SESSION 8C

NEW APPROACHES TO CROP PROTECTION BY EXPLOITING STRESS-RELATED SIGNALLING IN PLANTS

Chairman and Session Organiser:	Professor John Pickett *Rothamsted Research, Harpenden, UK*
Papers:	8C-1 to 8C-4

Exploring multi-trophic plant-herbivore interactions for new crop protection methods

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ABSTRACT

Biological control of arthropod herbivores in agricultural crops depends on antagonists or enemies of the pest organisms. To minimise damage to a crop, it is crucial that the biological control agents are able to find their prey efficiently. Here we discuss the finding that when herbivores feed, plants produce volatiles that are attractive to the predators. The effects of biotic, abiotic and genetic factors on volatile formation and the biochemical and molecular regulation of this indirect defence mechanism are reviewed. The opportunities to use genetically modified plants to further understanding this complex interaction and the possibilities of using our knowledge to improve biological control in agricultural crops are discussed.

INTRODUCTION

Environmentally benign protection of crops against arthropod herbivores can use either direct host plant resistance or biological control. These two approaches are fundamentally different in that host plant resistance depends on direct – constitutive or feeding-induced - defence traits of the crop plants (such as trichomes, toxic secondary metabolites or proteins, and repellents), whereas biological control depends on the use of antagonists or enemies of the pest organisms. For the latter to be effective, it is crucial that the biological control agents are able to find their prey efficiently enough to minimise damage to the crop.

More than a decade ago it was discovered that when herbivores feed, the plants produce volatiles that are attractive to the natural enemies of the herbivore (Dicke *et al.*, 1990a; Turlings *et al.*, 1990) (Figure 1). Thus, plants indirectly defend themselves by enhancing the effectiveness of the natural enemies of the herbivores. The use of predators and parasitoids for biological control is receiving more and more attention and for many years it has been common practice in a number of crops in glasshouse as well as open fields (Van Lenteren, 2000; Kfir *et al.*, 2002). Nevertheless, breeders and agronomists have so far paid little attention to the optimisation of biological control. This is probably due to the relatively recent discovery of the phenomenon of indirect defence, the more complex relationships involved and the difficulties associated with quantification of the effects.

Here we will discuss the principles of plant-mediated multitrophic interactions involved in biological control and our research into the possibilities of using this principle to design new control methods. We use a combination of scientific disciplines varying from ethological studies of predators to plant molecular biology. We focus our work on the induction of volatiles by spider mites in a number of plant species, such as cucumber and potato, and the effects of abiotic and biotic factors on this induction, and we study the effects of the volatiles on the behaviour of predators such as predatory mites. The biochemical and molecular regulation of volatile formation is investigated using enzymology and cDNA microarray technology and genes involved in volatile formation are cloned from a variety of plant species. These genes are then used to transform plants, including model plants such as tobacco and arabidopsis, to change their volatile profile and study the effects on the behaviour of parasitoids and predators.

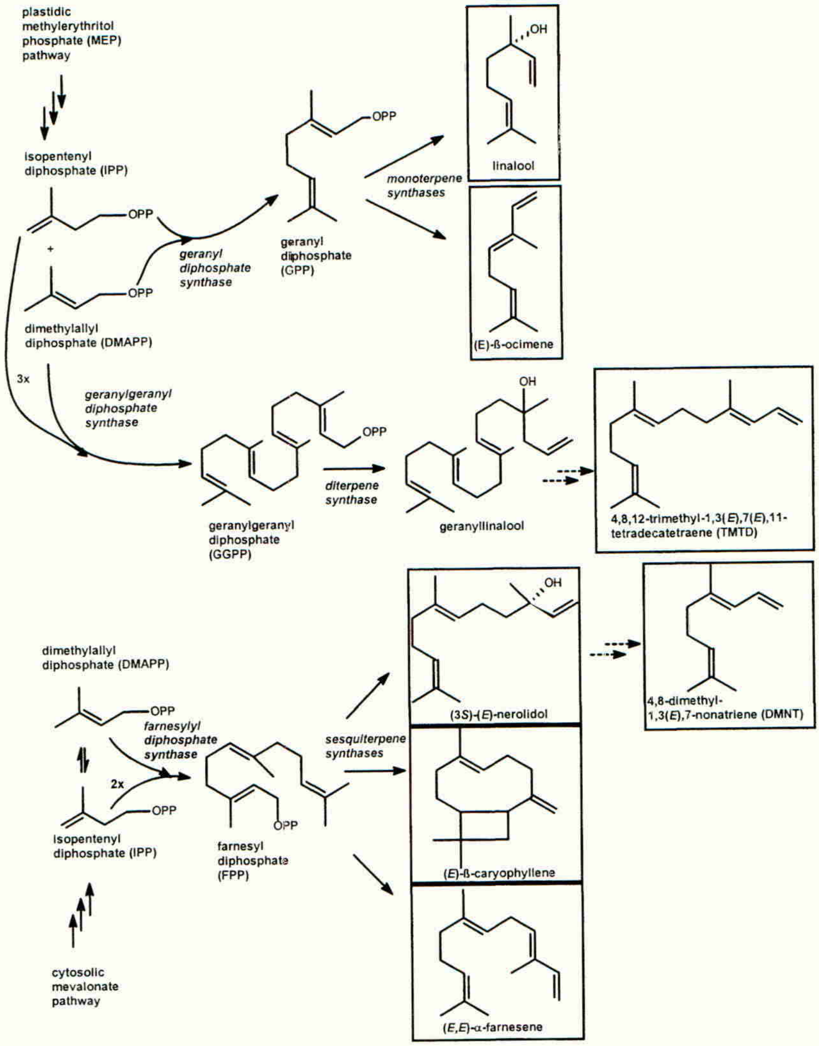

Figure 1. Examples of isoprenoid volatiles that have been shown to be induced in a range of plant species by a range of arthropod herbivores (boxed compounds) and schematic representation of biosynthetic pathways involved in their formation. (Broken) arrows indicate (putative) enzymatic steps.

VOLATILE INDUCTION AND EFFECTS ON PREDATOR BEHAVIOUR

The importance of the third trophic level for plant defence was first suggested by Price et al (Price *et al.*, 1980), and was followed by the discovery of the herbivory-induced volatiles that attract parasitoids and predators (Dicke *et al.*, 1990a; Turlings *et al.*, 1990). Upon infestation with two-spotted spider mites (*Tetranychus urticae*), lima bean plants responded by emitting a mixture of volatiles attracting the predatory mite *Phytoseiulus persimilis* (Dicke *et al.*, 1990b) that effectively eliminated local populations of the spider mites (Dicke *et al.*, 1990a). Similarly, corn plants respond to feeding damage of *Spodoptera exigua* caterpillars with the production of volatiles that attracted the parasitoid *Cotesia marginiventris* (Turlings *et al.*, 1990). Since then, it has been shown that this is a common mechanism employed by many plant species in the interaction with many different herbivores, and that these volatiles are not usually emitted in response to mechanical wounding (Dicke *et al.*, 2003).

Figure 2 shows a typical example of the induction of volatiles in cucumber and Figure 3 the results obtained in Y-tube olfactometer experiments on predator attraction by induced plants for a number of plant species. Although treatment with jasmonic acid mimics fairly well the effect of spider mite infestation, some differences (e.g. peak 5, (*E,E*)-α-farnesene) can be found in the volatile blend (Figure 2). Typical volatiles released from a multitude of species after herbivory are the so-called green leaf volatiles such as C6-alcohols, -aldehydes, and -esters, derivatives of the shikimate pathway such as methyl salicylate, and isoprenoids such as (*E*)-ß-ocimene, linalool, (*E*)-ß-caryophyllene, (*E,E*)-α-farnesene and the homoterpenes 4,8-dimethyl-1,3(*E*),7-nonatriene and 4,8,12-trimethyl-1,3(*E*),7(*E*),11-tetradecatetraene (Figure 1). The isoprenoids are by far the most important components of the induced volatile blend and hence this is the class we focus on.

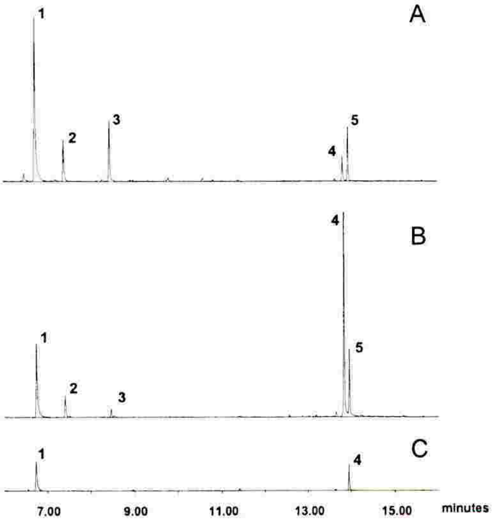

Figure 2. Induction of volatiles in cucumber by spider mite feeding (A) and jasmonic acid spraying (B). Panel C shows the response of control leaves. Peaks are: (*Z*)-3-hexen-1-yl acetate (1), (*E*)-ß-ocimene (2), 4,8-dimethyl-1,3(*E*),7-nonatriene (DMNT) (3), (*E,E*)- α-farnesene (4), 4,8,12-trimethyl-1,3(*E*),7(*E*),11-tridecatraene (5).

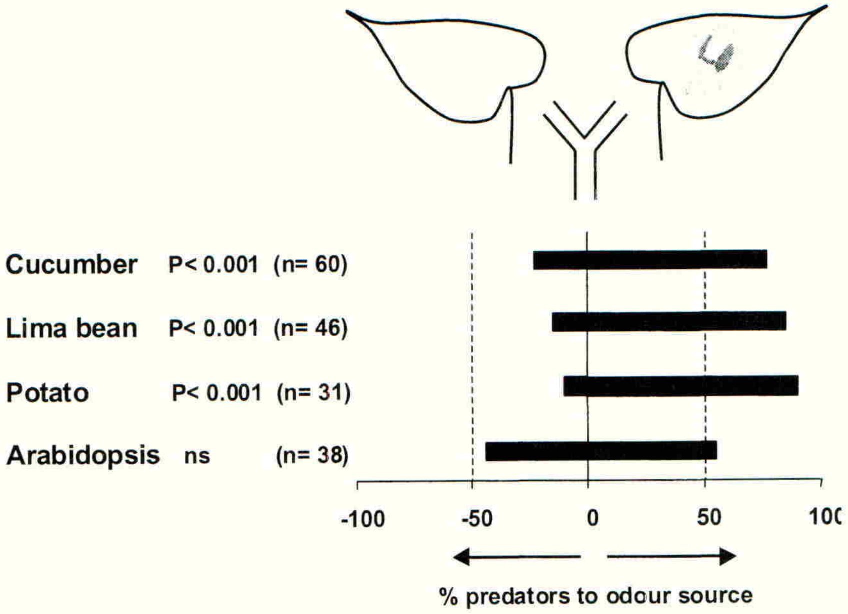

Figure 3. Predator attraction (*Phytoseiulus persimilis*) by spider mite infested cucumber, lima bean, potato and arabidopsis plants in a Y-tube olfactometer.

FACTORS AFFECTING INDUCED VOLATILE FORMATION

Biotic and abiotic factors

Plants have been shown to respond to different herbivore species with quantitatively and qualitatively different volatile blends allowing predators to respond to their specific prey (Sabelis & van der Baan, 1983; Takabayashi *et al.*, 1991; De Moraes *et al.*, 1998; Du *et al.*, 1998). Within a plant species, the quality of the volatile blend may be affected by the developmental stage of the herbivore (Takabayashi *et al.*, 1995), and the volatiles induced by insect egg deposition differ from those induced by feeding (Wegener *et al.*, 2001; Hilker & Meiners, 2002).

There are many reports that the production of non-herbivore induced secondary metabolites by plants is influenced by environmental conditions. It is therefore likely that this also holds for herbivory-induced volatiles and knowledge of these effects may be important for improvement of biological control, e.g. by using optimal conditions for efficient signalling. Some of the factors that have been shown to affect secondary metabolite production such as light, temperature and water availability have also been investigated for their effect on herbivore-induced volatile biosynthesis. High light intensity and water stress are generally reported to increase induced volatile production and/or predator attraction for example in Lima bean, kidney bean, maize and cotton (Loughrin *et al.*, 1994; Takabayashi *et al.*, 1994; Turlings *et al.*, 1995; Gouinguene & Turlings, 2002).

Genetic variation

If conventional plant breeding is to be used to improve biological control through enhanced volatile production there has to be genetic variation in the ability to produce herbivore-induced, predator-attracting volatiles. In gerbera, a number of cultivars differed in composition and amount of volatiles produced after spider mite feeding (Krips *et al.*, 2001) and there were differences between the cultivars in the odour-preference of predatory mites. The composition of the volatile blend seemed to be more important for this difference than the total amount of volatiles produced, and the terpenoids (*E*)-ß-ocimene and linalool were possible candidates in determining the difference in attractiveness between cultivars. Also maize cultivars and *Zea* spp. showed large differences in the composition of the volatile blend induced by the application of the oral secretion of *Spodoptera littoralis* to mechanically damaged leaves (Gouinguene *et al.*, 2001).

There are several problems associated with comparing genotypes for their production of induced volatiles, when other differences between the genotypes can not be controlled (Krips *et al.*, 2001). For example, there may be differences in direct defence causing differences in developmental rate of herbivores leading to differences in volatile formation. To circumvent this problem, in addition to spider mite infestation we also used jasmonic acid (JA) treatment in a comparison between seven cucumber genotypes. Earlier research had shown that JA treatment mimics the effect of spider mite infestation in lima bean, gerbera and cucumber plants (Dicke *et al.*, 1999; Gols *et al.*, 1999) (H J Bouwmeester *et al.*, unpublished data). Figure 4 shows that there are large differences in the response of cucumber genotypes to spider mite infestation. In addition, there are similar differences in volatile production among cucumber genotypes after JA treatment. There is a fair correlation between the response to spider mites and JA (e.g. high production in genotypes 1, 6 and 7 for both treatments and low for genotypes 2 and 3). In a preliminary Y-tube olfactometer experiment, the attractiveness of genotypes 1, 4, and 7 to predatory mites upon spider mite infestation was compared, showing a small, but significant preference towards genotype 1 (data not shown).

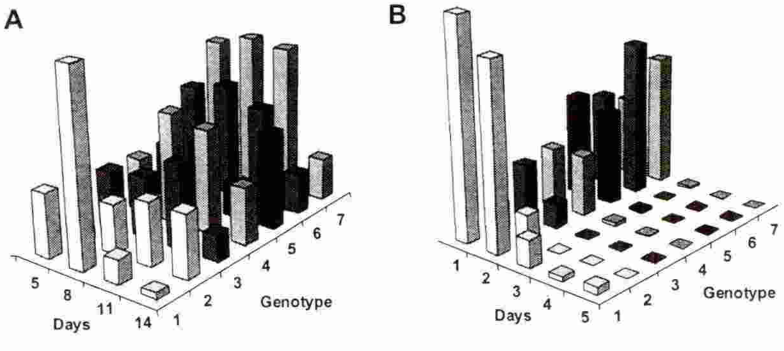

Figure 4. A, Time course of total volatile induction by spider mite feeding and B, jasmonic acid spraying in seven cucumber genotypes. A. 2-Wks old plants were infested with 50 spider mites on day 0. Leaves were sampled after 5, 8, 11 and 14 days for headspace analysis. B. 2-Wks old plants were sprayed with jasmonic acid on day 0. Leaves were sampled after 1, 2, 3, 4 and 5 days for headspace analysis.

HOW IS VOLATILE INDUCTION REGULATED?

Using $^{13}CO_2$ pulse-labeling experiments it was demonstrated that most of the induced volatiles are biosynthesised *de novo* in response to herbivory (Pare & Tumlinson, 1997). This has led to the search for the induced enzymes involved in the formation of these volatiles. The first herbivory-induced enzyme to be identified was (3*S*)-(*E*)-nerolidol synthase in cucumber and lima bean (Bouwmeester *et al.*, 1999). This enzyme catalyses the conversion of farnesyl diphosphate to (3*S*)-(*E*)-nerolidol, a sesquiterpene alcohol, likely an intermediate in the formation of 4,8-dimethyl-1,3(*E*),7-nonatriene (DMNT) (Donath *et al.*, 1994; Donath and Boland, 1995) (Figure 1). Indeed DMNT is one of the major induced volatiles of cucumber (Figure 2) and all the evidence suggests that (3*S*)-(*E*)-nerolidol synthase is the regulatory step *en route* to this compound. DMNT is also an important constituent of the induced volatiles of other plant species such as lima bean, maize and cotton (Dicke *et al.*, 1990a; Turlings *et al.*, 1990; Takabayashi *et al.*, 1991; Loughrin *et al.*, 1994; Takabayashi *et al.*, 1994) and seems responsible for the attraction of the predatory mite *P. persimilis* to Lima bean and cucumber plants infested with the spider mite *T. urticae* (Dicke *et al.*, 1990b). A synthetic mixture of volatiles, including DMNT, that mimics the blend of volatiles that is emitted by corn plants infested with *S. exigua* caterpillars, attracted the parasitoid *C. marginiventris* which parasitizes the caterpillars (Turlings *et al.*, 1991).

MOLECULAR TOOLS

Elucidation of mechanisms

As mentioned, the elucidation of genotype differences and the role of individual volatiles in herbivory-induced indirect defence, is hindered by the lack of genotypes that differ in one factor only (*e.g.* only differ in the level of volatile production, not in composition and/or direct defence level) (Dicke & Hilker, 2003), but see (Van Poecke & Dicke, 2002) for a first example). With the advent of molecular tools, the use of transgenic plants is now becoming a feasible and exciting way to unravel not only the importance of individual compounds but also the regulation of their induction. Research in our laboratories has focussed on the major components of the blends of induced volatiles, the terpenoids. We have isolated and characterised a multitude of genes involved in terpene biosynthesis such as the monoterpene synthases (+)-limonene synthase, ß-pinene synthase and γ-terpinene synthase from lemon (Lücker *et al.*, 2002), α-pinene synthase from strawberry (Aharoni *et al.*, 2003b), the sesquiterpene synthases amorpha-4,11-diene synthase from *Artemisia annua* (Mercke *et al.*, 2000; Wallaart *et al.*, 2001), two isoforms of germacrene A synthase from chicory (Bouwmeester *et al.*, 2002), (+)- and (-)-germacrene D synthase, α-gurjunene synthase and cascarilladiene synthase from *Solidago canadensis* (I G Altug, W A König & H J Bouwmeester, unpublished) and a combined mono-/sesquiterpene synthase linalool/nerolidol synthase from strawberry (Aharoni *et al.*, 2003b). Most of these enzymes are involved in the constitutive (or developmentally regulated) biosynthesis of essential oils or flavour compounds in these plant species. They were cloned using PCR with degenerate primers or cDNA microarray analyses from plant tissues that were shown to produce the corresponding products, and hence needed no induction by herbivores.

Some of these enzymes catalyse the formation of compounds that have been shown, in other plant species, to be produced upon induction by herbivores. Examples are limonene and linalool (Dicke *et al.*, 1990a; De Moraes *et al.*, 1998), germacrene A (Van den Boom *et al.*, 2002) and nerolidol. Nerolidol is found in the induced volatile blend of for example maize but is, more importantly, the first dedicated intermediate *en route* to DMNT (Bouwmeester *et al.*, 1999; Degenhardt & Gershenzon, 2000) (Figure 1). In addition, we have cloned two spider mite induced sesquiterpene synthases from cucumber, (*E,E*)-α-farnesene synthase and (*E*)-ß-caryophyllene synthase (P M Mercke, I F Kappers, F W A Verstappen, M Dicke & H J Bouwmeester, unpublished), two other major contributors to the induced volatile blend of many plant species (Dicke *et al.*, 1990a; De Moraes *et al.*, 1998) (Figure 1). In addition to the directed cloning of genes encoding enzymes involved in terpenoid biosynthesis, we and others use more generic approaches such as cDNA microarray analysis (Reymond *et al.*, 2000; Schenk *et al.*, 2000; P M Mercke, I F Kappers, F W A Verstappen, M Dicke & H J Bouwmeester, unpublished) or DDRT-PCR (Hermsmeier *et al.*, 2001) to explore genes which are regulated by herbivory.

Use of transgenic plants

With the cloning of the genes involved in volatile biosynthesis it is becoming feasible to produce transgenic plants with overexpression or antisense/silencing to produce a new volatile blend. Alternatively, eventually transcription factors may be cloned that could be used to upregulate entire indirect defence pathways. Such plants would be the perfect tools to study the importance of individual compounds, blends, timing and magnitude of the plant's response for predator behaviour. In our laboratories, we have used a number of terpene synthases to transform model plant species. One of the first studies reported the transformation of petunia with the *Clarkia breweri* linalool synthase (Lücker *et al.*, 2001). Instead of the expected emission of linalool, transgenic plants were demonstrated to contain appreciable amounts of linalyl-ß-D-glucoside, a non-volatile storage form of linalool, likely formed through the action of an endogenous petunia glycosyltransferase (Lücker *et al.*, 2001). In potato, transformation with a strawberry linalool synthase also led to the formation of glycosides of linalool, and also high amounts of free linalool were emitted, particularly from young plants (A Aharoni, H J Bouwmeester, M A Jongsma *et al.*, unpublished) (Figure 5). The difference between the transgenic and wild type plants could easily be detected with the (untrained) human nose.

More straightforward results, i.e. with less side effects caused by endogenous enzymes, were obtained with other monoterpene synthases in tobacco. Tobacco was transformed with three lemon monoterpene synthases that were combined into one plant by crossing (Lücker *et al.*, 2002; Lücker *et al.*, 2003). Transgenic tobacco plants were obtained that produced up to seven new monoterpenes in leaves and flowers (three major products (-)-ß-pinene, (+)-limonene and γ-terpinene plus some side products that had also been detected upon heterologous expression in *E. coli*) (Lücker *et al.*, 2003). Finally, the recent successful transformation of arabidopsis with a strawberry linalool/nerolidol synthase (Aharoni *et al.*, 2003a) brings the molecular advantages of arabidopsis into research on the effects of altered volatile blends on multi-trophic interactions. Arabidopsis has shown to be a good model plant for the investigation of induced indirect defence: feeding by the crucifer specialist *Pieris rapae* as well as other herbivorous arthropods results in the emission of volatiles that attract the parasitoid *Cotesia rubecula* (Van Poecke *et al.*, 2001; Van Poecke *et al.*, 2003). Preliminary Y-tube olfactometer experiments with the transgenic potato plants showed that the attractiveness to predators is enhanced by the introduction (or increase) of a single component in the volatile blend (Figure 5).

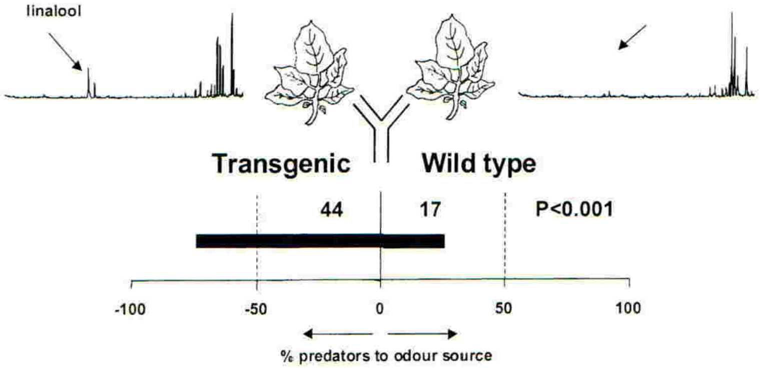

Figure 5. Attractiveness of non-damaged wild type and transgenic, linalool producing potato leaves to predatory mites. Bars represent choices of predatory mites reared on lima bean. Chromatograms show GC-MS analysis of the headspace of the odour sources.

CONCLUSIONS AND OPTIONS FOR NEW CONTROL METHODS

This review shows that our understanding of tritrophic interactions and the chemical signalling molecules and mechanisms involved is increasing rapidly. The use of this knowledge for the design of new control methods will depend on the agricultural system for which the method is intended. Biological control of herbivores in a glasshouse, for example, usually depends on the introduction of the predators, whereas in open systems natural enemies may be present or attracted from elsewhere (although also there it is feasible that introduction of natural enemies will become increasingly important). The introduction of enemies is of course costly and it is important that they survive for some time (which may mean a threshold level of their prey has to be accepted) and are able to find and control their prey quickly once it reaches a threshold level. The advantage of introduction is that the predators are reared under controlled conditions (constant quality) and can even be trained (Dicke *et al.*, 1990c). In open agricultural systems, the farmer in principle depends on the natural presence or invasion of predators, which also have to be kept alive, present and active when necessary. Now that many of the induced volatile blends have been identified, artificial mixtures could be used or alternatively, crops could be sprayed with jasmonic acid to induce volatile production that should lead to the increased presence of natural enemies (Thaler, 1999). However, there are examples where this approach has been unsuccessful (Chiri & Legner, 1983) and several authors have expressed the feeling that this approach will fail because the presence of the volatile cue and the prey are uncoupled (Dicke *et al.*, 1990a; Degenhardt *et al.*, 2003).

If the volatile cues and the presence of the prey are not uncoupled (*i.e.* attractive volatiles are only or mainly produced upon herbivory) then an adequate response of the crop to herbivory is most important. We have reviewed several studies in which the effect of environmental conditions on volatile production and herbivore attraction has been demonstrated and these results should be taken into account when designing future experiments. However, for a practical application, such as the optimisation of biological control, these factors may be important and it would be of interest to see whether environmental conditions that stimulate induced volatile formation actually improve biological control in a field situation. Another as yet completely ignored factor in the optimisation of biological control is the selection for genotypes with improved (faster, stronger) response. Our results on cucumber (Figure 4), and results with other plant species, demonstrate that genetic variation for this response is available. Further research should demonstrate the effectiveness and the best and easiest way to exploit this variation in breeding.

Finally, it is tempting to speculate about the opportunities that lie within molecular approaches. The knowledge about the molecular regulation of indirect defence volatile formation is rapidly increasing, a multitude of structural genes are already available, and we have shown ample evidence that expression of these structural genes in plants can lead to the production of the expected volatiles. Making this volatile production dependent on herbivore-feeding is only a small step away that will be facilitated by our hunt for herbivore-feeding induced gene expression and hence inducible promoters. There are several indications in the literature that even though the induced volatile blends are sometimes extremely complex mixtures, there are major contributors to the attractive effect of these blends, which increases the chances for success of a molecular approach. Such a molecular approach could include genetic modification or the use of genetic (gene) markers in the selection process. Finally, it is conceivable that changes in the (induced) volatile production in commercial crops could lead to the development of biological control packages in which biological control agents trained specifically for the modified crop are included. It will be exciting to see whether these approaches can lead to plants with altered (improved) predator behaviour and to crops with improved biological control.

ACKNOWLEDGEMENTS

We thank the Dutch Technology Foundation for their support (to IFK and LLPL) (STW project WPB.5479).

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Plant activation of barley by intercropped conspecifics and weeds: allelobiosis

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ABSTRACT

Experiments with components of a barley cropping system are summarised in a tritrophic context with reference to allelochemical communication between undamaged plants. The results are discussed with regards to different mechanistic explanations. With reference to the different roles of allelochemicals in plant/plant communication in a "winner-loser" perspective, it is suggested that the term "allelobiosis" is used to denote this type of interaction.

INTRODUCTION

The impression of peaceful coexistence in a eutrophic flowering meadow might be an anthropomorphic misinterpretation of the real situation. Even if attacks by herbivores are common, it is fair to assume that the most serious biotic challenge for an individual plant is sharing and competing for space and resources with other plants. Thus the meadow is a battlefield upon which all kinds of weaponry are used to gain ecological advantages, and similarities in resource patterns between plant individuals intensifies the struggle. Many aspects of this fight for survival and ecological success have been studied thoroughly and are described and discussed in most standard ecological textbooks.

Even though the existence of plant/plant competition is mentioned in ancient Greek literature (Plinius first century A.D.), an important step towards a mechanistic understanding was taken when it was shown that allelochemicals could assist in mediating plant dominance (allelopathy sensu Molisch 1937). Since then the literature on this topic has grown rapidly and there are several known examples of the potential competitive value for a plant individual of emitting allelopathically active semiochemicals. Effects of the emitted substances can be manifested as profound changes in the physiology and development of neighbouring plants (Rice 1984). Classic examples of chemo-dominance are the sage bush and the eucalyptus (Harborne 1993), and the literature on this and related topics is vast. Since investigations on chemical plant/plant communication are usually focussed on the rhizosphere and on non volatile soil borne substances (for a review see Rice 1984), it is not always obvious whether the substances are produced by the plants themselves – true exudates - or by associated micro-organisms in the rhizosphere.

The term "allelopathy" was originally formulated to denote plant/plant communication as a means by which one plant individual can suppress other plants. However, we hypothesize that it may, in the long run, be favourable for a "listening plant" individual to be able to detect the presence of its neighbours. For example, elegant studies have shown that when herbivores attack a plant, the wound responses in this individual can promote reduced herbivore acceptance of neighbouring plants (Bruin *et al.,* 1995; Bruin & Dicke, 2001; Farmer 2001) and promote searching behaviour of natural enemies of the herbivores (Dicke *et al.* 1999). To what extent similar effects can occur also between undamaged plants is still an open question. However, it is obvious that chemical plant/plant communication can favour both the emitting plant and the receiving plant.

There is some justification in speculating that allelobiotic plant interactions can affect susceptibility to herbivores. In many respects plant responses to different types of biotic stress are reasonably uniform (Lambers *et al.* 1998). For example, volatile messenger substances emitted by *Artemisia* have been shown to promote, via aerial allelopathy, induced resistance to herbivores (Farmer & Ryan 1990), supporting the thoughts on similarities between plant/plant and herbivore-induced stress.

The classical view of allelopathy as a term to denote plant-plant interactions via semiochemicals may be misleading. It implies that a plant individual emitting allelobiotically active substances benefits by doing so if it reduces competition with other individuals for available resources. In principle, this is a form of chemical warfare and the winner is the emitter of the active substances. Under certain conditions however, the winning concept may derive from perceiving stimuli from a neighbouring plant, if those stimuli induce favourable modifications in growth patterns, such as a reinforced water uptake capacity through a promoted root growth capacity as reported by Ninkovic (2003). Therefore we suggest using the term "allelobiosis" which is neutral with regards to the winner-loser perspective. This term would also be more appropriate to cover the situation in which volatiles from a herbivore-attacked plant promote defence in a neighbouring plant.

Superficially, the structure of agricultural monocultures invites competition for the same set of resources by plants with very similar resource need profiles in dense stands. However, human screening and breeding efforts have modified what may have been the original characteristics of the plants, and to what extent allelobiotically based mechanisms are still important in these cropping systems is unknown. Although mixed cropping is a traditional, and often successful, strategy to increase plant protection, data on the effects of allelobiosis on higher trophic levels are lacking (Vandermeer 1992).

Allelobiotic mechanisms operate on a time scale set by plant phenology, and herbivores closely adapted to this are likely to be more affected than others. Aphids are phloem feeders with a sophisticated capacity for host plant discrimination and feeding site evaluation (Dixon 1998; Pickett *et al.,* 1992). The importance of plant volatiles in the settling process, and of food quality in terms of free amino acids and secondary metabolites have been demonstrated (Pickett *et al.* 1992; Hardie *et al.* 1994; Pettersson *et al.* 1994; Pettersson *et al.* 1996). Thus aphids may be a group of herbivores likely to respond to allelobiotically-induced changes in host plant traits such as phloem transport and the pattern of emitted volatiles.

In this presentation we summarise the results of experiments in which a barley crop (*Hordeum vulgare*) is used as a model system for studies of tritrophic effects promoted by allelobiosis. We summarise the results from a series of experiments based on the common conditions in a barley crop in central Sweden. The overall objective is to contribute to a general understanding of how allelobiotic mechanisms can have effects spanning different tritrophic levels of the model system. Hypothetically these mechanisms may form a link between botanical and faunistic biodiversity, and contribute to the development of new approaches to plant protection.

MATERIAL AND METHODS

Four commercial barley cultivars (Frida, Alva, Hulda and Kara) were chosen from field experiments on intercropping to control powdery mildew (Wiik 1987). These genotypes are comparable, with regards to agronomic characters, but differ with respect to genes for resistance to powdery mildew. No differences with regards to aphid responses in olfactometer, preference or settling tests are apparent (Pettersson *et al.,* 1999; Ninkovic 2002).

Barley plants were exposed individually to air that had passed over another plant in a twin cage system with a one-way-airflow-system (Pettersson *et al*., 1999) (Figure 1). Each cage unit consisted of two chambers and air passed first through one of these and then through the second and out via a vacuum tank. The chamber units could also be rearranged to make exposures to root exudates. The treatments were randomly distributed and each twin unit was regarded as a replicate.

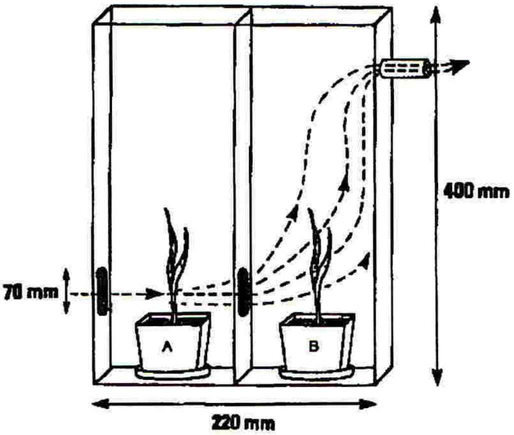

Figure 1. Twin cage system.

The herbivore chosen for the experiments was the bird cherry-oat aphid, *Rhopalosiphum padi* (L.), a key pest in spring-sown cereals. It is a host-alternating aphid that is monophagous on its winter host plant, *Prunus padus* L. but has a broad range of grasses as summer hosts (Wiktelius *et al*., 1990). A common predator of *R.. padi,* the seven spotted ladybird *Coccinella septempunctata* L. was chosen to represent the third trophic level.

Plant responses to treatments were evaluated through estimates of changes in biomass allocation (Ninkovic 2003) and changes in leaf temperature, measured with an infrared camera (Pettersson *et al*., 1999).

The following methods were used to evaluate aphid responses to plant treatment

1. preference in host plant choice experiments – initial laboratory experiments (Pettersson *et al.* 1999)

2. settling measured in a no choice test – in field and laboratory experiments (Ninkovic *et al.* 2002)
3. olfactometer responses (Ninkovic 2002; Glinwood *et al.* 2003;).

Ladybird responses were recorded from field observations and olfactometer experiments (Ninkovic *et al.*, 2001; Ninkovic & Pettersson 2003).

RESULTS AND DISCUSSION

Barley/barley allelobiosis

Changes in biomass allocation and leaf temperature. In specific cultivar combinations, plants exposed to volatiles from another cultivar allocated more biomass to roots compared with plants exposed to air from the same cultivar, or to clean air (Ninkovic 2003). There were no significant changes in relative growth rate (RGR, increase in biomass per unit biomass per unit time) and unit leaf ratio (ULR, increase in biomass per unit time and leaf area) but there was a significant increase in specific leaf area (SLA, leaf area per leaf dry weight). Thus allelobiotic interaction between two barley cultivars may not affect total biomass production, but rather alters biomass allocation in individual plants. This is in line with previous studies (Aerst *et al.*, 1991; Boot & Den Duddelden, 1990) showing that reduced biomass allocation to leaves can be compensated for by high SLA. The observed influence of one cultivar on another probably has implications for competition. More roots may be of advantage in dry situations, increasing stress tolerance. Obviously, such variation would increase the phenotypic stability of mixtures and thus be of competitive advantage.

Effects of allelobiosis on plant leaf temperature were measured by infrared imaging (Pettersson *et al.*, 1999). Comparisons were made between plants exposed to air passed over (i) a plant of a different genotype (ii) a plant of the same genotype (self-induced control) and (iii) no plant at all (clean air control). Significant decreases in temperature were recorded in response to air passed over a different genotype in eight of 12 cultivar combinations when compared to clean air controls. When compared to self induced controls, leaf temperature effects were found in four combinations. Significant self-inducing effects were found in two of four tested cultivars.

Effects of allelobiosis between barley genotypes on aphid olfactory responses, preferences and settling in laboratory bioassays

Preference test. Effects on aphid host plant preference were examined in a choice test with treated plants (exposure period 6 days) compared to one of two types of control treatment: (i) self inducing control, (ii) non self inducing control. Significant reduction of aphid preference was found in seven of the 16 possible combinations (Pettersson *et al.*, 1999), including the combination Alva-Kara (Figure 2). It is also interesting to note that plant individuals of two genotypes also showed a change with regards to aphid response when exposed to volatiles from the same cultivar. The results of the study indicate that this phenomenon is genotype specific, not only for responding cultivars but for the inducing cultivar.

Olfactometry Aphids did not show any intrinsic preference or aversion for any of the four tested cultivars, based on the inherent odour composition of unexposed plants. Two series of olfactometer tests were made with exposed plants, one in which the inducing plants were retained as part of the overall odour stimulus, and one in which they were removed. This was to test whether aphids responded to allelobiosis-induced changes in the exposed plant or merely to an increased complexity of volatiles in the mixture. The aphid visiting frequencies were significantly lower in arm zones with volatiles from cv Kara exposed to cvs. Alva or Frida when the inducing plants were retained (Figure 3). With the inducing plants removed, the response to the Frida-Kara combination disappeared (Figure 3). This indicates that exposure to volatiles from another cultivar may induce a systemic change in the volatile profile of a responding cultivar.

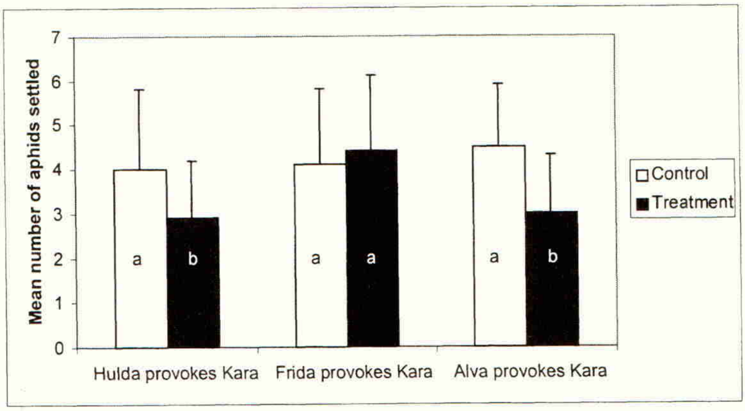

Figure 2. Changes of aphid settling in the preference test after exposure of one cultivar to another.

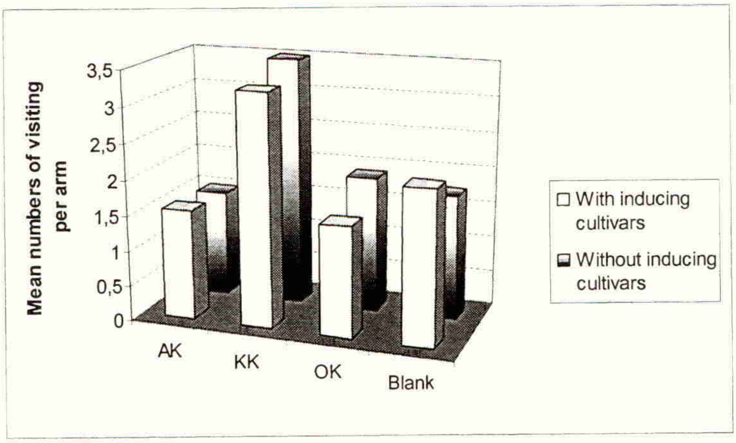

Figure 3. Olfactometer data for aphid responses. Change in aphid plant attraction of cv. Kara after exposure to cv. Alva. AK is a cultivar combination where cv. Kara was exposed to cv. Alva. KK is a cultivar combination where cv. Kara was exposed to the same cultivar (self-induced control). OK is a combination where Kara was exposed to no plant volatiles (clean air control).

Field experiments. All cultivars were sown in pair wise combinations in field plots under conditions identical to common agricultural methods. Careful weeding was done by hand. Aphid response to treated plants was estimated with a no-choice settling test when the plants were at the two leaf stage. Ten aphids were released in a clear plastic tube placed over the youngest leaf of the barley plants in the field plots. The number of aphids settling on the leaf was recorded after two hours and used as an estimate of settling. This estimate of the aphid response was used to make statistical comparisons. The same test of aphid settling responses was applied to a set of plant genotype combinations using the twin cage system for plant exposure.

When aphid responses in plots with only one cultivar were compared with plots with two cultivars, significant differences were found in three of the eight possible combinations. No difference was found between plots with one cultivar (Figure 4).

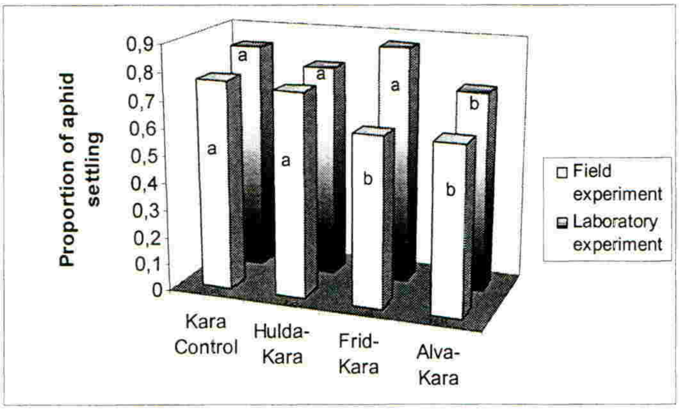

Figure 4. Settling data from laboratory and field experiments. Changes in aphid settling on cv. Kara when grown in mixture with another cultivar, in comparison with a pure stand (field experiment), and when Kara was exposed to another cultivar (laboratory experiment).

Weed-barley allelobiosis

Some common weeds were tested for their potential to cause, via allelobiosis, changes in aphid acceptance of barley. Couch grass (*Elytrigia repens*) is known to produce allelobiotically-active root exudates (Hagin, 1989; Hagin & Bobnick, 1991). When barley plants were exposed to either root leachates from living *E. repens* plants, or to solutions containing known *E. repens* root exudates, settling by *R. padi* was significantly reduced (Glinwood *et al.*, 2003) (Figure 5). Barley plants exposed to root exudates also became less attractive then unexposed plants in an olfactometer, suggesting that allelobiosis changed the volatile profile. Thistles in the genus *Cirsium* are also known to exert allelobiotic effects on other plants (Kazinczi *et al.*, 2001). Exposure of barley to root leachates from *Cirsium vulgare* or *C. arvense* did not affect aphid settling, however when plants were exposed to volatiles from either thistle species, settling by *R. padi* and *S. avenae* was significantly reduced (Glinwood *et al*, unpublished). *Cirsium*-exposed plants were also less attractive than unexposed plants to aphids in an olfactometer.

A further weed species, *Stellaria media*, exerted similar allelobiotic effects on aphid acceptance of barley via its root leachates but not via volatiles (Fig 5). However, the common aggressive stinging nettle, *Urtica dioica*, did not affect aphid responses to barley after exposure to either root leachates or volatiles (Figure 5). These results indicate that the expected effects of plant-plant allelobiosis on herbivores is likely to be dependent upon the specific combinations of plant species, and thus difficult to predict without experimental study.

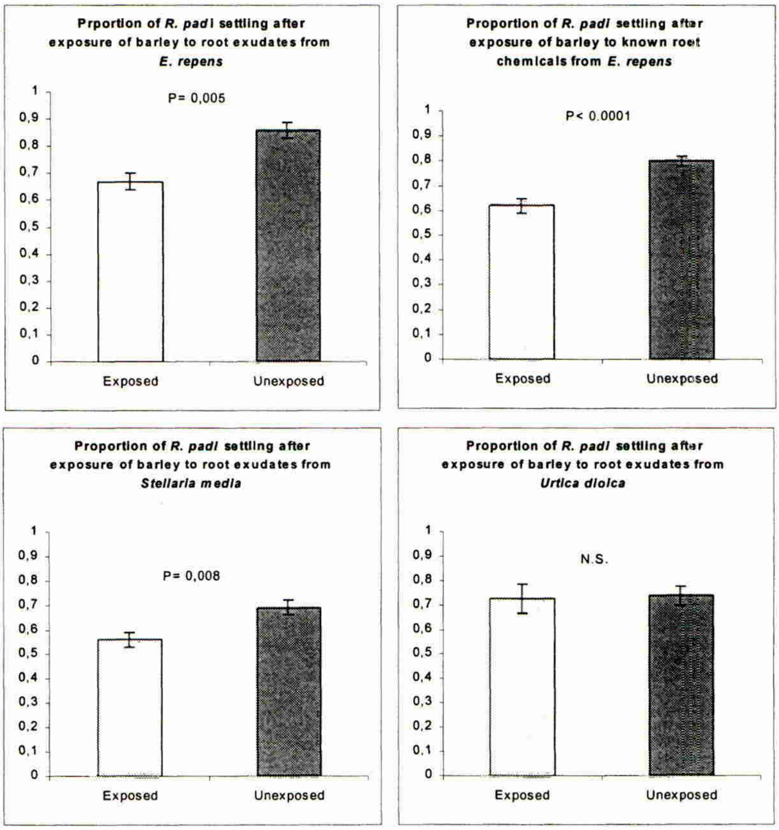

Figure 5. *R. padi* settling on barley exposed to allelobiosis from weeds.

Allelobiosis and natural enemies

Field observations. Observations in a barley field showed that *C. septempunctata* was significantly aggregated to patches with *Elytrigia repens* and *Cirsium vulgare* although no obvious food resource such as pollen or aphids or other small prey insects were abundant (Ninkovic & Pettersson 2003).

Olfactometry. In olfactometer experiments adult ladybirds showed no preference for either of the three plant species. However, a significant preference was shown for a mixture of barley odour together with each of the two weeds. The effect on barley exposed to volatiles from *E. repens* and *C. arvense* differed in that barley plants exposed to *C. arvense* remained attractive when the weed was taken away whereas those exposed to *E. repens* lost their attractiveness. This indicates that the positive effect of the barley/*E. repens* combination may merely be an effect of mixed volatiles, whereas the barley/*C. arvense* mixture is likely to represent a more complex mechanism involving allelobiosis.

Several experimental studies have shown how natural enemies respond to volatiles from plants attacked by herbivores. There is also convincing evidence showing that volatiles from attacked plants can induce responses in neighbouring plants that encourage searching behaviour of natural enemies. To what extent the volatile profile of the attacked plants and the affected neighbouring plants differ is still not known.

CONCLUSIONS

When two plant genotypes or species are mixed, there are at least three possible ways to explain how semiochemicals can interfere with host plant discrimination by herbivores. (i) Messenger substances from one plant affect another via allelobiosis, and cause changes that affect aphid plant acceptance. (ii) Behaviourally active components from one plant are enriched in, or on the surface of, an exposed plant and then released thus affecting herbivore responses. (iii) Volatiles from the different plants are mixed and this mixture offers new components or ratios of components. This would be in line with the concept of olfactory masking as suggested by Hardie *et al.* (1994) and Pettersson *et al.* (1994)

Further studies are necessary to clarify the mechanisms, but the results presented support the hypothesis that allelobiosis influences aphid host plant acceptance. The experiments with barley and known allelobiotic messenger substances from *E .repens* show a significant change in aphid host plant responses, although the substances are not themselves behaviourally active. The experiments with combinations of barley genotypes show that allelobiosis causes significant changes in growth patterns and leaf temperatures (Ninkovic 2003a; Pettersson *et al* 1999), and that the effects are limited to specific genotype combinations. Although no inherent differences in the preference of aphids to unexposed cultivars were found, differences were found after the cultivars were exposed to volatile allelobiosis.

Ladybird responses to volatiles from plant combinations appear to be complex. Olfactometer responses of *C. septempunctata* to volatiles from barley exposed to *E. repens* and *C. arvense* indicated attraction. However, when the weed plants were removed from the odour stimulus, barley plants exposed to *C. arvense* remained attractive while those exposed to *E. repens* plants lost their attractiveness. This indicates that the positive effect of the barley/*E. repens* combination is merely an effect of mixed volatiles (cf. iii above) while with the barley/*C. arvense* mixture may constitute a more complex mechanism in line with type *i)* or *ii)* as described above.

From an applied point of view, the findings contribute to two plant protection approaches. The first is an increased understanding of the mechanisms potentially active in different systems for intercropping and mixed cropping. Obviously, the positive effects of these cropping strategies are a combination of several mechanisms, of which allelobiosis has been less recognised until now. Increased knowledge should contribute to the search for more effective combinations of plants. The second contribution will be to increase our understanding of the extent to which active substances can be used to manipulate induced resistance in plants. Further knowledge might also favour breeding for cultivars that combine high yield with a capacity to allocate resources depending on prevailing conditions.

The results presented deal with experiments in which two plant species or genotypes have been combined. The extent to which the effects shown operate in natural plant communities is still an open question. If they do, it would be a contribution to our understanding of how plant community composition contributes to entomological diversity via allelobiosis.

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Plant-fungal interactions mediated by volatile signals

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ABSTRACT

Previous exposure of plants to attack by pests or pathogens can alter the response of such plants to subsequent challenge. The molecular basis of this induced resistance is now well documented and the signal networks are being dissected through the analysis of mutants in plant genetic models such as *Arabidopsis*. Circumstantial evidence is accumulating that plants neighbouring those subject to attack or infection may also be altered in response, implicating the involvement of volatile signals. Experimental work to test the hypothesis that volatile emissions from plants infected by pathogens can induce resistance in adjacent plants, using barley seedlings and the necrotrophic fungi *Rhynchosporium* and *Pyrenophora,* is described. Practical exploitation of this phenomenon in crop protection, for instance through chemical elicitors or the use of responsive crop genotypes in variety mixtures, will depend upon identification of the key signals involved and a better understanding of the molecular and physiological basis of the induced resistance.

INTRODUCTION

Higher plants have evolved a range of surveillance and defence systems that enable them to recognise and respond to pests and potentially pathogenic organisms. Recent molecular analyses have shown that defence genes may be expressed locally in the proximity of pathogen challenge as well as in tissues remote from the initial challenge. The involvement of a complex of signalling networks is implicated by genetic and molecular analyses (Dangl & Jones, 2001; Asai *et al.*, 2002). Identification of the key signals involved in the induction and regulation of defence networks is a major objective of current research to elucidate the mechanisms by which plants resist potential pathogens.

Plants exposed to stress, including attack by herbivorous insect or mites, emit a range of volatile chemicals (Koch *et al.*,1999). Some of these volatiles are known to act as signals that either repel the herbivore or attract natural predators of the pest. Such stress signals can also switch on so-called defence genes, the products of which have been implicated in plant resistance to both pests and pathogens (Pickett & Poppy, 2001). There is also the possibility that volatile stress signals might induce similar responses in neighbouring plants not yet exposed to attack (Farmer, 2001). To date, most of the experimental evidence concerns the effects of plant volatiles on insect pests. Much less is known about potential effects on plant pathogenic microorganisms.

Two main induced defence pathways have been identified in plants, in which either salicylic acid (systemic acquired resistance: SAR) or jasmonic acid and ethylene (induced systemic resistance: ISR) play key roles. These compounds are anticipated to serve as endogenous signals, although ethylene has long been recognised as a volatile hormone with potential effects on plant disease. Recent work has confirmed this, for instance in tomato exposed to an ethylene pre-treatment the expression of several pathogenesis-related (PR) protein genes was induced, and resistance to the necrotrophic pathogen *Botrytis cinerea* was increased (Diaz *et al.*, 2002). Volatile analogues of other signal molecules have also been implicated in the induction of plant defence. In tobacco plants forming local lesions in response to infection by tobacco mosaic virus, the emission of gaseous methyl salicylate was suggested to be at levels sufficient to induce PR proteins and to increase resistance in nearby healthy plants (Shulaev *et al.*, 1997). Methyl salicylate has also been detected in volatile samples from peanut plants infected with the white mould fungus *Sclerotium rolfisii* (Cardoza *et al.*, 2002). In this case direct effects of volatile compounds on the growth of the pathogen were reported.

Circumstantial evidence that interactions between infected and healthy plants mediated by external signals might influence resistance to pathogens comes from studies on crop variety mixtures in which the levels of disease observed in the mixture are often lower than would be expected from the resistance or susceptibility of the individual component genotypes. One mechanism might be the induction of resistance in genetically susceptible genotypes by volatile signals emitted from nearby infected plants. Testing this hypothesis under field conditions is problematical in the absence of information on the identity of the likely signal molecules and their effects on host-pathogen interactions at defined physiologically-active concentrations.

A MODEL SYSTEM

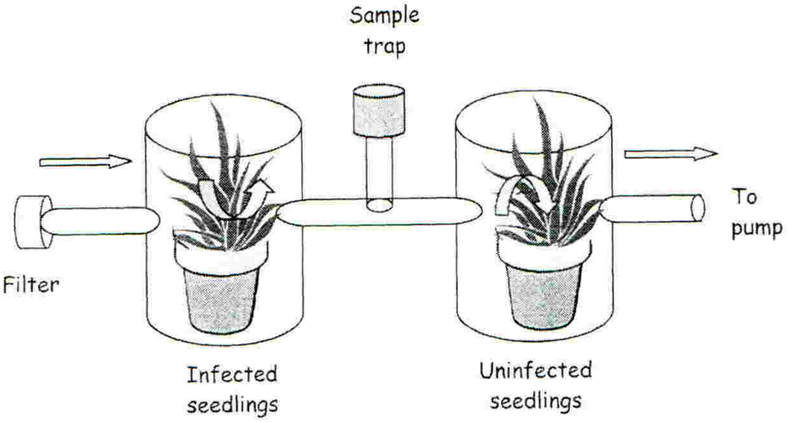

Figure 1. Two compartment system for testing emission and effect of volatile compounds.

We established a model system using commercial cultivars of barley (*Hordeum vulgare*) and two foliar fungal pathogens, leaf scald (*Rhynchosporium secalis*) and net blotch (*Pyrenophora teres*). Air from chambers containing susceptible barley seedlings inoculated with the pathogen was transferred to uninfected seedlings in a second chamber (Figure 1). The barley seedlings exposed in this way were then inoculated with spores of the same pathogen and disease severity on individual leaves scored after 7 and 14 days. Control plants were exposed to air from uninfected seedlings. Figure 2 shows disease severity data for leaf scald 14 days after inoculation. Disease severity on leaf 3 (the youngest leaf at the time of exposure) was reduced by around 50% compared with plants exposed to the air from healthy control seedlings. Furthermore, in exposed plants the onset of leaf scald symptom expression was also delayed by 48 hours. A similar reduction in disease severity was observed in barley plants inoculated with *P. teres* and scored at 7 days. These results suggest that volatile compounds emitted by infected barley plants can trigger changes in uninfected plants that induce or enhance resistance to subsequent pathogen attack.

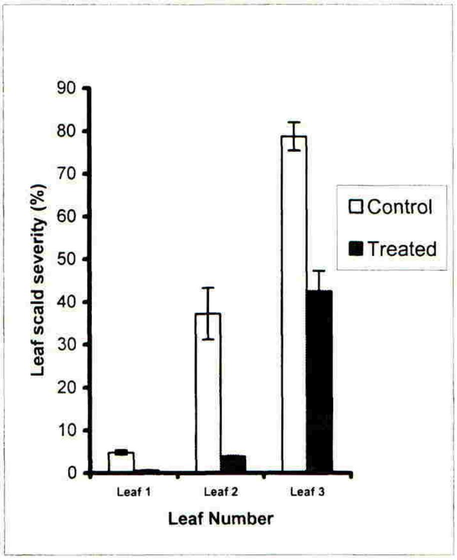

Figure 2. *Rhynchosporium* disease severity in control and treated barley seedlings 14 days after inoculation.

The volatile profiles of headspace samples from infected plants were analysed by high-resolution gas chromatogaphy-coupled mass spectroscopy (GC/MS) (Birkett *et al.*, 2000). Known signal molecules such as methyl jasmonate and methyl salicylate were at the limits of detection.

DISCUSSION

Communication between plants mediated by volatile signals is known to affect resistance to herbivorous insects, but effects on pathogens including fungi are less well documented. Volatiles released following wounding have been shown to induce changes in tobacco plants that reduce damage by insects under field conditions (Karban *et al.*, 2000). Petterson *et al.* (1999) demonstrated that volatiles from barley seedlings may affect aphid acceptance of neighbouring exposed plants. In the present experiments changes in leaf reaction type to two necrotrophic barley pathogens were observed after exposure to atmospheres from compartments containing diseased plants. The identity and activity of the potential signal(s) involved is not yet known. Further work is also required to establish whether plant to plant communication might be one mechanism underlying the enhanced resistance of mixtures of crop genotypes to pathogens, including barley leaf scald (Newton *et al.* 1997).

ACKNOWLEDGEMENTS

I thank Olu Latunde-Dada for constructive input to this paper. Rothamsted Research receives grant-aided support from the Biotechnology and Biological Science Research Council of the UK.

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Registration opportunities for natural products versus synthetic plant stress signals (or plant activators) for crop protection

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ABSTRACT

Policies to reduce the use of plant protection products are being developed in many Member States of the EU and elsewhere in the world. To meet such objectives, viable low risk alternative products need to be developed and authorised. Until now the regulatory procedures based on synthetic chemical active substances have been regarded as a barrier to the commercialisation of alternative products. The EU is now taking steps to encourage the development of natural plant extracts by proposing reduced data requirements.

INTRODUCTION

To gain any insight into the often difficult and constantly changing European regulatory requirements, an understanding of both the procedures and the wider political issues are necessary. Over the last 10 years, since the implementation of Council Directive 91/414/EEC (Council Directive, 1991), policies have been developed at both EU and Member State level in response to perceived and real risks both to the environment and to humans through the food chain. Initiatives such as the 6th Environment Action Programme (Commission Decision, 2002), the Precautionary Principle (Commission Communication, 2000) and the setting up of the European Food Safety Authority (Commission Regulation, 2002a) have wide reaching implications for the use and regulation of all plant protection products. Unfortunately they may not always keep pace with the rapidly developing sectors of science and technology and the development of plant protection by plant extracts seems a far cry from the situation found over a decade ago which prompted the need for EU-wide legislation.

Data requirements necessarily reflect the political climate of the day and much has evolved since the establishment of the regulatory framework for plant protection products. The European Commission has indicated that the Directive will have to be amended to reflect the current policies. The main issues were included in a report submitted to the European Parliament in 2001 (SANCO, 2001a). These were expanded and discussed with stakeholders at a meeting held in Corfu in 2002 (SANCO, 2002). Not all stakeholders attending this meeting supported the continued use of plant protection products on the current scale. For example, PAN Europe presented a position paper on behalf of a number of NGOs on Pesticide Use Reduction in Europe (PURE) (PAN, 2002). This paper outlined the position over the need, as they saw it, for mandatory Community-wide targets and a clear timetable for achieving the reduction in use of plant protection products throughout Europe.

Regulatory science and risk assessment practices also are evolving continuously. Data requirements together with risk assessment guidelines have been developed globally and have added hugely to the burden of the notifier. When drafting legislation there has always been a tendency to attempt to cover all regulatory eventualities. So the lists of data requirements have increased alarmingly and are based almost exclusively on synthetic chemical active substances and extreme worst-case scenarios. Of course the key to developing a data package to support any application is to navigate the most appropriate path through this list and provide the most relevant data. However, this assumes that Regulatory Authorities have the necessary experience and expertise to evaluate dossiers based on scientific argument rather than compliance with a list of requirements. No matter what size of enterprise involved, regulatory requirements now present a major financial hurdle to every applicant. Clearly SMEs will find this burden disproportionately large compared to the turnover expected from any product marketed.

Can there really be hope for SMEs involved with the development of plant extracts and niche products? This paper intends to present the current regulatory position and also to explore the wider issues in order to put the requirements into some context and offer some hope. It assumes some knowledge of Directive 91/414/EEC and the control of plant protection products.

PLANT EXTRACTS AND THE SCOPE OF 91/414/EEC

Plant extracts intended for use as plant protection products are covered by the legislation. In the Commission report to Parliament (SANCO, 2001a), plant extracts were identified as needing to be in the list of substances to be included in the 4th stage of the EU Review Programme. This recognised that plant extracts were already on the market and needed to be included in the scope of the legislation. The Commission stated that a host of new niche companies developing environmentally friendly alternative methods of plant protection were emerging. These were almost always SMEs adopting innovatory approaches to existing problems. Nurturing their continued development by not imposing impossible regulatory hurdles in their path, whilst guaranteeing health and environmental safety and effective crop protection, would be a big challenge for the future.

Subsequently, the Commission Regulation (Commission Regulation, 2002b), laying down the rules for the 4th stage of the EU Review Programme, included plant extracts in the list of substances under review. It stated that detailed provisions concerning the submission of dossiers and deadlines will be established by a further Regulation in due course. In the meantime a list of all of the existing active substances that were notified by companies willing to support their continued use has been published (SANCO, 2003). Although the list now includes many plant extracts such as essential oils, plant oils, seaweed and seaweed extract, it is far from exhaustive. Any active substance not on this list and which is being actively developed will need to be registered as a new active substance. One of the biggest challenges will be the recognition that the use of such technology differs fundamentally from the use of chemical plant protection products (A Hamer, personal communication). The main objective of most plant protection products is to eradicate a pest, disease or weed. The novel approach being developed is completely different with the aim, for example, to improve defence systems. This will require a completely different approach to both risk assessment and efficacy evaluation.

The attraction of plant extracts appears to be associated with the likely low adverse impact on crops or stored products themselves or on human health. They also appear to be biodegradable so the likely environmental impact is low (Velcheva, *et al.*, 2001). For some uses the results seem very promising and range from direct effects on pests and diseases (Boeke, *et al.*, 2001; Parimelazhagan, 2001; Thacker, *et al.*, 2002) to plant activators (J Pickett, personal communication).

The realisation that regulatory requirements need to be scaled down for certain groups of plant protection products with reduced risks has been picked up by two Member States. The Dutch have produced guidelines for plant strengtheners with low risk profiles (SANCO, 2001b) and the French Authorities have developed a draft working document on plant extracts (A Hamer, personal communication). Before the French proposals are discussed it might be helpful to explore the wider initiatives that have prompted the need to develop such guidance.

SIXTH ENVIRONMENT ACTION PROGRAMME

The 6th Environment Action programme (Commission Decision, 2002), whilst recognising Directive 91/414/EEC, goes further by proposing a two-track approach to minimise the risks due to the use and misuse of plant protection products. This initiative is interesting as it identifies the thinking in the Commission outside the group directly involved with Directive 91/414/EEC. It is clear that plant protection products are still regarded with suspicion and despite the huge data package developed to support safe use, the risks are still perceived to be high. The first track of the proposal is to ban or severely limit the placing on the market and use of the most *hazardous* and *risky* plant protection products. The words in italics are the actual words used in the proposal. It does not indicate how this would be done or whether it would be based on a risk assessment or on cut-off values related to the hazard classification of the active substance. The second track is to ensure that best practice is adopted regarding the use of those plant protection products that are authorised.

To assist with the general aims of the Action Programme, a Thematic Strategy on Sustainable Use of Pesticides has been developed (Commission Communication, 2002) which aims to provide a general overview of the risk reduction efforts and policies. The main objectives include the need

- to minimise the hazards and risks to health and the environment from the use and distribution of plant protection products;
- to reduce the levels of harmful active substances, in particular by replacing the most dangerous by safer (including non-chemical) alternatives;
- to encourage the use of low-input or pesticide-free crop farming particularly by raising the user's awareness, promoting the use of codes of good practices and consideration of the possible application of financial instruments.

The first of these aims has been taken up already by a number of Member States where national plant protection product reduction programmes have been introduced. In future a policy will be developed to cover a reduction plan and efforts to increase IPM, pest forecasting and biological control methods.

US EPA REQUIREMENTS

In the US the Environmental Protection Agency (EPA) has also recognised that "biopesticides", covering microbial products and biochemicals which include plant extracts, differ markedly from traditional synthetic chemical plant protection products. The EPA take the line that such products occur in nature and affect pests by means other than toxicity. Therefore, the extent of the data required to support a typical biopesticide is very much reduced compared to the requirements for synthetic chemicals. The key data requirements (Table 1) are very similar to those proposed by the European Union with a few notable exceptions. This list represents the requirements for active substances produced synthetically but based on natural products. Plant extracts would not be expected to trigger the need for higher tier testing regimes.

Table 1. Data requirements for "biochemicals"* – US EPA

1	Product Identity and Composition		
2	Analysis and Certified Limits		
3	Physical and Chemical Characteristics		
4	Toxicology data requirements	Tier I	Acute toxicity, skin sensitisation, hypersensitivity incidents, genotoxicity, immunotoxicity, 90-day studies, teratogenicity (1 sp)
		Tier II	Mammalian mutagenicity studies, immune response
		Tier III	Chronic exposure, carcinogenicity
6	Chemical Identity		
7	Directions for use		
8	Nature and Magnitude of Residues		
9	Impact on non-target organisms	Tier I	Acute toxicity on bird, fish, aquatic invertebrate, non-target plant, non-target insect testing
		Tier II	Environmental Fate
		Tier III	Terrestrial Wildlife (determined on a case-by-case basis)

* Details on US EPA website: www.epa.gov/pesticides/biopesticides/regtools/guidelines/biochem_gdlns.htm

OECD ACTIVITY

The Organisation for Economic Co-operation and Development (OECD), under the Pesticide Programme, has been working on the harmonisation of their plant protection product review procedures, sharing the evaluation of plant protection products and proposing policies for the reduction of risks associated with plant protection product use. The OECD together with the FAO held workshops on Integrated Pest Management and Pesticide Risk Reduction in Neuchâtel, Switzerland in 1998 (OECD, 1999). Rather than be restricted to a single definition of Integrated Pest Management (IPM), the workshop developed an approach which was to combine a variety of methods to control pests rather than relying on chemical plant protection products alone. Alternative methods included cultural strategies and biological methods. They discussed the role of IPM in plant protection product risk reduction. This theme was developed through reducing plant protection product usage and promoting reduced risk products. The group recognised that the take-up of IPM and plant protection product risk reduction would be slow because of the lack of viable alternative control methods and, in general, regulatory procedures not being flexible enough to deal with biological or reduced risk products. The rigid regulatory procedures developed for synthetic chemical products were seen as a barrier to the development of alternative strategies and achieving the aim of reduced plant protection product usage.

The OECD has maintained its active role in this area, developing a guidance document for the preparation of a dossier to support pheromones and other semiochemicals (OECD, 2002). They recognise that semiochemicals act by modifying the behaviour of pest species rather than killing them and can be target specific. They are used at very low rates, are non-toxic and dissipate rapidly. Therefore, they can be regarded as low risk and the data requirements are not as onerous as for synthetic chemical substances.

EU REQUIREMENTS

For the EU, the French Authorities have prepared a draft working document on the preparation of dossiers for plant protection products made from plants or plant extracts and have distributed it to Member States and Trade Associations such as the International Biocontrol Manufacturers Association (IBMA) for comment. The working document is intended to cover plant protection products made from plants or plant extracts, pheromones and [other] semiochemicals and commodity chemical substances. Although commodity chemicals have been included in the title, the French intend to cover this group in another document, which is in preparation. Also the French intend to use the guidance already in existence and developed by the OECD for pheromones and other semiochemicals (OECD, 2002).

The document now under discussion develops ideas on the data needed to support products made from plants or plant extracts. It is understood that all plant protection products must be authorised and must be supported by sufficient data to satisfy Articles 4 and 5 of Directive 91/414/EEC. These state that authorisation cannot be granted unless it can be demonstrated that there is no risk to human and animal health or the environment. The proposal is for the minimum data requirements to be applied to extracts obtained from plants included in a reference list (Table 2). This list contains plants authorised as herbal drugs in the European pharmacopoeia which are known to possess plant protection properties and would be expected to present a reduced risk. It is also the intention to up-date this list regularly.

Table 2. Proposed list of authorised plants

Common name of plant	Species	Part of plant used
Sweet chamomile	*Anthemis nobiilis*	Whole plant
Bladder wrack	*Fucus vesiculosus*	Thallus
Feverfew	*Chrysanthemum parthenium*	Whole plant
Lavender	*Lavandula officinalis*	Whole plant
Nettle	*Urtica spp,*	Whole plant
Rhubarb	*Rheum officinale*	Rhizome

The French have proposed that plant protection products made from plants or plant extracts should be defined as “mixtures or solutions comprising two or more substances intended for use as a plant protection product”. They define plants as being “live or dried plants and live or dried parts of plants including seeds and fruit” and a plant extract is “obtained by concentrating, through evaporation, distillation or some other process, a solution achieved by treating plants or parts of them with a liquid such as water and ethanol”.

It is proposed that there should be a tiered approach to the generation and evaluation of data. The data requirements proposed have been tabulated in a very brief format in Table 3. Two examples are given

- Case 1 - Plant protection products made from plants included in the reference list and mixed with water, possibly with formulants added, and used directly on the crop
- Case 2 - Water/ethanol extracts of plants included in the reference list and possibly with formulants added. In this example it is assumed that an extract would be prepared but not used directly as a plant protection product. Data would be expected to be generated for the initial plant extract in addition to the plant protection product

Table 3. Proposed data requirements for plant extracts

	Data		Case 1	Case 2	
				Extract	Product
	Plant nomenclature				
		description	✓	✓	
		origin	✓	✓	
1	Identity of water ethanol extract				
		Particulars of extraction		✓	
		Specification		✓	
	Identity of plant protection product				
		Particulars of preparation	✓		✓
		Specification	✓		✓
		Full list of ingredients	✓		✓
2	Physical and chemical properties		✓	✓	✓
3	Data on application		✓		✓
4	Further information on plant protection product		✓		✓
5	Analytical methods		✓	✓	✓
6	Efficacy data		✓		✓
7	Toxicological studies				
		Acute oral, dermal and inhalation		✓	✓
		Skin and eye irritation	✓	✓	✓
		Skin sensitivity	✓	✓	✓
		Other toxicity data from literature		✓	✓
		SDS on formulants if added	✓		✓
		Risk assessment for operator and workers	✓		✓
8	Ecotoxicological studies				
		From literature	✓	✓	✓
		SDS on formulants if added	✓		✓
9	Classification and labelling		✓	✓	✓

It is expected that the package could rely heavily on data in the published literature. The proposals have enough detail to determine that a flexible approach will be taken. For example, analytical methods should be developed as far as possible. If the active components of the extract are unknown, a representative marker chemical should be used. Also since natural products often do not act directly on harmful organisms, the initial efficacy package need not reach the same level as for chemical products. So the scale of all of the requirements is vastly reduced from those expected for active substances based on synthetic chemicals provided that the extract is from a plant that is included in the reference list.

The document also states that for plants or plant extracts not included in the reference list, the requirements of Annexes II and III of the Directive 91/414/EEC must be fulfilled. Similarly synthetic active substances based on plant extracts will need to satisfy the relevant Annexes. For both of these examples a flexible approach to the selection of studies will be acceptable to most authorities but the extent of the data package will depend on the perceived degree of risk.

CONCLUSION

Policies developed from within the EU and from groups elsewhere in the world have stated the need to reduce traditional plant protection product usage. One of the options available to achieve this aim is to use low risk alternatives. Most authorities now accept that such low risk active substances must be treated in a different manner compared with synthetic substances. The political climate is changing and alternative strategies are being sought. Hope and encouragement lies in the French proposals to reduce the list of requirements needed to support natural products. However, there has been very little experience gained with assessing the risk of these products. A few examples are needed to act as pilot compounds to develop the system and give confidence to the proposed procedures.

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