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ORIGINAL ARTICLE

Pollen-mediated gene flow in flax (*Linum usitatissimum* L.): can genetically engineered and organic flax coexist?

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Coexistence allows growers and consumers the choice of producing or purchasing conventional or organic crops with known standards for adventitious presence of genetically engineered (GE) seed. Flax (*Linum usitatissimum* L.) is multipurpose oilseed crop in which product diversity and utility could be enhanced for industrial, nutraceutical and pharmaceutical markets through genetic engineering. If GE flax were released commercially, pollen-mediated gene flow will determine in part whether GE flax could coexist without compromising other markets. As a part of pre-commercialization risk assessment, we quantified pollen-mediated gene flow between two cultivars of flax. Field experiments were conducted at four locations during 2006 and 2007 in western Canada using a concentric donor (20×20 m) receptor (120×120 m) design. Gene flow was detected through the xenia effect of dominant alleles of high α-linolenic acid (ALA; $18{:}3^{cis\Delta 9,12,15}$) to the low ALA trait. Seeds were harvested from the pollen recipient plots up to a distance of 50 m in eight directions from the pollen donor. High ALA seeds were identified using a thiobarbituric acid test and served as a marker for gene flow. Binomial distribution and power analysis were used to predict the minimum number of seeds statistically required to detect the frequency of gene flow at specific α (confidence interval) and power ($1-\beta$) values. As a result of the low frequency of gene flow, approximately 4 million seeds were screened to derive accurate quantification. Frequency of gene flow was highest near the source: averaging 0.0185 at 0.1 m but declined rapidly with distance, 0.0013 and 0.00003 at 3 and 35 m, respectively. Gene flow was reduced to 50% (O_{50}) and 90% (O_{90}) between 0.85 to 2.64 m, and 5.68 to 17.56 m, respectively. No gene flow was detected at any site or year >35 m distance from the pollen source, suggesting that frequency of gene flow was $\leqslant 0.00003$ ($P = 0.95$). Although it is not possible to eliminate all adventitious presence caused by pollen-mediated gene flow, through harvest blending and the use of buffer zones between GE and conventional flax fields, it could be minimized. Managing other sources of adventitious presence including seed mixing and volunteer populations may be more problematic.
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Introduction

With the introduction of genetically engineered (GE) crops, pollen-mediated gene flow has become easier to detect and important to mitigate. Detection of adventitious presence of transgenes at low frequency using real time PCR has become a routine testing procedure to detect GE seeds of major crops including canola (*Brassica napus* L.), maize (*Zea mays* L.) and soybean (*Glycine max* L.) and their products (Hubner *et al*., 2001; Pla *et al*., 2006). In addition, conventional products with minimal GE content are in demand in some regions, notably the European Union (EU), but worldwide, adventitious presence thresholds either vary or have yet to be established (Demeke *et al*., 2006). Concurrent with the commercialization of GE crops, there has been increased demand for organic products in which there is a zero threshold for the presence of transgenes. Pollen-mediated gene flow is only one source of adventitious presence. Contamination of seed for sowing, gene persistence in volunteer populations (Jhala *et al*., 2010; Dexter *et al*., 2010a, b) and seed mixing during transport must also be considered. Traditional isolation distances and other management practices designed to segregate crop varieties and production systems to ensure seed and product purity may be insufficient given these constraints. Quantification of pollen-mediated gene flow is required to determine if GE, conventional and organic crops can coexist without constraints to international trade and to develop practices for mitigation of transgene movement (Devos *et al*., 2004).

Flax, *Linum usitatissimum* L., an oilseed crop, is predominantly a self-pollinated species (Gill, 1987). Flax has a perfect flower with stamens and pistils present in the same flower. As the flax flower opens, the stamens bend inward, discharging pollen on stigma, usually resulting in self-pollination (Dillman, 1938). Gene flow in flax not only depends on the position of anthers in

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relation to stigma, receptivity of stigma, viability of pollen, availability of pollinators (Henry and Tu, 1928; Yermanos and Kostopoulos, 1970) but may also vary with genotype and environment (Dillman, 1938). Previous reports have indicated outcrossing rates in flax in the range of 1–5%, when flax plants were grown in close proximity (Howard *et al*., 1919; Graham and Roy, 1924; Bolley, 1927; Dillman and Stoa, 1935; Dillman and Goar, 1937; Joshi, 1994). However, pollen-mediated gene flow beyond 7.0 m was not documented. There is no recent information on pollen vectors, although insect pollinators have been reported to favor gene flow in flax (Dillman, 1938; Gubin, 1945).

Flax has been transformed with several novel genes (McSheffrey *et al*., 1992; Dong and McHughen, 1993; McHughen and Holm, 1995; Wijayanto and McHughen, 1999; McHughen, 2002) but currently no GE flax cultivars are commercially grown. Sulfonylurea-resistant flax (CDC Triffid) (Event PF967) was approved as safe for release in Canada and the United States but was withdrawn before full commercial release at the request of the Flax Council of Canada, primarily to avoid trade concerns with the EU, the primary export market. Approval of Event PF967 was not completed in the EU and there is a zero tolerance for contamination with an unapproved trait. Recently Event PF967 was identified in Germany in a shipment of Canadian flaxseeds (Bedard, 2009). This incident led to a disruption in the trade of Canadian flax to many countries including the EU.

The development of GE flax is under consideration in Canada and several parts of the world (Wrobel *et al*., 2004; Wrobel-Kwiatkowska *et al*., 2007). Development of these cultivars is predicated on the approval of food, feed and environmental biosafety. In the EU, crop products that contain approved GE events must be labeled if the content exceeds 0.9%. If thresholds can be met, GE flax could coexist along with conventional and organic flax.

Quantifying pollen-mediated gene flow before commercialization of GE flax allows trait developers and the flax industry to assess the risks (in part) of releasing a GE flax cultivar at a commercial scale. However, because pollen movement is dependent on the size of the donor and recipient populations, small trials may underestimate the distance and frequency of pollen-mediated gene flow. For example, Hanson *et al*. (2005) detected pollen-mediated gene flow in wheat (*Triticum aestivum* L.) up to a maximum of 42 m in a 45.7 m diameter pollen source, while Matus-Cadiz *et al*. (2007) reported occurrence of gene flow up to 2.75 km from a 33-ha pollen source.

As no GE flax cultivars were available to be used as the donor population, various methods have been used to identify gene flow. Dillman (1938) used flax flower petal color as a marker, but it was not reliable or accurate. Several markers have been used in other crops to detect pollen-mediated gene flow in donor–receptor experiments including molecular markers based on quantitative PCR (Weekes *et al*., 2005, 2007; Pla *et al*., 2006; Weber *et al*., 2007) or micro-satellite markers (Chaix *et al*., 2003; Dje *et al*., 2004; Isagi *et al*., 2004). Other techniques like green fluorescent protein (Halfhill *et al*., 2003) and blue aleurone seed color in wheat (Hanson *et al*., 2005) and triticale (Hills *et al*., 2007) have also been reported. Flax cultivars with high ALA (α-linolenic acid: $18{:}3^{cis\Delta 9,12,15}$) (Kenaschuk and Rashid, 1994) and low ALA 'Solin' (*<3% ALA*) cultivars have been developed (Dribnenki *et al*., 2003). Two independently inherited genes, *LuFAD3A* and *LuFAD3B* control the ALA trait in flax (Vrinten *et al*., 2005). Seeds formed from the hybridization between conventional and Solin cultivars express high levels of ALA.

A thiobarbituric acid (TBA) test has been described for rapid screening of individual seed of flax for the content of ALA (Bhatty and Rowland, 1990). The TBA test is sensitive in determining peroxides of ALA and arachidonic acid ($C20{:}4^{cis\Delta 5,8,11,14}$) because autoxidation of monoenes and dienes do not yield products that react with TBA, only trienes and more highly unsaturated fatty acids yield such products (Dahle *et al*., 1962). Polyunsaturated fatty acids form five-membered peroxide ring on oxidation (McGregor, 1974). In this study, the dominant high ALA trait in combination with the sensitive TBA test was exploited to determine pollen-mediated gene flow between two flax cultivars.

An additional constraint to detect pollen-mediated gene flow is sample size. Without testing every seed in a population, it is not possible to confirm the absence of transgenes. A strategy is required for testing the minimum number of seeds to provide a statistically meaningful conclusion. Power, broadly defined as the probability of getting a positive result when one is present, provides a decision-making framework. A sampling strategy was developed using power analysis and binomial distribution to define the minimum number of seeds required to measure the pollen-mediated gene flow for a specified null hypothesis between two crop cultivars. Three basic parameters affect power: proposed sample size, significance level used to determine whether or not to accept the study's hypothesis and theoretical effect size (Bausell and Li, 2002). Generally, a power value of 0.8 at 95% confidence interval is statistically accepted. However, for longer distances (>25 m in this study) it is advisable to screen more seeds to provide a higher power (>0.8). A larger sample size generally leads to parameter estimates with smaller variances, giving a greater ability to detect small differences.

The objective of this study was to measure pollen-mediated gene flow in flax under field conditions using high and low ALA flax cultivars in western Canada. Frequency of gene flow and distance from the pollen source was quantified up to a distance of 50 m. We also determined the minimum number of seeds required to provide a statistically meaningful conclusion on detecting gene flow at various distances by using the theoretical values of gene flow frequencies and various confidence intervals (α). This ensured that sufficient power ($1-\beta$) existed to measure the pollen-mediated gene flow.

Materials and methods

Plant material

Breeder seed of two flax cultivars, 'AC McDuff' and 'SP 2047' were provided by Vittera, Calgary, Alberta, Canada. AC McDuff, released by the Agriculture and Agri-Food Canada, is a late maturing cultivar with high ALA content (~70% ALA) (Kenaschuk and Rashid, 1994). SP 2047, is a solin cultivar with low ALA (<3%), developed by Vittera and registered in 2002 (Dribnenki *et al*., 2003). The reproductive biology of these two

cultivars was representative of flax cultivars grown in western Canada.

Field experiments

Field experiments were conducted at two locations, Edmonton Research Station (EdRS) and Ellerslie Research Station (ElRS), University of Alberta, Canada in 2006 and 2007. The soil at EdRS was a clay loam, which consisted of 34.2% sand, 37.5% silt, 28.3% clay, 12.9% organic matter with a pH of 5.6. Soil at ElRS was a clay loam with 28.2% sand, 41.1% silt, 30.7% clay, 11.2% organic matter with a pH 6.5. The high ALA flax cultivar AC McDuff was seeded in a 20×20 m area in the center, surround by the low ALA cultivar SP 2047, for a total area of 120×120 m in a concentric ring experimental design (Figure 1) at both locations and years. Flax was seeded on 16 and 18 May in 2006; and 26 and 28 May in 2007, at EdRS and ElRS sites, respectively. Fertilizers applied at each site were at the recommended rates based on soil tests (data not shown). Both flax cultivars flowered synchronously at each location-year. Just before harvesting, the field was divided into eight directional blocks and the crop between blocks was removed to facilitate accurate sampling (Figure 1). Harvesting took place on 17 and 21 September at EdRS and ElRS, respectively in 2006; and on 23 and 27 September at EdRS and ElRS, respectively in 2007. Harvesting began distal to the source and continued inward in a clockwise direction to reduce cross contamination. Samples were harvested with a Wintersteiger 2001 Elite research combine in 1.2 m wide swaths from each block from 50 to 3 m from the source in all blocks. A binder was used to harvest plants from 3.0 m to the source to reduce sample contamination. Samples were combined at longer distances to increase sample size. Seeds were threshed

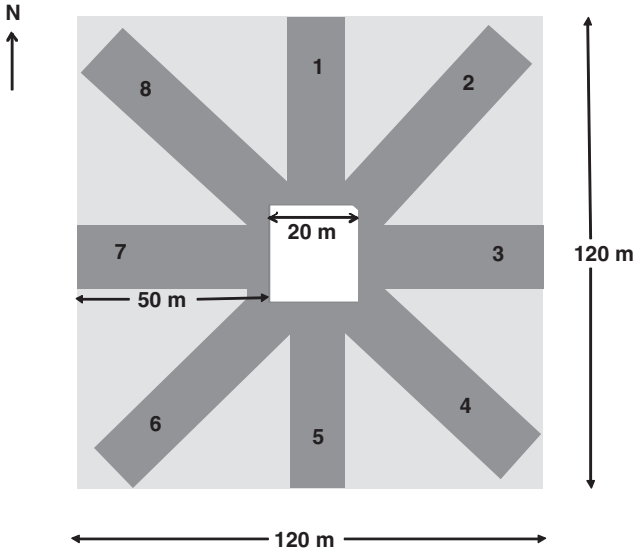

Figure 1 Design of pollen-mediated gene flow experiment in flax at all the locations and years. The pollen source flax cultivar (AC MCDuff) was seeded in the area of 20×20 m in the center of the field. The pollen receptor solin flax cultivar (SP 2090) was grown in the surrounding 120×120 m area. After flowering but before harvesting, the pollen receptor area was divided in eight blocks, crop between blocks removed and flax seed samples were collected at specific distances.

(if required), cleaned and stored at room temperature before screening.

Seed screening

The TBA test described in Bhatty and Rowland (1990) was modified to accommodate approximately 500 flax seeds on a 13×30 cm rectangle paper, facilitating the screening of nearly 20 000 seeds per day. Before planting in 2006 and 2007, 1.3 million SP 2047 seeds were tested for purity and five and three seeds, respectively were identified with high ALA content, a frequency of <0.00005, $\alpha = 0.05$, power $= 0.95$. This level of contamination was considered as a minor source of variance. The known standards of high and low ALA seeds were tested with every 500 seed lot and no false positives or negatives were identified. Seeds harvested from pollen receptor plots were screened in laboratory using TBA test and those containing high ALA were considered the product of gene flow from high ALA to low ALA flax cultivars.

Statistical analysis

A power analysis, using binomial probabilities was used to determine the minimum sample size required to accept the outcome of a statistical test with a particular level of confidence. If sample size is too low, the experiment will lack the precision and if sample size is too large, time and resources will be wasted, often for minimal gain. We estimated the minimum sample size for different theoretical frequencies at three different confidence intervals (α) and power values ($1-\beta$) (Zar, 1999; McPherson *et al*., 2008). The theoretical frequencies at the different α values served as the null hypotheses levels used to declare significance of gene flow. The observed frequencies from the study were then compared with the theoretical frequencies and if the observed frequencies were above the theoretical frequency, there is significant gene flow. Sample size was calculated with the following equation using software *R* (Chambers, 2008):

$$\beta = P\left(Z < \frac{p_0 - p}{\sqrt{\frac{pq}{n}}} - Z_\alpha \sqrt{\frac{p_0 q_0}{pq}}\right)$$

$$n = \frac{pq\left(\phi^{-1}(\beta) + Z_\alpha \sqrt{\frac{p_0 q_0}{pq}}\right)^2}{(p_0 - p)^2}$$

$$= \frac{(p_0 - \Delta)(1 - p_0 + \Delta)\left[\phi^{-1}(\beta) + Z_\alpha \sqrt{\frac{p_0(1-p_0)}{(p_0-\Delta)(1-p_0+\Delta)}}\right]^2}{\Delta^2} \quad (1)$$

where, n, minimum number of seeds required; β, power; Z, random variable following N(0, 1); q, $1-p$; $q_0 = 1-p_0$; p_0, true value in null hypothesis-hypothesized parameter; p, theoretical frequency of gene flow; Δ, effect size (p_0-p); Z_α, critical value for significant level α; ϕ^{-1}, anti-function of the normal curve.

Compared with other equations that also estimate the minimum sample size, such as the binomial estimator ($\ln(\alpha)/\ln(1-p)$) (Mcpherson, 2008), the above equation has two notable advantages: (i) the researcher/worker is capable of determining the effect size (Δ), thus the

sensitivity of the test and (ii) it affords the researcher the flexibility of determining (fixing) the ideal power (β) which, in the context of this study, is the probability of detecting gene flow when present. The sensitivity of the test is manipulated by varying p; and if for example high sensitivity, which is small Δ (the ability to detect low level gene flow), is required, the p is moved closer to p_0. The p_0 (the true value of the null hypothesis) could be a set frequency such as a threshold established by commodity importers and p the theoretical frequency of gene flow would be the frequency the researcher requires in a sample. Thus, moving p closer to p_0, which shrinks Δ (making the test more sensitive) enables the researcher/worker to detect the required gene flow frequencies, which could be those set by commodity importers. Hence the two advantages, ability to manipulate the sensitivity (Δ) and fix the power (β) give certainty to the tests and therefore confidence to markets, especially in which strict restrictions to certain levels of contamination in some commodities are applied. With the binomial estimator, such certainty is lacking. The assumptions with the binomial estimator being that once the type I errors are controlled, the statistical requirements have been met and the sample size is adequate. This is not the case with the equation above, in which the sample size is derived by controlling three important parameters, α, Δ and β, which enhances degree of accuracy by reducing assumptions.

Experiments were analyzed separately and as combined, with gene flow frequency calculated as the ratio of high ALA seeds to the total seeds analyzed divided by 100. A 95% confidence interval for the mean frequency of gene flow at each distance was calculated assuming a binomial distribution (McPherson *et al*., 2008). The mean frequency of gene flow at each mean distance was subjected to nonlinear regression using a mixed model (PROC NLMIXED) (SAS Institute Inc., 2007). The dependent variable was estimated using a binomial distribution by fitting the data to an exponential decay function (Hanson *et al*., 2005),

$$p = a\,e^{-bd} \tag{2}$$

where, p is the predicted frequency of gene flow; a the intercept; b the curve parameter; and d the mean distance from the source (m). Standard errors and 95% confidence intervals were calculated for each parameter estimate. The distances wherein frequency of gene flow was reduced by 50 and 90% were estimated from the exponential decay function following the equations (McPherson *et al*., 2008):

$$O_{50} = \frac{\ln(0.5\times a) - \ln a}{-b} \tag{3}$$

$$O_{90} = \frac{\ln(0.1\times a) - \ln a}{-b} \tag{4}$$

where, a is the intercept and b the slope.

To test for heterogeneity of gene flow between directional blocks for each experiment, a log-likelihood ratio test using the χ^2 distribution was calculated using the –2 log-likelihood ratio provided by the regression analysis in Statistical Analysis System (SAS). In measuring low frequencies of gene flow, care was taken to reduce seed contamination. However, potential sources of sample contamination must be considered, including low frequencies (<0.00005) of high ALA seed in the low ALA seed source, and potential for cross contamination of samples during combine harvesting.

Results

Uncertainty resulting from testing random samples can be reduced by combining binomial probability distribution and power analysis to define a sampling strategy (Table 1). Seeds were screened at each distance until the minimum power of 0.8 ($\alpha = 0.05$) was obtained. The objective of the sampling strategy was to allow researchers to confirm the sample size necessary to enable statistical judgments that are accurate and reliable. This strategy is useful wherein large samples are available but sampling costs are not insignificant. Alternatively, a sample size can be determined *a priori* if the theoretical frequency of gene flow can be estimated. To further interpret the data, samples containing zero outcrossing frequencies would be described as having a frequency equal to or less than the null hypothesis (*H*o) frequency level (Table 1).

To evaluate differences in the gene flow frequency among directional blocks at each experimental site, the maximum likelihood ratio was calculated (data not shown, see Supplementary material). Results suggest that frequency of gene flow between various blocks was relatively similar; indicating that wind or wind direction did not have a significant role in pollen dissemination from source to receptor plant population. Regular observations recorded during the growing season suggest that flowering of recipient and donor flax cultivars were synchronous and uniform at all the sites and years (data not shown). Therefore, it is unlikely that flowering time or flower density influenced gene flow frequency at different experimental sites.

Gene flow rates in flax followed an exponential decline with distance in these small plot experiments. However, maximum gene flow and gene flow distance was affected by differences between sites and years (Figure 2). Gene flow was lower in 2006 than 2007. At EdRS in 2006, a total 647 hybrids were identified by TBA testing 769 600 seeds (Table 2, Figure 2). A maximum gene flow of 0.0166% was observed at the minimum mean distance of 0.1 m from the pollen source. At 35 and 45 m, no positive seeds were detected in the sample, and therefore gene flow was <0.00005. At ElRS in 2006, the frequency of gene flow immediately adjacent to the source was 0.0145 (Table 3, Figure 2). Gene flow of a 0.00011 was detected at 35 m but was <0.00005 at 45 m. In some instances, the sample size exceeded the minimum requirements.

In 2007 at EdRS, a maximum frequency of gene flow 0.0242 was recorded at the mean distance of 0.1 m from the pollen source (Table 4; Figure 2). Gene flow of 0.000063 was detected as far as 25 m distance at this site in 2007 with 13 seeds with high ALA out of 460 600 seeds screened to achieve the power value of 0.85 (Table 4). Gene flow at 35 and 45 m was <0.00005. Immediately adjacent to the pollen source, at ElRS gene flow was 0.018646 (Table 5; Figure 2). Some rare gene flow was observed (0.000028) at a power value of 0.85 after screening 476 800 seeds at 35 m distance from the pollen source at ElRS site in 2007.

The exponential decay curve models for each site and year suggest that differences between locations are less

Table 1 Power analysis assuming a binomial distribution to determine minimum number of seeds required to detect at least one GE seed for different frequency of gene flow; four α values ($\alpha = 5$, 2.5, 1.0, 0.05%); and three power ($1-\beta$) values (0.80, 0.85, 0.95)

Ho[a] *frequency of gene flow p(x)*	*α Value (α)*											
	$\alpha = 5\%$	$\alpha = 2.5\%$	$\alpha = 1\%$	$\alpha = 0.05\%$	$\alpha = 5\%$	$\alpha = 2.5\%$	$\alpha = 1\%$	$\alpha = 0.05\%$	$\alpha = 5\%$	$\alpha = 2.5\%$	$\alpha = 1\%$	$\alpha = 0.05\%$
	Minimum sample size (n)[b] for power = 0.80[c]				*Minimum sample size (n)[b] for power = 0.85[c]*				*Minimum sample size (n)[b] for power = 0.95[c]*			
0.01	1989	2588	3383	3985	2242	2875	3710	4340	3128	3869	4829	5544
0.005	3996	5199	6797	8007	4503	5776	7454	8719	6282	7771	9700	11136
0.0025	8010	10422	13625	16051	9026	11577	14941	17477	12590	15573	19441	22319
0.001	20052	26090	34109	40183	22595	28980	37402	43750	31513	38982	48664	55870
0.0005	40122	52204	68248	80402	45209	57985	74837	87540	63051	77995	97368	111788
0.00025	80262	104431	136528	160841	90437	115996	149707	175120	126127	156023	194777	223624
0.0001	200681	261114	341367	402159	226123	290028	374319	437860	315356	390106	487005	559132
0.00005	401379	522251	682765	804355	452266	580082	748671	875759	630737	780244	974051	1118312
0.000025	802776	1044526	1365561	1608746	904551	1160190	1497376	1751558	1261500	1560519	1948144	2236672
0.00001	2006968	2611350	3413949	4021921	2261406	2900514	3743490	4378955	3153787	3901347	4870422	5591751
0.000005	4013954	5222724	6827930	8043879	4522833	5801054	7487013	8757949	6307600	7802726	9740884	11183549
0.0000025	8027925	10445471	13655891	16087794	9045685	11602134	14974060	17515937	12615225	15605485	19481810	22367146

Abbreviation: GE, genetically engineered.
[a]Null hypothesis: theoretical value of frequency of gene flow.
[b]Minimum sample size required to detect one or more GE seeds at a given theoretical frequency of gene flow and different values of α and power ($1-\beta$). Null hypothesis that the frequency $X \geqslant P$ is rejected at a given α value, when no GE seeds were found in a sample size of n or greater.
[c]Value of power to ascertain how much power ($1-\beta$) would be available for a given sample size and confidence interval.

important than differences between years, presumably because of the influence of weather on gene flow (Figure 2). The distance wherein gene flow was decreased by 50% ranged from <1 m (0.85 m at ElRS in 2007 to a maximum of over 2 m (2.6 m at ElRS in 2006) (Table 6). The variability of gene flow increased with distance from the source and this was reflected in increase in confidence intervals at the distance that 90% of the gene flow occurred (O_{90}). The O_{90} ranged from 5.7 m at ElRS in 2007 to 17.5 m at ElRS in 2006. Average across all locations and years, the O_{50} was 1.61 m and the O_{90} was 5.37 m (Figure 2). Although average values from small plot research are valuable, a more conservative approach of using worse case scenarios should be taken when regulations for isolation distances are established.

Discussion

Results indicate that maximum gene flow, ranging from 0.0144 to 0.024 was observed at the closest distance (a mean distance of 0.1 m). At 0.5 m from the source, average gene flow was reduced to 0.0077; and further reduced at 1.0 m to 0.0027. Similar results were obtained in earlier studies in short distance gene flow between flax cultivars. Henry and Tu (1928) studied the extent of gene flow in flax by growing blue and white flowered cultivars in adjacent rows. Frequency of gene flow was reduced from 0.0126 to 0.0033, when flax cultivars were grown 0.25 to 1.25 m apart, respectively. In a similar experiment, (Robinson, 1937) reported that frequency of gene flow in flax varied from 0 to 0.003, depending on the spacing between plants and climatic conditions. Kadam *et al*. (1938) reported the frequency of gene flow was in the range of 0–0.06, however, the average frequency was <0.03.

Flax gene flow did not differ significantly by direction (block), suggesting that wind may not have had an important role in flax gene flow. Flax is an indeterminate species and may keep flowering up to 33–63 days (Chopde and Thakre, 1969) during which time wind speed and direction may vary considerably. Early reports stated that flax flowers generally produce pollen in small quantities and it is relatively heavy and therefore, pollen dissemination by wind seems unlikely (Eyre and Smith, 1916). In several studies insect-mediated pollination was considered to determine the effect of insects on seed production or yield. Dillman (1938) reported that sticky flax pollen was primarily disseminated by honey bees and thrips. Subsequent experiments suggest that the honey bee was the most frequent visitor of flax flowers followed by bumble bees and thrips (Smirnov, 1956). Although insects visit flax flowers, the importance to gene flow has been disputed (Dillman, 1938; Gubin, 1945; Gill, 1987). Most recently, Williams *et al*. (1991) reported no increase in flax seed production in the presence of honey bees suggesting limited gene flow mediated by insects. In this study, pollinators were observed at each location and year but there role in promoting gene flow between flax cultivars was not quantified.

Gene flow declined exponentially with distance, with the value of O_{90} being <18 m at all site-year. No gene flow was detected beyond 35 m and from the power analysis we would accept the null hypothesis that the frequency of gene flow from these samples was equal to or <0.00005 at 95% confidence interval. Cultivar,

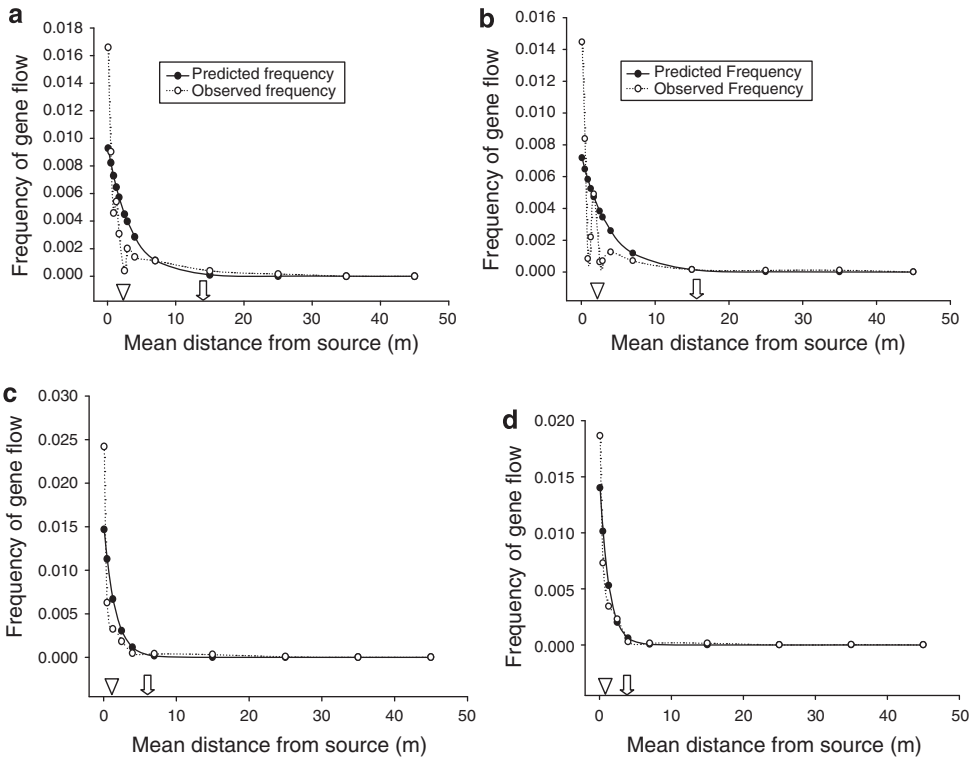

Figure 2 Pollen-mediated gene flow in flax at various locations and years (**a**) EdRS, 2006; (**b**) EIRS, 2006; (**c**) EdRS, 2007; and (**d**) EIRS, 2007. The triangle indicates the distance, in which 50% (O_{50}) reduction in the frequency of gene flow and the arrow indicates the distance in which 90% (O_{90}) reduction in gene flow from the pollen source (m).

Table 2 Frequency of flax gene flow from 0.1–45 m distance from the pollen source at EdRS in 2006

Mean distance[a] *(m)*	*Seeds screened*[b]	*Seeds with high ALA*	*Actual frequency of gene flow in sample*	H_o *frequency of gene flow p(x)*	*Power,* $(1-\beta)$*;* $x\alpha = 0.05\%$[c]
0.1	14 400	239	0.016597	0.005	0.95
0.5	14 400	130	0.009028	0.005	0.95
0.9	7200	33	0.004583	0.01	0.95
1.3	2400	13	0.005417	0.01[d]	<0.8
1.7	12 000	37	0.003083	0.005	0.95
2.5	4800	2	0.000417	0.005[d]	<0.8
2.9	14 400	29	0.002014	0.005	0.95
4	26 400	37	0.001402	0.0025	0.95
7	40 800	46	0.001127	0.0025	0.95
15	105 600	41	0.000388	0.001	0.95
25	244 800	40	0.000163	0.00005	0.95
35	138 400	0	0.000000	0.00005	0.95
45	144 000	0	0.000000	0.00005	0.95

Abbreviations: ALA, α-linolenic acid; EdRS, Edmonton Research Station.
[a]Mean distance from the pollen source was used in analysis for all observations.
[b]Total number of seeds screened from all (eight) blocks for a specific distance from the pollen source.
[c]Value of power $(1-\beta)$ was calculated for 95% confidence interval ($\alpha = 5\%$) using equation (1) (see text).
[d]$\alpha = 5\%$.
Frequency of gene flow was calculated from the number of seeds screened and the number of high ALA seeds. To determine gene flow significance, the null hypothesis frequency, based on the sample size and power, was compared with the sample frequency.

environment and their interaction may have effect on the gene flow and inferences cannot be made to field scale gene flow because the size of the pollen source may affect distance of pollen movement (Matus-Cadiz *et al*., 2004, 2007). A simple empirical modeling approach for these initial small-scale trials was chosen rather than the alternative of dispersal kernel modeling because of its ease of interpretation (Beckie and Hall, 2008). As part of a tiered approach to quantifying gene flow, it should facilitate decisions on the feasibility of continued development of GE flax, assuming a strategy of coexistence. Given the small size of the pollen source and receptor; inferences drawn from complex mechanical or determinist models would be suspect.

Although pollen-mediated gene flow has been studied in many crops including canola (Beckie *et al*., 2003; Knispel *et al*., 2008), maize (Messeguer *et al*., 2006; Goggi *et al*., 2007; Weber *et al*., 2007; Mercer and Wainwright, 2008), wheat (Gaines *et al*., 2007; Gatford *et al*., 2007; Matus-Cadiz *et al*., 2007), soybean (Yoshimura *et al*., 2006), safflower (*Carthamus tinctorius* L.) (McPherson *et al*., 2008) and other crops reviewed by Mallory-Smith

Table 3 Frequency of flax gene flow from 0.1–45 m distance from the pollen source at ElRS in 2006

Mean distance[a] *(m)*	*Seeds screened*[b]	*Seeds with high ALA*	*Actual frequency of gene flow in sample*	H_o *frequency of gene flow p(x)*	*Power, (1−β); α = 0.05%*[c]
0.1	24 000	347	0.014458	0.005	0.95
0.5	24 000	201	0.008375	0.005	0.95
0.9	4800	4	0.000833	0.005	<0.8
1.3	9600	21	0.002188	0.01	0.95
1.7	9600	47	0.004896	0.01	0.95
2.5	9600	6	0.000625	0.005[d]	<0.8
2.9	14 400	10	0.000694	0.005[d]	<0.8
4	48 000	60	0.00125	0.025	0.95
7	52 800	37	0.000701	0.001	0.85
15	148 800	22	0.000148	0.0005	0.95
25	168 000	17	0.000101	0.0005	0.95
35	100 800	11	0.000109	0.001	0.95
45	134 400	0	0.000000	0.0005	0.95

Abbreviations: ALA, α-linolenic acid; ElRS, Ellerslie Research Station.
Frequency of gene flow was calculated from the number of seeds screened and the number of high ALA seeds. To determine gene flow significance, the null hypothesis frequency, based on the sample size and power, was compared with the sample frequency.
[a]Mean distance from the pollen source was used in analysis for all observations.
[b]Total number of seeds screened from all (eight) blocks for a specific distance from the pollen source.
[c]Value of power $(1-\beta)$ was calculated for 95% confidence interval ($\alpha = 5\%$) using equation (1) (see text) and Table 1.
[d]$\alpha = 5\%$.

Table 4 Frequency of flax gene flow from 0.1–45 m distance from the pollen source at EdRS in 2007

Mean distance[a] *(m)*	*Seeds screened*[b]	*Seeds with high ALA*	*Actual frequency of gene flow in sample*	H_o *Frequency of gene flow p(x)*	*Power*[c]*, (1−β); α = 0.05%*
0.1	9600	232	0.024167	0.01	0.95
0.5	9600	60	0.00625	0.01	0.95
1.3	9600	31	0.003229	0.01	0.95
2.5	14 400	26	0.001806	0.005	0.95
4	91 200	40	0.000439	0.001	0.95
7	115 200	44	0.000382	0.0005	0.95
15	96 000	29	0.000302	0.0005	0.85
25	460 600	13	0.000063	0.0001	0.85
35	176 800	0	0	0.00005	0.95
45	176 800	0	0	0.00005	0.95

Abbreviations: ALA, α-linolenic acid; EdRS, Edmonton Research Station.
Frequency of gene flow was calculated from the number of seeds screened and the number of high ALA seeds. To determine gene flow significance, the null hypothesis frequency, based on the sample size and power, was compared with the sample frequency.
[a]Mean distance from the pollen source was used in analysis for all observations.
[b]Total number of seeds screened from all (eight) blocks for a specific distance from the pollen source.
[c]Value of power $(1-\beta)$ was calculated for 95% confidence interval ($\alpha = 5\%$) using equation (1) (see text) and Table 1.

Table 5 Frequency of flax gene flow from 0.1–45 m distance from the pollen source at ElRS in 2007

Mean distance[a] *(m)*	*Seeds screened*[b]	*Seeds with high ALA*	*Actual frequency of gene flow in sample*	H_o *frequency of gene flow p(x)*	*Power, (1−β); α = 0.05%*[c]
0.1	9600	179	0.018646	0.01	0.95
0.5	9600	70	0.007292	0.01	0.95
1.3	9600	33	0.003438	0.01	0.95
2.5	9600	22	0.002292	0.001	0.85
4	100 800	27	0.000268	0.001	0.95
7	76 800	11	0.000143	0.001	0.95
15	91 200	13	0.000143	0.001	0.95
25	176 800	0	0	0.0005	0.95
35	476 800	17	0.000028	0.00001	0.85
45	170 000	0	0	0.00005	0.95

Abbreviations: ALA, α-linolenic acid; ElRS, Ellerslie Research Station.
Frequency of gene flow was calculated from the number of seeds screened and the number of high ALA seeds. To determine gene flow significance, the null hypothesis frequency, based on the sample size and power, was compared with the sample frequency.
[a]Mean distance from the pollen source was used in analysis for all observations.
[b]Total number of seeds screened from all (eight) blocks for a specific distance from the pollen source.
[c]Value of power $(1-\beta)$ was calculated for 95% confidence interval ($\alpha = 5\%$) using equation (1) (see text) and Table 1.

Table 6 Parameter estimates and the distances where 50% (O_{50}) and 90% (O_{90}) reduction in frequency of gene flow occurred with their respective standard errors and confidence intervals from the regression analysis at various sites in 2006 and 2007

Experiment	*Parameter*[a]	*Estimate*[b]	*Standard error*	*Df*[c]	*95% Confidence interval*	
					Lower	*Upper*
Edmonton, 2006	*a*	0.009570	0.03162	60	−0.05368	0.07282
	b	0.3024	1.1767	60	−2.0514	2.6563
	O_{50}	2.29191	8.91769	60	−15.5461	20.1299
	O_{90}	15.2271	59.2478	60	−103.286	133.74
Ellerslie, 2006	*a*	0.007363	0.02665	63	−0.04589	0.06062
	b	0.2622	1.1557	63	−2.0473	2.5717
	O_{50}	2.64364	11.6528	63	−20.6427	25.9300
	O_{90}	17.5640	77.4198	63	−137.147	172.275
Edmonton, 2007	*a*	0.01566	0.05022	64	−0.08466	0.1160
	b	0.6588	2.1599	64	−3.6561	4.9737
	O_{50}	1.05209	3.44916	64	−5.83840	7.94258
	O_{90}	6.98994	22.9157	64	−38.7895	52.7694
Ellerslie, 2007	*a*	0.01518	0.05221	64	−0.08911	0.1195
	b	0.8106	2.8400	64	−4.8630	6.4842
	O_{50}	0.85513	2.99613	64	−5.13032	6.84058
	O_{90}	5.68136	19.9058	64	−34.0851	45.4478

[a]Parameters a and b were estimated using equation (2). The distances (O_{50} and O_{90}) where gene flow was reduced by 50 and 90% were estimated using equations (3) and (4), respectively.
[b]Estimates of the parameters for intercept (a), slope (b) and the estimates of the distances where gene flow was reduced by 50 and 90%.
[c]Degree of freedom.

and Zapiola (2008), it is difficult to directly compare the gene flow between crops because of study differences. Soybean is an annual, self-pollinating, oilseed species. Gene flow in soybean was measured in previous studies in the range of 0.03–0.44% with some rare gene flow events (0.004%) observed at 14 m distance from the pollen source (Caviness, 1966). In this study, gene flow in flax was higher than reported for soybean, but lower than reported in similar scale experiments for wheat (Hanson *et al*., 2005), and safflower (McPherson *et al*., 2008). In contrast, gene flow in the obligate outcrossing, insect-pollinated alfalfa (*Medicago sativa* L.) was detected as high as 0.2% at 1.5 km from the pollen source using a glyphosate-resistant trait (Van Deynze *et al*., 2004).

When developing a GE crop, one factor to consider may be the proclivity for long-distance gene flow. Although pollen-mediated gene flow contributes to gene flow model, for highly self-pollinated species such as flax, the seed-mediated gene flow (occurring primarily during harvest loss, transportation or trade) will have an important role and therefore require careful management operations to reduce admixture.

Strategies to reduce pollen-mediated gene flow in flax

As recent as 2009, flax international trade was interrupted and market prices reduced following the detection by the EU of an unapproved GE flax in a shipment of Canadian flax. The variety CDC Triffid had not been registered to grow in Canada since 2001 and the source of contamination has not been established (Bedard, 2009). Unapproved GE crops have a zero threshold for adventitious presence in the EU (Devos *et al*., 2004). In Canada, approved GE crops are considered substantially equivalent to non-GE crops and segregation is not required between two cropping systems or their products (Smyth and McHughen, 2008). However, Europe has defined a 0.9% labeling threshold for the EU approved GE seeds in organic or conventional crop seeds (European Commission, 2003). Adventitious presence above this threshold triggers product labeling as originating from GE material (Devos *et al*., 2008). Before the commercial release of GE flax, a strategy is required to mitigate transgene movement.

The current isolation distance for certified flax seed growers is 3 m (Canadian Seed Grower's Association, 2010). Although this research does not specifically address isolation distances, we suggest isolations be increased and routine testing of certified flax seed should be implemented (Jhala *et al*., 2008). For coexistence of GE, conventional and organic flax, the use of non-GE buffer zone around GE flax fields and discarding buffer zones around organic flax fields after flowering should be considered (Jhala *et al*., 2009). The maximum gene flow recorded in this research between cultivars was $<3\%$ immediately adjacent to the source and 90% of gene flow occurred within 6 to 18 m. In combination with harvest blending of fields, the adventitious presence through pollen-mediated gene flow could be reduced but not eliminated. Recent GENESYS modeling on gene flow containment in canola (*Brassica napus* L.) suggest that buffer zones are more effective than isolation zones in reducing harvest admixture because they increased the distance between GE and non-GE fields and diminished the proportion of GE pollen in the total pollen cloud (Colbach *et al*., 2009). These measures, in addition to practices designed to reduce seed-mediated gene flow (Jhala *et al*., 2010; Dexter *et al*., 2010a) may be useful to develop the best management practices to grow GE flax in western Canada.

Conclusions

Pollen-mediated gene flow alone should not prevent the coexistence of conventional, organic and GE flax if the novel trait is approved in the EU and consumers will

accept a non-zero threshold. However, pollen is only one possible source of adventitious presence. Other sources that must be mitigated include seed-mediated gene flow through certified flax seed; volunteer flax and inadvertent mixing of products within the transportation system. Risk mitigation through agronomic practices, product testing and channeled production, all impose costs that must be balanced by the benefits of GE varieties to flax growers.

Conflict of interest

The authors declare no conflict of interest.

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