SESSION 7A

THE CONTRIBUTION OF BIOTECHNOLOGY TO BREEDING FOR DISEASE AND PEST RESISTANCE

Chairman	Dr M J Dickinson *University of Nottingham*
Session Organiser	Professor G J Jellis *Plant Breeding International, Cambridge*
Papers	7A-1 to 7A-4

THE DISEASE RESISTANCE GENES: THEIR STRUCTURE, FUNCTION AND POSSIBLE USES

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ABSTRACT

The cloning and analysis of disease resistance genes has revealed a group of proteins that are essentially similar from a range of plants. Furthermore, the same types of proteins are used by plants to detect many types of pathogen, including bacteria, viruses and fungi. The plant probably uses a Leucine Rich Repeat structure to detect the pathogen. This LRR domain is extra- or intra-cellular depending on the nature of the infection process used by the particular pathogen and is highly variable suggesting an ability to be altered in order to detect new pathogen features. In general the LRR is associated with protein structures that could be involved in signal transduction. This suggests that the detection of the avirulence signal by the LRR is transmitted to the disease resistance response system of the plant. The cloning of genes from the model plant systems will lead to an understanding of the function of the resistance genes and will enable the cloning of many other genes from crop plants. Furthermore, studies on their mode of action and the evolution of new forms of the gene could enable strategies to be defined that create new resistance capability that the pathogens will have difficulty overcoming.

INTRODUCTION

Plant breeders have made very effective use of naturally occurring resistance, in crop species, to provide varieties that produce high yields in environments where disease pressure can be intense. In some instances, crop varieties are released that are resistant to the prevalent forms of the relevant pathogen, the presence of this variety results in the selection of new forms (or races) that are capable of infecting and reproducing on the plant. It is then necessary to identify new resistance specificities that work against this novel pathogen race. Such genes may be available in previous or current varieties or it may be necessary to obtain them from related species from the wild, in which case the breeding process may be quite complex. Hence, the genes involved in producing the resistance response have been the subject of great scientific interest over many years. These studies have resulted in the recent cloning of a number of resistance genes from several plant species. This paper will describe the nature of these genes and suggest ways in which they may function and how the cloned sequences could possibly be used to increase the pool of genes available to plant breeders.

THE "GENE FOR GENE" HYPOTHESIS

The key work that has formulated modern thinking concerning the nature of disease resistance genes was that of Flor (Flor, 1946; Flor, 1971) . In an elegant piece of work he formulated a simple concept, called the "gene for gene" hypothesis, that has stood the test of time and has been a central dogma of plant pathology in describing plant pathogen interactions. In this hypothesis he proposed that there was a gene in a plant that resulted in a resistance response when it was exposed to an appropriate pathogen (See Table 1). If this gene was missing or non-functional then clearly the plant would be susceptible to invasion by the parasite. However, the resistance genes only seemed to work against certain races of the pathogen. Therefore, the pathogen had to contain a gene, the product of which interacted in a direct or indirect manner with the plant resistance gene product. These pathogen genes were termed avirulence (*avr*) genes as their presence prevented pathogen development in the presence of a

resistance gene. Hence, a plant carrying a particular resistance gene would only express resistance when it was exposed to a pathogen race that carried a particular gene, the product of which caused the resistance response interaction. If the pathogen did not contain the appropriate avirulence gene then no resistance response would occur in the plant and pathogen reproduction would occur even if potential resistance genes were present. The concept of a resistance gene in the plant and an avirulence gene in the pathogen leading to a resistance response is the basis of the "gene for gene" hypothesis.

Table 1. The "gene for gene" hypothesis.

Pathogen	Host Plant	
	Resistance gene absent	Resistance gene present
Avirulence gene absent	Susceptible	Susceptible
Avirulence gene present	Susceptible	**Resistance Response**

The "gene for gene" hypothesis would suggest a mechanism by which the plant disease resistance gene detects the presence of a pathogen via an interaction with the product of an avirulence gene or a metabolite that it produces. Additionally, the resistance gene must have the ability to pass on the message represented by this detection mechanism to plant defence systems, probably involving a signal transduction mechanism resulting in the expression of genes that limit the ability of the pathogen to grow in the infected tissue.

CLONING OF THE DISEASE RESISTANCE GENES

Initially nothing was known about the structure of a disease resistance gene and, therefore, no method of cloning based on homology to known DNA or protein sequences was appropriate. This meant that there were two main approaches that could be used to clone disease resistance genes, namely chromosome walking and transposon tagging. Chromosome walking required a plant genome that was well mapped with molecular markers and the availability of extensive appropriate mapping populations. Initially such experiments were carried out in tomato, rice and *Arabidopsis* (Bent *et al.*, 1994; Debener *et al.*, 1991; Grant *et al.*, 1995; Martin *et al.*, 1993; Mindrinos *et al.*, 1994; Song *et al.*, 1995), resulting in the cloning of a range of resistance genes. Transposon tagging is a powerful technique as it makes use of a mobile genetic element that can insert into a gene rendering it non functional. Once such a mutant is identified the gene can be cloned directly based on the known sequence of the transposon. Such a method was successfully used to clone genes from tobacco, tomato and flax (Jones *et al.*, 1994; Lawrence *et al.*, 1995; Whitham *et al.*, 1994).

STRUCTURE OF THE DISEASE RESISTANCE GENES

The first gene: *Pto* - Bacterial Resistance

The gene *Pto* confers resistance in tomato plants to strains of the bacterial pathogen *Pseudomonas syringae* pv. *tomato* (the causative agent of bacterial speck) that express the avirulence gene *avrPto*. Martin *et al.* (1993) cloned *Pto* and showed that it encodes a protein kinase which specifically phosphorylates serine and threonine residues. Such kinases are commonly found as part of signal transduction pathways. Hence, the structure of *Pto* fulfils only part of the model for a disease resistance gene, in that it could transmit a signal to the disease response mechanism but, from structure alone, does not show a clear detection capability. Salmeron *et al.* (1994) showed that a second gene, *Prf*, closely linked to *Pto* , was required for recognition of *avrPto* . Recently, the structure of *Prf* has been determined and shown to contain a Leucine Rich Repeat (LRR) structure and a nucleotide binding site

(Salmeron *et al.*, 1996). LRR motifs have been implicated in protein/protein interactions (Kobe & Deisenhofer, 1994). Hence, potentially, the *Prf* gene product detects the *avrPto* signal and passes that signal to *Pto*, possibly via an interaction involving the nucleotide binding site. However, there is currently no proof for this model. Additionally, Zhou *et al.* (1995) have isolated a gene, *Pti*, that is specifically phosphorylated by *Pto*. *Pti* is another serine/threonine kinase, which is probably cytoplasmic located, reinforcing the possibility that *Pto* is part of a signal cascade resulting in a disease resistance response.

The Perfect Resistance Gene?: *Xa21* - Bacterial Resistance

Song *et al.* (1995) cloned *Xa21*, a rice gene which confers resistance to *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) race 6, the causal agent of rice blast. The gene is composed of several discrete domains the functions of which can be proposed based on homology to previously studied genes. Like *Pto*, the C terminal domain is a protein kinase and contains conserved sequences that would suggest that it has serine-threonine specificity. However, additionally *Xa21* contains 23 imperfect copies of a 24 amino-acid Leucine Rich Repeat, similar to *Prf*. Therefore, potentially, *Xa21* contains both the ability to detect the avirulence signal and to pass that message to a signal transduction pathway. Moreover the LRR is preceded by an N-terminal domain characteristic of a signal sequence, suggesting that the protein is targeted to an extracellular location and followed by a structure likely to be a membrane spanning helix, suggesting that the C-terminal, containing the kinase domain, is intracellular. Such a structure would suggest that *Xa21* detects the avirulence signal extracellularly but transmits the resulting signal intracellularly.

Incomplete resistance genes?: *Cf-2* and *Cf-9* - Fungal Resistance

The tomato genes *Cf-2* and *Cf-9* confer resistance to isolates of the fungal pathogen *Cladosporium fulvum* (grey mold) that carry the avirulence genes *Avr2* and *Avr9*, respectively. Dixon *et al.* (1996) and Jones *et al.* (1994) cloned these genes using both an elegant targeted transposon approach and chromosome walking. The structure of the *Cf-2* gene was highly similar to that of the *Cf-9* gene (Dixon *et al.*, 1996). As in *Xa21*, the N-terminal of*Cf-2* contains a putative signal peptide followed by an LRR domain with 33 perfect and five imperfect repeats. Therefore, like *Xa21*, the LRR domain is extracellular. Following the LRR domain is a stretch of hydrophobic amino acids consistent with a membrane spanning region, suggesting that the extracellular LRR region is anchored to the plant cell membrane. However, unlike *Xa21* and *Pto*, *Cf-2* and *Cf-9* do not contain an intercellular kinase domain or functional equivalent. This would suggest that they lack a direct connection to a signal transduction pathway that could lead to disease resistance. The *Avr9* gene, the product of which interacts with *Cf-9*, has been cloned (van Kan *et al.*, 1991) and shown to be a small, cysteine-rich peptide. When this product is purified and applied to plants it elicits the disease resistance response in tomato plants containing the *Cf-9* gene but not in susceptible lines. The extracellular location of these LRR domains is completely consistent with the structure and location of the *Avr9* product, which can be isolated from intercellular wash fluids from infected plants. This would imply a direct interaction between AVR9 and Cf-9, probably mediated via the LRR domain. Unless the AVR9/Cf-9 complex is able to interact directly with a disease response mechanism, there is likely to be another gene in the pathway that is involved with signal transduction, such as a serine threonine kinase. This could be via a *Pto* like gene interacting with the intercellular domain of *Cf-2* and *Cf-9* or by the AVR9/Cf-9 complex interacting with a gene such as *Xa-21*.

A third class of resistance gene: *N* - Viral Resistance

Whitham *et al.* (1994) reported the cloning of the tobacco gene *N* that confers resistance to the viral pathogen tobacco mosaic virus (TMV) . The *N* gene contains the LRR structure that appears to be a common feature of disease resistance genes. No signal sequence is present and, hence, it is likely that the gene product is cytoplasmically located. *N* does not contain a

serine-threonine kinase domain but, like *Prf*, does posses a neucleotide binding domain which is commonly found in proteins known to bind ATP/GTP. This domain could be involved in signal transduction and represent the route by which defence responses are initiated. An N-terminal domain of *N* showed homology to the cytoplasmic domains of the *Drosophila* Toll protein and the human interleukin-1 receptor (IL-1R). These proteins are involved in the activation of transcription factors that then cause the expression of particular genes. Hence, the presence of such homologies are completely consistent with the role of *N* being to promote the expression of genes involved in the resistance response when the LRR domain detects the presence of TMV. The cytoplasmic location of the gene would also be logical when considering the life cycle of TMV.

Arabidopsis: A Model System for Studying Disease Resistance Genes

Arabidopsis thaliana has proven to be an excellent model system for the molecular analysis of plant development and responses (Meyerowitz & Somerville, 1994). This will also be the case for plant pathogen interactions. To date the cloning of only two resistance genes, that recognise bacterial pathogens, have been reported: *RPS2* confers resistance to *Pseudomonas syringae* pv *tomato* expressing the avirulence gene *avrRpt2* (Bent *et al.*, 1994; Mindrinos *et al.*, 1994) and *RPM1* confers resistance to *Pseudomonas syringae* pv *maculicola* expressing the avirulence gene *avrRpm1* (Debener *et al.*, 1991; Grant *et al.*, 1995). Both genes contain LRR and neucleotide binding site domains and are probably cytoplasmically located. Hence, they are similar to the other genes described above, although each has its own unique features.

However, work with a range of pathogens is revealing a complex picture in which genes scattered around the genome are involved in recognising pathogens. Studies of the interaction between *Peronospora parasitica* (downy mildew) and *A. thaliana* have revealed the existence of more than 15 resistance specificities (Holub & Beynon, 1996). Resistance genes appear to be present on all five *Arabidopsis* chromosomes and in several cases these fall into regions each covering approximately 15 cM. This implies that large regions of *Arabidopsis* chromosomes are involved in specifying disease resistance and the clustering could imply evolution by duplication. However, cloning of these loci is still necessary to reveal the nature of the genome at these locations. Resistance genes that detect other pathogens, notably *Erysiphe cruciferarum* and *E. cichoracearum* (powdery mildew), *Albugo candida* (white blister) and *Plasmodiophora brassica* (club root) have been mapped in the *Arabidopsis* genome (reviewed by Kunkel, 1996). The cloning of many of these genes is at an advanced stage and analysis of their structure will be reported in the near future. This flood of information will be critical in the analysis of the roles of the particular protein domains revealed in pathogen detection and signal transduction.

POSSIBLE USES

The analaysis done to date will almost certainly allow the cloning of more genes known to be effective in particular crop plants. The comparison of all the known resistance genes may allow the identification of conserved regions that are specific to this class of genes. This enables molecular biologists to design primers that allow the amplification, using the Polymerase Chain Reaction, of this class of gene directly from genomic DNA from any crop plant. These DNA fragments can then be mapped using molecular mapping techniques to determine if they cosegregate with known resistance specificities. If this is the case they can be used as probes to identify appropriate genomic or cDNA clones. Such clones may be the resistance gene itself or part of the gene family containing the functional gene. Hence, the cloning of many more resistance genes from crop plants may become possible. In time this will place a large new resource in the hands of the plant breeders. It may be possible to respond to the emergence of a new pathogen race by the introduction of a specific resistance gene into an otherwise commercially successful variety without changing any other characteristic of the plant other than disease resistance. Furthermore, it may be feasible to

insert several resistance genes recognising a particular pathogen into a single variety, potentially making it harder for the pathogen to alter sufficiently to overcome the plants recognition capability.

Given the ubiquitous nature of the LRR it seems possible that the introduction of resistance genes from alien genera and species may provide new recognition capability to a plant. Such recognition capability might not be limited to the pathogen recognised in the original host. However, even if the gene is capable of detecting a pathogen in the new host it may not be capable of interacting with the signal transduction mechanisms of the plant. Some success has been shown by Rommens *et al.* (1995) where function of the *Pto* gene from tomato in *Nicotiana* was demonstrated. The overall practicality of such an approach can only be determined by experimentation.

New resistance specificities may be created by altering the structures of known resistance genes. Should it be proven that the recognition capability does lie in the LRR domain then changing its structure may create new recognition capability associated with a signal transduction mechanism that is known to work in the appropriate plant. Initial work along these lines will be carried out by swapping protein domains between related resistance genes. These experiments will define two things, firstly the role of particular domains in the functioning of the gene and, secondly, serve to define the extent of the functional domains. The latter information is critical to define those regions that are essential to gene function, and should be left unaltered, and those that can be changed to generate new recognition capability.

Further into the future other possibilities for engineering new resistance capabilities are now conceivable. Pathogens overcome resistance genes either by altering the structure of an avirulence gene or by preventing its expression. This means that many avirulence genes are not essential to the survival of the pathogen. If resistance genes could be altered to recognise gene products that are essential to the survival of the pathogen it would be harder for the pathogen to overcome such resistance by mutation. Such a strategy would first require the development of procedures that could monitor the binding of resistance genes to targeted avirulence products. LRR domains could then be altered by mutation or synthesised randomly and screened for interaction with the target. An effective LRR could then be attached to signal transduction components and introduced into appropriate plants. Much more speculatively, but along the same lines, antibodies could be selected that interacted with domains of an essential pathogen protein. Such antibodies could be attached to plant disease resistance signal transduction components and form a totally new class of resistance gene.

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IMMUNOTHERAPY OF PLANT DISEASES

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ABSTRACT

Antibodies perform an essential role in animal immunity where their prime function is to bind antigens in a highly specific manner. In recent years technologies have been developed that allow the production of specific antibodies, and antigen-binding fragments of antibodies, in a range of heterologous (naturally, non-antibody producing) hosts, including plants. Increasingly, the specificity of such recombinant antibodies is being used to exert an effect in the cells that produced them. Based on this approach, novel forms of plant disease and pest resistance are being developed.

INTRODUCTION

Antibodies are complex, multimeric proteins that function as fundamental components of the immune system of animals. Antibody proteins, the immunoglobulins, are typically composed of a basic unit of two identical 'heavy' polypeptide chains and two identical 'light' polypeptide chains. The heavy and light polypeptide chains are covalently linked together by several intermolecular disulphide bonds. Each of the polypeptide chains is folded into a series of discrete domains, stabilised by an intramolecular disulphide bond. (Fig. 1).

The recognition and binding of target antigens represents the primary function of all antibody proteins. The antigen-binding activity of an antibody, which can be highly specific for a particular target, is conferred by six peptide loops, the complementarity determining regions (CDRs), that are hypervariable in both amino acid sequence and length. Three of the CDRs are displayed by the domain located at the N-terminus of the heavy chain and the other three are displayed by the domain located at the N-terminus of the light chain (Fig. 1). These domains, which provide a protein scaffold to display the CDRs, are termed the variable heavy-chain domain (V_H) and the variable light chain domain (V_L). The specificity of antigen-binding is a consequence of the hypervariability of the CDRs and the immune system is able to generate antibodies to a great diversity of antigens including proteins, carbohydrates, small organic molecules and even metal ions.

A series of constant domains, dispensable for antigen-binding activity, make up the remainder of the antibody molecule. These domains mediate a number of antibody effector functions such as complement fixation, placental transfer and binding to immune system cell types. The constant region of each heavy chain carries one or more N-linked, complex glycan.

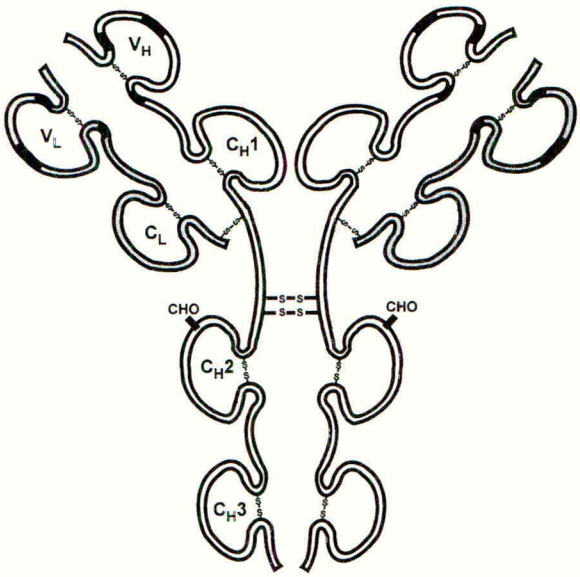

Fig. 1 Domain structure of a typical antibody (whole IgG molecule). Antigen-binding activity, solely a function of the variable domains of the light- and heavy chains (V_L and V_H), is determined by three 'hypervariable' loops, the complementarity determining regions (CDRs), shown in black, within each of the V domains.

Full antigen-binding activity is shown by a number of antibody fragments that possess the variable domains. One such fragment, that can be generated by recombinant DNA methods, is the single-chain Fv (Fig. 2). In the single-chain Fv (scFv) the V_L and V_H domains are joined together by a synthetic polypeptide linker.

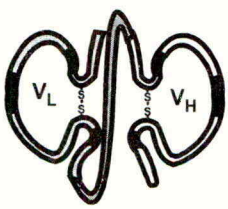

Fig. 2 Domain structure of an antigen-binding, recombinant single-chain Fv fragment.

Gene sequences encoding antibodies and antibody fragments can be expressed in a range of host cells that do not normally produce antibodies. Recombinant antibody production in such heterologous hosts is exploited in two different types of application in biology and medicine. In *'ex situ'* applications, antibodies, isolated from non-natural sources, are used as tools in diagnosis, therapy and purification. In *'in situ'* applications a recombinant antibody acts as an effector reagent in the cell in which it was synthesised. Here the objective is to modify the activity of the antigen *in vivo*.

ANTIBODY EXPRESSION IN PLANTS - 'PLANTIBODIES'

In recent years it has been shown that transgenic plants can produce intact antibodies and antibody fragments ('plantibodies'). The production of a functional, intact recombinant antibody in plants was first described by Hiatt *et al.* (1989). Gene sequences encoding either the full-length heavy- or light-chain polypeptides of a mouse monoclonal antibody directed against a synthetic phosphonate ester were transferred to transgenic tobacco (*Nicotiana tabacum*) plants. The sexual crossing of individual plants expressing each of the gene sequences produced F_1 progeny that expressed both. These plants accumulated assembled, functional antibody.

Antibodies are normally secreted proteins and Hiatt *et al.* (1989) found that co-secretion of heavy- and light-chain polypeptides was necessary in order to observe antibody protein accumulation in tobacco plants. The secreted antibody was found in the plant cell wall space, the apoplast. Attempts to produce the antibody in the cytoplasm of tobacco plant cells were unsuccessful. Secretion of proteins involves passage through the endoplasmic reticulum (ER), entry to which is mediated by signal sequence peptides located at the N-terminus of the protein. The lumen of the ER is an oxidising environment and contains a number of proteins that are required for disulphide bond formation and correct antibody protein folding (see Whitelam & Cockburn, 1996). Since the original work of Hiatt *et al.* (1989), several whole antibodies have been successfully produced in plant cells following passage of heavy and light chains to the ER (e.g. Voss *et al.*, 1995; De Wilde *et al.*, 1996).

Recently, an elegant extension of the cross-pollination strategy for combining the genes encoding the different components of a multimeric antibody has been described (Ma *et al.*, 1995). Here, the objective was to create a single transgenic tobacco plant producing and assembling a polymeric, secretory antibody. Secretory antibodies are composed of two antibody units covalently linked by a small joining (J) chain peptide and associated with a stabilising secretory component (SC) polypeptide. Ma *et al.* (1995) produced four separate transgenic lines. The first expressed a gene sequence encoding the antibody heavy chain, the second expressed a gene sequence encoding the antibody light chain, the third expressed a J chain gene sequence and the fourth expressed a gene sequence encoding SC. By a series of successive cross-pollinations amongst these lines Ma *et al.* (1995) were able to select progeny in which all four gene sequences were simultaneously expressed and in which an assembled secretory antibody accumulated.

Gene sequences encoding a range of different antigen-binding fragments of antibodies have been stably expressed in transgenic plants. For example, functional single-chain Fv (scFv) proteins with specificities for phytochrome, abscisic acid, fungal cutinase, artichoke mottle crinkle virus (ACMV) coat protein, oxazalone, human creatine kinase and nematode salivary

secretions have all been successfully produced in tobacco plants (Owen *et al.*, 1992; Artsaenko *et al.*, 1995; Schouten *et al.*, 1996; Tavladoraki *et al.*, 1993; Fiedler & Conrad, 1995; Bruyns *et al.*, 1996; Rosso *et al.*, 1996).

Recombinant scFv fragments have several advantages over whole antibodies for *in situ* applications. Perhaps the most significant of these is that scFv proteins are often able to fold more readily than whole antibodies into a functional conformation Thus, whereas the synthesis of whole antibodies requires passage into the ER, scFv fragments have the potential to be produced in a range of cellular compartments, including the cytoplasm. The use of a recombinant scFv protein to stably perturb the activity of cytoplasmic antigen in transgenic plants was first described by Owen *et al.* (1992). This study used polymerase chain reaction (PCR) amplification to isolate the VH and VL coding regions from cDNA derived from a mouse hybridoma line secreting an antibody directed against a conserved epitope on the plant regulatory photoreceptor, phytochrome A. Expression of the scFv-encoding gene in transgenic tobacco led to low level accumulation of the scFv protein in the plant cell cytoplasm. The anti-phytochrome scFv protein was found to display antigen-binding activity following its extraction from plant tissues (Owen *et al.*, 1992). Evidence that the scFv protein was functional *in vivo* was obtained from analysis of phytochrome-mediated developmental responses of the transformed plants. Homozygous seeds expressing the anti-phytochrome A scFv gene were found to show poor phytochrome-dependent, light-mediated promotion of germination (Owen *et al.*, 1992; Whitelam *et al.*, 1994). In addition, other aspects of the photomorphogenesis of the transformed plants were perturbed, including the photocontrol of hypocotyl elongation and the photocontrol of cotyledon expansion (Whitelam *et al.*, 1994). These observations provide validation of the use of *in situ* synthesised antibodies, or antibody fragments, for the modulation of cytoplasmic plant antigens.

More recently, Artsaenko *et al.*, (1995) transformed tobacco plants with gene sequences encoding an scFv fragment derived from a monoclonal antibody specific for the plant hormone abscisic acid. In this case, the gene construct was designed in such a way that the scFv protein was transported to and retained within the lumen of the ER. The scFv protein was found to accumulate to very high levels in the some of the transformed plants up to a maximum of 4.8% of total soluble protein (Artsaenko *et al.*, 1995). Plants producing anti-ABA scFv protein at levels around 1-2% of total soluble protein could only be grown under high humidity and were observed to undergo rapid wilting under normal greenhouse conditions. This is characteristic of the phenotype of ABA-deficient mutants and so suggests that antibody-binding to ABA *in vivo* has reduced the effective titre of the hormone. It was speculated that in the transformants synthesising the anti-ABA scFv the hormone was sequestered in the ER, thereby reducing its concentration in the cytoplasm.

PLANTIBODY-MEDIATED DISEASE RESISTANCE

Following the earliest demonstrations that functional antibodies could be produced in transgenic plants it was speculated that an important application of plantibody technology would be the creation of novel forms of disease resistance. Of course, plant cells lack the diverse array of mechanisms for antigen removal that result from antibody binding within the animal immune system. Nevertheless, simple agglutination, enzyme inhibition or the blocking of essential epitopes are effector mechanisms that could operate in plants to reduce

pathogenicity. So far, there have been two reports of the successful application of the plantibody approach to the development of resistance to plant viruses.

The first of these reports was the demonstration that *Nicotiana benthamiana* plants synthesising a scFv against recognising the coat protein of artichoke mottled crinkle virus (AMCV) exhibited enhanced resistance to the virus (Tavladoraki *et al.* 1993). *Nicotiana benthamiana* is a readily transformed, symptomatic host for ACMV. The scFv protein was produced in the cytoplasm of the plant cells, the compartment in which virus replication occurs.

When protoplasts, derived from transgenic plants synthesising the scFv, were challenged with ACMV virions they accumulated lower amounts of viral coat protein and showed a lower frequency of infection than protoplasts from control plants (Tavladoraki *et al.,* 1993). When challenged with an unrelated virus, cucumber mosaic virus (CMV), infection was the same in transgenic and control protoplasts, indicating that the reduced ACMV accumulation was related to the binding specificity of the scFv antibody. Significantly, when the leaves of intact plants were infected with ACMV by rubbing them with a suspension of virions, transgenic plants showed lower virus accumulation and a marked delay in symptom development compared with control plants (Tavladoraki *et al.*, 1993).

The mechanism by which the scFv affords protection to ACMV is unknown. The epitope on the coat protein recognised by the scFv antibody is involved in divalent cation-regulated swelling of the virus and it is speculated that antibody-binding could interfere with uncoating of the virus or assembly of progeny virus.

Subsequently, Voss *et al.* (1995) demonstrated virus resistance in intact transgenic tobacco plants following production of a secreted, whole antibody. Here, the transgenic plants synthesised a mouse-derived monoclonal antibody against a surface epitope of intact tobacco mosaic virus (TMV) virions. The transgenic plants were created from a tobacco line containing the *N*-gene such that they respond hypersensitively to virus infection and form clearly visible lesions. Upon infection with TMV, transgenic plants producing the intact antibody showed a significant reduction in the number of necrotic lesions compared with transgenic plants that did not produce antibody. The size of the necrotic lesions was similar for antibody-producing and non-producing plants. Plants producing higher amounts of antibody were found to develop fewer lesions, indicative of an inverse correlation between the level of antibody accumulation and lesion number. Antibody-producing and control plants were found to be equally sensitive to another virus, tobacco necrosis virus (TNV). This indicates that the observed protection was related to the specificity of the expressed antibody (Voss *et al.,* 1995).

Since the assembled, functional antibody was secreted to the apoplast of the transgenic plants, Voss *et al.* (1995) speculated that the antibody might be able to 'decorate' the TMV particles in this compartment and that this may in some way neutralise them. In earlier studies it had already been shown that binding of the monoclonal antibody to the virus prior to inoculation of plants reduced the infectivity (Voss *et al.,* 1995).

TARGETS FOR PLANTIBODY-MEDIATED RESISTANCE

The studies with ACMV and TMV have substantiated proposals that plantibodies could be used for plant protection. It is anticipated that, where suitable epitopes can be identified, and the cognate antibody isolated, *in situ* antibody expression will find many other applications in plant protection. So far the plant viruses have been the focus of attention for this form of 'therapy'. This probably reflects the fact that the pathogenicity of several important viruses is relatively well characterised and determined by a small number of proteins. Even for plant viruses, only antibodies directed against viral structural proteins have been tested. This is because of the ready availability of monoclonal antibodies to this class of antigens. Future antibody-mediated virus-resistance strategies will no doubt include, as targets, non-structural viral proteins, such as polymerases and the proteins involved in intra- and inter-plant virus transmission. The potential value of non-structural targets has recently been illustrated by the demonstration that production in the cytoplasm of cultured mammalian cells of an scFv antibody directed against the human immunodeficiency virus (HIV) reverse transcriptase makes the cells resistant to HIV infection (Maciejewski *et al.,* 1995).

The plantibody approach should be applicable to a wide range of other plant pathogen types, including bacteria and fungi as well as to insect and nematode pests. The determining factor will be the identification of suitable target antigens since simple binding of antibody to target will have to be sufficient to compromise pathogenicity. This could involve for example, inhibition of a crucial enzyme or other protein function by antibody-binding, sequestration of pathogenicity factors or agglutination of the pathogen or one of its components. Potential targets would include the cell wall modifying enzymes commonly involved in plant pathogenesis, phytotoxins and pathogen signalling molecules Some specific targets have already been identified. For example, Saunal *et al.* (1993) have characterised a monoclonal antibody that binds to the TMV coat and interferes with the *in vitro* co-translational disassembly of TMV particles. It has been suggested that expression of genes encoding this antibody in tobacco cells may provide resistance to the virus.

It has also been proposed that the *in situ* expression of antibodies that bind to, and inactivate, the salivary proteins of plant nematodes could provide a means of engineering resistance (Schots *et al.*, 1992). Salivary secretions of some nematodes are known to play a key role in the infection process. Recently, Rosso *et al.* (1996) have described the transient production, in tobacco leaf protoplasts, of a functional scFv antibody directed against salivary secretions of the root-knot nematode (*Meloidogyne incognita*).

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PROGRESS TOWARDS THE USE OF TRANSGENIC PLANTS AS AN AID TO CONTROL SOFT FRUIT PESTS AND DISEASES

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ABSTRACT

The cowpea trypsin inhibitor gene from cowpea (*Vigna ungulata*), which confers resistance to insect pests, has been inserted into strawberry cultivars by *Agrobacterium*-mediated gene transfer. Glasshouse trials in which transformants were challenged by vine weevil (*Otiorhynchus sulcatus*) larvae established that this heterologous gene may have value in the soft fruit industry and it is envisaged that plants containing this gene will be resistant to chewing insects. The identification and isolation of a gene encoding a polygalacturonase-inhibiting protein (PGIP) from raspberry gives the opportunity to evaluate it in soft fruit crops as a source of resistance to post-harvest grey mould (*Botrytis cinerea*). Field trials of genetically modified strawberries have started and the implications of such trialling are discussed.

INTRODUCTION

Arthropod pests and fungal and viral diseases are major problems in soft fruit crops such as strawberry, raspberry and blackcurrant. These problems have been tackled by a range of approaches including plant breeding, chemical and biological control. The major insect pests, fungal and virus diseases for which improved control is urgently required will be discussed briefly. The major arthropod pests are shown in Table 1, but those currently causing greatest concern for the soft fruit industry in the UK are wingless weevils (*Otiorhynchus* spp.) on strawberry, gall mite (*Cecidophyopsis ribis*) on blackcurrants, raspberry beetle (*Byturus tomentosus*) and moths (e.g. *Graphiphora augur*) on *Rubus*.

The vine weevil (*O. sulcatus*) is now the major pest of strawberry in Europe where it is endemic. The larvae damage root systems resulting in severe loss of yield or plant death. Horticultural intensification and the use of polythene mulches are regarded as the major reasons for the spread of this insect in strawberry and blackcurrant (Moorhouse *et al.*, 1992). Since the withdrawal of persistent organochlorine insecticides, control of adults and larvae has become difficult, as few of the currently available products are particularly effective under field conditions and no genetic resistance in commercial germplasm has been identified.

Blackcurrant gall mite is the most serious pest of blackcurrants and the vector of reversion disease (Gordon *et al.*, 1994). Chemical control in Europe relies on endosulfan and/or fenpropathrin applied in the spring during mite migration, but failures in control are now common in some areas. Large numbers of mites migrate from infested galled buds, often

by wind dispersal. Once a suitable host has been located, adult mites enter the buds and feed on the leaf primordia initiating gall formation. Genes *Ce* and *P* have been identified and have been successfully incorporated into blackcurrant breeding material (Gordon *et al.*, 1994). Other resistance genes are being sought for insertion into *Ribes*.

Table 1. Types of damage caused by important arthropod pests of temperate soft fruit crops in Europe

Crop	Pest	Plant parts damaged	Mode of feeding
Strawberry	Vine weevil (*Otiorhynchus sulcatus*)	Roots & foliage	Chewing
	Two-spotted spider mite (*Tetranychus urticae*)	Foliage	Sucking
	Aphids (*Chaetosiphon fragaefolii*)	Foliage	Sucking/virus vectors
Raspberry and other *Rubus*	Raspberry beetle (*Byturus tomentosus*)	Foliage & fruit	Chewing
	Aphids (*Amphorophora idaei/Aphis idaei*)	Foliage	Sucking/virus vectors
	Raspberry cane midge (*Resseliella theobaldi*)	Canes	Sucking
	Raspberry cane moth (*Lampronia rubiella*)	Buds & fruit	Chewing
	Clay-coloured weevil (*Otiorhynchus singularis*)	Foliage & roots	Chewing
	Other Lepidoptera	Buds/foliage/canes	Chewing
Blackcurrants and other *Ribes*	Blackcurrant gall mite (*Cecidophyopsis ribis*)	Buds	Sucking/virus vector
	Vine weevil (*O. sulcatus*)	Roots & foliage	Chewing
	Blackcurrant leaf curling midge (*Dasineura tetensi*)	Foliage	Sucking
	Aphids (*Hyperomyzus lactucae*)	Foliage	Sucking

Raspberry beetle and clay-coloured weevil (*O. singularis*) are the most serious pests in raspberry in the UK. Adult raspberry beetles damage flower buds and flowers causing malformation. Larval contamination renders fruit unacceptable for both the fresh and processing market. The pest can be controlled by insecticides applied at the pre-blossom stage or between the 80% petal-fall and first pink-fruit stage. Later sprays are becoming less acceptable to consumers (Jennings, 1988). Clay-coloured weevils feed on the petioles of leaves on the fruiting laterals causing them to break or wilt and they damage the developing flower buds. Control in raspberry plantations in Scotland relies on nocturnal sprays of organophosphorus insecticides applied in spring when adult weevils are feeding on buds or expanding lateral shoots (Gordon & Woodford, 1986), but recent experience suggests that the level of control given by fenitrothion, the only product now available, is inadequate.

The fungal diseases of greatest concern in raspberry and strawberry are grey mould (*Botrytis cinerea*) and root rots (*Phytophthora* spp.). *B. cinerea* is difficult to control because it has a number of pathways for infection and there is no source of strong resistance available to breeders. The fungus infects newly-opened flowers of soft fruits by germinating in the stigmatic fluid and colonising the transmitting tissues of the style (Bristow *et al.*, 1986, Williamson *et al.*, 1987). In raspberry, the pathogen also infects mature leaves on primocanes, spreading to the stem and retarding axillary bud growth and consequently reducing fruit production in the following year (Williamson & Jennings, 1986). At present, most soft fruit crops are sprayed at 7-10 day intervals from first flower until shortly before fruit ripening to control post-harvest grey mould, but raspberry flowers open daily over a 5-week period. Therefore, many flowers will be left unprotected and a resistance mechanism which prevents spread of the pathogen in mature drupelets is desirable. Despite the presence of infected styles, drupelets of immature raspberries are highly resistant to infection. This resistance is associated with high levels of a polygalacturonase-inhibiting protein (PGIP) (Johnston *et al.*, 1993,1994) and we have isolated genes encoding this protein from raspberry (Ramanathan *et al.*, 1995). The purified PGIP is active against two endo-polygalacturonases from *B. cinerea* thought to be involved in tissue degradation and the onset of grey mould.

Raspberry root rot (*Phytophthora fragaria* var. *rubi*), a major problem worldwide since the 1980s, has the potential to completely destroy plantations. Rapid spread of this disease was due to planting stocks from infected nurseries (Duncan *et al.*, 1987). Once introduced, the pathogen can survive in the soil for many years. Present application of fungicides based on metalaxyl or oxadixyl can be effective, but there is a risk of selecting fungicide-resistant strains. Resistance from North American cultivars could be incorporated into highly adapted European germplasm using conventional plant breeding, but the introduction of these genes could be speeded up if molecular markers can be generated to the resistance gene(s).

In raspberry, the virus currently causing great concern is raspberry bushy dwarf virus (RBDV), for which a resistance-breaking isolate (RB) has emerged (Jones, 1995). RBDV-RB isolates can overcome gene *Bu* which gives protection against the common S isolates and has the potential to cause great damage. Because RBDV is pollen-borne, its control can only be achieved through genetic resistance. At present, no suitable germplasm with resistance to RB isolates is available so that the only strategy is to use genetic based resistance through biotechnology.

BIOTECHNOLOGICAL APPROACH

The availability of pest and disease resistance genes from related and non-related plant species, coupled with the development of gene vector systems (methods to insert single desirable genes into the genome of the target plant), has the potential to enhance existing cultivars or accelerate resistance breeding. These technologies eventually should allow a cultivar to be transformed with a number of different resistances, used together or individually, to reduce the risk of increased virulence in the pathogen or pest, leading to erosion of resistance. Several potentially useful genes have been identified and cloned. In

soft fruits, the genes of current interest at SCRI include those for insect resistance, such as the cowpea trypsin inhibitor (CpTi) gene (Hilder *et al.*, 1987) and lectin genes from snowdrop (*Galanthus nivalis*) (Hilder *et al.*, 1995). It is envisaged that the CpTi gene will be effective against chewing insects, but will have little effect against sucking arthropods. Purified CpTi incorporated into artificial diets has shown anti-metabolic activity against a wide range of chewing insects (Hilder *et al.*, 1987). The PGIP genes with potential to confer fungal resistance, and those for virus resistance, including the coat protein genes of RBDV and arabis mosaic virus (Bertioli *et al.*, 1991) are also being evaluated.

Techniques for genetic transfer in soft fruit

Protocols for genetic transformation of some soft fruit crops have been developed based on *Agrobacterium tumefaciens* as a vector of single genes. This soil bacterium has the ability to transfer genetic material into individual plant cells which can be regenerated into whole plants containing the new gene. This approach has been used in strawberry (James *et al.*, 1990, Nehra *et al.*, 1990, Graham, 1990), *Rubus* (Graham *et al.*, 1990) and blackcurrant (Graham & McNicol, 1991, B. Millam-Mendoza, pers. comm.). At present, the greatest success has been achieved with strawberry due to its ability to regenerate whole plants easily in tissue culture after inoculation for gene transfer.

Strawberry: Transformation using the CpTi gene as an example of the use of biotechnology for pest and disease control

Plants of transgenic strawberry cvs Melody and Symphony expressing the CpTi gene (Axis Genetics) have been produced as described previously (Graham *et al.*, 1995) by inoculation of stem tissue with *Agrobacterium* isolate LBA4404 containing the CpTi+5 construct (Gatehouse *et al.*, 1988). After regeneration of shoots, putative transgenics were rooted on medium containing 50 mg/l kanamycin. Gene insertion was confirmed by the polymerase chain reaction (PCR). A brief trypsin assay was carried out on these plants to estimate the levels of enzyme activity as compared to non-transformed controls (Graham *et al.*, 1995). Glasshouse bioassays demonstrated that the damage caused to transgenic plants was significantly lower ($p<0.001$) than the non-transgenic controls.

Field release of genetically modified strawberries, and environmental considerations

Field trialling against vine weevil and other pests is essential to confirm the effectiveness of the CpTi gene in strawberries. Release of any genetically modified organisms into the environment can only be done in the UK with permission from the Department of the Environment, after scrutiny of the protocols by the Advisory Committee on Releases to the Environment. The field release design includes a number of containment measures to prevent escape of the introduced gene in pollen or seed and containment of the target pests species, vine weevil, in the confines of the experimental area. Further investigations on the biological effect of pollen feeding by bumblebees (*Bombus* spp.) are underway, and another study on the potential influence of transgenic plants on soil-inhabiting insects, such as ground beetles (Carabidae), has been initiated.

DISCUSSION

We see gene transfer technology primarily as a supplement to long-established breeding programmes for soft fruit species. In the short term, single genes will be inserted into commercial cultivars, as with the CpTi gene, to enhance certain quality characteristics or disease and pest resistance. Once these transformants have a proven track-record they can be used as parents in breeding programmes. Rapid selection for the gene in progenies can then be achieved using small seedlings from which DNA is extracted and specific primers used in PCR. The polymerase chain reaction involves synthesising multiple copies of a region of DNA using primers designed to the region of interest and this allows the inserted DNA to be easily detected without the need for growing the plant and screening for the trait. Extensive field trialling must be done on transformed plants to ensure that the genes transferred into the genome do not have any unforeseen detrimental traits which can adversely affect the environment or the consumer.

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USING MOLECULAR MARKERS TO BREED FOR PEST AND DISEASE RESISTANCE

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ABSTRACT

Molecular markers are finding widespread use in enhancing traditional plant breeding programmes. When tightly linked to a gene of interest, selecting for the marker can replace direct selection for the trait. This is particularly useful if the direct screen is difficult or inconvenient. Conversely markers can aid the identification of rare recombinants permitting selection against unwanted chromosome segments linked to a desirable gene. Thus markers form a tool kit available to improve the precision and speed of a breeding programme.

Theory and practice will be discussed drawing on practical examples, including some from the research and breeding programmes at Plant Breeding International.

INTRODUCTION

The use of naturally occurring resistances is a cost effective and environmentally benign approach to controlling pests and diseases. Most of these pathogens have parasitised crop species and their relatives prior to domestication, leading to the evolution of a range of resistance genes in the host plants. Some entered the crop gene pool on domestication, many others were left behind at this genetic bottleneck but have been more and more exploited since systematic breeding began.

Until comparatively recently selection for disease resistance has been by direct screening; exposing a segregating breeding population to natural or artificial inoculum, and selecting the resistant lines. Whilst this can be highly efficient in screening large numbers, there are circumstances where it is difficult or inconvenient. Examples would include recessive resistance genes that cannot be directly tracked though a backcrossing programme; where the screen is labour intensive, e.g., many soil borne pathogens; where the disease develops late in the crop's life cycle, but early selection is required; where a virulent disease must be kept away from other valuable stocks; and where multiple resistances are to be screened for. In these circumstances indirect selection via a linked molecular marker can offer a practical solution.

THEORY OF MARKER ASSISTED BREEDING

The genetic information for higher plants is distributed over several chromosomes which segregate independently during meiosis. Genes on the same chromosome are physically linked and would be inherited together except for crossing over between pairs of homologous chromosomes during meiosis. The practical effect is that genes that are close together will also

tend to be inherited together, the tightness of that association varying with distance. Thus a marker gene lying close to another of breeding interest, e.g. a resistance gene, may, with a certain probability, be used indirectly to track that gene.

TYPES OF MARKERS

Until recently most marker genes were phenotypic, frequently recessive mutants, with distinct and deleterious effects on plant morphology and vigour, and available only in special stocks. Molecular markers as a class avoid many of these drawbacks. Generally they are codominant, selectively neutral, having no overt phenotype except as revealed by the appropriate assay, and can be found throughout breeding populations.

The molecular markers currently in use fall into three basic types. The earliest to be developed were isozymes, enzyme systems that show the polymorphism necessary for use as markers. The enzyme is extracted from plant tissue, the differing forms separated on the basis of charge by gel electrophoresis and then visualised with a specific stain. In general they can be set up quite simply and are suitable for the large scale screenings that breeding requires. But being the product of a gene, an isozyme may be expressed only under certain circumstances, e.g. in a specific tissue, in response to a particular stress, or at one stage in the life cycle. Further, useful isozymes are comparatively few in number giving poor genome coverage.

With the advent of molecular biology new marker types have been developed that assay polymorphism in the DNA directly. Restriction Fragment Length Polymorphisms (RFLPs) (Beckmann and Soller, 1983) were the first type based on the presence or absence of a restriction enzyme site near a particular length of DNA identified by a radioactive probe. Their numbers are practically unlimited, leading to the development of quite dense maps of important crop genomes (O'Brien, 1993). This greatly improves the chances of finding a marker closely linked to a resistance gene. Being based directly on DNA, RFLPs can be produced from any tissue at any time. However, their preparation involves many steps and is rather labour intensive. This limits their extensive application.

More recently, marker systems based on the Polymerase Chain Reaction (PCR) (Saiki *et al.* 1988) which amplifies specific lengths of DNA, have been developed. These are faster and require much less DNA preparation. However, maps of PCR markers are presently much less dense than RFLPs as they require more development work, and some of the PCR markers are insufficiently robust to be used on a large scale. Fortunately there are techniques for converting RFLPs to a PCR format. Thus a frequent approach is first to identify a useful RFLP, then convert it to a PCR marker. PCR technology is developing rapidly and improvements in speed, simplicity and robustness are greatly extending the range of applications for molecular markers.

APPLICATION OF MARKERS

In order to be effective a molecular marker must lie on the same chromosome as the resistance gene of interest, and sufficiently close that they are unlikely to be separated by recombination.

One of the tightest, used extensively at PBI in its wheat breeding programme, is endopeptidase-1 (*Ep-D1b*), which is linked to *Pch1*, the gene for resistance to the eyespot pathogen (*Pseudocercosporella herpotricoides*) derived from the wild relative *Aegilops ventricosa* (Summers *et al.*, 1988). This is a soil borne pathogen for which direct screening is difficult and tedious. In contrast the endopepetidase screen allows up to 600 samples to be processed by one person in three days.

Another association has been exploited for many years by tomato breeders for resistance to the root-knot nematode *(Meloidogyne incognita*) conferred by a single gene, *Mi*, also introduced from a wild relative, *Lycopersicon peruvianum*. Rick & Fobes (1974) showed that an isoenzyme marker *Aps-1* (acid phosphatase-1) was linked to *Mi*, with the codominant allele, *Aps-1*^{1}, cosegregating with the resistance.

Later work has shown *Aps-1* is in fact a short distance from *Mi,* (Ho *et al.*, 1992). Whilst this does not detract from its practical utility, it means that germplasm carrying *Mi* such as Rossol or Motelle (Fig.1) in which a rare recombination event has taken place cannot be screened by isozymes. This is unfortunate as these lines carry a smaller segment of chromosome from the wild parent and consequently are less likely to bring over additional undesirable traits. To tackle this Williamson *et al.* (1994) screened a set of varieties with RAPD markers (Random Amplified Polymorphic DNA, see Waugh and Powell, 1992), which are PCR based, simple to apply, but dominant and not robust. They identified a particular band, REX-1, associated with *Mi*, even when *Aps-1*^{1} was absent. Additional cloning and sequencing work enabled them to convert this to a robust, codominant format.

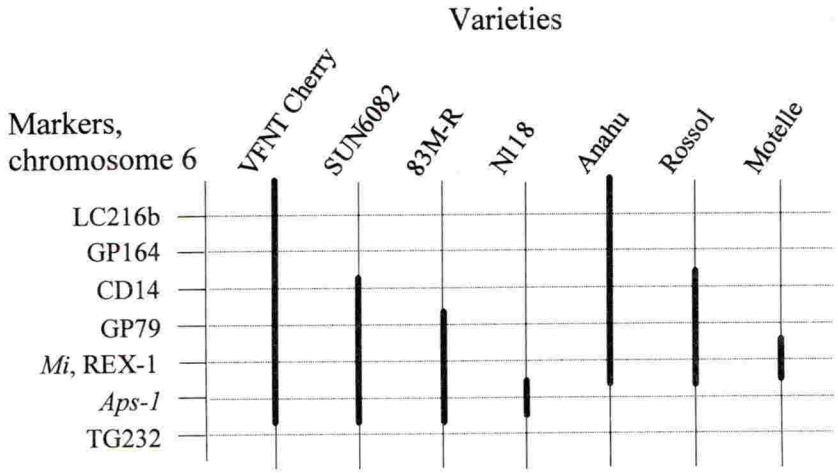

Figure 1. Introgressed chromosome segments from *L. peruvianum* into *L. esculentum* near the *Mi* locus (after Ho *et al.*, 1992)

Besides showing tighter linkage REX-1 has an additional advantage. *Aps-1* is not reliably expressed in older plant tissues whereas PCR based markers can be used at any stage provided

quality DNA can be isolated. Thus, REX-1 can be used to screen mature plants in the field following an initial breeder's selection when the numbers of plants are much reduced.

Resistance genes derived from the crop species itself are much more difficult to mark as the flanking chromosome segments will likely be very similar to those around the susceptible allele. In consequence the best identified linkages are likely to be much looser. An example of this is resistance to pea seed-borne mosaic virus conferred by *sbm-4*. This resistance is recessive and much of the present breeding material is susceptible making a general glasshouse screen unwelcome. Recently at PBI, Dhillon *et al.* (1995) identified a loosely linked RFLP marker, GS 185, with about 10% recombination. An extensive search for one more tightly linked has been fruitless. Even so, if used to screen an F_2 breeding population, about 80% of the plants homozygous for this marker will also be homozygous for *sbm-4* as opposed to 25% in the population as a whole, a useful enrichment pending later direct screening. If, however, a backcross population is underway there are opportunities for recombination at each generation. After five backcrosses and tracking the marker, there is only a 60% chance that the resistance allele has been retained. In such a case it would be necessary to organize several independent backcross lines in the expectation that one at least will segregate for resistant types on selfing. If this seems inefficient, it compares well with only a 3% chance of retaining resistance without the use of the marker or disease screening.

If a single tightly linked marker cannot be found, then two loosely linked flanking markers can serve instead. The probability of both recombining with the resistance gene is the product of their individual frequencies. For this purpose, then, two markers at 10% recombination are equivalent to one at 1% (Tanksley, 1983).

Where a marker cannot be found closely linked to a gene of interest, it may be possible to introduce one. Miklas *et al.* (1996) have suggested intercrossing lines where the gene and potential marker are in repulsion. A round of screening identifies the rare crossover event bringing the marker and gene into coupling. Such plants may then be used as breeding stock as outlined above.

Resistances derived from wild species are favourable subjects for marker assisted breeding since the resistance gene resides on a segment of alien chromosome that will likely have many differences from the corresponding crop species segment. This has its own drawbacks however, as the alien segment is also likely to carry additional genes for 'wild' traits unwanted by the breeder. This problem can be minimised by selection against flanking markers (Young & Tanksley, 1989). A backcross population is first screened for the resistance gene either by inoculation or with a tightly linked marker. Plants carrying the resistance are then rescreened with more loosely linked markers to identify close recombinants. A further backcross cycle will identify additional events on the other side of the gene. These 'double recombinants' carrying a minimal segment of the alien chromosome can then be incorporated in the conventional breeding programme.

Identifying markers linked to a resistance gene can be a considerable undertaking if it has not been previously mapped. Cultivars and lines with and without the resistance will also differ over other parts of the genome, and in screening a large number of markers for linkage, spurious associations will be found. Where available, near isogenic lines are very valuable, being bred

to differ only in the resistance gene itself. It follows that any differences in marker profiles are due to those markers being tightly linked to the resistance (Muehlbauer *et al.*, 1988).

Most resistances are not available in such favoured backgrounds, and screening many plants for many markers in a segregating population requires considerable effort. Michelmore *et al.* (1991) have developed an approach to circumvent this problem termed Bulk Segregant Analysis. Resistant and susceptible groups are established from a segregating population and two bulk DNA samples representing them are prepared. Because of the independent assortment of unlinked genes the two bulks will only differ for markers closely linked to the resistance gene, just as with near isogeneic lines. Apparent associations can then be verified with more intensive work on the candidate markers.

Finally markers for resistance offer a way of stacking resistance genes that classical selection procedures cannot. Resistance genes deployed singly by breeders are frequently matched by new pathogen races on the basis of a single rare mutation in their corresponding virulence genes. It follows that deploying resistance genes in multiples could be advantageous to the stability of resistance since simultaneous mutations in multiple virulence genes would be required by the pathogen, a much less likely event. However, stacking such resistance genes cannot easily be accomplished by traditional techniques as there would be no way of distinguishing breeding lines carrying any of several, or all resistance genes. Markers allow the differing resistance genes to be tracked individually and complex resistant lines to be constructed. Similarly it would ease the assembling of minor resistance genes each of small effect to produce a more highly resistant variety.

The present state of marker assisted breeding for pest and disease resistance must be seen as only an interim stage. Its application is presently restricted by the labour that must go into identifying and using markers. Over the next few years new classes of markers, more rapid procedures and automation can all be expected to reduce the burden of work and extend their application into more and more routine areas of plant breeding. At a more fundamental level a number of resistance genes have now been sequenced (see Beynon, J L, this volume), and the common motifs found indicate that very many more will be investigated soon. In terms of marker assisted breeding alone, the identification of polymorphisms within resistance genes, especially those that distinguish resistant and susceptible alleles will assist the development of appropriate markers and remove the constraints of loose linkage by making it absolute.

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SESSION 7B

PESTICIDE EFFICACY AS INFLUENCED BY FORMULATION AND ADJUVANTS

Chairman & Session Organiser	Dr P J Holloway *IACR-Long Ashton, Bristol*
Papers	7A-1 to 7A-4

ACHIEVING OPTIMAL BIOLOGICAL ACTIVITY FROM CROP PROTECTION FORMULATIONS: DESIGN OR CHANCE ?

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ABSTRACT

Until recently, the development of biologically optimised agrochemical formulations has involved a significant element of chance, because of essentially empirical testing procedures. Due to the pressures on the industry to produce low dose rate products with minimal impact on non-target sites, the role of formulation technology has now aquired a much higher profile. As the requirements for optimised targeting are becoming increasingly understood, the use of predictive design elements should increase. Examples are provided which show how the performance of an active ingredient can be modified significantly by altering its formulation type and composition. Whilst the use of tank-mix adjuvants may sometimes be necessary, the development of optimised one-pack formulations should be the commercial target for most active ingredients.

INTRODUCTION

The concept of designing agrochemical formulations for optimal biological activity is relatively new. Whilst there has been extensive consideration of the design of a.i.s for specific biochemical targets, the effort devoted to influencing the targeting of the a.i. has received considerably less attention. This is surprising in view of the known enhancement which can be achieved by tank-mixing some pesticides with adjuvants. The gaps in our understanding of formulation-activity relationships is, perhaps, largely attributable to the subdivision of expertise in R&D based agrochemical companies. Whilst the development of stable complex formulation systems is now a defined science to physical chemists, the impact of such systems and their "inert" components on activity is not considered to be the domain of the chemist. Conversely, research biologists often have a limited understanding of the formulation process. Frequently, development of biologically optimised formulations may be seen as a screening procedure conducted by biologists for the evaluation of many formulation versions. This view of the formulation development process is now being rapidly superseded by a drive to achieve optimal activity from new, as well as existing compounds, and to gain a scientific understanding of the processes involved. This more rational approach has been forced by several factors; the expense of developing new compounds means that the maximum biological activity per unit cost must be achieved to justify further development. In addition, the regulatory pressures to reduce doses and losses to non-target sites in the environment has forced the industry to consider closely the ecotoxicological impact of formulation design.

Role of formulation technology in discovery process

The whole subject of screening compounds for biological activity, irrespective of whether they are fungicides, insecticides or herbicides is inextricably linked with formulation issues. Within traditional screening methodology, compounds are sprayed onto the target at a range of dose rates using a particular delivery system, which may be considered to be a formulation. The nature of the formulation varies between companies but is either a model system, typically an aqueous acetone

solution (or suspension depending upon compound solubility and dose rate) containing a surfactant or surfactant blend, or alternatively a traditional agrochemical formulation. Within the limits of such systems, any defined activity per unit rate is inevitably a judgement on the formulation system in addition to that of the a.i. It would seem unreasonable to expect any formulation system to perform equally effectively for compounds of diverse physicochemical properties with differing sites of action within the target organism. Any screen of such inflexible design may preferentially select for compounds of a type which have been discovered previously.

Within one broad class of agrochemical products, fungicides, it is remarkable how commercial (organic) compounds are confined to a limited range of physicochemical parameters, with a few exceptions. Water-soluble fungicides are relatively rare, as are phloem mobile ones (fosetyl aluminium), whilst many are encompassed by a relatively narrow log P range compared with the range exhibited by all pesticides. Does this reflect the defined properties required for fungicidal activity or the historic limitations of the screening process ? In order to truly screen compounds, systems must be designed to determine the fundamental activity of compounds. Whilst biochemical tests exist, for example, mitochondrial assays for electron transport inhibition (ETI) compounds, it must be recognised that compounds will be stressed more rigorously in commercial use.

Design of formulations: critical compound criteria

In order to design formulations for optimal expression of biological activity, there are various criteria which must be satisfied. The design process should be an integrated research process involving inputs and feedback from numerous disciplines. Many questions must be answered and results relayed to the formulation chemist in a timely manner to ensure that an optimised product is taken to the market. This is becoming an increasingly difficult task if a compound is to be registered and launched as soon as possible after discovery to maximise its patent life. It may be surmised that the most appropriate delivery systems have not always been the first to reach the market. The benefits, in many situations, of tank-mixing with adjuvants may be interpreted as identification and correction of product failure. Compound-determined factors which must be considered during the design process can be categorised under the following headings:

(i) Pesticide mode of action

To achieve optimised and robust targeting of an a.i. the mode and/or site of action should be critical in the design process. Whilst the situation with herbicides is relatively simple, fungicide targeting is a complex process, with a large interacting matrix of parameters which must be interpreted. Factors to be considered include the host plant, target pathogen, sensitive stage(s) of the pathogen life cycle, biochemical target site, a.i. and the delivery system. Often at an early stage of investigation, the biochemical mode of action of a new a.i. is not fully understood. To aid in the formulation development process, empirical biological testing can be conducted with formulations of varying properties, for example, differing foliar penetration and target coverage. In parallel with biochemical studies, this focuses future development effort towards the formulation properties required.

(ii) Physicochemical properties

Whilst a compound may show good intrinsic activity, this must be realised within the constraints of a commercially acceptable delivery system. The physicochemical properties of the compound are pivotal in determining formulation opportunities (Stock & Davies, 1994). However, requirements for optimal biological activity and the opportunity to build such requirements into the formulation

system do not necessarily coincide. The task of maximising the activity of a compound, despite physicochemical limitations, has led the design of agrochemical formulations to converge with those of the pharmaceutical industry with regard to scientific input; whilst the basic activity of a compound is not in doubt, ever more complex formulation systems may be required to maintain this activity in the commercial product.

Critical physicochemical factors of a.i.s for consideration during biological optimisation include melting point, solubility in a range of materials, volatility, lipophilicity and photochemical stability. It is often found that, in order to achieve maximum biological activity, a solvent-based formulation system is required. Whilst such systems may be regarded as less preferable with regard to storage, transport and operator safety, they can permit significant dose reductions of the a.i..

Microencapsulation of an a.i., whilst lowering the toxic potential of a pesticide can be a useful tool for reduction of volatility and maintaining the a.i. in an available form for prolonged periods. A number of film-forming tank-mix adjuvants are claimed to have a similar effect with potentially volatile a.i.s. An alternative approach to the reduction of volatilisation is to enhance penetration of the a.i., although this is not a guarantee of enhanced activity; it may even reduce activity despite reduction of loss to the atmosphere. The a.i. may not be required inside a plant to exert its biological effect; this requirement has been identified for some non-systemic fungicides. In addition, metabolism subsequent to penetration may be a significantly greater route of loss than volatility. Such considerations show the need for close cooperation between formulation, biological and biochemical disciplines. The potential conflict between penetration and volatility is well illustrated by the aphicide, pirimicarb (Table 1). Loss of this compound by volatilisation can be significantly reduced by maximising penetration through surfactant addition.

Table 1. Recovery of radiolabel from ^{14}C-pirimicarb (0.05%) after foliar application to field bean in the presence of an alcohol ethoxylate surfactant

Time after application	Surfactant (% w/v)	Recovery of label (%)		Loss (%)
		Surface	Internal	
8h	0	39.8	28.9	32.1
8h	0.05	29.7	40.5	29.8
8h	0.1	13.6	68.0	15.7
8h	0.5	5.6	91.9	2.6
1d	0	29.7	31.9	38.4
1d	0.05	13.7	45.2	41.1
1d	0.1	9.1	67.7	23.2
1d	0.5	2.8	92.5	4.7

Adapted from Stock (1990).

Formulation-determined parameters relevant to activity and their prediction

There are numerous factors which can be modified by formulation in order to vary biological response. The importance of these factors and freedom to vary them depend greatly upon the nature of the pesticide and its intended site of action. Important parameters are summarised below.

(i) Impact of formulation on spray quality and retention

Of critical importance for any pesticide formulation is the need to ensure satisfactory spray performance to ensure delivery to the target site. Whilst additives, such as spray-modifier adjuvants, may be tank-mixed, it should be questioned whether this is a requirement, an avoidable shortcoming of the formulation process or a consequence of a particular market segment being of insufficient

value to justify development of a niche formulation. Much depends upon the intended target. For easily wetted plant species which do not possess a microcrystalline covering of epicuticular wax, wetting of the leaf surface often does not require significant quantities, if any, of surfactant materials.

Model track sprayer studies (Grayson *et al.*, 1991) have shown that the dynamic surface tension of the spray solution is a critical factor in determining retention efficiency, notably with difficult-to-wet plant species. The importance of this parameter has been reinforced by extensive statistical analysis of parameters relevant to spray atomisation (Hermansky & Krause, 1995). Of critical importance is not only the chemical nature of spray additives but also their concentration. The amounts required to improve foliar retention tend to be far in excess of those required to have an effect on equilibrium surface tension. Upon calculating the total required dose of spray additive from the spray volume applied to a crop, the quantity is often large. The amounts involved may be difficult to incorporate into a one-pack formulation system.

Having established that formulation components and/or additives can have a significant influence on foliar retention, it must be recognised that other effects may also occur. For example, the additives may have either beneficial or antagonistic effects on foliar penetration. In addition, there can be significant influences on spray quality, especially for surfactants. Holloway (1994) noted that lower alcohol ethoxylates caused a marked increase in spray droplet volume median diameter (VMD) compared with water alone. Similar studies by Miller *et al.* (1995) showed a variety of responses of spray quality to inclusion of a selection of adjuvants, with clear implications for the categorisation of spray quality from nozzles.

(ii) Foliar distribution as influenced by formulation

For many pesticides, foliar distribution can be important in influencing biological activity. This may be in relation to the foliar placement and size of droplets, for example, in the control of some insect pests (Sharkey *et al.*, 1987). As already discussed, spray atomisation and deposition processes can be influenced significantly by formulation and additives. In addition, overall coverage (percent leaf area covered) can be of importance, particularly in relation to non-systemic pesticides. Poor coverage may be overcome by vapour activity of pesticides, provided that the rate of vapour loss is not too great, this factor being particularly critical for herbicides !

(iii) Optimising penetration for biological activity

In order to optimise foliar penetration, the destination of the a.i. must be known. For herbicides, this is clearly within the living tissues of the plant. However, the optimal location of fungicides is not necessarily within the leaf.

For investigation of foliar penetration, a number of approaches have been used ranging from isolated cuticle studies to whole organism investigations. Such studies have been beneficial in helping to elucidate the mechanisms of the foliar penetration process and the impact of surfactant/adjuvants/coformulants on this process. Investigations with radiolabelled surfactants and intact plants suggest that foliar penetration of surfactants is required with that of certain a.i.s, examples being cyanazine and WL 110547 (Stock *et al.*, 1992). Investigations using isolated cuticles (Schönherr & Baur, 1994) would suggest that surfactants act to modify the diffusive properties of cuticular waxes and act as a solvent front which facilitates the subsequent penetration of the a.i..

The ability to predict foliar penetration phenomena precisely (which are distinct from biological activity) is not yet possible for realistic complex commercial formulations. Under commercial application conditions, it must be remembered that there are environmental effects which influence the penetration process. These include moisture stress of the plant (de Ruiter & Meinen, 1995), relative humidity (humectant effects) and temperature. All such factors may influence penetration and subsequent biological performance. It is possible to incorporate such parameters into penetration models for isolated cuticle/barrier systems. More importantly, however, agrochemical formulations are not ideal delivery systems applied to a uniform test surface. They are often complex mixtures of dispersants, structuring agents, emulsifiers, solvents and buffers which may interact in numerous ways to influence the penetration process, in addition to the previously mentioned retention and foliar distribution processes.

It is evident that the complexity of agrochemical formulations must be addressed if we are to understand the penetration process of complex interactive systems and how to adjust this process predictively. It is not only the physicochemical properties of an a.i. which are important, but the type of formulation presented to the leaf surface. In addition, the ratio of components can influence penetration, with differing results on a selection of plant species (Stock & Davies, 1994). An example of the differential response of prochloraz and fluquinconazole to a lipophilic alcohol ethoxylate surfactant, Synperonic A5 ($C_{13/15}$ alcohol, 5EO) is shown in Table 2.

Table 2. Influence of adjuvant rate and ratio on the foliar penetration of fluquinconazole into vine and prochloraz into wheat, 1 day after foliar application in aqueous acetone

		Compound on leaf surface (% applied)	
Compound (% w/v)	Synperonic A5 (% w/v)	Fluquinconazole	Prochloraz
0.005	0	88.0	91.0
0.05	0	98.7	92.8
0.5	0	98.7	102.5
0.005	0.005	87.2	62.4
0.05	0.05	88.5	24.4
0.5	0.5	79.7	6.5
0.005	0.25	11.9	5.0
0.05	0.25	26.9	4.6
0.5	0.25	96.2	4.4
		LSD 13.9	LSD 8.4

These data, for a model aqueous acetone system, show clear differences in response. Whilst a constant percentage penetration is achieved with fluquinconazole if the a.i.:adjuvant ratio is maintained, the absolute rate of adjuvant has a dominant effect with prochloraz. Synperonic A5 is of a chemical type which is know to penetrate rapidly. It may be that, for fluquinconazole there is an insufficient adjuvant:a.i. ratio to cause significant penetration, except for two of the treatments in which there is at least a five-fold excess; this compound penetrates relatively slowly but steadily under normal circumstances (Wilde, 1994).

Fate, behaviour and phytotoxicity of inert formulation components and adjuvants

When considering the formulation design process, it is important that the fate and behaviour of formulation components and tank-mix adjuvants at the target site are considered. This can have a significant impact upon the performance of such materials as biological activators, in addition to side-effects, such as phytotoxicity. It has already been mentioned that solvent-based formulation systems are often the most dose-efficient (depending upon a.i.) with regard to activity. However, the solvent-emulsifier system must also be considered in relation to the target. For example,

glasshouse studies have shown that high flashpoint solvents can be more damaging than low flashpoint ones. A matrix study was conducted in order to investigate the impact of solvent type and emulsifier content using xylene and a high flashpoint solvent (Stock, unpublished data). It was found that, within the range investigated, emulsifier content had no significant impact on phytotoxicity. However, the type of solvent was critical. Phytotoxicity could be described by the equation: $y = 67.2x - 4.5$ (coefficient of determination, $r^2 = 0.95$), where, y = percent damage to vine leaves and x = percent content of high flash solvent at field dilution. The solvent system and nature of the target crop is, therefore, a significant consideration, in addition to the proposed loading of the formulation per unit crop area.

In addition to the foliar penetration of formulation components, which can cause phytotoxicity, losses due to volatility must be considered, because they can have a significant impact on biological performance. Whilst many formulation materials may not be considered to be volatile, losses from the surface of a crop can be dramatic. This point is illustrated in Table 3 which shows volatility rates for a range of equivalent hydrocarbon (EH) values.

Table 3. Volatilisation rate for compounds of varying equivalent hydrocarbon (EH) values

Hydrocarbon	Vapour pressure (Pa)	Volatilisation (kg/ha per day)	$t_{1/2}$ on leaf surface at 1kg/ha
EH 10	400	68 000	<<< 1 min
EH 15	9	1 400	< 1 min
EH 20	0.1	30	30 min
EH 25	4×10^{-3}	0.5	1 day
EH 30	8×10^{-7}	0.01	50 days

From Briggs & Bromilow (1994).

As penetration into the cuticle is required for uptake enhancement for many materials, volatility of the uptake-enhancing material can be a significant competitive force for its activity. This may be particularly problematic for situations of slow penetration for which reasonable persistence of the uptake activator is required. For example, the EH values of methylated seed oils are typically in the range 13 (for $C_{8/10}$ methyl esters) to 21 (methyl oleate).

Tank mixing versus one-pack formulations

When deciding the approach to use for optimising the activity of a compound, it must be decided whether a complete product is required or whether tank-mixing with an adjuvant is appropriate. This decision depends largely upon the territory and market segment. In the USA, use of tank-mix adjuvants is extremely high in contrast with Europe (Uttley, 1995). Within Great Britain, an adjuvant is considered to be a tank-mix additive other than water which has no biological activity in its own right, but which enhances the activity of the a.i. This definition does not, therefore, include built-in adjuvants which may be considered as coformulants and accordingly treated differently with regard to regulatory requirements. That an adjuvant or any formulation component is truly inert, with no activity is also somewhat dubious; all materials, however benign, have an innate toxicity. Most formulation materials will, therefore, have some biological effect above a critical concentration. For example, surfactants can have both antagonistic and beneficial effects on plant growth (McWhorter, 1985), whilst workers in the fungicide field will be familiar with the effects of surfactants against many pathogens, such as powdery mildews. Irrespective of the anomalies in the way in which tank-mix additives are considered in some imperceptible manner to be different from other formulation components, there are a number of significant obstacles to the building of one-pack optimised formulations.

(i) Compatibility

If an activator material is to be included as part of a formulation, this does not detract from the need to produce a chemically and physically stable product. For example, inclusion of large amounts of surfactant materials may lead to crystal growth within suspension concentrate (SC) formulations, gelling or phase separation. The type of formulation into which an activator is to be included is critical. For example, solvent-based emulsifiable concentrate (EC) formulations usually contain significant quantities of surfactant; thus, an activator may be included here as part of the emulsification system. Whilst problems can occur for aqueous SCs as indicated above, oil-based formulations can offer the opportunity for high level incorporation of oil-type adjuvants, which are typically required at higher rates than surfactant activators. Such systems include oil-SCs, oil-ECs and suspoemulsion (SE) formulations, the latter offering the opportunity to incorporate oil adjuvants into a water-based system.

(ii) Spatial considerations

The space available within a formulation for incorporation of an adjuvant largely depends upon the dose rate of the a.i., formulation type and market-acceptable pack volume per unit crop area. Clearly for extremely low rate pesticides, especially sulfonylurea herbicides, which are active at several grams per hectare, it would seem bizarre to develop a product containing 90% activator. This would add significantly to packaging, storage and transport costs for a high value pesticidal product. In such circumstances, the use of a tank-mix additive may be unavoidable for maximising the field potential of a compound and/or enhancing the activity spectrum. The lower value biological activator which will normally be of low toxicological and environmental concern can be transported separately or sourced locally.

Approaches to formulation design

Rational consideration of the factors mentioned previously, such as optimising placement on or in leaves, depending upon compound mode and site of action, allows formulation components to be selected and tank-mixed with a basic formulation to evaluate their potential for inclusion within the final formulation. Often, certain inert materials can have the required properties for increasing activity without deliberate selection for this purpose. For example, the emulsification system within Galtak EC is effective at enhancing uptake of benazolin-ethyl, although it was not specifically selected for this purpose (Table 4).

Table 4. Foliar penetration of radiolabel from ^{14}C-benazolin-ethyl 4 days after foliar application to field bean in 50% acetone in the presence of 0.3% w/v emulsifiers from Galtak EC formulation

Benazolin-ethyl (% w/v)	Surface recovery (% applied label)	Calculated uptake (µg)	
		Unadjusted	Volatility-corrected
0.125	9.0	2.28	1.94
0.20	18.9	3.24	2.90
0.25	30.5	3.48	3.24
0.50	77.5	2.25	2.25

Stock, unpublished data.

Tank-mixing evaluation of potential formulation components has been extensively used, for example, the evaluation of biological activators for dimethomorph (Grayson *et al.*, 1996a,b). Certain fundamental problems exist with this approach; these are related largely to the ubiquitous problem of glasshouse to field transfer of compound activity. For fungicide evaluation, compound rates must generally be much lower than field rate if comparisons of activity are to be made. However, for beneficial effects of many adjuvants, they must be present as a certain percentage of the final spray dilution. The a.i.:adjuvant ratios of indoor tests are, therefore, often vastly different

from those which can be achieved for realistic outdoor conditions. It is known that both adjuvant rate and adjuvant:a.i. ratio can have a significant effect on foliar penetration and subsequent biological activity.

Examples of systematic formulation design

Whilst it is established that there are numerous obstacles to designing formulations for optimised activity, work within this area to achieve maximal activity from compounds is increasing. The instability of the strobilurin analogue BAS 490 F in plants has established the need to minimise penetration (Gold *et al.*, 1994). In addition, the studies by Grayson *et al.* (1996a,b) have shown the benefit of appropriate amounts of some alcohol ethoxylate surfactants within one-pack formulations of dimethomorph.

An example of the influence of formulation systems is shown for the low melting point acrylate, SN 618899 (Table 5). This compound was not suitable for formulation as a particulate system, a system which is appropriate for minimising penetration. In order to maximise the amount of a.i. on the leaf surface, three solvent-based formulations were evaluated. The choice of emulsification system was devised to minimise use of materials which were known to enhance the penetration of lipophilic materials. Using this procedure it was possible to significantly modify penetration properties.

Table 5. Surface recovery of SN 618899 (0.5% w/v) 5 days after foliar application to wheat as a 20% EW formulation and two types of 20% EC formulations

Formulation	Surface recovery as % of applied dose
20EW	97.2 (2.3)
20EC (Type A)	60.8 (4.2)
20EC (Type B)	43.5 (13.5)

Standard deviation for five replicates in parentheses. Recovery measurements made by HPLC.

An example of the benefits of enhanced formulation design is provided by the new quinazoline-based triazole fungicide, fluquinconazole. Triazole fungicides cover a range of physicochemical properties. They range from oils (e.g propiconazole) to high melting point solids, whilst the log P values vary from 2.7 (flutriafol) to 4.0 (difenoconazole). Amongst these compounds, fluquinconazole is extreme in both its high melting point and low vapour pressure, which can be accounted for by its compact and rigid molecular structure. These properties have been related to the long-lasting activity of this compound, coupled with its steady penetration. The early expression of activity can, however, be greatly enhanced by increasing the rate of initial penetration. Extensive screening of many materials has shown that it is possible to improve the initial penetration significantly (Table 6).

Table 6. Surface recovery of ^{14}C-fluquinconazole (0.05%) after foliar application to wheat in a model and commercial-type formulation system in the presence of three novel adjuvant materials

		Aqueous acetone		Experimental SC	
Adjuvant	Rate (%)	1 day	5 days	1 day	5 days
DV 1	0.05	40.7	18.5	92.2	86.2
DV 1	0.25	13.2	3.7	86.0	47.9
DV 2	0.05	27.7	16.5	88.2	81.7
DV 2	0.25	11.1	2.5	79.3	32.5
DV 3	0.05	25.7	4.4	99.9	92.5
DV 3	0.25	7.9	1.9	79.4	37.2

Values mean of four replicates. Adjuvants belong to the same chemical class and are of increasing chain-length from DV1 to DV 3.

For this fungicide, there is a considerable difference between the model aqueous acetone system and an experimental aqueous SC; the penetration potential of SC systems is considerably reduced. However, even with the SC system, there is considerable uptake enhancement of fluquinconazole when the adjuvants are present at 0.25% of the spray volume. Testing showed that there was good correlation between uptake enhancement and biological activity. In particular, the control of *Erysiphe graminis* on wheat, in addition to the curative activity against *Puccinia recondita*, was significantly enhanced in the presence of the most effective "adjuvant" without sacrificing long-lasting disease control. Biological enhancement materials which were initially selected for their likely beneficial effect on foliar penetration of lipophilic materials and were further evaluated by tank-mix bioassay, have been successfully engineered into active oil-based one-pack fluquinconazole products to give enhanced second generation formulations. These materials are not available as tank-mix additives.

Studies of this type have established that there is now greater scope for evaluation of new inert materials for beneficial effects on biological activity. Such materials are generally cheaper than pesticidal a.i.s and offer the opportunity for dose reduction and the realisation of the biological potential of the a.i.. This is not, therefore, true synergy but the overcoming of physicochemical and conventional formulation constraints on the expression of innate activity.

CONCLUSIONS

From this brief survey of recent work in the formulation-activity area, it is clear that there is an increasing appreciation of the implication of formulation parameters for the activity of foliage-applied agrochemicals. This work has been motivated by the ever increasing demands for reduced pesticide doses and more precisely targeted applications. Whilst the process of product optimisation has historically been a very empirical process, with a significant element of chance, there has been a considerable shift towards the rational design of agrochemical formulations.

It is probable that this greater emphasis on compound optimisation with newer products will significantly reduce the frequency with which tank-mix adjuvants are found retrospectively to enhance such products. However, tank-mixing will remain necessary for the enhancement of many older product formulations. In addition, their use will be necessary for market segments which do not justify development of specific tailored products. Application practices will also influence the need for tank-mix adjuvants. It must be recognised, however, that the future viability of tank-mix products will depend upon the outcome of decisions by relevant regulatory authorities.

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IMPACT OF FORMULATION PROPERTIES ON INSECTICIDAL EFFICACY

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ABSTRACT

The effectiveness of contact insecticides as crop protection agents is known to be influenced by formulation properties. These modify droplet formation, the in-flight behaviour of airborne droplets and the efficiency of retention by the crop surface. Formulation properties also influence deposit behaviour on the crop in ways that can have profound effects on the availability and transfer of a.i. to the target insect, thereby modifying the quantity of insecticide which needs to be applied to achieve a required level of control. This paper reviews some of the ways in which formulation can be adjusted to minimise the amount of insecticide sprayed and render broad spectrum insecticides more selective. These include changes to the surface properties of deposits and to formulation viscosity and cohesion. The importance of matching formulation and application procedures is emphasised.

INTRODUCTION

Growing concern about the use of agrochemicals as crop protection agents is resulting in a reassessment of the way these materials are formulated and applied. Research suggests that the choice of formulation components can have a profound influence on retention of a.i. by a crop and on the subsequent behaviour of the retained deposit (Stock & Davies, 1994), its transfer from plant surface to target insect (Ford & Salt, 1987) and, therefore, on the quantity of a.i. that must be applied to achieve field control (Salt et al.,1989). Understanding how deposit behaviour is related to formulation properties may therefore suggest ways of reducing the amounts of contact insecticides used. Such knowledge may also indicate how broad spectrum compounds can be targeted more effectively against the insect pest rather than non-target or beneficial species.

Recent research at the University of Portsmouth has investigated the use of adjuvants to modify formulation properties and improve insecticidal efficacy. These studies have been concerned primarily with processes taking place at or near the crop surface once impaction and retention have occurred. However, the properties required to maximise transfer to a target insect can sometimes conflict with those needed for droplet formation, delivery to and retention by the crop. Furthermore, to improve biological performance, residual, contact and systemic pesticides will all have different adjuvant or formulation requirements (Holloway, 1994). Ways must therefore be found to adjust deposit properties so that they meet these different specifications.

Liquid formulations

The formation of agrochemical sprays is usually achieved by forcing liquids under pressure through a fan nozzle. Fan nozzle sprayers are capable of producing the high volume throughputs required by conventional application systems but are restricted to using low viscosity carriers, such as water. Water, however, is a very polar, highly volatile solvent of low viscosity which evaporates rapidly during delivery to the crop surface and is repelled by the waxy plant surface unless surfactants are added to lower the surface tension. It is for these reasons that many agrochemical formulations are water-based ECs and WPs. Such formulations are retained by the plant surface and spread to provide effective (and sometimes coherent) cover before drying soon after impaction. The resulting deposit may be a slowly crystallising liquid, a residue of concentrated micro-droplets or an amorphous solid left in intimate contact with the surface waxes and plant epidermis. Such deposits are suitable for herbicides and systemic insecticides and fungicides which must penetrate into the plant before exerting a toxic action. Indeed, for such materials the presence of surfactants within the deposit may aid penetration through the cuticle by disrupting the surface waxes and/or aiding dissolution. However, these properties are inappropriate for contact insecticides which must be retained proud of the plant surface where they will be available for encounter by and transfer to mobile target insects.

These requirements can be met by hydrophobic carriers or adjuvants which adhere more strongly to the epicuticular waxes of arthropods than to plant cuticular waxes. Studies using mineral oils have established that the transfer of a deposit from plant to insect depend crucially on the its surface energy, its physical size and fluidity. Thus, highest transfer efficiencies from leaf surfaces of *Brassica oleracea* to the false legs of larvae of the Egyptian cotton leafworm (*Spodoptera littoralis*) were obtained using small deposits of cypermethrin dissolved in a viscous carrier of liquid hydrocarbon (Crease et al.,1987). For transfer to caterpillars, viscous carriers appear to have a number of advantages, *viz.*:

(1) a slow rate of surface spread resulting in deposits which remain proud of the plant surface and available for transfer;
(2) a high initial transfer efficiency to the surface of the caterpillar;
(3) a low redeposition potential which results in a high retained fraction.

For maximum transfer, the contacting surface area of an insect should exceed that of the deposit and the plant surface should be smooth and free of wax and blooms which lead to roughness. During contact, the mineral oil was observed to adhere strongly to the insect as the liquid deposit peels away from the plant surface. The adhesion forces must have been very high since the deposit was retained by the insect surface throughout the period of severe convulsions which followed shortly after transfer of toxicant to the larva (Salt & Ford, 1984). However, the proportion of deposit transferred to a leafworm following a single contact seldom exceeded 0.8, so that a proportion always remained on the leaf surface. This implies that the force of adhesion between the liquid carrier and the plant surface which it wets remains high and the oil deposit is unlikely to be entirely stripped from the leaf surface with which it is in such intimate contact (Hemberg, 1965). When a larval proleg is retracted from an oil deposit attached to a leaf surface, the liquid

film between the surfaces will normally break to leave films on both surfaces. To achieve transfer efficiencies above 0.8, the cohesion of the deposit must be high enough to resist the competing forces of adhesion to the plant and insect surfaces; adhesion must also be higher to the insect than to the plant surface.

The proportion transferred is determined by two further factors determining availability, both associated with deposit spread, *viz.*:

(1) the spread deposit may occupy a greater area than the proleg resulting in incomplete contact;
(2) the thinner film of oil resulting from a spread deposit will be in intimate contact with the leaf, its surface lying partly below the peaks of crystalline plant wax and less available for encounter by a passing insect (Crease, 1987).

Note that factor (1) suggests that the coherent cover approached by conventional high volume application will be inefficient for oil-based formulations since the deposit will always cover a much larger area than that contacted by the insect. Low volume application comprising smaller, discrete deposits may more effective for oil-based formulations. Note also that mineral oils applied at high volume application rates (high leaf cover) are often phytotoxic, whereas these same oils applied at low volume (low leaf cover) seldom are.

The capacity of the proleg of the leafworm to intercept and retain carrier oil is rate-limited. Crease (1987) reports, for example, that following a half second contact, the maximum volume of oil that can be transferred from an oil drop (2.4 nl) with a deposit diameter of 166 μm and retained by the proleg of a 25mg leafworm is 1.3 nl (i.e. the transfer efficiency, *pt*, = 1.3/2.4 = 0.54). This value, however, increased with duration of contact. Thus, the maximum volume of retained oil for the same sized larva (25 mg) and deposit (2.4 nl) rose to 1.9 nl (*pt* = 0.79) after a ten second exposure. These results suggests that carrier continues to flow onto and over the insect for several seconds, providing contact is maintained between the insect and the deposit.

During the transfer process, insecticide may move away from the point of interception as a result of surface tension effects arising from evaporation of volatile carrier oil components (Bascombe et al., 1964) and in response to deformations of the larval cuticle. It therefore becomes unavailable for subsequent redeposition onto the plant, a process further assisted by grooming and cleaning behaviour (Gratwick, 1957). Insects contact the surface over which they walk for periods varying from tenths to tens of seconds, with a distribution usually skewed to the right (large contact times) reflecting the higher probability of observing a short contact time; during rest, contact with the surface may last for several minutes. For contact efficacy, it is therefore important that the deposit remains fluid so that transfer and relocation can occur over the insect surface and the maximum quantity of a.i. can be retained (Crease et al., 1987). These considerations suggest that insects with different modes of locomotion and behaviour will be the subject of different rates of transfer and redeposition, and should therefore experience substantially different risks when walking over an insecticide-treated surface of given specification.

Does this result imply that by adjusting formulation and deposit characteristics, it might be possible to develop contact insecticide treatments that are selective for particular classes of arthropod? In order to obtain a preliminary answer to this question, the experiment investigating the effect of viscosity on transfer efficiency has been repeated using adult mustard beetles (*Phaedon cochleariae*) as the test insect. In contrast to leafworms, beetles walk across the underlying surface on the tips of their tarsi and contact the substrate only fleetingly and present a minimal surface area for interception and uptake (transfer) of insecticide. Interestingly, transfer efficiencies for mustard beetles (Ford, 1992) were highest for Sirius oil M40 (viscosity at 37.5°C is 38 - 43 cSt) and lowest for Sirius M350 (viscosity at 37.5°C is 345 - 365 cSt) in clear contrast to the earlier results for leafworms. Presumably, for mustard beetles the short times and small surface areas available for transfer per encounter lead to efficient uptake of the most fluid deposit. This intriguing observation requires further investigation before the implications for field practice can be properly assessed. However, the result does suggest that selectivity might be possible for broad spectrum insecticides by careful adjustment of deposit characteristics through formulation.

Seed oil adjuvants, developed to enhance herbicide performance, have recently been shown to enhance the insecticidal efficacy of EC formulations of the pyrethroid contact insecticide, α-cypermethrin (Ford & Loveridge, 1995), though not EC formulations of certain organo-phosphate insecticides (unpublished data). Thus, addition of ethylated esterified seed oils to FastacTM resulted in reduced response times of adult mustard beetles, following a short contact with treated glass surfaces. The effect was shown to arise from increased pick-up of a.i. by the insect, rather than enhanced penetration. Adjusting the quantity of oil adjuvant relative to the components of the EC modified the transfer efficacy in a manner that suggested an optimal ratio for maximum enhancement.

These preliminary studies suggest that effective treatments are possible using less insecticide. However, the ethylated seed oils used in this study are fairly volatile and may not persist for long on exposed plant surfaces (Briggs & Bromilow, 1994). This example illustrates the compromise that is often necessary when attempting to define optimal formulation properties for enhanced biological efficacy. Thus, decreasing volatility by increasing chain -length, for example, will also increase viscosity, possibly reducing any enhancement of activity to mustard beetles, though presumably not to cotton leafworm (see above). These considerations emphasise the dangers of making general predictions of enhanced efficacy from highly focused studies based on one species or test system. Combinations of adjuvant properties which are effective against one pest may be inappropriate against a second, resulting in the selective treatments already noted.

Solid state deposits

The observation that increasing adjuvant or carrier viscosity can modify the performance of contact insecticides raises the question whether solid or semi-solid hydrophobic adjuvants, such as greases, would improve or diminish the efficacy of contact insecticides. Solid formulations are not new. WP formulations of insecticides have been used in crop protection and public health for many years and, provided the forces of adhesion to the substrate are less than those to the target insect, can be very effective (Ford & Salt, 1987). Early work by Gratwick (1957) demonstrated that pick-up is

restricted if the intermediate surface has a high affinity for the solid, as was observed when a filter paper surface was covered by a thin film of oil which retained the insecticide deposit.

One very new and interesting development in solid formulation is the use of powders, granules or tablets in the form of a concentrate for ease of handling and transportation. Such formulations are diluted in water, disperse rapidly prior to application and on drying form a deposit comprising a stable, polymeric matrix containing a.i. plus adjuvants but without undesirable solvents. This type of deposit retains full biological activity but because of its polymeric character may be able to withstand mechanical detachment as a result of rain and wind action more effectively than conventional solid treatments, such as WPs. One such product recommended for use with contact insecticides, such as pyrethroids and acyl ureas, is the subject of a filed patent application by Cyanamid Agriculture Ltd (Wedlock et al.,1994).

CONCLUSIONS

This brief review of the relationship between adjuvant or carrier properties and the effective, safer use of contact insecticides as crop protection agents has presented examples to show how formulation properties may be manipulated to improve treatment performance. Adjusting key properties, such as physical state, fluidity, adhesion and surface energy, in order to increase pick-up of a.i. from plant surfaces by target insects, provides an effective strategy for optimising performance at reduced dosage. Adjuvants have an important role to play in this process. However, our understanding of insecticide transfer and performance remains sketchy and general principles which will allow confident prediction of performance from formulation properties have not yet been identified. Careful observation and measurement of how adjuvants interact with particular pest/crop control systems remains the most appropriate way to achieve optimised performance, selectivity and reduced environmental contamination.

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BEHAVIOUR OF ALKYL OLEATES ON LEAF SURFACES IN RELATION TO THEIR INFLUENCE ON HERBICIDE PENETRATION

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ABSTRACT

Alkyl oleates (AOs) increase the foliar penetration of the herbicides, phenmedipham and quizalofop-ethyl. When applied alone, the uptake rates of AOs (methyl to octadecanyl) into barley, cleavers and pea leaves decreased with increasing chain-length. However, these rates could not be related to their influence on foliar penetration of the two herbicides. The diffusion coefficient of AOs in isolated pea wax decreased sharply as their chain-lengths increased, from 6.1 x 10^{-17} m^2 s^{-1} for methyl oleate to 2.4 x 10^{-19} m^2 s^{-1} for octyl oleate. The diffusion coefficient of phenmedipham in this wax was 6.6 x 10^{-18} m^2 s^{-1} ; addition of AOs increased it by 7- to 8-fold. Similar results were found with quizalofop-ethyl (diffusion coefficient, 2.9 x 10^{-17} m^2 s^{-1}). This effect also could not be related to their influence on foliar penetration of either herbicide. The solubility of phenmedipham in AOs ranged from 1.0 to 3.4 g $litre^{-1}$, that of quizalofop-ethyl from 116 to 181 g $litre^{-1}$. In the case of quizalofop-ethyl, solubilisation of the herbicide by AOs on the plant surface may play a significant part in its penetration enhancement but not in the case of phenmedipham.

INTRODUCTION

In a previous work, we observed that addition of methyl, butyl, octyl, dodecanyl and octadecanyl oleates increased the foliar penetration of the herbicides, phenmedipham and quizalofop-ethyl, into plants with differing epicuticular wax structures, *viz.*, crystalline (pea and barley) or amorphous (cleavers, *Galium aparine*) (Serre *et al.*, 1993). For phenmedipham, the highest penetration increases were observed in the presence of butyl, octyl and dodecanyl oleates, the effect of methyl oleate being significantly lower (Serre, 1996). With quizalofop-ethyl, the enhancement differences between the various AOs were less conspicuous.

In the present study, we have attempted to determine the mechanisms by which AOs increase the foliar penetration of these herbicides. In this respect, Stock & Holloway (1993) have proposed four possible sites for adjuvant action : (1) on the cuticle surface, (2) within the cuticle, (3) in the outer epidermal wall underneath the cuticle and (4) at the cell membrane of internal tissues. We examined some effects of AOs at the first two putative sites of action. According to McCall *et al.* (1986) and Briggs & Bromilow (1994), the physical state of a pesticide is critical for its penetration into the plant cuticle. In particular, liquid or dissolved compounds are expected to penetrate to a greater extent than solid ones and among the latter, the amorphous state is more favourable than the crystalline state. Other authors have stressed that penetration enhancers may act inside the cuticle. For instance, ethoxylated alcohols increased the mobility of 2,4-D in isolated plant cuticles (Schönherr, 1993) and polyethoxylated nonylphenols decreased the fusion temperature of box-tree (*Buxus sempervirens*) waxes (Coret & Chamel, 1994). Hence, we examined, on the one hand, the solvent properties of AOs towards phenmedipham and quizalofop-ethyl and, on the other hand, the influence of AOs on the diffusion of the two herbicides in a typical plant wax. We also determined their intrinsic foliar penetration properties.

MATERIALS AND METHODS

Chemicals and plant material

[^{14}C] phenmedipham (648 MBq mmol^{-1}, AgrEvo, Germany) and [^{14}C] quizalofop-P-ethyl (78.3 MBq mmol^{-1}, Nissan, Japan) were uniformly labelled on the phenyl rings ; their radiochemical purity was higher than 98 %. AOs were synthesised by esterification of oleic acid (97% purity) with the corresponding primary alcohol in *n*-hexane at 60 °C under N_2 in the presence of 3% p-toluene sulfonate. [^{14}C] radiolabelled AOs were synthesized in the same way from [1-^{14}C] oleic acid (Amersham, UK). The AOs will be abbreviated hereafter as C1C18:1 (methyl oleate), C4C18:1 (butyl oleate), C8C18:1 (octyl oleate), C12C18:1 (dodecanyl oleate) and C18C18:1 (octadecanyl oleate). Cleavers, barley (cv. Plaisant) and pea (cv. Frisson) were grown under controlled conditions in vermiculite at 23 °C (light/dark), 16 h photoperiod (fluorescent light delivering 220 µE m^{-2} s^{-1} P.A.R.), 70 (±10) % relative humidity.

Foliar penetration of AOs

Uptake experiments were done with 3 replicates when the plants were at the 2-3 leaf or whorl stage. Twenty *c.* 0.2-µl droplets of ^{14}C-labelled AOs (10 mM in acetone-water (19:1), *c.* 170 Bq µl^{-1}) were deposited with a microsyringe onto the upper third of the second leaf. The plants were then returned to the same conditions as during their growth. Penetration was determined 0, 3, 9 and 27 h after treatment and evaluated by washing the treated area of each leaf with 0.25 ml acetone. The radioactivity in surface washes was determined by liquid scintillation counting. The solvent-washed treated leaf and the rest of the aerial parts were then combusted in oxygen for radioactivity assessment as [^{14}C]CO_2. Radioactivity found in the roots was negligible (data not shown). Radioactivity recovery was > 90 %, except for C1C18:1 27 h after deposition which was 55-70 % (volatility losses).

Herbicide solubility in AOs

Saturating quantities of [^{14}C] herbicide (*c.* 1700 Bq) were deposited in a conical centrifugation vial and 1 ml AO was added. After 60 min sonication, the suspension was centrifuged at 10 000 *g* for 30 min and the radioactivity in 0.5 ml of supernatant determined. Experiments were conducted at 23 ° with 4 replicates ; octadecanyl oleate was not examined because it is not liquid at this temperature.

Desorption experiments

The general procedures described by Schreiber & Schönherr (1993) were followed. Pea wax isolated from the adaxial leaf surface was dissolved in chloroform and mixed with [^{14}C] herbicide (*c.* 850 Bq). The solution was then spread into circular hollows (100 mm^2) pressed on an aluminium sheet. After evaporation of solvent, sheets were heated to 100 °C for 5 min then cooled down at room temperature. AO solutions in acetone-water (19:1) (20 x *c.* 0.2 µl) were deposited onto the wax and allowed to evaporate at room temperature for 168 h. The amount of deposited wax was 400 µg, which contained 1 % of herbicide and AOs. The sheets (6 to 10 replicates) were placed into glass vials and 4 ml of a liposome suspension of 1 % lecithin was added. The vials were rotated (60 rev min^{-1}) in the dark at 24 °. At various times, the lecithin suspension was withdrawn and replaced by fresh lecithin. Radioactivity of samples was determined, as well as the residual amount of radioactivity in the wax at the end of the experiment. This allowed the determination of the amount of herbicide desorbed from the wax into the lecithin suspension after different exposure times (Mt) and the calculation of the total amount of radioactivity at the beginning of the experiment (Mo). Mt/Mo was plotted against $t^{1/2}$ to calculate the diffusion coefficient (D), taking into account that only half of the wax surface

was covered with AOs. To determine the D of AOs in pea wax, the same procedure was used except that no herbicide was added to the wax ; the deposited AO was radiolabelled (*c.* 850 Bq).

RESULTS

AOs penetrated at different rates into pea leaves (Fig. 1), with uptake rates generally decreasing with increasing chain-length ; however, after 27 h, uptake was similar for all AOs. The same behaviour was observed on barley except that AO penetration was lower, ranging from 65 % (C1C18:1) to 15 % (C18C18:1) 9 h after application (data not shown). On cleavers, AO penetration was even slower but penetration of C1C81:1 was again the fastest (*c.* 50 % after 27 h, *cf.* < 20 % for other AOs) (data not shown).

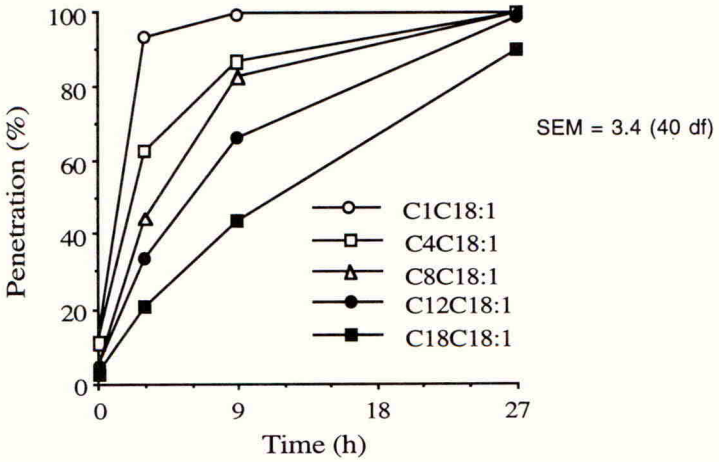

Figure 1. Penetration of AOs into pea leaves, expressed as % of radiolabel applied.

The D of AOs in isolated pea wax decreased sharply as their chain-length increased ; the decreases between C1C18:1 and C4C18:1, then between C4C18:1 and C8C18:1, were of more than one order of magnitude (Table 1). Under the test conditions used, however, the measurement of D for C12 C18:1 and C18 C18:1 was not reliable.

Table 1. Diffusion coefficients (D) of AOs in isolated pea wax

	D (10^{-17} m^2 s^{-1})	CI
C1C18:1	6.1	0.9
C4C18:1	0.49	0.15
C8C18:1	0.024	0.01
C12C18:1	< 0.05	nd
C18C18:1	< 0.05	nd

CI = confidence interval at $P = 0.05$ probability level; nd = not deterimined.

The D of phenmedipham in pea wax was 6.6 (± 0.9) 10^{-18} m^2 s^{-1} ; all AOs tested increased it by 7- to 8-fold (Fig. 2). However, the increase was 2.5-fold in a control experiment where acetone-water (19:1) droplets were deposited without AO. Similar effects with AOs were found with quizalofop-ethyl whose diffusion coefficient was 2.9 (± 0.5) 10^{-17} m^2 s^{-1} (data not shown).

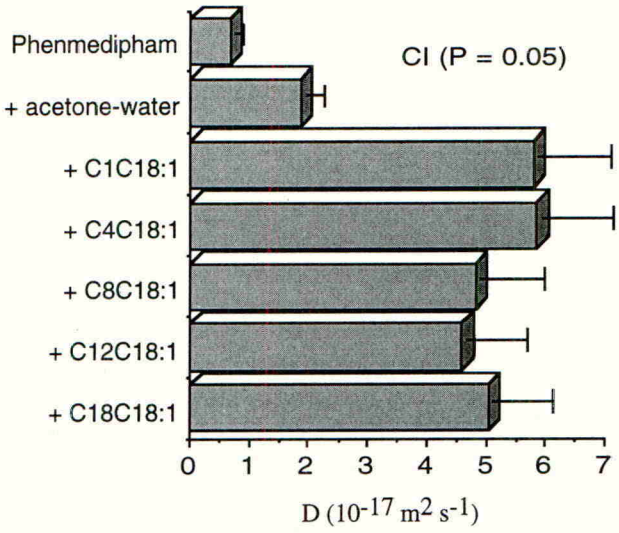

Figure 2. Influence of AOs on diffusion coefficient (D) of phenmedipham in isolated pea wax.

The solubility of phenmedipham in the AOs studied ranged from 1.0 to 3.4 g $litre^{-1}$. That of quizalofop-ethyl was higher, ranging from 116 to 181 g $litre^{-1}$ (Table 2). Herbicide solubilities decreased with increasing chain-length of the AOs.

Table 2. Solubility (g $litre^{-1}$) of phenmedipham and quizalofop-ethyl in AOs at 23 °C

	C1C18:1	C4C18:1	C8C18:1	C12C18:1
Phenmedipham	3.4 (0.2)	2.2 (0.2)	1.3 (0.1)	1.0 (0.1)
Quizalofop-ethyl	181 (19)	166 (32)	137 (30)	116 (14)

Confidence interval (CI) at P = 0.05 probability level given in parentheses.

DISCUSSION

Chain-length had an influence on AO penetration rates into barley and pea leaves, and to some extent those of cleavers. In all species, uptake rates decreased with increasing chain-length. For C1C18:1 - C8C18:1, this was correlated with a decrease of D in isolated pea wax. Similar relationships between chain-length and D were previously shown for aliphatic acids in barley wax (Schreiber & Schönherr, 1993). However, the differences we found between the diffusion coefficients of AOs were greater than those between their rates of penetration. For example, the ratios between D in pea wax were 12 and 20 for C1C18:1 *vs* C4C18:1 and C4C18:1 *vs* C8C18:1, respectively, whereas the corresponding ratios between their uptake rates into pea leaves after 3 h were 1.6 and 1.3. Consequently, diffusion in wax may not be the only factor determining the rate of foliar penetration of AOs from dried deposits.

The D of phenmedipham in isolated pea wax was of the same order of magnitude as those found by Schreiber & Schönherr (1993) for triadimenol and lindane in isolated barley wax. The D of quizalofop-ethyl was closer to those of benzoic acid, 2,4-D and pentachlorophenol determined by the same authors. All AOs increased the diffusion coefficients of phenmedipham and quizalofop-ethyl in pea wax, suggesting that AOs may also increase the mobility of phenmedipham and quizalofop-ethyl in pea cuticles. However, there was no apparent relationship with their influence on foliar penetration. For phenmedipham, all AOs increased diffusion coefficients to similar extents, whereas the effect of C1C18:1 and C18C18:1 on foliar penetration was lower than that of C4C18:1, C8C18:1 and C12C18:1 (Serre *et al.*, 1993, Serre, 1996). Hence, influences on herbicide mobility in wax only partly explain the observed effects of AOs on foliar penetration. It should be noted that acetone-water (19:1) applied alone also increased diffusion coefficients of phenmedipham and quizalofop-ethyl slightly. This factor would also be present in our penetration studies since acetone-water was used as the carrier solvent for herbicides and AOs.

From previous observations one can infer that AOs may act inside the cuticle but to do so they must first penetrate into it. Indeed, on wheat, wild oat (*Avena fatua*), field bean and pea, penetration of the surfactant, hexaethylene glycol tridecyl ether, appeared to be necessary to enhance the uptake of several compounds (Stock *et al.*, 1992). In the present study, however, there was no relationship between the rates of foliar penetration of AOs and their effects on the penetration of phenmedipham and quizalofop-ethyl. This may mean that penetration of AOs is not an essential requirement for their mode of action. A dose-response study of their effects would help clarify this point.

AOs can solubilize significant amounts of quizalofop-ethyl (120 to 180 g litre^{-1}). Under our experimental conditions (equimolar quantities of herbicides and AOs), 16 to 17 % of quizalofop-ethyl could be solubilized in the deposit on the leaf surface and, presumably, be available for penetration (McCall *et al.*, 1986, Briggs & Bromilow, 1994). This may play a part in the enhancement of quizalofop-ethyl penetration. However, the same reasoning does not apply to phenmedipham, of which less than 0.4 % could be solubilized by AOs in the deposit. The enhancement of phenmedipham penetration by AOs into pea and cleavers was roughly twice than that of quizalofop-ethyl ; enhancement rates were similar on barley (Serre, 1996). Hence, such effects cannot be related to the observed differences in solubilisation of the two herbicides. An analogous conclusion was reached by Holloway *et al.* (1992) using nonionic polyoxyethylene surfactants and the fungicides, ethirimol and diclobutrazol.

Finally, other seed oil derivatives than the currently used methyl esters may also be a source of adjuvants for pesticides. We have already shown that the butyl, octyl and dodecanyl esters of oleic acid performed equally well as or better than the corresponding methyl ester (Serre *et al.*, 1993, Serre, 1996). Additionally, some seed oil derivatives with polar moieties exhibit surface-active properties and were found to be good enhancers of herbicide uptake (Gauvrit & Mouloungui, 1996). Derivatives of seed oils are thus good candidates for use in agrochemical

formulations, especially as they are expected to be more biodegradable than mineral oils (Cornish *et al.*, 1993) ; this non-food use may constitute a niche for some agricultural products.

ACKNOWLEDGEMENTS

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ORGANOSILICONE ADJUVANTS TO TARGET AGROCHEMICALS TO THEIR SITES OF ACTION

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ABSTRACT

Organosilicone adjuvants can be employed to deliver agrochemicals preferentially to locations where their activity will be maximized. Their unique superspreading capabilities can be used to enhance spray coverage and carry insecticides into the cryptic habitats of pests. Biasing the deposition of fungicides to lower horizons within the canopy of cereal crops enables the pattern of disease control to be manipulated. In particular, substantial improvement in the control of stem-based disease is provided. By facilitating uptake into foliage, the performance of certain systemic pesticides can be improved using organosilicones. For example, promotion of the cuticular penetration of abamectin insecticide reduces weathering losses, making it a viable product because its activity is extended beyond the life-cycle of the pests it controls. The interactive effects of organosilicone concentration and spray volume to control stomatal infiltration are discussed. Data for a foliar nutrient applied to an arable crop are presented to illustrate that the utility of infiltration extends beyond the recognized rainfastening of herbicides.

INTRODUCTION

The organosilicone chemistry of utility for agrochemical adjuvants was first developed in the mid-1960s. Attracting primarily academic interest in the 1970s and early 1980s, it was only in the latter part of the 1980s that agricultural use on a commercially significant scale commenced. Since then, interest in these special surfactants has increased very rapidly. This is evidenced by presentations at the 1995 International Symposium on Adjuvants for Agrochemicals, 40% of which reported on organosilicones (Gaskin, 1995a). Nonetheless widespread awareness of the potential utility of these products remains restricted and has provided the stimulus for the present paper.

SPRAY COVERAGE

The 'superspreading' ability of organosilicones (Fig. 1), in fact is specifically associated with the trisiloxane structure (Murphy *et al.*, 1991). This makes trisiloxane adjuvants ideal tools to maximize the coverage of fungicides and insecticides.

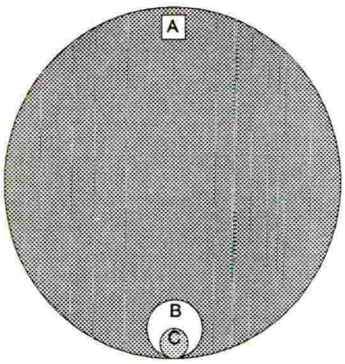

Figure 1. Relative spread areas of aqueous surfactants (10µl) on a waxy surface.

A = Silwet® 408, trisiloxane-8EO (0.1%); 1385 mm^2
B = Triton X-100, octylphenol-10EO (0.25%); 38 mm^2
C = water; 10 mm^2

For example, to control blackspot disease and various pests, including leafroller caterpillars and scale, a dicarboximide fungicide and two organophosphorus insecticides were applied on apple trees. The performance of these protectant chemicals was enhanced by the addition of Silwet L-77® adjuvant, but not of another organosilicone or either of two nonsilicone adjuvants (Stevens *et al.*, 1994). Activity of half rates of the a.i.s plus L-77 gave performance equivalent to full rates applied without adjuvant. Residues of a.i. in the fruit at harvest were reduced because they were a function of application rate which was not affected by adjuvant. Laboratory tests with one of the pests monitored in the field trials demonstrated that L-77 alone had no significant toxicity (data not shown), indicating that the increased activity it provided is likely associated with improved spray coverage. Because the benefits were achieved with high volume sprays (2000-2500 liters/ha) applied using commercial airblast equipment, improved coverage at the macroscopic level was excluded as the mode of action. The target pests are cryptic by nature and inhabit the multitude of crevices on fruit trees. It appears that the adjuvant improved coverage at the microscopic level, spreading spray deposits into these habitats to increase contact with pests.

A similar mechanism is implicit for improved performance in an insecticide trial on citrus in Spain during 1995 (Table 1). Despite L-77 being added only at a low concentration to spray applied at low volume (400 liters/ha), even half rates of the insecticide provided good pest control.

Table 1. Enhancement by Silwet L-77 (0.05%) of activity of abamectin (Vertimec) against citrus leafminer (*Phyllocnistes citrella*)

Vertimec (liters/ha)	Control* 14 DAT	
	Alone	+ L-77
0.375	46	91
0.75	63	95

* % reduction in number of insects from unsprayed.

SPRAY TARGETING

The most publicized example of the use of organosilicones to target agrochemicals is their application against eyespot disease infecting the stem-base of cereals. The spreading properties of an organosilicone can be used to target deposition of fungicide spray to the base of the stem (Green & Green, 1992), an effect which could not be reproduced using two conventional adjuvants (Stevens, 1993). Disease occurrence can be used as a bioassay to monitor the relocation of spray deposits to lower horizons in a crop canopy. Addition of L-77 to cyprodinil actually reduced control of mildew on the flag leaf of wheat, but had some beneficial effect on flag-1 (leaf immediately preceding flag) and a marked one on flag-2 (two leaves before flag), while also greatly improving the control of eyespot at the stem-base (Table 2). This altered pattern of disease control appears to be a gain as regards control of eyespot which is offset by the loss of mildew control on the flag leaf, a major contributor to photosynthate production for grain-filling. However, in keeping with the microclimate within the canopy, the level of mildew infection on flag-1 and flag-2 was two- and three-fold greater, respectively, than on the flag leaf. Thus overall, on a whole plant basis, addition of the organosilicone was also beneficial for the control of mildew.

Table 2. Influence of Silwet L-77 (0.15%) on control of wheat diseases by cyprodinil (Unix)

Disease	Location	Level of infection (% of unsprayed)	
		Alone	+ L-77
Mildew	Flag leaf	55	67
	Flag-1	56	42
	Flag-2	60	33
Severe eyespot	Stem-base	40	19

The increased performance of graminicides in the presence of organosilicone adjuvants also may be the result of spray redistribution to the base of the leaf, where uptake is maximized (Stevens *et al.*, 1995).

FOLIAR UPTAKE

The sites of action for herbicides, plant growth regulators and nutrients are within the plant tissues; many fungicides and insecticides are also systemic. The commercial development of organosilicone adjuvants was spurred by their ability to enhance foliar uptake (Stevens, 1993).

Cuticular penetration

Penetration of the protective, waxy cuticle is perhaps the major limitation on the activity of foliage-applied systemic a.i.s and enhancement of uptake is one of the most common reasons for the use of adjuvants. Despite this being probably the most researched aspect of adjuvant science, only a qualitative framework exists for the rational selection of adjuvants for this

purpose (Stock *et al.*, 1993). The interaction among a.i., plant and adjuvant tends to be highly specific and organosilicones can promote penetration of certain a.i./plant combinations (Gaskin, 1995b).

Spreading of spray into microscopic insect habitats was suggested as the primary mechanism for the enhancement of abamectin (Table 1). Additionally, effective use of abamectin depends on its being taken up into the cuticle to minimize the action of oxidative and photodegradative weathering processes on superficial deposits (Peterson, *et al.*, 1996). This extends its period of activity beyond the life-cycle (up to 2 weeks) of the mites which it controls (Beers *et al.*, 1990). Residual activity was enhanced by L-77 over that provided by the recommended mineral oil adjuvant in a trial conducted in Washington State, USA in 1995 (Fig. 2). The two- to four-fold higher amount of the oil needed restricts its use to a 'tank-mix' adjuvant. L-77 may be preferable not only because of its greater performance but also because its smaller quantity makes feasible its incorporation into the pesticide formulation as an 'in-can' adjuvant.

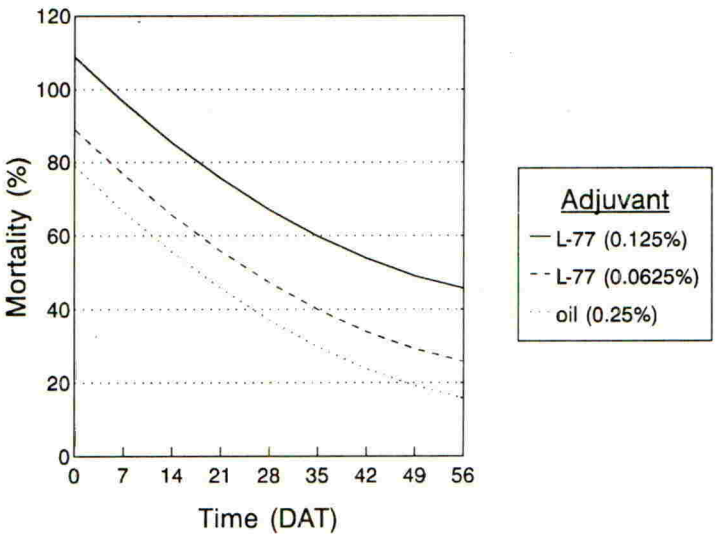

Figure 2. Effect of adjuvants on residual activity of abamectin (Agri-Mek) against twospotted spider mite (*Tetranychus urticae*) on apple foliage.

Stomatal infiltration

Organosilicones lower surface tension below the critical levels for the wetting of leaf surfaces (*c.* 25 mN/m) and, thus, can promote the infiltration of spray solutions into leaves via stomata, circumventing the cuticle (Stevens *et al.*, 1991). Because the cuticle is continuous from the exterior surface of leaves through the stomata into the substomatal cavities within the leaf, infiltration is partially a spreading phenomenon. Infiltration requires superspreading capabilities (Policello *et al.*, in press) and so, similarly, is also uniquely associated with the trisiloxane structure. Infiltration is determined by the pressure of the liquid meniscus across the stomatal pore (Schönherr & Bukovac, 1971). Accordingly, at constant surfactant

concentration, infiltration is expected to increase with increasing spray volume because of the hydraulic contribution to the process. Also, because the total amount of surfactant applied is increased, the effect of its dilution by adsorption at the expanding interfaces of spreading droplets is less deleterious (Buick *et al.*, 1993). The interaction between concentration and volume for stomatal infiltration is shown in Figure 3. It is clear from the uniformly high infiltration provided by 0.4% L-77, regardless of volume, that concentration is the most important controlling factor. These data demonstrate that the most cost-effective means to induce infiltration (i.e. using least amount of adjuvant/ha) is to use a high organosilicone concentration applied in a low volume. Nonetheless, to minimize risk of phytotoxicity, it may be preferable to use a lower concentration and maintain infiltration by application in a larger volume.

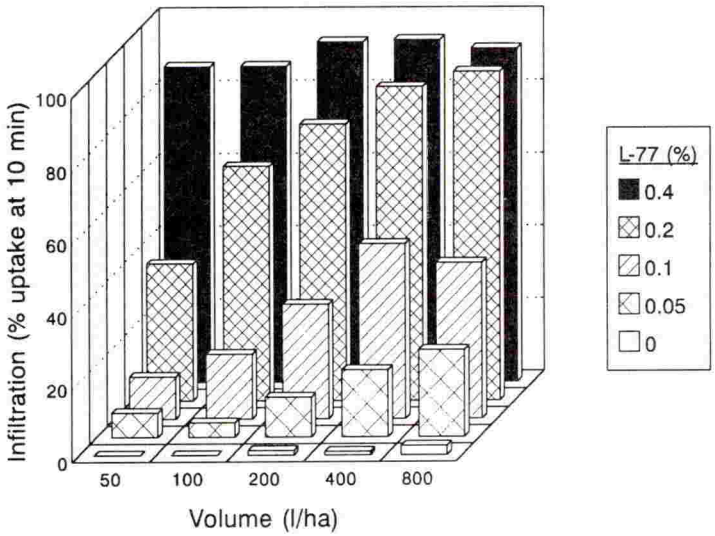

Figure 3. Stomatal infiltration of deoxyglucose into bean (*Vicia faba*) leaves as affected by Silwet L-77 concentration and spray volume (Gaskin *et al.*, 1996).

The data in Figure 3 suggest that the uptake which prolonged the activity of abamectin (Fig. 2) may have been by stomatal infiltration rather than by cuticular penetration, because the insecticide was applied in *c.* 950 liter/ha. However, experience of applying similar concentrations of the same adjuvant in similar volumes to the same crop (Stevens *et al.*, 1994) indicated that infiltration is minimal under these conditions. The apparent discrepancy is because the infiltration data (Fig. 3) were generated in the laboratory using microsyringe applications (i.e. 100% 'spray' retention) to bean plants with a leaf area index (LAI) of 1.6. In contrast, the apple orchard used for the trial in Fig. 2 will have had a LAI of 4 to 5 and only *c.* 30% of the airblast spray would have been retained on the trees (Herrington, *et al.*, 1981). As a result, the orchard presented a greater surface area per volume of spray retained for the solution to spread over. This will have minimized infiltration, both by reducing the hydraulic effect and by increasing adsorptive dilution of the surfactant.

There is increasing interest in the application of nutrients to foliage because soil applications may be inefficient or even ineffective and because responses to foliar applications are much more rapid, so that applications can be timed to coincide with critical growth events. However, the impermeability of plant cuticles to inorganic nutrient ions is notorious, making stomatal infiltration an attractive alternative route (Stevens, 1994). Promotion of uptake of foliar nutrients was a major part of the early research which elucidated the properties and behavior of organosilicones and contributed to their commercialization as agrochemical adjuvants (Stevens, 1993). The data in Table 3 illustrate the benefits that infiltration can provide and its concentration dependence. The decreasing response evident at 0.4% organosilicone (compare 0.4% versus 0.2% with 0.2% versus 0.1%) may be inherent in the process, but may be because this high concentration caused some run-off of spray.

Table 3. Promotion by Slippa (trisiloxane-based adjuvant) of infiltration of magnesium (applied as $MgSO_4$ in 200 liters/ha) into potato foliage

Slippa (%)	Mg in foliage 4 DAT (% increase over untreated)
0	3
0.1	29
0.2	42
0.4	52

CONCLUSION

Intensive agricultural systems require increasingly sophisticated approaches to maximize benefits from their agrochemical inputs. Organosilicones are adjuvants which can deliver chemicals preferentially, across external surfaces and into plant tissues, to optimal effect.

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SESSION 7C

VERTEBRATE PEST MANAGEMENT

Session Organiser	Dr D P Cowan *Central Science Laboratory, York*
Poster Papers	7C-1 to 7C-5

THE EFFECT OF CONTROLLED SCARING MEASURES ON GREY GOOSE DISTRIBUTION IN THE NORTH-EAST OF SCOTLAND

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ABSTRACT

The effectiveness of controlled scaring measures on grey goose distribution was assessed on grass fields around the Loch of Strathbeg Area in North East Scotland in 1995. Fields were protected by scaring on alternate weeks using "Clarratts Scarey Man" devices. Goose grazing was assessed at the end of each week by counting defecation rate. Nearby non-scared grass fields were assessed for goose grazing at the same time as the scared fields to determine whether scaring had resulted in their increased use. Geese did not appear to use nearby non-scared fields more heavily on scaring weeks. The presence of the Scarey Man device was found to be effective in reducing goose grazing. No evidence of habituation to the devices was found during the weeks when scaring was carried out. Geese return to the protected fields during non-scaring weeks but in much reduced numbers.

INTRODUCTION

The Royal Society for the Protection of Birds Reserve at the Loch of Strathbeg in the North East of Scotland is an internationally important site for waterfowl. It is a major roost for Pink-footed Geese (*Anser brachyrhynchus*) in the spring as they prepare for their migration to the breeding grounds of Iceland and western Greenland. Lesser numbers of the Greylag Goose (*Anser anser*) are found at this site. Large numbers gather here during March and April and peak counts of over 30,000 have been recorded roosting at the Loch and surrounding pools.

During this period, grazing on grass fields in the vicinity of the roost is intensive and considerable damage occurs by the removal of early spring grass. This delays the early spring bite, in turn delaying cattle turnout by up to three weeks, necessitating the production of extra fodder as grass silage. First cut silage on fields which are heavily grazed may be delayed by up to three weeks (often sward lengths are only 2cm at the end of April). The net effect of goose grazing is that limitations are placed upon grass production and the choice of enterprise may also be restricted (Ross 1990, Daw 1992, Patterson & Fuchs, 1993).

In an attempt to reduce the goose/agriculture conflict in the area, Scottish Natural Heritage (SNH) set up and funded a goose management scheme in 1994 and 1995, where farmers in affected localities were given payments to allow geese to graze on selected grass fields. Participating farmers were required to apply 50kg/ha of nitrogen fertiliser in early spring and to carry out scaring activities on nearby grass crops to encourage geese to use the refuge fields. Refuge fields would be kept free from disturbance from normal farm activities from the beginning of February to the end of April (Raynor, 1994).

The study presented in this paper was undertaken in the spring of 1995 to establish the effectiveness of scaring devices to protect vulnerable grass crops from goose grazing and to determine whether scared geese used nearby refuge fields as alternative feeding areas.

MATERIALS AND METHODS

The device used for the scaring experiments was the Clarratts "Scarey Man". This is an inflatable fluorescent orange scarecrow with a built-in high-pitch wailer, operated by a 12 volt motorised fan. A variable timer allowed inflations to be regulated to give a consistent interval of 20 minutes between displays. A photo-electric sensor enabled the scarer to be operated only during the hours of daylight. The device was tested by Stickley *et al.* (1995) and found to be successful in reducing bird predation on fish farms in Mississippi, USA.

Preliminary experiments in the Loch of Strathbeg area in 1994 had indicated that the Scarey Man device could be effective against geese, though there could be limitations to the areas and distances protected (Fuchs & Chapman, 1995). It was decided to use the same device in 1995 as it had given positive results in 1994 and the consistent scaring programme would allow for direct comparisons to be made between different fields.

The 5 fields selected for scaring were used regularly by geese in previous years and were free from external disturbance such as muck spreading, rolling and sheep grazing from February to April. Each selected field was "paired" with a nearby control field on a SNH refuge, to determine whether there was a significant increase in use of the control field during the week that scaring took place. Scaring was carried out on alternative weeks A week of control (i.e. no scaring) was alternated with a week when scaring operations were carried out in each of the five experimental fields. "Scared" fields 1 to 4 were subject to scaring operations over the same period. The experiment started two weeks later in field 5 than in the other fields.

Goose grazing was assessed by determining the density of faecal droppings. Defecation rate has been shown to be an acceptable method to estimate goose usage of an area (Owen, 1971, Patterson, 1994). Small wooden pegs were placed in rows in each of the fields to be assessed. Two pegs were placed per hectare. A five square metre quadrat as counted and cleared around each peg at weekly intervals; the number of droppings per seven day period per square metre was calculated.

RESULTS

There was an overall significant reduction in the use of the "scared" fields between weeks when the scarers were operated - scaring weeks - and weeks when there was no scaring activity - control weeks (ANOVA $P = 0.01$). See Table 1.

Table 1 Results of weekly counts of faecal droppings counts over a six week period in scared and refuge fields. (Droppings/m²/7 days)

SITE	Control week 1 22/3/95	Scare week 1 29/3/95	Control week 2 5/4/95	Scare week 2 13/4/95	Control week 3 19/4/95	Scare week 3 26/4/95
Field 1 -scared	0.67 ± 0.13	0.04 ± 0.02	0.00	0.00	0.00	0.00
Control field 1	0.35 ± 0.10	0.35 ± 0.09	0.26 ± 0.13	1.23 ± 0.16	1.74 ± 0.23	1.30 ± 0.13
Field 2 -scared	0.81 ± 0.10	0.04 ± 0.02	0.74 ± 0.12	0.24 ± 0.09	0.13 ± 0.04	0.00
Control field 2	0.09 ± 0.05	0.02 ± 0.02	0.31 ± 0.07	0.80 ± 0.10	3.16 ± 0.33	1.87 ± 0.16
Field 3 -scared	1.00 ± 0.14	0.00	1.46 ± 0.13	0.00	0.02 ± 0.01	0.00
Control field 3	0.13 ± 0.07	0.00	0.11 ± 0.05	0.85 ± 0.17	1.97 ± 0.22	0.00
Field 4 -scared	2.49 ± 0.27	0.00	0.00	0.00	0.73 ± 0.15	0.00
Control field 4	0.12 ± 0.04	2.25 ± 0.28	0.25 ± 0.06	1.29 ± 0.15	4.65 ± 0.32	0.64 ± 0.1
Field 5 -scared	+	+	0.90 ± 0.07	0.00	4.42 ± 0.24	0.00
Control field 5	+	+	0.03 ± 0.02	1.02 ± 0.16	2.80 ± 0.31	0.04 ± 0.02

+ not assessed

There was no significant increase in the use of control fields between scaring week 1 and control week 1 ($P = 0.46$). If geese which were deterred from the "scared" fields simply moved instead to adjacent control fields, then it would be predicted that there would be a corresponding significant increase in use of control fields between the control week and the scaring week. There was a significant increase in use of control fields between control week 2 and scaring week 2 ($P = 0.001$); however, there was also a significant increase between scaring week 2 and control week 3 ($P = 0.01$).

A significant decrease in the use of the control fields was detected between control week 3 and scaring week 3 ($P = 0.01$). The apparently contradictory difference between control week 3 and scaring weeks 2 and 3 (see Figure 1) reflects a steadily increasing number of geese arriving in the area and hence using the control fields from early to mid April. The decrease in goose use of the control fields at the end of the month in scaring week 3 was due to decreasing numbers of birds in the area as they departed further north on their migration to the Icelandic breeding grounds.

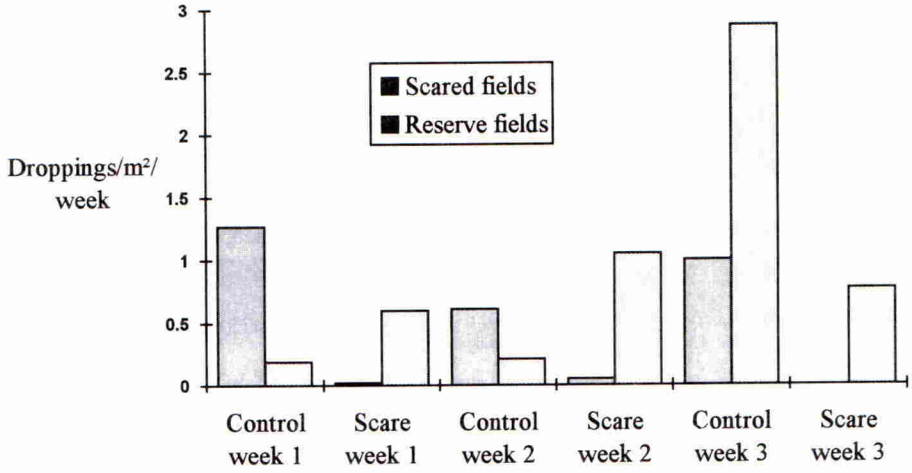
Small numbers of droppings (0.04/m² and 0.04/m²) were found in fields 1 and 2 at the end of scaring week 1 which could be due to geese landing between activation periods of the scarer. No droppings were found closer than 100 metres from the Scarey Man device. There was evidence of greater goose grazing (0.25/m²) in field 2 at the end of scaring week 2, most likely due to defective operation of the scarer, outlining the need for regularly monitoring of such devices. Again no droppings were found closer than 100 metres from the device. It would appear that even the non-operative presence of the scarer may have had some deterrent effect; geese appeared to be unwilling to graze within the immediate vicinity of the device.

The Scarey Man proved to be effective in field 3 : no faecal droppings were recorded during the weeks that scaring was carried out, though the field was used by geese in control week 2 when 1.46 droppings/m² were recorded. Control field 3 was only heavily used by geese in the middle of April (1.97/m²), consistent with recorded use for previous seasons.

Field 4 had been observed to be used frequently used before the start of the experiment but after scaring week 1 was not used again by geese except for control week 3, when 0.73 droppings/m² were counted. Geese were still common in the vicinity and control field 4 was heavily used during control week 3 (4.65 droppings/m²). Cattle were put into a grass field adjacent to field 4 in the middle of April and the disturbance caused by their presence could possibly have been a deterrent to geese landing in the vicinity. Cattle are commonly supposed by farmers to deter the birds from feeding in the same field. However, flocks of between 50 to 100 geese were observed grazing alongside cattle in a nearby, large grass field on a number of occasions.

Field 5 was in an area was heavily used by geese. Considerable numbers of Pink-footed geese (several hundred) were present on a nearby SNH refuge on every occasion that the site was visited. The "scared" field was used by geese during both control weeks that it was monitored. In control week 2 , this field was heavily used (4.42 droppings/m²) - the most heavily used of any "scared" field during a control week. The scarer was situated on a rise in the middle of the field but was a considerable distance - 200 metres - from the far end of the field. Inspection of the whole field at the end of both scaring weeks confirmed that the pegged area was representative. There were no signs of fresh droppings in other parts of the field, despite the presence of large numbers of geese on nearby SNH refuges. The field was a newly-sown ley and would be very attractive to geese.

Figure 1. Mean density of goose faecal droppings in control and scare weeks for "scared" and control fields



DISCUSSION

The use of the Scarey Man device prevented significant grazing damage to grass crops in March and April, even where the fields were in areas of heavy goose use. In "scared" fields during control weeks after scaring, counts of faecal droppings tended to remain low. No evidence was found of habituation to the scaring devices. Geese were subjected to the Scarey Man on only three separate weeks. Inglis (1980) reported that the likelihood of habituation to scaring devices by birds will increase with increasing length of exposure to the same device. If the devices had been used continuously over a longer period, it is possible that habituation would have occurred. The presence of refuge fields provided by the SNH Scheme enabled the geese to move to alternative areas to graze, thus they were not "forced" to use the protected fields.

Although geese used some fields where the scarers were not fully functional, grazing was restricted to at least 100 metres distance from the site of the device. It may be possible to operate a week on/week off system of rotating scarers between fields thus halving the need for these expensive scarers. The effectiveness of a non-operational substitute during the "off" week may not be 100% but could prove to be cost-effective. Stickley *et al.* (1995) suggest that scarers should be moved to different locations and that "look-alike" human shooters should be used to prolong the effective period of protection.

Strachan & Raynor (1995) reported that the SNH Goose Management Scheme had worked, with more use being made of the refuge fields by geese in 1995 than in 1994. No direct relationship was detected in the experiments between the use of the Scarey Man

and any direct increased use of adjacent control fields. It would appear that Pink-footed Geese will not necessarily simply fly to the nearest available refuge if deterred from landing in a favoured field, but may move further. This indicates that a network of reserve fields spread across an area used by geese dispersing from a roost site, accompanied by effective scaring, may be a useful approach to reducing overall damage to crops in that area.

ACKNOWLEDGEMENTS

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WHEAT LEAF SILICIFICATION: AN INDUCIBLE DEFENCE AGAINST VERTEBRATE HERBIVORES

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ABSTRACT

It is proposed that systemic application of sodium silicate may significantly enhance the level of leaf silicification in winter wheat (*Triticum vulgare* cv. Mercia) and reduce the palatability of these plants for vertebrate herbivores such as the European rabbit (*Oryctolagus cuniculus*). Concentrations of sodium silicate above 1ppm significantly increased the level of leaf silicification when compared with the controls. Treated plants (100 & 250ppm) also had significantly higher levels of leaf silicates than cv. Mercia grown in three geologically distinct locations around the UK. Scanning electron microscopy (SEM), showed that the treated plants also appeared to have a greater number of trichomes on the leaf tips when compared with the controls. We provide here evidence that leaf silicification is an inducible defence mechanism in wheat which has potential for use as a novel method of crop protection against vertebrate pests.

INTRODUCTION

Numbers of the European wild rabbit (*Oryctolagus cuniculus*) have been steadily increasing in recent years, returning in many areas of the UK to numbers unseen since the 1950's when myxomatosis became endemic to the UK (Trout *et al.,* 1986). The rabbit is now the major vertebrate pest of British agriculture, causing damage estimated at more than £100 million per year (Crawley, 1989). With regard to cereal crops, rabbits have been estimated to reduce yields by up to 1% for each rabbit grazing on each hectare of crop (Anon, 1992).

Traditional mammalian control methods for crop protection, such as fencing, poisoning or shooting, are either costly to initiate, require repeated treatments and/or need intensive maintenance. In addition, the use of lethal control techniques is increasingly giving rise to serious environmental and humanitarian concerns. It is therefore essential that a more cost-effective and environmentally benign approach is developed.

During the co-evolutionary arms race between plants and their predators, plants have evolved diverse ways of defending themselves. A number of studies have encouraged dicotyledonous plants to enhance their own defence in the form of secondary chemicals against both disease and insect pests (Carrasco *et al.*, 1978, Hartley & Firn, 1989). However monocotyledons, such as cereal crops are comparatively simple structurally and lack the diverse secondary defence chemical characteristics of the dicotyledons (McNaughton & Tarrants, 1983). They do however, incorporate a number of physical

defence strategies to deter their predators, e.g. subterranean hypocotyls, dense rosettes of leaves and leaf trichomes made of silica.

Leaf silicification is important in the defence of monocotyledons against herbivory. Vertebrate herbivores discriminate between high and low silica plants and preferentially feed on the latter (McNaughton & Tarrants, 1983). Avoidance of high silica plants is attributed to increased mechanical abrasion of the teeth (Baker *et al.*, 1959) and internal malaise caused by ingested silica (O'Neill *et al.*, 1980). The aim of this study was to determine whether physical defence characteristics can be elevated in winter wheat by the systemic application of sodium silicate.

MATERIALS AND METHODS

Growth and treatment of wheat plants

Wheat plants were cultivated at a constant temperature of 22±5°C in a greenhouse lit by Phillips Son-T-Agri lamps on a 12 hour light/dark photo period. Wheat seeds (cv. Mercia) were planted in 1-2mm perlag (Silvaperl Ltd, Gainsborough, Lincs., UK), in perforated seed trays (25 x 30cm) lined with landscaping fleece material (Netlon Sentinal, Sheffield, UK). Perlag was chosen as a growth medium as it is considered to be biologically inert (D Cook, pers. com.).

One hundred seeds were planted in each tray, which was placed in a slightly larger tray containing a nutrient solution of Hoagland and Arnon (Carrasco *et al.*, 1978). A total of twenty-five trays of wheat were planted, and arranged into five blocks, each block containing five trays. Emergence occurred after five days with comparable development in all the trays. Seven days after emergence, plants within each block were maintained on a nutrient solution containing either 1, 10, 100 or 250ppm sodium silicate (McNaughton & Tarrants, 1983). To maintain the silica in its soluble form, the pH of the nutrient solution was adjusted to 5.0. The plants were treated for two consecutive weeks. Controls were treated during the same period with nutrient solution only. Immediately after the treatment period the plants were analysed for levels of leaf silicification.

Sampling of plant material from various geological locations

In April 1996, three samples of wheat (100g; cv. Mercia) of similar post-emergence age and height as the greenhouse-grown plants, were collected from each of three field sites in Hampshire (silty clay), Warwickshire (loam over chalk, heavy clay interbedded with limestone) and Nottinghamshire (light sandy loam).

Silicate analysis of leaf material

Wheat plants were harvested by cutting immediately above the growing medium. Plant material within each tray was combined, roughly cut and dried in a convection oven (50°C for 72 hours or until fully dried). Once dried the plant material was milled in a hammer mill to pass through a 1mm mesh sieve and stored desiccated until required for analysis. Levels of silicification were determined by wet ashing and weighing as described in the MAFF/ADAS Reference No. 427.

Scanning electron microscopy (SEM)

Leaf tips obtained from 100ppm sodium silicate treated plants and also control plants were harvested and prepared for SEM. Leaf tips were fixed onto a metal SEM stub, coated in gold and the abaxial region scanned on a Cambridge Scanning Electron Microscope.

RESULTS

Comparison of sodium silicate-treated plants and control plants using a one-way ANOVA showed that the treated plants had a significantly higher level of leaf silicates ($F = 14.7$, 1, 4 df, $P < 0.001$; Fig. 1). Treatments of 1, 10, 100 and 250 ppm silica raised the levels of leaf silicates by 45%, 42%, 48% and 52% respectively when compared with the controls. Multiple-range comparison tests (Tukey-B test), however, failed to show any significant differences between the leaf silicifcation levels of the treated plants ($P > 0.05$).

Mean concentrations of silicates from the different geological locations differed by < 3%, enabling the mean quantity of silicates from the three field sites to be used in any calculations. Levels of leaf silicates observed in the field samples were significantly higher than levels in control plants (two-tail t-test; $P < 0.001$). A one-way ANOVA showed that there was also significant difference between the silicate levels in treated and field samples ($F = 6.68$, 1,4 df, $P < 0.001$). Further analysis revealed that the levels of leaf silicates in plants treated with 100 and 250ppm sodium silicate were significantly greater (Tukey B Multiple range test; $P < 0.05$) than the field samples. However, treatment of wheat plants with solutions containing 1 and 10ppm sodium silicate did not significantly increase the levels of silicification ($P > 0.05$) above those observed in the field sample.

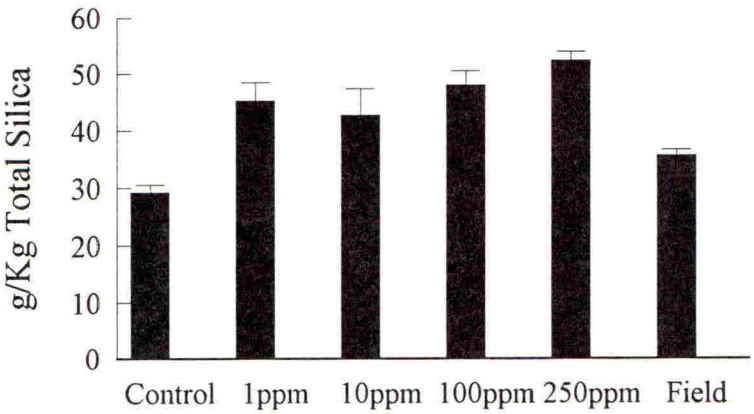

Figure. 1. Mean concentrations and standard errors of leaf silicates (g silica per Kg dry weight of wheat).

SEM of the abaxial region of the 100ppm treated and control leaf tips showed that the treated plants appear to have a greater number of silica trichomes than the control plants (Fig. 2 and Fig. 3).

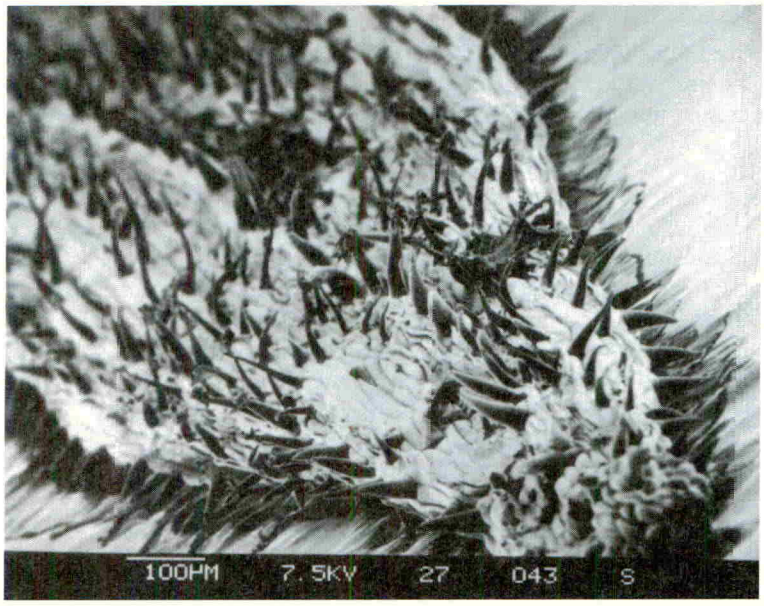

Figure 2. SEM of the abaxial region of the treated leaf tip from treated wheat (100ppm) plants.

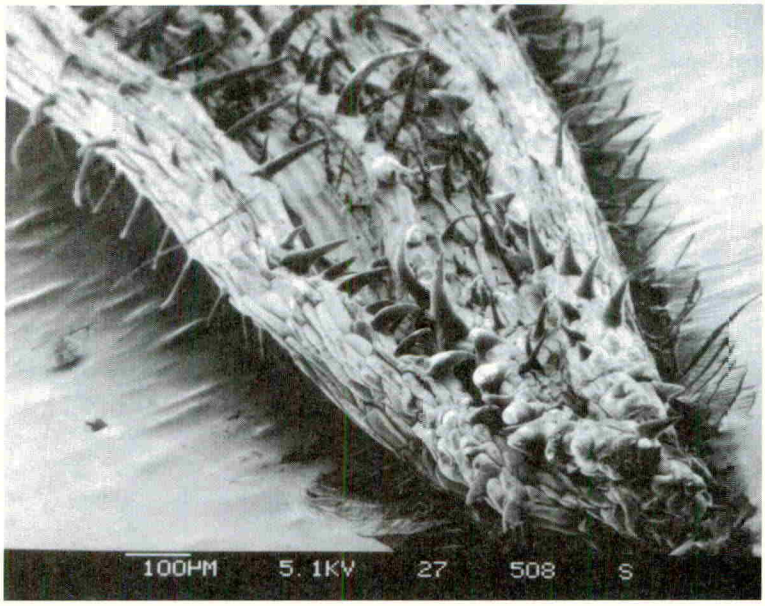

Figure 3. SEM of the abaxial region of the leaf tip from untreated (control) wheat plants.

DISCUSSION

McNaughton & Tarrants (1983) found that African grasses native to areas that were heavily grazed accumulated more silica in their leaves than plants from less heavily grazed areas, and that silica levels were higher in grasses that had been defoliated. The results described here show that this apparent defence mechanism can be manipulated and enhanced by the systemic application of sodium silicate. Indeed in winter wheat, this treatment of plants with 100 and 250ppm sodium silicate increased the concentration of leaf silicates to levels that were significantly higher than those observed in natural populations.

This approach may have considerable advantages over traditional methods of crop protection. For instance, the systemic application of soluble silica could, potentially, provide a simple treatment with which to increase the density of the silicate hairs and associated silicate bodies, thereby improving the plant's resistance to herbivore damage. Losses in the yields of winter wheat have been found to be greatest when grazing occurs immediately after germination (November and December in the UK). Exposure to rabbit grazing during the rapid growth phase (April to June) has less effect on the final yield (Crawley, 1989). Ideally in terms of cost-effectiveness, a single application of sodium silicate would be used to boost the level of leaf silicification in the plant during its most vulnerable period of growth. Thereafter, this effect would become diluted as the plant develops and has less need for this physical defence.

Increasing the levels of leaf silicification, and thereby the toughness of the plant, may provide the plant with a number of other advantages. The structural support provided by the silica may reduce lodging. The increased density of the silica hairs could also enhance the plant's resistance to environmental stress (e.g. drought) and, reduce the survival and reproduction of many small insects and mites by impeding their movement and feeding. Potential costs to the plant may include, provision of the metabolic resources needed to produce the silicate bodies and an increase in the number of micro-habitats for mycorrihizal parasites.

This approach may be applied to other cereal crops such as rye, barley and oats. However, dicotyledonous crops could also benefit from this application. The selection for improved, high-yield crop varieties has often also led to selection for plants that have few resistance characteristics to protect them against vertebrate damage. The systemic application of sodium silicate may improve the herbivore resistance of dicotyledonous plants, such as the new, low alkaloid cultivars of lupin, without adversely effecting the quality of the final product.

Further experiments are planned to determine longevity of the effect and whether this increase in leaf silica reduces rabbit damage. The pathway leading to the formation of leaf silicates still remains to be identified. However we provide here evidence that levels of leaf silicification can be raised in winter wheat, a technique which potentially may provide a novel and effective method of crop protection.

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FERTILITY CONTROL OF BADGER POPULATIONS AS A MEANS OF REDUCING BADGER DAMAGE TO GROWING CROPS

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ABSTRACT

Although badgers (*Meles meles*) are protected in the UK, they are generally common in south and south-west England. Badgers can cause extensive visible damage to agricultural crops, by both direct consumption and trampling. Damage may be reduced legally without a licence by physical exclusion with electric fencing or by the use of certain chemical deterrents. However, these methods may not be effective where damage is considered serious. Under these circumstances, a licence may be obtained to kill or remove the badgers. Recently there has been growing interest in the development of fertility control as a more humane method of reducing badger population densities, and this could provide an alternative means of controlling crop damage in certain circumstances. In this paper, the efficacy of fertility control as a means of badger population control is evaluated using a simulation model.

INTRODUCTION

Badgers (*Meles meles*) are protected in Britain under the Protection of Badgers Act 1992. This affords them protection equivalent to species listed on Schedule 5 of the Wildlife and Countryside Act 1981. However, badgers are not rare, and this level of protection is more a reflection of the high non-use value of the species than because of any immediate threat of extinction. Badgers are less common in upland areas, East Anglia and parts of northern England, but can be very common in the south and south-west of England where they may reach densities as high as 20 per km^2 in favourable habitats.

Badgers are omnivorous, consuming a wide variety of foods of both plant and animal origin. The composition of their diet varies between different localities according to habitat and food availability. However, in some parts of southern England, cereals (oats *Avena sativa*, wheat *Triticum vulgare*, barley *Hordeum sativum*, rye *Secale cereale* and maize *Zea mais*) are important, and may constitute up to 30% of the diet by volume (Skinner & Skinner, 1988, Roper *et al.*, 1995). Badgers may cause damage to growing crops either by direct consumption or by trampling. In a 1987 National Farmers Union survey, of the 83% of 1030 respondents who said badgers lived on or used their farms, 75% had experienced problems with badgers, and 30% perceived these problems as "severe". Structural damage (42%) was the most frequent complaint and crop damage (39%) came a close second (Symes, 1989). However, the area of the crop affected by badger-induced damage is generally small, being recorded as <4% for oats, 0.25% for wheat and 0.05% for barley (Wilson, 1993, Roper *et al.*,

1995). Roper *et al.* (1995) calculated that average grain losses for wheat were equivalent to 7.21 kg per ha, or <0.1% of the total crop.

Under the Protection of Badgers Act 1992, it is illegal to wilfully kill, injure or take any badger to prevent damage to crops. It is legal to use certain chemical repellents, such as those based on aluminium ammonium sulphate, but there is little information regarding the efficacy of these against badgers (Harris *et al.*, 1994). The only other method of damage control which can be used legally is physical exclusion. Electric fencing may be used to protect areas up to about 1 ha in size, but it is less effective for larger areas (Harris *et al.*, 1994). Furthermore, it is expensive, and in general the level of absolute damage is not large enough to make it a cost-effective strategy (Symes, 1989, Wilson 1993). Where legal methods are ineffective in reducing damage, licences to kill or take badgers or interfere with setts to reduce severe crop damage may be issued by Ministry of Agriculture, Fisheries and Food (MAFF) (England), the Scottish Office or the Welsh Office.

One possible future option for reducing the size of problem badger populations is fertility control, which is widely considered to be more humane and ethically acceptable than traditional methods of control (Tyndale-Biscoe 1991). In this paper, the potential efficacy of fertility control as a means of badger population control is evaluated using a simulation model. Some studies have shown that effective fertility control can be achieved by the use of contraceptive implants (Bickle *et al.*, 1991). However, any method relying on surgical intervention would be impractical for general use. In such circumstances, an oral bait-delivered contraceptive would be much more cost-effective. In addition, due to the badger's social organisation and mating system (Cresswell *et al.*, 1992), it would be most effective to concentrate on a technique that targeted females rather than males. For these reasons, an oral-bait delivered contraceptive directed at females is the type of fertility control considered in this paper.

METHODS

The model

Badger population control was evaluated using a spatial stochastic simulation model similar in structure to that used by White & Harris (1995) to investigate the dynamics of bovine tuberculosis in badger populations. The model operated on a square grid, within which each grid cell represented a single territory that could contain a single group of badgers, and shared its main boundaries with four other territories. This is a reasonable representation of the pattern of spatial organisation that occurs in reality (White & Harris, 1995). A main grid of 16 (4 x 4) cells was used, and this was surrounded by a further 20 boundary cells which served as a source of immigrants to and a sink for emigrants from the main grid.

The equilibrium badger population density was predetermined at the social group level, and was assumed to be dependent on the availability of resources. This was the level around which density-dependent processes operated. The model stored information in terms of the number of constituent individuals of each sex for each of the three age classes considered (adults >2 years old, yearlings 1-2 years old and cubs <1 year old). The model ran on a quarterly basis using the quarters January-March (winter/spring), April-June (spring/summer),

July-September (summer/autumn) and October-December (autumn/winter). These quarters were chosen to reflect the behavioural patterns of badgers throughout the year (White & Harris, 1995).

The model consisted of a main program and a series of subroutines which represented the biological processes of birth, dispersal, colonisation and natural mortality. These subroutines therefore constituted transitional processes through which the composition of each group could be altered. The dynamics of group size were controlled through relations between group size, fecundity and density-dependent mortality. Cubs were born at the start of the spring/summer quarter, and the number of cubs produced per social group was determined by the relationship $P_{cubs} = 0.60 + 0.63\ a_f$, where P_{cubs} is the number of cubs produced per social group per year and a_f is the number of adult females in the group at the start of the year (Cresswell *et al.*, 1992, White & Harris, 1995). No adjustments were made to skew the sex ratio of cubs born away from unity. Adult and yearling mortality was taken as 19% per annum (Cresswell *et al.*, 1992). Cub mortality varied with population density between a minimum of 19% and a maximum of 70% per annum which occurred when the adult and yearling group size was 100% above its equilibrium level. Dispersal probabilities were set at 15% and 5% per annum for adult males and adult females respectively, and colonisation probabilities at 4% per annum for both sexes (White & Harris, 1995).

Badger population control

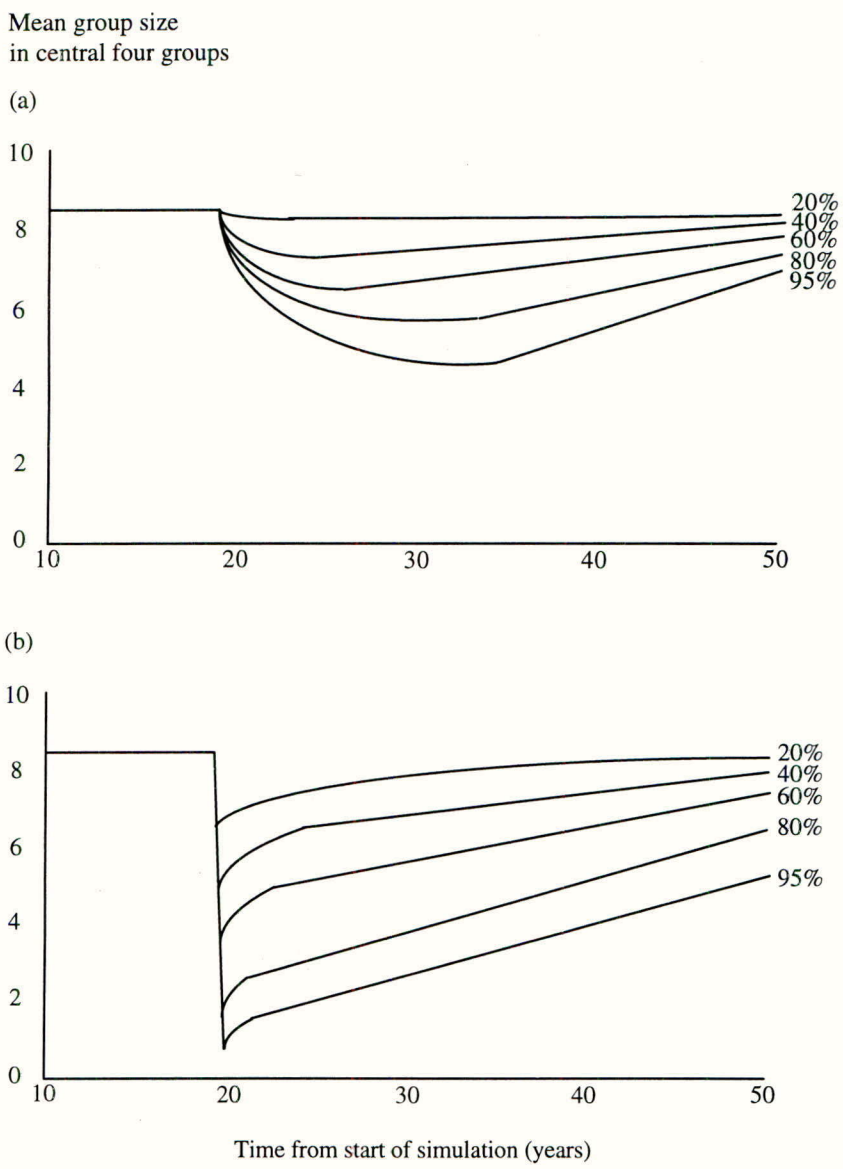
The model was run for a period of 50 years for each simulation. Population control took place in year 20, by which time the mean badger group size would have stabilised around the equilibrium. To simulate localised control, as appropriate for problems of crop damage, only the central four badger groups on the grid were exposed to control. Twenty simulations were run for each control scenario investigated.

Fertility control was a once-off operation that took place in the spring/summer quarter. The efficiencies (proportion of adult and yearling females reached and rendered infertile by the oral bait-delivered contraceptive) used ranged between 10-95%. For a particular control efficiency, each individual adult and yearling female badger was independently subjected to the specific equivalent probability of being reached by the contraceptive and rendered infertile. Thus for 60% fertility control, each badger was subjected to fertility control independently with a probability of 0.60. Animals rendered infertile remained so for the rest of their life. Culling was also a once-off operation that took place in the spring/summer quarter. As for fertility control, the culling efficiencies used were 10-95%. However, badgers of both sexes and all three age groups were subjected to culling.

RESULTS

Fertility control (Fig. 1a) reduced the badger population in the central four groups. However, the badger population was only significantly reduced at relatively high fertility control efficiencies. Furthermore, any impact on population size was relatively slow-acting. Higher efficiencies of fertility control resulted in a greater total impact on the badger population within the central groups and a faster rate of population decline. However, maximum population depression was not achieved until some time after the fertility control operation

Figure 1. The effects of once-off (a) oral contraceptive based fertility control and (b) culling operations of varying percentage efficiencies on mean group size of badgers within the central four groups of the grid. Control was conducted at the start of year 20 in all instances. For reasons of clarity the curves have been smoothed, standard errors omitted, and only efficiencies of 20, 40, 60, 80 and 95% are shown.



had been conducted, and the time period to maximum impact increased with higher control efficiencies. Even with the highest fertility control efficiency considered, the badger population in the central four groups was depressed by less than 50% below the equilibrium density.

Culling (Fig. 1b) was far more effective at reducing the badger population than fertility control, both in terms of depressing the badger population density, and in terms of the time period in which this was achieved. The impact of culling was immediate, and so the maximum depression of the badger population was generally recorded in the same year or very soon after the control operation was conducted.

DISCUSSION

The results of any modelling exercise need to be interpreted with caution, since they represent an idealised world rather than the complexities of reality. This is especially true in cases such as this where real field data against which to test the predictions are not available. However, spatial stochastic models offer a closer representation of reality than non-spatial deterministic ones for wildlife populations which display marked spatial organisation, as is the case for badgers. In this study, the model was used to examine the effects on control on four badger groups central within the grid. In the real situation, control might only be affecting one or two social groups rather than four. However, the relative impacts of the two types of control would be similar.

The results of the model showed that population recovery following either culling or fertility control could take 30 years or more, reflecting the fact that dispersal is infrequent and badgers are relatively poor colonisers. Field data from a single very high density population show that the time to recovery of the badger population following localised removal may be significantly less, in the region of ten years (Cheeseman *et al.*, 1993). There is some evidence that badgers do not realise their full reproductive capacity in high density populations, and these populations would therefore be capable of more rapid growth to compensate for removal (Cresswell *et al.*, 1992, Page *et al.*, 1994). These effects are not accounted for in the model, but it is likely that the recovery times for most real populations following localised removal would lie somewhere between these two figures.

This study has shown that fertility control delivered by an oral-bait contraceptive could be used to reduce badger populations locally where they are causing excessive damage to growing crops. However, it has also demonstrated that, in comparison with culling, fertility control would be a relatively ineffective means of population control for badgers. The same would be true for control over a wider area, although in this case the time to recovery in the control area would be greater for both methods.

The two main reasons for the relative ineffectiveness of fertility control are (i) it takes a long period of time before the full effects of control are realised, making it inappropriate for situations where immediate effects are required, and (ii) a high degree of efficiency is required for any significant impacts on the population to be detected. In contrast, a traditional culling approach has a far more immediate impact, and much greater reductions in population density can be achieved at relatively low culling efficiencies. In terms of total population reduction,

fertility control with an efficiency of 95% had an equivalent effect on population density as a cull with an efficiency of 50%, but the effect of the cull was immediate whereas the effect of the fertility control was not fully realised until 12 years after the control operation. Annually repeated fertility control in areas prone to damage problems would be likely to offer some improvement in efficacy over once-off operations. However, even in these circumstances, fertility control is not likely to be effective on its own unless high overall efficiencies can be reached. If there is a future for fertility control in the control of badger populations, it is likely to be as part of an integrated strategy alongside other, more traditional, population control methods.

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THE EFFECTIVENESS OF REPELLENTS TO DETER FIELD VOLES (*MICROTUS AGRESTIS*) FROM LIVING BENEATH MULCH MATS

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ABSTRACT

Field voles (*Microtus agrestis*) are known to colonise the areas under mulch mats, thereby compromising the effectiveness of mats, placed around newly planted trees, as weed control barriers. Five chemicals, that had previously been reported to be aversive to mammals, were tested over a seven-month period to evaluate their effectiveness as area repellents against voles in the context of mulch mats. Ziram and renardine proved highly successful in preventing voles recolonising the areas under the mats. Methyl-anthranilate, was effective only through regular application. Cinnamaldehyde and synthetic stoat odour failed to deter voles from colonising the underside of mulch mats.

INTRODUCTION

Mulch mats are currently used by foresters to maintain weed-free areas around newly planted trees. By reducing competition for moisture and light from the fast growing weed species, mulch mats greatly increase the survival and growth rate of young trees (Davies, 1985; Davies, 1987). This plant husbandry technique has the added advantage of avoiding the need for herbicide inputs.

Unfortunately, mulch mats are also an attractive refuge and nesting site for field voles (*Microtus agrestis*). These herbivores cause economically significant damage to trees on newly planted or replanted forest land by removing the bark at ground level and attacking the stems and roots (Gill, 1992). The presence of voles also attracts predators (e.g. foxes, *Vulpes vulpes*) which destroy the mats whilst in search of their prey. If voles could be deterred from living under the mats then damage to young trees may be reduced or prevented altogether. Chemical repellents are prime candidates for this application, providing an humane, non-toxic, cost-effective method of discouraging voles from colonising the underside of mats.

For a repellent to offer more than transitory effectiveness, the stimulus induced by the compound (e.g. taste/odour) must be strong enough to encourage the vole to seek alternative food/habourage and this effect must not wane with continued exposure. There are three main sources of potential repellents that are likely to act as repellents for mammals (Muller-Schwarze, 1994). Firstly, there are a number of materials that are known to cause temporary irritation of the mucosal membranes of the mouth, nose and eyes, avoidance of which is unlikely to habituate. Secondly, there are a variety of naturally occurring plant compounds to which animals have evolved an inherent aversion. These compounds are produced by plants to deter herbivores from feeding and may therefore be effective in this application. Thirdly, the odours left by predators and products derived from rendered animal tissues are known to

cause avoidance behaviour amongst small mammals. Opportunities for the development of repellents in each of these three categories were investigated in this study.

The aim of this study was to assess the efficacy and persistence of irritants (ziram, Myllymaki, 1977), plant defence compounds (cinnamaldehyde, Linhart *et al.*, 1977; methyl-anthranilate, Nolte *et al.*, 1993) and predator odours (synthetic stoat odour, Sullivan *et al.*, 1990; renardine, Atkinson & Macdonald, 1994) as repellents to deter field voles from living beneath mulch mats.

MATERIALS & METHODS

Study site

One ex-grassland study site was used at an experimental farm near Winchester. A 0.4 ha paddock were used for the trial. This paddock is part of a unique set of paddocks that are fenced to exclude wild rabbits and managed to encourage the establishment of large field vole populations. This is achieved by allowing the luxuriant growth of grass and weeds. Before the mats were put in place, an area of roughly the size of the mat (c. 0.5 m x 0.5 m) was strimmed in order to reduce the height of the vegetation. Prior to putting the mat in place all signs of vole occupation (e.g. runs) were removed from the area.

Experimental Layout

A randomised block design with 30 replicates was used. Each treatment was set out as a rectangle of 3 rows and 2 columns. The centre of each mulch mat was 2 m from the centre of neighbouring mats. In each block five different substances were tested: (1) Control (No odour); (2) Renardine; (3) Ziram; (4) Methyl-anthranilate; (5) Cinnamaldehyde; (6) Synthetic stoat odour (1:1 mixture of 3-Propyl-1,2-dithiolane and 2-Propylthietane).

These substances were first applied to the underside of the mats on 17 October 1995 and reapplied after 5 and 10 weeks. Formulations of renardine (Roebuck-Eyot Ltd., Bishop Auckland, Durham, UK), ziram (a.i. 32%; Dalgety Agriculture Ltd, Reading, Berkshire, UK) and methyl-anthranilate (a.i. 14.4%; PMC Specialities Group, Cincinnati, USA) were painted on the entire underside of each mat. Cinnamaldehyde (a.i. 99%; Sigma Chemical Co., Poole, Dorset, UK) was placed in ten 140µl capillary tubes under each mulch mat. The synthetic stoat odour (PheroTech Inc, Delta, British Columbia, Canada) was supplied encapsulated in an inert polymer dispenser. Initially two dispensers were placed under each mat. At the second application two extra dispensers were placed under each mat whilst the originals were left *in situ*. On the third application the first two dispensers were replaced with two new ones. No chemicals were applied after early January 1996 but the assessment was continued to determine the longevity of the repellent effect for each treatment.

Data collection

Signs of vole occupation were recorded under the mulch mats every two weeks initially (until late January) and thereafter every month. Each mulch mat was lifted up and the presence of nests, runs, cuttings and droppings were noted.

Assessment of small mammal populations

M. agrestis populations were assessed in November and April using Longworth traps. Sixteen trapping points were laid out in a line, with two traps per point. These were run for five consecutive days, with traps checked twice a day (Sibson, 1991). All small mammal species caught were recorded and fur-clipped so that recaptures could be identified. The number per hectare (minimum number alive) was calculated from the total number caught over the five days of the census.

Statistical analysis

Chi-squared analysis (contingency tables) was used to test for differences in the proportion of mats that had signs of vole occupation under them. Data from four of the assessments, were used in the analysis: those carried out one (20-11-96), three (10-01-96), five (21-03-96) and seven (22-05-96) months after the initial application of the chemicals. Each treatment was compared against the control (using 2 x 2 contingency tables) to ascertain which treatments significantly reduced the number of occupied mats.

RESULTS

Field vole densities remained relatively stable over the entire course of the trial at 40.3 ha^{-1} in November and 50.4 ha^{-1} in April.

The occupancy rates of the various treatments over time are described in Table 1. By the end of the seven months, overall almost two-thirds of the mats were occupied. The control mats were rapidly colonised: by the sixth week 56.7% of the mats had evidence of vole occupation under them. This figure remained relatively stable until the fifth month at which point the number of sites occupied began to increase (Table 1).

The proportion of occupied mats for both renardine and ziram treatments was significantly less than the control at all the assessment periods (Renardine: $\chi^2 = 8.44$, df = 1, $P < 0.01$ in November; $\chi^2 = 15.3$, df = 1, $P < 0.001$ in January; $\chi^2 = 7.04$, df = 1, $P < 0.01$ in March; $\chi^2 = 15.6$, df = 1, $P < 0.001$ in May. Ziram: $\chi^2 = 4.56$, df = 1, $P < 0.05$ in November; $\chi^2 = 5.88$, df = 1, $P < 0.02$ in January; $\chi^2 = 4.38$, df = 1, $P < 0.05$ in March; $\chi^2 = 12.1$, df = 1 , $P < 0.001$ in May).

Treatment of mats with methyl-anthranilate resulted in a more equivocal and less persistent effect on mat site occupation; only in January was a significant difference observed ($\chi^2 = 2.12$, df = 1, $P > 0.05$ in November; $\chi^2 = 4.68$, df = 1, $P < 0.05$ in January; $\chi^2 = 0.6$, df = 1, $P > 0.05$ in March; $\chi^2 = 0.93$, df = 1, $P > 0.05$ in May). Both the cinnamaldehyde and the synthetic stoat odour treated mats also showed relatively rapid rates of colonisation. There was no significant difference ($P > 0.05$) between the efficacy of these treatments and the control at any point during the trial (Cinnamaldehyde: $\chi^2 = 0.00$, df = 1, $P > 0.05$ in November; $\chi^2 = 0.26$, df = 1, $P > 0.05$ in January; $\chi^2 = 0.00$, df = 1, $P > 0.05$ in March; $\chi^2 = 0.14$, df = 1, $P > 0.05$ in May. Stoat odours: $\chi^2 = 0.00$, df = 1, $P > 0.05$ in November; $\chi^2 = 0.26$, df = 1, $P > 0.05$ in January; $\chi^2 = 0.26$, df = 1, $P > 0.05$ in March; $\chi^2 = 3.20$, df = 1, $P > 0.05$ in May).

Table 1. The number of mulch mats with signs of vole occupancy for the six treatments (30 mats per treatment) over the course of the experiment.

Assessment Date	Control	Renardine	Ziram	Methyl-anthranilate	Cinnam-aldehyde	Synthetic stoat odour	Total
03.11.95	4	0	0	2	5	2	13
20.11.95	11	0	3	5	12	10	41
07.12.95	17	1	3	5	11	14	51
19.12.95	18	1	5	6	13	14	57
10.01.96	15	1	5	6	12	12	51
24.01.96	15	1	3	8	11	13	51
07.02.96	17	2	5	9	13	13	59
23.02.96	18	4	8	11	13	14	68
21.03.96	17	5	8	13	17	14	74
29.04.96	25	8	12	20	22	17	104
22.05.96	26	10	12	22	25	19	114
27.06.96	28	12	12	26	26	22	126

DISCUSSION

The present studies have clearly demonstrated that both renardine and ziram effectively deter voles from investigating the underside of the mulch mats, at least in the medium term. The reduction of occupancy observed with the plant derivative, methyl-anthranilate, was short-lived. After the final application in January the proportion of sites occupied returned to levels comparable with the control. It therefore appears that a major constraint on the utility of this relatively benign compound (acute oral LD_{50} = 2910 mg/kg) may be the lack of persistence of the current formulation. This problem has been reported in previous studies (Vogt, 1992).

Both cinnamaldehyde and the synthetic stoat odour appear to have failed to deter the voles from colonising the mulch mats. It is possible that the method of delivery was not optimal or that the concentrations of the chemicals were too low to be effective. Ziram, renardine and methyl-anthranilate were all applied as a suspension concentrate formulation painted onto the underside of the mats, whilst cinnamaldehyde and the stoat odour were deployed in special dispensers. However, field observations indicated that the voles did not seem to be actively avoiding the dispensers. In many cases voles had made runs directly on top of the dispensers. The lack of an apparent aversion to the stoat odour is perhaps surprising considering its success as a repellent for other vole species (*Microtus pennsylvanicus* & *M. montanus*; Sullivan *et al.*, 1988). Predator odours appear to require reinforcement by encounters with live animals to have a long-term effect (Muller-Schwarze, 1994). The paddock used in this trial was free of mustelids and therefore this reinforcement of the signal was absent. This may account for the compound's poor performance. A further constraint on the effectiveness of methyl-anthranilate and cinnamaldehyde is that as they are secondary plant compounds that deter feeding rather than reduce vertebrate activity in an area. Their use in the context of deterring occupancy of mulch mats may thus be inappropriate.

The remaining question about the effectiveness of renardine and ziram is exactly how long does their repellent effect persist? Mulch mats have a useful life of over a year and repeating the repellent application several times during this period would be economically unacceptable. The decline in efficacy observed afterthe final application in January may be the result of the loss of the active ingredients or the habituation of the voles to their effects. We are currently extending the trial to ascertain the length of time these two chemicals continue to deter voles and are planning a second trial to determine the persistence of the compounds and their effects after a single application. Furthermore, we are investigating the potential use of encapsulated formulations to increase persistence and deliver, sequentially, a number of repellents to reduce the habituation to the overall treatment.

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MONITORING THE ACTIVITIES OF FREE-LIVING WILD RATS IMPLANTED WITH TRANSPONDERS

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ABSTRACT

Further improvements to the efficiency of rodent control could be made if the behavioural responses of free-living wild rats towards rodenticide treatments could be observed. Since visual observations of these largely secretive animals are impractical, remote sensing is the only viable technique. Passive integrated transponders (PIT tags) offer a safe and humane way of distinguishing many individual animals from each other and suitable detectors can be set out at key points such as bait stations and alternative food sources to log the presence of tagged rats and thereby help identify specific characteristics that enable some individuals to survive rodenticide use. A pilot trial is described in which 33 rats living on a farm were tagged and four detectors were set out around the main food source whilst a rodenticide treatment was carried out. This trial revealed certain problems which will need to be addressed in future studies, but the technique offers experimental opportunities that have hitherto not been possible.

INTRODUCTION

Norway rats (*Rattus norvegicus*) are significant pests of stored products and are also a threat to public and animal health. The main method of population control is to use toxic preparations, mostly in the form of cereal-based baits. A survey of rodenticide usage on farms growing arable crops in England, Wales and Scotland estimated that in 1992 1,487,870kg of bait were applied of which 99.5% contained an anticoagulant rodenticide as the only active ingredient (Olney and Garthwaite, 1994). However, rodenticide use does not always produce satisfactory results and Quy *et al.* (1992) showed how the presence of stored cereal significantly reduced treatment effectiveness. The main reason for a poor outcome was that many individuals had consumed little or no bait. A greater understanding of rat behaviour leading to poor bait consumption during control operations would thus help improve the efficiency of treatments. Previous behavioural studies have essentially been restricted to enclosures because of the relative ease with which such experiments can be carried out, but it is not clear how their findings relate to the activities of free-living wild rats. Since direct observation of small mammals living often within dense cover is practically impossible, remote sensing of rat behaviour may thus be the only viable technique, provided a satisfactory method is available to identify and track individual animals.

One technique for following the activities of Norway rats is radio-tracking (Taylor, 1978). However, tracking rats as they move among farm buildings is complicated by reflected signals giving misleading directional information. Moreover, transmitters can only be fitted

to rats big enough to carry them and then only to adults, because no suitable expandable collars have yet been devised. Passive integrated transponder (PIT) tags have been shown to be a safe and humane way of permanently marking laboratory rats (Ball *et al.*, 1991). Schooley *et al.* (1993) successfully implanted PIT tags into free-ranging ground squirrels (*Spermophilus townsendii*) and suggested that such tags were suitable for long-term demographic studies of small mammals. Implanted transponders offer the opportunity of giving unique identification codes to almost unlimited numbers of rats of any size. Each code is transmitted to a decoder when the transponder enters a magnetic field that is generated around a loop antenna. Codes can be read easily through most materials except metal and they can be stored or displayed by the decoder. Prentice *et al.* (1990) gave a brief technical description of the PIT system. With suitable equipment, automated, continuous monitoring of activity is possible.

We describe the method of implanting transponders into wild rats in the field and the use of a battery-operated detector for automated remote sensing of marked rats. The technique was employed in the field to monitor the activities of rats at a main food supply that supported an infestation around farm buildings. A rodenticide treatment was carried out to see if individuals could be attracted towards the bait, but the main aim was to discover which rats survived the longest in terms of sex and age (weight) class. A preliminary laboratory test established that rats could carry the implants without any adverse effects. Implanting the PIT tags in the field was carried out under conditions laid down in the Animals (Scientific Procedures) Act, 1986.

METHODS

The Trovan PIT (supplied by RS Biotech, Finedon, Northants.) used was encased in a sealed biocompatible glass tube that measured approximately 12mm long and 2mm in diameter. Each transponder came in an individually-packed sterilised needle ready for attachment to a trigger-operated implant tool. Thirty-three individual wild rats (19 males, 14 females) weighing 159-430g were trapped in wire-mesh cage traps set in and around farm buildings in February 1995. Each rat was transferred to an inhalation chamber via a cloth bag and then lightly anaesthetised with diethyl ether. We considered it would be undesirable to attempt to insert an implant into a fully conscious wild rat as the force required to restrain them runs the risk of causing injuries. When unconscious, the rat was removed from the chamber and laid on a flat surface. A pinch of skin between the shoulder blades was lifted up, the needle inserted pointing slightly downwards and towards the head and the trigger of the implant tool squeezed, forcing the transponder into the space between the skin and body wall. Other implantation sites such as the rump were ruled out in case the rat attempted to bite at the transponder and also because wild rats frequently suffer injury to the hindquarters during intraspecific fights. Before the rat recovered consciousness, the transponder's code was read and recorded using a hand-held detector (RS Biotech PI-101 Minireader) and the sex and body weight were also noted. Each rat was returned to the trap and kept under shelter to allow it to recover fully. Before being released, the rat was scanned through the mesh of the trap with the minireader to check that the tag had not come out; it was possible to detect an implanted tag when the rat's shoulders were close to the top of the trap and the mesh size was >=19mm (3/4 in.). Thus recaptured rats could be identified in the trap without handling them again; however, negative responses could not be trusted as the metal of the trap might

have interfered with the signal, hence all doubtful cases had to be transferred into a black bag for confirmation.

The site was an arable farm in the county of Hampshire. The infested area of about 0.3ha included the buildings and adjacent ground. The main building consisted of an L-shaped grain store within which were grain bins and bags of artificial fertiliser. All the bins were empty except for two which contained some wheat left over after the bulk removal of grain and which appeared to be the only food source on the farm. Rats gained access to this wheat through a metal pipe in the bottom of each bin that led to the outside and which was normally connected to a hot air blower used to dry the grain. These bins were also made of metal which would have desensitised any PIT tag detector put around the access pipe. Therefore, a pair of wooden bait boxes were placed in front of each of the two access pipes with the pairs 3m apart. Each box measured 360 x 260 x 140 mm, had an entrance at both ends and was covered with a metal lid to protect it and its contents from bad weather. The boxes of each pair were placed end to end (entrance to entrance) with no gap between them. Each one was filled with whole wheat and was regularly topped up to maintain a surplus throughout the experiment. Four self-contained detectors were installed, each one to monitor activity around the entrance on either side of each pair of boxes. Each detector consisted of a single point decoder coupled to a data logger and connected to a square-shaped loop antenna measuring 300 x 300 mm. Power was supplied by two 63Ah 12v rechargeable dryfit batteries connected in series that maintained the electromagnetic field continuously for at least 4 days before recharging was necessary. The batteries and the decoder/logger were enclosed in a weather-proof box. A 3m lead ran from the decoder to the loop antenna which was placed about 100mm from the entrance to the bait box. (The antenna had to be at least 50mm away from the metal lid of the bait box in order to avoid a reduction in sensitivity through induced eddy currents.) The bottom part of the antenna was buried in the soil, thus the best chance of detecting a tagged rat was when it passed through the loop on its way into the bait box, although approaches just outside the loop (within about 100mm) would also be detected.

Visits to the wheat supply by tagged rats were logged continuously between 13 February 1995 and 21 April 1995. The date and time of each detection was also logged. Each day the data collected by each logger was transferred to a laptop computer. During the week beginning 6 March, a census of the entire rat population was carried out using an established tracking plate method (Quy *et al.*, 1993). One week later 26 bait boxes, which had been set out before the census began to match the distribution of rat signs, were baited with 100g of a commercial whole wheat bait containing 0.005% bromadiolone. Each bait point was inspected on a Monday-Wednesday-Friday schedule for 18 days and the amount of bait remaining was recorded to the nearest 5g and then topped up to maintain a surplus until the next inspection. A second census was carried out beginning on day 14 of the treatment. On day 18 all bait was removed from the boxes and all rat burrows that could be found within the treated area were baited. Each baited burrow was lightly blocked and over the next 14 days any that were reopened were rebaited. In total, 78 burrows were found. A final post-treatment census was carried out after all baiting ceased. After 21 April when the detectors were switched off, an attempt was made to trap surviving rats.

RESULTS

Of the original 33 marked rats, 15 males and 10 females were known to be alive at the start of the treatment. The estimated size of the pre-treatment population was 146 rats (Fig. 1). Maximum bait take was attained by day 4 and thereafter it declined steadily until the end of the container-baiting phase. The second census indicated an estimated 42% reduction in the population. At the start of the burrow-baiting phase, 12 males and 6 females were known to be alive: the change in sex ratio between the two baiting phases was not significant (χ^2 = 0.02, 1 df). Using the body weights recorded when the animals were first tagged, the ratio of rats in two weight groups (<200g, >=200g) at the beginning of the treatment was 9:18 and after container-baiting was 7:9: the difference in the ratios was not significant (χ^2 = 0.13, 1 df). The post-treatment census showed that the population had been reduced by an estimated 97%. One tagged rat, a male, was found dead during the treatment and it showed signs typical of anticoagulant poisoning. Only one tagged rat, a male, was known to have survived and it was trapped close to the wheat-filled boxes on 26 April: it weighed 324g (174g when first tagged).

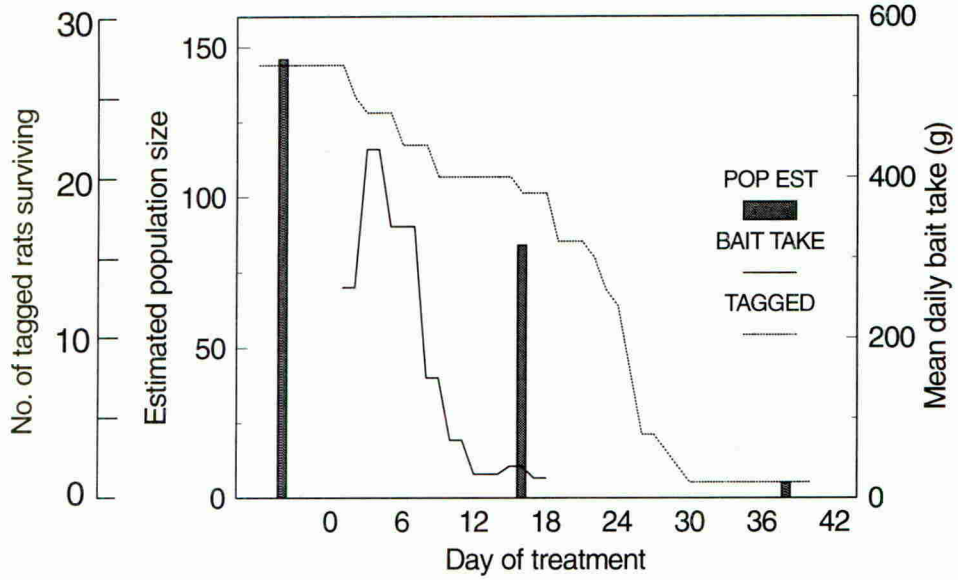

Figure 1. Progress of a rodenticide treatment in which the activity of individually marked rats around an alternative food source was monitored. Between days 18 and 32 the bait was applied directly into rat burrows from which takes could not be measured.

DISCUSSION

In this limited pilot trial there was no evidence to show that the sex or age (weight) of a rat increased its chance of surviving the rodenticide treatment, although there was a trend for

the heavier (older) animals to die first. Those rats that survived the longest could have been the most neophobic (Shepherd and Inglis, 1987) or those that were prevented from approaching the bait until other (dominant?) rats had died (Fenn and MacDonald, 1987). If social exclusion had been a more significant factor than neophobia, then the takes from the containers should not have declined to negligible amounts while there were still plenty of rats alive. Most rats died (including, presumably, all but one of the marked rats) when the bait was placed in burrows, suggesting that a degree of container neophobia was widespread in the rat population and was more significant than any wariness towards a novel food (Inglis *et al.*, 1996). The surviving marked rat was thought to have been living inside a building rather than a burrow which could be baited.

Of the original 33 tagged rats, which were trapped and released from all parts of the site, 31 were detected at one or more of the logging stations. Because the detection range is so short with current technology, careful siting of the antennae is essential and on this farm that was made easier by being able to control access to what was apparently the only food source available. Thus the regular pathways used by rats leading to the food supply could be easily identified and the antennae sited accordingly. However, the short detection range could be an advantage where the response of individuals to, say, a specific control measure must not be confused by other tagged rats located nearby. Loop antennae smaller than the ones used here can be made with a corresponding decrease in detection range which would be more appropriate for particular applications. For example, some rats survive attempts to kill them with cumulative poisons even though they have apparently eaten what should be a lethal dose (Quy *et al.*, 1992). Either the rats are resistant or their feeding patterns enable them to avoid accumulating a lethal dose. With tagged individuals and detectors attached to bait containers that detect only those animals entering the container, it is now possible to discover how free-living rats respond to the introduction of a poison bait in a variety of environmental conditions.

One serious shortcoming of the system used here was the lack of memory available in the data logger which effectively prevented more information on activity cycles being presented. The problem is created as much by the behaviour of individual rats as by any specification flaw. It was quite common to find a long succession of records one second apart (the smallest time resolution with this particular equipment) for one animal at one detector. The records were undoubtedly genuine and caused by the tagged rat moving in and out of the electromagnetic field. In this study where the antennae were just outside the entrances to the boxes containing the food supply, any hesitancy about entering because of, say, the presence inside of other rats, could have produced a multitude of records for what was essentially one visit. While many of these records could be discarded, they nevertheless fill up the logger with the result that subsequent data may be lost. It was apparent from this pilot trial and from later studies we have carried out that vast quantities of data can be generated by relatively small numbers (<30) of PIT-tagged rats. Handling this amount of data is a considerable task.

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