing varieties (Deokar and Patil, 1976).

The only study of safflower outcrossing conducted in the USA also used corolla color as a marker (Claassen, 1950). Using insect exclusion cages over safflower plants, they showed that wind and mechanical collisions among flowers did not move pollen beyond 1.2 m, suggesting that safflower pollen is not transferred extensively without insects. Outcrossing frequencies for different cultivated varieties grown in close proximity without insect exclusion ranged from 0 to 100%, with most between

0.5 to 40%, while higher oil-yielding safflower lines had outcrossing rates from 1 to 5%. The outcrossing rate of transgenic 'Centennial' safflower, the variety developed in the USA with high seed oil content intended for PMF in the Americas, was unknown. It readily produces seeds under greenhouse conditions when insects are excluded, suggesting it is highly self-pollinating.

This study quantified the frequency of outcrossing from transgenic safflower to non-transgenic safflower under field conditions in three different environments at El Bosque (Santiago, Chile) in 2002, Westwold (British Columbia, Canada) in 2002 and Lethbridge (Alberta, Canada) in 2004. Transgenic and non-transgenic safflower (cv. 'Centennial') were used as pollen source and recipient, respectively. Outcrossing from transgenic to non-transgenic safflower produced a hemizygous seed resistant to the herbicide glufosinate. Seed harvested from the non-transgenic plants at various distances and directions from the transgenic pollen source were grown in subsequent years and the seedlings screened for glufosinate resistance. Surviving and dead plants were counted and the presence of the transgene confirmed at the protein and DNA level using molecular techniques. Pollen movement as a function of distance was modeled using regression analysis. Heterogeneity of outcrossing by direction was assessed with a log-likelihood ratio test. To draw conclusions about samples that did not contain a transgenic seed, we conducted a power analysis using a binomial distribution to determine the minimum number of seeds (sample size) from outcrossing experiments to be screened to detect at least one or more transgenic seeds at different theoretical outcrossing frequencies and confidence thresholds.

RESULTS

Seeds harvested from non-transgenic recipient plots at different directions and distances from the transgenic pollen source were screened in the field with two applications of glufosinate; survivors were considered a product of outcrossing. The frequency of outcrossing was calculated as a proportion of the number of safflower seedlings surviving glufosinate and the total number that emerged (Tabs. 1 to 3). Of the 1258 herbicide-resistant seedlings observed in the field, 302 were from El Bosque, 902 from Westwold and 54 from Lethbridge (Tabs. 1 to 3). PCR analyses of DNA extracted from 539 survivor leaf samples confirmed they all contained the transgene, *pat*; immunochromatography of 99 leaf samples from survivors confirmed they all contained the transgenic protein, PAT.

Outcrossing frequency at increasing distances from the pollen source was modeled using an exponential decay function. Parameter estimates for intercept (a)

Table 1. Harvest distances and field screening results for the outcrossing experiment at El Bosque, Santiago, Chile.

Mean distance (m) ¹	Emergence ² total (n_o)	Survivors total	Outcrossing (%)	95% confidence interval ³	
				Lower	Upper
0.7	18 504	89	0.48098	0.38760	0.59433
2.1	18 404	27	0.14671	0.09643	0.21377
3.4	21 156	22	0.10399	0.06501	0.15755
4.6	21 100	21	0.09953	0.06146	0.15215
5.8	22 648	28	0.12363	0.08201	0.17891
7.0	20 660	7	0.03388	0.01361	0.06967
8.2	20 728	20	0.09649	0.05885	0.14885
9.4	17 284	8	0.04628	0.01995	0.09108
Unharvested (1 m) ⁴					
11.6	22 116	22	0.09947	0.06219	0.15072
12.8	23 844	4	0.01677	0.00457	0.04298
Unharvested (2 m) ⁴					
16.0	17 912	10	0.05580	0.02672	0.10251
17.2	15 864	9	0.05673	0.02591	0.10774
Unharvested (3 m) ⁴					
21.4	17 792	20	0.11241	0.06857	0.17340
22.6	18 840	0	0.00000	0.00000	0.02326
Unharvested (4 m) ⁴					
27.8	18 408	10	0.05432	0.02600	0.09975
29.0	15 260	2	0.01311	0.00159	0.04736
Corner samples ⁴					
52.1	5632	1	0.01775	0.00045	0.13089
60.5	6648	2	0.03008	0.00364	0.10867
Total	322 800	302	-	-	-
Mean	17 933	17	-	-	-

¹Mean distance was used in all analyses. The corner samples were harvested from the most distant corners of the rectangular-shaped experiment (Fig. 2).

²The mean and total number of plants that emerged during the field screening with glufosinate for each distance sampled. Seed from three plots in block 8 were not screened (808 at 13.2 m, 812 at 23.0 m and 816 at 36.8 m from the source plot).

³The 95% confidence interval using a relationship between F and binomial distributions, with a correction for the lower interval value when the number of survivors was zero as outlined in Zar (1999), p. 528.

⁴To reduce the amount of transgenic material to be transported back to Canada for screening, harvested material in the area between some samples was not retained.

and the rate of decline of outcrossing (b) were significant ($P < 0.0001$), indicating that the model was not over-parameterized. The rate of decline (b) was steepest (largest) at Lethbridge (Tabs. 1 to 4). The distance at which outcrossing frequency was reduced by 50% (O_{50}) ranged from 6.2 to 11.4 m; values for O_{95} ranged from 26.6 to 49.4 m (Tab. 4). The frequency of outcrossing was highest in the recipient plants closest to the source, 0.48% at 0.7 m in Chile, 1.67% at 3 m at Westwold, and 0.62% at 0.3 m at Lethbridge (Tabs. 1, 2 and 3, respectively). Over all three experiments, the outcrossing frequency ranged from 0.0 to 0.86% at 10 m and from 0.0 to 0.54% at 20 m from the transgenic pollen source. At distances of 50 to 100 m from the transgenic pollen source, the outcrossing frequency ranged from 0.0024 to 0.12% (Tabs. 1 to 3). In all experiments, safflower density was consistent with producer fields in similar environments and did not

differ significantly between transgenic source and non-transgenic sink populations.

Several of the blocks at each location were significantly heterogeneous, as determined from the maximum-likelihood ratio test (Tab. 5), suggesting wind direction or spatial aggregation of insect pollinators played a significant role in the pattern of safflower outcrossing. Bi-weekly observations at each site indicated that flowering of the transgenic pollen source plants and the non-transgenic recipient plants was synchronous and uniform at each site. Thus, it is unlikely that flowering time or flower density influenced outcrossing rates.

No field survivors were detected from all of the blocks at a mean distance of 22.6 and 300 m from the source at Chile and Westwold, respectively (Tabs. 1 and 2). At Lethbridge, survivors were not detected from any blocks at several distances from the source (Tab. 3). For samples

Table 2. Harvest distances and field screening results for the outcrossing experiment at Westwold, BC, Canada.

Mean distance (m) ¹	Emergence ² total (n_o)	Survivors total	Outcrossing (%)	95% confidence interval ³	
				Lower	Upper
3.0	12 514	209	1.67011	1.44739	1.93467
6.0	9707	154	1.58654	1.34945	1.87555
9.0	10 081	87	0.86301	0.69374	1.08351
12.0	11 215	106	0.94517	0.77346	1.16426
15.0	10 274	69	0.67159	0.49719	0.85012
18.0	8663	47	0.54252	0.39885	0.72119
21.0	8081	21	0.25987	0.14647	0.38827
24.0	12 157	33	0.27145	0.18658	0.38162
27.0	16 606	17	0.10237	0.05961	0.16422
30.0	14 396	15	0.10420	0.05823	0.17238
33.0	11 081	10	0.09025	0.04320	0.16566
36.0	5989	4	0.06679	0.01820	0.17096
39.0	6093	10	0.16413	0.07858	0.30109
42.0	10 155	11	0.10832	0.05419	0.19364
45.0	8766	13	0.14830	0.07893	0.25367
48.0	4376	1	0.02285	0.00058	0.12737
51.0	7455	3	0.04024	0.00830	0.11742
54.0	13 306	16	0.12025	0.06859	0.19584
57.0	14 978	6	0.04006	0.01473	0.08735
60.0	11 155	11	0.09861	0.00049	0.17628
63.0	8796	9	0.10232	0.04674	0.19423
66.0	11 499	8	0.06957	0.03000	0.13688
69.0	12 738	6	0.04710	0.01732	0.10270
72.0	15 441	9	0.05829	0.02662	0.11068
75.0	18 084	3	0.01659	0.00342	0.04842
78.0	16 457	5	0.03038	0.00987	0.07070
81.0	20 444	4	0.01956	0.00533	0.05012
84.0	19 354	6	0.03100	0.01140	0.06761
87.0	19 936	6	0.03010	0.01107	0.06564
91.78	20 265	1	0.00493	0.00012	0.02753
101.0	18 711	2	0.01069	0.00129	0.03863
Total	388 770	902	-	-	-
Mean	12 541	29	-	-	-
300.00 ⁴	85 239	0	0.00000	0.00000	0.00514

¹Mean distance was used in all analyses.

²The total and mean number of plants that emerged during the field screening with glufosinate for each distance sampled. Seed from three samples in blocks 2 and 3 were not screened (mean distance from the source of 45 m). Data for samples collected from the distance closest to the source (mean distance from the source 0.75 m) were excluded due to admixture during harvest.

³The 95% confidence interval using a relationship between F and binomial distributions, with a correction for the lower interval value when the number of survivors was zero as outlined in Zar (1999), p. 528.

⁴The single non-transgenic plot grown 300 m from the pollen source. These data were not included in the regression analysis but were screened in the field and are thus treated separately here.

where no transgenic seedlings were found, we employed binomial probabilities and sample sizes to conduct a power analysis to estimate the minimum detectable differences or limits of detection (Tab. 6).

Outcrossing was detected at all distances at the Chilean site except from the 18 840 seeds screened from samples taken at a mean distance of 22.6 m in eight

directions from the transgenic pollen source (Tab. 1). From the power analysis results (Tab. 6), we would accept the null hypothesis that the frequency of transgenic seeds screened from this sample was equal to or greater than 0.00025 only 1% of the time. Thus, it is likely that the frequency of transgenic seeds in this sample is less than 0.00025.

Gene flow from transgenic to conventional safflower

Table 3. Harvest distances and field screening results for the outcrossing experiment at Lethbridge, AB, Canada.

Mean distance (m) ¹	Emergence ² total (n_o)	Survivors total	Outcrossing (%)	95% confidence interval ³	
				Lower	Upper
0.3	5764	36	0.62454	0.43688	0.86318
1.3	6123	1	0.01633	0.00041	0.09107
2.8	5406	0	0.00000	0.00000	0.08103
4.3	5973	0	0.00000	0.00000	0.07333
5.8	4805	0	0.00000	0.00000	0.09116
7.3	5316	0	0.00000	0.00000	0.08239
8.8	6141	0	0.00000	0.00000	0.07134
10.3	4012	0	0.00000	0.00000	0.10916
11.8	7079	0	0.00000	0.00000	0.06188
13.3	7006	0	0.00000	0.00000	0.06253
14.8	11 312	0	0.00000	0.00000	0.03873
16.3	13 126	0	0.00000	0.00000	0.03338
17.8	15 390	0	0.00000	0.00000	0.02847
19.3	16 421	0	0.00000	0.00000	0.02668
20.8	16 962	0	0.00000	0.00000	0.02583
22.3	13 885	0	0.00000	0.00000	0.03155
23.8	15 501	1	0.00645	0.00016	0.03599
25.3	15 353	0	0.00000	0.00000	0.02854
26.8	18 490	1	0.00541	0.00014	0.03017
28.3	22 490	1	0.00445	0.00011	0.02481
29.8	20 510	0	0.00000	0.00000	0.02136
31.3	20 489	0	0.00000	0.00000	0.02139
32.8	22 506	1	0.00444	0.00011	0.02479
34.3	28 187	0	0.00000	0.00000	0.01554
35.8	18 177	0	0.00000	0.00000	0.02410
37.3	31 826	0	0.00000	0.00000	0.01377
38.8	28 791	1	0.00347	0.00009	0.01947
40.3	25 879	7	0.02705	0.01086	0.05563
41.8	25 894	0	0.00000	0.00000	0.01692
43.3	26 537	0	0.00000	0.00000	0.01651
44.8	33 958	3	0.00883	0.00182	0.02579
46.3	45 726	0	0.00000	0.00000	0.00958
47.8	35 213	1	0.00284	0.00007	0.01584
49.3	41 156	1	0.00243	0.00006	0.01356
Total	621 404	54	-	-	-
Mean	18 277	2	-	-	-

¹Mean distance was used in all analyses.

²The total and mean number of plants that emerged during the field screening with glufosinate for each distance sampled. Samples from the distances closest to the source for blocks 2, 4, 6 and 8 were not harvested to avoid potential admixture with the transgenic source plot.

³The 95% confidence interval using a relationship between F and binomial distributions, with a correction for the lower interval value when the number of survivors was zero as outlined in Zar (1999), p. 528.

Outcrossing was detected at all distances for the Westwold site except from the 85 239 seeds screened from the single non-transgenic plot 300 m from the transgenic pollen source (Tab. 2). From the power analysis, we would accept the null hypothesis that the frequency of transgenic seeds screened from this sample was equal to or greater than 0.00005 only 2.5% of the time.

Outcrossing was detected at very few distances at the Lethbridge site. All of the samples screened from the Lethbridge site at mean distances of 2.75 to 22.25 m from the transgenic pollen source did not contain a glufosinate-resistant seed (Tab. 3). From the power analysis (Tab. 6) and the range of seeds screened from each distance, 4012 to 16 962, we would accept the null hypothesis 2.5% of

Table 4. Parameter estimates and O₅₀ and O₉₅ values with their respective standard errors and confidence intervals from the regression analysis and equation 1, 2 and 3¹.

Experiment	Parameter ²	Estimate ¹	Standard error	Df ³	95% confidence interval	
					Lower	Upper
Chile	<i>a</i>	0.0025	0.0002	129	0.0020	0.0030
	<i>b</i>	0.0757	0.0078	129	0.0603	0.0912
	O ₅₀	9.1512	0.9421	129	7.2873	11.0151
	O ₉₅	39.5507	4.0716	129	31.4950	47.6065
Westwold	<i>a</i>	0.0168	0.0008	124	0.0152	0.0184
	<i>b</i>	0.0607	0.0020	124	0.0566	0.0647
	O ₅₀	11.4267	0.3838	124	10.6669	12.1864
	O ₉₅	49.3852	1.6589	124	46.1018	52.6687
Lethbridge	<i>a</i>	0.0011	0.0002	269	0.0007	0.0015
	<i>b</i>	0.1126	0.0118	269	0.0893	0.1360
	O ₅₀	6.1534	0.6469	269	4.8798	7.4270
	O ₉₅	26.5946	2.7959	269	21.0901	32.0992

¹Estimates of the parameters for intercept (*a*), slope (*b*) and the estimates of the distance where outcrossing was reduced by 50 and 95% (see text for regression equation). All predictions were significant ($P < 0.0001$).

²Parameters *a* and *b* were estimated from the regression analysis with equation 1. The distance that outcrossing was reduced by 50 and 95% were estimated using equations 2 and 3, respectively.

³Degrees of freedom.

Table 5. Outcrossing directionality: log-likelihood ratio test among blocks for each experiment.

Experiment	Block (s)	Direction ¹	Combined		Partitioned ⁴		X ² Value ⁵		Significance ⁶
			Value	Df ⁷	Value	Df ⁷	Value	Df ⁷	
Chile	All	All	693.2	16	571.3	2	121.9	14	****
	1 vs. 5	N and S	234.8	4	221.0	2	13.8	2	***
	2 vs. 6	NE and SW	128.9	4	125.4	2	3.5	2	<i>ns</i>
	3 vs. 7	E and W	143.5	4	95.0	2	48.5	2	****
	4 vs. 8	NW and SE	145.8	4	129.9	2	15.9	2	****
Westwold	All	All	722.7	8	687.0	2	35.7	6	****
	1 vs. 2	N and S ²	301.8	4	297.5	2	4.3	2	<i>ns</i>
	2 vs. 3	N and S ²	307.4	4	289.8	2	17.6	2	****
	3 vs. 4	N and S ²	398.5	4	389.5	2	9.0	2	*
Lethbridge	All (less 1, 3 and 8) ⁸	Subset of all	312.4	10	149.2	2	163.2	8	****
	2 vs. 6	NE and SW	21.3	4	19.9	2	1.4	2	<i>ns</i>

¹Direction from the transgenic pollen source where 'N' is north, 'S' is south, 'W' is west and 'E' is east.

²The Westwold blocks were in a North-South direction relative to the transgenic source plot, but are east and west of one another.

³The overall value is the -2 log-likelihood value calculated by SAS (2007) for the regression of the combined data set for the blocks being compared.

⁴The individual sum value is the sum of the -2 log-likelihood values calculated by SAS (2007) for each individual regression of the partitioned data sets for the blocks being compared.

⁵The chi-square value was calculated as twice the value of the log-likelihood ratio of the sum of the partial data sets and the combined data set.

⁶Significance is given for $\alpha \leq 0.05$, 0.01, 0.005 and 0.001 corresponding to *, **, *** and ****, respectively. When the value was not significant it was designated as *ns*.

⁷Degrees of freedom.

⁸At the Lethbridge site, the blocks 3 and 8 were excluded from the log-likelihood ratio test because the observed outcrossing frequency was zero at most distances for block 3 and all zeros for block 8, and the program could not converge during the analysis. When block 1 was analyzed by itself, the program gave a warning and did not calculate the standard error and confidence intervals. This may have occurred because the only survivors observed were at the distal portion (farthest from the transgenic source plot). For this reason block 1 was excluded from the log-likelihood ratio tests.

Table 6. Power analysis assuming a binomial distribution and using equation 2 (see text) to determine the minimum number of seeds to screen to detect at least one transgenic seed, for different levels of outcrossing and three alpha values.

Null hypothesis ¹ Frequency of $p(X)$	Alpha value (α)			
	0.05 (5%)	0.025 (2.5%)	0.01 (1%)	0.005 (0.05%)
	Minimum sample size (n_p) ²			
0.01	299	368	459	528
0.005	598	736	919	1058
0.0025	1197	1474	1840	2117
0.001	2995	3688	4603	5296
0.0005	5990	7376	9209	10594
0.00025	11982	14754	18419	21191
0.0001	29956	36887	46050	52981
0.00005	59914	73776	92102	105964
0.000025	119828	147554	184205	211931
0.00001	299572	368887	460515	529830
0.000005	599145	737775	921032	1059661
0.0000025	1198292	1475550	1842066	2119325

¹The theoretical value of outcrossing (p).

²The minimum value of n_p is the sample size required to detect one or more transgenic seeds given a theoretical frequency of transgenic seeds (p) and different values of alpha. Values for minimum sample size were rounded upward to ensure they are within the bound set by the alpha value. The null hypothesis that the frequency is $X \geq p$ is rejected at a given percentage (alpha value) of the time, when no transgenic seeds were found in a sample size of n_p or greater.

the time that the frequency of transgenic seeds in these samples would be equal to or greater than 0.001 and 0.00025, respectively. The samples taken at a mean distance of 46.25 m in eight directions from the transgenic pollen source had 45 726 seeds screened and no transgenic seeds were detected (Tab. 3). From the power analysis, we would accept the null hypothesis only 2.5% of the time that the frequency of transgenic seeds screened from this sample was equal to or greater than 0.0001.

DISCUSSION

Safflower outcrossing among plants within 1 to 3 m from the transgenic pollen source at all three sites ranged from 0 to 1.7% (Fig. 1), similar to the outcrossing rates reported for high-oil-yielding safflower lines in the USA (1 to 5%) (Claassen, 1950). Outcrossing declined steeply with distance, but was still detectable at very low frequencies at 49 m or more from the sources, ranging from 0.0024 to 0.03% at all three sites (Fig. 1). Outcrossing from transgenic safflower to non-transgenic plants was reduced more than 40% at 6 m and reduced 96–100% at 50 m (Fig. 1). The frequency of outcrossing from transgenic to non-transgenic Centennial safflower at distances greater than 3 m was ten times lower than that reported for safflower with different floral coloration in India (Deokar and Patil, 1976; Howard et al., 1915; Kadam and Patankar, 1942).

The power analysis, assuming a binomial distribution, allowed interpretation of samples with no transgenic

seeds detected, which sometimes occurred after screening of large numbers of seeds. The ability to change the alpha value (type 1 error) and incorporate the various sample sizes from the screening process facilitated a robust estimate of the probable transgenic seed frequency in each sample.

The frequency of outcrossing was as heterogeneous among blocks (replicates) as it was among the sites. The regions where the outcrossing experiments were conducted all had predominantly westerly winds; however safflower flowered over a period of weeks and the wind direction varied considerably over that time. The results of the likelihood ratio test did not indicate greater outcrossing on the leeward side of any of the experiments. Because wind does not facilitate significant safflower outcrossing beyond 1.2 m (Claassen, 1950), it is likely that the heterogeneity in outcrossing pattern observed at all three outcrossing sites was due to non-random pollen movement by insects.

Outcrossing rates were influenced by site environment. Outcrossing differed among the three experiments (Tabs. 1 to 4), being the highest at Westwold and lowest at Lethbridge (Tabs. 2 and 3). The observed outcrossing frequency nearest the transgenic pollen source at Chile was more similar to Lethbridge than Westwold (Tabs. 1 to 4). Differences between sites could have been influenced by experimental design, which differed in the size of the transgenic pollen source plot and shape and distribution of the non-transgenic pollen recipient area. The Chilean and Lethbridge experiments had similar

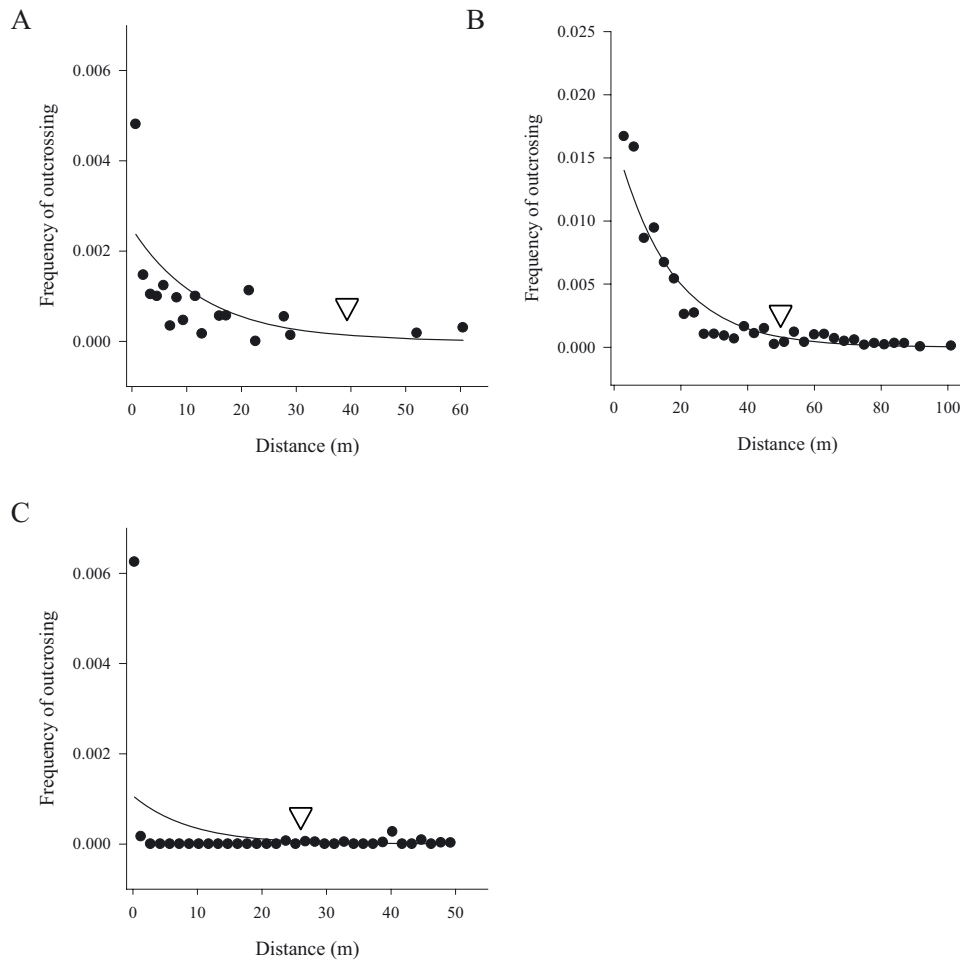


Figure 1. Frequency of outcrossing with increasing distance from the transgenic pollen source at (A) El Bosque Chile in 2002, (B) Westwold in 2002, and (C) Lethbridge in 2004. Arrows indicate O_{95} values (see Tab. 4 for regression equation and parameter estimates). Note the scale used for frequency of outcrossing differs among the graphs to prevent obscuring detail.

transgenic pollen source sizes and rates of outcrossing. The Westwold experiment had a pollen source three times greater in size than the others, and an outcrossing rate four times greater closest to the source, with a slower decline in outcrossing over distance as indicated by the O_{50} and O_{95} values (Tab. 4 and Fig. 1). The experiments at Chile and Westwold had barren zones (patches free of vegetation) between the transgenic source plot and the non-transgenic recipient plots, whereas the experiment at Lethbridge did not (Fig. 2). The lack of barren zones at Lethbridge may have reduced the rate of outcrossing observed relative to the other two sites. Barren zones between patches of insect-pollinated crop increased the distance of outcrossing in experiments conducted with *Brassica campestris* L. (Manasse, 1992) and *Brassica napus* L. (Morris et al., 1994; Reboud, 2003). The experimental designs would also provide different edge shapes

for pollinators. Previous research has shown pollinators entering large monoculture fields may settle first at the edge and then move inward, increasing outcrossing at the field margin (Ramsay, 2005). In addition, studies have shown that pollinators deposit pollen from the first plant visited to the next few visited plants, creating a pattern of outcrossing referred to as a paternity shadow (Cresswell et al., 1995). If pollinators at the Chilean and Westwold sites followed these patterns of behavior, cross-fertilization rates would depend on the direction they approached the safflower and where they would perceive the edge to be. At the Lethbridge site, the edge would have consistently been 50 m from the transgenic pollen source plot (Fig. 2).

Pollen movement may have been influenced by diverse-pollinating insects at the different sites. Butler et al. (1966) documented 40 species of non-parasitic bees

Gene flow from transgenic to conventional safflower

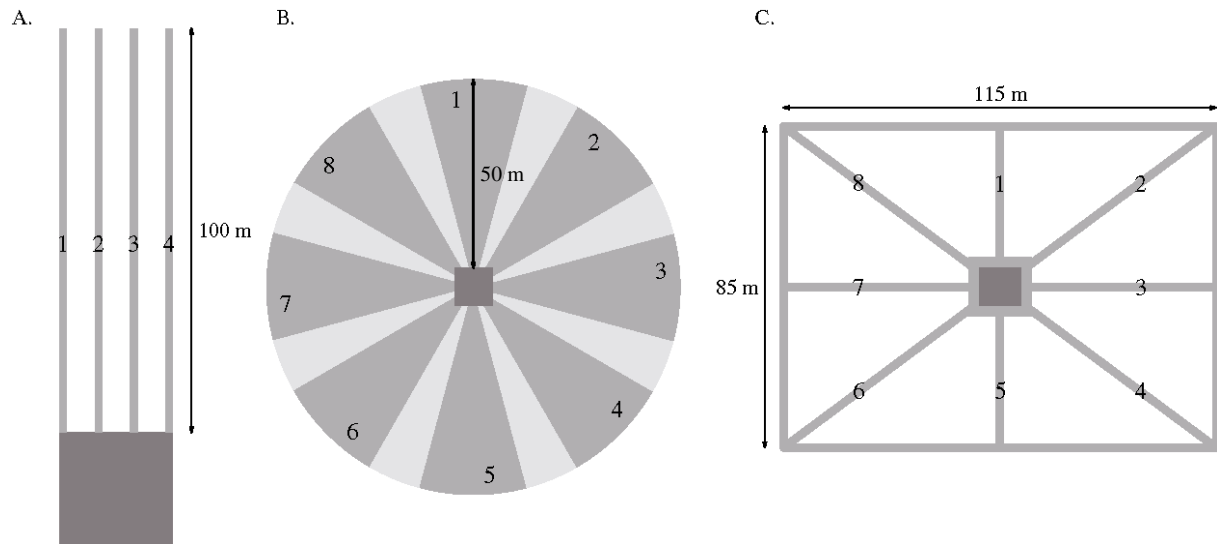


Figure 2. Experimental designs showing the spatial arrangement of the transgenic pollen source (darkest color), non-transgenic recipient plants (light and dark grey) and the area free of vegetation (white). (A) Westwold, British Columbia, (B) Lethbridge, Alberta, and (C) El Bosque, Chile. The light grey area was sampled and retained, whereas the dark grey area was not retained at harvest; numbers indicate blocks or replicates.

and their relative abundance on safflower experiments and commercial fields in Arizona. Native bees made up 10 to 15% of pollinators on safflower field experiments and 8 to 13% at the edge of commercial fields. Thus, pollinator diversity and movement patterns can vary greatly over short distances in similar agroecosystems.

The experiments presented here were designed to quantify pollen-mediated gene flow on a relatively small scale (50 to 100 m), and do not predict maximum distances of pollen movement by pollinators or the distance required to isolate fields of transgenic safflower from outcrossing with commodity safflower. Long-distance bee foraging has been documented up to 11.2 km from the hive on sweet clover pollen (Ramsay, 2005), and one of 2000 marked bees was found foraging 7.1 km from the hive on safflower (Gary et al., 1977).

A trap crop consisting of non-transgenic plants surrounding transgenic safflower may minimize long-distance outcrossing by insects. Currently in Canada, trap crops are used to reduce pollen movement from transgenic oilseed rape (*B. napus* L.) field experiments (CFIA, 2006). The efficacy of a trap crop to reduce outcrossing has been documented for transgenic oilseed rape (Morris et al., 1994; Reboud, 2003; Staniland et al., 2000) and cotton (*Gossypium hirsutum* L.; Kareiva et al., 1994). The O_{95} for the safflower experiment with the highest outcrossing (Westwold) was 37.9 m, suggesting a trap crop of this width around a transgenic safflower field could significantly reduce the risk of outcrossing to distant commodity safflower fields. Currently, seed growers maintain

certified and foundation level safflower seed purity with an isolation distance of 400 m from other safflower varieties in the USA and Canada (Anonymous, 2007a, 2007b; CFIA, 2005). By using both a trap crop and isolation distance, safflower intended for PMF may be grown without compromising conventional safflower growers. However, in addition to information on outcrossing distance, threshold levels for adventitious presence of transgenes, based on risk for food and feed must be established. The current requirements for a confined crop are zero and would be difficult to maintain under field production conditions. The results presented here should aid industry and regulators to designate best management practices to mitigate pollen-mediated gene flow from transgenic safflower intended for PMF to commodity safflower.

Pollen-mediated gene flow has been quantified in detail in several crops using transgenic and marker genes including wheat (*Triticum aestivum* L.) (Gustafson et al., 2005; Hucl, 1996; Hucl and Matus-Cadiz, 2001; Matus-Cadiz et al., 2007), oilseed rape (*B. napus*) (Beckie et al., 2003; Damgaard and Kjellsson, 2005; Rieger et al., 2002; Weekes et al., 2005) and maize (*Zea mays* L.) (Goggi et al., 2006, 2007; Gustafson et al., 2006; Weekes et al., 2007) (reviewed in Beckie and Hall, 2008). Because of differences in research design and source plot size, studies are difficult to compare directly. Safflower and wheat appear to have similar outcrossing at a 10 meter distance, and both have lower pollen-mediated gene flow than oilseed rape and much lower levels of outcrossing than maize. We anticipate that, similar to other crops,

seed mediated gene flow may be a more significant avenue for transgenic movement than *via* pollen.

MATERIALS AND METHODS

Source of plant materials

To quantify distance and direction of pollen-mediated gene flow from transgenic safflower intended for PMF to non-transgenic safflower under different environmental conditions, we conducted experiments at El Bosque, Chile and Westwold, BC in 2002 and Lethbridge, AB in 2004. Experiments were designed and implemented by different research groups and therefore varied at each site (Fig. 2). Non-transgenic seed cv. 'Centennial' safflower and cv. 'Centennial' safflower transformed using a construct encoding a seed-targeted protein and constitutive expressed *phosphinothricin acetyltransferase (pat)* were provided by SemBioSys Genetics Inc. (Calgary, Alberta, Canada). The seed lots were tested for cross-contamination between transgenic and non-transgenic safflower by screening with glufosinate in a greenhouse. Both seed lots were found to be true to type (described below under herbicide resistance of transgenic plants).

Environmental biosafety compliance

For all of the transgenic field experiments, confinement protocols were followed as outlined by the federal governments of Canada and Chile. These measures included appropriate isolation distances between transgenic and commodity safflower crops and other crop species, triple containment of seed to and from the site, cleaning and inspection of all equipment used at the sites, and frequent monitoring and control of safflower volunteers in the experimental sites and designated perimeters during the growing season and in post-harvest years.

Outcrossing experimental design and implementation

El Bosque, Santiago, Chile

Planting was conducted in the municipality of El Bosque, in the province of Santiago, Chile, near the Catholic University at Pirque (32° 47' 49.18" S; 70° 40' 01.43" W; elevation 732 m), on September 14, 2002 in a wheel and spoke design (Fig. 2C). The transgenic 10 × 11.2 m pollen source plot in the middle of the experiment consisted of 16 rows each 10 m in length. Four rows spaced 0.70 m apart of non-transgenic safflower were planted around the transgenic source plot for a total width of

2.8 m. Eight non-transgenic safflower blocks (replicates) with four rows of safflower 2.8 m wide were planted radially from the transgenic source plot. In addition, four rows of non-transgenic safflower were planted around the experiment in a rectangle of 115 × 85 m. Target seeding rates for both transgenic and non-transgenic cultivars were 30 kg.ha⁻¹. The nearest glufosinate-resistant safflower field was 573 m away from the experiment, and the nearest non-GM safflower was *ca.* 1000 m away. Neighboring crops included an alfalfa (*Medicago sativa* L.) field *ca.* 200 m to the north-east, maize (*Z. mays*) *ca.* 200 m to the south-east, sunflower (*Helianthus annuus* L.) *ca.* 700 m to the south-west and sugar beets (*Beta vulgaris* L.) *ca.* 50 m to the north.

Seeds were harvested on March 15, 2003 by hand at 16 distance intervals from the source plot in each of the eight radial blocks. The first two samples nearest the pollen source were 2.8 m wide. Subsequent samples were 1.2 m wide. To reduce the amount of transgenic seed collected and transported to Canada for screening, areas between some samples were not harvested (Tab. 1). A total of 16 samples were harvested per radial block (replicate). In addition, samples were taken at the outer corners, the farthest distance from the transgenic source (Tab. 1).

Westwold, British Columbia, Canada

Planting was conducted in Westwold, BC (50° 28' 04.66" N; 119° 45' 09.26" W; elevation 616 m), on May 17, 2002 in a comb design to maximize the distance of the pollen recipient plants to the source within available field dimensions (Fig. 2A). The transgenic pollen source plot was 30 × 30 m and the non-transgenic safflower recipient plots were planted in four blocks extending South from the source plot. The recipient blocks were 107 × 1.6 m in 16 rows. In addition, a plot of non-transgenic plants was 300 m from the experiment on the other side of an alfalfa field. Target seeding rates for transgenic and non-transgenic cultivars were 30 kg.ha⁻¹. The nearest glufosinate-resistant safflower was several kilometers away from the experiment. The farm site was surrounded by mixed boreal forest trees and its associated flora.

Seeds were harvested on October 16, 2002 with a Wintersteiger 2001 Elite research combine in an east/west direction with each sample consisting of a 1.2 m wide swath. Harvesting began furthest from the source plot to reduce cross-contamination of samples. Commingling of the non-transgenic samples closest to the transgenic pollen source at the Westwold site during harvest was suspected and these samples were removed from the analysis. The non-transgenic plot 300 m from the source was harvested separately after the harvester was cleaned and inspected.

Lethbridge, Alberta, Canada

Seeds were planted in Lethbridge AB (49° 41' 51.91" N; 112° 46' 24.82" W; elevation 909 m), on May 10, 2004 in a bulls-eye design. The transgenic source plot was 10 × 9.9 m consisting of 55 rows located in the center of the experiment. The recipient non-transgenic safflower was planted 50 m around the transgenic pollen source plot with row spacing of 18 cm. Target seeding rates for transgenic and non-transgenic cultivars were 30 kg.ha⁻¹. Once the safflower plants had bolted, the outside of the non-transgenic recipient plot was mowed into a circle with a 50 m perimeter around the transgenic pollen source. The closest transgenic and non-transgenic safflower was > 50 m. The experiment was conducted on a research farm with a diversity of crop species grown, including barley (*Hordeum vulgare* L.) to the east, west and north for over 50 m and peas (*Pisum sativum* L.) to the south for over 50 m.

Seeds were harvested on November 3, 2004. Eight wedges were removed (*ca.* 1/3 of the site area) between the blocks intended for harvest (Fig. 2B). The area removed between blocks had an outside arc distance of 14.4 m. The remaining area comprised eight blocks with an outside arc distance of 28.8 m and an inside arc distance of 2.5 m. Seeds were harvested in 1.2 m wide swaths from each block with a Wintersteiger 2001 Elite research combine and each swath was considered a plot. Harvesting began distal to the source and continued inward to reduce cross-contamination of samples.

Herbicide resistance of transgenic plants

A dose-response experiment with the herbicide glufosinate and homozygous transgenic and non-transgenic safflower plants was conducted in the greenhouse to determine a discriminating dose (Beckie et al., 2000). Experiments were conducted in the greenhouse at the University of Alberta, Edmonton, Alberta from 2004 to 2006 (data not shown).

Transgenic and non-transgenic seeds were planted in trays with six cells each (1 L per cell) containing soil-less vermiculite-peat mixture (Metro-Mix 290, The Scott's Company, 14111 Scottslawn Rd., Marysville, Ohio 43041). Twelve seeds per cell were planted and thinned to eight plants after emergence. Plants were exposed to natural light supplemented for 16 h by 400 W high pressure sodium, high intensity discharge bulbs and maintained at 21/18 °C day/night temperatures. Plants were watered as required and fertilized biweekly with complete 20-20-20 plus at a concentration of 200 ppm.

Glufosinate was applied to paired cells of transgenic and non-transgenic plants at 0, 0.25, 0.5, 1, 1.5 and two times the recommended field rate of 400 g ai.ha⁻¹ (grams

active ingredient per hectare) (Ali, 2003). Each herbicide dose-response experiment had three replicates arranged in a randomized complete block design and was repeated three different times. Herbicide was applied when safflower had two to six true leaves using a custom built track sprayer with a Billericay Air Bubble Jet 110015 nozzle tip calibrated to deliver 100 L.ha⁻¹ at 200 KPa. After herbicide application, trays were returned to the greenhouse and irrigated from above as required, commencing 24 h after application. Twenty-one days after herbicide application, plants were counted and cut at the soil surface, dried in paper bags at 60 °C for five days and dry weight measured.

Transgenic plants did not show signs of herbicide damage at rates of glufosinate as high as 800 g ai.ha⁻¹ with a water volume of 100 L.ha⁻¹ when the plants were at the two- to six-leaf stage, but occasionally a non-transgenic plant would survive. Further experiments were conducted with glufosinate applied at a rate of 800 g ai.ha⁻¹ and an increased water volume of 200 L.ha⁻¹ applied twice at a 4- to 7-day interval between applications. None of the 738 non-transgenic plants survived and all of the 738 transgenic plants showed no signs of herbicide injury.

Because pollen-mediated gene flow in the outcrossing experiments resulted in hemizygous seed, a second experiment was conducted to ensure the high rate of glufosinate used on the homozygous plants was an appropriate screening rate. Hand-pollination of homozygous transgenic 'Centennial' safflower plants with a non-transgenic 'S-317' safflower (and the reciprocal) was performed to generate hemizygous seeds. Seeds were planted in the greenhouse and screened as described above, except they were sprayed with glufosinate at 800 g ai.ha⁻¹ with double the previous water volume (200 L.ha⁻¹). All of the plants survived without herbicide injury. The F₂ seeds harvested from the surviving hemizygous parents were planted and sprayed as above. Chi-square analysis of the F₂ plants with and without herbicide damage fit the predicted three to one ratio, confirming the parents were hemizygous and not the product of self-fertilization. Results were used to establish the herbicide rate and water volume to discriminate between transgenic and non-transgenic safflower in the field screening.

Seed screening

Seeds from all sites were cleaned and stored at 10 °C until field screening was performed. Seed from Chile was grown in 2004, and from the Westwold and Lethbridge sites in 2005, on land in Edmonton, Alberta never utilized for safflower cultivation. Seeding rates were adjusted for a target of 250 plants.m⁻² based on seed germinability and kernel weight. An early fall frost at the Lethbridge

site reduced seed viability, and on some occasions the maximum number of seeds that could be planted with the seeding equipment still did not provide the target plant density.

Prior to planting samples from the Chilean outcrossing experiments in 2004, the site was first tilled and glyphosate was applied at a rate of 810 grams acid-equivalent per hectare ($\text{g ae}\cdot\text{ha}^{-1}$). Prior to planting samples from the Westwold and Lethbridge outcrossing experiments in 2005, the site was first tilled, packed with a spiral packer and glyphosate applied at $810 \text{ g ae}\cdot\text{ha}^{-1}$. Seeds from the Westwold and Lethbridge sites were treated with Helix Xtra[®] (an insecticide-fungicide mixture of thiamethoxam, difenoconazole, mefenoxam, and fludioxonil) as recommended for oilseed rape to increase seedling emergence and survival. Previous germination experiments with Helix Xtra[®] have shown that it does not inhibit germination (unpublished data). Each plot was planted with a seed sample from an outcrossing experiment to a depth of 1.3 to 3.8 cm and consisted of six rows 7 m long spaced 20.3 cm apart with a 1 m alley between plots. Fertilizer (0-45-0) was placed with the seed at a rate of $1.8 \text{ g}\cdot\text{m}^{-1}$ of row. Planting was performed with a reduced-disturbance Fabro air seeder equipped with Atom Jet double shoot openers. After seeding each plot, hoses and openers were checked to ensure all seeds were cleared. Several control plots of non-transgenic and transgenic safflower were randomly positioned among the outcrossing-sample plots to ensure the transgene was functional and verify herbicide efficacy and coverage.

When safflower seedlings were at the four- to six-leaf stage, plant number per plot was estimated based on three 0.25 m^2 quadrats per plot. Following emergence counts, the seedlings were sprayed with glufosinate at $800 \text{ g ai}\cdot\text{ha}^{-1}$ using low drift Billericay Air Bubblejet nozzle tips that delivered a water volume of $200 \text{ L}\cdot\text{ha}^{-1}$, and the application repeated 4 to 7 d later. A week later, surviving safflower plants were counted. The frequency of outcrossing was calculated as the number of survivors following herbicide application as a proportion of the number of emerged seedlings.

To reduce the area of land required to screen samples, pairs of adjoining plots from the Westwold experiment were combined, thereby increasing the effective range of the distance from the transgenic pollen source (Tab. 2). In addition, to increase the number of seedlings screened and reduce the area of land dedicated to transgenic confinement, seed from the Westwold and Lethbridge experiments were planted, seedlings screened and removed in each plot three times over the growing season. For each round of screening, the surviving plants were counted and sampled, and then the field area was treated with $810 \text{ g ae}\cdot\text{ha}^{-1}$ glyphosate to kill all remaining transgenic safflower plants and any weeds. Seed from the same

samples (outcrossing trial site and direction and distance from pollen source) were planted into the same plots to ensure any volunteers (late germinated seed) from prior screening tests did not confound the results.

Molecular confirmation of field survivors

A leaf from each plant surviving both applications of glufosinate in the field was harvested and frozen at -20°C . A subset of these samples were confirmed as transgenic using commercially available immunochromatographic lateral flow test strips (Strategic Diagnostic Inc.[®]) and event-specific end-point PCR. Immunochromatography was carried out on *ca.* 0.25 cm^2 leaf tissue ground in the supplied kit buffer. After 5 min, the lateral flow test strips were inserted and allowed to develop for another 5 min.

For the PCR confirmation of field survivors, total genomic DNA was extracted from *ca.* 0.25 cm^2 leaf tissue using a CTAB-based protocol described by Doyle and Doyle (1987). Multiplex PCR was conducted with primers specific to a non-coding region of the safflower genome to indicate the reaction was working and primers specific to the herbicide resistance gene (*pat*) to confirm the presence of the transgene. The primers JCH1 (CAC ACT AAG CCA CTC CAA CC) and JCH4 (TTG ACA ACT CCA ATC CCT GC) were specific to the non-coding region and produced a 900 bp product; the primers JCH5 (GAT CTG GGT AAC TGG TCT AAC TGG) and JCH6 (GTT GCA AGA TAG ATA CCC TTG GTT) were specific to *pat* and produced a 350-bp product. Each PCR was performed in $20 \mu\text{L}$ volumes using 10 to 40 ng of DNA template, 0.5 mM of each primer, 500 mM KCl, 100 mM Tris-HCl (pH 8.4), 3.0 mM MgCl_2 , 0.25 mM each dNTP and 2 units of *Taq* DNA polymerase. Cycling conditions for amplification consisted of 95°C for 10 min, followed by 35 cycles of 95°C for 20 s, 59°C for 30 s and 72°C for 45 s, followed by a final extension of 5 min at 72°C . Ten percent of each PCR reaction was loaded into 1.5% agarose gels; electrophoresis was conducted to separate the products and then visualized using ethidium bromide under UV light.

Statistical analysis

Experiments were analyzed separately (El Bosque, Westwold and Lethbridge) with outcrossing frequency (f) calculated as the ratio of glufosinate survivor(s) to estimated total seedlings emerged (n_o). A 95% confidence interval for the mean frequency of outcrossing at each distance was calculated, assuming a binomial distribution as described by Zar (1999), pp. 527–528 (Tabs. 1 to 3). This method is error-prone when a zero value is observed (no survivors in the

sample), thus an alternative method was used when this occurred, as described by in Zar (1999), p. 528 (Tabs. 1 to 3).

Mean frequency of outcrossing (f) at each mean distance were subjected to regression analysis using a non-linear regression mixed model (PROC NLMIXED) with Statistical Analysis Software (SAS, 2007). The binomial distribution (\sim binomial (n_o, f)) was employed to approximate the dependent variable. The data were fit to equation (1), an exponential decay function (Hanson et al., 2005):

$$p = ae^{-bd} \quad (1)$$

where p is the predicted outcrossing frequency, a is the intercept, e is the natural log, b is a curve parameter (rate of decline), and d is the mean distance (m) from the edge of the source plot. Model fit was evaluated by the significance of the parameter estimates and visual examination of the residual structure (Hanson et al., 2005). Several other equations were fitted to the data but the exponential decay provided the best fit to the data as assessed with AICC and visual inspection of residuals. The standard error and 95% confidence intervals were calculated for each parameter estimate.

Using the regression estimation of equation 1, we estimated the distance where outcrossing was reduced by both 50 and 95% (O_{50} and O_{95}):

$$O_{50} = \frac{\ln 0.5 * a - \ln a}{-b} \quad (2)$$

$$O_{95} = \frac{\ln 0.01 * a - \ln a}{-b} \quad (3)$$

where a and b are intercept and slope, respectively.

To test for heterogeneity of outcrossing among the blocks for each experiment, a log-likelihood ratio test or G-test using the chi-square distribution was conducted using the -2 log-likelihood score provided by SAS for regression analysis (as above) with outcrossing data sets partitioned by blocks compared to a data set containing all of the partitions (Zar, 1999, pp. 473–475) (Tab. 6).

In addition, a power analysis using binomial probabilities was conducted to determine sample size required to detect at least one transgenic seed for samples with different theoretical frequency (transgenic seed content) and three different alpha values (Zar, 1999, pp. 539–542) (Tab. 6). The minimum sample size to detect at least one transgenic seed at a given frequency and alpha value was derived from the following formula

$$n_p \geq \frac{\ln \alpha}{\ln(1 - p)} \quad (4)$$

where n_p is the minimum sample size required to detect one or more transgenic seeds with a set value of α for a sample containing a theoretical frequency of transgenic

seeds (p). The null hypothesis that the frequency is equal to or greater than (p) could be rejected when zero transgenic seeds were found in a sample of seeds (n_p) with a confidence value related to the type I error alpha value by $1 - \alpha$.

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