MONITORING LARGE SCALE RELEASES OF GENETICALLY MODIFIED CROPS (EPG 1/5/84)

INCORPORATING REPORT ON PROJECT EPG 1/5/30: MONITORING RELEASES OF GENETICALLY MODIFIED CROP PLANTS

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Final report of monitoring studies of field scale releases of GM oilseed rape crops in England from 1994 – 2000.
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1. SUMMARY

Background

In 1994 the National Institute of Agricultural Botany (NIAB) and the Laboratory of the Government Chemist (LGC) were commissioned by the Department of the Environment, Transport and the Regions (DETR) to monitor the first agricultural releases of genetically modified (GM) oilseed rape (OSR) for a three year period. Subsequently NIAB received a second contract in 1997 to continue monitoring releases of GM OSR including all the previously studied sites and any new sites over 1ha. The contracts also required NIAB to conduct studies of monitoring methods and of the flow of transgenes to crops and wild relatives. The monitoring terminated at the end of 2000.

The first crops monitored were seed production crops sown in spring 1995 and 1996. Two 5ha areas of GM winter OSR sown in the autumns of 1995 and 1996, were also monitored. The seed production crops monitored were for the production of the Plant Genetics System (PGS) GM hybrid oilseed rape and consisted of a GM male-sterile female parent line, interplanted with a pollinator containing a male fertility restorer gene, and both lines containing the *Bar* marker transgene conferring tolerance to the herbicide glufosinate-ammonium. The winter rape areas were PGS trials containing a mixture of GM and non-GM parent lines and hybrids. The transformations were similar to those in the spring rape.

From 1997 several new sites containing trials or crops of glufosinate (*Bar* and *Pat* genes) and glyphosate tolerant transgenic varieties were monitored. In 1998, several sites growing a high laurate transgenic spring OSR variety were included in the monitoring study. In 1999 the monitoring included the two first Farm Scale Evaluation (FSE) trials of GM OSR and provided an opportunity to study gene flow between two large adjacent blocks of spring OSR at these sites. By the year 2000, a total of 11 sites that had grown GM OSR were being monitored. All sites continued to be monitored in the years following the GM OSR crop or trial until the end of 2000.

The monitoring programme studied the characteristics of herbicide-tolerant transgenic rape which were most likely to effect the crop, the cultivated and the non-cultivated environment. These characteristics were assumed to be the same as those of non-transgenic rape, namely dispersal into and colonisation of these environments and gene flow into other crops, feral populations and wild crucifers. The following factors were studied and comparisons made between the behaviour of transgenic OSR and conventional OSR where possible.

Intra-specific gene flow.

Gene flow was monitored from GM OSR crops to adjacent crops, OSR volunteers and feral rape populations.

No intra-specific gene flow was detected at any of the sites monitored between 1994 and 1997. During this time none of the GM release sites were near to other synchronously flowering oilseed rape crops. No gene flow was detected to OSR volunteers and feral OSR growing near the GM releases monitored at any of the sites during this period.

In the period 1998 to 2000 gene flow was detected from GM trials into adjacent OSR crops. At one of the FSE sites gene flow decreased rapidly with distance from the pollen source. However at both FSE sites, levels of herbicide-tolerance in excess of 0.5% were found in some samples taken at 100m from the source while at one FSE site levels of herbicide-tolerance in excess of 0.5% were found in some samples taken at 200m, though the overall trend was for gene flow to decrease with distance. These could have resulted from several factors including, adventitious GM material in the original seed batch of Hyola 401, the possible presence of male-sterile individuals, weather conditions or a combination of these and other unknown factors.

Gene flow was also measured from 2 GM trials into adjacent fields of OSR in 2000. Gene flow levels were found to be substantially higher into a varietal association than a conventional variety, due to the male sterile component of these systems. Levels up to 3.2% herbicide tolerance were found at the edge of one field of the varietal association Gemini, at 105m distance from a small block of transgenic herbicide tolerant OSR. By contrast when a transgenic herbicide tolerant trial pollinated a neighbouring conventional crop of the variety Apex, at a different site, maximum levels of outcrossing at 100m were 0.2%. However at most sampling points less than 0.1% herbicide tolerance was found 70m from the pollen source.

Inter-specific gene flow.

Gene flow was monitored between GM OSR and related cruciferous species. In the first three years of the contract (1994 to 1997) a wider range of crucifers was monitored including *Capsella bursa-pastoris* (shepherd's purse) and *Sisymbrium officinale* (hedge mustard). When the contract was renewed in 1997 it was considered that resources should be concentrated on species considered to be important candidates for hybridisation with OSR. The species that continued to be monitored were *Brassica rapa* (wild turnip), *Raphanus raphanistrum* (wild radish), *Sinapis arvensis* (charlock) and *Sinapis alba* (white mustard).

No gene flow was detected from OSR into the related species examined in this study during the period 1994 to 1997. Between 1997 and 2000 hybridisation was detected with *B.rapa*. One site was examined where weedy *B.rapa* occurred in an agricultural field. Hybridisation frequencies varied between plants and were between 0.0% and 48.5%. When seeds were germinated from hybrid mother plants, some evidence of backcrossing in the direction of both parents (*B.napus* and *B.rapa*) was also found. Backcrossing to *B.napus* plants was identified by their ploidy level, however back-crossing to *B.rapa* plants could not always be determined by their ploidy level as in many cases this was the same as or very similar to the ploidy level of *B.rapa*. The co-existence of the *B rapa* populations with *B napus* crops and the numbers of hybrids found, suggested that gene flow has been occurring for some time between these populations.

Seed dispersal.

Seed dispersal was usually associated with spillage and distribution by agricultural machinery, particularly combine harvesters. In the contract from 1994 to 1997, it was found that combine harvesters were often not thoroughly cleaned after the harvesting of the GM crop, and the crop harvested subsequently flushed out the GM rape seed onto the ground causing contamination of this field (often winter barley). OSR volunteers were frequently found in these fields in stubble, however they were generally controlled in the same way as conventional volunteers. Outside the cultivated area establishment and survival of seedlings was very poor, and few feral transgenic OSR plants survived to maturity

Persistence of transgenic OSR volunteers.

The persistence of transgenic OSR volunteers was compared to existing data and observations of non-transgenic volunteers. The numbers of GM OSR winter and spring volunteers were generally low in subsequent crops. The presence of a herbicide-tolerance transgene or high laurate transgene did not appear to increase the weediness or persistence of volunteer OSR in this study.

Feral Oilseed Rape

Only one feral OSR population was found to persist for more than one year at any of the sites being monitored. The herbicide-tolerance *Bar* gene was not detected in any of the feral OSR plants so that effects on weediness and persistence of these populations could not be assessed.

Development of optimal methodology for monitoring.

A practical, effective and economical combination of monitoring methodology was developed to cover all the above aspects of monitoring. This included familiarity with the species and sites involved, combined with phenotypic and genotypic testing for the presence of the transgene. The combination of methods used ensured that any major impacts of the GM plants on the agricultural and local environment occurring at each site were likely to be observed.

Conclusions

The high levels of isolation from other OSR crops flowering synchronously, and the relatively small GM pollen sources and low levels of cruciferous weeds present at the sites limited potential gene flow at the sites monitored in the first 3-year contract (1994 to 1997). Larger trials or crops released during the second 3-year contract (1998 to 2000) and the closer proximity of pollen receptive crops and related wild species allowed greater opportunities for gene flow to be studied. The results from these larger trials and crops indicate that commercial scale releases of GM OSR in the future could pollinate other crops and *B.rapa,* the levels of cross pollination depending on the environmental, varietal and agronomic factors prevailing at the time. There may be a need to review isolation requirements in keeping with current legislation on contamination thresholds in crops, in light of this research.

2. INTRODUCTION

The first releases of GM OSR in the UK were transgenic herbicide-tolerant lines. Initially these consisted of oilseed rape genetically modified for tolerance to glufosinate-ammonium (Basta™ or Liberty™). The GM oilseed rape monitored in the contract was the Plant Genetic Systems (PGS) hybrid system containing three components. The first was a male sterility gene (*barstar*) controlled by a promoter, ensuring the gene is only activated in the anthers. A selectable marker gene, in this case conferring tolerance to glufosinate-ammonium (*bar* gene), allows male-sterile offspring to be selected without waiting until flowering. The third gene introduced to the system was a male fertility restorer gene (*barnase*) that ensures the hybrid progeny will produce pollen. To produce the hybrid seeds, the parent containing the restorer gene pollinates a male-sterile parent with the selectable marker gene. Rows or blocks of male-sterile and restorer plants are sown together in the seed production field. Seed is only harvested from the male-sterile parent, ensuring that all the seed harvested is hybrid.

It should be noted that the male line used in the breeding/ seed production is hemizygous for herbicide-tolerance (*bar*) so that while all the F_1 generation are heterozygous for herbicide-tolerance, a proportion (normally 1/3) of the male gametes (pollen) will not carry the *bar* gene. Thus outcrossing studies using herbicide-tolerance as an indicator of cross-pollination are only likely to detect 2/3 of actual outcrossing levels from this material. In addition a small proportion of plants in the F_1 may also be male-sterile (usually 8-9%). These plants in turn will be dependent on cross-pollination to set seed and may bias levels of outcrossing observed in these cultivars.

The original application by PGS for release into the environment in 1993/94 was supported by extensive documentation including an environmental risk assessment based on knowledge at that time. DETR subsequently conducted a review of this risk assessment in order to re-assess the environmental risks of commercial scale release as more data had become available. NIAB contributed data from the first contract to this review (Gray, 1998).

The general conclusions from the original risk assessment were:

- There are no indications to anticipate……altered weediness and/or invasiveness of the transgenic versus the non-transgenic oilseed rape in natural or agricultural environments.

- Under natural conditions chances for successful exchange of genetic information are extremely low and practically limited to closely related species *(B.campestris (syn. = rapa)* and *B. juncea……*The outcrossing frequency of *Brassica napus* to wild relatives has to be considered extremely low while incrossing from weed species to oilseed rape [seems] somewhat more likely to occur, though [is] still very low.

- In managed areas, the genetically modified oilseed rape plants will be part of the normal crop rotation. Since products based on phosphinothricin are not used today for the control of volunteer oilseed rape in subsequent cropping, no particular problems are anticipated.

- The oilseed rape plants carrying the gene coding for tolerance to phosphinothricin will only get a selective advantage where they are treated with herbicides containing the active ingredient. Under all other conditions the transformed plants have no competitive advantage over the untransformed plants in any way.

The 1999 DETR review concluded that research since 1994 supported the original risk assessment that the GM hybrid oilseed rape is not more weedy or invasive than untransformed varieties, and that none of the inserted transgenes appear to increase the spread or persistence of volunteer and feral OSR populations. The review also agreed that there was no evidence to indicate that the transfer of herbicide-tolerance to neighbouring crops by cross-pollination involves a greater risk to the environment than the Advisory Committee on Releases to the Environment (ACRE) anticipated from the data that was available in 1994. The review concluded that the generation of glufosinate tolerant weed populations from *B.rapa* hybrids is possible but unlikely if current agricultural practices are maintained. However the review stated a concern that hybridisation with *B.rapa* and some other wild crucifer species could be occurring at higher rates than was anticipated in the original risk assessment and that gene introgression may occur. While this may not be ecologically significant with herbicide-tolerance genes, other transgenes that confer fitness characteristics could be of greater ecological importance.

This monitoring programme was initially designed to study whether glufosinate-tolerant GM oilseed rape had the potential to have the environmental and agronomic impacts outlined in the PGS risk assessment. Later the programme also included glyphosate tolerance and high lauric acid transgenes. The monitoring

plan incorporated assessments of any obvious effects of these transgenes, or the transgenic rape that were measurable and consistent, and had not previously associated with conventional oilseed rape. The DETR review highlighted the need for more information on hybridisation and gene flow from OSR to *B.rapa* and so studies of this commenced in the second contract period (Objective 5 below).

3. OBJECTIVES

The objectives for each of the two contracts were similar. The objectives for the second contract were:

1. Consider options for the effective and appropriate monitoring of commercial releases of GM crop plants in the UK over a three-year period, and to present a work plan for consideration by the Department.

2. Carry out the monitoring strategy envisaged as above, and evaluate existing monitoring techniques when applied on a large scale.

3. Liaise with specialist HSE inspectors acting under EPA90 warrants with regard to the exercise of their monitoring duties.

4. Report to the Department, and to ACRE on how the observed behaviour of released crop plants equates with expectations.

5. Determine levels of hybridisation between *B.napus* and *B.rapa* and the likelihood of transgene introgression into weedy *B.rapa* populations.

4. MONITORING METHODS

Monitoring methods were refined throughout the duration of the two contracts. Familiarity with the sites was a major component of effective monitoring, which enabled unusual events or changes to be observed and acted upon. The scale of some of the sites and the volume of testing necessary to detect unlikely hybridisation events meant that some may have gone undetected, and there is no guarantee that results shown in this report are definitive. Very rare hybridisation events may not have been detected but were considered less likely to have short-term impacts on plant populations. The monitoring concentrated on major direct short-term impacts, which were likely to have environmental consequences in the short to medium term.

Before the second programme of monitoring commenced in 1998, a detailed review of the techniques used in the monitoring of gene flow was carried out. These were primarily phenotypic techniques used to detect transgene expression in whole plants, plant samples and seeds. This review is reported in Appendix 1.

4.1 Release site surveys

Each site was mapped and records acquired of previous cropping in the release field and neighbouring fields. Oilseed rape fields were recorded within approximately a 500m radius of the release area. Geographical features such as hedges, roads, watercourses etc. were also noted. Where these were considered to be corridors along which dispersal of transgenic material or gene flow could occur, then they were also monitored for distances up to 1km. Familiarity and observation at each site was important

to identify changes in weed or volunteer populations, and to gain information on the population dynamics of both feral and volunteer oilseed rape, and the cruciferous weeds most closely related to oilseed rape.

Consultation with the farmers was important, so that likely cultivation practices could be estimated in relation to each visit. Sometimes it was possible to liaise with the farmer to postpone cultivation for enough time to enable samples to be taken or results to be recorded, although farmers were never encouraged to deviate from their normal cultivation practices.

At sites where releases of GM oilseed rape were taking place, the site was visited from sowing of the crop and at regular intervals subsequently. Detailed notes were made of locations of crucifer populations, existing volunteer and feral populations and any nearby oilseed rape crops. Observations were made on the condition and growth stage of the crop and the presence or absence of weeds in the crop.

GM crops were visited at least once during the flowering period. The locations of related crucifers were detailed, and their synchrony of flowering with the oilseed rape was noted. The flowering times of other oilseed rape crops in the vicinity were compared to the GM crop, as were feral and volunteer plants within a 500m radius of the released GM crop.

After flowering, sites were visited on several occasions in order to monitor the ripening of seedpods and to estimate the most appropriate time to take samples of seed from crucifers and other oilseed rape.

Two or three weeks after harvest of the oilseed rape, each site was visited. Numbers of germinated OSR seedlings were observed prior to cultivation of the field. The areas around the field such as tracks and roadsides within 500m of the trial were observed for germinating rapeseed due to seed dispersal. A 500m radius from the field was examined for the presence of crucifer species known to form hybrids with OSR. Seed samples were collected from these plants if present.

After cultivation of the field and the sowing of the next crop (if the field was sown in the autumn), the site was observed again for all the above. If the field had not yet been treated with herbicides, OSR volunteer numbers were counted or estimated (see 4.7) and either sampled or spot tested for herbicide tolerance (see 4.4). Each site continued to be monitored in this way for intra-specific, inter-specific and temporal gene flow for the duration of the research programme.

Details of each site and the studies conducted at each site are described in 5.0 below.

4.2 Seed sampling

Seed samples were taken at maturity (growth stage 6,7, Oilseed Rape Manual, 1989) from feral, volunteer OSR plants and related crucifers growing within approximately 400 to 500m of the GM rape. Where volunteer OSR plants were growing in a nearby crop, all seed pods were taken from a random selection of plants at set distances from the release site. In the case of feral OSR and related weeds, not all seed pods were taken from individual plants, but a selection of pods from the upper, middle and lower raceme were sampled from all plants observed. This was so that a representative seed sample could be taken, while leaving a range of seeds on the plants in their habitats.

4.3 Sampling methods for herbicide spot testing

Herbicide spot testing for detection of gene flow from transgenic OSR was carried out on volunteer OSR, feral OSR and related cruciferous weeds growing within the sampling zone of the site of a GM OSR

release. Sampling methods were dependent on the site, the population size and distribution of the OSR being sampled. If small numbers of plants were found, all members of the population were tested. Where there were large, dense populations, not every individual could be sampled, so ten plants were tested at regular intervals in the population depending on the size and density.

Some populations were very uneven in their distribution, and where this occurred a sampling pattern was used to cover a representative proportion of the population. This involved testing samples in a 'W' formation across a field.

As a non-destructive phenotypic test for use in the field was not available for detection of glyphosate tolerance or for high lauric acid, the only method for detecting the presence or absence of these particular transgenes was by PCR (4.7 to 4.16). Leaf samples for PCR testing were taken in the same way as described for herbicide spot testing (4.6).

4.4 Herbicide spot testing

1995 to 1998 method

After an initial series of tests using different concentrations of herbicide on a range of oilseed rape and related weeds, an optimum concentration of herbicide was determined for spot testing. A 0.6cm antibiotic assay filter paper disc (Whatman grade AA) was soaked in either a 1%, or in temperatures of <10°C, a 2% solution of glufosinate ammonium (Basta/ Liberty), and placed on one true leaf of each test plant. Tested leaves were identified by a coloured card tag (Fisher Clark strung hardware tickets) placed around the leaf stem. Results were recorded as tolerant or susceptible after approximately one week. Tolerant leaves were not damaged by the herbicide, with only a slight brown superficial discolouration of the leaf whereas susceptible leaves gave a necrotic reaction within 3-7 days.

1999 and 2000 method

In 1999 and 2000, the glufosinate spot testing method was modified by using a pencil with a piece of gauze wrapped around the end, as an applicator for the herbicide. The use of the applicator made the herbicide easier to apply, and plants could be tested more quickly. The rest of the method was as in 1997 and 1998.

Both methods were generally used in temperatures of between approximately 6°C and 25°C. In temperatures below 10°C a 2% solution of glufosinate was used, as the herbicide is less effective in cool temperatures. Also during very hot weather (above 25°C) susceptible leaves were very quickly scorched by the herbicide and many were lost before scoring could take place. Results of the herbicide test were unreliable for mature plants, as often these only had very small upper leaves, which were easily damaged and lost. Flower buds were easily destroyed by contact with the herbicide, so testing flowering plants was avoided where possible. The test was therefore mainly used on small to medium plants during spring and early summer.

4.5 Herbicide testing of seedlings

100 oilseed rape seeds were sown in each plastic half tray (H. Smith Plastics Ltd) containing general potting compost (Shamrock). Glasshouse germination conditions were 16 hours daylight @ 18-22°C, 8 hours night @16°C. Supplementary lighting was provided in banks of six fluorescent tubes (5ft- 65 to 80W and 8ft- 100 to 125W). Positive controls of oilseed rape cultivars known to contain the glufosinate tolerance transgene, or glyphosate tolerance transgene, for glufosinate screening and glyphosate

screening respectively, were sown with each batch of samples. Negative controls were trays sown with a non-transgenic cultivar.

At growth stage 1.2 (Oilseed rape Manual, 1989), the seedlings were sprayed with either glufosinate-ammonium (200g/l) at 0.1gai/m^2 or glyphosate (360g/l) at 0.18gai/m^2, depending on the origin of the samples. A hand sprayer (Hozelock, Polyspray P2) was used to apply the herbicide.

Results were recorded for glufosinate tolerance or glyphosate tolerance after approximately 7 and 14 days respectively. Surviving plants were re-treated to confirm their tolerance and numbers recorded after a further 7 days.

4.6 Polymerase chain reaction (PCR) for transgene identification

Development of PCR

A PCR method was developed by NIAB in collaboration with LGC for detecting transgenes in oilseed rape and other related crucifers. A number of methods for the extraction of DNA from leaf material and seeds were initially evaluated in 1996. The sodium dodecyl sulphate (SDS)/ proteinase K method was found to produce DNA of good quality and high yield. This method was initially used until the Qiagen DNA extraction kit was found to produce DNA of equal quantity and yield. The kit method was less time consuming and less leaf material was needed. This method was used from 1998 to 2000.

A 307 base pair sequence of the *BAR* gene encoding glufosinate herbicide tolerance was amplified with *BAR* primers obtained from PGS, Belgium. Another set of PCR primers for a seed storage gene yielding a 394 base pair product in oilseed rape were used to provide an endogenous internal standard. Both these sets of primers generated different sized PCR products allowing the identification of transgenic and non-transgenic DNA obtained from oilseed rapeseeds and leaf material.

The effect of agrochemicals on the PCR reaction was also tested. Chemical residues from agrochemicals and a variety of natural compounds may cause inhibition of the PCR assay. Plants were sprayed with the agrochemicals carbendazim, iprodione, prochloraz, glyphosate and propyzamide, and then tested by PCR. The chemicals used did not affect the PCR assay.

The detection limit for transgenic oilseed rape DNA was determined in an increasing background of non-transgenic plant DNA for both leaf and seed tissues. The detection of transgenic oilseed rape DNA extracted from leaf discs was possible in a 1:500 ratio. Dilutions above this gave unreliable results.

The PCR assay was applied to field samples of wild *Crucifers.* Preliminary results in 1996 confirmed that this assay could be used to accurately monitor the fate of transgenes in natural populations of wild *Crucifers.*

Sample storage

Samples were taken for molecular testing by polymerase chain reaction (PCR), using sequence specific primers in order to confirm or detect the presence of a particular transgene. Ten young true leaves from individual plants of a species in a particular sampling group were placed together in a self-seal polythene bag (2.25 x 2.25inches) and labelled with a permanent marker pen. Where the plants had previously been tested in the field with glufosinate, these were grouped into tolerant and susceptible batches of ten leaves. Samples were placed in a freezer (-80°C) as soon as possible until DNA could be extracted. When individual leaf samples were required, these were placed in a self-seal polythene bag with a silica

gel desiccant sachet (5g) to remove all moisture. The two storage methods for samples produce DNA of similar quality and yield.

DNA extraction

Leaf tissue was ground to a powder under liquid nitrogen. DNA was extracted from a maximum of 100mg of ground plant tissue. DNA isolation was carried out using a Qiagen DNeasy plant mini kit (all chemicals supplied). The general principle of the kit is after the plant tissue is ground, it is lysed and precipitated. The precipitate is then centrifuged through the filter provided, and ethanol is added. The DNA is then bound to a membrane by centrifugation through a filter, and washed twice. Finally the DNA is eluted to the concentration required.

DNA quantification

Visual estimates were made of DNA quantity. DNA restriction digestion of the lambda bacteriophage with *Hin*d III yields fragments of known size. The fragments were used as a means of estimating genomic DNA concentration. Genomic DNA was electrophoresed alongside aliquots of λ*Hin*d III digested DNA markers on a 1% agarose (Seakem) gel. 2μl loading dye (bromophenol blue and xylene cyanol FF), 7μl sterile H_2O and 1μl DNA were loaded into each lane. Gels were subjected to electrophoresis 100V. Gels were stained with Ethidium Bromide at 1mg/ml and viewed on an UV transaluminator.

PCR amplification

The biotechnology companies responsible for producing the transgenic OSR cultivars supplied the primer sequences used in this research. The primers were synthesised by Sigma-Genosys Ltd., UK. The primers used are listed in Table 1. The sequences are confidential. Primers for a seed storage protein gene (CVZ) were used as an endogenous internal standard for oilseed rape.

Table 4.1: Primers used for detection of the presence of specific transgenes by PCR.

Primer name	Number of Bases	Gene	Trait
BAR	20	BAR	Glufosinate tolerance
PAT	25	PAT	Glufosinate tolerance
C1 & C2	24 & 28	CP4 EPSPS GOX	Glyphosate tolerance
LAUR1 & LAUR2	17	BTE, Uc FAT B1	High lauric acid
CVZ	22	CVZ	Seed storage protein

PCR optimisation

From the beginning of the contract commencing in 1998, the PCR assay was optimised to include new sets of primers for detection of the transgenes encoding glyphosate tolerance (*GLY*), glufosinate tolerance (*BAR* and *PAT*) and high laurate (*BTE, Uc FAT B1*). Optimisation involved finding a combination of temperature profile and DNA concentration that allowed more than one primer set to be used in each reaction batch, therefore making the analyses more economical. A reaction concentration and temperature profile was achieved whereby all primer sets could be used successfully under the same conditions.

PCR cycling conditions

A Primus 96 plus cycler MWG-Biotech was used, and programmed to carry out the following sequence of cycles:

1 cycle: Denature 5 minutes @ 94°C
30 cycles: Denature 45 seconds @ 94°C
Anneal 30 seconds @ 58°C
Extend 2 minutes @ 72°C
1 cycle: Extend 10 minutes @ 72°C

PCR reaction

25μl PCR SuperMix per reaction (Gibco BRL, Cat No. 10572-014) containing:
22mM Tris-HCl (pH 8.4)
55mM KCl
1.65mM $MgCl_2$
220μM dGTP
220μM dATP
220μM dTTP
220μM dCTP
22 U recombinant *Taq* DNA Polymerase/ml

Primer and template added to PCR Supermix

1μl each primer (20pmole/μl, or 50 pmole/μl for detection of BAR)
1μl genomic DNA (500ng)

A positive control consisting of DNA extracted from a transgenic OSR plant with the relevant construct, a negative control of DNA extracted from a non-GM OSR plant and a negative control of water were used in each batch. Where batches of ten leaves were used, one GM leaf and nine non-GM leaves were used as an additional positive control. The CVZ primers were used as a control to confirm that the PCR reaction was functioning correctly.

Visualisation of PCR products

PCR products were visualised on a 1% agarose (Seakem) gel containing 1mg/ml Ethidium Bromide. 2μl loading dye (bromophenol blue and xylene cyanol FF) and 8μl PCR product were loaded into each lane. A 100 base-pair ladder (Pharmacia Biotech Inc.) was loaded into lane 1 as a size marker.

PCR product sizes were: BAR, 307bp; PAT, 378bp; CP4 EPSPS GOX, 1036bp; CVZ, 394bp and BTE, Uc FatB1, 960bp.

The presence on the gel of a fluorescing band of the correct size for the transgene being detected, indicated a positive result for the presence of that transgene in the sample tested. The absence of a fluorescing band of the correct size showed a negative result.

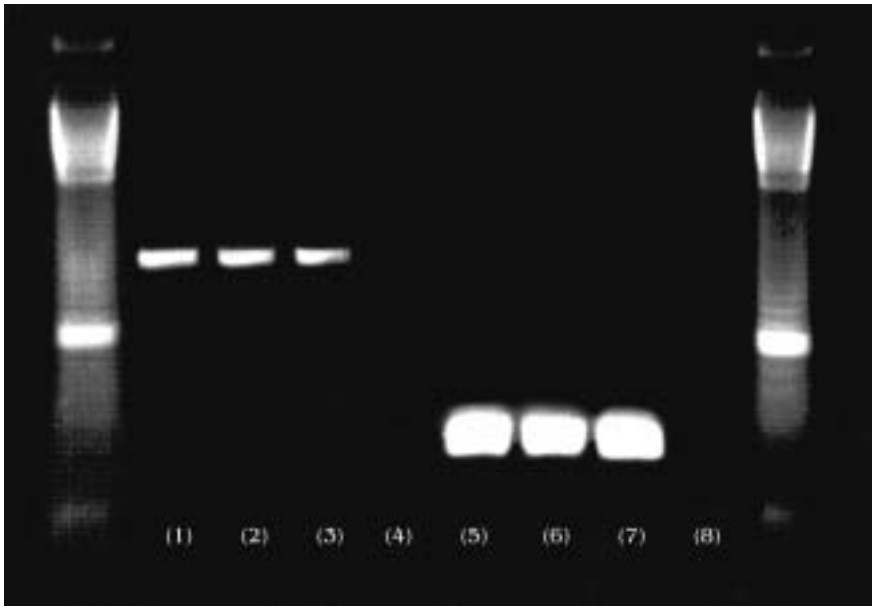


Plate 4.1: Agarose gel picture showing samples from plants expressing tolerance to either glufosinate or glyphosate with 100 base pair ladder.

Lane 1: Sample of oilseed rape expressing glufosinate-tolerance (*Pat* gene - 378bp) from NIAB (1998)

Lane 2: Sample of oilseed rape expressing glufosinate-tolerance (*Pat* gene - 378bp) from EAST WINCH (1998)

Lane 3: Positive control sample from glufosinate tolerant plant (*Pat* gene - 378 bp)

Lane 4: Negative control sample from non-GM oilseed rape plant

Lane 5: Sample expressing glyphosate-tolerance (*CP4 EPSPS* gene - 1036bp) from NIAB (1998)

Lane 6: Sample expressing glyphosate-tolerance (*CP4 EPSPS* gene - 1036bp) from East Winch (1998)

Lane 7: Positive control sample from glyphosate tolerant plant (*CP4 EPSPS* gene - 378 bp)

Lane 8: Negative control sample from non-GM oilseed rape plant

4.7 Estimation of volunteer numbers

Where large numbers of volunteers were present in a field, an estimation of their numbers was made. Estimation of volunteer numbers in a field was carried out by marking and measuring the area within which volunteers were visible. The marked area was then divided into plots of equal size. For each plot, the numbers of oilseed rape volunteers in 20 quadrats of 0.5m2 were counted. The quadrats were taken in a 'W' shape from each plot. Individual plot counts were averaged, and converted to numbers per metre^2 to give an estimate of the total number in the field.

4.8 Seed sampling

4.8.1 *Oilseed rape seed sampling*

Seed samples were taken as described in 4.4 from plants growing within 500m of the GM rape. The frequency of cross-pollination events beyond this distance is thought to be extremely low and large sample sizes would be required to detect out-crossing at greater distances. By focusing on a smaller area, more samples could be taken with a higher probability of detection of any out-crossing.

Where volunteer OSR plants were growing in a nearby crop, all seedpods were taken from a selection of plants. In the case of feral OSR, not all seedpods were taken from individual plants, but a selection of pods from the upper, middle and lower plant were sampled from all plants found. This was so that a representative seed sample could be taken, and also to preserve a good range of remaining seeds on the plants in their natural habitat for subsequent generations.

4.8.2 *Seed sampling from wild relatives*

Seed samples were taken at maturity from wild relatives of OSR growing within approximately 400m of the GM rape. Occasionally seeds had to be taken slightly before maturity if they were amongst an OSR that was going to be combined. Maturity was determined by the colour of the seeds. Samples were taken when the seeds were dark brown or black (depending on the species) and easily removed from their pods.

The sampling method was dependent on the spatial arrangement of the weed population being sampled. Care was taken not to deplete the number of seeds returning to the seed bank, so if populations were small, numbers of seeds sampled had to be low. If the plants were growing amongst an oilseed rape crop, all seeds were sampled, as they would mostly have been lost in the combine harvester under normal agricultural practices. In populations where there were a large number of plants, more seed was taken from many plants, but none of the individual plants had all their seeds removed, so that the natural balance of seed return to the soil seed bank was disturbed as little as possible.

4.9 Herbicide screening of seedlings

4.9.1 *Oilseed rape screening: for studies of gene flow to adjacent oilseed rape*

Oilseed rape seeds (100 per tray) were sown in plastic half trays (H. Smith Plastics Ltd) containing general potting compost (Shamrock). Glasshouse germination conditions were 16 hours daylight at 18-22°C, 8 hours night at 16°C. Supplementary lighting was provided in banks of six fluorescent tubes (5ft- 65 to 80W and 8ft- 100 to 125W). Positive controls of cultivars known to contain the glufosinate-tolerance transgene or glyphosate-tolerance transgene, for glufosinate screening and glyphosate screening respectively, were sown with each batch of samples. Negative controls were trays sown with a non-transgenic cultivar.

At growth stage 1.2 (Oilseed Rape Manual, 1989) seedlings were sprayed with either glufosinate-ammonium (200g/l) at 0.1gai/m^2 or glyphosate (360g/l) at 0.18gai/m^2, depending on

the origin of the samples. A hand sprayer (Hozelock, Polyspray P2) was used to apply the herbicide.

Results were recorded for glufosinate tolerance or glyphosate tolerance after approximately 7 and 14 days respectively. Surviving plants were re-treated to confirm their tolerance and numbers recorded after a further 7 days.

4.9.2 *Screening for herbicide tolerance in wild Crucifers*

Seeds from crucifer species were germinated on petri dishes in a 100 p.p.m. solution of gibberellic acid (Sigma) to break dormancy. This was very successful and gave over 90% germination of seeds in most cases. Shortly after germination, seedlings were transplanted into half trays containing general potting compost (Shamrock) in batches of 10. Seedlings were treated with the relevant herbicide and recorded as in 2.7.1.

4.10. Ploidy level testing by flow cytometry (to test for interspecific hybridisation in Brassica species)

Samples from the Humberside site were tested for ploidy level by Plant Cytometry Services, The Netherlands according to methods by Brown *et al.,* (1991). Proliferating cells pass through cell cycle components referred to as G1 (Gap 1), S (DNA Synthesis), G2 (Gap 2) and M (Mitosis). The frequency of each cell type can be calculated from the histogram of nuclear DNA levels, assuming that all cells are in proliferation. Cell nuclei are stained and passed through a cytometer, which produces a histogram of fluorescence intensities. Most plant cells differentiate mainly in the G1 state and histograms of DNA levels in developed tissue will be dominated by a Gaussian distribution whose mode reflects its ploidy level. Samples were initially prepared by placing a fresh leaf in a labelled self-seal polythene bag, and sending by courier in order to maintain the freshness of the plant material. Control leaves from *B.rapa* and *B.napus* were sent with each batch of samples.

4.11 Amplified fragment length polymorphism (AFLP) testing of *B.rapa* populations

4.11.1 *Digestion and ligation of adapters*

The restriction enzymes PstI and MseI were used for digestion of the genomic DNA. DNA was digested in a total volume of 50μl including: 5.0μl of reaction buffer (One Phor All Buffer, Pharmacia), 2.5μg DNA 12.5 units of restriction enzyme PstI, 12.5 units of restriction enzyme MseI and water to volume. The mixture was then vortexed, spun down and incubated for 2 hours 15 minutes at 37°C. While the DNA was digesting, the MseI and PstI adapters (synthesised by Bioline) were prepared to a concentration of 50pmoles/μl. The adapters have the following sequences:

PstI adapter

5'-Biotin-CTCGTAGACTGCGTACATGCA-3', 3'-CATCTGACGCATGT-5';

MseI adapter

5'-GACGATGAGTCCTGAG-3', 3'-TACTCAGGACTCAT-5'.

For the ligation of the adapter molecules a 10μl aliquot of a mixture containing:

5pmoles of PstI adapter, 50pmoles MseI adapter, 1.2μl 10mM ATP solution, 1μl reaction buffer (One Phor All buffer), 1unit of T4-ligase and H_2O up to 10μl was prepared. The total reaction volume was 60μl. This mixture was incubated for 3 hours at 37°C. At this stage, the mixture contained fragments with MseI restriction sites on both ends, fragments with PstI sites on both ends and those with a PstI site and a MseI site.

4.11.2 *Selection of biotinylated DNA-fragments*

Pre-washed Dynabeads (streptavidin) M-280 were used to select only those fragments of DNA with a PstI end. The beads were thoroughly washed in TE buffer before being added to the ligated DNA. The beads were separated from the sodium azide in which they are stored by using a magnet. The beads were then resuspended in the original volume of TE .

After the sodium azide was removed, 10μl of suspended beads was added to each DNA sample and left at room temperature for 30 minutes. The supernatant was then removed by holding the Eppendorf on a magnet, which attracts the beads to the side of the tube and separates them from the remainder of the liquid. The beads were then resuspended using 100μl of TE. This procedure was repeated 4 times, after which the DNA fragments attached to the beads were finally suspended in 100μl, and stored at -20°C.

4.11.3 *Radioactive labelling of the PCR- primer*

Primers were used at a concentration of 100ng/μl. The following volumes were used per DNA sample to be amplified: 30ng Mse primer, 0.1μl reaction buffer, 0.1μl [g- ^{33}P] ATP, 0.05μl T4 Kinase, and H_2O to give a total volume of 1μl. The mixture was then incubated at 37° C for 1 hour and then at 68°C for 20 minutes to stop the reaction.

4.11.4 *PCR*

PCR was carried out in a 20μl volume using the following reaction mixture: 1μl of bead-DNA template, 2μl 10´ PCR buffer (200mM Tris, 5mM KCL, 15mMMg Cl2, pH 8.4), 30 ng ^{33}P-labelled Mse primer, 30 ng of Pst primer, 1U (0.1μl) Taq DNA polymerase (BRC), 2μl 2mM dNTP solution and 13.6ml H_2O.

PCR amplification was carried out as follows: 12 cycles: denaturing 94°C for 30sec, annealing (lower annealing temperature by 0.7C each cycle)
65°C, 64.3°C, 53.6°C, 62.2°C, 61.5°C, 60.8°C, 60.1°C, 59.4°C, 58,7°C,
58,0°C, 57.3°C for 30 seconds
Extension 72°C from 60 seconds
23 cycles: denaturing 94°C for 30 seconds
Annealing 56°C for 30 sec
Extension 72°C for 60 sec
Final extension for 30 min at 65°C.

4.11.5 *Polyacrylamide gel electrophoresis (PAGE)*

Glass plates were washed and cleaned thoroughly with ethanol. The mould is carefully constructed by placing one glass plate on top of the other with 0.03mm spacers between them. The gel solution consisted of: 75 ml of sequencing gel solution; 70μl TEMED and 370μl 10 %

APS (ammonium persulphate). The solution was carefully poured between the glass plates. The gel was left for 45 minutes to polymerise. The bottom spacer was removed and 1xTBE buffer was injected into the space. The gel was then pre-run for 30 minutes at 65 watts before loading the samples. A comb was inserted to form the wells.

The samples were prepared by adding 10μl stop buffer (contains dye and formamide), and denaturing them for 4 minutes at 94°C, and holding them on ice while loading. 4μl was loaded into each well. The gel was run at constant power of 58 watts for approximately 2 hours. The glass plates were carefully separated and the gel transferred to 3mm Whatman paper, and dried for 40 minutes. The gel was then exposed to Kodak Biomax film. Film was developed manually after 1 to 4 days of exposure.

4.11.6 *Scoring and graphical presentation of AFLP results*

Gels were scored by eye for the presence or absence of bands and recorded where a 1 denotes the presence of a band and a zero its absence. Up to 100 bands were scored on each gel. Results were then analysed using STATISTICA for Windows (StatSoft, Inc., 1999) and principal co-ordinate (PCO) plots produced for each gel. The aim of a PCO plot is to create a two-dimensional plot of the samples, which represents as closely as possible the original distances or similarities amongst the samples.

5 GM CROP RELEASE SITE DETAILS

In the initial 3-year contract 4 sites were monitored, as these were the first larger scale releases to be grown in the UK. These were monitored from the first year of their release up to 2000. Monitoring visits were less frequent in the years following the crop after the GM release. New sites continued to be added to the monitoring programme during the second 3-year contract. By the end of 2000, 11 sites were being monitored, the newer sites more frequently than the older ones.

5.1 Berkhamstead 1998: Monitored 1998 to 2000

Primarily a cereal and grass farm. 38ha of high laurate (Uc FatB1) GM spring rape comprising 5 fields in close proximity or adjacent to each other was grown at this site in 1998. No other oilseed rape crops were grown in the surrounding fields. This was the largest release of transgenic oilseed rape in the UK and provided a basis for testing gene flow from a large source of oilseed rape pollen. In 1999 and 2000 no GM rape was grown in this area and cereals were grown in the release site fields (Table 5.1).

Table 5.1. The cropping regime at Berkhamstead, Hertfordshire from 1998-2000

1998	1999	2000
GM spring OSR	Spring wheat	Winter wheat

5.2 East Devon 1995: Monitored 1995 to 2000

This farm was a mixed arable and livestock farm with a shale/ brash soil. Crops grown on the farm were cereals, linseed, peas, forage rape (*Brassica napus*) and stubble turnips (*B.rapa*). The release field was a south facing slope with established hedges and a road bank. A disused quarry and gravel pit bordered the site. OSR had not been grown in the release site field.

The 1ha of a glufosinate tolerant (*BAR*) Canadian hybrid spring OSR seed crop was drilled in late April 1995, and re-drilled a month later because of severe flea beetle attack. There was a ratio of 1 pollinator: 3 male sterile plants. After the first application of glufosinate-ammonium, approximately 50% of the male sterile plants were killed because they did not possess the *BAR* gene. The crop was harvested with a conventional combine harvester, and harvested yields for this crop were very low (approximately 300kg/ha) due to the late drilling and the low plant density.
The cropping regime for the Devon site is shown in Table 5.2

Table 5.2. The cropping regime at Devon from 1995-2000

1995	1996	1997	1998	1999	2000
GM spring OSR	Split field of winter barley and winter wheat	Winter barley	Forage rape	Peas	Winter wheat

5.3 East Winch: GM crops from 1997; Monitored to 1999.

This was an experimental farm growing small area rotations for the demonstration of the efficacy of herbicides and other agrochemicals. The monitoring area consisted of 6 fields used for trials of all types of crops such as cereals, oilseed rape, potatoes, linseed and sugar beet. Trials of GM winter and spring oilseed rape ranging in size between less than 1 hectare and up to 2 hectares (1998/99 trial) had been grown over the last few years on these fields. Each trial contained glufosinate tolerant varieties (initially *PAT* and later *BAR* gene) grown for agronomy, seed rate, fertiliser and herbicide application timing purposes. In 1997/98 the farm also grew a trial comprising three small blocks of GM winter oilseed rape (30 x 40 m each), containing both glufosinate and glyphosate tolerant varieties, to study multiple herbicide tolerance. Because of this history of culturing GM OSR the whole farm was established as a monitoring area. The experimental farm was closed and sold, and monitoring ceased at the end of 1999.

5.4 Fakenham: GM Crops in 1997 and 1998: Monitored to 2000.

The 1997 release site was a 11ha area of high laurate spring OSR on a farm growing principally cereals and sugar beet. No other spring oilseed rape crops grew nearby. The 1998 release was 12ha of high laurate spring rape on another field on this farm. The two fields were approximately two miles apart and there was also no spring OSR grown in the area. OSR had not been grown in either of these fields previously. There were no other GM oilseed rape releases on this farm in 1999 and 2000. Cropping for Fakenham is shown in Table 5.3.

Table 5.3. The cropping regime at Fakenham, Norfolk from 1997-2000

	1997	1998	1999	2000
Field 1	GM spring OSR	Winter wheat	Winter barley	Spring OSR
Field 2	Winter cereal	GM spring OSR	Winter wheat	Winter wheat

5.5 Lincolnshire: GM crops in 1995 and 1996: Monitored to 2000.

This farm predominantly grows cereals, pulses and sugar beet. In late April 1995 a 1.38ha seed crop of a Canadian hybrid spring glufosinate tolerant (*BAR*) OSR was drilled into a dry seedbed on a level site on medium loam soil. There was a ratio of 1 pollinator: 3 male sterile plants. A turnip seed crop had been grown in an adjacent field to the GM release in the previous year and some shed seed regrew in 1995.

Germination was slow and uneven due to the dry conditions, and therefore establishment was thin. The crop flowered at the end of June and the pollinator was destroyed in late July. Pollen beetle attack was

severe and several sprays were applied to control this. The crop was harvested in mid September and yielded a total of 380kg.

The 1996 site on this farm was two fields away (approximately 700m) from the 1995 field with a similar soil composition. The release area was part of a large field previously cropped with sugar beet. 1.25ha was drilled with a seed crop of the glufosinate tolerant (*BAR*) spring oilseed rape variety PHY 22 in mid April into a moist seedbed at a ratio of 1 pollinator: 3 male sterile female parents. The crop established well and good ground cover was achieved quickly. The crop flowered in mid to late June and the pollinator was destroyed towards the end of July after flowering. The crop was harvested in mid September giving a yield of approximately 1000kg. Cropping for the two release sites at Lincs is shown in Table 5.4.

Table 5.4. The cropping regime at Lincs from 1996-2000

	1996	1997	1998	1999	2000
Field 1. GM spring oilseed rape seed crop in 1995	Sugar beet	Peas	Winter wheat	Winter wheat	Trees
Field 2	GM spring oilseed rape seed crop	Winter wheat	Winter barley	Winter OSR	Winter wheat

5.6 Melbourn: GM cropping from 1995 to 2000: Monitored to 2000

This site was a farm which grew spring and winter GMHT oilseed rape trials from 1995 –2000. The major crops grown on this arable farm were winter wheat, winter and spring barley, along with linseed and other break crops, on a light, chalky soil. The monitoring area at this site encompassed all of the fields in which GM rape has been grown, and any adjacent fields, which may have been affected by gene flow from the transgenic rape. Thus the monitored area was a substantial part of this farm that had a total of eight glufosinate tolerant winter and spring rape trials in fields situated in close proximity to each other since 1995. The size of the trials ranged from 3ha to 15ha. The trials were made up of a mixture of parental lines of both GM glufosinate-tolerant (*BAR),* non-GM lines and lines of male sterile plants and pollinator plants in varying proportions. The farm had not previously grown OSR in any of the fields used for the trials. This site was particularly useful for evaluating the effects of different cultivation and environmental conditions on subsequent volunteer incidence. Table 5.5 shows the crops grown in each of the fields where GM oilseed rape was grown.

Table 5.5. The cropping regime at Melbourn, Cambridgeshire from 1996-2000

	1995/6	1996/7	1997/8	1998/9	1999/2000
Field 1	GM winter OSR trial	Winter wheat	Winter barley	Winter barley	Peas
Field 2	Winter wheat	GM winter OSR trial	Winter wheat	Winter wheat	Winter wheat
Field 3	Winter wheat	GM spring OSR trial	Winter wheat	Set-aside and linseed	Winter wheat
Field 4	Winter barley	Winter wheat	GM OSR winter and spring trials	Winter wheat	Winter oats
Field 5	*	*	winter cereal	GM OSR winter and spring trials	Winter wheat
Field 6	*	*	Winter cereal	Winter cereal	GM OSR winter and spring trials

* Information unavailable

5.7 NIAB Headquarters (HQ) Cambridge: Monitored 1997 to 2000

The NIAB HQ trials ground has grown both winter and spring GM oilseed rape in demonstration trials and variety testing trials containing glufosinate and glyphosate tolerant varieties, for consecutive years from 1997. This included trials of approximately 5.5 ha (of which 4 ha was GM rape) and 2.0 ha (of which 1.4 ha was GM rape) drilled at this site in autumn 1998 for the project studying the Botanical and Rotational Implications of GM Herbicide Tolerance (BRIGHT). These trials included glyphosate tolerant, Imidazolinone tolerant (non-GM) varieties as well as F_1 hybrid varieties of glufosinate tolerant (*BAR*) oilseed rape. Due to the extensive trialling of GM varieties, the whole area of the NIAB trial ground was monitored, and several adjacent fields. The cropping details of the principal fields growing GM crop trials are shown in Table 5.6.

Table 5.6. The cropping regime at NIAB, Cambridgeshire from 1996-2000

	1996	1997	1998	1999	2000
Field 1	Winter wheat and winter beans	Winter and spring linseed	Winter wheat	GM winter OSR trial	Winter wheat
Field 2	Spring barley and cereal demonstration plots	Winter wheat and spring barley	Winter beans	GM maize trial	GM winter OSR trial
Field 3	Winter wheat	Winter beans	Winter wheat	GM winter OSR trial	Winter wheat
Field 4	Winter wheat	Winter wheat	Winter OSR trial	GM winter and spring OSR trials	Winter wheat and spring linseed
Field 5	Winter wheat	GM winter and spring OSR trials	Winter wheat	Winter beans	Winter wheat and triticale

5.8 Yorkshire 1995: Monitored 1995 to 2000

The site was part of a farm growing predominantly cereals, sugar beet and potatoes though some spring rape had been grown in the previous year in neighbouring fields. The release field was a gentle south-facing slope on a fine sandy loam soil.

The 3ha GM seed crop of the glufosinate tolerant (*BAR*) spring oilseed rape variety PHY 7 was drilled in mid April 1995 in a 5:1 ratio of male sterile to pollinator plants. Establishment of the crop was initially patchy. The crop flowered in mid-June and pollination was enhanced by the placing approximately a dozen beehives in the field. The crop was harvested by direct combining at the end of August giving a total field yield of 3500kg. Subsequent cropping at this site is shown in Table 5.7.

Table 5.7. The cropping regime at Yorks from 1996-2000

1996	1997	1998	1999	2000
Winter wheat	Winter barley	Winter barley	Winter OSR	Winter wheat

5.9 Humberside (experimental site in 1999 - 2000)

This site was on a farm that predominantly grew rotations of cereals with oilseed rape. Oilseed rape had been grown previously at the release site and historical records had been kept of all varieties grown in the field for the last decade. This was the only site where *B.rapa* was found growing as a weed in the field. It has been observed as a weed problem in this area for over 30 years and recorded growing in this field for at least 10 years. This site was used specifically for studying hybridisation and introgression between weedy *B.rapa* and oilseed rape. The *B.rapa* plants at the site grow in the cropped field and in field margins but were not found outside the cultivated area. The plants have morphology similar to turnip rape.

AgrEvo (now Aventis) drilled a trial area of glufosinate-ammonium tolerant *(BAR* construct) transgenic winter oilseed rape in autumn 1998 in an area of the field where *B.rapa* was known to be particularly abundant. The purpose of their trial was to examine the control of weedy *B.rapa* with glufosinate used as a selective herbicide in GM oilseed rape, since traditional herbicides give poor control under normal conditions. The opportunity was also taken to study interactions between oilseed rape and weedy *B.rapa* under agricultural conditions. The incidence of hybridisation and possible introgression between the weedy *B.rapa* and the individual non-GM varieties grown in the field in the last ten years was investigated by using the AFLP technique. The possibility of identifying bands specific to individual oilseed rape varieties that appear in *B.rapa* if introgression has taken place, was investigated. This technique may give an indication of the levels of gene introgression from *B rapa* to *B napus* and the likelihood of the long-term establishment of oilseed rape genes in weedy *B.rapa* populations.

5.10 Farm Scale Evaluation Sites

In 1999 DETR commenced the Farm Scale Evaluation (FSE) of GM crops to determine the environmental impact of the GM crops and the herbicides used on them. The study involved split field designs whereby the field is divided into two areas, one containing a conventional rape cultivar, and the other a GM oilseed rape cultivar. NIAB was requested to monitor two release sites in 1999 and to specifically measure gene flow between the GM and non-GM rape crops.

Farm Scale Site 1: Boothby Graffoe

This Lincolnshire site was sown on 28th April 1999 with 9.0ha of non-GM Hyola 401 and 8.2ha of the transgenic glufosinate tolerant variety PGS96-452. At this site the GM variety started flowering two to three days later than the Hyola 401.

Farm Scale Site 2: Watlington

This Oxfordshire site was also a split field design sown on 29th April 1999 with 10ha of Hyola 401 as the conventional crop, and 10ha of the transgenic glufosinate tolerant variety PGS96 – 452. The GM and conventional crops had a similar rate of development and the GM variety started to flower between two and three days later than the Hyola 401.

5.11 Site surveys and sampling

Table 5.8. Dates of site visits in 1995

Site	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Devon		✓		✓	✓		✓			✓
Lincs 1		✓		✓	✓	✓	✓	✓	✓	

Table 5.9. Dates of site visits in 1996

Site	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Devon		✓			✓			✓		
Lincs 1 and 2	✓		✓	✓		✓	✓	✓	✓	
Yorks	✓		✓	✓	✓	✓	✓		✓	
Melbourn	✓		✓	✓	✓	✓	✓	✓	✓	

Table 5.10. Dates of site visits in 1997

Site	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Devon		✓		✓	✓		✓			
Lincs 1 and 2		✓	✓	✓	✓	✓	✓			
Yorks		✓	✓	✓	✓	✓	✓			
Melbourn		✓	✓	✓	✓	✓	✓			

Table 5.11. Dates of site visits in 1998

Site	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Devon									✓	
Lincs 1 and 2	✓									
Yorks	✓									
East Winch	✓	✓	✓	✓	✓	✓	✓			
Fakenham				✓		✓				
Melbourn		✓	✓	✓	✓	✓	✓		✓	
NIAB	✓	✓	✓	✓	✓	✓	✓			
Berkhamstead					✓	✓				

Table 5.12. Dates of site visits in 1999

Site	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Devon			✓				✓			
Lincs 1 and 2		✓								
Yorks		✓								
East Winch		✓	✓		✓					
Fakenham		✓								
Melbourn		✓	✓	✓	✓	✓			✓	
NIAB	✓	✓	✓	✓	✓		✓			
Berkhamstead			✓							
FSE Sites*			✓		✓					

Table 5.13. Dates of site visits in 2000

Site	Mar	Apr	May	Jun	Jul
Devon			✓		
Lincs 1 and 2					✓
Yorks			✓		
East Winch #					
Fakenham				✓	
Melbourn			✓	✓	
NIAB		✓			
Berkhamstead	✓				
FSE sites *				✓	✓

Site closed in late 1999
* Sites primarily examined for hybridisation between GM and non-GM component of the trial and subsequent rape volunteers

6 GENE FLOW BETWEEN ADJACENT OILSEED RAPE CROPS

INTRODUCTION

Oilseed rape is predominantly self-pollinating, with about one third of seed produced by outcrossing. The outcrossing rate is greatly influenced by environmental factors and can vary between 12% and 47% (Becker *et al.,* 1992). The environmental influences include geographical location, weather conditions at the time of flowering, and within-plant position of the flowers. Among flowers at different positions on the same plant, outcrossing varies from 11% at the top of the inflorescence to 39% at the bottom. Outcrossing in the experiments by Becker *et. al.,* (1992) was estimated in plants grown in plots at a density normally found in an oilseed rape crop, in five geographical locations, by comparing isozyme patterns of mother plants with their progeny.

Oilseed rape is both wind and insect pollinated. Oilseed rape flowers contain nectaries, which make them attractive to honey bees (Free and Nutall, 1968), although good seed yields can still be achieved without insect pollination. Oilseed rape pollen grains are roughly spherical with a geometric diameter of about 25 μm (McCartney & Lacey, 1990). Fungal spores are of a similar size and are primarily dispersed by wind. A mathematical model from pollen trap data, produced by McCartney & Lacey (1990) predicted that more than 60% of pollen lost from an oilseed rape crop would still be airborne 100m downwind of the crop. The concentration at the ground at this distance, however, would only be 2 to 10% of the value at the edge of the crop. The conclusion from this work was that, although large amounts of pollen are released into the air from flowering oilseed rape crops, it seems unlikely that windborne pollen could play a significant role in cross-pollination at distances greater than a few tens of metres from that crop.

Plate 6.1: An oilseed rape flowerhead showing the four sepals, four petals and six stamens.

Although most wind-pollinated species are characterised by their smooth dry pollen grains, oilseed rape has sticky pollen which remains adherent to the anthers even under high wind velocities (Eisikowitch, 1980). Insects visit all stages of flowers, touching the anthers, which leads to pollination. It is thought that insects disturb pollen grains on dry days resulting in dispersal by wind. From Eisikowitch's work it appears that only the pollen grains initially released by insects can be
dispersed by wind. Pollination may also occur however by physical contact between neighbouring plants, and this may account for much of the local seed set occurring in the absence of insects.

Although these experiments have suggested that the majority of pollen from a source is likely to fall on the nearest plants, there are very rarely some long distance pollinations and therefore always a small risk of transgene movement outside isolation distances (Ellstrand & Hoffman, 1990).

The measured rates of hybridisation at distances from a pollen source in oilseed rape vary between different experiments. Part of this variation may be explained by the dual pollination modes of insects and wind. However, there are many other factors that can affect hybridisation rates, such as the shape and size of the source and sink, the density of the plants, flowering synchrony, weather conditions, environmental factors such as aspect and exposure, and the genotypes of the source and the sink. Lavigne *et. al.* (1997) measured the average pollen dispersal from single transgenic oilseed rape plants within a large plot. Results from this work suggest that approximately half the pollen from an individual plant falls within three metres, and that the probability of fertilisation decreases exponentially with increasing distance from that single plant. Lavigne *et. al.* (1997) also predicted that, as a result of insect movements among neighbouring plants and weak winds, a major proportion of pollen would fall around

the source plant and the rest would be distributed over the field as a result of long-distance insect flights or strong winds and turbulence.

Both honeybees (*Apis mellifera*) and bumblebees (*Bombus spp.*) play an important role in cross-pollination of oilseed rape. Ramsay *et. al.* (1999) made observations of honeybee colonies and found that bees may forage up to 2km in all directions from a hive, and when returning to the hive they carry large numbers of loose grains. If these are then picked up by other bees, then this can mean that some pollen transfer and fertilisation could occur up to 4km away from the pollen source by bee transport.

Timmons *et. al.* (1995) measured airborne pollen levels along a linear transect up to 2.5km from fields of oilseed rape. Measurements were taken using volumetric spore traps. Although wide day-to-day fluctuations in airborne pollen densities were found, pollen levels generally declined with distance and pollen did not remain airborne for significant periods of time. Low pollen densities were consistently recorded at 1.5-2.5km from the source, representing background levels (Timmons *et. al.* 1995).

The use of male-sterile bait plants has also shown how far pollen moves either by wind or by insects. Timmons *et. al.* (1995), in a further experiment detecting pollen at long distances, used emasculated and de-petalled oilseed rape plants and placed them in fields at various distances from an oilseed rape field. Seed set on plants 2km away from the source showed that pollen is able to move considerable distances. The experiment could not determine the method of pollen movement, although the removal of petals may have reduced the attractiveness of the plants to pollinating insects in this case. Timmons *et. al.* (1995) concluded that insect pollination cannot be entirely ruled out, but wind is likely to be the major component of pollen movement in this case. Although precise data does not exist on the amount of pollination that occurs through insects or wind, it is clear that both modes of pollination are important, and that insects may have a key role in long distance pollination events.

The various experiments that have examined gene flow from fields of oilseed rape undertaken in the UK have mainly concentrated on small blocks of transgenic oilseed rape as the pollen source. The use of herbicide-tolerance, in particular glufosinate-ammonium as a marker system, means that these transgenes can easily be detected in conventional rape cultivars by phenotypic and molecular methods.

Although experiments such as the one by Lavigne *et. al.* (1997) have suggested that the majority of pollen from a source is likely to fall on the nearest plants, long distance pollinations have been reported so that there is always a small risk of transgene movement outside set isolation distances (Ellstrand & Hoffman, 1990).

Detection of herbicide tolerance in seed of male sterile oilseed rape plants at distances of up to 400m from a transgenic pollen source (Simpson *et. al.*, 1999) show that there is potential for oilseed rape pollen to be dispersed by wind and remain viable over considerable distances. However over 1000 seeds from a population of feral rape plants flowering at the same time, and growing within 150m of the same transgenic pollen source were tested and no herbicide-tolerant plants were detected. This suggests that pollen competition is an important factor to consider when studying normally self-fertile oilseed rape.

In this study gene flow was monitored between GM OSR and adjacent crops, GM OSR and feral rape and between GM OSR and OSR volunteers in neighbouring fields. During the first 3 years of the contract (1994 to 1997) the monitoring of intra-specific gene flow was limited to seed production trials of spring GM OSR of between 1ha and just over 7ha. The trials contained between 1/3 and 1/5 male fertile pollinators and produced low seed yields. The fields were isolated from other rape crops by a minimum of 1km and the nearest crops were generally winter rapes with little synchrony of flowering. Harvested seed was bagged in the field and removed to limit seed dispersal. However all farms failed to clean their

harvesters after harvest and some seed was dispersed in this way. The trials monitored during this period gave very limited data on gene flow and gene dispersal to other rape crops and fields.

However during the period 1997 to 2000 more fully fertile GM spring and winter GM OSR was grown and this provided some of the first real opportunities in the UK for measuring outcrossing from transgenic OSR into adjacent fields of commercial OSR.

The introduction of the BRIGHT trials and FSE trials in 1998/99 meant that for the first time cross-pollination between large blocks of OSR could be assessed directly by phenotypic methods.

6.1 Farm Scale Evaluation Experiment: Oxfordshire and Lincolnshire

During the period 1999 to 2000, genetically modified (GM) spring and winter GM oilseed rape was grown in fields adjacent to 'commercial' oilseed rape crops for the first time. This provided some of the first real opportunities in the UK for measuring gene flow from a transgenic oilseed rape into adjacent fields of oilseed rape. The introduction of these Farm Scale Evaluation (FSE) trials meant that for the first time gene flow between large blocks of oilseed rape could be assessed directly by phenotypic methods. In 1999 two spring oilseed rape crops were grown for the purpose of evaluating GM oilseed rape and these were used to assess the gene flow between two large adjacent blocks of oilseed rape.

6.1.1 The sites

The sites were located at Watlington, Oxfordshire and Boothby Graffoe, Lincolnshire. Both sites were of a split field design whereby the field was divided into two areas, one of a conventional oilseed rape cultivar, and one of a GM oilseed rape cultivar. The transgenic variety was a hybrid produced from two transgenic lines; a female, male-sterile parent (containing a gene coding for the ribonuclease inhibitor protein barnase) hemizygous for glufosinate-tolerance (BAR gene) and a male pollinator line homozygous for glufosinate-tolerance and containing a fertility restorer gene (*barstar*). If the hemizygous female line has genotype H1- - - and the homozygous male has genotype - - H2 H2, then the F_1 will have two forms in a 1:1 ratio, H1- H2- and - - H2 -. Both are resistant to glufosinate and have gametes as follows: one F_1 form, H1 – H2 –, produces gametes which are tolerant or susceptible in a 3:1 ratio: H1 H2 (25%), H1 – (25%), -H2 (25%) and - - (25%). The other F_1 form, - - H2 -, produces gametes which are tolerant or susceptible in a 1:1 ratio: - H2 (50%) and - - (50%). Combining the ratios from the two F_1 forms gives an overall ratio of 5:3 tolerant to susceptible pollen produced by the F_1 hybrid crop (G. Ramsay, pers. comm). Therefore only 5/8 of the pollen rain produced from the crop will contain the herbicide tolerance gene.

i) The Lincolnshire site was sown on 28th April 1999 with 9.0ha of Hyola 401 on the conventional side of the field and 8.2ha of the transgenic glufosinate tolerant variety PGS96-452 on the GM side (Figure 6.1). At this site also the GM variety started flowering two to three days later than the Hyola 401.

ii) The Oxfordshire site was sown on 29th April 1999 with 10ha of Hyola 401 as the conventional crop, and 10ha of the transgenic glufosinate tolerant variety, PGS96 – 452 (Figure 6.2). The GM and conventional crops had a similar rate of development with the GM variety starting to flower between two and three days later than the Hyola 401.

The conventional rape and the GM rape were sown immediately adjacent to each other and were distinguishable by a slight difference in height and colour at the Oxford site (Plate 6.3). At Lincolnshire they were separated by a 5m gap of fallow (Plate 6.2).

No other spring oilseed rape was grown within 10km of either site. Winter oilseed rape crops were grown near to both sites. However there was no overlap of flowering between the spring rape and the winter rape.

6.1.2 Field sampling

Seed samples to test for gene flow were taken from the conventional variety. At the Oxfordshire site, three transects were taken across the conventional side of the trial perpendicular to the boundary line (Figure 6.1). Transect 1 was taken at 20m from the left-hand side of the conventional plot to avoid the influence of edge effects. Transect 2 was taken at the centre point of the conventional plot (205m from the edge), and transect 3 was taken at 20m from the right hand side of the conventional plot. Sampling points were measured from the boundary of the GM crop into the conventional rape at the following distances: 5m, 10m, 15m, 20m, 25m, 30m, 35m, 40m, 45m, 50m, 60m, 100m, 125m, 150m and 200m. Transect 1 only had sampling points up to 100m, as the field was shorter at this side.

Three transects were also taken at the Lincolnshire site (Figure 6.2). Transect 1 was taken at 20m from the right hand side of the conventional crop, transect 2 at 150m into the centre of the crop and transect 3 at 20m from the left hand side of the crop. Sampling points were measured from the boundary at distances away from the conventional rape at the following distances: 5m, 10m, 15m, 20m, 25m, 30m, 40m, 50m, 60m, 75m, 100m, 125m, 150m, 200m, 250m.

Seed samples were taken at growth stage 6.7, most seeds black but soft (Oilseed Rape Manual, 1989) on 15th and 16th August 1999 from Oxfordshire and Lincolnshire respectively. At each sampling point a 1m quadrat was placed over the oilseed rape, and 20 main racemes from within the quadrat cut and placed in a labelled cloth bag (Plate 6.1). Samples were left to dry for two weeks in the glasshouse, and seeds removed and thoroughly mixed by hand.

6.1.3 Screening of seed samples for herbicide tolerance

A total of 2000 seeds per sampling point were tested as in 4.5. Positive controls of a glufosinate-tolerant variety were sown with each batch of samples. The non-transgenic variety Apex was used as a negative control.

Plate 6.1: An example of a main raceme sample taken from a commercial crop of Apex for testing for glufosinate- tolerance
Foreground: a single main raceme sample of winter oilseed rape.
Background: complete sample of 20 main oilseed rape racemes taken from $1m^2$.

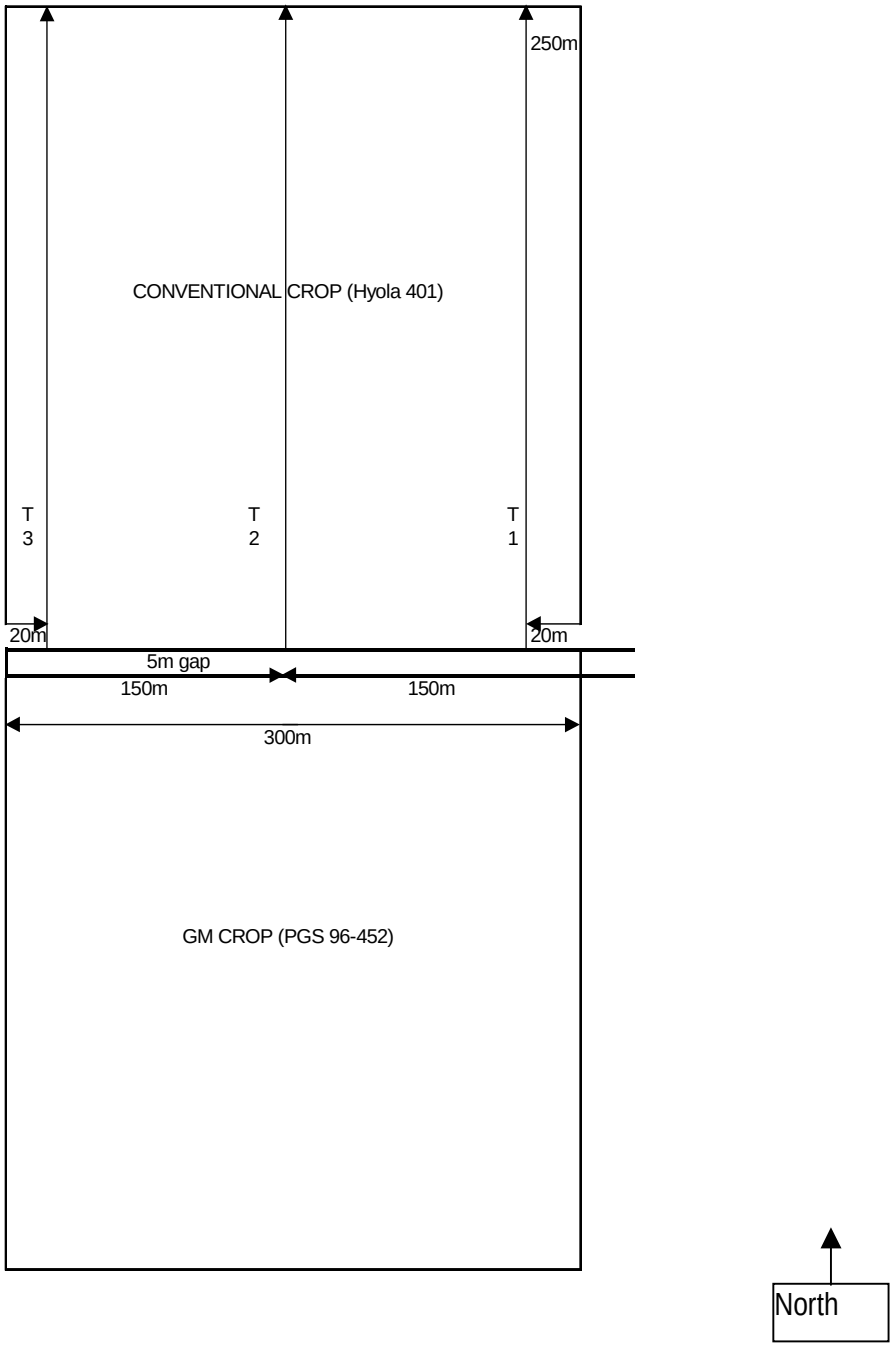


Figure 6.1: Layout of the FSE trial site at Lincolnshire in 1999, showing the GM variety (PGS 96-452) and conventional variety (Hyola 401).

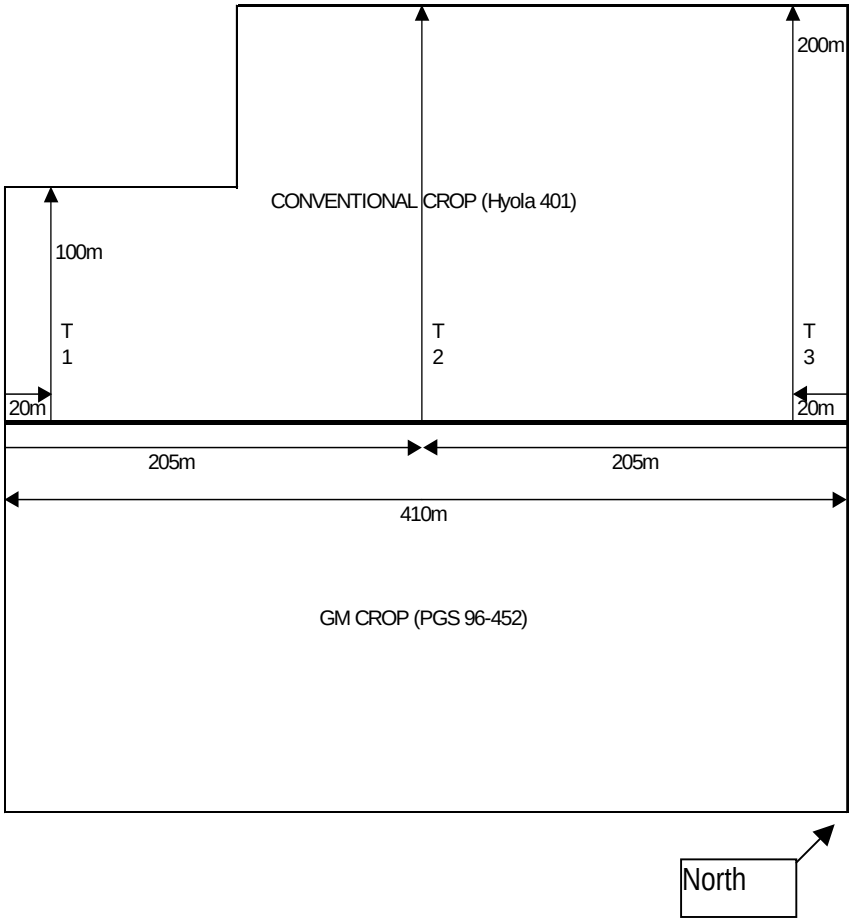


Figure 6.2: Layout of the FSE trial site at Oxfordshire in 1999, showing the GM variety (PGS 96-452) and conventional variety (Hyola 401).

Plate 6.2: The Farm Scale Evaluation site at Boothby Graffoe, Lincolnshire showing the five metre gap between the GM variety PGS 96-452 (left) and the conventional spring rape variety Hyola 401.

Plate 6.3: The Farm Scale Evaluation site at Watlington, Oxfordshire, Lincolnshire showing the interface between the GM variety PGS 96-452 (right) and the conventional spring rape variety Hyola 401.

6.2 RESULTS

All herbicide tolerant controls plants in 45 trays of 100 seeds and 42 trays of 100 seeds for Lincolnshire and Oxfordshire respectively, survived treatment with glufosinate. There were no survivors in any of the trays of negative controls sprayed with glufosinate (45 trays of 100 seeds for Lincolnshire and 42 trays of 100 seeds for Oxfordshire).

1) Gene flow at the Lincolnshire site

In all three transects the proportion of glufosinate-tolerant plants declined with distance into the conventional crop. The maximum frequency of tolerance was 3% at 5m (Transect 2), and rapidly declined to 0.5% or less (Figure 6.3). The pattern was consistent in all three transects. Since the pollen source was segregating for glufosinate-tolerance only 5/8 of the total gene flow would have been detected. Although there was a regular decline in detected cross-pollination in all transects glufosinate-tolerant plants were detected at 250m into the conventional variety in one of the three transects.

A slightly higher cross-pollination frequency was recorded in all transects at 20m than at the sampling points on either side. The reason for this is obscure but is site specific. Transect 1 shows lower numbers of tolerant plants than the other two transects. A total of 34,000 seeds were tested in each transect. Transect 1 had 137 tolerant seeds, Transect 2 had 246 and Transect 3 had 261.

Overall then the total number of tolerant seeds found was 644 in 102,000 tested (0.63%). This represents about 1.00% of pollen transfer from the GM source to the conventional variety sink, when accounting for the 3/8 non-transgenic pollen emitted.

2) Gene flow at the Oxfordshire site

As at Lincolnshire, outcrossing frequencies at the Oxfordshire site declined with distance into the conventional crop. A maximum of 2% glufosinate-tolerance was recorded at 5 m in Transect 1, and to a level of 0.5% or less at most sampling points after 25m (Figure 6.4).

Transect 1 showed higher levels of cross-pollination than the other two transects (0.52% cf 0.31% and 0.32%). In Transect 1, much of this is ascribable to cross-pollination frequencies declining only gradually between 5m and 25m. In the other two transects, the levels adjacent to the GM pollen source were low (0.5%), and generally remained so at further distances.

Transect 2 shows unusual ‘hot spots’ of 1.5% at 100m and 0.8% at 200m but this was not reflected in Transects 1 or 3. The total number of glufosinate-tolerant seeds found was 316 in 84,000 tested (0.38%), and this equates to cross pollination of 0.60% from the GM pollen source to the conventional sink. Overall, then, there is approximately half the pollen flow at the Oxfordshire site as at the Lincolnshire site.

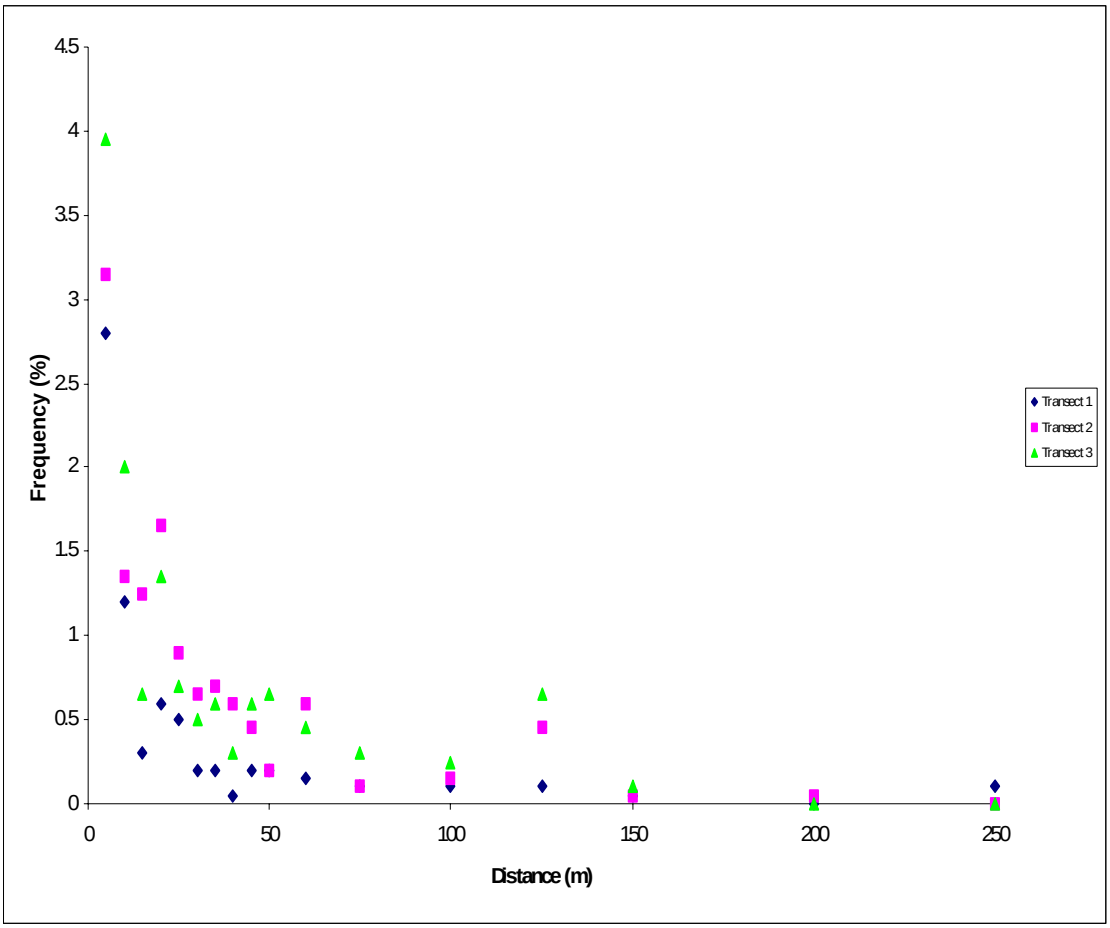


Figure 6.3: The frequency (%) of glufosinate-tolerant seeds recorded with distance into a block of conventional oilseed rape variety Hyola 401 along three transects at the Boothby Graffoe, Lincolnshire site.

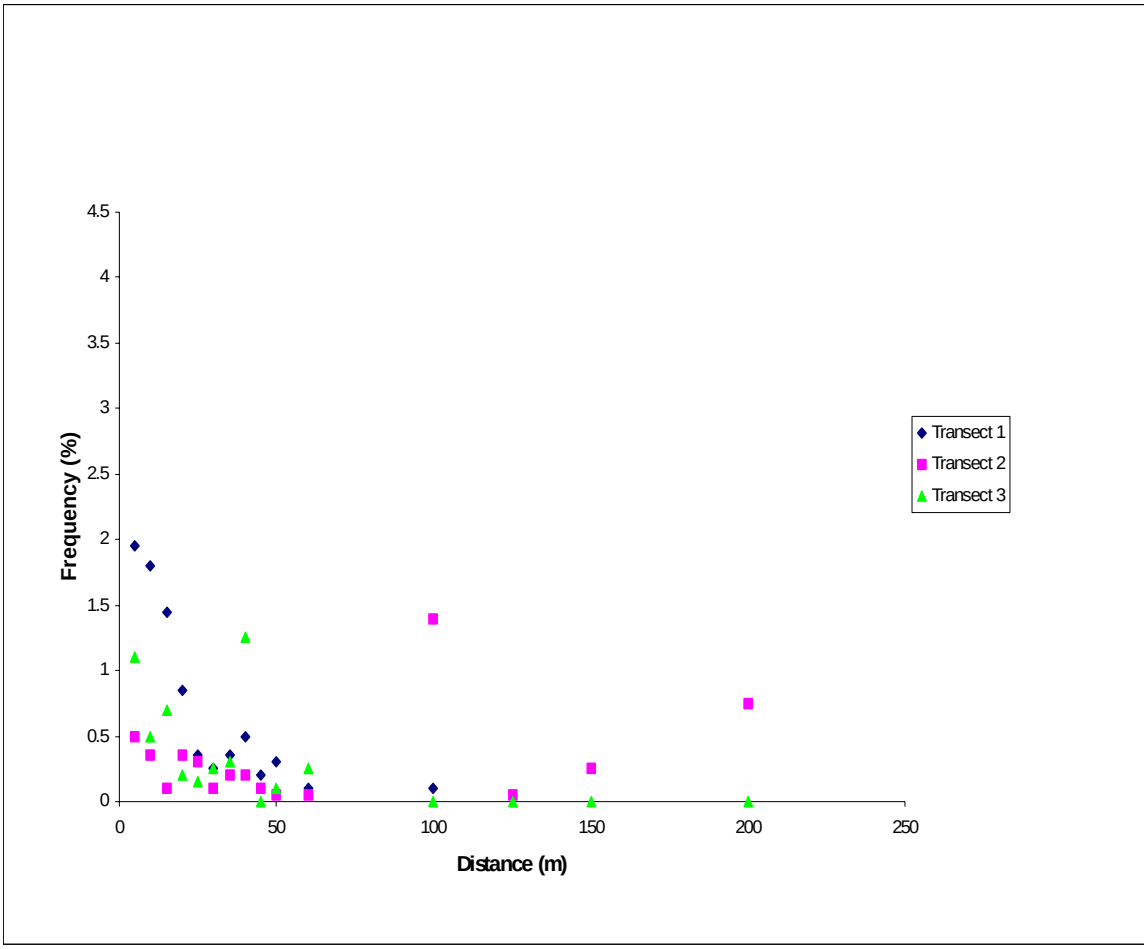


Figure 6.4: The frequency (%) of glufosinate-tolerant seeds recorded with distance into a block of conventional oilseed rape variety Hyola 401 along three transects at the Watlington, Oxfordshire site

6.3 DISCUSSION

The higher outcrossing levels at 5m distance recorded at the Lincolnshire site than the Oxfordshire site are most likely to have been caused by the gap between the GM crop and the conventional crop. Pollen would move either by wind or by insects directly over the gap and pollinated the first of the conventional plants at the 5m point. At the Oxfordshire site, there was no gap between the two varieties, and the plants located in the area between the GM crop and the 5m sampling point would have acted as a pollen trap. If the 5m and 10m results are excluded from the Lincolnshire totals then the rates of flow at these two sites become more similar so that at 15m - mean cross-pollination was 0.73% and 0.75% at Lincolnshire and Oxfordshire respectively.

The results from the Oxfordshire site indicate some long-distance outcrossing events, at 100m and 200m in Transect 2. There are several factors that may influenced these results:

i) The conventional variety Hyola 401 used in this experiment is a restored hybrid variety, which is the first generation of a cross between a female fertile, male sterile recipient line and a pollinator line. In the construction of this variety then, only the female rows carrying the F_1 generation of hybrid seed are harvested. However there may be a low proportion of contaminating male-sterile plants (<10%) amongst the F_1 generation sown as Hyola 401. These male-sterile plants in the conventional crop would be entirely outcrossing and thus more receptive to incoming pollen. If male sterile individual plants were sampled at 100 and 200m they were likely to have higher levels of outcrossed progeny.

ii) Advanta Seeds who supplied the Hyola 401 for these trials confirmed that some of the seed batches of Hyola from Canada were contaminated with GM glyphosate and possibly glufosinate tolerance in the 1999 and 2000 sowing years. If the conventional variety in trials contained a proportion of GM plants, this could have a major effect on the cross-pollination data obtained. If one of the sampling points had included a GM plant, or a GM plant was close to a sampling point and so likely to pollinate its neighbours, this might explain the anomalies seen at 100m and 200m in the Oxfordshire trial. Unfortunately the original seed batch of Hyola 401 used in these experiments was not available to test these hypotheses. However independent tests conducted at NIAB of Hyola seed accessions supplied by plant breeders since 1996 showed that several seed lots contained adventitious seed with both glufosinate and glyphosate tolerance up to levels of 0.5% (E. Simpson, pers. com.).

iii) An alternative possibility for the high cross-pollination levels observed at 100m and 200m from the GM pollen source may relate to the position of a small copse situated in the middle of the field (Plate 3.4). This copse may have disrupted the air currents over the field, thereby affecting pollen movement by wind. The flight paths of foraging bees may also have been disturbed by the position of the copse, leading to anomalous long-distance dispersal of transgene-containing pollen.

iv) A major cause of pollen movement at the Oxfordshire site may have been the invasion of the field by anti-GM demonstrators during the flowering period of the rape. Much of the GM crop was deliberately destroyed by this action. Human activity therefore may have carried some GM pollen into areas of the conventional crop where it might otherwise not have reached.

Plate 6.4 Demonstration in GM oilseed rape at Watlington Farm Scale site.

These results suggest that there are a number of unpredictable factors that may influence levels of outcrossing in agricultural systems. This study has shown some unusually high levels of gene flow at distances of 100m or greater. There is, therefore, some potential for significant levels of contamination between large agricultural fields. These data indicate, however, that effective gene flow in oilseed rape is limited in its extent. Only those plants within 5m of the boundary between varieties gave more than 5% cross-pollination. However, long-distance cross pollination at 100m or more from the boundary occurred in 5/6 transects, and attempts to prevent gene flow simply by distance seems an unlikely possibility.
The total number of tolerant seeds amongst the seeds of the conventional variety found at Lincolnshire and Oxfordshire falls well below 1% (0.63% and 0.38% respectively). The frequency of contamination is thus low but reflects only 5/8th of the total outcrossing frquency. More data from large-scale plantings are needed to assess patterns of gene flow between varieties of oilseed rape under agricultural conditions. Only when such studies have been carried out, will it be possible to establish the most effective isolation distances between transgenic oilseed rape crops and conventional crops in order to keep contamination levels within defined limits.

The possibility of contamination of conventional seed sources by transgenic material discussed here also suggests that total isolation or exclusion from GM varieties is not practicable in current farming systems.

6.4 Cross pollination of the conventional variety Gemini by GM winter oilseed rape at NIAB, Cambridgeshire

As part of the Botanical and Rotational Implications of growing GM Herbicide Tolerant Crops (BRIGHT) research project, plots of glufosinate and glyphosate tolerant winter oilseed rape were grown at NIAB, Cambridge in the 1999/00 growing season. The purpose of the trial was to study the agricultural implications of genetically modified herbicide-tolerant crops in arable rotations. In this trial the minimum isolation distance from the edge of the GM block to a commercial crop of oilseed rape had been set at 50m with an additional 'pollen barrier' of the conventional oilseed rape variety Apex between 10m and 15m wide surrounding the GM variety. In addition a commercial crop of the variety Gemini was sown on a farm adjacent to the NIAB site. This situation presented an opportunity to study gene flow from two contiguous sources (glufosinate and glyphosate-tolerant plots) into a nearby commercial crop. The distance between the trial and the crop variety was 75m at the nearest point, so this represented a likely scenario to occur in an agricultural situation. The pollen sources in this experiment were small with each plot only 12m by 48m, while the sink was much larger, approximately 50 ha. Therefore, gene flow might be expected to be lower than that between two full-size varieties, as reported in the Oxfordshire/ Lincolnshire trials above.

6.5 MATERIALS AND METHODS

i) The GM plants in this experiment were grown at the NIAB trial ground, Cambridge. The oilseed rape crop grown adjacent to the GM plants, from which samples for gene flow studies were taken, was a varietal association (VA) called Gemini. 'Varietal associations' were the first hybrids to enter official trials of oilseed rape varieties. This hybrid system uses a mixture of a male sterile hybrid with one or more male fertile cultivars to supply pollen for fertilisation (NIAB Oilseeds Variety Handbook, 2000).

ii) The commercial crop of Gemini was adjacent to the GM trial on two sides (Figure 6.5). On the Northwest side, a 10m oilseed rape barrier of the conventional variety Apex separated the trial and the Gemini crop, with a 65m gap between the edge of the Apex barrier and the crop. In this gap were a 2 to 3m tall hedge, a footpath and some bare ground. On the Northeast side of the GM trial was a 15m barrier of conventional oilseed rape (Apex). The distance between the Apex and the sink variety Gemini was 90m at one edge and 135m at the other edge. Within this area between the trial and the Gemini crop was a maize trial (approximately 20cm tall at the time of rape flowering), a hedge (height 2 to 3m) and some bare ground. The time of flowering of Gemini coincided with flowering of the GM plots.

6.5.1 Seed sampling

Seed samples were taken at growth stage 6.7 (Oilseed Rape Manual, 1989) from the commercial Gemini crop. Samples were taken from two areas of the crop (Figure 6.5) to the Northeast and Northwest. Eight samples were taken from one area (A), from the edge of the crop at 10m intervals corresponding to the width of the GM trial (78m). A further four samples were taken at each distance of 5m, 10m and 15m into the crop, away from the GM trial. From the other sampling area (B), 14 samples were taken directly from

the edge of the crop at 10m intervals, and again a further four samples were taken at distances of 5m, 10m and 15m into the Gemini crop, in the centre of the front facing the GM blocks.

At each of the sampling points a $1m^2$ quadrat was placed over the oilseed rape plants and 20 main racemes cut from within the quadrat with secateurs and placed in a cloth bag (Plate 6.2). Samples were left to dry thoroughly for two weeks in a glasshouse. Seeds were removed from the pods by hand threshing and mixed thoroughly. As with the previous study, a total of 2000 seeds per sampling point were sown in sub-samples of 100 seeds. Positive controls of cultivars known to contain the glufosinate-tolerance transgene were sown with each batch of samples. Negative controls were trays sown with the non-transgenic cultivar Apex.

At growth stage 1,2, second true leaf emerged (Oilseed Rape Manual, 1989), the seedlings were sprayed with a solution of glufosinate-ammonium (Liberty) (200g/l) at $0.1gai/m^2$ or a 1% solution of glyphosate (360g/l) at $0.18gai/m^2$. Survivors were scored as tolerant if still green after a period of 5 to 7 days.

6.6 RESULTS

There were no survivors in the 46 trays of 100 seeds of negative control plants sprayed with glufosinate or glyphosate. All plants in the 46 trays of positive HT controls survived spraying.

i) Glufosinate-tolerance was detected in 45 out of 46 seed samples of Gemini up to the maximum distance of 144m from the GM pollen source (Figure 6.5). At area A, there was some indication of decline in frequency with distance into the recipient crop. By 90m in area A, only 0.16% (a mean of 4 samples) was seen, equivalent to three seeds in 2000). At the sampling points nearest to the GM source (75m), the mean frequency of tolerant seeds found was 0.55% (Table 6.1). The highest frequency recorded in area A was 1.1%, and overall the mean was 0.38%. In area B, at a greater distance from the source, the overall frequency was 0.28%. The sample points closest to the source were again lower than that in area A: 0.38% versus 0.55%.

ii) Glyphosate tolerance was again detected in 44 of the 45 seed samples of Gemini up to a distance of 120m, the limit of sampling, from the GM pollen source (Figure 6.6). The results from glyphosate testing showed higher levels of tolerance in the offspring of Gemini than those from glufosinate testing in both areas A and B, with means of 0.85% and 1.32% respectively. Again there was some indication of a decline in cross-pollination frequencies with distance into the Gemini crop. In area A, the frequency at the furthest distance four sampling points was 0.11% on average and 1.31% nearest the pollen source. The highest cross-pollination from an individual sampling point was 3.2% in area B. Three sampling points had over 2% tolerant seeds, although mean frequencies at all distances were 1.32% or lower (Table 6.2).

In sampling area A, a total of 130 (0.33%) glufosinate-tolerant and 324 (0.81%) glyphosate-tolerant seeds were found in the 40,000 tested. In sampling area B a total of 144 (0.31%) glufosinate-tolerant and 527 (1.15%) glyphosate-tolerant seeds were found in 46,000 tested. Thus the total number of glufosinate-tolerant seeds found in all samples was 274 in 86,000 (0.32%). The total number of glyphosate-tolerant seeds found in all samples was 851 in 86,000 (0.99%). Therefore the total numbers of glyphosate-tolerant seeds detected were significantly higher than the number of glufosinate-tolerant seeds ($x^2_{(1)} > 300$, $P < 0.01$).

Table 6.1: Mean glufosinate-tolerance of all sampling distances in areas A and B from the GM source.

Area	Distance from GM plot (m)	% tolerance (mean)
A	75 (8)	0.55
	80 (4)	0.31
	85 (4)	0.39
	90 (4)	0.16
B	129 (14)	0.38
	134 (4)	0.19
	139 (4)	0.10
	144 (4)	0.19

Figures in parenthesis are numbers of samples.

Glyphosate testing

Table 6.2: Mean glyphosate-tolerance of all sampling distances in areas A and B from the GM source.

Area	Distance from GM plot (m)	% tolerance (mean)
A	75 (8)	1.31
	80 (4)	0.66
	85 (4)	0.66
	90 (4)	0.11
B	105 (14)	1.33
	110 (4)	0.58
	115 (4)	0.74
	120 (4)	0.39

Figures in parenthesis are numbers of samples.

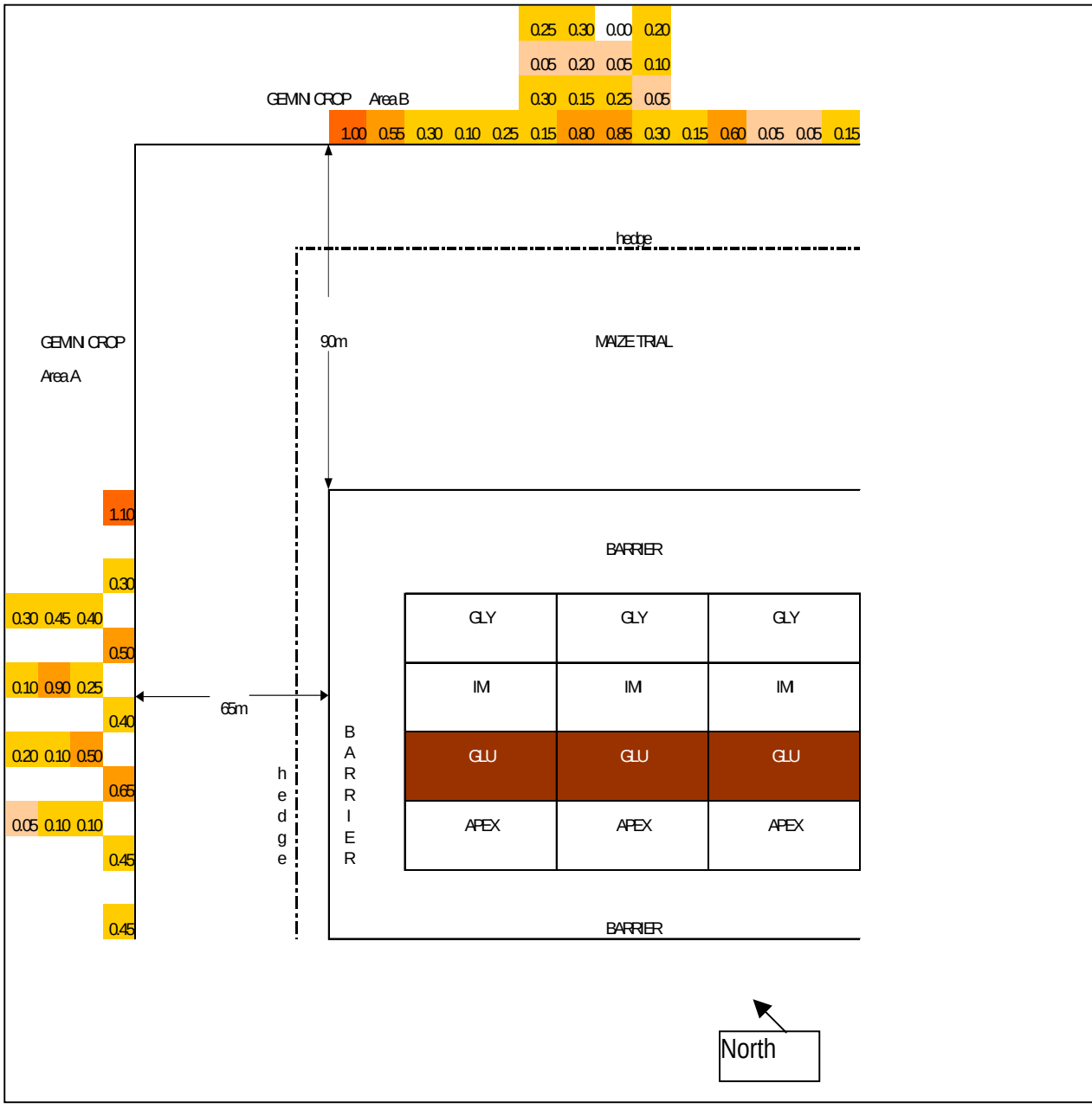


Figure 6.5: Frequencies (%) of glufosinate-tolerant seeds found in a commercial crop of Gemini adjacent to a GM oilseed rape trial at NIAB

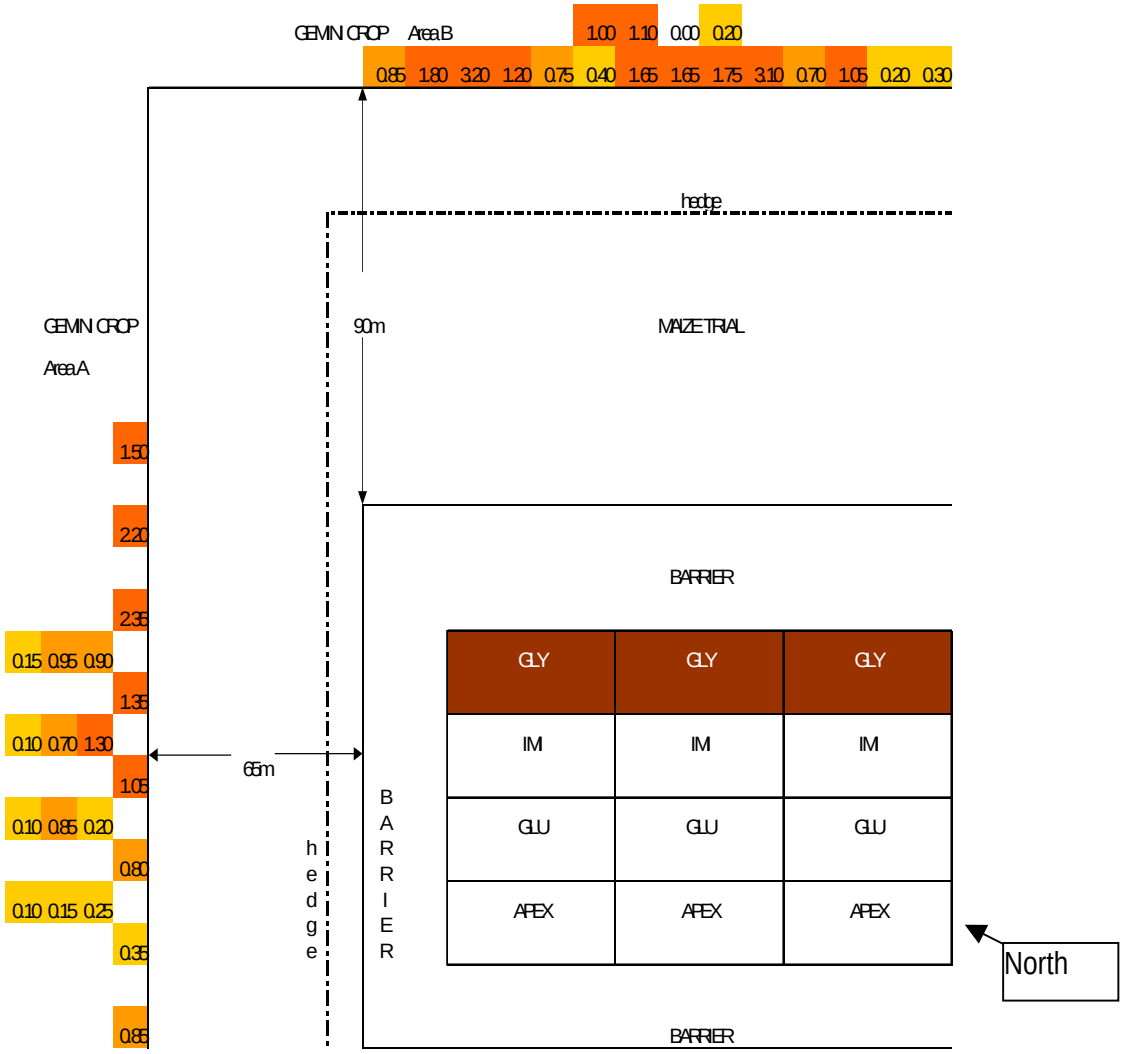


Figure 6.6: Frequencies (%) of glufosinate-tolerant seeds found in a commercial crop of Gemini adjacent to a GM oilseed rape trial at NIAB, Cambridge

6.7 DISCUSSION

The use of male-sterile or emasculated plants has provided information on pollen movement and can give a guide to what is likely to occur when varietal associations (VAs) including a male-sterile component are grown. Where male-sterile *B.napus* plants have been used to measure gene flow, outcrossing has been detected over long distances. Simpson *et. al.,* (1999) detected up to 8% outcrossing at 400m from a GM pollen source, and Thompson *et. al.* (1999) reported that 88.4% of male-sterile flowers were fertilised 1m from an oilseed rape field edge, 13-57% at 500m and 5% at 4km. In this study, there was up to 1% cross pollination between the GM pollen source and the edges of areas A and B in the Gemini crop, despite a hedge and emerging maize crop between source and sink. There was a slow decline in outcrossing rate with penetration into the crop. This suggests perhaps that with VAs there is a reduced pollen barrier effect of the VA crop itself due to reduced production of its own pollen. These

results confirm the findings of Simpson and Sweet (NIAB report to MAFF, 2000/01) who also concluded that VAs would have higher levels of outcrossing than conventional varieties pollinated from similar sources. The levels of cross-pollination in the current experiment were considerably higher than those found in similar situations with fully fertile rape, and perhaps reinforce the need for greater isolation requirements of GM releases from VAs (Ingram, 2000).

Physical barriers may also have an effect on the movement of pollen around oilseed rape crops, although evidence of this from experiments is difficult to obtain. Morris *et. al.* (1994) concluded that barren zones of 4-8m in width might actually increase gene flow between test blocks compared to the same area planted with a trap crop, (a border of non-transgenic plants of the same species surrounding the transgenic plot). They concluded that the most effective strategy for reducing the movement of genes between two varieties therefore is to devote the entire region between them to a barrier crop of fully male-fertile rape, which will produce pollen that dilutes the alien pollen.

In the NIAB experiment the pollen source of transgenic plots was separated from the Gemini variety by several physical barriers on both sides, such as a trap crop directly around the trial, an area of recently drilled maize, a hedge and a pathway. Woods and hedges may serve as potential barriers to wind-borne pollen and to flying insects (Ingram, 2000) and the amount of pollen delivered to a receptor field is also affected by topography. Despite the barriers at the NIAB site, the extent of outcrossing detected from the transgenic plots for both the glufosinate and glyphosate markers was surprisingly high.

Several factors may have influenced the degree of pollen flow occurring between the transgenic plots and the surrounding Gemini crop. The size of the pollen source has an effect on the dispersal of pollen and its attractiveness to pollinator insects. In addition, there was likely to be a high local concentration of pollen arising from a large source and it is possible that the effects of wind dispersal in this situation may be strong due to the overall size of the pollen mass from the whole trial. However the presence of a large non-GM source of pollen would tend to dilute the GM pollen. Scheffler *et. al.,* (1995) reported that the use of small experimental plots as pollen sources may cause bees to forage to more distant areas to collect a load of pollen or nectar. Although the glufosinate and glyphosate plots used in this experimental situation were small when considered individually, the GM trial as a whole represented a fairly large pollen source. Bees will generally collect pollen and nectar from within a small area and are unlikely to move to another crop of oilseed rape. Thus the size of the total area of oilseed rape in the GM trial may have discouraged them from foraging outside the area.

The total number of glufosinate-tolerant seeds found in all samples from the Gemini crop was about 0.32%, while the total number of glyphosate-tolerant seeds was considerably higher at 0.99%. The differences in outcrossing rate found between the two transgenic varieties probably arose from genetic differences in compatibility with the Gemini variety. The glufosinate and glyphosate varieties used here have different genetic backgrounds as well as having the different transgenes incorporated into them. The glufosinate and glyphosate plots flowered at the same time, so flowering time was no different between both sources and the sink. The differences in outcrossing with the two transgenic varieties reported here indicate that each transgenic oilseed rape line may produce different levels of outcrossing under the same conditions and sampling methods. Therefore when considering isolation distances between transgenic and conventional crops, each transgenic variety may behave differently, depending on its genetic background and the inheritance of the novel trait introduced. The hemizygous male and homozygous female parents of the hybrid glufosinate tolerant cultivar contain the *Bar* gene at two loci so that the F_1 hybrid produces glufosinate tolerant and non-tolerant pollen in a 5:3 ratio as described. This means that using herbicide tolerance in seedlings to measure cross- pollination from glufosinate tolerant cultivars will only measure 5/8ths of the true level of gene flow. In addition, the parents of the glufosinate tolerant hybrid contain the *barstar* male sterility gene and the *barnase* male-fertility restorer gene. These

segregate in the F_1 hybrid so that approximately 8% of the plants produce no pollen, reducing the amount of outcrossing from glufosinate-tolerant varieties even further. The glyphosate tolerant variety used in this experiment, however, does not produce any male sterile plants, and all of its pollen carries the herbicide-tolerance gene. This may account for the differences in outcrossing levels from the two varieties reported here.

6.8 Gene flow from a GM winter oilseed rape into an adjacent commercial crop of variety Apex

In the 1999/00 growing season, a commercial crop of Apex winter oilseed rape was grown in an adjacent field to the GM glufosinate tolerant winter oilseed rape trial at Melbourn, Cambridgeshire. The GM trial itself consisted of an area of GM and non-GM plots surrounded by a non-GM pollen barrier planting. The distance from the edge of the GM trial to the start of the commercial crop was approximately 70m at the shortest distance and 90m at the furthest sampling point. The total GM area of the trial was 4.9ha. The GM trial and the Apex crop were separated by winter barley nearest to the trial, a grass verge, a road and a ditch (Figure 6.7).

Seed samples for glufosinate testing were taken at growth stage 6,7 (Oilseed Rape Manual, 1989) from the commercial Apex crop. Samples were taken from the area at the edge of the crop indicated on Figure 6.7. Eight sampling points were used along a 78m length of the edge of the Apex field at approximately 10m intervals along the edge. In addition samples were collected at 5m, 10m, and 15m into the crop and away from the GM pollen source. The sampling strategy, seed handling and herbicide testing protocols were as described previously.

6.9 RESULTS

There were no survivors in the 32 trays of 100 seeds in the negative (non-HT) controls sprayed with glufosinate. All 32 trays of 100 plants survived spraying in the trays of positive HT controls.

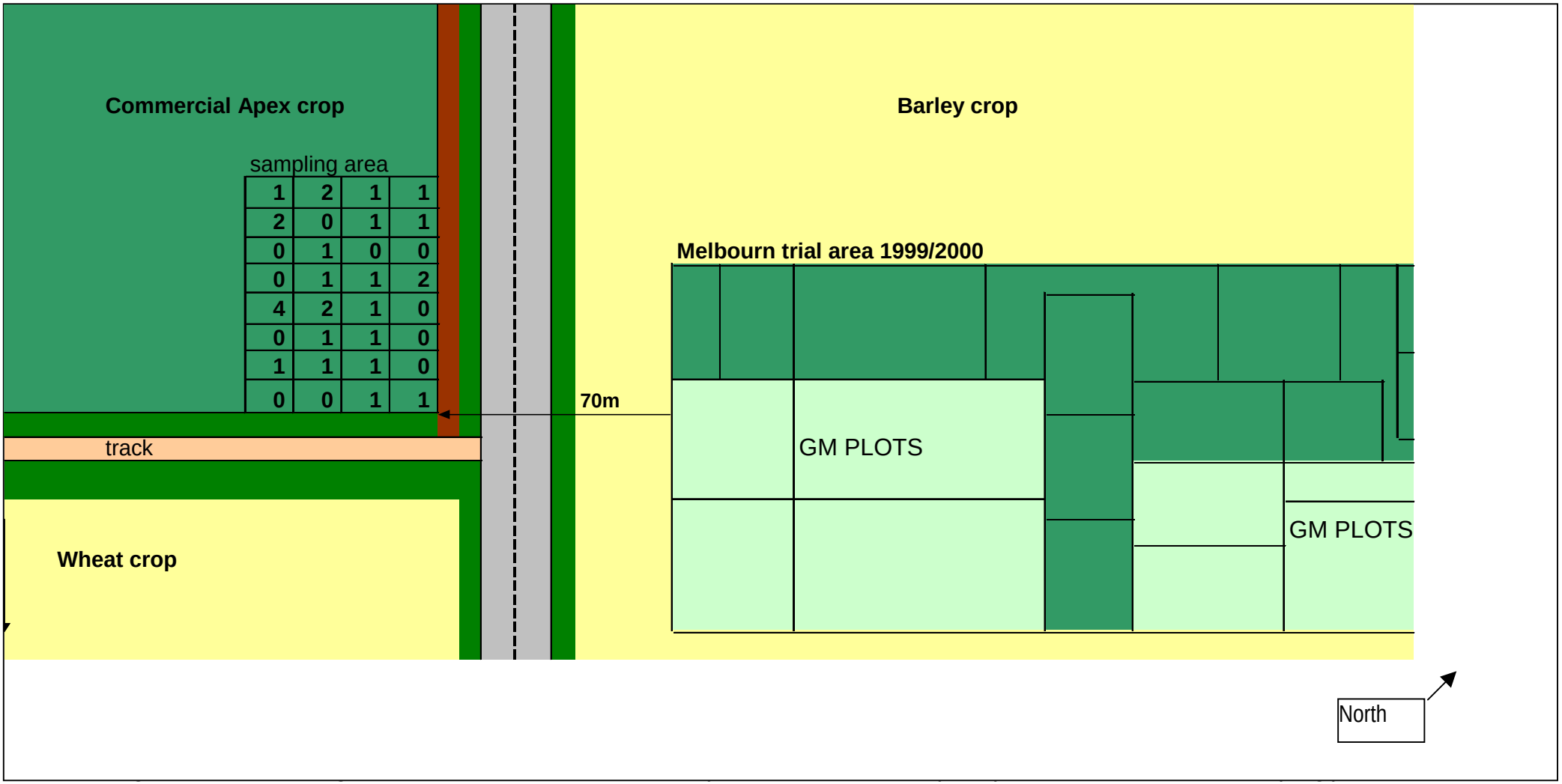
Cross-pollination levels determined from glufosinate tolerance in the commercial Apex crop were low (Figure 6.7). At eleven of the 32 sampling points, no herbicide-tolerance was detected amongst the 2000 seeds tested, and only five of the 32 sampling points had two or more tolerant seeds. The highest level of cross-pollination recorded was 0.2% (4 in 2000) which was at 90m away from the GM pollen source. The distribution of glufosinate-tolerant plants found in the Apex crop was apparently random throughout the sampling area. There was no indication of decline with distance. The total number of glufosinate-tolerant individuals found in all samples from the Apex crop was 28 in 64,000 (0.04%).

6.10 DISCUSSION

Where fully fertile *B.napus* plants are used as the recipient crop in outcrossing studies, much lower levels of outcrossing were detected than with male-sterile plants. Many experiments using fully-fertile sink varieties have been conducted under different environmental conditions and trial designs and in all of them low levels of outcrossing have been detected at various distances from the pollen source. A number of studies have detected outcrossing at low levels in fully fertile recipient crops of oilseed rape adjacent to the pollen source. Outcrossing was detected at up to 30m (Staniland *et. al.,* 2000), 47m (Scheffler *et. al.,* 1993), 100m (Manasse and Karieva, 1991), 115m (Champolivier *et. al.,* 1999) and 400m (Scheffler *et. al.,* 1995). However there is only one report of more than 1% outcrossing from a

source to a sink in fully fertile recipient oilseed rape plants at 137m (Stringham and Downey, 1982), using a chlorophyll deficient marker.

The low levels of glufosinate-tolerance detected in the receptor variety Apex at Melbourn (0.04%) compared to those found in the VA Gemini at NIAB (0.32% for glufosinate-tolerance and 0.99% for glyphosate-tolerance) suggest that fully fertile varieties are much less susceptible to outcrossing than varieties with a male-sterile component. At both sites, sampling points were at similar distances from the GM pollen source, although the two sites cannot be directly compared as they were in different locations with different topographical features. At the Melbourn site there were fewer physical barriers to wind and insect dispersal, such as hedges, than at the NIAB site, so the movement of pollen should perhaps have been less impeded. Remarkably a maximum of only 0.2% was found at 90m from the GM source at Melbourn, this was at only one sampling point of eight at this distance. The mean at this distance then is well below 0.1%. Samples were taken at distances up to 15m into the crop. It seems likely that further away from the GM pollen source, glufosinate-tolerance would not be detected amongst the 2000 seed samples.


Commercial Apex crop
sampling area
1 2 1 1
2 0 1 1
0 1 0 0
0 1 1 2
4 2 1 0
0 1 1 0
1 1 1 0
0 0 1 1
track
Wheat crop
Barley crop
Melbourn trial area 1999/2000
70m
GM PLOTS
GM PLOTS
North

6.11 OVERALL CONCLUSIONS

Oilseed rape pollen is transferred between flowers by wind and insects, especially by honeybees (*Apis mellifera*) and solitary bees. Insects play an important role in pollination over long distances. Ramsay *et. al.*(1999) for example, found that honeybees in a colony in Scotland flew to an oilseed rape source 5km away from their hive. Theoretically then there is potential for pollen to be transferred within a radius of at least 10km by the mixing and contact within the hive of bees foraging in different directions. Thompson *et. al.* (1999) concluded however, that the relative importance of wind and insect pollination was difficult to determine under field conditions. In their study, male-sterile oilseed rape bait plants were placed at selected distances between zero and 4000m from the nearest non-GM oilseed rape crop. They found that pollination of the male-steriles occurred at all distances. Although airborne oilseed rape pollen was detected at all sites, its concentration declined rapidly with distance from the source. This perhaps indicates that bees may be important pollen vectors over long distances. However, Squire *et. al.,*(1999) suggested that airborne pollen shows a rapid decline in pollen concentration near the source, followed by a very slow decline with increasing distance. They concluded that the absolute level of airborne pollen then is dependent on the size of the pollen source.

The use of male-sterile bait plants provides some information on pollen movement, but is not an accurate guide to the likely levels of effective intercrossing between fully-fertile oilseed rape crops, because it does not take into consideration the effect of competition from self pollen. Levels of outcrossing decrease with increasing distance from the source, and very low levels of outcrossing (0.0038%) were reported at distances up to 400m from the pollen source with fully fertile recipient crops by Scheffler *et. al., (1*995). The FSE trials were sampled up to maximum distances of 200m (Oxfordshire) and 250m (Lincolnshire) and at these distances outcrossing was near to zero. Levels fell to below 1% by 25m distance into the receptor variety.

The varying results obtained in the experiments described here can be partly explained by the differences in the trial size and therefore the size of the pollen source. In the Melbourn study that estimated gene flow into the commercial variety Apex, the transgene source trial was a complex mixture of transgenic and non-transgenic plots so it was difficult to determine the exact size of the total transgenic pollen source or the density of transgenic-bearing pollen in the source output. The pollen source in the Gemini experiment at NIAB was smaller than at Melbourn. However, gene flow detected was higher, perhaps because of the male-sterile component of the Gemini receptor crop. This indicated that pollen competition may be an important factor in the amount of outcrossing that occurs. Simpson and Sweet (2001) studied cross pollination with another VA variety, Synergy, and found that, where there was little pollen competition from self produced pollen due to the high proportion of male-sterile plants in the varietal association, there was a higher frequency of outcrossing than with conventional oilseed rape. However in this study there was competition from pollen from neighbouring plots and barrier plots and it appears from comparisons of these three studies that fully fertile barrier (trap) crops surrounding the source have less effect on outcrossing than similar barriers around the receptor.

Although it is not possible to compare the individual studies reported here in a statistical fashion, the results presented show that different situations can give very different results under natural field conditions and that it is not always possible to extrapolate from small-scale experiments to field conditions. The results provide valuable data towards determining isolation distances for both VAs and standard fully-fertile oilseed rape crops to ensure cross-contamination in field scale crops is kept below allowable thresholds.

7. FERAL AND VOLUNTEER RAPE: GENE FLOW, PERSISTENCE AND WEEDINESS

Volunteer and feral rape arises from seed shed both before and during harvest of an oilseed rape crop, from seed shed from existing volunteer and feral populations and from seed spilled during transport on and off the farm . Mature pods opening during harvesting (pod shatter) results in seed dispersal before harvest of at least 10% of the yield each year, with up to 50% yield losses in some seasons (Child and Evans, 1989). Seeds lost at harvest (Plate 7.1) are generally left to germinate on the surface for 2 to 3 weeks after which the field is cultivated (Plate 7.2). Any ungerminated seeds are subsequently buried when the field is ploughed, and can germinate when brought to the surface again by cultivation in subsequent years. This results in unwanted oilseed rape plants as volunteers in the subsequent crops (Plates 7.3 and 7.4). and can reduce yield and affect the composition of following crops if allowed to persist.

Plate 7.1: An area of ground immediately after the harvest of a GM oilseed rape crop at Melbourne in 1998, showing spilled seeds on the soil surface.

Plate 7.2: Oilseed rape seedlings germinating amongst crop debris two weeks after the oilseed rape harvest.

Plate 7.3: Oilseed rape volunteers in wheat stubble at Melbourn four weeks after harvest in 1998

Plate 7.4: Oilseed rape volunteers in a crop of linseed at Melbourn in 1999.

In broad-leaved crops such as sugar beet, volunteer rape is generally not well controlled. However, in cereal crops, control of volunteer rape is relatively easy to achieve using selective broad-leaf herbicides. Various studies have provided evidence that rape seeds can persist in the soil for at least five years (Lutman, 1993; Schlink, 1995) and perhaps as long as ten years. A potential concern with regard to the introduction of transgenic oilseed rape is that the presence of a transgene may change the way in which oilseed rape persists in the agricultural environment over time. It is possible that genetic manipulation may cause changes in the agricultural and ecological behaviour of transgenic crop plants. If a transgene produces a trait that gives the plant a fitness advantage, then oilseed rape could become more persistent as a weed in other crops or more invasive of uncropped and marginal land.

Possession of a herbicide-tolerance transgene by an oilseed rape plant would not in itself offer any fitness advantage to the plant, unless the herbicide was applied. However there may be pleiotrophic effects which enhance fitness. Conversely there may be a physiological cost to carrying a transgene that would reduce the fitness of a transgenic rape plant compared to its non-transgenic counterpart. It may be however that the costs of carrying a herbicide-tolerance transgene are relatively low, in which case there may be no measurable differences between transgenic and non-transgenic lines. In the case of the high laurate transgene, for example, there is a small amount of evidence to suggest that the presence of high lauric acid may affect germination rates and seedling mortality (Linder and Schmitt, 1995). This in turn could affect plant development and thus overall fitness of the adult plants.

Transgenic lines of glufosinate-tolerant, glyphosate tolerant and high laurate oilseed rape were studied for their persistence when occurring as volunteers in subsequent arable crops. The use of transgenic rape in this study also gave an opportunity to study the longevity of oilseed rape seed in the soil seed bank under arable conditions for the first time, since the presence of the transgene gives an absolute dating point. In addition some of the sites used had never grown oilseed rape on their land previously, and thus comparisons were made with non-GM rape also grown at these sites. Previous work on dormancy of oilseed rape and its persistence in the seed bank has concentrated mainly on germination and burial experiments and not on natural (agricultural) situations such as these (Pekrun and Lopez-Granados, 1995; Pekrun *et. al.,* 1997).

In addition cross-pollination between volunteers, feral populations and rape crops was studied to determine the significance of this for gene flow of transgenes.

7.2 MATERIALS AND METHODS

Fields where transgenic rape was grown were observed for the presence of OSR volunteers in the years following the harvest of the rape. Sites used for this study are described in Section 4. Volunteers found in fields where GM oilseed rape had previously been grown, were recorded and the type of crop in which they were found was noted. Methods for determining numbers of volunteers are described in 4.7. All OSR volunteers seen at the sites were included in the estimation of numbers, regardless of whether they were transgenic or not. Their transgenic status was later determined by herbicide testing (4.3, Materials and Methods) and by PCR (4.4, Materials and Methods). Dates of site visits are shown in Tables 4.8 to 4.10 (Materials and Methods).

7.3 RESULTS

7.3.1 Volunteer incidence

Overall numbers of volunteers found at sites where herbicide tolerant GM oilseed rape had previously been grown varied from zero to several thousand (Table 7.1). Numbers tended to be low or even zero in the crop immediately following the GM rape, and to be more prevalent in the second crop. Oilseed rape volunteers were generally very well controlled in cereal crops and were rarely seen to survive after they had flowered in these crops.

At the Devon site large numbers of oilseed rape volunteers set seed in a field of forage rape in 1998, further contributing to the seed bank. After this date however, no more volunteers were seen at this site. Very few volunteers were seen at either of the Lincs sites over a three-year period. At Lincs site 1 however, six fully-grown oilseed rape plants were found five years after the GM crop, when the original area was planted with trees. Only one volunteer was found at the Yorks site, and at Fakenham no volunteers were observed (although they were impossible to distinguish from the oilseed rape crop grown in 2000). At East Winch oilseed rape volunteers were seen in all areas of the farm where GM oilseed rape trials had previously been grown.

At the Melbourn site where oilseed rape had never previously been grown before the GM releases, volunteers were found in a crop of peas, four years after the original trial. Melbourn had more volunteers than at any other site studied and these were seen in all fields where GM oilseed rape was previously grown. Although the volunteers were mostly killed by herbicides before flowering, in one field 241 oilseed rape volunteers set seed in the crop of barley grown the year after the GM trial.

Table 7.1: Oilseed rape volunteers found at GM OSR release sites

SITE	YEAR OF GM RELEASE HARVEST	FOLLOWING CROPS	OSR VOLUNTEERS FOUND	COMMENTS
DEVON	1995	Barley (1996/97)	None	
		Forage rape (1997/98)	>600/ha	OSR volunteers set seed
		Spring barley (1998)	None	Farmer reported early OSR volunteers which were controlled by herbicide application
		Peas (1999)	None	
		Wheat (1999/00)	None	
LINCS SITE 1	1995	Wheat (1997/98)	None	
		Wheat (1998/99)	None	
		Trees (2000)	6 plants	In 2000 the area was planted with trees and fully mature OSR plants were found growing in a grassy area
LINCS SITE 2	1996	Barley (1997/98)	Some seen between crops	Plants found after harvest destroyed by cultivation
		Winter oilseed rape (1998/99)	None	
		Wheat (1999/00)	None	
YORKS	1995	Wheat (1997/98)	None	
		Winter oilseed rape (1998/99)	1 plant	Identified as GM by PCR
		Wheat (1999/00)	None	
EAST WINCH	All years since 1994	Trials plots of different crops	Volunteers seen in plots all around the farm	All volunteers sprayed off before seed set.
FAKENHAM	1997	Barley (1997/98)	None	
		Wheat (1998/99)	None	
		Spring oilseed rape (2000)	None	Unable to distinguish volunteers from crop
	1998	Barley (1999)	None	
		Wheat (2000)	None	
MELBOURN	1996	Barley (1997/98)	490/ha	241 volunteers in 5ha set seed
		Barley (1998/99)	20/ha	Volunteers observed post-harvest. Volunteers in crop sprayed off in April
		Peas (2000)	22/ha	Large numbers of volunteers seen between crops. Large OSR plants in pea crop damaged by insects.
	1997 (Spring rape)	Oats (1997/98)	None	

Table 7.1 continued: Oilseed rape volunteers found at GM OSR release sites

SITE	YEAR OF GM RELEASE HARVEST	FOLLOWING CROPS	OSR VOLUNTEERS FOUND	COMMENTS
MELBOURN	1997 (Spring rape)	Set-aside (1998/99)	73,000/ha	All plants sprayed with glyphosate before setting seed.
		Set-aside and linseed (2000)	39,000/ha	OSR volunteers sprayed off before seed set
	1997 (winter rape)	Wheat (1997/98)	None	14/ha seen between crops
		Wheat and set-aside (1998/99)	106/ha seen in set-aside. None in wheat	
		Barley (1999/00)	Small OSR seedlings seen in spring 2000	Sprayed off in Spring
	1998 (winter and spring rape)	Wheat (1998/99)	None	
		Oats (1999/00)	None	
	1999 (winter and spring rape)	Wheat (1999/00)	None	
NIAB	1997	Wheat (1997/98)	None	
		Beans (1999)	20,000/ha	All plants sprayed with glyphosate before setting seed.
		Wheat (1999/00)	None	
	1998	Wheat (1998/99)	None	
		Barley (1999/00)	None	
	1999	Wheat (1999/00)	None	
BERKHAMSTEAD	1998	Wheat (1998/99)	10,000 to 100,000/ha	Area too large to estimate numbers of volunteers. Volunteers sprayed off in Spring.

Table 7.2 Numbers of glufosinate-tolerant volunteers in crops following GM oilseed rape, determined by herbicide spot testing.

SITE	TOTAL AREA OF RAPE (ha)	APPROX % GM IN PARENTS	YEAR TESTED	CROP	NUMBER TESTED	%TOLERANT
LINCS 1995	1.4	100	1996 2000	Sugar beet Trees	4 8	25.0 25.0
LINCS 1996	1.3	100	1996 1997	Between crops Between crops	124 77	96.8 57.1
YORKS 1995	3.0	100	1996 1997	w.wheat w.barley	170 6	10.6 0.0
MELBOURN 1995/96	5.2	48	1997 1998 1998 1999 2000	w.wheat w.barley post harvest post harvest peas	77 90 142 602 23	6.5 23.3 16.9 19.1 26.1
MELBOURN 1997	1.0	90	1998 1998 1999	w.oats post harvest Linseed and set-aside	0 87 352	0.0 42.5 25.6
MELBOURN 1996/97	7.2	69	1998 1998 1999	w.wheat post harvest Set-aside	0 19 81	0.0 10.5 14.8
N.I.A.B. 1996/97	2.8	49	1998 1999	w.wheat Beans	16 667	0.0 1.3
EAST WINCH 1996/97	Various	Various	1999	w.wheat	216	41.2

Table 7.3 PCR testing of volunteers within GM crop fields of oilseed rape for the presence of transgenes.

SITE	TOTAL AREA OF RAPE (ha)	APPROX % GM IN PARENTS	YEAR TESTED	CROP	TRANSGENE	NUMBER TESTED	NUMBER POSITIVE
LINCS 1995	1.4	100	1996 2000	Sugar beet Trees	BAR BAR	4 8	1 2
LINCS	1.3	100	1996	Between crops	BAR	125	120
1996			1997	Between crops	BAR	162	51
YORKS 1995	3.0	100	1996 1997 1998	w.wheat w.barley WOSR	BAR BAR BAR	357 8 1	35 0 1
MELBOURN 1995/96	5.2	48	1997 1998 1998 1999 2000	w.wheat w.barley post harvest post harvest peas	BAR BAR BAR BAR BAR	77 90 * 140 * 200 * 200 *	7 3 * 3 * 1 * 1*
MELBOURN 1997			1998 1999	post harvest Linseed and set-aside	BAR BAR	80 * 300 *	4 * 1*
MELBOURN 1996/97			1998 1999	post harvest Set-aside	BAR BAR	10 * 50 *	1* 1*
N.I.A.B.	2.8	49	1998	w.wheat	BAR PAT GLY	10 * 10 * 10 *	0* 0* 1*
1996/97			1999	Beans	BAR PAT GLY	600 * 600 * 600 *	1* 0* 2*
EAST WINCH 1996/97	Various	Various	1999	w.wheat	BAR PAT GLY	200 * 200 * 200 *	4 * 0* 0*
BERKHAMSTEAD 1998	38.0	38.0	1999	w.wheat	Uc FatB1	150 *	10*

* Samples tested as bulks of 10 leaves. Results of positive samples are shown as numbers of batches of 10 showing the transgenes.

At NIAB oilseed rape volunteers were not seen in cereal crops, however, when a crop of beans was grown in a field where a GM oilseed rape trial had been grown the year previously, an estimated 20,000 volunteers per hectare were observed. In the year after the GM oilseed rape crop at Berkhamstead (1999), large numbers of oilseed rape volunteers were seen in the following wheat crop (an estimated

10,000 to 100,000 per hectare). However these were treated with herbicide in the spring and none were seen after they were killed.

Broad-leafed crops such as linseed and beans generally contained high numbers of oilseed rape volunteers, which sometimes persisted after they had finished flowering, and produced pods. Volunteers were often seen after harvest after the soil was disturbed before the following crop was sown. This was particularly evident if a spring-sown crop was next in the rotation, when the soil was left uncultivated over winter. Often under these conditions the OSR volunteers grew large with six or seven leaves but again rarely flowered. These were generally well controlled by herbicides or cultivation before the sowing of the next crop.

The OSR volunteers observed in this study were a mixture of transgenic (herbicide tolerant and high laurate) and non-transgenic rape. Their origin could not be directly determined unless oilseed rape had never previously been grown at a site (such as Melbourn, Devon and Berkhamstead). The volunteers found rarely matured to set seed as they were usually treated with herbicide or with desiccant such as glyphosate to kill them before they had reached the green pod stage.

7.3.2 Frequency of transgenic volunteers

Where volunteers were tested for glufosinate tolerance by herbicide spot testing in the field in the years following a GM crop, the result was always a lower proportion of plants expressing tolerance to the herbicide than in the original crop or trial (Table 7.2). At Lincs the volunteers tested five years after the GM crop were only 25% tolerant when the original crop was 100%. At Melbourn numbers of tolerant plants were only a proportion of the original. For example in the field where GM oilseed rape was grown in 1997, and originally had 90% transgenic plants, two years later only 25.6% (herbicide testing) were found. In another field where 69% transgenic rape was grown, 14.8% (herbicide testing) were found.

Glufosinate-tolerant oilseed rape volunteers were found outside the original trial area at Melbourn but at none of the other sites. The plants found were in set-aside and were sprayed with glyphosate and killed at the green pod stage before any viable seed was set. Some mature plants were removed before spraying and seeds left to mature so that they could be tested for tolerance to glufosinate. However the Melbourn site grew new lines of GM rape with varying proportions of male-fertile and male-sterile plants, together with some non-GM lines, making it difficult to estimate the proportion of volunteers likely to be herbicide-tolerant. Trials at NIAB and East Winch were also complex. Trials were made up of small plots of different varieties, some of which were not herbicide-tolerant, including some which were male-sterile and therefore produced no pollen but still contained the glufosinate transgene. This made it difficult to predict the proportion of GM volunteers likely to arise in subsequent crops. Accurate quantitative measurement of seed dispersed from these trials was impossible due to these factors. However persistence of transgenes in the environment could be determined by using herbicide testing and PCR.

7.3.3 Molecular testing

Oilseed rape volunteers were tested for the presence of transgenes by PCR. Where individual plants were tested, there were slightly higher proportions of tolerant plants than found by phenotypic testing (Table 7.2). Some individuals that were identified as susceptible by herbicide spot testing were found to be positive by PCR, indicating some degree of gene silencing.

7.4 DISCUSSION

In the UK winter oilseed rape is generally grown in rotation with two cereal crops. After harvest of oilseed rape the seeds dispersed or shed before harvest are allowed to remain on the surface for two to three weeks prior to cultivation. Weather conditions such as temperature and rainfall during this time determine the extent to which germination of the seeds occurs before the next crop is sown. Any rape seedlings emerging during this time are generally removed and the soil cultivated for the next crop, normally a cereal crop. Ungerminated oilseed rape seeds tend to be buried by mechanical cultivation forming the soil seed bank where they can remain ungerminated and viable for some time. This burial is known to induce secondary dormancy in rapeseeds (Lutman, 1993; Lutman and Lopez-Granados, 1998).

When oilseed rape seeds are first harvested or shed, they possess virtually no primary dormancy. Secondary dormancy can be induced in the absence of light when available moisture is sufficient for germination (Pekrun *et. al.,* 1997; Pekrun *et. al.,* 1998), or in response to water stress, oxygen deprivation or low temperatures (Pekrun *et. al.,* 1995). Seeds may then remain viable in the soil for at least 5 years (Schlink, 1994). Exposure to light due to further soil cultivation in subsequent crops may trigger germination of these rape seeds which then grow into unwanted volunteers in subsequent crops.

Pekrun and Lutman (1997) studied the susceptibility of different oilseed rape cultivars to induced dormancy. They subjected 47 cultivars of oilseed rape to an osmotic stress treatment known to induce secondary dormancy, and found considerable variation (2% to 50% dormant seed) between different genotypes. However only Pekrun and Lutman (1997) studied a small range of cultivars and those used at the sites monitored here have not been tested for their susceptibility to induced dormancy nor is there any no information available. Also at sites such as Melbourn, NIAB and East Winch, a range of new cultivars was grown in trial format. These cultivars are not genetically uniform being composed of male-sterile and fertility restorer lines. Differences between transgenic and non-transgenic plants arising from secondary dormancy may result from genetic variation between lines and cannot necessarily be attributed to the genetic construct itself.

The Melbourn site had a volunteer problem in some of the early release fields that grew GM OSR in 1996. Volunteers were still seen in 2000 in the first release field in which the GM rape was harvested in 1996, although these were controlled by herbicide. This indicates a persisting viable seedbank of GM oilseed rape in this particular instance. The site has a free draining, chalky soil and conditions post-harvest in 1996 were very dry. Substantial numbers of seed remained ungerminated on the surface and the farmer ploughed the field and buried the seed putting it into secondary dormancy (Lutman & Pekrun, 1998). This site was ploughed again in the following autumn, redistributing seeds in the soil and bringing them to the surface so that considerable numbers emerged that autumn (1997) creating a high volunteer population in the winter barley sown in that season.

All the sites used in this study followed normal cultivation practices for oilseed rape as described above, allowing the shed seeds to germinate for two to three weeks. These practices did not allow significant numbers of oilseed rape volunteers to become established as weeds in the crops at the other sites which followed GM trials. In many cases the crop directly following oilseed rape was winter barley which generally has fewer volunteers than the subsequent crops of winter wheat. This could either be due to the different herbicide treatments given to wheat and barley, or to the particular conditions in the years and sites observed.

There are several possible explanations for the reduced proportions of the transgene in volunteers in the years following the GM release. Firstly, seed losses from the GM varieties, before and during harvest,

may have been less than that from conventional varieties, due to differences in pod shattering or ripening characteristics. Secondly more GM plants than non-GM plants may have germinated initially in the period after harvest, leaving a smaller proportion of GM seeds to be buried and become dormant. Another possibility is that the GM plants may be less fit than conventional rape due to metabolic trade-offs caused by the presence of the transgene. Seedlings may have died, or fewer GM plants may have survived to maturity than the conventional plants. These factors were not investigated in this study. The conclusion that the proportion of plants with transgenes is lower in crops after the initial trial was made from only one site from which data could be collected. Other sites did not have large enough numbers of volunteers to investigate this further. The longevity of the seeds in the soil seedbank was also not investigated fully as this research was conducted over a three year period, and viable oilseed rape seeds can persist in the soil for several years (Lutman, 1993; Lutman and Lopez-Granados, 1998). The GM oilseed rape site studied at Melbourn first grew GM oilseed rape in 1995, and by 2000 volunteers were still seen in one field. A proportion of these carried the transgene although lower than in the original trial. It is possible that GM volunteers originating from the 1995 release in this field will emerge in future years. In some of the other fields monitored in this study (Devon, Yorks, Lincs 2 and Fakenham) volunteers have not been seen for several years. There may no longer be a seedbank of GM rape in these fields. However from this information alone it would be inappropriate to conclude that the seedbank has been depleted of rapeseeds carrying the transgene. The sites would need to be monitored for several more years and soil samples taken to establish this.

The results of PCR testing of individual plants that had previously been herbicide tested indicate that there may have been some silencing of the herbicide tolerance transgene in volunteers, although this was not investigated fully in this study. Other studies have concluded that environmental stress may induce transgene silencing. Krishna (2001), for example, subjected transgenic lines of *Arabidopsis thaliana* to stress treatments of 30°C followed by 4°C and found that the frequency of silencing of the transgene was higher in the stressed group than in the control group. He concluded that the environmental stress had caused changes in post-transcriptional methylation patterns and chromatin conformation. Ratcliff *et. al.* (1997) studied gene silencing in relationship to viral infection. They found that transgenes that are derived from viral cDNA are able to induce gene silencing, and also may suppress the accumulation of viruses that are similar in nucleotide sequence. Al-Kaff *et. al.* (1998) found that in oilseed rape containing a transgene with a cauliflower mosaic virus (CaMV) 35S promoter, the transgene was silenced upon infection with cauliflower mosaic virus. They concluded that post-transcriptional cosuppression of viral genes results in post-transcriptional cosuppression of transgenes sharing sequence homology with the virus.

Many factors influence the ways in which transgenes express, but a crucial factor is the presence of DNA sequences homologous to regions of transgene constructs such as the CaMV promoter with sequence homology to the CaMV virus itself. The transgenic oilseed rape grown at Melbourn, NIAB and East Winch contained the CaMV promoter and cauliflower mosaic virus is a common viral infection in oilseed rape. Oilseed rape volunteers would have been particularly vulnerable to aphid virus vector infestation. This may possibly explain the lack of expression of the transgene seen in some of the plants at Melbourn, NIAB and East Winch. Volunteers that are not growing in an oilseed rape crop are also more stressed due to crop competition and other agronomic factors. Any of these stresses could have caused silencing of the transgene to occur. The results from PCR testing therefore indicate that herbicide spot testing is likely to underestimate the true proportion of plants containing the transgene.

Control of herbicide-tolerant and high laurate volunteers by the non-selective herbicides used in cereal crops appeared to be no different from the control of non-GM rape. The transgenic lines of oilseed rape used in this study were no more persistent or weedy than conventional rape at any of the sites or in any of the years studied. It is not possible to conclude, however, that the GM lines studied were less

persistent than conventional lines. There were many variables that could have affected the survival and fitness of individual genotypes, whether they were transgenic or not. The main causes of mortality in rape volunteers were predominantly herbicide application, and the cultivation of the host crop. Transgenic traits affecting the plant's ability to overcome these threats may lead to an increase in volunteer numbers. Numbers of volunteers following GM oilseed rape are dependent on many factors including the weather conditions following harvest, the succeeding crop species, management of the crop, the soil type on which the crop is grown and the harvesting procedure of the original GM crop.

7.5 Gene flow in volunteer and feral rape

Oilseed rape volunteers commonly occur in other crops in fields adjacent to those cropped with oilseed rape. These volunteers may be pollinated by oilseed rape in neighbouring crops and, if these crops are transgenic, this means transgenes may move into other fields. Hence non-GM volunteers can become a source of GM contamination in fields that have never grown transgenic crops. Similarly oilseed rape carrying transgenes may persist over several generations and therefore pose a problem for future oilseed rape crops. This may have consequences for the subsequent cropping of these fields, particularly if seed crops of oilseed rape are to be grown. Concerns have been raised about the potential effects of the introduction of transgenic rape on oilseed rape volunteers growing in neighbouring fields, and the effects of transgenic volunteers on conventional oilseed rape crops. In this study the extent of gene flow from transgenic rape into oilseed rape volunteers in neighbouring fields and into feral oilseed rape populations was studied.

The movement of oilseed rape transgenes in space and time was investigated at all the sites where volunteers were found in fields adjacent to or within a 500m radius of a GM OSR site. The presence of transgenic rape at these sites allowed the year of sowing and original site of the GM plants to be determined, so that movement of seed and cross-pollination could be detected.

Oilseed rape is a common and widespread weed in field margins and roadsides where it is known as feral rape. It usually occurs as a result of seed shed during harvesting or lost during transportation. Oilseed rape is commonly seen flowering along roadsides from April to June.

Both volunteer and feral rape are interesting to study from the aspect of gene flow, as they can give indications about possible temporal gene flow through seed dispersal, and also may act as both source and sink for pollen mediated gene flow. In this study gene flow into feral and volunteer rape through pollen from a GM source was examined. By using GM trials or crops at several sites as the pollen source, it was possible to trace gene flow by phenotypic and molecular methods in nearby volunteer and feral plants

Sites were monitored as previously described (4. Materials and Methods) for the presence of individual oilseed rape plants growing outside the original GM area, or for the presence of feral OSR populations established on roadsides or verges near to GM trials. Where these plants were found, they were herbicide spot tested and samples taken for PCR analyses. If volunteer or feral plants were found to set seed after flowering synchronously with the GM oilseed rape, then seed samples were taken for growing in the glasshouse and screening with herbicide (4.7.1, Materials and Methods).

7.6 RESULTS

Only one persistent population of feral oilseed rape was found at any site in any year. This population was found at the Melbourn site and persisted for three years, and in 1998 was 150 m away from a GM oilseed rape trial. The feral population was present in 1997 but the numbers of plants were not recorded.

In 1998, 71 mature plants were found, and in 1999 45 mature plants. The feral plants were found along a roadside verge, which was mown in late 1999. No oilseed rape plants were seen in 2000. No glufosinate tolerant plants were found in this feral rape population, despite its proximity to the GM trial site.

Many oilseed rape plants were found along roadsides around the GM OSR areas at several sites (Table 7.3). Very little outcrossing into these plants in nearby fields or along roadsides was detected. At the two Lincs sites a total of 537 plants were tested outside the GM oilseed rape area and none contained the glufosinate-tolerance transgene. At Yorks, from 145 oilseed rape plants tested in an adjacent field to GM oilseed rape, none were tolerant. Herbicide tolerant plants were found at Melbourn in areas outside of the original growing area (Table 7.3), however from 1518 tested, only 161 contained the transgene. At all other sites, no herbicide tolerant plants were found outside the original trial areas.

When seeds were tested from oilseed rape plants flowering synchronously with GM oilseed rape trials, small numbers of tolerant seeds were found (Table 7.4). At Newbury 57 seeds contained the transgene from 14,421 tested from a sampling point at 10 m away from the GM trial. At Melbourn seven tolerant seeds from 6,758 were found on plants growing 20 m from a GM trial. No other tolerant seeds were found from those tested at other sites. These plants did not establish to become feral populations and died off before maturity and seed set.

Seed taken from individual oilseed rape plants that were identified as tolerant did not always give 100% tolerant progeny (Table 7.5). The three tolerant plants whose seeds were tested gave between 56.6% and 98.7% tolerant progeny. Susceptible individual rape plants growing near to these single tolerant volunteers showed very low levels of cross-pollination with tolerant plants, despite their close proximity (Table 7.5). The proportion of tolerant progeny from these plants ranged from 0.03% to 0.60%.

Table 7.3: Proportion of glufosinate-tolerant volunteer oilseed rape plants growing adjacent to release sites

SITE AND YEAR OF GM	LOCATION OF PLANTS	DISTANCE FROM LOCATION OF GM RAPE	YEAR TESTED	NUMBER TESTED	NUMBER TOLERANT
LINCS 1995	Roadside	200m	1996	15	0
LINCS 1996	Roadside	100m	1996	373	0
	Field margin	10m	1997	4	0
YORKS 1995	Field combined after GM OSR	1000m	1996	145	0
MELBOURN	Roadside	150m	1996	132	0
	Roadside	200m	1997	50	0
	Adjacent field	50m	1997	541	0
	Roadside	40m	1998	92	15
	Adjacent field	100m	1998	135	54

	Track	200m	1999	7	0
	Adjacent field	50m	1999	81	0
	Adjacent field	60m	1999	352	90
	Adjacent field	50m	1999	7	2
	Roadside	150m	1999	116	0
	Grass verge	50m	2000	5	0
EAST WINCH	Adjacent field	100m	1998	160	0

Table 7.4: Proportion of seedlings tolerant to glufosinate from seeds taken from feral and volunteer oilseed rape plants growing adjacent to GM rape.

SITE	LOCATION OF PLANTS SAMPLED FOR SEED	YEAR TESTED	NUMBER OF SEEDLINGS TESTED	NUMBER TOLERANT
NEWBURY 1998	In adjacent OSR crop 10m from GM trial	1998	14421	57
EAST WINCH	In adjacent field 50m from GM trial	1998	2004	0
MELBOURN	In adjacent field 20m from GM trial	1997	7998	0
	Along a roadside 20m away from GM trial	1998	6758	7
	From feral population 150m from GM trial	1998	3947	0
	Along a roadside 150m from GM trial	1999	4712	0
NIAB	Seed from susceptible volunteer, 20m from single plant, 400m from crop	1999	2366	0

Table 7.5: Numbers of glufosinate-tolerant seedlings from seeds taken from individual volunteers

SITE	LOCATION OF PLANTS SAMPLED FOR SEED	YEAR TESTED	NUMBER OF SEEDLINGS TESTED	NUMBER TOLERANT
Plant 1	Seed from tolerant volunteer, 2m from nearest GM plant, 700m from GM trial	1999	1360	1342
Plant 2	Seed from tolerant volunteer, 2m from nearest GM plant, 700m from GM trial	1999	2078	1176
Plant 3	Seed from tolerant volunteer, 3m from nearest GM plant, 700m from GM trial	1999	530	404
Plant 4	Seed from susceptible volunteer, 6m from nearest GM plant, 700m from GM trial	1999	2888	3
Plant 5	Seed from susceptible volunteer, 6m from nearest GM plant, 700m from GM trial	1999	6020	2
Plant 6	Seed from susceptible volunteer, 4m from nearest GM plant, 700m from GM trial	1999	664	4

7.7 DISCUSSION

Feral populations of oilseed rape were rare in this study, and where populations were found in one year, these did not generally persist to following years. Individual plants growing in roadsides and verges rarely survived to maturity and set seed. Many factors were involved in the mortality of these plants, the main ones being competition with perennial weeds (especially grasses), predation by animals (slugs, birds and insects) and susceptibility to diseases of oilseed rape.

Invasions of semi-natural habitats by oilseed rape would require the plant to be vegetatively and reproductively successful by transferring the genetic modification from generation to generation, creating a feral population. A study carried out in Scotland monitored the occurrence of feral oilseed rape populations over a large area of arable countryside (Charters *et al*, 1996). The study demonstrated that feral oilseed rape is already well established in Scotland as a weed in arable environments. It shows weedy characteristics that make it invasive on disturbed ground, such as asynchronous germination and flowering, the ability to flower as a small plant and over-winter as a mature plant. In addition, it has seed that has been known to persist in the soil seedbank for several years (Pekrun & Lutman, 1998 ; Lutman, 1993).

A study of the dynamics of feral oilseed rape on the M25 motorway (Crawley and Brown, 1995) concluded that the apparent permanency of roadside populations of oilseed rape was due to seed spillage from passing lorries. In the absence of soil disturbance, feral populations of oilseed rape usually become locally extinct within three years because of occupancy of space by perennial grasses. Local extinction can be followed by recruitment from passing traffic when the soil is disturbed, giving the impression of a permanent population. These conclusions were supported by observations of the feral population found at Melbourn, which appeared to die out after two years. This population was growing in a grass verge, and the plants surviving to maturity only became established in areas of ground, which had recently been disturbed by animals or by vehicles driving on the verge. Some plants grew at the very edge of the road where there was no competition and where the soil was frequently disturbed by passing vehicles. Plants growing in the undisturbed parts of the verge failed to compete with grasses and other invasive weeds. The lack of oilseed rape plants seen in 2000 may have been as a result of the verge becoming overgrown with perennial grasses. Mowing of the verge may also have had an effect on the feral oilseed rape population. Mature plants were present before mowing took place but these failed to return viable seeds to the seedbank.

Although the Melbourn feral population appears to have died out, there still may be oilseed rape seeds in the soil seedbank, which may germinate if the verge is disturbed again. Recruitment may also occur again from passing vehicles if there is little competition from perennial grasses.

Oilseed rape thus appears to be an obligate cultigen, and cannot persist in undisturbed habitats. The recent study by Crawley *et. al.,* (2001) showed that oilseed rape lacks the ability to survive in most natural and semi-natural environments, preferring disturbed habitats. The feral population observed at Melbourn in this study, and the individual oilseed rape plants found along roadsides and verges all behaved in this way.

If a GM crop plant becomes established as a weed in managed environments such as field margins or crops, control could become a problem. This may be so particularly if multiple herbicide-tolerance arises by cross-pollination between more than one GM herbicide-tolerant crop variety. Multiple tolerance has already been reported in Canada (Downey, 1999) and in UK (Simpson & Sweet 2001) . However, in order for crop plants to establish outside their agricultural environment, would require that transgenes give them a fitness advantage over competitor plants and other biotic factors that inhibit their colonisation of that environment. For example, without the selection pressure of the herbicide to which it is tolerant, a modified plant for herbicide-tolerance has no more of a fitness advantage than conventional oilseed rape. It is therefore very unlikely to spread outside the cultivated environment.

In a recent study Crawley *et. al.,* (2001) planted experimental plots of all GM crop plants currently available. They found that oilseed rape GM cultivars tolerant to herbicides did not become self-seeding, and self-sustaining populations nor did they spread into neighbouring areas. However Crawley *et. al.* (2001) did not test crops with transgenes conferring a fitness advantage such as tolerance to pests or diseases. Oilseed rape plants with a 'fitness transgene' may become more invasive of natural habitats, or change the equilibrium of other plant communities.

Cross-pollination of volunteer rape plants by GM rape varieties in adjacent fields was low in these studies. At the time that the GM oilseed rape flowered there was likely to be a large background level of rape pollen in the air and only a small proportion of GM pollen. There was also competition from the pollen of each individual volunteer plant. Environmental influences, geographical location and effect of within-plant position of the flowers are known to have an effect on outcrossing rate in oilseed rape. The latter can vary between 12% and 47% according to Becker *et. al.* (1992).

There was no evidence of cross-pollination from GM oilseed rape into the feral population at Melbourn despite the closest GM oilseed rape being only 150m away in 1998. In the same year Simpson *et. al.* (1999) placed some male sterile oilseed rape 'bait' plants 100m away from the GM trial in the same field and in the same direction as the feral population described here. Simpson *et. al.* (1999) found 8% of the seeds collected from the bait plants were glufosinate-tolerant. In the same experiment, cross-pollination by GM pollen was found up to 400m down wind from the pollen source. The comparison between the results from the male-sterile plants and the fully fertile plants in the feral population suggests that competition from other plants and from selfing is important in fertilisation. Where other oilseed rape plants are in close proximity, such as was the case in the feral population, individual plants are more likely to be fertilised by pollen from those plants, or even by self-pollination rather than from a GM pollen 150m away. In the case of the male sterile bait plant experiment by Simpson *et. al.* (1999), however, the nearest large pollen source was the GM trial itself, and there was of course no competition from self-pollination.

The results of screening of seed taken from individual tolerant oilseed rape plants at Melbourn show that segregation of the transgene takes place in generations after the initial GM crop. At Melbourn where the tolerant volunteers were tested (Table 7.5), the two GM lines producing the F_1 hybrid grown in the trials contained two transgenic loci, as described in Chapter 6. The F_1 generation will have two forms in a 1:1 ratio, which will both be tolerant. Plant 1 in Table 7.3 is likely to be an F_1 hybrid of the two parental lines as it has 98.7% tolerant progeny. If one of the forms of the F_1 crosses with another F_1 of the same genetic make-up then a 3:1 tolerant: susceptible ratio will result in the progeny. The 3:1 ratio is close to that seen in Plant 3 (76.2% tolerant progeny). Plant 2 produced just over half its seed as tolerant. It is likely to have arisen from a cross between an F_1 plant and a plant not carrying the transgene, which would result in a 1:1 tolerant to susceptible ratio.

These results show that when a glufosinate-tolerant plant is released into the environment, the likely outcome is for the transgene to diminish over generations through dilution from non-transgenic plants, and segregation of the transgene. This is likely to be the case for other herbicide-tolerance genes when selection does not play a part, however this may not happen if transgenes conferring a fitness advantage are introduced.

These results showed that the amount of cross-pollination from stands of GM oilseed rape into neighbouring oilseed rape volunteer plants and feral plants was low. Where it did occur, the HT gene was unlikely to become established as the plants and populations were transient and died out within a short period. Key factors for mortality of oilseed rape outside the arable environment appeared to be competition from perennial grasses, predation and disease. Of these competition appears to have the most effect, as wherever feral plants were seen, they never survived in undisturbed habitats where perennial grasses had become established. Thus for a genetic modification to give the feral populations the ability to persist it would have to confer the ability to compete with other competitor species such as perennial ryegrasses.

8 INTERSPECIFIC GENE FLOW: HYBRIDISATION WITH WILD RELATIVES AND OTHER CROP SPECIES

INTRODUCTION

Hybridisation between different species can only occur if the sexual compatibility of the two parental genomes is sufficient to allow the development of a mitotically and stable hybrid. Once seeds have been formed, the endosperm must be able to support normal hybrid development. If the endosperm does not function effectively, development of the hybrid embryo will be aborted (Dale, 1992).

The direction of the cross can also be important as the cytoplasm of one parent may support embryo and seed development more effectively than the other. Once viable seeds are formed, establishment is influenced by the adaptation and fitness of the hybrid to the habitat and the ecological conditions to which the plant is subject. Factors such as seed dormancy, the vigour of the hybrid plant in its habitat, competition with pests, other plants and diseases will determine the ability of the hybrid to survive.

Oilseed rape has several wild relatives with which it has been known to hybridise. In this research we have focussed on four of these species: wild turnip (*Brassica rapa*), wild radish (*Raphanus raphanistrum*), white mustard (*Sinapis alba*) and charlock (*Sinapis arvensis*).

Wild turnip (*B. rapa* ssp. *sylvestris*/ *B. campestris*)

Frequencies of hybridisation between oilseed rape and wild *B.rapa* have been reported in numerous studies. Frequencies vary between studies and appear to be much higher where *B.rapa* occurs as a weed in oilseed rape crops. Jørgensen *et. al.* (1996) measured frequencies of spontaneous hybridisation between oilseed rape and *B.rapa* and found that the frequency of interspecific hybridisation varied significantly with experimental design. Frequencies ranged from 9% in a 1:1 mix of *B.rapa* and *B.napus* with oilseed rape as the mother plant, to 93% where single isolated *B.rapa* mother plants were growing in an oilseed rape field. Hansen *et. al.* (2001) reported finding evidence of introgression in an organic field where *B.napus* and *B.rapa* coexisted.

Scott & Wilkinson (1998) also measured gene flow from oilseed rape into wild *B.rapa* populations growing outside field boundaries. Low hybridisation frequencies were found (between 0.4 – 1.5%). In addition to this they found that less than 2% of hybrid seedlings survived, and suggested that establishment of GM *B.napus* x *B.rapa* populations would be poor and that introgression of the transgenes into the wild population would be unlikely or very slow.

Wild radish (*R. raphanistrum*)

This species is the second most likely of the four species tested to form hybrids with oilseed rape. In an experiment by Darmency *et.. al.* (1995) hybridisation was detected at a rate of 1:625, wild radish:oilseed rape. In this experiment 0.2% of the seeds derived from wild radish were intergeneric hybrids. In a similar experiment by Chevre *et. al.* (1999), wild radish plants were transplanted at different densities into a field of the partly male sterile oilseed rape variety Synergy. They found very low frequencies of hybrids were formed with *R.raphanistrum* as the female parent. Only one hybrid was found in the 189,420 seeds tested. Darmency *et. al.* (1995) obtained 45 intergeneric hybrids from each male sterile oilseed rape plant used as the female parent. Another study be Darmency *et. al.* (1998) found up to 3 intergeneric hybrids per 100 plants when *R.raphanistrum* was the female parent and isolated from other *R.raphanistrum* plants in a field of oilseed rape. Seed production of the F_1 hybrids in this case was up to 0.4% of the wild species.

The use of a male sterile recipient and high pollen pressure from the wild species has enabled interspecific hybrids between oilseed rape and *R.raphanistrum* to be relatively easily produced. However under field conditions there would only be a very small chance of interspecific hybrids being produced with *B.napus* as the female parent because of the availability of pollen from rapeseed in large quantities. This pollen competition would only allow hybrids to be formed at very low frequencies. Studies to date have mainly used *B.napus* as the female as no male sterility is available in *R.raphanistrum*. However the presence of self-incompatibility in *R.raphanistrum* makes the reciprocal cross possible.

White mustard (*S.alba*)

Although hybrids between *S.alba* and *B.napus* have never been found under natural conditions, several methods have been used to overcome the interspecific barriers preventing hybrid production. Protoplast fusion, *in vitro* fertilisation of ovules, ovary culture and embryo rescue have all been used in the synthetic production of hybrids. 50 to 60 bud pollinations were made (Brown, *et. al.*, 1997) by applying pollen from parent A to the stigmas of parent B, directly after emasculation. Developing ovaries were then excised from the plant 8 days after pollination and surface sterilised. The ovaries were then rinsed and transferred to a sterile culture medium. Embryos were then rescued from the ovaries and placed on fresh media.

All hybrid plants resulting from this procedure were found to be sterile. To overcome sterility cuttings were taken from the sterile plants and treated with rooting powder and then immersed in a colchicine solution in water to induce chromosome doubling. Only two plants were obtained from this, both of which were fertile. Pollination appeared to be more successful with *S.alba* as the female parent.

Charlock (*S.arvensis*)

S.arvensis like the other weeds mentioned in this study, is self-incompatible and pollinated by both insects and wind. In the UK *B.napus* and *S.arvensis* often grow alongside each other in agricultural fields and the two species often flower simultaneously though *S arvensis* individuals will flower over a much longer period from spring to late summer.

Artificial hybrids have been produced by emasculation and *in vitro* embryo rescue techniques (Mathias, 1991; Kerlan *et. al.*, 1992). Kerlan *et. al.,* (1992) succeeded only in producing hybrids where *B.napus* was the female parent and this was only possible by ovary culture, embryo rescue. No hybrids were produced using *S.arvensis* as the female parent. A study by Lefol *et. al.* (1996) revealed no hybrids found among 2.9 million seeds produced by *S.arvensis* growing amongst herbicide tolerant transgenic oilseed rape. In the same study, when male sterile *B.napus* plants were grown in the presence of *S.arvensis* , no more than 6 hybrids were obtained from 50,000 flowers from the oilseed rape plants (Lefol *et. al.*, 1996). These results show that it is possible for a small number of hybrids to be produced under the most favourable conditions.

A more recent study (Chevre *et. al.*, 1998) also using male sterile oilseed rape plants produced 0.18 interspecific hybrids from 100 seeds. The experiment involved growing several replicates of male sterile transgenic oilseed rape alongside *S.arvensis* under natural field conditions.

Results of similar trials by Moyes *et. al.* (1999) with seed samples from populations of *S.arvensis* in the UK and France are still awaiting publication. No interspecific hybrids between *B.napus* and *S.arvensis* have as yet been reported under natural field conditions and Downey (1999) concludes that from the data

available so far there appears to be a general agreement that natural gene flow is unlikely to occur between *B.napus* and *S.arvensis.*

8.2 MATERIALS AND METHODS

Seed sampling from wild relatives

Seed samples were taken at maturity from crucifers growing within the GM crop and the monitoring zones of the GM rape releases. Occasionally seeds had to be taken slightly before maturity if they were amongst an OSR crop that was going to be harvested. Maturity was determined by the colour of the seeds. Samples were taken when the seeds were dark brown or black (depending on the species) or were allowed to ripen before seeds were removed from their pods.

The sampling method was dependent on the spatial arrangement of the weed population being sampled. Care was taken not to deplete the number of seeds returning to the seed bank, so if populations were small, numbers of seeds sampled had to be low. If the plants were growing amongst an oilseed rape crop, all seeds were sampled, as they would mostly have been lost at harvest and emerging seedlings destroyed in subsequent cereal crops. In populations where there were a large number of plants, more seed was taken from many plants, but none of the individual plants had more than 50% of their seeds removed, thus minimising the effect on seed return to the soil seed bank.

Testing seeds of Crucifer species

Seeds from crucifer species were germinated on petri dishes in a 100 p.p.m. solution of giberrellic acid (Sigma) to break dormancy. This was very successful and gave over 90% germination of seeds in most cases. Shortly after germination seedlings were transplanted into half trays containing general potting compost (Shamrock) in batches of 10.

Seedlings were treated with the relevant herbicide and recorded as in Methods (4.).

Herbicide spot testing of Cruciferous plants in the field

Plants of the four species of related cruciferous weeds were tested in the field in the years following release of the GM trial, in order to detect possible hybrids that would have been formed in the growing year of the GM OSR. Plants were spot tested with glufosinate herbicide as described in 2.5. Not every individual could be sampled where there were large, dense populations, so sub-samples of ten plants were tested randomly at regular intervals across the population depending on its density.

Some populations were very uneven in their distribution, and where this occurred a sampling pattern was used to cover a representative proportion of the population. This involved testing samples in a 'W' formation across a field. If any plants were seen whose morphology differed from others in the population, these were also sampled along with those of 'normal' morphology.

A non-destructive phenotypic test for use in the field was not available for detection of glyphosate tolerance or for high lauric acid, and PCR was used for detecting the presence or absence of these particular transgenes.

Molecular testing

Molecular testing was carried out as in 4.7. A proportion of samples was tested by PCR and in batches of ten leaves. As a control for crucifers, a positive oilseed rape DNA sample was mixed in a 1:1 ratio with DNA from the relevant cruciferous species.

8.3 RESULTS

Table 8.1 shows results of herbicide spot testing of all the weed species related to oilseed rape used in this study. No herbicide tolerance was found in any year and at any of the sites monitored, in *S.arvensis, S.alba* and *R.raphanistrum*. The PCR testing confirmed the negative results of the phenotypic testing. No plants with obvious hybrid morphology were identified by eye, although one *R.raphanistrum* plant with unusual appearance was sent for ploidy testing by flow cytometry. Results showed it had the same amount of DNA as a normal runch plant and was therefore not a hybrid with *B.napus*.

Table 8.4 shows the results of testing of the seeds taken from cruciferous weeds, which flowered synchronously with the GM oilseed rape and could therefore be capable of producing interspecific hybrids. Again no tolerant seedlings were found from any of the seed tested at any site in any year.

The results of the monitoring of hybridisation between *B.rapa* and oilseed rape at the Humberside site are discussed in Section 9.

Table 8.1. Results of herbicide spot testing of related crucifers for glufosinate tolerance

SITE	SPECIES	LOCATION OF PLANTS	YEAR TESTED	NUMBER TESTED	% TOLERANT
DEVON 1995	*Sisymbrium officinale*	In field margin (10m from trial)	1996	80	0
		In field margin (10m from trial)	1997	27	0
LINCS 1995	*S.officinale*	In field margin (20m from trial)	1996	24	0
	Sinapis arvensis	In field margin (20m from trial)	1996	144	0
		In field margin (20m from trial)	1997	16	0
LINCS 1996	*S.arvensis*	In release field	1997	3	0
YORKS 1995	*Capsella bursa-pastoris*	In release field	1996	27	0
	Alliaria petiolata	In field margin (10m from trial)	1996	123	0
	A.petiolata	In field margin (10m from trial)	1997	39	0
NIAB	*S.arvensis*	1-200m from trial	1998	301	0
	Raphanus raphanistrum	200m	1998	18	0
	S.arvensis	Adjacent to 1997/98 GM trial area	1999	222	0
	S.arvensis	Adjacent to 1996/97 trial area	2000	117	0
NEWBURY	*Brassica.rapa*	400m from GM trial	1998	35	0
	B.oleracea	400m from GM trial	1998	27	0
MELBOURN	*A.petiolata*	Adjacent to 1995/96 trial (100m to 200m away)	1997	404	0
	Sinapis alba	400m from 1995/96 trial	1997	40	0
	S.alba	200m from 1996/97 trial	1998	15	0
MELBOURN	*S.officinale*	100m from 1995/96 trial	1997	80	0
	S.alba	Surrounding the 1998/99 trial (between 10m and 50m away)	1999	1677	0
	S.alba	In field margins of the 1998/99m site	2000	60	0
EAST WINCH	*S.arvensis*	Growing amongst the 1998 trial	1998	122	0
	R.raphanistrum	Growing amongst the 1998 trial	1998	32	0
	S.arvensis	In wheat herbicide efficacy trial	1999	170	0
	R.raphanistrum	In wheat herbicide efficacy trial	1999	112	0

Table 8.2. PCR testing of related crucifers for the presence of transgenes

SITE	SPECIES	LOCATION OF PLANTS	YEAR TESTED	TRANSGENE TESTED FOR	NUMBER TESTED	NUMBER OF SAMPLES POSITIVE FOR TRANSGENE
DEVON 1995	*S.officinale*	In field margin (10m from trial)	1996	BAR	80	0
		In field margin (10m from trial)	1997	BAR	50	0
LINCS	*S.officinale*	In field margin (20m from trial)	1996	BAR	24	0
1995	*S.arvensis*	In field margin (20m from trial)	1996	BAR	144	0
		In field margin (20m from trial)	1997	BAR	16	0
LINCS 1996	*S.arvensis*	In release field	1997	BAR	4	0
YORKS 1995	*C.bursa-pastoris*	In release field	1996	BAR	41	0
	A.petiolata	In field margin (10m from trial)	1996	BAR	181	0
YORKS 1995	*A.petiolata*	In field margin (10m from trial)	1997	BAR	105	0
NIAB	*S.arvensis*	1-200m from trial	1998	BAR	100 *	0
				PAT	100 *	0
				GLY	100 *	0
NIAB	*R.raphanistrum*	200m	1998	BAR	100 *	0
				PAT	100 *	0
				GLY	100 *	0
	S.arvensis	Adjacent to area where 1997/98 GM trial was	1999	BAR	200 *	0
				PAT	200 *	0
				GLY	200 *	0
	S.arvensis	Adjacent to 1996/97 trial	2000	BAR	500 *	0
				PAT	500 *	0
				GLY	500 *	0
NEWBURY	*Brassica.rapa* (stubble turnips)	400m from GM trial	1998	BAR	300 *	0
	B.oleracea (kale)	400m from GM trial	1998	BAR	200 *	0
MELBOURN	*A.petiolata*	Adjacent to 1995/96 trial (100m to 200m away)	1997	BAR	461	0
	S.alba	400m from 1995/96 trial	1997	BAR	40	0
	S.officinale	100m from 1995/96 trial	1997	BAR	80	0

	S.alba	200m from 1996/97 trial	1998	BAR	100 *	0
	S.alba	Surrounding the 1998/99 trial (between 10m and 50m away)	1999	BAR	200 *	0
	S.alba	In field margins of the 1998/99m site	2000	BAR	600 *	0
EAST WINCH	*S.arvensis*	Growing amongst the 1998 trial	1998	BAR	500 *	0
				GLY	500 *	0
	R.raphanistrum	Growing amongst the 1998 trial	1998	BAR	300*	0
				GLY	300*	0
	S.arvensis	In wheat herbicide efficacy trial	1999	BAR	170*	0
				GLY	170*	0
	R.raphanistrum	In wheat herbicide efficacy trial	1999	BAR	600*	0

* Samples tested as bulks of 10 leaves; for 100 leaves, 10 bulks of 10 leaves were tested. Results of positive samples are shown as numbers of batches of 10 testing positive for the transgene.

Table 8.3. Background testing of cruciferous weeds prior to GM OSR

SITE	SPECIES	TRANSGENE TESTED FOR	NUMBER TESTED	NUMBER OF +VE PLANTS
BERKHAMSTEAD	*S.alba*	Uc FatB1	100	0
	R.raphanistrum		50	0
FAKENHAM	*R.raphanistrum*	Uc FatB1	30	0

Table 8.4 Herbicide screening of seeds taken from synchronously flowering cruciferous weeds

SITE	SPECIES	DISTANCE FROM GM RAPE	HERBICIDE USED	YEAR TESTED	NUMBER SCREENED	% TOLERANT
DEVON 1995	*R.raphanistrum*	200m	Glufosinate	1996	29	0
	*B.rapa (*turnip*)*	200m	Glufosinate	1996	282	0
EAST WINCH	*S.arvensis*	In crop following GM trial	Glufosinate	1998	170	0
	R.raphanistrum	In crop following GM trial	Glufosinate	1998	112	0
LINCS 1995	S.arvensis	Growing amongst GM rape crop	Glufosinate	1996	204	0
	R.raphanistrum	Growing amongst GM rape crop	Glufosinate	1996	1	0
	S.officinale	Growing amongst GM rape crop	Glufosinate	1996	2763	0
LINCS 1996	S.arvensis	Growing amongst GM rape crop	Glufosinate	1997	107	0
MELBOURN	B.rapa	100m from single GM plant, 700m crop	Glufosinate	1999	7700	0
	B.rapa	100m from single GM plant, 700m crop	Glufosinate	2000	500	0
	S.alba	Surrounding the 1998/99 trial (10 – 15m from GM trial)	Glufosinate	1999	1677	0
	S.alba	50m from crop	Glufosinate	1999	306	0
NEWBURY	B.rapa (stubble turnips)	400m from GM trial	Glufosinate	1998	1180	0
	B.oleracea (kale)	400m from GM trial	Glufosinate	1998	1180	0
N.I.A.B.	S.arvensis	Growing in GM plot of glyphosate tolerant rape	Glyphosate	1998	826	0
	S.arvensis	Adjacent to area where 1997/98 trial was	Glufosinate	1999	222	0
	S.arvensis	Growing in GM plot of Glufosinate tolerant rape	Glufosinate	1999	441	0

Table 8.4 contd……..

SITE	SPECIES	DISTANCE FROM GM RAPE	HERBICIDE USED	YEAR TESTED	NUMBER SCREENED	% TOLERANT
NIAB	S.arvensis	Growing in GM plot of glyphosate tolerant rape	Glyphosate	1999	392	0
	S.arvensis	Growing in GM plot of glufosinate tolerant rape	Glufosinate	2000	762	0
	S.arvensis	Growing in GM plot of glyphosate tolerant rape	Glyphosate	2000	676	0

8.4 DISCUSSION

These results suggest that if hybridisation occurs between oilseed rape and *R.raphanistrum, S.arvensis* or *S.alba* it is at a very low frequency and that there is poor survival of hybrids. The results this study supports previous work where hybrid formation only occurred under forced conditions or where male sterile oilseed rape was used as the female parent. Results of the work by Lefol *et. al.*(1996) where 6 hybrids were obtained from 50,000 flowers from male sterile oilseed rape growing amongst *S,arvensis* show that it is possible for a small number of hybrids to be produced under the most favourable conditions. In a natural situation with male fertile plants there would be pollen competition from the oilseed rape plants and *S.arvensis* pollen would be even less likely to be successful in fertilisation.

Despite the two species growing together in rapeseed fields for more than fifty years, no hybrids between *B.napus* and *S.arvensis* have yet been reported under natural conditions, so transgene movement from *B.napus* into *S.arvensis* seems highly unlikely. Likewise hybrids between *B.napus* and *S.alba* have never been found under natural conditions, and these results support the general agreement that natural gene flow is unlikely to occur between these two species.

No evidence of hybridisation between *B.napus* and *R.raphanistrumn* was found over six years at any of the sites monitored. However the numbers of populations of *R.raphanistrum* found were few, and therefore data on this species is limited. This weed was only found at two of the sites where glufosinate tolerant rape was grown, and at three sites where high laurate rape was grown. Often these *R.raphanistrum* populations were only found in the year the GM rape was grown, and were well controlled in following crops. Thus information on hybrids appearing in subsequent years is limited, and it would be necessary to carry out further monitoring at the sites where *R.raphanistrum* was found in subsequent crops of oilseed rape, in order to test for the presence of hybrids in these crops. As naturally occurring hybrids between *B.napus* and *R.raphanistrum* have been reported (Darmency *et. al.,* 1995; Chevre *et. al.,* 1999) this species is a more likely candidate for the possibility of hybridisation than either *S.arvensis* or *S.alba.*

There are strong reproductive barriers to inter-specific hybridisation between oilseed rape and most of its related cruciferous weeds. The probability of inter-specific gene flow is therefore very low, but nevertheless possible (Raybould & Gray, 1993). If these barriers are overcome and inter-specific hybridisation does occur, weedy characteristics may be transferred by introgression into the crop plant, making it fitter in natural or semi-natural habitats. Alternatively transgenes could be transferred to weeds making them more difficult to eradicate in agricultural environments or affecting their ecology in natural environments. The results of this research have shown that this is not likely to be the case with the species *S.arvensis* and *S.alba.* More data needs to be obtained on the spontaneous hybridisation between *B.napus* and *R.raphanistrum* under natural field conditions.

Rare hybridisation events occurring at very low frequencies would not necessarily be detected using these methods. It was not possible or desirable to test all plants in populations, since this would have artificially disrupted the monitoring sites. However the combination of tests and sites used in this would have allowed significant hybridisation events and their consequences to be detected.

Weedy *B.rapa* was found at only one of the sites monitored. Results of testing of this species are discussed in Chapter 10.

9. HYBRIDISATION BETWEEN OILSEED RAPE AND TURNIP RAPE

INTRODUCTION

In autumn 1998 a block of winter turnip rape (cv. Debut) was sown directly adjacent to a GM oilseed rape trial containing blocks of glufosinate and glyphosate tolerant varieties (Figure 9.1). The turnip rape was separated by approximately 5m on one side from a block of glufosinate tolerant rape and on the other side from a discard barrier plot of the conventional variety Apex. This study investigated gene flow from turnip rape into oilseed rape by screening seedlings grown from seed samples of oilseed rape by morphology. The reciprocal cross was also examined by measuring the proportion of GM seeds at set distances into the turnip rape plot..

9.1 MATERIALS AND METHODS

Sampling of oilseed rape seeds and Screening seeds for hybrids

20 main racemes at three intervals along the edge of a plot of glufosinate tolerant rape, and the discard barrier plot of Apex were taken at maturity. Both plots were adjacent to a plot of turnip rape (*B.rapa*), and sampling points were approximately 1m away from the edge of the turnip rape plot.
Seeds were removed from pods by hand and germinated in batches of 100 seeds per plastic half tray (H. Smith Plastics Ltd) in Shamrock general potting compost, under glasshouse conditions of 16 hours daylight @ 18-22°C, 8 hours night @16°C. Supplementary lighting was provided in banks of six fluorescent tubes (5ft- 65 to 80W and 8ft- 100 to 125W). Seedlings were examined individually by their morphology for hybrids. These were identified by eye as being a slightly paler green, less glaucous and more hairy (both leaf and stem) than oilseed rape. Putative hybrids were confirmed as such by flow cytometry. 100 individuals not showing hybrid morphology were randomly selected and examined by flow cytometry for the presence of hybrids as a control.

Sampling of turnip rape seeds and *Screening seeds for herbicide tolerance*

Prior to harvest of the turnip rape plot seed samples were removed by hand at set distances from the interface with the glufosinate oilseed rape into the plot of turnip rape. Seeds were sampled at distances (0m, 5m, 15m, 20m, 30m, 40m, 50m) along three linear transects.

Seeds were tested for glufosinate tolerance using the same procedures described in 4.6.

9.3 RESULTS

Results of the morphological identification of *B.napus x B.rapa* hybrids are shown in Table 9.1. All identified hybrids were confirmed as such by flow cytometry and none were wrongly identified. No hybrids were identified by ploidy analysis in the 100 random control samples not showing hybrid morphology. A higher frequency of hybridisation was detected in the Apex samples than in the glufosinate tolerant variety PGS W5.

With *B.rapa* as the female parent hybridisation between the 0.8ha plot of GM glufosinate tolerant oilseed rape and the 0.12ha plot of turnip rape averaged 0.25% at 1m (Table 9.2). Levels declined to an average of 0.008% at 41m, and no herbicide tolerance was detected at 51m.

9.4 DISCUSSION

The higher frequency of hybridisation detected in the Apex than the glufosinate tolerant variety may have occurred due to a slight difference in flowering times between the two varieties. The turnip rape was the first plot to start flowering, followed by the Apex and then the PGS W5 plot. A more likely explanation would be the establishment of the two varieties. The plot of Apex was badly damaged by pigeons and therefore had patchy and poor establishment with small plants. Pollen production from this plot therefore was not at its optimum, allowing turnip rape pollen to compete well. This could also explain the lower levels of cross-pollination occurring with *B.rapa* as the female parent. *B.napus* has been shown in previous studies to be the preferable parent for hybrid formation. However this study shows that hybridisation will occur between turnip rape and oilseed rape and thus there is a potential for gene flow and cross contamination between these crops. This study was not sufficient to indicate relative frequencies compared with outcrossing in oilseed rape varieties. However Downey (1999) suggested that inter-specific hybridisation between *B.napus* and *B.rapa* occurred at similar frequencies to within species outcrossing.

Figure 9.1 Hybridisation of oilseed rape by turnip rape – plan of experimental layout

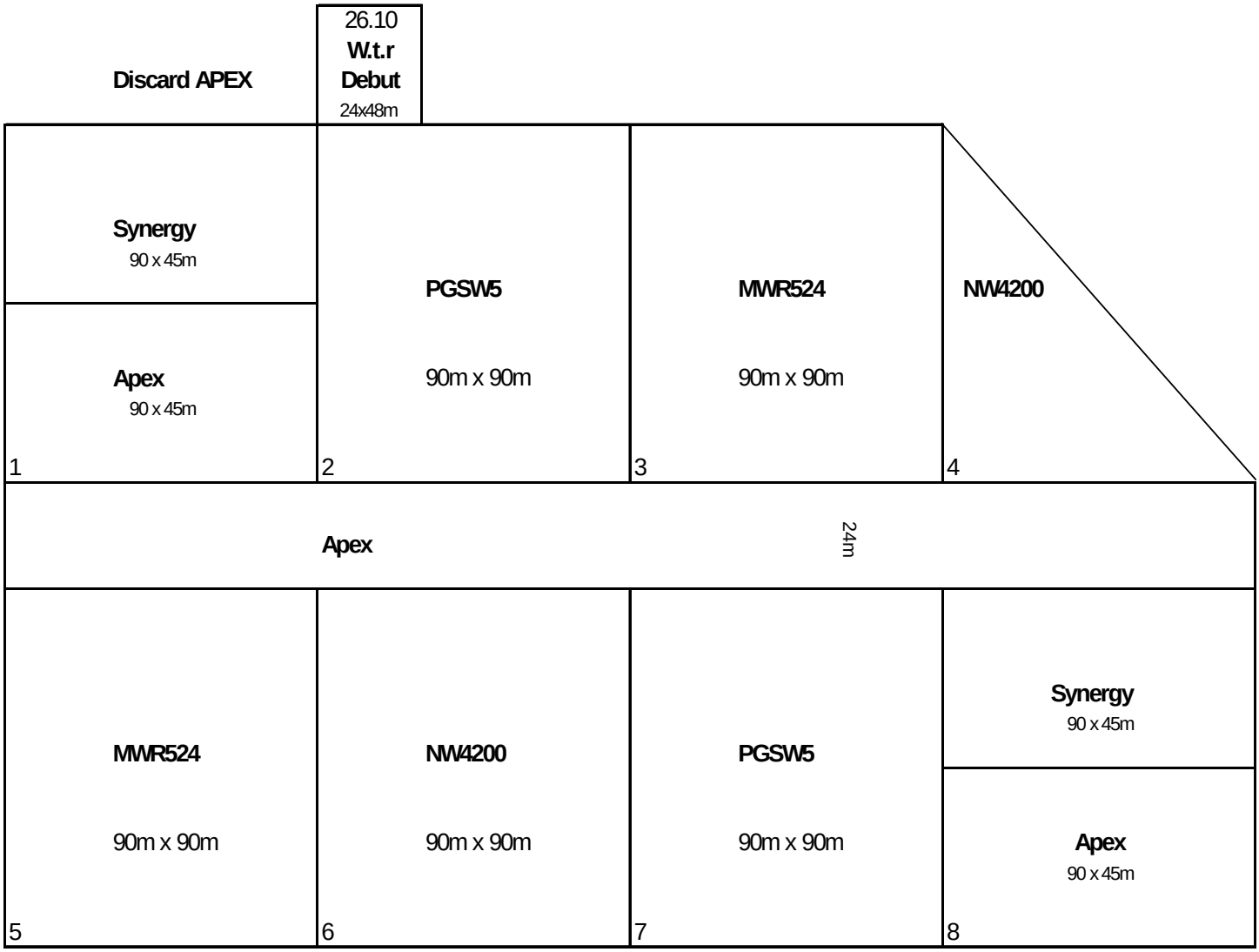


Table 9.1 Numbers of *B.napus x B.rapa* hybrids found from oilseed rape seeds

OILSEED RAPE VARIETY	NUMBER OF SEEDLINGS SCREENED	NUMBER OF HYBRIDS FOUND	% HYBRIDISATION
Apex	4800	47	0.98
PGS W5	9000	24	0.27

Table 9.2 Numbers of *B.rapa x B.napus* hybrids found in seed samples from turnip rape.

DISTANCE FROM EDGE OF OSR PLOT	TRANSECT	MEAN NUMBER OF TOLERANT SEEDLINGS IN 1000	% HYBRIDISATION
1	1	2.3	0.23
6		2.3	0.23
11		2.8	0.28
16		1.0	0.10
21		0.3	0.03
31		0.0	0.00
41		0.0	0.00
51		0.0	0.00
1	2	1.75	0.18
6		1.25	0.13
11		1.25	0.13
16		0.50	0.05
21		0.25	0.03
31		0.25	0.03
41		0.00	0.00
51		0.00	0.00
1	3	3.25	0.33
6		0.50	0.05
11		0.25	0.03
16		1.25	0.13
21		0.75	0.08
31		0.00	0.00
41		0.25	0.03
51		0.00	0.00

10 GENE FLOW AND INTROGRESSION BETWEEN *BRASSICA NAPUS* AND WEEDY *BRASSICA RAPA*

10.1 INTRODUCTION

B.rapa (wild turnip) is a self-incompatible annual or biennial plant which is reasonably common in weedy habitats throughout Britain and Ireland (Plates 6.1 and 6.2). It is locally abundant on roadsides, in arable fields, on waste ground and particularly along riverbanks. It has a chromosome number of 2n=20 and it is thought to be one of the progenitor species (AA) of oilseed rape (AACC) along with *B.oleracea* (CC). Perhaps because the genome of *B.rapa* is in common with part of the oilseed rape genome, the two species are sexually compatible and hybridise readily under certain conditions. The resulting hybrids are triploid with 2n=29, of genome constitution AAC. Oilseed rape can still be resynthesised by hybridisation of the two parental species (Prakash and Hinata, 1980).

B.rapa ssp. *rapa* (turnip) and *B.rapa* ssp. *oleifera* (turnip rape) are both grown as crop plants in the US and Europe. In addition, *B.rapa* ssp. *sylvestris* (wild turnip, Bastard turnip, Bargeman's cabbage or navew) is found in both agricultural and non-agricultural environments as a weed and as feral populations. It is the most likely of the cruciferous weed species found in the UK to form hybrids with oilseed rape, and is the species on which the largest number of interspecific hybridisation studies have concentrated (Jørgensen and Andersen, 1994; Landbo *et. al.,* 1996; Scott and Wilkinson, 1998).

Weedy *B.rapa* is smaller than *B.napus* and has open flowers overtopping the flower buds. In *B.napus* the open flowers are overtopped by the flower buds. *B.rapa* has brighter green leaves, which are hairier than those of *B.napus.* Interspecific hybrids are sometimes more vigorous than *B.rapa* and can be very close in appearance to *B.napus* but can be distinguished by their hairier leaves.

Plate 10.1: Weedy *B.rapa* growing in an oilseed rape field at Patrington

Interspecific hybrids with winter varieties of *B.napus* inherit a requirement for vernalisation. The *B.rapa* weedy parent however, does not need a cold treatment to initiate flowering (Jorgensen & Andersen, 1994).

Hybridisation between oilseed rape and wild *B.rapa* has been reported on numerous occasions (Jørgensen *et. al.*, 1996; Scott and Wilkinson, 1998). Hybrid frequencies in populations vary between studies and are much higher where *B.rapa* occurs as a weed in oilseed rape crops, rather than in its 'wild' habitat along riverbanks. Jørgensen *et. al.* (1996) measured frequencies of spontaneous hybridisation between oilseed rape and *B.rapa* in experimental fields in Denmark and found that the frequency of interspecific hybridisation varied significantly with experimental design. Frequencies of hybrids in progenies ranged from 9% in a 1:1 mix of *B.rapa* and *B.napus* with oilseed rape as the mother plant, to 93% where the mother plants were single isolated *B.rapa* plants surrounded by oilseed rape. *B.rapa* is highly self-incompatible a single plant will accept pollen from other plants and even pollen from other species more easily than its own.

Spontaneous hybridisation between *B.napus* and *B.rapa* has been observed in agricultural fields and also occasionally in nature (Jorgensen & Anderson, 1994; Jorgensen *et al,* 1996; Landbo *et al,* 1996). These observations show a large amount of variation in the frequency of hybrid seeds produced, depending on the spatial structure and flowering time of the two species. Interspecific pollination is more likely to occur where *B.rapa* is well spaced out in the field. Where *B.rapa* occurs in dense patches, hybrids are less likely to occur because of pollen competition.

Scott & Wilkinson (1998) measured gene flow from oilseed rape into natural populations of *B.rapa* growing outside field boundaries along the riverbanks of the Thames. A low level of hybridisation was found (between 0.4 – 1.5%) in populations growing adjacent to oilseed rape fields. However they found that less than 2% of the hybrid seedlings survived. Scott and Wilkinson (1998) therefore suggested that establishment of GM *B.napus* x *B.rapa* plants would be at very low frequencies and that introgression of transgenes from *B.napus* into wild *B.rapa* populations would be very unlikely or at most would be very slow.

Previous hybridisation studies between oilseed rape and weedy *B.rapa* have focussed on the hybridisation and introgression of transgenes into weedy *B.rapa* under controlled conditions, but have not examined the extent of historical introgression with conventional oilseed rape varieties under field conditions. However some studies in Denmark have recently examined introgression from organically grown oilseed rape into weedy *B.rapa* using AFLP markers (R.B. Jørgensen, pers. com.). L.B.Hansen and R.B. Jørgensen (pers. com.) screened *B.rapa* growing in an organic rape crop with 24 species-specific AFLP markers. From 102 plants screened, they found 44 appeared to be introgressed beyond the F_1 generation.

The review by Gray (1999) of the risk assessment on the PGS hybrid herbicide tolerant oilseed rape considered that the risk of gene transfer to *B.rapa* had been underestimated and that further research on the mechanisms, frequency and consequences of introgression of genes from *B.napus* to *B.rapa* should be undertaken. The second monitoring project was amended in order to gain more information on the extent or possibility of introgression from oilseed rape into weedy *B.rapa*, and to study crop and weed interactions under agricultural practices.

In 1998 it was brought to our attention by a consultant working in the North Humberside area that wild turnip or bargeman's cabbage was a weed problem in oilseed rape in this area and he suspected that hybridisation was occurring between the two species. We examined sites near

Plate 10.2: *B.rapa* seedlings growing in an oilseed rape field at Patrington

Plate 10.3: Mature *B.rapa* seedpods under the immature oilseed rape canopy at Patrington

Patrington, North Humberside and observed plants of intermediate morphology between oilseed rape and wild turnip. The wild turnip appeared to be poorly controlled by the standard herbicides used on oilseed rape and farmers were using additional Fortrol (cyanazine) treatments in an attempt to control the weed. The wild turnip appeared in patches in fields that remained in much the same place from year to year. At some sites field margin populations were observed but plants were only found in cultivated ground. One of the sites had been growing conventional oilseed rape for many years and historical records had been kept of all varieties grown in the field for the last decade. AgrEvo (now Aventis) expressed interest in this site for a study of the use of HT oilseed rape to control wild turnip.

In the autumn of 1998 a trial area of 200m x 50m of glufosinate tolerant transgenic oilseed rape (*BAR* construct) was established in an area of the field known to have a recurring problem with *B.rapa* as a weed. The purpose of the trial was to study the control of weedy *B.rapa* with glufosinate but the opportunity was also taken to examine the interactions between weedy *B.rapa* and oilseed rape under agricultural conditions. Hybridisation between glufosinate tolerant oilseed rape and *B.rapa* was examined and the morphology and survival of interspecific hybrids and other crosses was also studied. In addition the possible historical introgression between the weedy *B.rapa* and the individual varieties grown in the field in the last ten years was investigated by using the amplified fragment length polymorphism (AFLP) technique. Individuals from the *B.rapa* population were screened for bands specific to oilseed rape to try and detect any evidence of past introgression having taken place.

Previous hybridisation studies between oilseed rape and *B.rapa* have focussed on the introgression of transgenes into weedy *B.rapa* but have not examined the extent of past introgression with conventional varieties under field conditions. Any evidence of historical introgression may give an indication of the likelihood of long-term persistence of oilseed rape genes in weedy *B.rapa* populations.

10.2 MATERIALS AND METHODS

10.2.1 Site description: Patrington

B.rapa has given a localised weed problem in the Humberside area of Yorkshire in the UK for over 30 years and, according to local farmers has been growing in oilseed rape fields for at least 10 years. The site used in this study was an arable field at Patrington, North Humberside. The farm principally grows oilseed rape and cereal crops, and pigs are also reared. Records have been kept of all varieties of oilseed rape grown in this field for since 1988. These were: Cobra (1988), Rocket (1992) and Apex (1996).

B.rapa plants at the site grow only within the agricultural field, not outside it (Plates 6.1 and 6.2). *B.rapa* is poorly controlled by the standard herbicides used on oilseed rape and farmers use additional Fortrol (cyanazine) treatments in an attempt to control the weed. *B.rapa* appears in patches in the fields that remain in much the same place from year to year (B. Beeney, pers. com.).

A trial area of glufosinate-ammonium tolerant transgenic oilseed rape (BAR construct), variety PGSW5, was drilled in Autumn 1998 by AgrEvo in an area of the field where *B.rapa* was known to have been particularly abundant over the last ten years. As part of a herbicide efficacy trial, half of the field was treated with glufosinate–ammonium (Liberty™), and the other half was treated with the conventional herbicides; Butisan followed by the graminicide Laser. A central strip of the field was left untreated. Plots were marked out for sampling purposes as in Figure 6.1.

10.2.2 DNA amount testing by flow cytometry

DNA level testing by flow cytometry was used to confirm hybrid (2n=29) or backcross (2n=29-38) status of individual plants. Samples from the site were tested for DNA amount by Plant Cytometry Services, The Netherlands, according to methods of Brown *et al.,* (1991) (4.8 Materials and Methods).

Flow cytometry results are presented as DNA ratios derived by the *Brassica* sample by the peak median of the standard peak (*Lactuca sativa* leaf material). The DNA ratio of a *B.rapa* plant gave ratios between 0.17 and 0.20, *B.napus* between 0.39 and 0.44, and hybrids 0.29 to 0.33 (Figure 6.2). This enabled *B.rapa, B.napus* and hybrid types to be easily distinguished, and the method was used as confirmation both of morphological identification and the identification of hybrids by herbicide spot testing.

10.2.3 AFLP analysis

The AFLP technique (4.8, Materials and Methods) was used i) to try to establish differences in band patterns between individual oilseed rape cultivars, and ii) to identify whether specific varietal markers were present in the weedy *B.rapa* population, or in hybrids with *B.napus*. This might give some indication of whether introgression has occurred since 1988 when these specific varieties of oilseed rape had been grown in the presence of *B.rapa* as a persistent weed. Gels were scored as in 4.8.6, (Materials and Methods).

Reference samples were cultivars of *B.napus, B.rapa* (turnip rape) and *B.oleracea (*kale, Brussels sprouts and cabbages). Field samples were taken from both mature plants and from plants grown in the glasshouse from seeds derived from the soil seedbank. All plant samples taken from the field site were tested by flow cytometry in order to determine their DNA amounts before AFLP analysis.

10.2.4 Soil sampling

Soil cores were taken from the Patrington site using a soil gauge auger to investigate proportions of oilseed rape, *B.rapa* and hybrid seeds in the soil seed bank. 50 soil cores (2.5cm diameter, 35cm depth) were taken from each fixed quadrat within the six plots, CONV 1 and 2, LL 1 and 2 and Untreated 1 and 2 (Figure 10.1). Fixed quadrats were $3m^2$. The 50 cores from each quadrat were placed in plastic bags and weighed. Sampling was carried out on two occasions, the first was pre-GM rape (18/02/99) and the second was post-GM rape (03/11/99).

The bags of soil were divided into 4 trays each and mixed well. The soil was spread out, watered and placed in the glasshouse under clear plastic to prevent dehydration. Trays were kept moist for 10 days to allow seeds to germinate. Emerging seedlings were transplanted to pots and the soil was mixed again to allow more seeds to germinate. Proportions of oilseed rape, *B.rapa* and hybrid seeds in the soil seed bank were then estimated from germinated seeds. Samples were taken on four occasions: February 1999 (before harvest of the GM crop), November 1999 (immediately after harvest of the GM crop), February 2000 (in the following wheat crop) and November 2000 (after harvest of the wheat crop). These sampling dates were chosen to establish any changes in the proportions of *B.rapa, B.napus* and hybrid seeds in the soil seedbank over time and in different crops. Estimates of numbers of seeds per m^2 were carried out according to P. Lutman (pers. com.).

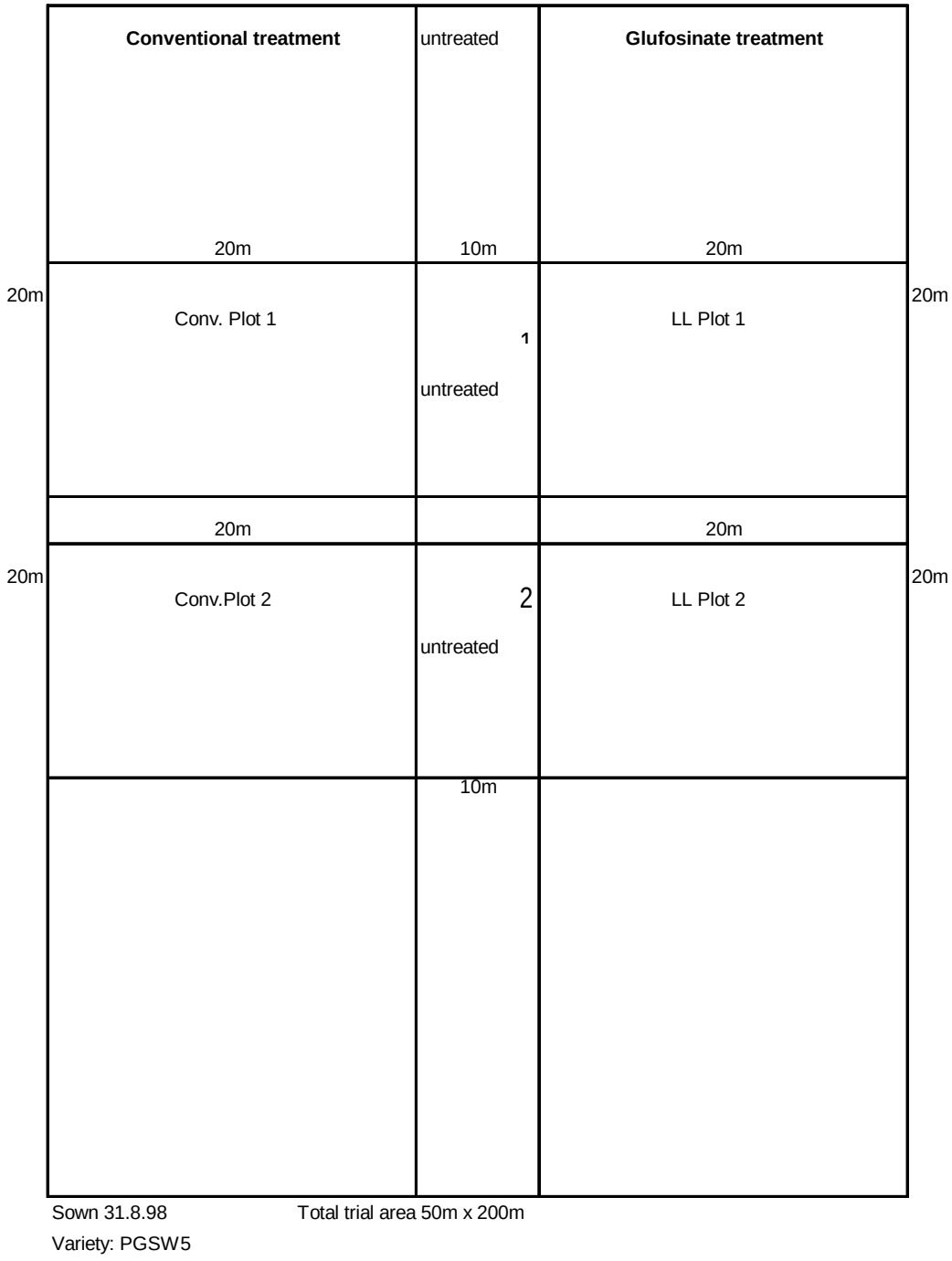


Figure 10.1: Trial plan of GM oilseed rape variety PGS W5 at Patrington in 1998/99. Sampling plots were CONV 1, CONV 2, LL 1, LL 2, untreated 1 and untreated 2.

10.2.5 Seed sampling from *B.napus* and *B.rapa*

Plants with *B.rapa* morphology growing in the oilseed rape crop were selected and tagged early in the growing season in November or December, so that seed samples could be collected from them at maturity. Leaf samples from these plants were examined by flow cytometry to confirm their identity. Morphology and growth habit of these plants was described on each visit. Notes were also made of the spatial distribution of the tagged plants in relation to other *B.rapa* plants and to the oilseed rape crop. All the mature seedpods were harvested from each selected plant. The seeds from each plant were germinated (4.7.2, Materials and Methods) and the progeny examined for hybrids by spraying with glufosinate (4.7 Materials and Methods).

All the seedpods from four *B.napus* plants growing in an area of the field densely infested by *B.rapa* were also sampled to look for reciprocal hybridisation. The seeds from the individual plants were mixed together and 7512 seeds were germinated in the glasshouse. The seedlings were examined morphologically for hybrids. Hybrids were identified as being a slightly paler green, less glaucous and hairier (both leaf and stem) than oilseed rape seedlings. One hundred seedlings with oilseed rape morphology were randomly selected and tested by flow cytometry to check that hybrids were not being missed by using morphology alone.

10.2.6 Post-harvest sampling in 1999

The crop following the trial of GM oilseed rape was wheat, which was drilled in late September 1999. When the wheat had started to establish in October 1999, ten 1m^2 quadrats were placed in areas where *B.rapa*, *B.napus* and putative hybrids were seen. Every *Brassica* plant was herbicide spot tested in each quadrat for tolerance to glufosinate. Leaf material from each plant was also sent for analysis by flow cytometry.

Sixty plants with the morphology of *B.rapa* and 60 putative hybrids were selected from one area of the field and transplanted to pots for further analysis. DNA levels of these plants were confirmed by flow cytometry for these plants.

10.2.7 Determination of DNA levels of the progeny of hybrid plants

Seed was collected from four *B.napus* x *B.rapa* hybrids whose identity had been checked by flow cytometry. This seed enabled an assessment to be made of the nature of the progeny produced by hybrids under natural conditions and the extent of backcrossing to *B.rapa.* The seeds were grown and spot tested for glufosinate tolerance to identify those with *B.napus* male parentage (ie those produced from backcrosses to *B.napus*). Susceptible plants were then tested by flow cytometry to determine their DNA levels.

10.2.8 Pollination of *B.rapa* bait plants by transgenic *B.napus*

This experiment was designed to establish differences between *B.rapa* individuals in their ability to accept pollen from different oilseed rape varieties and to examine their ability to produce viable hybrids when isolated from other *B.rapa* plants. The experiment simulated the effect of high pollen pressure from oilseed rape on individual *B.rapa* plants and so assessed interspecific hybridisation under optimum conditions. It was carried out at NIAB, Cambridgeshire in the BRIGHT project experimental plots.

Twelve *B.rapa* plants (confirmed by flow cytometry) were removed from the Humberside field trial on 26/04/99 and transplanted into pots. Plants with some unopened flower buds were selected, and any open flowers were removed. On 28/04/99 six plants were placed at 15m intervals between plots of glufosinate and glyphosate tolerant oilseed rape along the interface of the two varieties, and six were placed between plots of glufosinate and imidazolinone (non transgenic) tolerant rape. Plants were watered by hand in the field when necessary.

Flowering of the oilseed rape coincided with flowering of the *B.rapa* plants. When flowering was complete (08/05/99) the plants in pots were removed to a glasshouse and seeds allowed to develop. Seedpods were removed when mature and seeds per pod counted in 50 pods. Twenty-five seeds from each plant were germinated and tested for tolerance to the relevant herbicides. In order to test for tolerance to glufosinate and glyphosate in the same plant, it was necessary first to spot test with glufosinate, and then spray with glyphosate (Section 4.7).

Germination tests were carried out on healthy seed produced by the *B.rapa* bait plants, available after glufosinate testing. Four replicates of 25 seeds were germinated on petri dishes containing two filter papers (Whatman circles, Grade 1) and with 5 ml water. Samples were incubated in the dark at 25°C and scored for germination at intervals of 24 hours, 48 hours, 72 hours and 96 hours. After the final count, 1 ml of gibberellic acid (10ppm) was added to each petri dish and further germinated seeds counted after another 48 hours.

10.3 RESULTS

10.3.1 Flow cytometry

This method was successful in confirming the identity of *B.rapa* individuals and triploid hybrids were easily distinguished by flow cytometry due to their DNA amount being intermediate between *B.rapa* and *B.napus*.(see Fig 10.2). However backcross plants could not be identified easily by this method unless their DNA amount was different from either parent or the triploid hybrid. Backcrosses and hybrids resulting from unreduced gametes could also be identified by their DNA amount being higher than that of *B.napus.*

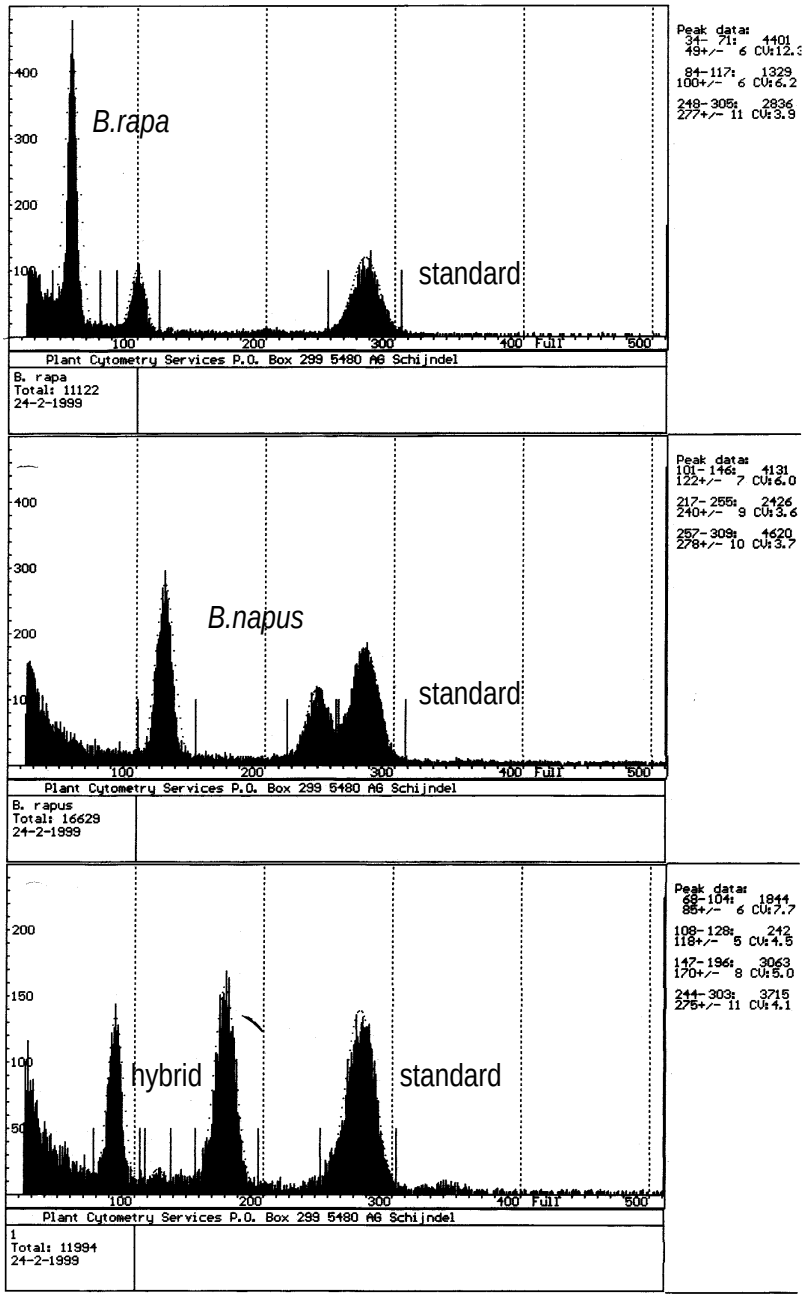


Figure 10.2: Histogram of DNA absorbances in nuclei of *B.rapa* (top), *B.napus* (middle) and hybrid (bottom), analysed by flow cytometry. The standard used was *Lactuca sativa.*

10.3.2 AFLP

The AFLP technique (4.8) was used as a means of detecting possible introgression of *B.napus* genes into *B.rapa.* Different primer sets were examined to establish the most effective combination of primers for the species used. The primer set MSEI1 PSTI1 gave the most polymorphisms between oilseed rape varieties, although the three varieties examined had over 90% of bands in common of the 100 bands scored. Despite this, discriminatory bands between oilseed rape varieties were observed (Plate 6.4). In Apex and Cobra 5% of bands were polymorphic while in Rocket 6% of bands differed from the other two oilseed rape varieties. Hybrids were identifiable due to bands in common with both parental species (Plate 6.4). In some cases it was possible to identify oilseed rape specific varietal bands. The *B.rapa* individuals were highly polymorphic, with 32% of bands polymorphic between only four individuals (Plate 6.5).

Three PCO plots from sample sets using the primer set MSE1 PST1 were informative. The first compares *B.rapa* (weedy and turnip rape), *B.napus, B.oleracea* and hybrids (Figure 10.3), the second more weedy *B.rapa,* hybrids and a backcross to *B.napus* identified by flow cytometry (Figure 10.4), and the third (Figure 10.5) a mixture of weedy *B.rapa* field samples, samples from the soil seed bank, hybrids and backcrosses to *B.napus.*

a) Weedy *B.rapa* samples taken from mature plants growing in the field, and turnip rape (*B.rapa*) show two distinct but closely related groups (Figure 10.3). *B.napus* varieties group together while *B.rapa* x *B.napus* hybrids taken from the field fall between the parents, although closer to *B.napus* than to *B.rapa*. *B.oleracea* also gave a distinct group.

b) The weedy *B.rapa* samples examined were a mixture of plants taken from the field and plants grown from the seedbank (Figure 10.4). In addition to *B.napus* and *B.oleracea*, two backcrosses to *B.napus* (identified by flow cytometry with DNA ratio of 0.39 or 0.40) are also shown. Again, the species separate distinctly. However two of the apparent weedy *B.rapa* plants from the seedbank fall close to the F_1 hybrid and backcross hybrids, which may indicate past hybridity.

c) In a third experiment, the weedy *B.rapa* individuals studied were much more polymorphic than in the other analyses (Figure 10.5). As a result much less clear-cut results have been obtained. Some *B.rapa* individuals plot with the hybrids and backcrosses to *B.napus*, similarly two *B.napus* individuals fall within this scatter of *B.rapa* and one within the *B rapa* area. It is likely however, that the sample of *B.napus* falling within the area of *B.rapa* was incorrectly identified.

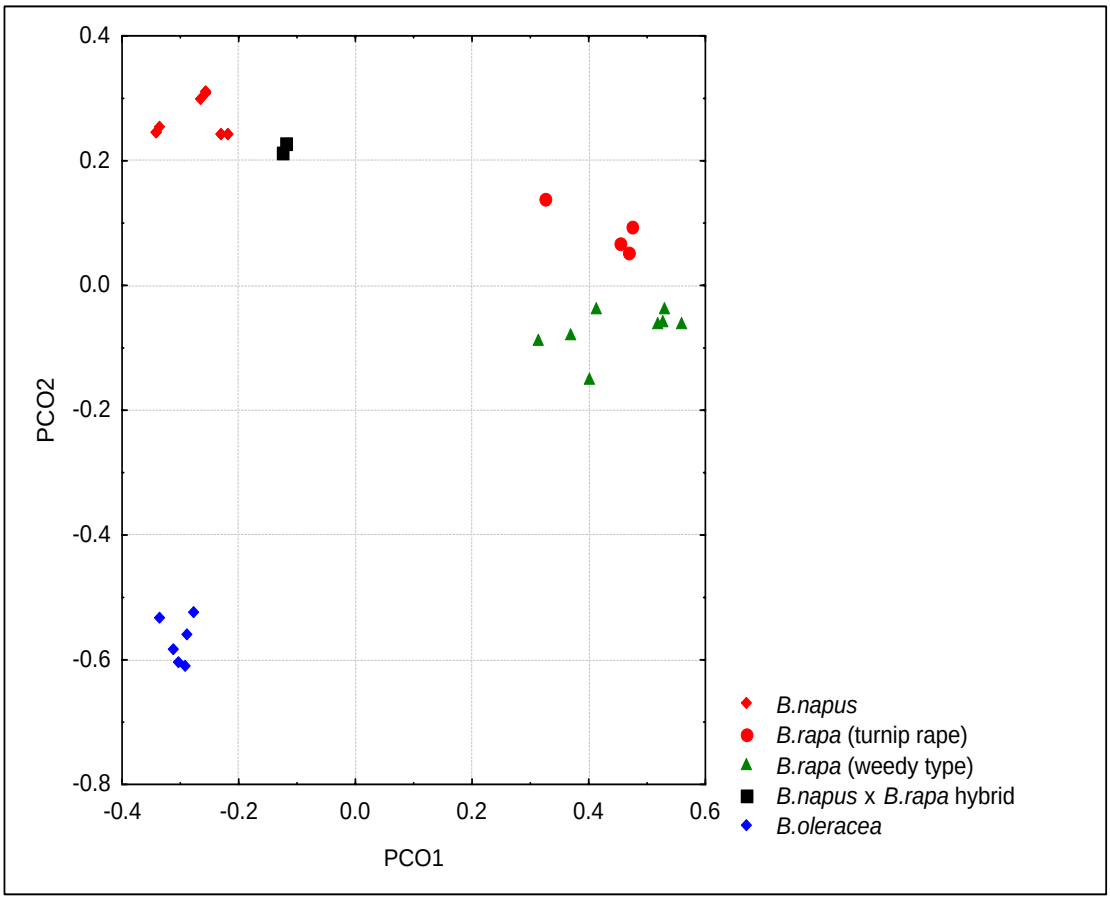


Figure 10.3: AFLP analysis of weedy *B. rapa*, turnip rape, *B.napus* and their hybrids. *B.oleracea* is also included.

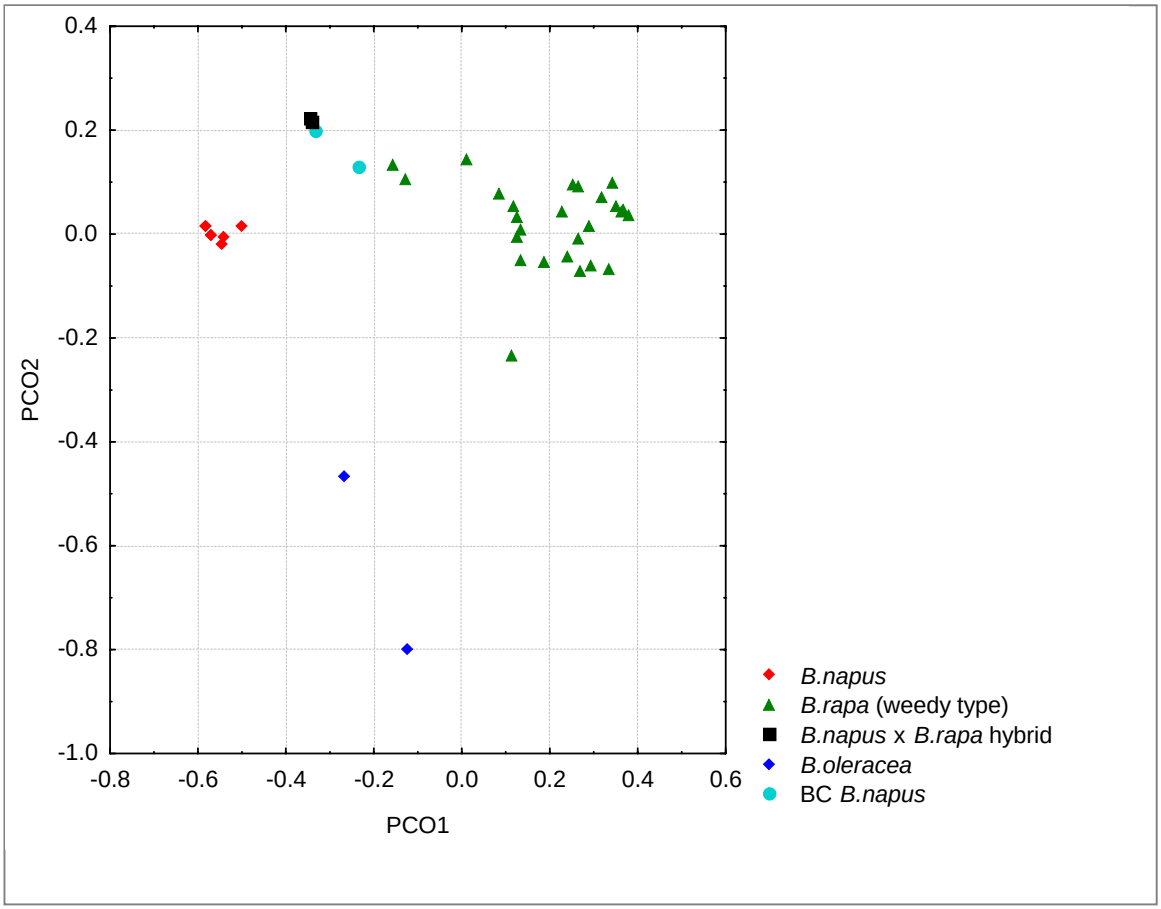


Figure 10.4: AFLP analysis of weedy *B. rapa*, *B.napus* and their hybrids and backcrosses to *B.napus*. *B.oleracea* is also included.

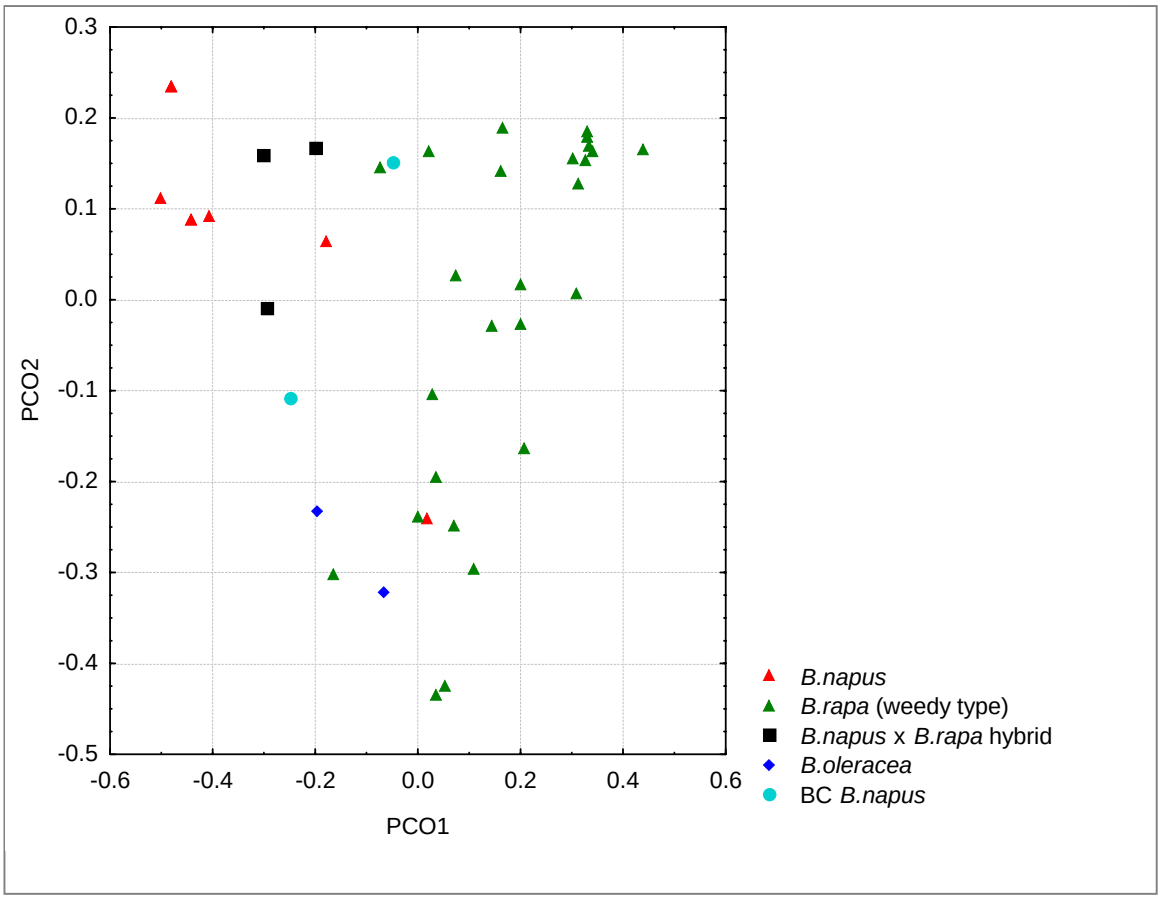


Figure 10.5: AFLP analysis of weedy *B. rapa, B.napus* and their hybrids and backcrosses to *B.napus. B.oleracea* is also included.

10.3.3 Soil Seed Samples

The 190 plants germinated from seed in soil cores from six plots (Figure 10.6), appeared, from flow cytometry, to represent *B.napus, B.rapa* and their F_1 hybrid. No plants were identified as backcrosses to *B.napus* from their DNA amounts. The number of oilseed rape seeds greatly increased in all cores following the oilseed rape harvest of 1999 but fell sharply by November 2000. The numbers of *B.rapa* seeds in the cores remained constant during the sampling period and were generally lower than oilseed rape. Hybrids occurred at a low frequency but apparently increased a little over time (Table 10.1). The proportion of hybrids ranged from 5.5 to 27.3, and apparently showed a marked increase over the sampling period. However, this may be a reflection of the associated crash in oilseed rape abundance in November 2000.

Table 10.1: Numbers of *Brassica* seeds found in 50 soil cores in plots at the Patrington site sampled at four times: February 1999 (F 99); November 1999 (N 99); February 2000 (F 00) and November 2000 (N 00)

LL Liberty treated plots
CON Conventional herbicide treated plots
UN Untreated plots

	B.rapa				*B.napus*				F_1 Hybrid			
Plot	F 99	N 99	F 00	N 00	F 99	N 99	F 00	N 00	F 99	N 99	F 00	N 00
LL1	1	6	4	0	2	13	14	1	1	1	1	0
LL2	2	1	3	0	6	21	10	1	0	1	2	1
CON1	2	0	1	1	2	7	3	0	0	0	0	2
CON2	0	1	0	1	2	8	14	2	0	1	0	1
UN1	3	4	2	3	3	7	5	2	0	1	2	1
UN2	3	0	1	5	5	1	0	0	1	0	0	1
TOTALS	11	12	11	10	20	57	46	6	2	4	5	6

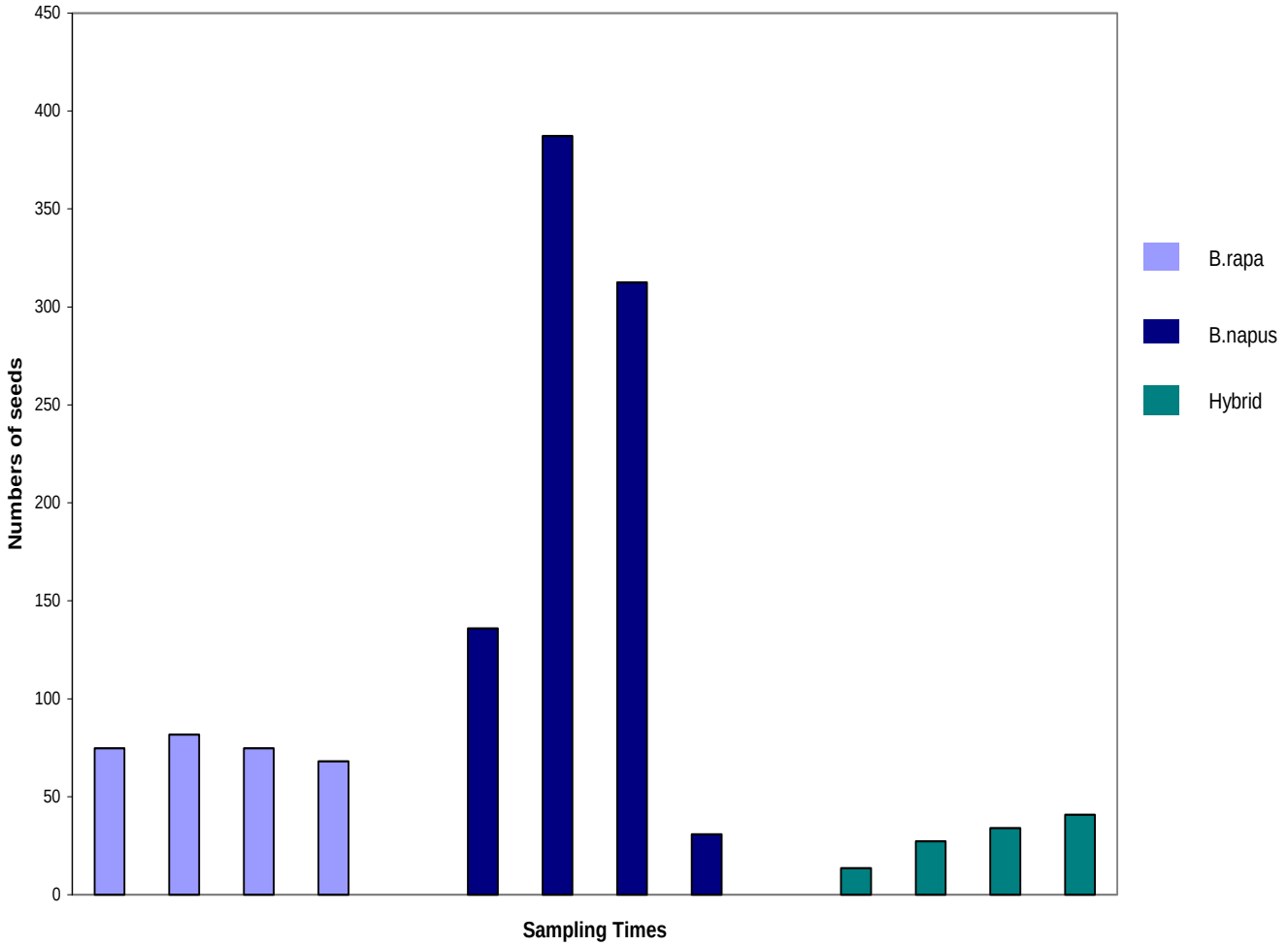


Figure 10.6: Numbers of *B.rapa, B.napus* and hybrid seeds in the soil seedbank per m^2 estimated from numbers found in soil cores at Patrington in February 1999, November 1999, February 2000 and November 2000 (mean of all plots).

Table 10.2: Numbers of hybrid seedlings from seeds harvested from individual *B.rapa* plants in the field at Patrington

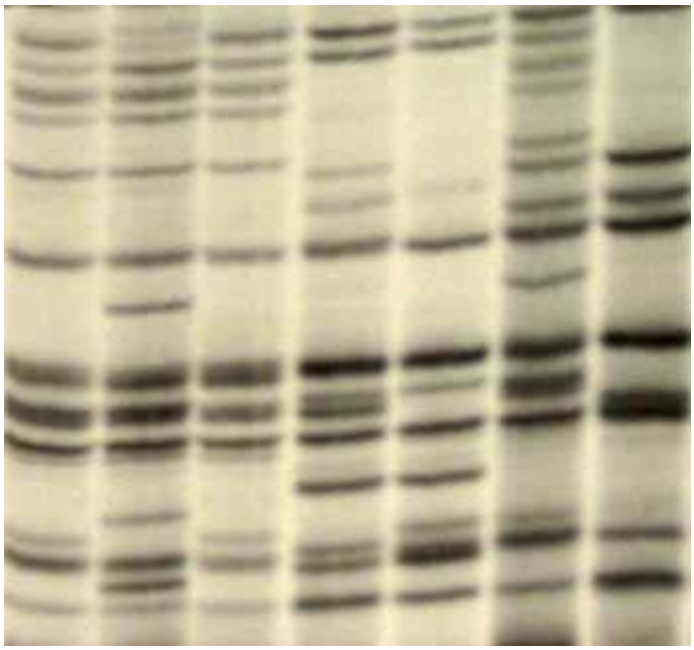
PLANT NUMBER	NUMBER OF PROGENY	NUMBER OF HYBRIDS*	PROXIMITY TO OTHER B.RAPA PLANTS
1	291	0	<1m
2	16	0	>4m
3	328	1	>1m
4	28	12	>2m
5	167	81	>2m

* Hybrids detected by their glufosinate-tolerance

Table 10.3: Quadrat counts and herbicide testing on post-harvest emerging *Brassica* plants at Patrington, November 1999

QUADRAT ($1M^2$)	NUMBER OF PLANTS TESTED	NUMBER OF *B.napus*	NUMBER OF *B.rapa*	NUMBER OF HYBRIDS
A	6	2	4	0
B	8	6 (2)	1	1
C	4	0	3	1 (1)
D	7	7 (3)	0	0
E	7	2 (1)	1	4
F	6	0	3	3
G	8	4	4	0
H	4	1	2	1
I	9	1	5	3 (2)
J	8	0	5	3 (1)
TOTAL	67	23 (6)	28 (0)	16 (4)

Figures in parenthesis are glufosinate-tolerant



Rocket Apex Cobra B rapa B rapa hybrid B rapa

Plate 10.4: A portion of an AFLP autoradiograph of the three oilseed rape varieties grown at Patrington (Rocket, Apex and Cobra), weedy *B.rapa* and a *B.napus* x *B.rapa* hybrid using the primer set MseI 1/ PstI 1. The hybrid shows bands in common with Apex, and also has bands in common with both parents.

10.3.4 Frequency of hybridisation between *B.rapa* and *B.napus*

The number of seeds collected and tested from individual plants of *B.rapa* and *B.napus* varied according to maturity at the time of seed sampling. Hybridisation frequencies in the field obtained from analysis of the progenies of individual *B.rapa* plants are shown in Table 10.2. The proximity of *B.rapa* plants to other plants did not appear to affect the extent of hybridisation. Plants 1, 2 and 3 for instance had only one hybrid in over 600 tested. Plants 2 and 3 produced no hybrids. By contrast, almost half the seeds taken from plants 4 and 5 were hybrid (93/195).

A total of 7512 seeds were germinated from *B.napus* plants growing adjacent to *B.rapa. N*o hybrids were identified on morphological criteria. Flow cytometry of 100 plants randomly chosen, from amongst them similarly indicated no hybrids.

10.3.5 Post GM harvest quadrat counts and hybridisation

A total of 67 plants in 10 quadrats germinated after the harvest of the GM oilseed rape crop, and after the following wheat crop was sown (Table 10.3). They consisted of 23 *B.napus*, 28 *B.rapa* and 16 hybrids as determined by flow cytometry. Glufosinate tolerance was detected in six *B.napus* plants and four hybrids. All these plants were found either on the side of the field that had been treated with glufosinate in 1999 or in the untreated strip. No volunteers or weedy *B rapa* plants were seen on the side of the field that had been treated with the conventional herbicide.

A total of 112 plants from an area outside the quadrats were selected on the basis of morphology as 60 *B.rapa* plants or 60 hybrids. Flow cytometry showed 59 plants had the DNA ratios of *B.rapa*, 36 of F_1 hybrids, and 12 a DNA ratio indicating backcrosses to *B.napus*. Five were probably *B.napus,* or

backcross hybrids to *B.napus* from unreduced gametes on F_1 hybrids. The two putative backcrosses to *B.rapa* were indistinguishable by morphology from *B.rapa* individuals, while the putative backcrosses to *B.napus* were indistinguishable from standard *B.napus*.

10.3.6 Determination of DNA levels of hybrid progeny

The numbers of tolerant offspring amongst the progenies of F_1 hybrids varied between plants (Table 10.4). The proximity of the sampled F_1 hybrid plant to *B.rapa* individuals did not appear to influence the level of its pollination by *B.napus.* Indeed, the hybrid that was furthest away from a *B.rapa* plant (more than 10m) carried a lower proportion of tolerant offspring than the other hybrids. The highest proportion of tolerant progeny (52%) was produced by a hybrid 2m away from the nearest *B.rapa* plant. A range of DNA ratios were found amongst 13 glufosinate-susceptible offspring of a *B.napus* x *B.rapa* hybrid indicating backcrossing to both parents (Figure 10.7). Eleven of the 13 fell between *B.rapa* and *B.napus.* The remaining two had DNA ratios higher than *B.napus* but not as high as would be expected from unreduced gametes on the F_1 hybrid pollinated by reduced *B.napus*. Offspring 1 had a DNA amount equal to that of *B.rapa,* while progeny 10 and 11 had similar DNA ratios to *B.napus***.** The plants varied greatly in morphology (Plate 6.6), and did not thrive under glasshouse conditions. Four survived to flowering (1, 2, 10 and 11) but the remainder died before reaching maturity.

Table 10.4: Frequencies of glufosinate tolerant seeds harvested from hybrid plants in the field

Plant number	*Proximity to other* B.rapa *plants*	*Number of progeny tested*	*Numbers tolerant (%)*
1	***<1 m***	***221***	***103 (46.6)***
2	***2 m***	***66***	***34 (51.5)***
3	***>10 m***	***29***	***8 (27.5)***
4	***1m***	***119***	***37 (31.1)***

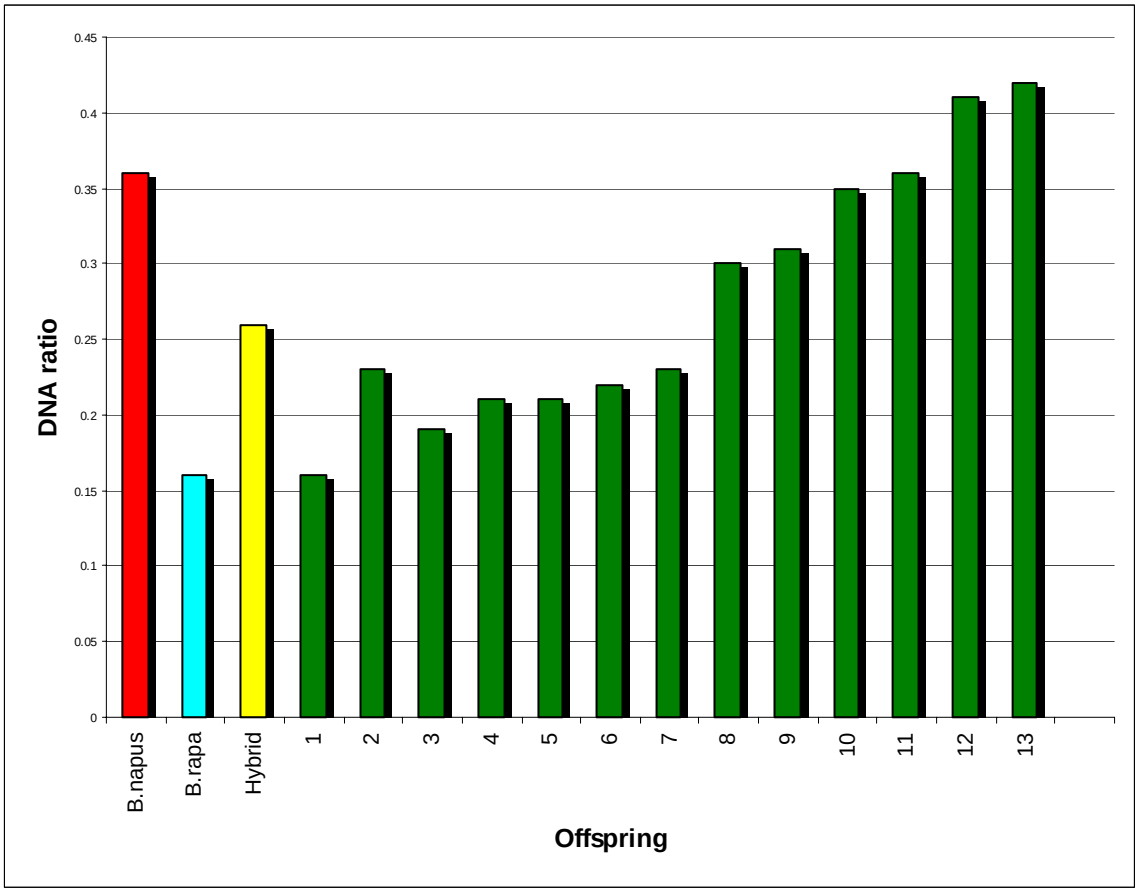


Figure 10.7: DNA ratios of glufosinate-susceptible offspring of a *B.napus* x *B.rapa* hybrid (Plant 1) under field conditions from Patrington.

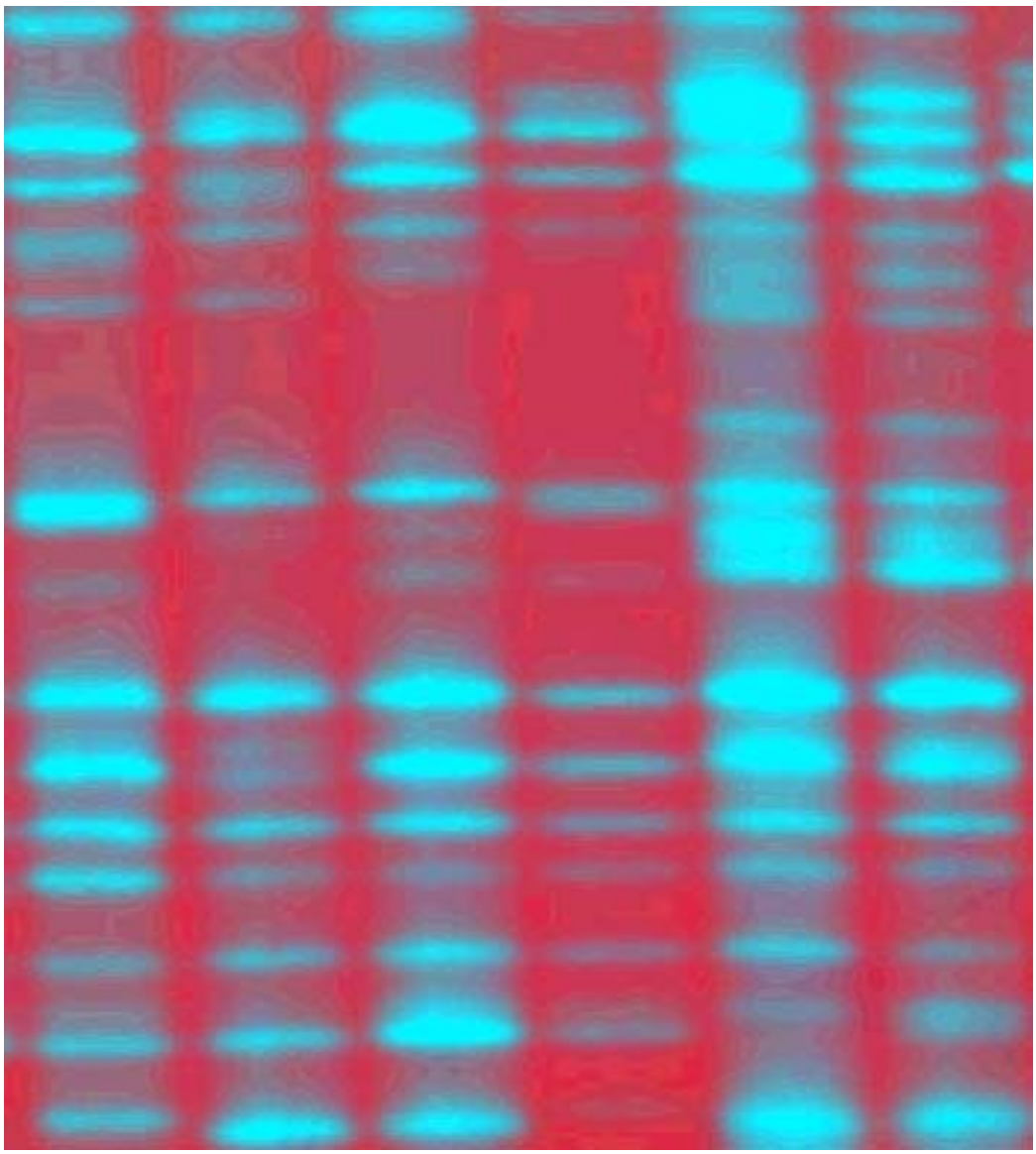


Plate 10.5: A portion of an AFLP autoradiograph of weedy *B.rapa* and two hybrids using the primer set MseI 1/ PstI 1. *B.rapa* shows polymorphic bands between individuals. The hybrids have many more bands than *B.rapa.*

Plate 10.6: Some of the progeny from a *B.napus* x *B.rapa* hybrid mother plant, showing phenotypic differences.

10.3.7 Pollination of *B.rapa* bait plants

Seed harvested from bait plants of *B.rapa* placed in between herbicide-tolerant plots of *B.napus* was variable in quantity and quality (Table 10.5). On three of the 12 bait plants the pods shrivelled and died and set no seed at all, despite having many flowers on these plants. Many of the remaining bait plants had pods that contained undeveloped, shrivelled or even germinated seeds, many of which were easily crushed during harvesting. However plant 4 produced large numbers of pods and also a high mean number of seeds per pod (13.6). The seeds from this plant appeared healthy. Plant 12 produced more pods than plant 4 but with a much lower number of seeds per pod (5.14). Most fertile plants had means of five or less seeds per pod compared to the average number of seeds per pod of *B.rapa* (15) and *B.napus* (20).

Table 10.5: Number of seeds per pods set on *B.rapa* bait plants in transgenic oilseed rape plots at NIAB, Cambridgeshire in 1999

PLANT NUMBER	TOTAL NUMBER OF PODS PRODUCED	NUMBER OF PODS USED FOR SCORED	MEAN NUMBER OF SEEDS PER POD
1	63	50	0.88
2	0	-	-
3	0	-	-
4	121	50	13.60
5	55	50	1.88
6	102	50	4.16
7	17	17	0.94
8	0	-	-
9	71	50	0.50
10	113	50	4.88
11	106	50	3.84
12	152	50	5.14

Table 10.6 shows the germination levels of the harvested seeds from seven of the bait plants of *B.rapa*. Plant 10 showed a tendency towards dormancy as indicated by the increase in germination after the addition of gibberellic acid. All other plants showed little response to gibberellic acid (0-4 seeds), while Plant 11 showed no response. Plant 6 showed very low germination compared to other plants. Germination varied considerably between plants with plant 12 showing the highest level and most rapid germination.

Table 10.6: Accumulative germination of seeds harvested from *B.rapa* bait plants (mean of 4 replicates of 25 seeds) in transgenic oilseed rape plots at NIAB, Cambridgeshire in 1999

PLANT NUMBER	24 HRS	48 HRS	72 HRS	96 HRS	AFTER ADDITIONAL GA_3 TREATMENT
4	3	12	13	15	17 (+2)
5	0	9	10	10	12 (+2)
6	0	0	1	1	2 (+1)
9	3	10	10	11	15 (+4)
10	1	1	1	2	12 (+10)
11	2	9	11	10	10 (+0)
12	9	19	21	21	24 (+3)

Increase in germination after addition of GA_3 is shown in parenthesis

Seedlings from the bait plants were tested for their susceptibility to glyphosate and glufosinate (Table 10.7). Some seedlings with *B.rapa* morphology were produced which were susceptible to both herbicides, and their identity as *B.rapa* was confirmed by DNA amounts obtained from flow cytometry. The remainder of the offspring were hybrids. Only a very low proportion of these hybrids showed herbicide-tolerance. *B.rapa* plants (Plants 9 and 11) were apparently prone to producing unreduced gametes. Hybrids from these unreduced gametes have DNA ratios similar to, or slightly higher than, *B.napus*.

10.3.8 Observations of the *B.rapa* population examined, and crop/ weed interactions

The GM oilseed rape at the Patringtron site examined in this study was surrounded by 'set aside' land in the growing year. The weedy *B.rapa* population was unevenly distributed over the whole area with only a few plants in the uncultivated set aside. The population was patchy, with densely grouped plants in a strip approximately 10m by 50m across the field, and was particularly concentrated in the area of the field receiving no herbicide. The population size was estimated at approximately 500 plants before herbicide application. Some parts of the field had isolated *B.rapa* individuals that were separated from others of the same species by more than 10 metres.

Most of the *B.rapa* germinated at the same time as the oilseed rape. Germination tended to be quite even with most plants germinating simultaneously, rather than being staggered over a period of months. However, a few individuals germinated later and were often at a disadvantage as they were by then over-shadowed by the developing crop canopy.

Flowering of *B.rapa* started before the stem extension of oilseed rape. The first individuals started to flower in early February, and flowering continued until the oilseed rape crop had finished flowering.

When flowering of the *B.rapa* began, most plants were taller than the oilseed rape. Many *B.rapa* individuals had almost finished flowering when the rape started to flower in April/ May. However, a large proportion of the Patrington *B.rapa* population had an overlap with the rape in their flowering duration. At the peak of oilseed rape flowering, the rape is about half a metre taller than *B.rapa*.

Most of the *B.rapa* individuals matured and shed seed two to three weeks earlier than the oilseed rape crop, so, by the time the crop was harvested, most *B.rapa* seed was already on the ground and escaped being harvested with the crop (Plate 10.3).
Interspecific hybrids also germinated at the same time as the oilseed rape. They were distinguishable by the two or three leaf stage, being intermediate in morphology between the two parental species. Stem extension and flowering occurred later than in *B rapa* and often at the same time as oilseed rape. The hybrid plants grew as tall as, or sometimes taller than, the rape. Anthers on hybrid plants were sometimes reduced in size or completely absent and the quantities of pollen produced on these plants were lower than either parent. Most hybrids however, were not generally completely male sterile. Seedpods on hybrids were often empty or contained very few seeds. Many of the seeds were aborted, shrivelled or malformed, although some filled, round seeds were formed and these were larger than those of either parent (Plate 10.7). Also seeds produced on hybrid plants often germinated in the pod. A characteristic of hybrid plants at a late stage of development was their tendency to grow new flowering shoots after pods had been formed and the rest of the plant was starting to senesce. This did not occur in either parent, but is a common feature of near-sterile hybrids in many species (Stace, 1974).

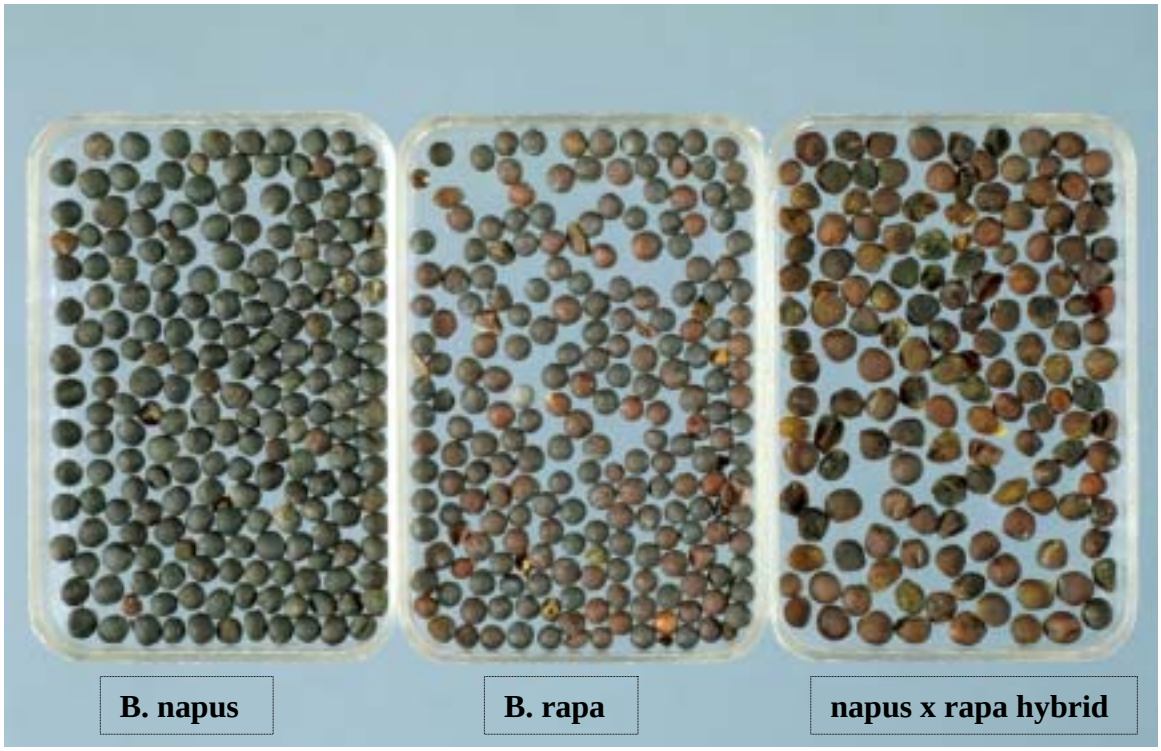


Plate 10.7: Seeds from *B.napus*, *B.rapa* and *B.napus* x *B.rapa* hybrid. The hybrid seeds are larger than seeds from either parent and are misshapen and deformed.

Table 10.7: Herbicide testing and flow cytometry testing of seedlings from *B.rapa* bait plants

PLANT NUMBER	NUMBER OF SEEDLINGS TESTED FROM 25 SEEDS	HERBICIDE USED	NUMBER TOLERANT	NUMBER OF HYBRIDS FROM UNREDUCED GAMETES OF *B.RAPA*	NUMBER OF TRIPLOID HYBRIDS	NUMBER OF B.RAPA
4	21	Glufosinate	5	1	20	0
5	11	Glufosinate	3	1	9	1
6	10	Glufosinate	5	0	10	0
7	3	Glufosinate	1	0	2	1
		Glyphosate	0			
9	12	Glufosinate	1	5	5	2
		Glyphosate	5			
10	12	Glufosinate	0	0	8	4
		Glyphosate	1			
11	14	Glufosinate	2	5	8	1
		Glyphosate	0			
12	17	Glufosinate	6			
		Glyphosate	5	0	17	0
TOTAL	100		34	12	79	9

10.4 DISCUSSION

Extensive hybridisation between oilseed rape and *B.rapa* has occurred at the Patrington site. This finding supports previous work carried out on weedy *B.rapa* in oilseed rape fields in Denmark (Jørgensen and Andersen, 1994; Jørgensen and Andersen, 1996). No other research has been carried out on hybridisation between the two species in arable fields in the UK, although Scott and Wilkinson (1999) found hybrids in seeds collected from sympatric riverbank populations of *B.rapa* in the Thames valley

The spatial distribution and growth habit of *B. rapa* at the Patrington site offers an explanation for the high frequency of hybridisation. *B. rapa* is a mostly self-incompatible species, so very high proportions of inter-specific hybrid seed are likely to be set on *B. rapa* mother plants when individuals are isolated from sources of intra-specific pollen and surrounded by oilseed rape. It has also been shown that when *B rapa* and *B. napus* pollen are applied to *B. rapa* stigmas, they have equal fitness and so are equally likely to fertilise *B. rapa* (Hauser, *et. al.*, 1997). The spatial distribution of *B. rapa* was patchy at Patrington, with many isolated plants surrounded by *B. napus* pollinators. Thus *B. rapa* pollen would have been under a great deal of competition from oilseed rape pollen in these fields.

Flowering time will have an influence on the probability of hybridisation between *B. rapa* and *B. napus.* Observations of *B. rapa* populations in Humberside showed that when growing amongst winter oilseed rape, *B. rapa* started to flower earlier than the crop. Some individuals finished flowering before the oilseed rape started. Flowering of a proportion of the *B.rapa* then continued throughout the period of the crop flowering, so there was some overlap. At Patrington the majority of the *B.rapa* population flowered synchronously with the oilseed rape. It is likely that the early-flowerers maintain a discrete *B.rapa* population despite a high degree of hybridisation in the population.

Differences in dormancy and germination patterns between *B.rapa* and *B.napus* may have an effect on emergence and growth of interspecific hybrids. *B.rapa* produces seeds with many and varying germination requirements. Seeds from the same mother plant can have different levels of dormancy, as can seeds from different plants in the same population. This contributes to making *B.rapa* a persistent weed in disturbed habitats by increasing the probability of germination at a favourable time in an unpredictable environment (Rees and Long 1992).

The ability of annual plants to survive in the environment is affected by certain environmental conditions and cues, and also by seed dormancy. Factors affecting the degree of dormancy include seed coat structure, light sensitivity, nutrient availability and cold stratification. Other factors, such as temperature and gaseous concentrations may also influence dormancy. Cold stratification in *B.rapa* has been found to induce light sensitivity and sensitise seeds to nutrient levels (Adler *et. al.,* 1993). Adler *et. al.* (1993) suggested that *B.rapa* seeds that have dispersed in the autumn will be subjected to cold stratification over the winter and are likely to germinate in the spring. This is in contrast to the findings at Patrington where most *B.rapa* germinated in the autumn at the same time as the crop. Late germinating plants were at a disadvantage due to light competition from the oilseed rape canopy.

B.rapa seeds are more likely to germinate at the same time as the crop seeds when the soil is fertile and cultivated, and crop cover is sparse and there would be advantages to this. However there may also be selection for plants in the *B.rapa* population to germinate later than the crop if herbicide is applied to the crop soon after emergence. Although conventional selective herbicides such as Laser and Butisan are not fully effective against weeds such as *B.rapa,* treated plants will nevertheless be affected in their development and may well be stunted.
In an agricultural environment, weedy *B.rapa* is subjected to frequent nutrient inputs. This may result in seeds becoming less responsive to changes in nutrient levels as a germination cue. In non-agricultural

environments, however, *B.rapa* will be more likely to germinate after nutrient flushes because these do not occur as regularly as in agricultural fields. Far-red shade caused by the presence of a canopy of established plants can suppress germination of *B.rapa* and other species (Gorski 1975; Rees & Brown 1991). However this sensitivity to the presence of established plants may be over-ridden when nutrient levels become ideal for germination in non-agricultural environments.

B.napus seed at harvest does not show tendencies toward dormancy and its seeds will germinate under most conditions (see Chapter 6). At harvest, however, certain environmental conditions may induce secondary dormancy. *B.napus* seeds regularly survive burial in the soil for two to three years (Lutman and Lopez-Granados, 1998) and sometimes even up to ten years, depending on the agricultural practice and whether they remain undisturbed. *B.napus* seeds tend to germinate after being brought to the surface by cultivation, but will remain dormant and viable if left buried.

The results of seed germination from bait plants (Table 10.6) showed variable dormancy since the seed from some plants responded well to the addition of gibberellic acid. However, these seed progenies would have been a mixture of hybrids with some *B.rapa* so dormancy would be expected to be variable.

Hybrids between *B.napus* and *B.rapa* have been shown to inherit the dormancy characteristics of the maternal parent. This could be due to seed-coat influences that are maternally inherited, but organelle inheritance and endosperm formation are also unequally inherited. However, there may also be some zygotic influence on dormancy (Adler *et. al.,* 1993) as the hybrid dormancy characteristics are closer to those of the maternal parent, but are not identical to it. Adler *et. al.,* (1993) reported that the hybrid produced when *B.napus* is the maternal parent is slightly less dormant than the hybrid produced when *B.napus* is the pollen donor. The parentage of the hybrids found in the field at Patrington was unknown. The lower degree of dormancy of hybrid seeds compared to the weedy *B.rapa* parent could reduce the number of hybrid plants reaching the adult stage as they will predominantly be shed in disturbed habitats where the crop parent is found. A larger proportion of hybrid seeds will germinate immediately compared with the staggered germination pattern of *B.rapa,* which allows it to germinate in years when conditions are optimum for its growth. Some degree of dormancy in interspecific crop/ wild hybrids will allow alleles to persist over time, therefore affecting the genetic structure of the population. This could allow repeated backcrossing to wild relatives many years after the initial introduction of a transgene (Linder and Schmitt, 1994; Linder and Schmitt, 1995).

If the hybrid seeds germinate immediately after harvest, as do a large proportion of shed *B.napus* seeds, then the normal agricultural practice of leaving seeds to chit for a minimum of two weeks before cultivation will ensure that the majority of hybrid plants do not survive. The uniformity of their germination requirement could reduce their fitness under field conditions. Alternatively, interspecific hybrids growing in agricultural conditions may maintain similar growth stages to the crop plant, thereby flowering and producing seed at similar times. F_1 interspecific hybrids vary considerably in their fitnesses, depending on parental genotypes (Hauser *et al* 1998). Some varieties of *B.napus* seem to produce hybrids more readily with particular *B.rapa* genotypes and the results of the bait plant experiment show that some *B.rapa* genotypes do not produce hybrids even when exposed to massive amounts of *B.napus* pollen.

For long-term survival, hybrid seeds must remain dormant until suitable conditions for *B.rapa* and its hybrids occur in the agricultural field when *B.napus* is grown. In normal agricultural rotations in the UK, this is likely to be every 4-5 years. Hybrid plants emerging in interim years will usually be growing amongst cereal crops and exposed to herbicides used to eradicate them. Thus few hybrids will reach maturity in years when cereals are grown. Because hybrids are thought to have dormancy characteristics somewhere between the two parental species, they are more likely to emerge when the soil is disturbed for cropping than are plants of *B.rapa.* Seeds germinated from the soil seed bank

(Figure 10.6) show that *B.napus* tends to germinate in a flush after the input of seeds from a crop. However, numbers of seeds in the soil then reduce dramatically in the following years until the next crop of oilseed rape. Numbers of *B.rapa* and hybrids, however, remained fairly constant over the sampling period, and no rapid reduction of their seeds in the seedbank was observed. Hence the proportion of both hybrids and *B.rapa* in the total *Brassica* seed bank population increased with time.

The first backcross generation to *B.rapa* produces seeds with a more *rapa*-like germination pattern. Amongst the seed produced there will be differing germination requirements and some seeds will exhibit dormancy. Interspecific gene flow in the first backcross generation would not therefore be limited by seed germination characteristics to the same degree as that in F_1 hybrids.

In a study where reciprocal crosses were made (Hauser *et al* 1998) those with *B.napus* as the female parent were found to produce more viable seeds than when *B.rapa* was the female parent. This is contrary to the results found when 7512 *B.napus* seeds from plants at Patrington were screened for hybridity. No hybrids were identified amongst these *B.napus* parents growing adjacent to *B.rapa.* It is a possibility that hybrids with morphology close to oilseed rape may not have been identified, although, from previous experience of hybrids, almost all are identifiable with a trained eye as having some intermediate traits.

The triploid (AAC) F_1 hybrid with 2n = 29 often shows sterility or reduced fertility. Interestingly in the bait plant experiment, it was found that hybrids formed from unreduced gametes were a common occurrence. These had the genomic constitution AAAC (2n = 39). A selection of plants with DNA ratios between 0,36 and 0,39 were found which is higher than expected for a triploid hybrid. Many of these expressed tolerance to one or other herbicide, showing that they were pollinated by *B.napus* and were therefore a form of hybrid. Some plants seemed more likely to produce this type of hybrid than others did, implying a genotypic tendency. These plants were not grown to maturity therefore there is no information about the comparative fertility of the two types of hybrid. It is possible though that some AAAC hybrids exist in the field as they appear to be commonly present as seed. However they would be difficult to distinguish by either flow cytometry, molecular markers or morphology from backcrosses to *B.napus.* Chromosome analysis would be required to assess genomic constitution. Plants that were identified as backcrosses to *B.napus* by flow cytometry, and then analysed by AFLP, group together with the F_1 hybrids, (Figures 10.4 and 10.5). This may indicate that these arose from F_1 hybrids with unreduced gametes.

Although *B.rapa* has always been thought to be a highly self-incompatible species, results from the bait plant experiment (Table 10.7), suggest that this may not be entirely the case. Under conditions of isolation from other *B.rapa* plants, some *B.rapa* individuals produced a small number of progeny that were *B.rapa.* The most likely cause of this would be selfing, as there were no other plants of the same species within 15 m and the plants were subjected to a massive excess of oilseed rape pollen.

Some plants in the bait plant experiment produced no seed indicating a genetic incompatibility between some *B.rapa* plants and oilseed rape. All the plants flowered and set seed under the same conditions, so environmental influences seem unlikely. A high proportion of the hybrids produced from all *B.rapa* bait plants growing between glufosinate and glyphosate plots were not tolerant to either herbicide. This indicates that the transgene, if present, was not being expressed in these plants. It is known that expression can be lost under certain conditions of stress in oilseed rape (see Chapter 6). Perhaps this is more likely to occur in the genomic background of the hybrid due to its genetic constitution. A PCR test on these susceptible plants would have confirmed the presence or absence of the transgene. An alternative explanation for the susceptible hybrids is that they were fertilised by non-GM oilseed rape growing in a nearby plot. This is unlikely, given the pollen pressure from the two GM plots adjacent to these *B.rapa* plants.

F_1 hybrids were found to be fertile under field conditions, although seed production was low compared to either parent species. Thus, F_1 hybrids may provide an avenue for introgression to take place. Pollen production from hybrids was not measured but is probably low due to the small anthers observed. The growth of new flowering shoots on hybrid plants after pod formation also indicates low fertility. Sterile hybrids, or those with low fertility, often produce many flowers throughout the life cycle (Stace, 1974). Normally annual plants die after seed production, as energy is diverted from other parts of the plant to the seeds. In hybrids that are sterile or with low fertility, few seeds are produced so energy resources are directed to continuing flower production to compensate. Many of the seeds produced on F_1 hybrid plants were inviable, had shrivelled or had germinated inside the pods. Hauser and Ostergard (1999) also reported germination of F_1 and F_2 seeds within pods, which may be an important loss of hybrid seeds. Hauser *et. al.* (1998) examined fitness of F_1 hybrids between weedy *B.rapa* and *B.napus* and remarkably found that seed set in hybrids was intermediate between its two parents, with *B.rapa* producing fewer seeds per plant than F_1 hybrids. This result is very different from that observed in the Patrington population where hybrids produced many fewer seeds per plant than *B.rapa.*

Backcrosses to F1 hybrids from oilseed rape plants were also found in the field but were less easy than the F_1 hybrids to identify by morphology alone, as they appear very similar to oilseed rape. Backcrossing to *B.rapa* was likely to occur only in areas of the field where there were F_1 hybrids and densely populated by *B.rapa.* Isolated hybrids from *B.rapa* were most likely to backcross to *B.napus* but the potential for self- pollination may exist.

Flow cytometry testing of twelve susceptible offspring from a hybrid parent showed a remarkable range of DNA ratios. Flowers on the hybrid parent could have been pollinated by *B.napus, B.rapa,* another hybrid or have been selfed. Offspring with DNA ratios between *B.rapa* and the hybrid were likely to have been pollinated by *B.rapa* - the first backcross generation. Seven plants fell into this category. One plant had a DNA ratio indistinguishable from *B.rapa* and in a field situation would be impossible to identify as a backcross plant due to its *B.rapa* morphology.

Two plants grown from the soil samples at the Patrington site, were shown by DNA ratio analysis to be backcrosses to *B.rapa*. These backcrosses appeared to be infrequent but were nevertheless possible, as shown by the putative backcrosses to *B.rapa* identified in the hybrid progeny discussed above. However, plants with intermediate DNA levels were not found growing in the field, which indicates that aneuploids do not generally survive to produce viable plants under natural conditions.

Backcrosses to *B.rapa* are likely to have the same, or very similar, chromosome numbers as *B.rapa,* so these may not have been identified by flow cytometry or by morphology. It is likely that individuals with a chromosome number close to *B.rapa* will be the most stable of the range of chromosome complements produced from backcrossing hybrids with *B.rapa*. They may occur at a higher frequency than other aneuploids due to selection taking place under field conditions. Hauser *et. al.* (1998) concluded that backcross and F_2 plants are often aneuploid with unbalanced C chromosomes (backcrosses: AA + 0-9 C; F_2 : AA + 0-18 C) and this may seriously reduce their fitnesses. Translocations between the A and C chromosomes of *B.napus* may also reduce fitness, because some backcross and F_2 plants may then inherit incomplete sets of genes from the original *B.napus* A-genome.

The proposed reduced fitness of backcross plants is supported by the fact that no backcrosses to *B.rapa* individuals with DNA amounts between *B.rapa* and hybrid were found growing in the field, but when progeny from known hybrids were grown under glasshouse conditions several were identified. For this reason, numbers of backcrosses to *B.rapa* plants identified in the field may be underestimated, as those with very similar DNA amount to *B.rapa* were indistinguishable from *B.rapa*. It was difficult to distinguish

between plants that were truly *B.rapa* and those that may have had some of the *B.napus* genome introgressed into them by using morphology and flow cytometry as means of identification.

This conclusion is supported by the AFLP results. Some plants were identified by morphology and flow cytometry as being *B.rapa*, but cluster together with F_1 hybrids. This suggested that these plants were either first generation backcrosses to *B.rapa,* or later generation introgressed individuals. A larger sample size was needed to make firm conclusions about introgression taking place in this population. However the evidence here from AFLP, hybrid progeny testing and field-testing pointed towards the presence of an introgressing population.

A further problem in attempting to draw conclusions about introgression arises from the original parentage of *B.napus* itself. *B.napus* (2n = 38, AACC) is an amphidiploid derived from *B.rapa* (2n = 20, AA) and *B.oleracea* (2n = 18, CC) and therefore evidence of introgression from *B.napus* into *B.rapa* would have to involve the C genome for it to be readily detected.

The results of the field herbicide spot testing and the soil sampling showed that the population found growing each year was only a snapshot of the true population in the seed bank. Only a proportion of the F_1 hybrids found were herbicide tolerant, suggesting that tolerant hybrids could still remain in the seed bank. Some of the non-tolerant hybrids may have arisen from hybridisation in previous oilseed rape crops several years before. This means that introgression may have occurred over a long period of time as oilseed rape crops are generally only grown on the same field every three or four years. Hybridisation and backcrossing between the two species can only take place in the years when oilseed rape is grown, as broad leaved weeds such as *B.rapa* are easily killed by the herbicides applied to cereal crops. Thus the long-term consequences of introducing a transgene into a weedy *B.rapa* population are as yet unknown, but these studies suggest that introgression could occur.

Familiarity with crop/ weed interactions has enabled predictions to be made about the fate of the herbicide-tolerance transgene under field conditions. Herbicide-tolerant hybrids have already been recorded post-harvest at the Patrington site, after a single GM crop. However, further data could be gathered when the next oilseed rape crop is grown and the opportunity will then arise to study the consequences of any hybridisation. Will the next stage of introgression (back crossing to the *B.rapa* parent) take place? The work discussed here took place mostly over one growing season and the results have given an insight into the complex relationships between the *B.rapa* weed population and the oilseed rape crop. The methods used have not been powerful enough to be able to distinguish with certainty between some backcross plants and F_1 hybrids. At the Patrington site, all possible combinations of crosses probably occurred in the field, whether at the seed or the mature plant stage. It has been impossible to distinguish the genomic make up and parental origin of each individual without detailed molecular analyses and chromosome study.

The offspring from any of these F_1 and backcross hybrids could potentially cross with any of the others: the number of combinations of possible crosses is thus enormous. The results of this study have categorised the plants into major classes only: the two parental species, F_1 hybrids and backcrosses. What is more important than the exact identification of each cross, perhaps, is the discovery of extensive hybridisation here, and the evidence of backcrossing in both directions. Thus a transgene is likely to persist past the initial F_1 hybridisation and will move into further generations in an unpredictable way. In addition, the way the transgene will move between generations may also be determined by the fitness it confers on the plant.

In an agricultural environment, the nature and extent of introgression will depend very much upon the type of cropping and the crop management. If weed management of cruciferous weeds is effective, there

may be a higher potential for hybridisation between *B.rapa* and oilseed rape, since individual *B.rapa* plants may escape herbicide treatment and thus become isolated within the crop. If weed management is poor, more *B.rapa* plants will be left in the field, and perhaps there will be less hybridisation. For high levels of backcrossing to occur the opposite scenario will apply. Backcrossing to *B.rapa* is more likely when hybrids are present in the field and there is an abundance of *B.rapa* plants (when weed management is poor).

The results presented here have shown that introgression between *B.napus* and *B.rapa* has been occurring at the Patrington site for many years. The introduction of a transgenic oilseed rape to the site has allowed the extent of initial hybridisation to be determined, and will enable further studies to be carried out on backcrossing events in the future. However these data are from only one population of *B.rapa* and occupy only two years so cannot be deemed to be representative of every population. Further sites similar to this one at Patrington need to be studied over several years in order to gain more information about the nature and extent of introgression of oilseed rape genes into *B.rapa.*

11. CONCLUSIONS AND GENERAL DISCUSSION

11.1 CONCLUSIONS

The conclusions from this study can be summarised as follows:

- Cross-pollination between areas of oilseed rape

  In fully fertile varieties outcrossing declined rapidly with distance from the source with the majority occurring within the first ten metres. However cross-pollination levels in excess of 0.5% were found at distances of 100 – 200m in some samples so that further studies are needed of the factors influencing cross-pollination in order to accurately predict cross-pollination between neighbouring crops.

  The levels of outcrossing found in samples from varietal associations (VAs) were considerably higher than those found in samples of fully fertile rape. This reinforces the requirement for greater isolation distances between GM releases and VAs. Any varieties of oilseed rape containing a male sterile component are more likely to outcross than fully fertile varieties. Pollen-mediated gene flow was also observed between oilseed rape and turnip rape at similar levels.

- Cross-pollination of volunteer plants in adjacent fields

  Pollination of volunteers in neighbouring fields by GM oilseed rape crops occurred at very low rates due to the volunteers being fully fertile and competition from selfing and other pollen. No volunteers with reduced male fertility were studied as outcrossing would be expected to be higher in these plants. (Simpson et al 1999).

- Feral populations of oilseed rape

  Feral populations of oilseed rape were rarely observed near GM release sites in this study, and were transient, not usually persisting for more than one growing season. Observations made on individual plants growing in roadsides and verges showed that only low proportions survived to maturity and set seed. There was no indication that herbicide tolerant transgenic oilseed rape would survive any better outside of cultivation since the herbicides are unlikely to be used in these environments and thus the GM plants would have no additional competitive advantage. High laurate oilseed rape does not appear to be better adapted for survival as a weed or feral plant than conventional oilseed rape, although more data is needed for this to be conclusive.

- Gene flow into wild crucifers

  Natural hybridisation under field conditions was not observed between GM oilseed rape and *S.arvensis, S.alba* or *R.raphanistrum.* If a rare hybridisation event were to take place, further introgression would be unlikely due to the reported lack of fitness in the hybrids and back crosses. Information on *R.raphanistrum* was scarce in this study and, as this is the most likely of the three species to hybridise with oilseed rape, more monitoring is necessary to make firm conclusions on the likelihood of hybridisation in other situations. Ongoing studies in France suggest that hybridisation and backcrossing occurs but that cytoplasmic incompatibility markedly reduces the vigour and viability of BC5 and BC6 and they become infertile (Anne-Marie Chevre, pers comm, 2001).

Extensive hybridisation was observed when oilseed rape and *B.rapa* grew together in fields. Hybrids were fertile although seed production was low. Evidence of backcrossing in the direction of both parents was found, although backcross plants of an unstable chromosome complement appeared not to survive well in the field. Some evidence of possible past introgression was found from AFLP analyses. Therefore it is possible that introgression of a transgene from oilseed rape into weedy *B.rapa* could occur, a result which confirms the work of Hansen *et. al.* (2001), who found oilseed rape markers occurring at different frequencies in populations of *B.rapa*. Only one site was used to study hybridisation and introgression between oilseed rape and *B.rapa* in this project, therefore more information is needed from other sites and other *B.rapa* populations. The population of weedy *B.rapa* growing in oilseed rape fields at Patrington showed:

- Extensive hybridisation occurs between oilseed rape and weedy *B.rapa* in the field;
- Hybrids seeds were found on *B.rapa* mother plants, but not on *B.napus*;.
- Some evidence of an introgressing population between *B.napus* and *B.rapa* was detected from AFLP analyses.
- Genetic incompatibility exists between some *B.rapa* individuals and oilseed rape.
- Hybrids were fertile under field conditions, despite their triploid nature, although seed production was low compared to the parents.
- Interspecific hybrids backcrossed with both *B.napus* and *B.rapa*; so that the progeny from a hybrid mother plant had a range of DNA amounts.
- Individuals backcrossed to *B.rapa* had DNA amounts similar to *B.rapa* and could not be distinguished by a combination of morphology and flow cytometry. Chromosome analyses and DNA studies are required to monitor the backcrossing.
- Aneuploids were found in soil seedbank samples but were not found growing in the field.
- Hybrids formed from unreduced gametes were relatively common.
- Hybridisation occurred between oilseed rape and turnip rape.

In the recently commissioned BBSRC/NERC Gene Flow Programme, NIAB, together with Reading University, CEH, HRI and other partners, is conducting a detailed study of gene flow from *B napus* to B *rapa* and *B oleracea.* This study is addressing some of the issues raised by this report and is hoping to gain more information on historical gene introgression from oilseed rape to these species using variety specific markers.

Weediness and persistence

In studies of volunteers of GM rape, weediness and persistence of did not appear to be enhanced by the presence of herbicide-tolerance transgenes or a high laurate transgene. This confirms the findings of Simpson and Sweet (2001) and Booth et al (1995).

11.2 GENERAL DISCUSSION

Crop-to-crop gene flow: Pollen

Although this study has given data on various components of gene flow in oilseed rape, it has also raised new questions about the extent of gene flow that potentially may occur in the agricultural environment. Many of the studies reported here gave the first opportunity in the UK to carry out gene flow studies in 'real' agricultural situations using transgenic markers to estimate the extent of gene flow.
However if transgenic oilseed rape is grown on a large scale in the UK, then gene flow will occur between fields, farms and across landscapes.

In the large scale gene flow studies between blocks of oilseed rape, such as those carried out on the FSE trials in Lincolnshire and Oxfordshire and the VA variety Gemini at NIAB, it was possible to demonstrate certain features of gene flow between varieties. However, quantification of the extent of gene flow remains problematic for a number of reasons, as described in Chapter 7 . The current FSE trials were established to investigate the effects on diversity of wildlife of the herbicides used in the herbicide-tolerant system of oilseed rape cultivation. They were not designed to study pollen related gene flow though they are contributing some useful information and will give an indication of gene flow on a large scale. Gene flow at this level should be investigated on a landscape scale using larger numbers of transgenic pollen sources, and examining different genotypes, (both of the transgenic plants and the conventional varieties), the extent of pollen flow at further distances from sources, a range of environmental conditions, geographical locations and patterns of cropping of GM and non-GM crops. It is only when these studies have been conducted under a range of UK conditions that farmers and seed producers will be able to accurately predict outcrossing levels and develop appropriate strategies for managing it.

Crop-to-crop gene flow: Seed

The persistence of GM oilseed rape volunteers reported here and observed in other studies (Simpson and Sweet, 2001, BRIGHT, Genesys etc..) indicate that they are likely to be able to persist for as long as conventional oilseed rape. However, to give more definitive information on specific GM types, further long term experiments are needed to study survival of seed banks of GM volunteers over years in a range of crops and rotational circumstances, and in different soil types and conditions. In addition, this study, Simpson and Sweet (2001) and the Genesys study in France, have shown that seed from GM rape is likely to become dispersed by normal agricultural practices so that it will become widely distributed on farms growing GM rape, on farms sharing equipment with GM rape growers, contractors machinery, bulk transporters etc.

It is likely that the EU will set standards of 99.7% purity for non-GM certified seed of oilseed rape. Tests of certified seed of a particular variety imported from N America since 1996, conducted by NIAB, detected GM contamination in c 40% of samples ranging from 0.05% – 0.5%. It is anticipated that contamination of this type will become more frequent as GM rape is commercialised globally.

Simpson and Sweet (2001) showed that one volunteer per square metre (which is approximately 1 plant in 100 depending on sowing rate), whether arising from seed or soil contamination, will result in crop contamination levels of between 0.6% in conventional varieties to 1.5 % in varietal associations. Thus even low levels of seed or seed bed contamination in some varieties will result in the establishment and persistance of contamination in fields and subsequent crops. However this study showed that there was a decline in glufosinate tolerance in subsequent volunteer populations, probably due to the segregation of the Bar gene in subsequent populations. Therefore it is not clear how transgenes will persist in subsequent generations of volunteers and feral plants.

As the commercialisation of GM oilseed rape proceeds, farmers growing non-GM rape will face contamination from several sources and will need to be able to accurately predict likely contamination rates and implement a range of practices in order keep below thresholds for GM contamination. Seed and seed bed contamination is likely to be just as important as the more widely discussed and publicised contamination from outcrossing.

Interspecific Gene Flow:

Further investigations are needed to determine the extent of spontaneous hybridisation between oilseed rape and certain wild crucifers and the production of backcrossed and introgressed populations. This study, plus those in Denmark (Hansen et al 2001) have shown that hybridisation with *B.rapa* and survival of hybrids occurs, though the extent in the UK is not yet known. Hybridisation with *R.raphanistrum* has been reported in France (Darmency *et. al.,* 1998) though recent research suggests that introgression is restricted by genetic/cytoplasmic incompatability (Chevre , pers comm). In this study *R raphanistrum* only occurred at a few sites and thus there were limited opportunities for hybridisation to occur. Further studies of hybridisation between *R.raphanistrum* populations and *B.napus* under UK environmental conditions are needed to determine the likelyhood of gene flow into this species.

This study is significant in that it revealed for the first time that there are weedy populations of *B rapa* co-existing and hybridising with oilseed rape in England, in situations similar to those reported in Denmark. It is likely that these populations will readily acquire genes from oilseed rape particularly if they enhance the survival or fitness characteristics of the *B rapa* growing as a weed in oilseed rape crops. These studies have revealed the evolutionary interaction between tetraploid *B.napus* and one of its diploid parents, *B.rapa.* This could serve as a model system in clarifying our understanding of the evolution and maintenance of polyploidy.

Further studies are needed to determine the extent of gene introgression from rape into weedy *B.rapa*. Molecular investigations of the historical introgression of *B.rapa* and oilseed rape are required and AFLP analyses on a further range of populations of *B.rapa* in other parts of the country are required to assess the extent of gene exchange between these species. The development and use of micro-satellites (simple sequence repeats) will allow gene flow and paternity to be studied by the use of co-dominant markers for clearly definable alleles. Chromosome analysis is also required to confirm the status and meiotic behaviour of backcross and hybrid individuals identified by flow cytometry. The fate of the herbicide-tolerance transgene in the *B.rapa* population at Patrington is intriguing. This marker will allow the extent of backcrossing to either parental species in following generations to be assessed.

BBSRC and NERC research projects started in 2001 are investigating the rate, extent and consequences of gene flow into *B.rapa*, by studying historical introgression with markers from conventional rape varieites and using herbicide tolerance, insect resistance and disease resistance as model systems.

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13. BIBLIOGRAPHY

Adler L.S., Wikler K., Wyndham F.S., Linder C.R., & Schmitt J. (1993) Potential for persistence of genes escaped from canola: germination cues in crop, wild, and crop-wild hybrid *Brassica rapa.* Functional Ecology 7: 736-745

Al-Kaff, N.S., Covey, S.N., Kreike, M.M., Page, A.M., Pinder, R. and Dale, P.J. (1998) Transcriptional and Post-transcriptional plant gene Silencing in Response to a Pathogen. Science, Vol 279: 2113-2115

Becker, H.C., Damgaard, C. and Karlsson, B. (1992) Environmental variation for outcrossing rate in rapeseed (*Brassica napus*). Theor. Appl. Genet. 84: 303-306

Booth E. J., Walker K. C., Whytock G.P., Sovero M. (1996) Assessment of the ecological consequences of introducing transgenic rapeseed. In: *4th ESA Congress, Abstracts* pp 144-145.

Brown J. Brown A.P Davis J. B. Erickson D. (1997) Intergeneric hybridisation between *Sinapis alba* and *Brassica napus*. Euphytica 93: 163-168.

Champolivier, J., Gasquez, J. and Messean, A. (1999) Crop management of transgenic rapeseed: risk assessment of gene flow. GCIRC 10th International Rapeseed Congress, Canberra, Australia: 6

Charters, Y.M., Robertson, A., O'Brien, E.T. & Squire, G.R. (1996) Investigation of Feral Oilseed Rape Populations. Department of the Environment Contract PECD 7/8/237 Final report

Chevre, A.M., Eber, F., Baranger, A. & Renard, M. (1998) Risk assessment on crucifer species. Proc. Int. Symp. on Brassicas. Acta Hort. 459: 219-224

Chevre, A.M., Eber, F., Renard, M. and Darmency, H. (1999) Gene flow from oilseed rape to weeds. In: Gene Flow and Agriculture, Relevance for Transgenic Crops. Proceedings of BCPC Symposium, University of Keele. 125-130

Child, R.D. and Evans, D.E. (1989) Improvement of recoverable yields in oilseed rape (Brassica napus) with growth retardants Aspects of Biology 23:135-143

Crawley, M.J., & Brown, S.L., (1995) Seed limitation and the dynamics of feral oilseed rape on the M25 motorway. Proc. R. Soc. Lond. B. 49-54

Crawley, M. J., Brown, S. L., Hails, R. S., Kohn, D. D. and Rees, M. (2001) Transgenic crops in natural habitats. Nature 409: 682 - 683

Dale, P.J,, (1992) Spread of engineered genes to wild relatives. Plant Physiol 100: 13-15

Darmency H. Fleury A. Lefol E. (1995) Effect of transgenic release on weed biodiversity: oilseed rape and wild radish. Brighton Crop Protection Conference 1995 (5A-2):433-438

Darmency, H., Lefol, E. and Fleury, A. (1998) Spontaneous hybridisations between oilseed rape and wild radish. Molecular Ecology 7: 1467-1473

De Block, M., Botterman, J., Vandewiele M., Dockx T., Gossele V., Movva N.R., Thompson C., Van Montagu M., Leemans J. (1987) Engineering herbicide resistance in plants by expression of a detoxifying enzyme. EMBO Journal 6: 2513-2518

Delourme, R., Eber, F and Renard, M. (1995) Breeding double low restorer lines in radish cytoplasmic male sterility of rapeseed (*Brassica napus* L.). Proceedings of 9th International Rapeseed Congress. Cambridge, UK 1: 6-8

Delourme, R., Foisset, N., Horvais, R., Barret, P., Champagne, G., Cheung, W.Y., Landry, B.S. and Renard, M. (1998) Characterisation of the radish introgression carrying the Rfo restorer gene for the Ogu-INRA cytoplasmic male sterility in rapeseed (*Brassica napus* L.) Theoretical Applied Genetics 97: 129-134

Eisikowitch, D. (1980) Some aspects of pollination of oilseed rape (*Brassica napus* L.) J. Agric. Sci. Camb. 96: 321-326

Ellstrand, N., and Hoffman, C. (1990) Hybridization as an avenue of escape for engineered genes. Bioscience 40: 438-42

Free, J.B. and Nutall, P.M. (1968) The pollination of oilseed rape (*Brassica napus*) and the behaviour of bees on the crop. J. Agric. Sci. Camb. 71: 91-94

Gorski T., (1975) Germination of seeds in the shadow of other plants. Physiologia Plantarum 34: 342-346

Hansen, L.B., Siegismund, H.R., R.B. Jørgensen (2001). Introgression between oilseed rape (*Brassica napus* L.) and its weedy relative *B. rapa* L. in a natural population. Genetic Resources and crop Evolution 48 (6): 621-627.

Hauser T.P. and Ostergard H. (1999) Precocious germination of Brassica rapa x Brassica napus seeds within pods. Hereditas, 130:89-93

Hauser, T.P., Jørgensen, R.B. & Ostergard H. (1998) Fitness of F_1 hybrids between weedy *Brassica rapa* and oilseed rape (*B.napus*). Heredity 81: 429-435

Ingram, J. (2000) The separation distances required to ensure cross-pollination is below specified limits in non-seed crops of sugar beet, maize and oilseed rape. Plant varieties and seeds. 13: 181-199

Jorgensen R.B., & Andersen B. (1994) Spontaneous hybridisation between oilseed rape (*Brassica napus*) and weedy campestris (*Brassicaceae*): a risk of growing genetically modified oilseed rape. Amer. J. Bot. 81 (12): 1620-1626

Jorgensen R.B., Andersen B., Landbo L., Mikkelson T.R. (1996) Spontaneous hybridisation between oilseed rape (*Brassica napus*) and weedy relatives. Acta Hort 407: 193-199

Krishna, P. J. (2001) Gene Silencing. Environmental Influences on Gene Silencing. [Online] 09/01/02 <http://www.biotech-info.net/influences.html>

Landbo, L., Andersen, B., Jorgensen, R.B. (1996) Natural hybridisation between oilseed rape and a wild relative: hybrids among seeds from weedy *B.campestris.* Hereditas 125: 89-91

Lavigne, C., Klein, E.K., Vallee, P., Pierre, J., Godelle, B., Renard, M. (1997) A pollen-dispersal experiment with transgenic oilseed rape. Estimation of the average pollen dispersal of an individual plant within a field. Theor. Appl. Genet. 96: 886-896

Lefol, E., Danielou, H., Darmency, H. (1996) Predicting hybridization between transgenic oilseed rape and wild mustard. Field Crops Research 45: 153-161

Linder, C.R., and Schmitt, J. (1994) Assessing the risks of transgene escape through time and crop-wild hybrid persistence. Molecular Ecology 3: 23-30

Linder, C.R., and Schmitt, J. (1995) Potential persistence of escaped transgenes: Performance of transgenic, oil-modified *Brassica* seeds and seedlings. Ecological Applications. 5(4): 1056-1068

Lutman, P.J.W., (1993) The occurrence and persistence of volunteer oilseed rape (*Brassica napus*). Aspects of Applied Biology 35: Volunteer Crops as Weeds: 29-36

Lutman, P.J.W and Lopez Granados, F. (1998) The persistence of seeds of oilseed rape *(Brassica napus*). Apsects of Applied Biology 51, 147-152

Manasse, R. and Karieva, P. (1991) Quantifying the spread of recombinant genes and organisms. In: Assessing Ecological Risks of Biotechnology. Ed L. Ginzburgh. 215-231

Mathias, R. (1991) Improved embryo rescue technique for intergeneric hybridization between *Sinapis* species and *Brassica napus.* Cruciferae Newsletter, 14-15: 90-91

McCartney, H.A., and Lacey, M.E. (1990) Wind dispersal of pollen from crops of oilseed rape (*Brassica napus* L.) Journal of Aerosol Science 22: 467-477

McNeilly, T. (1968) Evolution in closely adjacent plant populations. III. *Agrostis tenius* on a small copper mine. Heredity 23: 99-108

Metz, P.L.J., Jacobsen, E., Nap, J.P., Pereira, A., Stiekema, W.J. (1997) The impact on biosafety of the phosphinothricin-tolerance transgene in inter-specific *B.rapa* x *B.napus* hybrids and their successive backcrosses. Theor Appl Genet 95: 442-450

Morris, W. F. Kareiva, P. M. Raymer, P. L. (1994) Do barren zones and pollen traps reduce gene escape from transgenic crops? Ecological Applications 4 (1): 157-165

Moyes,C., Lilley , J., Casais C., Dale, P.J. (1999) Gene flow from oilseed rape to *Sinapis arvensis*: variation at the population level. In: Gene Flow and Agriculture, Relevance for Transgenic Crops. Proceedings of BCPC Symposium, University of Keele. 143-148

NIAB. (2000) Oilseeds variety handbook. Supported by HGCA Research & Development: 22

Oilseed Rape Manual (1989) Oilseed rape growth stages. Agricultural Division Advisory Service. 1-13

Pekrun, C. and Lopez-Granados, F. (1995) The effect of water stress and light conditions on the induction of secondary dormancy in seeds of *Brassica napus* L. In: 9th International Rapeseed Congress. Crop Protection: Forecasting and Chemical Control, pp. 1052-1054.

Pekrun, C. and Lutman, P.J.W. (1996) Population dynamics of volunteer rape and possible means of control. Second International Weed Control Congress, Copenhagen: 1223-1227

Pekrun, C., Lopez-Granados, F. and Lutman, P. J. W. (1997)a Studies on the persistence of rape seeds (*Brassica napus* L.), emphasizing their response to light. In: Basic and Applied Aspects of Seed Biology. Eds: R.H. Ellis, M. Black, A.J. Murdoch and T.D. Hong pp. 339-347.

Pekrun, C., Lutman, P.J.W. and Baemer K. (1997)b Germination behaviour of persisting oilseed rape seeds in relation to temperature. Weed Research 37: 419-431

Pekrun, C., Potter, T. C. and Lutman, P. J. W. (1997)c Genotypic variation in the development of secondary dormancy in oilseed rape and its impact on the persistence of volunteer rape. BCPC – Weeds, 4A-2, 243-248.

Pekrun C., Hewitt J.D.J., Lutman P.J.W. (1998) Cultural control of volunteer oilseed rape (*Brassica napus*). *Journal of Agricultural Science Cambridge* 130:155-163.

Pekrun, C. and Lutman, P.J.W., (1998) The influence of post-harvest cultivation on the persistence of volunteer oilseed rape. Aspects of Applied Biology 51: 113-118

Prakash, S. and Hinata, K. (1980) Taxonomy, cytogenetics and origin of crop *Brassicas,* a review. Opera. Bot. 55: 1-157

Ramsay, G., Thomson, C.E., Neilson, S. and Mackay, G.R. (1999) Honeybees as vectors of GM oilseed rape pollen. In: Gene Flow and Agriculture, Relevance for Transgenic Crops. Proceedings of BCPC Symposium, University of Keele. 209-214

Ratcliff, F., Harrison, B.D., and Baulcombe, D.C. (1997) A similarity between viral defence and gene silencing in plants. Science, Vol 276: 1558-1560

Raybould, A.F., and Gray, A.J., (1993) Genetically modified crops and hybridisation with wild relatives: a UK perspective. Journal of Applied Ecology 30: 199-219

Rees M. and Brown, V.K. (1991) The effects of established plants on recruitment in the annual forb, *Sinapis arvensis. Oecologia* 87:58-62

Scheffler, J.A., Parkinson, R. and Dale (1993) Frequency and distance of pollen dispersal from transgenic oilseed rape (*Brassica napus*). Transgenic Research 2: 356-364

Scheffler, J.A., Parkinson, R. and Dale, P.J. (1995) Evaluating the effectiveness of isolation distances for field plots of oilseed rape (*Brassica napus*) using a herbicide-resistance transgene as a selectable marker. Plant Breeding 114: 317-321

Schlink, S. (1995) Uberdauerungsvermogen und Dormanz von Rapssamen *(Brassica napus*) im Boden. Proceedings of 9th EWRS Symposium, Budapest. 65-72.

Scott, S.E. and Wilkinson, M.J. (1998) Transgene risk is low. Nature 393:320

Simpson, E., Norris C.E., Law, J.R., Thomas, J.E. and Sweet, J.B. (1999) Gene flow in genetically modified herbicide tolerant oilseed rape (*Brassica napus*) in the UK In: Gene Flow and Agriculture, Relevance for Transgenic Crops. Proceedings of BCPC Symposium, University of Keele. 72: 75-81

Simpson, E.C. and Sweet, J.B. (2001) Consequence analysis of the impact on agriculture and the environment of the release of herbicide tolerant oilseed rape. MAFF report RG0217

Squire, G.R., Crawford, J.W., Ramsay, G., Thompson, C. and Bown, J. (1999) Gene flow at the landscape level. In: Lutman, P.J.W. Gene flow and Agriculture: Relevance for Transgenic Crops. BCPC Symposium Proceedings no. 72: 57-64

Stace, C.A. (1975) Hybridisation and the Flora of the British Isles. Academic Press London. New York. San Francisco 4; 38

Staniland, B.K., McVetty, P.B.E., Friesen, L.F., Yarrow, S., Freyssinet, G. and Freyssinet, M. (2000) Effectiveness of border areas in confining the spread of transgenic *B.napus* pollen. Canadian Journal of Plant Science 80: 521-526

Stringham, G.R. and Downey (1982) Effectiveness of isolation distances in seed production of rapeseed (*Brassica napus*). Agronomical Abstracts. Madison. 136-137

Timmons, A.M., O'Brien, E.T., Charters, Y.M., Dubbels, S.J., & Wilkinson, M.J. (1995) Assessing the risks of wind pollination from fields of genetically modified *Brassica napus* ssp. Oleifera. Euphytica 85: 417-423

Thompson, C.E., Squire, G., Mackay, Bradshaw, J.E. Crawford, J. and Ramsay, G. (1999) Regional patterns of gene flow and its consequences for GM oilseed rape. In: Gene Flow and Agriculture, Relevance for Transgenic Crops. Proceedings of BCPC Symposium, University of Keele. 72: 95-100.

APPENDIX 1: TRANSGENE DETECTION ASSAYS

TEST	PRINCIPLE	PROS	CONS
Herbicide plate assay	Leaf tissue grown on selective media containing herbicide. Susceptible seeds fail to germinate. Chlorophenol red can be added to induce colour change if tolerant.	Laboratory method. Large batches of seed may be screened.	Not practical for field testing. Aseptic conditions required.
Kanamycin plate assay	Leaf tissue grown on selective media containing kanamycin. The presence of a callus indicates expression of the kanamycin resistance transgene NPT II. Uses leaf material.	May be used for all constructs with NPT II. Large numbers of plants may be screened.	Cannot be used on constructs not containing NPT II. Not practical for field testing. Aseptic conditions required.
Histochemical GUS assay	Leaf tissue incubated with X-gluc and examine under microscope for presence of colour. Colour indicates active copy of GUS transgene.	May be used for all constructs containing GUS.	Cannot be used for constructs that do not contain GUS. Leaf tissue must be kept fresh between field and laboratory.
NPT II Elisa assay	Double antibody sandwich immunoassay.	Reliable and accurate. Gives quantitative result. 96 samples can be tested at once.	Leaf tissue must be kept cool between field and laboratory. Can only be used for constructs containing NPT II
Herbicide spot testing	Glufosinate-ammonium wiped directly onto plant leaves in situ.	Allows rapid field testing of many plants with reliable results for OSR. Non-destructive.	Involves two site visits. Temperature dependent. Only detects expression of the glufosinate transgene. Specific to the glufosinate-ammonium tolerance construct.
Herbicide screening of seedlings	Seeds germinated and seedlings sprayed with herbicide. Tolerant seedlings survive.	Allows screening of large numbers of seeds with glufosinate and glyphosate.	Only gives an estimate of real levels of gene flow.
Polymerase chain reaction (PCR)	Specific primers are used to detext DNA sequences from the transgenic contructs.	Accurate and can detect specific transgenes. Long term storage of samples possible. Many samples can be run together.	Specific sequences for transgenes are difficult to obtain. Can be expensive so only used for confirmation of phenotypic tests.
Morphological observation	Familiarity with plant species enabling identification of hybrids.	Many plants can be screened at low cost. Observation may result in identification of unusual events regarding plant populations.	Not accurate and requires skill. Hybrids are not always intermediate in morphology so may not be detected.