Pollen-mediated gene flow in maize tested for coexistence of GM and non-GM crops in the Netherlands: effect of isolation distances between fields

C.C.M. Van De Wiel[1,*], R.M.W. Groeneveld[1], O. Dolstra[1], E.J. Kok[2], I.M.J. Scholtens[2], J.T.N.M. Thissen[1], M.J.M. Smulders[1] and L.A.P. Lotz[1]

[1] Plant Research International, Wageningen University and Research Centre, P.O. Box 16, NL-6700 AA Wageningen, The Netherlands

[2] RIKILT – Institute of Food Safety, Wageningen, The Netherlands

* Corresponding author (tel.: +31-(0)317-480839; fax: +31-(0)317-483457; e-mail: clemens.vandewiel@wur.nl)

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Abstract

In 2006 and 2007, field trials were performed to study the effects of the two isolation distances indicated by the Dutch Coexistence Committee, i.e., 25 m between GM (genetically modified) and conventional maize, and 250 m between GM and deliberately non-GM (e.g., organic) maize, on pollen-mediated gene flow (PMGF) under representative agricultural conditions in the Netherlands. Each isolation distance was tested at three different locations across the Netherlands in both years. For testing PMGF with the 25 m isolation distance, GM source fields of 100 m × 100 m (1 ha) were surrounded by four equally sized non-GM receptor fields at a distance of 25 m. For testing PGMF with the 250 m isolation distance, 1-ha GM source fields were surrounded by four 50 m × 50 m (0.25 ha) receptor fields in four different directions at 250 m. For the GM source field, the maize variety DKc3421YG containing the MON810 event was used with both distances. A maize variety near-isogenic to the GM variety was grown in the receptor fields to obtain good flowering synchronicity between GM and non-GM maize and thus optimal conditions for PGMF. Levels of the transgene in grain samples from the receptor fields were measured by a validated real-time PCR (polymerase chain reaction) quantification method for the MON810 event. Analyses showed the following levels of grain admixture as a consequence of PMGF, averaged over 12 fields for each isolation distance tested: at 25 m 0.084% (individual field averages ranged from 0.009% to 0.296%) in 2006 and 0.080% (0.002% to 0.318%) in 2007, respectively, and at 250 m 0.005% (individual field averages ranged from 0 to 0.040%) in 2006 and 0.007% (0 to 0.037%) in 2007, respectively. Although weather conditions clearly differed between 2006 and 2007 (a hot and dry summer in 2006 vs. a relatively wet one with about-average temperatures in 2007), outcrossing rates did not differ significantly between these years.

Additional keywords: outcrossing, pollen gene flow, qPCR, *Zea mays*

Introduction

Pollen-mediated gene flow (PMGF) has always been an important issue in sowing-seed production, since crop production and product quality critically depend on varietal purity, and in the past small-scale studies of PMGF have taken place in many crops (e.g., review by Eastham & Sweet, 2002). In more recent years, PMGF has become increasingly relevant for crop production too because of the interest to separate genetically modified (GM) and non-GM based production in order to fulfil GM labelling requirements. In 2003, the EU decided to enact this so-called coexistence by EU recommendation 2003/556/EC (Anon., 2003), which stipulates that member states should take measures to avoid inadvertent admixture of GMOs with products from organic or non-GM conventional farming. With regard to adventitious GM presence, a threshold of 0.9% above which products have to be labelled as GM was set by the EU. As of yet, no tolerance thresholds have been set for sowing-seed lots. The EU has transferred the decision on specific measures guaranteeing coexistence to the individual member states.

In the Netherlands, this need for drawing up practical measures crystallized into the inauguration of a Coexistence Committee by the Minister of Agriculture, Nature and Food Quality. The committee comprised representatives from the most relevant parties in the primary agricultural production sector, including conventional and organic farmers and representatives from the breeding industry. In 2004, the Dutch Coexistence Committee published a report proposing measures allowing coexistence between GM and non-GM crops in the Netherlands, based, amongst other things, on a review of the literature on gene flow in four relevant crops: oilseed rape, sugar beet, potato and maize (for this review see Van De Wiel & Lotz, 2006).

For maize, separate isolation distances to counter undesired PMGF were proposed for conventional non-GM and deliberately non-GM (e.g., organic) crops, i.e., 25 m and 250 m, respectively. Two different isolation distances were introduced, because in 2004 there was still uncertainty about whether the labelling threshold of 0.9% would also apply to deliberately non-GM crops. For instance, also a threshold of 0.1% was put forward as one being around the lowest quantification limit from a practical point of view. A threshold in the order of 0.1% would suggest an isolation distance of 250 m (compare the respective distances as modelled from the UK Farm Scale Evaluations (FSE) results in Henry *et al.* 2003). At the time, also additional research on the effectiveness of these distances was deemed necessary, as for the Netherlands there had been no specific studies performed on outcrossing in maize under normal agronomic conditions, and the scientific literature was limited to only a small-scale study (Meijers, 1937). Moreover, studies elsewhere at a scale representative for normal agricultural conditions had been mainly performed using GM and non-GM maize fields next to each other or a GM field completely surrounded by non-GM maize (comprehensively reviewed in Devos *et al.*, 2005). Therefore, the effect of 'empty' isolation distances, that is, without maize or any other tall crop being grown in the isolation space between two fields, had not been thoroughly tested, whereas an isolation space filled with non-GM maize clearly represents a most effective barrier by a twofold effect: (1) a mere mechanical shielding effect retarding pollen flow, and (2) its own production of pollen, which competes with incoming GM pollen for fertilization, thus reducing the rate of undesired hybridization with transgenic maize in the non-GM field.

In view of the above, field trials were performed in 2006 and 2007 to study the effects on PMGF of the two isolation distances proposed for maize in the Netherlands, i.e., 25 and 250 m. In this paper, the outcrossing levels occurring under the two distances as measured with a transgene as marker are described and discussed in relation to coexistence of GM and non-GM maize cultivation.

Material and methods

Plant material

The transgenic Bt event MON810 was used as a marker for the detection of pollen flow. MON810 has been allowed for cultivation and for use in food and feed in the EU since 1998. The maize (*Zea mays* L.) MON810 variety DKc3421YG was grown in the source fields. In the fields to be sampled as receptor, a nearly isogenic non-GM variety, DKc3420, was grown to obtain maximal synchronicity in flowering between GM source and non-GM receptor maize. With an FAO maturity rating of 240 for use as grain maize, this variety can be cultivated in the Netherlands and practically represented one of the best varieties with a MON810 variant that was available for growing under Dutch climatic conditions.

Experimental design

For each isolation distance, trials were performed at three different locations across the Netherlands, both in 2006 and 2007 (Table 1). The trials were mostly located on farmers' land, except for one located on land of Wageningen University and Research Centre at Lelystad, Province of Flevoland. For the 25 m isolation distance, the MON810 variety DKc3421YG source fields of 100 m × 100 m (1 ha) were surrounded at all four sides by equally sized non-GM receptor fields at a distance of 25 m. For the 250 m isolation distance, the 1 ha DKc3421YG (MON810) source fields were surrounded by 50 m × 50 m (0.25 ha) receptor fields in four different directions at 250 m. One ha is not an unusual field size in Dutch agricultural practice (although square fields do represent a simplification of the normal agricultural landscape with fields of mostly variable shapes). The receptor fields in the 250 m experiment were smaller as to better represent a worst case of small-scale production, such as may be found with sweet maize cultivation by farmers producing for an organic market. While planning the experimental locations, neighbouring farmers were consulted, according to the coexistence measures as laid down in the 2004 report by the Coexistence Committee. In this way, it was also possible to ensure that no other non-GM maize was grown within 250 m of the source fields in the 25 m trials and within 250 m of the receptor fields in the 250 m fields so as to minimize occurrence of competing non-GM pollen in the receptor fields from outside the trials. There was no soil cover or grass between the donor and receptor plots in the 25 m trials; crops between donor and receptor plots in the respective 250 m trials are specified in Table 1. According to GM cultivation rules, the locations of the GM source fields were published with maps on internet (http://www.vrom.nl/ggo-vergunningverlening).

Table 1. Location of field trials, their sowing date, soil characteristics, isolation distance and remarks on crop growth in 2006 and 2007.

Year	Location		Soil	Isolation distance (m)	Sowing date	Remarks
	Number	Place name				
2006	1	Eerste Exloermond, Community of Borger-Odoorn, Province of Drenthe.	Sand; 10% organic matter.	25	25 April	Higher level of weeds during initial crop growth; slight drought stress during flowering.
	2	Tweede Exloermond, Community of Borger-Odoorn, Province of Drenthe.	Sand; 10% organic matter.	250	25 April	Higher level of weeds during initial crop growth; slight drought stress during flowering; wheat between donor and receptor plots.
	3	Lelystad, Community of Lelystad, Province of Flevoland.	Clay; 2.5% organic matter.	250	26 April	Good crop; 25% of plants damaged at end of June, i.e., one month before onset of flowering; barley, flax or grassland between donor and receptor plots.
	4	Lage Zwaluwe, Community of Drimmelen, Province of Noord Brabant,	Clay; 47% clay; 7% organic matter.	25	4 May	Irregular initial growth due to variation in soil structure; good soil moisture regime during flowering; 20% of GM field (internal northern part) destructed during flowering.
	5	Blitterswijck, Community of Meerlo-Wanssum, Province of Limburg.	Sand; 2% organic matter.	25	27 April	Good initial growth; drought stress during flowering; 15% of GM field destructed after flowering.
	6	Wijnandsrade, Community of Nuth, Province of Limburg.	Sand; 27% clay; 2% organic matter.	250	3 May	Good crop; 20% of GM field destructed at end of flowering period; barley, wheat, potato or grassland between donor and receptor plots.
2007	1	Uithuizen, Community of Eemsmond, Province of Groningen.	Clay, 25% clay; 1.6 % organic matter.	250	1 May	Initial crop growth slow and variable due to variation in soil structure in combination with drought during germination and very wet weather later on; barley between donor and receptor plots.
	2	Eerste Exloermond, Community of Borger-Odoorn, Province of Drenthe.	Sand; 10% organic matter.	250	28 April	Initial crop growth slow and variable due to drought at sowing, but later good; sugar beet between donor and receptor plots.
	3	Tweede Exloermond, Community of Borger-Odoorn, Province of Drenthe.	Sand; 10% organic matter.	25	28 April	Initial crop growth slow and variable due to drought at sowing, but later good.
	4	Lelystad, Community of Lelystad, Province of Flevoland.	Clay; 25% clay; 2.5% organic matter.	250	26 April	Good crop; pea, wheat or flower bulbs between donor and receptor plots.
	5	Schaarsbergen, Community of Arnhem, Province of Gelderland.	Sand; 4% organic matter.	25	2 May	Irrigated before sowing because of drought; afterwards good crop growth.
	6	Tholen, Community of Tholen, Province of Zeeland.	Clay; 20% clay; 3.5% organic matter.	25	27 April	Good crop.

The maize was sown at the end of April – beginning of May at a normal density of 85,000 to 95,000 plants per hectare. Row distance was 0.75 m (for details see Table 1). The onset of both male and female flowering was assessed by scoring 40 plants in each field for the first appearance of open anthers and stigmata, respectively. Scoring was performed three times per week during the whole flowering period. Each year, the production of pollen was monitored by covering male inflorescences of 10 DKc3420 plants with bags on a field site in Wageningen, Province of Gelderland. Each day, bags were replaced and the daily pollen production was determined by weighing the dry pollen contents of each bag.

Sampling and sample analysis

For sampling, the 250 m receptor fields were divided into 16 sections and samples were taken from each section (Figure 1A), leading to a total number of 16 samples per receptor field. Within the 25 m receptor fields, samples were taken at pre-determined positions along three transects, starting at the border row closest to the GM source field and moving into the field perpendicularly to that border row (Figure 1B), leading to a total number of 21 samples per receptor field. The 25 m receptor fields were sampled more intensively (i.e., with shorter intervals) in the border region most closely to the GM source field (Figure 1B), because the highest and most variable PMGF results can be expected there (e.g., Henry *et al.*, 2003). With the considerably larger distance of the 250 m receptor fields, smaller differences between border and central parts and less regular patterns of decrease of PMGF across the fields can be expected. Therefore, samples were taken from sections covering the whole field. Because higher values could still be expected in border parts, border sections were of a smaller size than central sections to increase sampling intensity at the borders here as well (Figure 1A). Each sample consisted of five ears. The ears were dried and subsequently threshed. The total weight of the grain samples was up to 880 g in 2006 and up to 750 g in 2007. In all cases in which sample weight was more than 500 g, a subsample was taken for use in the MON810 quantification; in all other cases, the total sample was used.

For MON810 quantification, of each field sample approximately 500 g of grain, amounting to about 1500 kernels, were ground in a Retsch ZM200 mill over a 1 mm sieve and homogenized. Subsequently, DNA was isolated from 100 mg dry material of each ground sample, using the DNeasy Plant Mini Kit (Qiagen) according to the manufacturers' protocol and modifying the lysis step. The lysis step was carried out with CTAB buffer (20 g/l CTAB, 1.4M NaCl, 0.1M Tris, 20 mM Na_2EDTA, pH 8.0), instead of the manufacturers' AP1 buffer, as this improved the DNA yield. DNA contents were measured with a Thermo Scientific NanoDrop™ Spectrophotometer. Levels of the transgene in the grains were measured using a real-time Taqman PCR quantification method for the MON810 event (Anon., 2006). This method was scientifically assessed and validated by the Community Reference Laboratory for GM Food and Feed in collaboration with the European Network of National Control Laboratories, assembled in the European Network of GMO Laboratories (ENGL) and in-house validated at RIKILT – Institute of Food Safety (Wageningen, The Netherlands). For each sample, 3 separate polymerase chain reactions (PCRs) were performed using 50 ng of sample DNA per

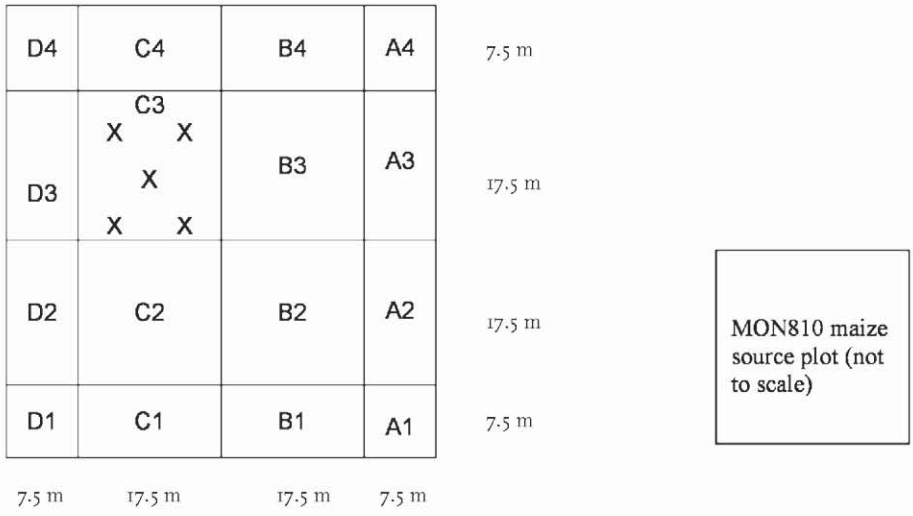

Figure 1A. Sampling scheme for each receptor field at 250 m isolation distance. There were 16 sections in total of which 4 central (C2, C3, B2, B3) and 12 border sections (A1, A2, A3, A4, B4, C4, D4, D3, D2, D1, C1, B1). X indicates locations where individual ears were taken within a section.

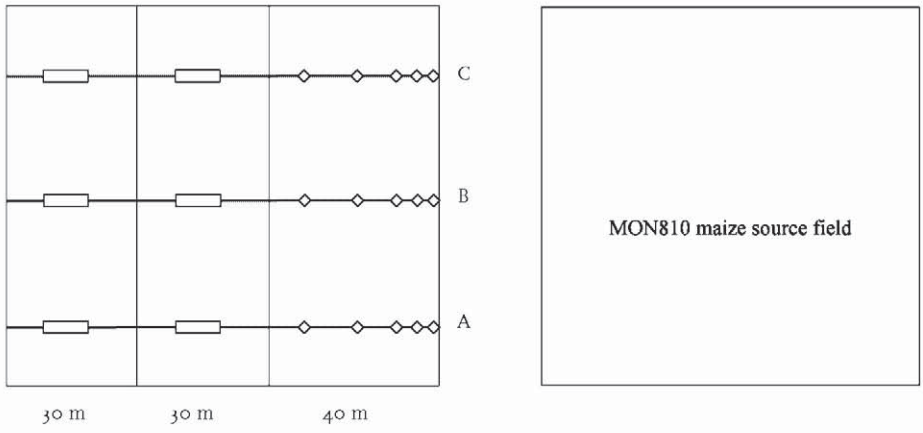

Figure 1B. Sampling scheme for each receptor field at 25 m isolation distance. Samples were taken in 3 transects (A, B, C) at distances of 0, 2.25, 5.25, 11.25, and 23.25 m from the border, and at regular intervals (6 m) across each of the last two 30-m parts of the field relative to the source field.

reaction. The reaction volume was 25 µl. Real-time PCR tests were performed on a Bio-Rad i-Cycler iQ (Bio-Rad) and analysed with Optical System Software version 3.1.

In this PCR test, a 92 bp event-specific fragment of the 5' boundary between the plant genome and the 35S CaMV (Cauliflower Mosaic Virus) promoter of the MON810 construct was amplified. A 79 bp fragment of the endogenous maize gene HMG (High Mobility Group Protein Gene) was amplified as a reference for the amount of crop DNA in a separate reaction on the same PCR plate as the respective 92 bp event, The two calibration curves were made with 5% (w/w) MON810 certified reference material IRMM ERM-BF413f (Geel, Belgium), with calibration points of 150, 37.5, 9.4, 2.3 and 0.6 ng DNA. Copy numbers of the standard curves were calculated by taking into account a 1C value for maize genomes of 2,725 pg (Arumuganathan & Earle, 1991), but without correcting for the hetero(hemi)zygotic state of MON810, with MON810 derived from the mother line, in the hybrid variety used in the preparation of the reference material (Anon., 2006). This means that the endogenous reference gene (HMG) calibration points were taken as containing 55,046, 13,761, 3,440, 860, or 215 copies and the MON810 calibration curve 2,752, 688, 172, 43, or 11 copies (that is, 5% of the reference gene copies). One calibration curve was used for the measurement of the number of MON810 copies and one curve for the measurement of the number of endogenous gene copies that were taken as representative for the number of reference assay haploid genome equivalents (HGE). The MON810 percentage was calculated as the ratio of these two copy numbers, the MON810 and the reference assay HGE, multiplied by 100. In the standard EU-validated test, a conservative estimate of the limit of quantification (LOQ) was 0.1%. With regard to reproducibility, the coefficient of variation, CV_R, was 32%. In our set-up, we did some additional tests and so were able to show that one heterozygous MON810 kernel in 1500 kernels (the approximately 500 g used in sample analysis) was readily detectable (data not shown).

Data analysis

For the quantification of field levels of GM presence in each of the 25 m receptor fields, the measurements of the samples in the three transects at the same distance were averaged after performing an analysis of variance. Next, an inverse power regression (Generalized Linear Model with Reciprocal Link and Gamma distribution) was applied with the GM level as response variable and the distance from the field border as the explanatory variable using Genstat version 10.2. The GM level in each row was estimated with the model parameters and the mean of all estimates was used as the average GM level of the receptor field (e.g., Henry *et al.*, 2003). For the 250 m fields no regression curve describing GM presence was expected, and hence the average value of a receptor field was calculated as a weighted average of the 12 section values (see Figure 1A) where the weights are the ratios between the areas of the sections and the total area of the receptor field.

Results

Flowering

The onset of flowering was recorded in all fields. Generally, there was a good synchronicity in flowering between GM and receptor fields. Female flowering started on average two days earlier than male flowering (Table 2). This was most probably a varietal characteristic, since it occurred in both 2006 and 2007, even though the climatic conditions differed considerably between both years: in the summer of 2006 the weather during flowering was warm and dry, whereas in the summer of 2007 it was relatively wet, and temperatures were about the same as the long-term averages of the Dutch maritime climate. Pollen production may have been influenced by the weather conditions. In 2007 pollen production lasted for a longer period than in 2006: 12 days in 2007 vs. 6 days in 2006. Total pollen production was a little higher in 2006: on average 1.19 g per plant in 2006 vs. 0.87 g per plant in 2007. As a consequence of the unusually dry weather in 2006, flowering suffered somewhat from drought stress at five of the six field sites. However, ear development was seriously hampered only in one of the receptor fields at one location (Blitterswijck). In 2006, severe drought stress not only occurred on the sandy soils found in that particular region; it was also recorded on plants in the GM fields (subsequently claimed by gene-technology opponents) at four of the six field sites. At two locations, damage occurred after the bulk of pollen production. Only at Lage Zwaluwe, damage occurred at the peak of flowering, so that one would have to take into account a somewhat lower pollen production when assessing the outcrossing results. At Lelystad, part of the plants were damaged well before the onset of flowering, so here it is not clear to what extent pollen production may have been compensated by a possibly better growth of the remaining plants (for further details on crop performance see Table 1). No damage by gene-technology opponents was encountered in 2007.

25 m isolation distance

The pattern of GM presence in the receptor fields at 25 m distance from the GM source field was in accordance with the PMGF to be expected in agricultural fields: the highest levels were found at the side of the field closest to the source field, and GM levels dropped quickly moving further into the field. No statistically significant differences were found between the values of the three sampling transects within a receptor field. Therefore, the measurements of the samples in the three transects at the same distance were averaged. These values in each receptor field are presented for both years in Figures 2A and 2B, together with the curve fitted using the regression model described above. GM levels in the border rows averaged per receptor field ranged from 0.03 to 4.4% in 2006 and from 0.01 to 5.3% in 2007. Estimates of the 12 field averages in each year are presented in Table 3, together with the averages per location. The estimated average GM levels across receptor fields ranged from 0.009 to 0.296%, with an overall average of 0.084% in 2006, and from 0.002 to 0.318%, with an overall average of 0.080% in 2007. The 95% confidence intervals of the averages per year were calculated on the basis of the location averages. The year averages were not significantly different (t-test, $P = 0.89$).

Table 2. Mean start of flowering per field, in 2006 and 2007. Data in body of table refer to day number (1 July = day number 1).

Male flowering

Year	Location		Receptor field					GM field
	Number	Name	North	East	West	South	Mean	
2006	1	Eerste Exloermond	31.2	28.9	28.4	28.4	29.2	30.2
	2	Tweede Exloermond	27.4	29.9	26.3	29.9	28.4	27.2
	3	Lelystad	26.4	27.4	26.0	30.0	27.4	27.6
	4	Lage Zwaluwe	27.7	27.6	29.0	33.7	29.5	32.3
	5	Blitterswijck	19.3	21.2	21.6	256	21.9	23.2
	6	Wijnandsrade	24.1	26.5	24.5	27.0	25.5	24.0
2007	1	Uithuizen	39.6	45.4	40.4	46.5	43.0	43.2
	2	Eerste Exloermond	38.1	34.3	39.7	35.8	37.0	38.7
	3	Tweede Exloermond	33.8	32.7	33.8	34.5	33.7	35.1
	4	Lelystad	29.3	32.8	33.8	33.4	32.3	33.2
	5	Schaarsbergen	32.0	32.3	29.1	30.2	30.9	31.7
	6	Tholen	32.0	29.1	30.4	30.4	30.5	31.6

Female flowering

Year	Location		Receptor field					GM field
	Number	Name	North	East	West	South	Mean	
2006	1	Eerste Exloermond	29.9	27.2	27.3	26.7	27.8	28.0
	2	Tweede Exloermond	26.1	29.0	24.3	28.6	27.0	25.6
	3	Lelystad	23.2	24.7	22.4	26.8	24.3	25.7
	4	Lage Zwaluwe	27.7	27.2	29.3	34.6	29.7	32.5
	5	Blitterswijck	19.4	28.0	33.9	35.2	29.1	30.4
	6	Wijnandsrade	24.4	26.2	24.5	25.9	25.2	24.1
2007	1	Uithuizen	36.8	42.5	36.9	43.3	40.0	40.6
	2	Eerste Exloermond	35.0	30.8	37.0	32.6	33.8	36.3
	3	Tweede Exloermond	30.2	29.2	31.0	31.6	30.5	33.0
	4	Lelystad	25.1	27.9	31.7	29.4	28.5	30.1
	5	Schaarsbergen	27.8	28.1	25.3	25.5	26.7	28.3
	6	Tholen	27.9	25.8	27.0	27.0	26.9	28.1

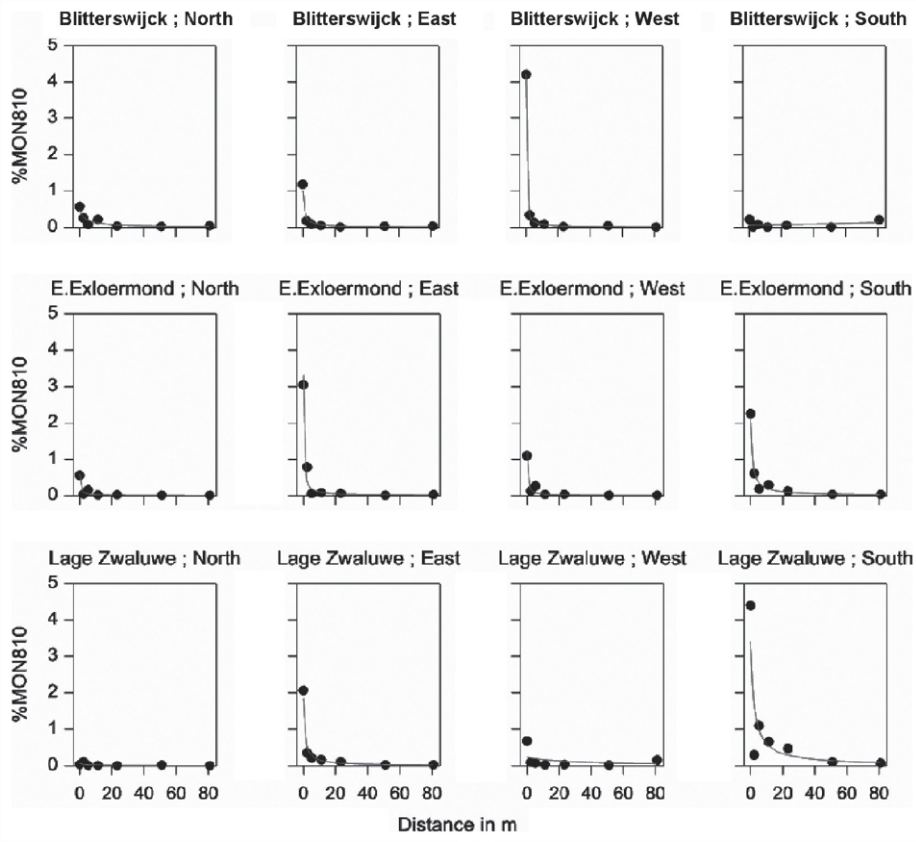

Figure 2A. Reciprocal functions fitted to MON810 content in grain samples from individual receptor fields against distance from the field border exposed to the GM source field at 25 m isolation distance, in 2006. Blitterswijck South could not be completely sampled due to poor ear formation as a consequence of severe drought during flowering.

250 m isolation distance

In the 250 m fields, MON810 was detected in 7 of the 12 fields in 2006 and in 10 of the 12 fields in 2007 (Table 4). In 2006, 17% of the samples from field border sections facing the GM source field were positive for MON810; and in 2007, 23% were positive. For the border sections not facing the GM source fields, these values were 8% in both years, and for the central sections they were 2% and 8% in 2006 and 2007, respectively. The maximum sample level of MON810 over all sections of the 12 receptor fields was recorded in 2006: 0.23% in a central section at Wijnandsrade South, whereas the maximum sample level in 2007 was 0.16% in a border section facing the GM source field.

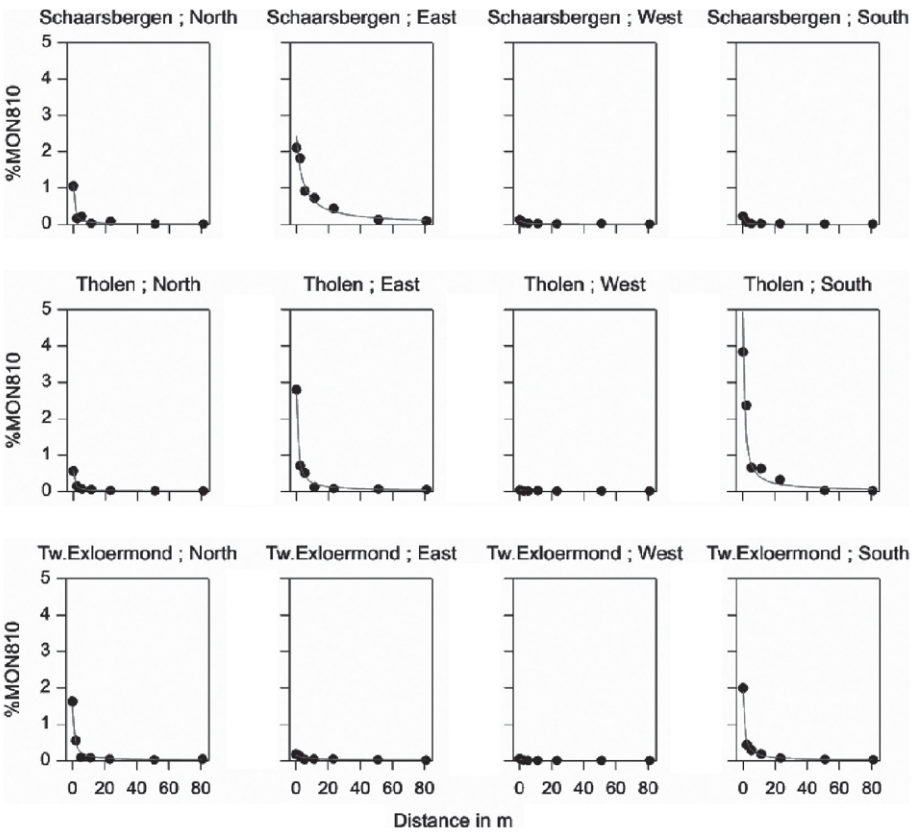

Figure 2B. Reciprocal functions fitted to MON810 content in grain samples from individual receptor fields against distance from the field border exposed to the GM source field at 25 m isolation distance, in 2007.

In 2007, an extremely high value of 13% MON810 was found in one of the corner sections (Uithuizen site, south field), which could not be realistically attributed to pollen flow from the GM source 250 m away. Seed admixture during sowing is considered as the most likely explanation of this high GM rate, as it was the first receptor field sown after sowing the GM field. Normally the GM field was sown last as a precautionary measure to prevent seed admixtures. At this site this procedure was not followed. However, the sowing machine first used for the GM field had been thoroughly cleaned before sowing the receptor fields. Nevertheless, a sowing error remains the most likely explanation, although admixture of the sowing seed lots could not be completely ruled out, since the supplier's specifications allowed for an admixture rate of ≤ 0.1%. With our sampling

Table 3. Estimated average levels of MON810 content (%) in grain samples from receptor fields at 25 m isolation distance, in 2006 and 2007. The 95% confidence intervals are based on the means per location

2006

Receptor field	Location			Overall mean
	Blitterswijck	Eerste Exloermond	Lage Zwaluwe	
	(%)			
North	0.060	0.017	0.009	
East	0.045	0.075	0.089	
West	0.073	0.031	0.096	
South	0.102[1]	0.110	0.296	
Mean	0.070	0.058	0.123	0.084
95% confidence interval				(−0.002, 0.169)

2007

Receptor field	Location			Overall mean
	Tholen	Tweede Exloermond	Schaarsbergen	
	(%)			
North	0.022	0.075	0.039	
East	0.131	0.029	0.318	
West	0.002	0.002	0.011	
South	0.236	0.092	0.008	
Mean	0.098	0.049	0.094	0.080
95% confidence interval				(0.014, 0.147)

[1] Results for Blitterswijck South are based on limited sampling (basically only a single transect) due to poor ear formation as a consequence of severe drought during flowering.

Table 4. Distribution of samples positive for MON810 among and within receptor fields at 250 m isolation distance in the years 2006 and 2007. Border section 'protected' refers to all border sections with another part of the same receptor field in between them and the GM source field, 'shielding' them from direct pollen flow, as opposed to Border section exposed to GM source field, where no maize was grown between them and the GM source field.

	No of sections with MON810 detected	% of sections with MON810 detected	Mean MON810 content (%)	Total No of fields or sections per year
2006				
Receptor fields	7	58		12
Border sections exposed to GM source field	13	17	0.0076	78
Border sections 'protected'	5	8	0.0044	66
Border section total	18	13	0.0061	144
Central sections	1	2	0.0048	48
2007				
Receptor fields	10	83		12
Border sections exposed to GM source field	19	23	0.0126	77 [1]
Border sections 'protected'	4	8	0.0037	66
Border sections total	23	16	0.0085	143 [1]
Central sections	4	8	0.0038	48

1 Data from one border section were discarded, since its high level of MON810 could only be explained by a sowing error and not by outcrossing with the GM field (see section 'Results 250 m isolation distance' for further details).

scheme, a grand total of 1920 plants were sampled in the 250 m plots over the two years, meaning that one transgenic seed among them would lead to an admixture rate below this 0.1% level. Other possible explanations, such as commingling during harvest and subsequent analysis have also been investigated, but could be ruled out. Even though MON810 was found in more border sections in 2007 than in 2006, the actual values were generally lower so that the overall averages differed little: 0.0085% in 2007 vs. 0.0061% in 2006. A similar situation was found in central sections: more were positive in 2007 than in 2006, but overall averages were similar: 0.0038% in 2007 vs. 0.0048% in 2006. Although there was a tendency towards higher percentages MON810 in border sections exposed to the GM source fields compared with non-exposed border and central sections, this difference was not statistically significant. Estimated whole receptor field averages of MON810 are presented in Table 5. These levels ranged from 0 to 0.04% in both years, with an overall average of 0.005% in 2006 and 0.007% in 2007.

Table 5. Estimated average levels of MON810 content (%) in grain samples from receptor fields at 250 m isolation distance, in 2006 and 2007. The 95% confidence intervals are based on the means per location.

2006

Receptor field	Location			Overall mean
	Lelystad	Tweede Exloermond	Wijnandsrade	
	(%)			
North	0.0037	0.0000	0.0000	
East	0.0087	0.0000	0.0017	
West	0.0023	0.0000	0.0000	
South	0.0037	0.0037	0.0401	
Mean	0.0046	0.0009	0.0104	0.0053
95% confidence interval				(-0.0018, 0.0125)

2007

Receptor field	Location			Overall mean
	Lelystad	Eerste Exloermond	Uithuizen	
	(%)			
North	0.0030	0.0044	0.0098	
East	0.0066	0.0000	0.0020	
West	0.0000	0.0003	0.0375	
South	0.0055	0.0124	0.0004 [1]	
Mean	0.0038	0.0043	0.0124 [1]	0.0068 [1]
95% confidence interval				(-0.0053, 0.0189)

1 Data from one corner section in Uithuizen South were discarded, since its high level of MON810 could only be explained by a sowing error and not by outcrossing with the GM field (see section 'Results 250 m isolation distance' for further details).

Discussion

In this study, carried out over a period of two years, we measured the effect of two different isolation distances on the levels of pollen-mediated gene flow (PMGF) in maize using the transgenic event MON810 as a marker. With an isolation distance of 25 m, the estimated MON810 levels in 1-ha receptor fields averaged 0.08% in both years, despite the large difference in weather conditions during flowering: warm and dry in 2006, and about normal temperatures in 2007 with more rainfall in comparison to the usual temperate climatic conditions encountered in the Netherlands. Under such different conditions, traits potentially influencing PMGF, like pollen viability, are expected to vary considerably, with pollen losing viability faster under more desiccating conditions (Devos *et al.* 2005). Nevertheless, we did not find an effect of these differences on our overall PMGF results.

Variation in GM presence among receptor fields was high, ranging from 0.01% to 0.32%, but was observed among the four receptor plots for each donor field. It is a typical feature of PMGF studies that can be ascribed to the variation in wind direction (Devos *et al.*, 2005; Van De Wiel & Lotz, 2006). The maximum values found were considerably below the labelling threshold of 0.9% set by the EU and thus also left room for other possible causes of adventitious GM presence in the final product, such as GM presence in the sowing-seed or commingling during down-stream processing. At an isolation distance of 250 m, PMGF could still be detected, but was considerably lower. The maximum sample level found was 0.2%, while the greater part of the positive samples remained below 0.1%. Eighty-nine percent of all samples were negative for MON810 and in 7 out of the 24 receptor fields none of the samples were positive for MON810.

Our results are in line with several recent studies on PMGF in maize, performed in relation to coexistence of GM and non-GM fields in the EU. Messeguer *et al.* (2006) determined outcrossing between GM and non-GM fields in situations representative of BT maize growing in Spain. On the basis of their data, using a relatively simple empirical model, they concluded that an isolation distance of 20 m between fields should be sufficient to keep admixture below the 0.9% labelling threshold. Also Gustafson *et al.* (2006) developed a relatively simple empirical model, in their case using data from several recent large-scale experiments in the USA and France and from an older small-scale study in the USA (Jones & Brooks, 1950) using various 'empty' (without maize plants) isolation distances. For a 'reasonably worst' case scenario (defined as covering 90% of the cases), this model also indicated an isolation distance of 20 m to be sufficient to keep admixture below 0.9%, but only if combined with removal of the non-GM field's first row of plants exposed to the GM source. However, in doing so, the model of Gustafson *et al.* (2006) also left room for a maximum adventitious transgene presence of 0.3% in the seeds while remaining below the 0.9% threshold. So removing the exposed outer row is expected to keep admixture for the whole field below 0.6%. As no tolerance threshold for seeds has yet been set by the EU, it is not yet clear how much room should be reserved for GM seed admixture.

The removal of the outer row for diminishing GM admixture levels as indicated by Gustafson *et al.* (2006) highlights the well-known double protective effect exerted by the receptor maize plants themselves, that is, by physical protection and by their

production of competing non-transgenic pollen. Thus, earlier results from large-scale field experiments in the UK Farm Scale Evaluations (FSE) with GM fields immediately next to non-GM fields implied that outcrossing levels dropped to below 1% after 25 m of maize plants (Weekes *et al.*, 2007). Even though 'empty' isolation distances should have a significant effect on PMGF in maize, since maize pollen is relatively heavy for a wind pollinator and thus tends to settle down relatively quickly (e.g., Raynor *et al.*, 1972), 'empty' isolation distances were shown to be less effective in a study by Pla *et al.* (2006). They showed this in an experiment using yellow-seeded maize as a colour marker for PMGF in white-seeded maize as receptor: an 'empty' isolation distance of 10 m led to 6% outcrossing in the outer row as opposed to 2% when these 10 m were filled by white maize. In our experiments, the 25 m isolation distance often led to outcrossing levels in the exposed border row that were above the 0.9% EU labelling threshold and that could be as high as 5% (Figure 2A). However, beyond the border row of the receptor field, outcrossing was found to drop quickly so that average values in the fourth row (2.25 m into the field) for the greater part were already below 0.9%, thereby leading to low average values for the receptor fields as a whole.

In our study, GM admixture was assessed in the grains, but in the Netherlands most maize is grown for silage, in which case any grain fraction containing transgenes from outcrossing would be 'diluted' with non-transgenic vegetative material. Generally, grains constitute about half of the dry weight of silage maize and for that reason several publications (e.g., Weekes *et al.*, 2007; Sanvido *et al.*, 2008) have simply suggested to divide proposed isolation distances into halves for silage maize. However, one of the very few reports on measurements in silage maize so far (Weber *et al.*, 2007) came up with outcrossing values for whole plants (silage) very much alike those found for grains. Unfortunately, the results for silage (Weber *et al.*, 2007) were obtained from other fields than those for grains. Nevertheless, similar results would be possible with the DNA extraction method and PCR quantification method used if the vegetative parts and the grains differ in DNA extractability and/or deliver DNA differing in PCR quality. This is not unlikely since vegetative tissues will have matured further than grains and may even already be subject to degradation at the time of harvesting, which will have a negative impact on DNA extractability and/or quality. These problems are the subject of an ongoing study at our laboratory.

In their modelling of the FSE results, Weekes *et al.* (2007) additionally used a correction factor of 0.58 on their qPCR outcomes to bring the FSE results into line with recommendation 2004/787/EC (in the context of EC Regulation 1830/2003), which prescribes the percentages to be simply the ratio between the number of copies of a transgene and the target species-specific DNA copy number in terms of haploid genomes (%HGE). This calculation was needed, because so far in the EU-validated method, and therefore also in the present study, the ratio is determined with reference to a calibration curve based on reference material prepared on a w/w basis by the IRMM (Institute for Reference Materials and Measurements, Geel, Belgium). This means that the nominal transgene percentage of 5% has resulted from adding ground hybrid maize grains to non-trangenic maize grains purely on a w/w basis. The hybrid GM maize added to attain the 5% GM is thus taken as 100% transgenic in practice, even though the hybrid used is hetero(hemi)zygotic for the MON810 event. For obtaining a quantification

result as % reference assay HGE, the number of MON810 events and reference genes, respectively, were subsequently calculated on the basis of the 1C value of maize genomes, with the number of MON810 copies simply taken as 5% of the number of reference gene copies. When calculating a correction factor for the actual number of MON810 copies relative to the number of reference gene copies (%HGE) as proposed by Weekes *et al.* (2007), one needs to take into account the fact that the heterozygotic state of the MON810 event in the reference variety has special consequences because of the way the triploid state of the endosperm is generated. As a consequence of the double fertilization, the maize endosperm contains two genomes derived from the mother and one from the father line; in the case of the reference variety, the MON810 came from the mother line. So the calculation of correction factors critically depends on the contributions of the various grain tissues (embryo, endosperm and seed coat/pericarp) to the DNA sample in the maize material at hand. Zhang *et al.* (2008) empirically established a formula for the correction factor, taking into account the contribution of the endosperm to the total DNA sample. However, Trifa & Zhang (2004), and Papazova *et al.* (2005; 2006) have shown that the contribution of different grain tissues varies among maize cultivars. So calculations of correction factors have to be treated with caution, and verification based on the more direct method with plasmids containing the reference genes used to measure the number of crop genomes as well as the event-specific fragments as reference materials appears to be highly recommendable. For instance, very recently the IRMM determined the correction factor to be 0.57 specifically for their 1% BF413d reference material on a w/w basis, by direct comparison with reference material consisting of such double plasmids (Charels *et al.*, 2007). In order to avoid introducing any possible additional sources of error, we did not apply a correction factor in the present study. Using the more direct method based on double plasmids for calibration will in all cases produce outcrossing levels that are lower than those presented here.

In our study the maximum values found for PMGF were considerably below the labelling threshold of 0.9%, indicating that an isolation distance of 25 m is sufficient for commercial maize cultivation. However, in commercial cultivation two aspects may differ from the conditions of our field trials: relative field configurations and extreme wind conditions. In the practice of GM cultivation, field configurations may substantially differ from those tested here and in other studies. For instance, non-GM fields may be surrounded by several GM fields or may have a considerably larger GM field as neighbour. Deterministic modelling using the MAPOD model (Messean *et al.*, 2006) indicated that an isolation distance of 50 m may be necessary for adhering to the 0.9% threshold if the non-GM field is three times or more smaller in size than the neighbouring GM field (5 vs. 15 ha in the modelled example in the Poitou-Charente region of south-west France). Thus, as suggested by Devos *et al.* (2008) flexible use of filling isolation distances with more effective buffer rows of maize plants, may improve feasibility of implementing co-existence in commercial maize cultivation, particularly in agricultural landscapes with a scattered distribution of small fields. Hoyle & Cresswell (2007) and Kuparinen *et al.* (2007) have recently indicated on the basis of their respective models that the limited number of large-scale empirical field studies that are feasible within realistic research budgets may well not cover more rarely occurring wind conditions that can have a relatively large influence on pollen flow distances and thus on

admixture rates downwind. Taking into account such rare climatic events, which would also depend on specific landscape characteristics, is also not easily incorporated in the modelling. Therefore, our study will be followed up by monitoring non-GM fields during the first three years following the actual start of commercial GM maize cultivation in the Netherlands, in line with the proposal by the Dutch Coexistence Committee.

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