Quantification of pollen mediated gene flow in maize

SORINA POPESCU*, FLORENCE LEPRINCE**, OANA IOJA-BOLDURA*, ANDREI MĂRUȚESCU***, IOAN SABĂU****, ELENA MARCELA BADEA*****

* Banat University of Agricultural Sciences Timișoara, Romania
** ARVALIS - Institut du vegetal France
*** Ph.D student Banat University of Agricultural Sciences Timișoara, Romania
**** GMO Consulting Bucharest
***** Institute of Biochemistry, Romanian Academy

Abstract

One approach to ensuring coexistence of genetically modified (GM) and conventional maize (Zea mays L.) is the understanding of pollen dispersal in order to keep GM-inputs below the regulated tolerance threshold of 0.9%. In defining science-based isolation distances between GM and non-GM maize fields, cross-fertilization rates have been studied both in experimental and commercial fields. Gene flow was followed based on Bt maize sequence information. Our results pointed out that cross-fertilization levels rapidly decrease with increasing distance from the pollen source and a 25 m isolation distance would be sufficient to keep GM-inputs from cross-fertilizations below the tolerance threshold of 0.9% in the harvest of neighboring non-GM maize fields.

Key words: Bt maize, gene flow, q-PCR

Introduction

In 2009, GM maize was the second biotech crop grown in the world, occupying 41.7 million hectares, which represent about 30% of global biotech crop area. Most GM maize hybrids cultivated globally exhibits two output traits, herbicide tolerance and insect resistance. GM maize event MON810, has been genetically modified (GM) through the insertion of a gene from the Bacillus thuringiensis (Bt) soil bacterium and thus produce an insecticidal protein (δ-endotoxin) for control of Lepidoptera pest insects such as Ostrinia nubilalis (Hbn.), the European corn borer.

In Europe, cultivation of Bt maize has increased constantly in recent years and reached 108 000 hectares in 2009, amounting to around 1 percent of the total area cropped with maize. Romania was the third European country, after Spain and the Czech Republic with a Bt area of about 3000 ha in 2009.

One approach in ensuring coexistence of genetically modified (GM) and conventional maize (Zea mays L.) is to better understand maize pollen dispersal in order to avoid the potential contamination of non-GM maize due to pollen-mediated gene flow from GM maize (A.S. GOGGI & al. [1], J. MESSENGUER & al. [2], C.M. SMITH & al. [3]). Since 2003, coexistence in Europe has been subject to Regulation (EC) 1830/2003 that sets a labeling threshold of 0.9% for unintentional or the technically unavoidable (“adventitious”) presence of GM material in harvested material or products from non-GM crops [ B.L. Ma & al. [4], A. MESSEAN & al. [5], [6]).

Given that pollen concentrations and thus cross-fertilization levels rapidly decrease with increasing distance from the pollen source, spatially isolating GM maize fields from non-GM maize fields is an effective on-farm strategy to reduce the extent of cross-fertilization. As maize pollen is fairly heavy, the vast majority is deposited within a maximum of 18–20
meters distance of the emission source, minimizing the chances of cross-fertilization occurring beyond this distance (Y. DEVOS & al. [7]).

In defining science-based isolation distances between GM and non-GM maize fields cross-fertilization rates have been studied both in experimental and commercial fields. Gene flow was followed based on Bt maize sequence information.

The construct used in the transformation of Bt maize includes the enhanced CaMV 35S-promoter, the maize hsp70 intron 1 and the synthetic δ-endotoxin cry IA (b) gene followed by the nos terminator (M. QUERCI & al. [8]) (Fig. 1).

Figure 1. The structure of the Bt maize transgenic construct (after M. QUERCI & al. [8])

For GMO screening, the 35 S promoter was targeted by specific primers p35S-cf3 and p35S-cr4. The expected amplicon was a 123 bp fragment where the primers have been positioned in the corresponding region of the CaMV 35S promoter sequence (F.E. AHMED & al. [9]). For a more specific detection, mg1 and mg2 primers were used. In this case, the expected amplicon was a 401 bp fragment where the primers have been positioned in the corresponding region of the CaMV 35S- hsp 70 intron sequence.

Conventional qualitative Polymerase Chain Reaction (PCR) assay to detect the presence of MON810 event and a real-time PCR analysis to quantify the amount of transgene present in positive samples were performed. The specific target for MON810, a 92 bp fragment of the single copy DNA integration-border region of the genomic sequence and the inserted sequence element originating from CaMV (35S promoter), was amplified in TaqMan PCR, compared with a reference gene [M. HARDEGGER & al. [10]].

Material and methods

Sampling process

In 2007 and 2008, samples were collected from experimental fields organized by Monsanto Company in plots with GM maize (300 x 250 m), surrounded by 36 rows with conventional maize. Cobs were sampled from each sixth row (6 samples for each geographical direction) (Fig. 2).

Figure 2. The sampling scheme of the 2007 and 2008 experimental field
The GM source was DKC4442YG hybrid containing the MON810 event. To obtain good flowering synchronicity between GM and non-GM maize and thus optimal conditions for pollen-mediated gene flow, a near-isogenic maize variety was grown in the receptor field.

In assessing the efficiency of co-existence measures established according to specific regulation, samples have been taken from the refuge area from commercial fields located in pedoclimatic conditions favorable for maize cultivation, in South (Chiciu and Chirnogi from Călărași county) and West (Gătaia from Timiș county) regions of Romania. According to the Insect Resistance Management strategy, the refuge is cultivated with conventional maize and represents 20% of the total area cultivated with GM maize.

For Chiciu farm, the refuge area has been interspaced within genetically modified maize strips and samples have been taken from rows 3, 9, 12, 18, 24 and 71 representing a total width of 50 m (Fig. 3A). Numbering begins from the row at separation point between the two maize types, in the direction of predominant wind according to the design below. Five ears from five consecutive plants at a distance of \( \frac{1}{4}, \frac{1}{2} \) and \( \frac{3}{4} \) of the total field length have been sampled. (F. LEPRICE – BENETRIX & X. FOUEILLASAR [11]).

For Chirnogi farm, samples were taken from the border row area of 200 m width surrounding the field, according to the design presented below (Fig. 3B).

For Gătaia farm, the refuge area consisted of 12 rows with conventional maize surrounding the Bt field. The high level of contamination in the samples collected from the refuge area suggests that the farmer did not properly apply the technology recommended by the seed producer.

In 2009, cobs were collected only from one farm (Chiciu, Călărași county), following four sampling schemes, from the refuge rows 43, 46 and 50. The methodology used was elaborated by the Co-Extra Project, developed in FP6 Program (Fig. 4).

![Figure 3](image-url)

**Figure 3.** The commercial fields sampling schemes for Chiciu (A) and Chirnogi (B), in 2008
For molecular analysis, maize samples collected from different locations previously described were used. For the standard curve, certified materials with known GM maize concentrations were analyzed (0.1; 0.5; 1; 2 and 5% GMO).

First, maize seeds were grinded, the flour was homogenized and the analytical samples were prepared (100 mg for each sample). The DNA was extracted using CTAB method. Each sample was extracted in two replicates; in addition, an extraction blank control (EB) and an environment control (EC) were prepared.

Before amplifications, extracts were assessed for total DNA yield using spectrophotometer. Each amplification was performed in two repetitions, thus each sample was analyzed in four amplification reactions (M. QUERCI & al. [8]).

The specie specific amplification.

The primers sequences: ZEIN3 \(5'\)AGTGCGACCCATATTCCAG\(3'\); ZEIN4 \(5'\)GACATTGTGGCATCATCATT\(3'\). The PCR mixture: PCR buffer 1x; MgCl\(_2\) 2,5mM; dNTP 0,2mM; ZEIN3 0,5μM; ZEIN4 0,5μM; Taq DNA polymerase 0,025U. The amplification program: denaturation 95°C -3 min; 50 cycles: denaturation 95°C -1 min; Primer annealing 60°C -1 min, DNA synthesis 72°C -1 min; Final extension 72°C -3 min.

The GMO specific amplification.

The primers sequences: \(p\)-35S-cf3 \(5'\)CCACGTCTTTCAAGCAAGTGG\(3'\); \(p\)35S-cr4 \(5'\)TCCTCTCCAAATGAAATG AACTTCC \(3'\). The PCR mixture: PCR buffer 1x; MgCl\(_2\) 2,5mM; dNTP 0,64mM; \(p\)35S-cf3 0,6μM; \(p\)35S-cr4 0,6μM; Taq polymerase ADN 0,1U. The amplification program: denaturation 95°C -10 min; 50 cycles: denaturation 95°C -25 sec; Primer annealing 62°C -30 sec, DNA synthesis 72°C -45 sec; Final extension 72°C – 7 min.

The GMO quantification.

For the GMO quantification each sample was analyzed in three repetitions using the following reagents:
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- the specie specific primers (hmg gene) ZM1-F 5´-TTg gAC TAg AAA TCT C gT gCT gA-3´ and ZM1-R 5´-gCT ACA TAg ggA gCC TTg TCC T-3´ and a TaqMan probe ZM 5´-FAM—CAA TCC ACA CAA ACg CAC gCg TA-TAMRA-3´
- the GMO specific primers Mail-F1 5´-TCg AAg gAC gAA ggA CTCTAACgT-3´ and Mail-R1 5´-gCC ACCTTCCTTTTCCAC TAT C TT-3´ and a TaqMan probe Mail-S2 5´-FAM-AAACATCTTTgCCATTTgCCAgC-TAMRA -3´

The content of the qPCR for hmg gene: TaqMan Universal Mastermix 1x; ZM1-F 300 nM, ZM1-R  300 nM, Taqman probe ZM 160nM; DNA 50 ng. The content of the qPCR for transgene: TaqMan Universal Mastermix 1x; Primer Mail-F1- 300nM; Primer Mail-R1-300 nM; Probe Mail-S2 180nM; DNA 50 ng.

The amplification program: Pre-PCR: decontamination 50°C -120sec; Pre-PCR: DNA polymerase activation and DNA template denaturation  95ºC - 600sec; PCR (45 cycles): Stage 1 Denaturation 95ºC - 15sec; Stage 2 Annealing and extension 60ºC - 60sec.

Results and discussions

To confirm the presence and quality of DNA extracted from maize samples collected from experimental fields, ZEIN 3 and ZEIN 4 primers, specific to the maize zein gene, were used (Fig. 5).

Figure 5. The agarose gel analysis of the amplification products derived from samples collected from 2 geographical directions (S- South and W- West), using the primers specific for zein gene
West -WP1 (4.2 m), WP2 (8.4m), WP3 (12.6 m), WP4 (16.8 m), WP5 (21 m), WP6 (25 m);
South - SP2 (8.4m), SP3 (12.6 m), SP4 (16.8 m), SP5 (21 m), SP6 (25 m);
EB - Extraction blank; EC - Environmental control; NDT - Negative DNA target; NTC - Non template control, PE - Positive extraction control

The gel analysis confirmed that cross-contamination was avoided in the DNA extraction and amplification procedures. All negative and positive controls were as expected and the amplified bands for the samples were similar (277 bp) to the positive control. All of the DNA samples were amplifiable and further analyzed.

Screening target commonly used for MON810 event is the 35 S promoter. For its specific detection, p35S-cf3 and p35S-cr4 primers were used (Fig. 6). The expected amplicon was a 123 bp fragment where the primers have been positioned in the corresponding region of the CaMV 35S promoter sequence.

In quantifying the amount of GM sequences present in conventional maize, the positive samples for the 35 S promoter have also been analyzed. The value was determined as a ratio between the number of GM maize specific target DNA sequences and the number of DNA sequences of an endogenous reference gene. The reference gene is species-specific, has a single copy per haploid genome and as amplifiable as the analysed transgene.

All samples collected from the experimental field in 2008 were analyzed and the GM content was determined by q-PCR technique (Table 1).
Figure 6. The agarose gel analysis of the amplification products derived from samples collected from 4 geographical directions (E-East, W-West, N-North and S-South), using the primers specific for 35 S promoter.

**East** - EP1 (4.2 m), EP2 (8.4 m), EP3 (12.6 m), EP4 (16.8 m), EP5 (21 m), EP6 (25 m);

**West** - WP1 (4.2 m), WP2 (8.4 m), WP3 (12.6 m), WP4 (16.8 m), WP5 (21 m), WP6 (25 m);

**North** - NP1 (4.2 m), NP2 (8.4 m), NP3 (12.6 m), NP4 (16.8 m), NP5 (21 m), NP6 (25 m);

**South** - SP2 (8.4 m), SP3 (12.6 m), SP4 (16.8 m), SP5 (21 m), SP6 (25 m);

EB - Extraction blank; EC - Environmental control; NDT - Negative DNA target; NTC - Non template control; PE - Positive extraction control.

Table 1. The GM content in conventional maize samples collected from 2007 experimental field correlated with the distance from the Bt source.

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Conc. (%)</th>
<th>Sample code</th>
<th>Conc. (%)</th>
<th>Sample code</th>
<th>Conc. (%)</th>
<th>Sample code</th>
<th>Conc. (%)</th>
<th>Distance (m)</th>
<th>Average conc. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E 1</td>
<td>6</td>
<td>W 1</td>
<td>2,2</td>
<td>N 1</td>
<td>2,9</td>
<td>-</td>
<td>-</td>
<td>4,2</td>
<td>3,70</td>
</tr>
<tr>
<td>E 2</td>
<td>8,6</td>
<td>W 2</td>
<td>3,8</td>
<td>N 2</td>
<td>0,5</td>
<td>S 2</td>
<td>1,7</td>
<td>8,4</td>
<td>3,65</td>
</tr>
<tr>
<td>E 3</td>
<td>2,4</td>
<td>W 3</td>
<td>0,9</td>
<td>N 3</td>
<td>0,4</td>
<td>S 3</td>
<td>1,3</td>
<td>12,6</td>
<td>1,25</td>
</tr>
<tr>
<td>E 4</td>
<td>1,8</td>
<td>W 4</td>
<td>0,6</td>
<td>N 4</td>
<td>0,1</td>
<td>S 4</td>
<td>1,0</td>
<td>16,8</td>
<td>0,88</td>
</tr>
<tr>
<td>E 5</td>
<td>0,8</td>
<td>W 5</td>
<td>0,4</td>
<td>N 5</td>
<td>0,2</td>
<td>S 5</td>
<td>0,2</td>
<td>21</td>
<td>0,40</td>
</tr>
<tr>
<td>E 6</td>
<td>0,5</td>
<td>W 6</td>
<td>0,1</td>
<td>N 6</td>
<td>0,1</td>
<td>S 6</td>
<td>0,4</td>
<td>25</td>
<td>0,28</td>
</tr>
</tbody>
</table>
The results obtained show that a distance of 21 m, in all four geographic directions, is sufficient for a 0.9% threshold. The GM content in conventional maize seed was different for each geographic direction, with the highest value of 8.6% and the lowest 0.1%. When the average concentration for each distance was analyzed, it can be observed that 16.8 m was enough to decrease the GM content below 0.9%.

The DNA extracted and purified from the samples collected from the experimental field in 2008, was amplified with the *zein* primers and all the results proved to be positive. The amplifiable DNA was further analyzed using p35S-cf3 and p35S-cr4 primers, but the reactions were not successful for any of the analysed samples, even for the positive controls. It is believed that a mutation must have taken place in the annealing sequences of these primers. Similar observation was done in different studies demonstrating a truncation event at the 3’ end of the *cryI(A)b* gene leading to the complete loss of the NOS terminator (M. HERNANDEZ & al. [12]). Other analysis revealed the absence of both T-nos and part of the 3’ (tail) end of the *cry1Ab* gene and also indicated a partial deletion of P-e35S (K. CHANDRA & al. [13]).

In this case, using a different primer pair, mg1 and mg2, positioned in the corresponding region of the CaMV 35S-hsp 70 intro sequence, the amplification reactions were possible and the positive samples could be visualized on agarose gel (Fig. 7).

The GM quantification reaction was carried out without any problems, emphasizing that the sequence from the integration-border region of the genomic DNA and the inserted sequence element originating from CaMV (35S promoter) have not been modified.

**Figure 7.** The agarose gel analysis of the amplification products from E-East samples, using the primers specific for 35 S promoter - hsp 70 intron sequence

**East** - E1 (4.2 m), E2 (8.4m), E3 (12.6 m), E4 (16.8 m), E5 (21 m), E6 (25 m);

EB - Extraction blank; EC - Environmental control; NDT - Negative DNA target; NTC - Non template control, PE - Positive extraction control

**Table 2.** The GM content in the conventional maize samples collected from the 2008 experimental field correlated with the distance from the Bt source

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Concentration (%)</th>
<th>Distance (m)</th>
<th>Average concentration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1</td>
<td>13.06</td>
<td>4.2</td>
<td>13.65</td>
</tr>
<tr>
<td>E2</td>
<td>2.74</td>
<td>8.4</td>
<td>2.12</td>
</tr>
<tr>
<td>E3</td>
<td>1.34</td>
<td>12.6</td>
<td>1.5</td>
</tr>
<tr>
<td>E4</td>
<td>0.44</td>
<td>16.8</td>
<td>0.9</td>
</tr>
<tr>
<td>E5</td>
<td>0.71</td>
<td>21</td>
<td>0.93</td>
</tr>
<tr>
<td>E6</td>
<td>0.48</td>
<td>25</td>
<td>0.53</td>
</tr>
</tbody>
</table>

The GM quantification results obtained showed that 25 m was enough to keep GM content below 0.9%, for all of the four geographic directions. In case of average concentrations, only a 16.8 m isolation distance was enough to decrease the GM content below 0.9%.
In the samples collected from the two commercial fields at Chiciu and Chirnogi, GM maize sequence was detected and quantified in conventional seed (Table 3). The highest concentration of GM presence in conventional seed was 9.62% (4.2 m) and the lowest 0.11%. These results demonstrate that 8.4 m is enough to keep the GM concentration below 0.9%.

Only in the case of Chiciu farm where the refuge was interspaced within two genetically modified maize strips, the samples collected from 50 m distance were positive (0.28%).

Table 3. The GM content in the conventional maize samples collected from 2008 commercial fields correlated with the distance from the Bt source

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Distance (m)</th>
<th>Conc. [%] Chiciu</th>
<th>Conc. [%] Chirnogi</th>
</tr>
</thead>
<tbody>
<tr>
<td>R 3</td>
<td>2.1</td>
<td>2.02±0.29</td>
<td>4.08±0.58</td>
</tr>
<tr>
<td>R 6</td>
<td>4.2</td>
<td>1.08±0.15</td>
<td>9.62±1.37</td>
</tr>
<tr>
<td>R 12</td>
<td>8.4</td>
<td>0.38±0.05</td>
<td>0.11±0.01</td>
</tr>
<tr>
<td>R 18</td>
<td>12.6</td>
<td>0.48±0.07</td>
<td>0.21±0.03</td>
</tr>
<tr>
<td>R 24</td>
<td>16.8</td>
<td>0.82±0.12</td>
<td>0.39±0.06</td>
</tr>
<tr>
<td>R 71</td>
<td>50</td>
<td>0.28±0.04</td>
<td>negative</td>
</tr>
</tbody>
</table>

The results collected from all the analysed samples demonstrate that in the commercial fields, the GM average concentrations in conventional maize were lower than in conventional maize sampled from experimental fields. It is possible that in the commercial fields flowering was not synchronized between GM and non-GM maize.

An average isolation distance of 16.8 m seems to be enough to assure a GM-input in conventional maize below 0.9% (with a maximum of 25 m for one samples) (Fig. 8).

Figure 8. The comparative analysis of the average GM content (%) in conventional maize seed collected from experimental and commercial fields

To confirm the results previously obtained, in 2009 a complex sampling procedure was used, following four schemes (S1, S2, S3 and S4). Each sample was analyzed twice and for each distance, the average concentration of GM-inputs in conventional maize was determined (Table 4).
Table 4. The GM content in conventional maize samples collected from 2009 commercial fields following four sampling schemes (S1, S2, S3 and S4) correlated with the distance from the Bt source

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>S1 / conv. 2</th>
<th>S1 / conv. 3</th>
<th>S2 / conv. 3</th>
<th>S3 / conv. 3</th>
<th>S4 / conv. 3</th>
<th>Distance (m)</th>
<th>Average conc. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R 43</td>
<td>0.3</td>
<td>0.5</td>
<td>0.6</td>
<td>0.4</td>
<td>0.3</td>
<td>30</td>
<td>0.42</td>
</tr>
<tr>
<td>R 46</td>
<td>0.37</td>
<td>0.9</td>
<td>0.8</td>
<td>0.5</td>
<td>0.3</td>
<td>32.5</td>
<td>0.9</td>
</tr>
<tr>
<td>R 50</td>
<td>0.4</td>
<td>0.3</td>
<td>0.2</td>
<td>0.4</td>
<td>0.2</td>
<td>35</td>
<td>0.33</td>
</tr>
</tbody>
</table>

Average value 0.45

In the interval of 30-35 m, the average GM concentration in conventional seed was 0.45%, with a maximum of 1.0% and a minimum of 0.2%. These differences are most probably caused by incorrect drilling techniques.

It is known that the most important factor in managing co-existence between GM crops and other cropping systems is geographical isolation of fields. These separation distances between GM and non-GM maize are widely accepted as an effective measure to reduce cross-contamination. Several EU countries proposed different separation distances ranging from 25 m to 800 m: Sweden, 15 m for forage and 25, for grain; France, 50 m for grain; UK, 80 m for forage and 110 m for grain; Germany, 150 m for grain; Denmark and Portugal, 200 m; Luxembourg, 800 m (Y. BRUNET [14]).

Assuming that coexistence regulations should be based on scientific criteria, our results, obtained both in experimental and commercial fields, demonstrate that in Romanian agricultural conditions, a 25 m isolation distance is enough to keep GM-inputs in conventional maize fields below 0.9%. Our results are in accordance with the EU recommendations which proposed separation distances of 20 m and 50 m for silage and grain maize, respectively, values determined based on statistical data of maize areas and aerial photographs with geographic information systems (A. RICHROCH & al. [15]).

Conclusions

In detecting the presence of MON810 event in conventional maize samples collected from experimental fields, it was necessary the use of two distinctive primer sets. For the samples collected in the second year of study, the amplification with primers specific for 35S promoter (p35S-cf3 and p35S-cr4) have not proved to be successful, probably due to base mutation in the annealing sequences. In this case, by using other pairs of primers, amplification reactions were possible, meaning that there was no modification in the CaMV 35S- hsp 70 intro sequence or in the integration-border region of the genomic sequence and the inserted sequence element originating from CaMV 35S promoter.

For experimental fields, the GM content in conventional maize seeds was different depending on the geographical direction studied. The maximum distance where the GM content was below 0.9% for all of the four geographic directions was 21 m, in 2007, and 25 m, in 2008. If the average value for each distance was analyzed, 16.8 m isolation appears to be enough to maintain GM –inputs in conventional maize field below 0.9%.

The results of GM quantification in the samples collected from commercial fields in 2009, demonstrated that an isolation distance of 8.4 m is necessary to decrease cross-fertilization rate below 0.9%. The results obtained in 2009 using a different sampling scheme, show an average for cross-fertilization rate of 0.47% for the rows 43, 46, 50 (at 30m – 35m from the pollen source).
The results obtained in both fields types demonstrated that cross-fertilization levels rapidly decrease with increasing distance from the pollen source and a 25 m isolation distance would be sufficient to keep GM-inputs from cross-fertilizations below the tolerance threshold of 0.9% in the harvest of neighboring non-GM maize fields.

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