DEFRA
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Quantifying landscape-scale gene flow in oilseed rape

Gavin Ramsay, Caroline Thompson and Geoff Squire
Scottish Crop Research Institute
Invergowrie
Dundee DD2 5DA

email: g.ramsay@scri.sari.ac.uk
telephone: 01382 562731
fax: 01382 562426

Final Report of DEFRA Project RG0216: An experimental and mathematical study of the local and regional scale movement of an oilseed rape transgene.

Contents

Executive Summary

1. All the methods required for this study were adapted and put into place. These consisted of protocols for the use of male-sterile plants to estimate potential cross-pollination, recording airborne pollen using suction traps and impaction on static surfaces, recovering pollen from bees, microscopic identification of pollen types, DNA extraction and fingerprinting methods for pollen and vegetative plant material, PCR detection of transgenes, and screens for marker genes giving resistance to antibiotics and sulphonylurea herbicides.

2. Male-sterile plants were used to generate large data sets of maximum landscape-scale gene flow. This method over-estimates gene flow found in more normal situations due to the lack of competing self-pollen. In one season, direct comparisons were made of frequencies of fertilisation of male-sterile recipients and the movement of a marker gene into the seeds produced by male-fertile recipients. The data from male-sterile plants over-estimated the flow of a marker gene into a normal male-fertile population by about one order of magnitude.

3. Gene flow into larger populations of male-fertile oilseed rape is less than that into smaller populations. In one experiment to investigate this effect, increasing recipient populations from 10 plants to areas of approximately 0.1 ha reduced the inflow of genes about 4-fold. This reduction was greater in internal parts of these larger blocks.

4. Rates of cross-pollination, measured either by pollination of male-steriles or by tracking a marker gene in the progeny of male-fertile plants, dropped rapidly over the first few tens of metres from the edge of a field, but beyond that the decline with distance was slight over long distances. The exact shape of the decline varied between seasons and did not follow one mathematical description. However, declines with increasing distance are best described by functions giving a sharp initial decline then a long tail.

5. Cross-pollination was highly variable from site to site and dependent on additional factors than simply distance to the nearest source. This applied whether gene flow

was measured by fertilisation of male-sterile plants, or the presence of marker genes in seeds from male-fertile plants.

6. Gene flow was detected over long distances. To facilitate a mathematical description of the decline in fertilisation and to characterise better the long tail in the distribution, sites with male-sterile plants were set out at 5 and 26 km from the nearest known source with little expectation of finding pollination. Low levels of fertilisation occurred at both sites. Although the origin of such events is hard to ascribe with absolute certainty, it appears that they were due to normal natural pollination via vectors which operate over long distances. A critical appraisal of this long-distance gene flow is presented in an appendix.

7. Gene flow over the landscape involving a specific gene reflects the proportion of competing donor fields carrying that gene within a radius of a few kilometres.

8. Pollen movement and fertilisation in oilseed rape have been assumed by some researchers to be due only or mainly to wind transport. Our data indicated that insects were predominantly responsible for pollination in the areas and years examined, even close to a source field. In one experiment, gene flow took place several km upwind of the source as effectively as downwind. Evidence was gathered which is suggestive of bee-to-bee contact in the hive being a major means of effective pollen dispersal through the foraging area of the colony. However, pollination of male-sterile plants took place over longer distances than those flown by worker honeybees. Of the many dispersive insects visiting OSR flowers, the pollen beetle was found on flowers at the most distant site and is already known to move over long distances.

9. Patterns of pollination in these experiments were relatively insensitive to airborne pollen deposition and therefore should not be modelled on that basis. Factors such as the numbers of fields being worked simultaneously by single bee colonies, the degree of mixing achieved, other insect activity, and the relative areas of sources and sinks will partially govern cross-pollination rates.

10. The data generated in this project permitted estimates to be made of cross-pollination into different types of recipient population over widely different distances. These are

presented along with a note of the assumptions made and uncertainties requiring consideration.

11. Increasing separation distance is an inefficient means of maintaining stringent crop purity, and complete freedom from impurity is unlikely to be guaranteed by geographical separation. However, even relatively small separation distances reduce impurity through cross-pollination in fields of fully-fertile oilseed rape varieties to a low level, around 0.1% or below.

Introduction

Gene flow is the process of achieving genetic exchange between populations, and in evolutionary terms is crucial to the maintenance of the integrity of natural biological species. In plants this exchange can be brought about via pollen or propagules such as seeds. However, in the context of crop purity, gene flow is seen in a different light. Concerns over the adoption of new crop types, particularly GM, have placed gene flow at the centre of the debate on the prospects for maintaining certain levels of purity in crop production systems.

Considerable literature exists on gene flow in natural populations. Such studies usually attempt to infer historical gene flow from genetic features of populations and are not particularly effective at guiding the prediction of single or multiple generation gene flow in crops. Since about 1990 there have been many studies focusing on gene flow in crops likely to be grown in GM form. Much research in the topic has concentrated on cases where gene flow and persistence are likely to be maximal, as in open-pollinated crops such as oilseed rape which can leave feral populations in and around fields. Early measurements with oilseed rape implied that gene flow was localised, diminishing to negligible values within 100 m of a source. By the mid-1990s, however, information had been accrued to indicate that gene flow was more of a regional process, in that a field or feral population can receive genes from several sources and over quite long distances, and the fraction of genes coming from one type of source (e.g. GM) compared with another (e.g. non-GM) may depend on the proportions of these sources in the landscape (Anon 1999; Timmons *et al*., 1996).

DEFRA commissioned the present study specifically to examine the regional nature of gene flow in oilseed rape. The study faced certain technical difficulties. Gene flow frequencies were likely to be very low, so highly sensitive techniques were required for detection. The detection techniques had to be deployed, not just around single fields or plots on experimental farms as had been done previously, but at a scale that encompassed fields and feral plants distributed across a real landscape and repeated over several seasons. Moreover, earlier work had assumed that wind was the main vector for pollen. As the work progressed it appeared that insects may have a more important role in gene flow and so research objectives changed to account for this. Therefore, DEFRA (as MAFF) commissioned the present study with the following main aims:

- to estimate the distances and frequencies of gene flow at a landscape scale,
- to investigate the relative roles of insects and wind,
- to summarise gene flow rates among blocks of different size and male fertility, and from this predict likely outcrossing rates in different situations, including typical feral colonies and typical OSR fields.

To achieve these aims, existing techniques were refined or new techniques developed for specific purposes, as follows:

1. Characterise the profiles of airborne pollen with increasing distance from a source.
2. Relate pollen density to hybridisation in bait populations (of male-sterile plants).
3. Use insect-exclusion cages to assess the relative contribution of pollen carried by insects and wind.
4. Collect pollen from bees to investigate whether bees are foraging from one or more sources simultaneously.
5. Assess genetic mixing within feral populations using DNA fingerprinting.
6. Devise tests for detecting the presence of GM in pollen.
7. Assess the competitive status of GM OSR pollen compared to non-GM pollen in glasshouse experiments.
8. Characterise the progeny from plants pollinated at long distances from known source fields using DNA fingerprinting and test crossing as appropriate.

Methods

Surveys and feral populations

In a previous grant, DEFRA (as DETR) funded a road-based survey of oilseed rape fields and feral populations in a >500 km^2 study area located in Angus and the Carse of Gowrie for three years between 1993 and 1995. The survey was conducted by SCRI for a further year, 1996. The findings in the four consecutive years were analysed to show typical distances between fields and ferals and the degree of persistence of feral populations. In 1998 a further survey was made of winter and spring OSR fields and feral plants, following part of the road route taken in the previous study. More limited surveys of field distribution were made in subsequent seasons for the purposes of identifying distances to fields and their spatial relationships to gene flow sites.

The previous project had also used DNA fingerprinting techniques to show the varietal origin of feral populations and their persistence over time. The present project extended that work to investigate specific populations that were either likely to be highly mixed or had been known to persist for several years already. At three feral sites, samples of maternal leaf tissue and mature seeds were taken and subjected to DNA fingerprinting to determine outcrossing rates. The three feral sites studied were Dundee Docks (NO 425307), Balgillo (NO 465324), and the Carse roadside site near Kingston (NO 298276). At Dundee Docks there was a large population of seedling and flowering OSR plants of mixed genotype which had formed on waste ground near the waterfront. It was likely that the origin of the seed was from grain lorries taking seed to a large warehouse, and probably seed blown from operations at the warehouse itself about 200 m away. At Balgillo, OSR plants were growing on heaps of agricultural soil at the edge of a field earmarked for housing development. The Carse roadside site supported a feral colony on the edge of an arable field. This last site had been monitored for several years prior to this project.

Biological pollen traps and field methodology

Male-sterile plants

To study the patterns of decline of cross-pollination over long distances, male-sterile plants were used as a highly sensitive detection system for pollination events. Male-sterile (MS) seeds were separated from the spring-sown, varietal association hybrid cultivar Triolo (kindly donated by CPB Twyford Ltd.), sown in 8 cm pots, raised to flowering in an insect-screened polythene house and individually verified as male-sterile. Groups of 10 MS plants, with a canopy area of approximately 0.15 m^2, were set out at a number of sites where cross-pollination was to be measured. Plants vulnerable to grazing were protected by a ring of 50 mm mesh wire netting 1 m high. Flowers open during transport of the plants to the site were removed. Sites were visited by SCRI staff at intervals of 2-3 days with the exception of sites outwith the main study area where alternative arrangements were made (see later). Alongside a group of MS plants, a static pollen trap was set out (described later) and in some instances a continuously recording pollen suction trap was installed. After being at sites for typically 14 or 15 days, MS plants were marked with plastic ties to delimit the portions of inflorescences exposed during the experiment. Plants were collected, returned to an insect-screened polythene house at SCRI and grown to maturity.

Seed set on the MS plants can only have been the result of pollination from cross-compatible pollen-producing plants. Numbers of flowers exposed and siliquae formed were counted for each inflorescence and plant. An average number of seeds per fruit was also determined for 2 siliquae at each of 5 evenly spaced positions on the primary stem. These data were used to calculate: i) the percentage of flowers forming fruits, and ii) an estimate of fertilisation expressed as the percentage of potential seed yield. This last variable was derived as (100 x seeds produced on MS flowers/22), where 22 is taken as the mean number of seeds that grow in well-filled siliquae in a normally-fertile crop. The first of these variables represents the frequency of flower pollination events and the second is related to the contamination rates likely in harvested male-fertile (MF) seed.

Field experiments using MS plants were conducted mostly in and around the Carse of Gowrie, a part of the larger study area referred to earlier. Three field experiments were conducted here in 1998 and 1999 using MS plants to measure cross-fertilisation on a regional scale. The first experiment was carried out in July 1998, when spring-sown crops were in flower. This experiment was repeated in 1999, but in May when autumn-sown oilseed rape fields were in flower. A third experiment was conducted in July 1999, again on a regional scale but this time using a known pollen source, unique in the area. Additionally, in 1998,

the opportunity was taken to examine cross-pollination from a GM trial crop that was being grown by a commercial company about 30 km to the W of the main study area.

July 1998 field work

In the study area to the W of Dundee, 52 sites were set out on 1 and 2 July 1998 for 14 days to study gene flow rates. Three fields within the study area were of *Brassica rapa* cultivars; all others were conventional spring *B. napus*. Ten male-sterile plants were located at each site, with permission from the landowners and tenants, in field margins, road and track-side verges, gardens, landscaped road interchanges, set aside and derelict land. Forty-two of these sites were located in a 21 km^2 area with a high density of rape fields (Figure 1). Each site had a static pollen trap, changed at every site visit. Burkard 7-day volumetric spore traps were used to record suspended airborne pollen density at four sites.

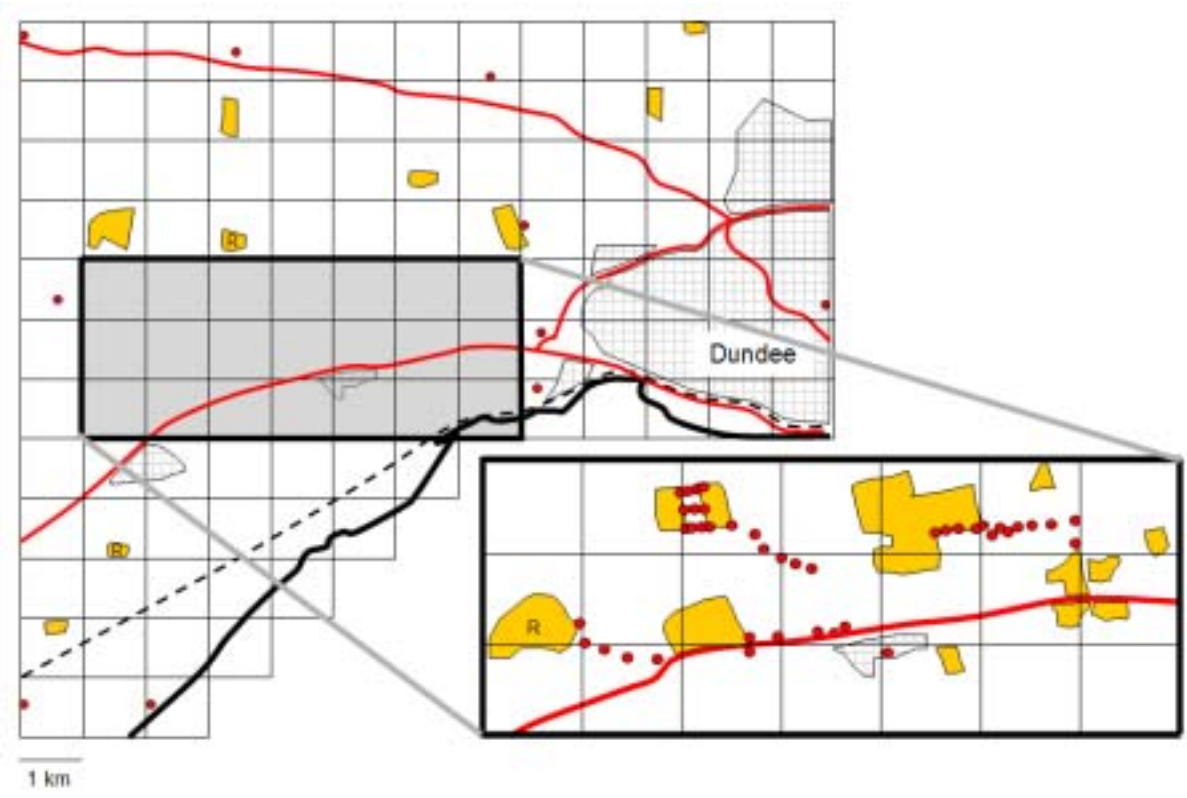

Figure 1. Layout of field experiment in July 1998 showing OSR and *B. rapa* fields (R), and male-sterile plant sites (red circles).

In a second area, 4 km to the S of Perth at Bridge of Earn (30 km to the W of the main study area), one 11 ha GM trial of high laurate spring rape was sown by John King Ltd. Groups of male-sterile plants and static pollen trap were placed at five sites in the vicinity of this field on 7 July for 15 days in roadside verges and a railway embankment, with the kind permission of Perth and Kinross Council and ScotRail (Figure 2). At the southernmost site, 800 m from the GM field, a bee hive was placed with a current year's queen and brood on six frames. Site visits and maintenance were as described above. This area was used for some of the bee work reported below, and was also used to verify that GM pollen behaves in a similar manner to non-GM pollen.

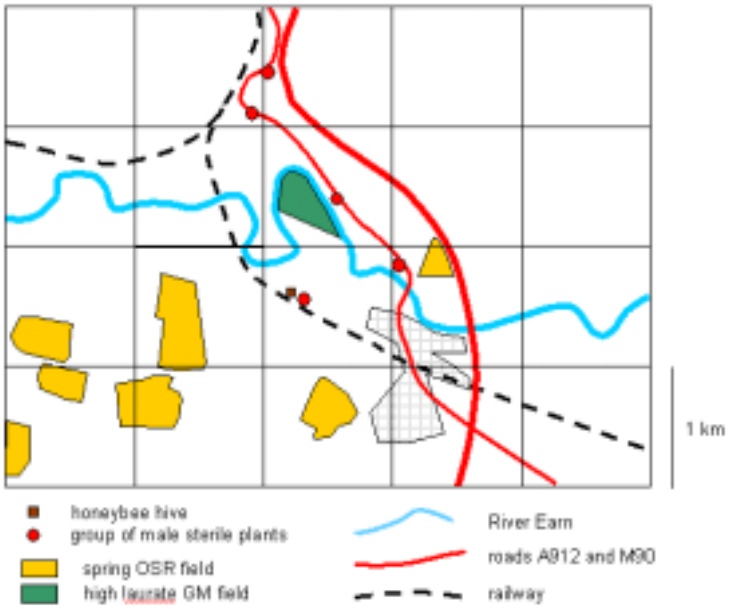

Figure 2. Layout of field experiment at Bridge of Earn in July 1998, showing GM and non-GM OSR crops and sites with male-sterile plants.

May 1999 field work

Male-sterile plants were raised as in the preceding July 1998 season and set out in groups of 10 plants along with static pollen traps across the region to the W of Dundee (Figure 3). The 14-day exposure periods began on 10 and 11 May for sites within this area.

In addition to these 34 sites, two additional sites were set out at extreme distance from known fields, one of them beyond the known flying distance of worker honeybees. One site was in Fife to the E of Tentsmuir Forest in the National Nature Reserve (NO 503275), set out with

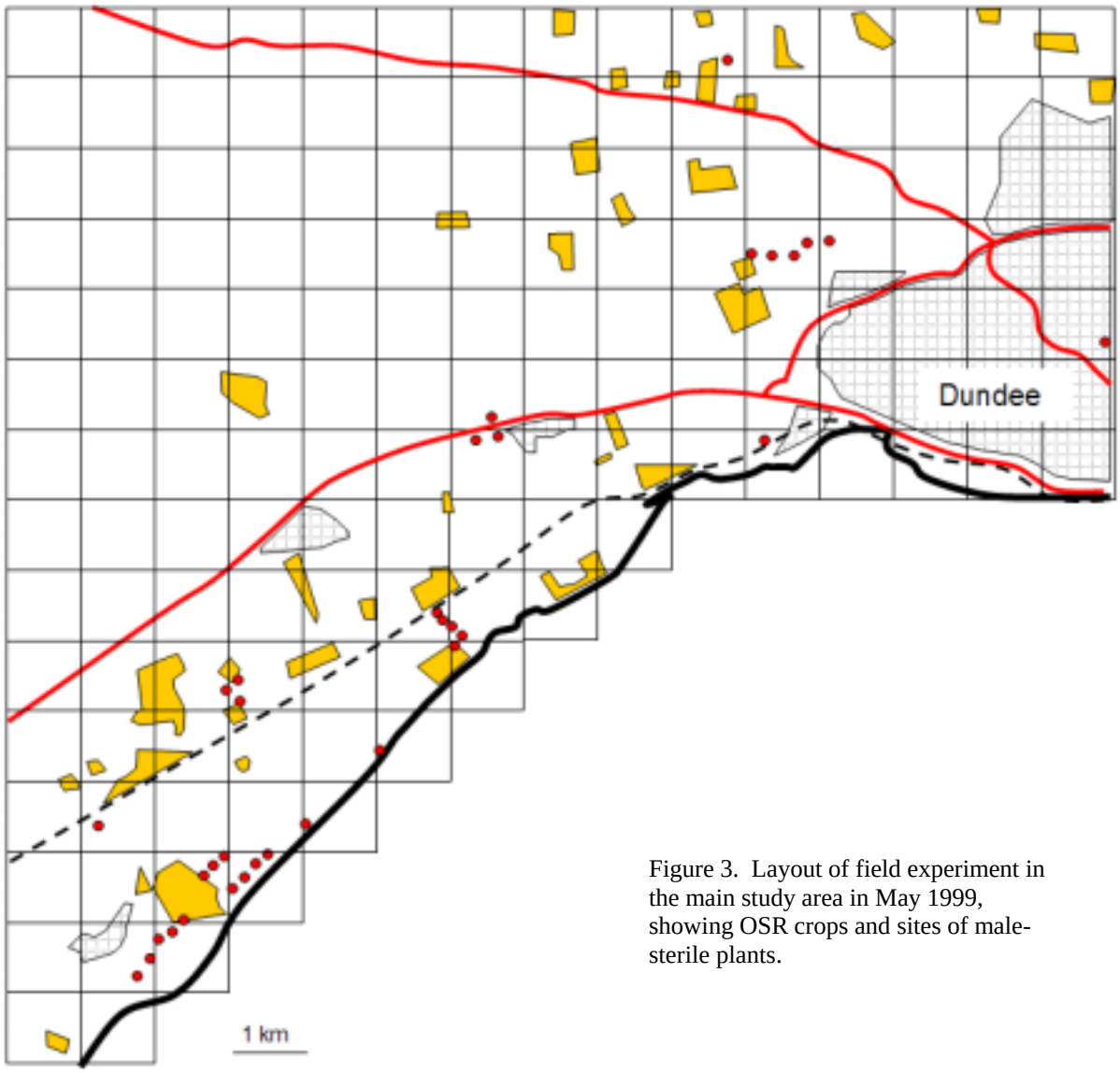

Figure 3. Layout of field experiment in the main study area in May 1999, showing OSR crops and sites of male-sterile plants.

the kind permission of Scottish Natural Heritage. The Fife site was re-started on 14 May, after damage to plants due to cold weather, and marked and brought back to SCRI on 25 May. In that time it was visited once by SCRI staff on a day when no fieldwork close to OSR fields had been planned, and otherwise watered by the local SNH warden. The site was in dune grassland and open scrub away from footpaths and about 10 m E of a conifer plantation. The NNR is separated from farmland by a band of plantation forest 3 km wide and is

bordered to the E by the North Sea. Only one field of winter OSR was grown that season in NE Fife, at Craigie, 5.3 km to the SW (NO 453255). The other site was at the meteorological station at Faskally House (NN 917599), with the kind permission of the Director of the Freshwater Fisheries Laboratory. The site was in a fenced area of mown grass in private grounds in a sheltered, steep-sided and largely forested valley in Perthshire. The Faskally site had 16 MS plants which were watered by local Freshwater Fisheries Laboratory staff living and working in an area with no winter OSR. SCRI staff did not visit the site between delivery of plants on 5 May and their recovery on 26 May and Freshwater Fisheries Laboratory staff removed open flowers from the plants on 6 May. No arable land occurs within 5 km of the site. Small areas of arable land are found in the valley floors between 5 and 24 km away, and beyond that to the S the landscape opens out to larger pasture and arable areas. A car-based survey was conducted at the end of the period. No fields or roadside feral plants could be found during an extensive search of all roads in the area closer than 26.25 km to the SSE at Balquharn near Bankfoot (NO 026358) and 30 km to the SE at Lethendy near Blairgowrie (NO 135420). For these long-distance sites at Tentsmuir and Faskally, no pollen traps were set out as it was not planned to visit them regularly. Subsequent plant care, data collection and collation were as described previously except that, for Tentsmuir and Faskally plants, the positions on stems were recorded for each fruit and seed.

July 1999 field work

In the previous experiments, with the exception of a few sites near the GM field in 1998, the source of pollen that caused seed to be set on the MS plants was uncertain. Therefore a defined and unique pollen source was established in the study area in July 1999. A 7 ha field was sown in April 1999 with an oilseed rape line containing a mutated acetolactate synthase gene, conferring resistance to imidazolinone and sulphonylurea herbicides. This non-GM line was kindly donated by Pioneer Hi-Bred. It is a true-breeding pure line and so does not segregate for this trait. In order to compare crossing frequencies to MS plants and to fully male-fertile plants, this experiment deployed the fully male-fertile, oilseed rape cv. Maskot, in similar groups of 10 plants and as larger field-sown blocks of 10x10 m and 30x30 m (Figure 4). Wooden cages covered in PE monofilament netting (5.4 mm x 3.5 mm pore size, light transmission > 80%, wind permeability > 85%) were used to exclude larger insects on a proportion of MS and MF groups. In addition, two cages were used which were covered in

fine netting (0.50 mm x 0.45 mm pore size). Additional groups of male-sterile plants were set out over the wider landscape (Figure 4). This experiment ran from 9 to 23 July 1999. A mini-met station was also set up near the SE corner of the herbicide-tolerant field. Hourly data on wind speed and direction over the period of the experiment were plotted as a wind rose using WRPLOT (Lakes Environmental Software).

For all MS plants, data were recorded as previously described. MF plants at replicated sites at three distances from the field were scored for numbers of flowers exposed and numbers of siliquae formed. In summary, this experiment provided the opportunity to investigate three outstanding issues: the balance of wind and insect input into cross-fertilisation; the comparison of gene flow into similar-sized and replicated groups of male-sterile and male-fertile plants; and the effect of the size of the recipient population.

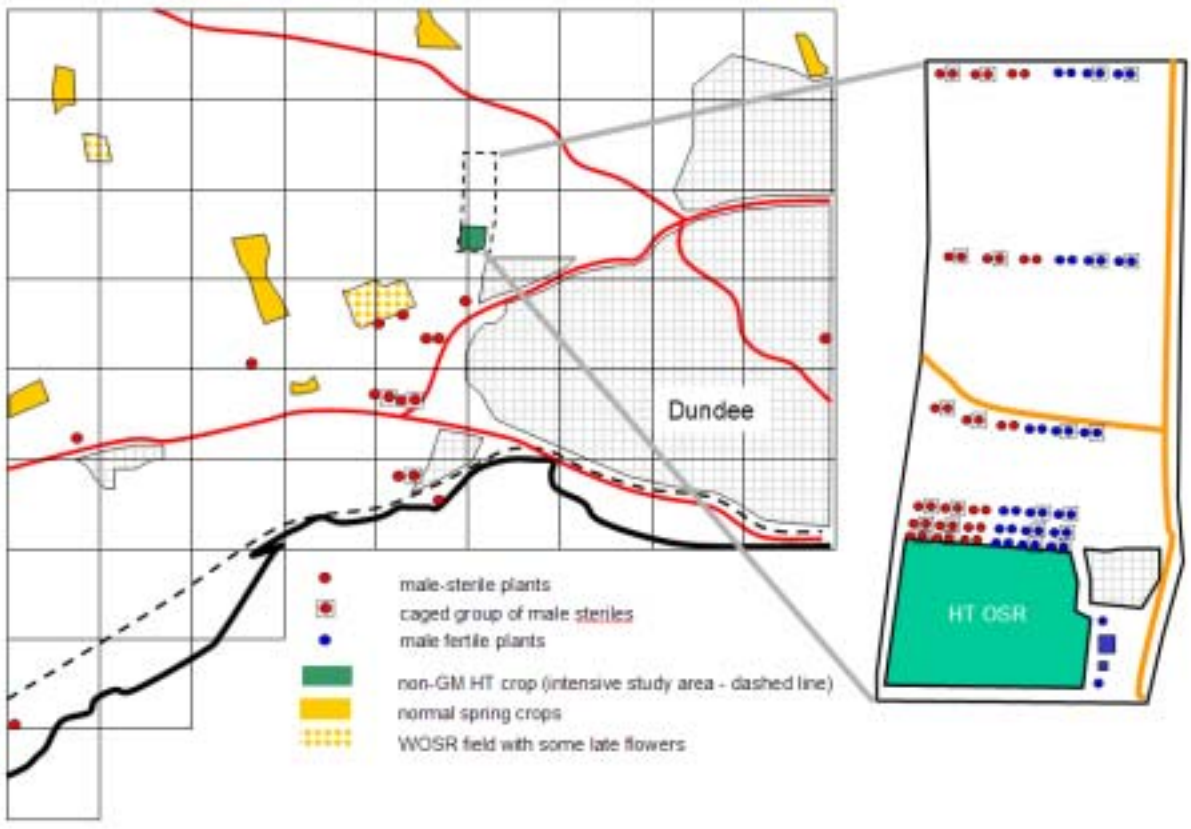

Figure 4. Layout of field experiment in July 1999, showing OSR crops, male-sterile and male-fertile bait plants. Inset shows detail of the layout surrounding the herbicide tolerant OSR crop.

Summary of MS bait plant populations

In the three flowering seasons, July 1998, May 1999 and July 1999, a total of 57, 34 and 58 MS plant sites, respectively, were positioned and maintained. All sites except one produced data in the first two seasons. In the last season, 49 sites produced data, of which 17 sites were covered with cages to prevent pollination by large insects. A further 9 were lost through random vandalism, theft or grazing.

Pollen sampling

Airborne pollen traps

Burkard 7-day volumetric spore traps were used to record suspended airborne pollen density at four study sites. Data were also used from an additional site at SCRI, the E. Scotland site for the national airborne pollen recording network, *Pollen UK*. Sites where MS plants were located, with very few exceptions, were also monitored for pollen depositing or impacting on microscope slides with double-sided adhesive tape attached to canes at 1 m above ground level. Slides were changed every 2-4 days.

Sampling pollen from insects

Pollen being carried by bees was sampled in several ways. Worker bees emerging from or returning to a hive placed 800 m from the GM site in July 1998 were trapped directly into clean plastic tubes and stored on ice. In July 2000 both honeybees and bumble bees were collected into clean tubes from an ornamental plant *Santolina* growing in the SCRI grounds. These trapped bees were held with clean forceps and brushed onto the stigmas of MS flowers growing in an insect-screened polythene house. Pollen loads, collected by worker honeybees, were recovered from beehives near the GM site in July 1998 and at SCRI during the summer of 1999 for analysis of pollen content by cytology and PCR tests. The pollen loads were collected near the GM site using a plastic hive-entrance pollen sampler, and at SCRI using a National hive floor pollen trap (Thornes, Tayport). To determine the effectiveness of two other possible pollen vectors, pollen beetles and seed weevils were collected from local OSR crops, transferred to a clean container, and released into a fine mesh cage over MS plants in a polythene house.

Pollen cytology

Tapes with pollen grains from Burkard or static traps were stained and mounted in basic fuchsin in 59% (w/v) glycerol, 1% (w/v) phenol and 0.9% (w/v) gelatin. OSR pollen was identified and counted under the supervision of staff accredited by *Pollen UK*. Pollen loads were dried and stored at –20°C until required, when they were humidified, broken open, and an uncontaminated sample from the central part of the load transferred to a slide and stained in the mixture above. This pollen was identified by comparison to samples prepared from species the bees were likely to be visiting.

Detection and screening

DNA fingerprinting and detection of transgenes

A rapid DNA extraction protocol was developed for the extraction of PCR-quality DNA from large numbers of leaf samples of OSR plants. Approximately 1 cm^2 of leaf tissue was ground with a micropestle in a 1.5 ml centrifuge tube and incubated at 37^oC for 1½ hrs with 400 µl extraction buffer (100 mM Tris-HCl, 50 mM EDTA, 500 mM NaCl, 1.25% SDS) and 10U proteinase K. The samples were treated with ribonuclease (2U RNase A per sample) before a single chloroform extraction (400 µl). DNA was precipitated using three volumes of 90% ethanol at room temperature. The resulting pellet was washed overnight in 70% ethanol, and resuspended in 50 to 100 µl of TE buffer. A 1/10 dilution of DNA was used for all following PCRs. The protocol was adapted to extract DNA from individual pollen loads by doubling the amount of proteinase K and ribonuclease used. The pollen DNA was precipitated by adding 70% ethanol in excess, incubating overnight at -20 ^oC and spinning briefly in a benchtop centrifuge. The inter-simple sequence repeat (I-SSR) PCR technique previously adapted in this laboratory (Charters *et al.* 1999) was used for DNA fingerprinting of samples. The following modifications were used to improve reliability and ensure a high-throughput of samples: use of the 1/10 sample dilutions mentioned above, a fast PCR cycle (15 sec), and Amersham Pharmacia Biotech's PlusOne DNA silver-staining kit. For pollen samples, PCR Ready-to-Go-Beads (Amersham Pharmacia Biotech) were used to ensure repeatability. The *npt*II and the thioesterase genes in GM material was detected by standard PCR at 62.5°C annealing temperature using published primers for the *npt*II gene and primers designed using

Primer (Whitehead Institute) to amplify the thioesterase gene (EMBL M94159; 5' GAGCTTGAAAAGGTTGCCTG, 3' GCTATCCCTCGTGCACTCTC).

Screening for antibiotic and herbicide tolerance

Seeds from MS plants near the GM field were sown in 9 cm Petri dishes containing agar-solidified Murashige and Skoog medium with 300 mg l^{-1} kanamycin sulphate to detect individuals expressing the *npt*II gene. Seeds from the plants from the July 1999 herbicide tolerant trial were screened for herbicide resistance in two ways. Samples of seed were sown in cellular seed trays in the glasshouse and sprayed with Ally (DuPont), a sulphonylurea-based herbicide, at the 4-leaf stage (150 mg l^{-1} using approx 1 l per 80 m^2 of foliage). Survivors were re-sprayed after two weeks and counted when a clear difference was visible between resistant survivors and susceptible plants with chlorotic and dying new growth and brittle leaf abscission zones. The time required for plants to reach this stage varied between batches, reflecting differing susceptibility of different batches of seedlings in different environmental conditions. Controls were added to batches of seed trays comprising both resistant homozygous Pioneer stock and samples of seed from sites known to have a high percentage of progeny from crosses between MS Triolo and the Pioneer Hi-Bred stock. The latter controls were heterozygous for the herbicide tolerance trait and so would respond in a similar manner to seeds under test. Larger batches of seed were coded and screened by sowing 550 12.5 m^2 field plots of approximately 1800 seeds each with an Oyjord 8-row seed drill. Control plots were interspersed among the test plots and comprised 1800 seeds of pure cv. Maskot, and Maskot spiked with 3 or 18 homozygous seeds of the Pioneer stock, and 18 or 90 seeds from July 1999's site 5 which was known to have a high percentage of crosses with the Pioneer line. Plots were sprayed at the two-leaf stage and again 3 weeks later with 15 g/ha (active ingredient) Ally, and scored on two separate occasions by different staff. Repeat scores within the field-grown plots were very similar, samples grown in both the glasshouse and field also gave closely matching values, and the control plots provided scores consistent with expectations.

Results

Spacing of crops and feral populations

Re-analysis of the previous four-year regional study of feral oilseed rape (DEFRA, 1999) showed a moderate number of populations re-occurred at the same sites over successive years. In a year, typically 20% of flowering feral populations were located within 100 m of a field simultaneously in flower, and about 90% within 2 km.

The more detailed surveys in the Carse area in 1997 and 1998 found a slightly different distribution from that in the larger study area. Oilseed rape fields occupied more of the arable land here, such that half of both winter and spring OSR fields were within 100 m of another field. Winter crops were 3-5 times more frequent than spring crops, and the two types were clustered in different parts of the study area. Around a third of feral populations were found within 100 m of a winter crop and some of these were adjacent to a crop; most ferals in the area were within 1 km of a field.

Variability and outcrossing in feral populations

Plant-to-plant outcrossing was explored at two main sites. At one roadside site bordering an arable field W of Dundee a population of cv. Rafal, last sown 12 years previously, continued to persist. DNA fingerprinting indicated that this population contained individuals of different types. Some had fingerprints consistent with cv. Rafal, others were variants (possibly hybrids) of Rafal, and one additional cultivar was present. Prior to a crop of Inca in the adjacent field in 1998, the only OSR to be grown here according to the farmer's records was Rafal in 1983 and 1987. The additional types in the field margin were therefore likely to have been imported (e.g. by farm machinery). In a sample of 206 seeds collected from individual feral plants with fingerprint patterns consistent with Rafal, 4% carried non-maternal bands, mostly consistent with a paternal origin in the adjacent field of cv. Inca. This population was therefore producing hybrid seeds with nearby fields, and receiving new genotypes presumably via farm machinery.

A mixed stand was investigated at Dundee Docks. Many genotypes were present among 109 individual plants subjected to fingerprinting. A selection can be seen in Figure 5.

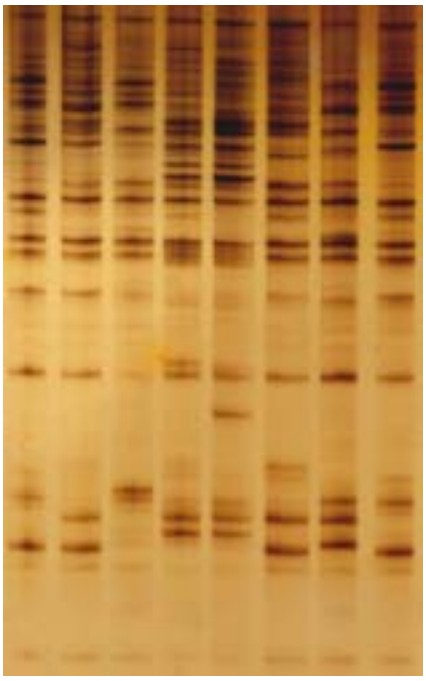

Figure 5. I-SSR DNA fingerprint for 8 individuals growing at Dundee Docks. Six distinct types are present in this random sample (l to r: 1 and 8; 2 and 6; 3; 4; 5; and 7).

Table 1. Outcrossing in two feral OSR populations.

Mother plant	No. seedlings	Seedlings with non-maternal bands	Estimated outcrossing rate (%)
Dundee Docks			
Plant 1	50	3	6
Plant 2	28	10	36
Plant 3	18	1	6
Plant 4	18	3	17
Plant 5	64	31	48
Plant 6	125	6	6
Plant 7	41	6	15
Plant 8	59	7	10
Plant 9	27	3	11
Balgillo			
Plant 1	20	6	30
Plant 2	53	0	0
Plant 3	126	0	0
Plant 4	55	2	4

Many plants grew to maturity, although seeds were often removed near maturity by feral pigeons at this site. In a sample of seeds collected from nine plants, plant-to-plant

outcrossing, as measured by non-maternal bands appearing in the offspring, lay between about 5% and 50% for individual plants (Table 1, Figure 6). At a second site, Balgillo, outcrossing rates were in the range 0% to 30% in progeny seeds from four mother plants.

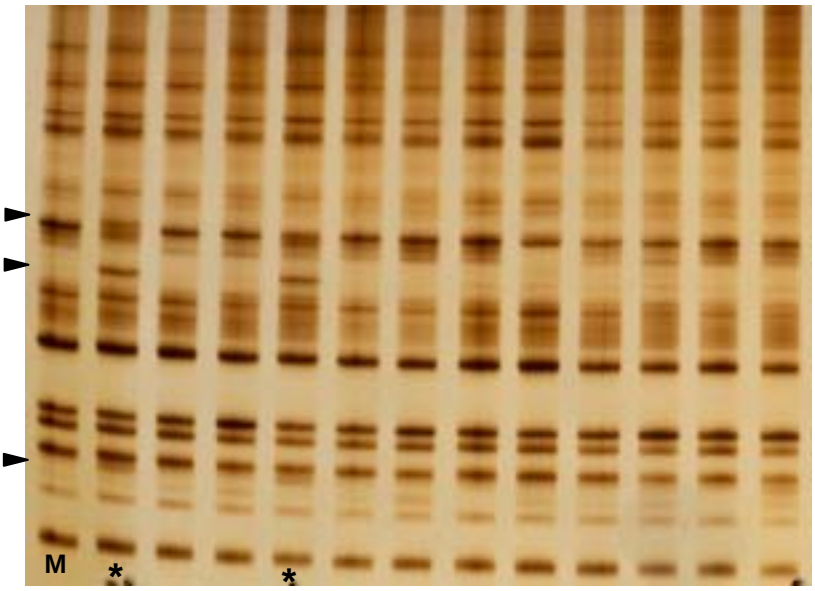

Figure 6. Outcrossing demonstrated in the progeny of one feral plant at Dundee Docks. (M – mother plant, * - progeny with new bands, arrows – diagnostic bands.)

Declines in hybridisation with distance using male-sterile plants as sensitive indicators

Graphs summarising the data collected in 1998 and 1999 are presented in Figure 7. The frequency of fertilisation events declined markedly over distances of a few tens of metres from field sources. Expressed as the percentage of potential seed set (percentage of ovules fertilised, assuming 22 in a fully fertilised crop), the decline is particularly pronounced. This decline therefore comprises a reduction in frequencies of floral pollination events and a reduction in the mean numbers of seeds set per siliqua. This marked decline slows and finally, over distances of several km, hardly reduces further. The profiles obtained by smoothing the data varied between seasons. Not just the height of the profile but its shape differs across seasons and no one mathematical description covers all seasons. The difference between seasons is more marked in the plot of percentage of potential seed set, where reduced flower pollinations and reduced numbers of events per fertilised siliqua combine to depress the height of the graph over intermediate distances. The winter OSR

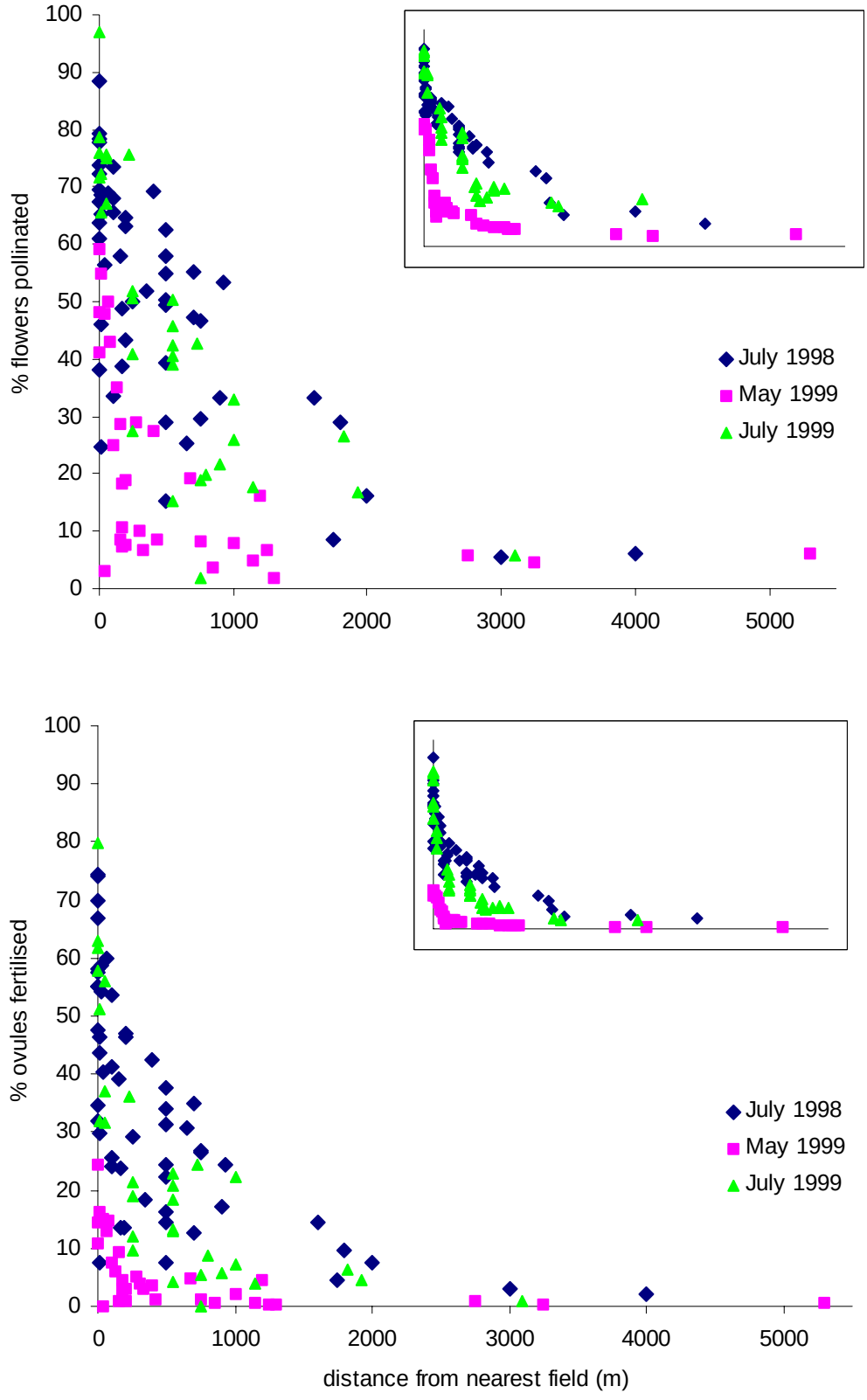

Fig. 7. Declines in fertilisation frequency on a per flower basis (above) and a per ovule basis (below, expressed as a percentage of the numbers of seeds expected from flowers within a crop) for male-sterile plants sited at different distances from oilseed rape fields. One site 26 km from the nearest field in May 1999 (3.1% of flowers and 0.15% of potential seeds) is omitted for clarity. Insets show data smoothed by 5-point moving averages.

flowering season in 1999 was notable for its particularly poor pollinating weather, with persistent cold E winds over this period. The counts of airborne pollen were low during this period (see below), and only brief weather windows were available for pollinator activity. Crops of Synergy, a popular varietal association hybrid cultivar consisting of MS and pollinator lines, had previously yielded well locally. Its composite nature leaves it vulnerable to poor weather at flowering and in May 1999 it was particularly poorly pollinated within fields locally. These weather factors mean that caution has to be exercised in ascribing differences to winter and spring OSR crop types in the data presented here, but do imply that the gene flow recorded during May 1999 is representative of the low end of the range. Smoothed curves, using a 5-point moving average, clarify the patterns of decline and their differences between seasons.

Pollination from a GM field trial

In July 1998, a GM trial field was grown 30 km to the W of the main area. All five sites placed between 100 and 930 m from this field produced progeny bearing the transgenes (Figure 8), and the total fertilisation frequencies on these male-sterile plants were within the range found in the main study area. At the site closest to the GM field for example, the seed set, at 26.3% of estimated normal production, was typical for a set of male-sterile plants 100 m from a source. Within that harvest of seeds, the percentage of GM progeny was high at 75%, reflecting its proximity to the GM field and the lack of competing self-pollen. The other four sites had lower percentages of GM seeds in their harvests (mean of 21%), and this figure was similar to the proportion of total OSR surface within a couple of km which was planted with the GM crop (i.e. the GM crop occupied about one-fifth of the total OSR crop area in that locality).

Pollen deposition

In addition to fertilisation frequencies, the OSR pollen depositing or impacting on static traps was recorded at almost all of the bait plant sites. Summaries of these data are presented in Table 2. As in previous studies, site-to-site and day-to-day variation was high. Sites with the highest mean levels of pollen on static traps over the 14-day period had about 300 OSR grains $cm^{-2}d^{-1}$. During one time period at one site adjacent to the main study field in July 1999, the count peaked at over 1000 grains $cm^{-2}d^{-1}$. Values declined rapidly over short

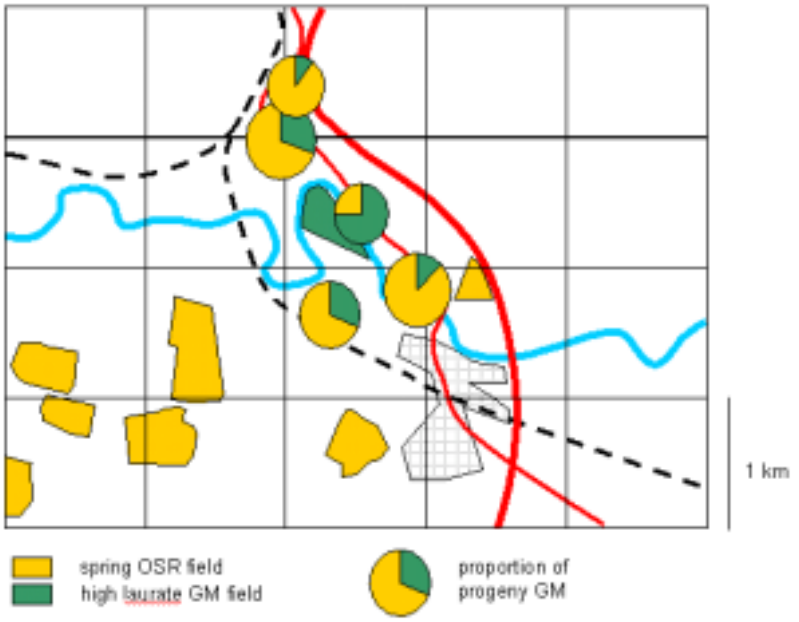

Figure 8. Results from screening the progeny of male-sterile recipient plants for antibiotic resistance in July 1998 experiment.

distances, then maintained low levels over long distances from fields. The background levels at those long distances were low during May 1999, a reflection of the poor weather over that period and of a reduced density of fields in 1999 compared to previous years. The low level at longer distances in July 1999 may be partially explained by the layout of the experiment,

Table 2. Summary data for pollen collected on static traps at different distances from OSR crops in three seasons.

		Pollen on static traps (grains cm^{-2} d^{-1})			
		Distance from nearest OSR crop (m)			
		0-50	51-200	201-500	501+
July 1998	mean	47.3	12.5	5.6	4.8
	n	18	13	8	13
	range	2.6-282	1.0-38.0	1.5-15.1	0.3-25.6
May 1999	mean	11.4	2.6	1.4	0.3
	n	5	11	5	10
	range	0.5-17.5	0-11.5	0-4.5	0-2.1
July 1999	mean	51.4	-	0.2	0.1
	n	12	-	8	17
	range	0.4-304.1	-	0-1.7	0-0.54

where most sites were not downwind of the only field in the immediate vicinity during the experimental period. Comparing these data to historical data collected on behalf of Pollen UK at the SCRI site, the July 1998 season was typical, but the May 1999 season had lower than average pollen counts of all types including OSR, with the exception of birch which was particularly abundant that season.

One original objective of the project was to relate pollen deposition to fertilisation frequencies. In a later section, evidence will be presented that clearly indicates that the levels of pollen deposition seen could only explain a small proportion of the cross-fertilisation on these plants. Pollen deposition data are, therefore, not appropriate for predicting gene flow in oilseed rape and are not considered independently of insect-mediated cross-fertilisation.

The effect of excluding larger pollinating insects with netted cages

In the July 1999 experiment, paired sites were included with plants fully exposed alongside plants in cages covered with an open-weave netting. At two of these sites, a cage with fine-weave netting was also used in order to give improved exclusion of insects but with an uncertain effect on airborne pollen and microclimate. As with other data sets, a high degree of variation in pollen deposition was found from day to day and site to site. However, the log-log plot given in Figure 9 shows that there was no detectable effect on pollen deposition of the cages used in this experiment. It is a reasonable assumption that the cages with coarse netting do not unduly impede airborne pollen flow. As there were only two cages with fine netting there is less certainty on their effect on airborne pollen.

A major reduction in pollination occurred when cages were used to exclude larger pollinating insects (Figure 10). Only very close to the field was there a significant apparent contribution to pollination from airborne pollen. However, it was clear that during the experiment the cage did not completely exclude smaller pollinators such as pollen beetles and smaller flies. The direct reduction in pollination resulting from the use of cages seen in Figure 10 is also associated with a reduction in the mean number of ovules fertilised per siliqua (at paired sites, 3.9 ± 0.8 per fertilised siliqua for plants in cages of coarse netting, and 11.7 ± 1.0 per siliqua for sites without cages).

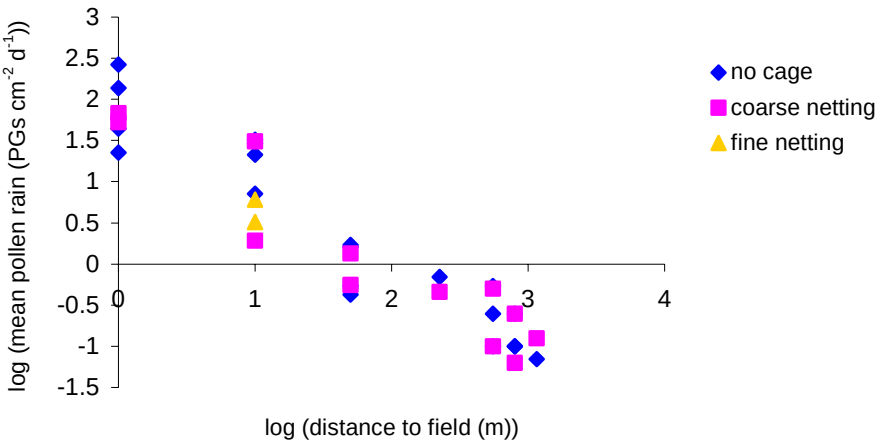

Figure 9. A log-log plot of distance from the field against pollen deposition outside cages and under coarse and fine netting.

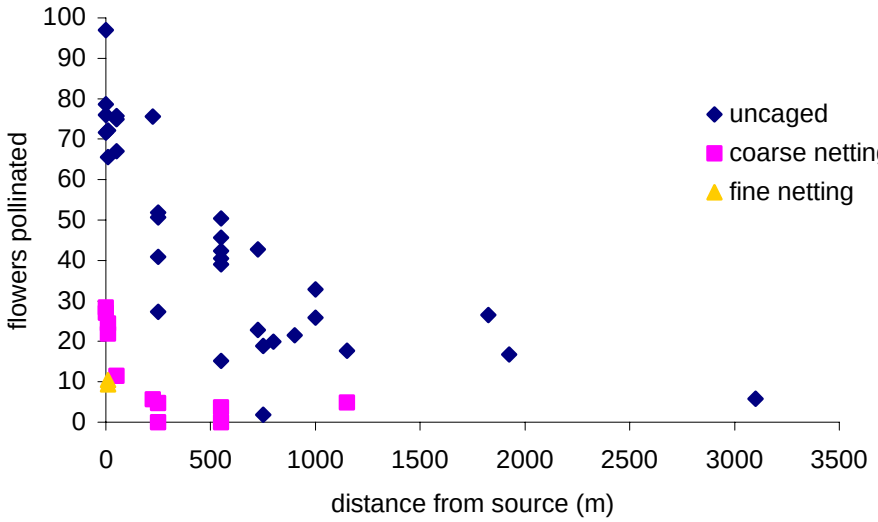

Figure 10. The effect of excluding larger pollinating insects on fertilisation rates of flowers on male-sterile bait plants.

For sites with caged and open MS plants the possible relationship between depositing pollen and fertilisation can be investigated. In Figure 11 pollen on static traps is plotted on a log scale against the frequency of flowers pollinated or ovules fertilised. A linear relation was found between log-transformed deposited pollen data and the fertilisation frequency. With the proviso that the cages are not completely excluding insects, this gives an approximate description of the effectiveness of wind-pollination in this crop. Extrapolation along this straight line for caged data, if justified, indicates that enormous quantities of airborne pollen

would be required for full pollination. A pollen rain of more than 10^7 pollen grains cm^{-2} d^{-1} would be required to achieve 80-90% fertilisation of flowers for example, and higher levels would be required to achieve full fertilisation within these flowers. In the same figure, a linear relationship also describes the data from uncaged sites. The difference between the two lines represents the pollination achieved by insects excluded by the netting.

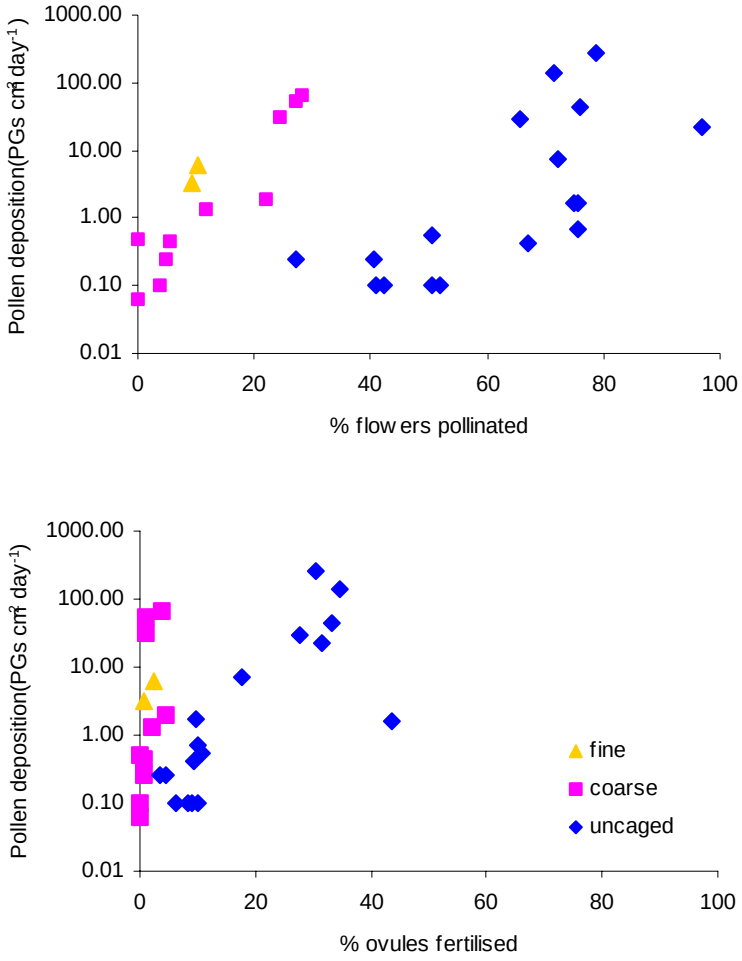

Figure 11. Relation between deposited or impacted airborne pollen and the percentage of fertilisation achieved on male sterile plants (as % of flowers, top, or % of ovules, bottom) inside and outside of netted cages in July 1999.

The two other points shown on the graph represent cages having a fine mesh covering which excluded pollen beetles and other small insects and prevented stigmas protruding through the cage where they may be exposed to insects feeding through the mesh. Airborne pollen impacted on slides in these cages in amounts which were not markedly different to the other cages at similar sites (Figure 9). The negligible entry of insects into these cages suggests that they may provide a better estimate of the relationship between airborne pollen and

fertilisation in oilseed rape. If this is indeed the case, then the results described above over-estimate pollination by wind-borne pollen.

Comparison of gene flow into MS and MF plants

Data on gene flow into MS flowers was recorded widely over three seasons. To be able to use this data to predict gene flow into populations of normally-fertile OSR, the July 1999 experiment included a comparison of MS and MF recipient plants together with a traceable source of pollen, a single 7 ha field of imidazolinone-resistant OSR. Comparing MS and MF data requires careful analysis, as different components of gene flow were measured from the different types of plant. The data were collected in a manner which allows the calculation of each component and Table 3 summarises these results. As can be seen from the table, the

Table 3. Comparison of gene flow from a herbicide-tolerant field into male-fertile and male-sterile OSR bait plants.

Distance from field (m)	Proportion of flowers fertilised (p_f) [1.]	Proportion of potential seed set (p_{ps}) [2.]	Proportion herbicide tolerant (p_{ht}) [3.]	No. sites scored for p_{ht} [4.]	Gene flow estimate (F) [5.]
Male-sterile recipients					
0	0.801	0.656	0.889	4	0.583
10	0.689	0.416	0.733	2	0.305
50	0.726	0.416	0.786	3	0.327
225	0.756	0.362	0.569	1	0.206
550	0.426	0.156	0.655	4	0.102
800	0.431	0.162	0.117	4	0.019
Male-fertile recipients					
0	0.82	0.82	0.145	4	0.119
10	0.812 [6.]	0.8 [6.]	0.049	4	0.039
50	0.812 [6.]	0.8 [6.]	0.019	4	0.015
225	0.812 [6.]	0.8 [6.]	0.024	4	0.019
550	0.755	0.738	0.002	4	0.001
800	0.861	0.841	0.034	4	0.029

[1.] No. fruits/no. flowers exposed during the period. [2.] $p_{ps} = p_f \times s/22$, where s = mean no. seeds per fruit at that site. The mean no. seeds per fruit from in-field plants is 22. [3.] The proportion of a sample of harvested seeds which gave rise to herbicide tolerant seedlings. [4.] A site is a set of 10 male-sterile or male-fertile plants which were placed in rows different distances from the source field. [5.] An estimate of gene flow from the herbicide-tolerant field, expressed as the proportion of the seeds, which would have been produced from these flowers in a normal crop, which carried herbicide tolerance, $p_{ps} \times p_{ht}$.
[6.] Not determined for these sites and so obtained by deriving the mean from other distances.

proportions of flowers fertilised declined with distance from the field for MS plants (from 80% to 43%), but did not do so for MF plants, remaining at about 80%. This reflects declining access to incoming pollen with increasing distance from the field for MS plants, but the predominance of self-pollen bringing about fertilisation on MF flowers. The proportion of potential seed set (the proportion of ovules fertilised compared to a fully-fertilised crop) achieved on MS flowers declined more steeply than the proportion of flowers pollinated, a result of the decline in seeds per siliqua. The proportion of harvested seeds which carried herbicide tolerance from the nearby field declined with increasing distance for MS flowers. However, that decline was largely due to the high value on the border of the field and the very low one at 800 m, with herbicide tolerance in the harvested seed otherwise varying between 57% and 79%. For MF flowers, the proportion of harvested seeds which were herbicide tolerant fell greatly in the first 10-50 m but after that variable levels were recorded. These percentages mask variability from site to site at each distance from the field.

Figure 7 presented data from three seasons for the proportion of flowers fertilised (p_f) and the proportion of potential seeds set, the product of p_f and an estimate of the seed set in each fruit (p_{ps}). To use this latter variable from these data sets to determine the likely proportion of herbicide tolerant seed set on MF plants over wide areas, it is necessary to relate p_{ps} for MS plants in the July 1999 experiment (mean = 0.302 excluding the value for 0 m) and p_{ht} for MS plants (mean = 0.025 excluding the value for 0 m). Comparing these values suggests that the recorded values for p_{ps} for MS plants will over-estimate p_{ht} for MF plants approximately 12-fold. The uncertainty in this figure is high, but it can be used tentatively to predict the response of MF plants where MS data are known.

Additional 10-plant groups of male-sterile plants were distributed more widely throughout the area in July 1999. These sites varied in distance to the nearest field from 550 to 3100 m, and to the herbicide tolerant field from 1100 to 7025 m. The proportion of herbicide tolerant seedlings was determined in samples of these seed (Figure 12), although seed numbers produced and tested at some sites were low. The percentage of those seeds which carried the herbicide tolerance trait varied from 0 to 27% (average value 13%). As in the July 1998 experiment using the GM field at Bridge of Earn, overall this proportion was similar to the proportion of OSR fields available to pollinators over a radius of a few km. A wind rose for the period of exposure of male-sterile plants is given in Figure 13, showing the direction from which the wind was blowing. The wind was largely from the W, NW and SW during the

period, with only brief periods of light E to S winds. The herbicide tolerance found in the progeny of MS plants to the SSW and WSW of the source field was therefore appearing at sites several km upwind.

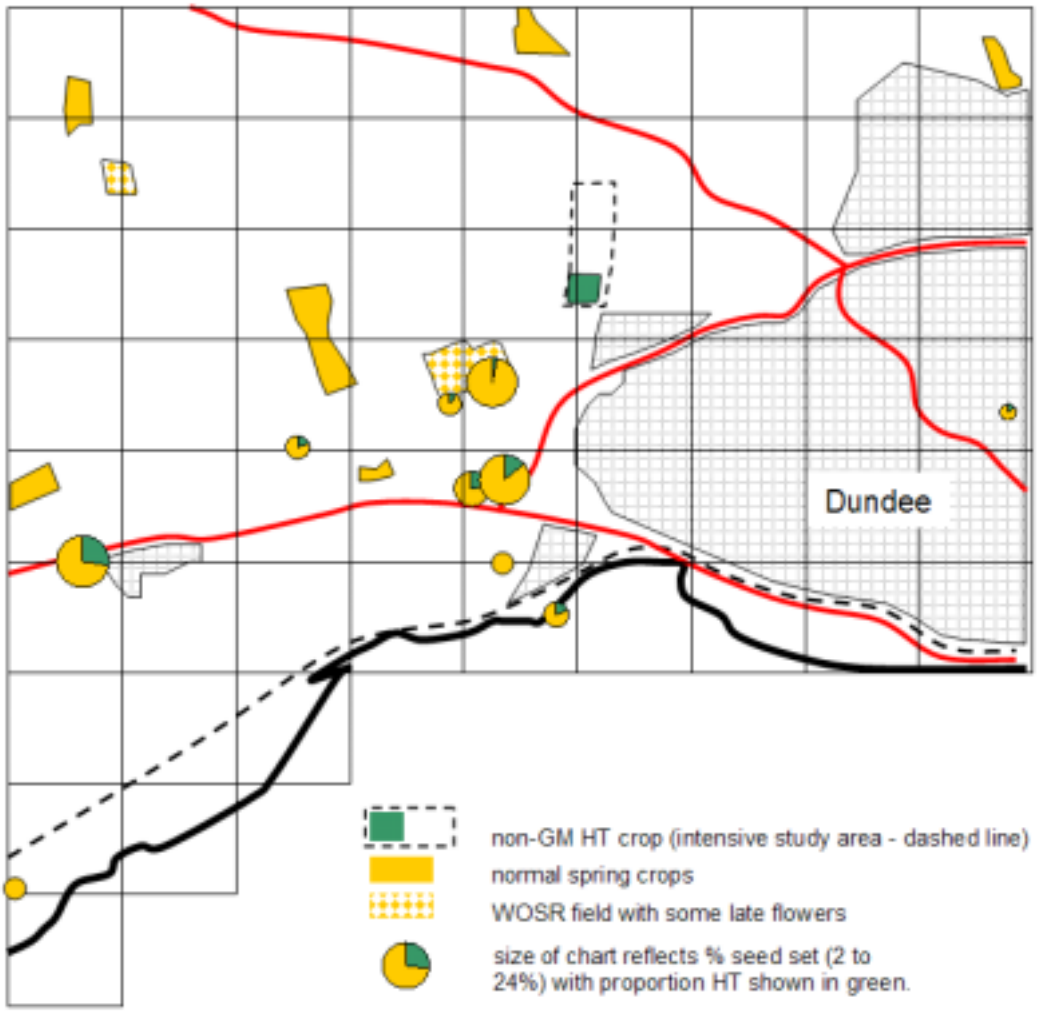

Figure 12. Results of screening male-sterile bait plant progeny for herbicide tolerance in July 1999.

Effect of size of recipient population

The field experiment described in the last two sections also included an element designed to investigate the effect of the size of the recipient population on gene flow. All previous work relied on the use of 10-plant groups, with the exception of the 16 plants at Faskally. It may be expected that larger groups of male-fertile plants would display lower rates of inward gene

flow due to the dilution of incoming pollen with self-pollen. To the E side of the herbicide resistant field, 10-plant pot-grown colonies and field-sown blocks of 100 m^2 and 900 m^2 of the male-fertile cultivar Maskot were located, all approximately 50 m from the edge of the field. The 10-plant colonies at this position produced seeds with a frequency of

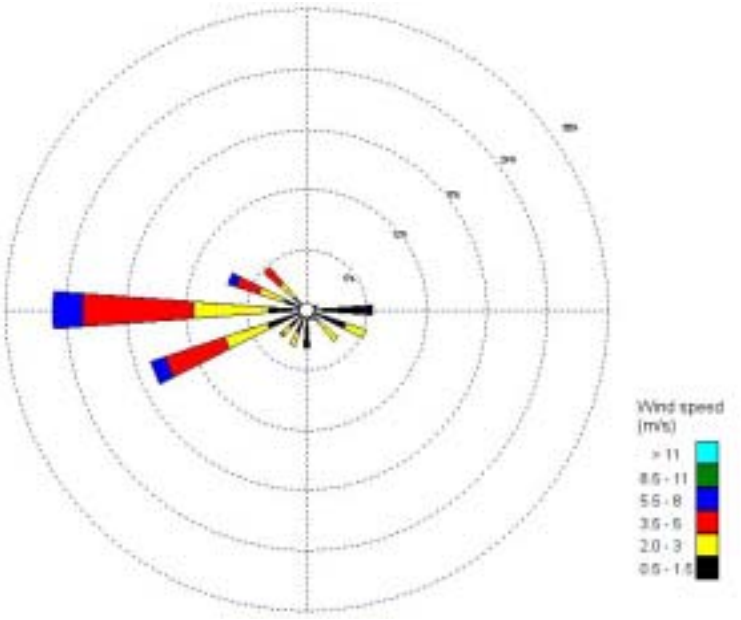

Figure 13. Wind rose indicating the strength and direction the wind was blowing from during the period of exposure of male-sterile plants in the experiment reported in Figure 12 above.

approximately 0.45% herbicide tolerance. Mean values from the 100 m^2 and 900 m^2 plots were 0.126% and 0.116% respectively. In other words, there was an approximately 4-fold reduction in cross-fertilisation as the sink population was increased from 10 plants to large blocks. The 100 m^2 and 900 m^2 plots were sampled across a grid, and the values found for each coordinate used to produce a contour map of cross-pollination frequency (Figure 14). Values are high to the W, facing the herbicide tolerant field 50 m away, but are also higher near the S and E edges. The highest values were not always at the edge, but sometimes a few metres back from the edge. Patterns were subjected to Mantel tests and found to be highly significantly different from random, and similar to each other across the two plots. We interpret the patterns as reflecting pollinator movements both away from the source and returning to the area following a visit to the home colony. The contour plots confirm an edge effect, although not a simple one, implying that the gene flow into larger areas would be lower than 0.1%, and that discarding the edge of seed production plots would reduce cross-contamination slightly.

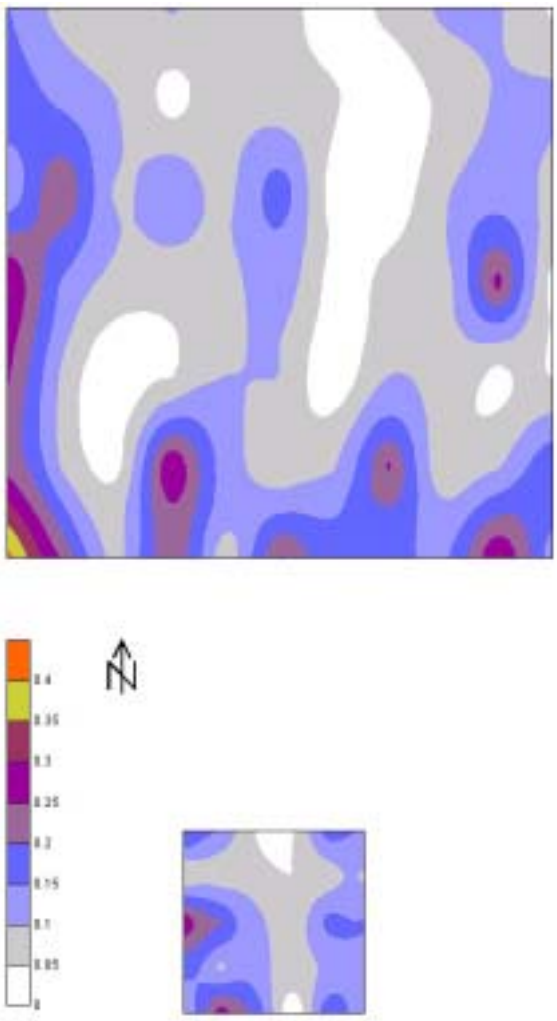

Figure 14. Contour plots of cross-fertilisation frequencies across a small and large block of normal male-fertile OSR 50 m to the E of a 7 ha crop of herbicide tolerant OSR

Studies on insect pollen vectors

Pollen may be delivered to the stigmas of oilseed rape flowers by several different types of insect. A number of uncertainties exist over the role of insects in this process. Honeybees repeatedly re-visit patches of flowers, and so there is uncertainty about the degree of landscape-scale mixing which they could achieve. To investigate mixing within colonies, pollen was identified and counted from pollen loads brought back to a hive working oilseed rape. Fingerprinting confirmed that pollen loads brought back to single colonies on single days were of several different OSR genotypes, consistent with observations of flight lines of foragers at hives. Sixty pollen loads selected from the bulk harvested were examined to represent the three main colour classes (Table 4). The yellow class contained pollen loads

comprised primarily of either of two species, *Brassica napus* and *Sinapis arvensis.* The brown class were clover, either *Trifolium repens* or *T. pratense*, and the blue-grey type all predominantly rosebay willowherb, *Chamerion angustifolium.* Approximately 70% of the pollen load harvest from this colony was of the yellow class. OSR pollen was found in every pollen load, including those predominantly of non-OSR pollen. A wide range of pollen types, representing the range of flowers likely to be visited by the colony, was present in low frequency in pollen loads of all types. Together, this implies that bees pick up pollen grains of all of the flower types visited by the colony from sister workers when inside the hive.

To determine whether bees exiting colonies carried viable pollen, a small sample of honeybees was collected emerging from a bee colony situated 800 m from the GM trial (Figure 2) and applied to the stigmas of MS flowers (Ramsay *et al*. 1999*).* From five bees brushed onto five flowers each, three bees gave seed set, a total of 62 seeds including 12 carrying transgenes. The spread of pollen around the foraging area of a colony was further verified by sampling bees working plants other than OSR. A sample of bees was collected from ornamental plants on the SCRI site in the summer of 2000, about 1 km from the nearest field of OSR which at that time was coming out of flower. Twenty six out of 40 honeybees gave seed set when brushed onto stigmas of male-sterile plants, and four out of five bumble bees also gave seed set when applied to stigmas of male-sterile OSR flowers, confirming a wide environmental distribution of viable OSR pollen by these pollinators.

The possibility of other insects contributing to gene flow in OSR was investigated by collecting pollen beetles and cabbage seed weevils from OSR plants, transferring them into a clean container in the laboratory to prevent the direct physical transfer of pollen from the original containers, and releasing them into fine-meshed cages within a polythene house containing male-sterile plants of oilseed rape. Seed was set on 13/30 flowers exposed to pollen beetles and 8/10 flowers exposed to seed weevils, confirming that these insects can act as effective pollen vectors. Pollen beetles were also discovered on the male-sterile plants set out at Faskally (see following section) when the plants were collected for return to SCRI. Given the very large separation distance of the plants at Faskally from other OSR it seems likely that pollen beetles rather than bees were the main pollen vectors at that site.

Table 4. Composition of pollen loads in the three predominant colour classes.

		Mean pollen composition within pollen loads of different types [1.]											
		Pollen types											
	no.	Bn	Sa	Can	Tr	Tp	Sj	Car	Hs	Tl	Ud	P	O
Bright yellow loads													
B. napus	12	99.90	0.03	0	0.02	0	0.01	0	0	0.03	0	0.02	0
S. arvensis	7	2.78	97.00	0.01	0	0	0	0.04	0.04	0.04	0	0	0.09
mixed *Bn/Sa*	1	37.81	61.83	0	0	0	0	0.27	0.09	0	0	0	0
Brown pollen loads													
T. repens	14	0.90	0.06	0.03	98.69	0.02	0.01	0.04	0.04	0.06	0	0.12	0.02
T. pratense	6	1.25	0.01	0.04	0.10	98.37	0	0.03	0.06	0.01	0	0.06	0.06
Dark blue-grey loads													
C. angustifolium	20	5.44	0.16	92.77	0.13	0.03	0.10	0.21	0.14	0.66	0.02	0.02	0.33

Bn - *Brassica napus*, Sa - *Sinapis arvensis*, Can - *Chamerion angustifolium*, Tr - *Trifolium repens*, Tp - *Trifolium pratense*, Sj - *Senecio jacobaea*, Car - *Cirsium arvense*, Hs - *Heracleum sphondylium*, Tl - *Tilia* species, Ud - *Urtica dioica*, P - *Poaceae*, O - other. [1.] At least 1,000 pollen grains scored per pollen load. Every one of the 60 pollen loads contained some *Brassica* pollen.

Pollination over long distance

In the experiment conducted in May 1999, two sites at extreme distance from known source oilseed rape plants were fertilised. The resulting plants inherited male-sterility from their mothers, as expected, and were fully female-fertile indicating that they were the result of pollination by *B. napus* and not turnip, *B. rapa*. At the site in an isolated area of Fife, 5.3 km from the nearest oilseed rape field, 5.8% of flowers were pollinated. The relatively few seeds forming in most of these fruits represented 0.5% of potential seed production (see methods for description). DNA fingerprinting of seeds from the larger fruits gave patterns which were consistent with the possibility that the nearest field contributed the pollen. The site at Faskally, 26 km from the nearest field of OSR, produced 42 seeds on 16 plants, representing 3.1% of total flowers pollinated and 0.15% of the production of normally-fertilised plants. Additional small fruits with shrivelled empty ovules were found on these plants. Judging by the position on the inflorescences, pollination events were scattered through the period of exposure of the flowers. Pollen beetles were found on these plants at the time of uplifting for return to SCRI and these insects may have pollinated the plants.

DNA fingerprinting was used to compare mother plants with their progeny seedlings. Three mother plants are shown in Figure 15. The progeny of the male-sterile plants set out at Faskally had in many cases one or more bands which were absent from the mother plants, confirming that they were derived from true fertilised ovules. This was also confirmed by the non-flowering rosette growth of the plants, thereby showing dominant winter OSR traits rather than the spring-type growth of the mother plants. Using 4 I-SSR primers, a total of 16 polymorphic bands were scored. Of these polymorphic bands 7 were informative, in that they were absent in some mother plants and present in progeny, and two of these bands were absent in all mother plants tested and present in progeny. Figure 15 shows a band in this latter category. Analysis of the DNA fingerprints of progeny seedlings places them into distinct clusters, implying different varietal origins. However, given the genetic heterogeneity within OSR cultivars, it was not possible to relate single pollination events directly to paternal cultivar.

All arable and roadside land within 30 km of Faskally was surveyed and no flowering crops or roadside feral plants of OSR were found closer than the fields at 26 and 30 km, as previously described. We investigated the possibility that garden sources of swede pollen

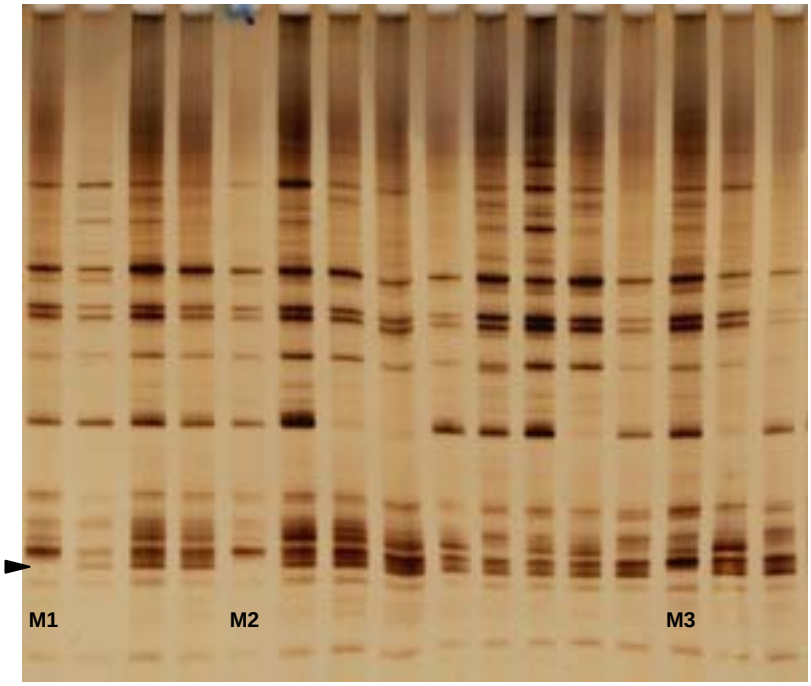

Figure 15. I-SSR DNA fingerprints of three MS mother plants (M1, M2 and M3) set out at Faskally and three, eight and two progeny plants respectively all showing a non-maternal band.

from bolting plants could have donated the pollen, even though none had been seen in the survey. Swede is also *B. napus* and is freely cross-compatible with *B. napus* OSR. Consequently, representatives of the progeny of the pollination events at Faskally and the Tentsmuir site in Fife were grown to flowering, pollinated with swede pollen, and the seeds germinated. The seedlings from the backcross to swede were compared to experimental seed from known (swede x rape) x swede backcrosses. The recessive yellow-fleshed hypocotyl trait present in common swede cultivars and segregating in the known swede test-cross was absent from the test-crosses made to Faskally and Tentsmuir progeny, confirming that the pollinations at these distant sites were unlikely to be short range events from local flowering swedes.

Discussion

This regional scale, intensive study of cross-fertilisation frequencies, mostly using male-sterile recipient plants, generated a comprehensive and unique data set. The results are particularly relevant to the flow of genes from large to small blocks of plants, but add considerably to knowledge of gene flow more generally between blocks of different size that occur throughout the arable landscape. There are several outputs from this work: an improved understanding of the vectors involved in the effective movement of OSR pollen around the landscape which indicates that airborne pollen can be relatively unimportant; a description of the pattern of decline in cross-fertilisation with increasing distance from the source; an understanding of the different levels of gene flow into different types of recipient population; and predictions beyond the bounds of the data-sets in this study to a range of realistic situations.

Distance and fertilisation frequency

There have been several previous studies of gene flow in oilseed rape. Some were based on small sources of pollen and large blocks of recipient plants, and found much smaller values of gene flow than in this study. Gene flow from realistically-sized areas have been studied using non-GM fields (Timmons *et al.* 1996; Bilsborrow *et al*. 1998) and GM fields (Champolivier *et al.*,1999; Downey, 1999; Simpson *et al.,* 1999). Many of these studies investigated gene flow into adjacent fields. Values of cross-pollination into the interior of adjacent fields are frequently around 0.1%. Simpson *et al.* (1999) have investigated gene flow into arrays in four directions up to 400 m from a source field, recording the proportion of the harvest from male-sterile plants which carried a transgene. These studies agree with those of the parallel project reported here in that transgenes can be found readily at some distance from sources, although the data were not quantified in a manner which allows direct comparison to the results described here, and the distances investigated were smaller. Timmons *et al.* (1995) also investigated longer range gene flow and reported fertilisation events on emasculated bait plants up to 2.5 km from the nearest source. The results of this study confirm and extend these findings. Confirmation was obtained that: i) gene flow from a large source into a substantial area of a fully-fertile form of the same crop nearby is of the order of 0.1%, with higher frequencies around the periphery and lower frequencies internally; ii) gene flow is intense immediately beside the source but rapidly declines over tens of

metres; and iii) beyond this initial phase, gene flow declines with distance in a gradual and erratic manner.

This study aimed at quantification of gene flow on a scale not attempted in previous work. Ensuring reliability in such data sets requires certain precautions. To attempt to prevent or reduce misleading accidental pollination by experimenters, flowers were not handled and sites were visited on alternate days, one day for sites relatively close to fields, and one for sites further away. Additional safeguards were in place for the two most distant sites. In these cases SCRI staff visited only once (Tentsmuir NNR at 5 km from the nearest field) or not at all (Faskally at 26 km from the nearest field) until the end of the experiment and the plants were watered by the staff of other organisations. Results from these last two sites are likely to receive attention when the significance of isolation by distance is being considered for oilseed rape crop purity, and so additional critical appraisal has been made of possible explanations for the seed set other than through natural pollen movement. This appraisal considered 10 possible alternative causes of seed set on these plants and is presented in Appendix A. The authors of this report believe that natural long-distance pollination is the most likely explanation for the observations on these plants. It is not clear whether the levels of pollination at these distances for these two sites are typical for isolated patches of plants, and this has been taken into account in the synthesis presented below.

Characterising the decline with distance was a major aim of this study. Mathematical description of the decline presented in Figure 7 cannot adequately describe the slope as each season's results differ in rate and pattern of decline. However, a number of points are clear. The initial steep fall soon changes to a gradual decline so that, for example, a doubling of distance has a small effect on the cross-pollination rate. It is also clear that there was high variation in the level of pollination at any one distance, within as well as across seasons.

In addition to the gene flow measured by pollination and fertilisation of male-sterile flowers, gene flow was recorded into a restricted set of similar populations of male-fertile plants, and into one small and one large population of normally-fertile oilseed rape. The male-sterile plants provide a straightforward means of discovering the patterns of decline in gene flow across the landscape. They are also of direct practical relevance as some cultivars use male-sterile plants in their production and also may leave MS feral and volunteer individuals in subsequent seasons. Crossing from fields to small blocks was a landscape-scale

phenomenon, in that the percentage of a specific gene in the progeny of the small blocks was proportional to the fraction of fields carrying that gene within a few kilometres. This finding was substantiated in two growing seasons using different source genes, and confirmed that mixing of gene flow from different sources is efficient over long distances.

The comparison of fertilisation in male-sterile and male-fertile plants in 1999 provided the means to use the extensive data sets for male-sterile oilseed rape to predict landscape-scale gene flow in male-fertile oilseed rape. The inclusion of larger blocks of oilseed rape also allowed preliminary extrapolations to predict gene flow into fields. Data from male-sterile bait plants overestimated cross fertilisation to similar male-fertile populations by about one order of magnitude. Crossing rates from large blocks into small blocks of oilseed rape scattered over the landscape of the order of 1% should therefore occur regularly. Crossing between large blocks is (as between the herbicide tolerant source and the fully fertile recipient fields in 1999) of the order of 0.1% or below, though variable spatially. This value is consistent with other recent estimates of cross-fertilisation into whole fields. The data on pollination frequency have been collated into one chart, presented at the end of this report together with notes on the assumptions and uncertainties which may affect these figures as Appendix B. However, further experimentation would be required to confirm that these extrapolations are valid.

Insect and wind pollination

A number of previous studies have commented that wind might be the most important vector in the movement of pollen across the landscape. This assumption has driven some of the modelling work on gene flow in this crop in the UK. It is certainly true that low or moderate quantities of oilseed rape pollen can be found in the atmosphere in some seasons many km from the nearest source. In May 1998 for example, a maximum daily count of 59 *Brassica* pollen grains m^{-3} was recorded at SCRI, about 2 km from the nearest source. Some pollen was recorded most days that month, whereas in May 1999 on most days no *Brassica* pollen was recorded. The assumption was made at the beginning of the project that airborne pollen was responsible for most of the pollination of flowers distant from fields, and plans were made to associate pollen collected on static traps with fertilisation frequencies. Data were collected on airborne pollen, but parallel studies indicated that insects were the most important pollen vectors transferring pollen from fields.

The main lines of evidence supporting insect pollination are: i) the result of excluding larger insects with netting, ii) the pattern of fertilisation events indicating multiple fertilisations per flower, even when few flowers were pollinated, iii) the lack of association between direction of gene flow and wind direction. Netting did not reduce airborne pollen deposition within cages but may have altered the microclimate around the plants. The principal effect of caging was that much less fertilisation occurred for a given level of deposited pollen. Further analysis of the data (Thompson *et al.* 1999) indicated that when few flowers were fertilised on male-sterile plants at long distances from sources, the mean number of seeds per siliqua was relatively high, and that several siliquae at these sites had many seeds. This pattern of pollination was considered to be unlikely if wind was the main pollen vector, but consistent with an insect visiting the plant and transferring several pollen grains to each stigma visited. Additional evidence was provided by the observation that as high a proportion of herbicide tolerant progeny seed was found at a range of distances upwind as was found downwind. There are previous publications which preferred to ascribe pollination events largely to airborne pollen (Timmons *et al.*, 1995; Simpson *et al.*, 1999). The first of these assumed that petal removal would deter insects, an assumption unlikely to be correct as apetalous mutant forms of oilseed rape are visited by bees as frequently as normal types. It also appears that Simpson *et al.* (1999) misinterpreted meteorological data, reversing the wind direction on which their comments were based. It remains possible however that in some seasons and on some days when airborne oilseed rape pollen is relatively abundant, significant pollination from airborne pollen may also occur. Nevertheless, an understanding of the vectors is important to the assumptions made in modelling or any form of prediction.

The most abundant pollinators visiting flowers were honeybees and bumble bees, while hover flies, pollen beetles, cabbage seed weevils, and other flies were also noted. Pollen beetles, cabbage seed weevils, honeybees and bumble bees were all directly demonstrated to be potential pollinators. Due to their density on the crops and their efficiency as pollinators, it is likely that honeybees and bumble bees are the major vectors of oilseed rape pollen over distances of a few km. As pollination events were seen beyond known worker honeybee foraging distances and were associated with the presence of pollen beetles, these insects and others may contribute to gene flow over even longer distances.

Each insect will have its own pattern of behaviour leading to different patterns of pollination. Bees for example are strong fliers, very effective pollinators and have foraging ranges limited by the need to return to a colony with the products of the trip. Foundling queen and drone bumble bees at certain times of year may not be restricted to a home range. Bumble bee workers may learn routes with regular foraging sites visited each trip. However, honeybees are regarded as faithful to specific patches of forage. There are several mechanisms by which pollen could be distributed around the foraging area of a colony of honeybees. Scout honeybees may move freely from one patch to another on a single trip. Workers may be recruited to exploit a different source, and thus carry pollen on their bodies from one field to a new one. One further mechanism which may permit more frequent cross-pollination across wide areas is nest-mate mixing. Bees return from rape fields with a large number of free pollen grains which are available for transfer to other workers through the intimate physical contact between bees in the hive. This mixing does occur, as was noted by observing contaminating pollen grains within pollen loads predominantly of one type. Pollen on the surface of the bee's body on leaving the hive is combed from the surface and incorporated into the pollen load while it is being collected. Although most pollen is from the type of flower being visited on the trip, the contaminating pollen is of mixed type reflecting the range of pollen being brought in by the whole colony. It is therefore available for the pollination of flowers met on the trip. As both observations of bee flight lines at hives and DNA fingerprinting of pollen collected at hives indicated that colonies frequently work several OSR fields at once, nest-mate mixing will contribute to the spread of pollen across the foraging range of a bee colony.

The patterns of gene flow across the landscape can be predicted to a degree according to the type of pollen vector. Most pollen will be deposited after a short flight of a pollinating insect to another flower, and hence the majority of cross-pollination events will take place over short distances. Over longer distances both honeybees and bumble bees are likely to be relatively insensitive to light winds in their foraging patterns, possibly preferring to travel upwind to a forage resource if odour is used as one means of locating the resource. Weakly flying insects may travel downwind and may therefore bring a directional component to gene flow, whereas others may exclusively travel upwind. Although no quantification has been attempted, it is likely that where bee populations are sufficient, these vectors will give the highest levels of gene flow and that this gene flow will be effective over distances up to several km. The gene flow seen at longer distance in this study is likely to be caused by one

of the less effective pollinating insects and will in most circumstances be at a very low level, especially in fully male-fertile recipients.

Implications of the findings

The research has implications for the achievement of purity standards in crops of oilseed rape and for genetic exchange between crop and feral oilseed rape.

Feral oilseed rape populations

The results of earlier studies during 1993-95 (Anon., 1999) were largely substantiated by later surveys, both in 1996 and during this project. The question of whether the feral populations that occupy roadsides, field margins and soil dumps lie within pollination distance of fields has been confirmed. Most of these populations are within 1 km of a field simultaneously in flower and will therefore be in potential pollen contact with fields. Many of these populations appears to be ephemeral, appearing in only one year, but around one quarter were found to occur in the same place over at least three years. One population found in the previous study was still in existence 12 years after the likely founding event, a shedding of seed into the field margin. Other such long-lived populations may exist but documenting them is not straightforward. Populations might consist of several source varieties. They probably change in genetic composition slowly over time through selection, further recruitment and, where the feral populations successfully shed viable seed, gene flow. It is feasible therefore that different genes from crops might accumulate in feral populations over time. However, as feral populations are usually small in area with respect to field crops, gene flow occurring back into field crops from feral plants is likely to be at a low level.

Crop purity

Impurities can arise in harvested seed either through their presence in the original sown seed, their introduction into the crop through the existence of volunteers from the seedbank, or through gene flow from other crops, ferals or volunteers.

OSR cultivars can be conventional (fully fertile), varietal association types usually with a dominant MS F1 hybrid component and a minor pollinator component, or restored hybrids of several types which may retain a low level of male sterility. Impurities in seed stocks due to cross-pollination will vary according to these characteristics as well as exposure to contaminating pollen. When the production of seed involves the planting of extensive blocks of male-sterile mother plants interspersed with male-fertile pollen donors, the long distance cross pollination seen in the experimental populations in this study will also occur, especially if the pollen input from their intended pollen donors is limiting. Any seed production system in oilseed rape which uses male sterility to generate F1 hybrids is therefore liable to pick up genes, including transgenes from GM field crops, over long distances. Cultivars retaining a degree of male sterility in the field crop will also be subjected to enhanced gene flow during the period of flowering of the crop, further enhancing impurity.

The results of this study indicate that if GM oilseed rape crops or other distinctive types of the crop are grown, the total exclusion of cross contamination to non-GM crops is unrealistic. Although cross-contamination declines with distance, even segregation according to bee flight ranges will be insufficient to prevent it. There are also important implications for the open trialling of seed-propagated GM oilseed rape with Part B consent, if regulations are brought in which do not permit any level of contamination of such GMOs in seed stocks. If, however, GM and non-GM crops were grown in the same farming region, the management of cropping systems to ensure purity at pre-determined levels should be possible, and the data and interpretations presented in this report will assist this process. Specified thresholds of impurity will be more difficult to achieve if the non-GM farm crops have impaired male fertility. Gene flow into these crops will be at higher levels than into conventional cultivars.

Possible future work

Several projects have investigated gene flow in oilseed rape. Oilseed rape gene flow occurs by self-pollination, airborne pollen (shown in this study to be a small component in the movement of genes), and insect vectors including social insects such as honey and bumble bees and free-living insects such as flies and pollen beetles. Each component will have its own pattern of decline over distance. The same components, modified by varying self-

pollination and varying attraction to insects, will contribute to gene flow in all seed crops. A fuller understanding of the relative importance of different pollen vectors, together with information on the intensity of gene flow and the patterns of decline with distance of each, would lay the foundations to predicting gene flow in and around other crops.

Gene flow to and among feral and wild species populations will occur, but its effect on their stability or decay is less well-known and is an appropriate subject for detailed study. The factors influencing feral life cycle biology and the relative importance of fresh seed spillage and the return of feral seed to the seed bank are relatively unknown.

The work presented here shows that impurities of one oilseed rape type in another will inevitably be introduced by gene flow if different types are grown in the same region. Reliable estimates of whole-field impurities are particularly needed for crops that are partly male-sterile.

Action resulting from the research

The results of this project need to be taken into account in discussions of segregation distances and the formulation of regulations affecting crop purity. Project staff have been involved in consultations on gene flow with government departments in the UK and have contributed expert opinion to EU committees concerned with GM regulations.

Conclusions

OSR gene flow out of fields has been shown to be due largely to insect-mediated pollination, contradicting some previous opinions. Male-sterile plants have been used as sensitive indicators for gene flow and factors have been derived to convert MS data to frequencies expected for normally-fertile plants. The size of sink populations and factors such as weather, pollinator populations and relative strengths of competing sources of pollen will affect levels of cross-pollination. A guide to expected frequencies of cross-pollination in different situations was presented. Cross pollination falls rapidly over the first few tens of metres, but the decline beyond a few hundred metres is gradual and erratic. Many of the feral

populations of OSR scattered around agricultural land are therefore in potential pollen contact with several fields of oilseed rape. Since most of these populations are fully male-fertile, when they have the opportunity to mature they are likely to shed seed containing a small percentage (around 1% in some situations) of seeds which are hybrids with the surrounding fields. Pollination of one field to the next is likely to be, as other studies have indicated, less than 0.1% averaged over the field. Two conclusions can be drawn from the results presented here. Firstly, the decline in cross-pollination to other fields may be gradual over long distances. Secondly, the results imply that greater than 1% crossing might occur in crops with impaired male fertility over hundreds of metres, perhaps even over km if many fields act as a source. A low level of cross-pollination was also detectable over very long distances from fields in a situation where no closer feral plants were found. Dispersive insects with the ability to pollinate OSR, such as pollen beetles, can move over distances greater than honeybee foraging ranges, and so distant pollination at low incidence will occur. Placing an upper limit on the distance over which this gene flow occurs is not possible.

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Appendix A. Long-distance pollination: critical appraisal of results.

In this study, male-sterile plants were set out at sites (Tentsmuir, 5 km, and Faskally, 26 km from the nearest field) which were expected to achieve pollination exceedingly rarely if at all. This was performed in order to characterise better the long tail of landscape-scale gene flow. Finding fertilisation readily if at low frequency at these sites was surprising. Possible explanations other than natural long-distance pollen transport for these pollinations are discussed below.

Cause	Relevant observations	Likelihood
Parthenocarpic seeds or leaky male-sterility	Progeny carried DNA bands absent in mother plants and all displayed winter OSR habit, unlike mothers.	Discounted
Pollination during transit to site	Flowers were removed on delivery (*Tentsmuir*) or next day (*Faskally*).	Discounted
Pollination during return journey to SCRI	Position of fruits indicated that the great majority of events were not from flowers receptive on return journey.	Discounted
Plants pollinated in polytunnels after return to SCRI	See above. Inflorescences were marked on return. Also a previous batch of 600 MS plants left in this tunnel during the flowering season had no pollination events.	Discounted
Surveyors or other staff pollinated flowers	SCRI staff made only one visit to *Tentsmuir* plants (on a day without work near OSR fields) and none to *Faskally*. An SNH warden (who commuted through an OSR-growing area) watered *Tentsmuir* plants and Freshwater Fisheries Laboratory staff (living and working in an area with no flowering OSR) watered *Faskally* plants. Staff avoided touching flowers.	*Tentsmuir*: unlikely. *Faskally*: discounted.
Male-fertile plant amongst batch	High levels of pollination would be seen, and progeny plants would have been male-fertile.	Discounted
Garden *B. rapa* (e.g. turnip) plants left to bolt and flower locally	Progeny plants had full female fertility indicating that they were not triploid hybrids.	Discounted
Garden *B. napus* (e.g. swede) plants left to bolt and flower locally	Progeny were vernalised and backcrossed to swede, and their progeny tested for recessive swede traits. None were found.	Discounted
OSR fields closer to site than survey indicated	*Tentsmuir*: the land was flat and roads do not completely cover the area. However, the survey was considered reasonably accurate and no arable land existed within the 3km wide forest bordering the site. *Faskally*: no arable land within 5 km. Beyond this arable land was restricted to easily surveyed valley floors. The landscape opened out with more extensive arable land after about 24 km.	*Tentsmuir*: possible but not likely and discounted within 3 km. *Faskally*: largely discounted.
Feral OSR plants closer than known fields	*Tentsmuir*: several field margins could not be adequately surveyed. *Faskally*: no recent history of OSR locally so no seed spillage at roadsides or field margins likely. Closer scrutiny and unsuitable habitat rule out plants within about 2.5 km. Use of agricultural soil containing OSR seeds in landscaping activities at longer distances cannot be fully ruled out. However, a car-based survey to 30km at the end of the period found only charlock (*Sinapis arvensis*) and no *Brassica* plants in field margins and roadsides.	*Tentsmuir*: possible. *Faskally*: possible but not likely, and largely discounted within a few km of the site.

Appendix B. Estimating rates of gene flow in oilseed rape over longer distances

Scope

The data generated in this study can be used to guide predictions of gene flow in oilseed rape in other situations providing that the assumptions and uncertainties listed below are understood. On the following page, two charts are presented. These show overviews of patterns seen in the data, extrapolations from these situations, and guesses to new situations.

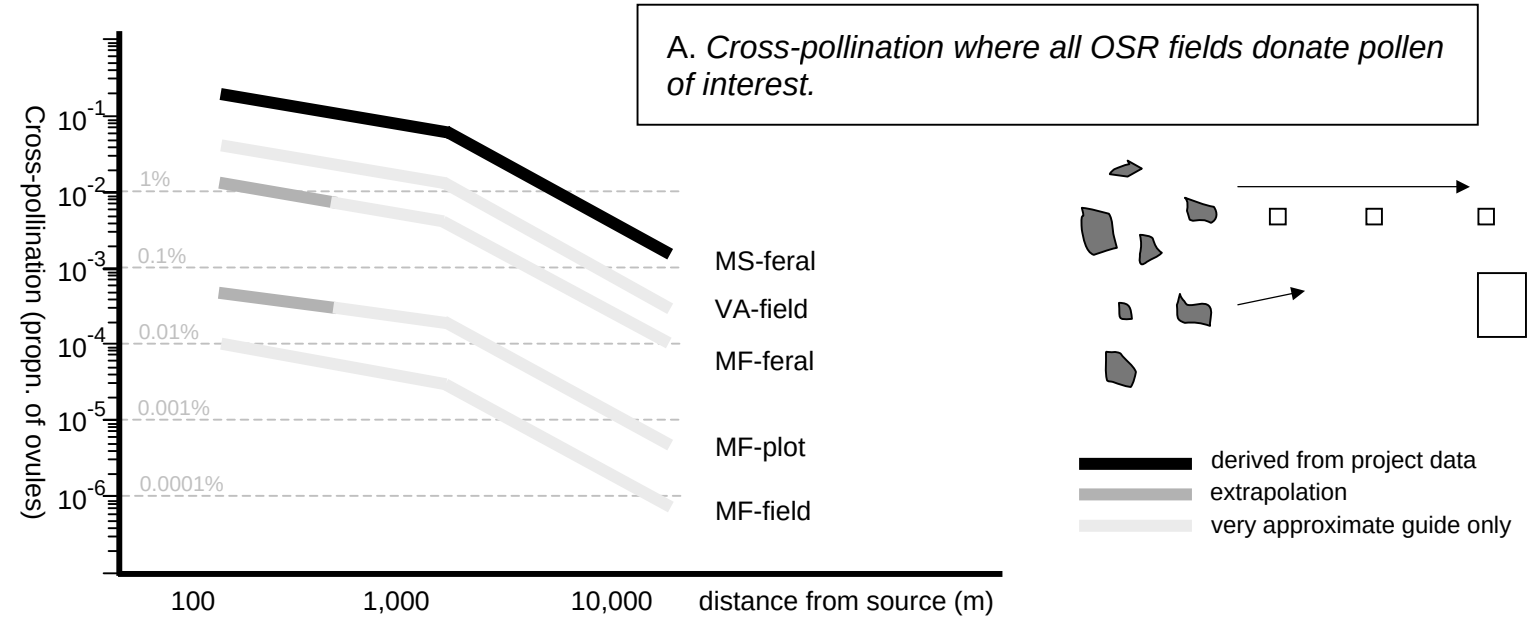
Assumptions

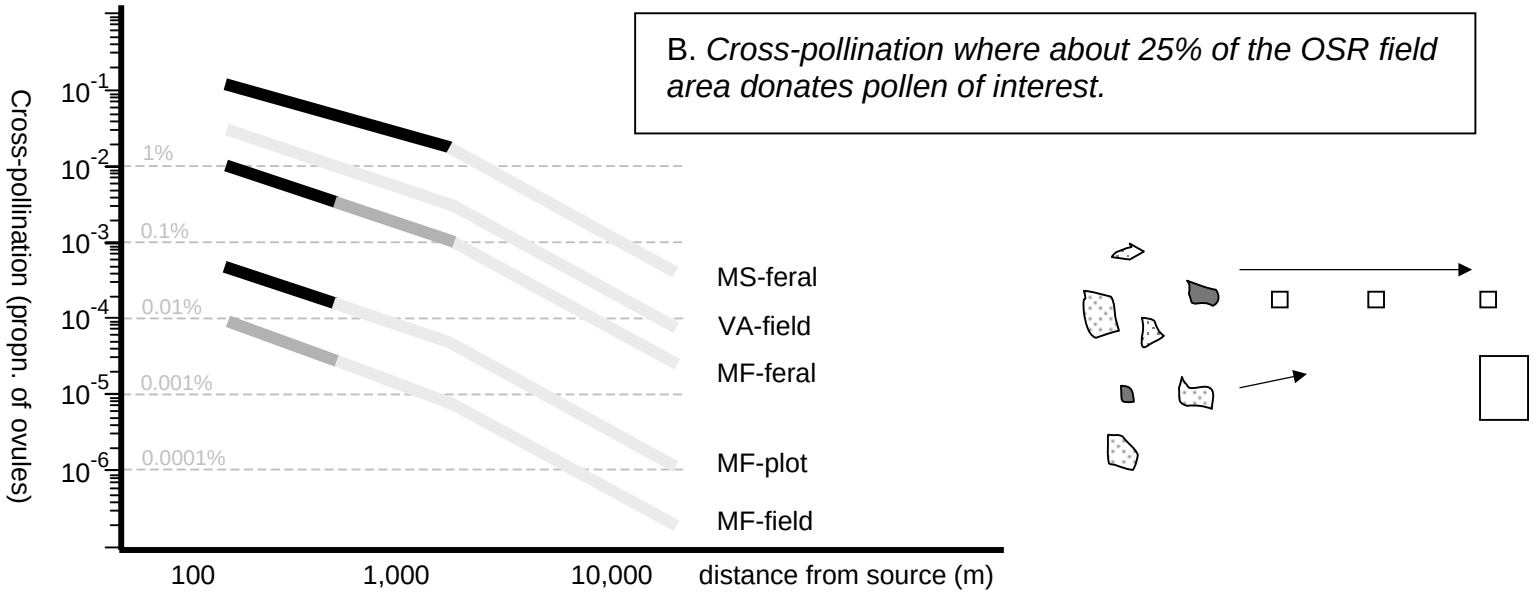
The following graphs and their interpretation make a number of assumptions.

1) That the pattern of decline with distance seen in small groups of male-sterile plants will also apply to larger areas of OSR plants. As the balance of types of pollinator may change with increasing distance, this may not be justified.
2) That the levels of gene flow seen in the experiments described in this report are typical of other OSR-growing areas.
3) That in a varietal association cultivar with 80% of plants male-sterile, an arbitrary 20% of flowers are pollinated in a manner similar to those on small groups of male-sterile plants and 80% are pollinated in a manner similar to those on male-fertile plants. This assumption is completely untested.
4) In environments with mixed sources, sites closer to the source carrying genes of interest may have a greater proportion of pollinations from that source, but this local influence will be diluted at sites further away from the source.

Confidence limits

Variation can be high between sites and between seasons. For example, in all available data sets using 10 male-sterile recipient plants between 500 and 2000 m from the nearest field, the mean proportion of normal seed set was 0.111 with lower and upper 95% confidence limits for the mean 0.081 and 0.147. The range was 0.001 to 0.377. Also, it should be borne in mind that the frequency of very long distance events was derived from only one site 26 km from the nearest known source which may not be typical. A number of factors may introduce greater variation in some circumstances. Landscape-scale features, such as topology, and competing insect forage crops will have an influence. The populations of different types of pollinators will vary between sites and seasons. Weather will also influence both the activity of insect pollinators and the quantities of pollen becoming airborne. For these reasons, and the inherent uncertainty in some of the extrapolations made here, the graphs presented can only be an approximate guide.


A. Cross-pollination where all OSR fields donate pollen of interest.
Cross-pollination (propn. of ovules)
10-1
10-2
10-3
10-4
10-5
10-6
1%
0.1%
0.01%
0.001%
0.0001%
MS-feral
VA-field
MF-feral
MF-plot
MF-field
100
1,000
10,000
distance from source (m)
derived from project data
extrapolation
very approximate guide only


B. Cross-pollination where about 25% of the OSR field area donates pollen of interest.
Cross-pollination (propn. of ovules)
10-1
10-2
10-3
10-4
10-5
10-6
1%
0.1%
0.01%
0.001%
0.0001%
MS-feral
VA-field
MF-feral
MF-plot
MF-field
100
1,000
10,000
distance from source (m)

Estimating rates of gene flow in oilseed rape over longer distances (cont.)

Notes on the graphs:

1) The following definitions of recipient population type apply:

    a. MS-ferals – a group of about 10 male-sterile plants
    b. MF-ferals – a group of about 10 normally male-fertile plants
    c. MF-plot – an area of tens or hundreds of square metres of normally fertile plants
    d. MF-field – a typically-sized field of normally-fertile plants
    e. VA-field – a typically-sized field of a varietal association cultivar with about 80% male-sterile individuals.

2) Cross pollination is presented as the proportion of normal seed set (assuming 22 fertilised ovules in each fruit). For male-fertile plants, this is assumed to be the same as the percentage impurity in the harvest. However, male-sterile plants have harvested seed numbers depressed to varying extents, hence the presentation of data involving male-sterile plants as the percentage of normal seed set.