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Reproductive phenology of transgenic *Brassica napus* cultivars: Effect on intraspecific gene flow

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Pollen-mediated gene flow in space is well documented and isolation distances are recommended to ensure genetic purity of *Brassica napus* seed crops. Isolation in time could also contribute to gene flow management but has been little investigated. We assessed the effects of asynchronous and synchronous flowering on intraspecific *B. napus* gene flow by seeding adjacent plots of transgenic spring canola cultivars, either resistant to glyphosate or glufosinate, over a 0–4 week interval and measuring outcrossing rates and seed-set. Outcrossing rates, evaluated in the center of the first adjacent row, were reduced to the lowest level in plots flowering first when the seeding interval > 2 weeks. Increasing the time gap increased outcrossing rates in plots flowering second up to a seeding interval of two weeks. Flowers that opened during the last week of the flowering period produced fewer seed (< 10% of total seed production) and a smaller fraction of outcrossed seed (–25%). Observed time gap effects were likely caused by extraneous pollen load during the receptivity of productive seed-setting early flowers. Clearly, manipulation of *B. napus* flowering development through staggered planting dates can contribute to gene flow management. The approach will need to be validated by additional site-years and increased isolation distances.

Keywords: assortative mating / canola / genetic purity / genetically modified organisms / herbicide resistant crops / oilseed rape / pollen-mediated gene flow / transgenic contamination

INTRODUCTION

Brassica napus L. canola (argentine canola, oilseed rape) cultivars with herbicide resistance (HR) traits were first commercialized in Canada in 1995. Ten years later, 96% of the 6.4 million hectares (Statistics Canada, 2008) of canola (including *Brassica rapa* L. canola: polish canola, turnip rapeseed) grown in Canada was herbicide resistant (Beckie et al., 2006). Three types of HR canola are available to producers. These include glyphosate resistant (commercialized as Roundup Ready [RR]), glufosinate resistant (commercialized as Liberty Link [LL]) and imidazolinone resistant (commercialized as Pursuit Smart or Clearfield) (Beckie et al., 2006). Except for imidazolinone resistance which was generated by mutagenesis (Tan et al., 2005), the other HR cultivars have bacterial transgenes. These transgenes segregate as single dominant genes (Hall et al., 2000).

Brassica napus is an allotetraploid (AACC, $2n = 4x = 38$) derived from *B. rapa* (AA, $2n = 2x = 20$) and *Brassica oleracea* L. (CC, $2n = 2x = 18$) (U, 1935). *B. napus* can hybridize with weedy *B. rapa* (bird rape) found in commercial fields (Simard et al., 2006) and introgression of an HR transgene into weedy *B. rapa* populations was observed (Warwick et al., 2008). Genetic material can also be exchanged between cultivated *B. napus* or *B. rapa* plants, volunteer plants growing from seed of both canola species shed at harvest (Gulden et al., 2003) and roadside populations of volunteer canola (Knispel et al., 2008). *Brassica napus* is predominantly self-pollinated but, in experimental plots, inter-plant outcrossing rates ranged from 12 to 55%, and averaged 30% (Cuthbert and McVetty, 2001; Rakow and Woods, 1987; summarized by Beckie et al., 2003). The majority of this cross-fertilization occurs within the first 10 m of receptor plants (Hüsken and Dietz-Pfeilstetter, 2007). *Brassica napus* fields can produce 9.3 ± 0.5 kg pollen. ha^{-1}. day^{-1} and pollen viability usually decreases over 4–5 days (see Hüsken and Dietz-Pfeilstetter, 2007). The species is pollinated by wind, insects and direct contact between flowers. The relative contribution of these factors to outcrossing is not known (Becker et al., 1992; Beckie and Hall, 2008).

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Potential gene flow is especially important to seed producers because pedigreed seed has to be 99.75% pure for the traits of the cultivar (Friesen et al., 2003). Contamination at this level or lower (foundation and registered seed) could disperse unwanted transgenes over a wide area as genetic admixture in certified seed planted by producers. Isolation in space is a standard management practice implemented to limit gene flow. The relationship between outcrossing and distance has been studied extensively (Beckie and Hall, 2008). Isolation distances ranging between 100 and 800 m (hybrid seed) from any other canola or rapeseed crop are required to produce pedigree canola seed (Ingram, 2000; Scheffler et al., 1995). Isolation in time could also contribute to gene flow management given that spring *Brassica napus* can be seeded over a four week period (in May), with the likelihood of some yield loss for later sown crops. The first *B. napus* flowers appear 40–50 days after planting and flowering will cover 3–4 weeks (Hüsken and Dietz-Pfeilstetter, 2007; Simard and Légère, 2004). In eastern Canada, Simard and Légère (2004) observed less than 25% flowering overlap between early (May 1) and late seeded *B. napus* (May 30). So far, to our knowledge, the potential of using relative planting dates as a gene flow management tool to reduce outcrossing rates in *B. napus*, or any canola species, has not been tested.

The objective of this study was to use relative planting dates to de-synchronize the flowering period of two transgenic *B. napus* cultivars that have the same relative maturity in order to evaluate the effect on outcrossing rates. In addition, the effect of flowering time on the outcrossing rate and seed-set per flower was assessed for synchronously flowering *B. napus* plants. We hypothesized that reducing flowering overlap would significantly reduce outcrossing rates in *B. napus* and that outcrossing rates would vary over the course of the flowering period of synchronously flowering cultivars.

RESULTS

Cultivar attributes

Commercially available transgenic hybrid cultivars were used. The information about the zygosity, or other attributes of these cultivars is private, and thus not published by the companies. Therefore, seed purity, zygosity and pollen production were evaluated. One out of 680 (0.15%) glufosinate resistant 5090 LL plants from the seedlot used in the field experiments tested negative for glufosinate resistance and none of 679 survivors tested positive for glyphosate resistance. None of the 920 glyphosate resistant 375 RR plants from the seedlot used in the field experiments tested negative for glyphosate resistance or positive for glufosinate resistance. Hybrid cultivars may be heterozygous for herbicide resistance if they result from a cross between two inbred lines, one of which not being herbicide resistant. Otherwise, if both lines are herbicide resistant, hybrids will be homozygous for herbicide resistance. Zygosity tests suggested that 5090 LL sired more transgenic offspring per silique (65,5%) than 375 RR (49,9%) ($p < 0.001$), irrespective of whether the receptor plant was transgenic or conventional ($p = 0.274$). All pollinated flowers produced 14–39 seeds per silique. Chi-square analysis specifying a fixed expected frequency of 5/8 (62.5%) for 5090 LL and 1/2 (50%) for 375 RR were not significant ($p = 0.97$ and $p = 0.99$, respectively). These frequencies are possible depending on the zygosity of the parental lines (Hüsken and Dietz-Pfeilstetter, 2007). Pollen production can also vary among *B. napus* cultivars (Kotowski, 2001; Singh et al., 2008). Pollen counts revealed that the 5020 LL cultivar potentially produced 2.5 times more pollen than 375 RR (8.3 ± 1.3 *vs.* $3.3 \pm 1.3 \times 10^5$ pollen grains per flower, $t = 2.677$, $p = 0.023$).

Seeding date interval experiment: de-synchronizing floral phenology to lower outcrossing rates

Outcrossing rates varied between cultivars in 2005 ($p < 0.001$). Cultivar 5020 LL was ca. 3 times less contaminated by transgenes from 375 RR (all plots: $2.1 \pm 0.4\%$ *vs.* $6.4 \pm 0.7\%$ – synchronous plots: $2.8 \pm 0.6\%$ *vs.* $8.7 \pm 1.1\%$). This difference can be explained by differences in zygosity for the transgene and pollen production. The pollen count combined with the zygosity results likely explained this 3 fold difference in outcrossing rates (also observed in 2007, see below) between cultivars, since 2.5 times more pollen and 1.25 times more transgenes would result in 3.125 more observed outcrossing. Both cultivars had similar flowering phenology (Fig. 1) and produced comparable total seed biomass ($p > 0.05$; data not shown). Temporal isolation (by seeding interval) did affect outcrossing rates and this effect varied with *B. napus* cultivar ($p < 0.001$) and seeding order ($p < 0.001$, preliminary analyses, see Materials and Methods). As predicted by flower counts, increasing the temporal isolation reduced outcrossing rates for the plots seeded first (before the adjacent plot) although the reduction was steeper than expected (Fig. 1). In plots seeded first, outcrossing rates reached their lowest value when the time interval was two weeks and greater. For plots seeded second, outcrossing rates increased up to a two week interval and decreased thereafter (Fig. 1). A second increase in outcrossing rate was observed for the LL cultivar at the four week interval (Fig. 1). The schedule factor (early seeded *vs.* late seeded intervals) was significant ($p < 0.001$) but did not interact with other factors for

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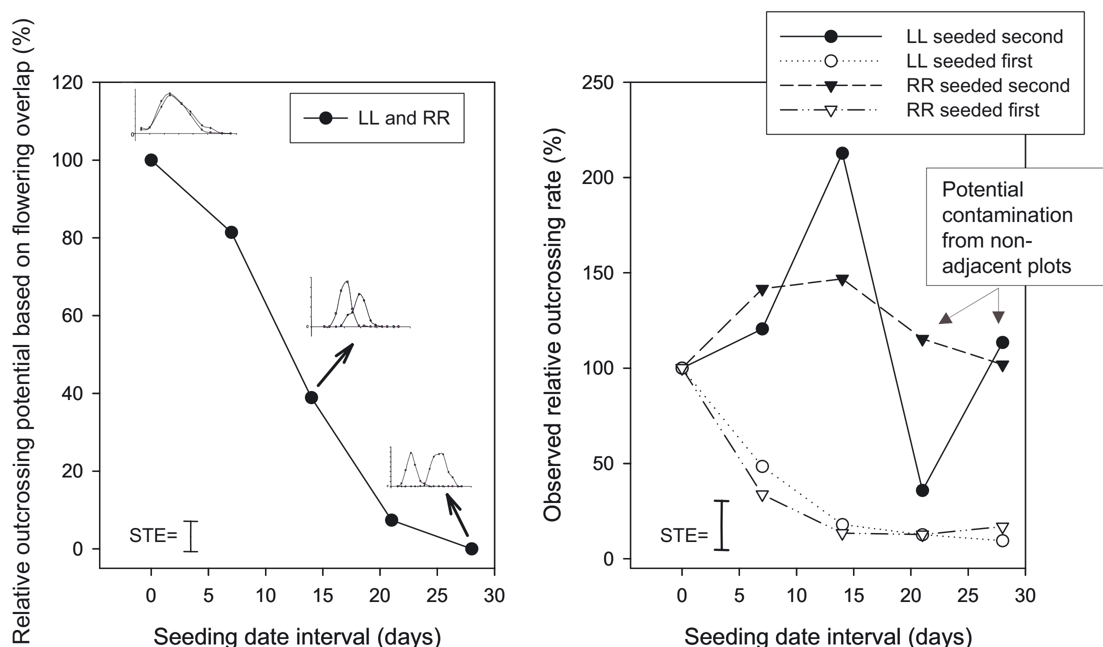

Figure 1. Relative outcrossing between two transgenic *Brassica napus* cultivars resistant either to glufosinate (LL) or glyphosate (RR) and seeded at different time intervals (in days) in different orders (either RR or LL cultivar seeded first) in 2005. Relative values are based on the highest expected value (synchronous flowering) per cultivar. Left: Expected rate based on flowering overlap (flower count curves are presented for three intervals). Right: Observed rate based on gene flow (values at 0 day interval are 2.8 for LL and 8.7% for RR). Bars indicate ± 1 mean standard error calculated across intervals and cultivars.

any variable ($p > 0.05$). Outcrossing rates for late seeded treatments were nearly twice that for early seeded treatments (all plots: early seeded 2.3 ± 0.5% *vs.* late seeded 4.3 ± 0.5%).

Reproductive ecology of individual flowers experiment: evaluating the outcrossing rate and seed-set during the flowering period

Although this experiment was done in 2007 at a different location, cultivar 5020 LL was again ca. 3 times less contaminated by transgenes from 375 RR than the converse ($p < 0.001$) (3.2 ± 0.4% *vs.* 9.2 ± 0.8%). There was a 25% reduction in outcrossing during the last week of the flowering period for both cultivars ($p = 0.012$, Fig. 2). No interaction between flowering date and cultivar on outcrossing was observed ($p = 0.611$). Both cultivars had similar total seed biomass ($p > 0.05$) and flowering phenologies (Fig. 3). Seed-set phenology differed between cultivars ($p = 0.004$) (Fig. 3). Seed production peaked and decreased earlier for the 357 RR cultivar while that of 5090 LL peaked later and was less skewed (Fig. 3). Ninety percent of the seed produced by both cultivars was sired by flowers that opened before the flowering peak (Fig. 3).

DISCUSSION

Observed outcrossing rates were mostly influenced by canola cultivar attributes (zygosity for the transgene, pollen production) and relative seeding order (first *vs.* second seeded). Data from one growing season indicates that increasing temporal isolation reduced outcrossing rates for the cultivar flowering first. This reduction reached the lowest values after a two week interval. Shifting time gaps during the growing season (early *vs.* late schedules) did not modify the temporal isolation effect, suggesting that these results would be consistent over different growing seasons. Accordingly, the few data points retrieved from a failed repetition of the experiment the following year (see Materials and Methods) confirmed the trends observed for outcrossing rates (data not shown). Absolute outcrossing rates can vary from year to year, but the effect of a time gap on outcrossing potential is likely more stable. Our outcrossing rates are also supported by actual flower counts. Moreover, rates for synchronously seeded plots in the experiment done in a different year-location are equivalent and the effect of the temporal isolation was similar for both cultivars when seeded first. Increased isolation by distance is however recommended to accurately evaluate outcrossing in plots seeded second (see below).

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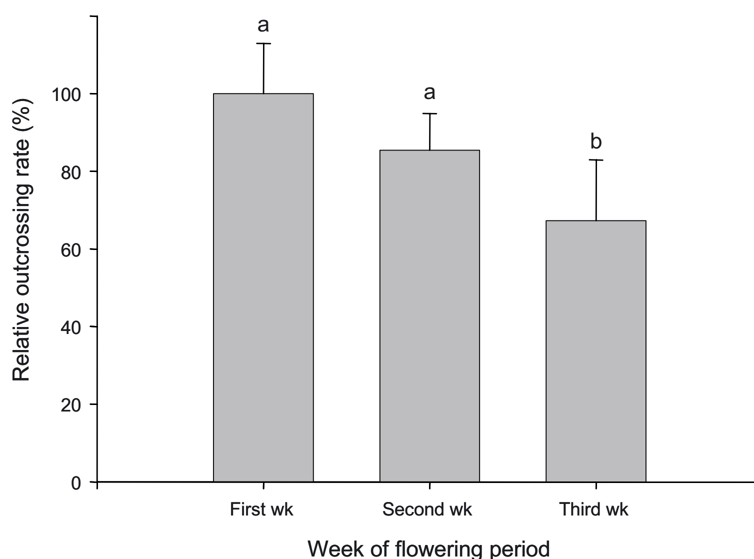

Figure 2. Relative outcrossing rate between two synchronously flowering transgenic *Brassica napus* cultivars resistant either to glufosinate (LL) or glyphosate (RR) during the first, second and third week of the flowering period in 2007. Different letters above bars indicate significant difference among weeks ($p < 0.05$). Average rate during the first week is 4.2% (LL) or 9.6% (RR). Bars indicate ± 1 standard error of the mean.

The important reduction in outcrossing of first-seeded plots can be explained by seed-set occurring mostly during the first two weeks of flowering. Only 40 to 55% of flowers produced on a canola (*B. napus* and *B. rapa*) plant develop into productive pods and these pods are produced by early as opposed to late flowers (Angadi et al., 2003; Canola Council of Canada, 2008). In a greenhouse experiment, > 80% of the seed produced by six *B. napus* cultivars occurred before the flowering peak (M.-J. Simard, unpublished data). Weis and Kossler (2004) observed that *B. rapa* (a species sharing a common genome with *B. napus*) flower production was 40% higher than seed-setting flower production and that seed-set per flower decreased in a logistic fashion during the flowering period. Alternatively, increasing the time gap increased outcrossing rates for the later seeded cultivar until the second week, followed by a variable reduction. This can be explained by the high pollen load from the first seeded cultivar relative to potential seed-set from the second cultivar at one and two week intervals. Outcrossing values for the second seeded cultivar never reached values as low as those of the first seeded cultivar even when flowering overlap was minimal, indicating that the second seeded plants also outcrossed with plants from non-adjacent plots. Barren zones are not ideal to limit plot to plot outcrossing and sowing a synchronously flowering crop between plots would probably have lowered potential plot to plot contamination (Morris et al., 1994; Reboud, 2003). Alternatively, isolation distances between adjacent plots should be greater than 100 m (Ingram, 2000; Scheffler et al., 1995). Nevertheless, this result still indicates that later flowering plots were more prone to genetic contamination.

A smaller fraction of the variation in asynchronous outcrossing rates is potentially explained by a decrease (−25%) in outcrossing during the flowering period. A 28% decrease in *B. napus* outcrossing rate on the main stem during the flowering period has also been observed by Becker et al. (1992). Damgaard and Loeschcke (1994) observed an increase in *B. napus* selfing rate during the flowering period. Becker et al. (1992) tentatively attributed this variation to uneven weather conditions during the flowering period. We propose an additional explanation. *Brassica napus* plants produce five (80 plants. m^{-2}: typical crop seeding rate) to nine branches (5 plants. m^{-2}) on the main stem and these branches can also produce tertiary branches (Angadi et al., 2003). Therefore, flowers on the main stem and first branches, *i.e.* those that contribute the most to seed production (Angadi et al., 2003), are increasingly surrounded by flowers located on higher order branches on the same plant, potentially increasing the selfing rate. Damgaard and Loeschcke (1994) also suggest that the density of self-pollen per ovule increases during the flowering period.

Implication for gene flow management

Isolation in time could be used in addition to isolation distances to minimize gene flow in *Brassica napus*

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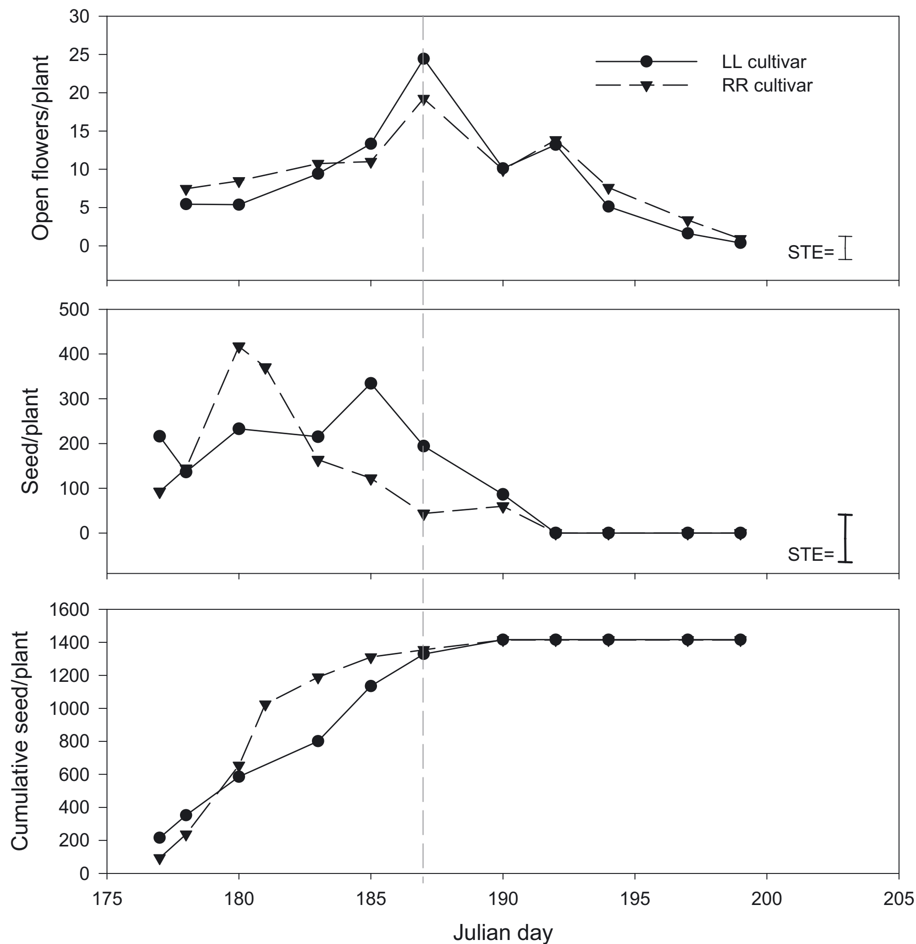

Figure 3. Flower and subsequent seed production of open flowers of two transgenic *Brassica napus* cultivars resistant either to glufosinate (LL) or glyphosate (RR) during the flowering period. Both cultivars have the same relative maturity and were seeded May 15, 2007 in adjacent plots. Bars indicate ± 1 mean standard error calculated across days and cultivars.

and other crops (Della Porta et al., 2008; Willenborg, 2009; Willenborg et al., 2009). Completely desynchronizing flowering periods between *Brassica napus* crops could be problematic in Canada because of the short growing season in the Prairie provinces where 99% of the canola growing area is found (Beckie et al., 2006). However, assuming that most of the seed-set occurs during the first half of the flowering period, then seeding two (or more) weeks earlier than other *B. napus* crop would significantly lower potential seed contamination from extraneous pollen dispersal. Wild or weedy relatives flowering after the crop's flowering peak or later (*e.g.* weeds that germinate after herbicide application) would also be less likely to generate hybrid seed on the crop. On the other hand, surrounding canola crops, canola volunteers, or weedy relatives that initiate flowering earlier than the crop warrant attention with regard to gene flow contamination risk. An important conclusion emerging from this research is that previous studies quantifying pollen-mediated gene flow from synchronous or later flowering pollen donors could have underestimated potential transgene contamination in *B. napus*. Currently, a similar conclusion can be inferred from pollination data in corn (*Zea mays* L.), a monoecious allogamous species (Della Porta et al., 2008).

MATERIALS AND METHODS

Cultivar attributes

Commercially available hybrid spring *B. napus* canola cultivars, with similar relative maturity, were used in our field experiments and included a glyphosate resistant cultivar (Hyola 375 RR, RR = Roundup Ready®, Monsanto Canada Inc.) and a glufosinate resistant cultivar (Invigor

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5020LL, LL = LibertyLink®, Bayer CropScience). Seed purity for the respective herbicide resistance transgenes was assessed by growing 680 and 920 seedlings of the glufosinate resistant 5020LL and glyphosate resistant 375RR commercial seedlots, respectively, and testing for herbicide resistance of both transgenes. Trays in which seedlings were grown were first sprayed with the herbicide appropriate to the resistance trait of the hybrid and sprayed two weeks later with the other herbicide. The zygosity of the cultivars for the transgene was evaluated in the greenhouse by growing 100 *B. napus* plants: 25 plants of each transgenic seedlots and 50 plants from a conventional cv. 46A65 (Pioneer Hi-Bred Production Limited), in individual pollen bags. Plants were grown at 16:8 photoperiod, 22 °C day, 17 °C night. Pots were filled with standard potting soil and watered once or twice a day according to need. Before anthesis, emasculated flowers of conventional or transgenic plants were fertilized with pollen from separate individuals of one of the two donor types (RR or LL). All other flowers were removed before hand pollination. After hand pollination, each flower was bagged individually. The seeds obtained from each silique were sown in the greenhouse and seedlings obtained were tested for herbicide resistance (see method below). Pollen production was estimated by growing nine plants of each transgenic seedlot in the greenhouse under the same conditions as above. Comparable flowers (same stage and location on plant) from each cultivar were collected just before anthesis and pollen production was evaluated using standard procedures for pollen count (Hinata and Konno, 1975). Five flowers from each plant were placed in a test tube containing 15 mL of distilled water and 0.02% of Tween 20. The solution was vortexed to break the anthers and stirred before each sampling (Rogers et al., 2006). Pollen grains were counted with a haemocytometer (American Optical, Scientific instrument division, Buffalo, NY).

Field study sites

Field trials were carried out at different farms near Lévis, (46.48 N, 71.11 W) in the canola growing region of Québec, Canada. The first experiment (2005) was conducted on a private farm (46.82 N, 71.07 W, 67 m above sea level) located on a Saint-Nicolas well drained schistose sandy loam with a 4% slope. The second experiment (2007) was set at the Harlaka Research Farm of Agriculture and Agri-Food Canada on a Saint-André well drained sandy-gravelly loam. In all trials, crops were grown according to standard provincial recommendations for canola (sowing rate: 5 kg.ha^{-1} in 17.5 cm rows), fertilization (based on soil analyses) and herbicide application (glyphosate: 445 g.ae.ha^{-1}, or glufosinate: 400 g.ai.ha^{-1}). Herbicides were applied at the four-leaf stage of *B. napus*.

Seeding date interval experiment: de-synchronizing floral phenology to lower outcrossing rates

Forty 3 × 3 m *B. napus* plots each separated by at least 7 m in a honeycomb display, were established in 2005. Each plot was subdivided in two (3 × 1.5 m) subplots seeded either with glyphosate (Hyola 375 RR) or glufosinate (Invigor 5020 LL) resistant *B. napus* (Fig. 4). Adjacent subplots were seeded with different herbicide resistant cultivars. The experiment included two schedules: early seeded (from May 9) and late seeded (until June 6) subplots, five time intervals between seeding adjacent subplots: 0, 7, 14, 21 and 28 days, and four replicates (Fig. 4). Twenty plots were seeded with the 5020 LL cultivar on the windward West side of the plots (block 1 and 2) and the other half was seeded with the 375 RR cultivar on the West side (block 3 and 4). Also, twenty plots were first seeded with 5020 LL (block 1 and 4) and the other twenty plots were first seeded with 375 RR (block 2 and 3). Therefore, wind-location (W *vs.* E) and seeding order effects on outcrossing were initially included in the variation. Statistical analyses revealed no interaction between wind-location effect on any variable ($p > 0.05$). Unexpectedly, seeding order did have a significant effect on outcrossing rates and interactions with other factors were significant. This factor was therefore included in the analysis using a split plot model with two replicates in which seeding interval was separated into nine categories (see statistical analyses). During the flowering period, open flowers were counted every fourth day on five randomly selected *B. napus* plants per subplot. Average cumulative degree-days (base 5 °C) ± SE for a seven day period in July 2005 was 111.1 ± 6.5 °C-days. When the seeds were ripe, *B. napus* plants were harvested on the center 1 m of the first adjacent row of both RR and LL subplot, at 8.75 cm (half the inter-row width) distance from the common border. Seed production per plant was assessed. Seeds were sown in a greenhouse and seedlings were sprayed for herbicide resistance. The entire experiment was repeated on another farm in 2006 but failed to produce a complete dataset because of poor *B. napus* establishment at certain dates due to unsuitable site and weather conditions during the spring.

Screening for herbicide resistance was achieved in the greenhouse by spraying seedlings at the two leaf stage with either glyphosate or glufosinate at 1.5× the recommended dose (glyphosate: 667.5 g.ae.ha^{-1}, glufosinate: 600 g.ai.ha^{-1}). Putative resistant plants were sprayed twice. Herbicides were applied in a single-blind fashion (seedling trays had numbers only) and included herbicide resistant and susceptible plants as controls. Herbicide resistance of each survivor was confirmed using test strips that detect the proteins produced by the transgenes conferring glyphosate or glufosinate resistance.

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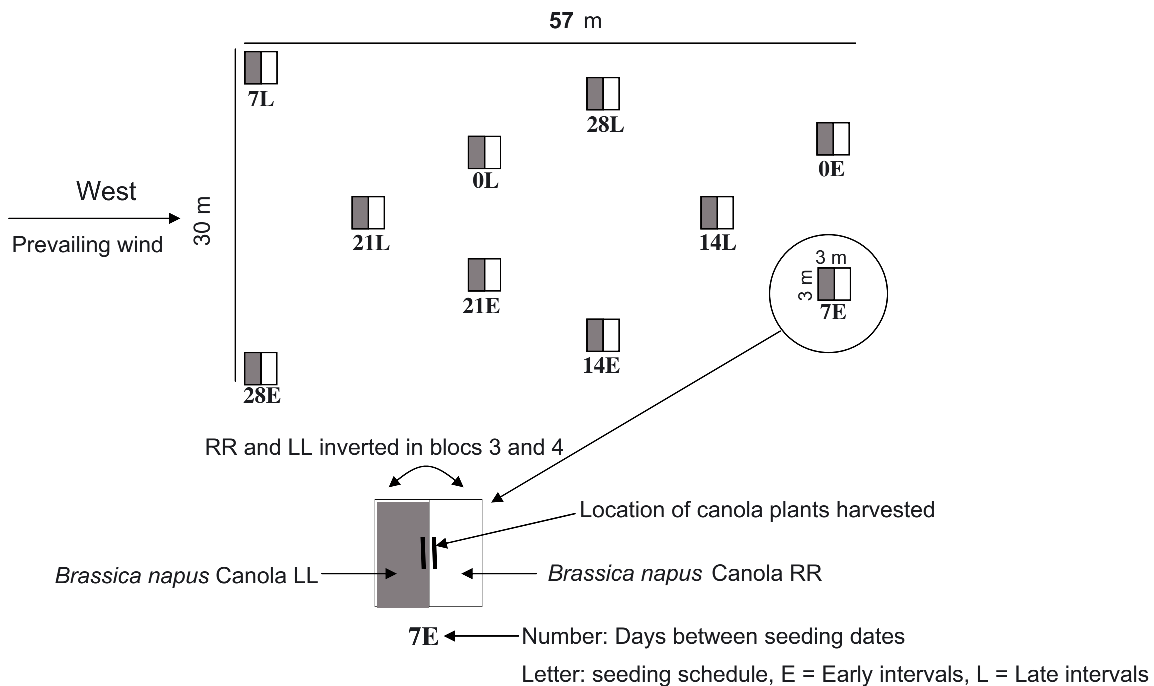

Figure 4. Diagram showing plot arrangement for one block of the seeding date interval experiment. Transgenic *Brassica napus* cultivars resistant either to glufosinate (LL) or glyphosate (RR) were seeded in adjacent subplots.

These test strips are commercially available as kits (Trait√RUR [Roundup Ready] or Trait√LL [Liberty Link] Flow Tests Kits: Strategic Diagnostics Inc., Newark, Delaware, USA). The RUR trait kit detects the CP4 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) protein produced by the introduced HOR gene whereas the LL trait kit detects the phosphinothricin acetyltransferase (PAT) proteins, PAT/pat and PAT/bar produced by the introduced pat and bar genes, respectively. Leaf samples were macerated in water and tests strips were dipped in the sample up to 10 min. Positive samples displayed two red coloured bands, a control band that tracks the capillary action and a secondary band that detects the protein (see Simard et al., 2006).

Reproductive ecology of individual flowers experiment: evaluating the outcrossing rate and seed-set during the flowering period

Six 3×3 m *B. napus* plots, each separated by at least 10 m in a honeycomb display, were seeded in 2007. Each plot was subdivided in two (3 × 1.5 m) subplots seeded either with the glyphosate or the glufosinate resistant canola cultivar as in the 2005 experiment. All plots were seeded on May 15. During the flowering period (July 2007), open flowers were counted on three randomly selected plants per subplot and two randomly selected plants located in the adjacent border row, every two or three days. Newly formed siliques were tagged on these two plants and five extra plants all located in the center 1 m, of the adjacent border rows of the subplots. Newly formed siliques were tagged instead of individual flowers because flower petioles are not strong enough to retain the tags in the wind. Average cumulative degree-days (base 5 °C) ± SE for a seven day period in July 2007 was 95.9 ± 9.1 °C-days. At maturity (August 17), tagged plants were harvested individually and siliques were separated by formation interval (*e.g.* all siliques located above the July 11 tags were grouped separately from those below the July 13 tags) for each plant. The flowering date of the siliques was determined by subtracting three days to the tagging date. This interval was estimated by tagging flowers and observing their development every day in the greenhouse, and from the literature (Pechan, 1988). Collected seeds were counted, grown and sprayed for herbicide resistance as above. Plants were also harvested in the center of the subplots (1 m^2) to evaluate total seed biomass production per cultivar.

Outcrossing probability based on flower frequency

The outcrossing probability (assortative mating probability: Φ_{ij}) of plants seeded at different time intervals

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(first experiment) was based on the frequency of open flowers during the flowering period as formulated by Fox and Pickering (1997) and Fox (2003):

$$\Phi_{ij} = \sum_t \frac{2p_i p_j \theta_i(t)\theta_j(t)}{\Sigma_k p_k \theta_k(t)} \quad (1)$$

where p = frequency of genotype i or j or both (k) in mating pool on day t and θ = frequency of type-i or j flowers or both (k) on day t.

Statistical analyses

The seeding date interval experiment was analysed as a split plot ANOVA. The dependent variable was outcrossing rate and fixed effects included seeding interval, schedule and *B. napus* cultivar. The seed interval (0, 7, 14, 21 and 28 days) factor was structured into nine categories (–28, –21, –14, –7, 0, 7, 14, 21 and 28 days) with negative values attributed for the plots seeded second. This allowed taking into account seeding order as well as the 0 interval when plots are seeded synchronously. The second experiment was analysed as a repeated measure randomized block ANOVA with heterogeneous compound symmetry (CSH) covariance structure for flowering dates. The dependent variable was outcrossing rate and fixed effects included plant, flowering date (repeated measure) and *B. napus* cultivar. Flowering dates were grouped as first, second or third week, in order to have sufficient observations per date (interval). A square root transformation was performed on outcrossing rates to satisfy statistical assumptions. Seed production (dependent variable) was analysed similarly. Analyses were conducted with the MIXED procedure of SAS (SAS, 2002). Differences in zygosity and pollen production between cultivars were tested using t-tests. Differences between observed and theoretical zygosity values were tested using Chi-square tests.

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REFERENCES

Angadi SV, Cutforth HW, McConkey BG, Gan Y (2003) Yield adjustement by canola grown at different plant populations under semi-arid conditions. *Crop Sci.* **43**: 1358–1366

Becker HC, Damgaard C, Karlsson B (1992) Environmental variation for outcrossing rate in rapeseed (*Brassica napus*). *Theor. Appl. Genet.* **84**: 303–306

Beckie HJ, Hall LM (2008) Simple to complex: Modelling crop pollen-mediated gene flow. *Plant Sci.* **175**: 615–628

Beckie HJ, Warwick SI, Nair H, Séguin-Swartz G (2003) Gene flow in commercial fields of herbicide-resistant canola (*Brassica napus*). *Ecol. Appl.* **13**: 1276–1294

Beckie HJ, Harker KN, Hall LM, Warwick SI, Légère A, Sikkema PH, Clayton GW, Thomas AG, Leeson JY, Séguin-Swartz G, Simard M-J (2006) A decade of herbicide-resistant crops in Canada. *Can. J. Plant Sci.* **86**: 1243–1264

Canola Council of Canada (2008) Canola Growers Manual. http://www.canola-council.org/canola_growers_manual.aspx

Cuthbert JL, McVetty PBE (2001) Plot-to-plot, row-to-row and plant-to-plant outcrossing studies in oilseed rape. *Can. J. Plant Sci.* **81**: 657–664

Damgaard C, Loeschcke V (1994) Genotypic variation for reproductive characters, and the influence of pollen-ovule ratio on selfing rate in rape seed (*Brassica napus*). *J. Evol. Biol.* **7**: 599–607

Della Porta G, Ederle D, Bucchini L, Prandi M, Verderio A, Pozzi C (2008) Maize pollen mediated gene flow in the Po valley (Italy): Source-recipient distance and effect of flowering time. *Eur. J. Agron.* **28**: 255–265

Fox GA (2003) Assortative mating and plant phenology: evolutionary and practical consequences. *Evol. Ecol. Res.* **5**: 1–18

Fox GA, Pickering CM (1997) Methods for estimating phenological assortative mating, with an application to Australian *Ranunculus*. Meeting of the Society for the Study of Evolution, Boulder, Colorado, USA, 12 p, available at http://chuma.cas.usf.edu/~gfox/papers/manual/ssepost.pdf

Friesen LF, Nelson AG, Van Acker RC (2003) Evidence of contamination of pedigreed canola (*Brassica napus*) seedlots in Western Canada with genetically engineered herbicide resistance traits. *Agron. J.* **95**: 1342–1347

Gulden RH, Shirtliffe SJ, Thomas AG (2003) Harvest losses of canola (*Brassica napus*) causes large seedbank inputs. *Weed Sci.* **51**: 83–86

Hall L, Topinka K, Huffman J, Davis L, Good A (2000) Pollen flow between herbicide-resistant *Brassica napus* is the cause of multiple-resistant *B. napus* volunteers. *Weed Sci.* **48**: 688–694

Hinata K, Konno N (1975) Number of pollen grains in Brassica and allied genera. *Tohoku J. Agric. Res.* **26**: 117–124

Hüsken A, Dietz-Pfeilstetter A (2007) Pollen-mediated intraspecific gene flow from herbicide resistant oilseed rape (*Brassica napus* L.). *Transgenic Res.* **16**: 557–569

https://doi.org/10.1051/ebr/2009013 Published online by Cambridge University Press

Ingram J (2000) The separation distances required to ensure cross-pollination is below specified limits in non-seed crops of sugarbeet, maize and oilseed rape. *Plant Var. Seeds* **13**: 181–199

Knispel AL, McLachlan SM, Van Acker RC, Friesen LF (2008) Gene flow and multiple herbicide resistance in escaped canola populations. *Weed Sci.* **56**: 72–80

Kotowski Z (2001) Results of the investigations into nectar secretion and pollen production of new cultivars of rape (*Brassica napus* L.). *Acta Hort. (ISHS)* **561**: 127–129

Morris WF, Kareiva PM, Raymer PL (1994) Do barren zones and pollen traps reduce genetic escape from transgenic crops? *Ecol. Appl.* **4**: 157–165

Pechan PM (1988) Ovule fertilization and seed number per pod determination in oil seed rape *Brassica napus*. *Ann. Bot-London* **61**: 201–208

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

Reboud X (2003) Effect of a gap on gene flow between otherwise adjacent transgenic *Brassica napus* crops. *Theor. Appl. Genet.* **106**: 1048–1058

Rogers CA, Wayne PM, Macklin EA, Muilenberg ML, Wagner CJ, Epstein PR, Bazzaz FA (2006) Interaction of the onset of spring and elevated atmospheric CO_2 on ragweed (*Ambrosia artemisiifolia* L.) pollen production. *Environ. Health Perspect*. **1146**: 865–869

SAS (2002) SAS/STAT User's Guide, Version 9.1. SAS Institute Inc., Cary, North Carolina, USA, 5180 p

Scheffler JA, Parkinson R, Dale PJ (1995) Evaluating the effectiveness of isolation distances for field plots of oilseed rape (*Brassica napus*) using a herbicide-resistance transgene as a selectable marker. *Plant Breeding* **114**: 317–321

Simard M-J, Légère A (2004) Synchrony of flowering between canola and wild radish (*Raphanus raphanistrum*). *Weed Sci.* **52**: 905–912

Simard M-J, Légère A, Warwick SI (2006) Transgenic *Brassica napus* fields and *Brassica rapa* weeds in Québec: sympatry and weed-crop in situ hybridization. *Can. J. Bot.* **84**: 1842–1851

Singh SK, Kakani VG, Brand D, Baldwin B, Reddy KR (2008) Assessment of cold and heat tolerance of winter-grown canola (*Brassica napus* L.) cultivars by pollen-based parameters. *J. Agron. Crop Sci.* **194**: 225–236

Statistics Canada (2008) Field and specialty crops (seeded area). Available at http://www40.statcan.ca/l01/cst01/prim11a.htm, 1 p

Tan S, Evans RR, Dahmer ML, Singh BK, Shaner DL (2005) Imidazolinone-tolerant crops: history, current status and future. *Pest Manage. Sci*. **61**: 246–257

U N (1935) Genome analysis in *Brassica* with special reference to the experimental formation of *B. napus* and peculiar mode of fertilization. *J. Jpn. Bot.* **7**: 389–452

Warwick SI, Légère A, Simard M-J, James T (2008) Do escaped transgenes persist in nature? The case of an herbicide resistance transgene in a weedy *Brassica rapa* population. *Mol. Ecol.* **17**: 1387–1395

Weis AE, Kossler TM (2004) Genetic variation in flowering time induces phenological assortative mating: quantitative genetic methods applied to *Brassica rapa*. *Am. J. Bot.* **91**: 825–836

Willenborg CJ (2009) Characterizing the factors contributing to pollen-mediated gene flow between cropped and volunteer spring wheat (*Triticum aestivum* L.). Ph.D. Thesis, University of Manitoba, Winnipeg, Manitoba, Canada

Willenborg CJ, Luschei EC, Brûlé-Babel AL, Van Acker RC (2009) Flowering phenology and synchrony between volunteer and cropped spring wheat: Implications for pollen-mediated gene flow. *Crop Sci.* **49**: 1029–1039

https://doi.org/10.1051/ebr/2009013 Published online by Cambridge University Press