SESSION 9A

THE ENVIRONMENTAL IMPACT OF GM CROPS: SAFETY TESTING, RISK ASSESSMENT AND REGULATION

Chairman and Session Organiser:	Dr Alan Raybould *Syngenta, Bracknell, UK*
Papers:	9A-1 to 9A-4

The use of ecological endpoints and other tools from ecological risk assessment to create a more conceptual framework for assessing the environmental risk of GM plants

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ABSTRACT

Genetically Modified (GM) plants are heralded as a "second green revolution", but in order to gain the benefits they can offer we must also understand the risks they pose in order to allow cost/benefit analyses to be undertaken. Assessing the risks of a biotic introduction to the environment is very complex, and has not been helped by the lack of information flow from traditional risk assessment to GM biosafety research. At present we lurch from one small study to the next and really don't have a robust framework or any form of conceptual model into which the studies can fit together to complete the jigsaw. The introduction of tiered risk assessment methodologies have helped address a number of key ecological and environmental questions but such schemes need to be considered within a broader perspective so that other areas of risk can be considered and we can adopt comparative risk assessment of GM technology compared with alternative agricultural practices. Ecological risk assessments have been widely developed for assessing the impact of "contaminants" (usually an abiotic chemical input). Such assessments involve problem formulation and the development of a conceptual model into which studies gathering data can be placed. The use of assessment and measurement endpoints allow clear integration between the collection of data and the management goal and thus allow risks to be characterised, assessed and managed. A similar approach for assessing GM plants would be a powerful advance on the current risk assessment framework, and may allow scientists involved in detailed laboratory studies to fit their research into the bigger picture. Using Bt maize as an example endpoints and a conceptual model are developed.

INTRODUCTION

The debate concerning the ecological impact of genetically modified (GM) plants still rages, frequently fuelled by the media. A recent comprehensive scientific review highlighted the need for more studies to ascertain the environmental impact of GM crops (DTI, 2003). One of the biggest challenges will be developing the best framework for assessing the past and future studies that are generating extensive data on the risks and impact of GM crops. Too many studies have been conducted without reference to a conceptual framework/model. This has led to numerous scare stories which in turn have obscured considerable research addressing the same questions but part of a more complete risk assessment framework. The best example relates to the risk posed to the Monarch butterfly by pollen from Bt maize. John Losey and colleagues at Cornell University showed that Monarch butterfly larvae could be killed by pollen from Bt maize in a classical first-tier "worst-case scenario" experiment (Losey *et al.*,

1999). The identification of this hazard generated tremendous media and public attention and even speculation that Monarch migrations may be a thing of the past. A comprehensive risk assessment was then undertaken by many scientists in North America resulting in a series of papers published in Proceedings of National Academy of Sciences (PNAS, 2001). These concluded that the risk to Monarch butterflies from Bt maize is very low and that there are ways to manage the risk One could ask the simplistic question as to why it took a preliminary scientific study and tremendous media attention to initiate a comprehensive risk assessment of Bt maize and Monarch butterflies. However, it should be noted that the Bt maize had been through a risk assessment prior to commercial release but this did not appear to cover many of the questions which were now being asked.

There have been other similar cases to the "Monarch story" and there will be more unless we are more careful in how we interpret and most importantly frame scientific Biosafety studies on the environmental and ecological impacts of GM plants (Poppy, 2000). One way in which progress has been made is the use of tiered risk assessment schemes similar to that used for assessing pesticides. There is not room in this paper to comprehensively outline such a scheme (see previous BCPC proceedings paper by Schuler *et al.*, 2000), but such a framework does allow hazards to be identified and subsequently risks quantified. Such a tiered approach can also be extended to address other risks such as that posed by geneflow (Wilkinson *et al.*, 2003) This has offered a major advance to the risk assessment of GM plants and is allowing studies to be placed in context and risks to be compared. However, there are still many discrepancies in how this process is completed and at what thresholds (trigger values) different tiers are adopted. There is too little interaction between many scientists working on GM biosafety and the wider risk-assessment community. This may be because of the terminology being used and the differences between principally abiotic changes by man as opposed to the biotic change (a living GM plant) but I still think we have a lot to learn and would benefit from "borrowing" methods and approaches from the broader risk assessment community. This paper will take some of these concepts and try to incorporate them into the assessment of GM plants.

ECOLOGICAL RISK ASSESSMENTS (ERA)

The US Environmental Protection Agency (EPA) defines ecological risk assessment as "an evaluation of the likelihood that adverse ecological effects could result from exposure to one or more stressors" (USEPA, 1998)). An ecological risk assessment does not consider the impacts to humans or domesticated species. The goal of the risk assessment is to evaluate actual and predicted potential effects on plant and animal populations by principally addressing the following three questions:

1) Do current stressor levels pose a current or future ecological risk?
2) What portions of the site should be monitored or be subjected to remediation?
3) Have past activities adversely affected biodiversity?

The number of steps in the process vary according to degree of splitting and/or clumping of components. EPA recommend an eight-step process, but for clarity for non-specialists in risk assessment, this paper will only highlight three basic parts to the ecological risk assessment: Problem Formulation, Analysis Phase and Risk Characterisation.

Problem formulation

As the name suggests this is a systematic planning step that identifies the major factors to be considered in the risk assessment. For example, if considering the impact on a site, it reviews all the existing data relating to that site. The process will deliver a conceptual model that identifies the characteristics of the stressors (an abiotic or biotic entity that can cause an adverse effect), the ecosystems potentially at risk, and the ecological effects to be evaluated. During this process, the assessment and measurement endpoints (see next section) for the ecological risk assessment are identified.

Analysis phase

This phase can usually be considered in two parts.

The first is called an exposure assessment which quantifies the exposures of ecological receptors (animals, plants, microorganisms) to the stressor. Key aspects in this assessment involve quantification of the "substance" acting as the stressor, its migration and fate in the environment, and determining which organism are exposed and at what levels. There are numerous factors which influence exposure which relate both to the nature of the contaminant or ecology and behaviour of the receptor (ecological entity being exposed).

A second phase called the ecological effects assessment attempts to create a dose response relationship by linking concentration of contaminant to adverse effects in receptors. A range of lab and field tests are conducted to establish links between cause and effect (contaminants causing ecological effects).

Risk characterization

The final phase of the assessment compares the results from the exposure and ecological effects assessments. This allows hazards to be identified and the risks they pose to be quantified. If the process is successful it will allow a risk description to be developed in both numerical and descriptive terms. It is important to be able to link this back to the endpoints developed in the problem formulation part of the process. This will identify thresholds for adverse effects and provide an indication of the confidence the risk assessor has in the results.

ECOLOGICAL ENDPOINTS

Ecological endpoints are critical both in the problem formulation at the start of the assessment and in the risk characterisation stage at the end of the ecological risk assessment when risks are related back to the assessment endpoints. Ecological endpoints are thus critical in ecological risk assessment and warrant detailed consideration in a risk assessment of GM plants. The concept of Assessment and Measurement endpoints was initially described by Suter (1989, 1990) and appeared shortly after in the USEPA Guidance in the Framework for ERA (USEPA 1992).

Assessment endpoints

"Explicit expression of the actual environmental values that are to be protected, operationally defined by an ecological entity and its attributes" (USEPA 1992, 1998).

These are critical in establishing the rigour of an ecological risk assessment and should be selected in light of goals and methodology to be used in the assessment. They are also subject to public perception since there is little point establishing endpoints for unfavoured groups of organism (e.g. soil microorganisms), unless they are considered as part of an ecosystem attribute which is to be measured. A critical factor relates to the site management goals and objectives which can guide and influence the assessment endpoints and thus need to be identified and developed prior to selection of assessment endpoints in the problem formulation stage of the assessment. A significant problem relating to GM crops is the lack of agreement about site management goals which is discussed in more detail later in this paper.

Assessment endpoints are typically identified at the population, community or ecosystem level of biological organisation (USEPA 1996) in contrast to measurement endpoints which tend to focus on the individual level of organisation. A range of criteria are used in selecting the endpoints which can have either ecological or societal relevance. However, they all must be unambiguous, accessible to prediction and susceptible to the stressor. Commonly used assessment endpoints include variables relating to biodiversity, sensitive species and important ecosystem functions.

It is worth remembering that specific clearly defined assessment endpoints allow answers to be determined for specific questions, something critical for a good risk assessment framework. By developing specific assessment endpoints, it is easier to produce robust measurement endpoints and thus allow the two classes of endpoint to fully integrate, thus providing confidence in the risk assessment.

Measurement endpoints

"Measurable responses to a stressor that are related to the valued characteristic chosen as the assessment Endpoints" (Suter, 1989, USEPA 1992)

If the appropriate measurement endpoints are selected, these can be used to infer a measure of protection or evaluate risk to the assessment endpoints. A number of subclasses have been defined which include "Measures of effect", "Measures of Exposure" and "Measures of ecosystem and receptor characteristics) but these are beyond the scope of this paper (see USEPA 1998), but are used in Figure 1 which outlines management goals, assessment and measurement endpoints for Bt maize.

In some cases the measurement endpoint can be the same as the assessment endpoint, but usually they are measurable responses which relate to the assessment endpoint. They are usually at a lower level of biological organisation, principally focussing at the individual physiological, morphological or anatomical levels of organisation. Again a range of criteria are used in selecting the measurement endpoints including: relevance to assessment endpoint, high signal to noise ratio, sensitivity and response time, practicality and of high diagnostic ability.

USING ENDPOINTS FOR ASSESSING GM PLANTS

Although members of national risk assessment committees for GM crops may be familiar with the ideas associated with generic risk assessment such as ecological risk assessment as outlined above, not all practicing research scientists are familiar with the framework, let alone the terms. One could criticise those scientists who have failed to read the extensive literature, although this may be explained by the ecologists struggling to understand details relating to the science and terminology of GM biotechnology. However, it should be noted that the literature on ecological risk assessment abounds with acronyms, flow charts and diagrams which can make the topic unapproachable. Hopefully, such barriers will breakdown and the useful aspects of conventional ecological risk assessments can be adopted and translated for GM plants, allowing more time to focus on fine tuning the system for the stressor (GM plant) and the receptors (ecological entities being exposed to GM plant) at risk.

During the problem formulation process of an ecological risk assessment, the ecological endpoints are determined and a conceptual model developed. It is this stage which appears to be lacking in GM risk assessment and is why there has been little consensus into acceptability of the risks associated with GM. There has been considerable effort in the analysis phase, but how can one characterise the risks using the data from the analysis phase if the problem has not been formulated.

A conceptual model is a written description and visual representation of predicted relationships between ecological entitities and the stressors to which they are exposed. It is thus possible to develop such a model for a GM risk assessment, but the harder aspect is determining the endpoints around which the whole assessment will be based. (See Figures 1 and 2 for examples of ecological endpoints and a conceptual model relating to the risks posed to parasitoids and predators by Bt maize).

In order to generate a series of management goals for GM crops, it is important to be clear about what type of environment you require during the problem formulation part of the ecological risk assessment. In agroecosystems, there are conflicting interests and much uncertainty about what we want from our agricultural environment. As consumers, we require high quality cheap food, whereas as land users, we demand a beautiful countryside which is full of wildlife. A recent discussion meeting at the Royal Society tried to explore the impact of farming on the environment and the implications of adding GM crops into the equation (Royal Society, 2003). A principal area of discussion related to how we want farming to be like in the future and the roadmap for getting to that point. This was in contrast to the approach of describing the current baseline and looking for perturbation from that point. Unfortunately what we require from Agriculture is not clear due to conflicting issues and agendas, but it does seem that we wish to have food security with a minimum ecological footprint. There are various ways of achieving this involving a number of factors relating to productivity, scale of production area and biodiversity. For example one question we may need to consider relates to whether it is better for biodiversity to produce more food from less land or farm less intensively on greater land areas?

GOAL: Sustain populations of natural enemies (predators and parasitoids)

Assessment endpoint:

- Survival and reproduction of generalist and specialist predators/parasitoid

Measurement endpoints:

a) **Measure of effects:**
   - Analysis of adverse effects to adult parasitoid/predators (specialist/generalist on European cornborer and other non-target herbivores e.g. *Trichrogramma* spp., *Chrysoperla, Cotresia margineventris, Aphidius rhopalosiphi*)
   - Reproductive success of above species of parasitoid/predator
   - Population structure of above species of parasitoid/predator
   - Parasitoid/Predator Community analysis in Bt fields and margins

b) **Measures of ecosystem and receptor charcteristics**
   - Abundance and distribution of prey/hosts and other food sources (nectar, pollen)
   - Quality and size of habitat (floral/faunal diversity, refugia size, spatial arrangement)
   - Environmental conditions (e.g. temp, management regime – sprays etc.)

c) **Measures of exposure**
   - Bt expression levels in the plant tissue (e.g. leaves, pollen, nectar) and in the tissue of hosts/prey

Figure 1. Management goal, assessment endpoint and measurement endpoints for Bt maize and insect natural enemies.

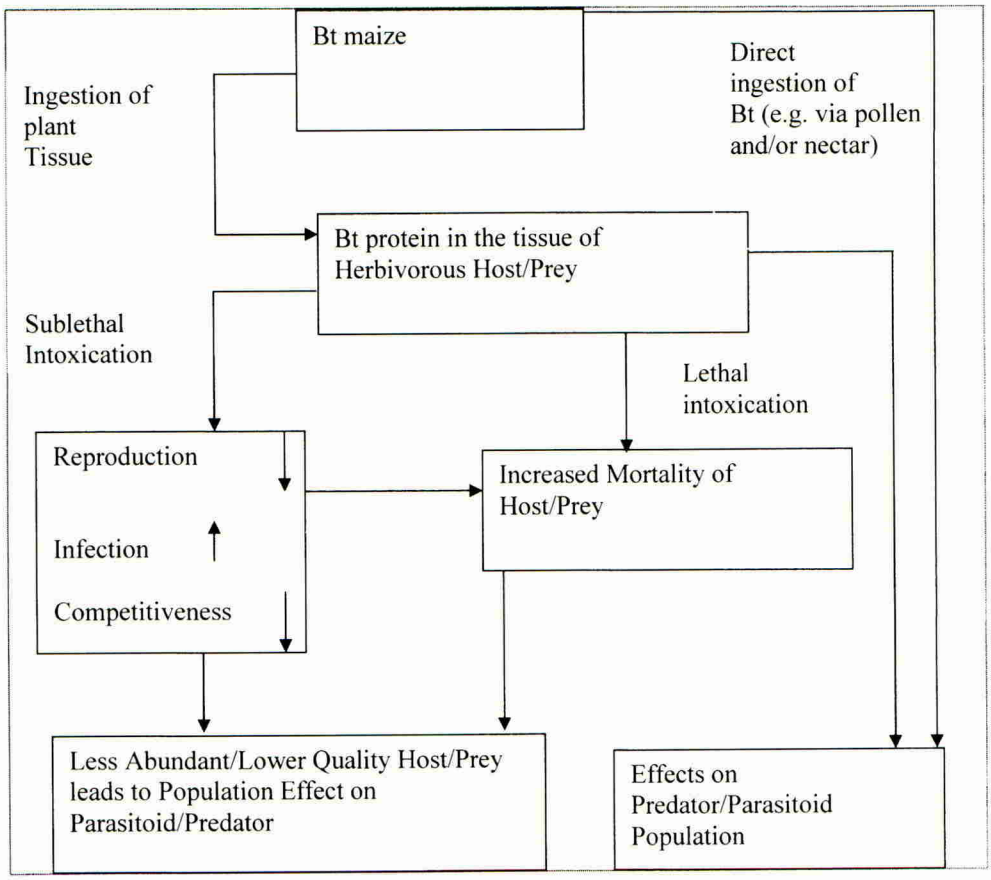

Figure 2. A conceptual model for assessing the impacts of Bt maize on insect natural enemies

CONCLUSIONS

The risk assessment of GM crops is a young science which has made progress, but it is important that it does not reinvent the wheel. Much can be learnt from more generic risk assessment, especially that used for assessing pesticides and other stressors which affect ecological systems. Although the terminology can seem difficult, the use of conceptual frameworks and endpoints allow quantitative assessments and decisions to be made about safety. If we ignore this approach, we are in danger of going round in circles and not really differentiating between degrees of risk and thus wasting our resources focussing on issues which don't require the degree of testing of another trait. We all speak of the need for case by case analysis, but we don't seem to have the necessary conceptual frameworks, endpoints or trigger values (values which mean further testing is required) to allow for joined up decision making. If the regulators and company regulatory specialists have produced such a system, then let's ensure that the practising scientists utilise it rather than continue to mechanistically take systems apart without really knowing the question for which they seek the answer.

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Rethinking the herbicide development and regulation process post GM crop environment impact studies

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An assessment of the level of crop to crop gene flow in forage maize crops in the UK

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ABSTRACT

Farm-scale evaluation (FSE) trials were established to assess the effects on farmland biodiversity of weed management methods associated with the use of genetically modified herbicide tolerant (GM HT) crops compared with conventional crops. Gene flow was monitored at the FSE sites of fodder maize over the duration of the 3-year trial. The trial sites had a split field layout; one half of the field was planted with the GM HT crop, the other half with a conventional equivalent of the same crop. Maize samples were collected from the conventional crop halves of FSE sites at a range of distances from the GM crop. GM/non-GM hybridization was detected and quantified using molecular methods. Additional data on wind direction and landscape were also collected for each trial site. The results show that the rates of cross-pollination decreased over distance and followed the expected pollen dispersal curve. Evidence of cross-pollination was found up to 200m away from the GM/non-GM junction in the crop. There was significant variation in levels of cross-pollination between sites in each year ($p < 0.01$), although the variation between years across all sites was not significant ($p > 0.05$). The importance of isolation distances in contributing to reducing adventitious pollen intrusion will be discussed with respect to sustainable co-existence of GM, conventional and organic crops.

INTRODUCTION

There is currently (August, 2003) a moratorium on the commercial planting of genetically modified (GM) crops in the UK. The government will make a decision on whether or not GM crops should be cultivated following publication of the results of the Farm-Scale Evaluations, reviews of the costs and benefits of GM crops (Strategy Unit of the Cabinet Office, 2003) and a review of the science relevant to GM crops and food based on interests and concerns of the public (King *et al.*, 2003).

In 1999, the Department for Environment, Food and Rural Affairs (Defra) established farm-scale evaluation (FSE) trials to assess the effects on farmland biodiversity of the weed management methods associated with the cultivation of GM herbicide tolerant crops compared with conventional (non-GM) crops. In conjunction with these trials, a study of gene flow from the GM to conventional crops was also established, using the FSE sites of winter and spring oilseed rape and fodder maize, genetically modified to be herbicide tolerant (HT). This paper reports the results of the forage maize trials.

A review, which addressed the issue of separation distances between GM and other crops, was published in 2000 by the Ministry of Agriculture, Fisheries and Food (Ingram, 2000). Currently the minimum separation distance required in the European Union is 200m for all categories of seed production, which is believed to be sufficient to maintain inbred lines at 99.9% purity (Ingram, 2000). The recommended separation distances for non-GM crops from the Supply Chain Initiative on Modified Agricultural Crops (SCIMAC) guidelines for growing GM HT crops are 200m for sweetcorn and 80m for forage maize. However, whilst no maize seed is currently produced in the UK fodder maize is grown and harvested for silage, and sweetcorn is also grown in some areas.

A number of factors affect pollination rates in maize. Most of the pollen is shed from the plants before the silks are receptive, but there is some overlap, resulting in up to 5% self pollination (at least 95% of ovules are fertilised by pollen from other plants). Pollen viability can vary between 2h and 8 days, depending on environmental conditions. The impaction rate (settling velocity) of maize pollen is 30-40 cm s^{-1} so the pollen normally only travels short distances. Pollination rates can also be affected by competition from pollen from other sources. Finally, wind speed, wind direction and surface turbulence can also affect pollination rates. These factors make it difficult to predict the effect of one maize field on another. A higher wind speed will cause the pollen to travel further downwind but the impaction rate of the pollen will also increase. Other factors that will affect the rate of cross-pollination between fields are synchronisation of flowering, the relative concentration of the pollen in the donor and receptor plot (the protective strength of the field pollen cloud), the levels of selfing and the density of the stands.

Although hybrid corn production practices have remained basically unchanged for the last thirty years (Burris, 2002), there is limited literature available on gene flow from pollen. Reports of outcrossing rates range from 40% at 2.5m (Bateman, 1947), 4.5% at 3m (Jugenheimer, 1976), 1.11% at 200m (Burris, 2002) and 2.47% at 200m (Jones & Brookes, 1950). Under very arid, calm conditions, outcrossing was not detected beyond 200m (Baltazar and Schoper, 2002). Previous studies on gene flow from maize have not been carried out on a commercial scale (with the exception of Burris in the USA). The FSE offered the opportunity to sample a large number of fields in a wide range of locations and environments in England. This paper presents quantitative results from years 2000 to 2002 for the extent of transfer of the GM herbicide tolerance gene to conventional fodder maize at different distances from the GM crop unit.

MATERIALS AND METHODS

The gene flow study commenced after the FSE biodiversity study had been established. Sites consisted of a split field design, half planted with Liberty Link™, line T25 (containing the *pat* gene), which is tolerant to Liberty™, a broad spectrum, non-residual, glufosinate ammonium herbicide and the other half with a conventional equivalent maize variety. A total of 55 trial sites were used in the maize study (Figure 1). Samples were taken from the conventional crop halves of the fields, along transects, at 2, 5, 10, 20, 50 and 150m from the division between the crop units. Transect positions were determined to be approximately one quarter, one half and three quarters of the distance across the width of each field.

Covariate data were collected for each of the fields sampled. The FSE dataset was used to provide data on the size, aspect, orientation and slope of the fields. Field boundary attributes describing the type, height and completeness of boundaries round the sampled fields were also extracted from the dataset. These covariate data were used in conjunction with the PCR data to explain patterns of hybridisation and gene flow from the GM crop to the conventional.

The maize samples were tested for the presence of the *pat* gene using real time (TaqMan) PCR. Briefly, a reporter dye and a quencher dye are attached to the 5' and 3' ends of a TaqMan probe. When both dyes are attached to the probe, reporter dye emission is quenched. During each PCR extension cycle, the *Taq* DNA polymerase cleaves the probe when bound to the template ahead of the *Taq*, which separates the dyes. Once separated from the quencher, the reporter dye emits its characteristic fluorescence. The fluorescence is detected using an ABI Prism 7700 sequence detection system. Results are in the form of Ct values, which represent the PCR cycle at which an increase in reporter fluorescence can first be detected. Primers (and probe) were designed using published sequence data for the *pat* gene for maize (T25) (referred to as the target genes). In addition to the GM detection primers (and probe), primers and a probe were also required to detect an endogenous reference for relative quantification of the target. The rationale behind the design of these primers was to pick a single copy gene that was specific to that species of plant. For maize the endogenous control *Zea mays cdc*2 was used.

Genomic DNA was extracted from maize using the Promega Wizard Magnetic DNA purification system and the Labsystems KingFisher ml Magnetic Particle Processor. DNA standards (used for quantification of unknown samples) were produced using known quantities of genomic T25 DNA and making a dilution series in DNA extracted from *Nicotiana tabacum*. Standard curves were created by plotting the Ct values of the known standards against the log of the concentration of DNA. Data for the unknown samples was then calculated from the standard curves. A normalized amount of target DNA was obtained by dividing the target value by the endogenous control reference amount. The normalized TaqMan data was expressed as a GM: non-GM ratio. The T25 maize was heterozygous for the transgene and this was taken as being 100% (i.e. 1:1 ratio) reference material.

To stabilise variances all results of the proportion of GM DNA (*pat* gene) detected in the field, samples were subjected to a probit transformation (Armitage, 1983). To determine the effect of year and site on proportion of GM cross-pollination, field sites that had been sampled in more than one year were chosen. The transformed results were analysed using General Linear Model (GLM) Analysis of Variance (ANOVA) (McCullagh & Nelder, 1989). To determine the spatial spread of the *pat* gene, results collected at different distances along transects established in the fields were used. The results were subjected to non-linear regression analysis to estimate the extent of gene flow with distance from source.

RESULTS

A total of 55 FSE sites were sampled during the three years of the study. The sites were at a range of locations across England (Figure 1) and were representative of some of the main maize growing regions. Evidence of gene flow by cross-pollination was detected at all the sites that were sampled. Overall the results showed a decrease in the rate of cross-pollination with increasing distance from the GM crop unit (Figure 2). There was a rapid decrease in the

rate of cross-pollination within the first 20m from the donor crop and beyond this distance the rate of decrease was much slower. The average GM: non-GM ratio was 0.06 (6%) in the first 2m of the crop, this decreased to approximately 0.0003 (0.03%) at 200m. At two of the sites a number of samples were taken (with the kind permission from the grower) from the facing edge of forage maize fields nearest to the trial sites. One positive result was obtained at a distance of 650m from the GM source.

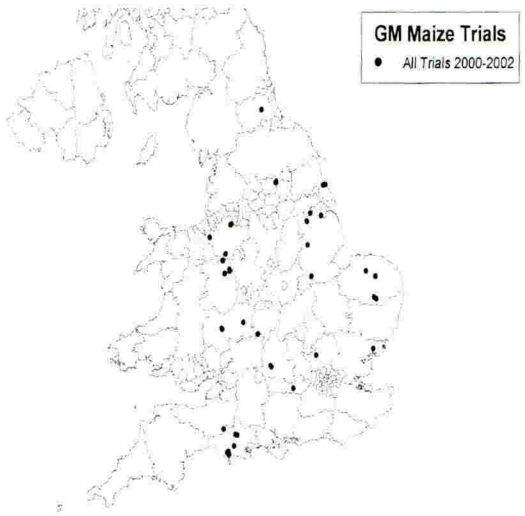

Figure 1. Location of maize FSE sites from 2000-02. A total of 54 sites were used, some of the sites were used for more than one year and other sites were relatively close to each other.

Taking the 99.9% purity threshold for seed production and a 50m-isolation distance for forage maize, the fields were scored according to how many had GM: non-GM levels greater that 0.1% at a distance of 50m or more. Evidence of cross-pollination was found beyond the 50m-isolation distance in 42 out of 54 fields. Of these 34, were at a level of $\geq$0.1% and 23 of these fields were found to have levels $\geq$0.3%. At 150m from the GM source there was evidence of cross-pollination in 19 of 44 fields and of these 12 were $\geq$0.1% and 7 were $\geq$0.3%. In all cases where there was evidence of cross-pollination at 150m there was also cross-pollination at 50m.

Results of the GLM ANOVA indicated that GM: non-GM cross-pollination was significantly different between sampling locations on the field transects with distance ($t = -5.67$, d.f. = 65, $p < 0.001$) and between field sites ($t = -3.32$; d.f. = 65; $p = 0.001$) but not between years ($t = -1.18$; d.f. = 65; $p = 0.241$). A comparison of different non-linear equations indicated that a negative power regression explained most of the variation in the experimental results and thus, was chosen for subsequent analysis. Results of the non-linear regression analysis further indicated that gene flow was highly dependent upon distance from the source of GM DNA ($F = 30.4$; d.f. = 2,8; $p < 0.001$; Figure 2).

The regression equation was validated against field results not used in its derivation. The model predicted that at 650m from a source of GM maize, cross-pollination would be 0.04 %, whereas a mean value of 0.02 % was recorded. Further examination of the predicted equation

indicated that at a distance of 80m cross-pollination levels would be less than 0.3%. To ensure contamination levels of less than 0.9% and 0.1% crops would need to be located at distances greater than 24.4m and 257.7m, respectively.

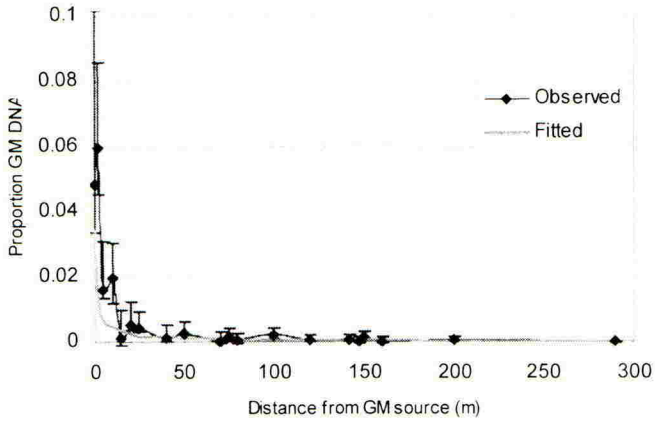

Figure 2. Comparison of fitted and observed GM DNA cross-pollination against distance from GM source in metres.

DISCUSSION

To our knowledge this is the first study on crop-to-crop gene flow in maize at a farm-scale level in the UK. This study is unique both in the size and range of the trial sites and in the molecular approach to quantification of gene flow. The FSE trials were set up to compare effects on biodiversity of GM and conventional weed control practices and not explicitly to determine the extent of gene flow. However, these trials represent the potential for gene flow under realistic agricultural conditions rather than either small-scale trial plots or a number of GM plants in the middle of a conventional field.

The original aim of this project was to validate assumptions made in risk assessments concerning gene flow by pollen from the farm-scale evaluations and to ensure that the guidelines issued by SCIMAC stipulate an effective separation distance for the crop. It is evident from the results that cross pollination events occurred not only beyond the 80m isolation distance recommended for forage/fodder crops by SCIMAC, but also beyond the 200m distance recommended for sweetcorn and organic crops. Although these trials did not use sweetcorn, it is reasonable to assume that pollen distribution from the two crops would be similar. It is important to emphasise that the whole of the plant is harvested in forage crops and thus any cross-pollination events will be 'diluted' out in the final product. Sweetcorn presents more of a problem in that individual cobs will be consumed. So, even if a field was well below the threshold (currently at 0.9% for the labelling of GM food and feed), individual cobs may not be.

If the aim is to maintain a 99.9% purity level then an 80m-separation distance will not be enough. The current proposed threshold for the adventitious presence of GM seeds in

certified seed lots is now 0.3% for authorized events and 0.1%-nil for unauthorized events (under part C of Directive 2001/18/EC). A recent report published by the European Commission (Block *et al.*, 2002) suggested that for maize, a threshold of 0.1% would be extremely difficult to achieve for any farming scenarios (conventional and organic farms). The report also pointed out that in less intensive maize growing regions it would be possible to meet a 1% threshold providing some changes were made to farming practices (assuming a GM adventitious presence of 0.3% or less). Maize seed is not produced in this country, therefore it is more important to consider the threshold for food and feed, which is currently set at 0.9 %. Based on the results presented here it would be possible to meet this threshold with an increase to the current isolation distances.

A more in depth analysis of the landscape characteristics of individual fields will be carried out using the data collected. It is hoped that this huge data set can be used to give us a better understanding of how typical UK farming conditions and landscape can be utilised to limit the extent of gene flow and maximise the potential for co-existence of the two farming practices.

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Containment and mitigation of transgene flow from crops

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ABSTRACT

There are many ways to prevent transgene introgression from crops to other varieties, or to related weeds or wild species (containment strategies), as well as to preclude the impact should containment fail (mitigation strategies). The needs are most acute with rice and sunflowers, which have con-specific weeds, and with oilseed rape, sorghum, barley, which have closely related weeds. Containment and mitigation are critical for pharmaceutical crops, where gene flow from the crop to edible varieties must be precluded. Some gene flow (leakage) is inevitable with all containment mechanisms and once leaked, could then move throughout populations of undesired species, unless their spread is mitigated. Leakage even occurs with chloroplast-encoded genes, a >0.03% pollen transmission was found in the field. We focused on mitigation, which should be coupled with containment as a last resort. A mechanism for mitigation was proposed where the primary transgene (herbicide resistance, etc.) is tandemly coupled with flanking genes that could be desirable or neutral to the crop, but unfit for the rare weed into which the gene introgresses. Mitigator traits include dwarfing, non-bolting, no secondary dormancy, no seed shattering, and poor seed viability, depending on the instance. We demonstrated the potential utility of the concept using tobacco as a model, and dwarfing as the mitigator with herbicide resistance as the primary gene. Hybrids with the tandem construct were unable to reach maturity when grown interspersed with the wild type. Such mitigation should greatly decrease risk of transgene movement especially when coupled with containment technologies, allowing cultivation of transgenic crops having related weeds. As the number of transgenic plants being released is increasing, and the problems of monitoring such genes increases geometrically, we suggest that a uniform biobarcode™ system be used, where a small piece of non-coding DNA carrying an assigned variable region is used to mark transgenic crops, allowing monitoring.

INTRODUCTION

Farmers in most of the world have begun to realize the benefits that accrue from cultivating transgenic crops, whether to prevent soil erosion by using post-emergence herbicides or use less expensive/toxic insecticides while contributing to farmer and environmental health and safety. Herbicide resistant crops are especially useful for controlling crop-related weeds where there had been no herbicide selectivity. Several crops (e.g., wheat, barley, sorghum, rice, squash, sunflower, sugarbeets, oats, and oilseed rape) can naturally interbreed with closely related weedy relatives under field conditions, in both directions (Ellstrand *et al.*, 1999; Gressel, 2002). There is a concern that transgenes may escape from engineered crops into related weedy species by hybridization and backcrossing. This could potentially result in hybrids and their progeny with enhanced invasiveness or weediness (Ellstrand *et al.*, 1999).

Many of the engineered genes such as those conferring resistance to herbicides, diseases, and to stresses may grant a fitness advantage to a weedy species growing in the same agricultural ecosystem. There is also the rather emotive issue of transgene flow from crops such as maize bearing transgenes encoding pharmaceuticals to other varieties. Farm produced pharmaceuticals especially enzymes and antibodies, can be produced inexpensively in plants, without the need for animal tissue culture cells grown in a medium of expensive serum albumin that is all too easily contaminated with pathogenic mycoplasms, prions and viruses. Still, there is reason not to want the pharmaceutical transgenes in other varieties of the crop.

Two general approaches are discussed below to deal with the problems of transgene flow: containment of the transgenes within the transgenic crop; transgenic mitigation of the effects of the primary transgenic trait should it escape and move to an undesired target. While most containment mechanisms will severely restrict gene flow, some gene flow (leakage) is inevitable and could then spread through the population of undesired species, unless mitigated.

CONTAINING TRANSGENE FLOW

Several molecular mechanisms have been suggested to contain the transgene within the crop (i.e. to prevent outflow to related species), or to mitigate the effects of transgene flow once it has occurred (Gressel, 1999, 2002; Daniell, 2002). The containment mechanisms include utilization of partial genome incompatibility with crops such as wheat and oilseed rape having multiple genomes derived from different progenitors. When only one of these genomes is compatible for interspecific hybridization with weeds, the risk of introgression could be reduced if the transgene was inserted into the unshared genome where there is presumed to be no homeologous introgression between the non-homologous chromosomes. It has not been reported if this mechanism works in wheat, and it was modeled to be ineffectual for oilseed rape (Tomiuk *et al.*, 2000) due to considerable recombination between the A and C genomes.

Another containment possibility is to integrate the transgene in the plastid or mitochondrial genomes (Maliga, 2002). The opportunity of gene outflow is limited due to maternal inheritance of these genomes. This technology does not preclude the weed from pollinating the crop, and then acting as the recurrent pollen parent. The claim of no paternal inheritance of plastome encoded traits (Bock, 2001; Daniell, 2002), was not substantiated. Tobacco (Avni & Edelman, 1991) and other species (Darmency, 1994) often have between a 10^{-3}–10^{-4} frequency of pollen transfer of plastid inherited traits in the laboratory. Pollen transmission of plastome traits can only be easily detected using both large samples and selectable genetic markers. A large-scale field experiment utilized a *Setaria italica* (foxtail or birdseed millet) with chloroplast-inherited atrazine resistance (bearing a nuclear dominant red leaf base marker) crossed with five different male sterile yellow- or green-leafed herbicide susceptible lines. Chloroplast-inherited resistance was pollen transmitted at a 3×10^{-4} frequency in >780,000 hybrid offspring (Wang *et al.*, 2003). At this transmission frequency, the probability of herbicide resistance form plastomic gene flow is orders of magnitude greater than by spontaneous nuclear genome mutations. Chloroplast transformation is probably unacceptable for preventing transgene outflow, unless stacked with additional mechanisms.

A novel additional combination that considerably lowers the risk of plastome gene outflow within a field (but not gene influx from related strains or species) can come from utilizing male sterility with transplastomic traits (Wang *et al.*, 2003). Introducing plastome inherited traits

into varieties with complete male sterility would vastly reduce the risk of transgene flow, except in the small isolated areas required for line maintenance. Such a double failsafe containment method might be considered sufficient where there are highly stringent requirements for preventing gene outflow to other varieties (e.g. to organically cultivated ones), or where pharmaceutical or industrial traits are engineered into a species. Plastome-encoded transgenes for non-selectable traits (e.g. for pharmaceutical production) could be transformed into the chloroplasts together with a trait such as tentoxin or atrazine resistance as a selectable plastome marker. With such mechanisms to further reduce out-crossing risk, plastome transformation can possibly meet the initial expectations.

Other molecular approaches suggested for crop transgene containment include: seed sterility, utilizing the genetic use restriction technologies (GURT) (Oliver *et al.*, 1998), and recoverable block of function (Kuvshinov *et al.*, 2001). Such proposed technologies control out-crossing and volunteer seed dispersal, but theoretically if the controlling element of the transgene is silenced, expression will occur. Another approach includes the insertion of the transgene behind a chemically-induced promoter so that it will be expressed upon chemical induction (Jepson, 2002). However, there is a possibility of an inducible promoter mutating to become constitutive. Schernthaner *et al.* (2003) proposed an impractical technology using a "repressible seed-lethal system". The seed-lethal trait and its repressor must be simultaneously inserted at the same locus on homologous chromosomes in the hybrid the farmer sows to prevent recombination (crossing over), technology that is not yet workable in plants. The hemizygote transgenic seed lethal parent cannot reproduce by itself, as its seeds are not viable. If the hybrid could be made, half the progeny would not carry the seed lethal trait (or the trait of interest linked to it) and they will have to be culled, which would not be easy without a marker gene. The results of selfing or cross pollination within the crop and leading to volunteer weeds where 100% containment is needed, would leave only 25% dead and 50% like the hybrid parents and 25% with just the repressor. Thus, the repressor can cross from the volunteers to related weeds as can the trait of choice linked with the lethal, and viable hybrid weeds could form. The death of some seed in all future weed generations is inconsequential to weeds that copiously produce seed, as long as the transgenic trait provides some selective advantage.

None of the above containment mechanisms is absolute, but risk can be reduced by stacking containment mechanisms together, compounding the infrequency of gene introgression. Still, even at very low frequencies of gene transfer, once it occurs, the new bearer of the transgene can disperse throughout the population if it has just a small fitness advantage.

MITIGATING FURTHER FLOW OF 'LEAKED' TRANSGENES

If the transgene has a small fitness disadvantage, it will remain localized as a very small proportion of the population. Therefore, gene flow should be mitigated by lowering the fitness of recipients below the fitness of the wild type so that they will not spread. A concept of "transgenic mitigation" (TM) was proposed (Gressel, 1999), in which mitigator genes are added to the desired primary transgene, which would reduce the fitness advantage to hybrids and their rare progeny, and thus considerably reduce risk. This TM approach is based on the premises that: 1) tandem constructs act as tightly linked genes, and their segregation from each other is exceedingly rare; 2) The TM traits chosen are neutral or favorable to crops, but deleterious to non-crop progeny due to a negative selection pressure; and 3) Individuals bearing even mildly harmful TM traits will be kept at very low frequencies in weed populations

because weeds typically have a very high seed output and strongly compete among themselves eliminating even marginally unfit individuals (Gressel, 1999). Thus, it was predicted that if the primary gene of agricultural advantage being engineered into a crop is flanked by TM gene(s), such as dwarfing, uniform seed ripening, non-shattering, anti-secondary dormancy, or non-bolting genes in a tandem construct, the overall effect would be deleterious after introgression into weeds, because the TM genes will reduce the competitive ability of the rare transgenic hybrids so that they cannot compete and persist in easily noticeable frequencies in agroecosystems (Gressel, 1999). Weeds are usually copious pollen producers and set large numbers of seeds, many of which germinate during the following season.

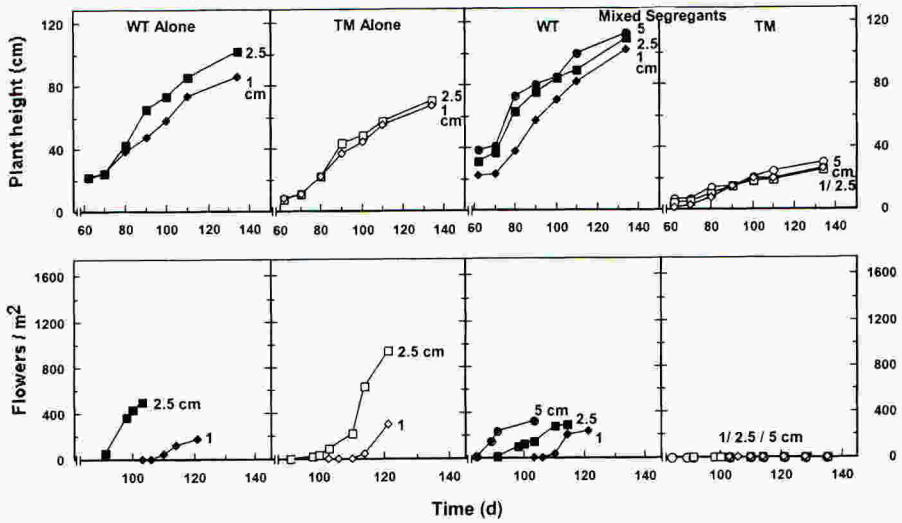

Figure 1. Suppression of growth and flowering of TM (transgenic mitigator) bearing tobacco plants carrying a dwarfing gene in tandem with a herbicide resistance gene (open symbols) when in competition with the wild type (closed symbols) (right panels), and their normal growth when cultivated separately without herbicide (left panels). The wild type and transgenic hemizygous semi-dwarf/herbicide resistant plants were planted at 1, 2.5, and 5 cm from themselves or each other, in soil. See Al-Ahmad *et al.* (2003) for further details.

We used tobacco (*Nicotiana tabacum*) as a model plant to test the TM concept: a tandem construct was made containing an *ahas*^{R} (acetohydroxy acid synthase) gene for herbicide resistance as the primary desirable gene, and the dwarfing Δ*gai* (gibberellic acid-insensitive) mutant gene as a mitigator (Al-Ahmad *et al.*, 2003). Dwarfing would be disadvantageous to the rare weeds introgressing the TM construct, as they could no longer compete with other crops or with fellow weeds, but is desirable in many crops, preventing lodging and producing less straw with more yield. The dwarf and imazapyr resistant TM transgenic hybrid tobacco plants (simulating a TM introgressed hybrid) were more productive than the wild type when cultivated alone. They formed many more flowers than the wild type, which is an indication of a higher harvest index (Figure 1). Conversely, the TM transgenics were weak competitors and highly unfit when co-cultivated with the wild type in ecological simulation competition experiments (Figures 1, 2). The lack of flowers on the TM plants in the competitive situation (Figure 1) led to a zero reproductive fitness of the TM plants grown in a 1:1 mixture with the wild type at the spacings used, which are representative of those of weeds in the field (Figure 2). The highest vegetative fitness was less than 30% of the wild type (Figure 2). Thus, it is clear that transgenic mitigation should be advantageous to a crop growing alone, while

disadvantageous to a crop-weed hybrid living in a competitive environment. If a rare pollen grain bearing tandem transgenic traits bypasses containment, it must compete with multitudes of wild type pollen to produce a hybrid. Its rare progeny must then compete with more fit wild type cohorts during self-thinning and establishment. Even a small degree of unfitness encoded in the TM construct would bring about the elimination of the vast majority of progeny in all future generations as long as the primary gene provides no selective advantage while the linked gene confers unfitness. Further large-scale field studies will be needed with crop/weed pairs to continue to evaluate the positive implications of risk mitigation. We have inserted the same construct into oilseed rape and are testing the selfed progeny, as well as hybrids with the weed *Brassica campestris=B.rapa.* The rare hybrid offspring from escaped pollen bearing transgenic mitigator genes would not pose a dire threat, especially to wild species outside fields, as the amount of pollen reaching the pristine wild would be minimal. Pollen flow decreases exponentially with distance, belying the unbased 'demographic swamping' by 'Trojan genes' giving rise to 'migrational meltdown', as predicted by Haygood et al. (2003).

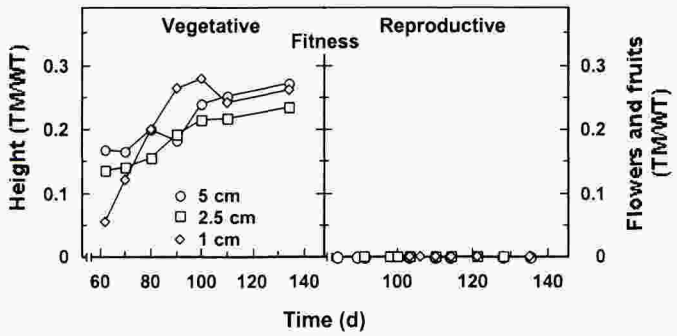

Figure 2. Suppressed vegetative and reproductive fitness of TM transgenics in competition with wild type tobacco. The points represent the calculated ratio of data for TM to wild type plants in Figure 1.

The containment of pharmaceutical transgenes has been physical, and as evidenced by recent human error that allowed temporary volunteer escape of "Prodigene" maize with such genes. The biological containment strategies described above may be preferable to depending on humans, and the mitigation strategies should work as well. Maize pharmaceutical transgenes are expressed in embryo tissues, and a potential tandem mitigating gene could be any dominant gene that affects the endosperm, e.g. the various "shrunken seed" loci, especially those where sugar transformation to starch is inhibited. Such shrunken seeds, with their high sugar content, are somewhat harder to store than normal maize but are extremely unfit in the field, and are unlikely to over winter. Because the endosperm of corn is 67% pollen genes, it is important that expression of pharmaceutical encoding genes be only in the embryo.

MONITORING TRANSGENE MOVEMENT

Using the various containment and mitigation strategies it should be possible to keep 'leaks' below risk thresholds, which should be mandated by science-based regulators on a case-to-case basis. As the numbers of transgenic species being released is increasing, and the problems of monitoring for such genes increases geometrically, we suggest that a uniform biobarcode^{TM} system be used, where a small piece of non-coding DNA with uniform recognition sites are at the ends (for single PCR primer pair amplification) with an assigned variable region in

between. Thus, PCR-automated sequencing could be used to determine the origin of 'leaks', contamination, liability, as well as intellectual property violations (Gressel & Ehrlich, 2002).

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