SESSION 8A

SYSTEMIC ACQUIRED RESISTANCE FOR CROP PROTECTION

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Session Organiser	Mr A J Leadbeater *Ciba Agriculture, Whittlesford*
Papers	8A-1 to 8A-5

SAR - THE POTENTIAL TO IMMUNISE PLANTS AGAINST INFECTION

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ABSTRACT

Control of fungal, bacterial and viral diseases through stimulation of either systemic or localised acquired resistance (SAR and LAR) in plants offers new potential for disease control. SAR was first discovered through inoculation of plants with plant pathogens but can now be activated through spray application of natural and synthetic chemicals that do not in themselves possess antimicrobial activity. SAR and LAR-inducing chemicals can be applied alone or combined with low dose applications of fungicides. These chemicals should be compatible with biocontrol agents in an integrated disease control strategy.

INTRODUCTION

Systemic acquired resistance (SAR) was first described by Ross (1961b) using tobacco mosaic virus to induce systemic resistance of tobacco not only to TMV but also to other viruses. Ross (1961a) used the term localized acquired resistance (LAR) to describe the resistance induced in inoculated leaves. SAR has since been described in many plant/pathogen interactions of economic importance including tobacco, cucurbits, potato, soybean, tomato, millet, alfalfa, rice, wheat, barley (Kuć, 1982, Kessmann *et al.*, 1994) as well as in Arabidopsis (Uknes *et al.*, 1992).

SAR was initially considered to be an induced response requiring necrosis at the site of initial infection (Ross, 1961b) but this was later shown to be unnecessary to induce a systemic response (Roberts, 1982). SAR is distinct not only from pre-existing resistance mechanisms but does not involve other inducible mechanisms such as phytoalexin biosynthesis, the hypersensitive response, and ethylene induced physiological changes. SAR is not induced by responses such as wounding or osmotic stress (Ryals *et al.*, 1994) but can be induced by chemicals (Kessmann *et al.*, 1994).

Mechanisms of induced resistance

The mechanisms associated with SAR have been correlated with systemic activation of several plant responses (Kessmann *et al.*, 1994, Ryals *et al.*, 1994, Kombrink *et al.*, 1996). For example, in tobacco, SAR correlates with a co-ordinated activation of at least nine families of genes (Ryals *et al.*, 1994). Proteins encoded by these genes include 1,3,β-glucanases, chitinases, chitin binding proteins, thaumatin-like proteins, and other pathogenesis-related (PR) proteins that have antifungal activity. The same set of genes are activated by chemical inducers such as salicylic acid regarded as a putative endogenous signal (Malamy & Klessig, 1992).

SAR is usually associated with an increase in PR proteins (particularly PR-1a), and transgenic tobacco plants expressing high levels of PR-1a show increased resistance to plant pathogens (Alexander *et al.*, 1993). However, using *Pseudomonas fluorescens* to induce resistance in radish, Hoffland *et al.* (1995) suggested that accumulation of PR proteins is not a prerequisite for

the expression of SAR, and induction of resistance to *Cercospora beticola* in sugar beet by INA was reported to be independent of chitinase and β-1,3-glucanase (Nielsen *et al.*, 1994).

Studies on the signal transduction pathway involving induced resistance are being carried out in several laboratories. These include studies on a salicylic acid receptor (Chen & Klessig, 1991, Chen *et al.*, 1993), protein phosphorylation (MacKintosh *et al.*, 1994, Ye *et al.*, 1995), and the role of SA (Ryals *et al.*, 1995). For example a protein receptor for salicylic acid has been reported in tobacco with sequence similarity to known catalases (Chen & Klessig, 1991, Chen *et al.*, 1993), and *nim1* (non-inducible immunity) mutants of Arabidopsis do not exhibit SAR suggesting that the *nim-1* gene lies downstream of salicylic acid accumulation (Delaney *et al.*, 1995).

RESISTANCE ELICITORS

A small number of compounds have been tested for their ability to induce resistance to diseases in laboratory, glasshouse and field experiments, the results demonstrating the potential of the techniques as well as the current problems. Extensive screening for new compounds is expected to improve on the level of disease control attainable.

Salicylic acid

The effects of salicylic acid (SA) and its derivative acetyl salicylic acid on plant defence responses have been reviewed by Malamy & Klessig (1992). Application of SA to plants induces systemic resistance against a range of viral, fungal and bacterial pathogens in both dicotyledonous and monocotyledonous plants including tobacco, bean, tomato, potato, cowpea, rice, cucumber, asparagus, and barley (Dempsey & Klessig, 1994, Walters *et al.*, 1993). SA is thought to act as an endogenous signal in induction of PR proteins and some components of SAR (Raskin, 1992) and can induce polygalacturonase-inhibiting protein (Bergmann *et al.*, 1994). Using a number of chemicals to induce SAR, SA and SA glucoside in tobacco, Malamy *et al.*, (1996) suggested that multiple pathways, one of which is independent of SA, lead to defence responses.

Jasmonic acid

Methyl jasmonate and jasmonic acid cause an increase in the expression of defence response genes in cell suspension cultures (Gundlach *et al.*, 1992) and in intact plants (Farmer & Ryan, 1990) as well as other changes in plant metabolism not related to disease resistance (Sembdner & Parthier, 1993).

Application of jasmonic acid or its methyl ester to potato and tomato plants in the glasshouse induced local protection against *Phytophthora infestans* at 62.5 µg/ml, and systemic protection at 1 mg/ml (Cohen *et al.*, 1993). At high concentrations (>1 mg/ml) the jasmonates are phytotoxic causing slight chlorosis in potato and tomato plants 2-3 days after spray application. When jasmonates at 100 and 1000 µg/ml were applied to the adaxial surface of potato leaves two hours before inoculation with *P. infestans*, they gave 92 and 100% control respectively. There was no accumulation of sesquiterpene phytoalexins or increased chitinase or β-1,3 glucanases in treated leaves of potato or tomato and the nature of the induced resistance is therefore not known (Cohen *et al.*, 1993).

Jasmonic acid applied as a topical spray application (1 mg/ml) to barley plants in a growth chamber gave protection from mildew (*Erysiphe graminis* f. sp. *hordei*) (Schweizer *et al.*, 1993). However, the level of protection was short-lived and declined from over 80% protection when plants were inoculated immediately after treatment to just over 20% when inoculated 3 days after treatment. At 1 mg/ml jasmonic acid caused weak chlorosis but at 10 mg/ml caused necrosis of leaf tips. Application of methyl jasmonate vapour for 30 minutes to first leaves of barley plants caused a 99% reduction in mildew on second leaves (Mitchell & Walters, 1995). In a field study on barley cv. Golden Promise, two sprays of methyl jasmonate (412 g/ha) controlled powdery mildew similar to that achieved by fungicides and increased yield although plant height was reduced.

Jasmonic acid induces the formation of a number of new proteins collectively referred to as jasmonate induced proteins (JIPs) which include the antifungal thionins (Andresen *et al.*, 1992), proteinase inhibitors (Farmer & Ryan, 1990), and a ribosome-inactivating protein (Reinbothe *et al.*, 1994).

Fatty acids

The fatty acids arachidonic and eicosapentaenoic acids were first shown to be elicitors of phytoalexins in potato tubers after being extracted from mycelium of *P. infestans* (Bostock *et al.*, 1981). Several fatty acids induce systemic resistance of potato plants to infection by *P. infestans*, including arachidonic, eicosapentaenoic, linoleic, linolenic and oleic acids (Cohen *et al.*, 1991). Arachidonic and eicosapentaenoic acids applied without any adjuvants to leaves 1-3 of potato plants cv. Bintje at a dose of 1 mg/ml, induced 94 and 97% protection in leaves 4 to 11 respectively, whilst linoleic, linolenic and oleic acid gave 82, 39 and 42% protection as assessed by a reduction in lesion number and diameter. Systemic protection was maximal in plants challenged 5 days after induction and lasted for at least 12 days. Arachidonic and eicosapentaenoic acids at 1 mg/ml induced necrotic spots and chlorosis of surrounding tissue and finally abscission of the treated leaves (leaves 1-3). Linolenic acid caused necrotic spots whilst linoleic and oleic acids caused no visible symptoms.

Arachidonic and eicosapentaenoic acids have a limited host range in their ability to act as elicitors and do not, for example, induce phytoalexins in broad bean, French bean, pea, soybean, tobacco, tomato, carrot and parsnip (Bloch *et al.*, 1984). However, arachidonic acid did provide some control of powdery mildew on barley and did not cause any symptoms of phytotoxicity (Reglinski *et al.*, 1994b).

Yeast-derived elicitors

Reglinski *et al.*, (1990) showed that elicitor-active yeast cell wall extracts induced localised resistance in proportion to their ability to elicit phytoalexin accumulation in soybean cotyledons giving up to 90% reduction in mildew infection on barley (Reglinski *et al.*, 1994a). In field trials, elicitor treatment reduced mildew infection, although less effectively than in laboratory tests, and enhanced yield in several spring and winter barley cultivars (Reglinski *et al.*, 1994b). The levels of disease control and yield achieved using elicitors rarely approached those obtained using full rate fungicide. Mixtures containing reduced rate fungicide with yeast-derived elicitor controlled mildew and enhanced yield more effectively than either of the component parts alone and sometimes achieved levels approaching those obtained with full rate fungicide. Unlike some of the other resistance elicitors no phytotoxic or otherwise detrimental effects were observed

following application of any of the formulations containing yeast-derived resistance elicitors to plants.

Yeast-derived elicitors reduced the severity of infections caused by *Botrytis cinerea* and *Rhizoctonia solani* on lettuce (Reglinski *et al.*, 1995). On leaf discs *B. cinerea* infection was reduced by up to 90% whilst on whole plants in the glasshouse *B. cinerea* and *R. solani* infection were reduced by 50-70%.

The activity of the yeast-derived elicitor is influenced by the choice of adjuvant employed suggesting that formulation optimisation is important for improving efficacy (Reglinski *et al.*, 1994a, Lyon *et al.*, 1995).

Synthetic elicitors

The synthetic elicitor 2,6-dichloroisonicotinic acid (INA) has been used to induce resistance in several plant/pathogen interactions (Neuenschwander *et al.*, 1995, Delaney *et al.*, 1995, Vernooij *et al.*, 1995, Hijwegen *et al.*, 1996). Some groups have reported problems with phytotoxicity of this compound and it is therefore unlikely that it will be further developed as a commercial product. It's most useful attribute will probably be as an inducer of SAR to understand the molecular pathways involved in SAR induction. INA does not induce SA accumulation in plants and can induce both disease resistance and SAR gene expression in transgenic tobacco and Arabidopsis plants that cannot accumulate SA (Vernooij *et al.*, 1995).

Recently, Ciba-Geigy has developed a new product based upon the active ingredient benzyl(1,2,3)thiadiazolcarbothioic acid S-methyl ester (BTH) (Görlach *et al.*, 1996) also called CGA 245704, which is structurally related to salicylic acid. BTH was detected by its ability to induce the protein PR1a in tobacco in a biological screen (Neuenschwander *et al.*, 1995). This compound is reported to give excellent control of mildew through induction of SAR on barley and wheat with a single spray application at 30 g ai/ha early in the season and is already commercially available in Germany as Bion®. BTH acts to stimulate the SAR signal transduction mechanism either at the same step as salicylic acid or downstream of it (Ryals *et al.*, 1996).

PRACTICAL APPLICATION OF ELICITORS

The ability to utilise 'novel' approaches to control plant diseases in the laboratory has existed for a number of years. Naturally occurring and synthetic compounds able to induce resistance locally or systemically are being given serious consideration as a practical method of disease control either as an alternative to fungicides, in combination with low dose rate fungicides, or alternating sprays application.

Several reasons have been cited as to why inducing resistance as a method of disease control would not work (Sequeira, 1983). These have included, (1) Resistance is not sufficient to improve yields significantly, (2) Induced resistance may cause substantial losses in yield, and (3) induced resistance may result in accumulation of undesirable secondary metabolites. All these fears have proven to be unfounded. High levels of disease resistance can be induced, particularly with the latest chemical elicitors, yields of treated crops are comparable to fungicide treated crops, and there is no evidence of accumulation of undesirable secondary metabolites.

Protectant type fungicides are normally highly effective when applied to crops before diseases such as potato blight are widespread but are ineffective against some diseases if the pathogen has progressed to where 5-10% of the foliage is infected. In this respect elicitors of SAR are similar, they will be most effective when applied before the pathogen becomes well established and may have little eradicant capability although they should restrict further pathogen development.

The advantages of resistance elicitors are numerous; they can be used where existing fungicides are ineffective or inappropriate, and as a combined application with fungicides they may enable low dose fungicide levels to be applied or extend the lifetime of existing fungicides before pathogen tolerance develops. This method of disease control is still very much in the early stages of development but is expected to expand in the forthcoming years. The transition from chemical to chemical/biological pest and disease control, though gradual, is inevitable. The application of non-antimicrobial resistance elicitor to crops will be compatible with the use of biocontrol systems.

The success or otherwise of SAR inducing agents, whether natural or synthetic, will be dependent upon their perception by farmers and growers. If they expect the levels of disease control and eradication achieved by conventional pesticides, then they may fail. However, in the context of environmental protection demands and integrated crop protection, they may offer a highly cost effective option. The non-synthetic resistance elicitors derived from fungal biomass, may enable produce to be labelled 'organic' and thus command a higher price which may more than compensate for any yield loss. Where buyers demand very low or zero levels of residues such as lettuce for supermarkets, such products may be used to achieve these objectives either alone or integrated with reduced dose fungicides.

Another factor little investigated with SAR inducing products is the relationship between disease and yield loss. Although the relationship is normally linear where disease levels vary naturally or are controlled by fungicides, when resistance elicitors are used to control disease levels the regression accounts for far less of the variation and may lose significance entirely. Many of the effects of such resistance elicitors are upon metabolic processes which will be involved in other cellular functions either directly or indirectly. Inevitably there will be knock-on effects on assimilate partitioning. There is therefore potential to manipulate this relationship in favour of disease tolerance, i.e. less yield loss than should result from the residual level of disease.

A further difference in approach which should be made when considering SAR inducers is their interaction not only with different host - pathogen systems, but also cultivar differences within host species and the environment. Again, it is the step(s) of mediating control through the host plant rather than directly against the pathogen which must be addressed. Not only the host genotype but also its physiological status will have a significant effect. Thus factors such as fertiliser level, temperature and water status will all interact with both the SAR inducer's efficacy and the final yield response. However, this should be seen as an opportunity to optimise and tailor disease control packages to individual crops on farms rather than as a source of uncontrollable variation introducing unpredictability.

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SUPEROXIDE GENERATION AT INFECTION SITES IN RESISTANT AND SUSCEPTIBLE NEAR-ISOGENIC BARLEY LINES ATTACKED BY THE POWDERY MILDEW FUNGUS (*ERYSIPHE GRAMINIS* F.SP. *HORDEI*)

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ABSTRACT

We have microscopically analysed the pathogenesis-related generation of superoxide anions (O_2^-) in near-isogenic barley (*Hordeum vulgare*) lines, carrying the powdery mildew resistance genes *Mla12*, *Mlg*, or *mlo5*, by the detection of nitroblue tetrazolium (NBT) reduction to the dark-blue dye formazan. The inoculation of primary leaves with the powdery mildew fungus (*Erysiphe graminis* f.sp. *hordei*) evokes the production of superoxide anions at the sites of attempted infection. The response occurs in two phases, the first of which occurs as a rapid and transient burst (approximately at 5 hours after inoculation [hai]) in all barley genotypes. The second response is unique to hypersensitive response (HR) - causing reactions mediated by the powdery mildew resistance genes *Mla12* and *Mlg*. Formation of effective papillae (penetration resistance) controlled by the *Mlo* resistance locus is not associated with O_2^- generation as revealed by a significant decline of O_2^- production right at the time when formation of papillae beneath appressoria proceeds (8 to 15 hai).

The data suggest an involvement of O_2^- in those defence responses of barley against the powdery mildew fungus causing HR but not in the response resulting in papillae resistance.

INTRODUCTION

The role of active oxygen species (AOS) in plant resistance to microbes has been widely discussed in the last few years (Mehdy, 1994; Baker & Orlandi, 1995; Tenhaken *et al.*, 1995). Doke (1983) was the first who showed O_2^- generation during a plant - pathogen interaction and its possible involvement in the hypersensitive response (HR) triggered in potato cells after penetration by an avirulent isolate of *Phytophthora infestans*. Among other compounds, Doke used nitroblue tetrazolium (NBT) to detect O_2^-. Since then, many investigations have been addressed to elucidate the role of superoxide and other active oxygen species in defence responses and especially in programmed cell death (PCD). At the time being, it is quite well accepted that AOS are involved in membrane deterioration (Ádám *et al.*, 1989), cell wall structural protein cross-linking (Brisson *et al.*, 1994), development of necrotic lesions (Doke and Ohashi, 1988), elicitor signal transduction (Nürnberger *et al.*, 1994), induction of cell death (Levine *et al.*, 1994) and probably in induced resistance responses (Chen *et al.*, 1993; Neuenschwander *et al.*, 1995). A plasma membrane NAD(P)H oxidase like that responsible for the oxidative burst in mammalian phagocytes (Morel *et al.*, 1991) has been implicated in

the hypersensitive response triggered in plant cells by pathogens. Subunits with considerable homology to those of the mammalian enzyme do probably exist in plants and (suicide) inhibition of the enzyme by diphenylene iodonium affects production of superoxide anions, hydrogen peroxide as well as gene activation in plant cell cultures (Levine *et al.*, 1994; Auh & Murphy, 1995; Tenhaken *et al.*, 1995). Thus, NAD(P)H oxidase might participate in signal transduction processes of plant - pathogen interactions (for review see Mehdy, 1994; Jones & Dangl, 1996), although such a broad claim is questioned by other authors. A recent publication of Glazener *et al.* (1996) disclosed *Pseudomonas hrmA* mutant strains which were unable to mount a hypersensitive response in tobacco but nevertheless retained the ability to elicit the oxidative burst, indicating that AOS production is not sufficient to trigger the HR.

Investigations of the role of AOS in plant defence were mostly performed with microbial elicitors in league with cell- or tissue-cultures of dicot plants. The knowledge of its role in true host - pathogen systems is fragmentary, and this is especially the case for monocots. Therefore, it was our intention to analyse the generation of O_2^- in the interaction of barley with the powdery mildew fungus.

The stages of development of *Erysiphe graminis* f.sp. *hordei* are well defined: germinating conidia produce a primary germ tube (PGT) 1-2 hours after inoculation (hai). A second, appressorial germ tube is produced shortly afterwards. The end of the appressorial germ tube swells to form a mature appressorium some 10-12 hai. The fungus attempts to breach the epidermal cell wall by developing an infection peg from the appressorium. If this fails, additional lobes may be produced from the appressorium, each of which in turn is capable of producing an infection peg in an attempt to penetrate the cell wall. In response, the plant deposits secondary metabolites, including callose, silicon, calcium, and phenolic-based compounds, in papillae beneath PGT and appressorial lobes some 12-15 hai (Aist & Bushnell, 1991; Carver *et al.*, 1995). If an appressorial infection peg successfully breaches the cell wall, it swells to form a haustorium, through which the fungus derives nutrients from the plant.

In the present work, we used the isolate *Egh*-A6 that triggers resistance responses in near-isogenic backcross lines bearing the *Mla12*, *Mlg* or *mlo5* gene for powdery mildew resistance. The genes interfere with different stages of fungal development thereby conferring distinctive infection phenotypes: While *Mlg* mediates resistance through a papilla response followed by a HR of the attacked epidermal cell (Görg *et al.*, 1993), functional alleles of the *Mla* locus mediate fungal arrest by mounting a HR after fungal penetration of the papilla (Koga *et al.*, 1990; Kita *et al.*, 1981) and *mlo* alleles control a penetration resistance solely due to effective papilla formation (Stolzenburg *et al.*, 1984; Zeyen *et al.*, 1993; Skou, 1982). We have shown that inoculation of barley leaves with the powdery mildew fungus results in the generation of O_2^- at sites of attempted infection and that O_2^- accumulation temporally and spatially depends on the plant resistance genotype.

MATERIAL AND METHODS

Plants, pathogens, and inoculation.

The barley (*Hordeum vulgare* L.) cultivars Pallas and the *Mla12*, *Mlg*, *mlo5* backcross lines in

Pallas (BCP) were obtained from Lisa Munk, Kopenhagen (Denmark). Their generation has been described previously (Kølster *et al.*, 1986). Plants were grown in a growth chamber at 16°C, 80% relative humidity and a photo period of 16 h (100 $\mu E\ s^{-1}\ m^{-2}$). Inoculation was done with conidia from *Erysiphe graminis* f.sp. *hordei*, race A6 (Wiberg, 1974).

Detection of the superoxide anions

The solution for the histochemical detection of O_2^- contained 0.1 % (w/v) nitroblue tetrazolium (NBT) and 10 mM NaN_3 in 10 mM potassium phosphate buffer, pH 7.8. At the indicated time points, the solution was injected into the primary leaves of barley seedlings. NBT-treated plants were kept for 30 min under day light and subsequently harvested by cutting the infiltrated region of the leaves and placing them in a clearance solution [0.15 % (w/v) trichloroacetic acid in ethyl alcohol:chloroform 4:1 (v/v)]. The solution was exchanged once during the next 48 h of incubation. Subsequently, leaves were stored in 50 % glycerol. To stain fungal structures for bright field microscopy, leaves were incubated in blue ink containing 5 % (v/v) acetic acid for 1 min and then washed to remove the excess of ink. Whole cell and papilla autofluorescence was observed by fluorescence microscopy (wavelength = 310 nm) using an Axioplan microscope (Zeiss, Jena, Germany). A hundred or more attacked short cells (type A and type B of the adaxial epidermis, Koga *et al.*, 1990) were scored per primary leaf. For each data point in Figure 1, three leaves were evaluated.

RESULTS

Reduction of NBT by O_2^- to formazan or further to diformazan was microscopically analysed at sites of attempted infections after inoculation of barley leaves by the powdery mildew fungus. The dark-blue formazan dyes were clearly distinguishable from the light-blue color of the ink used to stain fungal structures. Due to the substantial decrease in solubility, the formazans were not washed out during preparation of leaves for microscopy.

In order to distinguish between inoculation-related stress symptoms and those reactions specifically related to processes governed by powdery mildew resistance genes, we comparatively analysed pathogenesis-related O_2^- generation in *Mla12*, *Mlg* and *mlo5* backcross lines of cv. Pallas and the recurrent parent line Pallas in response to attack by race *Egh*-A6. The race possesses the *avrMla12* and *avrMlg* avirulence gene and additionally is incompatible with the line carrying the race-unspecific *mlo5* resistance function.

A kinetic analysis of NBT reduction by the detection of formazan accumulation at early stages of the interactions (time range 5 to 30 hai) is presented in Figure 1. All the barley lines show a significant dye accumulation at the sites of attempted infection at 5 hai, when the primary germ tube is formed. This response is followed by a pronounced decline of formazan formation at 11 hai, when papillae formation proceeds. A second phase of formazan accumulation with a peak at 24 hai is observed only in the barley lines BC-P*Mla12* and BC-P*Mlg*. At this time point, about 60 % of all sites in these lines are associated with enhanced formazan accumulation. At the same time range, the percentage of sites in *mlo5* plants showing formazan accumulation remains on a plateau of approximately 20 to 30 %. In the

susceptible parent line Pallas, a small increase in formazan formation occurs at 18 hai, followed by a decline at 24 hai.

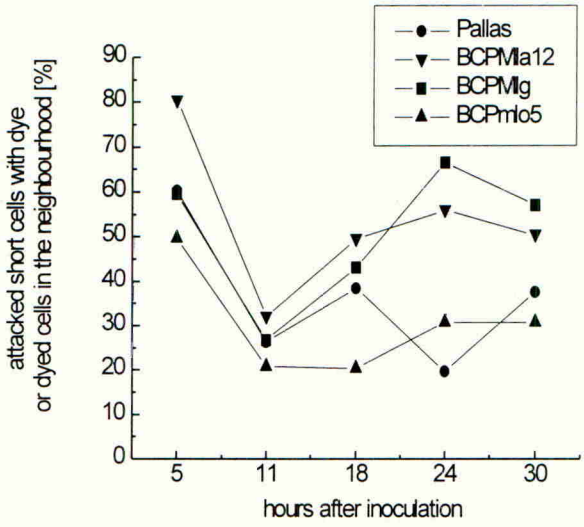

Figure 1. Time course of the localized activation of NBT reduction in cells of barley primary leaves at sites of attempted infection in response to inoculation with *E. graminis* f.sp. *hordei*, race A6. The isolate carries avirulence genes corresponding to the resistance genes *Mla12* and *Mlg*, and is also avirulent on genotypes with a functional *mlo* allele. Each data point represents the average of triplicates.

Because formazan formation resulting from NBT reduction is indicative for the presence of O_2^- (Auclair & Voisin, 1985) and because the second O_2^- burst is exclusively found in BC-P*Mla12* and BC-P*Mlg*, the data may indicate that the second response is associated with the HR governed by the *Mla12* and the *Mlg* gene. Furthermore, the results suggest that O_2^- is not involved in those defence processes resulting in papillae formation.

DISCUSSION

Early in the 1980s, Doke (1983) reported for the first time the possible involvement of AOS in the processes resulting in a hypersensitive resistance response. Inoculation of potato discs with an avirulent race of *Phytophthora infestans* was accompanied by enhanced reduction of both cytochrome c and nitroblue tetrazolium. Inclusion of superoxide dismutase in the bathing solution not only reduced these reactions but also the HR. Since then, several worker have shown that NBT reduction can be used as a suitable probe for O_2^- generation (Doke & Ohashi, 1988; Adam *et al.*, 1989).

We have shown in this paper that the resistance responses in barley governed by the powdery resistance genes *Mla12* and *Mlg* are associated with a two-phased generation of superoxide anions at sites of attempted fungal penetration. While the first phase of increased O_2^- production early after inoculation (5 hai) was evoked in all barley genotypes tested including those lacking a known resistance gene, the second phase production (18 to 30 hai) was observed only in lines bearing the *Mla12* or *Mlg* gene. Interestingly, infection phenotypes caused by these genes are associated with HR (Koga *et al.*, 1990; Görg *et al.*, 1993; Kogel *et al.*, 1994). Thus, because the second phase of increased O_2^- generation temporally coincides with HR formation, an involvement of O_2^- in this process is suggested.

The first phase of O_2^- accumulation is probably related to the inoculation process because it occurs independent of the barley resistance genotype either in compatible or incompatible interactions. The temporal coincidence of the first burst with the contact of the primary germ tube with the host cuticle (and probably with the epidermal cell wall) is evident and corroborates previous data suggesting that this fungal structure induces defence processes in the host without penetrating the host cell (Thordal-Christensen & Smedegaard-Petersen, 1988; Bushnell & Liu, 1994; Carver *et al.*, 1995; Beckhove *et al.*, 1996).

The lack of the second phase of O_2^- burst in *mlo5* resistant plants indicates that these oxygen radicals are not involved in the process mediated by this gene. It was previously shown that the *mlo* mechanism is based on the formation of effective papillae precisely at the sites of attempted penetration. Papillae formation commences with an aggregation of the cytoplasm right beneath the tip of the fungal appressorium approximately at 11 hai and is usually completed between 13 h and 15 h of infection (Aist & Bushnell, 1991). At this time, we find a significant decline of O_2^- generation. Moreover, papillae formation is not completely dependent on resistance gene function and to a certain extent occurs in all barley cultivars (Koga *et al.*, 1990; Görg *et al.*, 1993; Kogel *et al.*, 1994). Thus, the decline of O_2^- generation, just at the time papillae formation is to be expected, strongly suggests that O_2^- is not required for this highly abundant and substantial plant response.

A recent landmark study of the group of Lamb suggested that H_2O_2 from the oxidative burst drives several plant defence responses including the cross-linking of cell wall structural proteins (Brisson *et al.*, 1994). Accumulation of oxalate oxidase has recently been reported in interactions of barley with the powdery mildew fungus (Zhang *et al.*, 1995). By catalysing oxalate oxidation this enzyme produces H_2O_2. Thus, further work must show whether AOS species different from O_2^- could be involved in papillae formation.

The results additionally give a hint for the role of AOS in chemically induced resistance. It was previously demonstrated that resistance induced by chemical treatment of barley or wheat seedlings with either *Bacillus subtilis* culture filtrates (Steiner *et al.*, 1988) and more recently with chemical inducers like 2,6-dichloroisonicotinic acid (Kogel *et al.*, 1994) or benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (Görlach *et al.*, 1996) is closely associated with papillae formation. Hence, there are indications for a secondary role of O_2^- in chemically induced resistance of cereals.

Figure 1 combines data on O_2^- generation (*i*) in cells directly attacked by the pathogen and (*ii*) in cells neighbouring the attacked cells. In the present study a pronounced accumulation of

formazan was found in the chloroplasts of mesophyll cells in the neighbourhood of those cells directly attacked by the fungus. However, preliminary data suggest the existence of additional locations of O_2^- generation in close association with fungal infection structures (unpublished results). Because of the critical differences in the infection types governed by the different powdery mildew resistance genes, a more detailed analysis of subcellular locations of O_2^- generation in directly attacked cells is required for the elucidation of a causal or a secondary role of O_2^- in those interactions.

Interestingly, the two-phased profile of O_2^- generation follows the suggested activation of defence-related genes in barley genotypes bearing a functional allele of the *Mla* locus (Clark *et al.*, 1993; Boyd *et al.*, 1994; Walther-Larsen *et al.*, 1993). Thus, these findings are consistent with the suggestion that, in *Mla* plants, O_2^- generation is a signal for triggering defence. The two-phased O_2^- burst is also reminiscent of those events described in plant - bacteria interactions: A first phase response in compatible and incompatible *Pseudomonas* - tabacco and *Pseudomonas* - soybean interactions was followed by a second phase response only in incompatible interactions associated with HR-type reactions (Baker *et al.*, 1993, Baker & Orlandi, 1995). These data support a more general role of the AOS response in plant - pathogen interactions.

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SALICYLIC ACID: A FACTOR IN SYSTEMIC RESISTANCE OF CACAO TO *PHYTOPHTHORA PALMIVORA*

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ABSTRACT

The effect of salicylic acid (SA) on systemic resistance of cacao to *Phytophthora palmivora* was investigated using two clones, ICS 1 and IMC 67. The number of lesions per leaf were significantly reduced ($P<0.05$) in salicylic acid treated plants compared to those in the control. In addition, lesion area and the percentage of leaf area infected with *P. palmivora* were significantly reduced after salicylic acid treatment. Seed treatment, plant spray and soil drench induced systemic resistance in cacao, with seed treatment being most effective. While resistance was induced in both IMC 67 and ICS 1, the former had a comparatively better performance. These results indicate that SA induces systemic resistance in cacao and should be considered as a potential chemical for the control of *P. palmivora*.

INTRODUCTION

Resistance of plants to pathogenic invasion can be acquired by a number of mechanisms which could be local or systemic, constitutive or induced (Kessmann *et al.*, 1994). Induced systemic resistance has been achieved by prior treatment of plants with pathogenic or non-pathogenic micro-organisms (Kuc, 1983, Cloud & Deverall, 1987, Roby *et al.*, 1988). Systemic resistance has also been induced by non-pathogenic agents such as treatment with chemicals. Thus, oxalates, phosphates, EDTA, silicon, benzothiadiazole etc. have been used to induce resistance in cucumber (Doubrava *et al.*, 1988, Gottstein & Kuc, 1989, Cherif *et al.*, 1992), broad beans (Walters & Murray, 1992) and wheat (Görlach, 1996).

Among the various chemicals used as inducers of resistance in plants, salicylates are perhaps the best known. These compounds have been reported to induce resistance in a number of plants: tobacco (White, 1979, Pennazio & Roggero, 1984), broad bean (Pierpoint, 1994, Chen *et al.*, 1995) and asparagus beans (Pennazio *et al.*, 1987).

Rathmell (1983) claimed that direct antifungal activities of these compounds could affect the development of the pathogen within the host plants, thus making them less vulnerable to fungal invasion. Also, the alteration of metabolic activities of plants which results in the synthesis of pathogenesis related (PR) proteins has been reported as a crucial role of these chemicals in inducing resistance (Van Loon & Antoniw, 1982, Metraux *et al.*, 1990, Malamy *et al.*, 1990, Niderman *et al.*, 1995).

While the phenomenon of systemic induced resistance is becoming popular in the control of other plant diseases, such as those of cucumber, wheat, watermelon, tobacco, beans etc., its application on cacao with regard to the control of *Phytophthora* diseases has not been given adequate attention. However, Okey *et al.* (1995) associated cacao stem resistance to *P. palmivora* with salicylic acid. The present study reports on the utilisation of salicylic acid as an inducer of systemic resistance of cacao to *P. palmivora.*

MATERIALS AND METHODS

Plant and fungal materials

Ripe pods of ICS 1 and IMC 67 were collected from cacao fields at the St. Augustine campus of the University of The West Indies, Trinidad. An isolate of *P. palmivora* obtained from infected IMC 67 pods was used as the pathogenic organism. It was cultured in 20% V8 juice agar. Zoospores were obtained from ten-day old cultures as earlier described (Okey *et al.*, 1996) and used as the inoculum. The concentration of zoospores in the suspension was adjusted to 1.5×10^5 ml $^{-1}$.

Salicylic acid treatment

Eighty seeds of each genotype were processed by removing the testa. These were divided into two batches of 20 and 60 respectively. Seeds in the first batch were soaked in a 3 mM aqueous solution of salicylic acid while those in the second were soaked in sterile distilled water. This concentration (3mM) of salicylate was adopted since Pennazio *et al.* (1987) found it to be effective in inducing resistance in asparagus beans. Three hours after soaking, the seeds were transferred into different trays lined with wet paper towels. The trays were covered with polyethylene sheets and the seed allowed to germinate for three days. After germination, seedlings were planted in plastic pots containing 600 ml of Pro-mix general purpose growing medium. The greenhouse temperature varied from 25°C to 28°C.

Four weeks after germination, plants in the second batch were further divided into three groups of 20 plants per group. Plants in the first group were treated by spraying once dily with 3 mM of salicylic acid using a Chromist atomiser (Laboratory Spray Unit, Gelman Sciences Inc. Ann Arbor, MI 48106 USA) while those in the second group were treated by soil drenching each plant with 60 ml of sterile distilled water. This group served as the control. All treatments were continued for a period of 10 days after which plants were inoculated.

Plant inoculation and assessment of induced resistance

Each plant was sprayed with 20 ml of *P. palmivora* zoospore suspension (conc. 1.5×10^5 ml) using a Chromist laboratory spray unit. Plants were incubated in moist plastic polyethylene bags for 3 days. Induced resistance was assessed by (I) counting the number of lesions formed per leaf, 4 days after inoculation (ii) measuring lesion area with a Delta T Leaf Area Meter (Model MK2, Burwell, Cambridge UK) 7 days after

inoculation and (iii) estimating the percentage of leaf area infected, 14 days after inoculation. All experiments were replicated twice.

RESULTS

The number of lesions per unit area on both clones were significantly reduced ($P<0.05$) in salicylic acid treated plants compared to the controls (Table 1). In IMC 67, lesion number reduced from 40 to 20, 25 and 30 when seeds were soaked, plants sprayed or soil drenched respectively. A similar trend in reduction of lesion number was observed for ICS 1. In both clones, soaking seeds in 3 mM aqueous solution of salicylic acid was most effective.

Table 1. Effect of salicylic acid treatment on number of lesions.

	Lesion number per leaf			
Clone	T1	T2	T3	Control
IMC 67	20	25	30	40
ICS 1	34	40	45	52

LSD ($P<0.05$) = 3. T1 represents seed treatment, T2 plant spray, T3 soil drench and control, treatment with water. Data are means of five leaves per plant. Lesions were counted 4 days after inoculation with zoospores of *P. palmivora*.

The size of lesion was also influenced by salicylic acid treatment (Table 2). In IMC 67, lesion size reduced from 22 to 10, 15 and 16 mm^2 for seed treated, sprayed and soil drenched plants respectively. In ICS 1 the reduction was from 28 to 18, 20 and 23 respectively. Seed treatment again proved to be most effective.

Table 2. Effect of salicylic acid treatment on lesion size.

	Mean lesion size (mm^2)			
Clone	T1	T2	T3	Control
IMC 67	10	15	16	22
ICS 1	18	20	23	28

LSD ($P<0.05$) = 3.2. T1 represents seed treatment, T2 plant spray, T3 soil drench and control, treatment with water. Data are means of 30 leaves. Lesion size was measured 7 days after inoculation with zoospores of *P. palmivora*.

Salicylic acid treatment also affected percentage leaf area that was infected (Table 3). The percentage of lesion area in IMC 67 was reduced from 62.9% to 30.0% for seed treated, 36.9% for sprayed and 42.8% for soil drenched plants, while in ICS 1, leaf area infected was reduced from 49.5% to 26.1% (seed treatment), 30.0% (sprayed), and 36.2% (soil drenched). Seed treatment was most effective compared to spray or soil drench application.

Table 3. Effect of salicylic acid treatment on % of leaf area infected.

	% leaf area infected (mm^2)			
Clone	T1	T2	T3	Control
IMC 67	50(30.0)	60(36.9)	68(42.8)	89(62.9)
ICS 1	44(26.1)	50(30.0)	59(36.2)	76(49.5)

LSD ($P<0.05$) = 4.0. T1 represents seed treatment, T2 plant spray, T3 soil drench and control, treatment with water. Data are means of 30 leaves. Values in parentheses represent Arc sine transformed data. The percentage of leaf area infected was estimated 14 days after inoculation with zoospores of *P. palmivora*.

DISCUSSION

Different methods of chemical application have been employed for the induction of systemic resistance in plants. Pennazio *et al.* (1985) reported plant watering (soil drench) as an effective method of salicylate application on tobacco while Walters & Murray (1992) found spraying to be more effective. In the present study, salicylic acid applied by either seed treatment, spraying or soil drench was found to give disease protection by inducing systemic resistance in cacao. In both IMC 67 and ICS 1, seed treatment was found to be most effective in protecting these genotypes against *P. palmivora*. The fact that seed treatment as well as soil drenching resulted in a significant reduction in number of lesions, lesion size and % leaf area infected is an indication that induced resistance is a mechanism of disease protection in cacao plants.

The effect of salicylic acid on the reduction of lesion number could be associated with its effect on stomatal resistance as reported on tobacco leaves (Pennazio & Roggero, 1984). Stomata constitute openings for easy penetration of fungal pathogens. Salicylic acid effect on lesion size could be due to antifungal activities of PR proteins which accumulate around infection sites (Nidermann *et al.*, 1995). It is therefore logical to reason that with the reduction in lesion number and size, the percentage of leaf area infected is bound to decrease following salicylic acid treatment. Similar observations were made on broad bean leaves following prior treatment with phosphates and EDTA (Walters & Murray, 1992).

Although salicylic acid and its derivatives have been reported as inducers of systemic resistance (White, 1979, Pennazio & Roggero, 1984, Pennazio *et al.*, 1987, Pierpoint, 1994, Kessmann *et al.*, 1994, Chen *et al.*, 1995) their mode of action is not clearly

understood. Kuć (1983) noted that a stress systemically sensitises plants to respond rapidly to additional stress. Thus, salicylic acid could act as a sensitising stress which enables the plant to respond immediately to pathogen invasion. The results of the present study indicate that salicylic acid, like other chemical inducers, can stimulate defence mechanisms in cacao against *P. palmivora* infection.

A more specific role of salicylic acid as an inducer of disease resistance has been linked with physiological and biochemical changes which result in the synthesis of pathogen related (PR) proteins (Van Loon & Antoniw, 1982, Pennazio *et al.*, 1987, Malamy *et al.*, 1990, Metraux *et al.*, 1990). In these reports, exogenous application of salicylic acid induced PR genes and resistance which suggests that salicylic acid could act as a natural transduction signal. The role of this acid in cacao resistance to *P. palmivora* may be fully understood when proteins involved are extracted and analysed.

Comparatively, IMC 67 had a better performance than ICS 1 in terms of systemic resistance induction with salicylic acid. This suggests that cacao genotypes possess different responses to exogenous applications of salicylic acid. Thus, a screening trial is recommended using seed treatment in order to identify induced resistant genotypes.

This study indicates that salicylic acid can induce systemic resistance in cacao against *P. palmivora*. Since many of the chemicals previously used in the control of this pathogen are relatively toxic to humans, the concept of systemic resistance acquired through salicylic acid for the control of this pathogen is an exciting prospect. It would also be interesting to test the effect of exogenous application of salicylic acid on cacao pods on *P. palmivora* infection.

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CGA 2455704: MODE OF ACTION OF A NEW PLANT ACTIVATOR

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ABSTRACT

Plants, when locally infected with a necrotizing pathogen or a non-pathogen, often develop a long lasting, broad-spectrum "immunity" against subsequent infection. This natural phenomenon - called "systemic activated (or acquired) resistance" (SAR) - has been known for almost a century. However, naturally induced SAR is not predictable in timing and level of expression and therefore it could not be used for agricultural practice. Using special screening procedures, we were able to discover small molecules which activate the SAR response ("plant activators"). CGA 245704 (benzo[1,2,3]thiadiazole-7-carbothioic acid S-methyl ester) was developed for commercial use in a wide range of crops.

Plant activators protect the plant against the same spectrum of diseases as the natural response after localized infections. They do not exhibit direct activity against pathogens but instead cause the same biochemical changes in the plant as observed after biological activation.

Salicylic acid plays a central role in the SAR signalling pathway upon biological activation. Plant activators like CGA 245704 activate the SAR response by acting as functional analogues of salicylic acid in the pathway leading to SAR.

INTRODUCTION

Plants have evolved both permanent and inducible disease resistance mechanisms that together form an effective defence against pathogen infection, with disease resulting as the rare outcome in the spectrum of plant - microbe interactions.

The ability of a plant to respond to an infection is determined by genetic traits in both the host and the pathogen. Some resistance mechanisms are specific for plant cultivars and certain strains of pathogens. In these cases, plant resistance genes recognize pathogen derived molecules resulting from expression of so - called avirulence genes which often triggers a signal cascade leading to rapid host cell death (hypersensitive response, HR, for review: Dixon & Lamb, 1990, Pryor & Ellis, 1993). Such "gene - for - gene" interactions usually lead to highly efficient, but very specific, plant resistance.

Another level of inducible host resistance, commonly called "systemic activated (or acquired) resistance" (SAR) is expressed throughout the plant after localized necrosis induced by pathogens or non-pathogens. SAR provides broad-spectrum and long lasting "immunity" and is most likely present in all plant species.

This paper will summarize recent work on SAR and will focus on the mode of action of CGA 245704, a compound discovered and developed by CIBA - GEIGY which is able to activate the SAR response in plants ("plant activator").

SAR: THE BIOLOGICAL PHENOMENON

It has been known for about a hundred years that plants are able to develop resistance following minor infections. In 1933, K. Chester reviewed many descriptive studies on inducible plant resistance and concluded that "immunity in plants ... may play an important role in the preservation of plants in nature" (Chester, 1933). However, it took almost 30 years before Frank A. Ross conducted some classical experiments with tobacco (Ross, 1961) demonstrating that SAR can be activated by local infections e.g. with tobacco mosaic virus (TMV). This resistance was not limited to the site of infection but spread throughout the entire plant.

At about the same time Cruickshank & Mandryk (1960) showed that stem infection of tobacco with bluemold (*Peronospora tabacina*) can lead to an enhanced resistance to foliar pathogens. Interestingly, the effect of SAR induction on general plant health depends on the inoculation procedure. When conidia are injected into the cambium the SAR response was linked to severe dwarfing and premature senescence. In contrast, infection external to the cambium leads to an increase in plant weight and leaf number (Madamanchi & Kuc, 1991, Tuzun & Kuc, 1985).

In addition to tobacco, cucumber has been developed as a biological model for SAR (for review see Madamanchi & Kuc, 1991). SAR in cucumber can be induced by various microorganisms (e.g. TNV, *Pseudomonas lachrymans, Colletotrichum lagenarium*). After an incubation period of a few days, plants are protected against at least 13 diseases for up to 4-6 weeks.

Meanwhile, green bean, cotton, potato, soybean, tomato, alfalfa, barley, wheat, rice, and *Arabidopsis thaliana* (for review see Kessmann *et al.*, 1994), have also been shown to develop a SAR response.

MECHANISM OF SAR

Plants often respond at the site of attempted microbial infection with a localized cell death (hypersensitive response) followed by a wide range of additional defence responses, including phytoalexin and callose formation, lignification, and cell wall cross - linking (for review see Dixon & Lamb, 1990). These mechanisms are strictly localized and are not induced during the maintenance state of SAR (Ryals *et al.*, 1994, Kessmann *et al.*, 1994).

However, if a preinfected (immunized) plant is attacked by a pathogen the plant often responds faster with these mechanisms (priming effect, Kessmann *et al.*, 1994).

Van Loon *et al.* as well as Gianinazzi *et al.*, (for review see Bowles, 1990) showed that pathogen infection of tobacco with tobacco mosaic virus (TMV) leads to the accumulation of a set of so called "pathogenesis - related" (PR) proteins. Acidic, extracellular forms of these PR-proteins accumulate during the onset of resistance indicating that they may play a role in SAR. Ward *et al.*, (1991) showed that nine gene - families are coordinately induced both in infected, as well as in distal, untreated leaves after local infection of tobacco with TMV (Figure 1). Some of these "SAR genes" have been characterised as β-1,3 glucanases and chitinases (Ward *et al.*, 1991, Ryals *et al.*, 1994, Friedrich *et al.*, 1996). The role of the PR genes in the maintenance of SAR was further supported by results with transgenic plants in which some of the genes were constitutively expressed resulting in resistance against certain pathogens (for review: Ryals *et al.*, 1994).

INDUCTION OF SAR BY CGA 245704

The SAR system presents some interesting opportunities for the control of plant diseases in agricultural practice, and for enhancing our basic knowledge of disease resistance in plants. First, disease resistance that results from the SAR response is a natural defence phenomenon. Second, the biological models of SAR in cucumber and tobacco have demonstrated that SAR can lead to long lasting and broad spectrum disease control. Furthermore, there is ample evidence that SAR is based on multiple mechanisms which makes it less likely that pathogens can readily develop resistance to this control measure.

Using a special screening system we discovered that CGA 245704 (benzo[1,2,3]thiadiazole-7-carbothioic acid S-methyl ester) is a potent inducer of SAR since:

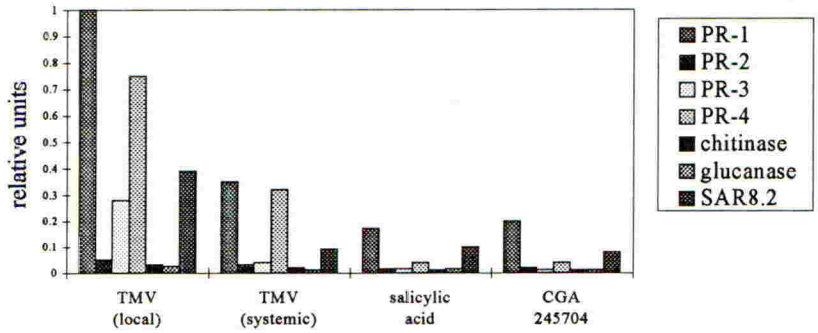
- the treated plants are resistant to the same spectrum of diseases as plants activated naturally (Kessmann *et al.*, 1996, Friedrich *et al.*, 1996)
- it requires a lag-time between application and protection
- the compound and the metabolites exhibit no direct antimicrobial activity towards plant pathogens tested (Friedrich *et al.*, 1996)
- it induces the same biochemical processes in the plant that are characteristic for systemic plant tissues after natural activation of SAR (Friedrich *et al.*, 1996, Lawton *et al.*, 1996a, Figure 1)
- it is inactive in plant mutants with a defective SAR-signalling pathway (Lawton *et al.*, 1996a,b)

In tobacco CGA 245704 induces the same set of SAR genes that is induced by biological induction (Figure 1). PR1a, a protein with unknown function, is the most prominent protein which accumulate after infection or chemical activation. Other proteins include chitinases and glucanases.

In wheat, CGA 245704 induces resistance to fungal pathogens in a very similar process (Görlach *et al.*, 1996). It induces a number of wheat genes that also accumulate during

pathogen infection. A close relationship exists between the induction of some of these genes and the resistance. These findings indicate a homology in the molecular events leading to the expression of SAR in monocot and dicot plants.

Figure 1: Relative Steady-State Levels of mRNA in tobacco induced by various activators.



The bars represent the approximate amount of mRNA corresponding to seven of the pathogenesis-related (PR) proteins. Values were normalized to the maximum level of PR-1 transcript detected. Levels of PR-gene expression in control samples were not detectable at this scale. In the case of activation with TMV, data are presented separately for the inoculated (=local) and the systemic leaf. mRNA was extracted 6 days after TMV treatment and 2 days after salicylic acid (SA) and CGA 245704 application (for experimental details see Ward *et al.*, 1991, Friedrich *et al.*, 1996).

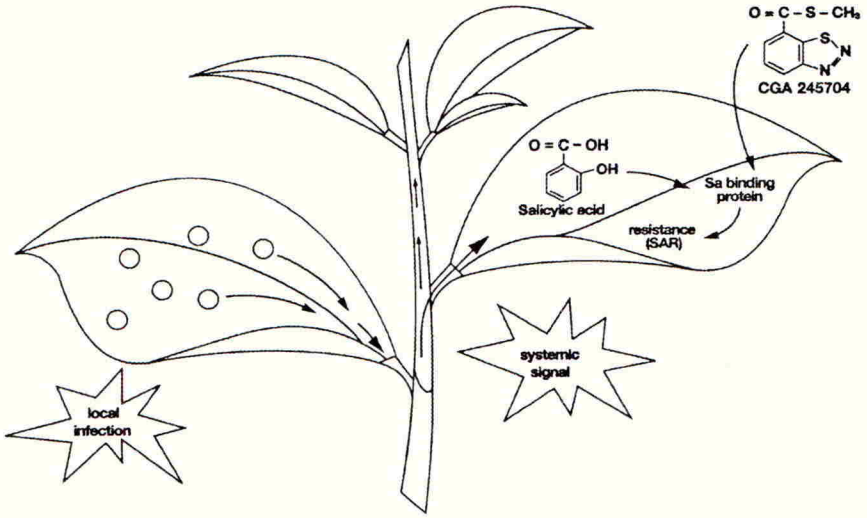
CGA 245704: A FUNCTIONAL ANALOGUE OF THE PLANT'S ENDOGENOUS ACTIVATOR SALICYLIC ACID

Ray White (Rothamsted, UK) showed in 1979 that exogenous application of salicylic acid (SA) or Aspirin® results in the accumulation of PR - proteins and protection of tobacco against TMV (White, 1979). However, the amount of salicylic acid required for efficient disease control is quite high which is most likely due to its rapid metabolism in the plant.

Further studies with tobacco and cucumber have shown that SA accumulates *endogenously* throughout the plant after induction of SAR by local infection with pathogens (Malamy *et al.*, 1990, Metraux *et al.*, 1990. In order to evaluate the role of salicylic acid in more detail, tobacco was transformed with the *nahG* gene from *Pseudomonas putida* which encodes a salicylate hydroxylase, an enzyme that catalyzes the degradation of SA to the non - inducing metabolite catechol (Gaffney *et al.*, 1993). The *nahG*-transgenic plants were shown to express the *nahG* - gene and did not accumulate SA after the onset of SAR nor exhibit the SAR response. However, CGA 245704 is still able to protect these plants by activation of the SAR response. As shown for *nahG*-tobacco (Friedrich *et al.*, 1996) CGA 245704 induces PR-protein accumulation and protects the plants against *Peronospora tabacina* and TMV to the same degree as wild type plants. These results confirm that SA plays an important role in the signal transduction pathway that leads to the induction of SAR and that CGA 245704 stimulates the pathway downstream of salicylic acid (Figure 2).

Recent studies with *Arabidopsis thaliana* mutants further confirmed (Lawton *et al.*, 1996a, Lawton *et al.*, 1996b) that CGA 245704 and biological activators share parts of the same signalling pathway.

Figure 2. Mode of action of CGA 245704 (hypothesis)



Local infection with a necrogenic pathogen triggers the release of a systemic SAR signal. In the systemic tissue salicylic acid (SA) is required for transmitting the signal to the resistant state. CGA 245704 seems to replace SA in this process inducing the resistance without prior infection (modified from Kessmann *et al.*, 1996; with permission).

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SYSTEMIC ACQUIRED RESISTANCE SIGNAL TRANSDUCTION

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ABSTRACT

While the systemic acquired resistance (SAR) response has been well characterized in tobacco and cucumber, the signal transduction pathway leading to the resistant state is not understood. The small crucifer, *Arabidopsis thaliana* (Arabidopsis) is a useful model system for molecular genetic experiments aimed at understanding signal transduction pathways. Therefore, we have developed Arabidopsis as a model for SAR.

The SAR hallmarks of broad-spectrum disease resistance, coordinate expression of pathogenesis-related (PR) genes and accumulation of salicylic acid (SA) are induced in Arabidopsis following inoculation with a necrogenic pathogen. Treatment with SA or the chemical activators INA and BTH also induces PR-gene expression and disease resistance. Transgenic Arabidopsis that are unable to accumulate SA, do not establish an SAR response following inoculation with a biological inducer and exhibit enhanced susceptibility to pathogens. Thus, SA is critical in SAR signal transduction and may play a role in restriction of disease symptom development.

Mutants have been isolated that constitutively express PR-1 mRNA. These plants are resistant to pathogens and are called *cim* for constitutive immunity. Some of these constitutive immune plants also show lesions simulating disease (*lsd*). Another class of mutants exhibit the non-inducible immunity (*nim*) phenotype. SA, INA or BTH treatment does not induce SAR in these mutants, indicating that these molecules act upon the same signal transduction pathway.

INTRODUCTION

With its rapid generation time and small genome, the tiny cruciferous weed, *Arabidopsis thaliana* (Arabidopsis), provides a useful model system for molecular genetic studies. The five chromosomes of Arabidopsis have been extensively mapped with molecular and phenotypic markers making it relatively simple to position a locus on a chromosome arm using PCR-based mapping technologies. Gene isolation is further facilitated by availability of yeast and bacterial artificial chromosomes (YACs and BACs, respectively)

that contain large segments of the Arabidopsis genome and that, in many cases, have been anchored to markers on the plant chromosomes.

To take advantage of this powerful technology we have established Arabidopsis as a model plant to understand systemic acquired resistance (SAR) signal transduction. Local inoculation of Arabidopsis leaves with a necrogenic pathogen results in broad-spectrum disease resistance, accumulation of salicylic acid and coordinate expression of pathogenesis-related (PR) proteins in the infected leaves as well as in the uninfected leaves of infected plants (Uknes *et al.*, 1993, Cameron *et al.*, 1994, Mauch-Mani; Slusarenko, 1994, Lawton *et al.*, 1995). Application of the SAR chemical activators salicylic acid (SA), 2,6 dichloroisonicotinic acid (INA) and benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (benzothiadiazole, BTH) induces the same broad-spectrum disease resistance and PR gene expression as observed with the biological induction of SAR by pathogen infection (Uknes *et al.*, 1992, Vernooij *et al.*, 1995, Lawton *et al.*, 1996). Thus, Arabidopsis exhibits the responses that are characteristic of SAR and as such can be a useful model system for SAR.

A WORKING MODEL OF SAR SIGNAL TRANSDUCTION

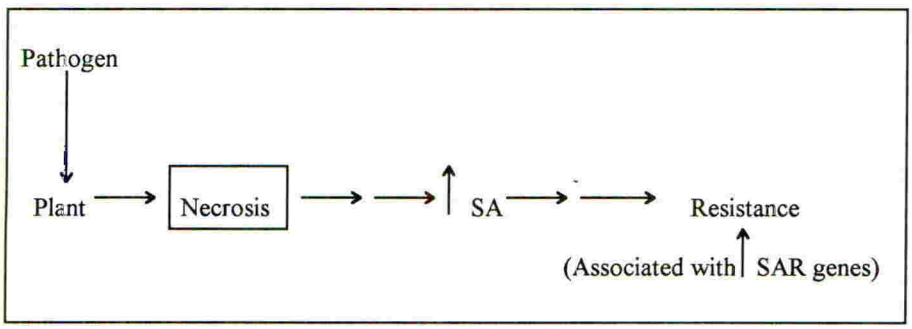

Figure 1. A working model of the SAR signal transduction pathway.

Figure 1 shows a schematic outline of the defined steps involved in SAR signal transduction. When a plant-pathogen interaction results in a necrotic lesion in the host plant, events are triggered that lead to the accumulation of SA. The SA accumulation in turn results in disease resistance both locally in the infected tissue as well as systemically in uninfected plant tissues. This disease resistance is associated with the expression of the genes encoding PR proteins and other SAR genes. Transgenic plants expressing the bacterial enzyme salicylate hydroxylase encoded by the *nahG* gene of *Pseudomonas putida* (i.e. NahG plants) are unable to accumulate high levels of SA (Gaffney *et al.*, 1993, Lawton *et al.*, 1995). These plants are severely compromised in the ability to resist diseases (Delaney *et al.*, 1994) and mount the SAR response, indicating that SA plays a crucial role in mediating SAR signal transduction (Lawton *et al.*, 1995). Thus, NahG Arabidopsis plants are blocked at steps downstream of SA accumulation and provide useful tools to study the events in SAR that are mediated by SA accumulation. Using these NahG plants we have shown that the chemical activators INA and BTH both induce the SAR signalling pathway independently of SA accumulation (Uknes *et*

al., 1992, Vernooij *et al.*, 1995, Lawton *et al.*, 1996) suggesting that these chemicals activate steps of the pathway downstream of SA accumulation.

ISOLATION OF SAR MUTANTS IN ARABIDOPSIS

Isolation of mutants with constitutive SAR

Of the PR genes that are expressed systemically following infection with necrogenic pathogens, accumulation of PR-1 is the most tightly correlated with SAR. Expression of this gene is also induced by application of SA, INA and BTH. Thus, we have found accumulation of PR-1 mRNA to be a useful molecular marker for SAR and have utilized PR-1 in screens for SAR mutants of Arabidopsis. We have taken two approaches to identifying SAR mutants using PR-1 as a marker. In the first approach we isolated RNA from a population of chemically (i.e. ethylmethane sulfonate, EMS) mutagenized Arabidopsis (M2 plants) and assayed PR-1 mRNA accumulation. Plants that had not been treated with chemicals or pathogens but showed high levels of PR-1 were identified. Progeny from these plants were screened again for high PR-1 expression and positives were tested for disease resistance. This screen resulted in identification of two classes of mutants with high levels of PR-1. The first class is designated as *cim* for constitutive immunity. These plants have high level SAR gene expression in the uninduced state, show enhanced disease resistance and are wildtype in morphology. SA levels are elevated in the *cim* mutants that have been analyzed. A second class of mutants has been termed the *lsd* class for lesions simulating disease (Dietrich *et al.*, 1994, Weymann *et al.*,1995). In addition to having high levels of *PR-1* gene expression and enhanced disease resistance necrotic lesions form spontaneously on the leaves of these plants.

In a second screening approach using the *PR-1* gene we have made transgenic plants that express a reporter gene (e.g. luciferase) fused to the *PR-1* promoter sequence that regulates expression of the *PR-1* gene in response to chemicals and pathogens. Transgenic plants transformed with a luciferase reporter construct have been characterized. Luciferase expression can be visualized *in vivo* by applying the luciferin substrate and viewing using a Hamamatsu photoimager or *in vitro* using a luminometer. A plant line with high level luciferase activity that correlates with activity of the endogenous PR-1 gene has been mutagenized and screened for plants that "glow" in the absence of an inducing treatment. This approach has yielded mutants of both the *cim* and *lsd* mutant classes. The advantages of this approach are the ability to screen large numbers of plants easily and rapidly. Furthermore, the sampling can be done *in vivo* which minimizes damage to the plant and further reduces sample handling. A similar approach using the *PR-2(BGL2)* promoter fused to the GUS reporter gene has been successful in identifying mutants with constitutive *PR* gene expression and enhanced disease resistance (*cpr*; Bowling *et al.*, 1994).

Isolation of mutants compromised in SAR induction

We have also taken the approach of identifying mutants that cannot induce the SAR response (non-inducible immunity; *nim*). In this screen mutagenized plants were sprayed with the chemical activator INA then challenged with the fungal pathogen *Peronospora parasitica* three days later. Plants that supported growth of the fungus

were selected as *nim* mutants. Characterization of a mutant identified in this screen, *nim1*-1, has been reported (Delaney *et al.*, 1995). Molecular analysis of RNA isolated from these plants showed that PR genes were not induced by INA in these plants. The plants also accumulate SA following pathogen infection. In other respects the *nim1* mutant plants appear phenotypically normal. Map-based cloning of the *nim1* gene is underway.

DISSECTING SAR SIGNAL TRANSDUCTION

As noted above experiments with transgenic NahG plants have been useful in showing that SA is required for SAR induction by a pathogen. Further, these plants have shown that the chemical activators act as SA analogs or activate SAR signal transduction downstream of SA accumulation. These results have led to a more refined model of SAR signal transduction, as shown in Figure 2. NahG plants have also been useful in understanding the mutants. Using crosses between the NahG and *lsd* plants we have determined that the *lsd* mutants fall into two classes; those that require SA for lesion formation (i.e. lesions are suppressed when *nahG* is expressed in the plant) and those that form lesions independently of SA (i.e. crosses to NahG retain lesions). Interestingly, *PR* gene expression and resistance are abolished in both classes when crossed to NahG plants. This indicates that necrosis is upstream of SA accumulation in the signaling pathway but that events downstream of SA may also play a role in lesion formation, perhaps by activating cell death pathways. Crosses between NahG and the *cim* mutants are in progress and will be helpful in determining the requirement for SA for the various *cim* phenotypes.

Arabidopsis mutants that are compromised in the ability to respond to the phytohormone ethylene or to methyl jasmonate have been identified by a number of labs (Bleecker *et al.*, 1986, Guzman and Ecker, 1990, Staswick *et al.*, 1994). Both ethylene and methyl jasmonate have been implicated as signal molecules in plant-pathogen interactions. We have used these mutants to demonstrate that neither ethylene (Lawton *et al.*, 1994) nor methyl jasmonate (Lawton *et al.*, 1996) are required for induction of SAR by chemical activators. Furthermore, we have shown that ethylene insensitive mutants are not compromised in SAR following infection with a necrogenic pathogen (Lawton *et al.*, 1995). However, ethylene may have a role of enhancing tissue sensitivity to low concentrations of SA (Lawton *et al.*, 1994).

The *nim1*-1 mutant has been useful in characterizing the SAR chemical activators. This mutant is blocked in the ability to respond to SA and INA (Delaney *et al.*, 1995) as well as to BTH (Lawton *et al.*, 1996). Thus, it appears that all three of these molecules act on the same signal transduction pathway. Crosses between *nim1*-1 and the *cim* and *lsd* mutants are underway and will provide the basis for interesting epistasis studies that may give insight into the interactions between various steps in the SAR signal transduction cascade.

SUMMARY

Arabidopsis has been established as a useful model plant for SAR. This work has led to a more detailed model of the steps involved in transduction of the SAR signal as shown in Figure 2. Mutants with enhanced disease resistance have been identified.

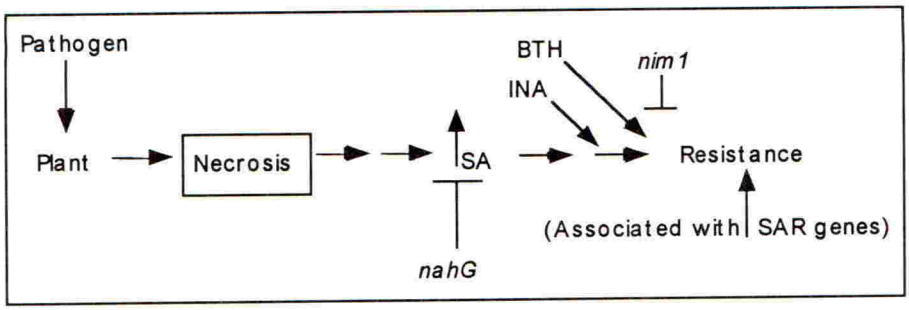

Figure 2. Refined working model of SAR signal transduction pathway. Experimental analysis has enabled placement of chemical activators and *nim1* mutant on working model of SAR signal transduction. BTH and INA may activate the same step of the pathway. For discussion see text.

In addition to increased disease resistance these plants have elevated levels of the PR genes associated with SAR. Some of these mutants are morphologically indistinguishable from wildtype Arabidopsis (e.g. *cim*) while others develop spontaneous necrotic patches that resemble lesions that are associated with infectious diseases (e.g. *lsd*). Isolation of the genes responsible for these mutant phenotypes is underway. Mutants that are non-responsive to SAR activators have also been identified (e.g. *nim*). Experiments with *nim1* plants have shown that the activators INA and BTH act on the same signal transduction pathway as SA, an endogenous plant molecule integral to SAR. Other Arabidopsis mutants have been used to demonstrate that jasmonates and ethylene, two other endogenous signal molecules, are not required to mediate SAR signal transduction. Thus, Arabidopsis is indeed a powerful tool to understand SAR.

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SESSION 8B

PESTICIDE RESIDUE ANALYTICAL TECHNIQUES – NOW AND IN THE FUTURE

Chairman	Dr C J Griffiths *Scottish Agricultural Science Agency, Edinburgh*
Session Organiser	Dr R Elliott *Zeneca Agrochemicals, Bracknell*
Papers	8B-1 to 8B-4

LC/MS: AN INCREASINGLY IMPORTANT TOOL FOR THE ANALYSIS OF AGRICULTURAL RESIDUES

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ABSTRACT

Many of the new agrochemical products under development are not amenable to traditional analytical techniques. The current generation of environmentally friendly, lower use rate, biochemically derived compounds are often more polar and less thermally stable than their predecessors. One of the most powerful techniques currently available to analytical chemists working with these compounds in both qualitative nature-of-the-residue and quantitative magnitude-of-the-residue studies is the combination of liquid chromatography and mass spectrometry (LC/MS). This paper will present a layman's overview of LC/MS technology, how it has evolved, and how it is applied to agrochemical research today and in the future.

FUNDAMENTAL BACKGROUND

The combined technique of liquid chromatography interfaced to mass spectrometry (LC/MS) is one of the most powerful techniques available to analytical chemists working in the area of residue analysis. Liquid chromatography is a widely used tool which separates complex mixtures of molecules into groups of like components so that the individual species in the mixture can be measured. A liquid is used to move the mixture through a column filled with a material that affects the separation such that, under ideal conditions, the components of the mixture come out of the column one at a time. Mass spectrometry is a very sensitive analytical tool that has many qualitative and quantitative applications including determining the structure and molecular weight of an analyte. Under a vacuum, compounds in the vapor state are subjected to some sort of energy transfer to apply an electric charge to the analyte creating an ion. The electric charge is used to move the ions through a mass filter which can be scanned to determine the mass of the ions or set such that only ions with a specified mass are detected. In many cases the energy transfer causes the analyte to explode into a variety of fragments which are then separated by their mass. The resulting fragmentation pattern or mass spectrum can be used as a chemical "fingerprint" to identify the analyte. In recent years researchers have successfully combined these two tools by using mass spectrometry as a detector for liquid chromatography. Commercial LC/MS instruments are now available which can be used for nature-of-the-residue studies to identify unknown metabolites, magnitude-of-the-residue studies to measure very low levels of residues, and other important agrochemical research.

The combination of chromatography and mass spectrometry is not new. The tremendous ability of gas chromatography (GC), which was developed in the 1950s, to separate and analyze complex mixtures is widely appreciated. GC/MS instruments have been used for many years in residue research. The mass spectrometer adds specificity of identification, selectivity, the ability to deconvolute compounds that are not completely separated, and the use of stable isotopically labeled internal standards to the chromatographic analysis. However, many samples simply are not amenable to gas chromatographic analysis without chemical manipulation. They are either thermally unstable and decompose under the conditions of separation, are insufficiently volatile and cannot pass through the column, or are so polar that they stick to columns and yield ineffective separation. There are many techniques available to force fit compounds to GC analysis, all of which involve changing the analyte to another more compatible compound. It has been estimated that only 20% of known organic compounds can be analyzed by GC. LC though is not limited by volatility or thermal stability. Therefore, LC is ideally suited for the separation of a wide variety of biochemicals, labile natural products, high molecular-weight molecules, and less stable or more polar organic compounds.

In recent years there has been a shift in the type of agricultural chemicals under development. The new compounds are more environmentally friendly, have lower use rates, and are often targeted at specific biochemical pathways. The new compounds are similar to drugs developed by pharmaceutical companies and have different chemistries than their predecessors. The new agricultural compounds are more polar, less volatile, and more thermally labile than the organophosphates, and chlorinated compounds of the past. These new molecules require new analytical chemistry technologies, such as LC/MS, for their development and registration.

LC/MS is applicable to several different parts of the analytical phase of agrochemical compound development. LC/MS can be used for nature-of-the-residue studies to identify metabolites in plant and animal metabolism studies and to identify degradates in environmental fate studies. Determining the structure of polar metabolites has always been one of the biggest challenges facing the residue chemist. LC/MS provides another technique that can assist in solving this problem. Many metabolites and degradates of agricultural compounds are more polar than their parent species and are often only suited to LC analysis. The mass spectrometer is used in a qualitative mode to directly determine the structure and/or molecular weight of metabolites separated by LC. Used in the same manner, LC/MS can also be useful in characterization studies to determine the identity of trace level impurities present in technical grade products and formulations. LC/MS can be applied to magnitude-of-the-residue studies to quantify residues in either raw agricultural commodities or environmental samples from dissipation studies. For these targeted analyses, the mass spectrometer is used in a quantitative mode as a very sensitive and very selective detector for the LC which is often optimized for extremely fast separations. LC/MS can also be used as a quick screening method to determine if a more rigorous analysis of a particular sample is required. The mass specificity of the MS detector can be used advantageously on sample extracts which have undergone considerably less wet chemistry preparation than is usually needed for traditional LC detectors. The recent infusion of combinatorial chemistry to new compound discovery can also utilize LC/MS to identify synthesis products and to screen for common analyte substructures.

Historical Perspective

LC/MS, although a relatively new analytical technique, has been around for several years. It has only been in the last several years that it has become robust enough to be useful for a wide variety of applications. The marriage of LC to MS is not an easy one. Like human marriages, it relies on compromise from both techniques to make a union in which the combination is more powerful than either of the single units on their own. There are three important factors to be considered in trying to combine LC and MS. Unlike the much older GC/MS interface in which the gas phase effluent from the GC can be directly inserted into the mass spectrometer's vacuum, the liquid effluent from an LC column must be manipulated before it enters the mass spectrometer. The liquid mobile phase effluent leaving the LC column at a typical rate of 1 mL/minute would generate approximately 4 liters of gas when introduced into a mass spectrometer. This volume of gas completely overwhelms the instrument's vacuum system. The analytes must be vaporized and ionized so that the mass spectrometer can measure and detect them. The vaporization and ionization must be accomplished without destroying the analyte, or all the advantages of using LC will be lost. And finally, the transfer of the analytes from the LC to the MS must be done in a way that retains the chromatographic performance of the LC. The interface will be of no use if the separated components of a mixture are mixed together again before entering the detector.

The first LC/MS interface techniques that overcame these obstacles were awkward, difficult to use, unreliable, and had poor sensitivity. The direct liquid introduction (DLI) technique developed by McLafferty in 1973 was very simple, but could only handle an LC flow rate of a few microliters per minute and was therefore not widely accepted. The moving belt interface introduced by McFadden in 1976 and then the thermospray interface developed by Vestel in 1983 were two other early LC/MS interfaces, each of which had their merits, but that have since been replaced by newer techniques. The continuous flow fast atom bombardment (CF/FAB) interface introduced by Ishi in 1986 is a variation of the DLI interface best suited for the analysis of large molecules. This technique is still in use in some laboratories, primarily applied to peptide and protein research.

There are three LC/MS interfaces or techniques that were developed in the mid 1980s that are still popular today. They are the particle beam interface (developed by Browner), atmospheric pressure chemical ionization (APCI; developed by Thompson et al.), and electrospray (developed by Fenn). The sum of these three techniques have made LC/MS a usable tool to the analytical chemist. The techniques are much more robust, predictable, and user friendly. Each of the techniques has its own advantages and disadvantages. There is no single LC/MS technique that is applicable to all situations. Taken together, these three techniques can detect a wide range of organic species, can be used with high and low LC flow rates, accept a wide variety of mobile phases including 100% water, volatile and non-volatile buffers, and take advantage of many available mass spectroscopic techniques.

The techniques are still not, however, as simple to use and the data generated not as easy to understand as GC/MS techniques. To get the most out of LC/MS requires a trained mass spectroscopist. To paraphrase Jack Henion, one of the leaders in LC/MS research, operating an LC/MS is not like driving a car in which all one has to do is turn on the key and drive away. It is more like flying a plane in which one has to understand what all the controls and

dials are for and how they all interact to make a successful flight. LC/MS is still not as easy as taking a developed HPLC method and adding a mass spectrometer. If it is known that LC/MS is going to be employed, it is best to develop the chromatographic conditions incorporating the limitations that the MS can impose. There are still compromises that must be made. LC/MS interfaces can not handle non volatile mobile phase modifiers and buffers such as phosphoric acid. Instead, organic acids such as formic or acetic acid should be substituted. Certain interfaces can not handle high concentrations of water in the mobile phase and therefore the chromatographic separation parameters need to be adjusted to avoid this situation. In all cases it is important to understand what the effect of these modifications will have on the separation and how they can effect the mass spectral data as well.

TYPES OF LC/MS INTERFACES AND MASS SPECTROMETER TECHNIQUES

There are two processes common to each of the LC/MS interfaces or techniques; a vaporization process and an ionization process. Each of the three popular interfaces use a nebulizer to remove the liquid LC effluent (solvent) and vaporize the analyte. There are three basic types of nebulizers; pneumatic, thermal, and electrostatic. Pneumatic nebulizers work under the same process used by old fashioned insect sprayers and paint sprayers in which a stream of air or gas is used to aspirate a liquid without using heat. Thermal nebulization is similar to a whistling tea kettle and uses heat to vaporize the liquid as it is forced through a small orifice. Electrostatic nebulization uses a high voltage to disperse the liquid into a vapor and is used by ink-jet printers. In their current state of development, most of the LC/MS interfaces use a combination of two of the nebulization methods. A discussion of the ionization methods available to LC/MS is beyond the scope of this paper, as they are all not yet completely understood by the scientific community. There is however one important distinction between the ionization processes employed. Ionization can either be accomplished by removing the solvent and then ionizing the analyte with traditional MS ionization techniques, or by ionizing the analyte while in the liquid and removing the analyte from the solvent as an ion.

Particle Beam

The particle beam interface is probably the easiest of the three interfaces to use. It uses pneumatic nebulization to strip away the solvent and introduce a stream of neutral analyte molecules into the mass spectrometer where traditional mass spectrometer ionization techniques can be employed. The ionization process used most often is electron impact (EI) which is a hard ionization process that often produces many ion fragments. The mass spectra that are produced are full of information that can be used to deduce the structure of the analyte. Because the mass spectra obtained from a particle beam are similar to mass spectra obtained by GC/MS techniques, they can be matched against computer databases or libraries of spectra which contain hundreds of thousands of spectra as an aid in determining the identity of, or substructures contained in, unknown analytes. Particle beam interfaces are not very sensitive and do not work for all types of analytes. It works best for relatively non-polar analytes that are easily separated from the solvent. Because most particle beam interfaces do not use a heated nebulizer, they can not handle high concentrations of water in the LC effluent or non-volatile inorganic buffers. They need a high concentration of organic solvent

for efficient solvent evaporation. Particle beam techniques are best suited to situations in which the analyst has a lot of sample to work with, such as synthesis confirmation, characterization studies, and identification of unknowns that are not sample limited.

Atmospheric Pressure Chemical Ionization (APCI)

APCI uses a pneumatically-assisted thermal nebulizer to aspirate the LC effluent past a corona discharge needle to affect a soft proton-transfer chemical ionization in the liquid state before complete desolvation and mass analysis. The vaporization and ionization take place at atmospheric pressure outside the mass spectrometer vacuum system. The ionized analyte is transferred into the mass spectrometer through a series of very small orifices. APCI has good sensitivity and is fairly universal for all LC type compounds. This interface can handle high LC flow rates, a wide range of LC effluents including 100% water, and is not hindered by many non-volatile buffers. APCI can be useful for determining the molecular weight of unknown compounds and for targeted quantitative analysis which often use rapid sample introduction methods. APCI is probably the most useful interface for agrochemical research.

Electrospray

Electrospray and the closely related pneumatically-assisted electrospray techniques are also soft ionization techniques which are applicable to compounds that exist as pre-formed ions in solution. Electrospray uses electrostatic nebulization to put an electric charge on droplets which then expel ions as the droplet evaporates. This technique is good for organic salts and compounds with acidic or basic functional groups that can be ionized by controlling the solution pH. The limitation that electrospray has for only working with slow LC flow rates has been overcome by the introduction of techniques which use pneumatically-assisted electrostatic nebulizers. These can handle normal LC flow rates and a wider range of LC effluents. Electrospray techniques are sensitive and useful for determining the molecular weight of unknowns. These techniques are also well suited to targeted analysis. Another feature of electrospray ionization is that if the analyte has more than one ionic site on the molecule, more than one charge can be attached. In effect, this reduces the apparent mass of the ion which the mass spectrometer measures. Large molecules such as small proteins with molecular weights in the tens of thousands have an ionizable site on each amino acid and can accept many charges and can thus be analyzed with mass spectrometers which have an upper mass limit of only a couple thousand.

Tandem Mass Spectrometry (MS/MS)

The one draw back to the soft ionization techniques is that they usually only produce one ionic species. This can be acceptable if one knows what compound was analyzed, but often more information is needed to accomplish the experimental goals. More than one characteristic ion is usually needed to confirm the identity of targeted analytes and many fragment ions are needed to elucidate the structure of unknown compounds. MS/MS techniques use collisions between the ions and an inert gas to induce fragmentation and are, therefore, often used in conjunction with soft ionization techniques.

There are two basic types of MS/MS used for LC/MS applications. The "in-source" fragmentation technique is available on instruments which have only a single mass filter, and collision cell fragmentation techniques are available on instruments which have two mass filters. In-source fragmentation is accomplished by accelerating the ions through a flow of inert gas before they enter the mass filter region of the mass spectrometer. Some of the ions collide with the atoms in the gas flow and are fragmented into smaller product ions which are then measured by the mass filter. When used with clean samples or very good chromatography, this technique can be used to generate a limited amount of additional information about the original precursor ion. The collision cell instruments can be operated in several different modes and can produce a wealth of information about a compound. These instruments are comprised of two mass filters connected in series by a collision cell which is filled with the inert gas. Precursor ions selected by the first mass filter enter the collision cell, collide with the gas atoms, fragment into product ions, and are then measured by the second mass filter as it is scanned. The product ion spectrum obtained can be similar to the spectrum obtained from in-source fragmentation and is useful in determining the structure of unknown metabolites, or unknown trace impurities. The instrument can also be operated by scanning the first mass filter and fixing the second mass filter such that only a single product ion can pass through to the detector. In this mode of LC/MS/MS operation all molecules in the sample that have a common substructure can be detected. For example, the second mass filter could be set to pass the glucose ion and then any glucose conjugates in the sample could be detected or the second mass filter could be set to pass the ion representing a central ring substructure of a parent compound and then any metabolites containing that ring structure could be detected in one LC analysis. A third mode of operation is to scan both mass filters at the same time with a fixed mass difference. In this mode all compounds that lose the same weight substructure during fragmentation can be detected. This technique is useful in screening analyses and can be used for example to detect all compounds containing chlorine, or all the acids in a mixture.

The MS/MS techniques are some of the most powerful analytical techniques available. The knowledge that one ion came from another ion is much more informative than just observing the two ions together in the same spectrum. This same relationship provides an extremely selective detector for targeted analyses. Because this detector can be so selective, residue methods can be developed in which the wet chemistry is reduced to a single extraction step. The LC provides the sample cleanup and the MS/MS system provides an unequivocal detection. Depending on the application and the nature of the compounds under investigation, MS/MS can be either more or less sensitive than MS techniques. MS/MS method development is often not routine, and can require a lot of sample, especially for identification of unknowns. But once a targeted analysis is established, it can be very simple and extremely sensitive.

CONCLUSIONS

There are several drivers that are expanding the use of LC/MS in agrochemcial analytical research. The compounds that are currently being developed are biologically oriented, more polar, non-volatile, and thermally unstable and are, therefore, not amenable to the GC based

analytical techniques used in the past. The development of low use rate compounds preclude the use of common LC detectors because they are not sensitive enough. LC/MS can be used for qualitative analyses to assist in the identification of metabolites, degradates, or trace impurities. LC/MS can also be used for quantitative analyses in both single and multi residue methods.

The current approaches to LC/MS are much more robust and useful than earlier instruments. There is still ongoing research in this area of analytical chemistry and the techniques will continue to improve. There is no single universal LC/MS technique that is applicable to all the types of analyses conducted in the agrochemical laboratories. To be fully functional, an LC/MS instrument has to have more than one interface. Over time however, the information provided by LC/MS techniques can simplify and reduce costs for agrochemical research.

SIMULTANEOUS DETERMINATION OF AN INSECTICIDE AND SEVERAL OF ITS METABOLITES IN PLANT MATERIAL USING ELECTROSPRAY HPLC/MS/MS

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ABSTRACT

High performance liquid chromatography (HPLC) separation coupled to a tandem quadrupole mass spectrometer (MS) via an atmospheric pressure electrospray ionisation interface provides an extremely powerful tool for rapid, selective and sensitive analysis of plant protection products in crops. A residue analytical method was developed using this technique for the quantitative determination of the insecticide triazamate and all of its major metabolites in sugar beet and apple in one single chromatographic run. The limit of quantification (LOQ) proved to be at 0.01 or 0.02 mg/kg.

INTRODUCTION

Triazamate is a carbamoyl triazole insecticide with systemic activity (Anon., 1994). It is very rapidly metabolised in all biological systems studied so far giving rise to several metabolites (cf. Figure 1). The small UV absorption coefficients of the compounds do not permit their quantification at trace analytical levels by HPLC/UV and a determination by GC/NPD is ruled out for some metabolites since they cannot be vaporised without degradation.

During the development phase of triazamate, the original, very laborious and time-consuming residue method allowed the GC/NPD determination of the parent compound and one of the metabolites (CL 900056), the latter after derivatization. Two further GC methods were then developed for the analysis of metabolite CL 900057 and for the determination of an apple metabolite (CL 900100). Subsequently, a method with HPLC/MS/MS determination was developed by a contract laboratory to cover metabolites CL 900057 and CL 900101 in plants.

It was thus considered necessary to develop a combined and simplified method for the simultaneous determination of the active ingredient and the above metabolites as well as two additional metabolites found in sugar beet.

MATERIALS AND METHODS

Sample processing and extraction

Plant material is ground using a food mincer. A 30 g representative aliquot of sample homogenate is extracted with 140 ml of a methanol/acetonitrile/water/glacial acetic acid mixture (75/19/5/1 V/V/V/V) in a glass suction filter using a blender. The extract is made up to 200 ml with methanol.

Purification

After filtration through a disposable 0.45 µm filter, a 5 ml aliquot of the extract is subjected to automated gel permeation chromatography (GPC). The GPC system consists of a solvent delivery system, an autoinjector system, a chromatographic column packed with TosoHaas Toyopearl HW-40S (length 28 cm, i.d. 2.6 cm, particle size 20 - 40 µm, pore size 5 µm, exclusion limit 3000 dalton) and a fraction collector. A methanol/water/glacial acetic acid mixture (97.2/2.5/0.3 V/V/V) is used as a solvent system at a flow rate of 3.3 ml/min. The 29 to 46 min fraction is reduced to nearly dryness by rotary evaporation and the residue redissolved in 2 ml of acetonitrile/1% acetic acid (15/85 V/V) for HPLC determination.

Determination

After filtration through a disposable 0.2 µm filter, 100 µl of the sample solution are injected by an autosampler into a high pressure gradient system with two binary HPLC pumps and a high pressure mixing chamber. The separation is performed on a 250 × 4.6 mm reversed-phase HPLC column (Phenomenex 00G-3300-E0: particle size 5 µm) using a water/acetonitrile mobile phase gradient (acidified with 0.1% formic acid) at a flow rate of 1 ml/min (t = 0 min: 85% water, t = 11 min: 85%, t = 14 min: 55%, t = 20 min: 55%, t = 22 min: 0%, t = 27 min: 0%, t = 30 min: 100%). The column effluent is split by a 'zero dead volume' tee piece so that 100 µl/min are directed to the electrospray probe of a Fisons VG Quattro tandem quadrupole mass spectrometer operated by VG MassLynx software. Positive ionisation MS data are acquired in multiple reaction monitoring (MRM) mode for quantitative analysis using the LCQuan module of the VG MassLynx software.

Evaluation

Evaluation is carried out using calibration curves. Calibration solutions in the concentration range of 2.5 to 100 ng/ml (compounds dissolved in acetonitrile/1% acetic acid (15/85 V/V)) are injected into the HPLC/MS/MS system. The peak areas obtained are then plotted against the corresponding amount of substance in the calibration solution.

RESULTS AND DISCUSSION

The insecticide triazamate (IUPAC name: ethyl(3-*tert*-butyl-1-dimethylcarbamoyl-1*H*-1,2,4-triazol-5-ylthio)acetate) is metabolised in plants to a number of metabolites of which the most frequent ones are depicted in Figure 1.

Based on already existing extraction methods which only allowed extraction of either polar or non-polar compounds from Figure 1, several solvent mixtures were tested to identify one which assures the simultaneous extraction of both the polar and the non-polar substances from plant material. The efficiency of extraction of all relevant compounds with an acidified methanol/acetonitrile/water mixture was proven with sample material derived from the respective plant metabolism studies which were carried out using ^{14}C-labelled substances.

The conditions required to extract the analytes from crop samples resulted in a solution containing considerable amounts of coextractives. However, it could be shown that the only clean-up step necessary was to subject an aliquot of the crude extract to gel permeation chromatography

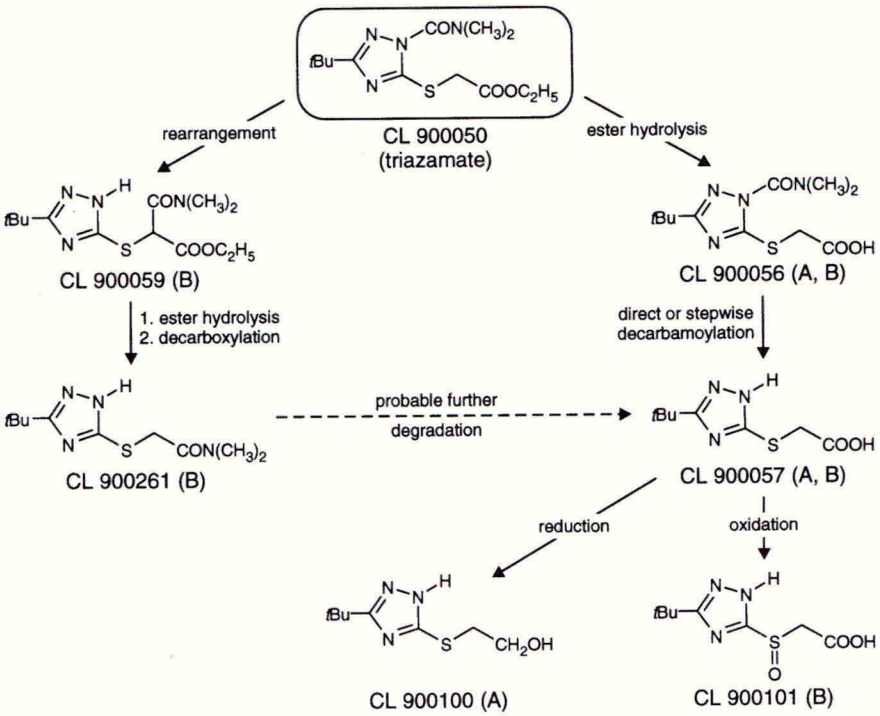

Figure 1. Metabolism of triazamate in apple (A) and sugar beet (B).

with aqueous methanol as eluent using a semi-rigid size-exclusion chromatography resin. A hydroxyethyl methacrylate gel with a exclusion limit of 3000 dalton allowed coextractives of higher molecular weight to be separated satisfactorily from the analytes.

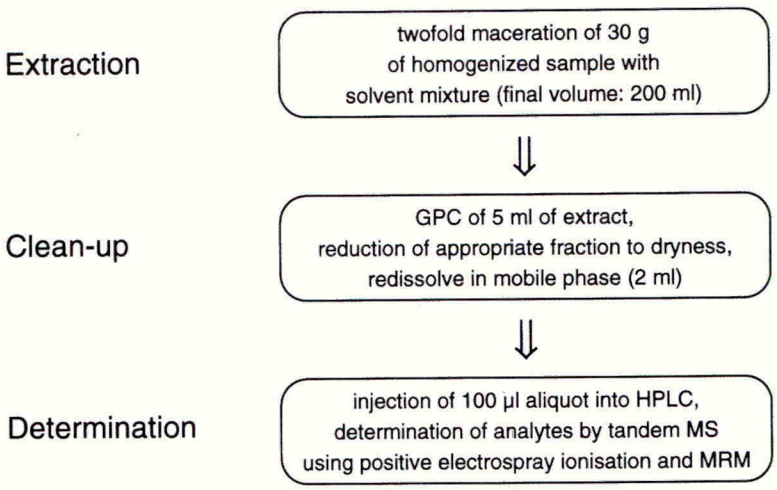

Figure 2. Procedure for the analysis of triazamate and metabolites in crops.

The final HPLC separation was performed with a reversed phase packing based on spherical silica using a water/acetonitrile mobile phase gradient. The column packing is completely end-capped (C18 carbon load: 18.4%) to help prevent silanol interaction with the analytes which have both acidic and basic functional groups. The column effluent was split to the tandem mass spectrometer at a 1:10 ratio. The positive electrospray ionisation MS data were acquired in multiple reaction monitoring (MRM) mode for quantitative analysis.

Figure 2 summarises the very compact analytical procedure.

The soft atmospheric pressure positive electrospray ionisation of small polar molecules generally leads to singly charged protonated molecular ions. For triazamate, this pseudo-molecular ion $[M+H]^{\oplus}$ is observed at m/z 315 together with the 'sodiated' molecule $[M+Na]^{\oplus}$ at m/z 337 and the 'potassiated' $[M+K]^{\oplus}$ at m/z 353. In the collision cell of the tandem mass spectrometer, selected (parent) ions undergo fragmentation induced by a neutral gas species at low energies (normally a few µbar of argon at some eV are sufficient). This process produces daughter ions that can be mass analysed by the second quadrupole giving rise to a daughter ion scan. In our example, only one daughter ion of triazamate is observed at m/z 72 which is $[CON(CH_3)_2]^{\oplus}$. Parent and daughter mass spectra are both shown in Figure 3.

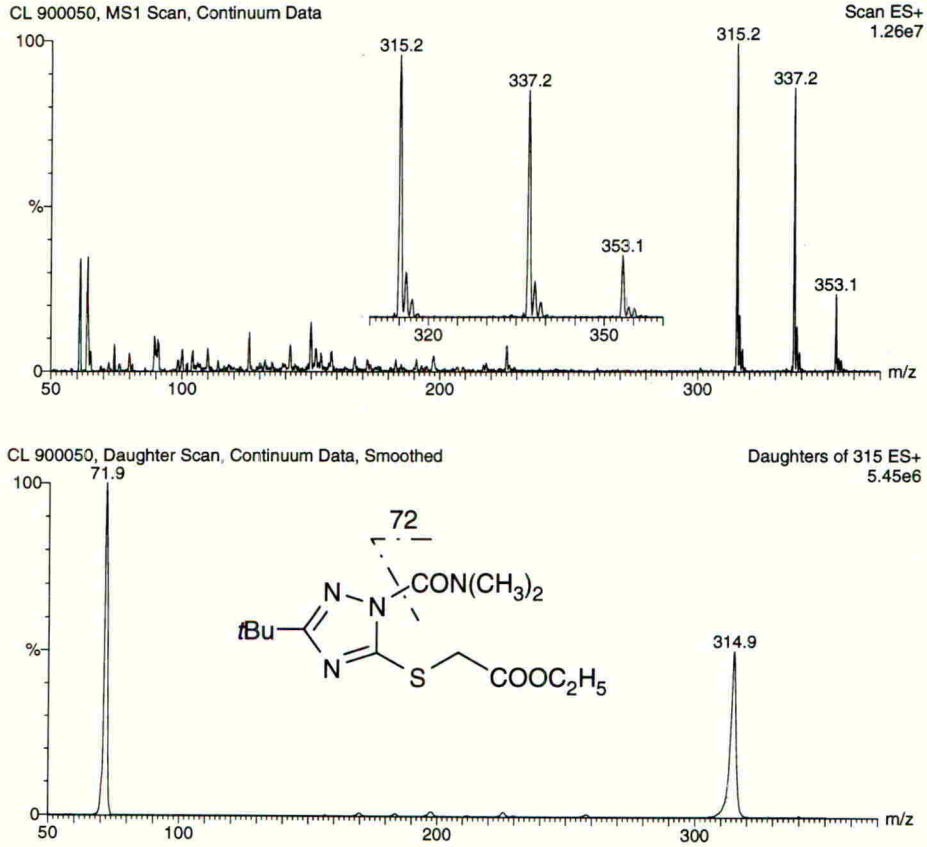

Figure 3. Parent and daughter electrospray mass spectra of triazamate.

The observed transition from the parent ion m/z 315 to the daughter ion m/z 72 can be used for monitoring triazamate during an HPLC run (hence 'reaction monitoring'). For each compound to be monitored, a suitable transition has to be found. The respective masses of all the compounds are given in Table 1 (for formulae cf. Figure 1).

Table 1. Parent and daughter ions of triazamate and metabolites.

Compound code	Parent ion (m/z)	Daughter ion (m/z)
CL 900050	315	72
CL 900056	287	197
CL 900057	216	170
CL 900059	315	223
CL 900100	202	156
CL 900101	232	172
CL 900261	243	170

If the transitions listed above are monitored while a standard calibration solution containing the compounds of interest at a concentration of 5 ng/ml is chromatographed, the result shown in Figure 4 is obtained.

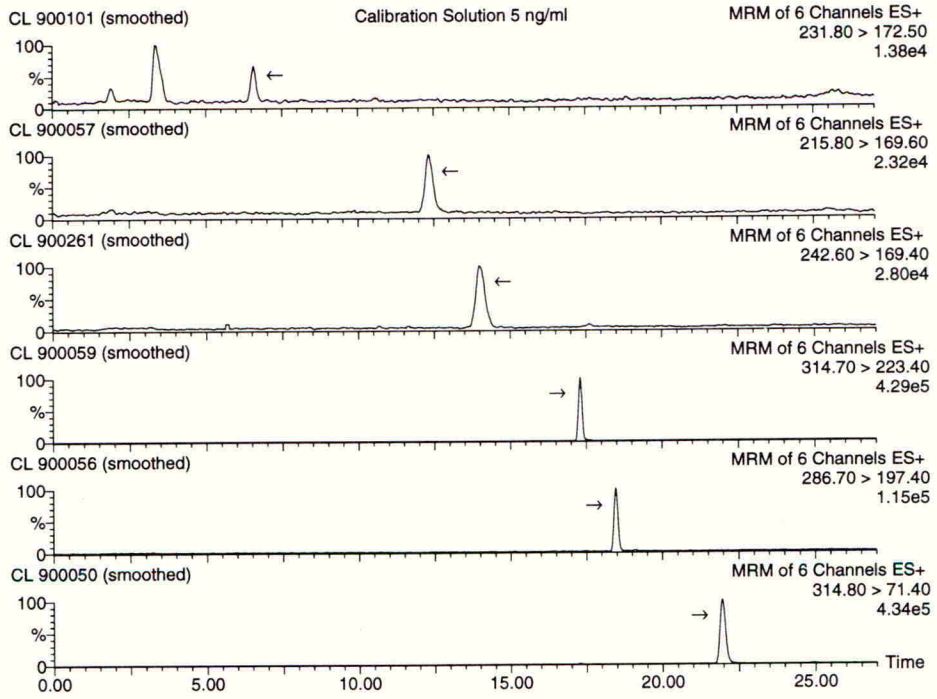

Figure 4. MRM mass chromatograms of triazamate and five metabolites.

For quantification, the concentration of each compound is determined by comparison with a set of at least four calibration standards. The peak areas of (external) standards obtained from the chromatograms of the MRM experiments are plotted against the concentration and the calibration curves are constructed covering the range from 2.5 or 5.0 ng/ml to 100 ng/ml. A typical calibration curve is shown in Figure 5.

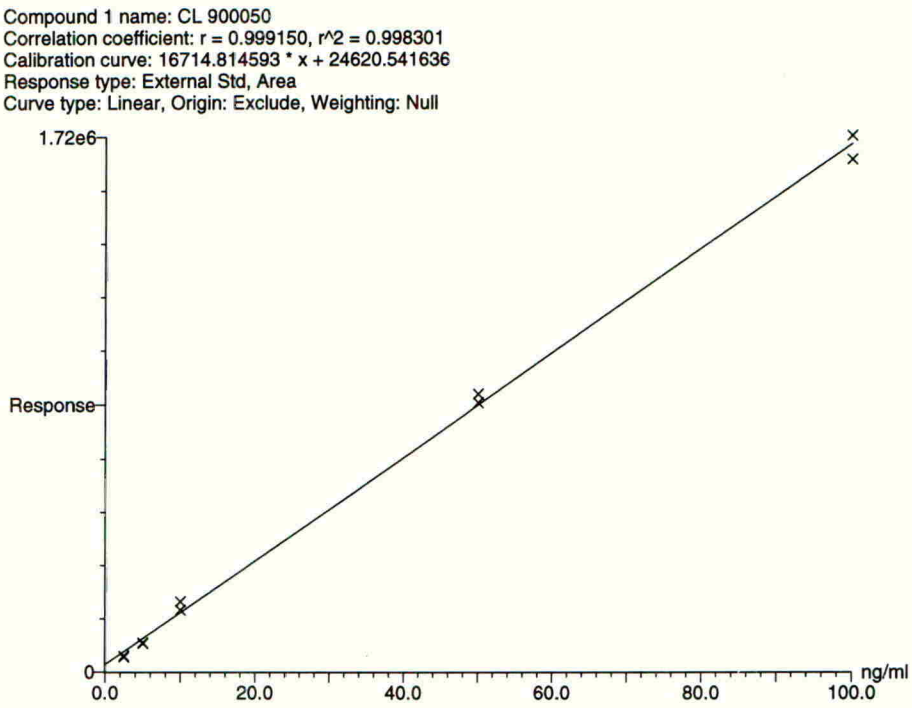

Figure 5. Calibration curve for triazamate (CL 900050).

Linear least squares regression (no weighting, origin excluded) yields calibration lines that are used to calculate the concentration of each of the six analytes in e.g. sugar beet. Correlation coefficients greater than 0.995 are obtained over nearly two orders of magnitude of concentration for all compounds.

Figure 6 shows the chromatograms obtained from the analysis of an untreated control sample of sugar beet roots. Due to the selectivity of the technique, no endogenous components, i.e. no interfering peaks originating from the matrix, are generally observed, thereby proving the specificity of the analytical method.

The validated sensitivity (limit of quantification, LOQ) of the method proved to be at a level of 0.02 mg/kg for CL 900057, CL 900059, CL 900100, CL 900101 and CL 900261 and even at 0.01 mg/kg for both triazamate (CL 900050) and metabolite CL 900056. The chromatograms from an untreated sugar beet root sample spiked with a mixture of all six analytes at a level of 0.02 mg/kg is given in Figure 7.

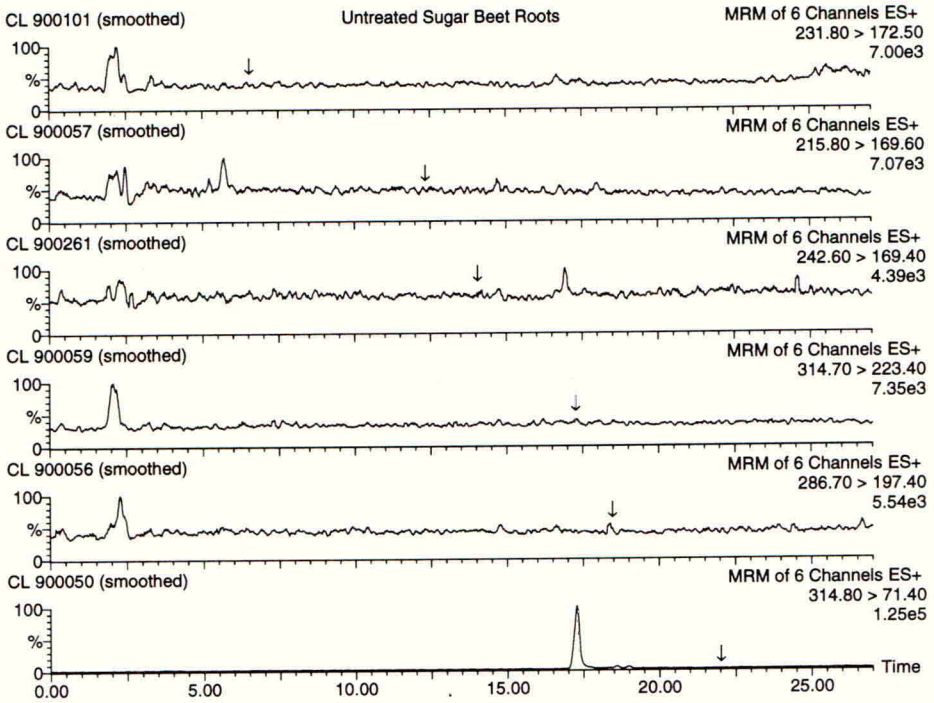

Figure 6. MRM mass chromatograms of untreated sugar beet roots.

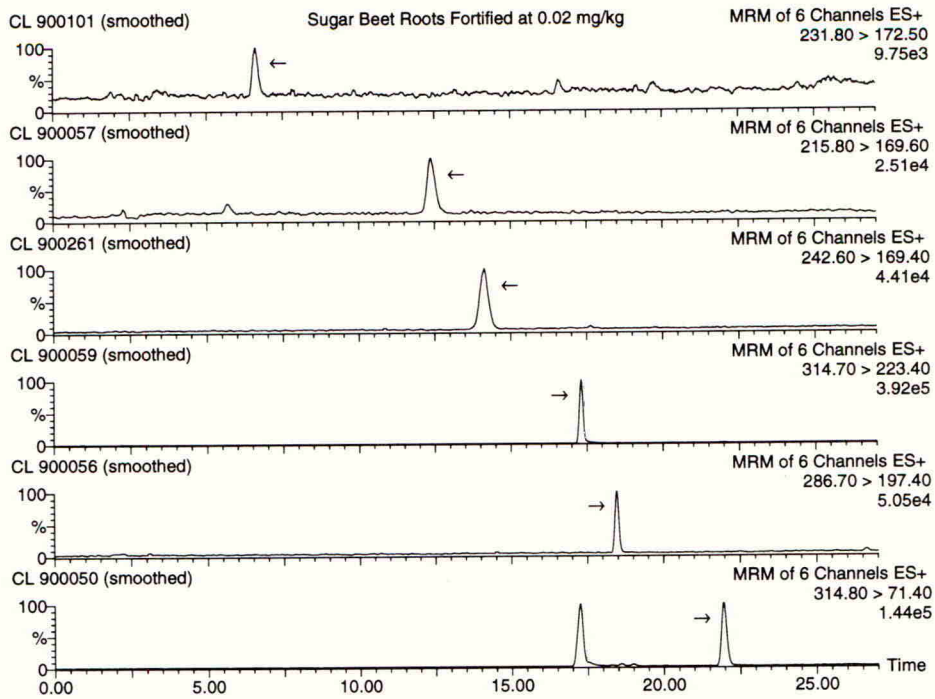

Figure 7. MRM mass chromatograms of sugar beet roots fortified at 0.02 mg/kg.

Recovery experiments carried out at concentration levels between the LOQ and 1.0 mg/kg during method validation or during analyses of real field-derived samples give values typically ranging between 70 and 110% for all compounds (except CL 900059: 60 to 100%) with a relative standard deviation (coefficient of variation, cv) of less than 20%. The methods are thus considered valid for the determination of triazamate and its metabolites in plant material in accordance to current registration requirements (Commission Directive 96/46/EC of 16 July 1996 Amending Council Directive 91/414/EEC Concerning the Placing of Plant Protection Products on the Market; Analytical Methods).

CONCLUSION

HPLC/MS/MS is a highly specific and sensitive method for the detection and determination of plant protection agents. In the residue analysis procedure described here, only one clean-up step is required after the initial extraction of plant material in order to provide sample solutions ready for quantitative measurement. The relatively mild ionising nature of the atmospheric pressure electrospray interface is suited in particular for polar, thermolabile substances or if the analytes of interest exhibit small UV absorption coefficients. To summarize, the use of electrospray HPLC/MS/MS allowed us to develop a rapid, robust, sensitive and selective residue method for the insecticide triazamate and all of its major metabolites in sugar beet and apple down to a limit of quantification of 0.01 or 0.02 mg/kg.

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PERFORMANCE AND APPLICATION OF PESTICIDE IMMUNOASSAYS IN CROP RESIDUE ANALYSIS

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ABSTRACT

The use of pesticide immunoassays in residue analysis is increasing in popularity. Most applications to date have been concerned with the quantitative determination of pesticide concentrations in soil and water. Recent work by a small number of researchers has applied the technology to crop residue analysis. This talk will review the current status of pesticide analysis in crops by immunoassays and their regulatory acceptability. The effectiveness of immunoassays as well as their limitations will be demonstrated using the example of the insecticide spinosad on fruit and vegetable substrates. Future application areas for the technique which could potentially support and improve the quality of regulatory packages will also be discussed.

INTRODUCTION

Immunoassays, have been utilised, particularly in clinical analysis, for nearly 40 years (Yallow and Berson 1960). Researchers have been developing pesticide immunoassays for some 18 years with more recently developed assays providing good performance characteristics. The literature is now abounding with pesticide immunoassay methods. These methods are mainly concerned with the determination of pesticides in surface or ground water. It is quite clear why immunoassays have gained in popularity; the promise of low capital and running costs, high sample throughput, excellent sensitivity and specificity all of which have been previously highlighted in numerous reviews (Hock 1993; Van Emon and Mumma 1990). Therefore, for the neutral observer, it may seem difficult to understand why today pesticide immunoassays are not routinely used in all residue laboratories. Some of the reasons for this position are clear, others nebulous. For example, during the late '80s immunoassays tended to be "oversold" with far too much promised and little delivered in terms of reproducible data of regulatory standard. Conventional residue analysis on the other hand has been well established over many years, nearly always with chromatographic separation and detection of residue components. As a new technology, immunoassay has no chromatographic system, is generally single component specific, is laced with foreign immunochemical terminology and has complex analytical formats. Consequently immunoassays have tended to be viewed cautiously by the residue chemist, particularly those who run multi-residue methods within their laboratories looking to determine a broad range of pesticide chemistries. However, it is less clear why some of the technical arguments persist. The variables in an immunoassay are controllable and if the correct hapten design is selected and subsequently good quality antibodies produced there is every chance a robust pesticide immunoassay can be developed.

This paper describes the approach taken by DowElanco in developing pesticide immunoassays to support a number of functional areas but particularly crop residue analysis.

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COMPANY STRATEGY AND PLANNING CYCLE

DowElanco has fostered a policy of developing immunochemical technology in a number of diverse areas such as pesticide discovery, manufacturing, biological and environmental sciences. It is viewed within the company as a core technology having short term and long term potential to aid its research and development programmes. There are obvious benefits from this multi-disciplinary approach in terms of costs and improved synergy.

Generally within the company antibodies will be raised to all new chemistries. For existing molecules these may or may not be subject to antibody production depending on intended needs and cost-benefit. In all cases the design phase is crucial. Hapten synthesis and the subsequent protein conjugation site are chosen to elicit either highly specific or generic antibody properties. In both cases it is important to consider all closely related structures to the pesticide and their potential for cross-reactivity. Either monoclonal and polyclonal antibody systems are selected depending on the degree of specificity and sensitivity required.

Following antibody production either a micro-titre plate assay is devised within our laboratories or more commonly a kit manufacturer is identified who will combine all components of the assay into a "user friendly" format for the analytical chemist. During kit development close liaison is maintained between kit manufacturer and sponsor so that strict quality performance is ensured. In addition to measuring the degree of cross-reactivity, the effect of matrix interference, pH, temperature, solvent content, drift and shelf life of the kit performance is evaluated. Only when a kit prototype has been fully tested by both parties, including an interlaboratory evaluation, will a full manufacturing run be instigated.

APPLICATIONS IN RESIDUE CHEMISTRY

Initially within our residue chemistry laboratories a number of applications for pesticide immunoassays were identified (Table 1). Of these, analysis of water and soil samples derived from various large field studies were considered a priority due to the obviously favourable economics. Consequently, over the years, soil and water methodology has been developed successfully for a wide range of pesticides and metabolites. More recently we have extended applications to milk, animal tissues and crop residue analysis.

Table 1. Potential Application Areas for Immunoassays in Residue Chemistry

- Crop residue analysis
- Soil analysis from field fate trials
- Soil analysis from carry over trials
- Sample analysis from operator exposure studies
- Water analysis from ground water and run-off studies
- Animal and biofluid analysis
- Ecotox study support analysis
- Stewardship issues

PESTICIDE IMMUNOASSAYS FOR CROP ANALYSIS

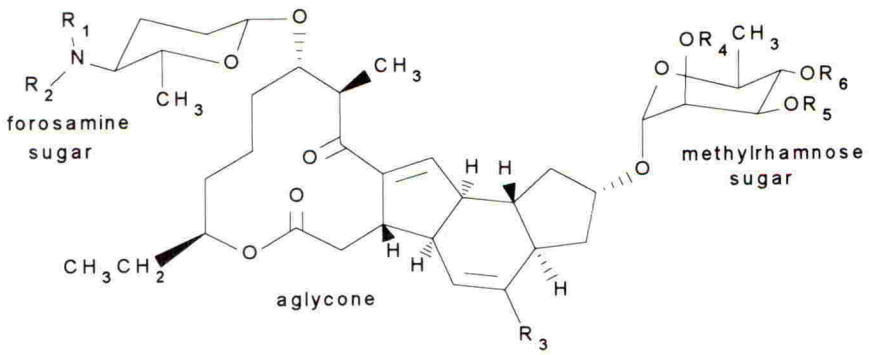
Examples of utilisation of pesticide immunoassays for crop analysis are emerging (Skerritt et al., 1992; Matthews et al., 1996). However, due to reasons outlined earlier in this paper, very little methodology has been applied to data generation for regulatory purposes from supervised residue trials.

At present a number of immunoassay crop data sets, derived by a number of companies, are being evaluated by the relevant regulatory bodies. Data generated by an immunoassay of the novel insecticide spinosad is presented here as an example of good quality of data that can be produced by this technology. This data is currently under evaluation by various government agencies.

SPINOSAD ASSAY

Spinosad is a naturally derived product which has shown activity against a variety of insect pests on cotton, tree crops and vegetable crops. The product is a mixture of two structurally related compounds, Spinosad A and Spinosad D (Figure 1).

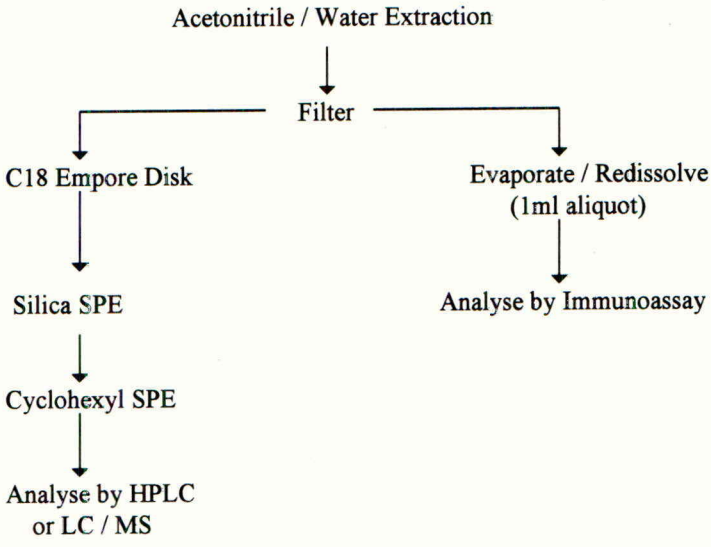
Figure 1.



Factor ID	R_1	R_2	R_3	R_4	R_5	R_6
A	CH_3	CH_3	H	CH_3	CH_3	CH_3
B	CH_3	H	H	CH_3	CH_3	CH_3
K	CH_3	CH_3	H	CH_3	CH_3	H
D	CH_3	CH_3	CH_3	CH_3	CH_3	CH_3
B of D	CH_3	H	CH_3	CH_3	CH_3	CH_3

Plant metabolism studies have shown that the total toxic residue consists of spinosad factors A,D,B,K and B of D. Polyclonal antisera raised against spinosad have shown affinity constants approximately equal to all spinosad factors and major metabolites. Thus utilisation of these antisera has produced a generic assay quantifying all total toxic residue. The antibodies have been incorporated into a magnetic particle assay kit system which has been applied to the development of a number of crop residue methods. For all crop substrates the same basic analytical procedure has been applied using both the immunoassay kit and the standard HPLC procedure (Figure 2). Both techniques have a limit of quantitation of 0.01 mg/kg.

Figure 2. Analytical Procedures for Determination of Spinosad in Crops

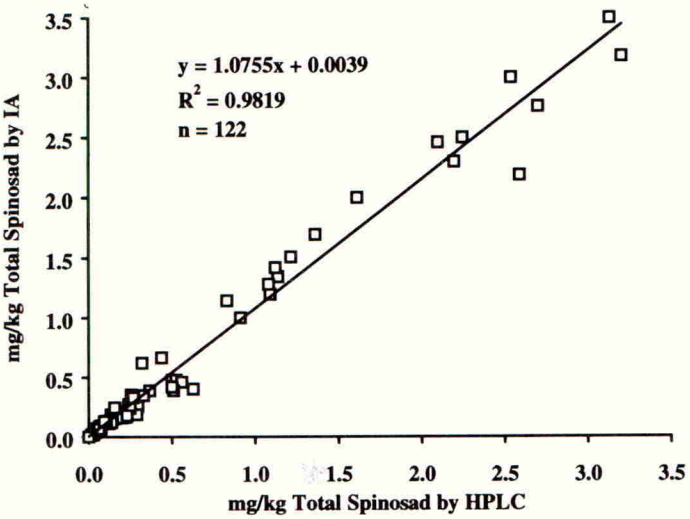


Accuracy and precision data generated at three fortification levels for nine substrates using the immunoassay procedure are shown in table 2. Data from the standard HPLC method gave similar recovery and precision values.

Table 2. Immunoassay Accuracy and Precision Data Generated from the Analysis of Spinosad in Various crops by Immunoassay.

	0.01 mg/kg		0.10 mg/kg		1.00 mg/kg	
	% Recovery	RSD	% Recovery	RSD	% Recovery	RSD
Apples	100	9	98	5	105	6
Broccoli	96	12	110	10	114	9
Cabbage	103	16	114	4	118	3
Tomatoes	105	14	106	2	107	6
Peppers	95	11	103	1	115	3
Spinach	106	10	108	7	77	7
Lettuce	100	4	105	3	77	11
Celery	104	16	98	5	77	6
Mustard	76	14	106	2	110	6

Figure 3. Comparison of Immunoassay and HPLC Data from Residue Trials



Samples taken from actual field trials covering eight different crops were successfully analysed using the immunoassay kit and HPLC. Immunoassay results produced no false positives and no false negatives. Correlation of determined concentrations against the HPLC method was excellent (Figure 3). The time taken to complete analysis by the immunoassay was 0.5 of a day compared to 2 days by the HPLC methodology for these samples.

PRACTICAL OBSERVATIONS

The main observations and experiences on the performance of a number of different pesticide immunoassays are :

1. Matrix effects happen fairly often and are not always predictable. For example sometimes drinking water gives rise to matrix effects, though often we see little or no such effects with many crop substrates. Generally matrix effects manifest themselves by producing overestimates of determinable concentrations. This is not uncommon in other analytical techniques such as GC/MS. Regardless of the technique, this effect is a variable which can be controlled by use of matrix standards, standard addition or by the introduction of a clean-up step in the analytical procedure.

2. The linear range of analyte concentrations in immunoassays is very narrow, often less than one order of concentration. This often requires a lot of re-dilutions of sample extracts. Although not ideal this can be achieved within work schedules due to the speed of operation of the whole procedure.

3. Kit storage shelf life is finite and therefore it is essential that work schedules are planned carefully with the right stock control of kits to be aligned with project timelines.

4. When developing an immunoassay kit it is essential to have detailed information about all the relevant closely related structures e.g. metabolites which may make the final assay prone to interference or cross-reactivity. However, for molecules in early phase development such details may not be available. In such cases waiting for this information could mean losing the benefits of the technology at the time of maximum sample generation during the registration schedules. This problem should not be of concern for existing molecules where all relevant chemistry profiles should be known at the time of planning antibody production.

5. It is often stated that immunoassays are best performed on hydrophilic non volatile molecules. In practise the performance of immunoassays for lipophilic volatile chemistries have often been equivalent or even better.

6. Since utilising this technology it has been striking to see the reduction in solvent usage and disposal costs.

REGULATORY ACCEPTANCE

A number of country, European and North American EPA guidelines are in existence covering performance requirements of analytical methods for residue analysis.
The important fundamentals of these performance requirements can generally be distilled into the following criteria (Table 3).

Table 3. Main Performance Requirements by Government Agencies Reviewing Pesticide Residue Data.

- Method specific for analyte/s in residue definition
- Recoveries 70 - 110% (120%)
- Precision $\leq$ +/- 20%
- Equipment and reagents used to be commercially available
- Confirmatory method needed
- Independant laboratory validation

At present there is an absence of any formal regulatory working documents or guidelines on use of pesticide immunoassays and subsequent interpretation of data generated by this technique.
The nearest to such a document is a report by the International Union of Pure and Applied Chemistry (Krotsky 1995a) and a paper produced by the Analytical Environmental Immunochemical Consortium. The latter paper outlines under what circumstances the technology is most appropriate and how a tiered approach to using immunoassays may be

used for analysing sample sets. This type of approach has been taken by the US EPA's Office of Pesticide Programmes and today a number of pesticide immunoassays for use on soil and water have been given approval (Telliard 1996). In all cases the registrant has to meet all the performance criteria outlined in Table 3. Additionally there has to be demonstrated reproducibility of the antibody pool and proof of a sufficient supply of antibody. The spinosad pesticide immunoassay has been well received by the EPA and has been granted approval for use in determining residues on various crops. Further, the methods supplied may be used for enforcement purposes.
In Europe regulatory agencies have been slowly changing their views on pesticide immunoassays from a position of conservatism to one of cautious optimism. Immunoassay methodology developed within BASF (Krotsky 1995b) for the analysis of quinmarec in various crops has been accepted by a number of these agencies. The German BBA and UK MAFF have both sponsored their own research programmes on the potential use of immunochemical techniques in residue analysis.

Fundamentally if the immunoassay has attained the performance criteria described for analytical methods in residue analysis and performs equal to or better than conventional methods, then regulatory acceptance should be granted.

CONCLUSIONS

After a slow start pesticide immunoassays are becoming widespread particularly when applied to soil and water systems. Application to crop substrates can be made if, as with any other analytical method, the performance of the methodology meets regulatory standards.

Research and development costs for agrochemical companies are always under pressure. Re-registration programmes of old products, line extensions and the development of new actives into the market place all add up to high cost and the need for greater efficiency. Today we are seeing many growers complain that support for "minor crops" is absent by most manufacturers as the companies consolidate on the major crop markets. The European Crop Protection Agency claim it costs approximately $200,000 for a residue package to support a minor crop (Buys et al.; 1994), it is obvious immunoassay technology within the residue laboratory must be utilised to impact on these costs with subsequent benefit to all.

With recent regulatory hurdles overcome and further development in methods for antibody production and new detection systems, immunoassays should become a valuable complementary analytical technique routinely used within the residue laboratory.

ACKNOWLEDGEMENTS

I would like to thank and congratulate Charles Mihaliak, Debbie Young and co-workers in DowElanco, Indianapolis, USA for their efforts in generating the Spinosad data used in this paper.

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ON-LINE ANALYSIS OF RAW RIVER WATER FOR TRACE LEVELS OF PHENOLIC COMPOUNDS BY AUTOMATED SOLID PHASE EXTRACTION AND COLUMN LIQUID CHROMATOGRAPHY

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ABSTRACT

This paper describes a modified SAMOS system employed by Northumbrian Water at the Horsley Water Treatment plant near Newcastle as an on-line monitor for the determination of phenolic compounds in the humic acid rich water taken from the River Tyne. Analyte preconcentration takes place using two styrene divinylbenzene copolymer precolumns utilising the break-through characteristics of phenol and cresols. Phenol and both o-cresol and p-cresol, breaking through the first precolumn after sampling 52 ml are quantitatively collected on a second precolumn in 16 ml. On desorption the humic substances are present at low levels and do not interfere with the analysis of phenol and the cresols. Other phenolic compounds (di-, tri- and pentachlorophenol) are analysed in the second part of the analysis process by desorption from the first precolumn. Analysis is by hplc with u.v. detection. The method is sensitive to phenols at less than 0.1 ppb (parts per billion) and operates 24 hrs per day, seven days a week.

INTRODUCTION

Northumbrian Water abstracts up to 80 million litres of water per day from the River Tyne for treatment in its Horsley Treatment Works to supply drinking water to the City of Newcastle upon Tyne and the surrounding area. In the past, isolated problems have arisen due to low levels of phenolic compounds present in the raw water being converted into chlorinated phenols during the treatment process imparting an unacceptable taste and odour to the final drinking water.

Pesticides and herbicides are not generally a problem in the River Tyne due to the upland nature of the water source and the very limited arable farming in the Tyne Valley. However, as the river catchment area is essentially in peat moorlands, the water contains

high levels of humic substances which have similar chromatographic and u.v. characteristics to phenol and cresols and make the analysis of low levels of phenol very difficult.

The Drinking Water Regulations (1989) specify a limit of 0.5 ppb for phenol in drinking water and it is, of course, prudent to monitor for other impurities which might give rise to treated water quality problems.

Simple solid phase extraction followed by liquid chromatography (hplc), Figure 1, demonstrated that the co-concentration of humic substances and polar phenols took place but that phenol could not be reasonably detected in the broad humic acid band in the resulting chromatogram.

In the Rhine Basin, the emphasis has been directed towards monitoring herbicides and pesticides moving down the River Rhine and its tributaries and crossing international boundaries. The methods developed are based upon solid phase extraction followed by chromatographic separation. These developments have led to the establishment of so-called **SAMOS** systems, (**S**ystem for the **A**utomated **M**easurement of **O**rganic micropollutants in **S**urface water). In general the analytes of interest were relatively non-polar and amenable to both High Performance Liquid Chromatography (hplc) (Brinkman *et al.*,1994) and High Resolution Gas Chromatography (hrgc) (Mol *et al.*,1995).

In the SAMOS approach, analyte preconcentration is carried out using on-line solid phase extraction on a C18 modified silica or styrene divinylbenzene copolymer precolumns followed by column liquid chromatography with diode array detection. The problem of coelution of humic substances with polar analytes was solved by using two styrene divinylbenzene precolumns (Brouwer & Brinkman, 1994); the first used to preconcentrate the humic substances and the rather apolar analytes; polar components breaking through from this precolumn were then concentrated on a second precolumn. Desorption was carried out in two parts, The second precolumn was desorbed and the polar analytes separated in 5 to 10 minutes. Then the first precolumn was eluted using the continuing hplc gradient programme.

The present paper describes this adaption of the SAMOS approach to analyse polar phenolic compounds and the raw water filtration system which was essential to the success of the project.

EXPERIMENTAL

Chemicals and Standards

Standard solutions are prepared using Gold Label or ACS standard grade samples of the following compounds; phenol, *p*-cresol, *o*-cresol, 2,5-dichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol. Approximately 20 mg of each compound was accurately weighed into a suitable stoppered container and dissolved in 20 ml of hplc

grade acetonitrile. These solutions should be stored in a refrigerator and are stable for three months.

200 µl of each of these standard solutions was transfered to a 10 ml volumetric flask and the resulting solution diluted to volume with acetonitrile. This solution contains approximately 20 ppm of each analyte, calculated from the original weight of analyte. This solution should also be stored in a refrigerator and is stable for three months.

200 µl of the 20 ppm standard solution is transfered to a 2 ml volume injection vial and diluted to 1.00 ml with hplc grade water. This provides a 4 ppm standard solution which is used to prepare spike samples and is also used for the routine chromatography check. Spiked raw water samples at 1 ppb level are prepared by diluting 125 µl of the 4 ppm standard with 500 ml of filtered raw water.

Equipment

The equipment comprised a Hewlett-Packard HP1090 HPLC fitted with Diode Array Detection coupled to a Spark-Holland Prospekt sample preparation unit and Solvent Delivery Unit. The complete system is controlled by a Hewlett Packard Vectra XM2 personal computer. The complete system was supplied by Hewlett-Packard Analytical Products Group,UK.

Procedure

Figure 2 shows the flow diagram of the sample preparation unit. Precolumn P1 can be changed after every analysis, P2 is fixed and fitted across one of the built-in Rheodyne valves and is only changed during routine maintenance. Styrene divinylbenzene precolumns, 10 mm x 2 mm internal diameter, are used throughout.

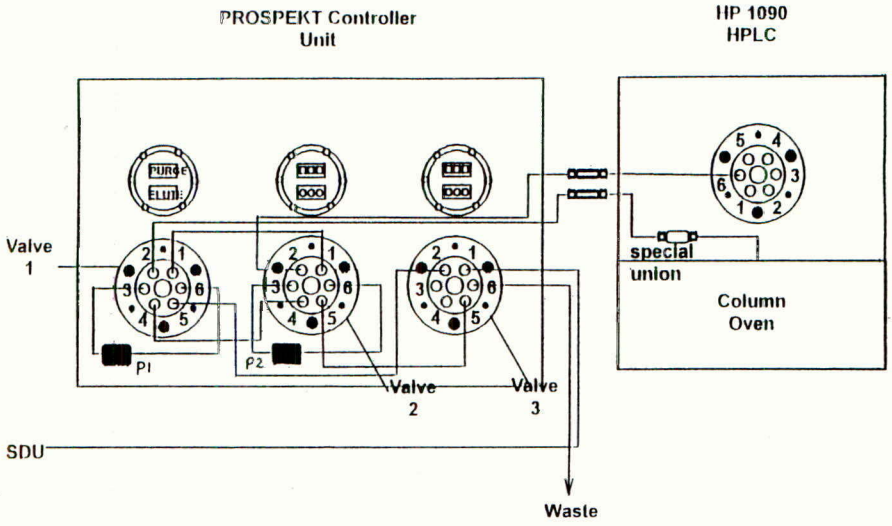
Operation of the system is as follows. Precolumns P1 and P2 are preconditioned with acetonitrile and then hplc grade water prior to analyte concentration. Water samples taken from either one of the sample bottle lines or from the flowing raw water line is initially pumped to waste through Valve 3 to flush the lines. P1 is switched into line and 52 ml of sample water pumped through the precolumn at 3 ml/min prior to switching P2 into line in series with P1 and pumping a further 16 ml of water through the system.

On completion of the preparation phase, P2 is switched into the hplc analytical mobile phase flow line and the hplc gradient programme commenced. After sufficient time to complete elution of the phenol and cresols from the second precolumn, P2 is switched out of line and P1 into the mobile phase line.

The sequence of sample preconcentration, elution of the precolumns and the initiation of the chromatography is controlled by the system software.

Figure 2

HP 1090 Win/SPE System
Capillary Connections



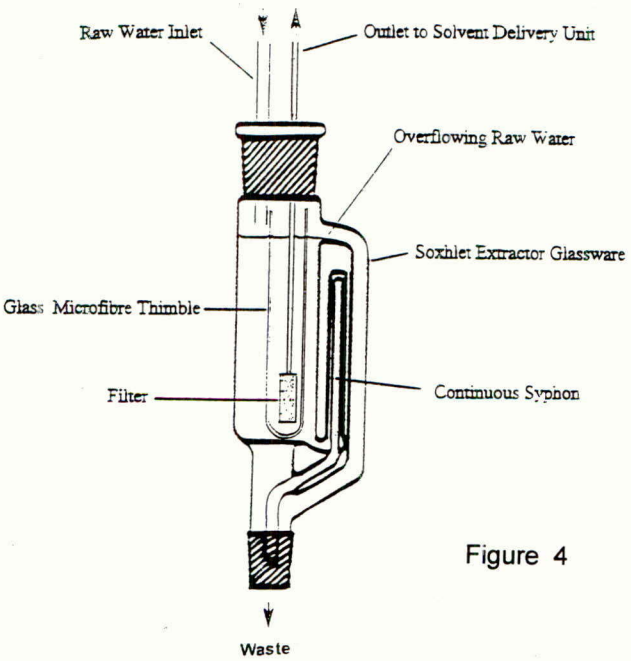


Figure 4

Figure 3 shows the resulting chromatogram obtained from raw water spiked with phenols at the 1 ppb level where the phenol and cresols concentrated on the second precolumn are eluted first followed by the bulk of the humic substances and the chlorinated phenols which were concentrated on the first precolumn eluted second.

Separation of the concentrated analytes is carried out on a Hewlett-Packard Hypersil BDS-C18, 5 μm particle size cartridge column, 250 mm x 4 mm internal diameter cartridge fitted with a 4 mm x 4 mm Lichrospher 100 RP-18 guard cartridge. A gradient mobile phase is generated from a mixture of 0.005 M, pH 3.0 phosphate buffer and hplc grade acetonitrile increases the organic modified content from 20% to 95 % over a period of 35 minutes. The analytical mobile phase flow rate is 1.0 ml/min during analysis and 0.1 ml/min during stand-by periods. Extended solvent reservoirs have been incorporated into the system for long term operation. The column oven temperature is set at 45°C.

Raw Water Filtration

Raw water entering the Treatment Works is unfiltered apart from a coarse screen at the riverside intake to remove leaves, twigs and other floating objects. Samples taken from this liquid line for use in the preparation of spiked standards are filtered through a glass fibre disc prior to analysis on the Prospekt.

The filtration method employed at Northumbrian Water is based upon readily available Soxhlet Extraction Glassware and uses a Glass Microfibre Thimble (Whatman Cat No 2814199) as the filter element, Figure 4. Incoming water is allowed to flow round the outside of the thimble, draining through the syphon and overflowing through the vapour by-pass tube, thus continuously flushing the surface of the thimble with fresh water. The incoming water flow rate is typically 38 l/hour. Water percolates through the thimble which contains the inlet to one line of the Solvent Delivery Unit of the Prospekt.

RESULTS AND DISCUSSION

Long term retention time repeatability is excellent and this provides considerable confidence in component identification during the automatic analysis of raw water.

Chromatographic performance is checked regularly by direct injection of 25 μL of a 4 ppm mixture of phenols in acetonitrile/water. Any degradation in column performance can be corrected by replacing the guard cartridge and pumping out the system with acetonitrile followed by the initial mobile phase mixture.

The chromatograms are monitored at two wavelengths, 195 nm for phenol and the cresols and 210 nm for the chlorophenols. 195 nm provides a sensitivity enhancement of six for the phenol and cresol peaks over the response at 210 nm.

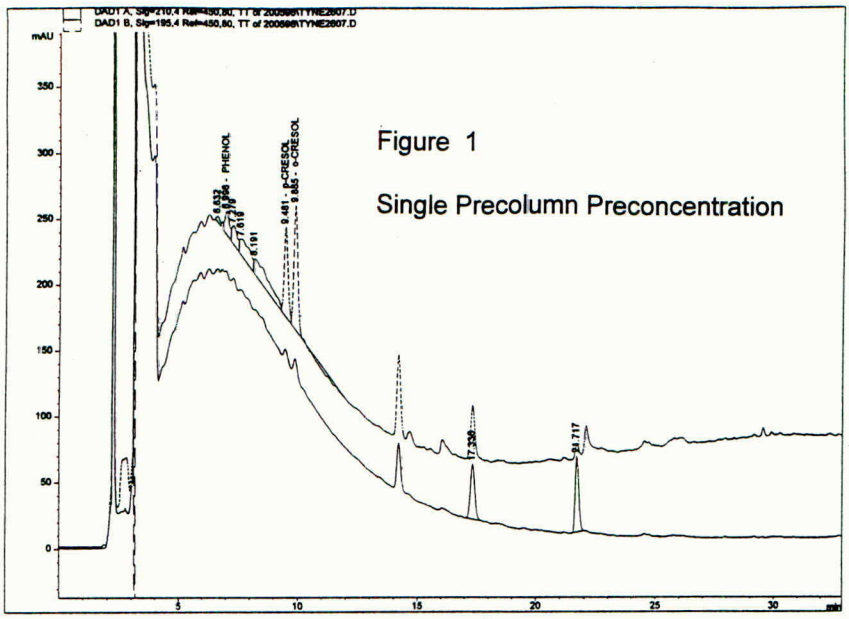


Figure 1

Single Precolumn Preconcentration

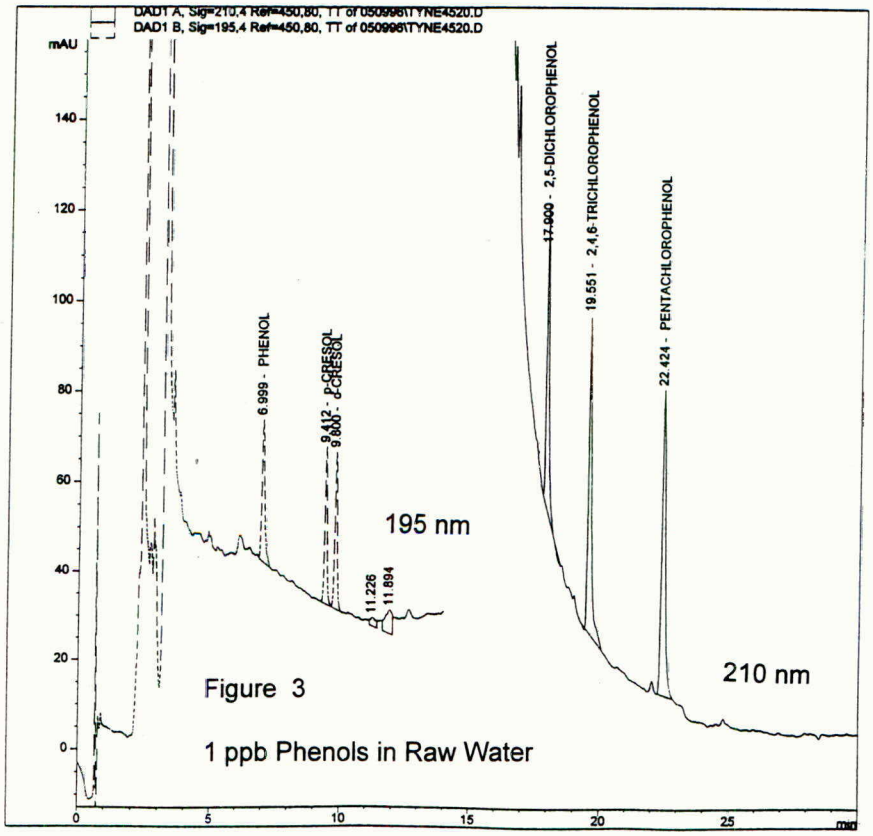


Figure 3

1 ppb Phenols in Raw Water

The phenol monitor is routinely calibrated using raw water spiked with a mixture of phenols at 1 ppb prepared as described above. Raw water is used in preference to hplc grade water since the integration parameters have to be set very carefully to take into account the overall profile of the chromatogram, Figure 3. Compound response in the range 0.25 ppb to 3 ppb is essentially linear. Duplicate processing of the same spike solution is not valid since phenol and cresols are lost from solution quite rapidly due to biodegradation and evaporation at room temperature.

Table 1 shows the recovery data for the 1 ppb spike standard mixture analysis over a period of several weeks.

Table 1. Spike recoveries at 1 ppb.

Date	Run	Phenol	p-Cresol	o-Cresol	2,5-DCP	2,4,6-TCP	PCP
31/5	2848	1.00	1.01	1.01	1.09	1.08	1.03
03/6	2881	1.02	0.93	0.77	1.09	1.06	0.91
05/6	2901	0.98	1.09	1.09	1.02	1.01	0.93
06/6	2925	0.82	1.06	1.03	1.10	1.08	1.03
10/6	3011	1.03	1.17	1.05	1.10	1.04	0.97
11/6	3035	0.93	0.97	1.26	1.10	1.02	1.00
13/6	3084	0.98	0.77	0.70	1.13	1.07	0.93
14/6	3100	0.84	0.84	0.92	1.02	0.95	0.91
17/6	3176	1.21	1.26	1.54	1.16	1.00	0.99
17/6	3177	1.20	1.06	1.23	0.93	0.75	0.87
25/6	3200	0.93	1.09	1.19	1.09	1.08	1.00
27/6	3249	1.00	1.11	1.23	1.04	1.06	0.93
01/7	3333	1.05	1.04	1.00	1.11	1.08	1.05

The precision of measurement of phenols is less than that of the chlorophenols, partially a reflection of the fact that only 16/68 of the water sample is being used in the phenol determination. Water very high in humic substances due to flooding on the River Tyne or during periods when water is being released from Kielder Water gives rise to a greater interference making analysis more difficult. Where this problem can be anticipated, the situation can be improved by reducing the sample volumes and recalibrating.

Analyte hold-up in the thimble at the ppb level has been shown to be in the range 0 - 10% by comparing a 1 ppb spike solution sampled from a bottle or through the thimble.

The operation of the system is not limited to raw water intake monitoring, samples taken from a number of points within the treatment works are routinely analysed together with samples from local reservoirs in order to establish a data base for future investigations.

In addition, it is possible, by simply changing the sample preparation program to monitor for the presence of uron and triazine herbicides in the raw water.

It has proved possible to provide routinely in excess of 90% cover on-line monitoring with 95% being an achievable objective.

Alarms have been established which alert key personnel should phenol and cresols be detected in the raw water at levels greater than 1 ppb thereby providing the opportunity to shut down the intake pumps to the treatment works. The system is also modem linked for off-site monitoring and control.

Successful on-line monitoring requires the establishment of a routine maintenance program which allows sufficient margins for the system to be able to cope with unanticipated changes in water quality and also to ensure that the quality of the chromatography does not degrade to such an extent that the analytical results are of no value.

CONCLUSIONS

The phenol monitoring system described, based upon the Prospekt sample preparation unit and the Hewlett-Packard HP1090 gradient elution hplc is now an established part of Northumbrian Water's treatment facility, safeguarding the supply of drinking water to the City of Newcastle upon Tyne. Levels of phenol and cresols in the range 0.1 to 1.0 ppb can be detected, if present, with confidence, on a 24 hour/day, 7 days/week basis but, in addition, the system provides the potential to monitor other possible river pollutants and the opportunity to study the progress of organic species through the water treatment process.

System performance has exceeded expectations and has provided the safeguards required by the Company and the Regulatory Authorities.

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SESSION 8C

INSECT GROWTH REGULATORS – CURRENT DEVELOPMENTS AND FUTURE PROSPECTS

Chairman	Dr K B Wildey *Central Science Laboratory, York*
Session Organiser	Professor J P Edwards *Central Science Laboratory, York*
Papers	8C-1 to 8C-4

NEW JUVENILE HORMONE ANALOGUES AND THEIR ACTION ON *TENEBRIO MOLITOR* PUPAE

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ABSTRACT

6-[1-(2-phenoxyethoxy)ethoxy]-1,3-benzoxathiole (I) and three new derivatives, differing from it by the introduction of a iodine atom in the positions 2-, 3-, or 4- of the phenyl ring, were synthesised and applied to five groups of ten 0-24 h old *Tenebrio molitor* pupae, to evaluate their juvenilizing action. These compounds were topically applied at doses of 100, 10, or 1 μg/pupa in 1 μl of acetone. The results of these tests were compared with those obtained with the parent compound and with the derivatives of (I) having either a fluorine or a chlorine atom substituted in the same positions. The maximum juvenilizing action of the above mentioned compounds was reached with the fluorine atom in position 4- at the dose of 100 μg/pupa. At this dose, this compound was significantly more active than either (I) or its 4-chloroderivative.

INTRODUCTION

For over twenty years we have been conducting research on juvenile hormone analogues (JHA) with the aim of obtaining new compounds that are both active on pests, and environmentally considerate. Our investigations have focused on the synthesis of new derivatives of 5-hydroxy-1,3-benzodioxole (sesamol) and 5-hydroxy-1,3-benzoxathiole. Over 200 compounds (mostly ethers and acetals) have been synthesised, together with some esters, carbonates, and sulfonates. All of the new compounds have been tested on 0-24 h old *Tenebrio molitor* pupae. In previous studies, acetal and ether derivatives of 5-hydroxy-1,3-benzoxathiole were, respectively, more active and less active than the analogue derivatives of sesamol (Arzone & Dolci, 1980a, 1980b, 1980c, 1983, Marletto & Dolci, 1980, Dolci & Arzone, 1982, 1990, Arzone *et al.*, 1985).

The most active compounds in this series have also been tested on older (>24 h old) *T. molitor* pupae, as well as on fully grown larvae of *Calliphora vomitoria*, on both larvae and pupae of *Bombyx mori*, and on pupae of *Apis mellifera*. In addition, some of these JHA, have been tested as synergists for pyrethrum and synthetic pyrethroids against adults of the WHO (World Health Organisation) susceptible strain of *Musca domestica*. Finally, we have determined the oral and topical toxicity of several of the compounds in adult honeybees and in mice (Degani *et al.*, 1982).

More recent investigations with this series of JHA have been undertaken to test the variations in juvenilising activity resulting from the substitution of halogen atoms in various positions of the heterocyclic system. In this paper, we present the results of preliminary studies with novel iododerivatives of 6-[1-(2-phenoxyethoxy)ethoxy]-1,3-benzoxathiole.

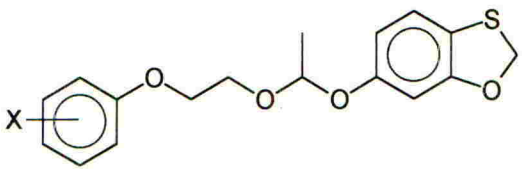
MATERIALS AND METHODS

6-[1-(2-phenoxyethoxy)ethoxy]-1,3-benzoxathiole (I) and three new derivatives, differing from it by the introduction of an iodine atom into the phenyl ring in positions 2- (II), 3- (III), and 4- (IV), respectively, were synthesised.

The new (iodinated) compounds II, III and IV, together with (previously synthesised) analogues containing either fluorine (V-VII) or chlorine (XIII-X) atoms substituted in the same positions, and the parent compound (I) were each applied topically to 0-24 h old *T. molitor* pupae bred on wheatmeal and Indian meal (1:1v/v) and kept in a darkened environmental chamber at 25-26 °C and 55-60 r.h.

The compounds used are shown in Table 1.

Table 1. Structure of the new derivatives of 6-[1-(2-phenoxyethoxy)ethoxy]-1,3-benzoxathiole and of the derivatives used as a comparison.



X	Compounds	X	Compounds
H	I	3-F	VI
2-I	II	4-F	VII
3-I	III	2-Cl	VIII
4-I	IV	3-Cl	IX
2-F	V	4-Cl	X

Each compound was tested on five groups of ten pupae at each of the doses (100 µg, 10 µg, and 1 µg) contained in 1 µl of acetone. The solutions were topically applied to the last three segments of the ventral side of the abdomen with a Hamilton microapplicator. Untreated pupae and pupae treated with 1 µl of acetone only were used as controls for each dose level. Both treated pupae and controls were placed in Petri dishes with food and reared as described above. Assessments of the JHA activity of the compounds on morphogenetic characters were made two days after the emergence of controls, and assessments of fertility were made after three months. The JHA activity (rating) of the compounds was scored as follows:

0 = Adult without retention of any pupal characters.

1 = Adultoid with urogomphi and sometimes small gin traps on the last abdominal segments.

2 = Adultoid with urogomphi and well-developed gin traps on the last abdominal segments. Such adultoids often remained enveloped in the pupal cuticle.
3 = Adultoid with urogomphi and well-developed gin traps on all the abdominal segments, enveloped in the pupal cuticle; head, thorax, and appendages displaying patches of pupal cuticle.
4 = Second pupa.

The average rating of the different doses of each compound was calculated by multiplying the number of pupae (excluding dead pupae) in each activity category by the activity rating, and dividing the sum by the number of pupae treated (Redfern *et al.*, 1970). The results were submitted to analysis of variance and the Duncan test. The comparisons of activity were made between the activity of compound (I) at the different doses compared to those of its derivatives substituted with a iodine, fluorine, or chlorine atoms in positions 2-, 3-, or 4- of the phenyl moiety.

RESULTS AND DISCUSSION

The results of the tests for morphogenetic (juvenilising) activity of the compounds on *T. molitor* are shown in Table 2.

Table 2. Average rating of the juvenile hormone activity of novel JHA compounds topically tested on five groups of ten *Tenebrio molitor* pupae.

Dose µg	Compounds			
	I	II	V	VIII
100	2.6a	0.2c	0.0c	2.2b
10	2.1a	0.0c	0.0c	0.8b
1	1.1a	0.0b	0.0b	0.0b
	I	III	VI	IX
100	2.6a	0.2d	1.2c	1.6b
10	2.1a	0.2c	1.2b	1.2b
1	1.1a	0.1c	0.4b	0.3b
	I	IV	VII	X
100	2.6b	0.8c	2.9a	2.5b
10	2.1b	0.3d	1.5c	2.4a
1	1.1a	0.2c	0.3c	0.8b

In each line values followed by a different letter differ significantly ($P \leq 0.05$) according to the Duncan test.

In all tests, normal adults emerged from pupae treated with 1 µl of acetone and from untreated pupae and, in all cases, adultoids (i.e. adults retaining of pupal characters) resulting from the JHA treatments failed to reproduce (data not shown). The substitutions in positions 2- and 3- of the phenyl with all three halogens generally reduced the activity compared to the parent compound (I) at all doses. However, the substitution of the parent compound in position 4- with a fluorine atom appeared to significantly increase the activity compared to (I) and its chloroderivative at the 100µg/pupa dose rate. By contrast, at 10µg/pupa, the 4-chloroderivative (X) was the most active compound and, at 1 µg/pupa, the most active compound was the unsubstituted parent molecule (I). In general, the new iododerivatives were less active than either the non-substituted compound or the chloro- or fluoro- analogues (Table 2).

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NOVALURON (MCW-275), A NOVEL BENZOYLPHENYL UREA, SUPPRESSING DEVELOPING STAGES OF LEPIDOPTERAN, WHITEFLY AND LEAFMINER PESTS

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ABSTRACT

Novaluron, a novel benzoylphenyl urea, acts both by ingestion and contact. As such it is highly active against lepidopteran larvae (by ingestion) and against larvae of the cotton whitefly, *Bemisia tabaci* (by contact). The LC_{50} value of novaluron against 3rd-instar *S. littoralis* larvae fed on treated caster bean leaves was ~ 0.1 mg a.i. litre^{-1}. This value was similar to that obtained with chlorfluazuron and was about 10-fold lower than that of teflubenzuron. An application rate of 25 mg a.i. litre^{-1} in cotton fields resulted in ~100% mortality of both *Spodoptera* and *Helicoverpa* larvae fed treated leaves removed from plants up to 8 days after application. Novaluron was much more active against eggs and larvae of *B. tabaci* than either chlorfluazuron or teflubenzuron. At a concentration of 1 mg a.i. litre^{-1}, novaluron reduced adult emergence by ~ 90% when 1st instar larvae were exposed to treated cotton seedlings. Novaluron was more active in suppressing developing stages of the leafminer *Liriomyza huidobrensis* than teflubenzuron and chlorfluazuron. Suppression of about 80% adult formation was obtained at a concentration of 0.8 mg a.i. litre^{-1} and a similar inhibition of pupation and mine formation at a concentration of 20 mg a.i. litre^{-1}. These results indicate that novaluron has considerable potential for controlling lepidopteran pests, whiteflies and agromyzid leafminers in field crops, vegetables and ornamentals.

INTRODUCTION

Novaluron, 1-[3-chloro-4-(1,1,2-trifluoro-2-trifluoro-methoxyethoxy) phenyl]-3-(2,6-difluoro-benzoyl) urea, a novel benzoylphenyl urea acts, like other Insect growth regulators (IGRs) of this class, by inhibiting chitin formation (Ishaaya and Casida, 1974; Post *et al.*, 1974; Sowa and Marks, 1975; Hajjar and Casida, 1979). These compounds cause abnormal endocuticular deposition and abortive molting (Mulder and Gijswijt, 1973). While most of the benzoylphenyl ureas act by ingestion (Ishaaya, 1990), novaluron seems to act by both ingestion and contact. As such, it is active against lepidopteran larvae feeding on leaves, as well as sucking pests such as whiteflies. In these studies, we have evaluated the biological activity of novaluron against two lepidopteran pests, (*Spodoptera littoralis* and *Helicoverpa armigera*), the sweetpotato whitefly, (*Bemisia tabaci*) and the agromyzid leafminer (*Liriomyza huidobrensis*). Both *S. littoralis* and *H. armigera* are important pests in cotton, vegetable and ornamentals (Avidov and Harpaz, 1969; Horowitz *et al.*, 1996). *B. tabaci* is a cosmopolitan pest of many field and greenhouse crops (Byrne *et al.*, 1990; Horowitz *et al.*, 1994). *L. huidobrensis* is very important pest of vegetables and ornamentals. The larvae feed on the mesophyl producing mines beneath the epidermis resulting, in some cases, in the total destruction of various field

crops (Weintraub and Horowitz, 1995). All the above insect species can be serious pests of cotton in Israel.

MATERIALS AND METHODS

Insects

S. littoralis was collected in 1990 from a cotton field in the Bet Shé'an area of Israel, and subsequently reared on caster bean leaves under standard laboratory conditions (Ishaaya *et al.*, 1995). *H. armigera* larvae were from a standard laboratory colony reared on artificial diet for at least 10 years (Navon *et al.*, 1990). *B. tabaci* and *L. huidobrensis* were reared on cotton and Bulgarian bean seedlings, respectively, under standard conditions of 25±1°C, 65±5% r.h., and a photoperiod of 14:10 (light : dark) (Ishaaya and Horowitz, 1995).

Assays with *S. littoralis* under standardized laboratory conditions

Bioassays were done with 3rd-instars (0-24 h after ecdysis, weighing 12±1 mg). Larvae were offered caster bean leaves treated with aqueous solutions (a range of concentrations for determining mortality curves) of the test compound or with deionized water only, as a control. The treatment was accomplished by dipping the leaves in the appropriate dilutions of test compound. After 2 h of air drying at room temperature, the treated leaves were transferred separately to ventilated plastic boxes (8x5x3 cm, containing a layer of sawdust to avoid excess humidity) and given to 3rd-instar larvae for 2 days. Subsequently, the larvae were fed for an additional 4 days on untreated leaves. Mortality was assessed at 6 days after the start of assay, when all the untreated larvae (controls) had molted to the 4th instar. Each concentration was tested using 6-10 replicates of 10 larvae, and each mortality curve was based on at least 5 concentrations. Probit regression using the POLO analysis procedure (LeOra Software, 1987) was used for determining the slope and LC values of the test compounds.

Assays with *S. littoralis* and *H. armigera* using field-treated cotton leaves

Cotton plants were sprayed, on July 21, 1994, until runoff (2 litre/10m row) with solutions containing 25 mg a.i. litre^{-1} novaluron, chlorfluazuron, or with water as a control. Leaves were collected periodically after treatment and given to 3rd-instar *S. littoralis* larvae for a period of 2 days. The larvae were then transferred to untreated leaves, and mortality was assessed after six days. Treated leaves were also offered to 1st-instar *H. armigera* larvae for 4 days, after which time, mortality was determined. Data for both *S. littoralis* and *H. armigera* were based on averages ± SEM of 10 replicates of 10 larvae each.

Assays with *B. tabaci* under standardized rearing chamber conditions

Cotton seedlings (20-25 cm high) were dipped in various concentrations of the test compounds or in deionized water (control). The plants were allowed to dry for 2 h. Twenty *B. tabaci* females confined in leaf cages (Ishaaya *et al.*, 1988; Ishaaya and Horowitz, 1992) were exposed to treated plants for 48 h under controlled conditions of 26±1°C, 65±5% r.h., and a photoperiod of 14:10 (light : dark). The effects on adult mortality, fecundity, egg viability and cumulative larval mortality (expressed as percentage pupation and emergence) were determined. In other assays, cotton seedlings treated with various concentrations of the test compounds, were subsequently infested with 0-1-d-old eggs, or with 1st or 2nd instar larvae.

Probit regression using POLO analysis was used for determining the slope and LC values. Each concentration of the test compound was replicated 5 times (20-80 insects) and each mortality curve was derived from the results obtained with at least 4 different concentrations.

Assays with *L. huidobrensis*

Bulgarian bean seedlings with two true leaves, infested with 0-20-h-old *L. huidobrensis* eggs, were dipped in various concentrations of the test compound or in deionized water (control). The effects of this treatment on pupation and adult emergence were then determined. Each concentration was replicated 4 times, and each replicate used 20-40 eggs.

Statistical analysis

All results were subjected to one-way analysis of variance (ANOVA) and means were separated by Scheffé's multiple range test (P=0.05) (Day and Quinn, 1989). Angular transformations for percentage egg hatch, and adult emergence were done before statistical analysis. POLO-PC (LeOra Software, 1987) was used to estimate probit regression.

RESULTS AND DISCUSSION

Comparative toxicity of novaluron, chlorfluazuron and teflubenzuron on *S. littoralis* larvae

The potency of novaluron was compared with two other benzoylphenyl urea chitin synthesis inhibitors (chlorfluazuron and teflubenzuron) both of which are considered to be among the most potent IGRs used against lepidopteran pests. According to the LC_{50} and LC_{90} values obtained in the present experiments (Table 1), the potency of novaluron is similar to that of chlorfluazuron, and both compounds are over 12-fold more potent than teflubenzuron. The LC_{90} values of novaluron, chlorfluazuron and teflubenzuron were 0.43, 0.62 and 7.64 mg a.i. $litre^{-1}$ in the treatment solutions, respectively (Table 1).

Residual toxicity of novaluron and chlorfluazuron on *S. littoralis* and *H. armigera* on cotton plants

Reflecting the results obtained under laboratory conditions (Table 1), the residual toxicity of novaluron on cotton leaves treated in the field, was similar to that of chlorfluazuron on both *S. littoralis* and *H. armigera* larvae (Table 2). Up to 8 days after treatment, over 96% and 90% mortality was obtained with larvae of *S. littoralis* and *H. armigera*, respectively. At day 15, both compounds had lost some activity, resulting in a mortality of about 60% with *S. littoralis* larvae and about 30% with *H. armigera* larvae (Table 2). The results obtained under both laboratory and field conditions indicate that both novaluron and chlorfluazuron are potential compounds for controlling *S. littoralis* and *H. armigera* larvae in cotton and, possibly, in other field crops.

Effects of novaluron on *B. tabaci*

The effects of novaluron on adult mortality, fecundity, egg viability and progeny formation of *B. tabaci* are shown in Table 3. At treatment rates of up to 100 mg a.i. $litre^{-1}$, no effects on adult mortality, fecundity or egg viability were noticed. However, considerable larval mortality (98% suppression of pupation and adult emergence) was obtained at 1 mg a.i. $litre^{-1}$ (Table 3).

Table 1. Comparative toxicity of novaluron, chlorfluazuron and teflubenzuron against 3rd-instar *Spodoptera littoralis* larvae under standard laboratory conditions.

Compounds	n	Slope ±SEM	LC values in mg a.i. litre^{-1}		
			LC_{10}	LC_{50}	LC_{90}
Novaluron	520	2.89±0.22	0.06 (0.01-0.10)[a]	0.16 (0.08-0.28)	0.43 (0.25-2.21)
Chlorfluazuron	380	2.06±0.22	0.04 (0.02-0.05)	0.15 (0.12-0.18)	0.62 (0.46-0.94)
Teflubenzuron	290	2.93±0.36	0.76 (0.45-1.14)	2.42 (1.66-3.48)	7.64 (5.18-12.5)

[a]95% confidence limits in parentheses.

Table 2. Residual toxicity (% mortality) of novaluron and chlorfluazuron against 3rd-instar *Spodoptera littoralis* and 1st-instar *Helicoverpa armigera* larvae under field conditions

Compound and concentration (mg/litre^{-1}) .	Days after treatment 1	4	8	15	27
S. litoralis					
Untreated control	0	0	0	0	0
Novaluron, 25	99±1	98±2	99±1	58±9	15±7
Chlorfluazuron, 25	96±2	96±2	98±2	66±8	8±5
H. armigera					
Untreated control	0	0	0	5±2	0
Novaluron, 25	96±2	100	90±2	31±7	12±8
Chlorfluazuron, 25	93±2	98±2	90±2	25±7	10±6

Data are averages ± SEM of 10 replicates of 10 larva.

In other assays, cotton seedlings infested with 0-1-d-old eggs or with 1st-instar larvae, were treated with various concentrations of novaluron, chlorfluazuron or teflubenzuron. Cumulative larval mortality, expressed as reduction in percentage pupation, was determined (Tables 4 and 5). Novaluron was the most effective compound resulting in 4% and 0% pupation at concentrations of 0.25 and 0.5 mg a.i. litre^{-1} when tested on 0-1-d-old eggs, and 26% and 12% pupation when tested on 1st-instars. At similar concentrations, chlorfluazuron showed a moderate effect, while teflubenzuron had no detectable effect (Tables 4 and 5). On the basis of the LC_{90} values (Table 6) novaluron, applied to cotton seedlings under standard laboratory

conditions, was most effective against 0-1-d-old eggs (0.35 mg a.i. litre^{-1}), but less effective against 1st- (0.68 mg a.i. litre^{-1}) and 2nd- (2.2 mg a.i. litre^{-1}) instar larvae.

Table 3. Effect of novaluron on adult mortality, fecundity, egg hatch and progeny formation of the whitefly *Bemisia tabaci.*

Concentration, mg a.i. litre^{-1}	No. of adults	Adult mortality, %	Egg per female	Egg hatch, %	Pupation %	Emergence %
Untreated control	75	0	6.1±0.8	99±1	98±1	98±1
1	75	0	8.6±1.0	99±1	2±1	2±1
10	75	0	7.9±1.6	99±1	1±1	1±1
100	75	0	7.8±1.4	96±2	2±1	2±1

Data are averages ± SEM of 5 replicates of 15 females each.

In other preliminary experiments using a field strain of *B. tabaci* showing a high level resistance to pyriproxyfen (>500 fold) and a moderate resistance to buprofezin (> 5 fold) showed susceptibility to novaluron that was similar to that obtained with the laboratory strain (data not shown). These results, although preliminary, are of agricultural significance and indicate that (at present) no cross resistance exists between novaluron and the leading IGRs currently used against *B. tabaci*. The results obtained thus far indicate that novaluron has considerable potential for controlling one of the most important insect pests of cotton (*B. tabaci)*. Furthermore, this novel IGR could be used in alternating treatment programmes in combination with other leading compounds (e.g. pyriproxyfen and buprofezin) for controlling whiteflies (Horowitz and Ishaaya, 1994) as part of insecticide resistance management programmes that aim to reduce the development of resistance to novel IGRs.

Table 4. Comparative toxicity of novaluron, chlorfluazuron and teflubenzuron applied on cotton seedlings infested with 0-1-d-old *B. tabaci* eggs.

Concentration, mg ai litre^{-1}	Pupation, % ± SEM		
	Novaluron	Chlorfluazuron	Teflubenzuron
Untreated control	97±2 a	95±1 a	94±3 a
0.0625	19±4 b	-	-
0.125	9±3 c	-	-
0.25	4±2 cd	78±8 a	97±1 a
0.5	0 d	16±5 b	96±2 a
1.0	-	4±4 c	94±2 a

Data are averages ±SEM of 5 replicates of 20-80 eggs each. Within groups, means followed by the same letter are not significantly different at P=0.05.

Table 5. Comparative toxicity of novaluron, chlorfluazuron and teflubenzuron applied on cotton seedlings infested with 1st-instar *B. tabaci* larvae.

Concentration, mg a.i. litre^{-1}	Pupation, % ± SEM		
	Novaluron	Chlorfluazuron	Teflubenzuron
Untreated control	97±1 a	99±1 a	94±2 a
0.0625	56±6 b	98±2 a	95±2 a
0.125	33±4 c	94±3 a	83±4 a
0.25	26±5 cd	44±7 b	87±3 a
0.5	12±4 d	28±4 b	87±5 a
1.0	7±2 d	7±3 c	80±10 a

Data are averages of five (chlorfluazuron and teflubenzuron) and ten (novaluron) replicates of 50-150 larvae each. Within column means followed by the same letter are not significantly different at P=0.05.

Table 6. Comparative toxicity of novaluron applied on cotton seedlings infested with eggs, or with 1st- or 2nd-instar larvae of *B. tabaci* under standardized growth chamber conditions.

Stage	n	Slope ±SEM	LC values in mg a.i. litre^{-1}		
			LC_{10}	LC_{50}	LC_{90}
Egg	915	5.76±0.44	0.01 (0.001-0.022)	0.05 (0.01-0.13)	0.35 (0.13-1.23)
1st-instar	5443	1.35±0.06	0.01 (0.002-0.020)	0.08 (0.04-0.11)	0.68 (0.46-1.31)
2nd-instar	1984	1.12±0.11	0.04 (0.006-0.115)	0.30 (0.11-0.59)	2.20 (1.17-5.26)

Comparative toxicity of novaluron, teflubenzuron and chlorfluazuron on the agromyzid leafminer *L. huidobrensis*

Bulgarian bean seedlings infested with 0-20-h-old eggs of *L. huidobrensis*, were treated with various concentrations of novaluron, teflubenzuron or chlorfluazuron. The effect on the rate of pupation and on adult emergence was then determined (Table 7). Novaluron was far more active in suppressing developing stages of *L. huidobrensis* than either teflubenzuron or chlorfluazuron. Suppression of about 80% adult formation was obtained at a concentration of 0.8 mg a.i. litre^{-1} and a similar suppression of pupation and mine formation at 20 mg a.i. litre^{-1}. Teflubenzuron showed a mild effect on adult formation at 4 and 20 mg a.i. litre^{-1}, but no appreciable effect on pupation and mine formation. Chlorfluazuron had no apparent effect at any of the concentrations tested. The leafminer *L. huidobrensis* is an important pest of vegetables and ornamentals in California, Israel and Western and Central Europe (Parrella and Bethke, 1984; Weintraub and Horowitz, 1995). Whiteflies and lepidopterans are additional

pests of these crops. Our results indicate that novaluron has considerable potential for the simultaneous control of several important pests of cotton, and other crops.

Table 7. Effect of novaluron, teflubenzuron and chlorfluazuron on pupation and emergence of the leafminer *Liriomyza huidobrensis*.

Compound and concentration in mg a.i. litre^{-1}	No. of eggs used	Pupation, % ± SEM	Emergence, % ± SEM
Novaluron			
Untreated control	130	83±16 ab	43±11 a
0.16	112	94±2 a	13±4 b
0.8	122	94±3 a	12±3 b
4	113	72±5 b	0 c
20	120	14±4 c	0 c
Teflubenzuron			
Untreated control	124	90±8 a	57±7 a
0.16	107	92±5 a	37±5 b
0.8	120	91±7 a	53±13 ab
4	117	70±8 b	28±11 bc
20	111	56±10 b	4±2 c
Chlorfluazuron			
Untreated control	98	100 a	71±6 a
0.16	106	99±1 a	70±7 a
0.8	106	94±4 a	54±7 ab
4	148	100 a	61±4 ab
20	106	95±4 a	34±12 b

Bulgarian bean seedlings infested with 0-20-h old eggs of *L. huidobrensis* were treated with various concentrations of the test compound or in water (control). Effect on pupation and emergence were then determined. Data are averages of 4 replicates of 20-40 eggs each.

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ECTOPARASITOID VENOM AS A REGULATOR OF LEPIDOPTERAN HOST DEVELOPMENT AND MOULTING

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ABSTRACT

Parasitism by the gregarious ectoparasitoid wasp *Eulophus pennicornis* affects the survival, growth and development of penultimate and final larval instars of the tomato moth (*Lacanobia oleracea*). Nonparasitized fifth instar larvae gain weight rapidly, moult to the sixth stadium and pupate. By contrast, parasitized hosts stop feeding, neither moult nor pupate, and eventually die. These symptoms of wasp attack also occur in nonparasitized *L. oleracea* larvae which have been penetrated by wasps' ovipositors, or which have been injected with extracts of *E. pennicornis* venom glands. This suggests that the wasp's sting (rather than the act of oviposition), disrupts normal host endocrinology to cause developmental arrest. Radioimmunoassay confirms that the timing of the increase in ecdysteroid titre in the penultimate instar is significantly altered in parasitized hosts, and in hosts inoculated with venom extracts.

INTRODUCTION

By definition, parasitoid Hymenoptera inevitably cause the death of the arthropod hosts which they use as oviposition sites. However, hosts do not necessarily die immediately after attack, and may undergo a variety of pathological changes before they finally succumb to the effects of parasitism. Host-pathologies include disrupted development, growth, food consumption, immuno-responsiveness and morphology, and paralysis (Vinson & Iwantsch, 1980). Different symptoms reflect the various mechanisms which parasitoid wasps use to manipulate hosts' reactions towards their offspring. Some of these mechanisms involve interactions between developing parasitoid larvae and host tissues, but in other cases adult wasps introduce regulatory secretions into hosts before or during the act of oviposition. These maternal substances can have profound, even lethal, effects on the parasitized host's physiology and development (Vinson & Iwantsch, 1980). Investigations of the effects which adult wasps have on their hosts, and studies of those factors which play a role in causing pathologies, therefore provide important components in our understanding of parasitoids as sources of naturally occurring insect growth regulators.

Attack by the gregarious ectoparasitoid wasp *Eulophus pennicornis* (Hymenoptera: Eulophidae) on larvae of the pest *Lacanobia oleracea* (Lepidoptera: Noctuidae), causes dramatic weight loss, rapid reduction in feeding, reduced mobility and a failure to pupate (Marris & Edwards, 1995). Although *E. pennicornis* is an external parasitoid, during host-inspection the ovipositor frequently penetrates the host's cuticle, and wasps therefore have the opportunity to introduce host-regulatory substances in this interval prior to actual oviposition. Comparisons between the survival of nonparasitized *L. oleracea* larvae and the fates of hosts which have been subjected to differing degrees of wasp exposure, show that when stabbed but

nonparasitized caterpillars are isolated before any eggs are laid, they still suffer many of the developmental disruptions normally associated with parasitism. Since these debilitating effects cannot be entirely attributed to the demands placed on the host's constitution by developing wasp larvae, their existence suggests that adult female *E. pennicornis* plays a role in the ultimate state of parasitized *L. oleracea*.

Although several maternal substances may contribute to observed effects, products of the wasp venom gland have been implicated in other parasitoid/host associations (Beard, 1978; Vinson & Iwantsch, 1980; Shaw, 1981; Beckage, 1985; Stoltz, 1986). The first of the two experiments described below was therefore designed to ascertain how injection with *E. pennicornis* venom affects *L. oleracea's* larval survival, development and food consumption. Since the symptoms of parasitism suggest that wasp attack influences the underlying endocrinology of host development (Lawrence, 1986), a second experiment sought to make an initial comparison between levels of moulting hormones, ecdysteroids, in nonparasitized, parasitized or venom-injected *L. oleracea* respectively, at various stages during their lifespan.

MATERIALS AND METHODS

Experimental organisms

Tomato moth larvae and *E. pennicornis* adults were obtained from their respective cultures, held at CSL. Since *E. pennicornis* preferentially attacks newly-ecdysed late stadia hosts (Marris & Edwards, 1995), only fifth instar *L. oleracea* larvae were used in experiments. Before and during experiments, larvae were fed on a maize-based Noctuid food medium (Korano, France). Prior to use, parasitoids were provided with a honey solution food source (50 % V:V), and were maintained without access to hosts for 48 h. This delay allowed mating to occur, but prevented wasps from premature distribution of any eggs or venom gland components products. All experiments were carried under standard conditions of 25°C, r.h. 70 %, L:D 16 h:8 h.

Preparation of venom extract

Adult female wasps were immobilised by exposure to CO_2 (30 s), and placed into 0.5 ml sterile insect saline (50 mM sodium phosphate, 0.15 M NaCl, pH 7) on the stage of a light microscope (x 20). Pulling their stylets with fine forceps yielded entire reproductive systems, each including a poison gland and a pair of ovaries, still attached to the ovipositor. Ovaries were discarded, and remaining tissues were placed into chilled insect saline. This procedure was repeated until the whole poison glands (plus debris) of 250 female *E. pennicornis* had been collected in 100 µl saline. Glands were macerated, releasing their contents into solution, and the sample was spun (13,000 rpm, 1 min), to separate superfluous tissue debris. Supernatant was aspirated and stored at -20°C until required.

Experiment 1: The effect of *E. pennicornis* venom on *L. oleracea*'s larval survival, development and food consumption

Three groups of *L. oleracea* (25 larvae/group) were immobilised by water-immersion (30 min). Larvae in one group were individually injected with 300 nl of venom (equivalent to

0.7 gland/caterpillar), using a glass capillary needle mounted on a nanolitre injector; larvae in the second group were each injected with 300 nl sterile insect saline; the remaining 25 caterpillars were not subjected to any injection. Individual larvae from each group were weighed, and placed into separate plastic containers (150 x 150 x 75 mm), containing a pre-weighed volume (25 cm^3) of Noctuid diet. At 48 h intervals, all larvae were re-weighed, and any developmental changes or deaths were noted. This was repeated every 2 d for 14 d.

Each time larvae were inspected, all diet remaining in each container was collected, re-weighed, and any change in its mass was recorded. Each diet volume was then returned to the same *L. oleracea* individual from which it had been removed. In this way, the total amount of diet supplied to each venom-inoculated, saline-inoculated or non-inoculated larva respectively, was known, and any change in weight of this diet could be calculated. Prior to evaluating the feeding activity of *L. oleracea*, it was necessary to ascertain what proportion of the total diet weight was lost incidentally, through evaporation of its water content during the experiment. For this reason, a fourth set of 25 pre-weighed portions of diet was prepared, to serve as controls. These diet cubes were not exposed to *L. oleracea*, but they were weighed at 2 d intervals, and the average weight loss was calculated for each successive 48 h period. These correction values were then subtracted from the average weight losses measured in similar diet volumes which had been presented to tomato moth larvae. Resulting net weight losses from diets were assumed to be equivalent to the amount of food actually eaten by treated *L. oleracea* during the 2 w period.

Experiment 2: Radioimmunoassay (RIA) of ecdysteroid titres in venom-injected, saline-injected, parasitized or non-parasitized *L. oleracea*

Lacanobia oleracea were subjected to one of 4 treatment regimes: 1) exposure to and parasitism by *E. pennicornis*; 2) injection with 300 nl *E. pennicornis* venom (see above method); 3) injection with 300 nl sterile insect saline (see above method); 4) no injection and no wasp-exposure (= control). All larvae were supplied with food medium and maintained under standard conditions. At selected time intervals, 25 *L. oleracea* were harvested from each regime, and prepared for haemolymph extraction: Each caterpillar was immobilized in water, dried on sterile tissue, and positioned on its dorsal surface. Using an ultrafine glass capillary needle, each larva was pierced in the proximal region of one of its prolegs, releasing fresh haemolymph. This was collected using microcapillary tubes, and 10 µl volumes were immediately placed into 60 µl methanol. Each sample was vortexed for 5 s to ensure mixing, before being subjected to centrifugation (7,500 g, 10 min). The resulting supernatant was aspirated, and the pellet was re-extracted in a further 60 µl 80 % methanol and re-centrifuged. Both supernatants were then pooled to give a final volume of 120 µl. Extracts were dried by vacuum evaporation (50°C, 1 h), and frozen at -20°C. For RIA, samples were brought to 4°C, and resuspended in 0.1 ml borate buffer. To each assay tube was added 10,000 dpm [^{3}H]-ecdysone (100 µl), and 300 µl ecdysteroid antibody DBL-II (Trifolio-M, Germany). Incubations were conducted at room temperature (2 h), and antibody/radiotracer complexes were then precipitated with 400 µl ammonium sulphate, prior to incubation at 4°C (30 min). Following centrifugation (14,000 g, 5 min), supernatants were aspirated, and pellets were dissolved using Solvable (NEN-Dupont), with Formula 989 (Packard) as scintillant. Radioactivity was measured by liquid scintillation counting, and the respective ecdysone content of each sample was calculated by reference to a pre-prepared standard displacement curve.

RESULTS

Experiment 1: The effect of *E. pennicornis* venom on *L. oleracea*'s larval survival, development and food consumption

No significant difference existed between the zero % mortality observed in saline-inoculated caterpillars and the small number of deaths recorded in water-immersed hosts ($P > 0.05$). However, a significantly higher proportion of larvae (> 90 %) died after injection with venom ($P < 0.001$) (Figure 1). While non-injected or saline-injected larvae gained weight, moulted to the sixth stadium (3-4 d) and eventually pupated (12 d), venom-injected larvae scarcely grew at all beyond the date of injection (Figure 2). Moreover, these treated individuals did not moult and they did not pupate.

Non-injected or saline-injected caterpillars showed a steady, rapid rate of diet consumption until 6 d into the trial (Figure 3). By this stage they had moulted into their final instar, and were entering the "wandering" phase which precedes pupation (Corbitt *et al.*, 1996). Although feeding activity was subsequently reduced, as larvae approached metamorphosis, some diet continued to be consumed for 10 d. By contrast, *L. oleracea* which had been inoculated with *E. pennicornis* venom ate consistently less food than their non-poisoned counterparts. Their food consumption was reduced within 48 h of treatment. After 2 d there was no further change in the daily total diet consumed by venom-injected larvae, suggesting that feeding activity was effectively halted. On average, each saline-injected larva had consumed 2.43 g diet by the time of pupation, whereas each venom-treated individual ate 0.23 g diet over the same time interval. This difference was highly significant ($P < 0.001$), and corresponded to an overall reduction in food consumption of 90.5 %.

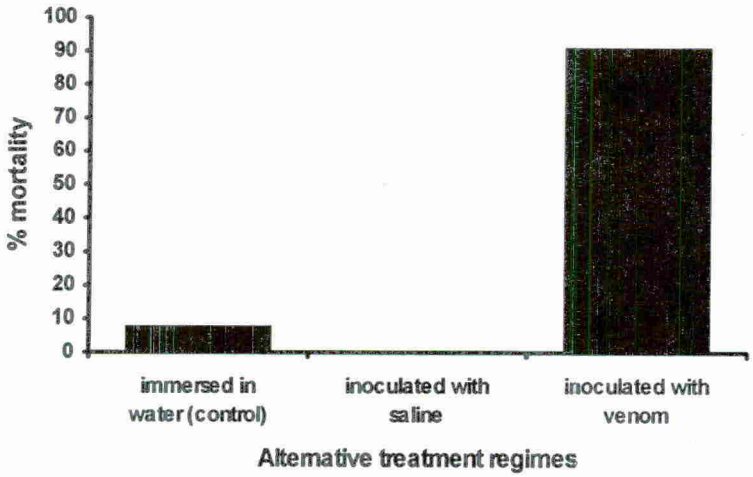

Figure 1. % mortality recorded in *L. oleracea* following inoculation with *E. pennicornis* venom. Bars = 95% confidence limits for percentages.

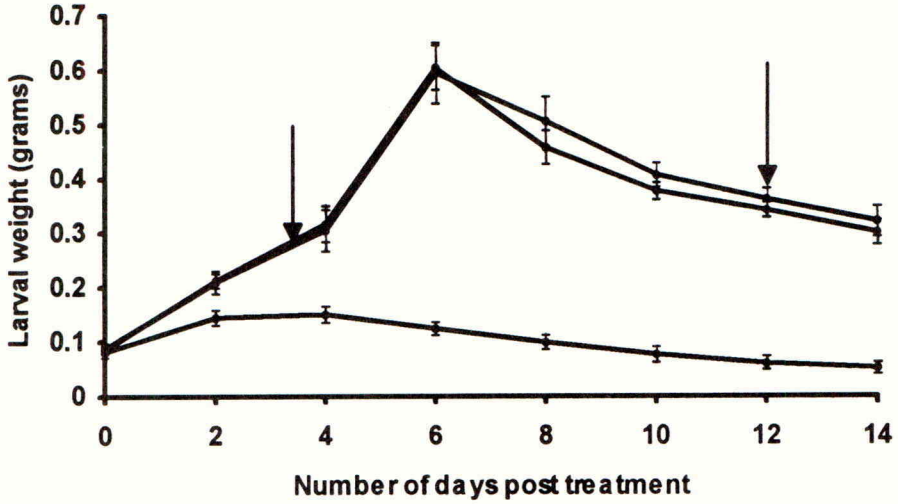

Figure 2. Growth and development of fifth instar *L. oleracea* after **a**: no treatment, **b**: saline inoculation, **c**: inoculation with *E. pennicornis* venom; **M** : untreated or saline-inoculated larvae moulted to sixth instar; **P**: untreated or saline-inoculated larvae pupated. Bars = 95% confidence limits.

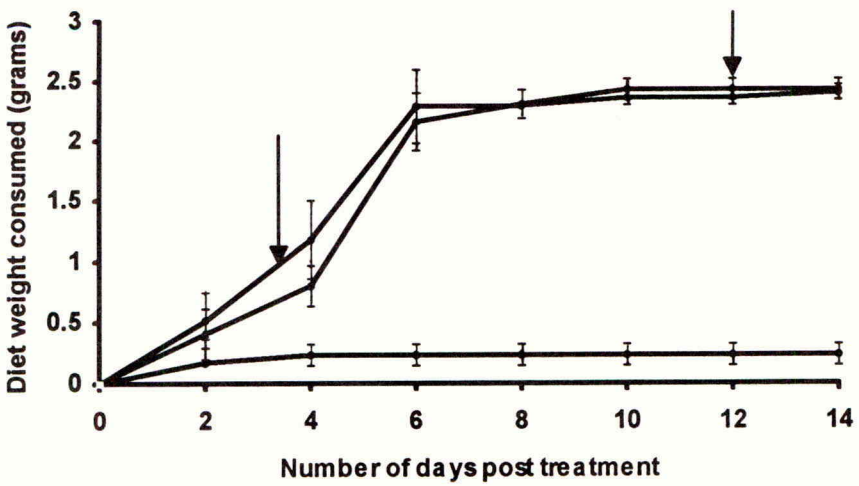

Figure 3. Cumulative diet-consumption by fifth instar *L. oleracea* after **a**: no treatment, **b**: saline inoculation, **c**: inoculation with *E. pennicornis* venom; **M** : untreated or saline-inoculated larvae moulted to sixth instar; **P**: untreated or saline-inoculated larvae pupated. Bars = 95% confidence limits.

Experiment 2: Ecdysteroid titres recorded in venom-injected, saline-injected, parasitized or non-parasitized *L. oleracea*

Figures 4 and 5 show mean levels of ecdysteroid recorded in healthy, parasitized, saline- or venom-injected *L. oleracea* larvae respectively, over 5 d. While non-parasitized individuals attained an ecdysone peak 48 h after their moult from fourth to fifth stadium, caterpillars of the same age which had been parasitized by *E. pennicornis* 48 h earlier, showed only a slightly raised hormone level (Figure 4). Haemolymph samples collected from non-parasitized individuals at or after 72 h showed no further rise in ecdysteroid titre. By contrast, parasitized caterpillars showed a marked increase in ecdysteroid level at 5 d post-parasitism.

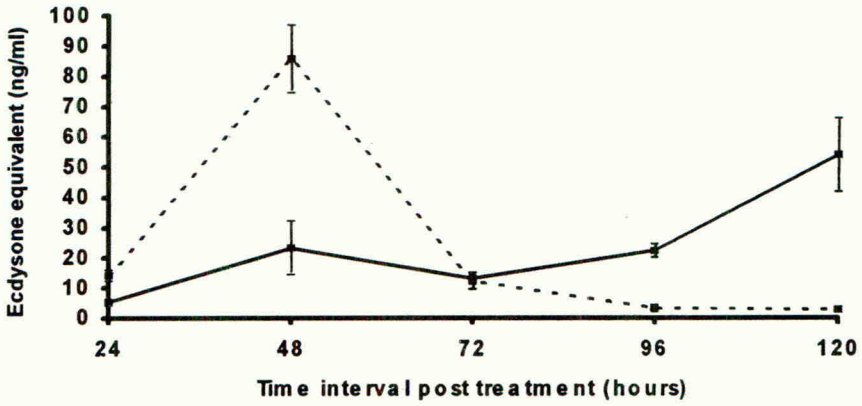

Figure 4. Haemolymph ecdysteroid titres recorded in non-parasitized (- - - - -) or parasitized (———) *L. oleracea* over a 5 d period. (Bars = +/- s.d.).

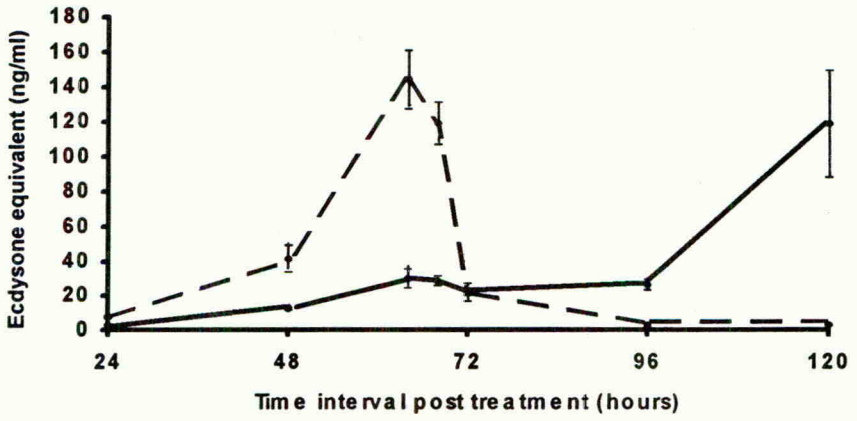

Figure 5. Haemolymph ecdysteroid titres recorded in saline-inoculated (— — —) or venom-inoculated (———) *L. oleracea* over a 5 d period. (Bars = +/- s.d.).

Differences in the comparitive endocrinology of saline-injected or venom injected larvae appeared to echo the differences which existed between the ecdysteroid levels recorded in nonparasitized or parasitized *L. oleracea* (Figure 5): Saline-injected caterpillars displayed a pre-moult ecdysone surge at 64 h after initial treatment. The titre in these control individuals then dropped rapidly, and remained low for the remainder of the experimental period. By contrast, venom-injected caterpillars (which do not moult) showed no significant ecdysteroid surge at 64 h, displaying a later peak at 120 h post injection.

DISCUSSION

The injection of *E. pennicornis* venom into nonparasitized *L. oleracea* had a rapid and profound effect on the survival, growth, development and food consumption recorded in fifth instar caterpillars. Since the symptoms shown by artificially stung caterpillars were the same as those normally associated with parasitism, but occured in the absence of any wasp brood, these findings support the hypothesis that maternal poison glands, rather than immature *E. pennicornis*, are the source of a powerful host-regulatory substance. The results of our second experiment reveal that haemolymph ecdysteroid content differs significantly between healthy *L. oleracea*, and similar larvae which have been parasitized by *E. pennicornis*. Moreover, the physiology of venom-injected individuals was directly comparable to that of their parasitized counterparts. When considered together, these results not only confirm that parasitism by *E. pennicornis* has a marked effect on host ecdysone levels, but also demonstrate that disruptions to host endocrinology are attributable to the action of venom.

During the normal course of insect development, a surge of ecdysone must take place before moulting can take place (Riddiford, 1980). Our findings show that this pre-ecdysis peak occurs in both healthy, nonparasitized *L. oleracea* (fifth instar + 48 h), and in similar saline-injected caterpillars (fifth instar + 64 h). However, while control larvae achieve an apparently normal hormone titre profile, parasitized or venom-inoculated *L. oleracea* do not exhibit any significant rise in their respective ecdysone levels until 120 h post-treatment. This means that when treated larvae reach the age at which they would usually be expected to moult to their sixth instar (fifth instar + 72 h), their hormonal constitution will not be compatible with ecdysis. This absence of the required peak at the appropriate time is therefore likely to account for the developmental arrest observed in poisoned and/or parasitized hosts.

Many ectoparasitoid species oviposit onto the cuticle of the larval stages of holometabalous insects. Unless these hosts are prevented from moulting, then wasp offspring must either complete their entire preadult development during the course of a single host stadium, or risk expulsion at the next host ecdysis. Although the most widely-documented effect of ectoparasitoid venom is undoubtedly host-paralysis (Beard, 1978; Vinson & Iwantsch, 1980; Quistad *et al.*, 1994), it has been shown that some wasp species inflict stings which inhibit further larval-larval host moulting and prevent subsequent host pupation (Shaw, 1981; Uematsu, 1986; Uematsu & Sakanoshita, 1987; Coudron *et al.*, 1990). The findings of the present study suggest that adult female *E. pennicornis* are similarly equipped with a venom which disrupts host endocrinology and development, thus enabling vulnerable wasp offspring to remain on the outside of the parasitized host long enough to complete their larval life cycle. Further characterization of *E. pennicornis* venom is in progress (Weaver *et al.*, in submission).

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ANTI JUVENILE HORMONES: FROM PRECOCENES TO PEPTIDES

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ABSTRACT

In this paper we shall attempt to review the historical development of, and current prospects for, novel insect control agents that act by inhibiting the production or action of insect juvenile hormones. The idea of anti juvenile hormone agents derived from the realisation that juvenile hormone analogues, although in some ways ideal alternatives to conventional neurotoxic insecticides, had some inherent disadvantages for use against a wide range of agricultural and horticultural pests. Despite considerable research efforts aimed at identifying or synthesising anti juvenile hormone agents, until recently (with the discovery of naturally-occurring allatostatic neuropeptides) this area has not fulfilled its undoubted promise. Currently however, research in this area is vibrant, and there is renewed hope that the present interest in allatostatic peptides will culminate in the development of a new generation of insect control techniques that are both highly effective and environmentally compatible.

INTRODUCTION

The discovery (Röller *et al.,* 1967) of the chemical structure of the first of a series of chemically related, naturally-occurring, insect juvenile hormones (JH) led to the search for compounds that mimicked the action of these molecules. The impetus for this search originated in the belief that such juvenile hormone analogues (JHA) could be used as safe insecticides (Williams, 1958). JHA act by disrupting insect metamorphosis and reproduction, thereby reducing or preventing subsequent generations. Despite the fact that juvenile hormone analogues have indeed found use as pest control agents (Staal, 1975), their widespread use in pest management programs has been limited by a number of factors. First, the early analogues were insufficiently stable in the outdoor environment to be useful against an acceptably wide range of horticultural and agricultural pest species. Second, JHA do not have toxic action even in target species, and therefore cannot be used to rapidly eliminate an existing pest population once it has become established. Lastly, JHA exert their morphogenetic effects at or around the time of metamorphosis (i.e. at the larval-pupal or pupal-adult transformation in holometabolous insects, or at the nymphal-adult moult in hemimetabolous insects). While this means that JHA can be extremely effective against pest species in which the larval stages are not responsible for economic damage or do not act as vectors of disease (e.g. ants, mosquitoes, fleas, certain flies, etc.), this factor precludes the use of JHA against many important pest groups (e.g. lepidopteran or coleopteran larvae) where larvae may cause significant crop damage before the time at which JHA compounds are effective. This latter problem was recognised early, and resulted in the search for effective anti juvenile hormone agents (AJH). The rationale behind this search was that such molecules, instead of replacing

endogenous hormones at the time of metamorphosis, would reduce or negate the effects of endogenous hormones much earlier in larval or nymphal development. Such action would (theoretically) disrupt embryogenesis, induce premature metamorphosis, and disrupt reproductive physiology. Such effects have long been recognised as a result of surgical removal (allatectomy) of the corpora allata (CA), the retrocerebral endocrine glands which synthesise and release juvenile hormone. Thus, there was every reason to believe that an agent capable of effecting a "chemical allatectomy" would be useful as an insect control agent.

Precocenes

The first group of AJH were discovered through the random screening of natural plant extracts. Bowers *et al.* (1976) reported that extracts from the plant *Ageratum houstonianum* contained biologically active principles that induced premature metamorphosis and adult sterility in the hemipteran *Oncopeltus fasciatus*. The active molecules were identified as mono- or di-methoxy substituted 2,2-dimethyl chromenes (precocenes I and II, Figure 1). These were subsequently shown to act by cell-specific toxicity in the CA, as a result of the formation of highly unstable epoxide alkylating agents (Pratt *et al.*, 1980). Despite the fact that the precocenes were effective AJH agents, their activity appears to be limited to certain hemipteran and orthopteran insects and to some ticks (Staal, 1986). Thus, the precocenes show an insufficient spectrum of activity against economically important pest groups to be considered for development as practical AJH. Nevertheless, the discovery of the precocenes did demonstrate that the concept of anti juvenile hormone agents was a viable one and, that such molecules occurred naturally.

Synthetic inhibitors of juvenile hormone biosynthesis

Following the impetus provided by the discovery of the precocenes, the search for other AJH agents centred around the opportunity to inhibit key steps in the biosynthetic pathway leading to the production of juvenile hormone from acetate and other simple precursors. Since it was suspected that HMG-CoA reductase was the rate limiting step in the JH biosynthetic pathway, several groups investigated the potential of inhibitors of this enzyme as AJH agents. In these early studies (Monger *et al.*, 1982; Edwards and Price, 1983) the fungal metabolite compactin (Figure 1) was shown to be a highly potent inhibitor of juvenile hormone biosynthesis *in vitro* (EC_{50} ~ $2x10^{-9}M$). However, these and subsequent researchers failed to demonstrate sufficient *in vivo* activity of this, or any other inhibitor of HMG-CoA reductase, for practical development against insect pests. (Compactin was shown to have *in vivo* AJH activity in *Mamestra brassicae*, but only after repeated injection (Hiruma *et al.*, 1983)). A variety of synthetic compactin analogues have been developed by the pharmaceutical industry as effective medicines for use in the treatment of human hypercholesterolemia. The structures of HMG-CoA reductase inhibitors are generally characterised by a number of sites that are highly prone to oxidative metabolism, which may explain their lack of *in vivo* activity in insects. However, industrial enthusiasm for this approach to AJH agents was dampened by the report from Merck scientists that the HMG-CoA inhibitor mevinolin (Figure 1) had teratogenic effects in rats, which could be reversed by treatment with mevalonic acid (Robertson *et al.*, 1981).

Using a similar rationale (i.e. inhibition of JH biosynthesis) Quistad *et al.* (1981) investigated the effects of another inhibitor of cholesterol biosynthesis (fluoromevalonolactone (FMev) Figure 1) and demonstrated convincing *in vivo* AJH activity in the moth *Manduca sexta*.

These studies represented the first demonstration of true AJH activity (i.e. the induction of premature pupation) in a lepidopteran species, and the same compound was also shown to be mildly effective in delaying the JH-regulated production of oothecae in *Periplaneta americana* (Edwards *et al.*, 1985). Unfortunately, any change in the chemical structure of this molecule caused a reduction in its (already modest) activity. A similar attempt at designing antimetabolites of intermediates of JH biosynthesis (Quistad *et al.*, 1985) resulted in the discovery of 3,3-dichloroallyl hexanoate (Figure 1). This material had only weak AJH activity on *M. sexta* and *Heliothis virescens.* However, later Henrick (1991) reported the discovery of a modified allylic ester ("ZR-7223", Figure 1) with strong AJH activity on a variety of lepidopteran insects, including *M. sexta, H. virescens,* and *Spodoptera exigua.* Foliar application of a 0.25% spray of this material gave premature development in 100% of second stadium *S. exigua* placed on treated foliage (Henrick, 1991). Further attempts to increase the inherent level of activity of this substance were unsuccessful. Regrettably, there appear to be no other published accounts of the biological properties of this active and extremely interesting chemical, and its precise mode of action remains unclear.

Kuwano *et al.* (1983;1984) discovered anti juvenile hormone activity on *Bombyx mori* in a series of 1-citronellyl-5-substituted imidazoles (e.g. citronellyl phenyl imidazole (CPI, Figure 1). These compounds may act by inhibition of the cytochrome P-450 which is necessary for epoxidation of methyl farnesoate and its homologues to juvenile hormones (Kuwano *et al.*, 1983). Unfortunately, these compounds have not been shown to be active *in vivo* in any other species of insect (Staal, 1986).

Miscellaneous anti juvenile hormone agents

Interestingly, some early studies also indicated that the application of JH or of the JHA hydroprene (Figure 1) to intact insects was highly effective in reducing JH biosynthesis (Tobe and Stay, 1979) and endogenous JH levels (Edwards *et al.*, 1983). This *in vivo* action of externally applied JH compounds was assumed to be the result either of feedback inhibition of juvenile hormone biosynthesis or of stimulation of the production of juvenile hormone esterases or other JH metabolising enzymes, since there was little convincing evidence that JHA affected JH biosynthesis by isolated corpora allata *in vitro.* Although this observation was scientifically interesting, it became clear that significant feedback-mediated reductions in endogenous JH levels were only apparent with highly active juvenile hormone compounds (Edwards, 1987) and, as a result, any reduction in endogenous hormone levels was normally compensated for by the biological activity of the externally applied analogue. Thus, no true AJH effects were manifest *in vivo,* and it is highly unlikely that analogues of JH could produce reductions in endogenous JH without the attendant replacement JH activity. Nevertheless, these experiments produced clear indication of the existence of feedback regulation of endogenous juvenile hormone levels.

Staal (1986) reported the discovery of two compounds, ethyl 4-[2-(tert-butylcarbonyloxy) butoxybenzoate (ETB) and ethyl 3-methyl 2-dodecenoate (EMD, (Figure 1) which have mixed JH agonist/antagonist activity. Both of these compounds have relatively low JH activity in a wide variety of species, and both have AJH effects which are manifest at only a few intermediate doses in the sensitive species; at higher doses the intrinsic JH agonist effects override the antagonist effects. Presumably these compounds exert this limited AJH effect by suppressing endogenous JH titres through a stronger effect on those receptors which cause the

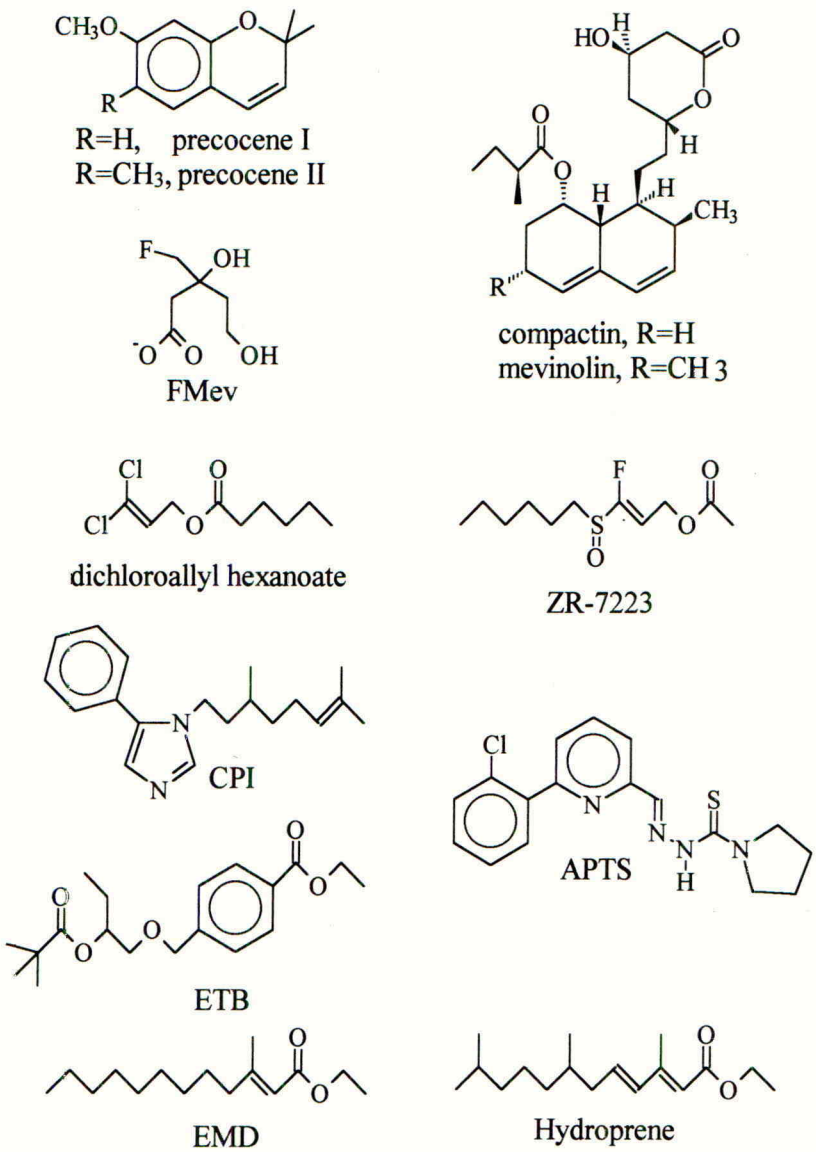

FIG 1. Chemical structures and trivial names of non-peptide anti juvenile hormone agents described in the text.

feedback inhibition *vs.* those receptors responsible for the morphogenetic effects. Unfortunately, efforts in synthesis of related structures failed to reveal any in which the antagonist and agonist effects could be separated. ETB has been claimed to have direct effects on inhibition of JH biosynthesis by CA of *M. sexta in vitro* (Kramer and Staal, 1981); however, its dose/response curve exhibits a steepness for "inhibition" of JH biosynthesis

resembling a chemical volumetric titration. In contrast, the dose/response curve for FMev is far shallower, covering a hundred-fold concentration range between 80% and 5% effect (Kramer and Staal, 1981). We believe that the curious dose/response curve for ETB is reminiscent of a physico-chemical process (such as reaching a critical micelle concentration value) that may have occurred during the *in vitro* assay procedure. Thus, it seems doubtful that these results have any bearing on the partial antagonist effects observed.

A group from Rohm and Haas (Barton *et al.,* 1989) reported anti juvenile hormone effects of a number of thiosemicarbazones, especially 6-(o-chlorophenyl) pyridyl-2-carboxyaldehyde pyrrolidinothiosemicarbazone (APTS, Figure 1). These substances are active on *M. sexta, H. virescens,* and the cutworm *Agrotis ipsilon,* showing a variety of effects including precocious pupae. Curiously, these substances were only active when fed in the diet and not upon topical application. Their AJH effects could be "rescued" by treatment of larvae with the juvenoid fenoxycarb (Barton *et al.,* 1989). Again, the mode of action of these compounds remains unclear.

Naturally occurring anti juvenile hormones - allatostatins

For many decades researchers have been interested in the control of juvenile hormone secretion by the corpora allata. Classically, hormone titres in vertebrates are regarded as being controlled by the rate of synthesis. While a similar situation has seemed likely in insects, evidence has also accumulated that at least part of the regulation in JH titres may be attributable to the rate of hormone degradation (Hammock, 1985). A large number of studies over several decades showed that severance of the nervous connection between the brain neurosecretory cells and the CA altered rates of JH production, as did cautery of certain brain neurosecretory cells (Feyereisen, 1985, Stay and Woodhead, 1993). More direct proof of the existence of factors that stimulate the CA (allatotropins) or inhibit these glands (allatostatins) have come from maintaining isolated glands *in vitro* and monitoring their rate of JH synthesis by radiochemical assay or radioimmunoassay for the JH (reviewed by Feyereisen, 1985). A great deal of the physiological literature regarding control of activity of CA is controversial and even contradictory; this may be due in part to differences in control mechanism between insect species of different orders. Once *in vitro* systems for studying control of corpus allatum activity by extracts of insect neuroendocrine tissue were adopted, significant progress was made (Feyereisen, 1985). The first endogenous factor identified that affects corpus allatum activity was an allatotropin from *M. sexta* (Kataoka *et al.,* 1989). To date this is the only insect allatotropin to be identified; its physiological role remains unclear as it is active only on the CA of adult lepidopteran insects, with no activity on larval *M. sexta* CA (Kataoka *et al.,* 1989).

Shortly after this discovery, the sequences were published of four closely related allatostatic peptides isolated from brains of the cockroach *Diploptera punctata* (Woodhead *et al.,* 1989). These are octa- to tridecapeptides containing the conserved structural motif Tyr-Xxx-Gly-Phe-Leu-amide. Subsequently a tyrosine-rich allatostatin (AS) containing eighteen residues was discovered containing the same conserved C-terminus (Pratt *et al.,* 1991). More recently, the sequence of a 370-amino acid pre-pro-peptide was deduced from a cDNA sequence derived from *D. punctata*; this allatostatin precursor contains thirteen allatostatin-type peptides (Donly *et al.,* 1993). Biological studies performed when only five of these peptides were available showed that the Tyr-rich allatostatin of Pratt *et al.* (1991) seemed to be the most potent in *D.*

punctata (Stay and Woodhead, 1993). However, the degree of activity of these peptides changes with the physiological age of the animal. Curiously, the CA of *D. punctata* seem to be least sensitive to these peptides when the rate of JH biosynthesis is highest, and to be most sensitive when the rate of JH biosynthesis is lowest (Stay and Woodhead, 1993). This paradoxical difference raises suspicions as to whether or not these molecules are the sole (or even the primary) regulators of JH levels in *D. punctata.* In fact, immunohistochemical data show neurones innervating the CA which do not stain for AS, suggesting the possibility of other undiscovered regulators of JH biosynthesis in this species. Interestingly, *in vivo* treatment of *P. americana* with each of four different Dip-AS causes a reduction of JH titer, although Dip-AS IV was most potent (Weaver *et al.*, 1995). However, these results illustrate that, if allatostatic peptides can be introduced into insects in sufficient quantities, they do indeed result in the lowering of endogenous JH levels.

Mas-AS	pQ V R F R Q C Y F N P I S C F
Dip-ASB2 (AS V)	A Y S Y V S E Y K R L P V Y N F G L-NH2
Dip-AS I	A P S G A Q R L Y G F G L-NH2
Dip-AS II	G D G R L Y A F G L-NH2
Dip-AS III	G G S L Y S F G L-NH2
Dip-AS IV	D R L Y S F G L-NH2
Dip-AS VI	D G R M Y S F G L-NH2
Dip-AS VII	Y P Q E H R F S F G L-NH2
Pea-AST 1	S P S G M Q R L Y G F G L-NH2
Pea-AST 2	A D G R L Y A F G L-NH2
Cav-AS 1	D P L N E E R R A N R Y G F G L-NH2
Cav-AS 2	L N E E R R A N R Y G F G L-NH2
Cav-AS 3	A N R Y G F G L-NH2
Cav-AS 5	G P P Y D F G M-NH2

FIG. 2. Sequences of the chemically isolated allatostatins of *D. punctata* (Dip), *P. americana* (Pea) *M. sexta* (Mas) and *C. vomitoria* (Cav).

P. americana has been shown to contain at least two AS related to those from *D. punctata* (Weaver *et al.*, 1994). Recently, a cDNA was isolated encoding a sequence specifying a preproallatostatin precursor from *P. americana;* it is highly similar to that from *D. punctata,* except that it encodes 14 separate allatostatin-like peptides rather than 13 as in *D. punctata* (Ding *et al.*, 1995). In addition, *Calliphora vomitoria* has been shown (Duve *et al.*, 1993) to contain at least five Dip-AS-like molecules. However, these have no allatostatic effects on CA of *C. vomitoria,* and immunohistochemical studies show them to be present in nerves projecting into parts of the body other than the CA. These observations suggest that the Cav-AS have an (as yet) unknown function in this animal. Lange *et al.* (1995) have shown that the Dip-AS inhibit both myogenic and proctolin-induced contractions of the hindgut of *D. punctata*, indeed revealing another possible mode of action for these compounds. However, these peptides do not affect the contractile properties of the oviduct of either *D. punctata* or *L. migratoria* (Lange *et al.*, 1995), suggesting a tissue specificity in their effects.

Kramer *et al.* (1991) identified an allatostatin from heads of pharate adult *M. sexta.* This single peptide is unrelated to the multiple allatostatins of *D. punctata;* it contains a disulphide bond and 15 amino acids. Whereas the C-terminal portion of the Dip-AS appears important for activity, limited data suggest that the amino terminal portion of Mas-AS is important for activity. This peptide is highly potent (EC_{50} ~ $2\text{-}5\times10^{-9}$ M) in larvae of early fifth stadium *M. sexta,* that point in this stadium at which JH titer is at its peak (Baker *et al.*, 1987). This is in marked contrast to the low activity of the *D. punctata* allatostatins in stages where JH biosynthesis is maximal. This allatostatin was reported to completely inhibit biosynthesis of JH by CA of adult female *M. sexta* at 0.1×10^{-6} M, and to inhibit CA of adult *H. virescens* by 77% at 0.5×10^{-6} M; however, it had no effect on CA of two species of orthopterans or on the CA of the coleopteran *Tenebrio molitor* (Kramer *et al.*, 1991). Thus, the two unrelated types of allatostatin appear to have activity only in species with a close phylogenetic similarity to those from which they were isolated. The activity of Mas-AS has not been thoroughly studied in other developmental stages as with the *D. punctata* allatostatins. As is the case with the latter allatostatins, the effects of *M. sexta* allatostatin are reversible; inhibited CA placed in medium lacking the peptide regain full activity (Kramer *et al.*, 1991). At present, it is not clear whether or not the AS isolated from *M. sexta* is indeed the lepidopteran allatostatin, although Jansons *et al.* (1996) have reported the molecular characterisation of a cDNA from brains of the noctuid moth *Pseudaletia separata* that codes for the Mas-AS peptide. In addition, other researchers have characterised a factor from *M. sexta* termed allatinhibin, an irreversible inhibitor of CA activity (Bhaskaran *et al.*, 1990; Unni *et al.*, 1993). Unfortunately, this material has yet to be isolated and sequenced.

It should be noted that virtually all studies on the *D. punctata* allatostatins have concentrated on the gonadotropic role of JH (its production by adult females) rather that the morphogenetic role in regulating metamorphosis, while the *M. sexta* allatostatin was isolated using larval CA as an assay. Similarly, research on allatinhibin has concentrated on the morphogenetic function of JH in larvae. It is tempting to speculate that this might explain why entirely different types of peptides have been identified.

Cusson *et al.* (1991) have published a preliminary report on photoaffinity labelling of receptors of *D. punctata* AS in membrane preparations of tissues of this species. The radioactivity allatostatin analogue labelled proteins of 59 and 39 kDa in the CA, 41 kDa in the brain, and 38, 41 and 60 kDa in the fat body. No information has been reported on receptors for other

allatostatins. The actual characterisation and cloning of a receptor for the allatostatins is likely to be a formidable task given the minute size of the CA. However, a receptor for the diuretic hormone of *M. sexta* has been characterised and cloned (Reagan, 1994) and over-expressed in a baculovirus system (Reagan, 1995). With the availability of a recombinant receptor, the stage would be set for development of extremely quick, high through-put screens to locate low molecular weight compounds that bind to and activate the allatostatin receptor. This seems clearly the best way to develop an agricultural pest control agent based on our current knowledge of the insects' own mechanisms for lowering JH levels. Thus, there is renewed optimism that several decades of research into the regulation of insect endocrine physiology, has now reached the point at which the results of these studies can be utilised in the development of a new generation of biologically active, insect specific and environmentally sensitive insect control agents.

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