Factors affecting cross-pollination in oilseed rape growing under UK conditions

Defra Project RG0125

Final Report

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Authors' Preface

This report is intended as a summary of the progression of the study and of the main general findings, particularly as they relate to the current debates on the coexistence of GM and other types of crop. The detailed methods, results and analysis of the experiments in this project will be submitted to refereed international journals for peer review. Further information on highly technical aspects of the work, for example on the genetics of oilseed rape, the diagnostic techniques and statistical modelling can be obtained on request from the authors.

Acknowledgement of contributions

Many people from the collaborating organisations helped in field and laboratory or gave specialist expertise or advice. They include: from SCRI, J Anderson; RRes, J Perry, A McCartney; CSL, J McMillan, R Weekes; CEH, KH Hodder, M. Heuner; NIAB, C Pratt, J Thomas, C Norris.

The SIGMEA project (www.sigmea.go.dyndns.org) provided the means to compare data and results with other research in Europe on cross pollination.

None of the work in the final two years of the project would have been possible without the cooperation of the company supplying HEAR seed and the farmers who provided information on the cropping history of fields and allowed access for sampling plant material.

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EXECUTIVE SUMMARY

Experiments were carried out over four years to measure cross pollination between fields of oilseed rape over distances up to 5 km in farmed landscapes. The study provided unique data on cross pollination between fields of oilseed rape in natural configurations. The general conclusion is that cross pollination, though widespread, mostly occurred at too low a frequency to reach by itself the present GM labelling threshold of 0.9%. The only exceptions occurred in fields close to (50 m) a large field of 'donor' pollen.

The study began by examining pollination of surrounding crops by GM herbicide tolerant crops in the Farm Scale Evaluations in 2003. Very low levels of the GMHT trait, mostly well below 1 in 10,000 or 0.01%, were found up to 4 km in some surrounding crops. The circumstances of the FSE, in which a single source of GM pollen lies in a landscape of many other flowering fields would occur only in the first stage of commercialisation. A surrogate was therefore sought to represent GM cropping some time after commercialisation, when large areas of GM crops would be present.

High erucic acid rapeseed (HEAR) was chosen as this surrogate. HEAR produces oil for industrial uses, characterised by a high percentage of the fatty acid, erucic acid. It is distinguishable, genetically and biochemically, from the majority of oilseed rape grown for food and animal feed (LEAR or low erucic acid rapeseed). The oil from pure HEAR seed has around 50% erucic acid (%EA), that from pure LEAR around 0.02% EA, while HEAR-LEAR hybrids resulting from cross pollination have intermediate levels. There is an EU limit of 2% erucic acid in oil intended for use as food. The two types are grown in the same landscape in some parts of the UK.

In the first season (2003), techniques to detect HEAR pollen and hybrids between HEAR and LEAR were devised and tested in both laboratory and field. A method known as ‘quantitative PCR’ was applied to measure the difference in DNA in pollen, leaf and seed. An additional method of detecting cross pollination – by measuring the change in %EA of seed by gas chromatography – was also found to be suitable. The qPCR method detected and quantified HEAR pollen on bees and in samples of air, and HEAR-LEAR hybrids were found in receptor plots, all up to 1 km from the source (the limit of the test). The HEAR surrogate was therefore found suitable, in principle.

The reliability and logistics of the techniques were examined in 2004, when LEAR fields were grown next to, and 1 km from, a HEAR donor field on experimental farms in the north and south of the UK. The test operated under stringent conditions in that the fields had not contained HEAR varieties previously, so no HEAR volunteer weeds (adventitious HEAR plants) were likely in the field, and the LEAR seed sown was free of high erucic impurities. However, the DNA-based, quantitative PCR method was judged to be not reliable for detecting hybrids at a low frequency in seed of some LEAR genotypes at the scale of operations envisaged. In contrast, the biochemical method of gas chromatograph for detecting change in %EA in seed was reliable. A statistical basis was developed for estimating whole-field cross pollination by this method at frequencies between 0.01% (1 in 10,000) and 0.1% (1 in 1000).

In 2005, the project moved up a scale to commercial farms and rural landscapes in four experimental domains, two in the west and two in the East of England, in which HEAR donor fields and LEAR receptors were both present in the landscape. All fields examined were winter oilseed rape. The landscapes were characterised, fields mapped and insect activity recorded. The receptor fields were sampled comprehensively, first when plants were in early leaf to check for volunteer impurities and then at seed maturity to estimate cross pollination. HEAR volunteers were found in moderate or small numbers at some sites, and HEAR impurities were found in some LEAR sown seed. Some of the HEAR volunteers contained seeds of 50% EA, indicating they were pure HEAR plants, either introduced in sown seed or the result of self pollination of existing HEAR volunteers. It was nevertheless possible to estimate that cross pollination was mostly around or below 0.1%, except at one site which was next to a large block of HEAR.

More fields of winter oilseed rape were sampled in 2006, with the addition of insect exclusion cages in the receptor fields. The seed lots and fields had fewer impurities than in 2005, cross pollination was mostly within the range found the previous year and insects were not abundant, mainly because of the early flowering of the winter crops. Cross pollination was reduced within the exclusion cages at sites close to blocks of HEAR; otherwise the cages had little influence on crossing.

Lessons of up-scaling for GM coexistence

Cross pollination in HEAR-LEAR landscapes was generally higher than around the single GM herbicide tolerant fields. Based on the results from this project, and from previous assessments using the half fields at FSE sites, three general situations can be defined for oilseed rape crops of full pollen fertility. Levels of cross pollination averaged over the whole field were -

1. below 0.01% in receptor fields sited 1 km or more from a single donor and within a landscape containing other LEAR fields;
2. around or just below 0.1% when receptors and donors were in similar proportions and the receptor no more than a few hundred metres from the nearest donor;
3. mostly below 0.9% for fields adjacent or close to a donor field of similar size, but rising to near or above this value for a small field close to a much larger block of donor.

The EU labelling threshold for GM in commercial production is 0.9%. In the first and second categories above, cross pollination was at least five times lower and mostly ten times lower than the 0.9% threshold. All of such fields in these categories could be harvested - there would be no need to remove or destroy any strip of crop at the edge of a field facing the nearest GM field in order for the harvest to be below the threshold.

Are there circumstances in which cross pollination would be greater than measured here? The single most important factor would be if the fields receiving donor pollen were varietal associations or other varieties that have low self pollen production. In the experiments in 2003, cross pollination to varietal associations was more than twice that to fully pollen-fertile crops. Few fields of varieties with low self pollen are now grown in the UK, however. More pollinating insects at the time of flowering would also probably raise cross pollination slightly. Otherwise, no normal circumstances could be envisaged for winter oilseed rape given the present landscape, climate and varieties where the impurity brought by cross pollination alone was greater than indicated for the 3 categories above.

If GM cropping became widespread, then cross pollination would not be the only and may not be the main means by which the genes of interest would be present in the crops. Volunteers would arise from sown seed impurities and from transport of seed into the field. In these circumstances, cross pollination would add a low amplitude ‘wave’ of impurity to fields simultaneously in flower, but the content of GM volunteers and seed impurities would primarily determine whether a non-GM field remained below the labelling threshold. Cross pollination could contribute to raising GM content of a non-GM field to above 0.9%, but to keep content below 0.9 %, all sources of impurity would need to be considered and minimised.

This study with HEAR demonstrated without question that the systems put in place by seed companies and growers to segregate HEAR from non-HEAR crops had worked, in that seed produced in all fields of LEAR examined was below the accepted limit for erucic acid.

While cross pollination alone is unlikely to breach labelling thresholds in most fields, it is argued that it could have ecological effects. Cross pollination frequencies of the order of 1 in 1000 or lower could be important in altering the properties of volunteer populations and thereby the seedbank, particularly if the transmitted gene had a selective advantage.

BACKGROUND, OBJECTIVES AND RATIONALE

The interest in cross pollination and other sources of impurity in crops

Consumers, retailers, producers and the public increasingly require that crops and food have a known standard of purity and are grown in ways that minimise environmental harm. Impurities arise from three sources: the seed sown by the farmer, crop-derived volunteer weeds in the field and cross pollination from other crops during the flowering period. Such impurities are of little consequence if they have the same qualities as the crop in question, but are becoming more important through the increasing diversification of crops for specific foods and industrial yields. In some instances, it may be essential to keep impurities below specific, low thresholds to ensure the food is safe for people or stock animals. In others, consumers may simply prefer the food they buy to contain minimal quantities of a specific product even if that product is shown to be of no harm. Generally, plant breeding and seed-bulking in the UK have so far achieved acceptable levels of varietal purity as traditionally assessed by morphological or biochemical characters of the crop plants (Moyes & Dale, 1999; Ingram, 2000). However, the labelling threshold for GM presence in the yield of non-GM crops in Europe is relatively low, at 0.9% or 9 in 1000 (EC, 2000) and refers to impurities from all sources. There is little verified experience in the UK as to whether farmers would be able to produce crops to this threshold in the general run of agriculture. This project was therefore commissioned to measure the contribution, primarily of one of the sources of impurity - cross pollination, at a scale of operations as near as possible that in commercial agriculture.

The scientific context

In an examination of the information on impurities likely to be brought by GM crops, the EC Scientific Committee on Plants, in 2001, came to the opinion that a threshold of 1% impurity in food and food ingredients could be met by rigorous but achievable standards that kept impurities in the sown seed, through volunteers and through cross-pollination of the farmer's crop, to 0.2-0.3% each. These figures applied to oilseed rape, which among common European crops, is the one most likely to give rise to volunteers and to cross pollinate over distance. It was also recognised that if a specific variety of a crop has been grown widely and produced volunteers whose seed was transported between fields on farm machinery, then its potential to introduce impurity into another crop would be inherently larger and more uncertain, so that the practical target level of impurity through cross-pollination might have to be nearer 0.1% (1 seed in a thousand).

Oilseed rape is self-compatible, so that a plant's pollen can fertilise ovules of flowers on the same plant, but is also cross-compatible with other oilseed rape plants, so is cross-pollinated by wind and insects, primarily bees. Knowledge of cross pollination between crops of oilseed rape has been gained from experiments using a range of donor and receptor blocks (ACRE/Defra, 1999; Damgaard & Kjellsson, 2005; Eastham & Sweet, 2002; Moyes & Dale, 1999; Norris & Sweet, 2000; Ramsay et al., 2003). The results converge to indicate that % cross-pollination declines steeply with distance from the crop, reaching less than 1% after tens of metres from the edge of the source crop and becoming less than 0.1% well into an adjacent field (order of magnitude values are here cited). Nevertheless, uncertainties restrict the ability to predict what would happen at greater distances and after commercialisation of GM crops. Perhaps the greatest uncertainty arises because most measurements of cross pollination between fields had been made using single, often small sources of pollen and relatively larger areas of receptors, whereas after commercialisation, the different sorts of fields would be distributed in the landscape and in some areas, the donor fields might be more numerous than receptors. Indeed, there are no cases in UK, such as that for maize in Spain (Messeguer et al., 2006), where cross pollination was measured in landscapes of mixed commercial GM and non-GM crops. Additionally for oilseed rape in UK, some exploratory measurements have indicated much higher rates of cross pollination to crops of the type known as 'varietal associations', in which only around 20% of the plants produce the pollen, the rest being male-sterile (Eastham & Sweet, 2002; Ramsay et al.,

2003; Simpson et al., 2006). Therefore a combination of a high density of donor crops, such as GM crops, coincident with male-sterile receptor fields, might well cause cross pollination at frequencies high enough to challenge a 0.9% labelling threshold in oilseed rape. The project therefore needed to measure and understand cross pollination in realistic farmed landscapes, a task in which major obstacles of method had to be overcome.

Formal objectives as set out in 2002

The main requirements of the project can be condensed into three:

1. Methods - a statistical sampling methodology coupled with rapid, high throughout, molecular or biochemical techniques for detection of low level impurity in the field.
2. The understanding of processes - notably the mechanisms of cross pollination (e.g. pollen carriers, plant phenology, pollen-fertility) and the contributions of nearby (up to 100 m) and regional (e.g. 4 km radius) pollen sources.
3. Synthesis and recommendations - leading to a definition of the conditions under which whole-field cross-pollination is likely to exceed the labelling threshold and recommend practices that will maintain cross pollination below the threshold.

The emphasis on partially male sterile varieties at the conception of the project was later reduced in view of their decreasing usage by farmers after 2002. Otherwise, the original objectives were achieved. The methods and results of the project are described chronologically, beginning with studies on a GM herbicide tolerant marker in 2003, followed by a series of experiments in the four years from 2003 to 2006 at increasing scales from small field plots to farmed landscapes using high erucic acid markers.

SCOPE CHRONOLOGY AND PROGRESSION

Estimating cross pollination in landscapes requires a system of markers by which cross pollination can be judged to have occurred, a representative landscape over which donor and receptor fields are located and which contains typical farms and other land use and a statistical framework for assessing the percentage of cross pollination in receptor fields. Such a programme of work has to balance the need for control of experimental conditions with the need for sampling in situations representative of agriculture. Moreover, the landscapes should be sufficiently ‘clean’ to allow what is likely to be a feint but pervasive ‘signal’ of cross pollination to arise from the ‘noise’ brought about by impurities in sown seed or volunteers. Finding and characterising such landscapes in commercial farming proved to be one of the most difficult tasks facing this study, and in this respect, the project is indebted to the growers who were willing to allow access to and measurements on their land, while still continuing their normal farming operations.

The project began during the final year of the Farm Scale Evaluations (FSEs) of GM herbicide tolerant crops (Bohan et al., 2005; Firbank et al., 2003; Perry et al., 2003; Squire et al., 2003), in which winter oilseed rape crops sown in autumn 2002 flowered and matured in 2003 (Table 1). The GM fields in the FSEs provided a unique marker for cross pollination, and had already been used to measure short-range cross pollination to the adjacent half-field at each FSE site (Weekes et al., 2005), while the surrounding fields offered receptors that were most likely to be free of any GMHT (glufosinate ammonium) genes. The value of the FSE fields in estimating crossing at a commercial scale was limited because the single donor half-fields of GMHT plants generally represented only a small proportion, such as 5%, of the total GM oilseed rape within a radius of a few kilometres. The final year of the FSEs nevertheless provided the opportunity to assess the likely lower limit of crossing between fields 1 km or more apart.

A more representative system of commercial cropping was then needed. After careful consideration of all possible donors and receptors, the type of oilseed rape known as high erucic acid rapeseed (HEAR) was chosen as the donor type (Bilsborrow et al., 1998). The fatty acids in the seed of HEAR crops contain a high percentage (e.g. 50%) of one particular fatty acid - erucic acid (EA). The receptor crops were low erucic acid rapeseed or LEAR, which produce oil with a low percentage of erucic acid (around 0.02%) and form the majority of oilseed rape crops grown

in the UK. The product of HEAR is used as a light industrial oil, and that of LEAR in food or animal feed. The farming and food processing industries accept that HEAR impurities will arise in LEAR oil (by cross pollination, volunteers, etc.) and work to a EU upper limit of 2% erucic acid in oil from LEAR crops destined for human and animal consumption. In certain areas, HEAR comprises as much as half of the surface area of oilseed rape. HEAR and LEAR crops have coexisted in many areas for several decades, and as such, they are a surrogate for the coexistence of commercialised GM and non-GM oilseed rape. Box 1 summarises definitions for cross pollination, HEAR and the EU labelling threshold for adventitious GM presence.

Box 1. Definitions of percentages and thresholds for cross pollination and erucic acid

Cross pollination

- The primary aim of the project is to estimate cross pollination in a field by pollen grains originating outside the field. Cross pollination is given as a percentage. For example, if 1 seed in 1000 is the result of cross pollination from outside the field, then cross pollination is 0.1%.
- Cross pollination is measured here using one of two markers or indicators – tolerance to a herbicide transferred from a GM herbicide tolerant field; and raised erucic acid content of seed transferred from a non-GM variety known as HEAR (high erucic acid rapeseed). Cross pollination can be measured by detecting the transfer of DNA or the presence of the phenotypic trait (tolerance to herbicide or raised erucic acid). The presence of the phenotypic trait was the primary method used here in high throughput screening.
- The occurrence of erucic acid in a sample of seed or in an individual seed is itself expressed as a percentage (%EA). This %EA is not the same as % cross pollination but is used to derive the measure of % cross pollination in the sample.

The EU labelling threshold for GM adventitious presence

- The EU has set a labelling threshold for GM in non-GM product of 0.9%, which refers to adventitious presence of any origin, i.e. seed impurity, volunteers and cross pollination. In this study we concentrate mainly on the contribution of cross pollination, but we also comment on the levels of admixture due to impurities in sown seed and volunteers.
- The units of the 0.9% GM threshold in the legislation have not been defined by the EU and are open to interpretation. The EU guidance interprets the units as %GM DNA, the minimum "GM unit" being a transformed haploid genome (EU legislation EC1829/2003 and the associated guidance, recommendation 2004/787,II(h))
- In this study, we concentrate on the biological process of cross pollination. The %GM DNA can be derived from a value of cross pollination for any defined transgenic donor and non-transgenic receptor.

There was, however, no off-the-shelf method for measuring cross-pollination from HEAR to LEAR types. Such a method would have to detect as few as one or two cross pollination events in, a sample of, say 500 or 1000 seeds, and be able to process thousands of such samples reasonably quickly, and do this for seed harvested from the field which might not be in pristine condition for analysis. Accordingly, a graded series of experiments was carried out from small field plots to agricultural landscapes, in which methods were developed and iteratively tested at increasing scales of operation (Table 1). It was accepted that this progression would bring with it increasing complexity and ‘noise’ and increasingly porous boundaries through which unwanted seed and genes might penetrate.

An important factor examined was the relative contributions of wind and insects to cross-pollination between fields, a topic that has been much debated (Ramsay et al., 2003; Ramsay, 2005; Hayter & Cresswell, 2006). Two approaches were taken, sometimes combined in the same experiments. In 2003 and 2006, cages of dimensions 3m x 3m x 2m, were placed over the crop during flowering to exclude large insects. In addition, the quantity of pollen in the air and on insects, the proportion of this that came from donor HEAR fields, the insect activity in fields and the meteorological conditions, were variously measured in the four years of field experiments.

Table 1. The main phases of the study from the GM trial sites in the Farm Scale Evaluations (FSEs) to a graded progression through increasing scale using the high erucic acid marker.

Year	geneflow marker	Purpose	locations	Receptor fields
2003	GM herbicide tolerance gene	To establish minimum geneflow frequency	Domains based on 7 FSE sites in England and Scotland	16
2003	high % erucic acid	Establish erucic acid DNA and biochemical markers and factors affecting its transfer by pollen	Experimental farm: Rothamsted, Hertfordshire	Large plots
2004	high % erucic acid	Test marker system in realistic farmed landscape	Experimental farms: Rothamsted and SCRI, Tayside	4
2005	high % erucic acid	Extend scale to two contrasting landscapes	Farmed landscapes: Shropshire and Cambridgeshire	8
2006	high % erucic acid	Widen range of landscapes; compare outside and inside insect exclusion cages	Farmed landscapes: Herefordshire and Cambridgeshire	8

FIELD TO FIELD GENEFLOW IN THE FSEs (2003)

The opportunity was taken in 2003 to measure cross pollination from Farm Scale Evaluation sites of winter oilseed rape to some of the surrounding fields. Fields in 7 domains were examined, each based around an FSE site, and together spanning 90% of the total latitudinal range of all FSE sites in the UK. Fields within, usually, a 5 km radius of the FSE site were recorded (Fig. 1), those simultaneously in flower with the FSE site were noted, and at least two of these fields were later sampled for seed according to a standard sampling scheme. In total, 16 fields were sampled and tested.

Method for detection of GM herbicide tolerance genes in fields around FSE sites

Previous work on cross pollination from the GM half to the non-GM half of FSE sites detected transfer of DNA using a ‘real-time PCR’ method to quantify %GM (DNA) in samples relative to the sown GM crop as a reference (Weekes et al., 2005). This method is limited to a sample size of say, 1000 seeds, which makes the method unfeasible for detecting cross pollination in a large operation in which several million seeds would have to be processed. Therefore, tolerance to applied herbicide, rather than a DNA-based technique, was employed as a primary screening tool for large seed samples.

The GM variety used in FSEs carried the *bar* gene conferring resistance to the herbicide, glufosinate ammonium. Seed sampled from a receptor field was germinated, sprayed with glufosinate ammonium according to manufacturer's instructions and surviving plantlets were scored as individual cross pollination events. Large samples were handled in this way, typically 250,000 seeds per field. However, the benefits in effort and cost bring several disadvantages to using herbicide tolerance alone: environmental factors may affect resistance of seedlings, expression of resistance may vary, and high density planting in seed trays may increase background seedling mortality and prevent the even application of herbicide. Therefore each survivor-seedling was dried and its DNA extracted and tested by standard PCR in duplicate to confirm the *bar* gene was present. To define a standard negative control, a field, which was at the farthest possible distance from any FSE site - in southern Scotland - and so likely to contain no GMHT seed, was sampled and indeed no positive individuals were found.

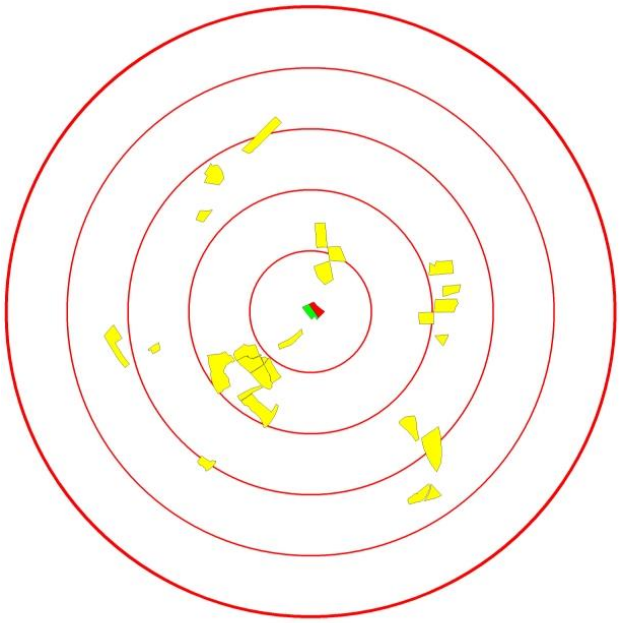

Figure 1 One of the seven domains based around FSE sites in 2003. FSE fields are at the centre (red GM, green conventional); oilseed rape fields simultaneously in flower are shown as yellow polygons. Typically two of these fields were sampled for presence of herbicide tolerance. Red circles show 1, 2, 3, 4 and 5 km radii from the FSE site.

Three million seeds were tested in total from the 16 sites spread over the 7 domains (Table 2). For ease of comparison, the percentage cross pollination, estimated for the whole field, is assigned to a range (e.g. 0.01 to 0.001%). Even the highest percentage was 48 times lower than the present GM labelling threshold of 0.9%, and at most sites the percentage was hundreds or thousands of times lower than the threshold. In the 9 fields where no *bar* genes were detected (shown in the column 'below limit' in Table 2), cross pollination, if it occurred, must have been below the detection limit of around 1 in 100,000. As indicated earlier, the circumstances of a single donor field among many other sources of oilseed rape pollen in the landscape would occur only in the early years of commercialisation, and should not be taken to indicate the frequency of cross pollination if GM (donor) fields occupied much more of the total area sown to oilseed rape. The main contribution of this experiment for the development of the project was to indicate the lower limit of the range of cross pollination that would likely be found at different distances from the source, and from that, the level of sampling that would be needed to ensure crossing could be estimated accurately in future work.

Table 2. Percentage cross pollination in non-GM receptor fields from GMHT winter oilseed rape fields in the FSE in 2003: measurements based in 7 domains (A to G) each located around one FSE field (general location indicated) in which a total of 16 receptor fields were sampled for seed at harvest; areas of donor and receptor fields, and distances between the nearest edges of each pair of fields and between their centres (estimated as the centroid of each polygon), are shown.

region	domain	field	% cross pollination 0.01% is 1 in a 10,000 0.001% is in 100,000 0.0001% is 1 in 1,000,000				distance in metres between		area in hectares of		receptor / donor area ratio
			0.1 – 0.01	0.01 – 0.001	0.001 – 0.0001	below limit	edges	centroids	GM field	receptor field	
north	A	1		x			1541	1930	4.49	13.76	3.06
-		2				x	3718	3981	-	7.59	1.69
-	B	3		x			336	646	2.79	3.78	1.35
-		4	x				334	612	-	7.69	2.76
-		5				x	2540	2741	-	1.84	0.66
-		6				x	2589	2957	-	15.12	5.42
central	C	7			x		1883	2641	10.82	45.35	4.19
-		8				x	3678	4476	-	26.72	2.47
-	D	9				x	2903	3392	7.74	15.79	2.04
-		10				x	2651	3007	-	7.15	0.92
south	E	11			x		2934	3292	4.88	6.38	1.32
-		12			x		3848	4275	-	13.83	2.83
-	F	13				x	830	1210	2.32	15.28	6.59
-		14				x	1892	2187	-	26.2	11.29
-	G	15		x			1551	1849	2.13	7.16	3.36
-		16				x	2191	2558	-	27.61	12.96

ESTABLISHING A METHOD BASED ON HIGH ERUCIC RAPESEED (2003)

Methods to detect the presence of high erucic acid rapeseed (HEAR) in leaf, seed and pollen were developed using HEAR DNA and plant material diluted in a LEAR sample, then tested on plant material from field experiments. The latter aimed to demonstrate whether HEAR pollen was distributed by insects and wind to produce HEAR-LEAR hybrids in sufficient number to be detectable in future studies at much larger scales.

Sites and methods

Development of the HEAR qPCR assay

A range of high erucic acid (HEAR) varieties was examined as possible sources of donor pollen, and low erucic acid varieties as possible receptors. A method known as real-time (TaqMan) quantitative PCR (qPCR) was examined to see if it could be used to test for the presence of donor genes in receptor pollen, seed and plants.

The following is a technical summary of the method. The genes to be detected were those controlling the production of erucic acid. The key enzyme in erucic acid biosynthesis (KCS; β-keto-acyl-CoA synthase) is the product of the Fatty Acid Elongation 1 (*FAE*1) genes in *Brassica* species. The trait in *B. napus* (oilseed rape) is controlled by two highly homologous genes Bn-FAE1.1 and Bn-FAE1.2, corresponding to the parental species *B. rapa* and *B. oleracea FAE*1 genes. Mutations observed in these genes were responsible for the low erucic acid trait; 2-base and 4-base deletions were identified in the FAE1.2 gene, and a 1-base substitution identified in FAE1.1. Primers were designed to amplify selectively either the FAE1.1 or FAE1.2 genes, and in agreement with the literature, the same two allelic forms (2-base and 4-base deletions) were identified in the FAE1.2 gene of the tested LEAR types.

Several primer (6 pairs) and probe combinations (5) were optimised and tested in parallel using both winter and spring oilseed rape donor and receptor types. PCR assays were developed using the deletions identified in the LEAR type as markers based on the following:

- The forward and reverse primers were specific to the FAE1.2 gene (*B. oleracea*) i.e. prevented amplification of FAE1.1 gene (*B.rapa*) due to a base difference at the 3' end of each primer sequence.
- Reverse primer discriminated against amplification of the 2-base deletion LEAR allele (-AA bases) on FAE1.2. i.e. only sequences with this +AA base sequence would be amplified.
- TaqMan probe discriminated against the 4-base deletion LEAR allele (-TCAG bases) on FAE1.2, and therefore a signal would only be generated with a sequence with this continuous 4-base marker.

The combination of primers TqF3C + TqR2 with probe TqP2 resulted in the required specificity using the HEAR diagnostic marker system and a three step cycling protocol (95^{o}C/15s + 56^{o}C/1.00m + 72^{o}C/30s). High throughput methods of extracting DNA from OSR seed material, leaf, and pollen collected from insects and pollen traps were developed and optimized for each substrate.

A slight reduction in the sensitivity of detection was first encountered with seed and leaf samples during the optimization of the real-time PCR assay. In summary, however, it was determined that a LEAR DNA background of the 4-bp deletion allele lowered PCR efficiency and sensitivity compared to 2-bp deletion allele types, resulting in limits of detection of between 0.5% to 0.3% and 0.2% to 0.1% respectively. Additionally, a method was needed to distinguish the situation when brassica DNA was present but HEAR absent from that when DNA was simply not amplified (e.g. because the plant sample was in poor condition). An endogenous control and normaliser assay was therefore designed for real-time PCR based on the published *S-glucosyltransferase* (Sgt) gene to exclude such false-negative results, and normalise all the quantitative values for the HEAR DNA by amount of brassica DNA present in the extract; this was successfully used with all

seed, leaf, and pollen samples and could reliably detect target DNA down to a level of 0.01% (1/10000) regardless of the LEAR genotype. The method used for detecting HEAR DNA in seed is described by Cullen et al. (in press).

Estimating percentage erucic acid (%EA) in OSR seed by gas chromatography (GC)

As an alternative or backup, gas chromatography (GC) was examined as a means for estimating cross pollination frequencies based on the HEAR phenotype. This method measures the %EA in the seed rather than the presence of the genes responsible. A procedure was used for the rapid preparation and GC analysis of fatty acid methyl esters from individual OSR seeds or homogenates of bulked OSR seed in DNA extraction buffer. Fatty acid methyl esters are prepared without isolation of the seed oil, and the GC conditions minimise analysis time while maintaining adequate separation of erucic acid. Fatty acids are identified by comparison with standards or known oils. The GC programme uses a fast temperature ramp to speed the analysis, resulting in incomplete separation of the earlier eluting C_{16} and C_{18} components. C_{22} methyl esters elute during the isothermal stage giving good separation of C_{22} monoenes (including erucic acid) and the C_{22} saturate (behenic acid). There is near baseline separation of erucic acid (22:1 13c) and minor amounts of the 11c isomer.

The data are normalized by expressing the amount of erucic acid in each sample as a percentage of the total content of fatty acids. The method was tested and optimized using a dilution series of HEAR seed in a background of LEAR seed (100% to 0.1%) for both winter and spring varieties of oilseed rape. This method could reliably detect erucic acid levels down to 0.1% (1/1000) regardless of the LEAR varietal background. Further information on the method can be obtained from the authors.

Experimental site - 2003

The efficacy of HEAR as a pollen donor was assessed by varying three factors: the distance from a HEAR source, the exclusion of large flying insects such as bees, and the degree of male sterility of the receiving flowers. Male sterile plants can only be fertilised by external pollen and so would ensure enough HEAR-LEAR hybrids for developing and testing the detection technique. The set up was as follows: using varieties that were satisfactory in the laboratory tests as described above, one donor field of HEAR was placed next to 3 receptor plots of differing proportions of male sterility - 100% male sterile (MS), 50% mixture of MS/MF, 100% male fertile (MF) in Great Knott field at Rothamsted. Three similar recipient plots were placed about 800 m away in Stackyard field (Fig. 2). Twelve sample points were positioned in each recipient plot. Three of these sample points were covered in insect-proof cages. Crop-stage, flower development and counts of insect flower visits (to areas of one square metre over 10 minutes) were assessed at all sample points throughout flowering. Yellow insect sticky traps were set up over 2 weeks at the six central sample points (3 inside cages, 3 outside). Rotorod traps to catch airborne pollen were set up at the 6 central sample points (3 inside cages, 3 outside). The pollen on the tape from these traps was counted, and analysed for the presence of erucic acid DNA marker. Bees on the plots were sampled: the pollen on their bodies was washed off and tested for erucic acid DNA. At harvest, plants were sampled to estimate the proportion of flowers setting seed and the percentage which were hybrids.

Findings from 2003 season and associated detection methodology

Transport of HEAR pollen and cross pollination

The future use of the HEAR–LEAR combination in this project relied on HEAR pollen being picked up by bees or released to the air, carried to surrounding fields, landing on stigmas and fertilising ovules on LEAR plants to produce HEAR-LEAR hybrids. To test for pollen transport, pollen was washed from bees caught in the receptor plots and from pollen traps, both the ‘sticky trap’ type and a spinning device called a rotorod, located there. The qPCR method was sensitive enough to determine both the presence and percentage of HEAR in total pollen. HEAR pollen was detected on bees, even at the far plots 900 m away from the donor. The rotorod pollen traps

showed no effect of the cage on the density of pollen grains in the air above the plants. Sticky traps showed that 5-15% of the very small insects (primarily flies and solitary wasps) in the field were also present inside the cages. The cages therefore allowed wind pollination, self pollination and a (probably) small amount of pollination by small insects. Outside the cages, all the above occurred, plus pollination by all other insects, particularly bees. Tests for HEAR-LEAR hybrids showed higher proportions of HEAR in the uncaged areas – for which the large insects are likely to be responsible – and HEAR-LEAR hybrids were also found in the far plots. Details of this study will be presented in peer reviewed papers.

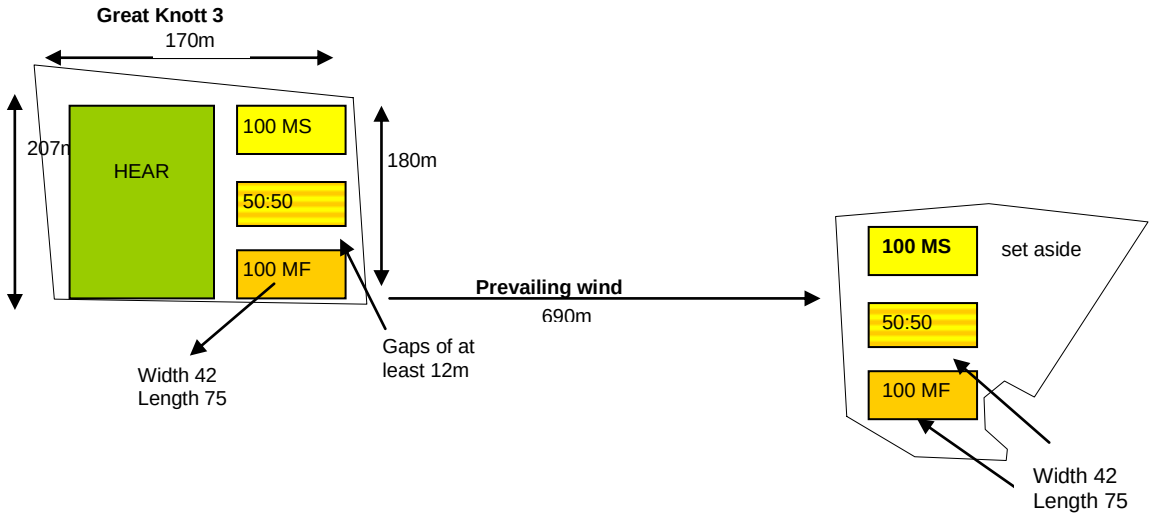

Figure 2 Arrangement of plots in 2003, showing the HEAR donor (green) and a range of receptor plots (yellow, brown) having % male sterility (MS) and male fertility (MF) indicated.

Summary 2003

- The qPCR method was sensitive enough to measure HEAR DNA at low frequency in pollen, leaf and seed, while gas chromatography could detect the raised %EA in seed resulting from as little as 1 HEAR seed in 500.
- HEAR pollen was carried by bees and in the air to both near and far receptor plots over a distance of 900 m. HEAR-LEAR cross pollination was evident in the near and far plots.
- The decision was taken to assess the logistics and robustness of the high throughput detection methods using fields of HEAR donor and LEAR receptor in 2004.

TESTING METHODS AND LOGISTICS USING A SINGLE DONOR FIELD (2004)

In the 2004 season, experiments were set up on the research farms at SCRI in Tayside and Rothamsted in Hertfordshire in which a single field of spring HEAR was grown in juxtaposition to a single field of spring LEAR immediately adjacent to it and to another field of spring LEAR around 1 km distant (Fig. 3). The purpose of the experiment was to test the methods on small fields of oilseed rape at a realistic scale in the landscape and to make a first assessment of the decline of cross pollination with distance, comparing the quantitative PCR and %EA methods. The LEAR fields were of low male fertility (80% male sterile, 20% male fertile pollinator) so as to increase the chance of cross pollination being detected at a high enough frequency in the far field to allow a comparison of techniques. A degree of control was possible in the siting of the fields, in

the choice of donor and receptor varieties and by the fact that no HEAR crops had been grown previously on the land. The main specific aims of the experiment were to assess

- qPCR and erucic acid techniques over a range of geneflow frequency;
- the number of samples that would need to be taken to estimate cross pollination accurately;
- whether it was logistically feasible to progress with the techniques to real arable landscapes.

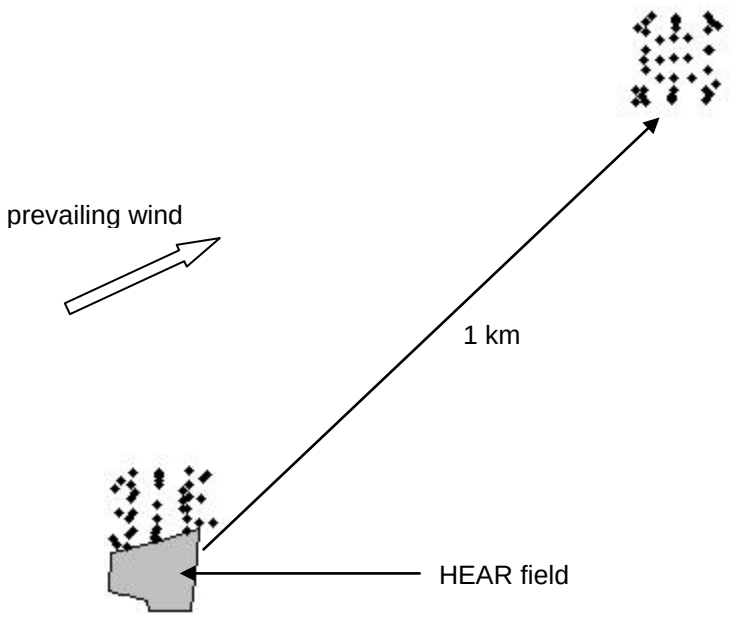

Figure 3 Layout of HEAR donor field and sample points in near and far low erucic receptor fields in Tayside, one of the two sites used in 2004; each donor and receptor field is about 2.5 ha.

Comparison of real-time PCR and GC analysis for estimating cross pollination frequencies

The real-time qPCR, which estimated the number of 'HEAR genes' per sample, and GC assays, estimating %EA, were compared using shared extracts of the sown seed varieties: *Sheila* (HEAR) and *Concept* (LEAR, 2bp allele). A dilution series of standards from 0.2% to 10% of HEAR in the 2bp LEAR background were extracted in 250 or 500 seed lots. There was a very high correlation (r^2 = 0.99) between the percentage of pure HEAR seeds in the mixture and the %HEAR content (by qPCR) and the %EA (by GC).

The comparison was repeated using mature seed collected from the near receptor fields. Cross-pollination measured by real-time qPCR was very high (around 30% in some samples) close to the donor, declined steeply to around 1% at 50m, then persisted at or below that level to the far edge of the near field. The qPCR and GC estimates of %EA were highly correlated down to around 1% cross pollination (Fig. 4). However, they diverged at lower crossing frequencies which are likely to occur far from a source. At the farther end of the near field, the GC method was more sensitive than real-time PCR in detecting low frequency crossing (Cullen et al., in press).

The results indicated that real-time PCR was unable to detect % HEAR content in field-harvested seed samples (500 lots) at the frequencies detected in laboratory-mixed samples of pure HEAR (~50% EA) and LEAR seed. The reason for this discrepancy is probably due to the heterozygous nature of the F1 HEAR-LEAR seed at both loci of the FAE1.1 and FAE1.2 genes, thus reducing the amount of target DNA by half compared to the pure HEAR seed used to produce standards. In addition, subsequent work using GC analysis (see results for 2005 later) indicated that field-harvested seed can have %EA lower (<25%) than that associated with an F1 phenotype,

indicating that the seed could be homozygous for the LEAR genotype at either loci of the FAE1.1 and FAE1.2 genes. If such a genotype occurred on the FAE1.2 gene, the qPCR assay would be unable to detect a signal. The GC analytical method is unaffected by the HEAR genotypic background of seed in the determination of %EA levels, and therefore was selected as the most reliable method for determining cross pollination of HEAR to non-HEAR plants in the field in batches of 500 seeds.

Derivation of cross pollination frequency from %EA in 500-seed samples

Seed from near and far fields was then processed for quantification of erucic acid by GC. The LEAR seed used had low %EA, 0.03% or less, while the HEAR seed had a mean %EA of 42%. Seeds that are assumed to have resulted from a geneflow event (F1, i.e. HEAR-pollen to non-HEAR stigma) had a percentage erucic acid very much higher than LEAR seed but lower than pure HEAR seed. If all the F1 seed had a single value of erucic acid, then detecting the number of F1s in lots of 500 seeds would be relatively simple. The no-event (LEAR) seeds could be distinguished and those with %EA above this would fall into discrete groups corresponding to 1, 2, 3, etc., F1 seeds in the sample. If the value of %EA in the F1 seed were known, then the categories could be defined.

However, variation in %EA among seeds was found in all seed lots. The only way to find the %EA of F1 seed was to process harvested seed individually until these F1s were found – they were easily recognisable above the LEAR background of <0.03% by having %EA around 25%. There was, however, variation in this %EA of the single F1s, which caused corresponding variation in the %EA of a 500-seed sample, manifest as a spread of values for each of 1/500, 2/500, etc. cross pollination events in a processed seed lot. The variation could have arisen as a result of genetic variation in the pollen donor or of the maternal conditions. (Certainly the %EA varied among seeds of this and all donors subsequently examined.)

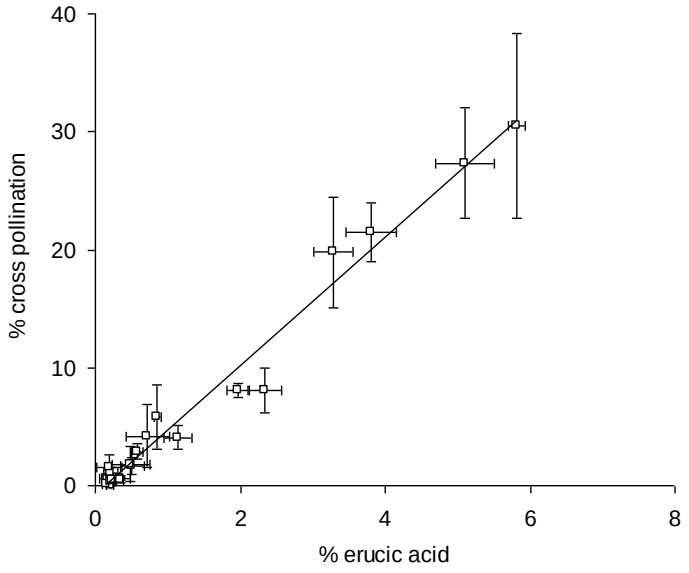

Figure 4. Comparison in a near field in 2004 of cross pollination estimated by real-time PCR and % erucic acid measured by GC on the same seed samples; standard errors based on three replicates from each sample locus.

Mixture model to estimate % geneflow from erucic acid level

A statistical method of assigning determinations to 0, 1, 2, etc., cross pollination events was then developed (Cullen et al., in press). Normal mixture models were fitted to the erucic acid estimates obtained for the seed samples from both the Rothamsted and SCRI far fields (Fig. 5). The high peak at the far left of each part of Fig. 5 at about 0.02%EA consists of the LEAR values (no cross pollination). Peaks for the 1-F1 event were estimated at 0.06%EA, for the 2-F1 event at 0.105%. Above that, the numbers of samples were quite small, but the F1-events were still estimated. Using the parameter estimates above, the posterior probabilities for 'number of geneflow events' for a given %EA were derived and hence an estimate of the most likely number of geneflow events in 500 for that % erucic value. The number of geneflow events implied by the erucic acid value for every sample was calculated for both sites. These were then compared assuming a poisson distribution for 'number of geneflow events'. The estimated means of cross pollination for the two sites were significantly different (P=0.001) at 0.31% for Rothamsted and 0.15% for SCRI, using a two-tailed test. (Full details of the method will be given in peer reviewed papers.)

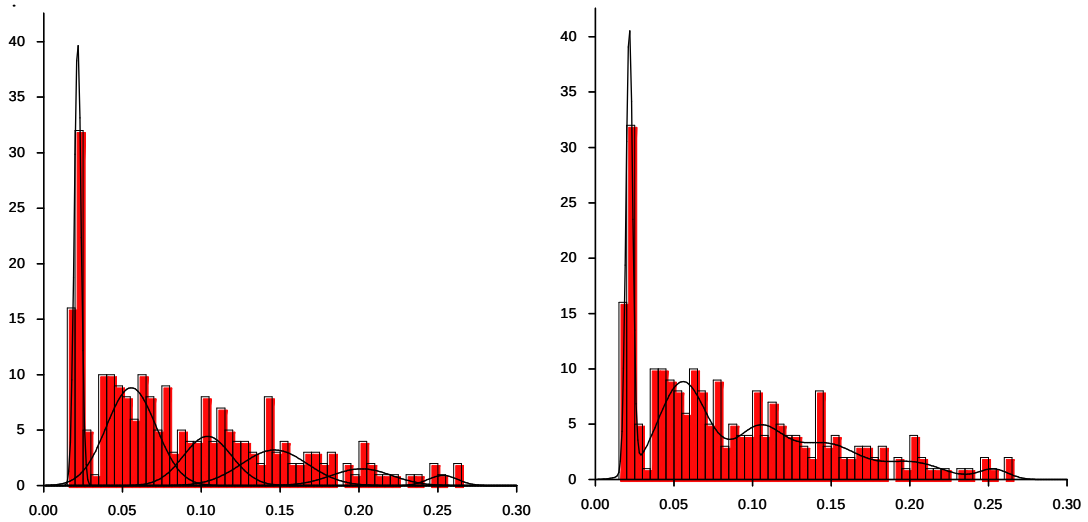

Figure 5. Frequency distribution of measured values of % erucic in 500-seed samples from a single field at 1 km from the donor, fitted with mixture models, showing left, the individual components, and right, the fitted mixture distribution. After Cullen et al., in press.

Findings 2004

- The method of quantitative PCR to detect the genetic difference between HEAR and LEAR, while working to high specifications under controlled mixing of DNA and seed in the laboratory, proved not sensitive enough to detect low frequency pollination in seed harvested from field crops.
- However, the GC method of detecting change in erucic acid content of seed was sensitive enough to detect cross pollination at 1 in 500 seeds, provided that 'zero' and F1 values were obtained from determinations of individual seed. A statistical mixture model was applied to assign %EA values to cross-pollination events.

- The GC method provides an accurate estimate of the effect of cross pollination on the oil quality of the crop to which the HEAR pollen moves. This is at least as important in the context of this project as knowing the actual frequency of cross pollination events.
- The decision was made to progress to the landscape scale using the %EA assay and at the same time explore other DNA based methods for detecting HEAR genes in seed on LEAR parent plants. A higher sampling intensity than used this year would be needed for adequate estimate of cross pollination frequency in fields consisting or fully pollen-fertile varieties (i.e. most if not all fields).

UPSCALING TO NATURAL EXPERIMENTAL DOMAINS (2005)

The methodological work in this project and the increasing body of knowledge on OSR seedbanks brought attention to two problems that had to be considered when selecting recipient fields in a farmed landscape. These are that LEAR commercial varieties might contain HEAR impurities and that LEAR recipient fields might contain HEAR volunteers in the seedbank. Volunteers might occur even if HEAR had not been grown in a field, if for example HEAR seed was brought in on machinery. The % presence of HEAR in both these instances might well be at levels 0.1% to 1% or higher. Accordingly, further testing for the presence of HEAR in seed and in young emerged plants was necessary. Given that each bag of seed to be sown would have to be tested rigorously, and that impurities could still be introduced through the machinery used during ground preparation and sowing, it was decided that the emerged crop in the recipient fields would provide the most appropriate indicator of the presence of HEAR from all possible sources before cross pollination occurred. This doubled the sampling and diagnostic work in each receptor field.

Choice and characterisation of landscapes in the experimental domains - 2005

By 2003/04, crops with low male fertility, such as varietal associations, had dropped out of usage due to poor performance, and most sown oilseed rape on farms were varieties with complete or almost complete male fertility. The factors most likely to influence regional pollination beyond about 100 m from a donor were expected to be distance from the donor, the ratio of the areas of donor and recipient fields and the activity of regional pollen carriers such as bees.

Criteria for selecting domains

Over the two seasons, 2005 and 2006, the project aimed to work in domains which have a range of LEAR/HEAR area ratios. For expediency, in the first instance, the boundary of a domain was defined as a line drawn 5 km outside all donor or receptor fields under study. Through liaison with the company which contracted HEAR growers, it was possible to make contact with the owners or managers of farms that would be growing HEAR. The main criterion for selecting a domain was that it was within and representative of one of the main HEAR-growing areas. By November 2005, two domains had been found in the west of England, representing mixed arable/grass farming, and two in Cambridgeshire, representing mostly arable farming.

Within the domains, specific donor and receptor fields were chosen from among the potential HEAR and LEAR fields in the landscape. In the first instance, two receptor fields were selected from each domain which would be sampled for leaf and seed. Conditions for selecting a recipient field were:

- the grower was willing to cooperate, give information on the history of the field and allow access to fields;
- the field had never grown a HEAR variety;
- field-to-field contamination (i.e. movement of seed from a HEAR field to this field) was likely to be very low because the grower had not used machinery that had also been used on a HEAR field;
- no HEAR has been grown immediately adjacent to the field.

The landscapes in each domain were characterised by ground surveys for two purposes: one, to quantify features that might affect cross-pollination between donor and receptor crops (e.g. Fig. 6), and two, to enable the representativeness of the domains to be assessed against general landscape data (e.g. the areas of the various crops). Superimposed on these features were the effects of weather, which influence the release of pollen from anthers into the wind, the direction along which it is carried by wind and the activity of insect pollinators. More specific objectives were as follows:

- to map each HEAR donor field and LEAR receptor field and all other oilseed rape fields within 3 km radii of the receptor fields, so as to determine competing rape pollen sources in the area;
- to map land use in fields and areas within 3 km radii of the receptor fields, so as to identify additional pollen sources for insect pollinators and habitats where insects might live and nest;
- to characterise the landscape in greater detail between the donor field and receptor fields, mapping in addition to land use, the boundaries and physical features, in order to determine barriers to wind flow and factors affecting insect activity;
- to determine the degree of flowering synchrony among the oilseed rape fields;
- to enable correlation of the seed test results from the receptor fields with co-variate factors such as distance from HEAR donor, intervening land use and barriers between donors and receptors, other local pollen sources, phenology (e.g. flowering), altitude, meteorological data, position of local apiaries, bee flight lines and insect activity.

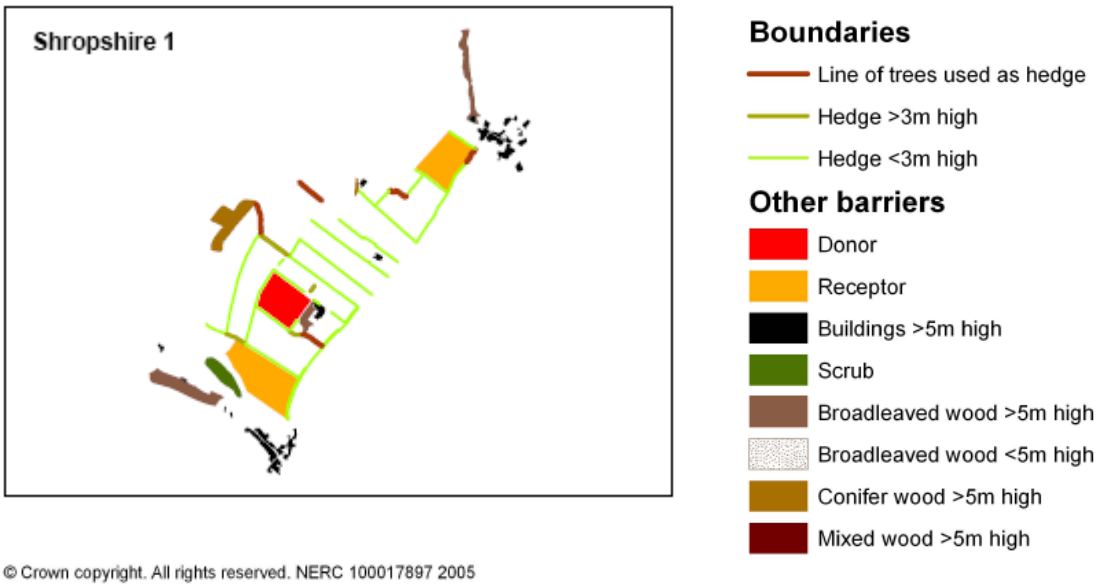

Figure 6 Example of field configurations of a HEAR donor field and two LEAR receptors. The donor field is 270 m from the edge of the receptor to the south and 1350 m from that to the north east.

The donor and receptor fields provided a wide range of configurations in 2005. Given the nearness of some donor and receptor fields, the distance between edges rather than centroids is used to show the range. Distances between donor and receptor fields ranged from 50 m to 1351 m (Table 3), and distances from sampling loci to the nearest HEAR ranged between 50 m and 2165 m. Configurations varied from a single donor field of 12 ha, giving a receptor/donor area of around one, to a relatively large block of several donor fields, comprising 83 ha, and giving a receptor/donor ration of much less than one (Table 3). All of the receptor fields were fully pollen-fertile.

Sampling receptor fields for HEAR in leaf and seed

Once potential donor and receptor fields were identified, the detection methodology was first tested on leaf samples from young plants taken from the fields. The method worked in all but one pair of donor-receptor varieties. (The reason for such varietal incompatibility is not fully understood.) Different donor and receptor fields were chosen in that instance. The donor fields at harvest had a high %EA of around 50%, typical of HEAR varieties.

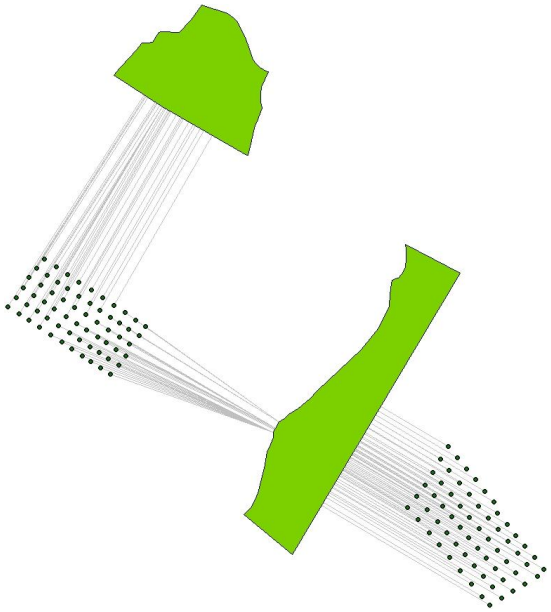

Figure 7. Example from 2005 of donor fields of high erucic acid rapeseed (green) and sampling loci (64 points) covering the area of two LEAR receptor fields; lines link each sample locus to the nearest point on the edge of a donor field.

Analysis of the variation in %EA in the 2004 samples indicated that more samples should be taken per field than in 2004 to obtain a reliable estimate of %EA. A grid of about 64 loci, for example in an arrangement of 11 x 6 or 8 x 8 (Fig. 7), was sampled to test for the presence of HEAR impurities. Leaves from 20 plants were examined per locus (totalling 1200 plants per field) and processed in batches by qPCR for detection of HEAR and in parallel using the SINE assay (see below). For illustration, fields were classified in three categories in terms of the likelihood that HEAR volunteers in them might contribute to %EA in seed: low, where HEAR was detected at less than 2% of loci; medium where it was detected at between 2 and 5%; and high where it was detected at >5%. Two of the fields were in this high category, the highest being HEAR present at 25% of the sample loci (though only a small percentage of total plants), despite there being no record of a HEAR crop in this field. The decision was made to continue with these fields since they would present an opportunity to study impurities from several sources. The loci were re-sampled for seed at maturity: 20 branches were taken from each locus, threshed and the seed pooled and mixed using a riffle divider for analysis in 10 batches of 500-seed per locus, amounting to around 320,000 seeds per field. (Any variation between these 10 batches is caused by incomplete mixing of the seed and does not relate to variation at the sampled locus.) The relation between the identified level of HEAR volunteers and the measured HEAR in seed is considered later (see Table 3 and associated text).

SINE (Short Interspersed Repetitive Element) qPCR assay

An additional qPCR assay, the SINE (Short Interspersed Repetitive Element) assay (Prieto et al., 2005, Allnutt et al., 2005) was developed and tested as a possible alternative to the HEAR DNA-marker in seed. SINE markers which showed differences between HEAR and LEAR varieties were selected for detailed study. Two candidate markers that were present in HEAR and absent in LEAR varieties were cloned and sequenced (GenBank DQ825762 and DQ825764). This sequence was then used to design a qPCR primer and probe set, to allow the quantification of HEAR in LEAR samples. When tested on actual field samples in 2005, however, the assay detected HEAR SINE alleles in seven out of eight fields tested. The SINE alleles were present due to either HEAR volunteers, seed impurity, genetic variability in the sown LEAR seed, or any combination of these, and so could not be used to estimate cross pollination with appropriate certainty. Nevertheless, the SINE markers were able to distinguish the different LEAR varieties, thereby confirming the information provided on the sown seed, and also detected spatial variation in the fields, including patches likely to consist of mixed crop and volunteer plants. The assay is described by Allnutt et al. (2008). Its contribution to defining the genetic composition of sampled loci will be reported in further refereed papers.

%EA and cross pollination – 2005

Spatial variation of %EA in receptor fields occurred both as a trend from one end of the field to the other, and as outcrops or hot spots of much higher %EA than the surrounding loci. Fig. 8 gives examples based on mean %EA per locus: in the left hand field, there is a covering of low %EA at the edge nearest the donor, decreasing in intensity across the field to zero at the far end of the field; in this are about five outcrops of relatively high %EA. In the right hand field, the %EA at the leading edge is higher than it was in the left hand one and the decline from top to bottom more pronounced. It is argued that the outcrops are HEAR volunteers and their hybrids, whereas the low values of raised %EA (usually directional) are cross pollination from outside the field.

The first step in analysis was to define samples representative of pure LEAR seed, i.e. seed without any HEAR impurity. (These values are analogous to the far left peak in the distribution in Fig. 5.) Such samples were identified by a %EA that was very much lower than the %EA of the outcrops, and discernibly lower than samples having even one or two HEAR-LEAR hybrid seed in 500 (as described later). They were found in greatest numbers in areas most distant from the nearest donor, such as those coloured blue in Fig. 8, where commonly all ten replicate 500-seed samples appeared to be pure LEAR. Site averages ranged between 0.01 and 0.02% EA; the distribution around the means was narrow, standard errors being less than 1% of the mean (Table 3). Three LEAR varieties were sown in the 8 receptor fields: one was grown only in field 2 which had the highest LEAR %EA of 0.0203%; another was grown only in field 6 which had the lowest %EA of 0.0130%; and the third was grown in the other 6 fields which ranged in %EA between those two. (It was fortuitous that the three LEAR varieties had different %EA.)

The second step was to investigate the outcrops. Seed from the outcrops was analysed individually for %EA. Giving a specific example - a locus having a mean %EA of around 3%, that is 100 to 200 times higher than the LEAR value, depending on variety, contained 11% of individual seeds with raised erucic acid values. More generally, up to 20% of the seeds in an outcrop could have high %EA, values for single seeds ranging from full HEAR (41-53% EA) down to around 10% EA. At most sites, the mean %EA at these high loci was typically 10 or more times greater than the other loci having %EA above the LEAR value. It was considered that seed from such outcrops indicated HEAR volunteers.

These measurements on harvested seed were mostly, but not always, consistent with earlier measurements of plants in the emerged crops. The two fields, 2 and 8, at which leaf sampling indicated HEAR volunteers were present at several loci, had EA values between 0.2 and 0.3%, caused by around 11% of loci being outcrops of raised %EA. One of these, field 8, had all the volunteers near one end. The other had them distributed over the field. Those fields designated as having low and mid volunteer presence did not have consistently different %EA in seed. Field

7 was anomalous, however, in that leaf samples indicated no HEAR volunteers, whereas seed samples had the highest in %EA of all sites. Indeed, some individual harvested seeds were full HEAR. This field had not previously grown HEAR. The harvested seed was germinated and tested by PCR to show HEAR DNA in some seedlings, confirming the raised %EA of the seed. This discrepancy has not been resolved. Despite the large sample size at the leaf stage, the sampling might simply have missed HEAR plants. Possibly the qPCR assay did not detect HEAR in leaf samples due to factors related to the variety or the condition of the plants. Alternatively, volunteers might have emerged later than the crop and grew on after the leaf samples were taken. Care was taken at seed harvest at this and all sites to exclude crucifer weeds such as *Sinapis arvensis*. However, field 7 was not included in further analysis.

Accordingly, three summary measures of %EA were derived for each receptor field (Table 3): the pure LEAR value for that field; the field average including the outcrops, determined simply as the mean %EA of all (64x10) samples, which in all instances was lower than the 2% threshold for %EA in LEAR yields; and the average for each field of those loci that were not outcrops. This latter average was taken to represent the rise in %EA due to cross pollination. Where there were few outcrops (fields 1, 3, 4, 5 and 6) or outcrops localised to one part of the field (field 8), and where a spatial trend was evident, the most likely pollen donor was considered to be the nearest HEAR field. For field 2 and the anomalous field 7, the contributions of internal and external HEAR plants to cross pollination were uncertain. The analysis therefore moved on to estimate % cross pollination for fields 1, 3, 4, 5, 6 and 8 among which %EA (all loci) ranged from 0.022% to 0.116%.

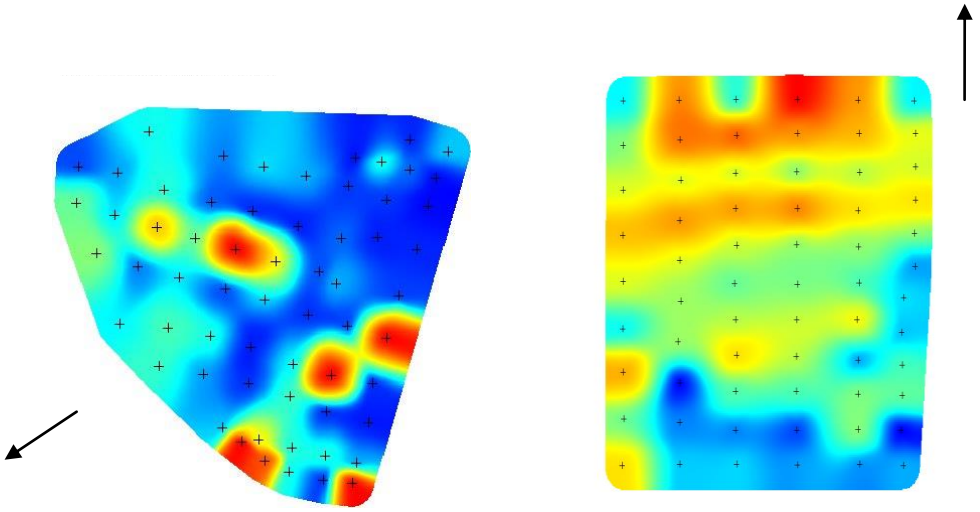

Figure 8. Examples of %EA in seed of mature plants at two receptor fields. Arrows indicate the direction to the nearest donor. Colour thresholds set low to emphasise low frequency cross pollination: deep blue – no detectable HEAR above the % in the receptor seed; green and yellow, one or two cross pollinations in 500 seeds; red, values above 0.1%EA caused by a higher frequency of cross pollination or volunteers.

Table 3. Fields used in 2005 and 2006, showing areas, distances, the likelihood (determined at leaf sampling) that HEAR volunteers would contribute to seed (see text for definitions), and three measures of mean % erucic acid in seed: first, for samples in which %EA did not rise above the LEAR value (implying no cross pollination from HEAR plants); second, for all loci in the field, to show the relative proximity to the 2% EA limit; and third, for loci except those having high percentage erucic acid attributed to volunteers and therefore showing raised %EA due to cross pollination (CP), with standard error in parenthesis; and % cross pollination derived from the latter data (see note f below).

			area of fields (ha)					mean % erucic acid in seed			
year	county	field	receptor	nearest HEAR donor	receptor / donor area	distance between edges (m)	HEAR volunteers	'LEAR' equivalent[d]	all loci	CP only	% CP [(f)]
2005	Shropshire	1	13.4	12.3	1.09	268	low	0.0200	0.048	0.025 (0.0096)	0.036 (0.0051)
2005	Shropshire	2	11.1	12.3	0.91	1351	high	0.0203	0.259	([e])	
2005	Shropshire	3	12.2	18.1	0.68	578	low	0.0170	0.108	0.033 (0.0066)	0.093 (0.036)
2005	Shropshire	4	22.4	18.1	1.24	1025	low	0.0170	0.024	0.020 (0.0009)	0.019 (0.0047)
2005	Cambridgeshire	5	14.0	20.8	0.67	490	mid	0.0160	0.064	0.032 (0.0026)	0.10 (0.016)
2005	Cambridgeshire	6	12.9	20.8	0.62	221	mid	0.0130	0.042	0.042 (0.0023)	0.099 (0.008)
2005	Cambridgeshire	7	14.5	83.1	0.17	986	low	0.0189	0.784	([e])	
2005	Cambridgeshire	8	11.9	83.1	0.14	50[b]	high	0.0192	0.220	0.116 (0.0090)	0.516 (0.050)
2006	Herefordshire	9[a]	10.3	8.1	1.27	297	low	0.0160	0.323	0.032 (0.0066)	0.083 (0.032)
2006	Herefordshire	10[a]	10.2	8.1	1.27	372	mid	0.0144	0.123	0.020 (0.0021)	0.033 (0.011)
2006	Cambridgeshire	11	15.1	64.6	0.24	941	low	0.0135	0.017	0.017 (0.0014)	0.024 (0.007)
2006	Cambridgeshire	12	10.7	64.6	0.17	50 [b]	low	0.0129	1.469	0.459 (0.1670)	1.56 (0.585)
2006	Cambridgeshire	13	26.7	64.6	0.41	497	low	0.0137	0.237	0.057 (0.0017)	0.029 (0.009)
2006	Cambridgeshire	14	27.0	64.6	0.42	1340	*[c]	0.0120	0.075	0.014 (0.0012)	0.011 (-)

[a] Fields 9 and 10 each consisted of two fields combined.
[b] HEAR crop sprayed before flowering to ensure there was a distance of 50 m between the edge of the HEAR crop and the nearest LEAR crop.
[c] The qPCR method for detecting HEAR in leaf samples did not work adequately for the crop variety in this receptor field.
[d] The standard error for the LEAR equivalent was typically <1% of the mean.
[e] Because of the widespread volunteers at these sites, the %EA attributable to cross-pollination alone could not be determined reliably.
[f] This column is an estimate of total cross pollination (HEAR plants to LEAR plants) after loci containing HEAR volunteers are removed from the analysis. Local pollination, i.e. from HEAR volunteers within the receptor field, might have contributed to these values at several sites, including 8 and 12, and led to a small overestimation of the %CP from external HEAR fields.

Estimation of cross pollination where there were no or few HEAR volunteers

Cross pollination was estimated based on sets of samples from which 'volunteer' loci were excluded. The decline with distance from the sample locus to the nearest HEAR field, such as that in Fig. 8, was statistically significant, despite local spatial variation. For illustration, trends are shown of %EA (mean of the 10 replicate seed samples) for loci having at %EA values below an arbitrary cut off 0.1% (Fig. 9). Beyond about 700 m, loci had low %EA which was no longer systematically affected by distance in this example.

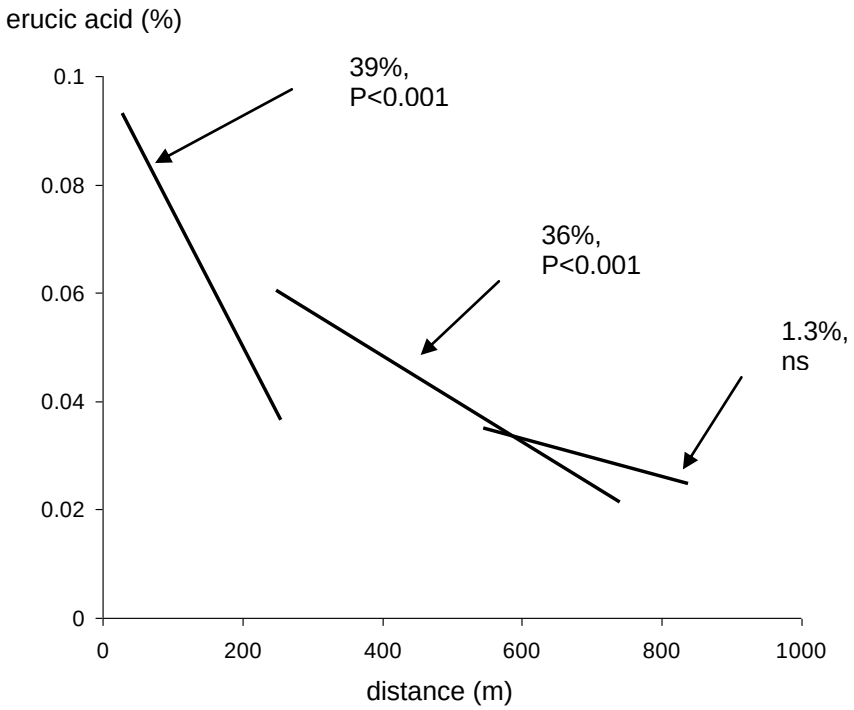

Figure 9. Linear regression lines summarising the change in %EA in 3 receptor fields in the east domain in 2005 with distance to the nearest point on a HEAR donor field, showing for each line the % of the variance accounted for by distance and that the regression was highly significant (P<0.001) or not significant (ns). Sample loci having %EA above 0.1% were excluded.

To estimate cross pollination in a sample, the LEAR value (no cross pollination) was defined and the F1 value (i.e. the %EA in a F1 HEAR-LEAR hybrid) was found by analysis of individual seeds from some of the loci. A relation between %EA and cross pollination was then constructed for each site. Values for 500-seed samples were averaged to give cross pollination for each locus (e.g. Fig. 10) and then for the whole of a field. The mean, whole-field, cross pollination ranged between 0.019% and 0.516% (Table 3). The values for 2005 are combined with those for 2006 later in the report to examine both the relation with distance (Fig. 12), and to indicate the potentially dominant contributions of other sources of impurity.

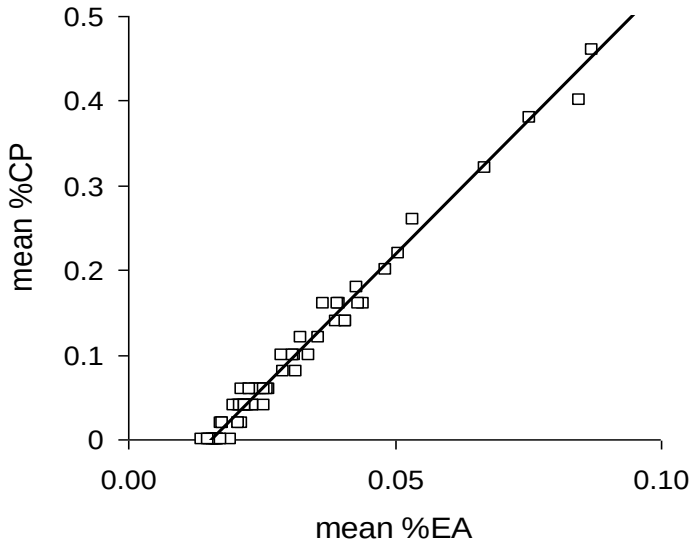

Figure 10 Example of mean percentage cross pollination (%CP) at loci in one of the fields estimated from measured % erucic acid (EA) content (each point is the mean of 10 x 500-seed samples): the intercept on the horizontal axis is determined by the %EA in samples where there was no cross pollination (i.e. equivalent to pure LEAR), and the slope by the measured %EA in hybrid seeds resulting from HEAR-LEAR cross pollination.

Findings 2005

- Samples showing high %EA (hybrid or full HEAR), arising most likely as volunteers or impurities in sown seed, occurred in most fields.
- The mean %EA of fields without substantial volunteers was low at 0.06%, and %EA in the field having most impurities was only 0.78%, less than half the accepted threshold of 2% EA in LEAR yield.
- The following evidence was taken for raised %EA being due to cross pollination: (a) no or few volunteers were found when sampling young plants, (b) few high %EA loci were present at seed sampling and could be excluded, and (c) the distribution of %EA was directional, decreasing away from the nearest donor or else the values were very small throughout the field.
- Given these conditions, cross pollination from HEAR fields was typically around or below 0.1% averaged over the whole LEAR field, except for one field that was 50 m from a large HEAR block and which had cross pollination of 0.52%. These estimates are among the first ever for whole fields in typical arable landscapes.
- The relative area of receptor to nearest donor was typically much smaller than for the FSE fields (Table 2, Table 3) and cross pollination much greater. Distance to the nearest HEAR field had the most influence on cross pollination in each domain (Fig. 9).

UPSCALING CONTINUED WITH EMPHASIS ON THE ROLE OF INSECTS (2006)

The approach taken in 2005 was repeated in 2006 in order to increase the range of configurations between donors and receptors. A more specific aim was to examine the contribution of insects by using large exclusion cages that prevented the ingress of bees and other large flying insects, paired with uncaged areas of crop.

Sites, sampling and general findings

New domains were selected in west (Herefordshire) and east (Cambridgeshire) regions and characterised as in 2005. Eight further receptor fields were identified. The four fields in Herefordshire were combined for sampling as pairs of fields. The pairs and the four fields in Cambridgeshire were equipped with insect exclusion cages. The fields were leaf-sampled before stem extension for detection of HEAR volunteers and seed-sampled at maturity. The procedure for assessing HEAR presence by qPCR worked well for all fields except field 14. As for the likelihood of HEAR volunteers contributing to yield: one field was classed as medium (some HEAR detected) while the rest were low (Table 3).

Six cages were placed in each of the receptor fields before crops had developed stems: one of the orientations is shown in Fig. 11 (upper). The alternate placing of the caged plots can be seen in the photograph in Fig. 11 (lower). The distance between caged and uncaged plots varied with the size of the field but was at least 10 m. A further 12 uncaged areas were sampled to give a better estimate of %EA in the whole field. The distance from the edge of a HEAR field to the plots ranged 50 m to up to 2 km. Crop phenology, flowering synchrony and insect activity were measured as in 2005. Leaf and seed were collected at each sample location as in 2005. During flowering, insect activity in the fields was measured using 8 x 100 m transect walks per field per week, pollen from the air was sampled on rotorods (1 pair per field in an open and caged locus) and sticky traps were used to estimate small insect activity inside and outside the cages at 3 loci in each field.

Donors, receptors and cross pollination

As before, the HEAR donor seed itself had a mean %EA around 50%, and three classes of sample were again found in the receptor fields - those having %EA the same as the sown LEAR seed at around 0.015% (which were the majority of samples), those having %EA above this value and indicating several cross pollinations per 500 seeds and those having %EA ten or twenty times higher than the previous category and indicating the presence of HEAR plants (arising from volunteers or impurities in seed). The areas of high %EA were highly localised and typically had a mean %EA of 3-4%. Analysis of single seeds at these loci showed that around 7% of seeds were high erucic, either full HEAR or hybrids. The general picture was of a low incidence of volunteer patches in an otherwise largely LEAR background.

When areas of high %EA were again omitted from the analysis, evidence of cross pollination was found at all sites. The following figures refer to only the uncaged loci. The sampling loci nearest to the HEAR donors again had the highest frequency of cross pollination in each domain, and because of the greater range of distances and fewer volunteers this year, the range of %EA attributed to cross pollination was wider than in 2005. Two examples show the range. The lowest cross pollination was measured in a field whose leading edge was about 1 km from the donor and which was close to other LEAR fields: the total number of cross pollinations from HEAR was estimated at 9 in 60,000 seed analysed, or 0.014%. These nine occurred in six of 120, 500-seed samples, spread over different sample plots. Since no volunteer patches were found, the EA in the whole field was hardly above the value for pure LEAR seed. At the other extreme was a receptor field whose southern corner was close to a much larger block of HEAR donor of 65 ha in area, about six times the size of the receptor (field 12, Table 3). Cross pollination was detected from the HEAR block to all sampled parts of this field. Volunteers and the widespread cross pollination together raised the mean EA over the whole field to 1.47%, the highest found but still below the %EA limit of 2%. The mean cross pollination for this near field was estimated to be

above 1% (see later).The other fields (at the uncaged loci) had estimated cross pollination frequencies within or below the range found in 2005, that is mostly below 0.1%

21	**22**	**23**	24
17	**18**	**19**	20
13	**14**	**15**	16
9	**10**	**11**	12
5	**6**	**7**	8
1	**2**	**3**	4

Figure 11. Schematic (upper) of the numbering and relative positions of sampling plots in a receptor field in 2006 showing plots covered by insect exclusion cages (red), the respective uncovered control or comparator (blue on the row) and additional sampled plots (no colour); and a photographic example (lower) showing four of the six caged plots in a field. Photograph by C. Boffey.

The influence of the cages

The cages inevitably altered the microclimate around the plants inside them. They might have reduced the movement of plants against each other in wind (though this was not measured), and might therefore have restricted the amount of local pollination caused through physical contact with neighbours. They allowed airborne pollen through: the average %HEAR grains on rotorods ranged from 0-0.02% (with one outlying value in a cage of 0.2%). These values were typically 10 times lower than in 2003 – probably because these were commercial fields, not plots, so they

would be saturated with their own local pollen. There was no clear relation between the % HEAR grains found on rotorods and distance from donor, and no difference in deposited pollen between caged and open plots. The cages allowed in small insects, but not large insects such as honeybees and bumblebees. In general, there were fewer bees and other insects recorded in the fields in 2006 than in 2005.

The most consistent effect of the cages measured at both domains was that they increased the mean mass of individual seeds by typically 1.5 times. This is likely due to the lack of large insects within the cage resulting in much lower seed set per plant, as was found in the HEAR experiment in 2003. Moreover, the %EA in the absence of cross pollination, i.e. the LEAR value, was slightly higher, by 1.1 times, inside than outside the cages. The reason for these differences due to the cages is still not clear.

Larger differences emerged at intermediate and high frequencies of pollination, but not in the same direction in each case. At intermediate frequencies (e.g. 1 to 3 cross pollination in 500-seed samples), the means at several sites were higher inside the cages, while at high frequencies – here represented by the site nearest the donors in each domain – the means were higher outside. The difference inside the cages at intermediate frequencies might have been the result of smaller frequencies of self pollination inside due to the restriction on wind-movement of the plants, while at the higher frequencies, the values might have been dominated by bee traffic. The investigation of these effects will be continued in a paper to be submitted for peer review. Insect abundance in the different years of the project is compared later in the report.

Findings 2006

- Cross pollination averaged over whole fields (outside the insect exclusion cages) was again generally low – between 0.01 to 0.1% - and consistent with the fields in 2005, except at one field located close to a large donor block where it was above 1%.
- Insect exclusion cages reduced cross pollination of HEAR only to those LEAR receptors that were closest to the donor in the respective domains. Several other effects of the cages were found on seed mass and %EA at low and intermediate crossing frequencies, denying any simple interpretation of the contributions of insects and wind as effective carriers of pollen.

IMPLICATIONS OF THE RESULTS FOR ECOLOGICAL PROCESSES AND COEXISTANCE

Frequencies and mechanisms of cross pollination

This study provided the first information on cross pollination between oilseed rape fields of different type occurring in real configurations in the farmed landscape. The values for average cross pollination in the fields studied in 2005 and 2006 are combined in relation to distance to the nearest donor in Fig. 12. The values declined with distance between field edges over a hundred-fold between 1% and 0.01%. There was still substantial variation in cross pollination (e.g. between 300 and 600 m in Fig. 12) that was not explained by distance alone. Further analysis is in progress to examine whether such variation is linked to the relative sizes and configurations of the donor and receptor fields.

These percentages can now be compared with previous measurements which were all at smaller experimental scales. Hüsken & Dietz-Pfeilstetter (2007) tabulated and summarised known experimental data for cross pollination in oilseed rape. They distinguished two configurations: continuous, for example, where a receptor plot or field surrounds a small donor plot, and discontinuous, where the receptor plot is to one side of or at a distance from the donor. Too few measurements had been made from which to generate reliable averages beyond about 75 m, but at this distance average cross pollination was around 0.1% in the discontinuous configuration, slightly lower in the continuous. The type of curve used in Fig. 12 to fit the HEAR data also provided a good fit to their data up to 75 m. There is no information as to whether the same fitted curve would continue to represent cross pollination at longer distances, but for illustration, the

curve is extrapolated to 750 m and shown as the lower dotted line in Fig. 12. The whole-field averages of a few of the fields in the HEAR study lie close to this extrapolated curve, but the HEAR-curve as a whole and values for most of the fields are higher. There have been few other measurements at distances of several hundred metres or more except in the FSEs as described in this report (Table 2) and at the French inter-institute platforms where the donor blocks were around 0.4 ha in area and the receptor fields around 10 ha (Devaux et al., 2008; C. Sausse, personal communication and exchange of data through the SIGMEA project). The highest percentage cross pollination of any receptor field in the FSEs was similar at a given distance to the curve for the HEAR study, but most of the assessments were lower. Similarly, a few of the sample-sets between 500 m and 1000 m in the French study were similar to values on the HEAR curve but measurements from most fields were again much lower.

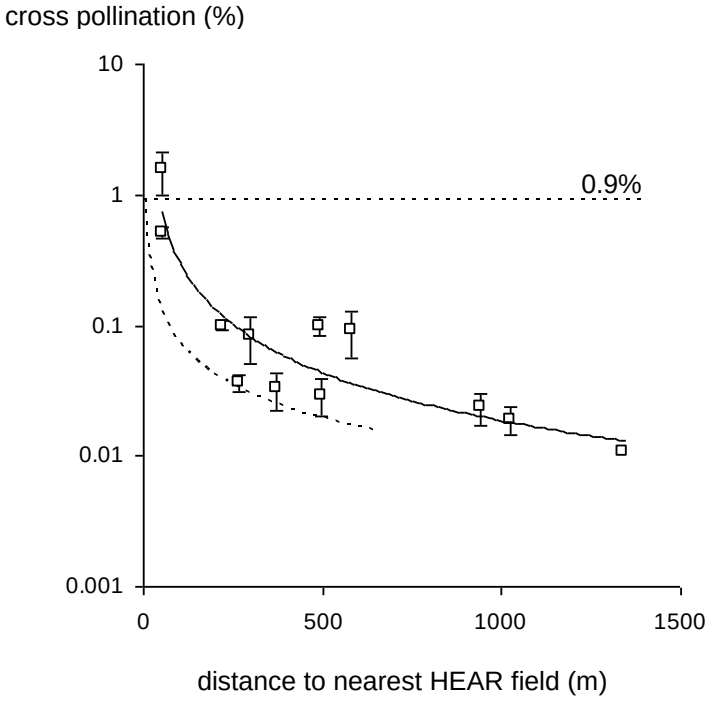

Figure 12. Percentage cross pollination, as whole-field averages (+ and – standard error) measured in 2005 and 2006 with distance from the nearest HEAR field, omitting two fields that had widespread HEAR volunteers. The fitted geometric curve (log-log) described 82% of the variation in %CP with distance. The lower dotted line is an extrapolation based on data up to around 100 m at the scale of the plot or small field (see text) summarised by Hüsken & Dietz-Pfeilstetter (2007).

Cross pollination from HEAR to LEAR fields in the real agricultural configurations studied here was therefore, with some exceptions, higher than found previously in experimental plots and fields of oilseed rape. In most previous studies, the area of donor crop was similar to or smaller than any one nearby receptor and was much smaller than the combined area of receptors within a radius of 1 to 2 km. It is likely that the higher cross pollination in this HEAR-LEAR study was due to the large areas of donor pollen (fields of 8 to 83 ha) and the similar areas of donor and receptor fields in the local landscape. It is also possible that HEAR volunteers in the receptor

fields could have raised the whole-field average slightly by local cross pollination. Even given the large number of samples processed (more than in most previous work), some HEAR volunteers might have been undetected and could have raised the HEAR-LEAR cross pollination within the field. The potential contribution of low-frequency impurities in this way is not confined to this study; it could have occurred in many previous experiments and is a general problem when estimating events of 1 in 1000 or lower.

The highest values of HEAR-LEAR cross pollination (Table 3) were measured in configurations where a receptor field was almost surrounded by or was a few tens of metres from a much larger block of donor. There are parallels here with the results from landscape-scale studies in commercial agriculture with maize in Europe. In Switzerland, for example, where pollen donors and receptors were >100 m to several kilometres apart, cross pollination was typically less than 0.01% (Bannert & Stamp, 2006, 2007). In Spain, where donor and receptor fields were occasionally grown next to each other, cross pollination was generally low but increased to around or above 1% in specific instances where a small receptor field was surrounded by donor fields (Messeguer et al., 2006). In neither that study, nor the work described here on the HEAR-LEAR combination, were specific measures used to achieve separation or restrict pollen movement. They nevertheless show that arrangements of fields do arise in commercial agriculture in which cross pollination from one type to another exceeds 1%.

In summary, the results at the landscape scale described here, and the previous estimates of pollination from GM to adjacent non-GM half fields in the FSEs (Weekes *et al*., 2005) enable three broad configurations to be defined for oilseed rape crops of full pollen fertility in the UK.

1. Receptor fields sited 1 km or more from the nearest donor and within a landscape containing other receptor but no other donor fields of oilseed rape: cross pollination was around or below 0.01%, that is fewer than 1 seed in 10,000 (and probably more like one in 100,000). Cross pollination at this frequency would have negligible influence on raising total impurities towards the 0.9% GM labelling threshold.
2. Receptors and donors in similar proportions and receptor within a few hundred metres of the nearest donor (e.g. the group of symbols between 200 and 600 m in Fig. 12): cross pollination was generally around or just below 0.1% or 1 in 1000; in these field configurations, cross pollination would not itself get near to challenging a labelling threshold of 0.9%, but might be high enough to 'top up' impurities from other sources.
3. Receptors adjacent or close to a donor field: cross pollination was mostly well below 0.9% when receptor and donor were similar in size, but increased to near or above this value where the receptor was much smaller than the donor.

The situations described above, including the configuration of 'small receptor - large donor', were found in these landscapes and were not created for the benefit of research. There is no information on how frequently the various configurations occur where HEAR and LEAR are grown together. However, blocking of crop types is a common feature of arable landscapes – further examples of fields sown in large blocks are given by Squire et al. (2005).

The placing of small receptor fields near to large blocks of donor can be avoided on the same farm by segregating donors and receptors to ensure that all receptors were in category 2, and it would be not much more difficult to ensure all receptors were in category 1 above, receiving negligible cross pollination. Segregating crops in this way on different farms is also achievable but requires close liaison between neighbouring farmers and good forward planning.

These conclusions are restricted to the conditions of the study in 2005 and 2006 – the main ones being that all receptor varieties were fully male fertile and all were winter oilseed rape, flowering before the main period of insect activity. The trend of growers to use 'varietal associations' consisting of a proportion of male sterile plants did not continue beyond about 2002. Rather, such varieties declined in area and were uncommon by 2006. All evidence to date, both here and in other studies, points to a much greater frequency of crossing in such varieties. They should simply be avoided if crossing to them is to be minimised.

Care should be taken, however, to consider the consequences of moving from winter to spring crops if there was ever reason to do this. The type of oilseed rape predominantly grown in the UK is winter oilseed rape, sown in late summer/autumn. Only if a drought in autumn causes widespread failure of crops will spring varieties be widely grown, as happened in 2003/04. The general varietal type – winter or spring – potentially affects pollination in two opposing ways. Flowering lasts longer in winter varieties – 6 weeks, allowing more opportunity for overlap between varieties - but insect activity is reduced by the cooler weather at the time of flowering. Flowering lasts for about 3 weeks in spring crops, usually beginning in early summer, after winter crops have stopped flowering, thereby giving less opportunity for overlap between fields, but insect activity is usually much greater in the later flowering spring varieties. The result of these opposing influences is uncertain.

The contribution of insects

The project provided new information on the process by which insects transfer pollen between fields. The DNA-based method for detecting HEAR genes in pollen washed from bees made it possible to quantify for the first time, the proportions of different types of pollen carried on their bodies. Bees that had visited a HEAR field had LEAR and HEAR pollen mixed on their bodies. They transported this pollen to LEAR fields in the neighbourhood, where some of the HEAR pollen gave rise to HEAR-LEAR hybrids. Two important parameters – the proportion of bees carrying donor (HEAR) pollen, and the proportion of HEAR pollen on those bees - were both here quantified for the first time in oilseed rape, or any other crop, to our knowledge. These values could be used to make more realistic predictions of gene flow from the models of bee pollination which have so far applied a worse-case scenario of assuming all pollen on a bee arriving at a receptor is from the donor in question (Cresswell et al., 2002).

Table 4 Representative examples of the range of insect activity in the three main field seasons in which either spring (SOSR) or winter (WOSR) oilseed rape was grown as donor and receptor crops. The transect length is 8 x 100 m; values are means of 3 or 4 walks over the season.

Year	*Domain*	*Crop type*	*Comment*	*Mean Insects / transect*	*Mean bees / transect*
2004	Hertfordshire	SOSR	Late flowering, coinciding with peak insect activity	130.3	130.3
2004	Tayside	SOSR	Late flowering, coinciding with peak insect activity	111.4	110.8
2005	Shropshire	WOSR	A donor field – highest number of bee hives at any field in 2005 and 2006	26.4	25.2
2005	Shropshire	WOSR	A receptor field – highest number of bee hives at margin of any receptor field	16.9	15.9
2005	Shropshire	WOSR	A second receptor field – few bee hives in the vicinity	3.2	2.9
2005	Cambs	WOSR	A receptor field – lower activity than Shropshire domain	1.4	1.3
2005	Cambs	WOSR	A second receptor field	0.3	0.2
2006	Herefordshire	WOSR	A typical receptor field – few hives in the domain	0.5	0.4
2006	Cambs	WOSR	A typical receptor field – few hives in the domain	0.8	0.8

More generally, it was difficult to separate the contributions of airborne and insect-borne pollen to cross pollination, mainly because of low insect activity in the east domains in 2005 and in both east and west domains in 2006 (Table 4). Such low activity is likely to be typical of winter-sown oilseed rape fields flowering in April and May. Insect activity was 100 times greater in the spring crops grown in 2004 than in the later two years, However, these receptor varieties were also part male sterile (20% plants producing pollen), so that contributions of male sterility and insect activity were potentially confounded. Overall, factors such as distance between donor and receptor, and the proportion of male sterility in the receptor, have a more pronounced effect on cross pollination than insect activity in these landscapes. The complex interactions between meteorological conditions, insect activity and crossing will be examined in peer-reviewed papers.

The contributions of HEAR in sown seed, volunteers and cross pollination

A main finding of this project is that HEAR impurities were widespread in fields that had not grown HEAR in the previous 6 years, and as far as could be ascertained for most fields, not at all. Project staff later discovered that HEAR had been grown in one field, initially designated as not having grown any, but volunteers there must have been well controlled because little HEAR was found in emerged plants or seed. In some fields, the HEAR impurities made a much greater contribution to %EA than did cross pollination. Mostly, with the exception of one or two fields where they occurred widely, they were confined to discrete locations, but they commonly occurred at such locations in quite high proportions, even up to 10 to 20% of the seed. The spatial distribution of volunteers was unlike cross pollination in having no directional distribution. The volunteer patches also sometimes differed by containing seed of high %EA that could only have come from self-pollination of full HEAR volunteers or from full HEAR seed impurities.

The sources of such volunteers and other impurities were not investigated in detail, and indeed other studies have been resourced to do this (Begg et al., 2006a; 2008; Gruber et al., 2004, 2005; Lutman et al., 2005, 2006; Pekrun et al., 2006; D'Hertefeldt et al., 2008). Most fields in the HEAR study had grown oilseed rape at least once in the previous six years. If volunteers persisted from HEAR impurities in those crops, they exhibited no consistent decline over time. The investigations referred to earlier in this paragraph showed populations of volunteers to depend more on field management than on time *per se.* They could also have persisted over much longer time scales than six years or been brought to the field in machinery or in sown seed.

Has coexistence worked for high and low erucic acid varieties?

The sampling of seed from loci systematically positioned over the fields in this study provided the most detailed account so far of the presence of an impurity in crops of oilseed rape. The accepted upper limit for erucic acid in low-erucic oil used for food is 2% (i.e. erucic acid should not constitute more than 2% of all fatty acids in the oil yield). All 16 fields examined in 2005 and 2006, even those very close to large fields of HEAR, were below this threshold, and most fields were hardly more than $1/10^{th}$ of it. Given that HEAR and low erucic acid varieties have both been grown in the landscapes for many years, coexistence - in the sense of keeping a product to stated limits - has worked. Farmers not wishing to grow HEAR and either never having grown it (15 fields) or grown it once previously (1 field), produced crops of low erucic acid varieties that were well within the accepted range. No particular additional forms of management seemed to be in place to achieve this other than good agricultural practice for standard control of volunteers and standard care over the ingress of HEAR in machinery. However, it should not be assumed from this evidence that, where GM and non-GM crops were grown in the same landscape, farmers would be able to keep GM content below 0.9% in all non-GM fields. This lower threshold will be much harder to achieve than the 2% for erucic acid content. The results of the SIGMEA project (SIGMEA, 2004) to be published in 2008 will consider the feasibility of GM coexistence in a range of European landscapes.

Caution over the purity of seed lots

It was found during the study that HEAR-type impurities existed in some sown LEAR seed lots. Their presence in stocks used as LEAR background during the development of the qPCR technique was at first undetected, but gave rise to some unreproducible results that set the project back several months. For instance, a determination by qPCR or GC on a seed lot that contained, say 1 HEAR seed in 1000 (for the sake of argument) would result in a %EA value hardly distinguishable from pure LEAR, but the presence of such an occasional HEAR or HEAR-LEAR hybrid when making up a standard for analysis would interfere with the result. It was only when a particular LEAR line was analysed by GC, seed by seed, that the existence of HEAR types in a LEAR background was detected.

Subsequently, LEAR standards and certified seed of varieties used in the experiments were tested in this way. Not all such seed contained HEAR-type impurities. For example, in the 2006 season, seed of all the receptor types tested negative. It is unlikely however, given the widespread existence of cross pollination, that farm-saved seed from fields in landscapes such as were studied here, would be free from HEAR impurities.

Our recommendation to investigators wishing to explore or use the HEAR-LEAR system (or by extension any donor-receptor combination) is not to assume any receptor seed or standard is pure, even if bulk determinations indicate %EA values in the range expected of a LEAR variety.

A statistical (genetics) model for separating cross pollination from other impurities

As described earlier, where volunteers or other forms of HEAR impurity were widespread in a field, the simple approach to estimating cross pollination developed in 2004 was not possible, since hybrids do not necessarily have %EA of around 25%. A more comprehensive statistical model of crossing in these complex populations was considered necessary and was developed late in the project, primarily as a potential tool for future analysis. A complicating feature with oilseed rape (*Brassica napus*) for any system of donor and receptor is its polyploid genome, arising from a combination of *B. rapa* and *B. oleracea* genomes. The basis of the model representing the HEAR-LEAR combination is that erucic acid levels are controlled by the genetic loci described earlier, one from each of the two genomes in oilseed rape. The individual seeds may be a mixture of genotypes, ranging from the HEAR double homozygote AABB through all genotype possibilities, such as AaBb, to the LEAR double homozygote aabb. A single harvested seed can be any one of 9 genotypes, each having a different value of %EA:

AABB, AABb, AAbb, AaBB, AaBb, Aabb, aaBB, aaBb or aabb.

Coincidentally, progeny from crosses from the GM glufosinate herbicide tolerant Ms8xRf3 variety also have 9 genotypes (Begg et al., 2008). Nevertheless, the model for the HEAR-LEAR system gives the probability of seed having one or other of these genotypes based on the proportions of the genotypes in donor and receptor plants. The full model and its application in the data collected here will be described in peer reviewed papers. In principle, however, the means by which the impurities arise – sown seed, volunteer weeds and cross pollination – are the same whatever the genetic nature of the impurity. A statistical model of this type could in principle be applied to other donor-receptor systems.

Questions unanswered and future work

Lessons for future experimentation – the balance between naturalness and control

The project team purposely and knowingly chose to conduct their main studies in rural landscapes rather than on experimental farms. The fields sown with HEAR and LEAR varieties were fields that farmers would have used whether or not measurements were made on them. The scope for the growers or their neighbours to introduce artificiality was therefore very small, and providing the field configurations and landscapes were characterised and shown to be typical – and the analysis so far is that they are - the results can be taken to represent general situations. On the other hand, the chance of the researchers encountering and having to explain unexpected

occurrences was certainly great, as was found particularly in 2005 with the range of HEAR impurities. On balance, and to our advantage, the configurations gave rise to a very wide range of cross pollination that was unlikely to have been achieved if the donors and receptors had been placed in a regular arrangement on experimental farms. The configurations at each extreme of the range were particularly useful in defining likely maximum and minimum frequencies of crossing. They showed, clearly, that coexistence measures should take account of the relative size of donor and receptor, not just distance to the nearest donor.

Distinguishing the different forms of impurity

The central aim of the project was to measure and understand cross pollination and not the origins and fate of the various kinds of impurity. High throughput systems of the type used here, whether DNA-based or biochemical, have to target fairly specific differences between donor and receptor, and by doing so are likely to miss other information that might distinguish sources of impurity. More generally, the various crop varieties can be distinguished by other genetic marker systems (e.g. Charters et al., 1996; Tommasini et al., 2003), so it should be possible, for example, to tell whether impurities in a field arose from varieties that had been grown previously, or from impurities in the sown seed. The SINE marker used here succeeded in doing that for some volunteers (Allnutt et al., 2008). But even if a marker system were to distinguish breeders' varieties, it would still not be possible to tell if a current variety brought an impurity through several routes at the same time, the same route in different years or a combination of these. Defining the origin and then tracing all forms of impurity would be a massive task requiring a much greater effort and cost than incurred in this project. Volunteer detection and dynamics is itself well researched (Begg et al., 2006b; Begg et al., 2008; D'Hertefeldt et al., 2008; Pekrun et al., 2006) and it would be more expedient and economical to combine data from this and other projects, using models of regional geneflow and population dynamics (e.g. Colbach et al., 2001; 2004; 2005) to predict cumulative impurities in various scenarios that could then be tested. An EU FP6 project (SIGMEA 2004) is attempting to do this for oilseed rape and other crops in Europe.

Ecological effects of cross pollination

While for most fields, cross pollination brings only small degrees of impurity, it occurs repeatedly in volunteer (in-field) and feral (wayside) plants and so might affect their micro-evolution by altering the range of traits within them (Devaux et al., 2007; Garnier & Lecomte, 2006; Timmons et al., 1996). The effect of repeated cross pollination of HEAR to LEAR volunteers and ferals has not been measured, but could be altering populations if plants with HEAR traits are selectively favoured. Similarly, the movement of a trait such as GM glyphosate tolerance (if GM glyphosate tolerant oilseed rape were grown in the UK) could bring an advantage to volunteer populations that were regularly sprayed with glyphosate, which would be likely since this herbicide has become one of the two most widely applied herbicides in the UK (PUS). A detailed study of the ecological effects of cross pollination on volunteers, ferals and any compatible wild relatives would have not only scientific but also economic interest since *B. napus* volunteers are now among the most widespread weeds.

OUTPUTS FROM THE PROJECT

Papers accepted for publication in peer reviewed journals

Allnutt TR, Roper K, Henry C J (2008) Development and application of SINE multilocus and quantitative genetic markers to study oilseed rape (*Brassica napus* L.) crops. *Journal of Agricultutral and Food Chemistry* 56(2), 426-432, doi: 10.1021/jf072047a.

Cullen DW, Squire GR, McNicol JW, Jacobs JH, Osborne JH, Ford L, Ramsay G, Scrimgeour S,Young MW. (in press) Development and validation of gas chromatography and real-time

quantitative PCR for the quantification of landscape-scale gene flow from varieties of high erucic acid (HEAR) oilseed rape. *Journal of the Science of Food and Agriculture*.

In preparation *(provisional titles)*

- Regional cross pollination from GMHT fields in the FSEs. Squire GR, et al.
- Mechanisms of cross pollination in relation to distance, male fertility and insect exclusion. Osborne J, Ramsay et al.
- Within-field and regional variation in cross pollination of oilseed rape estimated using the high erucic acid marker. Squire GR, Kilpatrick J, Kightley S, Cullen D, et al.
- A statistical model for estimating cross pollination from external fields and in-field impurities using the HEAR marker in oilseed rape. McNicol J, Ramsay G, Cullen D et al.
- Landscape features and bee activity. Osborne J, Boffey C, et al

Conferences, presentations, internal reports

Cullen DW, Anderson JN, McNicol J, Ramsay G, Squire GR. 2004. Field to field geneflow in oilseed rape. Annual Report of the SCRI 2002/03, 120.

Ramsay G, Squire GR, Thompson CE, Cullen D, Anderson JN, Gordon SC. 2003. Understanding and predicting landscape-scale geneflow in oilseed rape. In: *GMCC-03, GM Crops and Co-existence*, 13-14 November 2003, pp. 102-104. Danish Institute of Agricultural Sciences

Squire GR. 2005. Contribution to geneflow by seed and pollen. In: GMCC-05: Coexistence between GM and non-GM based agricultural supply chains, 73-77. Ed. Antoine Messean, Agropolis Productions, Montpellier, France.

Squire GR, Bohan DA, Brooks DR, Champion GT, Dewar AJG, Firbank LG, Haughton AJ, Hawes C, Heard MS, May MJ, Perry JN, Rothery P, Scott RJ, Woiwod IP. Ecosystem effects of Novel Living Organisms: Perspectives from the UK Farm Scale Evaluations (FSE) and other studies. (2004). Invited presentation at *Ecosystem Effects on Novel, Living Organisms (EENLO).* Workshop at Montebello. Quebec, Canada, 4-6 Fenruary 2004. Organised by *Environment Canada.*

Squire GR, Ramsay G. 2003. Dispersion du pollen sur les longues distances et role des insects: quantification du flux de genes a l'echelle du paysage dans du colza oleagineau. *In Impact sur l'environnement des cultures de colza genetiquement modifie tolerant a un herbicide*, pp. 2 - 9. Eds Marc Fellous, Antoine Messean. Paris: Commision du Genie Biomoleculaire

Invitations to present progress and results to government commissions, workshops and universities: given on behalf of the project team by the Project Coordinator (GR Squire)

- Danish GM Working Group workshop on Coexistence, Middelfart Conference Centre, Denmark, May 2003.
- SEERAD Coexistence Workshop, Edinburgh, 14 September 2004.
- BBSRC/NERC - *Gene Flow in Plants and Micro-Organisms Initiative Workshop, Initiative overarching talk 1: 'Crop to crop plant'*, London, 23 & 24 June 2005.
- Defra - meeting to discuss separation distance for gene flow in relation to coexistence, London, 24 May 2005.
- University of Aberdeen, National Science Week, to speak on *Genes and food webs in the GM crop trials,* Aberdeen, 10 March 2006.
- Josef Stefan Institute, Slovenia to speak on *GM crop risk assessments in the UK*, at a meeting of Slovenia Ministries of Agriculture, Environment and Health, Ljubljana, Slovenia. March 2006.

- University of Nottingham Seminar Series - *Risk assessment without connectivity?* Sutton Bonington Campus, 17 May 2006.
- Open University workshop on 'New technologies and scientific developments: exploring better ways to support farmers' decisions', to speak on *Technological developments and scientific innovation past, present and future*. Stoneleigh-park Exhibition and Conference Centre, 16 November 2006.
- Seminar of ANR-OGM - presentation on *Le changement d'échelle conduit à des effets inattendus : l'estimation des risques engendrés par le mise en culture des OGM*. Meeting on *Organismes genetiquement modifies: aspoects socio-economiques, alimentaires et environmentaux. Ministere delegue a l'Enseignement Superieur et a la Recherche, Amphitheatre Gay-Lussac*, Paris,14/15 December 2006.
- Persistence of oilseed rape. Presentation to *European Enforcement Project GMO conference*, The Hague, The Netherlands on 26/27 April 2007.
- Should the ecosystem or the GMO be the focus of risk assessment? Presentation at *2nd meeting of European committees on Biosafety in the field of the deliberate release of GMOs*, on 14-16 May 2007, Ljubljana, Slovenia
- Systems approach to biosafety. Presentations to Chinese Delegation from CAAS at Bioforsk, Norway on 22 June 2007

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Begg GS, Elliott MJ, Squire GR, Copeland J. (in press). Prediction, sampling and management of GM impurities in fields and harvested yields of oilseed rape. *Final Report of Defra Project VS0126,* Defra. London.

Begg GS, Elliott MJ, Iannetta PPM, Cullen DW, Squire GR. (2008) Heterogeneity in the distribution of genetically modified and conventional oilseed rape within fields and seed lots. *Transgenic Research*, doi:10.1007/s11248-008-9166-7

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