Outcrossing frequencies and distribution of transgenic oilseed rape (*Brassica napus* L.) in the nearest neighbourhood

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Abstract

The outcrossing of transgenic oilseed rape in the neighbourhood is of major concern with regard to the actual EU labelling threshold of 0.9% for transgenic contamination in food and feed and the regulations for the co-existence of cultivation of genetically modified crops, conventional and organic farming. In a two-year field trial, the outcrossing frequencies and distribution from plots with different ratios of transgenic plants (100%, 1.0% and 0.1%) containing the *pat*-gene for resistance towards the broad-range herbicide glufosinate-ammonium were determined in surrounding acceptor plots within a distance of 3–11 m. Randomly distributed outcrossing with isolated pollination events became apparent, and an average gene flow of 0.28%, 0.01% and 0.0065% was detected for 100%, 1.0% and 0.1% transgenic donor plots, respectively. Significant effects on the distribution of outcrossing were found for distance, but not for the prevailing wind direction. The random distribution in combination with the behaviour of honey-bees and bumble-bees gives strong evidence that insects play an important role for short distance gene dispersal. A curve fit assuming an exponential decline was performed with the experimental outcrossing data as a function of distance and was applied to the actual EU labelling threshold. The contamination limit of 0.9% in food and feed could be kept without cultivation distances to the transgenic source. The impact of effective detection limits of analytical methods on the establishment of specific rules for the co-existence is discussed.

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1. Introduction

Since the introduction of genetically modified (GM) plants for commercial production in 1996, the global area of GM crops has continued to grow for the seventh consecutive year to 67.7 million hectares in 2003 (James, 2003). Most of the GM plants were grown in six countries (United States: 63%, Argentina: 21%, Canada: 6%, Brazil: 4%, China: 4% and South Africa: 1%), whereas in the European Community the cultivation was hindered by a de facto moratorium on new GM crop approvals. Recently, new EU regulations (Nos. 1829/2003 and 1830/2003) concerning the traceability and labelling of GM food and feed products were passed by the European Parliament and the Council. Labelling is not obligatory for food and feed products with GM proportions below 0.9% of the ingredients considered individually or products consisting of a single ingredient, provided

that this presence is adventitious or technically unavoidable during seed production, cultivation, harvest, transport or processing.

In addition, a recommendation on guidelines for the development of national strategies and best practices was published by the European Commission involving cultivation distances between GM crops and non-GM crops, buffer zones, cropping intervals, the control of volunteer plants, etc., to ensure the co-existence of GM crops with conventional and organic farming. Particularly for oilseed rape, which can be described as a high-risk crop for crop-to-crop gene flow due to cross pollination by the vectors insects and wind, specific rules for cultivation are discussed.

A number of studies already examined the relation between distance and outcrossing of oilseed rape in the neighbourhood (Table 1). In the case of pollination of male sterile ‘acceptor plants’ the outcrossing rates are always significantly higher than in the case of male fertile ‘acceptor plants’. Timmons et al. (1995) found a 0.08% gene flow at a distance of 2500 m from the transgenic source using emasculated bait plants. These studies, however, only present the potential

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Table 1
Outcrossing rates from oilseed rape at different distances to the pollen source

Distance (m)	Outcrossing rates (%)											
	Male sterile acceptors		Male fertile acceptors									
0												
0.3												
0.6												
1												
2.5												
3												
4												
4.6												
5												
6												
6.5		28.50										
7.5		3.75										
8												
10												
12												
14												
15												
16												
20												
24												
25												
30												
32												
34												
36												
42												
47												
50												
54												
56												
100												
200	1.2–21.0	0										
150		.83										
400												
References	Pfeilstetter et al. (1998)	Feldmann (2000)	Scheffler et al. (1993)	Morris et al. (1994)	Pauk et al. (1995)	Scheffler et al. (1995)	Wilkinson et al. (1995)	Brown et al. (1996)	Downey (1999)	Simpson et al. (1999)	Feldmann (2000)	Staniland et al. (2000)

of pollen-mediated gene flow. Differing in the extent of the outcrossing rates, most of the small-scale release experiments usually showed a dramatic decrease with increasing distance from the transgenic pollen source. Both insects and wind can play a role as vectors in dispersal of oilseed rape pollen, although their contribution on transgenic gene flow is discussed controversially. Particularly honey-bees and wild bumble-bees are important pollinators of oilseed rape (Ramsey et al., 1999). By removing the petals from male sterile trap plants in order to make them less attractive to honey-bees, Timmons et al. (1995) showed a significant influence of the wind on the frequency of pollination.

The main objective of this study was the examination of short distance outcrossing of transgenic oilseed rape in the neighbourhood. The experimental design allowed the detailed determination of the effects of distance and wind direction on pollination frequencies and distribution. In addition, outcrossing from plots containing only 1.0% and 0.1% transgenic plants (simulation of transgenic seed contaminations) was investigated in view of co-existence of GMO, non-GMO and organic production.

2. Materials and methods

2.1. Plant material

The oilseed rape (*Brassica napus* L.) varieties used in the field trial were the conventional winter variety ‘Falcon’ and the isogenic transformation line ‘Falcon GS40/90’ which is tolerant towards broad-range glufosinate-ammonium herbicides (trade names BASTA® or Liberty®) due to the integration of the synthetic phosphinothricin acetyltransferase (*pat*) gene derived from *Streptomyces viridochromogenes*. The transgenic plants were used in ‘contamination plots’ as source of transgenic pollen (‘donors’). The non-transgenic variety ‘Falcon’ was used in surrounding plots (‘acceptors’) to determine the outcrossing frequencies from transgenic oilseed rape caused by transgenic pollen. Seeds were kindly provided by NPZ (Norddeutsche Pflanzenzucht, Holtsee, Germany).

2.2. Experimental design

The field experiments were carried out on an experimental station near Munich (Germany) in the vegetation periods 2001/2002 and 2002/2003. Each transgenic donor plot was surrounded by eight acceptor plots with non-transgenic plants. Plot size was 6 m × 6 m with 50 plants/m². The distance between donor and acceptor plots was 1.5 m (Fig. 1).

In 2002/2003, the donor plots containing 100% transgenic plants were grown in two replications. In 2002/2003, the donor plots contained different ratios of transgenic plants (100%, 1.0% and 0.1%) in order to simulate transgenic seed contaminations.

Wind direction was recorded permanently during the whole flowering time by an anemometer (Model 1482, Lambrecht GmbH, Göttingen, Germany). The field trial was located on plane farmland. The predominant soil type was sandier clay. In the vicinity of the field trial, there were several bee hives. In the north (distance 1 km) about 10 bee colonies, in the south

(distance 500 m) 8 bee colonies and in the west (distance 1 km) 5 bee colonies.

2.3. Seed sampling and testing

A grid with 25 sampling points was laid over each of the surrounding non-transgenic acceptor plots. 30 mature pods were collected randomly at each sampling point resulting in a total of 750 pods per plot. For each sampling point, 320 seeds were sown in plastic trays (29 cm × 46 cm × 5 cm) containing a peat-sand substrate in the greenhouse. Considering a germination capacity of 95%, the technically detectable outcrossing frequency at each sampling point was 0.33% (1 transgenic out of 300 seedlings).

After one week, the seedlings were sprayed with a 1% BASTA® solution (200 g glufosinate-ammonium/l). Resistant plants could be distinguished after two weeks from non-transgenic plants. In order to exclude false-positives (i.e. non-transgenic survivors), all putative transgenic seedling were retested by PCR. Genomic DNA from seedlings was extracted and purified with a NucleoSpin Multi 96 Plant Kit (Macherey-Nagel, Düren, Germany) as described by the manufacturer. PCR was carried out on a Perkin-Elmer 9600 thermocycler (Applied Biosystems) in a 20 µl reaction volume containing 10 ng of genomic DNA, 0.5 units of Taq DNA polymerase (Qiagen), 200 µM of each dNTP, 0.2 µM of each oligonucleotide and 1.5 mM MgCl₂ in a 1 × PCR buffer (Qiagen). Cycling conditions were as follows: 2 min initial denaturation at 95 °C, 35 cycles of: 30 s at 95 °C, 1 min at 60 °C and 72 °C for 2 min, terminated by a final extension step at 72 °C for 10 min. PCR amplicons were separated on 1.5–2.0% agarose gels and visualised on an ultra-violet transilluminator after staining with ethidium bromide.

For the construct-specific detection of the *pat*-gene the primer pair *pat.f* 5′-CACAATCCCCTATCCTTCGC-3′ and *pat.r* 5′-TGCTGTAGCTGGCCTAATCTCA-3′ were used targeting the 35S-*pat* junction region. PCR control reactions were performed with the primer pair *s.gt.f* 5′-CAA-AGACGATAAAGGCTACGGC-3′ and *s.gt.r* 5′-TAATGCTCC-GATCAGAGCTTCC-3′ for the *Brassica* specific nucleotide sequence of the *S*-glucosyltransferase gene according to Block and Schwarz (2003).

2.4. Data analysis

The outcrossing frequencies from the particular transgenic plots in the respective surrounding non-transgenic plots were calculated by dividing the number of transgenic seedlings by the total number of tested seeds.

ANOVA for the comparison of the means of outcrossing frequencies in replicated plots, χ^2 -tests, and correlation analyses were performed using SPSS software Version 11.5.1 for Windows (SPSS, Chicago). The χ^2 -test values were calculated as follows:

$$\chi^2 = \sum_{i=1}^k \frac{O_i - E_i^2}{E_i}$$

Fig. 1. Schematic representation of the experimental design. One transgenic donor plot is surrounded by eight non-transgenic acceptor plots. Plot size is $6\text{ m} \times 6\text{ m}$, distance between plots is 1.5 m. Dots within the acceptor plots indicate the sampling points. Circles illustrate the distances to the donor plot.

O, observed values—outcrossing events detected in each of the eight surrounding acceptor plots (corresponding to wind directions); *E*, expected values—theoretical values of outcrossing events due to the wind distribution during flowering time in each of the eight surrounding acceptor plots (corresponding to wind directions); *k*, number of classes—number of surrounding acceptor plots (corresponding to wind directions).

Curve fitting for the relationship between outcrossing frequency and distance was performed by linear least-squares regression using SigmaPlot Version 6.0 for Windows (SPSS, Chicago).

3. Results

In 2001/2002, outcrossing frequencies and distribution from two 100% transgenic plots were investigated. Transgenic and non-transgenic plants flowered synchronously during a period of approximately 20 days.

In total, 120,000 seedlings from surrounding acceptor plots were tested in the greenhouse for glufosinate-ammonium resistance, and 409 individuals were found to be *pat*-positive. In order to exclude seedlings which had survived the application of BASTA[®] without being transgenic, a subsequent PCR analysis was performed with *pat*-specific primers. PCR reactions for the exclusion of false-positives carried out with DNA from 347

seedlings resulted in the amplification of a *pat*-specific DNA fragment with the expected size of 141 bp, while 62 seedlings (15%) showed no signals and were therefore classified as false-positives. The average gene flow from each of the two 100% transgenic plots was 0.29%. The majority of measuring points (60.5%) showed outcrossing rates below 0.33% (Table 2). Fig. 2A and B illustrates the spatial distribution of the outcrossing events around the two 100% transgenic plots. The peaks represent the extent of outcrossing in the surrounding acceptor plots. The maximum number of transgenic progeny at one single measuring point was 5.0% (Fig. 2B). For both 100% transgenic plots, a random distribution with isolated pollination events became apparent.

In 2002/2003, the experimental design was repeated with a single 100% transgenic plot due to labour and space limitations. In contrast to 2001/2002, the period of flowering of approximately 14 days was considerably shorter. Out of 60,000 seedlings from the acceptor plots 154 transgenic plants were confirmed by the application of BASTA[®] and subsequent PCR analysis. The discrepancy between the results of greenhouse and molecular tests was even higher than the year before (35%). The high number of false-positive seedlings is not surprising because due to the high throughput of tested seedlings great variances are inevitable. Biotests are subject to many influencing factors like plant density, temperature or herbicide concentration. Therefore,

Table 2

Cumulative outcrossing frequencies from the transgenic donor plots with the number of observed single outcrossing rates

Transgenic donor plots (%)	Replications	<i>n</i>	Single outcrossing rates (%)	Cumulative (%)
100 (2001/2002)	2	1	>5.00	100.0
		21	<5.00	99.7
		20	<1.00	94.5
		116	<0.67	89.5
		242	<0.33	60.5
100 (2002/2003)	1	1	>5.00	100.0
		8	<5.00	99.5
		10	<1.00	95.5
		54	<0.67	90.5
		127	<0.33	63.5
1.0 (2002/2003)	2	0	>5.00	0.0
		0	<5.00	0.0
		1	<1.00	100.0
		9	<0.67	99.75
		390	<0.33	97.5
0.1 (2002/2003)	2	0	>5.00	0.0
		1	<5.00	100.0
		1	<1.00	99.75
		3	<0.67	99.5
		395	<0.33	98.75

the additional verification by PCR was essential. An average gene flow of 0.26% was calculated with the majority of measuring points (63.5%) being below 0.33%, and thus similar to the year before. The distribution of pollination is shown in Fig. 2C. To simulate transgenic seed contaminations, outcrossing from each of two plots with 1.0% and 0.1% transgenic plants was also examined. Regarding the 1.0% and 0.1% transgenic plots, a marginal average gene flow amounting to 0.01% and 0.0065%, respectively, was determined (Table 3). The rare outcrossing events detected in the acceptor plots were randomly distributed and are illustrated in Fig. 2D–G.

One-way analyses of variance were conducted to compare the means of outcrossing frequencies from replicates of 100%, 1.0% as well as 0.1% donor plots. Between the means of the replications of 100% ($p=0.477$) and 0.1% ($p=0.833$) donor plots, respectively, no significant differences were found, whereas for replication of 1.0% donor plots outcrossing frequencies were different at $p=0.012$.

In order to test the significance of wind direction on the distribution of outcrossing events in neighbouring plots, a χ^2 -test for goodness-of-fit was performed by comparing observed data with the theoretically expected number of transgenic progeny, which were derived from the wind distribution during flowering time (Tables 4 and 5). With respect to a χ^2 -value of 14.07 for eight classes of wind directions at a probability level of 0.05, no significant influence of wind on the distribution of transgenic outcrossing was found.

The overall means of outcrossing events from the 100% transgenic donor plots were plotted against the distance. The pollination declined rapidly with distance, and the highest standard deviations were observed at a distance of 3 and 4 m (Fig. 3). The effect of the distance on the number of outcrossing events was found to be highly significant ($p<0.0001$) by correlation analysis. Assuming an exponential decrease of outcrossing, a curve fit was created. Nonlinear least-squares regression for the equation $y=a \cdot e^{-bx}$ resulted in the best fit

Table 3

Outcrossing rates from transgenic donor plots with different ratios of transgenic plants in 2001/2002 and 2002/2003

Transgenic donor plots (%)	2001/2002		2002/2003	
	Outcrossing rates from single plots (%)	Average outcrossing rates (%)	Outcrossing rate from single plots (%)	Average outcrossing rates (%)
100	0.29 0.29	0.29	0.26 –	0.26
1.0	– –	–	0.002 0.018	0.01
0.1	– –	–	0.005 0.008	0.0065

Fig. 2. Distribution of the outcrossing events in non transgenic acceptor plots. (A and B) 100% transgenic donor plots 2001/2002; (C) 100% transgenic plot 2002/2003; (D and E) 1.0% transgenic donor plots 2002/2003; (F and G) 0.1% transgenic donor plots 2002/2003.

($R^2 = 0.58$) for the parameters $a = 0.01828$ and $b = 0.2422$. The fitted curve is displayed in Fig. 3.

4. Discussion

Pollen dispersal from transgenic oilseed rape can lead to unintended gene flow to neighbouring non-transgenic oilseed rape crops. This is of major concern especially if outcrossing

causes quality losses in conventional crops by exceeding the actual threshold of 0.9% for transgenic contaminations in food and feed. This study focussed on the determination of outcrossing rates from transgenic oilseed rape in the nearest neighbourhood. The experimental design and the extensive drawing of samples in the acceptor plots allowed the detailed analysis of the outcrossing pattern with respect to the param-

Table 4

Wind distribution during flowering time, observed and expected outcrossing rates, and χ^2 -test values for the single transgenic plots with the respective ratios of transgenic plants in 2001/2002

Outcrossing events in 100% transgenic plots 2001/2002							
Wind		Replication 1			Replication 2		
Wind direction	Wind distribution (%)	<i>O</i>	<i>E</i>	χ^2	<i>O</i>	<i>E</i>	χ^2
NW	2.2	23	3.8	305.4	5	3.7	317.1
N	2.3	7	4.1		34	4.0	
NE	7.9	7	14.0		13	13.6	
E	36.8	37	64.9		24	63.0	
SE	24.1	9	42.5		14	41.3	
S	8.7	31	15.3		27	14.8	
SW	11.4	10	20.1		23	19.5	
W	6.5	52	11.4		31	11.1	
Sum	100	176	176		171	171	

O: number of observed outcrossing events; *E*: number of expected outcrossing events; if the calculated χ^2 -value exceeded $\chi^2_{7;0.05} = 14.07$, then there was no statistical evidence for a relation between the number of observed and expected outcrossing events.

Table 5

Wind distribution during flowering time, observed and expected outcrossing rates, and χ^2 -test values for the single transgenic plots with the respective ratios of transgenic plants in 2002/2003

Outcrossing events 2002/2003																
Wind		100%			1%			0.1%								
Wind direction	Wind distribution (%)				Replication 1			Replication 2			Replication 1			Replication 2		
		<i>O</i>	<i>E</i>	χ^2	<i>O</i>	<i>E</i>	χ^2	<i>O</i>	<i>E</i>	χ^2	<i>O</i>	<i>E</i>	χ^2	<i>O</i>	<i>E</i>	χ^2
NW	6.2	12	9.3	311.6	0	0.1	15.0	5	0.7	45.6	0	0.2	9.0	0	0.3	15.0
N	5.6	19	8.4		0	0.1		0	0.6		0	0.2		0	0.3	
NE	26.4	6	39.4		0	0.3		1	2.9		0	0.8		0	1.3	
E	24.9	27	37.1		0	0.2		1	2.7		3	0.7		1	1.2	
SE	16.6	12	24.8		0	0.2		0	1.8		0	0.5		0	0.8	
S	2.7	30	4.0		0	0.0		2	0.3		0	0.1		0	0.1	
SW	11.3	6	16.8		0	0.1		0	1.2		0	0.3		3	0.6	
W	6.2	37	9.3		1	0.1		2	0.7		0	0.2		1	0.3	
Sum	100	149	149		1	1		11	11		3	3		5	5	

O: number of observed outcrossing events; *E*: number of expected outcrossing events; if the calculated χ^2 -value exceeded $\chi^2_{7;0.05} = 14.07$, then there was no statistical evidence for a relation between the number of observed and expected outcrossing events.

Fig. 3. Averaged outcrossing events as a function of distance from the 100% transgenic plots. The best linear least-squares regression curve fit for the relationship is shown. Standard deviations are represented with error bars.

eters wind and distance. In this study, the replicated average outcrossing rates within a distance of 3–11 m from 100% transgenic plots were consistent and ranged from 0.26 to 0.29%. The transgenic contamination in neighbouring oilseed rape crops therefore was clearly below the EU labelling threshold of 0.9%.

The combination of biotest and subsequent PCR analysis was found to be very suitable for the detection of outcrossing events. Despite the high number of false-positives the screening in the greenhouse by spraying the seedlings with the herbicide BASTA® was very effective because large numbers of seeds could be tested. Without the subsequent PCR testing the detected outcrossing rates from the 100% transgenic plots would have been 0.34% in 2001/2002 and 0.4% in 2002/2003, respectively. The reason for the occurrence of false-positives might be the covering of non-transgenic seedlings through leaves of other seedlings during spraying.

Isolated and randomly distributed cross-pollination events were found to be irrespective of the wind direction during

flowering time. Random and undirected gene flow can be explained by insect activity, since honey-bees (*Apis mellifera* L.) and bumble-bees (*Bombus terrestris* L.) play an important role for cross-pollination. Our observations are supported by results of other outcrossing studies with oilseed rape (Scheffler et al., 1993, 1995), which observed no directional effects that could be ascribed to wind activity. Thompson et al. (1999) found the occurrence of pollination events in the absence of high levels of airborne pollen and concluded that insects play an important role also for gene flow over greater distances. The isolated outcrossing events found at particular sample points of the acceptor plots especially from the 1.0% and 0.1% donor plots can therefore be traced back to insect behaviour.

The distance from the central 100% transgenic donor plots was proved to affect the outcrossing rates significantly. We observed an exponential decline within a distance of 3–11 m. These findings are in accordance with the results of several pollen dispersal studies with transgenic oilseed rape (Lavigne et al., 1996, 1998; Thompson et al., 1999; Staniland et al., 2000; Reboud, 2003) and would therefore rather indicate that wind is the predominant vector for pollination. But as wind direction was shown to have no significant influence on the distribution of outcrossing events there is strong evidence that outcrossing events in short distances are mainly due to insect activities.

Several studies showed that pollen will predominantly be deposited by bees on plants close to the pollen source (Thomson and Thomson, 1989; Cresswell, 1994). Therefore, bees are likely to forage within a small area in and around the donor plots. A hitherto disregarded factor for pollination is the widespread pollen beetle, whose behaviour can also be assumed to contribute particularly to short distance cross-pollination.

No negative exponential decrease was found when gene flow was examined between commercial canola fields in Australia at a landscape level (Rieger et al., 2002). The randomness of long distance pollination events was explained by multiple pollination vectors of oilseed rape and the large sizes of the donor fields.

In order to predict the gene flow from the 100% donor plots, we performed a curve fit with the experimental data of pollination as a function of distance. The highest standard deviation in gene flow was detected in the direct proximity of the source plot. The variation of outcrossing within a few meters and exponential decline can also be ascribed to the bee behaviour during foraging as mentioned before.

For regulations of co-existence of GM crop cultivation with conventional and organic farming, the relationship between distance and outcrossing is of major interest. In order to reduce outcrossing, barren zones or gaps and the cultivation of trap crops are discussed. For short isolation distances, trap crops between transgenic and non-transgenic varieties were found to be most effective for the reduction of transgenic gene escape (Morris et al., 1994; Reboud, 2003). Another, so far neglected factor for the establishment of specific rules concerning co-existence between GM and non-GM crops is the effective detection limit of analytical methods for quantitative GMO diagnostics. This limit is caused by the amount of DNA that is introduced into the PCR reaction and depends on the genome size of the investigated species.

The DNA content of the haploid complement of an organism is defined as the 1C value. In case of oilseed rape, up to 81,633 copies of the haploid genome are present in PCR, given from the haploid genomic weight of 1.225 pg (Arumuganathan and Earle, 1991) and a typical 100 ng genomic DNA sample. A single copy of the haploid genome is present at a level of 0.001%. Therefore, using the curve fit equation derived from outcrossing data from the 100% transgenic plots in this study, transgenic contaminations cannot be reliably detected at distances greater than 30 m, making recommendations postulating cultivation distances of 200–500 m questionable. Applying the fitted curve, a distance as low as 1.1 m would already be sufficient to comply with the actual threshold of 0.9% for GMO contaminations of food and feed.

Many studies on outcrossing frequencies were conducted using extensive fields. In this study, the main focus was on the determination of outcrossing frequencies under small-scale field conditions in the direct vicinity of a transgenic field, as this aspect is still politically discussed. The rapidly decreasing pollen concentration with increasing distance results in a dilution of outcrossing events in larger fields. Therefore, our results cannot be transferred to conditions in large-scale experiments. Mathematical models offer a potential tool to quantify pollen-mediated gene flow between neighbouring oilseed rape fields at greater distances (Baker and Preston, 2003; Walklate et al., 2004). Provided a single source field is surrounded by non-herbicide resistant fields and outcrossing follows a Poisson distribution or a log decay curve, the average estimated frequencies of outcrossing of all models are likely to be below the current threshold for contamination of 0.9%. Nevertheless, as shown in this study the effects of the pollination vectors wind and insects are often interacting especially at short distances and can therefore not be predicted completely by models. Therefore, field trials providing real data are still necessary for the final determination of the gene dispersal of transgenic crops.

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