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Case study

Cross-fertilization between genetically modified and non-genetically modified maize crops in Uruguay

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The cultivation of genetically modified (GM) Bt maize (*Zea mays* L.) events MON810 and Bt11 is permitted in Uruguay. Local regulations specify that 10% of the crop should be a non-GM cultivar as refuge area for biodiversity, and the distance from other non-GM maize crops should be more than 250 m in order to avoid cross-pollination. However, the degree of cross-fertilization between maize crops in Uruguay is unknown. The level of adventitious presence of GM material in non-GM crops is a relevant issue for organic farming, *in situ* conservation of genetic resources and seed production. In the research reported here, the occurrence and frequency of cross-fertilization between commercial GM and non-GM maize crops in Uruguay was assessed. The methodology comprised field sampling and detection using DAS-ELISA and PCR. Five field-pair cases where GM maize crops were grown near non-GM maize crops were identified. These cases had the potential to cross-fertilize considering the distance between crops and the similarity of the sowing dates. Adventitious presence of GM material in the offspring of non-GM crops was found in three of the five cases. Adventitious presence of event MON810 or Bt11 in non-GM maize, which were distinguished using specific primers, matched the events in the putative sources of transgenic pollen. Percentages of transgenic seedlings in the offspring of the non-GM crops were estimated as 0.56%, 0.83% and 0.13% for three sampling sites with distances of respectively 40, 100 and 330 m from the GM crops. This is a first indication that adventitious presence of transgenes in non-GM maize crops will occur in Uruguay if isolation by distance and/or time is not provided. These findings contribute to the evaluation of the applicability of the "regulated coexistence policy" in Uruguay.

Keywords: Bt / Bt11 / gene-flow / MON810 / out-crossing / *Zea mays*

INTRODUCTION

Several transgenic maize (*Zea mays* L.) hybrids have been developed with pest resistance or herbicide tolerance (Devos et al., 2005). In Uruguay, cultivation of genetically modified (GM) maize is permitted since 2003 for event MON810, and since 2004 for Bt11. These events carry a *Bacillus thuringiensis* transgene coding Cry1Ab protein, which is toxic to larvae of some *Lepidoptera* maize pests. For GM maize cultivation, governmental resolutions mandate that a minimum of 10% of the area should be grown with non-GM maize as biodiversity refuge. These resolutions also establish a minimum isolation distance of 250 m between GM and non-GM maize crops in order to avoid cross-fertilisation (Ministerial resolutions RM 236A/2003, RM 276/2003 and RM 292/2004).

Coexistence implies that farmers can make a practical choice between organic, conventional, or GM crop production (Devos et al., 2005). The Uruguayan state aims to develop a national strategy to ensure coexistence (Presidential decree 353/08). Currently, the single measure aiming to apply this policy is the 250 m isolation distance mentioned above. Thereafter, the authorities have not yet established threshold levels for labeling GM food and feed, nor for organic products.

Cross-fertilization between nearby crops is a critical issue in the case of transgene flow from GM to non-GM maize crops. Maize is a monoecious species, with wind

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as the major vector of pollen dispersal (anemophily). This reproductive system leads to a high degree of cross-pollination between plants, and also between neighbouring crops. Several studies showed pollen-flow from elite varieties to local ones, as well as between varieties and hybrids (Burris, 2001; Doebley, 1990; Sanou et al., 1997).

Studies based on small-scale models have attempted to characterize the dispersal of maize pollen (Jørgensen and Wilkinson, 2005). Wind speed is the main variable determining the amount of pollen that will disperse in the air, and the release of pollen grains from anthers. Once in the air, the timing and distance for pollen grain decantation depends on opposed forces: gravity in one side, and turbulence and convective air currents in the other. The density of air-borne pollen decreases at short distances from the source but values are asymptotically maintained at longer distances. Emberlin et al. (1999) estimated that relative pollen density falls to approximately 2% at 60 m from the crop edge, but remains at about 0.5–0.75% at 500 m from the crop edge.

Pollen viability is another critical point determining the frequency of out-crossing between neighboring crops. Maize pollen grains are viable for one or two hours after emerging from the cob (Luna et al., 2001). However, pollen can often remain viable for more than 24 hours, a period that depends on temperature, humidity and atmospheric water potential (Ma et al., 2004).

After release of GM maize, the issues of pollen dispersal and out-crossing acquired a higher profile. Studies on gene-flow by cross-fertilization were reviewed by Devos et al. (2005) and Sanvido et al. (2008). Many of them aimed to provide information to define the necessary isolation distances, so that the presence of transgenes in non-GM crops could be maintained below certain threshold levels. Differences in research approaches, analytical methods and experimental designs over studies, hinders comparison of results, and make also complex the definition of appropriate measures to limit cross-fertilization in the field (Devos et al., 2005). However, studies comparing results of these experiments found that cross-fertilization patterns are similar, notwithstanding differences in experimental approaches (Riesgo et al., 2010; Sanvido et al., 2008). In spite of similar patterns for cross-fertilization events, isolation distance between GM and non-GM maize mandated by different EU countries differ greatly, ranging from 25 to 600 m (Riesgo et al., 2010), even though they share the guidelines on GM foods labeling (EC N° 1829/2003) (Devos et al., 2009).

Besides the distance between crops and synchrony of flowering, gene-flow is also affected by size and orientation of fields (Langhof et al., 2010). Most gene-flow studies were founded on a single sources of pollen, often smaller or equal to the size of the receptor crop (Sanvido et al., 2008). However, as the acreage of GM maize continues to extend in Uruguay, a non-GM crop can receive GM pollen from various large sources (Devos et al., 2005).

Maize cultivation in Uruguay is performed in farms that markedly differ in size (MGAP, 2008). In the season 2007–2008, a total of 2821 maize growers sowed 80 500 ha. Among these growers, 86% sowed less than 20 ha each, providing 7% of the total maize area. There is no official data for the GM maize area. Regarding the volume of imported GM seeds (INASE, 2008), it is roughly estimated that 66% of planted corn in the season 2007–2008 and 80% in the season 2008–2009 was GM corn.

The degree of cross-fertilization between GM and non-GM maize crops in Uruguay has not been previously studied. This knowledge is important for *in situ* conservation of local genetic resources, for organic farming systems, for production of high quality maize seeds, and as a whole for the evaluation of the applicability of the co-existence policy. This research aimed to evaluate the occurrence of transgene flow from commercial GM to non-GM maize crops in Uruguay, by analyzing the presence of transgenes in the progeny of the non-GM crops.

RESULTS

The analysis of plants sampled in GM and non-GM fields (mother plants) by DAS-ELISA and PCR confirmed in all cases the information obtained from the farmers about GM or non-GM identity of their maize crops. Therefore, in all sampling sites where the transgene was detected in the non-GM offspring (seedlings), it was inferred that transgenes were acquired by cross-fertilization with a GM crop. Transgenes were detected in the offspring of the non-GM maize crops, in three out of five cases analyzed (Cases 1, 2 and 4) (Tab. 1). For these three sampled fields, the detected transgene corresponded to the event present in the putative GM source of maize pollen (Tab. 2). No transgene flow was detected in two cases (Cases 3 and 5), maybe due to the lack of synchrony between GM and non-GM crops in flowering time, preventing cross fertilization or leading to frequencies not detectable with the used sample size.

The adventitious presence of GM material in the non-GM maize crops was expressed as the observed and estimated percentages of seedlings carrying transgenes in the offspring of non-GM crops (see Materials and methods). These estimations were based on results generated by DAS-ELISA (Tab. 2). Observed percentages were 0.56% for site 1.1, 0.83% for site 2.3, and 0.13% for site 4 (sampling sites with distances of 40 m, 100 m and 330 m respectively, from the source of GM maize).

Table 1. Information on the paired-field cases of GM and non-GM commercial maize crops studied in Uruguay.

Case	Non-GM maize field			Distance (m) and orientation[b]	GM maize field		Difference in planting date[d]
	Cultivar	Area (ha)	Site[a]		Event	Area (ha)	
1	IPB 871 CL	9.0	1.1	40 S	MON810 + Bt11	60.0	0
			1.2	190 S			
2	IPB 871 CL	9.5	2.1	380 NE	MON810	40.0	−2
			2.2	180 E			
			2.3	100 E			
3	IPB 871 CL	0.8	3.1	20 N	MON810	20.0	−3
4	Breeding line	4.5	4.1	330 N	Bt11	30.5	+1
5	Landrace	3.5	5.1	30 SW	GM[c]	0.6	+14

[a] For Case 1, two sites within the same non-GM field were sampled at different distances from the neighbouring GM field. For Case 2, three sites were sampled.
[b] Orientation of the GM field from the non-GM field.
[c] No information was obtained.
[d] Days. Negative values mean that the GM was planted before the non-GM field.

Table 2. Analysis of the progeny of non-GM plants sampled from commercial maize crops studied in Uruguay.

Case	Site[a]	DAS-ELISA		p^c (%)	PCR[d]			Nearest GM crop	
		Nr of positives[b]	Observed frequency		35S	MON810	Bt11	Distance (m)	Event
1	1.1	1/180	0.56%	1.7	+	−	+	40	MON810 + Bt11
	1.2	0/120	0	< 2.5	−			190	MON810 + Bt11
2	2.1	0/120	0	< 2.5	+	+	−	380	MON810
	2.2	0/120	0	< 2.5	−			180	MON810
	2.3	1/120	0.83%	2.5	−			100	MON810
3	3.1	0/180	0	< 1.7	−			20	MON810
4	4.1	1/764	0.13%	0.39	+	+	−	330	Bt11
5	5.1	0/180	0	< 1.7	−			30	unknown

[a] For Case 1, two sites within the same non-GM field were sampled at different distances from the neighbor GM field. For Case 2, three sites were sampled.
[b] Number of positive plants on the total number of tested plants.
[c] Estimated frequency in the population with 95% confidence for probability of detection, P_d, based on DAS-ELISA observed frequencies and sample sizes (see Materials and methods).
[d] Specific primers for 35S were used as a marker for transgenicity, primers VW01/VW03 for MON810, and PatB/IVS2-2 primers for Bt11. (+) indicates detection of the transgene.

For Cases 1 and 2, where more than one site was sampled within the non-GM crops, the presence of transgenes was found only in those sites closer to the neighbor GM crop (site 1.1 for Case 1 and site 2.3 for Case 2, see Fig. 1). For Cases 3 and 5 only one site was sampled in the non-GM crops, being at the edge closer to the GM crops, and in these cases no transgene flow was detected. For Case 4, the harvested grains of the non-GM crop were sampled and for this case the adventitious presence of GM material (0.13%) corresponds to the entire non-GM field instead of a particular sampled site within the crop.

In Case 1, two sites were sampled within the non-GM maize crop (sites 1.1 and 1.2). The adventitious presence of GM material was detected in the sampling site 1.1 (Fig. 1) located at the border of the non-GM crop and 40 m away from a GM field. The latter crop was sown with different commercial maize hybrids, one carrying MON810 and the other carrying Bt11. The GM crop area was 60 ha while the non-GM field size was 9 ha, and both were sown at the same date. Within the 180 seedlings (organized in six bulks of 30) produced from the 45 cobs collected in site 1.1 one positive seedling was detected by DAS-ELISA. Further analysis by PCR confirmed that

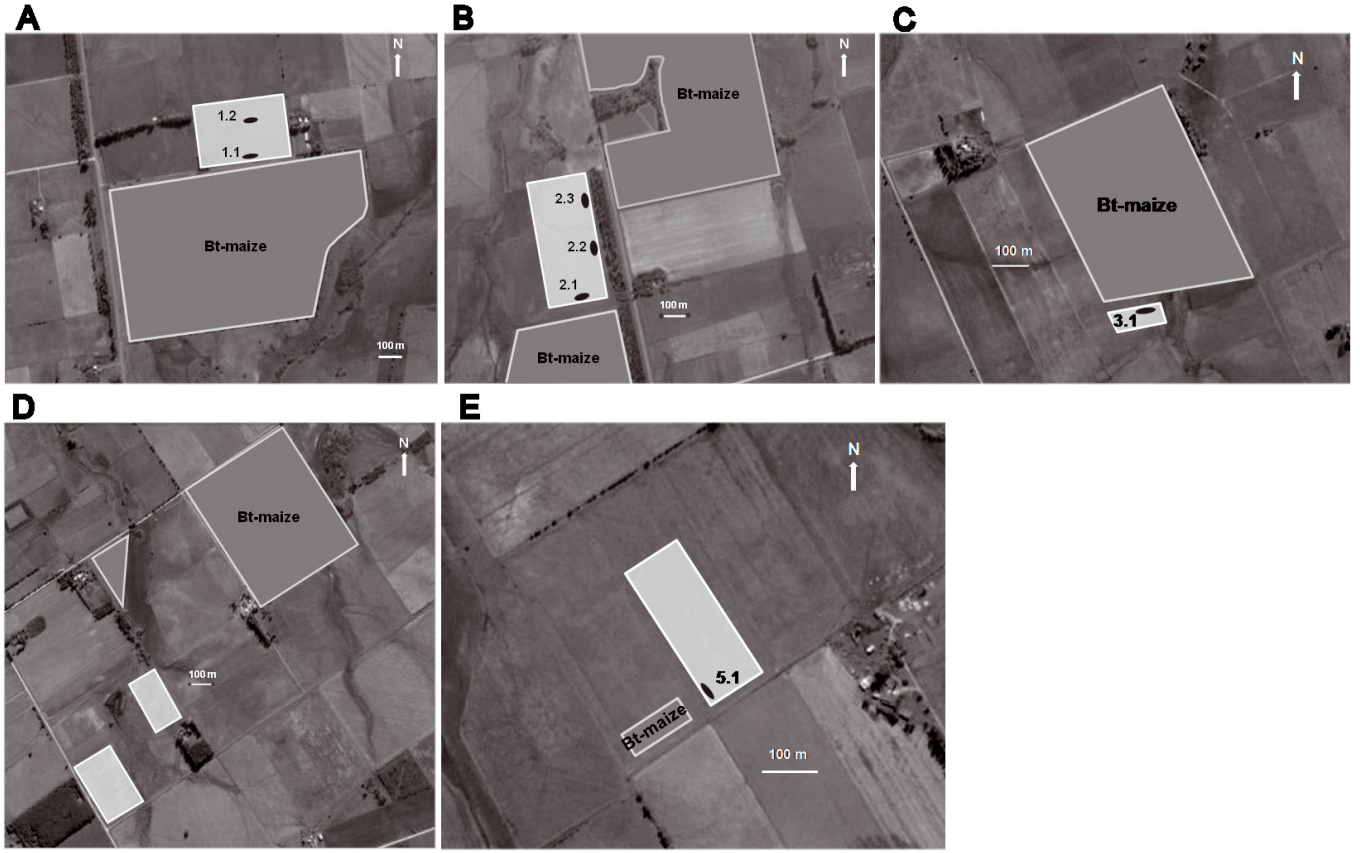

Figure 1. Schematic representation on satellite pictures of field cases in which cross fertilisation between commercial GM and non-GM maize was studied. GM fields are indicated as Bt-maize, non-GM maize as light grey areas, and sampling sites as black ovals within the non-GM maize crop area. White bars indicate 100 m. (**A**) Field Case 1. (**B**) Field Case 2. A non-GM crop had GM neighbouring fields in South and North-East borders. An area of tall trees 30 m depth was in between the non-GM and the North-East GM maize fields. (**C**) Field Case 3. (**D**) Field Case 4. GM maize crop was grown in two pieces of land, and a non-GM breeding line was also grown in two pieces of land. A distance of 330 m was estimated as the shortest distance between GM and non-GM fields. For this situation, the non-GM progeny was analyzed by sampling kernels already harvested. (**E**) Field Case 5.

the transgene corresponded to the event Bt11. The percentage of seedlings carrying transgenes for this site was 0.56%. Regarding the sample size, the frequency in the population (p) for which there is a 95% probability of detecting at least one seedling carrying the transgene is 1.7%. It has to be mentioned that for one analyzed bulk, the analysis by DAS-ELISA was negative for the presence of the transgenic protein, while PCR revealed the presence of the Bt11 event. For the site 1.2, located 190 m away from the next GM source, no transgenes were detected. For this site p was < 2.5% (see Materials and methods).

In Case 2, a non-GM crop (9.5 ha) was surrounded by two GM crops (Fig. 1). A field in direction NE (40 ha) was sown with a maize hybrid carrying MON810 event, two days before the non-GM field. These fields were 100 m apart, including a barrier of Eucalyptus trees 30 m width and 8–12 m high between them. A second GM crop was sown 20 m away from the non-GM crop, though more than two weeks later. Three sites were sampled within the non-GM maize area (Tab. 1, Fig. 1). The transgene was only detected in the sampling site 2.3, which was the closest to the synchronic GM crop. It is interesting to point that cross-fertilization occurred in spite of the *Eucalyptus* barrier. A total of 120 seedlings (organized in four bulks), obtained from 30 cobs, were analyzed from this sampling site and only one seedling was positive for the transgene. Then the observed percentage of transgenic seedlings was 0.83% for this site. Event MON810 was identified by PCR in this sample. Taking into account the size of the sample, the frequency p for which there is a 95% probability of detecting at least one seedling carrying the transgene is 2.5%. As well as for Case 1, no transgene was detected in the other sampling sites (sites 2.1 and 2.2) located at longer distances from the GM source. For the latter two sites p was < 2.5% (see Materials and methods).

In Case 3 a small area of a non-GM crop (0.8 ha) was sown next to a larger GM maize crop (20 ha). The distance between crops was 20 m and the non-GM maize was sowed three days after GM maize. Only one site was sampled (site 3.1) on the edge of the non-GM crop closer to the GM area. From 45 sampled cobs, 180 seedlings were produced and analyzed, and no transgenes were detected. For this site p was < 1.7%.

Case 4 consisted of a field, divided in two areas, devoted to the production of maize seeds of a non-GM maize line (4.5 ha), and two areas cultivated with a hybrid maize carrying Bt11. One of these areas (2.5 ha) was located 330 m North from the non-GM field, and the second area (28 ha) located 550 m NE. Sowing dates of non-GM and GM crops differed by only one day. The non-GM crop was sampled after harvest, and harvested seeds were sampled for progeny analysis. A larger sample size was analyzed, because this approach was equivalent to randomly sampling the entire field. A total of 764 seedlings were produced and analyzed by DAS-ELISA. Only one seedling was positive, and the presence of Bt11 event was confirmed by PCR. The observed percentage of transgenic seedlings in the non-GM corn was 0.13% and the frequency p was calculated as 0.39% with 95% confidence.

In Case 5 a small area of a GM crop (0.6 ha) was planted adjacent to a bigger area of non-GM maize crop (3.5 ha). The distance between crops was 30 m and the non-GM maize was sowed 14 days before the GM crop. Only one site (site 3.1) was sampled on the edge of the non-GM crop closer to the GM area. From 45 cobs, 180 produced seedlings were analyzed and no transgene was detected. For this site, frequency p was < 1.7%.

DISCUSSION

This research assessed the frequency of transgene events in the progeny of non-GM crops by sampling and analyzing commercial fields neighboring GM crops. To our best knowledge, this is the first report of cross-fertilization between GM and non-GM maize in South America. Assessing commercial fields may have advantages and constraints to establish general trends. Field situations may differ in a range of conditions, such as acreage, topography, number and direction of pollen sources. Flowering time may also differ, and differences in wind speed and the occurrence of storms during flowering will vary from one case to another (Langhof et al., 2010). As a consequence, results from commercial fields may not be easily comparable, and this information may not be useful to analyze a single factor, for instance, the effect of the distance between GM and non-GM crops on cross-pollination.

Diverse strategies have been used to test cross-fertilization between maize crops (reviewed by Devos et al., 2005). Despite limitations due to variations in an array of factors, sampling commercial field situations may estimate conditions in which cross-fertilization occurs better than experimental designs. For instance, maize plots have been established to be evaluated as recipients of pollen at regular distances from a pollen source plot (Luna et al., 2001). Results obtained following this approach may not be representative of commercial situations in which recipient and sources are of much greater size. Furthermore, another constraint is that many studies measured variation of gene flow at regular distances into maize crops from the pollen source (Weber et al., 2007). Such layouts may underestimate cross-fertilization in real commercial situations, as the first maize plants make a barrier for pollen dispersal to plants further into the crop (Weekes et al., 2007).

In this study, transgene flow was detected in three out of five cases with potential for gene flow considering the distance between GM and non-GM crops and synchrony between sowing dates. The detected GM events corresponded to the putative source of GM pollen in all cases. These results suggest that cross-fertilization may be a common situation in Uruguay, when isolation between crops either by distance or time is not provided. The presence of transgenes in the non-GM crop was detected even in situation 4, where isolation distance (330 m) was longer than the minimum of 250 m legally required in Uruguay. However, until now, no specific threshold level in harvested kernels for labeling non-GM conventional and/or organic maize was defined in Uruguay.

In Cases 3 and 5, no transgene was detected in the non-GM crop. Sowing dates in Case 3 were synchronized, but GM hybrid plants grew significantly larger and faster, thus flowering times were poorly overlapped. In Case 5, the area of GM-crop was six times smaller than the non-GM crop, and sowing dates had almost two weeks difference. Synchrony between flowering times seems to be a key factor for cross-pollination (Messeguer et al., 2006). In agreement with these previous studies, this research suggests that isolation by distance between crops or by the presence of physical barriers in between may quantitatively reduce cross-fertilization, but would not completely prevent it.

Detection techniques used in this research provided the frequency of transgene in the progeny of non-GM recipient crops, which is half the actual frequency of cross-fertilization. GM maize hybrids are usually hemizygous for the transgene (Devos et al., 2005). Thus, only half of the pollen grains produced by GM crops carry the transgene, and therefore actual cross-fertilization rates could be double the frequencies reported for the transgenes.

A progeny plant from site 1.1 and one from site 2.1 were negative for DAS-ELISA but positive for PCR analysis, which is not easy to explain. Detection by DAS-ELISA implies that the transgene must be biologically functional to express the Cry1Ab protein, which may not always be the case, though recombination events affecting the inserted transgene are unlikely to occur in one generation. This result is neither attributable to stochastic variation, nor to a low sensitivity of DAS-ELISA. The test was repeated twice for each sample, and we achieved a detection threshold of (at least) one positive in 100 bulked plants with this method, whereas 30 plants were bulked for this study.

Whenever two or more sampling sites were defined within the non-GM maize field, cross-fertilization was only detected in the site closest to the GM source. This finding is in line with previous studies in which cross-pollination frequencies were usually found higher for the outer parts of the recipient field, and declined towards the inner parts due to a higher density of and competition by own pollen of the recipient crop (Devos et al., 2005). Langhof et al. (2010), however, evaluated Bt adventitious presence at different depths into the recipient field, and found that cross-fertilization did not follow a regular pattern deep into the field. Therefore, discard of the rows facing Bt source may help to maintain cross-fertilization below a threshold level, but would not completely prevent it.

This study constitutes a first approach to estimate cross-pollination between GM and non-GM maize fields in actual commercial situations in Uruguay. Forthcoming research should expand the investigated regions in the country, and increase both the number of sampled pools and plants within pools in order to diminish the detection thresholds and confidence of estimators for Bt adventitious presence (Montesinos López et al., 2010). The use of real-time PCR will help by providing quantitative results for a single composite sample (Langhof et al., 2010). As the GM maize acreage in Uruguay is tending to increase, updated information about transgene flow and adventitious presence of transgenes in non-GM crops will contribute to the evaluation of the feasibility of the regulated coexistence policy.

MATERIALS AND METHODS

Sampling and production of seedling

In the season 2007–2008, 40 maize farm fields were visited in Colonia and San José (Uruguay) to detect appropriate sampling cases. Nine pair-field cases with non-GM crops adjacent to a GM crop were identified, five of them with potential cross-fertilization between GM and non-GM maize due to suitable distances between crops and synchrony between planting dates (less than two weeks). Geographic coordinates and the distances between fields were determined using GPS technology. Table 1 summarizes information for the five selected cases (data not shown for other cases).

The following sampling strategy was employed. Firstly, leaves were collected from each field, in order to confirm the respective GM and non-GM identity. Four bulked leaf samples (each one composed by leaves from 15 plants), from each non-GM crop were sampled. In addition, two bulks from each GM crop were sampled. Leaves were kept at −20 °C until processed and analyzed. Secondly, the non-GM maize crops were sampled for progeny analysis: when plants had cobs with mature grains, one cob per plant was collected, forming bulks of 15 cobs. Sampling sites were defined within the non-GM crop in function of the distance to the neighbor GM crop (see Fig. 1), and at least two bulks of 15 plants were

randomly selected from each sampling site. For Case 1, two sampling sites were defined, 1.1 and 1.2, at distances of 40 m and 190 m from the neighbor GM field, respectively. For Case 2, three sampling sites were defined as 2.1, 2.2 and 2.3, at distances of 380 m, 180 m and 100 m from the GM pollen source, respectively. For Cases 3 and 5 only one sampling site per case was defined (identified as sites 3.1 and 5.1 respectively), located in the edge of the non-GM field next to the neighbor GM crop. For Case 4 no sampling sites were specifically defined within the non-GM crop, because grains harvested were randomly sampled instead of cobs from the plants.

A total of seventeen bulks of fifteen cobs each were collected: three bulks from the sampling site 1.1 and two bulks from the site 1.2 (Case 1), two bulks from each of the sampling sites 2.1, 2.2 and 2.3 (Case 2) and three bulks from each of the sampling sites 3.1 and 5.1 (Cases 3 and 5). For Case 4, grains already harvested were randomly sampled.

To get leaf tissue of the progeny for DNA isolation and protein extraction, four seedlings were grown from grains taken from each cob. At least 120 seedlings per sampling site were produced for analysis. For Case 4, 764 seedlings were produced from randomly sampled grains.

Transgene detection

Leaf extracts for DAS-ELISA (Double Antibody Sandwich Enzyme-Linked ImmunoSorbent Assay) and PCR (polymerase-chain reaction) analyses were prepared from bulked material of either 15 mother plants or 30 seedlings. All samples were tested by DAS-ELISA. A total of 480 plants (32 bulks) from GM and non-GM fields (mother plants) were analyzed. In addition, 1020 seedlings (34 bulks) grown from collected cobs and 764 seedlings from grains collected in Case 4 (23 bulks of 30 seedlings and four bulks with a variable number) were analyzed.

DAS-ELISA was performed using PathoScreen kit for Bt-Cry1Ab/1Ac protein (Agdia Inc, Indiana, USA) following manufacturer instructions. Aqueous extracts were prepared by treating plant tissue with PBS-T buffer pH 7.4, and centrifuged. A volume of 100 µL of each clear supernatant was placed into a well coated with the Cry1Ab/1Ac specific antibody in the microtiter plate, mixed with 100 µL of the enzymatic conjugate and incubated during two hours at room temperature. Plates were carefully washed and TMB was added as substrate. The presence of the protein was detected by color development measured at 450 nm in a Multiskan RC (Labsystems). All assays were performed by duplicate and including positive and negative controls (Agdia Inc, Indiana, USA). Samples that had positive DAS-ELISA result were further analyzed by PCR to confirm transgenicity and identify the involved event.

Maize DNA was extracted using the method described by Dellaporta et al. (1983) and modified as follows: 100 mg of leaf tissue was ground under liquid N_2. DNA was re-suspended in 100 µL of mQ water. DNA concentrations were estimated by UV spectrophotometry, and adjusted to 100 ng.µL^{-1}. Samples were kept at –20 °C until use. All extractions were performed by duplicate. A first polymerase chain reaction test for transgenicity was based on CaMV promoter 35S-1/35S-2 primers (FAO/WHO, 2002). Thereafter, positive samples were tested using Cry05/Cry06 specific primers for event Bt176, VW01/VW03 primers for MON810, and IVS2-2/PAT-B primers for Bt11 (FAO/WHO, 2002). All reactions were run in 250 µL PCR tubes containing 1 X PCR buffer (Fermentas), 0.2 mmol.L^{-1} deoxynucleotide triphosphates (dNTPs), 2 mmol.L^{-1} $MgCl_2$, 0.5 µmol.L^{-1} primers, 1 U Taq DNA polymerase (Fermentas) and 1 µL (100 ng) of DNA. PCR was performed in Perkin Elmer Gene Amp PCR System 2400 with the following program: a denaturing step during 3 min at 94 °C, followed by 35 cycles of 45 s at 94 °C, 55 s at annealing temperature, and 45 s at 72 °C, and a final extension step at 72 °C for 7 min. All PCR reactions always included positive and negative controls, and were performed in at least three independent experiments. A volume of 5 µL of each reaction-tube was analyzed in a 6% PAGE electrophoresis and further stain using a silver protocol (Sanguinetti et al., 1994).

Data analyses

Bulk samples of 30 seedlings (progeny plants) with positive results for DAS-ELISA were subdivided into three sub-samples (10 individuals each) in order to investigate the number of positive seedlings per sample, but not further subdivided. In each positive bulk sample, only one of those three sub-samples was positive. This indicated that there was a probability > 99% that only one plant per positive bulk-sample was positive.

The frequency of transgene detection in the non-GM progeny was calculated in two ways: (i) the observed frequency, as the percentage of progeny plants that expressed the transgene (Cry1Ab protein) on the total number of progeny plants analyzed per site; and (ii) the frequency (p) for which, according to the sample size per site, the detection probability (P_d) of a rarely occurring allele is 95%. P_d is defined as the probability to detect at least one positive plant per site in a sample of n plants, according to the formula $P_d = 1 - (1 - p)^S$ where S is defined as the number of progeny plants (n) analyzed per site, considering that all positive ones are distributed with uniform frequency p (Piñeyro-Nelson et al., 2009). For instance, if sample size (n) is 120 plants, finding one GM

positive plant results in an observed frequency of 0.83% (1/120 = 0.00833), and an estimated frequency in the population (p) of 2.5% (1/40) with 95% of confidence. Whenever no GM positive plant is detected, adventitious presence of transgene in non-GM plants is considered below a threshold detection frequency defined by the sample size (n).

As it is assumed that non-GM plants do not contain the transgene, S is equivalent to n (instead of $2n$). For a sample composed of n individuals, P_d is 95% for $p = 1 - \sqrt[n]{0.05}$. For those sampling sites where no GM positive result was detected, it was inferred that cross-fertilization frequency with the transgene is below the detection threshold $p < 1 - \sqrt[n]{0.05}$ with 95% of confidence.

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