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Effect of a gap on gene flow between otherwise adjacent transgenic Brassica napus crops

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Abstract Gene flow resulting from cross pollination becomes an issue when transgenic crops are involved and the genetic modification carries a trait of ecological importance. As crop fields are often separated by a barren gap, such as an intervening roadway or unplanted area, I measured cross contamination between two herbicide-resistant transgenic fields (canola, *Brassica napus*) across a gap of up to 12 m. I focused on pollen exchange from the field border up to 7 m inside each field over two seasons. In the absence of a gap, I found that gene dispersal diminished rapidly with distance, with more than 40% of transgenic progeny found within the first meter from the edge of the adjacent crop. Cross contamination between fields declined more rapidly when there were intervening plants, however. Plants separated from the transgenic source by a gap of 3–4 m, yielded the same level of transgenic progeny as those separated by 1 m of crop. Both insects and wind pollinate canola, and so the explanation for my observations could involve the influence of gaps on wind patterns or on the behaviour of pollinators. The gap effect does not seem to depend only upon the variation in the density of neighbours that surrounds those plants at the crop edge versus those in the crop matrix. On the basis of this study, it is recommended that economic profit would be maximised by removing field borders after flowering rather than by leaving a surrounding gap, which would need to occupy up to threefold as much field surface to achieve the same level of containment.

Keywords Transgenic oilseed rape · Pollen dispersal · Discontinuity · Gene escape · Contamination

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Introduction

Advances in biotechnology now make it possible to transfer genes from diverse organisms into crop plants to produce genetically modified crop varieties expressing new traits of agronomic, technological or medical interest (hereinafter referred to as transgene and transgenic plants). Thus far, this technology has found application in crop varieties with improved plant protection against pests. However, transgene technology has raised some concerns, both agronomic and ecological. Gene flows are natural phenomena and of common occurrence but because of potential ecological risk linked to the transgene, some have argued that the short-term economic benefit of transgenic crops could, over longer periods, be counter balanced by: (1) a problem in eliminating the transgenic plants when used in crop rotation, or as a transient naturalised weed population, (2) a problem of gene flow (contamination) whereby the transgene is naturally transferred to plants of the same species growing in other fields, (3) introgression of the transgene via hybridisation to wild (often weedy) relatives of the crop or via horizontal transfer to micro-organisms, (4) a modification of some important element of the cropping system resulting from the use of the transgene, thereby creating a new recurrent selective pressure on any populations interacting with the crop.

With respect to these concerns, at least the first three directly depend upon the dispersal capacity of viable pollen grains that could cross-pollinate nearby plants. Thus, it is crucial in studies assessing the ecological risks associated with the release of transgenic crops to establish the conditions whereby viable pollen flow results in cross pollination between crops (Skogsmyr 1994; Jørgensen et al. 1996; Timmons et al. 1996). This necessity to develop sound guidelines to limit the impact of gene flow has led to studies that have analysed and described a leptokurtic decrease in pollen exchange related to the distance between donor and recipient plants. These studies have identified that :

- Most gene flow occurs over very short distances with a long-tailed stochastic distribution with no absolute threshold upper limit for same-species gene exchange (Kareiva et al. 1994; Scheffler et al. 1995; Timmons et al. 1995; Wilkinson et al. 1995).
- There can be involvement and possible interaction between several pollen transport mechanisms such as passive diffusion, wind and pollination vectors, dependent on the particular plant species' reproductive system and agro-ecosystem (Kunin 1993; Morris et al. 1994a, b, 1995; Cresswell 1997; Cresswell et al. 1995).
- Between nearly adjacent fields, gene flow is expected to occur preferentially at the immediate border. Between fields further removed, this excess border-gene flow would be replaced by rare and randomly distributed cross-pollination events, notwithstanding the field position of recipient plants (Hall 2000).
- The long-distance pollen (seed) dispersal events are both rare and unpredictable. However ecologists and population geneticists have established that these rare events had a considerable impact on the population genetic structure or demography over time, especially in a patchy environment (Ellstrand et al. 1989; Ouborg et al. 1999; Cain et al. 2000).
- Wind-pollinated tree species seldom seem to follow the leptokurtic pollen dispersal described above but are prone to more stochastic long distance events (Adams et al. 1997; Pakkanen et al. 2000).

Within a discrete crop field, the natural gene flow that can occur between individual crop plants is not regarded as a problem. Conversely, at the landscape or agro-ecosystem level, gene "escape" from one crop field to another of the same species is an issue, especially if field purity at harvest is of economical importance. One situation that is common in agro-ecosystems is where a field border is devoid of plants, as in the case of a roadway between two crop fields. This empty area can be defined as a gap separating two fields. The influence of a gap between two otherwise adjacent crop fields on pollen movement is generally unknown, i.e. there could be increased pollen transfer causing more cross pollination from one field to another across this barren zone. Studies are required that unambiguously address how the common occurrence of a width gap between fields of a few meters affects gene flow between them. I conducted my study in almost commercial-sized canola fields within the normal European agro-ecosystem over two entire flowering seasons. Three transgenic cultivars were used in the experiments, and the presence of herbicide resistance was used as a convenient screen to identify inter-cultivar hybrids.

Three possibilities on how a gap between crops could influence the decline of cross pollination with distance from field borders are presented in Fig. 1. Hypotheses 1–3 can be considered as the principal hypotheses among several.

The aim of the investigation reported here, was: (1) to test the effect of a barren gap between canola fields that represents the situation where roadways, for example, separate fields and (2) to detect whether any effect of a gap between canola crops would be limited to the edges of the fields or would extend into the crop.

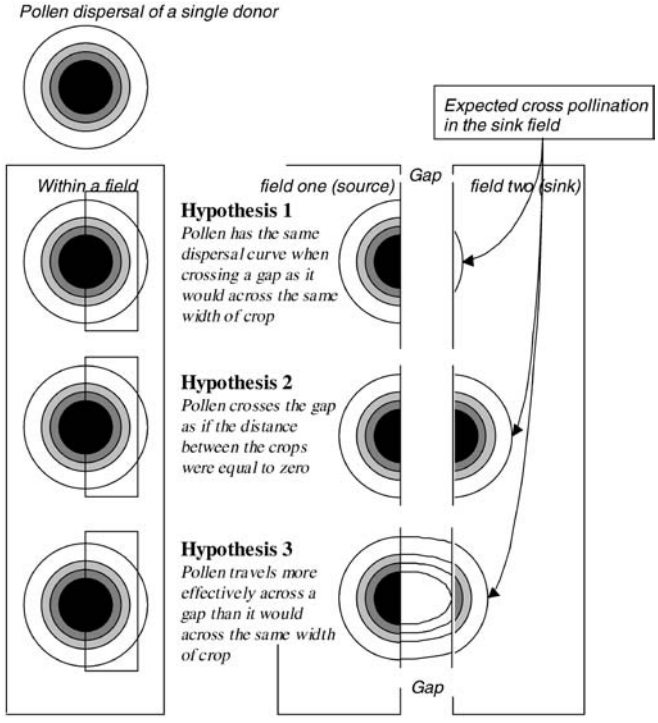

Fig. 1 Three theoretical expectations, presented as hypothesis 1–3, for gap effect on pollen dispersal. The annulus fit into each other symbolizes the varying levels of effective pollination of recipient plants by a single donor located at the central position within the circles. Illustrated here are three of the possible resulting patterns of cross pollination when a gap occurs within the pollination area. Each hypothesis leads to different amounts of cross-contamination between sink and source fields

Materials and methods

Experiments were conducted in 1998 and 1999, and since they were identical in their objective, they are presented in parallel, notwithstanding the unavoidable differences in geographic, field shape (detailed hereafter) and seasonal climatic variables.

Transgenic crop varieties utilised

Three transgenic varieties of canola, each expressing resistance to a particular herbicide (glyphosate, glufosinate or bromoxynil), were provided by commercial sources as "high-purity transgenic homozygous seeds". Hereinafter we will utilise the terminology GlyR, GluR and BroR to denote these glyphosate, glufosinate and bromoxynil herbicide-resistant transgenic canola cultivars respectively. These winter-growing canola varieties flower at very similar times with more than 92% synchrony over the total flowering period (data not shown). A slight flowering asynchrony observed during the 1998 season (experiment 1) with BroR led to its exclusion in the 1999 field experiment. This enabled a more complex field trial in 1999 (experiment 2) with only the GluR and GlyR varieties.

Concurrent with the field experiments, we conducted controlled cross pollinations across the three transgenic canola variet-

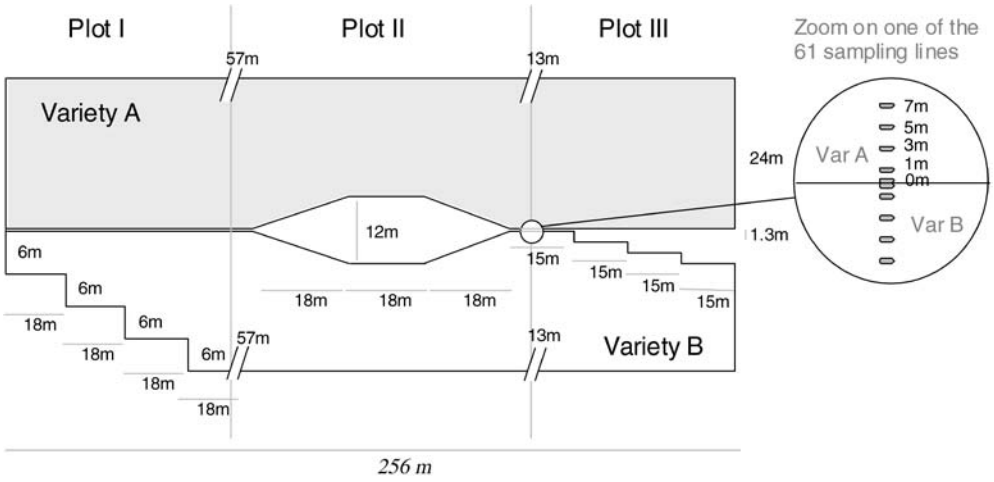

Fig. 2 Experiment 2 field trial. A 256 × 48-m experimental field sown with two varieties is divided into three plots. Plot I is devoted to testing the effect of a change in donor/recipient ratio on cross pollination between the two transgenic canola varieties. Plot II tests a continuous gap from 0 m to 12 m between two crop fields. It is identical to the repetitions from experiment 1 (except Exp. 1 had three varieties). Plot III tests a discrete three-step gap between two crop fields. The sampling shown in the amplified zoom area was conducted 61 times, giving a total of 736 sample points. This graph is not to scale, but principal distances are noted

ies. Segregations in the progenies confirmed that the varieties used were homozygous for their respective transgene herbicide resistance trait. The heterozygote progeny plants obtained were then tested for their phenotypic response to a discriminating herbicide dosage (details below). Each of these herbicide resistance traits thus served as a simple dominant Mendelian marker of parental origin. During the 2 years of the field trials, none of the varieties used in the experiment were commercially grown in Europe, thereby excluding the possibility of contamination of the experimental fields by any resistant volunteers. Therefore, the origin of any plant found to express a transgene could be unequivocally attributed as a pollen donor or recipient to or from an adjacent experimental field.

Experimental field design

The experimental field was partitioned into three blocks, each seeded with a different transgenic canola variety. The contact distance between these adjacent areas was varied, ranging between immediate contact (no gap) up to a 12-m gap between varieties. The gap was a barren ground surface kept free of vegetation by a combination of herbicide (Gramoxone, 2.5 l/ha) and hand weeding.

As depicted in Fig. 2, the field experiments (experiment 1 and experiment 2 – part II) were arranged with a progressive (triangular shape) gap with the maximum gap distance of 11.5 m and 12 m, respectively. Part III of experiment 2 consisted of three contiguous plots at least 15 m long, each with a gap width of 1.7, 3.0 and 4.4 m, respectively. This enabled measurements ranked from south to north, so different width gaps were not randomised but were in fact arranged in order of size. These measures were still considered to be replicates since samples were on a centrally positioned line within its respective block to limit the bias of cross contamination of convarietal pollen between neighbouring experiments. As different gap sizes could modify the size of pollen sources, part I in experiment 2 was added so that the donor to recipient ratio was changed, thereby enabling estimation of the part of any potential bias this side effect may have introduced.

Having plots with both immediate crop contact between fields and a gap between fields made it possible, with appropriate sampling (described hereafter), to measure gene flow with distance, as illustrated by the different annulus in Fig. 1 (left). I could then use this information to test which of the three theoretical situations (hypotheses 1–3, Fig. 1, right) best fit the experimental measures of reciprocal cross pollination between the two transgenic fields separated by a gap. Conducting this experiment over 2 years allowed me to test the consistency of this result over factors of year or crop variety.

As cross pollination between adjacent crops is expected to be quantitatively important only over short gap distances and likely to depend on the size of the source field, I focused on commercial fields of a relatively large size (1.1 ha and 1.4 ha in 1998 and 1999, respectively). The seeding rate was adjusted to provide a final plant density of 50 established canola plants per square meter present during the spring flowering period. The density of the canola plants was thus agronomically representative of canola cropping in France. All weed control and other treatments were identical in each plot.

Field sampling methodology

The choice of an internal comparison control in each field trial, while nearly doubling the sampling number, enabled me to minimise any potentially inexplicable variables such as preferential wind direction, year or variety effect (these "zero gap" samplings were used as references in all the statistical analyses). Additionally, although the phenotypic herbicide resistance status of individual plants could be easily and accurately identified, each field sample point was individually identified. I opted for a high number of survey points in each field (268 and 737, respectively) rather than high precision with a limited number of survey points. This choice confined my investigation to a relatively small individual sampling area (usually less than 0.2 m^2) from which two sampling sets of 600 seeds were taken. To remain statistically valid in detecting effective cross pollination, my field sampling mainly focused on relatively short distances from each field border row in contact with a gap; i.e. samples were taken at 0, 1, 2, 3, 5 and 7 m within each adjacent transgenic crop variety (see amplified zoom in Fig. 2). Except for the sampling areas, the transgenic crop had to be destroyed after flowering but before any seed maturity – a matter of regulatory requirements. At crop maturity, the canola seeds at each survey point were manually harvested.

Screening procedure

From each sampled field point, I obtained clean sets of 600 seeds using a Pfeuffer Contador E seed counter. A direct counting control on 20 samples showed that the counting error in mechanical

counting was less than 0.3% with clean sets of seeds. Particular care was taken to avoid any possible contamination between consecutive samples by opening the bags individually and monitoring the seed counter and seed counting area carefully. For germination, each sample of 600 seeds was placed on vermiculite and watered with nutrient solution under controlled conditions in a glasshouse maintained at a day/night regime of 16/8 h 20/15 °C. In both experiments, replicate sampling was conducted (by sub-sampling) to determine the seed germination rate. All three herbicide-resistant canola varieties had rapid (within 10 days) and high rates of germination with only slight differences between GlyR-(94.2%), GluR-(90.7%) and BroR-(90.6%)-resistant canola varieties. There was no variety effect on seed germination in experiment 1 (ANOVA, 2 *df*, *F* ratio = 2.443, *P* = 0.126). When pooled over the 2 years for GlyR and GluR varieties, I found a significant variety effect (ANOVA, 1 *df*, *F* ratio = 8.243, *P* = 0.008) but neither a year (*F* ratio = 2.912 *P* = 0.101) nor a variety × year interaction effect was evident (*F* ratio = 0.975, *P* = 0.333).

To establish whether gene flow had occurred between any two transgenic canola varieties, the relevant herbicides were sprayed when canola seedlings were at the three-leaf stage and then re-sprayed two weeks later. For example, to examine pollination from a GlyR crop on a GluR crop I treated the canola samples collected in the GluR crop with glufosinate to be certain of their Glu-resistant status and with glyphosate to identify whether any gene flow had occurred from the adjacent GlyR crop. Thus, each plant was treated with both herbicides and re-treated 14 days later. The dosages were calibrated so as to accurately identify heterozygotes (i.e. glyphosate 1,080 g/ha; glufosinate ammonium 600 g/ha; bromoxynil potassium salt 75 g/ha). Herbicide treatments were carried out using a precision sprayer delivering herbicide in a total volume of 300 l/ha.

Measurement precision and statistical analysis

The use of herbicide survival as a distinct marker for the presence of a herbicide-resistant transgene requires verification of the accuracy of this method by direct measurement of the transgenic protein with an ELISA antibody test. Only glufosinate resistance could be checked in this manner as an antibody test for glyphosate or bromoxynil was not available. The error rate for discriminating "real" from "false" positives was tested over 88 samples, and 100% concordance between the herbicide treatment and the ELISA antibody test was obtained. This confirmed confidence in the use of the herbicide treatment to identify herbicide-resistant individuals. Following glufosinate treatment, the surviving plants were visually classified 2 weeks after the first herbicide spray into three different groups – healthy, vigorously growing plants; poor growth with orange-to-red-coloured leaves; almost dead plants. Following the pooling of the results of the two experiments, there was only one incorrect classification over 55 plants, 10 over 52 and 0 over 43 for these healthy, visually affected and near-death classes, respectively. Thus, taking into account the respective proportions of these three classes, false positives comprised approximately 1.5% and even in the most unfavourable cases, less than 3%. This justified the systematic application of a second glufosinate treatment that efficiently killed the class containing most of the false-positive plants. The false-positive error value was then considered to be sufficiently small in regard to the number of samples analysed in each experiment. Double herbicide treatment was extended to the three herbicides in response to the lack of similar ELISA information for glyphosate and bromoxynil resistance.

Additionally, a large sub-sample (220 and 654 for experiments 1 and 2, respectively) was treated again, giving two replicate values for each field point on which a paired sample *t*-test was performed. As no significant difference between the two measures was observed, further analyses were performed either on the single value or on the mean between replicates. In all cases (single or replicate measures), the variable used to follow gene flow between fields was the number of surviving plants from the 600 sown seeds. This value may be divided by 6 and corrected by the mean germination rate (values given above) to give an estimate of the outcrossing rate between fields. Such transformation may only be of limited "absolute" value for it may first reflect the selfing rate, a biological parameter which may vary due to varietal differences in canola as well as climatic conditions during the flowering period (Becker et al. 1992). The experimental design always referred to the internal control of direct contact between two crops (no gap) so that the relative gap effect could be estimated with precision within each variety.

Statistical analysis

It has been suggested that when deriving parameters from discrete positive probability events, such as when detecting rare events, a bias correction may be necessary (SAS 1993; Kareiva et al. 1994) that will account for an expected relationship between mean and variance. This is especially worthwhile in assessing a probability risk if an observation of no resistant plants could simply reflect the sampling size when there is a low probability of cross pollination. When the sampling area was limited to a maximum of 7 m inside each field, gene flow was observed, with at least one resistant plant in 988 of the 1,004 sets of 600 seeds tested. Therefore, I considered the orthodox analyses both appropriate and, *a posteriori*, suitable. The analysis of the "gap effect" was mainly conducted using ANOVA or General Linear Model. In experiment 1 and in part II of experiment 2, where there were no replicate conditions for gap size, the distance to the border row was used as a covariate. Categorical factors are variety, gap size or ratio between varieties for parts I and III of experiment 2. The homogeneity of variance assumed by the ANOVA analysis was tested earlier using Levene's test. As this test sometimes led to the rejection of homogeneity of the non-transformed counting data, a $\sqrt{(Value+1)}$ transformation for "counts per unit" data (Underwood 1997) was applied that always restored variance homogeneity. A non-linear regression process was also used to incorporate the gap effect and other parameters estimates, under the Gauss-Newton option, into three commonly used model equations (power, negative exponential and Weibull) to describe the decrease of pollen dispersal with distance. Although other methods could have been used, R^2 was the measure of best fit. To test that the gap effect could be expressed as the relative decrease of pollen over the gap compared to the situation above the crop, the distance above crop was replaced by distance + gap/C where gap represents the distance covered by the gap and C its relative contribution. For example, a value of four for C would mean that a gap 4 m wide would be required to induce the same decrease in gene flow occurring in comparison to 1 m of crop. Thus, the smaller the C value, the more efficient is the gap in limiting gene exchange. I further checked how robust the parameter estimates were to several changes such as the arbitrary choice of the equation, the data set or the path that the pollen could have followed. Indeed, as the pollen had to traverse two different "environments" (i.e. the gap and the crop), the direction of pollen travel may have changed according to the angle α (represented by the triangular shape gap). I compared the actual measured distances (path A), the cosine α corrected gap width distance as if pollen had always traversed the gap from the nearest pollen donor (path B) and, lastly, the cosine α corrected distance for both gap and above crop distance (path C) as if an insect had directly traversed between donor and recipient plant instead of first reaching the gap and then changing direction.

Results

Analysis of different source effects on gene flow

In experiment 1 (Table 1), in the absence of a gap between fields, cross pollination between canola fields rapidly declined with distance. This effect was highly sig-

Table 1 ANOVA applied to experiment 1 data set – keeping the 212 field points within the range of 0 m to 7 m to their own border row. Number of seeds resulting from cross pollination/600 seed tested (CP) – the data was successfully transformed prior to analysis (Levene's test $n = 212$, $P = 0.971$). Gap size without replicate was introduced as a covariate

Dependant variable: $\sqrt{(CP+1)}$ n: 212 Squared multiple R: 0.704					
Source	Analysis of variance				
	Sum of squares	*df*	Mean square	*F* ratio	*P*
Distance[a]	165.634	4	41.408	82.884	0.000
Don_Rec[b]	12.660	3	4.220	8.447	0.000
Distance × Don_Rec	5.669	12	0.472	0.946	0.503
Gap as covariate	44.953	1	44.953	89.978	0.000
Error	95.423	191	0.500		
Test of hypothesis using Contrast, effect called: Don_Rec					
Hypothesis	11.074	1	11.074	22.165	0.000

[a] Distance: Range of 0–7 m from the border row
[b] Don_Rec: Each variety as pollen donor or receiver

Table 2 ANOVA applied to experiment 2, plot II data set – representing 336 field points. Number of seeds resulting from cross-pollination/600 seed tested (CP) data were successfully transformed prior to analysis (Levene's test $n = 336$, $P = 0.243$). Gap size without replicate was introduced as a covariate

Dependant variable: $\sqrt{(CP+1)}$ n: 336 Squared multiple R: 0.613					
Source	Analysis of variance				
	Sum of squares	*df*	Mean square	*F* ratio	*P*
Variety	0.020	1	0.020	0.092	0.762
Distance	75.961	5	15.192	70.620	0.000
Variety × Distance	2.388	5	0.478	2.220	0.052
Gap as covariate	31.854	1	31.854	148.070	0.000
Error	69.485	323	0.215		

Table 3 ANOVA applied to experiment 2, plot III data set – representing 149 field points. Number of seeds resulting from cross pollination/600 seed tested (CP) – the data was successfully transformed prior to analysis (Levene's test $n = 149$, $P = 1.000$). The gap has four categorical values of 0, 1.6, 3.2 and 4.4 m

Source	Sum of squares	*df*	Mean square	*F* ratio	*P*
a. Dependant variable: $\sqrt{(CP+1)}$ n: 149 Squared multiple R: 0.864					
Gap	11.826	3	3.942	24.990	0.000
Distance[a]	56.854	5	11.371	72.082	0.000
Variety	0.000	1	0.000	0.002	0.967
Gap × Distance	18.819	15	1.255	7.953	0.000
Gap × Variety	0.566	3	0.189	1.197	0.315
Distance × Variety	3.169	5	0.634	4.018	0.002
Gap × Distance × Variety	2.944	15	0.196	1.244	0.252
Error	15.933	101	0.158		
b. But removing distance = 0: n: 124 Squared multiple R: 0.651					
Gap	2.601	3	0.867	6.000	0.001
Distance	12.708	4	3.177	21.987	0.000
Variety	0.200	1	0.200	1.385	0.243
Gap × Distance	2.284	12	0.190	1.317	0.224
Gap × Variety	0.495	3	0.165	1.141	0.337
Dist × Variety	1.875	4	0.469	3.244	0.016
Gap × Distance × Variety	2.498	12	0.208	1.441	0.164
Error	12.137	84	0.144		

[a] Distance, Distance to field margin

nificant ($P < 0.0001$) and took place over the first centimeters in the control area with the number of cross-pollinated plants dropping from 9.1% hybrid seedlings on immediately adjacent plants (zero gap, 0 cm) to 5.6% hybrids and 3.7% hybrids at 10 cm and 30 cm, respectively, within a field. A gap between fields strongly influenced gene flow levels (gap as covariate, $P < 0.0001$). I also found a significant donor/receiver effect: the GlyR canola variety as a pollen source gave higher cross pollination on both GluR and BroR recipient canola varieties than the reverse (hypothesis using contrast test: $P < 0.0001$). This result may have been due to the GlyR variety either producing more pollen or, conversely, having a higher (non tested) selfing rate. This varietal effect difference in gene flow did not modulate the distance effect, with each donor/receiver pair still having a similar pattern of gene flow decline with distance ($P = 0.503$).

In plot II of experiment 2 (Table 2) with the progressive triangular gap (Fig. 2), as for experiment 1, the pat-

Table 4 ANOVA applied to experiment 2, plot I data set – representing 124 field points. Number of seeds resulting from cross pollination/600 seed tested (CP) – the data was successfully transformed prior to analysis (Levene's test $n = 124$, $P = 0.525$). The distance of 7 m has been dropped from the analysis to keep symmetry with the 4:1 ratio which has a maximum depth of 6 m

Dependant variable: $\sqrt{(CP+1)}$ n: 124 Squared multiple R: 0.874

Source	Sum of squares	*df*	Mean square	*F* ratio	*P*
Variety	11.258	1	11.258	36.163	0.000
Ratio[a]	1.485	3	0.495	1.590	0.198
Distance	152.741	4	38.185	122.658	0.000
Variety × Ratio	0.322	3	0.107	0.345	0.793
Variety × Distance	9.979	4	2.495	8.014	0.000
Ratio × Distance	2.419	12	0.202	0.648	0.796
Variety × Ratio × Distance	3.012	12	0.251	0.806	0.643
Error	26.151	84	0.311		

[a] Ratio: Donor/recipient ratio has four categorical values from 1:1 to 4:1

Table 5 Fitting of several non-linear models using Gauss-Newton option. R^2 has a different digit number in order to distinguish close estimate models. In part c, parameter C is given between brackets because it is of different nature and should not be compared to the other parameter C estimates

Data origin	Sub-sample	Total *df*	Model equation[a]	A	B	C[b]	D	R^2	C 95% Wald Conf. Inter.[b]
a. Test of different models without gap									
Experiment 2	Gap=0	328	Y = 10^(a – b*X^d)	1.45	0.46	/	0.34	0.8846	/
Experiment 2	Gap=0	328	Y = a*(1+b*(exp(–X))^d)	4.83	4.82	/	1.38	0.8843	/
Experiment 2	Gap=0	328	Y = a*(1-exp(–b*X^d))	28.15	0.44	/	–0.57	0.8851	/
b. integrating gap relative contribution									
Experiment 2	All	737	Y = 10^(a–b*(X+Gap/C)^d)	1.45	0.45	3.74	0.39	0.88282	2.85 to 4.62
Experiment 2	All	737	Y = a*(1+b*(exp(–X-Gap/C))^d)	4.19	5.60	3.10	1.20	0.874	2.54 to 3.65
Experiment 2	All	737	Y = a*(1–exp(–b*(X+Gap/C)^d))	28.09	0.44	3.46	–0.61	0.88280	2.67 to 4.24
c. Comparison to Pedersen (1969)									
Experiment 2	All	737	Y = a^(1–b*X)/(X^c+exp(d*Gap))	25.83	0.05	(0.41)	0.18	0.862	/
d. Year and variety consistency									
Experiment 1	Glufosinate	67	Y = a*(1–exp(–b*(X+Gap/C)^d))	41.74	0.26	3.78	–0.59	0.976	2.72 to 4.84
Experiment 1	Glyphosate	134	Y = a*(1–exp(–b*(X+Gap/C)^d))	63.17	0.27	4.92	–0.56	0.919	3.12 to 6.72
Experiment 1	Bromoxynil	67	Y = a*(1–exp(–b*(X+Gap/C)^d))	43.01	0.31	6.02	–0.70	0.914	3.38 to 8.65
Experiment 2	Glufosinate	369	Y = a*(1-exp(–b*(X+Gap/C)^d))	34.42	0.39	2.72	–0.65	0.923	2.03 to 3.42
Experiment 2	Glyphosate	368	Y = a*(1–exp(–b*(X+Gap/C)^d))	21.72	0.54	5.10	–0.59	0.895	3.49 to 6.72
e. Modelling different pollen paths									
Experiment 1	Path A	268	Y = 10^(a–b*(X+Gap/C)^d)	1.73	0.57	4.33	0.39	0.89666	3.12 to 5.54
Experiment 1	Path B	268	Y = 10^(a–b*(X+Gap/C)^d)	1.73	0.57	4.22	0.39	0.89654	3.04 to 5.40
Experiment 1	Path C	268	Y = 10^(a–b*(X+Gap/C)^d)	1.73	0.57	4.22	0.39	0.89658	3.04 to 5.40

[a] X = Distance to field margin
[b] /, Unnecessary measure

tern of a highly significant effect of distance and gap introduced as a covariate was also clearly evident. The variety effect was non-significant, while the interaction between variety and distance, accounting for varietal difference in pollen deposition, was just at the limit of acceptance ($P = 0.052$).

For experiment 2, plot III (Table 3) with three distinct gap widths (Fig. 2), the ANOVA tests for the gap, within field distance and interaction effects were all highly significant. There was no canola variety effect with the exception of a variety cross distance interaction ($P = 0.002$), showing that each variety behaved differently according to the sampling distance to its own field edge. The gap × distance interaction disappeared when applied to distances different from zero ($P = 0.224$) and also steeply declined after removing the single "gap 0 distance 0" class ($P = 0.066$), showing that the pollination on the very first crop row, when two varieties are in direct contact, contributes mainly to the interaction between distance to field edge and gap size.

Test that the gap effect is not a side-effect of a change of the source to recipient ratio between two crop varieties

The ANOVA for experiment 2, plot I (Table 4) varying the GlyR area depth (Fig. 2), enabled me to test for a significant effect of changing the donor-to-recipient ratio on gene flow levels. Changing the crop area depth of the GlyR variety from 24 m to 6 m did not result in increased pollen movement from the GluR variety to the GlyR variety as recipient (test hypothesis $P = 0.626$); nor did it decrease gene flow in the reverse direction from GlyR as the source to GluR as the recipient variety (test hypothesis $P = 0.736$).

Incorporating the gap effect into models linking gene flow to isolation and position within field

On the immediately adjacent, no-gap, control crops for both experiments, the R^2 values all exceeded 0.88 whatever the equation used to model the decrease of cross pollination with distance (Table 5, part a). Introducing the gap effect into the models maintained the R^2 within a range of 0.87 to 0.98. Parameter C, measuring the contribution of the gap, was insensitive to changes in the equation (Table 5, part b) and data set (part e) as none of the differences were substantial enough to diminish overlap between respective 95% Wald confidence limits. With a single exception, all models contained the value 3.4 for parameter C. They out-performed the single reference model incorporating both above crop and gap distances (Table 5, part c). Finally, the parameter C was robust to the simulated changes of possible paths followed by pollen (part d).

Discussion

Theoretical consideration of the effect of a gap between otherwise adjacent canola crops on gene flow

Partitioning a gap between otherwise adjacent canola fields into several distinct effects

One of the reasons this study was conducted was to obtain data on the effect of a gap on gene flow between otherwise adjacent fields. It is self-evident that a gap between flowering plants capable of cross pollination will affect gene flow for at least two reasons. Firstly, a barren gap enables free passage of both passive pollen transport and all pollinators (e.g., bees, etc.). This facilitate access to flowers fronting the gap may not necessarily increase exchange as pollinators may preferentially visit neighbouring flowers (bearing convarietal pollen) as opposed to those across the gap. Secondly, as a barren gap is also a different micro-environment compared to the crop, this micro-environment change may itself indirectly modify cross-pollination probabilities via three distinguishable elements. (1) Convarietal pollen load will be smaller at and near the field margins (bordering the gap). Whilst this is not limiting seed set, plants will be prone to compensate with more self pollination or to receive pollen from further away. This effect could thus interact with field plot size. (2) Direct modifications to microclimatology may occur so that air movement and thus pollen transport is affected (McCartenay 1997; Tufto et al. 1997; Nurminiem et al. 1998; Reynolds 2000) irrespective of field plot size. (3) Obstacles to foraging movements of any insect pollination vectors visiting the flowers may occur; the gap may either enhance or reduce exchange depending on whether the insect stops or moves across when fronting the gap. As these effects possibly combine or interact in some way, it is speculative whether direct versus indirect, local versus extended, passive versus active facets of the pollen transport, according to the presence of the gap, preclude any clear and general expectation on cross-contamination between adjacent margin sites.

Where total pollen amount is limited, a gap between crops may enhance pollen exchange because the gap may enable, and even favour, long-distance pollination events as observed by Manasse (1992) in *Brassica campestris*. His "unexpected" increase in gene flow with a 4-m isolation distance (instead of 0.5 m) could be explained by pollinators having to travel longer distances to complete their foraging tour. This effect should thus disappear when flowers become locally abundant to pollinators. Another mechanism that may influence cross pollination has been pointed out by Hokanson et al. (1997) when analysing the effect of varying source plant to recipient plant ratios. Using *Cucumis sativus*, they found that as source/recipient ratios increased, more pollen escaped – a result of an overloading of the sink plants with source pollen. Accordingly, there could be an indirect effect of the gap that only depends upon its associated modification of the local source/recipient ratio. Here again, this effect would be enhanced as the field plot size becomes smaller and isolated.

With two fields of normal agronomic size where pollen is abundant, predictions as to gene flow could markedly differ (compared to the pollen-limited situation as described above). However, the gap will still first influence the relative importance of convarietal (local) over intervarietal (across the gap) pollinations. The presence of a gap is expected to cause increased pollination between plants on the same side and, therefore, decreased pollination from plants across the gap. With respect to the behaviour of insects, the predictions of their movements in response to the presence of a gap between crops depends on a major question: what is the threshold gap distance that will be an impassable barrier for a particular pollinator species? Beyond such a gap threshold, insects would be expected to remain within one field. Should this occur, pollen transport across a gap would be drastically reduced. Conversely, in a situation where the gap distance is much below the threshold, insects would be expected to continue their foraging tour and, by

"jumping" the gap, move pollen to the adjacent field. Thus, via insects, gap size may either enhance or reduce gene flow irrespective of the abundance of flowers. Literature on this subject exists, but it is focused mainly on insect foraging strategy rather than on the plant reproductive perspective with no cited threshold estimate. In general, we may expect an increased cross pollination across small gaps and a decreased exchange across large gap distances. Therefore, the final outcome of the physical and biological repercussions of a gap effect on the level of expected gene flow between two fields is complex, depending upon possible interactions between factors (e.g., wind reducing insect foraging activity while increasing pollen flow across a gap) and on the stability over time of the factors themselves.

Experimental results of the effect of a gap on gene flow between otherwise adjacent canola crops

Despite the complexities discussed above, a gap effect can be easily studied and experimentally tested for consistency over years, sites and crop varieties. Canola may be considered as a good model crop plant having intermediate selfing and mixed wind and insect pollination (ambophily), thus embracing most of the complexities described above. Using the herbicide-resistant transgenes as easy selectable markers of effective cross pollination between two otherwise adjacent canola fields, I found that a gap between fields significantly increased gene flow between plants bordering a gap above the level observed at comparable distances within a continuous crop. Figure 3 illustrates that, within the range of values tested, the gap partly tends both to "reset" the zero point of the gene dispersal profile to the end of the gap zone and slightly increase cross-contamination on more internal sites. Even if observed pollen movement mix two or more distinct effects, resulting gene flow best follows the intermediate pattern evident in hypothesis 3 (Fig. 1). Changing the gap distance over the range 0 m to 12 m did not change the bias introduced by such a barren zone on the amount of gene flow. Thus, the direct outcome is that the isolation introduced between two crops by the presence of a gap could therefore be less efficient than actual real distance predicts. From Fig. 3 it is also clear that the effect of a gap is not restricted to the first immediate border row but also affects internal crop rows as I measured up to 7 m inside each field. If this slow decline when there is a gap also affects the crop beyond 7 m, it may induce an absolute rather than a negligible increase of cross-contamination when the seed production of the entire field is taken into account.

Still, the interaction between the gap size and the position of the sink plant inside the sink field on cross-pollination levels tends to depend largely upon the first crop rows. Consequently, the interaction effect decreases if the first row is removed from the analysis (see Table 4). Such a pattern would best fit an explanation of both a direct, gap-induced, increased access to external pollen, visits by pollinators and/or a more complex general gap effect encompassing a larger area within each field. The gap effect is thus not linear because of the short-distance and limited interaction. The interaction between gap size and distance to border was observed with both crop varieties but not over both years. The geographic distance between the field sites was only 15 km and thus the environment was similar and did not cover a wide range of factors such as wind direction, general climate conditions or pollinator types and availability, thereby limiting wide extrapolation to other crops or situations. The significant interaction between varieties and distance, as analysed in Tables 3 and 4, may be considered the consequence of some biological characteristics varying according to variety – i.e., total pollen production, viability over time and the size of individual or clustered pollen grains.

The range of gaps taken into account in this study were chosen to represent the types of gaps introduced by paths or small roads separating crop fields. Over this medium range of gap distances, our data fitted the patterns of gene flow decrease with distance that are evident in the literature. Of similar importance in decreasing cross pollination was the gap effect. I have presented a simple way of taking the gap into account by adding a parameter describing the relative transport achieved above the gap compared to above the crop. As I may, by chance, have limited my investigation to conditions where only an increase could be expected to happen, I emphasise that over longer distances this could be misleading (Lavigne et al. 1996, 1998).

As gene flow experiments of the type presented here are difficult to conduct, there are few comparisons in the literature. Analysing different methods designed to limit gene exchange between adjacent fields, Morris et al. (1994a, b) also found that barren zones – gaps 4–8 m in width – could increase gene flow over the levels expected if the intervening ground was a trap crop. However, their data were equivocal on the effect of "trap crop" finding opposite tendencies according to size of the trap. Pedersen et al. (1969) with both experimental alfalfa fields and computer simulation also addressed, within other factors, the question of the rate of gene flow with respect to isolation distance. Their model clearly predicted that edge contamination over different isolation distances would be higher than over similar distances between donor and recipient plants within a field. Our data correctly fitted their equation (Table 5) although we could also slightly outperform this fit ($R^2 = 0.862$ against 0.882, respectively) and take the gap into account with higher accuracy (confirmed by the lower asymptotic standard error/parameter percentage of 7.095 on our parameter C compared to 15.602 for Pedersen's parameter d).

It has been suggested that isolation by distance may be an unreliable method of controlling pollen-mediated gene flow from plots (Downey 1999). My work suggests that a gap is enough to induce increased exchange, even in situations of no general (but perhaps still local) pollen

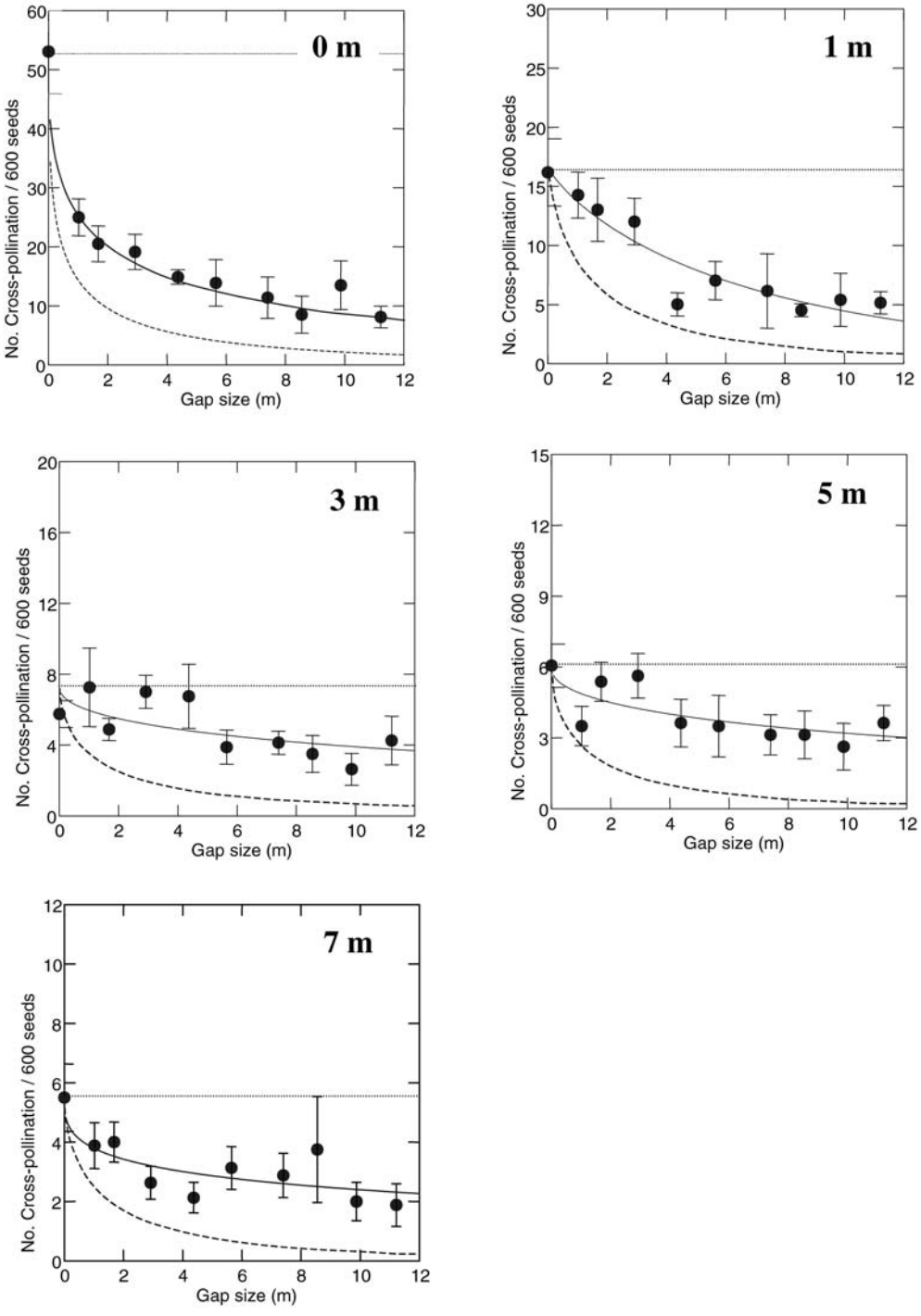

Fig. 3 Gap effect on cross pollination at different positions within the field from the immediate margin up to 7 m inside the crop. Each graph represents a set of samples taken at a fixed distance (0, 1, 3, 5 and 7 m) from the edge of the field margin between the two varieties, notwithstanding the existence of a gap. Three curves are presented in each graph and represent the expected results based on hypotheses 1, 2 and 3. The *dashed line* fits hypothesis 1 where samples taken at the same distance to the nearest donor should give similar cross rates. The *dotted line* fits hypothesis 2 where samples taken at the same distance to their own field edge would give similar rate of outcrossing no matter how large the gap. The *solid line* fits hypothesis 3 with contamination rates being intermediate between the expectations for hypothesis 1 and 2. Parameters used to fit the curves are as given in Table 5

limitation. The lowest observed decline of cross contamination linked to the presence of the gap is not, in the present investigation a side effect of changing the source-to-sink ratio. In contrast to Hokanson et al. (1997), the part of the experiment specially devoted to test the change in trap receiver/donor ratio denoted no significant effect, while the range of change of the ratio was rather substantial. An explanation for the inconsistency of my data with that of Hokanson's could be related to the experimental design. Contiguous plots varying in size are not equivalent to a series of isolated experiments: cross contamination (of convarietal pollen) between neighbouring experiments would remain a possibility, although the central position of the samplings within each large (at least 15 m) block would reduce this risk. Rather, the absence of a donor/receiver ratio effect was consistent with my expectation that the change in the ratio would only have a detectable effect on final cross-pollination rates in a situation where pollen becomes limited. Measurements with male-sterile plants

dispersed around the field gave the 100% expected amount of seed according to the development of their flowering architecture (data not shown). Thus, with respect to this aspect of pollen limitation, the data are among the very first to examine what could be considered a "real" agronomic situation with a clear-cut answer on gene flow across a gap. Therefore, the increased exchange observed in other experiments on small isolated plots used as receivers (Klinger et al. 1991, 1992; Rognli et al. 2000), which was also observed here between entities of greater size, is not a straightforward observation. It could be believed that as this increase is, on absolute value, mainly reflected in the border rows of the crop, the impact is not great. I have not attempted to obtain a whole-field estimate of cross-pollination frequency to see if what was measured up to 7 m inside each field held true at longer distances. However, a first approximate value derived from Fig. 3 lets me conclude that about twice the amount of exchange may be expected; a slight increase when very low rates are expected. As such "excess" cross induced by a gap could perhaps be extended to any non-pollen-limited situation, the first possible recommendation to limit contamination between fields would be to remove the first rows after flowering completion, rather than requiring the same empty space to ensure isolation. This previously documented strategy should remain the most efficient means to limit gene flow by favouring exchanges over the shortest distances. This will statistically limit the long-distance, random rare gene flow induced by the necessity for a cross-pollinated plant to find a partner in order to produce seed (Taylor et al. 1999; With and King 1999; Richards et al. 1999). My data suggests that, although remaining imperfect, the strategy of avoiding gaps between donor and receiver fields seems to be, for short distances, the most efficient "trap" to limit gene flow. The data describing the decrease of pollen exchange with distance for many crop species could be used to determine, by empirical law between benefit in seed purity and crop loss cost, the range of rows to be removed.

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