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Sampling and Modeling for the Quantification of Adventitious Genetically Modified Presence in Maize

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The coexistence of genetically modified (GM) and non-GM crops is an important economic and political issue in the European Union. We examined the GM content in non-GM maize crops in Spain in 2005. Both the standing crop and the harvest were tested, and the %GM DNA was quantified by real-time polymerase chain reaction. We compared the level of GM as a function of distance from known GM source fields in a 1.2 km^2 landscape. The distribution of GM was compared to predictions from previous studies, and good agreement was found. Control and monitoring of adventitious GM presence in non-GM crops can only be achieved by fit-for-purpose sampling and testing schemes. We used a GM dispersal function to simulate non-GM crops in the studied zone and tested the accuracy of five different sampling schemes. Random sampling was found to be the most accurate and least susceptible to bias by GM spatial structure or gradients. Simulations showed that to achieve greater than 95% confidence in a GM labeling decision of a harvest (when treated as a single marketed lot), 34 samples would be needed when the harvest was outside 50% of the GM threshold value. The number of samples required increased rapidly as the harvest approached the GM threshold, implying that accurate labeling when the harvest is within ±17% of the threshold may not be possible with high confidence.

INTRODUCTION

Cross-pollination between fields is considered one of the main mechanisms for avoidable adventitious GM (genetic modification) presence (AGMP) in out-crossing crops such as maize (*1, 2*). Importantly, this is one of the routes for AGMP that can be controlled and minimized by modification of farming practices and regulation. For this reason, a considerable research effort has been made to attempt to measure and model crop-to-crop pollen flow in maize. If we define GM coexistence as the ability for the production chain to maintain two streams of crop, non-GM and GM (under and above a labeling threshold, respectively), then it is clear that the ability to accurately predict AGMP in a landscape due to pollen flow is important.

Data gathered from field experiments and subsequent computer simulations have allowed the estimation of separation distances to avoid pollen AGMP exceeding prescribed thresholds. Between 2000 and 2003, in the United Kingdom, a large set of fields trials, the United Kingdom Farm-Scale Evaluations (FSEs), were conducted using GM and non-GM maize. Geneflow between over 50 GM and non-GM field halves was measured and was used to derive a probabilistic GM dispersal function (*3*). The FSE function's only variable was the orthogonal distance from the GM pollen source. Using the same data, similar functions were used to derive advice on United Kingdom GM separation distances (*1*). A large European Union synthesis of GM coexistence studies has used mechanistic models of GM geneflow to estimate coexistence measures, such as separation distances (*6*). Other studies have examined effects in addition to distance, such as GM field size, wind, and type of gap between fields. Weber et al. (*4*) found that wind did not play a major role in the degree of GM pollination, because no predominant wind direction was found, and that GM field size did not have a large effect. Flowering overlap time can produce significant effects, and it can be minimized to reduce GM pollination (*6*), but it is difficult to implement in non-Mediterranean regions (*4*). However, factors that are biological, meteorological, or a combination of these, while undoubtedly important for determining the degree of maize cross-pollination vs distance, cannot be reliably predicted for a specific farming area of interest. Therefore, separation distance in addition to good agricultural practices (e.g., machinery cleaning) and regulated AGMP in seed remain the primary AGMP on-farm control measures. Purely mechanistic modeling, for example, Hoyle and Cresswell (*7*), can provide valuable biological insights into the process, but environmental factors used in such models cannot be applied to general coexistence measures or be used

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Table 1. Mean % GM DNA Measured in Harvest ($n = 30$, except Field C, $n = 22$) and Field Samples ($n = 28$ for Fields A, B, and D; $n = 36$ for Field C)[a]

field	field mean[b]	harvest mean	FSE prediction	JM estimate[c]
A	1.14 (271)	0.84 (136)	1.51 (163)	0.45
B	1.13 (227)	0.62 (64)	0.63 (87)	0.54
C	0.76 (251)	0.31 (237)	0.50 (134)	0.34
D	0.52 (222)	0.03 (154)	0.46 (116)	0.157

[a] The FSE prediction is the mean field value obtained using the UK FSE maize GM dispersal function only. The JM estimate is the mean obtained for the whole field using the weighted field samples according to Messeguer et al. (*5*). (% CV among samples is shown in brackets). [b] Field samples were edge biased; this is not a representative mean. [c] % CV values are the same as field means' from which the JM value was calcualted.

Table 2. Heterogeneity, H_{95}, of Field and Harvest Sampling

field	field	harvest
A	64.4	39.7
B	50.5	18.2
C	78.0	61.1
D	65.3	51.1
mean	64.6	42.5

to predict AGMP in specific situations in advance. There is now a large amount of data available from field experiments to give robust functions and probability distributions for GM pollen flow vs distance. We can therefore begin to apply this information to study questions such as the economic impact of GM coexistence at the landscape and country level (*2, 8*). The critical output for realistic landscape coexistence studies is the probability that GM pollination will significantly affect the AGMP in a whole field harvest, that is, considering a whole field as a minimum marketable crop lot, which would require GM labeling and separated downstream processing.

In future GM coexistence European Union landscapes, monitoring of AGMP may be necessary at the farm level to ensure that regulation measures are effective. In this paper, we describe the measurement of maize AGMP through pollination in Spain in 2005. There were three aims: (i) to collect data in a real agricultural landscape and to compare it to geneflow predictions from the FSE function and others' experiments, (ii) to measure and compare AGMP in the standing crop field and in the corresponding harvest, and (iii) to examine the possibility of using standing crop AGMP measurements to predict harvest AGMP and to devise minimal, economical, sampling schemes to achieve this. To our knowledge, an analysis of the efficiency of different sampling schemes for the field determination of a crop impurity has not been performed. We hope that this work will assist stakeholders and field workers in GM monitoring or research where accurate measurement of whole field AGMP or other crop impurities is required. We compare AGMP vs distance for several other studies with this one and predictions from the FSE GM dispersal function.

MATERIALS AND METHODS

Study Area. A 1.2 km^2 zone was chosen for study through the EU FP6 project "SIGMEA". This zone was also studied in previous years by Messeguer et al. (*5*). The zone contained 10 maize fields growing GM maize (Monsanto event Mon810). Four non-GM grain crop maize fields were selected for study (A−D). These fields were chosen because they were adjacent to GM maize fields and they had varying sizes, shapes, and orientations. Previous studies (*3*) suggested that only juxtaposed non-GM fields would have AGMP near the EU GM threshold of 0.9% [Regulation (EC) 1829:2003] and the chosen fields would therefore be most likely to represent problematic fields with respect to AGMP monitoring.

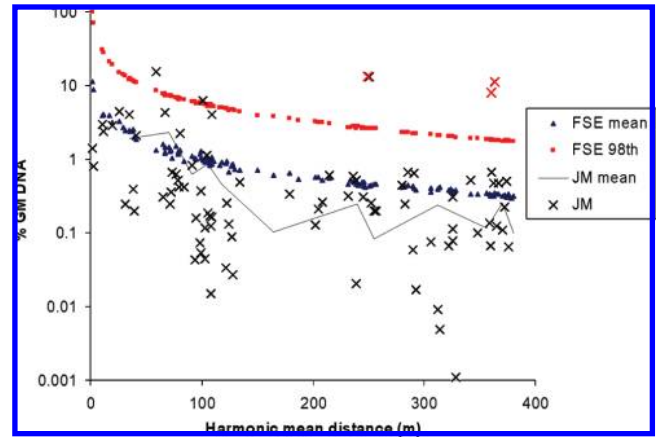

Figure 1. Graph showing % GM DNA vs distance for FSE maize GM dispersal function prediction (mean, triangles; 98th percentile, red squares) and field maize samples (crosses). The solid line indicates the mean values, taken for every 10 field samples when sorted by distance. All four fields are included. Field distances to each sample point were the harmonic mean distance nearest edge for all GM fields. Zero values are not shown. The red crosses are three outlying values excluded from the mean line.

Sampling. Two stages of sampling were performed. First, as soon as mature cobs had formed, samples of three cobs were taken from single plants at 0, 3, and 10 m from the field edge on eight equidistant transects (two on each edge where fields were approximately square) and four central equidistant samples (fields A, B, and D). For field C, because of its elongated shape, two extra transects were sampled on the longest two edges and six central samples were taken. These samples are referred to as "field". Second, during harvesting, at 30 regularly separated intervals, 1 kg grain samples were taken from the harvest machinery flow for each field. The approximate location of the harvest sample was noted according to the segment of field being traversed at that time. These samples are referred to as "harvest". All grain samples were dried (on the cob for field samples) and stored at 4 °C prior to analysis.

GM Quantification. Quantification of GM event Mon810 was performed by real-time polymerase chain reaction (PCR) as described in Weekes et al. (*3*) with the following differences: Taqman primers and probes specific for Mon810 were used as described in Pla et al. (*9*); an ABI 7900 SDS machine was used to run reactions. All GM quantifications are presented here as % GM DNA, as defined in European Union recommendations accompanying Regulation (EC) 1829/2003 (*10*), that is, allowing for an average F1 maize kernel content of 58% (*11*). Where necessary, data from other studies were also converted to % GM DNA (from % GM organisms w/w) to allow comparisons.

Statistics and Simulations. For each field, the mean field sample and harvest % GM DNA were calculated. The between sample variability was estimated as coefficient of variation (% CV = $\sigma/\mu \times 100$). Predictions of harvest % GM DNA were made for comparison to the observed harvest values. These were estimated by two methods: a model derived from the United Kingdom FSE data (see below) and by weighting field samples' values by their encompassed area (*5*). Heterogeneity among samples was calculated as 1 − the proportion of samples that contain 95% of the total GM × 100 (% H_{95}) (*14*).

The field data obtained in this study and the data for three other GM maize geneflow studies (*4*, *9*, *12*) were compared to an updated version of the GM dispersal function based on the FSE data described in Weekes et al. (*3*). This function consisted of a two-step probability distribution. First, the probability, p, that a plant will be pollinated by GM pollen as a function of distance, x:

$$p = -0.282 \times \log(x) + 1$$

and second, given that the first step occurs, the number of pollinations that will occur as a γ-distribution with parameters k (shape) and θ (scale), as a function of distance, x, where k is a constant = 0.462 and

$\theta = 10^{[-0.647 \times \log(x)] - 0.927}$.

$$\mathrm{GM}(x) = p \times \Gamma(k, \theta)$$

$$\therefore \text{ mean GM at distance } x = p \times k \times \theta$$
$$= x^{-0.647} \times 0.055 \times \log(10x^{-0.282})$$

with GM(x) being the probability density function for the % of GM pollination. Note that this function cannot be used when x >3475 m, after which negative values are obtained and pollination is assumed to be effectively zero. This function was found to best fit the empirical dta and its statistical distribution following exaustive fitting of different potential curves and distributions. The minimum mean value for % GM DNA pollination from F1 maize, that is, if a non-GM plant is crossed exclusively with an F1 GM plant is 21%, that is, half the pollen will contain a GM gamete and the resulting seed genotype will be a heterozygous GM embryo with monozygous GM triploid endosperm ($50 + 33\% \div 2 \times 0.5 = 21\%$). This was therefore set as the maximum obtainable mean % GM DNA value. However, GM(x) allows individual values >21%, according to the γ-distribution.

For each square meter of the studied zone, the shortest distance to the edge of all GM fields was calculated, and the sum of GM(x) and its upper 98th percentile from each source field was calculated. These values were compared to the actual observed field data at the location of each field sample point. The mean, whole field value expected under GM(x) for each studied non-GM field was also calculated and compared to that obtained in the harvest samples.

Five field sample plans were examined for their ability to predict harvest GM levels: (i) two orthogonal transects (a cross-pattern), (ii) four orthogonal transects, (iii) two orthogonal and two diagonal transects, (iv) random sampling, and (v) edge intensive sampling as used in ref *5*. The transect designs were chosen because they were simple to follow in the field and were expected to capture the gradient of GM across the studied fields. The theory behind edge intensive sampling (*5*) is that it has a higher density of samples where the variance and mean are higher, when AGMP is expected to be caused by gene flow from adjacent fields. A "W" walk scheme was also examined, but it was found that without clear definition of the walk angles and start and end points, its performance was difficult to assess and very variable and inaccurate (not shown). It was not examined further. For each sampling plan, the number of samples was incrementally increased (symmetrically for transect plans) and the accuracy of the % GM DNA estimate was measured as compared to the known, simulated, whole field (i.e., harvest) value. In each case, 1000 replicates of the studied field were simulated using the GM(x) distribution. The actual distances and shapes of the landscape and fields A−D were used. Over all replicates, the probability of obtaining a correct GM labeling decision for the harvest was taken from the observed frequency of harvest GM estimates being the same side of the GM threshold as the fields' real harvest sample estimates. For the random sampling plan only, the probability of obtaining a correct GM labeling decision with increasing number of samples was examined with thresholds set progressively closer to the known simulated harvest value.

RESULTS AND DISCUSSION

Field and Harvest Data. The AGMP in all of the harvests studied was below the European Union threshold of 0.9% GM DNA (**Table 1**). Heterogeneity among field samples was high (H_{95} = 64.4−78%) but was lower in harvest samples (H_{95} = 18.2−61.1%). This reduction in variation showed that homogenization of AGMP occurred during the harvesting process, but it was not sufficient to remove effects of stratified AGMP distribution in the field: There was a high correlation between heterogeneity in field and harvest samples ($R^2 = 0.9232$, **Table 2**). The variability and degree of harvest heterogeneity were of the same order as previously seen in large grain lots (*13*). This high harvest heterogeneity implies that subdivision of the harvest could lead to large deviations from the predicted total harvest AGMP.

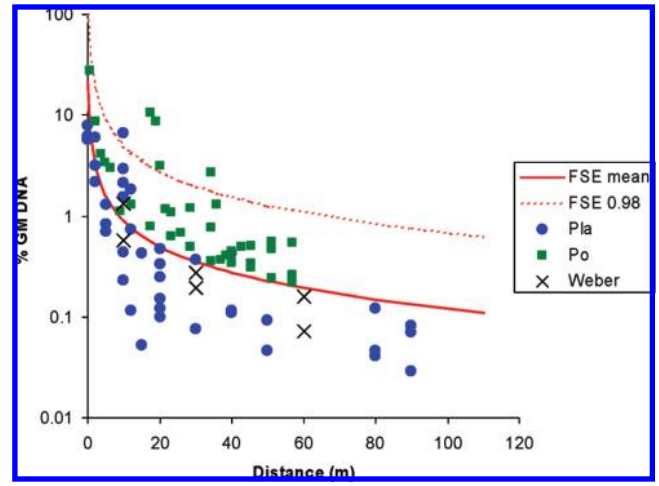

Figure 2. Comparison of FSE maize GM dispersal function to other published data. Solid line, mean predicted % GM DNA; dashed line, upper 98% percentile. Pla, Pla et al. (*9*); Po, Della Porta et al. (*12*); and Weber, Weber et al. (*4*).

Because of the edge-biased distribution of the field samples, it was expected that they would overestimate AGMP, as measured in the harvest. This was true, with an average bias of +81% (when used without any weighting). The harvest AGMP for field D was much lower than predicted by any method (**Table 1**) and was excluded from this calculation. Field D had the largest area, and it is likely that this is the reason for its much lower than expected AGMP, but the mechanism that led to field samples with much higher AGMP than observed in the harvest is unknown. Previously derived pollen dispersal predictions (*3, 5–7, 9–12*) cannot explain the very low overall AGMP of field D; unsynchronised flowering may have been responsible. The method of weighting field samples' AGMP values according to the area that they encompass, the JM method (*5*), gave the most accurate prediction of harvest AGMP but in one instance underestimated AGMP (field A). The FSE function prediction, using the distances to GM sources alone with no field measurements, gave reasonable predictions, and importantly, none exceeded the harvest AGMP estimate; it would, therefore, appear to be a good method for conservative estimation of compliance with European Union AGMP thresholds in the absence of field samples, and it can be applied in advance, without field sampling.

Comparisons of Model, Field Data, and Other Studies. The JM weighting method and sampling scheme were accurate in fields A−C, which were all in close proximity to GM pollen sources. However, it may not be efficient where AGMP is not expected to be higher in field edges than in the center of the field, for example, where AGMP has arisen largely from seed or volunteers rather than pollen. This is because it has an unequal probability of detecting AGMP toward the center of fields, due to lower sampling density. We would expect regular or random sampling plans to be more reliable in such circumstances. To test different sampling plans, simulated data were required that fitted the observed AGMP distribution in this and previous studies. The FSE function was compared to field data to test if it was suitable for such simulations.

The observed field samples' AGMP and the FSE function showed a good general agreement (**Figure 1**), with a linear correlation $R^2 = 0.637$. Two field samples (from total of 117) had % GM DNA values above the 98th percentile of the FSE function. Three field samples were outliers of the expected GM vs distance relationship (shown in **Figure 2**), and they were

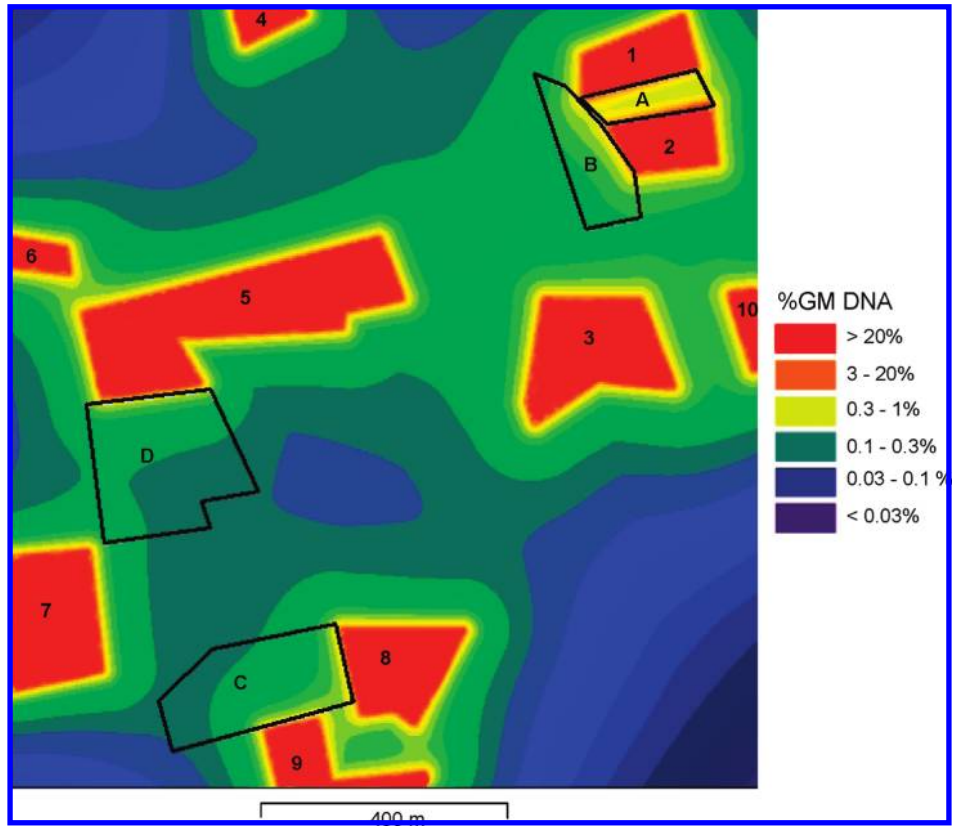

Figure 3. Maize study zone with color gradient showing the FSE maize GM dispersal function predicted mean % GM DNA. Areas 1–10 are Mon810 maize fields. A−D are non-GM sampled fields. Only outlines of Mon810 GM and sampled non-GM fields are shown.

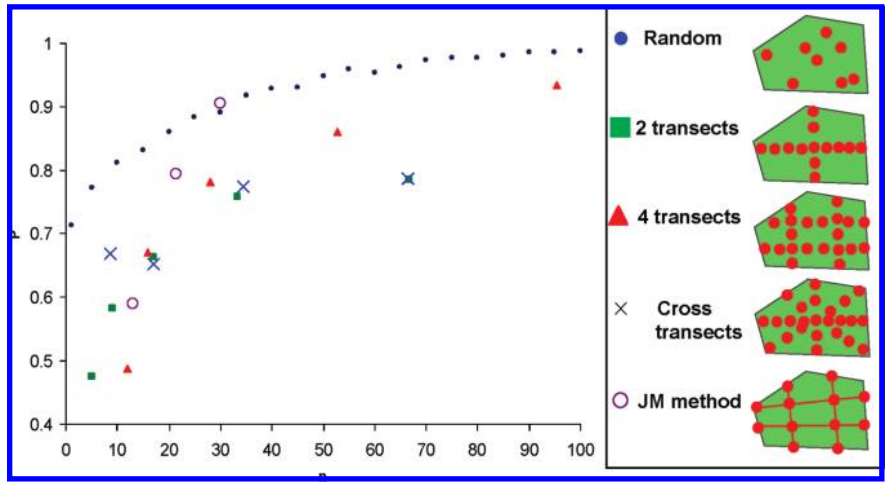

Figure 4. Probability of correct GM labeling decision vs number of field samples for different sampling schemes. Data are means of the four simulated fields.

excluded from this comparison; they were not excluded from other calculations shown in **Table 1**, which were not distance-dependent. These outliers may have been due to AGMP in the maize seed, which was not separately measured in this study. When the outliers were included, they did not raise mean values (pooling 10 samples sorted by distance from GM) over the FSE 98th percentile, but they did cause a poorer FSE vs field value correlation ($R^2 = 0.249$). The FSE function also showed good agreement with other studies of maize GM geneflow (**Figure 2**) with R^2 values of 0.69, 0.58, and 0.73 for linear correlation with data from Weber et al., Pla et al., and Della Porta et al., respectively (*4, 9, 12*). We therefore expected simulations using the FSE function to provide a reliable test for field sampling schemes. **Figure 3** shows the entire study zone with the mean FSE function % GM DNA. Note that the FSE function curves appear different in **Figures 1** and **2** due to the former accounting for multiple GM sources and the latter single sources.

It was not possible to examine sample number in the same increments for each scheme and maintain symmetry in the regular schemes. However, a realistic range of sample numbers was examined for each scheme. **Figure 4** shows the mean probability (over all four fields) of a sampling scheme providing accurate results, that is, a result the same side of the threshold (0.9% GM DNA) as the known harvest values for the simulated field. The random sampling scheme was consistently the most accurate. Two orthogonal transects were the least accurate. The JM method with weighting according to area performed at least as well as random sampling when $n \geq 30$. The regular sampling

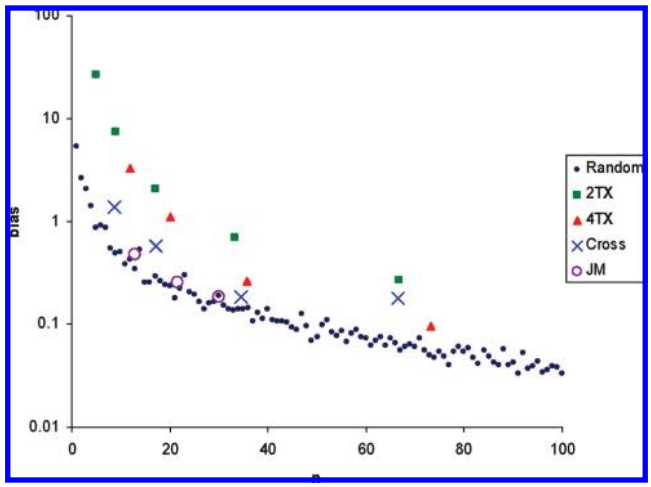

Figure 5. Bias of whole field GM estimate vs number of field samples for different sampling schemes (sum of squared differences). 2TX, two orthogonal transects; 4TX, four orthogonal equidistant transects; Cross, two orthogonal and two diagonal transects; JM, weighted area scheme after ref *6*. Data are means of the four studied fields.

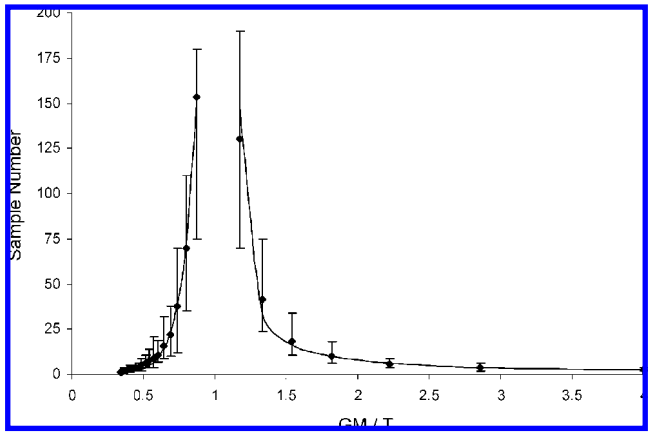

Figure 6. Number of random samples required to obtain ≥95% probability of a correct GM labeling decision (above or below threshold value) as the simulated fields approach threshold value. Vertical bars show variance among the four simulated fields. The horizontal axis, GM/T, is the proportion of the actual field mean % GM DNA to the threshold labeling value.

schemes (all bar random) were consistently less accurate in terms of absolute bias (**Figure 5**). It seems likely that any sampling scheme with spatial structure, for example, grids or transects, is prone to alignment with the AGMP gradient present in the fields adjacent to GM source fields (evident in **Figure 3**). While this may not be such a problem at greater distances from GM sources, it is only at close proximity that the quantification of samples is critical, being close to AGMP threshold. Weighting of samples by the area that they enclose can alleviate bias, but it provides an extra layer of complication to the procedure and subjectivity in outlining the areas to be contained by the sample points. Therefore, the best approach is a random sampling scheme. We examined the number of random field samples needed to obtain ≥95% probability of a correct GM labeling decision. **Figure 6** shows how the number of samples required increases as the actual (simulated) field value approaches the AGMP threshold. The distribution of number of samples needed was not symmetrical: For example, when the "true" field AGMP value was +50% of the threshold (1.35% GM DNA), $n = 14$; when it was −50% of the threshold (0.45% GM DNA), $n = 4$. This is an important consideration when assigning producer's and consumer's risks to test results based on field sampling. Another important outcome of this analysis is that impractical numbers of samples (>177) are required when actual field values are within approximately 17% of the threshold. It is therefore not possible to obtain reasonable certainty in crop labeling decisions when this close to the threshold. **Table 3** gives the number of random samples required based on the fields and landscape structure examined in this study. A range of non-GM field sizes and proximity to GM sources was included, which should lend confidence to the figures. If a landscape is to be sampled and tested in this way, simulations of geneflow such as the FSE function used here could be applied in advance to better plan field sampling schemes. The random sampling scheme recommended here, while ostensibly one of the most simple to implement, is not trivial. As with all of the sampling schemes, it is very sensitive to variation at the field edges. The location of randomly selected sampling points therefore needs to be very accurate (≤1 m), especially near field edges where the AGMP gradient may be steep and small differences in location may make large differences in AGMP. Accurate mapping and random selection of sample point coordinates are therefore required, and accurate GPS and/or surveying to locate the precise location of sample points would be required. In this study, we have not examined the further contribution to AGMP measurement uncertainty of the testing process. This has been attempted elsewhere (*14*) and must also be considered in addition to sampling error in AGMP measurements and harvest and field GM quantifications.

Table 3. Using a Random Sampling Scheme, The Number of Samples Required To Acheive ≥95% Correct GM Labeling Decisions[a]

% difference from threshold	no. of random samples required ($P \geq 95\%$)		
	minimum	maximum	mean
+50	14	**34**	19
−50	4	11	6
+33	24	**75**	41
−33	10	38	22
+18	70	**190**	130
−18	35	110	70

[a] The maximum, minimum, and mean are over all four fields. The maximum observed number of necessary samples, and therefore that recommended for field use, is shown in bold. The % difference from threshold is the fields' true mean % GM DNA value relative to the set threshold.

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