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Molecular Ecology of Weeds

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MOLECULAR ECOLOGY IN WEED SCIENCE – THE STORY SO FAR

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ABSTRACT

Molecular ecology is a newly–defined discipline, derived from a marriage between the fields of molecular biology and ecology. It combines the well–established methodologies of allozyme electrophoresis with more recently developed nucleic acid technologies such as Restriction Fragment Length Polymorphism and the Polymerase Chain Reaction. This paper briefly describes the principles of some of the molecular techniques that are particularly applicable to ecological studies and describes how they have been used, or may potentially be used, in the field of weed science.

INTRODUCTION

Molecular biology is a rapidly evolving science. This evolution has accelerated enormously in recent years due largely to the invention of the Nobel prize-winning technique of the Polymerase Chain Reaction (PCR; Mullis and Faloona, 1987), in which sections of DNA can be amplified exponentially, thus negating the need for large amounts of material for analysis.

Molecular ecology covers the interface between molecular biology and ecology. It involves the use of existing molecular biological techniques in the investigation of ecological problems, and the development of novel approaches arising from the evolving synergism between the two fields. Thus it is a discipline that can be approached from two very different angles, that of the laboratory–based molecular biologist, and of the field–based ecologist.

This paper outlines another, more specific angle to molecular ecology; namely, how can it be used to best advantage in the highly 'applied' field of weed science? In the first section, some of the molecular techniques most applicable to ecological studies are outlined. More detailed reviews are given by Hoelzel (1992), Finch (1994) and Avise (1994). In the second section, the previous uses and potential future target areas for these techniques within the field of weed science are discussed.

TECHNIQUES CURRENTLY AVAILABLE TO MOLECULAR ECOLOGISTS

Population genetics studies undertaken within the last thirty years using allozymes as markers should now be classified as 'molecular ecology'. In addition, within the last decade, it has become feasible to look at the nucleic acid composition of organisms directly. This trend has been facilitated by the development of two types of technique. Firstly, those based on DNA restriction, such as RFLP (Restriction Fragment Length Polymorphism) analysis, and secondly those based on PCR. This section discusses the functions, rationale and specific applications of allozyme electrophoresis, RFLP analysis and PCR.

Allozyme electrophoresis

The terms 'isozyme electrophoresis' and 'allozyme electrophoresis' are often used synonymously, but there is a subtle distinction between them. Isozymes are variants of an enzyme which arise from multiple loci, whereas allozymes are allelic variants occurring at a single locus. The latter are thus more appropriate for population dynamics studies. The aim of allozyme electrophoresis is to visualise allozyme variants. If differences in the nucleic acid

code lead to changes to the amino acid composition of enzyme subunits, then there will be a change in net molecular charge. This will alter the electrophoretic mobility of allozymes, thus revealing underlying allelic variation.

Restriction Fragment Length Polymorphisms (RFLPs)

Restriction endonucleases are enzymes, purified from bacteria, which have the capacity to cut or 'restrict' DNA at specific recognition or 'restriction' sites. Restriction endonucleases can be exploited in molecular ecology studies through the technique of RFLP analysis. The aim of this process is to visualise and compare restriction patterns. Differences between the DNA code of individuals may be revealed in two ways: (i) in the numbers of restriction sites present (created or lost through insertion/ deletion/inversion/substitution/duplication of bases in the DNA), which will be revealed by the number of bands on a gel, and (ii) in the distance between restriction sites (fragment length will vary due to insertion/deletion/duplication events), revealed by the differing mobilities of restriction fragments through the gel.

RFLPs of simple DNA molecules can be visualised by staining the fragments in the electrophoresis gel. Detection of RFLPs of more complex molecules usually requires the fragments to be denatured, transferred to a solid substrate, such as a nylon membrane, and hybridised to a radiolabelled DNA 'probe' which reveals homologous fragments on an autoradiograph.

Specific applications of RFLP analysis

(a) Variable Number of Tandem Repeat (VNTR) or 'minisatellite' loci

Many animals and plants contain tandem arrays of 15–60 nucleotide repeat units scattered throughout their chromosomes (Bruford *et al.*, 1992). The number of repeats between restriction sites is highly variable (e.g. Bachmann, 1994). These repetitive regions are known as "VNTRs" or "minisatellites".

A restriction enzyme is used to cleave the DNA outside the repeat arrays and the fragments are separated on a gel. A specific radioactive probe is used to locate the DNA fragments. If the sample is homozygous in its number of tandem repeats at a given locus, one band will show up on an autoradiograph. If it is heterozygous, 2 bands will be revealed (Avise, 1994). In a multilocus approach, identification of specific alleles is not easy, reducing its potential in molecular ecology to identification of individuals and parentage assessments. An alternative is to fingerprint single loci, and then combine information from several single locus analyses. However, for many plant ecology applications, difficulty and expense of designing specific primers may be too great (Bachmann, 1994).

(b) Simple Sequence Repeat (SSR) or 'microsatellite' loci

These are similar to minisatellites but the repetitive units are much shorter; typically 2–4 bp in tandem arrays. These segments are more abundant than minisatellites in vertebrates, so look promising as markers for population studies (Bruford *et al.*, 1992).

Polymerase Chain Reaction (PCR)

The polymerase chain reaction is a process which repeatedly copies a specific target section of DNA fragment present in a mixed background of nucleic acids. Two primers flank the target DNA sequence. By repeated thermal cycling (heating and cooling) in the presence of excess free nucleotides and a heat-stable polymerase enzyme, the target sequence is amplified exponentially by the repeated production of DNA, using each strand of the target DNA as a template, between the primer sites.

Specific applications of PCR analysis

(a) Arbitrarily Primed PCR (AP–PCR) / Random Amplified Polymorphic DNA (RAPD) analyses

These assays require no prior knowledge of nucleotide sequence for primer design. The RAPD reaction (Williams *et al.*, 1990) uses short oligonucleotide primers, typically 10 bases in length, whereas AP–PCR may use longer ones (Welsh *et al.*, 1991). Typically only one primer is used per reaction, thus one end of each amplified section will have the inverse 10 base sequence from the other end. PCR products may be visualised after gel electrophoresis by ethidium bromide staining, and banding patterns of different samples compared. Dyer (1991) predicted that RAPD analysis would be useful for characterisation of weedy populations. Visualisation of differences between PCR products may be enhanced by the use of TGGE or DGGE (Tempature/Denaturing Gradient Gel Electrophoresis; Lessa & Applebaum, 1993). DGGE of RAPD products has been used in pedigree assessment of crops (He *et al.*, 1992; Dweikat *et al..*, 1993). Note that the term DNA amplification fingerprinting (DAF; Caetano-Anollés *et al.*, 1991) is sometimes used to describe a technique that is essentially the same as the RAPD approach but uses shorter primers, and DGGE and silver staining of the amplification products to enhance the detection of polymorphisms.

RAPD analysis has probably been most widely tested as an alternative to allozyme electrophoresis for population genetics studies, due to its speed, lack of technical difficulty, and ability to use tiny specimen samples. However, its reproducibility has been questioned.

(b) DAMD (Directed Amplification of minisatellite DNA and SSR-PCR

DAMD (Heath *et al.*, 1993) employs a similar approach to the RAPD technique but the primer sequences are based on known VNTR (minisatellite) loci. SSR-PCR utilses primer sequences based on SSR (microsatellites) loci (Zietkiewicz *et al.*, 1994). Mitchelson *et al.* (1995) have illustrated the use of these assays.

Combined approaches

Since the invention of PCR, many studies have combined PCR and RFLP analysis techniques. For example, a DNA sequence may be amplified by PCR prior to digestion with restriction enzymes.

Amplified Fragment Length Polymorphisms (AFLP) analysis

This technique was developed by Keygene Co. (The Netherlands). No prior knowledge of nucleotide sequence is needed. As with RFLP analysis, the aim is to visualise restriction patterns. However, after the DNA is restricted, it is amplified by PCR (using radiolabelled nucleotides) following the attachment of synthetic adaptors to fragment ends. Fragments are separated using a sequencing gel and visualised by autoradiography. The products are mostly dominant, caused by mutations in restriction sites. Due to this fact, and because it is a time–consuming and technically complicated technique, it may be unsuitable for population genetics screening (Morgante, 1994).

Target DNA

Nuclear DNA (nDNA)

Specific areas of the nuclear DNA commonly targeted for analysis are repeat sequences (discussed above), single–copy nuclear DNA (scnDNA) and genes encoding ribosomal RNA (rDNA). Single–copy nuclear DNAs are those sequences that occur just once in a haploid genome. Restriction analysis of such sequences gives data akin to that from allozyme

electrophoresis, although with the potential to identify many more genetic variants. However, the time and expense involved in scnRFLP analysis may have prevented it from becoming popular in this field (Avise, 1994). Specific primers have been designed to detect rDNA variation. The sequences coding for the ribosomal subunits themselves are highly conserved, but the 'internal transcribed spacer' regions (ITS1 & ITS2) are variable. Although DNAs coding for ribosomes are found in mtDNA and cpDNA (see below) as well as nDNA, the sequences are not similar enough for the specific primers to match those from mitochondria or chloroplasts (Bachmann, 1994).

(b) Cytoplasmic DNA

Mitochondria are usually maternally inherited, and contain DNA that is independent from the main nuclear genome. Palmer & Herbon (1988) found that mitocondrial DNA (mtDNA) is relatively invariant in primary sequence in *Brassica* species, but undergoes much rearrangement, predominantly due to inversions. Chloroplasts, like mitochondria, contain independent DNA. Chloroplastic DNA (cpDNA) is typically maternally inherited in angiosperms, although it is inherited biparentally in about 14% of flowering plant genera (Corriveau & Coleman, 1988). Sequence comparisons have shown that the chloroplast genome changes slowly, though rates of evolution of genes are variable (Palmer, 1987). Development of universal primers to amplify polymorphic non–coding regions of plastid DNA (Demesure *et al.*, 1995) will undoubtedly make the study of cpDNA more accessible to molecular ecologists.

USE OF MOLECULAR ECOLOGY IN WEED SCIENCE

Weed science encompasses a wide range of disciplines and the ultimate aim of these is to enable land managers to be able to maintain weed populations below threshold levels. Some of the weed science disciplines can benefit from molecular biology techniques (Table 1). Molecular *ecology* will be most applicable in those areas such as population dynamics (reproductive biology, dispersal, diversity), herbicide resistance, GMOs and environmental impact. Whilst several problems in weed science have been examined using allozyme electrophoresis, relatively few have been tackled so far with the more novel nucleic acid technologies.

Table 1. Broad categorisation of weed science disciplines into predominantly chemical/ biochemical or ecological research areas

Weed Science discipline		Ecology	Chemistry / Biochemistry
Competition / yield loss		√	
Genetically modified organisms (GMOs)*		√	√
Herbicide formulation* and chemistry			√
Herbicide trials (crop and weed impacts)			√
Herbicide resistance*		√	√
Pesticide residues / environmantal implications*		√	√
Physiology of response to herbicides*			√
Population dynamics -	dispersal*	√	
	diversity*	√	
	reproductive biology*	√	

* denotes areas that could benefit from a molecular biology approach

Origin of infestations, plant dispersal, and reproductive biology

Whenever weed control measures become insufficient, it becomes essential to know how a population is maintaining or increasing its numbers. This requires a knowledge of the proportion of successfully established plants derived from seeds, versus those that have regenerated vegetatively. It is also useful to know whether establishing propagules have been produced at the site of the infestation, or have been introduced from another site.

The method of establishing the origin of an infestation is dependent upon the breeding system. For annual outcrossing species, genetic diversity of potential source populations and field infestations can be compared using allozymes. This approach was used by Theaker *et al.* (1995) to ascertain whether hedgerow populations of *Bromus sterilis* could give rise to field infestations. These workers could not distinguish hedgerow and weed populations from the same field, but could separate populations from different fields. This suggested that the more stable hedgerow populations of *B. sterilis*, could act as reservoirs for re–infestation of fields. Rieseberg *et al.* (1988) examined cpDNA, rDNA and allozyme variation to test the hypothesis that the weedy race of *Helianthus bolanderi* had originated by introgression of *H. annuus* genes into a serpentine race of *H. bolanderi*. Their results opposed this hypothesis.

For perennial species with clonal growth strategies, possible sources of an infestation can be identified by characterising individuals and looking at their spatial distribution. Sheffield *et al.* (1989) used allozyme electrophoresis to look at the spread of bracken. They found genet size varied between <30 m diameter to >390 m., with larger clones probably being around 1000 years-old. The mixture of different size classes of the individuals identified also provided evidence for multiple sporeling establishment.

Characterisation of individuals is one area to which the new DNA technology is well suited. Looking directly at the genetic code eliminates problems caused by effects of developmental stage and environment on gene expression (which may affect allozymes). Ryan (1995) used both RAPD and allozyme analyses to identify the source of hydrilla discovered in a freshwater lake in California. Isozymes suggested the population was of a monoecious rather than a dioecious type, and RAPDs showed that it differed from another monoecious plant tested. Okoli *et al.* (1995) found no variation in RAPD patterns between samples of purple nutsedge (*Cyperus rotundus*) from different geographic locations. They suggested that purple nutsedge plants may be a large global clone. Miller *et al.* (1995) used RAPD analysis on plants from a field infestation of onion couch (*Arrhenatherum elatius* ssp. *bulbosum*) and found most clones to be fairly localised. They suggested that more widely dispersed clones may have been transported by cultivation machinery.

It is possible to show conclusively that two individuals are different, but one can only obtain a probability that two individuals have the same genotype. Miller *et al.* (1995) identified 33 RAPD phenotypes amongst 65 ramets of *A. elatius* ssp. *bulbosum*, showing clearly that the sexual phase of onion couch is important in its perennation. Okoli *et al.* (1995) identified a difference in mode of reproduction between yellow nutsedge (*Cyperus esculentus*) and purple nutsedge (*C. rotundus*) using RAPD analysis. As mentioned previously, purple nutsedge plants appeared to be derived asexually, but yellow nutsedge was found to be extremely variable, suggesting an effective sexual reproductive strategy.

Pollen and seed dispersal

In order to fully understand the dynamics of populations, the pattern of gene flow should be identified. Parts of the genome are solely maternally inherited in many angiosperms, such as cpDNA and mt DNA, whereas the nuclear DNA is biparentally inherited. This means that there are two means of gene flow in plant populations (Ennos, 1994). The first is via pollen fertilising an ovule in a different population. The second is by seed dispersal and establishment. There may be no correlation between the amount of cpDNA variation and

morphological or allozyme variation, but this may provide a new insight into evolutionary relationships (Soltis *et al.*, 1992). Models on pollen and/or seed migration have been published by Asmussen & Schnabel (1991), Adams *et al.* (1992) and Ennos (1994). McCauley (1994) estimated the contribution of seeds and pollen to gene flow in white campion (*Silene alba*) by using a PCR–RFLP technique to examine cpDNA. He compared this with seven polymorphic allozyme loci, and concluded that both seeds and pollen were significant contributors to gene flow in *S. alba*.

Diversity

Genetic diversity is a property of species that greatly affects their response to different environmental conditions. It is particularly important to be able to assess diversity in weedy species, both in general terms, and in relation to specific traits, in order to develop effective control measures.

Breeding system tends to influence diversity, with predominantly selfing weeds having less intrapopulation diversity but more differentiated populations, and outcrossing species having less interpopulation differences with most of the species variation occurring in each population (e.g. Warwick 1991b). However, it is not always straightforward. Navas & Gasquez (1991) reported that "genetic diversity of clonal species is not clearly related to their dependence on sexual vs. asexual reproduction". However, they hypothesised that major weeds are associated with low levels of genetic variation due to the great selection pressures found in highly productive agricultural environments, but that this pressure may not be as strong for ruderals and minor weeds.

King & Schaal (1990) used RFLP of rDNA, cpDNA, and the alcohol dehydrogenase genes *Adh1* and *Adh2*, to demonstrate the occurrence of nonmeiotic recombination in the asexual (agamospermic) species *Taraxacum officinale*.

It must be realised that genetically variable weed populations do not always represent a problem (Warwick 1991b). For example, in the case of herbicide resistance, the presence of susceptible plants in the population will dilute the resistance gene(s), so yield losses might be reduced by strategically managing the evolution of weed populations (Darmency & Aujas 1992).

Herbicide resistance

Resistance to herbicides is an area of weed science that is becoming increasingly important. Since the first case of triazine resistance was reported 25 years ago (Ryan, 1970), herbicide resistance has been discovered in numerous plant species across the world. The issue is further complicated by the variety of mechanisms by which herbicide resistance is brought about.

Several studies have looked at allozyme variation between resistant and susceptible weed populations, mostly for triazine–resistance (e.g. Darmency & Gasquez, 1983; Warwick, 1991a). Warwick & Black (1993) found that the distribution of inter– and intrapopulation genetic variation for *Brassica rapa* ssp. *sylvestris* agreed with predictions for predominantly allogamous species. Although levels of alleleic diversity and heterozygosity were lower in resistant than in susceptible populations, they were less different than for previously documented autogamous species. Chauvel & Gasquez (1994) compared resistant and susceptible populations of *Alopecurus myosuroides* with four isozyme systems, but could not find differences relating to herbicide tolerance.

Relatively little work has been done on identifying DNA markers for herbicide resistance in weeds (Marshall & Finch, 1995) although Lopez–Martinez *et al.* (1995) used the RAPD technique to look at intraspecific variation in *Echinochloa crus–galli* with respect to quinclorac resistance. Another study revealed that chlorsulfuron resistance in *Lactuca serriola* (prickly

lettuce) and *Kochia scoparia* (Kochia) may in some instances be caused by a point mutation in a particular region of the ALS genes (Guttieri *et al.*, 1992). A result like this could potentially lead to a quick diagnostic test for this kind of resistance.

Genetically modified organisms (GMOs)

There is a clear concern about the safety of genetically modified organisms, particularly with respect to the flow of modified genes into weed populations. The danger is that introduced characters such as herbicide resistance might create "super weeds", which would be extremely difficult to eradicate. By using molecular techniques, hybridisation between crops and weeds can be assessed.

Lefol *et al.* (1991) reported the results of allozyme electrophoresis on an artificial hybrid between transgenic *Brassica napus* and *B. adpressa* (not known to hybridise naturally). The hybrid isozyme patterns were a combination of the parental ones, and occasionally showed a supposed new heterodimeric band. Colosi & Schaal (1994) used RAPD analysis to detect hybridisation between weed and crop biotypes of proso millet (*Panicum miliaceum*). Gene flow has also been studied in wild and cultivated *Setaria* spp. (Till–Bottraud *et al.*, 1992). They looked at RFLP of cpDNA of *S. italica* and *S. viridis*, to see if one species would be the natural female parent in a cross, but found that either species could be maternal in such a hybridisation event.

WHERE ARE WE NOW?

There are clearly many areas in the field of weed science that can benefit from a molecular ecology approach. Molecular ecology has evolved through a convergent evolution of ideas from molecular biologists and ecologists. The selection pressure steering this evolution is a quest for understanding the detail behind complex ecological processes. As shown above, some researchers have already applied the philosophy of molecular ecology to their studies, but there is still a long way for us to go. Detailed knowledge of the ecology of most weed species is limited and it is imperative that we try to understand the biology of this diverse group of plants if we are to achieve any form of integrated weed management.

Molecular ecology enables us to take a retrospective look at events that have occurred in the history of populations. The data produced should give us an insight into inter–plant relationships and the evolution of populations. This in turn may facilitate the implementation of weed management strategies which take account of parameters such as mode of reproduction and gene flow. This must surely be a positive development.

One of the goals of molecular ecology is to be able to achieve a synergy between ecologists and molecular biologists, but this is something that will, at least initially, develop slowly, as experience in the field progresses. Such development will be considerably helped by weed scientists using existing molecular biology techniques to solve ecological problems. From the range of topics covered above, it is clear that there is no shortage of questions that can be tackled using the techniques available through the science of molecular ecology. The problem is in knowing where to begin!

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EFFECT OF TRANSGENIC RELEASE ON WEED BIODIVERSITY: OILSEED RAPE AND WILD RADISH

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ABSTRACT

The release of herbicide resistant crops and other transgenic cultivars may lead to increased gene flow between crops and related weeds. As a consequence, weed populations may exhibit new advantageous or detrimental characteristics that may make them more, or less, troublesome in farmer's fields. Shifts in ecological balance outside the fields may also be expected. An example is given that concerns interspecific hybridisation between oilseed rape and wild radish, survival of hybrids and progeny, and introgression of oilseed rape traits into wild radish.

INTRODUCTION

New plant biotechnologies offer the possibility to transfer new traits of agronomic importance into crop species. These include resistance to diseases, pests and herbicides, male sterility, modification of fatty acid and protein composition and production of pharmaceuticals (Dale *et al.*, 1993). The need for weed control makes herbicide resistance desirable in crops (Gressel, 1993).

Crop cultivars with resistance to Bromoxynil, 2-4,D, Glufosinate, Glyphosate, Imidazolinone, Sulfonylurea and other herbicides are now available. Some have official agreement for commercial release in EC and Canada. Recent advances in this area are discussed in a forthcoming book (Duke, 1995). There is, however, one area for which little is known because of a lack of fundamental knowledge on plant biology; namely, gene flow from crop to weeds and its consequences on weed populations. This issue has seldom been seriously considered although gene flow within populations is now a well documented phenomenon (Levin & Kerster, 1974; Darmency, 1995a).

Gene flow between crops and weeds, or 'introgressive hybridisation', is sometimes difficult to demonstrate as morphology varies as a continuum within a gene pool, and intermediate forms may originate both from interspecific crosses and species variability. Molecular markers, which are now generally available, are necessary to investigate further and validate the suggestions of botanists. Several cases of introgression have been confirmed occurring both in allogamous and autogamous groups of species. Descendants may have become established successfully as weeds (Darmency, 1995b) or, in contrast, have expressed non-adaptive traits and contributed to detrimental changes within populations of the wild parent (Darmency, 1995a).

There are several crop species potentially affected by introgression in Europe (Raybould & Gray, 1993), but this number would probably increase with the use of transgenic crops. Firstly, the fact that few cases of introgression have been documented is perhaps due to the lack of field and population surveys dedicated to identifying introgression, and a lack of genetic markers which can be used to detect it. Transgenic crops will provide unambiguous genetic markers and material for numerous environmental safety studies. Secondly, failure in producing interspecific hybrids that are fit enough to survive in fields will be overcome

because of the high adaptive value of the new traits conferred to the crop, especially herbicide resistance, which was not the case in the past. Of course, this is not a special feature of transgenic cultivars and may also occur with cultivars derived from classical breeding, but genetic engineering deals with genes of major phenotypic effect that are generally not found in the botanical family of the crop. Most of the genes used for herbicide resistance in crops originated in micro-organisms, which means that a higher plant would never have acquired them even through mutation. Thirdly, where gene exchanges between crops and weeds provide weed populations with new adaptive traits, selection for weedy individuals with an enhanced ability to interbreed with related crops (i.e. fewer genetic barriers for interspecific crossing) will occur, thus increasing the potential of present weed populations for introgression.

An interesting case is the potential for gene escape from transgenic oilseed rape (*Brassica napus*). Various herbicide resistant cultivars have been released, as well as others with male sterility and modified proteins. Oilseed rape is a major crop grown on large areas, producing millions of flowers, each having 60,000 pollen grains. Naturally occurring hybrids were reported in the British Isles with *B. rapa* (Stace, 1975) and recent studies indicate that introgression in that species could occur (Jorgensen & Andersen, 1994). Crosses with other wild Brassiceae are also possible as indicated by hand crossing experiments and culturing plants *in vitro* (Kerlan *et al.*, 1992). Spontaneous hybridisation using a male sterile oilseed rape cultivar was shown to be frequent with hoary mustard (*Hirschfeldia incana*) and wild radish (*Raphanus raphanistrum*) (Eber et al., 1994), although very rare with wild mustard (*Sinapis arvensis*) (Lefol et al., 1995a). Spontaneous hybridisation of hoary mustard as the female was shown to occur in an oilseed rape field at a rate equivalent to 800 hybrids per ha (Lefol *et al.*, 1995b). Here, we report and discuss some data on interspecific hybridisation between oilseed rape and wild radish, the reproduction of hybrids and some characteristics inherited in the progeny.

MATERIALS AND METHODS

Spontaneous crosses were investigated in experimental designs approved by the French Commission of Biomolecular Engineering (Darmency & Renard, 1992). All plants used for this purpose were germinated in a growth cabinet at 10 to 15°C (wild radish seeds were dehulled) and transplanted at the rosette stage on dates as simultaneous flowering warranted.

(1) CMS Oilseed rape cv. Brutor (material provided by INRA, Rennes) was grown in the presence of wild radish in cages (2x2 mm mesh containing a bee-hive) at a 18:18 ratio in 1990 (3 cages). Seeds were collected from the male sterile cv., then sown in the greenhouse and analysed for leaf isozymes (esterases and acid phosphatases as described in Gasquez & Compoint, 1981) to confirm their hybrid status. Chromosome number was estimated by flow cytometry, following the procedure described by Akinerdem (1991), with a Partec CAII instrument (Chemunex, France) and using pea as an internal control.

(2) Wild radishes were planted at a density of 1 plant per 12 m^2 in a 200 m^2 plot of chlorsulfuron resistant Brutor (provided by INRA, Rennes) near the laboratory in 1994 (50 plants m^{-2}, inter-row: 0.33 m). The resistant cv. was cut off after flowering. Seeds were collected from the wild radish only, then dehulled and sown in trays filled with vermiculite in a regulated greenhouse (16-h day at 25°C, night below 20°C). Seedlings (3-4 leaves) were sprayed with 2.5 g a.i. ha^{-1} chlorsulfuron (Glean®). Surviving seedlings were analysed as in experiment 1 (above).

(3) Cuttings of artificial hybrids (partly provided by INRA, Rennes) rescued *in vitro* after crosses between a Canadian spring oilseed rape cv. Westar containing the *bar* gene conferring resistance to the herbicide glufosinate (material provided by Plant Genetic Systems, Gent), and wild radish, were grown in cages with the wild parent at a 10:62 ratio in 1990 (1 cage), and at a 5:19 ratio in 1991 (3 cages).

(4) Seeds collected from hybrids in experiment 3 (above) were germinated in Petri-dishes, transferred to Giffy 7 pots in the greenhouse, planted in cages in an experimental garden with the wild parent at a 4:30 ratio in 1992, and observed for reproduction. Resistance to the herbicide Basta® was checked in each plant by the deposit of a 40 µl drop of 0.5 % of Basta® onto a leaf.

(5) Hybrids obtained using the male sterile rape cv. in experiment 1 were grown in the experimental garden together with wild radish plants in 1992. Seeds collected from the hybrids were grown in the experimental garden in 1993, and observed for leaf morphology, flower colour and shape, oidium attack and sterility.

RESULTS

Seeds from the male sterile oilseed rape plants of one cage of experiment 1 were assayed for hybridity. Most of the seeds had a diameter less than 1.2 mm. In comparison, seed diameter is around 2 mm for both oilseed rape and wild radish. The germination rate was only 38%, indicating a high frequency of aborted seeds. Of the viable seeds, 81% were shown to be hybrids with wild radish (Table 1). The remaining seeds were hybrids with oilseed rape plants growing in other cages in the experimental garden. Indeed, pollen may travel across the net of the cage, exit from one cage and enter another as already stressed in safety studies (Darmency & Renard, 1992). As each male sterile rape plant produced around 400 flowers in the cages, the rate of hybrid production may be estimated as 0.1 per flower.

Table 1. Production of interspecific hybrids between oilseed rape and wild radish, and reproduction of hybrids and progeny in experiments described in the text.

No. of plants	Ratio female/male	No. of seeds collected	No. of seeds germinated	Total no. of hybrids	No. of hybrids per plant
Experiment (1) - Male sterile oilseed rape					
18	1:1	2600	988	806	45
Experiment (2) - Wild radish					
15	1:625	1421	956	2	0.1
Experiment (3) - Hybrids					
10	1:6	0	0	0	0
15	1:4	10	7	4	0.3
Experiment (4) - BC1					
4	1:8	70	50	50	12.5

The reciprocal cross carried out in experiment 2 resulted in very low seed production per wild radish plant (Table 1). In fact, only one wild plant of the 15 tested produced the two hybrids obtained, i.e. from 0.2% of the seeds collected. These two seedlings survived the chlorsulfuron treatment and were free of symptoms although they grew slowly in comparison to the resistant oilseed rape and unsprayed wild radish.

Hybrids were first identified with the isozyme markers. Slow migrating esterases and fast migrating acid phosphatases provided specific markers for each parent species. Other markers could be used but were variable within the population of the wild radish used for the crosses. DNA content, as estimated by flow cytometry, was 1.14 pg for wild radish (2n=18), 1.87 pg for the hybrids, and 2.58 pg oilseed rape (2n=38). This indicated that the hybrids all had half

the sum of the DNA content of the two parent species, which corresponds to 2n=28. Young hybrid plants exhibited leaf shape and hairiness intermediate between those of their parents. However, as these traits are variable within and among populations of wild radish, it would be hard to distinguish hybrids from wild radish on these bases in the field. With vegetative growth and at flowering, the leaves increasingly resemble those of oilseed rape (colour, hairiness, structure). The hybrids grew as big as wild radish plants and all flowered. Hybrids had either yellow or white flowers due to the variability at the locus encoding the flower colour within the wild radish population used for the experiments (white being dominant over yellow). Anthers were small and had very few pollen grains.

Cuttings of artificial hybrids rescued *in vitro* and used in experiment 3 were all triploids (2n=28) and were resistant to Basta® – just a necrotic disk developed one week after the deposit of the drop of herbicide solution on the leaf, whereas the whole leaf became necrotic in the case of susceptible plants. They produced very few seeds, of which only four germinated (Table 1). This corresponds to 0.16 descendants per plant. By comparison, one wild radish plant in the same cage produced nearly 2200 seeds.

The four BC1 seedlings from experiment 3 were susceptible to Basta®. They grew well but three of them were nearly sterile. Most of the BC2 descendants were collected from one plant only, resulting in an average production of 12.5 seeds per plant (Table 1).

Since experiment 5 consisted of growing BC1 collected from hybrids obtained from crosses between a male sterile oilseed rape and wild radish, and because we cannot be sure the cytoplasmic sterility system is overcome in hybrids, we do not present any results on seed yield. A total of 52 BC1 were produced in the experimental garden. Leaf shape was very variable, but close to that of the wild radish, and three plants had no fully expanded leaves. Flowers had colour ranging from the intense yellow of oilseed rape to pure white. A natural fungal attack allowed a distinction of the two groups of plants; one with white powdered leaves = oilseed rape (21 plants), the other with leaves free of oidium, like the wild radish grown as the control (31 plants). Pod shape was intermediate between those of the two parents or typically like wild radish, i.e. constricted in several segments. A majority of plants were sterile (33). Some plants (9) had leaves and pods similar to wild radish, were fertile, but had white powdered leaves, indicating a combination of the characteristics of both parents .

DISCUSSION

The rate of hybrid production with the male sterile oilseed rape (0.1 F_1 per flower), corresponds to findings in other experiments carried out using the same 1:1 species ratio in open fields, but at higher plant density (Eber *et al.*, 1994). In contrast, the hybrid yield per unit area (90 F_1 m^{-2}) appears to be very low in comparison to other studies in the open field that showed 3700 F_1 m^{-2} (Eber *et al.*, 1994), and even 5100 F_1 m^{-2} (Darmency *et al.*, 1995). This last value is equivalent to the release of 5.6 million hybrids per ha! This illustrates the ease of gene flow in seed production areas for hybrid varieties and in areas grown with composite female+male varieties. In those fields grown partly with male sterile oilseed rape, the fact that pollen from fertile rape is limited would provide the opportunity for pollen of wild radish to fertilise some flowers of oilseed rape. The presence of a few wild radish plants would be enough to release some interspecific hybrids which could shed seeds at harvest and remain in the field. In addition, in the case of certificated seed production, the seed dealer might export hybrids to other fields. Consequently, there may be a need to revise the regulations for seed production.

The reciprocal cross also appears to be possible in field conditions. This is the first report of such a natural event. Wild radish is a self-incompatible species (Sampson, 1964). Therefore, plants isolated within an oilseed rape field receive very few pollen from other wild radish plants and behave as male sterile plants, which provides an opportunity to cross-fertilise with

oilseed rape. The fewer the wild radish plants present, the higher the probability to produce hybrids! It is difficult to destroy all wild radish plants in a field grown with oilseed rape. Isoxaben is the only suitably selective herbicide that has been used recently to control wild radish in oilseed rape. Herbicides used with transgenic herbicide resistant varieties will obviously be selective also. However, nobody can be sure of killing 100% of the wild radish population, including those in the immediate vicinity of the field – road borders, waste ground, and fields of other farmers growing other crops.

The interspecific hybrids grew as well as normal wild radish but their reproduction was very low (0.16 seeds per plant). This is of similar magnitude as the results reported by Eber *et al.* (1994). Embryo formation probably occurred through back-crossing with wild radish. The 2n=28 nature of the hybrids means they have far from normal meiosis. However, in a study of more than 400 hybrids, 1% were shown to be amphidiploids, 2n=56 (Chèvre *et al.*, 1995). These amphidiploids had better chromosome pairing and produced both fertile ovules and pollen, which could result in a high risk class of fertile hybrids. The chromosome number has not been checked for the progeny of hybrids described in our work, but a study of 15 BC1 (Chèvre *et al.*, 1995) showed that it evolved towards high values instead of reverting back to the diploid level of the wild radish. This does not preclude further introgression with the wild radish, but rather shows that various cytogenetic routes may result in viable plants.

The relative fitness of hybrids compared to wild radish, in term of viable seeds produced, was nearly 0.01%. This gives hybrids little chance of producing progeny that can become successfully established in habitats where competition for space and nutrients is high. Recombination of characteristics of both parents occurred among hybrid progeny. Some oilseed rape traits disappeared (e.g. glufosinate resistance), whereas others remained in the descendants that resembled wild radish (e.g. fungal susceptibility). Thus, detrimental traits were transferred, not the advantageous herbicide resistance. This leads us to suggest that some chromosomal locations are safer than others in preventing the transfer of foreign genes through recombination. It is worth investigating this question, especially when one may observe that the progeny of the first generation produces 80 times more seeds than hybrids. The relative fitness of hybrid descendants was 0.6 %, and one may expect that further backcrossed progeny will exhibit increasingly greater fitness, thus becoming close to normal wild radish. At that point of the introgression, a wild radish population may display herbicide resistance. Examples of field infestations by herbicide resistant weeds are now well known and illustrate what may occur in the future (Darmency, 1994).

The results reported above show that spontaneous interspecific hybridisations between oilseed rape and wild radish are possible. Hybrids produce progeny which may exhibit the characters of both parents. The consequences of this phenomenon are still not clear, but it would be wise to undertake careful studies in this area before planting large areas of herbicide resistant crops. Further investigations have been carried out at our laboratory, but they often deal with large amounts of seeds which are not yet completely assayed. Experiments using fields of normal agricultural size (at least 1 ha) are planned in collaboration with farmer's associations, but their interpretation will be time consuming.

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GENETIC DIVERSITY IN SUSCEPTIBLE AND HERBICIDE RESISTANT *SINAPIS ARVENSIS*

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ABSTRACT

The characterisation of herbicide resistance in plants has traditionally been carried out using *in vivo* and *in vitro* assays. The advent of molecular based techniques makes study at the molecular level possible. A development of the polymerase chain reaction, random amplified polymorphic DNA (RAPD) analysis, was assessed for its utility in differentiating between *Sinapis arvensis* biotypes resistant to either dicamba or chlorsulfuron and a susceptible biotype.

INTRODUCTION

Molecular-based techniques are becoming widely used in the study of plant genetic diversity (e.g. Tinker *et al.*, 1993; Yang & Quiros 1993; Chalmers *et al.*, 1992; Vierling & Nguyen, 1992) and as tools to investigate and characterise plant resistance to pathogens (e.g. Haley *et al.*, 1993; Klein - Lankhorst *et al.*, 1991) complementing traditional physiological and biochemical studies. Such information, whilst facilitating the selection and production of improved crop lines, could also provide valuable diagnostic information on the potential occurrence of herbicide resistance amongst weed species. Traditionally, determination of herbicide resistance in weeds is made using *in vivo* or *in vitro* assays.

Restriction fragment length polymorphism (RFLP) analysis is widely used for the study of genetic linkage and genetic diversity. It provides large amounts of information for use in the production of genetic maps and hence cloning and sequencing of genes, and in the identification of molecular markers linked to traits of interest, such as disease resistance (Paran & Michelmore, 1993). Changes in RFLP patterns in a herbicide resistant biotype, caused by the creation or loss of a restriction enzyme recognition site/s due to mutation, can also provide valuable diagnostic information and this approach has already been applied to weed species (Guttieri *et al*, 1992; deCastro & Youmans, 1990).

Disadvantages of RFLP analysis include a requirement for relatively large amounts of DNA and the laborious nature of the technique. Random amplified polymorphic DNA (RAPD) analysis (Welsh & McClelland, 1990; Williams *et al.*, 1990) is an alternative technique widely reported for its utility in the study of plant genetic diversity. It requires much smaller amounts of DNA than RFLP analysis and no prior knowledge of the genome under study. The RAPD technique, based on a modification of the polymerase chain reaction (PCR), provides genetic fingerprints based on amplification of DNA using short arbitrary primers. Consequently, it generally produces simpler fingerprint patterns than RFLP analysis containing less genetic information, being based only on those regions of the genome that are amplified. Moreover, unlike RFLPs, RAPD markers are usually dominant and therefore cannot resolve heterozygous genotypes. However, the simplicity and speed of the technique, and the lack of a requirement for radioisotopes, have made it a useful and widely used approach, particularly when large numbers of samples or species are to be screened. The use of RAPDs to study plant resistance to pathogens has been mainly concentrated on crop species and the identification of RAPD molecular markers related to genes conferring known resistance traits (Klein - Lankhorst *et al.*, 1991; Paran & Michelmore, 1993; Haley *et al.*, 1993). The use of RAPDs to characterise herbicide resistance in weed species has not previously been reported but has been used to examine genetic variation in herbicide resistant *Echinochloa* spp. (Lopez-Martinez *et al.*, 1995).

Wild mustard (*Sinapis arvensis*) is a self incompatible, outcrossing, common annual weed (Wall, 1995), indigenous throughout most of the temperate regions of the world (Mulligan & Bailey, 1975). It can be a serious problem in cultivated land, particularly in cereal crops, requiring chemical control measures to be implemented (Mulligan & Bailey, 1975). Canadian biotypes of *S. arvensis* have been found that are resistant to the auxinic herbicide dicamba (Heap & Morrison, 1992) and to the ALS inhibitor, chlorsulfuron (I. Morrison, pers. comm.)

ALS inhibitor resistance rapidly developed in weed species with initial reports of its occurrence appearing shortly after the introduction of such herbicides. Subsequently, mutation in a single dominant gene has been found to confer resistance in several plant species, including weeds (Devine *et al.*, 1993; Guttieri *et al.*, 1992, 1995)

Resistance to auxinic herbicides is not widespread, despite their extensive use. This has been attributed to the unsubstantiated hypothesis that such herbicides have multiple modes of action, requiring mutations at several genetic loci for the expression of resistance (Jasieniuk *et al.*, 1995). However, a recent report on the inheritance of dicamba resistance in wild mustard has found that resistance was determined by a single, completely dominant nuclear allele (Jasieniuk *et al.*, 1995).

The apparent dominant nature of resistance to chlorsulfuron and dicamba makes RAPD analysis a feasible technique to screen for markers linked to herbicide resistance in large populations. The objectives of this study were to assess the utility of RAPDs in identifying genetic variation in three Canadian biotypes of wild mustard: an ALS-inhibitor resistant line, an auxin resistant line and a susceptible line.

MATERIALS AND METHODS

Plant Material

Seeds of *Sinapis arvensis* plants resistant to either of the herbicides dicamba or chlorsulfuron or susceptible to both, were germinated and grown until the two-leaf stage in compartmentalised seed trays containing potting compost (Fisons M3) in a glasshouse. The first leaf was removed from each plant and stored at -20°C until subsequent DNA isolation. The day following first-leaf removal, seedlings were sprayed with either dicamba (100 g ha^{-1}) or chlorsulfuron (40 g ha^{-1}) at a rate of 170 l ha^{-1} using an automatic sprayer system (Teejet 8002 nozzle). Subsequent plant growth was recorded and used to confirm resistance or susceptiblity. Leaves previously collected from these authenticated plants were used for DNA extraction.

Plant DNA Extraction

DNA was extracted from frozen *S. arvensis* leaf material using an adaptation of the method of Peterson *et al.*, (1993). Each leaf sample (approximately 0.1 g) was washed in H_2O (Gibco BRL, molecular biology grade), ground to a fine powder in liquid nitrogen using a pre-cooled mortar and pestle, and incubated in 500µl DNA extraction buffer on ice for 10 min. After centrifugation (3000 rpm, 20 mins), the pellet was resuspended in 600µl nuclei lysis buffer and the mixture incubated at 65°C for 30 mins. After reducing the temperature to 35°C, 5µl RNase (10 $mgml^{-1}$) was added and the incubation was continued for 10 mins. An equal volume of chloroform-isoamyl alcohol was added and after thorough mixing and centrifugation (3000rpm, 5 mins), the aqueous layer was transferred to a fresh Eppendorf tube. The latter step was repeated and then 450µl ice-cold isopropanol was added to the resultant supernatant to precipitate the DNA which was pelleted (13000 rpm, 5 mins) and washed briefly in 70 % ethanol. After a further centrifugation (13000 rpm, 5 mins), the ethanol was decanted and the pellet allowed to dry thoroughly before resuspension in TE buffer by heating (65°C, 30 mins). Samples were stored at -20°C.

RAPD Reactions

Amplification was carried out in a total volume of 25 µl containing 1 ng template DNA, 0.5 Units DNA polymerase (Ampli*taq*, Perkin Elmer), 2 mM Magnesium chloride (Perkin Elmer), 2.5 µl 10X *taq* buffer (Perkin Elmer), 100 mM each dNTP (Perkin Elmer), 5 pmols primer (Operon Technologies) and sterile water. The reaction mix was overlaid with 2 drops of mineral oil and the following amplification protocol carried out in a Techne PHC-3 thermal cycler: initial incubation at 95°C for 5 mins followed by 45 cycles of 1 min at 95°C, 1 min at 35°C and 2 mins at 72°C, and then a final incubation at 72°C for 10 mins. Amplification products were resolved by gel electrophoresis in 2% (w/v) agarose gels (Seakem LE agarose), stained with ethidium bromide and visualised with UV light.

Analysis of RAPD Fragments

Bands from DNA profiles generated for each sample by each of 7 random primers, previously found to generate reproducible and clearly scorable polymorphic markers, were scored as either present or absent. These data were used to create a similarity matrix using Jaccard's similarity coefficient, and cluster analysis was performed using the resulting data. Principle co-ordinates analysis of the transformed data was also performed, using Genstat 5 v2.2, to provide a measure of genetic distance between samples.

RESULTS AND DISCUSSION

The data discussed here concern results obtained from screening three physiologically distinct groups of samples with seven random 10-mer primers to obtain an initial impression of the utility of RAPDs in diagnosing herbicide resistance in *S. arvensis*. The average number of polymorphic loci per primer was 25 within a range of 18 to 33 and the total number of loci examined was 178 (Table 1).

Table 1. List of primers (and their nucleotide sequence) used in RAPD analysis and the number of polymorphic loci obtained with each following amplification of each susceptible, dicamba resistant and chlorsulfuron resistant *S. arvensis* individual tested.

Primer	Primer sequence 5' - 3'	No. of polymorphic loci
OPR 02	CACAGCTGCC	29
OPR 03	ACACAGAGGG	33
OPR 08	CCCGTTGCCT	20
OPR 09	TGAGCACGAG	26
OPR 10	CCATTCCCCA	25
OPR 15	GGACAACGAG	27
OPR 16	CTCTGCGCGT	18
	Total	178
	Mean	25

Note that thorough air drying of the DNA samples (1 hour +) was found to be essential for reproducible amplification. An example of profiles obtained from a typical RAPD analysis of the DNA from *S. arvensis* individuals is shown in Figure 1. Primer OPR 02 was used to amplify *S. arvensis* DNA from susceptible and herbicide resistant individuals. Multiple banding patterns were obtained with OPR 02 and the other primers tested with the *S. arvensis* samples.

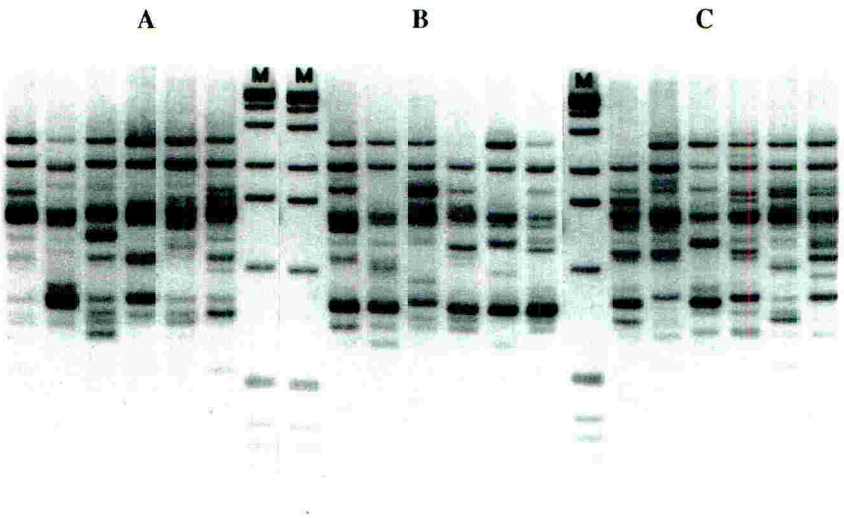

Figure 1. Agarose gel of RAPD amplified *S. arvensis* DNA using primer OPR 02. A - herbicide susceptible, B - dicamba resistant, C - chlorsulfuron resistant individuals. Lanes marked M = 1 kb size marker (Gibco BRL).

Results from the cluster analysis of RAPD data generated from amplification of all *S. arvensis* samples tested with each of the primers OPR 02, 03, 08, 09, 10, 15, & 16 are represented as a dendogram (Fig. 2). Although several groupings were observed, the 3 phenotypic groups of *S. arvensis* individuals tested were not clearly distinct. However, within the chlorsulfuron resistant individuals, two subgroups representing 9 of the 14 individuals in this group were observed. One group of 4 individuals (nos. 26, 27, 28 & 29) clustered at 45 % similarity and a second group of five individuals (nos. 19, 20, 21, 22, & 23) clustered at 35 % similarity. Both these groups were unrelated to each other until the 15 % level, when all individuals were related.

From the cluster analysis, all samples had 15% similarity, differences not appearing until the 20 % level, where 2 broad groups were observed. The smaller of these contained only one susceptible *S. arvensis* individual (no. 1), 4 of the dicamba resistant individuals (nos. 11, 12, 13 & 15) and the first group of chlorsulfuron individuals mentioned previously (nos. 26, 27, 28 & 29). The latter two of these groups clustered at the 30 % level. The second of the broad groups at the 20 % similarity level contained the remainder of the susceptible and other herbicide resistant individuals.

Principle co-ordinates analysis (data not shown) of the RAPD data did not differentiate any more groupings than those identified by cluster analysis but confirmed the presence of the two sub groups within the chlorsulfuron resistant samples.

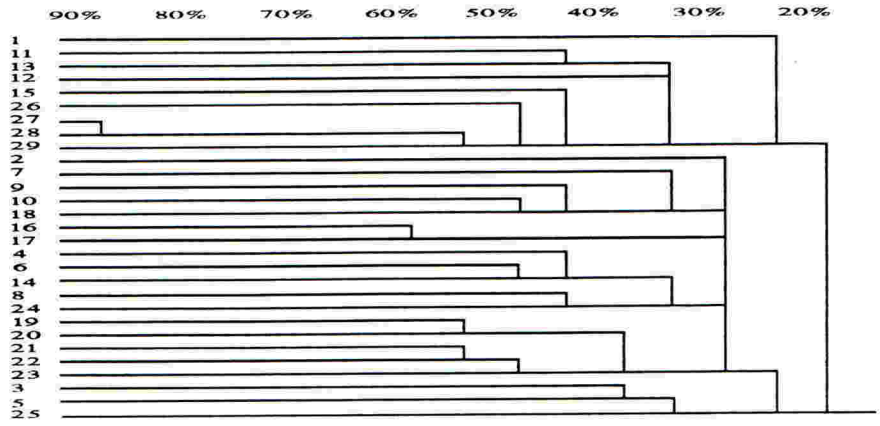

Figure 2. Dendogram showing cluster groups, based on percent similarity, between *S. arvensis* individuals based on cluster analysis of RAPD data generated from amplification of *S. arvensis* DNA by the primers listed in Table 1. Samples 1-7 - susceptible; samples 8 - 15 dicamba resistant; samples 16 - 29 chlorsulfuron resistant.

Resistance to chlorsulfuron has been attributed to a point mutation in a single (*als)* gene (e.g. Guttieri *et al,* 1992, 1995). Thus it is perhaps not surprising that we were unable to discriminate between chlorsulfuron resistant and susceptible plants using RAPD analysis, particularly bearing in mind the limited number of loci examined. The fact that the dicamba resistant plants were also indistinct from the susceptible plants suggests that dicamba resistance may also be attributed to a small, but significant, genetic change. This would concur with the conclusions of Jasieniuk *et al.* (1995) that dicamba resistance can be a single-gene trait. The lack of distinct groupings within and between the *S. arvensis* populations, collected from adjacent geographic areas within Manitoba, Canada, reveals a broad genetic diversity within this outcrossing weed species which, according to our data, exhibits only 15 % similarity amongst the individuals tested. Similar levels of genetic similarity have been detected in *S. arvensis* collected in the UK and screened using more than 60 RAPD primers (results not shown) and such underlying variation would tend to inhibit detection of minor genetic changes associated with herbicide resistance. However, we intend screening large numbers of additional RAPD primers, using pooled DNA samples, in order to detect polymorphism associated with dicamba resistance in *S. arvensis* with the aim of gaining an inroad to the identification of the target locus in this species. The *als* locus, associated with chlorsulfuron resistance, will be further examined using an RFLP-PCR approach (Guttieri *et al.*, 1992).

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A MOLECULAR ASSESSMENT OF GENETIC DIVERSITY IN *ECHINOCHLOA* SPP.

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ABSTRACT

Echinochloa spp. are weeds of maize and rice that cause serious yield losses and require to be controlled. In this study, RAPD (random amplified polymorphic DNA) analysis was used to assess interspecific variation in the *Echinochloa* genus, particularly in relation to the development of herbicide resistance. DNA was isolated from nine plants of five *Echinochloa* species (*E. crus-galli, E. oryzicola, E. orydoides, E. hispidula* and *E. colonum*) and a range of primers were screened for their suitability in generating reproducible DNA profiles. Interspecific variation was assessed using pooled DNA samples from each *Echinochloa* species and presence / absence data were recorded for 238 loci. Hierarchical cluster analyses of these data revealed that the five *Echinochloa* species formed only three discrete groups: (i) *E. crus-galli* + *E. hispidula*, (ii) *E. oryzoides* + *E. oryzicola* and (iii) *E. colonum*. Herbicide resistance was confined to group (i). This study demonstrates the utility of a genetic fingerprinting approach for confirming the identity of weed genotypes during studies of physiological adaptation and the potential for the development of molecular diagnostic assays for *Echinochloa* classification and quinclorac resistance.

INTRODUCTION

The genus *Echinochloa* spp. includes the most troublesome weeds in rice and maize fields. Classification and identification of *Echinochloa* spp. is notoriously difficult because morphological variation is often great within biotypes of one species. Establishing the identity of herbicide susceptible/wild biotypes of *Echinochloa* is essential when undertaking comparative mode of action studies with putative herbicide-resistant biotypes of the same species. Accordingly, we employed molecular techniques to make a genetic comparison of test biotypes of *Echinochloa*, free from the vaguaries of morphological variation and subjective assessment. In this study, RAPD (randomly amplified polymorphic DNA) analysis was used to assess interspecific variation in the *Echinochloa* genus, particularly in relation to the development of herbicide resistance.

MATERIALS AND METHODS

Plant material and growing conditions

Seeds of 28 biotypes of *Echinochloa* spp. were collected from rice fields subjected to the application of quinclorac. Four additional biotypes were collected from maize fields treated with atrazine over several years. The 32 biotypes were morphologically classified (Carretereo, 1981) as *E. colonum* (1), *E. crus-galli* (11), *E. hispidula* (10), *E. oryzoides* (7) and *E. oryzicola* (3) .

Seeds were placed in Petri-dishes containing filter paper moistened with 2 g l^{-1} KNO_3 solution and germinated under continous illumination of 350 µmol m^{-2} s^{-1} PPFD at 25°C and 80% relative humidity. Pre-germinated seeds were sown at five seeds per pot in a 1:2 peat:soil (sand/loam) mix. Plants were maintained in a growth chamber with a 16 h. photoperiod at 350 µmol m^{-2} s^{-1} PPFD. Day/night temperatures were 25/18°C and relative humidity was maintained at a constant 80%. Plants were watered as required.

Physiological assessment

To assess herbicide resistance, pots containing four biotypes, morphologically clasified as *E. crus-galli ('E. crus-galli')*, at the second leaf stage were treated with formulated herbicide: quinclorac (0.05 to 3.0 kg ha^{-1} a.i.), atrazine (0.1 to 10.0 kg ha^{-1} a.i.), propanil (0.2 to 2.0 kg ha^{-1} a.i.) and molinate (0.5 to 4.0 kg ha^{-1} a.i.). Plants were maintained for 21 days in the controlled-environment chamber under the conditions described above. After this time plants were harvested and growth was evaluated by determining shoot fresh weight. The herbicide dose that caused a 50% reduction in the shoot fresh weight (ED_{50}) was calculated for each herbicide as previously described (Menéndez *et al.*, 1994). Each treatment consisted of four plants with three replicates. The biotypes of the other *Echonochloa* species were treated with quinclorac (0.5 kg ha^{-1} a.i.) to assess their susceptibility/resistance to this herbicide.

Molecular assessment

DNA was isolated from nine plants of each bioytpe using a standard phenol:chloroform:isoamyl alcohol procedure, except centrifugation of ethanol-precipitated DNA was avoided prior to washing since co-precipitants were found to inhibit RAPD reactions. In each case the DNA samples were mixed in equal quantities to give pooled samples for analysis of intraspecific genetic variation.

Sixty primers were screened for their suitability in generating reproducible DNA profiles. An initial survey showed that 18 RAPD primers gave clear amplification products that could be readly scored. Presence / absence data were recorded for some 238 loci. For the statistical analysis of genetic variation, a similarity matrix was constructed from the presence/absence data using Jaccard's coefficient. Hierarchical cluster analysis was performed using a furthest-neighbour clustering algorithm. A principal coordinates analysis was conducted to provide a graphical summary of similarities between samples.

RESULTS

Physiological studies

The results of the activity studies with the '*E. crus-galli*' biotypes are presented in Table 1. These studies included the application of propanil and molinate in addition to quinclorac and atrazine since they provide alternative herbicides for the selective control of *Echinochloa* spp. in crops. The levels of resistance to quinclorac differed clearly between the test biotypes with ED_{50} ratios (ED_{50} resistant biotypes/ED_{50} susceptible biotypes) varying from 6 to 26 (Table 1). One biotype, designated as resistant (R), showed high resistance to atrazine (ED_{50} cross-resistant/ED_{50} susceptible values of 80). In addition, this biotype demonstrated cross resistance to quinclorac, being ten-times more tolerant than its susceptible counterpart (Table 1). A biotype designated as intermediate (I) was also observed. It was apparent that propanil controlled all the biotypes successfully. By contrast, molinate was required at significally higher rates of application to control the quinclorac susceptible (S) biotype as compared to the other biotypes.

The biotypes of the other *Echonochloa* species were treated with quinclorac (0.5 kg ha^{-1} a.i.). The response to the herbicide treatment depended on the species treated. *E. colonum, E. oryzicola* and *E. oryzoides* exhibited great susceptibility with this dose, showing symptoms after three days of the treatment. By contrast, *E. crus-galli* and *E. hispidula* showed resistance in many cases. Six of the eight quinclorac-treated *E. crus-galli* showed different levels of tolerance. Three of these were derived from atrazine-treated maize fields. One biotype showed cross-resistance to quinclorac. Note in particular, that the mode of action of the two herbicides is different. The *E. hispidula* showed great genetic similarity to *E. crus-galli*. The herbicide response also was similar to the '*E. crus-galli*'. The non-treated *E. hispidula* biotypes were susceptible but seven of the eight quinclorac-treated *E. hispidula* survived the herbicide treatment. In some cases, the susceptible *E. crus-galli* and *E. hispidula* showed the symptoms later than the other species. This resistance to quinclorac has appeared in Spain after three years of treatment, and we have no knowlegde of any other cases of resistance except in Korea. The maximun dose used in Asia, America and Australia is 0.5 kg ha^{-1} a.i. and at this rate quinclorac sucessfully controls the Echinochloa spp. However, in Spain, many *Echinochloa crus-galli* and *E. hispidula* escape when this dose is used. Our results induce us to think that in Spain there are different types of *E. crus-galli* and this may explain the early development of resistance to quinclorac.

Table 1. Effect of different herbicides on ED_{50} of '*E. crus-galli*' biotypes.

	ED_{50} following applications of the following (kg ha^{-1}):			
	Quinclorac	Atrazine	Propanil	Molinate
Resistant	2.6	0.1	0.4	1.1
X-resistant	1.0	8.0	0.4	0.8
Intermediate	0.6	0.1	0.5	1.1
Susceptible	0.1	0.1	0.5	2.0

Molecular analysis

The principle coordinates analysis of the RAPDs data revealed that the five *Echinochloa* species form only three discrete groups: (i) *E. crus-galli* + *E. hispidula,* (ii) *E. oryzoides* + *E.oryzicola* and (iii) *E. colonum.* It was not possible to distinguish between *E. crus-galli* and *E.hispidula* or between *E. oryzoides* and *E. oryzicola* with the primers used. Herbicide resistance was confined to group (i). The susceptible biotypes, morphologically classified as *E. crus-galli*, clustered with group (ii) biotypes and were thus reclassified as *E. oryzoides.*

Primer OP-A04 (3'AATCGGGCTG5') was found to be diagnostic of the three *Echinochloa* groups described above. A major 800 bp product classifies the sample as group (i) and a 1600 bp product classifies the sample as group (ii). *E. colonum* (group iii) which is readly identifiable by its morphology, gives rise to distinct profiles that lack both of these specific RAPD products. This is illustrated in Figure 2 which shows a RAPD analysis of 9 individual plants from three species using primer OP-A04.

This primer was tested in all of our 32 biotypes. One of the main problems for those working with *Echinochloa* is to classify the test material. In many cases it is not possible to distinguish morphologically between *hispidula* and *oryzoides.* However primer OP-A04 was very helpful in classifying the species.

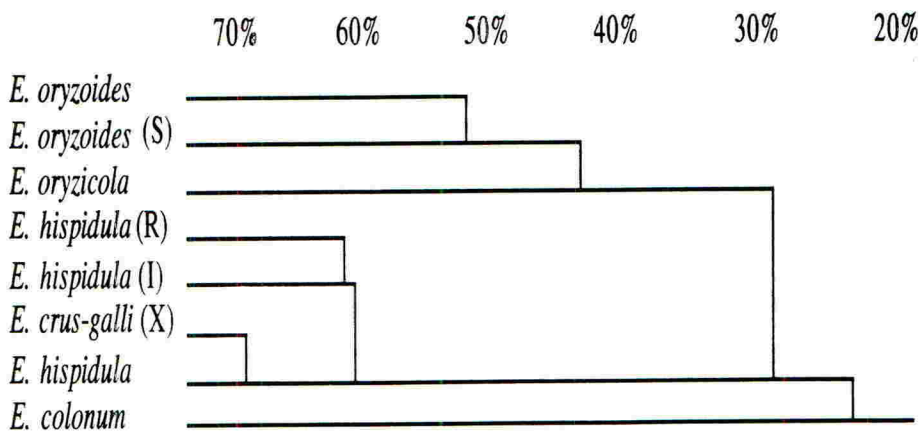

Figure 1. Dendogram constructed from furthest-neighbour cluster analysis showing three discrete groupings of *Echinochloa* based on percent similarity. S = susceptible, R = resistant, I = intermediate, X = cross resistant.

In summary, these studies have shown:

1. The importance of molecular characterisation of *Echinochloa* spp. to verify the study material. In this case there was a clear correspondance between the *Echinochloa* species and the development of resistance to quinclorac.

2. Quinclorac is very effective for the control of *E. colonum, E. oryzoides and E. oryzicola,* however many populations of *E. crus-galli* and *E. hispidula* escape herbicide control.

3. One biotype resistant to atrazine has been found in a maize field. The resistance is due to target site mutation. This biotype also showed cross-resistance to quinclorac.

4. The resistant biotype showed negative cross-resistance to molinate. This factor could be used as a tool to prevent or delay resistance to quinclorac and /or atrazine. The control of *E. crus-galli* and *E. hispidula* could be achieved using a mixture of herbicides.

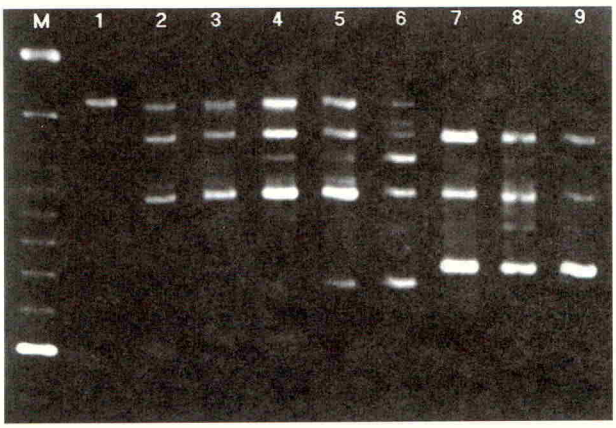

Figure 2. Amplification products from RAPD primer OP-A04. Lane M = 100 bp marker, Lane 1: *E. colonum,* Lanes 2-6: *E. oryzoides,* Lanes 7-9 *E. crus-galli.*

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MOLECULAR MARKERS FOR GENETIC DIVERSITY IN CLEAVERS (*GALIUM APARINE*)

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ABSTRACT

Cleavers (*Galium aparine*) is an annual weed with economic significance because of adaptive invasion of agricultural fields. The adaptation to growth on arable land is transmitted genetically, although presently few markers of adapted populations are known. Genetic diversity in cleavers was studied by random amplified DNA polymorphism (RAPD) analysis, by directed amplified minisatellite DNA (DAMD) analysis, and by simple-sequence-repeat PCR (SSR-PCR). Limited differences were detected between arable-adapted and wild plants from sites across western Europe by RAPD analysis. DAMD analysis and SSR-PCR both showed some genetic variation which may be linked to adaptation to growth on agricultural land.

INTRODUCTION

Cleavers or 'goose grass' is an economically important annual weed which can compete effectively with crop plants such as rape and cereals, and can interfere with combine harvester operations. Environmental plasticity has been proposed to aid cleavers populations in adapting to competition from other plant species within different ecosystems. Intraspecific differentiation allows facultative invasion of arable fields by weed forms which show physiological adaptations to accommodate modern agriculture practices. These adaptations include a stratification requirement for seed germination and a strong light inhibition of germination, as well as a requirement for nitrogenous supplements (van der Weide, 1992). Together the adaptations prevent the autumn germination seen in woodland populations and avoid the destruction of the seedlings of cleavers during autumn ploughing (van der Weide, 1992; Froud-Williams, 1985; Wilson & Froud-Williams, 1988). Although the physiological adaptations appear to be genetically inherited (van der Weide, 1992; Hill, 1991), molecular genetic markers for these adapted populations have not been previously investigated.

Recently, many studies of molecular genetic diversity in wild plant populations have been reported using polymorphic DNA markers to permit examination of several genomic regions simultaneously. These include the random amplified polymorphic DNA (RAPD) assay which involves PCR-mediated amplification of genomic loci using short arbitrary oligonucleotide primers (Williams *et al.*, 1993), the SSR-PCR assay which employs primers based on anchored microsatellite loci and is carried out using comparatively stringent primer annealing conditions (Zietkiewicz *et al.*, 1994) and the DAMD assay which involves amplification of minisatellite-like regions, also at high stringency (Heath *et al.*, 1993). RAPD analysis has been used widely to

detect genotype variation within ecotypes, cultivars and wild plant populations (Kaemmer *et al.*, 1992; Brauner *et al.*, 1992). The DAMD assay has detected intraspecific variation in populations of fungi (Stenlid *et al.*, 1994; Kuhls *et al.*, 1994).

Recently, several reports noted that the RAPD assay could be influenced by experimental conditions (Penner *et al.*, 1993; Meunier & Grimont, 1993). We also observed that the amplification of RAPD products was found to be affected by experimental factors which could influence the efficiency of the amplification reaction (Cheng *et al.*, 1994). When amplification factors were optimised for each primer, limited RAPD band variation was detected between arable-adapted and wild cleavers populations from sites across Europe. The DAMD assay uses higher stringency PCR conditions and was less influenced by the efficiency of the PCR reaction. Although the products generated by the DAMD assay also showed limited band variation, one major DNA product appears to be linked with the germination requirements of cleavers. SSR-PCR analysis of adjacent hedgerow and arable field populations of cleavers showed higher levels of band sharing within each environmental group.

MATERIALS AND METHODS

Nomenclature.

PCR, polymerase chain reaction; M13 minisatellite, *bacteriophage* M13 protein III gene repeat DNA element; EDTA, ethylenediaminetetraacetic acid; dATP, dTTP, dCTP, dGTP, deoxynucleotide triphosphates; *Taq* DNA polymerase, thermostable DNA polymerase from *Thermus aquaticus*; TBE, Tris-Borate-EDTA buffer; TE, Tris-EDTA buffer.

Isolation of genomic DNA.

Genomic DNA was isolated from cleavers using the Nucleon DNA extraction kit (Scotlab Bioscience, Ltd., Glasgow) as recommended by the manufacturer. The DNA pellet was washed with 1 ml of 70% ethanol at 4°C, drained thoroughly and air dried. The DNA was redissolved in 100 µl of TE.

RAPD, SSR-PCR and DAMD primers.

The 10-mer RAPD primers were purchased from Genosys Ltd. The SSR-PCR primer (5'-$(CA)_8$RG-3') and the M13-based DAMD primer (5'-GAGGGTGGCGGCTCT-3') were produced by Cruachem Ltd. The *Taq* DNA polymerase and the incubation buffer were provided by Boehringer-Mannheim and were used according to the manufacturer's instructions. Amplification was undertaken using a Techne PHC-3 thermal cycler.

DNA amplification conditions.

PCR amplification was carried out in a total reaction volume of 25 µl containing 1 µM single oligonucleotide primer for RAPD and 500 ng for DAMD / SSR-PCR; 200 µM each of dATP, dCTP, dTTP and dGTP, 1.0 Unit of *Taq* DNA polymerase and 1x reaction buffer from the supplier. The quantity of template DNAs were varied as described in the results section. The reactions were undertaken in 0.5 ml Eppendorf tubes and the aqueous solution was overlaid with 30 µl of mineral oil. For RAPD amplification the thermal cycling conditions were:- 93°C/1 min, followed by 30 cycles of 36°C/40 sec, 72°C/1.5 min and 93°C/10 sec with the maximum transition

rate between temperatures, and a final extension at 72°C for 5 min. The DAMD amplification conditions were:- 35 cycles of 95°C/1 min, 55°C/1 min and 72°C/1.5 min. The SSR-PCR amplification conditions were:- 92°C/2 min, followed by 35 cycles of 51°C/1 min, 72°C/2 min and 94°C/1 min with the maximum transition rate between temperatures. A final extension was performed at 72°C for 7 min.

Electrophoresis.

All RAPD and DAMD samples (20 µl) were analysed by electrophoresis in 1.5% agarose gels (15 x 15 cm) using glycine buffer-E (Maniatis *et al.*, 1982) and were visualised by ethidium bromide staining. SSR-PCR amplification products were electrophoresed on native 9% polyacrylamide gels using TBE buffer (Maniatis *et al.*, 1982) at 80 mA for 9.5 hr and DNA products were visualised by silver staining (Caetano-Anollés *et al.*, 1991).

RESULTS AND DISCUSSION

Ecological and physiological studies by many researchers (van der Weide, 1992; Froud-Williams, 1985; Hill, 1991; Hill & Courtney, 1991) have established that arable-land adapted populations of cleavers have heritable differences to wild (woodland or hedgerow) populations despite a degree of morphological and physiological plasticity within populations from the two environments (Groll & Mahn, 1986; Niemann, 1988; Auge & Mahn, 1988). Despite the wide variation in seed dormancy within populations, seed from hedgerow populations were generally less dormant than those from field populations. One physiological character strongly associated with differentiation of the two environmental populations is a requirement for seed stratification for successful germination of arable-land adapted populations (Froud-Williams, 1985; Wilson & Froud-Williams, 1988).

We undertook an investigation of RAPD, SSR-PCR and DAMD polymorphic markers in populations of cleavers described previously (Hill & Courtney, 1989; van der Weide, 1992) from different geographic origins, collected from two environmental populations: arable-land and hedgerow. Two experiments were undertaken to investigate possible relationships between DNA polymorphism and either the geographic origin of the cleavers samples, or the environmental adaptation of the populations. Seed and plant populations from across continental Europe, and more local populations from within the British Isles, were studied to provide an index of polymorphism that might be due to genetic isolation of the population groups. Where possible, populations from arable-field and adjacent wild terrain were also compared.

In addition, the conditions required for efficient germination of the seed populations (approx 100 seeds per test) were investigated under two different conditions. When cleaver seeds were incubated at 10°C in complete darkness over filter paper saturated with 12.5 mM KNO_3, all populations germinated with an average success of 70%. However, when seed was incubated on distilled water held at room temperature (19-21°C), seeds from wild populations germinated (average success 80%), while seeds from arable-adapted populations failed to germinate (data not shown).

RAPD primers have been used widely to investigate polymorphism between cultivars and closely related lines of many crop species. The general conclusion of these studies was that the degree of polymorphism detected by RAPD assay relates inversely to the degree of genetic relatedness and breadth of the genetic base of the plant species examined (Williams *et al.*, 1993). Detailed analysis of RAPD assay

suggests that low polymorphism can be expected per band. Based on an average of 5 bands per primer, we calculate polymorphism at approximately 6% per band for those studies (Williams *et al.*, 1993). We investigated twenty-seven short oligomers ranging from 10 to 23 bp for their utility as RAPD primers, as well as eight simple repeat di- and tetranucleotide oligomers for the amplification of potentially polymorphic simple repeat sequences in cleavers (*see* Table 1). A low degree of polymorphism was detected with a majority of the twenty-two 10-mer primers. Although a limited set of primers was tested, the average polymorphism was 0.9% per band. These polymorphisms could not be linked with either the geographic origins of the cleavers, the environmental population conditions (arable-field or wild-field), or the experimental germination requirements (stratification). Among the twenty-seven different RAPD primers used to test populations, only one (primer 20:-5'-GCCAATCCTG-3') detected a moderate degree of polymorphism (Fig. 1) which, similarly, did not relate consistently to these variables. While these data do not indicate a unique origin of the arable-adapted populations of cleavers throughout Europe, the low degree of RAPD polymorphism does suggest a limited mutation within populations.

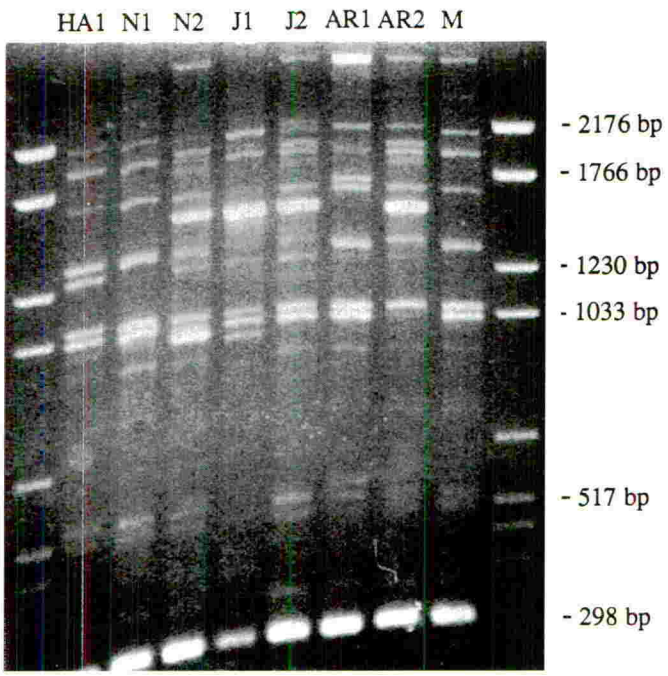

Fig. 1. Random amplification polymorphic DNA (RAPD) patterns of cleavers generated using primer 20 (see Table 1). Samples were electrophoresed as described in the Methods. Molecular size markers (bp) were Marker VI (Boehringer). Sample HA1 was from the arable-land in Holland. Samples N1 and N2 were from a hedgerow, Nottingham, UK. Samples J1 and J2 were from wild-fields in Northern Ireland, UK. Samples AR1 and AR2 were from hedgerow and wild field respectively, Ayr, UK. Sample K was from a hedgerow, Aberdeen, UK.

We also examined polymorphism of M13-like repeat loci in cleavers, by the DAMD assay (Heath *et al.*, 1993; Cheng *et al.*, 1994). Two oligonucleotide primers were used, one with homology to the abundant M13-repeat motif (see Materials & methods) and the second a mixture of four primers with homology to all variants of the repeat (data not shown). Because of the novelty in application of the DAMD assay to plant molecular genetics and because of reports of variation in the reproducibility of the related RAPD amplification assay between laboratories (Penner *et al.*, 1993) we tested the fidelity of the DAMD profile stringently. The reproducibility of the DAMD profile is high, in marked contrast to the RAPD assay which is sensitive to each of these reaction variables (Fig. 2).

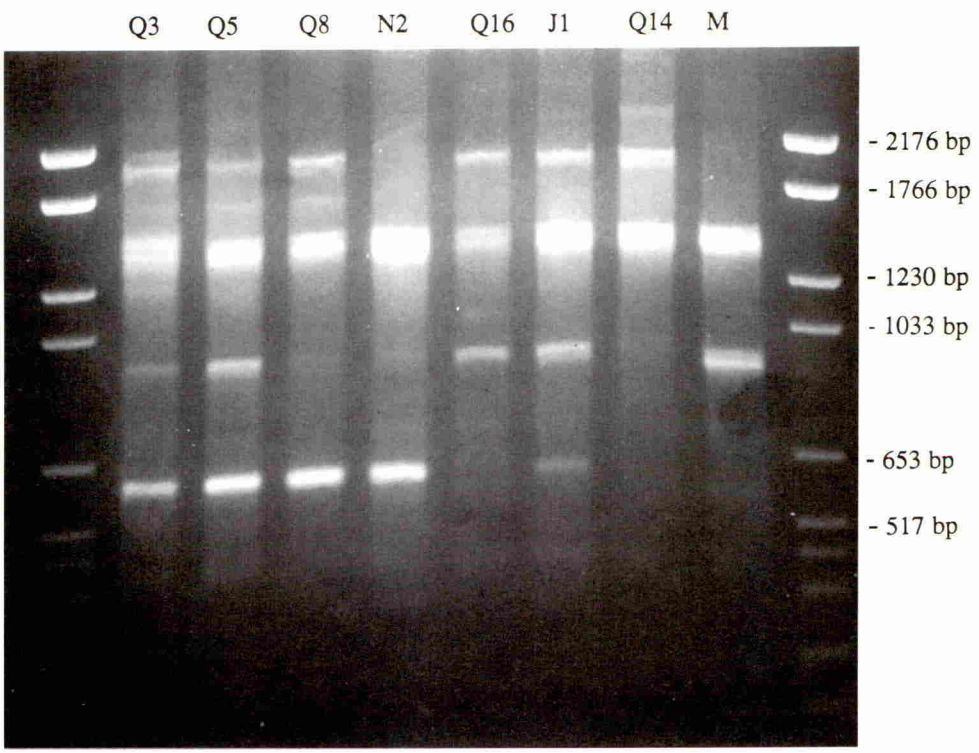

Fig. 2. DAMD fingerprinting of cleavers. Isolates Q3, Q5, were from arable fields and Q8, N2 and J1 were from wild-terrain, but each population was associated with a strict requirement for stratification and darkness for germination. Each of these populations show an amplified band of about 620 bp. DNA obtained from seedlings which had less rigorous germination requirements lacked the 620bp band. The seed populations Q14, Q16 and M were each from wild terrain. The size marker (bp) was DNA Marker VI (Boehringer).

TABLE 1. The random oligonucleotide primers used for the RAPD analysis. Polymorph(ism) indicates whether RAPD bands showed length variation between populations of cleavers.

	Sequence	Polymorph		Sequence	Polymorph
1	5'-ATTGCGTCCA-3'	No	19	5'-CGGCCCCTGT-3'	No
2	5'-CTGTTGCTAC-3'	No	20	5'-GCCAATCCTG-3'	**Yes**
3	5'-CGGTCACTGT-3'	No	21	5'-CCACGACGAT-3'	No
4	5'-CGGCCCCTGT-3'	No	22	5'-GGCCTTGAGT-3'	No
5	5'-CCGAACGGGT-3'	No	23	5'-GAGGGTGGCGGCTCT-3'	No
6	5'-GGTGGGTGCT-3'	No	24	5'-CCAAGCTCAGGGCAGG-3'	No
7	5'-TCGTAGCCAA-3'	No	25	5'-CCTCGATGTCGGCTCTTC-3'	No
8	5'-CGGTCTGCAT-3'	No	26	5'-CCAAGAGTTTCACCTCTGAC-3'	No
9	5'-TCACCGAACG-3'	No	27	5'-AGTCCGGTGCTCTAACCAACTGAG-	No
10	5'-GTGCGGACAG-3'	No	28	5'-$(AT)_8$-3'	Smear
11	5'-GGACCACCAT-3'	No	29	5'-$(CA)_9$-3'	Smear
12	5'-CTGTTGCTAC-3'	No	30	5'-$(CG)_9$-3'	Smear
13	5'-CGGTCTGCAT-3'	No	31	5'-$(TAT)_5$-3'	Smear
14	5'-TGGCCCCTGT-3'	No	32	5'-$(TGA)_7$-3'	Smear
15	5'-TGGTCACTGA-3'	No	33	5'-$(GACA)_4$-3'	No
16	5'-TGCTCACTGA-3'	No	34	5'-$(GATA)_4$-3'	Smear
17	5'-CGGGAGACCC-3'	No	35	5'-$(CACA)_4$-3'	Smear
18	5'-GCATGGAGCT-3'	No	36	5'-$(CA)_8$RG-3'	**Yes**

TABLE 2. Comparison of DAF assay bands shared/ unshared from adjacent arable-field and hedgerow populations from one site (Cotterel, Oxon). A single outgroup sample (Belcher) was included in the assay. (**A**):- Right: Fraction of shared bands [$F = 2X_{1,2} / X_1 + X_2$]. Left: Genetic distance [$D = -ln\ (F)$]. (**B**):- geographic origin and environmental population base.

A

				Fraction of shared bands			
Sample	**1**	**3**	**4**	**18**	**19**	**20**	**21**
1		1.0	0.82	0.55	0.78	0.71	0.40
3	0		0.82	0.55	0.78	0.71	0.40
4	0.20	0.20		0.50	0.84	0.88	0.36
18	0.60	0.60	0.69		0.30	0.50	0.80
19	0.25	0.25	0.17	1.20		0.84	0.16
20	0.34	0.34	0.13	0.69	0.17		0.36
21	0.92	0.92	1.02	0.22	1.83	1.02	
	Genetic distance						

B

Sample	**Location of Population**	**Environment**
1	Cotteral, Near Wantage, Oxon.	Arable field
3	Cotteral, Near Wantage, Oxon.	Arable field
4	Cotteral, Near Wantage, Oxon.	Arable field
18	Cotteral, Near Wantage, Oxon.	Hedgerow
19	Belcher, Near Thame, Oxon.	Hedgerow
20	Cotteral, Near Wantage, Oxon.	Hedgerow
21	Cotteral, Near Wantage, Oxon.	Hedgerow

In Fig. 2 it can be seen that a band of about 620 bp occurs among the amplified products with both types of primer, which was present only in cleavers populations that require stratification/ dark germination and was absent from cleavers populations which could germinate under natural day-night cycle condition for germination. The detection of the 620 bp band showed no correlation with the geographic origin of samples, and cleavers from different European countries share the same DNA banding pattern as far as they demand the same germination conditions. This suggests that the genetic variation detected by DAMD is dependent on the germination requirements of the cleavers populations and therefore may indirectly reflect whether the plant is potentially arable-land adapted. UK populations of cleavers from adjacent arable-field and hedgerow were studied by SSR-PCR analysis of polymorphism in inter-microsatellite regions (Table 2). Seven different SSR-PCR microsatellite-linked primers were examined for amplification products over an annealing temperature range of 7°C. Only two of the seven generated amplification products (data not shown). Of the two amplifiable primers, bands generated using primer 5'-$(CA)_8$RY-3' were identical for all cleavers populations. However, multiple polymorphic bands were reproducibly detected when SSR-PCR products of primer 5'-$(CA)_8$RG-3' were electrophoresed on native polyacrylamide gels (not shown). Average similarity indices were calculated by comparison of SSR-PCR bands shared between isolates. A higher degree of band sharing was detected among seedlings from the field population than with seedlings from an adjacent hedgerow population. Band sharing between seedlings from the hedgerow population was also high. The SSR-PCR analysis therefore offers potential as an intrapopulation marker revealing greater apparent polymorphism than the DAMD assay.

Morphological variation among populations of cleavers from crop infestations and adjacent hedgerow areas have been reported, with longer internodes (Froud-Williams & Ferris-Kaan, 1991; Bain & Attridge, 1988) and less elliptical cotyledons (Niemann, 1988; Hill & Courtney, 1991) in hedgerow plants. There are a number of factors that may have a role in the the determination of genetic variation within weed populations. Cleavers inbreeds and appears to establish clonal populations (Hill, 1991). The gene flow that occurs between weed populations might be expected to be enhanced by the intervention of humans, or could be carried by animals over great distances. The seeds of arable-land adapted cleavers can contaminate seed crops or agricultural machinery, which would aid their distribution into agricultural land. The 640 bp DAMD band was found to be common among European cleavers populations that displayed germination characteristics associated with spring emergence. While the distribution and origins of biotypes of cleavers in Europe are not known, arable-field adapted populations are known to occur in many countries (van der Weide, 1992; Froud-Williams, 1985). The data presented here is consistent with a small genetic base for cleavers populations. The 640 bp DAMD band may also represent a mutation associated with the physiological attributes for spring germination.

ACKNOWLEDGMENTS

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Session 5B

Management of Herbicides to Minimise their Impact on Water Quality

Chairman	Dr C J Swinnerton
Session Organiser	Dr A D Carter
Papers	5B-1 to 5B-4

PESTICIDE ECONOMIC AND ENVIRONMENTAL TRADEOFFS DECISION SUPPORT SYSTEM FOR PEANUT PRODUCTION

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ABSTRACT

A decision-support system was developed to evaluate both economic and environmental impacts of different pest management practices. A weed competition model capable of estimating crop yield for any mixture of weed species in combination with treatment efficacy data form the basis of the economic module. Pesticide movement to groundwater is simulated for each potential treatment using site specific soil properties, weather, and management practices. The groundwater hazard incorporates the amount of pesticide leached and its toxicity. PEET was implemented for weed control in peanut production in Florida, North Carolina, and Oklahoma. The interactive software is written so it can be used at new locations and with new crops with little or no additional programming. Information for the area and crop are stored in databases created by a team of local experts.

INTRODUCTION

The Pesticide Economic and Environmental Tradeoff, PEET, decision-support system was designed to aid farmers in selecting pesticides and minimizing groundwater quality degradation. Most farmers are interested in earning money without harming natural resources. When selecting pesticides, they often have several options available to control weeds. PEET is a decision support tool which allows them to see which pesticides, if any, will decrease their economic loss from the pest. PEET also evaluates the potential impact of each pesticide upon groundwater quality. In some cases, a pesticide can be used which minimizes both economic loss and groundwater quality degradation. In other cases, a tradeoff between economics and groundwater quality must be made. PEET is designed to make decision-makers aware of these tradeoffs so they can make informed decisions. In this paper we explain how PEET was designed and how it can be used to evaluate alternative weed control strategies for cost-effective peanut production.

SYSTEM DESIGN

The following design requirements were established for PEET:

1. It must provide economic and water quality information for specific sites and management practices specified by the user.
2. It must incorporate uncertainties in the information and convey that to the user.
3. It must operate on standard MS-DOS computers with a hard disk.
4. It must be convenient to enter information and to interpret results.
5. Its speed must be satisfactory for use in interactive mode.
6. It must be usable at different locations by changing data used, without reprogramming.

To meet these requirements, the PEET program must carry out four major tasks. Those include getting inputs from the user, calculating economic impacts for each treatment, evaluating the groundwater hazard posed by each treatment, and presenting results of the analysis in different forms. Details of the economic and groundwater hazard calculations are given on the following pages.

TASK 1: OBTAINING USER INPUTS

The user interface in PEET was designed to be easy to use and to assist the user in making entries. It enables the user to select answers from menus in many cases. The options are often reduced by other selections already made. For example, the soils displayed in the soil menu are only those for the county already selected. When numerical values are expected, default values are provided. The user can replace these values with more appropriate ones when known. Although five input screens are used, the user can page forward or backward as desired to change current values. Help messages are provided for each entry. The user can get these help messages by pressing the <F1> function key or by configuring the system to automatically display all help messages. Since we expect users to explore tradeoffs for different conditions, responses entered for one situation become the default values for the next one. That means the user must only make the changes of interest rather than redefine all the values.

To facilitate adoption of use in new locations, all options which can be selected, default values displayed, help messages written to the screen and credits for local developers are stored in databases and external files. These items can be changed by the team of local experts implementing PEET for a particular area. No programming skills are required.

TASK 2: CALCULATING ECONOMIC IMPACT

Yield loss due to weeds

Yield loss can be computed for any combination and density of weeds. In turn, yield savings can be calculated for any pesticide based on its efficacy on each weed type. The

yield reduction, $Y_{Loss\text{-}Abs}$ due to weed population and infestation is given by

$$Y_{Loss\text{-}Abs} = Y_{Loss\text{-}Rel} Y \quad (1)$$

where $Y_{Loss\text{-}Rel}$ is the relative loss in yield and Y is the projected weed-free yield. PEET displays economic loss for low, normal, and high weed free yield values to provide the user with insight into the impact of yield upon the economics of herbicide use. The relative yield loss is given by

$$Y_{Loss\text{-}Rel} = \frac{AID}{A + ID} \quad (2)$$

where D is the total competitive load, A is the maximum relative yield loss, and I is the yield loss per unit competitive load for low loads (Coble and Mortensen, 1992). The total competitive load D is the sum of the competitive loads for the different weed species. It is calculated using the equation

$$D = \sum_{j=1}^{n} c_j d_j \quad (3)$$

where n is the number of weed species infesting the field, c_j is the competitive index for weed j and d_j is the density of weed j.

Economic loss due to weeds

The economic loss, E_{Loss}, due to weeds is given by

$$E_{Loss} = Y_{Loss\text{-}Abs} V + C_{Appl} + C_{Scout} + \sum_{Herb=1}^{m} C_{Herb} R_{Herb} \quad (4)$$

where V is the expected value of the crop per unit harvested, C_{Appl} is the cost of herbicide application, C_{Scout} is the cost of scouting for weeds, C_{Herb} is the cost of herbicide Herb per unit applied, R_{Herb} is the rate of application of herbicide Herb, and m is the number of herbicides used in the treatment. If no herbicide is applied, the last three terms in the equation are zero.

Economic loss with treatments

The density of each weed species after treatment with a herbicide is calculated using the equation

$$d_{jk} = d_j (1 - e_{jk}) \quad (5)$$

where d_{jk} is the density of weed j after treatment k, d_j is the density of weed j before treatment, and e_{jk} is the efficacy of treatment k for weed j. Equations 1-4 are used to calculate economic loss for each treatment by replacing d_j with d_{jk} in equation 3.

Computing time requirements for economics

Computing times for economic analysis is on the order of 1 second. This component of the project provides no problem for interactive use.

TASK 3: EVALUATING GROUNDWATER HAZARD

Definition of groundwater hazard

In PEET, the groundwater hazard, GWH, associated with a particular pesticide is defined as the ratio of the estimated concentration, C, of the active ingredient in groundwater to the U.S. EPA lifetime health advisory level, HAL, (United States Environmental Protection Agency, 1989) for the active ingredient. That is

$$GWH = \frac{C}{HAL} \qquad (6)$$

If a treatment contains more than one active ingredient, the GWH for the treatment is taken as the sum of the GWH values for all active ingredients. This index, introduced by Hoag and Hornsby (1992), is useful in that it incorporates both the predicted concentration of the pesticide in groundwater and the toxicity of the pesticide. As defined here, values of GWH less than 1 correspond to active ingredient concentrations below the lifetime health advisory level for the chemical. Thus, those products in those conditions would not be thought to pose a significant risk to human beings.

Calculating groundwater hazard

With this definition, calculating the groundwater hazard requires that we calculate the concentration of each active ingredient in the groundwater below the soil of interest. In general, the amount of chemical leaching to groundwater depends upon soil properties, chemical properties, management practices such as irrigation management and tillage, application dates, application amounts, application depths, weather, water infiltration and runoff, and plant use of water. Various models exist which could be used to estimate the leaching and degradation of pesticides. *Any model capable of estimating mass emission of pesticides from the bottom of the plant root zone can be used with PEET.*

Since PEET is intended to be a decision-making tool, we are interested in predicting the concentration of chemicals in groundwater. However, we do not know what the future weather at a site will be. Large differences in leaching due to weather are common (Haan et al., 1994). Therefore, PEET incorporates this uncertainty into groundwater hazard estimates. That is done by simulating movement for many weather records at a site and obtaining distributions of expected concentrations. This provides the probability of exceeding different concentrations. These probability distributions for each active ingredient were used to obtain a distribution of GWH values for each treatment (Figure 1). PEET is used to display results for the probability of interest.

Ranking treatments

One of the types of output presented to the user of PEET is a ranking of treatments by groundwater hazard. This ranking is more complex because GWH is a probability distribution, not a simple number. Two forms of ranking are supported in PEET. In

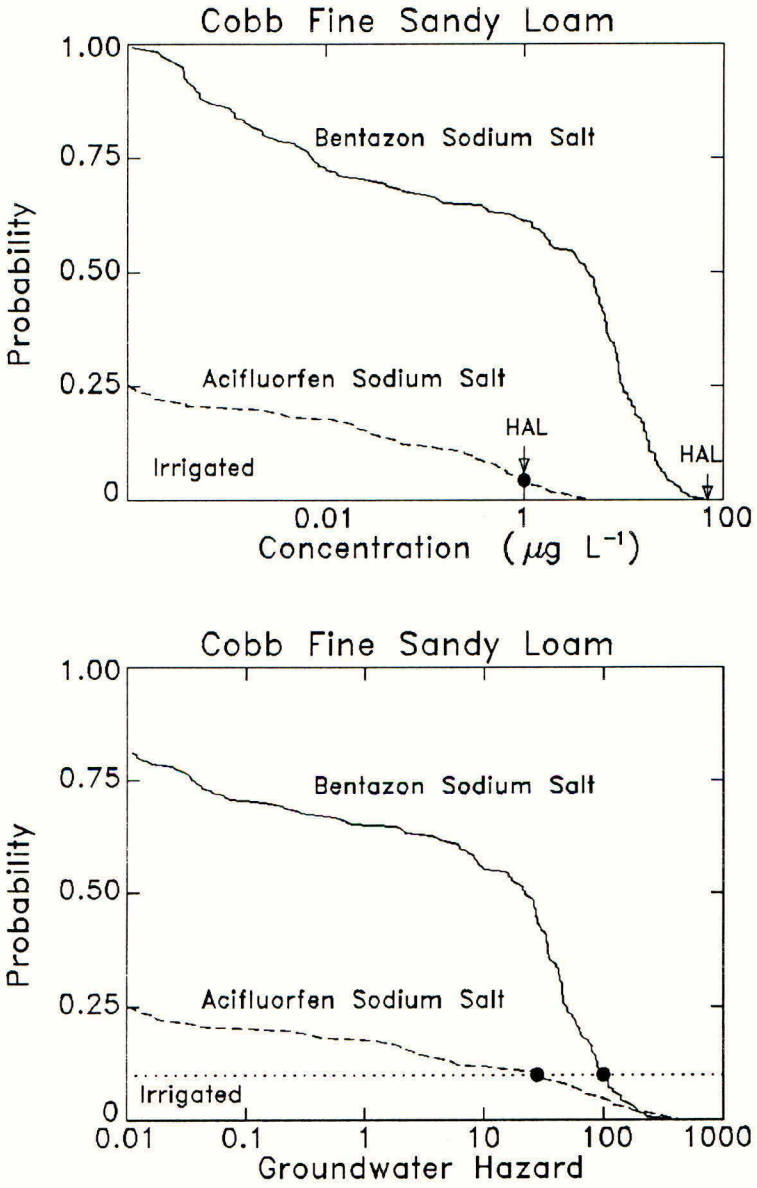

Figure 1. Probability of exceeding different concentrations (upper graph) and groundwater hazards (lower graph) due to differences in weather at the site in Caddo county, Oklahoma. Each chemical was applied at a rate of 0.56 kg ha^{-1}.

the first case, treatments are ranked on the basis of their GWH values at the probability level specified by the user. This ranking is easily understood. However, we observed that the probability distributions for different treatments sometimes intersect and cross-over one another. In those conditions the simple ranking at a specific probability level may not truly represent the overall placement of the treatments. A second ranking scheme based on principles of stochastic dominance is also supported in PEET. This ranking process considers the probability distributions for their entire range in making the ranking.

Model used to estimate GWH

The CMLS94 model of Nofziger and Hornsby (1994) was used to estimate the amount of each active ingredient leaching below a depth of 1 m. We assumed degradation below that depth was negligible and hence that amount represents the amount entering the groundwater. The concentration, C, in Equation 6 was estimated by mixing the calculated amount of pesticide leached in an aquifer with porosity f and mixing depth t. Values of f and t can be specified by the user.

CMLS94 enables users to assess uncertainty in predictions due to unknown future weather at a site by simulating chemical movement for many different weather sequences for that site. Probability distributions can then be obtained for any outputs of interest.

Computing time requirements

Obtaining probability distributions for GWH provides a great deal of important information, but it requires a large amount of computing resources. A typical analysis for a user could require more than 10,000 simulations. This would require more than 120 minutes on personal computers. Clearly, real-time simulation on these machines will not be satisfactory for interactive use.

To overcome this time limitation, groundwater hazards were calculated for each soil and chemical using sets of management practices which span the practices used by farmers. Those values along with treatment ranks by stochastic dominance were stored in a groundwater hazard database. That database is queried by the interactive PEET program. This reduces the time for a response to only a few seconds. The database for peanuts in Oklahoma occupies approximately 4 MB of disk space and contains approximately 40,000 records. This represents more than 25 million simulations. Several Sun SparcStations were used to make the simulations in approximately one week.

TASK 4. DISPLAYING RESULTS

Information on the economic and environmental consequences of using each weed control treatment are presented three ways. Once again, the user can move among the

three screens as desired. Help messages are available here also to explain the meaning of different parts of the output. An example output screen for "Cost and Potential Groundwater Hazard by Herbicides" is shown in Figure 2. This screen indicates that the use of 'Pursuit' at 4 fluid oz per acre returns $312 over no treatment for an expected yield level of 4000 pounds. Futhermore, the resulting potential GWH associated with application of this product is negligible, making it an excellent economic and environmental choice. All other choices are economically less efficient and some are potentially more hazardous to drinking water supplies.

COST AND POTENTIAL GROUNDWATER HAZARD BY HERBICIDES

Herbicide	Potential Loss to Weeds by Yield (lb/ac) 3600	4000	4400	Potential Groundwater Hazard Rank	Index (% of HAL) Preferred <
	($/acre)				1% 10% 100% 1000%
NO TREATMENT	461	513	564	NA	
Post-Emergence					
BUGLE 0.60PT	409	453	497	1	<1
BUGLE 1.20PT	403	445	488	1	<1
POAST PLUS 36.00FL O	418	462	506	3	<1
POAST PLUS 48.00FL O	412	454	497	4	<1
BLAZER 0.50PT	424	469	515	5	<1
BLAZER 1.00PT	383	424	465	6	<1
PURSUIT 4.00FL OZ	183	201	218	7	<1
BUTYRAC 175 1.80PT	330	365	400	8	3
BUTYRAC 200 1.60PT	330	365	399	9	3
BLAZER 1.50PT	352	389	426	10	4
+BUTYRAC 200 1.00PT					
BLAZER 2.00PT	324	358	391	10	4
+BUTYRAC 200 1.00PT					
STORM 1.50PT	307	339	371	12	90
BUTYRAC 200 8.00FL O	332	367	401	13	90
+STORM 1.50PT					
BUTYRAC 200 16.00FL	302	333	364	14	90
+STORM 1.50PT					
BASAGRAN 1.50PT	340	375	411	15	125
BASAGRAN 1.50PT	331	365	399	15	125
+BUTYRAC 200 8.00FL O					
BASAGRAN 2.00PT	304	335	366	17	166
BASAGRAN 2.00PT	293	323	352	18	167
+BUTYRAC 200 8.00FL O					

Figure 2. Example Output Screen of Cost and Groundwater Hazard for Post-Emergent Application of Herbicides And Periodic Irrigation of Peanuts on Cobb FSL Soil in Caddo County, OK. Product names preceded by the plus symbol indicate a mixture with product listed immediately above.

ADDITIONAL FEATURES

Special software was developed for horizontal and vertical scrolling of selected portions of the output screen. This enables the user to scroll vertically through all of the treatment options while keeping header information in place. Horizontal scrolling is used to replace the column with the heading "Potential Loss to Weeds by Yield" (see Fig. 2) to "Weed Density and Competitive Load." Using the right and left cursor keys,

the user can examine the total weed load (no treatment line), and the reduction of weed density and competitive load of each weed selected for each herbicide product listed in the first column. This feature indicates to the user the degree of expected control of each weed for each herbicide based on explicit or implied assumptions inherent in the input choices made and efficacy of the herbicide products. In this way, the user can see tradeoffs between the groundwater hazard and weed density for any particular weed species. PEET provides the user with a printout of the choices made on the input screens and the output results. This feature is accessed by pressing the <F2> key. This is particularly useful for reviewing several alternative scenarios and for record keeping.

USER EXPERIENCE

Although PEET was developed for use by farmers, cooperative extension agricultural agents and crop consultants, it has thus far been introduced only to agricultural agents and area and statewide specialists for testing. Initial feedback is very positive, particularly the ease in which the program can be used and visual impact of the graphics in the output screens. Although the program is self-explanatory on screen with available help messages, a users manual is available detailing the features, concepts, and algorithms used in the program. A developers guide is also available to assist those desiring to implement PEET technology at a new site (local, regional, or statewide). This guide provides detailed information about the data needed to implement the program, as well as database management formats for the requisite files.

NOMENCLATURE

'PURSUIT' S-benzyl 1,2-dimethylpropyl(ethyl)thiocarbamate

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PRACTICAL CONTROL OPTIONS FOR MINIMISING THE OCCURRENCE OF AGRICULTURAL PESTICIDES IN WATER

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ABSTRACT

Low levels of pesticides, primarily herbicides, are routinely detected in UK environmental waters. Concentrations found commonly exceed the EC "Drinking Water" standard and, rarely, environmental quality standards or health related standards for drinking water. Unlike mains water supplies, where pesticide removal treatment is possible, the only option to protect the aquatic environment is to minimise pesticide inputs to water. Case studies are described of catchments where practical control measures have been introduced for agricultural herbicides. Research in progress to investigate the need to target control on "approved usage" or on small point sources such as spillages, overspray or disposal is discussed. As a minimum, pesticide users should be aware of the necessity of always adopting "best practice". Where user cooperation and good practice are insufficient, additional controls may be required and options available to the National Rivers Authority and, subsequently, the Environment Agency are presented.

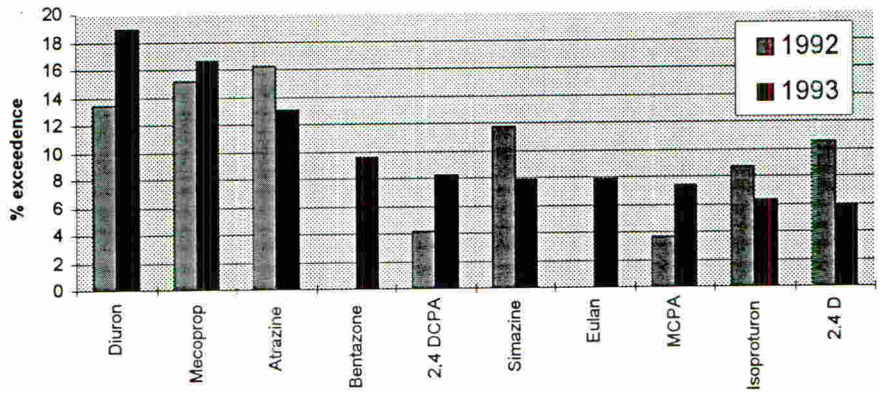
INTRODUCTION

Routine monitoring by the National Rivers Authority (NRA) (200,000 pesticide determinations a year) commonly detects the presence of low levels of pesticides in surface freshwaters, groundwaters and marine environments. Apart from pollution incidents concentrations rarely exceed 1 ug/l, although for some pesticides even these levels can be environmentally significant. Herbicides used for amenity, non-agricultural use are most frequently detected, e.g. diuron was found above 0.1 ug/l in 19% of samples throughout England & Wales in 1993 (NRA, 1995) . Atrazine and simazine are starting to decline following the ban on non-agricultural use in September 1993 with 16% and 12% of samples respectively exceeding 0.1 ug/l in 1992 reducing to 13% and 8% in 1993. Agricultural herbicides also feature strongly in the top ten list of pesticides detected routinely (Figure 1). The EC "Drinking Water" Directive standard of 0.1 ug/l applies legally only to treated drinking water (DoE *et al*, 1989) but is used as a convenient reference point for NRA data from untreated environmental waters. Water Company investment approaching £1 billion (Water Services Association, 1994) and annual running costs of £50 - 100 million ensure that treated drinking water has better than 98% compliance with the limit (DoE *et al*, 1994).

To assess the significance of pesticides in environmental waters NRA and the Department of the Environment (DoE) have an R&D programme to develop Environmental Quality Standards (EQSs) for common chemicals, including pesticides. Comparison of NRA monitoring data with EQSs shows that failures were recorded at only 3.8% of sites in England & Wales (NRA, 1995). The pesticides causing EQS failures (Figure 2) are markedly

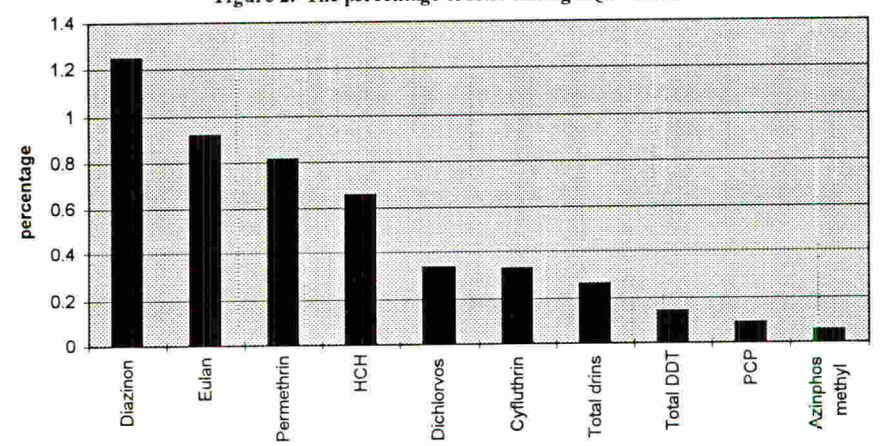
different from those most commonly detected (Figure 1), all but two being insecticides. Also the sources of many of the EQS failures are found to be industrial discharges from wool processing, carpet manufacture, timber treatment etc. Monitoring for Eulans (moth proofers) is targeted at sites downstream of known discharges, hence the occurrence in both Figures 1 & 2.

Figure 1. Pesticides exceeding 0.1 µg/l in Controlled Waters in 1992 & 1993



The relatively low level of EQS failure is encouraging but there is no place for complacency; only 120 pesticides are routinely monitored by NRA, although around 450 are approved for use. Only 20 pesticide EQSs were available to assess compliance in 1992 and 1993, but more have been proposed since. In particular no analytical methods or EQSs are available for the majority of agricultural fungicides which, because of their toxicity, might be expected to have an impact on the aquatic environment.

Figure 2. The percentage of sites failing EQS in 1993



To illustrate how control of pesticides can operate in practice three case studies of agricultural herbicides are presented together with a discussion of the precautions that users should take and some of the controls available to ensure that they do.

CASE STUDIES

1. Asulam in Yorkshire/Northumbria

"The Problem"
Much of the upland moorland in Yorkshire/Northumbria is used primarily as grouse moor and rough grazing. Bracken is an invasive, poisonous weed that competes with grass and heather and needs to be controlled. In recent years bracken control has commonly involved aerial spraying with asulam. This can result in spray drift and runoff reaching moorland streams some feeding moorland reservoirs, or draining into lowland rivers with drinking water abstractions lower down the catchment. In addition, some streams contain rare ferns that are highly sensitive to asulam.

Monitoring has shown that normal aerial spraying practice i.e. spraying whole areas of bracken without avoiding moorland streams, can result in exceedences of the 0.1 ug/l standard in small reservoirs. As these water supply sources had previously been unpolluted they often have little water treatment except simple filtration and chlorination. Installation of pesticide removal plant (usually some form of activated carbon treatment) in these situations is expensive and difficult to justify. If possible, prevention of pesticide pollution is preferable.

In the case of protecting rare ferns or other aquatic organisms, water treatment is not an option and pollution prevention is the only choice available.

"A Practical Solution"
Most moorland areas have a dense network of streams and drainage ditches. All parts of the moor can therefore be considered to be "near water". In these circumstances aerial spray operators need to consult the NRA prior to spraying to ensure that water pollution is avoided.

To avoid contamination from spray drift and direct overspray but still allow adequate bracken control, one solution is to establish 150m buffer zones. Work by English Nature (Cook, 1993) has shown that only around 1% of spray drift falls more than 150 metres from the sprayed area and that this is sufficient to avoid water pollution and give a safe "no effect" response for sensitive plants.

In detail, the moorland is first divided into catchments; those with no uses likely to be affected by asulam need no further control than that provided by the aerial certificate and the label recommendations. Those catchments that contain water supply reservoirs, drain to water supply rivers or have rare ferns needing protection are highlighted. Significant watercourses and reservoirs are identified and no aerial spraying is accepted within 150 metres of these. As this still leaves significant areas of bracken that would allow rapid re-colonisation of the moor, tractor spraying is allowed up to 6 m from the identified waters, up to a total of 20% of the total area within the 150 m protection zone.

This procedure protects against aerial spray drift in the 150 m zone; spray drift from tractor spraying is avoided by the 6 m buffer zone, and the risk of surface runoff is significantly reduced by only allowing 20% of the 150 m zone to be sprayed. On a 5 year rolling programme of bracken control this allows all bracken areas accessible to tractors to be sprayed. Any sizable remaining areas of bracken can be controlled by knapsack spraying or cutting.

This procedure is being assessed in summer 1995 and, when the monitoring results have been collated, it can be refined for future years by altering the width of the buffer zones or amending the percentage of the 150 m zone area that can be sprayed. There is every expectation that these measures will achieve the aim of preventing exceedances of the 0.1 ug/l pesticide standard in the drinking water reservoirs, protect the rare ferns and allow the landowners to control bracken without undue expense or inconvenience.

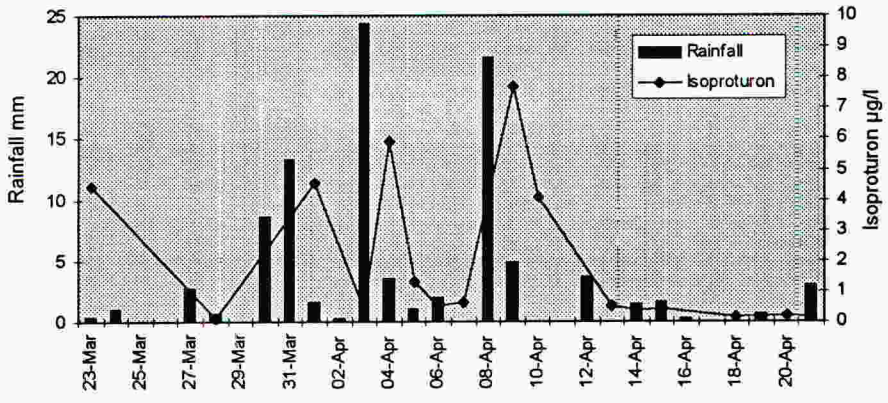
2. Isoproturon on the Isle of Wight

"The Problem"
The river Eastern Yar on the Isle of Wight has a water supply abstraction that provides around a third of the drinking water needs for the island. Its small catchment is largely arable with mainly silty soil, underlain by chalk. Heavy rain frequently causes soil erosion with roads being blocked and houses inundated in the worst cases. The high silt load in the river at these times causes difficulties with water treatment.

The autumn of 1993 was, in common with much of England, very wet on the island. This made it impossible to apply the normal autumn herbicides on winter cereals. Consequently, the use of isoproturon (IPU) was delayed until Spring 1994. This in itself was a wet season and most of the IPU used in the catchment was applied during a single two week period.

Further heavy rain following this application resulted in significant runoff of soil and IPU. The resultant IPU concentrations at the abstraction are shown in Figure 3.

Figure 3. The concentration of isoproturon and corresponding rainfall at Burnt House on the River Eastern Yar, Isle of Wight 1994



In the absence of pesticide removal treatment the Water Company closed the abstraction, to prevent pesticides entering the water supply. The alternative drinking water sources available are local boreholes which can be used only on a short term basis or a bulk supply from the mainland via an undersea pipeline. This latter source is expensive to use and at the time of the incident had its own water quality problems with *Cryptosporidium*.

"A Practical Solution"
Provision of alternative water resources would be expensive and installation of treatment, time consuming. It was therefore necessary to implement pollution prevention and control procedures to avoid repetition of the problem.

The immediate response was for the NRA to undertake a detailed survey of farmers in the catchment. This was done and, as far as could be ascertained, no one had had any spillages, used excessive amounts of IPU or disposed of pesticide washings, containers or surplus spray incorrectly. The conclusion, therefore, was that the pollution arose from normal, approved use of IPU followed by heavy rain onto already saturated soil.

A collaborative approach was adopted and at a meeting involving local NRA and TAPS staff, NFU and Rhône Poulenc (representing the IPU manufacturers), practical control options were discussed. These included;

- avoiding pre-emergence use of IPU,
- double sowing crops adjacent to water courses to provide maximum protection against soil erosion and surface runoff,
- avoiding use of IPU on saturated soils and when heavy rain is forecast,
- possible use of buffer zones alongside streams.

Subsequently the options available were put to farmers at a meeting in Autumn 1994. General agreement was reached that maximum care would be taken using pollution prevention measures as defined in relevant Codes of Practice (MAFF *et al*, 1990, MAFF *et al*, 1991, MAFF *et al*, 1993). It was noted, however, that use of IPU, particularly for blackgrass control was necessary and consistent with good agricultural practice in the catchment.

During the following 1994/5 winter no exceedences were recorded for IPU at the abstraction point. There were some failures for chlorotoluron, however, and it is possible that in minimising use of IPU more farmers have used chlorotoluron!

It will be interesting to follow the situation in further years to see if the reduction in IPU pollution can be maintained. This will be particularly significant in view of the 1995 review of IPU approval (MAFF, 1995) which introduced many of the controls considered for the Yar catchment; restriction to 2.5 kg/ha total IPU use (including mixtures), ban on pre-emergence use and consideration of water protection zones.

3. Atrazine in Southwestern Region

"The Problem"
Historically atrazine has been widely used for non-agricultural amenity weed control and has also had some use in agriculture. Following its ban from non-agricultural use approval was

modified and its remaining agricultural use is for weed control in maize. Effective weed control is essential for successful maize growing in England and atrazine is ideally suited to this purpose. Maize, unlike the majority of the weeds it competes with, has the ability to de-toxify atrazine which can therefore be applied pre and post emergence.

Ironically there has been a significant increase in the area of maize grown for silage in England as its nutritional advantages over grass silage have become apparent. The nutritional benefit, coupled with a significant subsidy under the Arable Area Payments Scheme (AAPS) resulted in a major increase in maize growing in 1994. The AAPS advantage has effectively been removed in the short term because the increased area grown in 1994 generated a massive setaside requirement for 1995. However, the advantages of maize over grass are still such that many farmers are continuing to plough up grass to plant fodder maize. This trend is prevalent in the South Western Region of NRA, particularly in Devon, Dorset, Wiltshire and Hampshire. Consequently, there has been a marked increase in atrazine exceedences in a number of surface and groundwater sources. Notably in the catchments of the Avon in Wiltshire/Hampshire and the Otter in Devon.

"A Practical Solution"

As with the IPU case, the first stage of finding a solution involved investigating the cause and finding the source. In the Avon catchment this involved field inspection with Water Quality staff effectively surveying the catchment by car and marking maize fields on a map. Obviously this was labour intensive and the next stage was to obtain satellite images of the catchment, "ground verify" the location of obvious large maize fields and then search the digital images for all fields with the same infra red colour signature. An 80% confidence analysis using this technique is relatively inexpensive. Further refinement is possible to give greater than 90% confidence but only at substantially increased cost. For NRA purposes the lower level of accuracy was sufficient to identify certain sub-catchments such as the Nadder had a very high density of maize fields. This mapping work provided useful guidance to optimise river sampling programmes and confirm the likely origin of much of the atrazine pollution. Monitoring then confirmed the occurrence of atrazine e.g. in the Nadder sub-catchment .

Although the atrazine exceedences were first noticed in surface waters one obvious first conclusion was that much of the maize growing was in aquifer areas. Sampling confirmed the presence of groundwater pollution. In some cases atrazine in farm wells was associated with maize growing in the immediately surrounding fields. In other situations with public water supply sources, intensive surveys of the groundwater protection zones revealed bad operational practice amongst atrazine users. In one location, for example, the spray tank mixing area was sited directly on chalk outcrop, all mixing was done on the same site and washings and small spillages of neat concentrate were allowed to drain away - into a small ditch, terminating in a swallow hole! This site has now been substantially improved but it is suspected that many similar situations still exist. It is too early to say whether this site was the sole or main cause of atrazine failure at the borehole but water quality does seem to be improving.

In surface water catchments atrazine peaks are noted following rain. If the number of maize fields is low and the intensity of rainfall is high then small amounts of atrazine will be diluted in the river and not reach the 0.1 ug/l limit. Problems arise when the density of maize growing is high or the rainfall is sufficient to provide runoff but not enough to give adequate

dilution in small catchments. A further factor is the geographic location of the maize fields in relation to the abstraction. In some situations large numbers of maize fields were found immediately upstream of water intakes. In these cases there is no time for dilution water to arrive from the rest of the catchment and atrazine peaks from local field runoff are common.

One obvious solution is to limit the amount of maize grown in particular areas. The NRA, however, has no direct powers to control land use and so has to use persuasion and cooperation as its main tools. In many cases this proves very effective.

Most farmers, when notified that they are polluting their own, or a neighbours, well by growing maize in adjacent fields will voluntarily change their practices. Options include using alternative fields away from the well, reverting to grass silage production or using alternative herbicides such as pendimethalin that are more expensive but less likely to leach.

On large farms, particularly those in close proximity to rivers and water supply intakes selection of maize fields away from the river is possible. To a limited extent it is also possible to stagger applications of atrazine so that rainfall events do not wash residues off all maize fields simultaneously.

The "solution" in this case study is at best only a partial one. It relies heavily on farmer co-operation and willingness to change planned land use or weed control practice. It remains to be seen over coming years whether the strategy is effective. Further increases in maize growing may necessitate controls on atrazine use or installation of pesticide removal plant at water treatment works.

BEST PRACTICE

Each of the above cases assumes that pesticides are used in accordance with label restriction and that the only residues reaching water are those arising from approved use i.e. true "diffuse" pollution.

On all farms close attention needs to be paid to preventing pollution arising at the storage, mixing, rinsing and disposal stages. Advice is available from NRA staff on pesticide pollution prevention and control centres on use of "best practice". To a large extent this is defined in Codes of Practice. Specifically, the "Green Code" (MAFF *et al*, 1990) for agricultural pesticide users and the "Yellow Code" (MAFF, 1990) for pesticide suppliers. Much useful guidance is also provided in the Codes of Good Agricultural Practice for Water, Soil and Air (MAFF *et al*, 1991, MAFF *et al*, 1993, MAFF *et al*, 1992). Further codes for non-agricultural pesticide users, timber treatment etc are also available. In addition, many organisations including NRA, MAFF, BAA etc have produced guidance leaflets highlighting the main ways in which pesticide pollution of water can be avoided. Much of this guidance is based on commonsense but is more obvious when it is pointed out!

Forward planning is the key to most successful pollution prevention measures. Pesticide stores should be designed to retain any spillages and to meet the requirements of the HSE guidance for farm stores, CS19, or the Food & Environment Protection Act, via BASIS registration and inspection for suppliers stores. Contingency plans should be devised for actions in the event of a major spillage or serious fire. Fire water retention systems are

essential in high risk areas. Stores should only contain sufficient pesticide for planned uses thus minimising the risk of retaining large stocks of chemicals with a high pollution hazard. Good stock control should ensure that only currently approved pesticides are held in store.

Careful use of pesticides includes selecting sensible sites for mixing that are adequately sited to avoid water pollution. Spillages should be minimised. If they occur, collection and addition to the mixing tank is the best option, if this is not feasible they should be soaked up with absorbent material such as sand, soil or "cat litter" type material. Container rinsings should be added to the spray tank. Wherever possible advantage should be taken of modern induction mixing, direct injection and automatic rinsing systems that minimise waste and reduce operator and environmental contamination. Spraying operations should involve care to avoid contamination of water with spray drift or direct overspray. Weather conditions should be suitable not just for the crop or pest control needs but also to minimise risk of pesticides being washed into water by imminent heavy rain.

When spraying is complete washing of spray equipment should be carried out away from water. Washings should ideally be sprayed back onto the crop if this can be done within the label restrictions. If not then dispose of onto a suitable area of uncropped land.

ENVIRONMENTAL CONTROL OPTIONS AVAILABLE

The main legislative powers available to the NRA are contained in the Water Resources Act 1991. Additional controls will also be introduced under the Environment Act 1995.

Water Resources Act 1991 (WRA)

Under the WRA it is an offence to discharge "poisonous, noxious or polluting matter" into any "controlled waters". Since all pesticides are to some extent "polluting" and as "controlled water" includes all groundwaters, estuarine, marine and surface freshwaters (except some small ponds) this should give very effective control over pesticide pollution. These powers are only retrospective, however, and can only be used to prosecute. Control of discharges is achieved by means of "consenting" with consent limits set as strictly as necessary to protect the aquatic environment. Increasingly the NRA is adopting programmes of pro-active site visits to advise on ways of avoiding pollution and to warn of the consequences of causing pollution.

Powers to "remedy or forestall pollution" are imperfectly drafted in the WRA and have only been used to recover costs of investigating and dealing with pollution once it has occurred.

Two further sections of the WRA allow the possibility of preventative control measures. At the discretion of the Secretary Of State, regulations can be introduced specifying particular control measures. So far these have only been used for control of storage of slurry, silage and fuel oil on farms. The second possibility is the use of Water Protection Zones (WPZs), again at the discretion of the Secretary of State. At the time of writing no WPZs have been introduced although a public enquiry has been held to consider a zone to protect water abstractions from the River Dee in North Wales. In view of current Government policy on de-regulation introduction of Regulations or WPZ measures will have to be fully justified and show a clear environmental benefit, outweighing any costs incurred.

Environment Act 1995

The main purpose of the Environment Act 1995 is to create Environment Agencies for England & Wales and for Scotland. These Agencies will come into being on 1 April 1996 and have powers and duties relating to protection of pollution of air, land and water. The Agencies are still in the process of being established and so as yet no pesticide control strategies or policies have been developed. It is anticipated that the current National Centre for Toxic And Persistent Substances (TAPS) in the NRA will continue in the Agency for England and Wales.

The Act contains relatively few new powers but one provision is the ability to serve "notices". As with any new legislation it is unclear precisely how the notice provision will work but in principle it could be used to require action to forestall pesticide pollution e.g. by bunding or re-siting high risk pesticide stores.

In addition to NRA powers, many other pieces of legislation are used to regulate pesticides, notably the Food and Environment Protection Act 1985 and the Control Of Pesticides Regulations 1986. One practical control measure that has already been introduced for some pesticides is a requirement for buffer strips alongside watercourses. These have been brought in at the recommendation of the Advisory Committee on Pesticides in the form of "no-spray zones" designed to protect against accidental over-spray. Since overspraying of water would be an illegal act there is concern that protection measures have been introduced in this way. The whole issue of "buffer strips" is currently being reviewed by the ACP and hopefully the review will revise the concept to take into account spray drift and runoff as additional routes for pesticides to reach water.

Future Pesticide Control Strategy

In its report "Pesticides in the Aquatic Environment" (NRA, 1995) the NRA have identified a series of recommendations for actions to reduce pesticide pollution in water. These centre on continuing co-operation between users and regulators. Emphasis is given to the development of buffer zones and larger WPZs if these can are found to be beneficial economically.

Development, by the TAPS Centre, of the risk assessment tool POPPIE (Prediction Of Pesticide Pollution In the Environment) will facilitate identification of catchments at risk from pesticides. Satellite image mapping (as used in the Avon catchment) coupled with the computer mapping facilities in POPPIE will further enhance targeting of pesticide monitoring and controls.

CONCLUSIONS

Low levels of pesticides are routinely detected in environmental waters in England and Wales. With respect to agricultural herbicides these rarely pose a threat to the environment, although more work needs to be done to confirm this. The main issue is low levels of pesticide in surface and groundwaters used as sources of public water supply. If the EC Drinking Water standard of 0.1 ug/l is likely to be exceeded then Water Companies have to install expensive pesticide removal treatment.

Practical options for reducing pesticide levels in water by controls on use and adoption of best practice have been discussed. In three case studies, two have shown significant reductions in pesticides in water whilst the third offers the possibility of fully effective control obviating the need for pesticide removal treatment plant.

In reality the need for absolute compliance with the Pesticide Parameter means that Water Companies are unlikely to rely solely on external controls. Any reduction in pesticide concentrations however could reduce the capacity required for capital plant and allow significant savings in operating costs. A combined approach of installed treatment providing insurance against accidental or unknown pesticide entries to water, together with rigorous field control is likely to be the best option. In the longer term greater use of buffer zones and introduction of selective water protection zone measures could provide additional environmental controls.

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REDUCING HERBICIDE RUNOFF: ROLE OF BEST MANAGEMENT PRACTICES

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ABSTRACT

Agricultural Best Management Practices can reduce runoff of herbicides to surface water, reducing chances of exceeding drinking water standards and avoiding the need for water treatment by utilities. Conservation tillage systems reduce herbicide runoff, as surface crop residue reduces erosion, slows runoff, and increases infiltration. When all historical data (1970-1990) are summarized, no-till systems reduced herbicide runoff by an average 70%, when compared to the moldboard plough. Studies we have conducted have confirmed these results, with herbicide runoff sometimes totally eliminated by no-till, due to complete water infiltration. Vegetative buffer strips reduce herbicide runoff by trapping sediment and increasing water infiltration. Historical data show that buffers can reduce herbicide runoff by up to 90%. Our studies have shown that the efficiency of herbicide removal by buffers varies depending on antecedent moisture, runoff volume,

and herbicide concentrations. Considering all available data, buffers removed 48% of herbicides contained in runoff, ranging from 9 to 91% removal.

INTRODUCTION

Several soil-applied herbicides (alachlor, atrazine, cyanazine, metolachlor, metribuzin, and simazine) are detected in surface waters in the United States, primarily in the Midwestern and Chesapeake Bay states. Historical monitoring data indicate detections and concentrations are highest in the spring, after the first major runoff event following herbicide application, and lower during other periods of the year.

Since 1993, United States' drinking water utilities on surface water test quarterly for several pesticides under the Safe Drinking Water Act. If the annual average concentration exceeds the Maximum Contaminant Level standard, the U.S. Environmental Protection Agency and the States can direct the utility to treat the water or reduce the concentration in the source water.

Agricultural Best Management Practices (BMP) can reduce herbicide entry into surface water and avoid the need for water treatment by utilities. This paper summarizes historical (1970-1990) and recent (1991-1994) tillage and buffer strip research in reducing herbicide surface runoff losses.

CONSERVATION TILLAGE

Historical data

Studies published through 1990 comparing herbicide runoff under conservation tillage systems to conventional tillage have previously been summarized and analyzed (Fawcett et al., 1994) . Many studies comparing herbicide runoff under different tillage systems have utilized rainfall simulation techniques on small plots. Almost always very heavy rainstorm events are simulated (such as once-in-50-year or once-in-100-year events) within about a day of herbicide application. Under these conditions, herbicides are washed from surface crop residue and may become a part of overland flow before infiltrating into the soil. Higher concentrations of herbicide in runoff may offset lower runoff volumes so that total herbicide runoff is sometimes similar or greater with conservation tillage than within conventional tillage utilizing the moldboard plough. When published rainfall simulation study data up to 1990 were summarized (Fawcett et al., 1994), all conservation tillage systems reduced runoff of pesticides by an average of 23%, compared to ploughing (99 treatment-site-years of data). Considering only no-till studies, pesticide runoff was reduced by an average 34% (29 treatment-site-years of data).

Conservation tillage systems have usually been shown to have greater benefit in reducing herbicide runoff in natural rainfall studies. Under natural rainfall conditions, usually small rains occur first after herbicide application, washing herbicides off crop residue and into the soil, before heavier runoff -producing rains occur. Natural rainfall studies are also more likely to be conducted on watersheds with more than a one year history tillage treatment, unlike simulation studies which are often short term. Benefits of greater water infiltration with conservation tillage are more likely to occur over several years. For example, in a Maryland study (Glenn & Angle, 1987), atrazine runoff was reduced by 29% by no-till in the first year of the study, but by the third and fourth year runoff was reduced by 100% due to elimination of any water runoff.

The summary of natural rainfall studies conducted up to 1990 (Fawcett et al., 1994) showed that on the average, no-till reduced herbicide runoff by 70%, compared to moldboard ploughing (32-treatment-site-years of data). No-till reduced herbicide runoff in 29 out of the 32 cases.

Current studies

In 1994 herbicide runoff under natural rainfall conditions was studied at locations in Iowa, Nebraska, and Missouri (Franti *et al.*, 1995). At the Iowa and Nebraska locations, tillage treatments were accomplished by one pass of a shallow tillage tool in sub-basins having a long-term history of no-till. At the Missouri location, long-term tillage sub-basins were compared to long-term no-till sub-basins.

At the Missouri location, no water or herbicide runoff occurred from the no-till treatments with the first two rain events producing runoff from tilled treatments. A small amount of runoff occurred from all treatments with a third rainfall event. Totaled over the 3 events, no-till reduced total herbicide runoff by 94 and 91% for cyanazine and atrazine, respectively. Total runoff volume was reduced by 72%.

At the Iowa site, the single tillage pass on long-term no-till did not significantly change water infiltration or herbicide runoff. Total runoff was similar for both tillage systems although herbicide concentrations were higher from no-till. At the Nebraska site where only one small runoff event occurred, no-till reduced runoff by 65% and 67% for cyanazine and atrazine, respectively.

These studies confirm conclusions made in the previous studies. Conservation tillage usually reduces herbicide runoff due primarily to increases in water infiltration. Benefits of conservation tillage may take several years to become evident, and one shallow tillage operation may not eliminate improvements in water infiltration gained from long-term no-till. On soils where water infiltration is limited due to a claypan or high clay soil, conservation tillage may not increase water infiltration or reduce herbicide runoff. Site conditions will need to be considered to determine if conservation tillage is an appropriate surface water BMP. Alternative BMPs such as incorporation of herbicides may be more appropriate on some soil types.

BUFFER STRIPS

Historical Data

Attempts to use computer models to predict the effectiveness of vegetative buffer strips in removal of contaminants have assumed that buffer strips only remove sediment without affecting water infiltration (Flanagan *et al.*, 1986, Nicks *et al.*, 1991). Thus these models predict that removal of moderately adsorbed pesticides would be minimal. However, recent studies have shown that buffer strips have significant impacts in increasing water infiltration, thus trapping dissolved pesticides within the strips. This phenomenon explains why controlled field studies have shown reductions in runoff of herbicides such as atrazine by buffer strips despite the fact that sediment-bound herbicide accounts for only a small percentage of herbicide contained in runoff.

A Pennsylvania study (Hall *et al.*, 1983) used a 6 m-long area seeded to oats at the base of 22 m-long plots planted to maize and treated with atrazine. Season-long runoff of atrazine was reduced by 91 and 65% by the oats strip at application rates of 2.2 and 4.4 kg/ha. In studies where runoff from a small watershed was directed down a 24 m-foot grassed waterway (Asmussen *et al.*, 1977, Rhode *et al.*, 1980) trifluralin runoff losses were reduced by 86 to 96%, and 2,4-D runoff was reduced by 70%.

Runoff losses of metolachlor and metribuzin were reduced by 50 to 75% by a grass buffer strip in a Mississippi study (Webster *et al.*, 1993). The buffer strip was 2 m wide, and the plots were 23 m long. Much of the reduction in herbicide runoff was attributed to greater water infiltration in the grass strip. In a later Mississippi study (Murphy *et al.*, 1995), buffer strip widths were varied from 0.5 to 4 m below a 22 m long soybean plot. Total annual runoff losses of metolachlor and metribuzin were reduced by an average 56%. There were no clear trends with changing buffer widths.

In France (Anon., 1994) runoff of isoproturon from wheat on a 6-10% slope was reduced 98% by a 6 m ryegrass buffer and 100% by a 12 m buffer. Runoff of diflufenican was reduced by 90% and 99% by the 6 m and 12 m buffer, respectively.

A Nebraska study compared pesticide concentrations in storm runoff from agricultural land in four first-order tributaries within a watershed (Langan *et al.*, 1994). These tributaries were characterized as to riparian cover. Cover ranged from no trees or shrubs and only 25% herbaceous cover to 65% tree cover and 73% herbaceous cover. Although pesticide use in the watershed was not documented, there was a strong trend of detecting the highest concentrations of pesticides, including atrazine, in tributaries with the least riparian cover.

Buffers and riparian zones may not only reduce surface runoff of herbicides, but also intercept pesticides and nitrate in subsurface flow, preventing entry into surface water. In Iowa, multispecies riparian buffer strips were constructed

along a stream (Schultz *et al.*, In press). Four or five rows of fast growing trees were planted adjacent to the stream bank; 2 rows of shrubs were planted next; and a 7 m strip of switchgrass was seeded adjacent to the agriculture field. Piesometers were installed to measure atrazine and nitrate concentrations in the vadose zone in the corn field and within each species of the buffer strip. On June 29, atrazine concentrations were 4.0, 3.5, 2.1, and 1.8 ppb in the corn field, switchgrass, shrub strip, and tree strip, respectively. On August 23, atrazine concentrations were 2.6, 0.9, 0.4, and 0.2 ppb in the corn field, switchgrass, shrub strip, and tree strip, respectively.

Current studies

Studies were conducted in Iowa and Texas to investigate the effectiveness of buffer strips in reducing runoff of atrazine and other herbicides and to determine the mechanisms of herbicide removal.

The effectiveness of bermudagrass and wheat buffer strips in reducing herbicide runoff has been studied at Temple, Texas for the past 3 years (Hoffman, 1995). Three 9.1 m wide strips of either bermudagrass or winter wheat were established 0, 43, and 88 m uphill from the base of the slope within a 133 m wide watershed planted to maize and compared to similar watersheds with no buffer strips. Studies conducted in 1992 indicated that herbicides were being intercepted by the buffer strips, with highest concentrations of herbicide detected in soil in the strips at the bottom of the slope adjacent to the catchment wier. Hydrologic data in 1993 showed that total water runoff volume was reduced over 57% by bermudagrass strips and 50% by wheat strips. Total atrazine loss in 3 events was reduced by 30% by bermudagrass and 57% by wheat. In 1994 two runoff events occurred during the same day with the second event generating greater runoff volumes. Atrazine runoff was reduced by 44-50% by the buffer strips.

Effectiveness of grass buffer strips were studied in Iowa under natural and simulated rain conditions. In a 1992 simulation study (Mickelson & Baker, 1993) field runoff was simulated by adding known concentrations of atrazine to water, based on previous measurements of actual field runoff. Runoff calculated to simulate
runoff from an area 46 m long was applied to the top of 4.6 m and 9.1 m long grass buffer strips. Thus ratios of treated area to filter strip were 10:1 and 5:1, respectively. A rainfall simulator was used to apply rainfall to the filter strip as simulated runoff was added. The 4.6 m buffer strip reduced atrazine runoff by 35%, while the 9.1 m strip reduced runoff by 59.5%.

In a 1993 simulation study using similar techniques (Misra *et al.*, 1994) runoff with concentrations of either 0.1 or 1.0 mg/liter atrazine was applied to filter strips in amounts calculated to represent relative drainage area to buffer strip areas of 15:1 and 30:1. Atrazine removal by the 15:1 ratio strip was 31.2% for 0.1 mg/liter inflow and 49.8% for 1.0 mg/liter inflow. Atrazine removal by the 30:1 ratio strip was 26.4% for 0.1 mg/liter inflow and 47.8% for 1.0 mg/liter

inflow. Removal was due both to infiltration of water and herbicide adsorption. An average 38% runoff water infiltrated into filter strips with the 15:1 ratio, while 32% of water infiltrated into strips with 30:1 ratio.

A 1994 simulation study (Misra, 1994) applied atrazine, cyanazine, and metolachlor at concentrations of 0.1 or 1.0 mg/liter to bromegrass buffer strips in amounts calculated to represent drainage area to buffer strip areas of 15:1 and 30:1. Rainfall at a rate of 6.35 cm/h was applied to the strips during simulated runoff. Considering both added simulated runoff and applied rainfall, 39.2% of added water infiltrated into the strips at the 15:1 area ratio, and 33.3% infiltrated at the 30:1 area ratio. Infiltration of water accounted for most of the reduction in herbicide runoff. At the inflow concentration of 0.1 mg/liter the 15:1 area ratio reduced herbicide runoff by 31.2%, 31.5%, and 30.1% for atrazine, metolachlor, and cyanazine, respectively. At inflow concentrations of 0.1 mg/liter, the 30:1 area ratio reduced herbicide runoff by 26.4%, 27.4%, and 25.6% for atrazine, metolachlor, and cyanazine, respectively. At the 1.0 mg/liter inflow concentration, the 15:1 area ratio reduced herbicide runoff by 49.8%, 46.8%, and 46.6% for atrazine, metolachlor, and cyanazine, respectively. At the 1.0 mg/liter inflow concentration, the 30:1 area ratio reduced herbicide runoff by 47.5%, 41.8%, and 42.4% for atrazine, metolachlor, and cyanazine, respectively.

A separate study (Misra, 1994) using similar techniques was conducted in 1994 to compare bromegrass vegetated buffer strips with bare ground buffer strips. Herbicides were applied in simulated runoff at the concentration of 1.0 mg/liter. Soil was added to some simulated runoff at a concentration of 10,000 mg/liter to represent eroded sediment. Simulated runoff was added to the filter strips in amounts calculated to represent a 15:1 drainage area to buffer strip ratio.

Infiltration of simulated runoff water and rainfall was 49.1% into bare soil strips and 83.1% into vegetated strips when no sediment was included. When sediment was included in runoff, total water infiltration was 29.8% into bare soil strips and 54.3% into vegetated strips. Thus more infiltration occurred into vegetative strips, while sediment in runoff reduced water infiltration.

In absence of sediment, vegetated strips removed 85.2% of atrazine, 82.6% of metolachlor, and 84.1% of cyanazine. With sediment, vegetated strips removed 53.6% of atrazine, 53.3% of metolachlor, and 57.5% of cyanazine. In absence of sediment, bare strips removed 50.7% of atrazine, 45.2% of metolachlor, and 50.1% of cyanazine. With sediment, bare strips removed 33.5% of atrazine, 27.5% of metolachlor, and 35.6% of cyanazine.

In an Iowa natural rainfall study (Arora *et al.*, 1993), runoff from a maize field was collected and distributed to 20 m grass buffer strips in amounts equaling drainage area to buffer area ratios of 15:1 and 30:1. Results for the first runoff event after herbicide application showed that atrazine removal was 12.5% for the 15:1 area ratio and 9.3% for the 30:1 area ratio. Wet antecedent soil conditions encountered during this study may have reduced water infiltration into buffer strips, diminishing effectiveness in reducing herbicide runoff. Average infiltration

of rainfall and inflow for the 15:1 and 30:1 drainage area ratio were only 13% and 3.9%, respectively.

Analysis of soil within the filter strips at this site (Hatfield, 1995) confirmed that the herbicides were being trapped and held by soil within the strips. After the first runoff event, 20% of the atrazine and cyanazine and 25% of metolachlor from runoff was recovered in the soil and surface organic matter layer. There was a decline in the herbicide concentrations in the buffer strip as the season progressed, presumably due to degradation. Concentrations of herbicide in the upper 2 cm of soil in the treated cornfield on June 15 were 4,800 ppb atrazine, 11,800 ppb metolachlor, and 4,800 ppb cyanazine. Within the filter strip on June 15 (following the first runoff event) herbicide concentrations in the upper 2 cm of soil were 750 ppb atrazine, 2,300 ppb metolachlor, and 730 ppb cyanazine. These concentrations of herbicide did not have a deleterious effect on the bromegrass vegetation.

The natural rainfall filter strip study was repeated in 1994, but data on filter strip efficiency are not yet available. Soil samples within the filter strip were analyzed as in 1993, with samples taken at upslope and downslope positions within the filter strip (Hatfield, 1995). Herbicide concentrations were higher in the upslope samples. Concentrations of atrazine collected on plant residue within the filter strips ranged from 80 ppb to 740 ppb, depending on date. These concentrations were similar to those found in upper soil. This suggests that organic plant residue on the soil surface contributes to the effectiveness of the filter strip.

SUMMARY

When all published studies through 1990 investigating herbicide runoff under natural rainfall conditions were summarized, no-till, ridge-till, and chisel plow tillage reduced herbicide runoff by 70%, 42%, and 69%, respectively. More recent studies have produced very similar results. When all published data for all conservation tillage systems in natural rainfall studies are averaged, conservation tillage reduced herbicide runoff by 60% (Table 1). The effectiveness of surface crop residue in reducing herbicide runoff will depend on site and weather conditions. Low soil permeability due to conditions such as claypans and heavy rains soon after application will reduce the efficiency of herbicide removal.

Buffer strip studies have also consistently shown reductions in herbicide runoff. Efficiencies of herbicide removal have varied depending on soil type, soil water content, runoff volume, buffer width, and buffer vegetation. When all published data in buffer studies are averaged (46 data points), buffers reduced herbicide runoff by an average 48% (Table 1) . The range of removal was 9% to 91%. Because many of the buffer studies were small scale, they may overestimate herbicide removal.

It is not known to what extent reductions in herbicide runoff caused by BMPs are additive. However, use of conservation tillage and buffers, along with other conservation practices in a systems approach, should produce greater reductions in herbicide runoff than would be expected with a single practice. These reductions in herbicide runoff should reduce concentrations of herbicides detected in surface water and reduce the need for treatment of water to meet drinking water standards.

Table 1. Summary of data from published studies comparing herbicide runoff with conservation tillage systems (greater than 30% crop residue cover) to moldboard ploughing under natural rainfall conditions, and investigating herbicide removal by buffer strips under natural or simulated rainfall.

Practice	Date Points Number	Herbicide Range % Removal	Herbicide Average % Removal
Conservation Tillage	54	-98 to 100	60
Buffer Strips	46	9 to 91	48

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MANAGEMENT PRACTICES FOR REDUCING MOVEMENT OF PESTICIDES TO SURFACE WATER IN CRACKING CLAY SOILS

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ABSTRACT

The most important mechanism for movement of pesticides from non-point sources to surface water in cereal-growing areas of the U.K. is rapid movement through the soil profile via macropore flow with drainage into nearby ditches and other surface-water bodies. Phase III of the Brimstone Farm experiment was established to develop management practices that reduce these losses. This four-year study is a collaborative government- and industry-funded research programme being conducted on the extensively instrumented Brimstone Farm facility developed jointly by ADAS and Rothamsted. Studies conducted during the first two years have examined the effect of drainage restriction, application rates, soil tillage, soil sealants, and pesticide sorption properties. Losses of triasulfuron were similar in the first and second years although isoproturon losses were quite different (whether this was the result of different soil moisture conditions at the time of application will be investigated further). The sorption coefficient of the pesticide to soil also influenced losses. Losses between full- and half-rate isoproturon applications, expressed as a percent of applied, were not significantly different when the variability of the drainflow was taken into account. Drainage restrictors reduced losses of less mobile pesticides by about 25 %. Current work is not sufficient to draw definite conclusions about the effects of soil sealants and soil tillage, but results are promising enough to warrant additional investigation.

INTRODUCTION

Macropore flow can rapidly transport pesticides to sub-surface drainage (Harris, 1995). This appears to be the most important mechanism accounting for the appearance of crop protection chemicals from non-point sources in surface water in cereal growing areas of the U.K. (Jones,

1993). A joint four-year research programme is being sponsored by MAFF Pesticides Safety Directorate and the British Agrochemicals Association to develop effective management practices for reducing losses due to this mechanism. Principal researchers for the project are ADAS and IACR-Rothamsted with water analyses conducted by CSL MAFF and BAA member companies. This paper presents the results from the first two years of experiments.

MATERIALS AND METHODS

Brimstone Farm

The ADAS/Rothamsted collaborative facility at Brimstone Farm, Oxfordshire (Cannell et al., 1984; Harris et al., 1994) was established in 1978 to study drainage in clay soils. The soil at the site is a heavy, structured clay (60 % < 2 μm) of the Denchworth series, having pH ~ 7.0 (0.01M $CaCl_2$) and containing 4.5 % organic matter. The site is at an altitude of 100-106 m OD, receives an average annual rainfall of 686 mm, and is representative of many cereal-growing regions of central England. There are 20 hydrologically isolated and highly instrumented plots of 0.19 ha each, 16 of which are being used for this study. Concurrent with the pesticide study, there is a companion study on the behaviour of soil nutrients.

Drainage in this soil is provided by pipe drains at 0.9 m depth with permeable backfill to within 0.35 m of the ground surface. A secondary drainage system is drawn at right angles to the pipe drains at 0.55 m depth and 2 m spacing on each of 12 plots (termed the core plots). On eight of these plots, this secondary system consists of conventional mole drains, drawn on different plots in 1992, 1993, and 1994; two more plots (numbered 1 and 15) have gravel-filled moles and two (7 and 9) have close-spaced pipes, all installed in 1988. On four more plots (termed the pilot plots) drainflow is collected only from two pairs of mole drains; because water flowing in cracks between mole drains is not collected on these plots, the flow is only approximately 60 % of that recorded in the collector drains on the core plots.

Drainflow and surface flow were measured continuously using V-notch weirs (Cannell et al., 1984). The water table was monitored in each plot using capacitance probes. All data were collected on a data logger and automatically transferred by remote telemetry to a base station.

Experimental Design

Different experiments were conducted on the core and pilot plots. The 12 core plots, with four-fold replication, were used to test treatments not expected to influence the crop nor affect the nutrient studies. The four pilot plots were used to test a range of more speculative ideas with less replication and a wider range of pesticides. Pesticides were chosen (Table 1) to span a range of sorption properties, expressed as Kd and Koc representing sorption to soil and to soil organic carbon respectively. In order to facilitate interpretation of results, only pesticides of at least moderate persistence were utilised.

The treatments are described in Table 2. Drainage restrictors were expected to encourage the closure of cracks in the soils and reduce pesticide losses due to increased sorption and water storage. The half-rate treatment examined whether pesticide losses are decreased at least proportionally to the decrease in application rate. Soil tillage (surface and deep) disturbs existing macropores and changes the soil structure, decreasing the rate at which water flows to

Table 1. Pesticide sorption properties, application rates and usage.

Pesticide	Koc (l/kg)	Full rate (g/ha)	Applied to core plots 1993/94	Applied to core plots 1994/95	Applied to pilot plots 1993/94	Applied to pilot plots 1994/95
Triasulfuron	9	7.5	no	yes	yes	yes
Isoproturon	125	2500	yes	yes	yes	yes
Propiconazole	568	250	no	no	no	yes
Prochloraz	895	405	no	no	yes	no
Pendimethalin	5000	2000	yes	no	yes	no

Note: Due to the use of a different formulation, the full rate for isoproturon in the core plots in 1993/94 was 2438 g/ha.

the mole drains. The soil sealant had the potential to decrease macropore flow by plugging soil cracks and decreasing infiltration of water into the soil. Incorporation of pesticides into surface soil was expected to decrease their contact with rapidly-infiltrating water.

Methods

Normal agricultural practices were followed in the development of the seed bed and the maintenance of the crop. The soil sealant was Vinamul 3270, a water-based emulsion of a vinyl acetate-ethylene copolymer, applied at a rate of 370 l/ha. A Paraplow was used to provide deep tillage in the layer 10-35 cm below ground surface.

Table 2. Treatments used in the experiments.

Year	Plots	Treatment
1993/94	Core	Restricted drainage, full-rate pesticide application
		Control: full-rate pesticide application, normal drainage
		Half-rate pesticide application, normal drainage
	Pilot	Control: cultivate in straw, prepare seedbed, drill, spray
		Cultivate in straw, prepare seedbed, drill, spray pesticides then soil sealant
		Cultivate in straw, prepare seedbed, spray, incorporate, drill
		Plough in straw, prepare fine seedbed, drill, spray
1994/95	Core	Restricted drainage, normal tilth
		Control: normal drainage, normal tilth
		Fine tilth, normal drainage
	Pilot	Deep loosen, plough, spray
		Deep loosen, plough, spray pesticides then soil sealant
		Plough, spray pesticides then soil sealant
		Control: plough, spray

Drainage restrictors installed on the 12 core plots consisted of rotatable U-bends, which prevented drainage leaving the plots until the water table was raised to the height of the U-bends (10 cm below ground surface during the trials conducted to date). For plots where the water table was raised using the drainage restrictor, its position was measured immediately upslope of the drainage restrictor, in addition to the normal water-table measurements in the plot.

EPIC programmable samplers collected flow-related water samples from the plot drains on the control plots and from mole drains on the pilot plots, and delivered them through Teflon-lined tubing to darkened-glass bottles. Samples were stored at 4°C until extracted and analysed. Those from the core plots were analysed individually to provide information on concentrations during drainage events while samples from the pilot plots were combined in volumes proportional to the amount of drainflow to provide a single sample for each drainage event.

RESULTS AND DISCUSSION

Limitations of space do not permit a detailed presentation of the data, so this section presents the most relevant data and a general discussion of the results. In order to keep the discussion as generic as possible, the pesticides are characterized by Koc and pesticide losses are expressed as percent of applied. Amounts lost and the mean concentrations can be derived from the application rates (Table 1) and the drainflows (Tables 3 and 4).

Weather Patterns and Drainage Events

Autumn rainfall patterns during the two years were quite different, although wet conditions were experienced during both of the years. In 1993 the onset of drainflow in mid-November was unusually early and occurred shortly after the pesticide applications on November 2 and 4. Rainfall of 10.6 mm on November 13-14 created the first drainage event. This and three further drainage events (starting on December 8 and 17 and January 1) were sampled.

Autumn 1994 was notable for the wet period from October 19, in which rain fell on 20 out of 25 days, giving little opportunity for drying of the soil surface and so causing difficulty in preparing an adequate seedbed. Occasional drainflow occurred from some of the plots before the pesticides were applied on November 17. A period of relatively dry weather followed for 16 days until rain on December 4 and 5; more rain on December 8 and 9 then produced further drainflow. Three major drainage events were sampled, occurring December 8-9, 28-29, and January 21-22.

Drainflows and Pesticide Losses

A summary of the drainflows and pesticide losses is presented in Table 3 for the core plots and Table 4 for the pilot plots. Since mole flow, as measured and sampled on the pilot plots, contributes only approximately 60 % of total drainage, drain flows and pesticide losses are not directly comparable between core and pilot plots. In spite of extensive efforts to ensure hydrologic similarity among plots before the imposition of the experimental treatments, considerable variation in drainflow complicated interpretation of the results. This variation could have resulted partly from the different ages and types of mole drains.

Table 3. Summary of experimental results from the core plots. Seasonal drainflows and surface flows are the totals for the period November to March. Event drainflows and pesticide losses are for the three or four drainage events that were sampled from the plot drains.

Year	Treatment	Measurement	Values of plot replicates				Mean
1993/94							
	Drainage restrictors		plot 1	plot 6	plot 7	plot 10	
		Seasonal drainflow (mm)	310	164	150	202	206
		Surface flow (mm)	9.8	8.5	7.4	9.7	11.8
		Event drainflow (mm)	95	63	42	74	68
		Isoproturon losses (%)	2.73	2.31	0.96	3.21	2.30
	Control		plot 5	plot 9	plot 15	plot 20	
		Seasonal drainflow (mm)	235	203	364	278	270
		Surface flow (mm)	3.8	2.9	2.5	13.6	7.6
		Event drainflow (mm)	70	70	111	90	85
		Isoproturon losses (%)	1.99	3.80	2.91	4.53	3.31
		Pendimethalin losses (%)	0.016	0.040	0.039	0.039	0.033
	Half rate		plot 4	plot 16	plot 18	plot 19	
		Seasonal drainflow (mm)	329	158	215	264	241
		Surface flow (mm)	6.4	49.8	11.2	1.3	22.9
		Event drainflow (mm)	95	45	71	77	72
		Isoproturon losses (%)	1.78	1.89	1.54	1.32	1.63
		Pendimethalin losses (%)	0.022	0.026	0.013	0.023	0.021
1994/95							
	Drainage restrictors		plot 1	plot 6	plot 7	plot 10	
		Seasonal drainflow (mm)	354	135	264	77	208
		Surface flow (mm)	19.8	10.0	13.8	70.7	28.6
		Event drainflow (mm)	74	38	52	16	45
		Triasulfuron losses (%)	7.51	1.13	4.55	1.07	3.56
		Isoproturon losses (%)	0.35	0.084	0.15	0.0001	0.15
	Control		plot 5	plot 9	plot 15	plot 20	
		Seasonal drainflow (mm)	184	228	270	80	191
		Surface flow (mm)	4.4	4.0	5.8	4.0	4.6
		Event drainflow (mm)	36	55	58	16	41
		Triasulfuron losses (%)	3.01	4.42	4.53	2.28	3.56
		Isoproturon losses (%)	0.17	0.34	0.24	0.061	0.20
	Fine tilth		plot 4	plot 16	plot 18	plot 19	
		Seasonal drainflow (mm)	168	221	243	194	207
		Surface flow (mm)	39.7	4.1	27.1	5.6	19.1
		Event drainflow (mm)	32	40	44	36	38
		Triasulfuron losses (%)	2.91	13.39	6.51	3.37	6.55
		Isoproturon losses (%)	0.05	0.36	0.28	0.21	0.23

Table 4. Summary of experimental results from the pilot plots. Values for seasonal drainflows are the totals for the period November to March, whereas event drainflows and pesticide losses are for the three or four drainage events that were sampled from the mole drains.

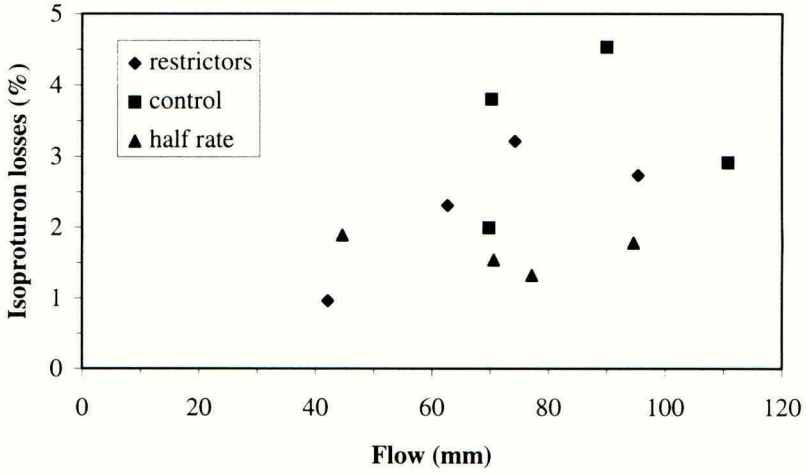
Year	Measurement	Plot 2	Plot 8	Plot 11	Plot 12
1993/94	Treatment	fine seedbed	soil sealant	control	incorporation
	Seasonal drainflow (mm)	128	77	116	160
	Event drain flow (mm)	66	37	54	67
	Triasulfuron losses (%)	5.2	2.9	4.8	5.2
	Isoproturon losses (%)	2.4	2.1	3.2	2.6
	Prochloraz losses (%)	0.05	0.02	0.05	0.05
	Pendimethalin losses (%)	0.02	0.009	0.02	0.04
1994/95	Treatment	control	deep plough, soil sealant	deep plough	soil sealant
	Seasonal drainflow (mm)	158	110	95	86
	Event drain flow (mm)	39	24	21	17
	Triasulfuron losses (%)	3.3	2.6	2.0	1.7
	Isoproturon losses (%)	<0.005	<0.003	<0.003	<0.003
	Propiconazole losses (%)	0.012-0.013	0.005-0.007	0.012	0.0007-0.006

Management practices could decrease the amount of pesticide losses either by reducing drainflow or by reducing the pesticide losses without influencing drainflow, or by a combination of both effects (such as might be achieved by soil-tillage). As shown in Figures 1 and 2, increased drainflow generally resulted in increased pesticide losses. In 1993/94 the relationship between drainflow and isoproturon losses (Figure 1) seems the same for the plots with the restricted drainflow and the control plots, but the drainflows and hence isoproturon losses are significantly less for the former. Figure 2 presents an example of decreased pesticide losses without a change in drainflow between the restricted and control plots. Although drainflow is similar, the relationships between flow and isoproturon losses on these two sets of plots are slightly different.

Effects of Variables

The sorption properties of the pesticides (as described by the Koc and Kd values in Tables 1 and 5) affected their losses in drainage water (Table 5). Results from the core and pilot plots in 1993/94 indicated that pesticide losses in drainage decreased with increasing Koc, although the losses of triasulfuron were only about twice those for the more strongly sorbed isoproturon. However, in the 1994/95 trials on the core plots, triasulfuron losses were about 20 times greater than the losses of isoproturon. The core plots had nearly twice the drainflow of the pilot plots and similarly greater losses of triasulfuron in the second year; this is consistent with the smaller amount of drainage water recovered directly from the moles on the pilot plots. However, the reason for the considerable differences between core and pilot plots in 1994/95 in isoproturon

Figure 1. Effect of management practices on drainflow and losses of isoproturon for the four sampled drainage events on each of the core plots during 1993/94.



losses is unknown. Analytical problems are suspected as the most likely cause, although there is a possibility that the differences result from the different collection point for water samples in core and pilot plots. Different laboratories and procedures were used for pesticide analyses on the core and pilot plots. However an inter-laboratory comparison of four duplicate spiked samples at different concentrations in 1994/95 showed no substantial difference between the two laboratories.

Figure 2. Effect of drainflow on losses of isoproturon in the first drainage event on each of the core plots in autumn 1994.

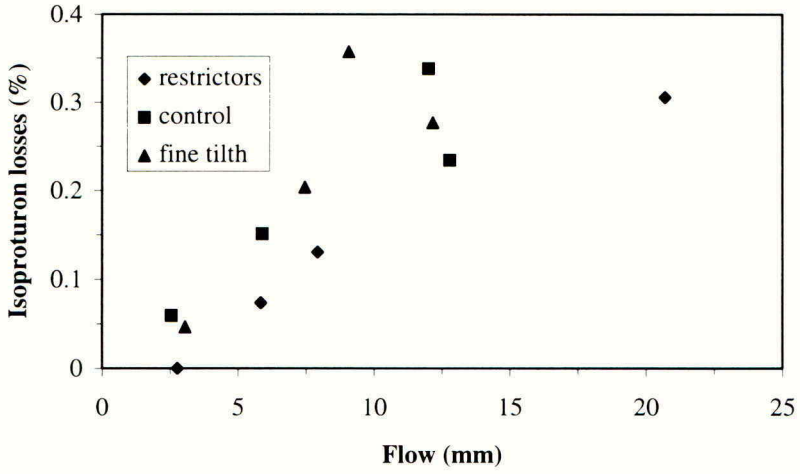


Table 5. Effect of sorption on pesticide losses in drainage. Kd values are measurements on Brimstone soil. NA indicates that the pesticide was not applied.

Pesticide	Kd (l/kg)	Pesticide losses in drainage (% of applied) 1993 - 1994 Core	1993 - 1994 Pilot	1994 - 1995 Core	1994 - 1995 Pilot
Triasulfuron	0.43	NA	2.9-5.2	5.7-9.4	1.7-3.3
Isoproturon	2.9	1.7-3.3	2.1-3.2	0.17-0.29	<0.005
Propiconazole	19.2	NA	NA	NA	0.0007-0.013
Prochloraz	75	NA	0.02-0.05	NA	NA
Pendimethalin	88	0.02-0.02	0.009-0.04	NA	NA

The differences in the pesticide losses between the 1993/94 and 1994/95 trials indicate considerable year-to-year variation. Little difference occurred between 1993/94 and 1994/95 for triasulfuron, since percentage losses were similar on the pilot plots during both years. However, losses of isoproturon decreased by tenfold on the core plots between 1993/94 and 1994/95. This year-to-year variation is at least an order of magnitude greater than any of the effects attributed to the different management practices evaluated during this two-year study period.

The distribution over time of the pesticide losses was also different in 1993/94 compared to 1994/95. During 1993/94, the applied compounds were present in all sampled drainage events; additional samples collected over the whole winter period from two plots indicate that the losses reported for the core plots in Tables 3 and 5 for 1993/94 were about half of the total losses for the season. During 1994/95 triasulfuron was present in all of the sampled drainage events but the losses of isoproturon in the core plots occurred mostly in the first drainage event. This timing of the 1994/95 losses on the core plots was similar to that observed in companion lysimeter experiments, in which all of the applied pesticides appeared in drainflow from the first drainage event but only a weakly sorbed compound appeared subsequently in appreciable amounts.

The cause of the differences in the isoproturon losses from the core plots between 1993/94 and 1994/95 has yet to be determined. One difference between the two years was the wetter surface soil conditions at the time of application in 1994/95 resulting from the differences in rainfall patterns. Further work is planned to investigate whether this and other possible causes of the year-to-year variations can be developed into effective management practices.

As discussed above, the effect of the drainage restrictors was different in 1993/94 from 1994/95. In 1993/94 the decreased losses of isoproturon on the plots with drainage restrictors (compared to the control plots) appeared to be the result of reduced drainflow while no effect of the drainage restrictors on drainflow was observed in 1994/95. During 1994/95, losses of isoproturon were about 25 % less than on the control plots (Table 3), but the restrictors had little effect on losses of triasulfuron. Differences in isoproturon losses between the restrictor and control plots were statistically significant in both 1993/94 and 1994/95. Differences in drainflows and isoproturon losses (expressed as a percent of applied) between the restrictor and

half-rate plots in 1993/94 were not statistically significant indicating a possibility that high drainflows on the control plots were the cause of variations between treatments on core plots in 1993/94.

The half-rate applications resulted in losses of isoproturon and pendimethalin (expressed as grams) which were statistically significantly less than half of those of the control plots. However, drainflows in the plots receiving the half-rate applications were also statistically significantly less than the control plots. Since the half-rate applications should not have affected the drainflow, we cannot conclude whether the half-rate applications or the reductions in drainflow were the cause of the proportionately smaller pesticide losses.

The results of the 1993/94 pilot-plot experiments indicated that tillage to produce a fine seedbed was promising enough to warrant additional investigation, but the lack of replication precluded definite conclusions. The 1994/95 core-plot experiments showed no significant effect of a fine tilth compared to a standard seedbed, but achieving a significantly finer tilth was difficult given the wet surface conditions at the time of cultivation. In lysimeters collected at Brimstone Farm as part of a separate research programme, isoproturon losses from those cultivated by hand to produce a fine tilth were only a third to a half of those from control lysimeters (C. D. Brown, 1995; personal communication).

The 1993/94 pilot trials with a soil sealant were also promising, indicating that the sealant might reduce drainflow. Follow-up trials with the sealant conducted in 1994/95 on the pilot plots were more difficult to interpret since only traces of isoproturon and propiconazole were detected in drainage from any of the pilot plots. Drainage from the first event on the control plot was five to seven times greater than on the three other pilot plots, two of which received an application of the soil sealant. Because drainflow on the control plot was about 50 % greater than the other three plots during the November to March period (Table 4), we cannot conclude that the soil sealant decreased the drainflow.

Deep ploughing was tested on two of the pilot plots in 1994/95. As discussed for the soil sealant, drainage from the first event on the control plot was much greater than on the two plots receiving deep tillage. So again we cannot conclude whether the deep tillage was effective in reducing drainflow and pesticide losses.

The 1993/94 trials on the pilot plots indicated that incorporation of pesticides into soil had little effect on pesticide losses in drainage. This is possibly because pesticides were still present on the surfaces of the soil aggregates and thus readily accessible to water moving through the soil. In order for incorporation to be successful, pesticides would have to be located within the soil aggregates so that they would be less accessible to infiltrating water.

FUTURE PLANS

Based on the results of the first two years, the experimental programme for 1995/96 includes further studies on drainage restrictors, deep tillage, and the effect of surface soil moisture at the time of pesticide application. Studies will be initiated on the effect of increased distance between mole drains and some exploratory experiments will be conducted with drains filled with sorbent material. Plans for the 1996/97 studies will be developed after results from the 1995/96 trials are available.

CONCLUSIONS

The results from the first two years of Phase III of the Brimstone Farm experiment show that pesticide losses in drainage can be influenced by management practices, though other factors are also important. The largest difference observed in the two years was the ten-fold difference in isoproturon losses between 1993/94 and 1994/95 on the core plots although losses of the less sorbed triasulfuron on the pilot plots did not vary significantly between the two years. One difference between the two seasons was the wetter soil conditions at the time of application in 1994/95, the year in which isoproturon losses were lower; this aspect will be investigated further in 1995/96.

The effects of the management practices tested were less than those resulting from both Koc and the difference between the two seasons. Incorporation of pesticides into the surface soil did not significantly reduce pesticide losses. Drainage restrictors reduced losses of moderately to strongly sorbed compounds in both years of the experiments relative to control plots. In the second year, the drainage restrictors reduced isoproturon losses by at least 25 %. Reducing the application rate was also successful in reducing pesticide losses. Losses between the different application rates expressed as a percent of applied were not significantly different when the variability of the drainflow was taken into account, indicating that reductions were proportional to the reductions in application rate. Promising results were sometimes obtained with soil sealants and fine seedbeds, but additional work is needed before definite conclusions can be drawn about the effectiveness of these management practices.

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