

## GIMI 2: A Tool for Fast Estimation and Prediction of GMO Maize Contents in Real Coexistence Situations

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Coexistence of GM and conventional maize in locations where the two types of varieties are commercially grown would benefit from a tool to estimate and predict the GMO contents of conventional fields that are surrounded by GMO crops. We present GIMI 2 as software to address this need.

Based on experimental data collected along a number of seasons in two regions where GM and conventional maize was concurrently grown, an index (GI) was established to calculate the GMO percentage in a given conventional field based on flowering coincidence and distances between fields. Upon validation (four seasons), we found sub-optimal agreement between *in silico* and experimentally calculated GM contents in long and narrow fields, so we quantified the effect of the field sizes on cross-pollination.

A new index was developed as an evolution of the GI that additionally compiles the field sizes and shapes, as well as the wind intensity and direction. GM contents calculated with the new index show close correlation with those obtained experimentally both in small and large fields.

To facilitate application of the new index in real agricultural field conditions, we developed the GIMI 2 software. It calculates the GM contents of conventional fields in an interactive and fast manner on the basis of the following data, which can be easily introduced by the user: field vertex coordinates, flowering date and variety (GM/conventional) of each field, and wind. We propose GIMI 2 as user-friendly software to coordinate coexistence in agronomic regions.

**Keywords:** coexistence, GIMI software, GMO, maize, prediction.

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## Introduction

Transgenic crops have been cultivated worldwide since 1996 in a progressively growing area that reached a total of 51.2 million hectares for maize in 2011 (James, 2012). Nevertheless, only 114,490 ha were cultured with transgenic maize in Europe in 2011. Bt-maize MON810, expressing the *Bacillus thuringiensis* (Bt) insecticidal protein Cry1Ab, is the only genetically modified (GM) maize currently grown in the European Union (EU). It confers resistance against larvae of certain lepidopteran pests such as *Ostrinia nubilalis* Hüber and *Sesamia nonagroides* Lefèbvre.

GM crops have very low adoption rates in the EU; this is generally attributed to social and political opposition towards agro-food biotechnology. This situation has forced policymakers to elaborate a complex legislation that regulates the cultivation of GM plants and the coexistence between transgenic and conventional cultures. Commission Recommendation 2010/C200/01/CE

(European Commission, 2010) defines coexistence as the principle according to which farmers should be able to freely cultivate the crops of their choice, whether GM, conventional, or organic. Each country in the EU is required to establish its own regulation to ensure coexistence, and it has to be based on scientific criteria. Moreover, consumers' freedom of choice is guaranteed through mandatory labelling and traceability of GM products (European Commission, 2003a; 2003b).

A number of different factors have been described to determine the adventitious presence of GM material in conventional or organic crops, including accidental seed impurity; carrying over through the sowing equipment due to inappropriate practices; cross-pollination from GM to non-GM crops; the presence of volunteer plants; and accidental product admixture at harvest, transport, and/or storage. Among these, cross-fertilization causes the highest concern because it is difficult to keep under

control and depends on many factors, including crop management, type of cultivar, and climatic conditions.

Maize is a monoecious plant. It has two types of flowers that develop at different parts of the plant—the male flower forms at the top of the plant and the female flower emerges from the leaf node on the side of the plant. Pollen release typically begins before the silks of the same plant are receptive, followed by an overlapping period. In general, self-pollination represents up to 5% of total fertilization (Purseglove, 1972). Numerous trials have been carried out to investigate maize pollen dispersal (reviewed in Emberlin, Adams-Groom, & Tidmarsh, 1999; and Treu & Emberlin, 2000). They clearly show that, even though maize pollen is relatively large and heavy, it can travel long distances on the airflow when suitable meteorological conditions occur. Therefore, some degree of cross-fertilization is almost inevitable.

The adventitious presence of GM grains in fields sown with conventional maize—occurring as a consequence of cross-fertilization from plants from other fields sown with GM maize—has been largely studied by several authors (reviewed in Devos, Reheul, & De Schrijver, 2005; Devos, Dillen, Reheul, Kaiser, & Sanvido, 2009; and Sanvido et al., 2008). In parallel, great efforts have been made to construct simulation models able to predict gene flow and, on this basis, establish reliable coexistence rules (reviewed in Beckie & Hall, 2008). These models must be validated on the basis of experimental data, both collected in specifically designed field trials and in real agricultural situations of coexistence.

We previously carried out several field trials with the aim of identifying the most relevant factors affecting gene flow from transgenic towards conventional maize fields. We could clearly establish a quick decrease in the gene flow with the depth of the receptor field (Melé et al., 2004; Palau-delmas et al., 2012; Pla et al., 2006). This also has been observed by others (reviewed in Devos et al., 2009).

In recent years, several studies have estimated the effectiveness of physically separating the GM donor and the conventional receptor maize fields to minimize undesired cross-fertilization (Bénétrix & Bloc, 2003; Brookes et al., 2004; Della Porta et al., 2008; Henry, Morgan, Weekes, Daniels, & Boffey, 2003; Ma, Subedi, & Reid, 2004; Melé et al., 2004; Ortega Molina, 2006; Weber, Bringezu, Broer, Eder, & Holz, 2007). From these studies, it can be concluded that a separation distance of 20–25 m is, in general, enough to achieve GM levels below the 0.9% threshold in the yield of conventional fields. Occasionally, and in particular for very

small fields (with areas below 0.5 ha) and for fields with a long and narrow shape, the isolation distance may need to be extended up to 50 m.

However, the separation distances can be reduced by sowing a buffer of non-GM maize plants surrounding the GM ones. We previously demonstrated (Pla et al., 2006) that adventitious presence of GM maize was significantly lower when a buffer zone of 10 m of non-GM maize plants connected the transgenic and conventional fields than in the absence of any plant barrier. These results confirmed those reported by Jones and Brooks (1950) and raised a question on the approach assumed to establish the coexistence regulation, which is based only in physical distance between fields.

Moreover, we confirmed that flowering coincidence is crucial for cross-pollination, and flowering asynchrony (or flowering delay) can be used as a tool to control cross-pollination rates (Palau-delmas et al., 2008). The size of the donor field has lower impact on cross-pollination than the size of the receptor field (Bannert, Vogler, & Stamp, 2008; Ireland, Wilson, Westgate, Burrell, & Lauer, 2006; Palau-delmas et al., 2012). Finally, a study performed from 2004 to 2006 (Palau-delmas et al., 2009) demonstrated the scarce contribution of possible GM maize volunteers to the GM contents of the yields of conventional fields. Only very high densities of volunteers (i.e., above 1,000 volunteers/ha) can significantly contribute on adventitious GM levels.

We further evaluated to which extent the results obtained in field trials can be extrapolated to agricultural fields by monitoring a series of real fields along several years. They were located in Catalonia, Spain, where growers can freely choose to cultivate conventional, organic, or GM (Bt) maize. In fact, 97,346 ha out of 114,490 ha cultivated in Europe in the 2012 cropping season were cultivated in Spain (85%); and from these, 29,632 ha were cultivated in Catalonia. We chose a number of selected fields in different cropping areas where the corn borer pest causes serious economic damages; as a consequence, GM and conventional fields coexist. The study of the first season (2004) showed that, as predicted by experimental field trials, the distance between fields and the flowering coincidence were the main factors influencing the cross-fertilization rate in real fields (Messeguer et al., 2006). On the basis of these results, a predictive index was developed that calculated adventitious GM contents as a function of the values of these two parameters (Messeguer et al., 2006). The expected cross-pollination index (ECP) for each conventional maize field placed in the proximity of a GM field was calculated by dividing the flowering syn-

chronicity (expressed in days) by the squared distance (expressed in decameters) plus one. The effect of fields more than 150 m distant or with flowering synchronicities below one day was neglected. For each conventional field, a global index (GI) was calculated by totalling its individual ECPs from every possible GM donor field. A high correlation ( $y = 0.068x$ ;  $R^2 = 0.9499$ ) was found between the *in silico* calculated GI and the experimentally obtained GM values (i.e., GM contents in the receptor field as determined by real-time PCR, qPCR). Thus, the GI correctly predicted the adventitious presence of GMOs in conventional fields due to cross-fertilization.

Based on the GI, the Global index Module Interactive (GIMI) software was designed to predict—graphically and in real time—the GMO contents of real conventional fields. It was specifically prepared for use in a particular region in Catalonia—Foixà. On executing GIMI, a map of the region is displayed and the user can introduce (for each field) data on the type of culture (transgenic or conventional) and flowering dates. The estimated GM content (in percentage) is immediately displayed on the screen. Moreover, the GM percentages that can be attributed to the different possible donor fields are displayed as well.

The software was initially implemented with an additional input that considered the effect of the wind direction and speed. This was calibrated on the basis of our previous results obtained in experimental fields consisting of a field sown with GM maize completely surrounded by conventional fields. The percentages of cross-pollination were measured in different directions around the donor field and analyzed, taking into account the measured wind speed and direction (Melé et al., 2004; Pla et al., 2006).

The GIMI software was successfully validated through comparison of the predicted and the experimentally obtained cross-pollination rates in up to 11 conventional fields in the Foixà region along 3 seasons (three fields in 2005, five fields in 2006, and three fields in 2007). It had adequate accuracy (Messeguer et al., 2009).

GIMI is a user-friendly and fast software. This allowed carrying out a number of simulations to predict the GMO contents of conventional fields under different scenarios considered, e.g. various degrees of GMO pressure. In this way, those fields with the highest risk of attaining adventitious GM contents above 0.9% in a particular scenario could be identified.

It should be noted that the experimental data supporting the GI design were obtained in two regions that are characterized by relatively small (around 1.3 ha) and uniform field sizes. In these regions, flowering coincidence and distance between the donor and the receptor fields can explain the experimentally determined cross-pollination rates. Thus, the field shape and size were not considered in calculating the GI, and the estimated effect of a GM field on a conventional one were considered the same, irrespective of its shape (square or irregular) and size (very small or relatively large).

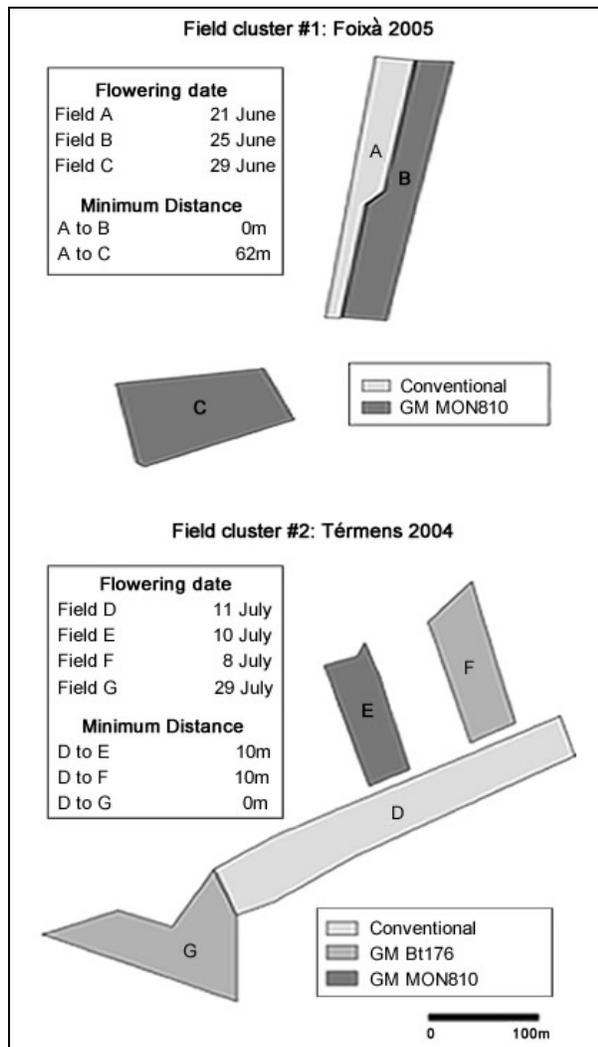
However, extreme field shapes and sizes were predicted to influence adventitious GM contents due to cross-pollination, and the flaw of GI in predicting the GM contents in long and narrow fields was mentioned in the initial GI publication (Messeguer et al., 2006). As an approach to overcome this problem, we initially proposed to virtually divide the irregular field into various regular subfields and to apply GIMI individually. Large fields are also predicted to have experimental cross-pollination values divergent from those calculated through the GI. Cross-pollination occurs mainly in the outer portion of the conventional fields, while the inner part of the fields receives mostly pollen from the same field. The ratio between the outer and the inner part of the field is obviously higher in small fields than in large fields (the perimeter increases linearly whereas the area increases exponentially).

Here we present a modification of the GI that includes the effects of the size and the shape of the fields on the adventitious GMO contents of conventional fields in real situations of coexistence. We show the evaluation of this new tool by comparison of predicted and experimental GMO percentages in (i) large fields and (ii) irregular fields for which the former GI did not accurately predict the adventitious presence of GMOs. Finally, we propose GIMI 2 as user-friendly software to coordinate coexistence in agronomic regions.

## Materials and Methods

### Selection of Small Quadrangular Fields

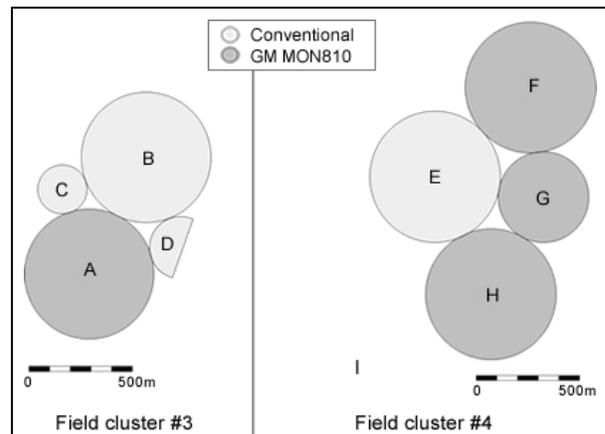
As mentioned, a number of agricultural fields were previously analyzed along four seasons to experimentally validate the GI and GIMI software simulations (Messeguer et al., 2006, 2009). Even though correlations were globally considered satisfactory, there was a small number of fields showing disagreement between experimental and *in silico* values above 20%. Here, we carefully examined the ECP values attributed to each



**Figure 1. Schematic representation of the groups of small quadrangular fields analyzed in this work. They are located in Foixà (2005 season) and Térmens (2004 season), Catalonia. Flowering dates and distances between fields are indicated.**

neighboring GM field and identified a few conventional fields with shapes and sizes clearly different from most fields in the region. Two of them were selected as candidates to validate the improvement of the GIMI software presented in this article (Figure 1).

Field Cluster #1 was in the Foixà region and comprises two fields (A=conventional and B=GM) with a long adjacent edge and very small depth. The estimated GI value was clearly below the experimentally obtained adventitious GMO content in Field A. Field Cluster #2 was in Térmens and was comprised of a long and narrow conventional field (D) with an adjacent MON810 field (E) and two adjacent Bt176 (F and G) fields. The



**Figure 2. Schematic representation of the groups of large circular fields analyzed in this work (located in Almacelles, Catalonia).**

effects of MON810 and Bt176 cross-pollination were considered separately both *in silico* and experimentally. For the two GMOs, the GI gave higher values than qPCR analyses.

### Experimental Determination of GMO Contents in Large Fields

The region of Almacelles (Lleida) is characterized by large extensions of maize fields mostly watered by pivots. Conventional and GM maize fields coexist in this region. Two field groups were identified (Figure 2) that included both conventional and transgenic fields in close proximity. Note that the fields were circular and most had diameters above 500 m. They were selected to exemplify pollen flux in large fields. Field Cluster #3 had a GM field (Field A, >30 ha) placed next to a conventional field (Field B, >30 ha) and two additional small fields (2 ha) sown with the same maize variety (Fields C and D). Field Cluster #4 had a large conventional field (Field E, 32 ha) surrounded by up to 4 large GM fields (Fields F, G, H, and I).

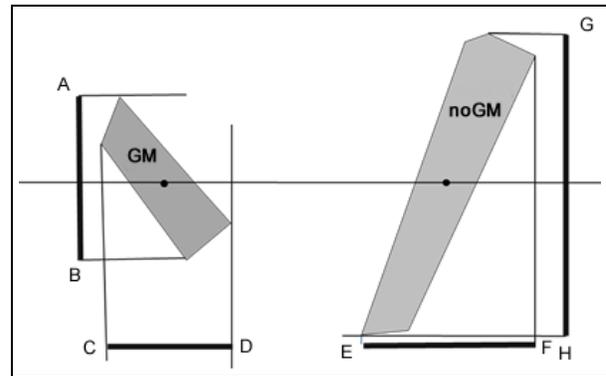
General maize development and culture parameters were monitored in all fields in the two selected clusters, with special emphasis on the flowering period, the presence of GM volunteers in conventional fields, and the wind speed and direction during the flowering period. Flowering monitoring was carried out in 20 plants per GM field (placed next to the conventional field) and 60 plants per conventional field (placed in three different zones of the field, close to the field borders and the proximal GM fields). Visual observations were performed three times a week and recorded using the scale reported by Fonseca and colleagues (2003).

Grain sampling was carried out prior to harvest. Each conventional field was divided into 12 equidistant radiuses, and samples of 3 cobs were taken on each radius at 0, 3, 10, 30, 100, and 200 m from the perimeter towards the center of the field. This is an asymmetric distribution of sampling points, which is an adaptation of the standard sampling procedure previously used in rectangular fields in the Foixà region. It intensifies sampling in the external zones of the field, i.e., where cross-pollination from neighboring fields is expected to be more intense. Additionally, during harvest each truck-load was sampled five times with a 1.5m-high sampling probe, allowing sampling at different depths.

Fields B, C, D, and E had been sown with GM maize the previous season. Culture of GM and conventional varieties in subsequent seasons by the same farmer is very common in the region. Volunteers were observed in all three fields. To assess the possible impact of GM volunteers, a 100 m<sup>2</sup> area was thoroughly monitored in Fields B and E.

For each sample, all seeds (approximately 1 kg) were pooled and grinded using a GRINDOMIX GM 200 knife mill (RetschGrain GmbH, Haan, Germany) and 1 g was subsequently used for genomic DNA extraction using a CTAB-based (Cetyltrimethyl ammonium bromide) protocol and qPCR analysis as reported (Pla et al., 2006). Specific qPCR assays targeting the endogenous maize *adh1* gene (Hernández et al., 2004) and the MON810 and Bt176 event-specific sequences flanking the transgene were used (Hernández et al., 2003; Shindo et al., 2002). Quantification was performed by interpolation in a standard regression curve of cycle threshold (Ct) values generated from genomic DNA isolated from powdered MON810 or Bt176 maize (certified reference material from Fluka in Buchs, Switzerland). DNA extractions from all samples were performed at least in duplicate and real-time PCR reactions in triplicate.

Values obtained from the qPCR analyses were used to estimate the total GMO content of the studied fields using an approach similar to that previously reported in small fields (Messeguer et al., 2006). Fields were divided into circular trapezoid portions on the basis of the sampling radiuses and perimeters. The partial GMO content of each portion was calculated by averaging the four samples that delimited the surface. The averages of these local values were weighted by their corresponding area to obtain a representative estimation of the global field value.



**Figure 3. Graphical representation of relative widths and depths of two adjacent fields as they are calculated in the modified GI.**

### **Modification of the GIMI Tool**

It is very important to consider the relative width and relative depth of a field when it is interacting with another field. In fields with an approximate rectangular shape, the length and width are located at the longest and shortest sides, respectively. However, this idea is not valid when considering cross-pollination between two fields. In general, fields do not have a rectangular shape and it is difficult to define the longest and shortest sides of the field. Moreover, the field depth we are interested in is not the one that derives from the field shape, but the one that result when facing the two fields in the direction of the pollen flux.

In order to capture these effects, we consider a GM field and a non-GM field (See Figure 3) and the line that joins their center of mass. By projecting both fields into this line, we can find the relative depth of each field, i.e., the segment  $\overline{CD}$  for the GM field, and the segment  $\overline{EH}$  for the non-GM field. Similarly, the relative width is found by projecting each field into a line perpendicular to the previous one. This gives a relative width for the GM field of  $\overline{AB}$  and  $\overline{GH}$  for the non-GM field.

There are three different corrections that depend on the relative width and depth that we just defined. From now on, and for clarity, we will simply use width and depth to refer to these relative quantities.

**Correction Due to the Receptor Field Depth.** It is known and accepted that the receptor field depth is a very important factor in determining the average percentage of GM material (%GM) within a field. This is because when one gets further inside the receptor field it also gets away from the transgenic pollen source. Moreover, as it is a competition phenomenon, the amount of

non-transgenic pollen of the receptor field protects the plants against the foreign one.

In a previous work that Pla et al. (2006) made with transgenic yellow (4 ha) and white conventional maize, we studied the reduction of the adventitious pollination inside the conventional field. We found that the %GM (namely  $F$ ) at a distance  $d$  of the field border followed reasonably well the expression

$$F(d) = F_0 \frac{1}{1+d}, \quad (1)$$

where  $F_0$  represents the %GM at zero distance, i.e., at the field border.

The average %GM as a function of the field depth,  $T$ , is found by integrating the last expression and dividing by the field depth  $d$ . This yields

$$T(d) = F_0 \frac{\ln(1+d)}{d}. \quad (2)$$

The correction to the global index, which was based solely on the flowering days and the distance between fields, has to adjust to the previous expression. Moreover, it will have a value of 1 for fields of 100 m depth, like the ones studied in Foixà. This gives a conventional correction index of

$$I_C(d) = 21.668 \frac{\ln(1+d)}{d}. \quad (3)$$

**Correction Due to the Donor Field Depth.** In the same publication, we also studied the effect of the transgenic field depth. We saw that when the depth of the donor field was duplicated, the amount of %GM in the conventional field increased only by 7%. This gives a transgenic correction index of

$$I_T(d) = 1.07^{\frac{\ln d}{\ln 2} \frac{d}{100}}. \quad (4)$$

In order to be consistent with the results found in Foixà, this index has a value of 1 for a transgenic field depth of 100 m, which was the average depth of the Foixà fields, and makes the conventional %GM increase 7% when the donor depth duplicates.

**Correction Due to the Field Widths.** Finally, we also have to consider the effect of the widths of both conven-

tional and transgenic fields. We define the relative width between fields as

$$W_{rel} = \frac{W_C}{W_T}, \quad (5)$$

where  $W_{C(T)}$  is the conventional (transgenic) width of the field. We distinguish two different situations: when  $W_{rel} < 1$  and when  $W_{rel} \geq 1$ .

For  $W_{rel} < 1$ , a good approximation for the correction index is

$$I_W = 1 - (1-d)^3. \quad (6)$$

This index has a value of 1 when both fields have the same width, and then decreases to 0 in the limit  $W_T = 0$ . However, it gives a value close to 1 for transgenic fields with widths up to  $\frac{1}{2}W_C$ , and then decreases much faster.

For  $W_{rel} \geq 1$ , the behavior of the correction index is completely different. Clearly, it has to start at 1 when both fields have the same width, but it also has an asymptotic behaviour; the effect of the transgenic field width cannot increase indefinitely. For example, the effect of a transgenic field of  $W_T = 500$  m on a conventional field of  $W_C = 100$  m has to be the same if the transgenic field has  $W_T = 1,000$  m, since the extra 500 m will be so far from the conventional field that makes the effect negligible. Therefore, the width correction index is

$$I_W = 2.56 - (1.56 / W_{rel}^2). \quad (7)$$

The three partial indexes  $I_C$ ,  $I_T$ , and  $I_W$  were designed so that they equal 1 upon application to regular fields of the same size as those in Foixà and Tèrmens originally used to design the GI. They can be grouped in a single correction index—the vulnerability index ( $I_V$ )—that corresponds to their product

$$I_V = I_C \cdot I_T \cdot I_W. \quad (8)$$

## Results and Discussion

### Development of the GIMI 2.0 Tool

A new index was designed to estimate the adventitious GM contents in conventional fields in real coexistence conditions. It is based on the GI and keeps flowering coincidence and physical distance between the receptor and the donor fields as the main parameters determining

**Table 1. Estimated cross-pollination (ECP) from transgenic to conventional fields (transgenic → conventional) according to GIMI and GIMI 2.0 tools. Experimental qPCR-based analysis of every conventional field is shown (total). The total GM contents of conventional fields estimated by summing the ECPs from the different donor fields are also represented, together with the experimental qPCR values. FC is the flowering coincidence and distance is de minimum distance between fields.**

Field cluster	Donor GM event	Pollen flux direction	FC (days)	Distance (m)	qPCR (%)*	GIMI (%)	GIMI 2.0 (%)
<b>Foixà 2005: Cluster #1 (small fields)</b>	MON810	ECP B → A	6	0	-	0.410	0.980
	MON810	ECP C → A	2	62	-	0.002	0.002
	MON810	Total	-	-	1.12	0.412	0.982
<b>Térmens 2004: Cluster #2 (small fields)</b>	MON810	ECP E → D	9	10	0.02	0.153	0.031
	Bt176	ECP F → D	7	10	-	0.119	0.062
	Bt176	ECP G → D	9	0	-	0.612	0.473
	Bt176	Total	-	-	0.51	0.731	0.515
<b>Almacelles: Cluster #3 (large fields)</b>	MON810	ECP A → B, C, and D	0	0	0.02	0	0
<b>Almacelles: Cluster #4 (large fields)</b>	MON810	ECP F → E	0	0	-	0	0
	MON810	ECP G → E	8	0	-	0.544	0.082
	MON810	ECP H → E	1	0	-	0.068	0.011
	MON810	ECP I → E	3	348	-	0	0
	MON810	Total E	-	-	0.04	0.612	0.093

\* Values corresponding to field sampling following the standard approach. RSD values were consistently below 20%

gene flow. The effects of these parameters are estimated as in the GI. However, it additionally includes a correction factor to consider other topographical features that can have an influence on adventitious cross-pollination. The depth of the receptor and the donor field and the relative width of the two fields are evaluated separately and grouped into a single correction factor (vulnerability index,  $I_V$ ) that represents the vulnerability of the receptor field to receive pollen flux from the donor as a function of the relative position and shape of the two fields. It expresses the degree of ease with which a GM field can have an effect on a conventional field due to their shape and spatial distribution. This refinement of the ECP estimation should allow more accurate predictions in geographical areas with different landscapes, field types, and distributions.

Detailed information on the reasoning and deduction of the basic GIMI 2.0 algorithm is given in the ‘Material and Methods’ section.

### **Validation of the GIMI 2.0 Tool Using a Selection of Small Quadrangular Fields**

Two field clusters were selected in Catalonia (Field Clusters #1 and #2, Figure 1) in which especially narrow conventional fields were placed next to transgenic fields. The adventitious GMO contents in these conventional fields had been previously experimentally determined using the standard method (Messeguer et al.,

2006, 2009) and *in silico* estimated with the original GI (GIMI software). We used the new GIMI 2.0 tool, which integrates the  $I_V$  correction to take into account the field shapes and relative position, to predict the GMO contents in these conventional fields. Table 1 shows the experimental values and the predicted values calculated on the basis of the GI and the GIMI 2.0.

In Field Cluster #1, the ECP from the transgenic Field B over the conventional Field A was clearly underestimated by the GIMI tool. Field A has extremely short depth, thus the pollen flux along the long edge close to the transgenic field is not compensated by a distal portion of the field essentially lacking transgenic pollen. Note that the effect of a narrow donor field is not as strong as the depth of the receptor field. The estimated value obtained by applying the GIMI 2.0 tool is substantially better adjusted to the experimental results than the former GI (Table 1), with just 12% difference, which is in the range of the accepted experimental standard deviation/error.

Conversely, the former GI overestimated the cross-pollination from transgenic Fields F and G towards the conventional Field D (Field Cluster # 2). In this case, donor fields are located at the narrow edges of a long and narrow receptor field. The new GIMI 2.0 prediction gave the same GM value as obtained experimentally (Table 1).

Cross-pollination from transgenic Field E to conventional Field D shows the importance of the relative width of the two adjacent fields. Field E has a strong influence on the central area of the receptor field; however, a large portion of the receptor field is quite distant from the donor. This effect was also efficiently corrected by the GIMI 2.0 algorithm, giving a much better estimation than the former tool.

### **GMO Contents in Large Fields and Validation of the GIMI 2.0 Tool in These Fields**

Two clusters of coexisting GM and conventional maize fields were selected in the region of Almacelles (Figure 2). Field Cluster #3 had a single GM field (A). Its mean flowering date was August 2<sup>nd</sup>. Three conventional fields (B, C, and D) showed mean flowering dates one month earlier, July 3<sup>rd</sup>. These fields had been sown with GM maize the previous season, thus the possible presence of GM volunteers was monitored. Up to  $217.2 \pm 60.5$  volunteer plants were found per ha, which is below 0.3% of total plants in the field and thus, it is considered not to hinder the yield. Nevertheless, cross-pollination from GM volunteers to conventional plants in the same field might occur at levels experimentally detectable by qPCR (see our previous results in Palaudelmàs et al., 2009). Most samples taken in the fields had GMO contents below the limit of detection, and just two samples were positive (0.01%). The global yield at harvest had a mean value (corresponding to the total yield of Fields B, C and D) of  $0.02 \pm 0.007\%$  GMO. This was an expected result, considering the lack of flowering coincidence (mean flowering dates are 31 days apart, thus cross-pollination is highly improbable) and the presence of GM volunteers. The residual GMO contents detected could be explained by the mentioned volunteer plants, pollen flux from distal fields, and/or seed impurities. We would tend to discard the latter since seed companies produce conventional seed in large areas where there is no culture of GMOs. Fecundation with pollen from distal fields seems not probable due to the strong competition that the pollen of the same field poses to a (putative) small amount of transgenic pollen from a different (proximal or distal) field. The massive presence of pollen from the same field is the main reason why foreign pollen has better chances of pollinating plants in the receptor field border than in the inner part of the field, where most own pollen accumulates. On the other hand, the fact that the detected adventitious GM presence in Fields B, C, and D are not located along the field borders but are distributed throughout the fields seems to

indicate that it arises from GM volunteers in these fields.

Field Cluster #4 had a single conventional field (E) surrounded by GM Fields F, G, H, and I. The mean flowering dates were July 23<sup>rd</sup> (for Field E) and July 25<sup>th</sup> and 30<sup>th</sup> (Fields G and I, respectively) and August 1<sup>st</sup> and 14<sup>th</sup> (Fields H and F, respectively). Thus, Field G was highly coincident with the receptor field; and Fields I and H had intermediate coincidences. Field F is considered not coincident with the transgenic field (i.e., about 3 weeks apart). The calculated density of GM volunteer plants in conventional Field E was  $175.3 \pm 17.9$  volunteers per ha.

The global adventitious GM content in Field E was 0.04%, with higher values obtained in samples taken next to the transgenic fields (above 0.13%) and lower values in samples taken far from the donor fields (about 0.1%). Having GM percentage values above the limit of detection in areas as far as 600 m from the donor fields are most probably explained by the presence of GM volunteers. This is similar to the values obtained in samples taken along harvesting, with a mean value of 0.06% GMO and higher values (up to 2.25%) in the loads corresponding to the area next to the transgenic donor fields.

According to our results, there is no need to establish separation distances between conventional and GM fields to assure coexistence in this type of landscape. Large conventional fields produce enough conventional pollen to be protected from external pollen (up to the limit established by the labelling regulation in the EU). The very low GM values in the large central part of the field compensate the cross-pollination in the border zones.

Field Cluster #3 and Fields F and E in Cluster #4 clearly illustrate the effectiveness of controlling flowering coincidence to facilitate coexistence of GM and conventional maize. Remarkably, in these specific climatic conditions, there is the real possibility of sowing GM and conventional fields on very different dates without reducing the yields; however, this results in extremely reduced levels of adventitious GMO in conventional fields. Thus, coexistence strategies based on different sowing dates is feasible and efficient in these conditions.

Both the former GI and the improved GIMI 2.0 algorithm were used to *in silico* predict the GMO contents in conventional large fields in Field Clusters #3 and #4. The lack of flowering coincidence resulted in the two tools predicting the absence of adventitious GMO in conventional fields in Cluster #3. Conversely, GIMI

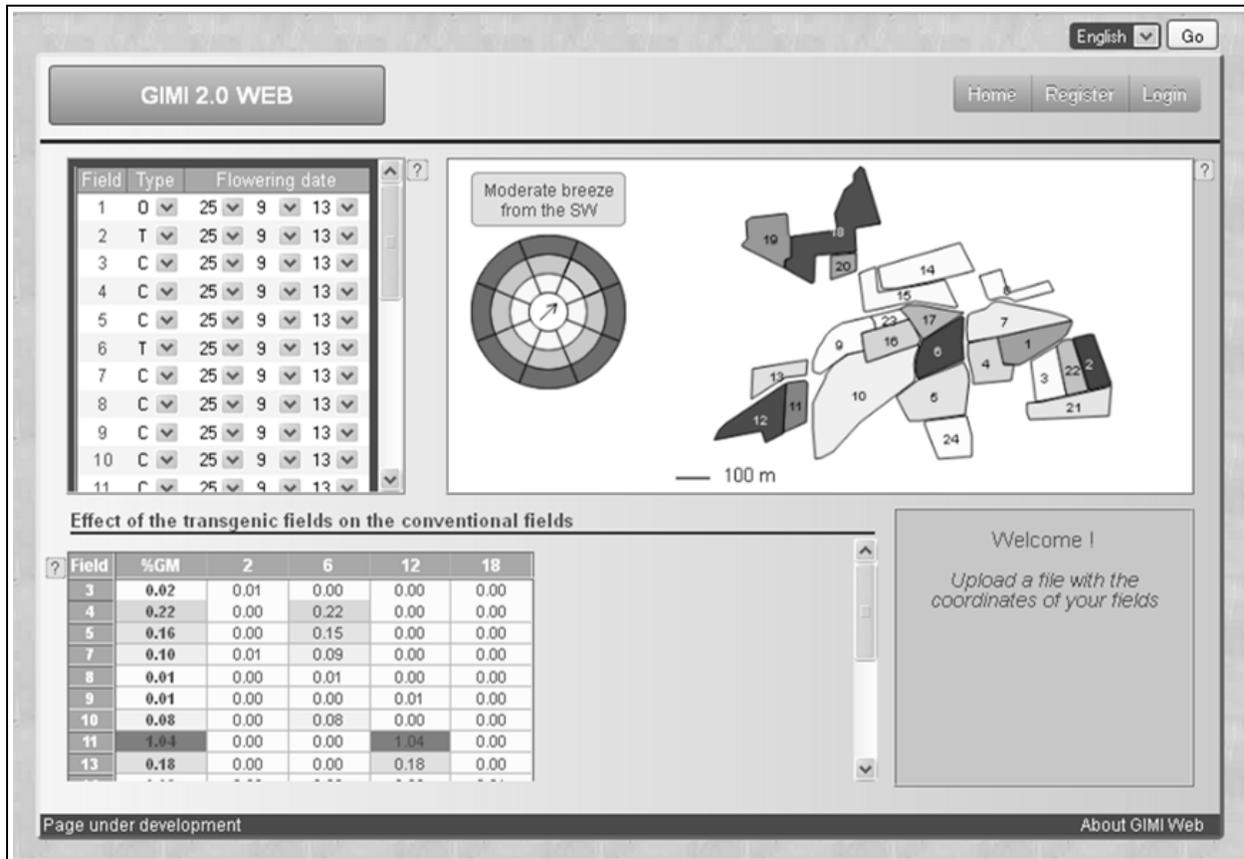


Figure 4. View of the GIMI web 2.0 interface.

largely overestimated the GMO values of Field E in Cluster #4 (i.e., 15-fold). This was obviously due to the small size of fields used to design GIMI. GIMI 2.0 produces a substantially more accurate estimation, which is still somehow above the experimental data obtained both by thorough field sampling (standard sampling method; 0.04%) and by sampling each truckload along harvesting (0.06%). With these values close to the limit of quantification of the analytical methods, the GIMI 2.0 estimation can be considered adequate.

## Conclusions

We designed and validated an improvement of the GI that accurately predicts the experimental data on adventitious GMO contents of conventional maize fields in real agricultural environments. It includes a correction index to normalize for the shape and size of the fields. Thus, it can be successfully applied to different landscapes, including those with small and relatively regular fields and those with vast or extremely narrow fields. The drawback of the improved index is the complexity

of calculation. The former GI was easily calculated with just two inputs, i.e. flowering coincidence and distance between fields. In contrast, the improved index requires calculation of the vulnerability index ( $I_V$ ), which is based on the minimal distance between fields but also the relative situation of all vertices of the considered fields. Therefore, user-friendly software to automatically calculate these indexes and display the resulting information in a clear way was necessary. GIMI 2.0 was designed to apply the algorithm according to the inputs set by the user. GIMI 2.0 has proven a tool suitable for quick and accurate prediction of adventitious GM contents in real coexistence situations.

GIMI 2.0's main goal was to contribute to better understand the many different factors influencing adventitious cross-pollination in maize. Its quick response to a change in any given factor (e.g., flowering date, wind speed, etc.) allows visualization of the effects of this specific factor and estimation of the possible consequences.

GIMI 2.0 has a user-friendly interface (Figure 4) where the fields of interest can be directly drawn on a

canvas, either with a background of choice that can be extracted e.g., from Google maps, or a plane of the land registry. Obviously a reference measure has to be included (a rectilinear segment with the correspondence in meters). Alternatively, geographical coordinates of the field vertices can be uploaded through a text file as indicated in the help tab. Datasets can be downloaded to the user's computer.

With the aim of making it available to all interested users, an online version of the GIMI 2.0 software is freely accessible upon registration. Thus, GIMI 2.0 can be used in an interactive way. A prototype can be found at <http://gimi.pythonanywhere.com>. Further improved versions of the software are currently being developed that include new functions and additional factors such as stacked events.

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