Chemical Ecology of the Carrot Fly, *Psila rosae* (F.): Laboratory and Field Studies

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ABSTRACT

The carrot fly (*Psila rosae* F.) is an important pest of the cultivated carrot (*Daucus carota*) and other crop species in the family Apiaceae, since the larvae burrow into and feed on the developing roots. Current *P. rosae* control relies heavily upon the use of chemical insecticides, but these are inadequate. The aims of this study were to investigate the chemical ecology of *P. rosae*, particularly with regard to long range attractant and repellent semiochemicals suitable for incorporation into integrated pest management strategies; the incorporation of attractant host plant extracts, or semiochemical attractants, into the monitoring programme; and the development of an autodissemination trap for release of the pathogenic fungus *Entomophthora schizophorae* in the field for biological control. A number of techniques for the extraction of volatile semiochemicals from a wide range of host and non-host plant species, and *P. rosae* adults themselves, were employed and compared. Samples were analysed by gas chromatography (GC), and the biologically active components in these complex natural product extracts were located by coupled GC-electrophysiological techniques and identified by coupled GC-mass spectrometry (GC-MS). Responses to the electrophysiologically active compounds were compared using electroantennographic (EAG) analysis: one unusually high EAG response was observed to a toxic component in hemlock (*Conium maculatum*) leaf extract (γ-coniceine). Of the forty-two EAG active components identified from common crop species and *C. maculatum*, eight had not previously been reported. A range of bioassay techniques (including four-arm star olfactometers, Y-tube olfactometers, and oviposition bioassays) were employed to determine behavioural activity of the samples and identified compounds, but only the oviposition bioassay showed significant behavioural discrimination to γ-coniceine. Further studies of longer range behavioural responses to volatile semiochemicals were performed in the field. Significant responses were seen to a known field attractant (combined *trans*-asarone and hexanal) and, for the first time, to a microwave assisted solvent extract of celery (*Apium graveolens*) leaf. A prototype autodissemination trap for *E. schizophorae* was produced and evaluated.

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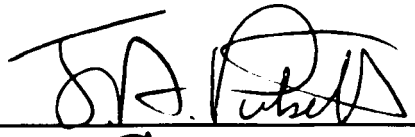
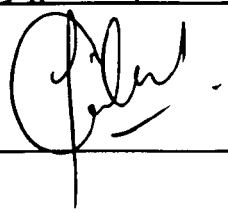
Supervisor 1: 

Supervisor 2: 

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1 GENERAL INTRODUCTION

1.1 THE CARROT FLY AS A PROBLEM

Throughout history, people from many different civilisations have used members of the family Apiaceae. A few species such as the carrot, celery, and parsnip are staple foods, whilst other species are grown as herbs and spices used as flavourings in food and drink. These apiaceous crops have a few important pests, as well as numerous occasional or minor pests (Ellis and Hardman, 1992).

The carrot fly (*Psila rosae* F.) (Diptera: Psilidae) is the most serious pest of carrot crops in the UK and northwest Europe (Ellis, 1993; MAFF and ADAS, 1980), and it frequently causes serious damage to this crop as well as to parsnips and some times celery and parsley, both on farms and in gardens (MAFF and ADAS, 1983). The UK is largely self sufficient in carrots; production figures for 1993/94 indicate that 840,000 tonnes (17,000 ha) of carrots with a market value of £93million were grown in the UK. Exports from the UK are small (8,000 tonnes) and imports (34,000 tonnes) are largely confined to May and June before the early UK crop becomes available (MAFF - PSD, 1995a). UK crops may be attacked by up to three generations of the fly each year (Coppock, 1974), but only the first two cause significant crop damage (Finch, Collier and Davies, 1996). It is the feeding activity of the larvae which makes this insect a pest. Larval feeding causes considerable economic losses annually. This is not so much due to a reduction in yield, but rather a reduction in quality, with many retailers placing heavy emphasis on blemish free produce (Parker, 1991). First generation carrot fly adults normally emerge in May or early June. The larvae initially feed on the side roots near the base of the taproot and carrot seedlings can be killed by early carrot fly attack. The second generation adults generally emerge between late July and September, and adult activity may continue though to November, either as a greatly extended second generation or partial third generation. Larvae from this second generation are responsible for the most severe damage on carrots, as although some larvae pupate in the late autumn, others continue feeding throughout the winter and damage becomes progressively, worse allowing entry of secondary microbial infections. Very high levels of damage from the pest can occur, with some trials reporting over 90% of unprotected roots with damage (MAFF and ADAS, 1983).

These economic factors and increasing problems with controlling this pest have stimulated increasingly detailed studies of its biology and control measures in recent years, see general summaries by Whitcombe (1938) and Dufault and Coaker (1987). A detailed bibliography of the multitude of carrot fly related papers published up to 1985 has been compiled by Hardman, Ellis and Stanley (1985). At present, carrot fly damage is maintained at low levels in conventional crops by application of a small range of synthetic insecticides. Insecticides commonly used to control carrot fly in carrots in the UK include chlorfenvinphos, phorate, triazophos and pirimiphos-methyl. Control of the larvae from the first generation is most effectively achieved by the use of granular treatments applied at drilling. Granules approved for this use include carbofuran, carbosulfan, chlorfenviphos, disulphoton and phorate. In areas of low risk, where the intensity of attack is less and the need for such robust and persistent treatments is reduced, triazophos is approved (MAFF - PSD, 1995a). Soil insecticides act against the carrot fly by being picked up and translocated to feeding sites on the roots at concentrations sufficient to kill larvae (Wheatley and Hardman, 1967). Granules applied at drilling are not sufficiently persistent to control second-generation attack (Wheatley, 1969). The protracted nature of this later generation means that effective control is required over an extended period, often involving from one to three applications (with up to nine applications reported in some instances) of organophosphorus insecticides. These supplementary treatments are applied as liquid insecticide sprays, and are most effective when directed at the crown of the carrot plant and surrounding soil for larval control (Mowat and Martin, 1984), although flies on the foliage may also be killed (Maskell and Gair, 1973). The performance of OP and carbamate insecticides recently became erratic in some regions (Maskell and Gair, 1973), and lead to suggestions that *P. rosae* was becoming resistant to these insecticides (Wheatley and Percivall, 1974; Thompson and Harris, 1982). Subsequent investigation concluded that the main reason for the poor performance of these treatments was, in fact, due to the development of microbial populations in the soil capable of rapid degradation of these compounds (Suett, 1986; Harris *et al.* 1988). Further problems with the use of insecticides have resulted from the carrot roots' ability to take up pesticides from the surrounding soil. The pesticides accumulate in the roots within ducts containing essential oils, and are not metabolised by the plant (Schupan and Boek, 1960; Suett, 1971), leading to a build up of residues in the crop. As these compounds can cause both acute and chronic toxic effects on the human body, resulting from inhibition of the enzyme

cholinesterase, composite samples (normally consisting of ten roots) from harvested crops are tested to monitor pesticide residue levels and ensure they are below acceptable safety limits. However, a recent study testing residue levels of organophosphorus insecticides in individual carrots found that accumulation varied widely (even between carrots grown in the same row in a field), with some carrots containing multiple and unexpectedly high residues (up to 25 times or higher) compared with that from the composite sample (MAFF - PSD, 1995a). Although consumption of these high residue carrots would result in a much higher intake of pesticides than was previously believed to occur, toxicological data indicated that these levels did not represent any immediate health risk and any effects would be mild and rapidly reversible. Investigations of domestic processing of carrots indicated that cutting the top 2-3mm from the roots i.e. 'topping', removed about one-half, and peeling and 'topping' removed about four-fifths of the OP insecticide residues. However, it was considered that the margins of safety had been eroded to a level where action was required to restore them. As a result, restrictions in the use of organophosphorus insecticides were introduced by the Pesticide Safety Directorate (MAFF - PSD, 1995c), along with changes to the monitoring methods for pesticide residues in carrots (MAFF - PSD, 1995b). The new regulations on OP insecticides applied to carrots limit applications to a maximum of 3 full rates per crop per year on mineral soils (up to 10% organic matter), or 4 full rates on organic (peat) soils (greater than 10% organic matter), not including OP seed treatments.

As a result of these problems, carrot growers in the UK are looking for ways to reduce their reliance on OP insecticides for carrot fly control. In order to stay within these new limits and obtain a reasonable degree of pest control against *P. rosae*, farmers have been forced to turn to the supplementary applications of pyrethroids, including tefluthrin and lambda-cyhalothrin (ADAS, HRI and PSD, 1995). However, these insecticides are far less effective (Andrews *et al.*, 1998) and have a lower persistence than OP insecticides, resulting in the need for frequent supplementary spray applications in order to remain effective during the long flight period of each generation of flies (Davies and Collier, 2000).

There are a number of alternative non-insecticidal methods of reducing carrot fly damage that could be incorporated into both conventional and organic carrot farming, as summarised by Collier and Finch (2000). However, these currently available alternative methods are insufficient to provide a fully effective or practical alternative to pesticides, and their use would also require that the current demand for

high quality blemish free carrots be adjusted to accommodate any lowering in quality that might result from a change in pest control and storage practices. Although no completely integrated approach to the control of this pest is available at present, a wide range of methods used in combination with limited insecticide application could be incorporated into a partially integrated strategy for reducing damage incurred from this pest. The use of partially resistant carrot varieties can reduce levels of carrot fly damage and improve control, particularly where less effective insecticides are used (Thompson *et al.*, 1994). The most promising cultural methods of control include the use of crop covers, crop isolation, the use of exposed sites, manipulation of drilling and harvesting dates, and the use of forecasting and monitoring to determine the timing of fly attack.

Many techniques of managing carrot fly require precise knowledge of when the adults emerge, reach peak numbers, or how long they remain active for each generation. Forecasting methods based on meteorological data have been developed to predict the timing of carrot fly activity, but they cannot predict the numbers or the severity of subsequent attack. However, they are useful for indicating when to deploy carrot fly monitoring traps (ADAS, HRI and PSD, 1995). In recent years, monitoring programmes have been established to reduce applications of insecticides against carrot fly, and monitoring is recommended by ADAS, HRI and PSD (1995) to all growers of susceptible apiaceous crops.

As the abundance of *P. rosae* varies widely from field to field, monitoring of individual fields has been employed rather than regional monitoring. Münster-Swendsen (1983) found that the number of flies caught on traps was related to activity, density and trapping efficiency, whilst Esbjerg *et al.* (1983) found that wind, rain and trap height influenced catches. The uneven distribution of flies in fields and inconsistent thresholds for the different generations have made the establishment of thresholds difficult (Philipsen, 1986). There are no valid thresholds for *P. rosae* in the UK. Nevertheless, the traps aid in optimum timing of first spray applications and can indicate the need and frequency of applications. In low risk areas, traps may indicate where later sprays can be omitted. However, current traps are not very efficient, particularly at low population densities. ADAS and HRI Wellesbourne have evaluated the most effective means of monitoring carrot fly. This has resulted in the adoption of the Swiss REBELL ® orange sticky trap as the standard monitoring tool for monitoring services (i.e. ADAS and other private consultants). These traps are used in sets of three per field and should be changed and checked twice

weekly. As these traps also catch other flying insects, some expertise is required to separate carrot flies from the rest (Parker, 1991). Trapping efficiencies are so low that economic thresholds are essentially based on the presence or absence of captured flies. Dufault and Coaker (1987) urged additional studies aimed at improving the monitoring system, by aiming to develop more efficient traps that are more selective for *P. rosae*, and further work on evaluating trap catch / crop damage relationships, in order to improve the current insecticide treatment recommendations based on presence or absence of flies.

There is a growing interest within farming to develop and exploit naturally occurring beneficial organisms such as predators, parasitoids, bacteria, viruses and fungi for pest, weed and disease control. The use of these organisms is advantageous as they may offer an environmentally friendly alternative to the use of chemical pesticides. Such an alternative is particularly important in farming systems where pest species have developed resistance to currently available pesticides, and where pesticides have been banned (e.g. organochlorides) or are being phased out (as in carrot production). Although a number of predators, parasitoids, nematodes and microbial pathogens are known to attack *P. rosae*, most seem unsuitable for use as biological control agents against this pest. This is often because they only cause low levels of mortality in the field, often far below 20%.

Fungal pathogens play a major role with regard to natural mortalities of *P. rosae* in the field, as epizootics (outbreaks of high levels of infection and mortality) tend to occur in most years. Two fungal pathogens, *C. apiculatus* and *E. muscae*, are known to cause significant mortality in field populations of *P. rosae*. Eilenberg (1988) suggested that both species were worth considering for use as biological control agents, but of the two pathogens, *C. apiculatus* seemed the least promising as it generally causes low levels of infection, while high mortalities have only been recorded during moist humid conditions in the autumn (Eilenberg, 1988). During five years of study on the occurrence of fungal pathogens in populations of *P. rosae* in carrot fields (Denmark), Eilenberg (1988) concluded that *E. muscae* should be regarded as the most promising agent for biological control. This pathogen was by far the most common species found to infect *P. rosae*, causing up to three epizootics per year, and was highly virulent towards this pest. Eilenberg suggested that the development of a biological control product, possibly using in vitro produced spores of one or several strains of *E. muscae*, could be used for control of *P. rosae*. He also suggested that this product could be modified, giving it a wider market for the control of several other

important agricultural pest species acting as hosts for this pathogen (e.g. *Delia radicum* and *Delia. antiqua*).

Ignoffo (1978) urged the exploration of novel approaches to targeting pest species with emtomopathogens, and defined the relatively new technique of autodissemination as the use of insects to introduce and spread entomopathogens in the ecosystem. The hypothesis behind this technique is that pest behaviour can be manipulated using semiochemicals to encourage the spread of pathogens to susceptible populations earlier in the season than would normally occur. In this way, disease epizootics could establish and decimate small early-season pest populations before the crop was damaged (Pell *et al*, 2001). This strategy has been applied to the biological control of the diamondback moth (*Plutella xylostella*) using *Zoophthora radicans* (Pell *et al*, 1993; Pell and Wilding, 1994; Furlong and Pell, 1995), and an adaptation of this strategy would seem ideal for the control of *P. rosae* with *E. muscae*. This strategy involved male moths being attracted into specially designed 'fast-entry / slow-exit' traps in response to synthetic female pheromone. Whilst inside the trap, moths were exposed to a sporulating source of *Z. radicans* and became infected with conidia. After becoming habituated to the pheromone, the infected moths left the trap and returned to the crop, spreading the pathogen into the field population. The benefits of this strategy are that the trap can be designed specifically to attract the target pest, thereby reducing the exposure of non-target organisms to the inoculum. As only small quantities of fungal inoculum are required for this method, some of the problems associated with mass production, formulation and storage can be overcome. As the inoculum is housed within the trap, it can be protected from the damaging effects of UV radiation and be provided with a microenvironment favouring conidial production and transmission of infection to the host (Pell *et al*., 2001).

A number of steps would be involved in the development of an autodissemination trap for the biological control of *P. rosae* using *E. schizophorae*, the first of which would be to design a trap capable of drawing flies into an enclosed area suitable for infection to occur. This could possibly be achieved for *P. rosae* using a combination of visual and semiochemicals attractants.

1.2 CHEMICAL ECOLOGY AND SEMIOCHEMISTRY

Although it is difficult to generalise about behaviour-controlling chemicals, due to their great diversity and the range of chemical groups into which they fall, it is well known that many of these semiochemicals, such as pheromones and plant kairomones, are perceived at a considerable distance from their original source. This is achieved by transmission of the semiochemicals through the air, requiring compounds that are volatile and have low molecular weights (typically <1000 Da) and relatively high lipophilicity (non-polar). Such compounds have also been found to act as semiochemicals in aquatic communication systems, e.g. algal pheromones (Jaenicke and Boland, 1982). Semiochemicals are generally small organic molecules, with between five and twenty carbon atoms and a maximum of two or three hetero-atoms, usually oxygen, but occasionally nitrogen or sulphur (Pickett and Woodcock, 1991). The great diversity found in semiochemical compounds results from the necessity of the signal to stand out as a clear, unambiguous message. Although compounds as simple as ethanol may be semiochemical components, larger molecules give greater possibilities for unique structures. This is often enhanced in many semiochemical compounds by the incorporation of asymmetric carbon atoms, which give rise to enantiomeric uniqueness in their structures. Many semiochemical signals are also composed of a mixture of chemical components, where the ratios of the individual compounds are important in imparting information to the receiving organism (Pickett *et al*, 1991). This semiochemical diversity amongst different species helps to avoid signal confusion, against the broader spectrum of chemicals present in the environment.

There are a number of approaches to the interpretation and classification of insect behaviour, ranging from the classical 'kineses-taxes' classification of Fraenkel and Gunn (1961) to a cybernetic concept of orientation (see Visser, 1988). In this study, definitions of chemicals eliciting behavioural responses have been limited to 'attractants, repellents and arrestants' in terms of the organisms' response patterns, as defined by Dethier *et al.* (1960):

Arrestant: A chemical which causes insects to aggregate in contact with it, the mechanism of aggregation being kinetic or having a kinetic component. An arrestant may slow the linear progression of the insects by reducing actual speed of locomotion or by increasing turning rate.

Stimulant: A chemical which causes, by kinetic mechanisms, insects to disperse from a region more rapidly than if the area did not contain the chemical.

Attractant: A chemical which causes insects to make oriented movements towards its source.

Repellent: A chemical which causes insects to make oriented movements away from its source.

Deterrent: A chemical which inhibits feeding or oviposition when present in a place where insects would, in its absence, feed or oviposit.

Host plant selection by phytophagous insects is generally divided into two somewhat overlapping aspects of behaviour. Firstly host plant finding, which relates to the insects spatial movements while on the ground or in the air, involving search patterns and orientation to hosts, which are affected by the plants distribution and other host characteristics (i.e. long range visual and olfactory cues). Secondly, host plant acceptance, which refers to the insect's decision to feed and/or oviposit on host plants, and to leave non-host plants (i.e. due to short range or contact cues).

A particularly important and complex behaviour for flying insects is that involved with finding a distant odour source (e.g. pheromones used in mate location or host plant volatiles utilised for host location). The mechanisms involved in this behaviour have primarily been studied in the attraction of male moths to calling females in response to their sex pheromones. The flight behaviour of male moths has been found to depend on a number of inputs from its internal state, including a circadian rhythm and age (male moths usually exhibit a peak of responsiveness at a particular time of day and at a particular age) (Shorey, 1973). Inputs from pheromone receptors also vary with factors such as temperature and previous exposure to sex pheromone, and these factors can have a significant effect on the flight behaviour of male moths to the pheromone (Baker *et al.*, 1988; Linn *et al.*, 1988). The sex pheromones of most species of moths consist of a specific blend of chemicals (Arn *et al.*, 1992), and each component is generally perceived by a single type of neurone on the antenna (Kaissling, 1986). In most cases, the complete blend of chemicals is processed as an integrated whole and mediates most or all of the behaviour from activation of flight, through landing and copulation (Linn *et al.*, 1987; Baker, 1989b). It appears that male moths use an integrated combination of behavioural mechanisms to maintain contact with, and thereby locate the source of, the sex pheromone. Two main behavioural mechanisms are involved, optomotor anemotaxis and a program of self-steered counterturns (Baker, 1989a). Optomotor anemotaxis in male moths requires

both chemical (sex pheromone) and visual inputs in order that it steers an upwind track, whilst the program of self-steered counterturns (internal input) is modulated by pheromonal input (concentration and blend quality) (Baker, 1989a). Flight altitude is also controlled by an optomotor response, requiring the integration of both olfactory and visual inputs (Preiss and Kramer, 1986). When nearing the pheromone source, pheromonal and visual inputs continue to be integrated and influence where the male lands in relation to the source. Even after landing, males probably need continued pheromonal stimulation, when visually orientating and moving towards the pheromone source (Charlton and Cardé, 1990; Foster and Harris, 1992).

Host finding behaviour of female onion flies (*Delia antiqua*) has been found to involve different mechanisms to those involved in sex pheromone location by moths. Many individuals are thought to locate onion fields by chance (Martinson *et al.*, 1989). However, some of the population have been found to exhibit anemotaxis from up to 100m downwind of a source of host volatiles (*n*-dipropyl disulphide) (Judd and Borden, 1988 and 1989). Female onion flies typically fly upwind to the host odour source in a series of short flights, each flight punctuated by the female landing on the ground, orientating in an upwind direction and again taking flight. There are probably two distinct types of odour-mediated flight behaviour involved: anemotaxis, involving mechanical stimuli whilst the fly is in contact with the ground, and optomotor anemotaxis involving visual stimuli whilst the fly is in flight (as also described for the cabbage root fly, Nottingham (1988).

During host plant location, phytophagous insects have been observed to switch between two different search patterns (Visser, 1988). The first primarily involves straight line locomotion 'ranging' which is most efficient for encountering resources at a distance (i.e. crossing distances between patches of host plants); this pattern of locomotion is under allothetic control (controlled by external information, i.e. wind). After encountering a resource (i.e. direct contact with a host-plant or encountering host odours), insects change to a convoluted pattern of locomotion 'local search', which is most effective for encountering nearby resources (i.e. host-plants within a patch or those giving off host odours); this pattern of locomotion is under ideothetic control (controlled by internal information). The transition between ranging and local search is caused by shifting the equilibrium between idiothetic circling and allothetic control, probably a result of the CNS changing its sensitivity to external stimulation. Ideothetic circling

occurs when external stimulation is very low (i.e. no wind), and also predominates after encounters with a resource (i.e. contact with a host plant or host odours) or when the insect is satiated. The return to ranging involves the gradual increase in allothetic course control. In situations where resource encounters occur frequently, insects remain in local search; where encounters are less frequent, insects alternate between ranging and local search; where few encounters are made, insects respond primarily by ranging. This ability to switch between search patterns allows insects to respond to variations in resource density, increasing their searching efficiency.

A mechanistic general theory of host plant selection has recently been proposed by Finch and Collier (2000), in which they discount a number of previously proposed hypotheses. This theory is primarily based on behavioural observations of pest insects of cruciferous plants, in which, during host plant finding, the searching insects landed indiscriminately on green objects such as leaves of host plants (appropriate landings) and non-host plants (inappropriate landings), but avoided landing on brown surfaces such as soil. This theory explains why fewer phytophagous insects are found on cultivated (or wild) host plants growing in a diverse background of plant species than on similar plants growing in bare soil (i.e. as in conventional cropping systems).

This theory of host plant selection can be divided into a chain of actions involving three links. In the first link, volatile chemicals emanating from plants indicate to flying receptive insects that they are passing over suitable host plants. The primary action of these volatile chemicals is to stimulate the insect to land. Under suitable weather conditions, these chemicals may also provide some directional information (Finch and Skinner, 1982), but this is of secondary importance. They suggested that the amounts of volatile chemicals released from plants that impinge upon the insect's receptors are sufficient to arrest, but rarely sufficient to provide accurate directional information to flying phytophagous insects. Insects that fly over plants growing in bare soil will be stimulated to land on host plants, the only green objects available to them, as most phytophagous insects avoid landing on brown surfaces such as soil (Kostal and Finch, 1994). When host plants are growing in bare soil, most landings will be on suitable host plants, classified as 'appropriate', and so the host plants will effectively concentrate the insects (Root, 1973). In contrast, insects flying over host plants surrounded by clover land in proportion to the relative areas occupied by leaves of host and non-host plants, as specialist phytophagous insects do not discriminate between the two

when both are green (Kostal and Finch, 1994). Hence, any landings made on the non-host plant are classed as 'inappropriate'. The amount of time the insects spend on the leaves of the non-host plants before taking off again is governed by whether the insects receive acceptable or antagonistic stimuli through their tarsal receptors. Once the insect is again airborne, if it is stimulated to land after flying only a relatively short distance, it could land on a host plant. In all situations, however, the plant on which the insect first lands, even if it is a 'host plant', may not stimulate the insect sufficiently, via its contact chemoreceptors on the tarsi or head appendages, to arrest it, and the overall process will be repeated. If this represented the complete system, then under 'no-choice' situations in the field, it could just be a matter of time before the numbers of eggs laid on host plants growing in diverse backgrounds were similar to those laid on host plants growing in bare soil. However, this does not occur, as there is a second phase to host plant finding.

This second phase, as illustrated for the cabbage root fly, involves the fly making a number of spiral flights (typically four) before laying eggs alongside the plant (Kostal and Finch, 1994). Thus, the insect stands a much greater chance of 'losing' the host plant in a diverse background as, it repeats the initial appropriate / inappropriate landing procedure a further three times. Fewer flies manage to oviposit in this situation, because following each short flight a proportion of the flies land on the leaves of the surrounding non-host plants. This failure to re-contact a host leaf after any spiral flight prevents the female from accumulating, within the allotted time, sufficient stimulation from the host plant to be induced to lay. Hence, the barrier that the fly faces when its hosts are growing in diverse backgrounds is not chemical (Dover, 1986) or mechanical (Theunissen and Schelling, 1996) but behavioural, simply because, during the innate series of spiral flights, the fly must continue to accumulate more positive host plant stimuli each time it lands.

The amount of stimulation the female picks up on each landing is crucial and this is where the phase of host plant finding (link 2) becomes truly integrated with host plant acceptance (link 3). In essence, the complete system really involves finding and re-finding the host plant. The female cabbage root fly may only have to visit two leaves of a highly stimulating plant, compared to six leaves on a poorly stimulating plant, before finding it an acceptable site to oviposit. Other insects, however, may accumulate sufficient stimuli to keep them searching, but not sufficient stimuli to induce oviposition and so will fly away. A similar outcome results when insects visit several leaves but do not manage to accumulate

sufficient stimuli in the allotted time to be induced to stay. Two other variations occur when the insects land initially on a stimulating leaf but subsequently on a non-stimulating leaf. It does not matter whether this leaf is from a host or non-host plant, as anything that interrupts the rate of accumulation of positive stimuli causes the insect both to abort its attempt to oviposit and to move elsewhere. In addition, interspecific competition may also become important (Jones and Finch, 1987).

The physiological status of the insect, which depends partly on its age and also on how long it has been deprived of a suitable oviposition site, also has to be superimposed upon this already complex system (Barton Browne, 1993). With time, phytophagous insects tend to become less discriminating in their choice of oviposition sites (Kostal and Finch, 1996). The condition of the plant is also important, as some species are more highly preferred than others, as well as some growth stages of the plant (Finch, 1980). However, even when the insect and the plant are both in the appropriate physiological state, it counts for nothing the moment the insect makes a wrong choice and alights on any green object other than a leaf of a host plant. This, however, is tempered by the fact that when the host plant is highly stimulating, the insect has to visit fewer leaves and so has less chance of making an inappropriate landing. In addition, highly stimulating plants invariably induce individual insects to lay more eggs (Roitberg *et al.*, 1999). Detailed descriptions of a multitude of other factors involved during host plant acceptance can be found in Simpson *et al.* (1999). Differences in the effect that diverse backgrounds have on host plant selection by different species may simply reflect the number of contacts / re-contacts the insect has to make to accumulate sufficient positive stimuli to oviposit.

The 'appropriate / inappropriate landing' theory can be used to explain why certain aspects of host plant finding by phytophagous insects, supposedly regulated by volatile plant chemicals, have proved intractable in the past. The evidence that volatile chemicals are the main regulatory stimuli in the central link of host plant finding is weak, as the maximum distance recorded for insect orientation to host plant volatiles in the field is only a few metres (Hawkes, 1974; Finch, 1980). In wind tunnel experiments, cabbage root flies flew upwind to cruciferous plant odour, released at a rate which was at least 10^5 times higher than the amount of chemical released from a healthy plant (Finch., 1980), although less than 10% of flights lasted more than 0.5m. Similarly, in traps releasing large amounts of chemical to provide directional cues in the field, many insects missed the trap and subsequently failed to enter (Finch and

Skinner, 1982). These findings suggest that the volatile host plant chemicals were acting as arrestants rather than true attractants (Dethier *et al.*, 1960; Kennedy, 1978).

Finch and Collier (2000) expressed considerable doubts about whether host plant volatile chemicals are truly attractants, or simply arrestants, for receptive insects, a debate that has been ongoing for many years. Most of the detailed experiments with host plant volatile chemicals have been done by releasing plant odours from a point source sited at one end of a wind tunnel, introducing supposedly responsive insects and then recording whether the insects move upwind to the source odour. However, many of the results from such experiments have provd disappointing (Hawkes, 1974; Finch, 1980). Often the only way to obtain data is to place a visual stimulus, normally a green coloured object, alongside the site from which the volatile chemical is being released (Rojas and Wyatt, 1999). To prove that the odours are arrestants rather than attractants, Finch and Collier suggested using a set-up with a wind tunnel in which the flow rate of odour-free air was balanced, to keep receptive insects flying above a green object. If a pulse of odour then stimulated the insect to land on the green object, it would help prove that the volatile chemical behaved primarily as an arrestant.

From a crop protection standpoint, the more non-host plants are removed from any crop area, the greater the chance an insect has of finding a host plant. Hence, our current cultural methods are exacerbating our pest control problems, as 'bare-soil' cultivation ensures that crop plants are exposed to the maximum pest insect attack possible in any given locality.

From a practical point of view, research into semiochemicals and the behaviours they mediate provides a basis for their successful use in pest control, as an alternative to broad-spectrum toxicants. To date, the most successful uses of semiochemicals in pest control utilise lepidopteran sex pheromones and the aggregation pheromones of Coleoptera (Ridgeway, Silverstein and Inscoe, 1990; Howse, Stevens and Jones, 1998). A number of commercially developed systems currently use lepidopterous sex pheromones either in monitoring programmes, or slow release formulations to disrupt normal mate location. In forest pest management, bark beetle aggregation pheromones are used in trap-out procedures. Numerous other semiochemical pest control measures are currently under investigation, and many of these are reviewed by Wadhams *et al.* (1999).

1.3 HOST PLANT FINDING IN *P. rosae*

Oviposition flights of female *P. rosae* have been found to take place during differing weather patterns, when temperature, humidity and light intensity were very different. Wakerley (1963) noted that female flies returned to hedges in the evening, despite no change in temperature or humidity, and concluded that only light intensity had changed from that required to stimulate oviposition flight. The optimum light intensity for oviposition appears to be within the range 100-1000 lux (Städler, 1975), and is found in the shade of plant foliage (Overbeck, 1978). Bohlen (1967) described a sequence of behaviour leading to oviposition, in which females flies landed on carrot leaves and immediately began to run over the upper and lower sides of the leaves and extending the ovipositor, without it contacting the plant surface. The haustellum was also repeatedly extended, and the labella briefly contacted the leaf surface. After at least 20 seconds on the leaf, the females moved down the leaf petiole to the soil, where they oviposited. Similar patterns of oviposition behaviour have also been described and quantified by Degen, Städler and Ellis (1999a). The choice of an oviposition site was determined by tactile stimulation of the ovipositor, roughness of the soil surface, negative phototaxis and the humidity of the substrate. Trichoid sensilla on the cerci of the ovipositor are probably mechanoreceptors, although some may possibly function as olfactory or contact chemo-receptors (Behan and Ryan, 1977; Dufault and Coaker, 1987).

In the laboratory, mating usually occurs during the afternoon; with pairs remaining coupled for at least 40 minutes (Wainhouse, 1975). Copulating pairs attract other males, which climb on to them without dislodging the male. In the field, mating is thought to occur in the shelter of the hedgerows, and to date, there has been no evidence presented for the involvement of sex- or aggregation pheromones; however, high-pitched sound produced by males may play some role (Städler, 1977). The males also vibrate their bodies with their legs, very rapidly for about one second; these vibrations are repeated after short runs, regardless of the substrate.

A large number of electrophysiological and behavioural studies have been undertaken for *P. rosae* and a fairly comprehensive summary of semiochemicals known to influence the behaviour of *P. rosae* has been compiled by Degen (1998a). The olfactory receptor cells in the antennal sensilla have been shown to be highly sensitive to certain fairly host-specific volatile compounds such as *trans*-asarone (Guerin *et al.*, 1983), possibly allowing it to perceive host plants at some distance. The same antennal

receptors are probably also used to perceive volatiles (and less volatile compounds) accumulated in the boundary layer of leaves, once the fly has alighted (Städler and Roessing, 1991). The outcome of ablation experiments and contact elctrophysiological recordings suggests that contact chemoreceptors in the tarsal D-hairs may be involved in the perception of host plant stimuli (Städler, 1977; Städler, 1982).

Host finding in larvae has been shown to involve both non-specific primary metabolites and host-specific secondary metabolites (Jones and Coaker, 1972). Larval attractants include: carbon dioxide, methyleugenol (Jones and Coaker, 1977 and 1979), falcarinol (and possibly other electrophysiologically active polyacetylenes, including falcarindiol and falcarindiol monoacetate), (+/-)-2-methoxy-3-sec-butylpyrazine (Maki *et al.*, 1989), bornyl acetate, 2,4-dimethylstyrene, α-ionone, β-ionone and biphenyl (Ryan and Guerin, 1982; Guerin and Ryan, 1984). *trans*-2-Nonenal was found to be a strong repellent, which also immobilised and killed larvae on contact (Guerin and Ryan, 1980).

The semiochemicals known to influence adult *P. rosae* are divided into those involved in host plant finding, which are detected by olfactory antennal sensilla (olfactory cues), and those involved in host plant acceptance, probably detected by olfactory antennal sensilla in the boundary layer of the foliage and possibly also by tarsal contact chemoreceptors (as close range stimui and contact chemicals). Semiochemical attractants involved in host finding include components of the 'green leaf volatiles' complex, primarily the aldehydes hexanal, (*E*)-2-hexenal and heptanal (Guerin and Visser, 1980; Guerin *et al.*, 1983), and the propenylbenzenes *trans*-asarone and *trans*-methylisoeugenol (Guerin *et al.*, 1983). So far no repellents to host finding have been identified.

A wider range of semiochemicals have been shown to influence the host acceptance of *P. rosae*. Identified oviposition stimulants include: the propenylbenzenes, *trans*-asarone and *trans*-methylisoeugenol (Guerin *et al.*, 1983 Städler and Buser, 1984); the allylbenzenes, 4-allylanisole and methylchavicol (Städler, 1972), and anisaldehyde; the furanocoumarins, bergapten, xanthotoxin, isopimpinellin, imperatorin and athamantin (Städler and Buser, 1984; Städler, unpublished); the substituted coumarins, osthol and ostruthin (Städler and Buser, 1984; Städler, unpublished); the polyacetylenes, falcarindiol, falcarinol, falcarinone, and 1,8-pentadecadiyne (Städler and Buser, 1984; Städler, unpublished); the flavanoid, apigenin-7-O- β-D-glucoside (Städler, unpublished); oleic acid (Städler and Buser, 1984). A number of possible oviposition deterrents or repellents have also been

identified; these include p-anisic acid, isoeugenol and methylnonylketon (Städler, 1972). However, a number of studies have suggested that as yet unidentified non-volatile oviposition stimulants and deterrents probably also play a part in the oviposition behaviour of *P. rosae* (Degen, 1998a; Degen and Städler, 1998; Degen, Buser and Städler, 1999). As can be seen from this list of known compounds, some host plant attractants also function as oviposition stimulants in this insect, and the distinction between host finding and acceptance may be somewhat artificial. The conformity of the responses of males and females to volatile plant constituents suggests that they may provide an additional aggregation cue for both sexes (Guerin and Visser, 1980; Dufault and Coaker, 1987).

Degen's in-depth investigation into the mechanisms involved in host plant acceptance by *P. rosae* (Degen, Buser and Städler, 1999; Degen, Städler and Ellis, 1999a, b, and c) suggested that a mixture of the identified oviposition stimulants act as a 'chemical search image' (Atema *et al.*, 1980) and enable *P. rosae* to distinguish between host and non-host species, while the as yet unidentified polar stimulants and deterrents mediate the oviposition preferences between particular species within the Apiaceae. The combination of propenylbenzens, furanocoumarins and the C17-polyacetylenes is characteristic of the Apiaceae (Städler, 1986); however, in all host species analysed, at least one of these groups of stimulants was present in very low amounts, and probably imperceptible to the fly. This led him to suggest that, unless the unidentified stimulatory compounds were also typical of the Apiaceae, host recognition must rely primarily on the C17-polyacetylenes as positive host-specific stimuli (Degen, Buser and Städler, 1999). Although Degen's studies did not investigate host plant finding in *P. rosae*, his results did lead him to question whether *P. rosae* was able to locate (precisely) its hosts using anemotactic flight from a distance. He pointed out that the quantities of phenylpropenes required to increase trap catches in the field were several orders of magnitude higher than the levels emitted naturally by undamaged host plants (Städler, 1992). He suggested that, in *P. rosae*, characteristic volatiles possibly enhanced the likelihood of encounters with hosts by attracting the flies towards vegetation that comprised of patches of host plants, while the landings on hosts interspersed within non-hosts probably occurred more or less at random. He corroborated this theory with evidence from the results of his observations of caged flies, in which flies were not found to alight more frequently on leaf mimics treated with host plant extracts than

on those without (Degen and Städler, 1997b). Foliar odour of the partially resistant carrot cultivar 'Sytan' was more attractive than that of the susceptible cultivar 'Danvers' (Guerin and Städler, 1984).

Degen's studies into the non-chemical traits involved in host acceptance (Degen and Städler,1996 and 1997b) showed that the *P. rosae* were able to discriminate between different leaf shapes before landing and showed a preference for compound leaf shapes. This leaf shape is typical, but not unique, to apiaceous host plants and may allow *P. rosae* to distinguish roughly between broad plant categories and increase the probability of 'correct' host plant recognition. This may enhance their efficiency in host plant finding by allowing them to avoid alighting on plants with non-pinnate leaves, or those with narrow blades (e.g. grasses). However, Degen found no evidence that *P. rosae* was able to distinguish between the more subtle differences among apiaceous host plants.

1.4 AIMS AND OBJECTIVES OF THIS STUDY

The overall aims of this study were to investigate the chemical ecology of *P. rosae*. One component of the thesis was to survey a range of extracts and compounds, particularly with regard to long range attractant and repellent semiochemicals, suitable for incorporation into integrated pest management strategies for this pest.

The first objective was to try to increase the efficiency of *P. rosae* population monitoring, primarily by increasing sticky trap catches at low population densities, and ideally increasing catch numbers by 4-5 times. To do this, host plant extracts or semiochemical attractants would need to be identified and incorporated into the monitoring trap. They would need to stand out as particularly strong points of attraction against the general background of attractive *D. carota* volatiles and the visually attractive stimuli associated with the large expanse of surrounding carrot foliage. It would also be advantageous to increase the selectivity of the monitoring traps by reducing the number of non-target insects caught, as this would increase the efficiency of the traps as well as making counting *P. rosae* more accurate.

The second objective was the development of an autodissemination trap for release of the pathogenic fungus *Entomophthora schizophorae* (Zygomycetes: Entomophthorales) into *P. rosae* populations in the field, to bring about the biological control of this pest by the early season inoculative

release of the pathogen. The development of this prototype pathogen-dispensing trap could utilise visual attractants, and any identified attractant compounds, to draw *P. rosae* into a central enclosed region suitable for their infection with the specific entomopathogenic fungi.

Further studies were also aimed at identifying possibly repellent extracts or compounds, as these might also be useful in future carrot fly control measures, such as incorporation into an integrated pest management (IPM) 'push / pull' strategy (Wadhams *et al.*, 1999).

The general process of semiochemical isolation and identification for a phytophagous insect colonising its host crops can be summarised as a series of steps (Wadhams *et al.*, 1999). The first step is to locate the sources providing volatile cues for the insect; the volatile semiochemicals mediating such interactions can then be isolated using various extraction techniques, to provide a liquid extract for further investigations. Many of the compounds present in extracts are of no relevance to the insect, so to identify all the components and to evaluate individually their ability to elicit behavioural responses would be a long and tedious procedure. This process can be made more efficient by using electrophysiological recording techniques. Insects perceive volatile semiochemicals via olfactory receptors (sensory cells) primarily on the antenna which, when stimulated, pass information to the brain in the form of an electrical signal, or action potential, which can be measured. Electrophysiological activity can be assessed either by electroantennography (EAG), in which, by placing an electrode at each end of antenna, the overall responses of the olfactory cells can be measured, or by recording from individual olfactory receptors within the sensilla (single cell recording, or SCR). By linking this system with high-resolution gas chromatography (GC), i.e. splitting the effluent from the GC column and presenting it simultaneously to the flame ionisation detector (FID) of the GC and the antennal preparation, it is possible to locate compounds within a complex extract which have biological activity. Active compounds are identified using coupled gas chromatography-mass spectroscopy (GC-MS) and confirmed by co-injection on GC with authentic compounds. Electrophysiological activity for a compound, although suggesting that the material is of importance to the insect, gives no indication of its behavioural role, nor whether it is active only at a particular concentration, or only in combination with other components. Other signals, including visual cues, may also be necessary for behavioural activity. Identified compounds therefore require assessment in laboratory bioassays such as olfactometer or wind-tunnel studies, using insects at the correct

physiological stage to elicit the behaviour of interest. Promising compounds, or mixtures of compounds, can then be tested in the field for their ability to influence the behaviour of naturally occurring populations of the pest and its natural enemies. If field trials are successful, the process of formulating and commercialising the compounds into usable pest control products or strategies can then be initiated.

During this study into the chemical ecology *P. rosae*, my investigation proceeded in line with this general strategy for investigating semiochemicals. The outline of this thesis consists of six research chapters, five involved primarily with different aspects of the investigation into the chemical ecology of *P. rosae*, and a sixth dealing with investigations into the development of an autodissemination pathogen trap for the release or *E. schizophorae* into populations of *P. rosae*. These six experimental chapters deal with the following areas of investigation:

Chapter 2. Investigations into extraction techniques for the production of host plant extracts suitable for use in further investigations of *P. rosae* semiochemistry.

Chapter 3. Investigations to identify electrophysiologically active compounds in the extracts of host plant species, using GC-EAG and GC-MS techniques.

Chapter 4. Investigations into the electrophysiological responses of *P. rosae* to the identified compounds, and the conformation of their electrophysiological activity using EAG techniques.

Chapter 5. Laboratory investigations into the behavioural activity of host plant extracts and electrophysiologically active compounds, using bioassay techniques.

Chapter 6. Field investigations into the behavioural activity of host plant extracts and electrophysiologically active compounds.

Chapter 7. Field investigations into the development of a prototype autodissemination trap for the release or *E. schizophorae* into populations of *P. rosae*.

2 EXTRACTION AND ISOLATION OF SEMIOCHEMICALS FOR *P. rosae*

2.1 INTRODUCTION

Chemical ecology is "the study of structure, function, origin and significance of naturally occurring compounds that mediate inter- and intra-specific interactions between organisms" (Miller, 1998), particularly focusing on determining the role of semiochemicals in their natural context.

Preparation of a biologically active extract is the crucial first step in the identification of semiochemicals. In the case of semiochemicals produced by living organisms, this may be achieved by producing an extract that approximates, as closely as possible, the authentic composition of compounds present in either the tissue or the air surrounding the organism (headspace). In practice, there are many problems characteristic of producing extracts for chemical ecological studies. These arise from the small quantities of natural products often released, coupled with the sheer number and range of concentrations of the individual components present. This is further complicated by the wide range of volatilities and stabilities of these component compounds. An efficient extraction method is one in which all or most of the desired active compounds are extracted, with the minimum of extraneous material included. It should also produce a sufficient quantity of material to facilitate further studies, including identification of biologically active components, compound identification and possibly behavioural studies. Whilst producing such extracts, it is essential to minimise the risks of accidental incorporation of contaminants into the extract, as well as minimising the degradation of the least stable organic compounds present. This may occur as a result of many physical and chemical factors inherent in the extraction process. As extracts for semiochemical work often yield only small quantities of material, they can become swamped by even low levels of contaminants, which would be insignificant in conventional chemistry. These contaminants are especially problematic where characteristics of the whole sample are used for identification, as in nuclear magnetic resonance (NMR), direct-insertion-probe MS or optical rotation. Where identifications are carried out by methods incorporating a further separation step (such as GC-EAG, GC-MS or coupled GC-Fourier transformed infrared spectroscopy (GC-FTIR)), or in bioassays, minor contamination is less crucial, although such contaminants are still liable to complicate identifications and may influence

bioassay results. Many organic compounds exhibit instabilities and are prone to chemical change or degradation, especially the terpenes (Hunter and Broyden, 1963 and Wrolstad and Jennings, 1965). These reactions may result from environmental conditions, as in light-sensitivity, sensitivity to pH or oxidation. Alternatively, they may be the result of enzyme action due to microbial growth or enzyme release from disrupted cells. Where such changes occur, quantities of authentic compounds will be lost, with corresponding increases in artefacts produced. The route and final products of these reactions depend primarily upon the structure and functional groups present in each case.

As a wide range of methods are available, each capable of producing biologically relevant extracts, this chapter is dedicated to the comparison of the most suitable, with an evaluation of their acceptability for subsequent identification and bioassay techniques relevant to the project. These include gas chromatography (GC), coupled gas chromatography-electroantennography (GC-EAG), coupled gas chromatography-mass spectrometry (GC-MS), olfactometer assays and fieldwork.

Hydro-distillation was included in this study as it is the standard method for the production of commercial essential oils, in bulk, from aromatic plant materials. However, it was not expected to produce suitable extracts for the study of host plant derived semiochemicals for *P. rosae*, for a number of reasons. Firstly, in a study by Guerin *et al* (1983), all of the commercially available apiaceous essential oils tested (anise, caraway, carrot, celery, coriander, fennel and parsley) were found to be unattractive when released from visually attractive sticky traps in the field. Secondly, the acidic conditions (typically pH 4-7) produced when plant material is distilled, combined with the presence of water and oxygen, and prolonged heating (from hours to days) at around 100°C, often results in major distortions to the profile of essential oils extracted by this method (Pickett *et al*, 1975), making them non-representative of those from the intact living organism (Teranishi *et al*, 1993).

The three other extraction methods (microwave assisted distillation, microwave assisted solvent extraction and air entrainment) were chosen primarily because they were more likely to produce extracts with profiles more representative of the intact living plant material, as they incorporated fewer factors responsible for the occurrence of contaminants and artefacts (i.e. exposure of the plant material to heat during the process was either brief or absent). The two microwave-assisted extraction methods were of interest, as they are relatively novel techniques, and recent studies suggest that their extracts may be of

significant importance with regard to the study of host plant semiochemicals for *P. rosae*. The microwave-assisted solvent (MAS) extraction method was of particular interest, as in oviposition bioassays it was found that artificial leaves treated with hexane MAS extract from host foliage were almost as acceptable to female *P. rosae* as real host leaves (Degen and Städler, 1998; Degen, Poppy and Städler, 1999). These MAS extracts were also found to elicit twice the level of oviposition found for the previously best extracts tested (a dichloromethane extract, and the diethyl ether fraction of a boiling water immersion extract). Air entrainment (dynamic headspace extraction) was chosen as this method was the most likely to produce extracts which contain volatiles in ratios similar to those actually released by plants, and found in the air surrounding them.

There were three aspects to this investigation:

1) Preliminary studies using parsley (*Petroselinum crispum*), to determine appropriate extraction conditions for apiaceous plant material for the relatively novel microwave-assisted distillation (MAD) technique.

2) The comparison of the different extraction methods, carried out using wild hemlock (*Conium maculatum*), as it was readily available locally in sufficient quantities for all the different extraction methods to be carried out. The methods compared and evaluated for acceptability, for subsequent identification and bioassay techniques relevant to the project were: microwave-assisted distillation, microwave-assisted solvent extraction, air entrainment and hydro-distillation.

3) With the microwave-assisted solvent extraction method, direct contact of solvent with the plant material, and considerable indirect heating, resulted in the extraction of large quantities of low volatility leaf surface waxes. Consequently, to solve this problem, a range of techniques for removal of the less volatile leaf waxes from this extract were also investigated. The methods employed included: precipitation, column chromatography, vacuum distillation and air entrainment.

2.2 METHODS

2.2.1 Source of Apiaceous Plant Material

For the comparison between the four most suitable extraction methods, wild hemlock (*Conium maculatum*) was used, as it was readily available locally (with kind permission from Redbourne Golf Club), in sufficient quantities, for all the different extraction methods to be carried out. Mature hemlock foliage was collected in June, prior to flowering (with the exception of that used to produce the microwave-assisted solvent extract (b), which was harvested from the same spot in late September). Composite samples from this bulk of foliage material (with the main stems removed, were extracted by each of the different extraction methods (microwave assisted distillation, microwave assisted solvent extraction, air entrainment, and hydro distillation) on the same day as harvesting. These extracts were then dried and concentrated to 25g/ml fresh weight equivalent (with the exception of the air entrainment sample). Samples were then analysed by GC (10m and 50m HP1) for comparison of their GC profiles, and by human organoleptic analysis (details of these processes are given later in these methods).

2.2.2 Extraction Methods

Microwave-assisted distillation (MAD)

Microwave-assisted distillation is a very rapid method for extracting small quantities of plant material in the region of 20-40g. Whilst giving similar extraction products as a conventional steam distillation, the quantity and ratio of these products may differ significantly from conventional steam distilled essential oil (Craviero, 1989). The plant material is heated in an enclosed system by microwaves as a stream of air or inert nitrogen gas is passed through the system. The microwaves cause the vascular and glandular cells of the plant to burst due to internal heating and build-up of pressure. This liberates steam and volatile compounds, which are drawn out of the vessel by the carrier gas. This passes through a condenser and into a cold trap where the condensed water, along with the volatile extract, is collected. This method has the advantages that few low volatile contaminants (i.e. leaf surface waxes) are extracted, making it a suitable method for preparation of extracts for GC analysis. Extraction is very rapid (typically 1 min), which

reduces the likelihood of volatile degradations occurring. The main drawback of this method is that it can only be used to extract relatively small samples of plant material.

During MAD, the apparatus was assembled as indicated in Fig. 2.1, based on the method of Craviero *et al.* (1989). 30g of *C. maculatum* foliage was placed inside a 500ml Florentine flask within a 800W microwave oven (Panasonic), adapted by the fitting of a piece of brass tubing 130 mm x 45 mm diameter extending through the centre of the oven to its exterior. This was capped with a brass lid through which two holes allowed the passage of the two glass tubes of the wash bottle head. This configuration was engineered in order to prevent leakage of microwaves during heating. PTFE tubing (3mm ID) was used to connect the component parts, and joints were sealed using PTFE tape. During extraction, purified nitrogen was flushed through the system (to reduce the risk of oxidation of volatiles) at a rate of 600ml/min, and the plant material was heated by the microwave for 1 min at medium power output. As volatiles were released from the plant material, they were forced into the stream of nitrogen. On exiting the system, the nitrogen passed through a sintered glass delivery tube into a Drechsel wash bottle, where it bubbled through 50ml of cooled solvent (hexane). The extract was then dried and concentrated to 25g/ml fresh weight equivalent. The optimum conditions for the MAD extraction of apiaceous foliage (as used for C. *maculatum* in this experiment) was determined by preliminary studies using parsley (*P. crispum*), as described below.

Preliminary Studies

As this method was relatively novel, preliminary investigations were carried out to establish suitable extraction conditions for volatiles from apiaceous foliage. For this investigation, trays of parsley (*P. crispum*) cv. 'Champion Moss Curled' were grown from seed (Cat. No 1781, Chiltern Seeds Ltd.). These were initially germinated in the glass house, then relocated outdoors and harvested after two months growth. Composite samples (30g) from this bulk of foliage material, with the main stems removed, were extracted on the same day as harvesting for all these preliminary experiments. The validity of using composite samples from the bulk parsley foliage was confirmed by making a further three extractions, using the optimum extraction conditions identified during this investigation. These showed no significant

differences in the extract's compositions, profiles, or the total quantity of material extracted, by GC analysis.

In order to evaluate a variety of heating conditions for extraction of volatile semiochemicals by MAD, 30g portions of fresh *P. crispum* leaf were extracted by the above method. They were heated for different periods (30 sec, 1 min, 2min, 3 min and 5 min) at each of the three microwave power outputs (low, medium and high), with an arbitrary nitrogen flow rate of 300ml/min. The extracts were then dried and concentrated to 25g/ml fresh weight equivalent, for comparison with a microwave-assisted solvent extract (details of this method follow in this chapter) of the same plant material (25g/ml fresh weight equivalent) and fresh plant material, by human organoleptic evaluation (see 2.2.3).

An experiment was also performed to assess the effect of varying the flow rate of nitrogen passing through the system during extraction on the concentration of volatile components subsequently trapped in the solvent. 30g portions of *P. crispum* were extracted by the above method at medium power for 1 min (found to be optimum in the previous preliminary experiment) and the volatiles released were collected in 50g of cooled hexane. This procedure was repeated for each of five flow rates of nitrogen: 50, 200, 400, 600 and 800 ml/min. The extracts produced were then dried and concentrated to 25g/ml fresh weight equivalent. The relative concentration of individual volatile components was then assessed by GC analysis (10m HP1 column).

Microwave-assisted solvent (MAS) extraction

Microwave-assisted solvent extraction is a recent adaptation that produces rapid extracts with very high yields of volatile components from plant materials (Gangler, 1986). Extraction is achieved using a microwave transparent (or semi-transparent) solvent with a low dialectic constant, through which microwaves pass without being absorbed. Consequently, the microwaves are preferentially absorbed by the vascular and glandular regions of the plant material (which contain water), generating a sudden rise in temperature and internal pressure within these localised cells and causing them to swell and explode. Steam and volatile organic components are released and, on contact with the relatively cooler surrounding solvent, the volatiles are dissolved (Pare, 1991). The main drawback of this method is that, with longer

extraction times for bulk extraction, considerable indirect heating of the solvent occurs, resulting in the extraction of large quantities of low volatility leaf surface waxes.

During MAS extraction, the apparatus was assembled as shown in Fig. 2.2, based on the method of Paré *et al.* (1991). Approximately 300 g of *C. maculatum* foliage was placed in a two-litre culture vessel (Fisher Scientific). This was immersed in solvent (hexane), and placed within a 800 W microwave oven (Panasonic), which was adapted by the fitting of a piece of brass tubing 130 mm x 45 mm diameter extending through the centre of the oven to its exterior. The plant material was then heated by microwaves at high power, until the solvent began to reflux in the distillation column (approximately 3 min). Upon cooling, the extract was filtered into a 5 litre conical flask, dried, and concentrated to 25g/ml fresh weight equivalent. As this extraction process is known to extract large quantities of non-volatile leaf surface waxes, a number of methods were employed in attempts to remove the less volatile components, while retaining the more volatile components of the extract.

(a) ***Column chromatography***

Column chromatography was performed based upon the method of Städler and Buser (1984, appendix 10), using the MAS extract in hexane, rather than a solvent dip extract in pentane. Dry silica gel (70-230 mesh) was mixed thoroughly with hexane to produce a slurry, which was used to fill a 2cm diameter chromatography column to a height of 22cm. A 2cm diameter filter paper disc was placed upon the surface of the settled gel column to reduce disturbances to the surface layer when fluids were added, and the excess hexane was run off. 10 ml of *C. maculatum* MAS extract (a) (25g/ml fresh weight equivalent) was evaporated down to 1ml. This concentrate was dripped onto the surface of the column and fed into the surface of the gel, by removal of an equal quantity of hexane. The column was then eluted with successive 200ml portions of 0%, 3%, 8%, and 20% diethyl ether in hexane; 100% diethyl ether; 20% methanol in diethyl ether and finally 100% methanol; with each fraction collected separately. Fractions were evaporated down to 1ml each and analysed by GC (10m -HP1).

(b) ***Precipitation***

5ml of *C. maculatum* MAS extract (a) (25g/ml fresh weight equivalent) was evaporated down to dryness. This dried extract was then re-dissolved in 1ml of acetone and stored for 24 hours at -5 °C, resulting in the

formation of a precipitate which was removed by filtration. The resulting extract was made up to the original 5ml volume with hexane and analysed by GC (10m-HP1).

(c) ***Air entrainment***

The air entrainment apparatus was assembled as indicated in Fig. 2.3, based upon the method of Blight (1990). This technique is explained in more detail later in this chapter (2.2.2). The purified air flow was split into two and entered two 50ml glass entrainment chambers (Quickfit), sealed with PTFE tape. The 'treatment' chamber contained 1ml of *C. maculatum* MAS extract (a) (25g/ml fresh weight equivalent) in a glass specimen tube, which was supported on a wire spring. The 'control' chamber contained an identical specimen tube with 1ml of hexane solvent. Volatiles released into the entrainment chambers (i.e. from the MAS extract in the treatment chamber) were carried into the adsorbent traps (100mg Porapak Q, 50-80 mesh) and adsorbed onto the Porapak. Entrainment was carried out over a 4-day period, and the Porapak traps were replaced with fresh ones daily. Desorption of the volatiles was carried out by eluting each of the Porapak tubes with 500 µl of freshly distilled diethyl ether. Extracts from like treatments were then combined, dried, and concentrated to the original volume of the MAS extract (1ml, 25g/ml fresh weight equivalent).

(d) ***Vacuum distillation***

The vacuum distillation apparatus (Soham Scientific) was assembled as indicated in Fig. 2.4. A low vapour pressure grease (Apiezon-M) was used minimally to seal the ground glass joints in the apparatus. 'Tap 1' was closed and 15 ml of *C. maculatum* MAS extract (b) (25g/ml fresh weight equivalent) was placed in the round-bottomed flask. The system was then evacuated using a vacuum pump, which was isolated from the apparatus by a trap cooled with liquid nitrogen. This prevented any back flow of oil vapour. The U-tube portion was then heated strongly with a hot air gun to remove any less volatile contaminants from its inner surfaces. After cooling, the U-tube section was immersed in liquid nitrogen and the round-bottomed flask was cooled with liquid nitrogen. Once the extract had frozen, both the taps were opened and the round-bottomed flask evacuated. As soon as the air had been removed, 'Tap 2' was closed and the solvent allowed to distil over, where it froze in the region of the pear-shaped vessel. 'Tap 1'

was then closed and the liquid nitrogen removed allowing the solvent to melt, removing it as an obstruction in the lower U-tube section of the apparatus. The U-tube was then completely immersed in liquid nitrogen and both taps were opened. The solvent extract residue was then distilled under a vacuum of <0.05 mm-Hg for 24 hours. The distillate collected in the upper portion of the U-tube marked '*' in the figure (Fig. 2.4). Both taps were then closed and the liquid nitrogen removed, allowing the U-tube to warm to room temperature. The distillate was then washed into the pear-shaped vessel by differential heating (20°C / -196°C), which allowed the solvent to reflux onto the region of the tubing marked '*'. 'Tap 1' was then opened and the round-bottomed flask removed, allowing air into the system. The stopper was removed and the vacuum distilled extract was removed from the pear-shaped vessel with a drawn Pasteur-pipette containing a small quantity of magnesium sulphate between two plugs of glass wool, which removed any water from the sample. The vacuum distillate was then made up to the original volume of the MAS extract (15ml, 25g/ml fresh weight equivalent).

Air entrainment (AE)

Porapak Q (ethylvinyl benzene - divinyl benzene co-polymer) was chosen as the best compromise between the three main adsorbents used to trap entrained volatiles (Porapak Q, Tenax GC, and Charcoal) (Golub, 1984). This adsorbent has the following advantages: a relatively high trapping capacity, it can be eluted with a relatively non-polar solvent (in this study diethyl ether), and it does not readily adsorb water, which can cause problems during subsequent GC analysis. The drawbacks of this adsorbent include its poor adsorbance of low molecular weight alcohols, resulting in their lower ratios in the Porapak extract than would actually be produced by the plant. Porapak Q has also been shown to contain large numbers of contaminants, which were carefully removed in this study by pre-use purification (see below). For more detailed information on trapping capacities, breakthrough volumes and drawbacks of adsorbent materials, see Núnez and González (1984) and Nordqvist (1997).

Prior to air entrainment, all glassware was scrupulously cleaned and baked in an oven for 12 hours at 200°C, to remove volatile contaminants. Molecular sieves and charcoal filters were pre-conditioned by baking in an oven for 12 hours at 200°C, while being continuously flushed with a stream of oxygen-free nitrogen. Adsorbent traps for the collection of volatiles were prepared by packing

approximately 100 mg of Porapak Q (50 – 80 mesh) into 10cm Pyrex glass tubes (3mm ID), blocked at either end with plugs of silanised glass wool. Traps were conditioned at least three times prior to use by elution with 2ml of re-distilled diethyl ether, followed each time by baking in a thermostatically controlled metal heating block for 12 hours at 140°C, whilst being continuously flushed with a stream of oxygen-free nitrogen (0.1 l/min).

The air entrainment apparatus was assembled as indicated in Fig 2.5, based upon the method of Blight (1990). PTFE tubing (2mm ID) was used to connect the component parts, which were joined to the glassware using brass dramllim unions fitted with PTFE-faced/silicone septa. Two glass bell jars (10 litres) with glass plate bases, sealed with PTFE tape, formed the two entrainment chambers. The 'treatment' chamber contained a 500ml conical flask of distilled water, sealed at the top with PTFE tape. The stems of approximately 200g of *C. maculatum* foliage were pushed through this tape into the flask of water. The 'control' chamber contained an identical flask of water, but the PTFE tape was punctured to mimic the stalk holes in the 'treatment' flask. This extra entrainment chamber was used as a blank control, enabling the identification of any contaminants entering the entrainment system. A diaphragm pump (Charles Austen Ltd. Model B100 SE) drew air through the system, which was dried and purified as it entered by passage through an activated 3Å molecular sieve (Aldrich), followed by an activated charcoal filter (Aldrich). As the purified airflow passed through the enclosed system it was split into two, with half passing through each of the two entrainment chambers, before passing through the adsorbent trap tubes, at a regulated flow rate of 1 l/min per chamber (controlled via flowmeters). Here, any volatiles (i.e. those released from the plant material into the treatment chamber) were adsorbed onto the large surface area of the adsorbent Porapak material. Entrainment of the plant material was carried out over a 48 hour period, and the Porapak traps were replaced with fresh ones daily. Longer entrainment times were precluded, because the cut foliage began to yellow after three days. Desorption of the volatiles was carried out by eluting each of the Porapak tubes with 500µl of freshly distilled diethyl ether. Extracts from like treatments were then combined, dried and finally concentrated to 50µl.

Hydro-distillation

The hydro-distillation apparatus was assembled as indicated in Fig. 2.6. Approximately 2 kg of plant material and 5 litres of boiling water were added to the 20 litre round-bottomed flask, and 12 ml of nonane were added to the 'Dean and Starks' receiver (for heavy entrainers). The flask was then heated and the contents were allowed to simmer for 4 hours. Upon cooling, the nonane (containing the dissolved essential oil distillate) was decanted off of the water that had condensed in the receiver. The extract was then dried, filtered, and made up to 25g/ml fresh weight equivalent by the addition of diethyl ether.

2.2.3 Analysis of Extracts

Water was removed from all extracts by addition of anhydrous magnesium sulphate, followed by filtration. Where concentration of the extract was required, it was carried out by reduced pressure distillation at 30°C (Büchi-Rotavapor), followed by evaporation under a slow steam of purified nitrogen at room temperature. Extracts were stored in appropriately sized glass screw cap vials sealed with silicone/PTFE septa (Chromacol Ltd) or, where long-term storage was envisaged, under nitrogen in sealed glass ampoules. All samples were stored in a freezer at –20°C.

Initial gas chromatographic analysis (using non-polar 10m HP-1)

All samples were initially analysed on a Hewlett Packard 5880A GC fitted with a 10 m, 530μm ID, HP-1 (2.65μm film thickness) capillary column, with a split/splitless injector operating in the splitless mode (at 250°C) and a flame ionisation detector (FID). The oven temperature was maintained at 40°C for 1 minute, and then programmed at 10°C /min to 250°C, followed by 25min isothermal final time (250°C). The carrier gas was hydrogen.

Non-polar gas chromatographic analysis (50m HP-1)

Detailed analysis of samples was carried out on a Hewlett Packard 5890 series2 GC, fitted with a 50m, 320μm ID, HP-1 (2.65μm film thickness) capillary column, with a temperature-programmed on-column injector (3°C above the column temperature). The oven temperature was maintained at 40°C for 1min,

programmed at 5°C /min to 150°C, then 10°C /min to 250°C, with an isothermal final time of 25min. The carrier gas was hydrogen.

Human organoleptic analysis

Human organoleptic analysis was used as a complementary way of estimating the suitability of extracts. This method was thought to be suitable, as general odorants for insects belong to the same chemical classes as strong odorants for vertebrates. Typical examples include aliphatic alcohols and aldehydes of medium size (6 to 10-carbon atoms), which are strong odorants for vertebrates and produce strong electrophysiological responses with most insects. Some compounds such as dipropyldisulphide and β-bisabolol are also exceptionally strong odorants for both insects and humans. This assumption, on the other hand, does not hold true for pheromonal compounds (of insects and vertebrates), which are mostly odourless for vertebrates (Pelosi and Maida, 1995).

Organoleptic analysis was achieved by an olfactory comparison of the emissions from the original plant foliage and a MAS extract with those of the test extracts. The concentration of volatiles in each extract was firstly standardised to a similar total content as was found in a MAS extract (25g/ml fresh weight equivalent); this was accomplished using the peak area counts (minus solvent area counts) from GC profiles of each extract. A similar quantity (20μl) of each standardised extract was applied to a separate filter paper disc (2cm diameter) and left for 20 seconds for the solvent to evaporate off. The sensoric qualities of the volatiles evaporating from the discs were then compared with those of a MAS extract and the original plant material, paying particular notice to any 'off notes' or pyrolitic odours present in the extracts.

Figure 2.1: **Microwave-assisted distillation apparatus**

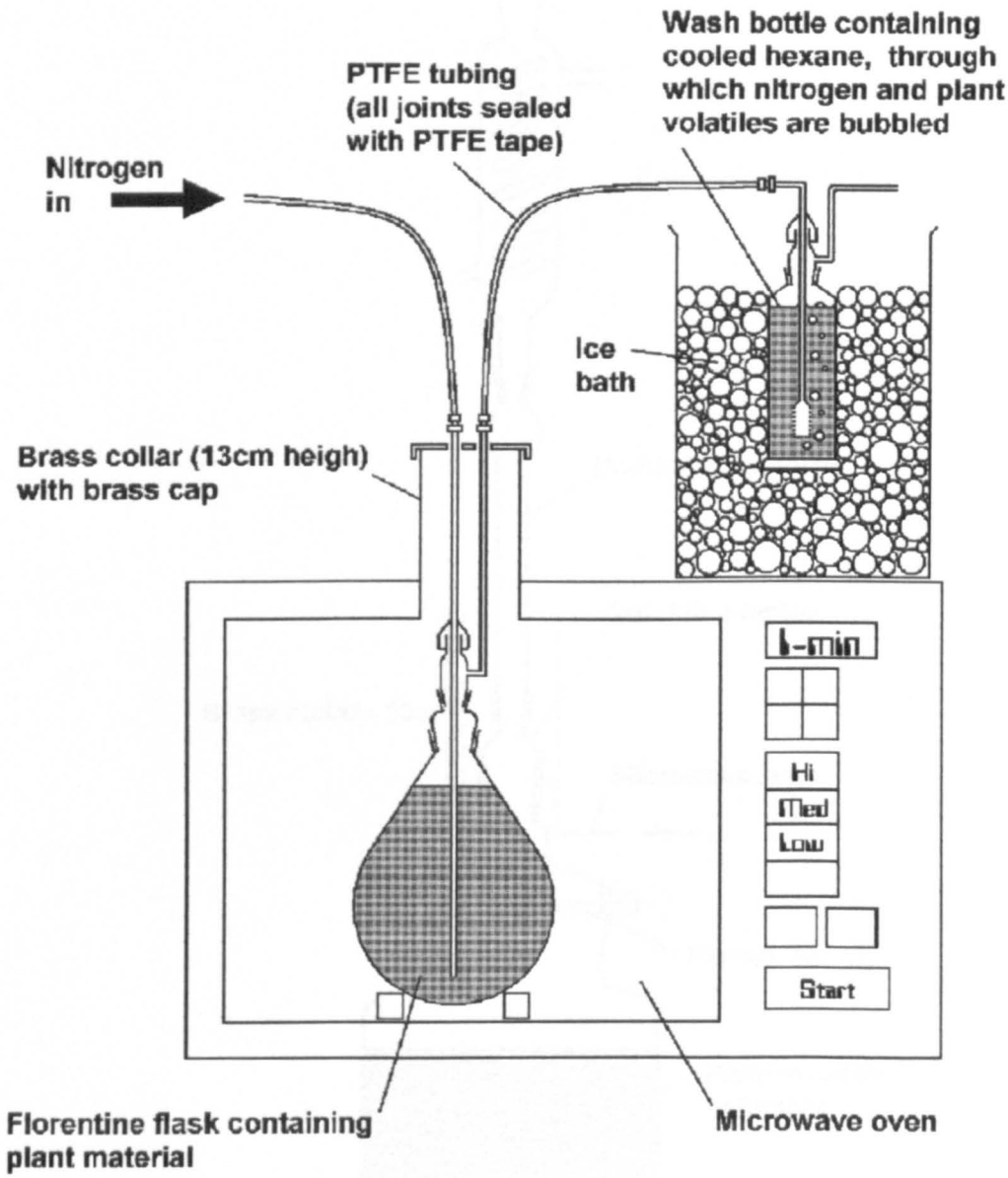


Figure 2.2: **Microwave-assisted solvent extraction apparatus**

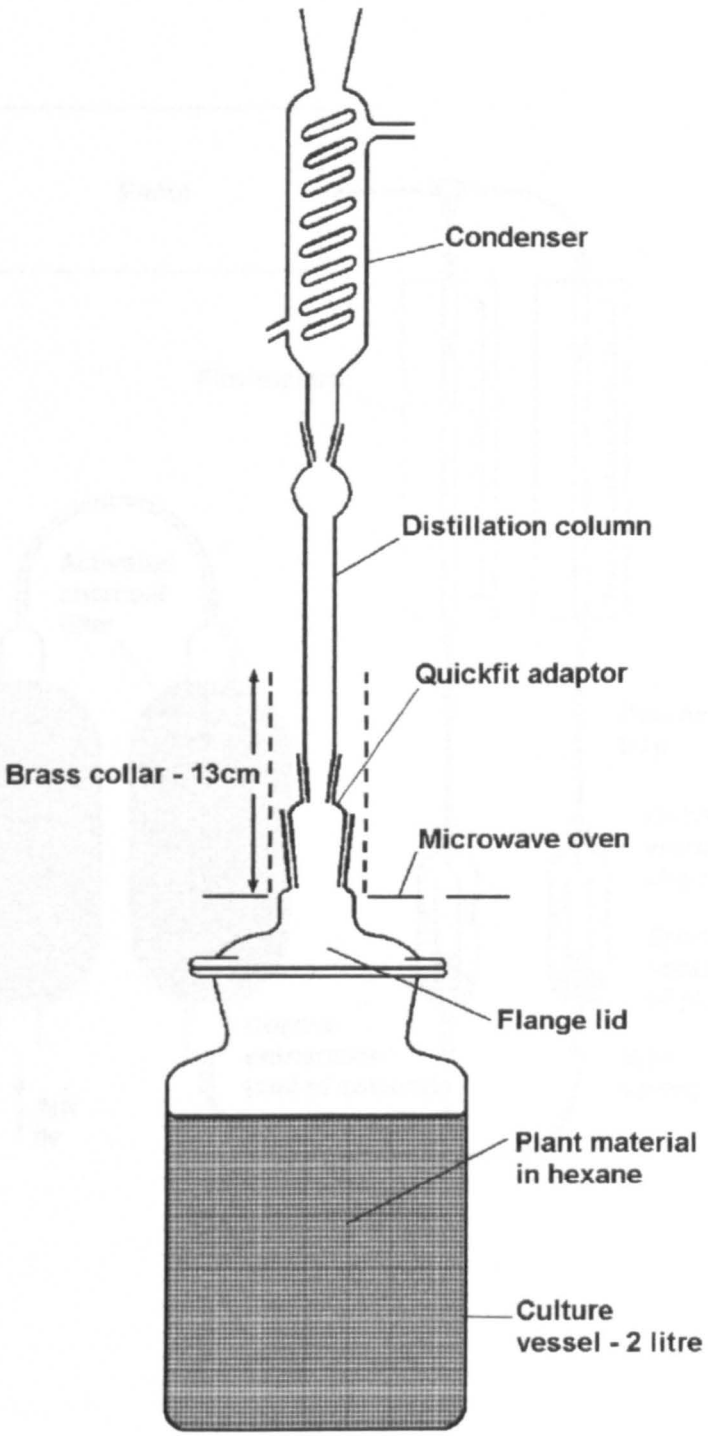


Figure 2.3: **Apparatus for the air entrainment of MAS extract**

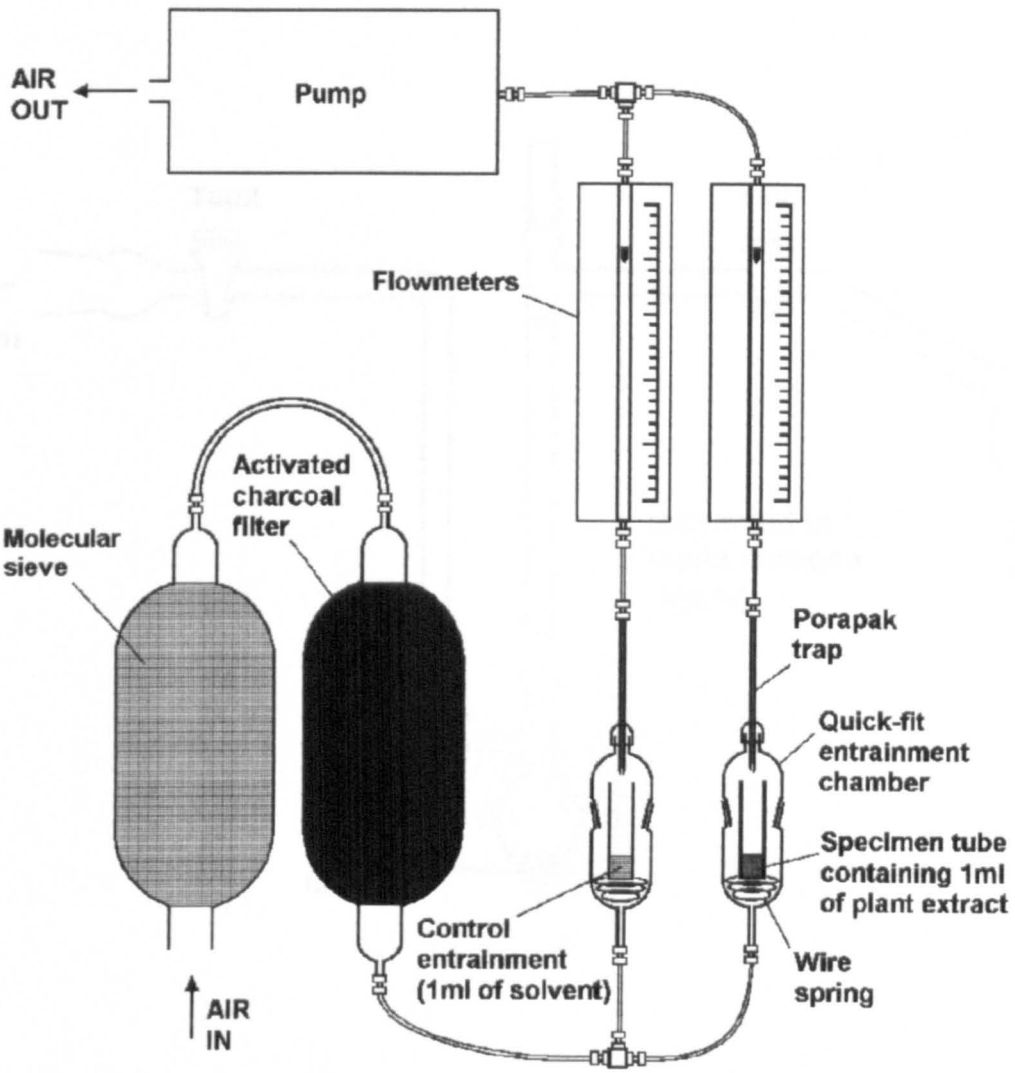


Figure 2.4: **Apparatus for the vacuum distillation of MAS extract**

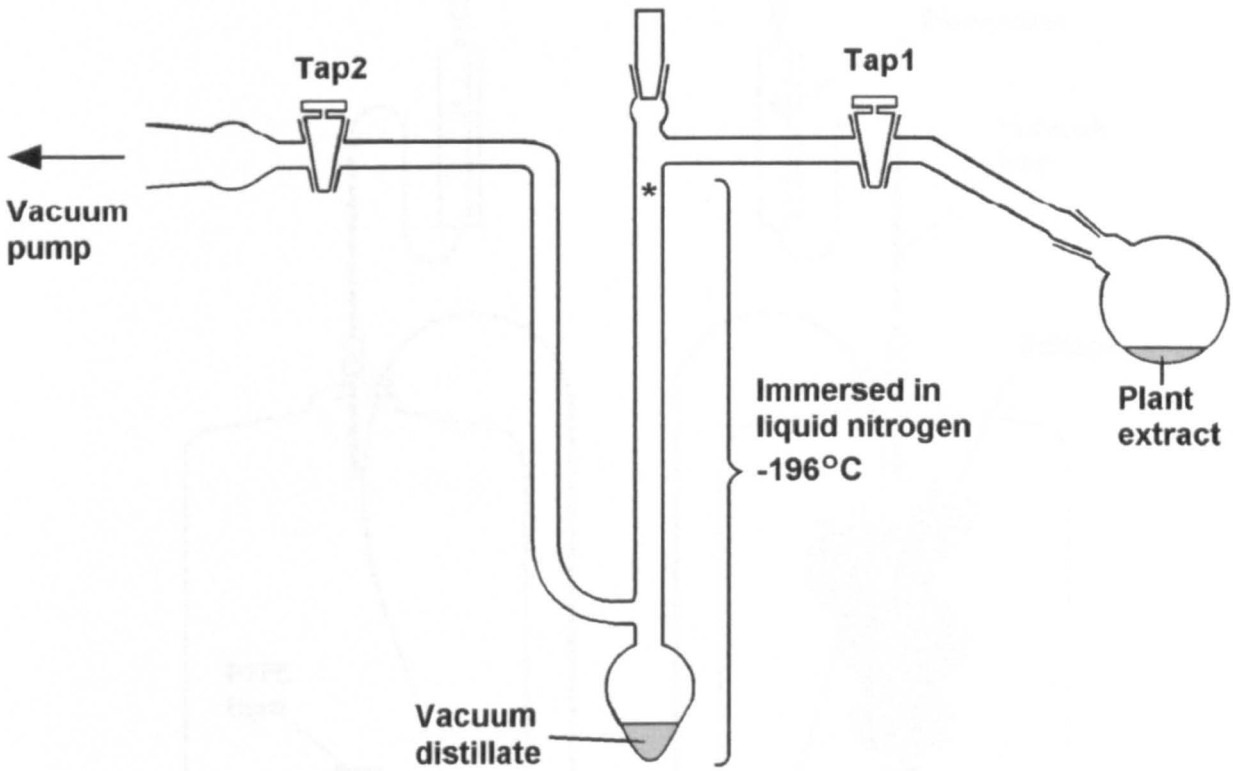


Figure 2.5: **Air entrainment apparatus for plant foliage**

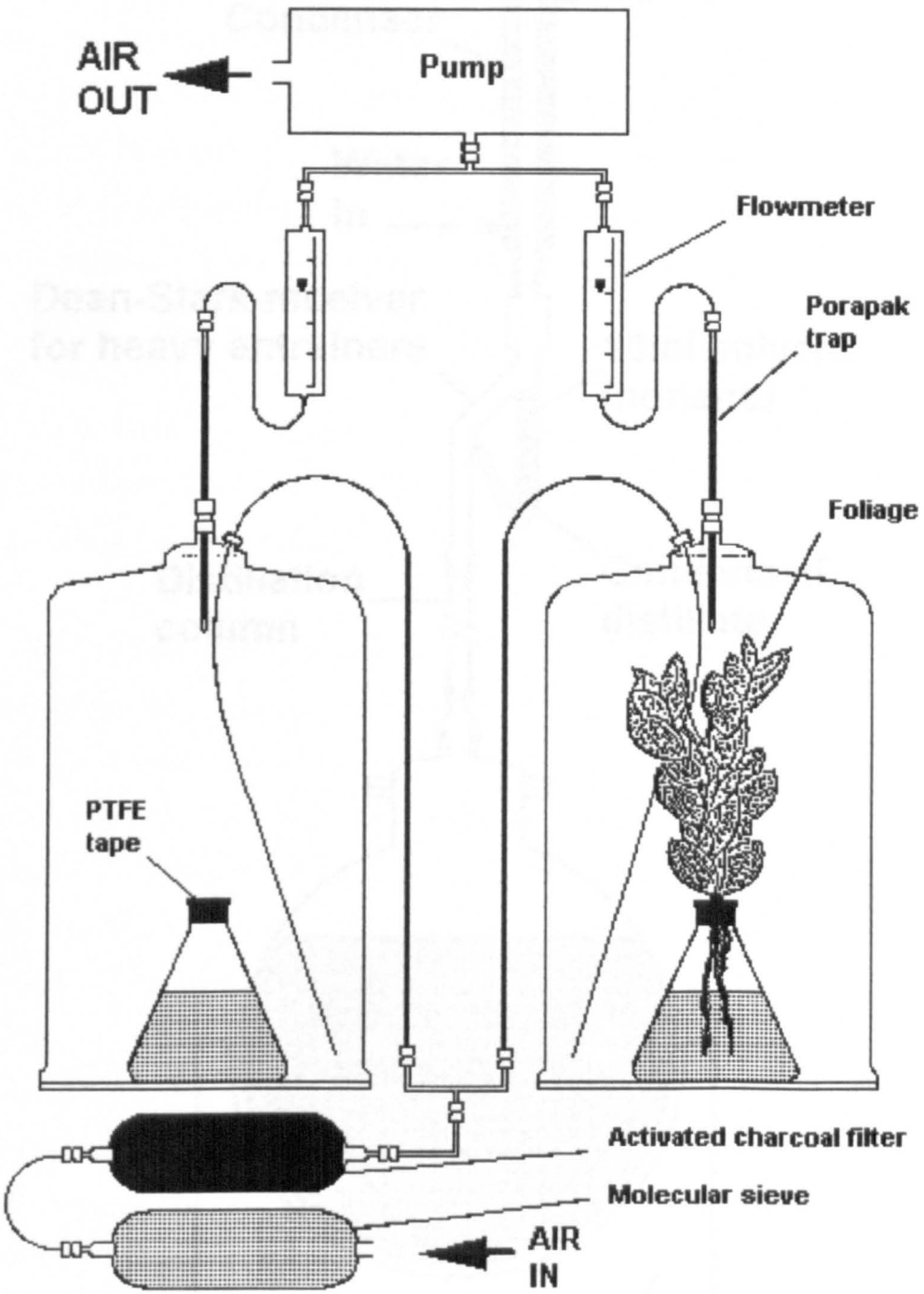
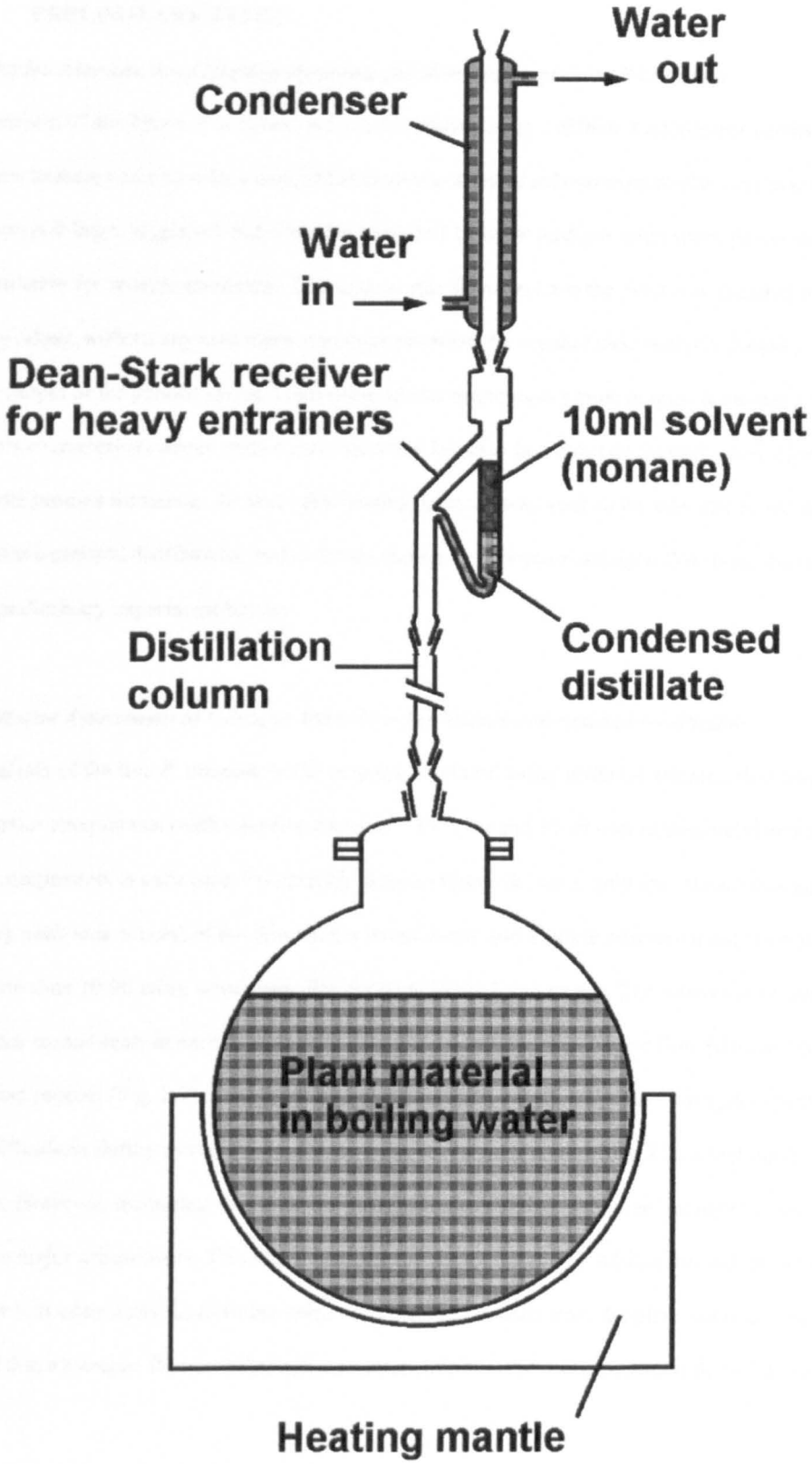


Figure 2.6: **Hydro-distillation apparatus**



2.3 RESULTS

2.3.1 PRELIMINARY STUDY

Qualitative Assessment of Heating Protocols for Microwave-Assisted Distillation

Comparison of the fifteen *P. crispum* extracts (produced using 3 different microwave power outputs and 5 different heating periods) with a standard microwave-assisted solvent extract of *P. crispum* and fresh *P. crispum* foliage, suggested that a heating period of 1 min at medium microwave power output was the most suitable for volatile extraction. This extract was found to have the most concentrated characteristic parsley odour, without any noticeable pyrolysis products, by organoleptic analysis. Heating on the high power output or for periods above 3 min (even at low microwave power output) produced a loss in the extract's characteristic odour, with a corresponding increase in a burnt or charred smell, characteristic of pyrolysis product formation. As such, this heating protocol was used as the standard in subsequent microwave-assisted distillations, and in the assessment of optimum nitrogen flow rates during extraction, in the preliminary experiment below.

Quantitative Assessment of Nitrogen Flow Rate for Microwave-Assisted Distillation

GC analysis of the five *P. crispum* MAD extracts, produced using different nitrogen flow rates, showed three major components (with retention times of 7.12, 8.14 and 14.09 min respectively) and numerous minor components in each case. To simplify these complex GC data, only the relative concentrations (in arbitrary peak area counts) of the three major components and a single arbitrary minor component (retention time 10.00 min), were quantified for each of the five extracts. The relative concentrations of these four components in each extract were then plotted against the nitrogen flow rate used during the extraction process (Fig. 2.7). This figure shows that, as nitrogen flow through the system increased from 50 to 600ml/min during extraction, so did the relative concentrations of the four compounds in the extracts. However, increasing the flow rate to 800ml/min caused a decline in the relative concentration of the three major components. This suggested that the rate of nitrogen bubbling through the solvent was too great for it to adequately dissolve the entrained volatiles released from the plant material. This evaluation showed that a nitrogen flow rate through the system of 600ml/min was optimum for volatile extraction

when using a medium microwave power output and heating period of 1 min during extractions. As such, this nitrogen flow rate was used as the standard in subsequent microwave-assisted distillations.

2.3.2 COMPARISON OF EXTRACTION TECHNIQUES BY GC ANALYSIS OF *C. maculatum* EXTRACTS

In order to establish the most appropriate methods of extraction, GC profiles of the four different types of *C. maculatum* extract were compared, see Figs. 2.8a-e, which show the GC profiles of each extract with the largest (non-solvent based) peak at full scale. The GC profiles of the two most successful methods for removing the less volatile 'leaf surface wax' contaminants from *C. maculatum* MAS extracts are also shown in Fig. 2.9a and b. Differences between the GC profiles of the two *C. maculatum* MAS extracts 'a' and 'b' (Fig. 2.9b and Fig. 2.9c, respectively) are due to differences in harvest date and qualities of the plant materials extracted. MAS extract 'a' was produced from the same batch of foliage used for the comparison of the four extraction methods. MAS extract 'b' was produced from a later batch of foliage and was re-extracted by vacuum distillation, and is only included for comparison with this extract Fig. 2.9b.

In order to simplify the complex GC data for evaluation, five regions of interest were identified within the GC profiles:

a) The solvent front, which is the band of closely associated off-scale peaks originating from a retention time of approximately two minutes. The solvent front varied in length depending on the solvent incorporated in the extract (i.e. hexane or diethyl ether).

b) The early retention time volatiles, producing peaks from approximately 12 - 16 min in the GC profiles.

c) Two very variable peaks with retention times of approximately 17 and 18 minutes. The relative heights of these two peaks, compared to the other regions of interest, varied widely between the GC profiles of the original *C. maculatum* MAS extracts and the MAS extracts which had been re-extracted to remove leaf wax contaminants.

d) The later retention time volatiles, producing peaks from approximately 26 - 32 min in the GC profiles.

e) The high molecular weight, less volatile, components appearing after 32 minutes in the GC profiles. These were associated with peaks which continued to appear after the end of the GC run, appearing as ghost peaks in subsequent GC injections, and which required removal by numerous solvent clean-up runs.

Differences in the GC profiles of the extracts were summarised according to these five regions of interest, and tabulated to aid comparison (Table 2.1 and 2.2). Comparison of the GC profiles of the four types of extracts showed major differences between the relative proportions of volatiles extracted from these five regions of interest (Table 2.1). As expected, only the MAS extracts contained high molecular weight 'leaf surface wax' contaminants, as this was the only extraction method in which the solvent was in direct contact with the extracted foliage. All four types of extract contained medium to high levels of the later retention time volatiles (rt. 26-32min), low to medium levels of the two peaks at approximately 17 and 18min, and relatively high levels of the early retention time volatiles (rt. 12-16min), with the exception of the hydro distillate, in which only low levels of the early retention time volatiles were extracted. The hydro distillate also contained nonane as solvent, which produced a peak at approximately 11min, plus numerous contaminant peaks with retention times ranging from 7 – 15min (as shown in the GC profile of nonane, Fig. 2.8f).

Comparison of the olfactory emissions from the four types of *C. maculatum* extract with fresh *C. maculatum* foliage, by human organoleptic analysis, found that the MAD and MAS extracts had odours which closely resembled the original fresh foliage (having an odour often described as 'mousey'). The odour from the air entrainment extract was also relatively similar to that of the fresh foliage. However, the hydro distillate had a strongly atypical odour (smelling of cooked leaves, with a sharp/sweet overtone) and no 'mousey' smell.

Comparison of Methods for Removing Less Volatile Leaf Surface Waxes from MAS Extracts

Attempts to remove the high molecular weight contaminants from the MAS extracts by column chromatography (as described by Städler and Buser, 1984), and by precipitation of waxes, failed to remove them completely and resulted in considerable losses of extracted material due to the high levels of concentration required in the early part of these procedures. Further attempts, using air entrainment (Fig. 2.9a) and vacuum-distillation (Fig. 2.9b) to re-extract the MAS extract, successfully removed the less volatile contaminants, although they also resulted in major distortions to the volatile profile when compared to the original MAS extracts used in their production (Fig. 2.8b and 2.8c, respectively). In both cases, very few of the later retention time volatiles were recovered (see Table 2.2) and possible

degradation of the extract was noticed, as seen by the large relative increase in the two peaks with retention times of approximately seventeen and eighteen minutes, which were only present as minor peaks in the original MAS extracts.

Table 2.1: **Summary of features of interest in the GC profiles of *C. maculatum* extracts produced using a range of different extraction methods.**

Type of Extract	Relative peak quantities and heights in five regions of interest in the GC profiles				
	Solvents used and solvent front characteristics	Early retention time volatiles (rt. 12-16 min)	Two very variable peaks (rt. 17 and 18 min)	Later retention time volatiles (rt. 26-32 min)	High m.w. contaminants (rt. longer than 32 min)
MAD	Hexane, long rt. 2-8 min	High	Medium	Medium	None
MAS extract (a)	Hexane, long rt. 2-8 min	High	Low	High	High
MAS extract (b)	Hexane, long rt. 2-8 min	High	Low	High	High
Air entrainment	Diethyl-ether, short rt. 2-4 min	High	Low	High	None
Hydro distillation	diethyl ether short rt. 2-4 min, plus nonane 7-15 min	Low	Low	High	None

Table 2.2: **Summary of features of interest in the GC profiles of *C. maculatum* MAS extracts after further processing to remove less volatile 'leaf surface wax' contaminants.**

Type of Extract	Relative peak quantities and heights in five regions of interest in the GC profiles				
	Solvents used and solvent front characteristics	Early retention time volatiles (rt. 12-16 min)	Two very variable peaks (rt. 17 and 18 min)	Later retention time volatiles (rt. 26-32 min)	High m.w. contaminants (rt. longer than 32 min)
MAS extract (a) re-extracted by air entrainment	Diethyl ether, short rt. 2-4 min	High	High	Low	None
MAS extract (b) re-extracted by vacuum distillation	Hexane, long rt. 2-8 min	High	High	Low	None

Figure 2.7: **Concentrations of four components of interest in the GC profiles of *P. crispum* MAD extracts (in arbitrary peak areas) against the nitrogen flow rate used during their extraction (single replicate of each extract; line fitted by eye).**

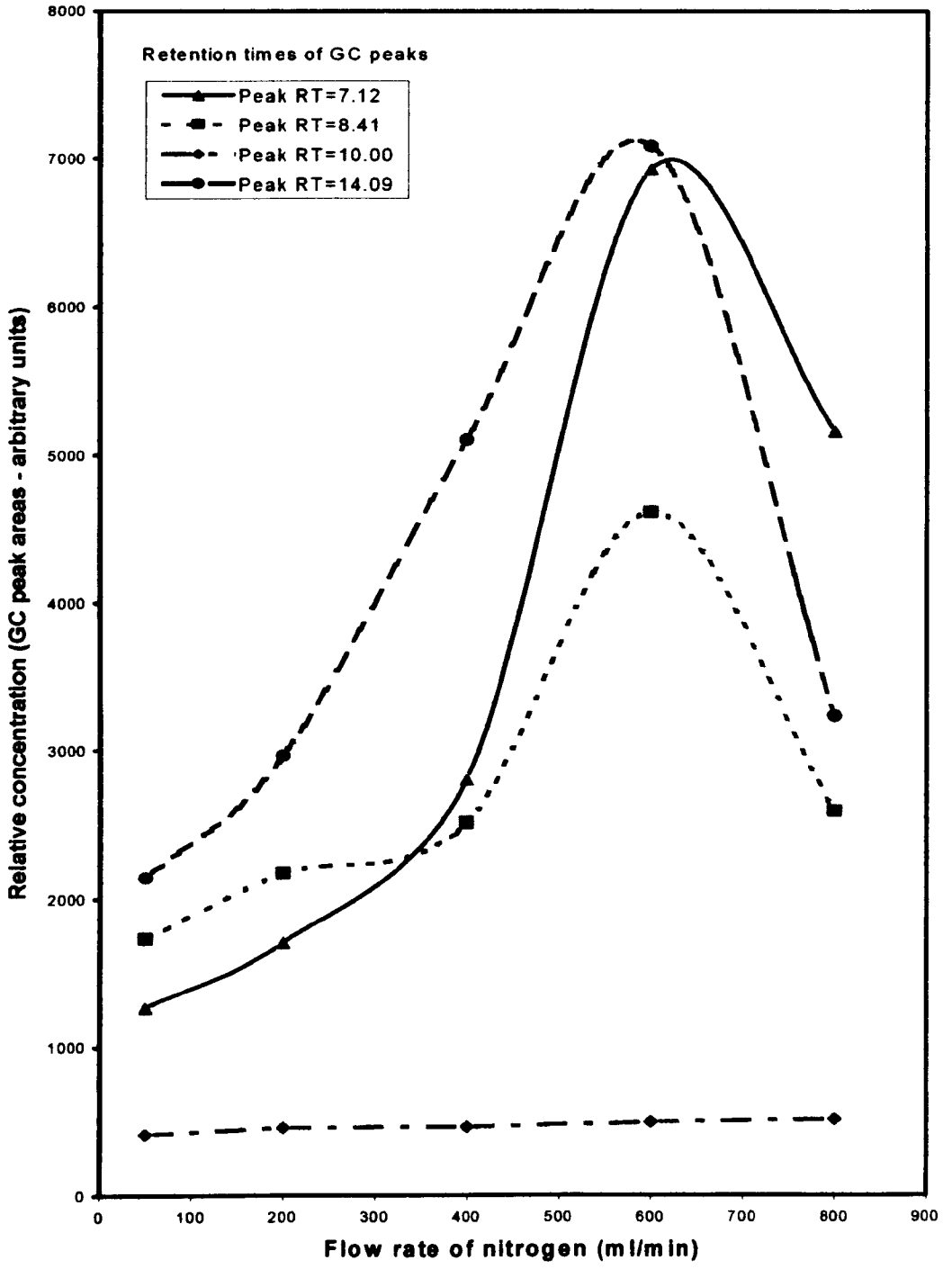
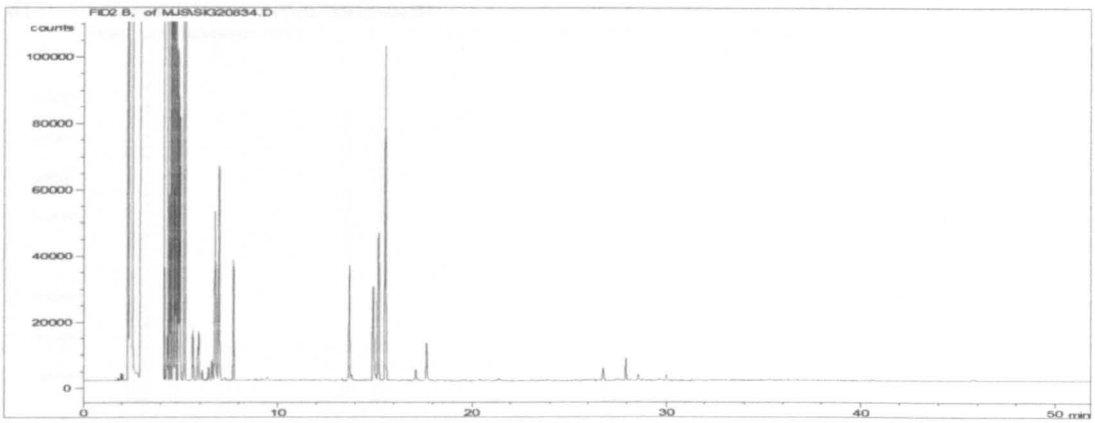
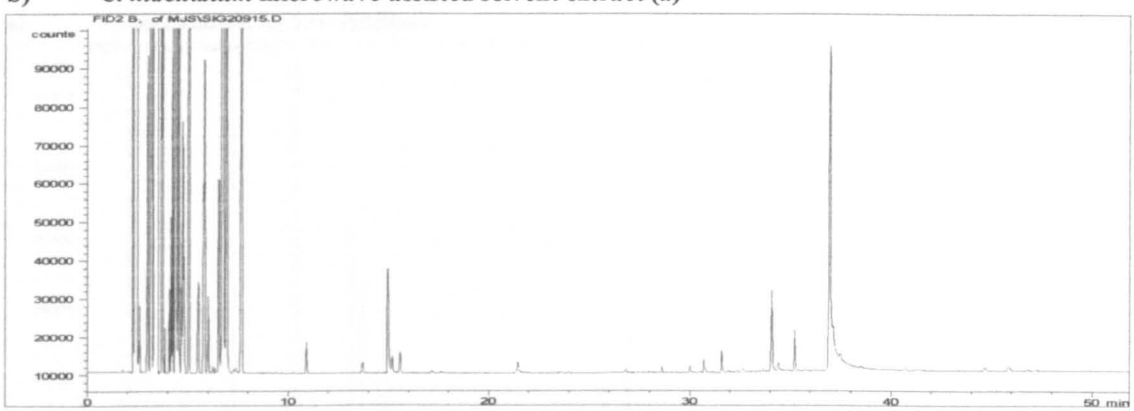


Figure 2.8: **GC profiles of *C. maculatum* foliage extracts, prepared using a range of different extraction methods: major component peaks shown at full scale.**

a) ***C. maculatum* microwave-assisted distillate**



b) ***C. maculatum* microwave-assisted solvent extract (a)**



c) ***C. maculatum* microwave-assisted solvent extract (b)**

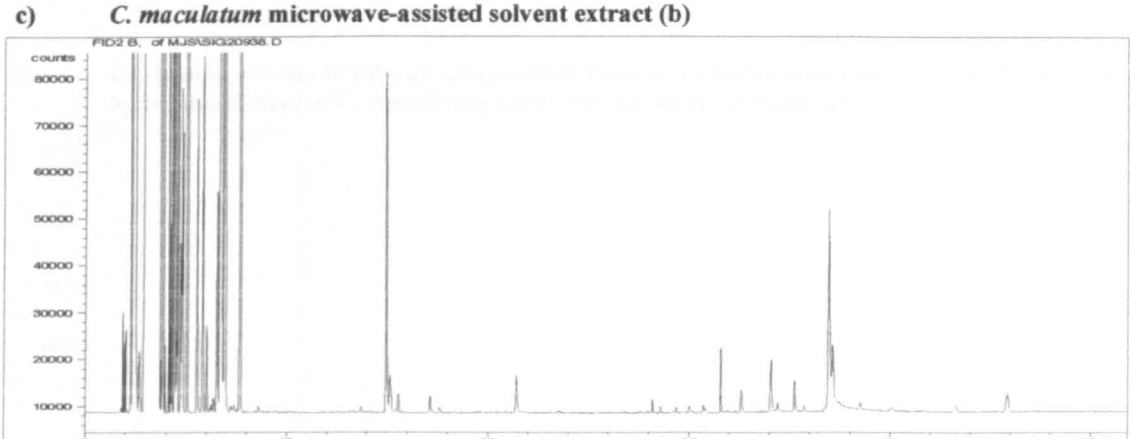
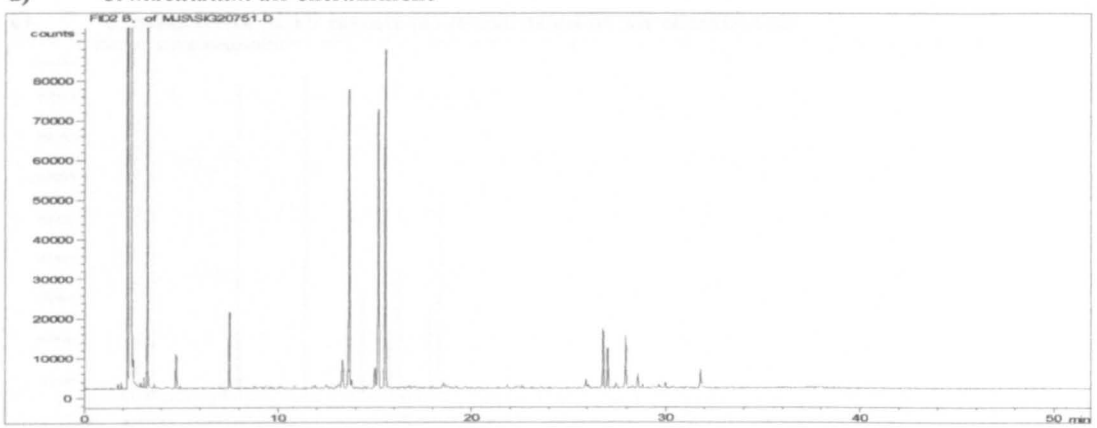
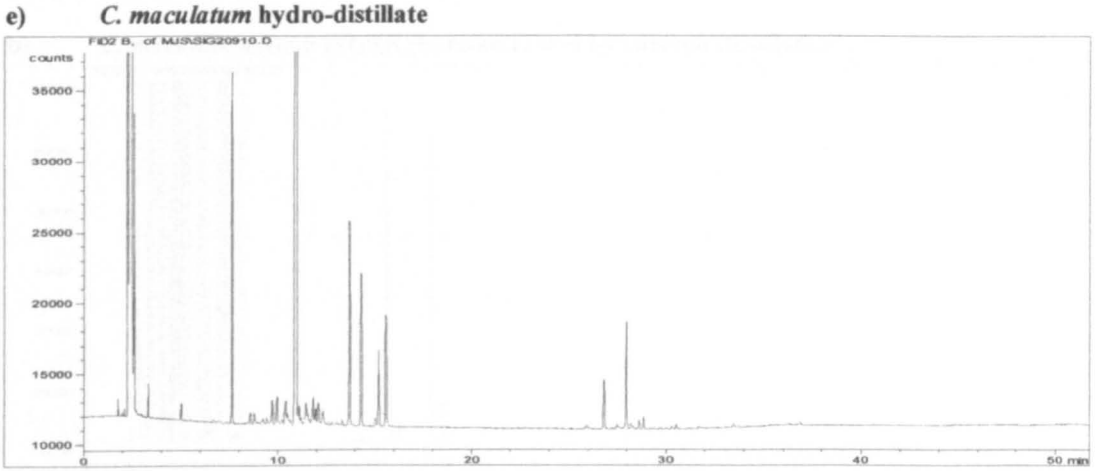


Figure 2.8: **GC profiles of *C. maculatum* foliage extracts, prepared using a range of different extraction methods: major component peaks shown at full scale (Continued).**

d) ***C. maculatum* air entrainment**



e) ***C. maculatum* hydro-distillate**



f) **GC profile of nonane solvent - evaporated down to a similar extent as occurred during the hydro-distillation of *C. maculatum* above, for comparison purposes**

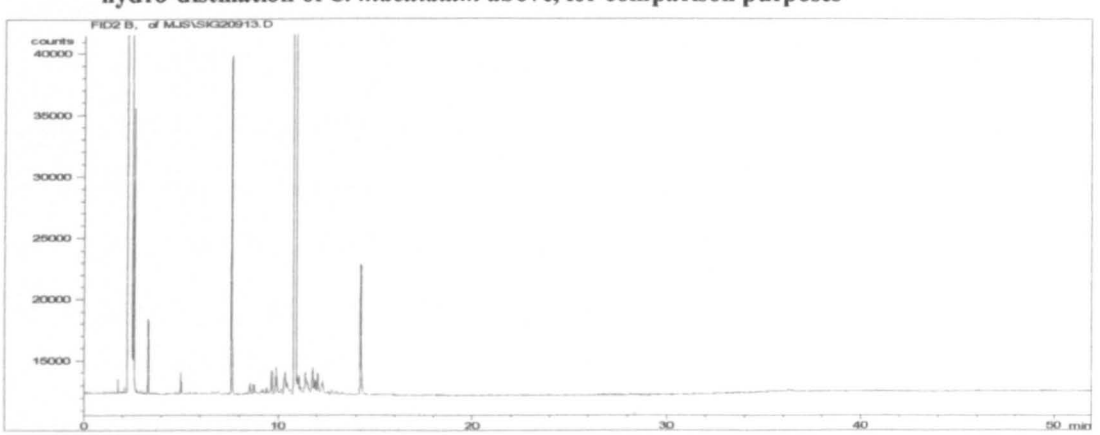
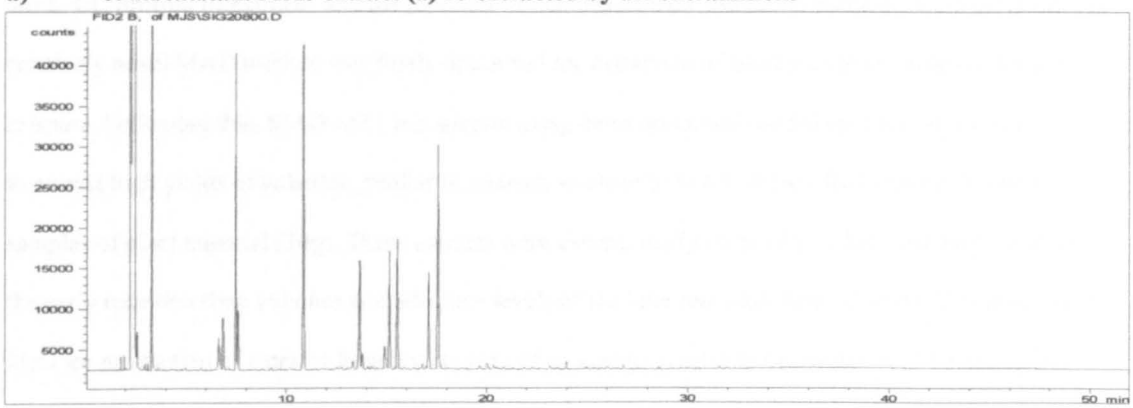
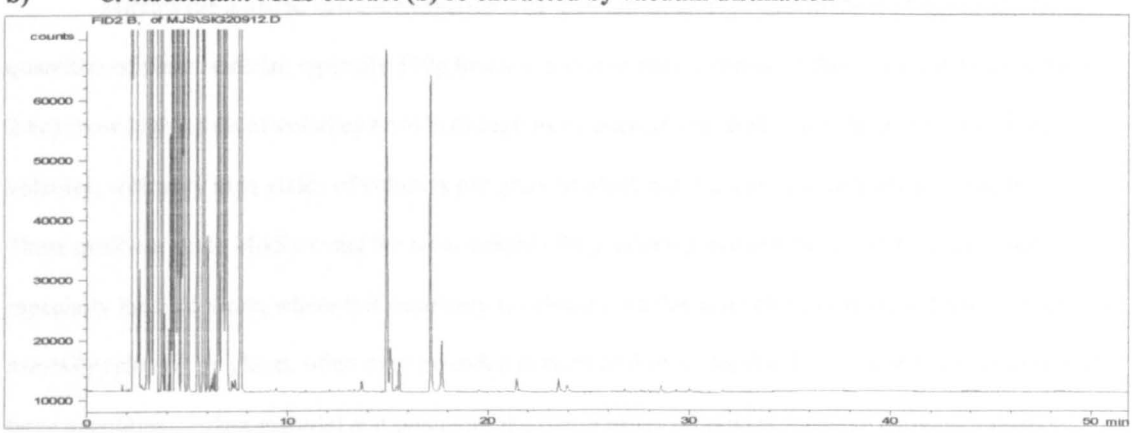


Figure 2.9: **GC profiles of *C. maculatum* MAS extracts after attempts to remove low volatility 'leaf surface wax' contaminants by re-extraction with air entrainment and vacuum distillation: major component peaks shown at full scale.**

a) ***C. maculatum* MAS extract (a) re-extracted by air entrainment**



b) ***C. maculatum* MAS extract (b) re-extracted by vacuum distillation**



2.4 DISCUSSION

In order to determine the most suitable extraction methods for subsequent identification and bioassay work, GC profiles of extracts, and the practical limitations of their extraction methods, were assessed. The relatively novel MAD method was firstly optimised for extraction of apiaceous plant material using *P. crispum*. Following this, MAD of *C. maculatum* using these optimised conditions showed it was possible to extract high yields of volatiles, similar in quantity to those of MAS extract, from relatively small samples of plant material (30g). These extracts were exceptionally clean (Fig. 2.8a), with high levels of the early retention time volatiles and adequate levels of the later retention time volatiles. This made MAD ideal for production of extracts from a wide rage of apiaceous species to be investigated for biological activity using GC-EAG, as well as providing sufficient material for identifications by GC-MS, and identification confirmation by GC peak enhancement.

Microwave assisted solvent extraction was found to be a very rapid method of extracting large quantities of plant material, typically 300g batches in one minute. Profiles of these extracts (Figs. 2.8b and 2.8c) show high levels of volatiles from both regions of interest, the 'early' and 'later' retention time volatiles, with very high yields of volatiles per gram of plant material (see quantifications, Chapter 3). These qualities made MAS extract the most suitable for producing extracts for use in bioassays and especially for field work, where it is necessary to release volatiles at levels typical of, or higher than, those naturally released by plants, often over extended periods of days to weeks. This requires the extraction of large quantities of plant material and precludes the use of many otherwise adequate extraction methods. Because this is a direct solvent extraction method, it has the drawback that the samples contain large quantities of less volatile and non-volatile contaminants, especially leaf surface waxes. These contaminants render this type of extract unsuitable for GC analyses on a routine basis, as introduction of such material into a GC system leads to the accumulation of non-volatile residues in the injector and column inlet. These residues may slowly degrade to produce volatiles which emerge as ghost peaks in subsequent analyses, or they may catalyse the decomposition of subsequent samples which are injected (Mitzer, 1964). This build up of non-volatile material, which results in a dirty injector, may also produce additional adsorption effects, leading to changing retention times, tailing peaks and reduced system efficiency (Grob and Grob, 1979). Attempts to remove the high molecular weight contaminants by column

chromatography (as described by Städler and Buser, 1984) and precipitation failed to remove them completely and resulted in considerable losses of extracted material, due to the high levels of concentration required in the early part of these procedures. Further attempts, using air entrainment (Fig. 2.9a) and vacuum-distillation (Fig. 2.9b) to re-extract the MAS extract, successfully removed high molecular weight contaminants, although they also resulted in major distortions to the volatile profile when compared to the original MAS extract used in their production (Fig. 2.8b and 2.8c, respectively). In both cases, very few of the later retention time volatile compounds were recovered and possible degradation of the extract was noticed, as seen by the large relative increase in the two peaks with retention times of approximately seventeen and eighteen minutes, which were only present as minor peaks in the original MAS extract. Although the air entrainment of the MAS extract resulted in a distortion of the GC profile, it was chosen for further identification work by GC-EAG, because it would contain compounds likely to be released from MAS extract lures used in subsequent field trials. Because re-extraction resulted in large changes to volatile profiles, and because these two-step processes greatly increased the risk of contamination, other methods of extraction were also investigated.

Air entrainment of *C. maculatum* foliage was found to produce extracts that were very clean (Fig 2.8d), but which also contained the highest proportions of the later retention time volatiles, compared to other methods. Unfortunately, air entrainment was prohibitively time-consuming to perform (48-hour entrainment) and resulted in very small yields of volatile material. It was, however, useful for the determination of approximate release rates of volatile components from living plants, as AE extracts are known to resemble most closely the composition of volatiles actually released. Such natural release rate information was important in the development of release systems for both extract and synthetic chemical based lures.

Extraction of *C. maculatum* by hydro-distillation produced high yields of both 'early' and 'later' retention time volatiles. This method was also capable of bulk extraction of plant material, typically two kilograms in four hours. Unfortunately, the hydro-distillation extract had to be rejected because of its strongly atypical odour characteristics, as assessed by human organoleptic analysis. In addition, the nonane solvent necessary to retain highly volatile compounds in the receiver (and its associated contaminants), had a similar volatility to the early retention time volatiles (see Figs. 2.8e and 2.8f),

making it impossible to remove without great losses in extracted components. The lack of suitability of hydro-distillation was not unexpected, as previous studies found that commercially produced host plant essential oils were not attractive to *P. rosae* in the field (Guerin *et al*, 1983). The low levels of the highly volatile 'early retention time' compounds in the hydro-distillate, probably resulted from their evaporation from the relatively warm Dean-Starks receiver during the extraction process. From this study, it seems likely that the low levels of the highly volatile compounds were, in part, responsible for the atypical odour of this distillate, and its lack of a 'mousey odour' typical of fresh *C. maculatum* foliage. Low levels of these highly volatile compounds, combined with chemical changes and decomposition occurring during the extraction process as a result of prolonged heating in the presence of water and oxygen, probably contributed strongly to its lack of attraction for *P. rosae* in previous field experiments.

3 IDENTIFICATION AND QUANTIFICATION OF SEMIOCHEMICALS FOR *P. rosae*

3.1 INTRODUCTION

Having established the most suitable methods of extracting plant material from the Apiaceae, it was necessary to locate the biologically active components in the extracts, amongst the large quantities of chemically similar but biologically inactive material also present. This was achieved by use of directly coupled GC-EAG, which allowed the GC column effluent to be monitored continuously and simultaneously by both GC and EAG detectors, providing an extremely sensitive detection system. The complete antennae of female *P. rosae* were used for the EAG detectors, as these have previously been found to produce identical relative responses to host compounds as males, but with more than twice the absolute amplitude, except for higher responses to the leaf aldehydes in males (Guerin and Visser, 1980). Tentative identification of biologically active compounds was made by coupled GC-MS. Confirmation of tentatively identified structures was then achieved by peak enhancement with authentic standards, where available, on two GC columns of differing polarity (non-polar HP-1 and semi-polar SPB-35). As many semiochemical compounds can exist in two or more enantiomeric forms, due to the presence of one or more chiral carbon atoms in their structure, a protocol for their separation (which cannot be achieved on normal non-chiral GC columns) and identification was established. This involved the selection of a chiral GC column capable of resolving the separate enantiomers into individual peaks, followed by peak enhancement with authentic chiral standards.

The first section of this investigation focused particularly upon *C. maculatum*, a preferred wild host species for *P. rosae*, with a briefer investigation into a range of cultivated host plants and some of the more common or interesting wild host species in the Apiaceae. This is followed by a brief investigation into possible repellent or masking compounds thought to be present in certain non-host species, such as the onion, *Allium cepa* (Liliaceae), (Uvah and Coaker, 1984) and the medic, *Medicago litoralis*, (Fabaceae = Leguminosae), (Rämert, 1993 and 1996), which have been shown to reduce *P. rosae* attack in field intercropping trials. Finally, an investigation was carried out to identify any pheromonal components, such as sex or aggregation pheromones, which may be released by the carrot flies themselves.

3.2 METHODS

3.2.1 Source of Plant Material

Plant material for extraction was collected from a variety of sources, depending upon its availability. Onion bulbs (*Allium cepa*) cv. 'Renate' were obtained commercially from J.S. Sainsbury plc. Giant hogweed (*Heracleum montegazzianum*) was obtained as a seed oil as a kind gift of English Hop Produce Ltd. All other plant material was collected as mature foliage harvested prior to flowering in May-June, (with the exception of the hemlock foliage used to produce MASE extract (b), which was harvested in late September). Plant foliage was extracted the same day as harvesting, or stored overnight at 5°C in a polyethylene bag for extraction the following day. The following plant materials were collected from wild populations in the local vicinity (Harpenden, Hertfordshire), taking care not to remove more than 20% of the leaves from any one plant: cow parsley (*Anthriscus sylvestris*), hogweed (*Heracleum sphondylium*), rough chervil (*Chaerophyllum temulentum*), wild carrot (*Daucus carota* ssp. *carota*) and hemlock (*Conium maculatum*) were collected, with kind permission, from Harpenden Golf Club; long leaf (*Falcaria vulgaris*) was collected from the High Wycombe area. The following agricultural crop species were collected from cultivated field crops in the Cambridgeshire and Norfolk region: celery (*Apium graveolens*) cv. 'Victoria' was a kind gift of Bourne Salads (Norfolk); parsnip (*Pastinaca sativa*) cv. 'Javalin' was collected, with kind permission of Dr. Jennie Blood-Smyth, from the ADAS – Arthur Rickwood site, whilst carrot (*Daucus carota* ssp. *carota*) cv. 'Nirobi' was collected with kind permission of Mr Bruce Butcher (Graves and Graves Ltd), Ely. The following species were grown outdoors from seed: fennel (*Foeniculum vulgare*) cv. 'Sweet Fennel' (cat. No 1384), parsley (*P. crispum*) cv. 'Champion Moss Curled' (Cat. No 1781) and garden chervil (*Anthriscus cerefolium*) cv. 'Curled Chervil' (Cat. No 1380), all purchased as seed from Chiltern Seeds Ltd. The medic (*Medicago litoralis*) was grown in the glass house and obtained as seed, as a kind gift of Dr. Birgeitte Rämert, Swedish University of Agricultural Science.

3.2.2 Extraction Techniques

Hemlock extracts

Two of the most suitable hemlock extracts, as determined in the previous chapter (Ch.2) as containing no low volatility leaf wax contaminants, were analysed for electrophysiological activity. These were microwave-assisted distillation and microwave-assisted solvent extract, which was re-extracted by air entrainment to remove leaf wax contaminants (for methods see Ch. 2.2.2).

Other host and non-host plant extracts

A wide range of other host and non-host plant species were also extracted, using the optimised MAD method as determined in chapter 2, and analysed for electrophysiological activity. This MAD involved heating 30-40g of fresh foliage (with the main stems removed) in an adapted 800W microwave oven for 1min at medium power, with a nitrogen flow rate of 600ml/min. Released volatiles were carried by the nitrogen into a wash bottle containing 50ml of chilled hexane solvent, where they were dissolved. Extracts were dried using anhydrous magnesium sulphate and concentrated to 25g/ml fresh weight equivalent. For a more detailed description of this method, see Ch. 2.2.2.

In the case of the *A. cepa*, 40g of onion bulb (rather than foliage) were diced and extracted by MAS extraction, followed by re-extraction by vacuum distillation (see method in Ch. 2.2.2) to remove low volatility contaminants. Giant hogweed extract was obtained as distilled seed oil from English Hop Produce Ltd.

Air entrainment of *P. rosae*

The air entrainment apparatus was assembled as indicated in Fig. 3.1, based upon the method of Blight (1990). The purified air flow was split into three and entered three 1 litre entrainment chambers (Quickfit), sealed using PTFE tape. The 'treatment' chamber contained a vertical brass mesh which acted as a roost for the flies, a water source (a glass specimen tube of water with a cotton wool wick) and approximately 1/8 of a white sugar cube, held in an aluminium foil basket, as a food source. The food and water sources were attached to the mesh roost by stainless steel wires. At the start of the entrainment, 25 male and 25 female *P. rosae* (1-2 days old, laboratory reared) were introduced into the treatment chamber.

The 'control' chamber was assembled identically to the 'treatment' chamber, but no flies were added, and the 'blank' chamber was left completely empty. Volatiles inside the chambers were carried into the trap tubes, where they were adsorbed onto the Porapak Q (50 mg) at a regulated flow rate of 1 l/min via flow meters. Entrainment of the flies was performed over ten days and the Porapak traps were replaced every 2 days. During this period, the 'treatment' and 'control' vessels were opened briefly, with the pump off, to facilitate topping up the water source and removal of any dead flies. At the end of the experiment, the volatiles from each Porapak tube were eluted with 300µl of freshly distilled diethyl ether, dried with anhydrous magnesium sulphate, and like extracts were combined and concentrated to 50µl.

3.2.3 Analysis of Extracts

Coupled gas chromatography – electroantennography (GC-EAG)

The coupled GC-EAG system was based on that of Wadhams (1990), and was assembled as shown in Fig. 3.2. As GC-EAG analysis is a highly specialised technique, it was performed by a skilled operative, Christine M. Woodcock, of the Biological and Ecological Chemistry Department, IACR-Rothamsted.

Gas chromatography

Chromatography was carried out on an AI 93 gas chromatograph (AI Scientific, Cambridge, U.K.) equipped with a cold on-column injector and flame ionisation detector (FID). The carrier gas was hydrogen, and the column (50 m x 0.32 mm ID, HP-1) was maintained at 40 °C for 1 min and then programmed at 5 °C/min to 100 °C and then at 10°C/min to 250°C. The capillary column effluent was split equally between the FID and the transfer line to the insect preparation by a low volume splitter constructed from glass-lined stainless-steel tubing and deactivated fused silica tubing. One length of tubing lead to the FID, whilst the other was taken through the oven wall and directed the remainder of the effluent into a purified airflow passing continuously over the antennal preparation. Components eluting from the capillary column were monitored simultaneously by the GC and the insect detector. Nitrogen was added as a make-up gas to reduce residence time in the transfer lines and to maximise FID response.

Electrophysiology

Electroantennograms (EAG) for plant extracts were recorded from recently emerged female flies (2-3 days old) using silver-silver chloride (Ag-AgCl) glass electrodes filled with saline (composition as in Wadhams 1990; NaCl - 7.55g, KCl - 0.64g, $CaCl_2$ dihydrate - 0.22g, $MgCl_2$ - 1.73g, $NaHCO_3$ - 0.86g, and NaH_2PO_4 - 0.67g per litre of distilled water). The insect was momentarily anaesthetised by placing it in a specimen tube in an ice bucket; the head was excised and mounted on the indifferent electrode. This was performed by guiding the tip of the electrode through the occipital opening to the base of the antenna, and just inside the pedicellus. The tip of the recording electrode was placed over the excised tip of the arista on the same antenna (see Fig. 3.2). This preparation reduced movement artefacts and desiccation to a minimum. The signals generated by the antenna were passed through a high-impedance amplifier (Syntech UN-03b) and displayed on an oscilloscope. The output from the oscilloscope and GC were monitored on a chart recorder. The effluent from the gas chromatograph was delivered into an air stream (600ml/min), purified but not humidified, flowing continuously over the preparation. Electroantennograms for the air entrainment of *P. rosae* (mixed sex) were recorded from both male and female antennal detectors, in order to compare the responses of the two sexes to any pheromone components possibly present in this extract.

Coupled gas chromatography – mass spectrometry (GC-MS)

Electrophysiologically active components in the sample were tentatively identified by GC-MS. A capillary column (50 m x 0.32 mm ID HP-1) fitted in a Hewlett Packard 5890 gas chromatograph was directly coupled to a mass spectrometer and integrated data system (70-250 VG Analytical or VG Autospec, Fisons Instruments). Ionization was by electron impact at 70eV, 230°C. The gas chromatograph was maintained at 30°C for 5min then programmed at 5°C/min to 180°C. Tentative identifications made by GC-MS were confirmed by comparison of mass spectral data with those of authentic samples and by peak enhancement when coinjected with authentic compounds using GC (Pickett, 1990). As GC-MS analysis is a highly specialised technique, it was performed by a skilled operative, Professor John A. Pickett, of the Biological and Ecological Chemistry Department, IACR-Rothamsted.

Analysis of extracts by gas chromatography

Internal standards

In order to quantify individual components within the volatile profile of an extract, the internal standard technique was used. The internal standard selected had to elute relatively close to the peaks of interest, yet elute separately from other components in the sample. For the purposes of this work, *n*-tridecane was found to fulfil this role adequately in the majority of extracts under analysis.

Confirmation of identifications by coinjection

Tentative identifications made by GC-MS were confirmed by peak enhancement. Extracts were coinjected with authentic standards in quantities that approximately doubled the peak height for the observed compound in question. The criterion for identification of a compound, as being the same as the standard, was taken to be where peak enhancement of the compound was obtained on two GC-columns of differing polarity (non-polar HP-1 and semi-polar SPB-35).

Non-polar gas chromatographic analysis (50m HP-1) of samples was carried out on a Hewlett Packard 5890 series2 GC, fitted with a 50m, 320μm ID, HP-1 (2.65μm film thickness) capillary column, with a temperature-programmed on-column injector (3°C above the column temperature). The oven temperature was maintained at 40°C for 1min, programmed at 5°C /min to 150°C, then 10°C /min to 250°C, with an isothermal final time of 25 min. The carrier gas was hydrogen.

Polar gas chromatographic analysis (30m SPB-35) of extracts was carried out on a Hewlett Packard 5890 series 2 GC, fitted with a 30m, 320μm ID, SPB-35 capillary column, with a split/splitless injector operating in the splitless mode (at 150°C). The oven temperature was maintained at 40°C for 1min, programmed at 5°C /min to 150°C, then 10°C /min to 250°C, with an isothermal final time of 25 min. The carrier gas was hydrogen.

Chiral gas chromatographic analysis of host plant extracts

Selection of a suitable column and conditions for chiral GC analysis

In a preliminary experiment, three different chiral GC columns were evaluated to determine their suitability for separating enantiomers of a group of chiral compounds identified in the host plant extracts.

The three columns evaluated were 30m, 250μm ID columns (Supelco), each with a 0.25μm film coating of α-cyclodextrin (α-DEX 120), β-cyclodextrin (β-DEX 120) or γ-cyclodextrin (γ-DEX 120) respectively. The enantioselectivity of these three stationary phases depends upon the size of the chiral cavity produced by the 6, 7, or 8 glucose units of the α-, β-, or γ-cyclodextrin rings respectively. Evaluation of the columns and subsequent chiral analysis of extracts was carried out on a Hewlett Packard 588A GC, fitted with a split/splitless injector operating in the splitless mode (at 250°C) and a flame ionisation detector (FID). The oven temperature was maintained at 40°C for 1 min, programmed at 5°C /min to 150°C, then 10°C /min to 200°C, with an isothermal final time of 25 min, and the carrier gas was hydrogen. In the preliminary investigation, a mixture of synthetic chiral standards, typical of the Apiaceae [(+) and (-)-α-pinene, (+) and (-)-limonene and (+) and (-)-fenchone], was injected into the GC fitted with each of the three different columns in succession, and the resulting traces were compared to assess separation of the individual enantiomers.

Enantiomeric composition of chiral compounds in host plant extracts

Chiral GC analysis was performed in order to elucidate the enantiomeric composition of specific chiral compounds (having two or more enantiomeric forms, due to the presence of one or more chiral carbon atoms in their structure), identified in host plant extracts. Chiral GC analysis was performed using a β-DEX 120 (Supelco) chiral column and suitable GC conditions, previously determined in preliminary investigations (see above). Confirmation of enantiomer identities [i.e. (+)- or (-)- form] was accomplished by coinjection with synthetic chiral standards, where available. As a result of limited time and availability of few enantiospecific compound standards, analysis focused on only a few chiral compounds from a limited number of apiaceous extracts, these included: β-pinene in hemlock MAD, fenchone and limonene in fennel MAD, and linalool in carrot MAD.

Figure 3.1: **Air entrainment apparatus for the entrainment of *P. rosae***

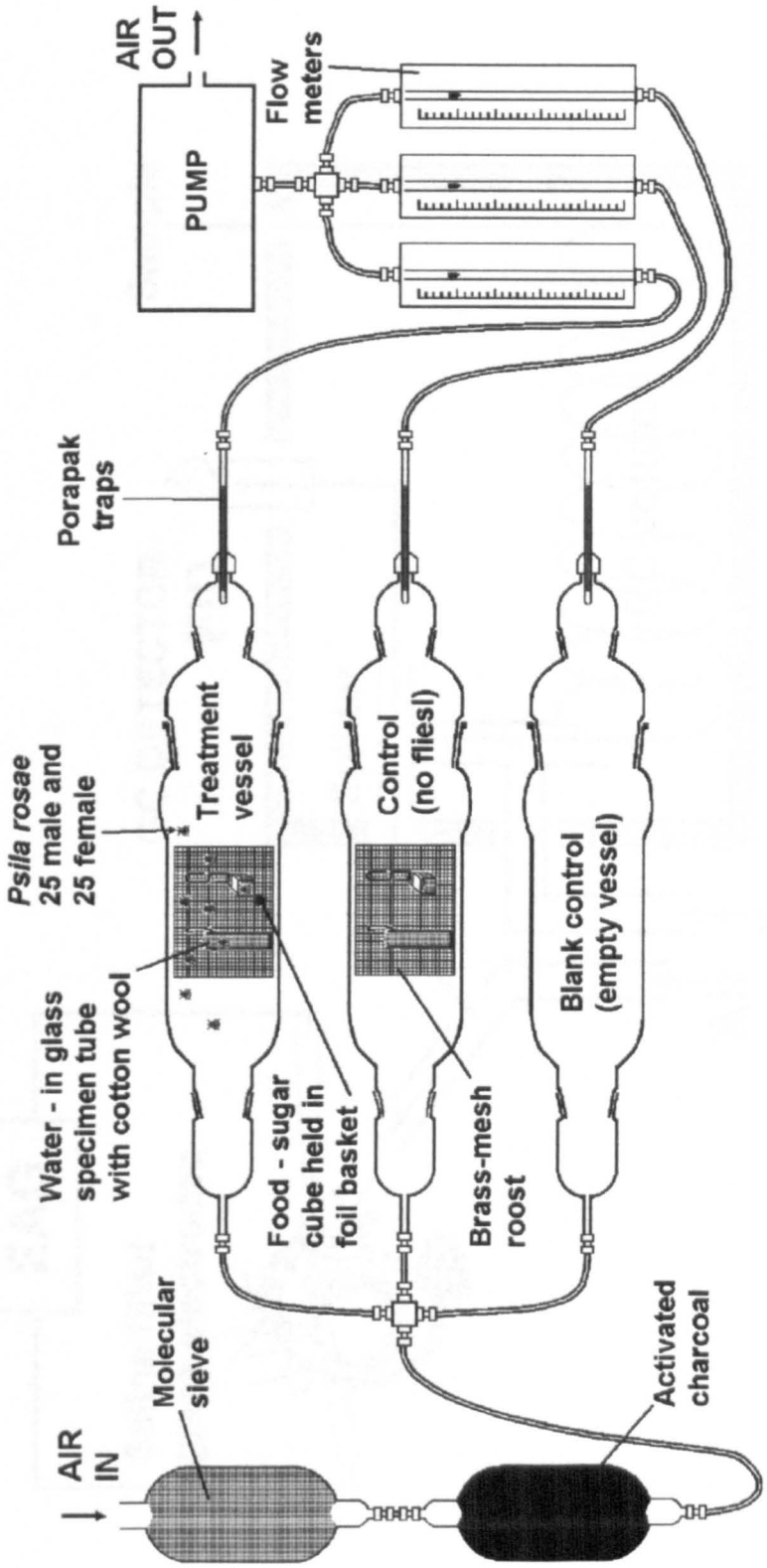
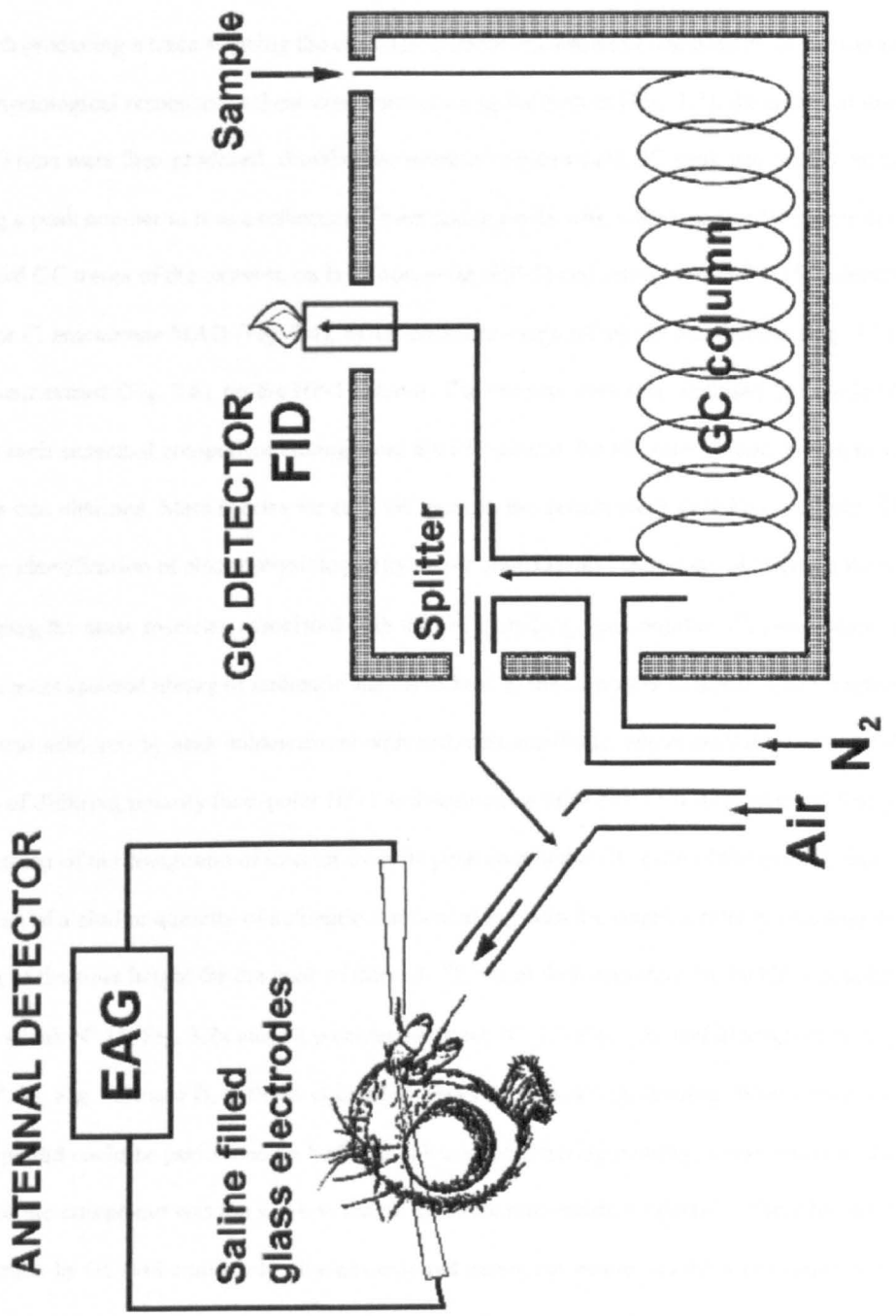


Figure 3.2 **Schematic representation of coupled GC-electrophysiological recording**



3.3 RESULTS

3.3.1 Biologically Active Compounds from Host Plant Species

The *C. maculatum* extracts from Ch.2 were first analysed by performing a number of coupled GC-EAG runs, each producing a trace showing the chromatographic separation of compounds on the top and the electrophysiological responses to these components along the bottom (Fig. 3.3). Summarised traces of the GC-EAG runs were then produced, showing the number of times each GC peak was seen to be active and ascribing a peak number to it as a reference. These active peaks with reference numbers were then located in standard GC traces of the extracts, on both non-polar (HP-1) and semi-polar (SPB-35) columns, as shown for *C. maculatum* MAD (Fig. 3.4), MAS extract re-extracted by air entrainment (Fig. 3.5) and air entrainment extract (Fig. 3.6), on the HP-1 column. The extracts were then analysed by coupled GC-MS, whereby each separated component eluting from the GC entered the MS inlet system, where its mass spectrum was obtained. Mass spectra for each GC peak in the extract were stored on computer file. Tentative identification of electrophysiologically active components (and peaks of interest) was achieved by matching the mass spectrum associated with the GC peak [e.g. peak number 17, γ-coniceine (Fig. 3.7)] against a mass spectral library of authentic standards held in the computer database. Confirmation of identity was achieved by peak enhancement with authentic standards, where available, on two GC columns of differing polarity (non-polar HP-1 and semi-polar SPB-35). This was achieved firstly by quantification of the compound of interest from its peak area in the GC trace of the extract, followed by coinjection of a similar quantity of authentic standard along with the original extract, resulting in a doubling of the peak height for the peak of interest. This is as demonstrated, on the HP-1 column, for myrcene (Peak N°.15, Fig. 3.8a and b), γ-coniceine (Peak N°. 17, Fig. 3.8c and d) and β-caryophyllene (Peak N°. 28, Fig. 3.8e and f), with the coinjected peaks emphasised by shading. Where peak enhancement of a compound could be performed on both GC columns of differing polarity, it was assumed that the identity of the compound was the same as that of the authentic standard injected. Where tentative identification by GC-MS could only give a compound name, but where a number of isomers existed for the compound (i.e. *cis* and *trans*), structures could sometimes be elucidated during the coinjection step if authentic standards were available for the different isomers. This was the case for the EAG-active peak

number 24, tentatively identified as limonene-1,2-epoxide and found to be the *trans*-isomer, (*E*)-limonene-1,2-epoxide, during coinjections. Finally a summary table was compiled, which showed the arbitrarily ascribed GC peak reference numbers, tentative compound identifications made by GC-MS analysis, which of these compound identifications had been confirmed by peak enhancement (on two GC columns of different polarity), and the number of times each peak was found to be active over a number of GC-EAG runs (with dots representing activity in one GC-EAG run) (see Table 3.1).

Where the quantities of active components in extracts were calculated (i.e. for *C. maculatum* extracts, Table 3.2), this was calculated from the individual peak area counts in the GC traces of extracts and the response factors for the individual compounds; response factors were calculated from coinjections of known quantities of each compound (100ng) with a known quantity of an internal standard (100ng, n-tridecane). Where the percentages of active compounds were calculated (i.e. for *C. maculatum* extracts, Table 3.3), this was done by calculating individual peak areas as a percentage of the total GC trace area, excluding areas in the solvent front (i.e. the first 7 minutes of the GC run).

Regrettably, there is a reversal in GC direction between the various analyses utilising this technique, with the GC trace of the GC-EAG analysis running in the reverse direction to all other methods.

Other host plant extracts

The same GC-EAG and identification procedures were performed for a wide range of other host plant extracts (generally MAD). These results are summarised with a sample GC-EAG trace for each plant extract, with the electrophysiologically active peaks ascribed reference numbers. This is followed by a table showing peak reference numbers, along with compound identifications, and dots representing the number of times each peak showed activity over a number of GC-EAG runs. The apiaceous extracts tested are presented in phylogenic order [following the general classification system of Drude (1897-98) and Heywood (1971)] and include: carrot (*Daucus carota* ssp. Carota) Fig. 3.9a, Table 3.4a.; wild carrot (*Daucus carota* ssp. *Carota*) Fig. 3.9b, Table 3.4b; celery (*Apium graveolens*) Fig. 3.9c, Table 3.4c; longleaf (*Falcaria vulgaris*) Fig. 3.9d, Table 3.4d; fennel (*Foeniculum vulgare*) Fig. 3.9e, Table 3.4e;

giant hogweed (*Heracleum montegazzianum*) Fig. 3.9f, Table 3.4f; hogweed (*Heracleum sphomdylium*) Fig. 3.9g, Table 3.4g; parsnip (*Pastinaca sativa*) Fig. 3.9h, Table 3.4h.

Host plants investigated only by GC-EAG

The following apiaceous extracts were investigated by GC-EAG, but compound identification by GC-MS was not performed due time limitations and the large number of electrophysiologically active compounds already identified in the earlier extracts. These results are summarised with a sample GC-EAG trace for each plant extract annotated with dots above the active peaks, the number of dots representing the number of times each peak showed electrophysiological activity over a number of GC-EAG runs. These extracts included: garden chervil (*Anthriscus cerefolium*) Fig. 3.9i; cow parsley (*Anthriscus sylvestris*) Fig. 3.9j; rough chervil (*Chaerophyllum temulentum*) Fig. 3.9k; parsley (*Petroselinum crispum*) Fig. 3.9l.

Chiral gas chromatographic analysis of host plant extracts

Selection of a suitable column and conditions for chiral GC analysis

In a preliminary experiment, the suitability of three chiral GC columns (α-, β-, and γ-cyclodextrin) for separating enantiomers of a test mix of three chiral compounds [(+)- and (-)-α-pinene, (+)- and (-)-limonene and (+)- and (-)-fenchone] was assessed. The results of this preliminary investigation are summarized in Table 3.5, which shows the GC-retention times of the six enantiomers when run on the three different GC-columns, under similar chromatographic conditions. On both the α- and γ-DEX 120 columns, the enantiomers of the three different compounds were not separated, and eluted as single compound peaks. The most suitable separation was obtained on the β-DEX 120 column, with a chiral cavity size corresponding to a seven glucose unit cyclodextrin ring. On this column, the enantiomers of all three compounds eluted as separate peaks (see Table 3.5), and as such the β-DEX 120 column, under these chromatographic conditions, was used for all subsequent chiral analyses.

Enantiomeric composition of chiral compounds in host plant extracts

Chiral analysis of the *C. maculatum* extract (MAD) revealed that both enantiomers of β-pinene are present, with 85-90% of the (+)-β-pinene and 10-15% of the (-)-β-pinene forms occurring. Analysis of the

fennel extract (MAD) revealed only (+)-fenchone with no (-)-fenchone occurring, whilst both enantiomers of limonene are present, in the ratios 75% (-)-limonene and 25% (+)-limonene. Analysis of the carrot extract (MAD) revealed only one enantiomer of linalool, (eluting first on this column), but its identity could not be determined as separate chiral standards were not available.

3.3.2 Biologically Active Compounds from Non-Host Plant Species (Repellent Intercrops)

The following non-apiaceous extracts were investigated by GC-EAG, but compound identification by GC-MS was not performed due time limitations and the low levels of electrophysiological activity in these non-host extracts. The results show the GC-EAG traces for *Allium cepa* Fig. 3.10a, and *Medicago litoralis* Fig. 3.10b, annotated with dots above the electrophysiologically active peaks, for these single GC-EAG runs.

3.3.3 Biologically Active Compounds from Air Entrainment of *P. rosae*

The air entrainment of *P. rosae* (mixed sex) was investigated by GC-EAG; however, compound identification by GC-MS could not be performed due to insufficient total material extracted in the AE sample. In order to compare the antennal responses of the two sexes to possible pheromone components in the air entrainment, GC-EAG analysis was performed using both male and female antennal preparations. The results are summarised with a sample GC-EAG trace from both a female (Fig. 3.11a) and a male (Fig. 3.11b) antennal preparation, with the electrophysiologically active peaks ascribed reference letters. This is followed by a table showing the number of times each peak showed activity, for each of the sexes, over a number of GC-EAG runs (Table 3.6).

Table 3.1: Summary of coupled GC-EAG activity found in *C. maculatum* extracts

The first column shows the arbitrarily ascribed GC peak reference number (as shown in the GC traces, Fig. 3.4 - 3.6). The second shows tentative compound identifications made by GC-MS analysis. The third shows which compound identifications were confirmed by peak enhancement (on two GC columns of different polarity). In the last two columns, electrophysiologically active peaks identified by GC-EAG experiments have been ascribed dots to represent the number of times they were found to be active over a number of GC-EAG runs, for two different types of extract.

Peak N°	Compound identification by mass spectra	Compound identities confirmed by GC peak enhancement	Type of extract and summary of GC-EAG activity found	
			Microwave-assisted distillation (n=3)	MAS extract re-extracted by air entrainment (n=8)
1	3-Hexanol	Yes		●●
2	2-Hexanol	Yes		●●
3	Octane	Yes		●●●●
4	(*E*)-2-Hexenal	Yes	●●	
5	1-Hexanol	Yes	●	
6				●●
7	Nonane	Yes		●●
8				●●
19				●●●●
10	4-Ethyl toluene	Yes		●●
11			●●●	●●●●●●●●
12			●●●	●●●●●●●●
13			●●	●●●●●●●●
14	β-Pinene	Yes		●●
15	Myrcene	Yes		
16	(*Z*)-3-Hexenyl acetate	Yes		
17	γ-Coniceine	Yes	●●●●	●●●●●●●●●●
18	(*Z*)-β-Ocimene	Yes		
19	(*E*)-β-Ocimene	Yes	●●●●	●●●●
20				●●●●
21				●●
22	Conhydrine			●●●●
23	Conhydrinone		●●	●●●●●●●●●●
24	(*E*)-Limonene-1,2-epoxide	Yes		●●
25			●	
26			●●	
27			●	
28	β-Caryophyllene	Yes	●●●●	
29	α-Humulene	Yes	●	
30			●	
31			●	

NB. A total of 4 GC-EAG runs were carried out on the microwave assisted distillation, and 10 on the microwave assisted solvent extract re-extracted by air entrainment.

Table 3.2: **Quantities of identified compounds in the extracts of *C. maculatum* produced by a wide range of extraction methods (per gram of fresh plant material).**

GC Peak N°	Compound	Microwave assisted distillation (µg)	Microwave assisted solvent extract (a) (µg)	Microwave assisted solvent extract (b) (µg)	Air entrainment **(ng/day)	Hydro-distillation (µg)	MAS extract (a) re-extracted by air entrainment (µg)	MAS extract (b) re-extracted by vacuum distillation (µg)
1	3-Hexenol	0.02	0.17	0.09	0.01	0.13	0.01	0.09
2	2-Hexenol	0.07	0.41	0.54	t	0.14	0.01	0.44
3	Octane	2.52	199.89	211.03	0.04	71.12	0.22	176.36
4	(*E*)-2-Hexenal	0.01	t	0.13	0.03	0.39	t	t
5	1-Hexanol	t	t	t	0.04	1.63	0.02	t
6								
7	Nonane	0.01	2.87	0.07	0.18	4583*	1.39	0.07
8								
9								
10	4-Ethyl toluene	0.02	0.01	0.02	0.10	0.81	0.01	0.02
11								
12								
13								
14	β-Pinene	0.04	0.04	0.09	2.72	1.56	0.04	0.05
15	Myrcene	3.12	1.08	0.53	21.54	51.29	0.50	0.46
16	(*Z*)-3-Hexenyl acetate	0.19	0.10	0.06	0.78	t	0.09	0.07
17	γ-Coniceine	4.73	19.89	45.50	2.88	3.79	0.24	21.92
18	(*Z*)-β-Ocimene	4.25	2.04	4.60	21.36	20.90	0.52	2.89
19	(*E*)-β-Ocimene	9.82	2.17	1.48	25.44	32.33	0.60	1.13
20								
21								
22	Conhydrine***	0.60	0.73	2.34	0.27	t	0.70	20.49
23	Conhydrinone***	1.93	0.25	0.71	0.17	0.46	1.71	4.09
24	(*E*)-Limonene-1,2-epoxide	0.02	0.08	0.08	0.07	t	0.01	0.07
25								
26								
27								
28	β-Caryophyllene	0.38	0.37	0.17	4.23	12.34	0.02	0.12
29	α-Humulene	0.04	0.06	0.11	0.53	2.31	t	0.02
30								
31								

NB. 't' - Trace defined as less than 0.1µg/g fresh weight of plant material, down to a visible peak on the GC base line (except in air entrainment).

'*' As nonane was used as a solvent in the water distillation, no extracted quantity could be calculated.

'**' Due to low recovery of compounds, quantity shown in nanograms for air entrainment (trace <0.01ng/g fresh weight/day)

'***' Quantity of conhydrine and conhydrinone, calculated from γ-coniceine response factor, as no authentic standards were available.

Table 3.3: **Percentage of identified compounds in *C. maculatum* extracts produced by a wide range of exraction methods**

Peak Nº	Compound	Microwave assisted distillation	Microwave assisted solvent extract (a)	Microwave assisted solvent extract (b)	Air entrainment	Hydro-distillation	MAS extract (a) re-extracted by AE	MAS extract (b) re-extracted by vacuum distillation
1	**3-Hexenol**	t	t	t	t	t	t	t
2	**2-Hexenol**	t	0.1	0.1	t	t	t	0.1
3	**Octane**	9.8	61.1	63.5	t	17.6	3.4	82.2
4	**(*E*)-2-Hexenal**	t	t	t	t	t	t	t
5	**1-Hexanol**	t	t	t	t	0.3	t	t
6		t	t	t	t	t	t	t
7	**Nonane**	t	0.9	t	t	*	20.8	t
8		t	t	t	t	t	t	t
9		t	t	t	t	t	t	t
10	**4-Ethyl toluene**	t	t	t	t	0.2	t	t
11		t	t	t	t	0.4	t	t
12		t	t	t	t	0.3	t	t
13		t	t	t	0.4	0.2	0.2	t
14	**β-Pinene**	t	t	t	3.1	0.4	0.7	t
15	**Myrcene**	12.5	0.3	0.2	23.2	13.1	8.1	0.2
16	**(*Z*)-3-Hexenyl acetate**	0.6	t	t	0.7	t	1.1	t
17	**γ-Coniceine**	11.7	3.9	8.7	1.9	0.6	2.3	6.5
18	**(*Z*)-β-Ocimene**	16.2	0.6	1.4	21.9	5.1	8.0	1.3
19	**(*E*)-β-Ocimene**	37.4	0.7	0.4	26.1	7.9	9.3	0.5
20		t	t	t	t	-	t	t
21		t	t	t	t	-	0.2	t
22	**Conhydrine**	1.5	0.1	0.45	t	t	7.0	6.1
23	**Conhydrinone**	4.8	t	0.13	t	t	17.0	1.2
24	**(*E*)-Limonene-1,2-epoxide**	t	t	t	t	t	t	t
25		t	t	t	t	-	t	t
26		t	t	t	t	-	t	t
27		t	t	t	t	-	t	t
28	**β-Caryophyllene**	1.5	0.1	t	4.5	3.1	0.3	t
29	**α-Humulene**	t	t	t	t	t	t	t
30		2.5	t	t	3.8	7.1	t	t
31		t	t	t	0.3	0.7	t	t

NB. 't' - Trace defined as less than 0.1% of the total area counts in a GC run, down to a visible peak on the GC base line.

' - ' No visible peak on the GC base line.

'*' Nonane was used as solvent in this extract, and was not included in the calculations of percentages.

Table 3.4: Summaries of coupled GC-EAG activities found in host plant extracts

The first column shows the arbitrarily ascribed GC peak reference number (as shown in the coupled GC-EAG traces, Fig. 3.9). The second shows tentative compound identifications made by GC-MS analysis. The third shows which compound identifications were confirmed by peak enhancement (on two GC columns of different polarity). In the last column, electrophysiologically active peaks identified by GC-EAG experiments have been ascribed dots to represent the number of times they were found to be active over a number of GC-EAG runs.

a) Carrot (*Daucus carota* ssp. *carota* L.) - MAD foliage extract

GC Peak N²	Compound identification by mass spectra	Compound identities confirmed by GC peak enhancement	Summary of GC-EAG activity in extract (n=5)
3	Octane	Yes	●●●
32			●●
7	Nonane	Yes	●●●●
33	2,3,4-Trimethyl heptane		●●
34	1-Octen-3-ol	Yes	●●●●●
35	Sabinene	Yes	●●●●●
15	Myrcene	Yes	●
36	Terpinolene	Yes	●●●
37	Linalool	Yes	●●●●
38	α-Terpinene	Yes	●●
39			●●
40	Methyl salicylate	Yes	●●
41	α-Terpineol	Yes	●●●●
42			●●
43			●●
44			●●
28	β-Caryophyllene	Yes	●●●●●
29	α-Humulene	Yes	●●●●
45	*trans*-Methyl isoeugenol	Yes	●●●●
46	(*E*,*E*)-α-Farnesene		●●●
47			●

b) Wild carrot (*Daucus carota* ssp. *carota* L.) - MAD foliage extract

GC Peak N²	Compound identification by mass spectra	Compound identities confirmed by GC peak enhancement	Summary of GC-EAG activity in extract (n=1)
125			●
126			●
127			●
36	α-Terpinolene	Yes	●
128			●
129			●
130	Isoeugenol	*	●
28	β-Caryophyllene	Yes	●
131			●
132			●
133			●
134			●
135			●

'*' Tentative confirmation by GC retention time only.

Table 3.4: Summaries of coupled GC-EAG activities found in host plant extracts (continued)

c) Celery (*Apium graveolens* L.) - MAD foliage extract

GC Peak N°	Compound identification by mass spectra	Compound identities confirmed by GC peak enhancement	Summary of GC-EAG activity in extract (n=2)
107			●
108			●
3	Octane	Yes	●●
109			●
4	(*E*)-2-Hexenal	Yes	●●
110			●●
49	Limonene	Yes	●●
111	Carvyl acetate	Yes	●
112	Copaene	Yes	●
28	β-Caryophyllene	Yes	●
113	*trans*-Asarone	Yes	●●

d) Long-leaf (*Falcaria vulgaris* Bernh.) - MAD foliage extract

GC Peak N°	Compound identification by mass spectra	Compound identities confirmed by GC peak enhancement	Summary of GC-EAG activity in extract (n=4)
54			●
55			●
56	Benzaldehyde		●
57	α-Pinene	Yes	●
58			●●●
35	Sabinene	Yes	●●●●
14	β-Pinene	Yes	●●●
59			●●
60			●
61			●●
62			●
41	α-Terpineol	Yes	●●
63			●
64			●●●●
65			●
28	β-Caryophyllene	Yes	●●●
29	α-Humulene	Yes	●
66	Germacrene-D	*	●●●●
67			●
68			●●

'*' Compound unavailable for coinjection, but MS and GC retention times were confirmed from previous authentic standard.

Table 3.4: Summaries of coupled GC-EAG activities found in host plant extracts (continued)

e) Fennel (*Foeniculum vulgare* Mill.) - MAS extract re-extracted by air entrainment

GC Peak Nº	Compound identification by mass spectra	Compound identities confirmed by GC peak enhancement	Summary of GC-EAG activity in extract (n=2)
3	Octane	Yes	●●
48			●●
49	Limonene	Yes	●●
50	Fenchone	Yes	●●
51	4-Allylanisole (estragole)	Yes	●●
52	*trans*-Anethole	Yes	●●
53			●●
45	*trans*-Methyl isoeugenol	Yes	●●

f) Giant-hogweed (*Heracleum mantegazzianum* Somm. & Lev.) - Seed oil (English Hops Ltd.)

GC Peak Nº	Compound identification by mass spectra	Compound identities confirmed by GC peak enhancement	Summary of GC-EAG activity in extract (n=5)
82			●
83			●
84			●●●
85			●
86			●
87			●
88			●
89			●
90			●●●
91			●●●●
92			●●
93			●
94			●●
95	Octyl acetate	*	●●●●●
96	Hexyl butanoate	*	●●●●●
97			●●
98			●
99			●
100			●
101			●
102			●
103			●
104			●●
105			●
106			●●

'*' Tentative confirmation by GC retention time only.

Table 3.4: Summaries of coupled GC-EAG activities found in host plant extracts (continued)

g) Hogweed (*Heracleum sphondylium* L.) - MAD foliage extract

GC Peak N²	Compound identification by mass spectra	Compound identities confirmed by GC peak enhancement	Summary of GC-EAG activity in extract (*n*=5)
69			●●
3	**Octane**	**Yes**	●●●●●
70			●
71			●●●
72			●●●●●
35	**Sabinene**	**Yes**	●
16	**(*Z*)-3-Hexenyl acetate**	**Yes**	●●●
73			●
19	**(*E*)-β-Ocimene**	**Yes**	●●
74			●
37	**Linalool**	**Yes**	●●
75			●
76			●
77			●●
78			●
79			●
80			●●
81			●●

h) Parsnip (*Pastinaca sativa* ssp. *sativa* L.) - MAD foliage extract

GC Peak N²	Compound identification by mass spectra	Compound identities confirmed by GC peak enhancement	Summary of GC-EAG activity in extract (*n*=2)
114			●
3	**Octane**	**Yes**	●
4	**(*E*)-2-Hexenal**	**Yes**	●●
115	**(*Z*)-3-Hexen-1-ol**	**Yes**	●●
116			●
117			●
34	**1-Octen-3-ol**	**Yes**	●●
36	**α-Terpinolene**	**Yes**	●
118			●
119			●
120			●
121			●
122			●
28	**β-Caryophyllene**	**Yes**	●●
123			●●
124			●

Table 3.5: Retention times of a test mixture of enantiomers (of three typical host plant compounds) run on three different chiral GC columns

Compound	Retention time (min)		
	α-DEX 120 column	β-DEX 120 column	γ-DEX 120 column
(-)-α-Pinene	6.51 *	11.23	10.84 *
(+)-α-Pinene	6.51 *	11.39	10.84 *
(-)-Limonene	6.90 *	13.67	15.10 *
(+)-Limonene	6.90 *	13.79	15.10 *
(-)-Fenchone	7.37 *	17.39	22.56 *
(+)-Fenchone	7.37 *	17.44	22.56 *

'*' = No separation

Table 3.6: Summary of coupled GC-EAG activity found in an air entrainment extract of mixed sex *P. rosae*

The first column shows the arbitrarily ascribed GC peak reference letter (as shown in the coupled GC-EAG traces, Fig. 3.11). In the next two columns, electrophysiologically active peaks identified by GC-EAG experiments have been ascribed dots to represent the number of times they were found to be active over a number of GC-EAG runs, using female and male antennal preparations respectively. Three GC-EAG runs were performed using a female antennal preparation, and two were performed using a male preparation; the air entrainment sample was 10x more concentrated for these male runs.

Peak N²	Female GC-EAG responses (*n*=3)	Male GC-EAG responses (*n*=2)
a	●●●	●●
b	●●	●●
c	●●●	●●
d	●●●	●●
e	●●	●
f	●●	●
g	●●	●
h	●●	
i	●●●	●
j	●●	●
k	●●	●
l	●●	
m	●	
n		●
o		●

Figure 3.3: ***P. rosae*** **coupled GC-EAG: (a) gas chromatogram of** ***C. maculatum*** **extract (MAD), and (b) corresponding EAG response.**

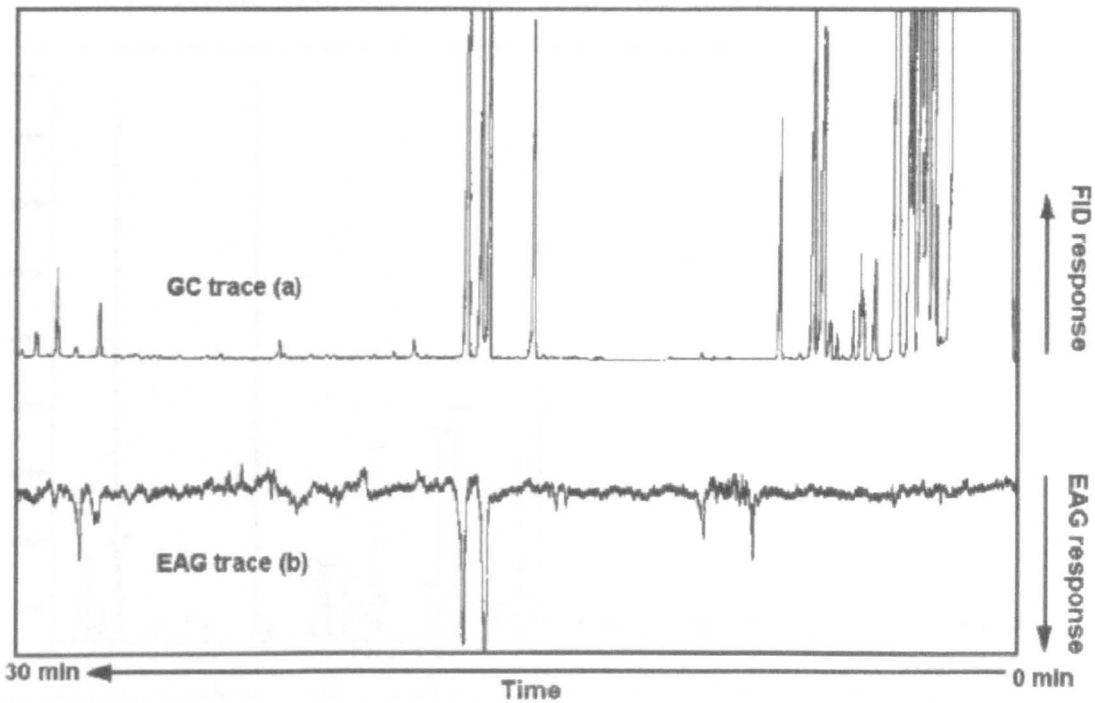


Figure 3.4: **Annotated GC trace (HP-1) of** ***C. maculatum*** **MAD extract, showing electrophysiologically active compounds (and major peaks of interest).**

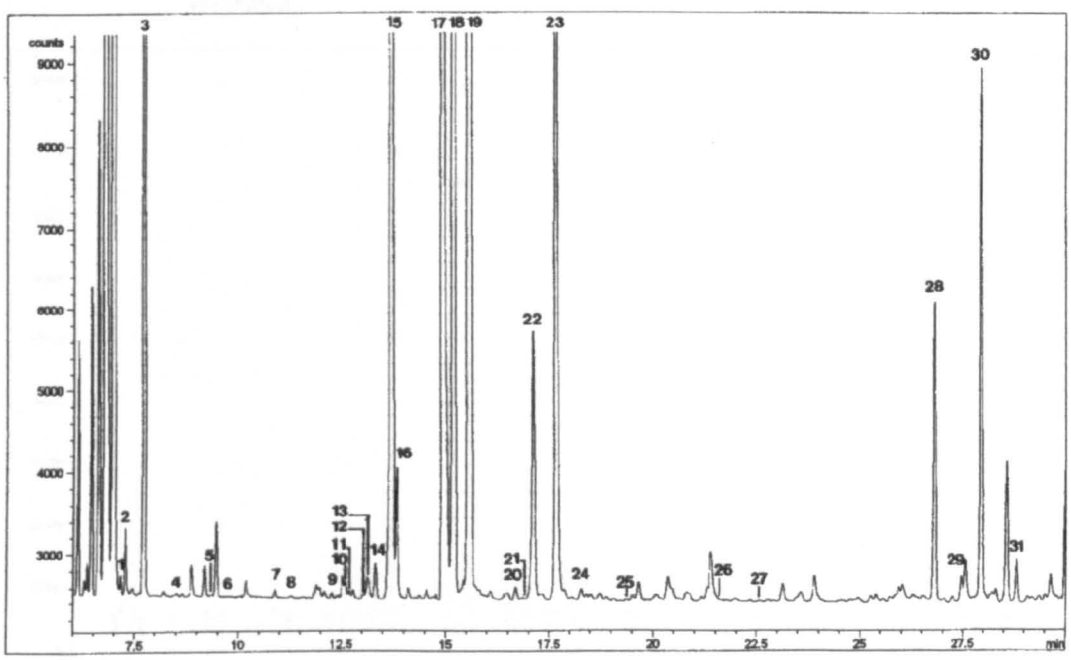


Figure 3.5: **Annotated GC trace (HP-1) of *C. maculatum* MAS extract re-extracted by air entrainment, showing electrophysiologically active compounds (and major peaks of interest) which were identified.**

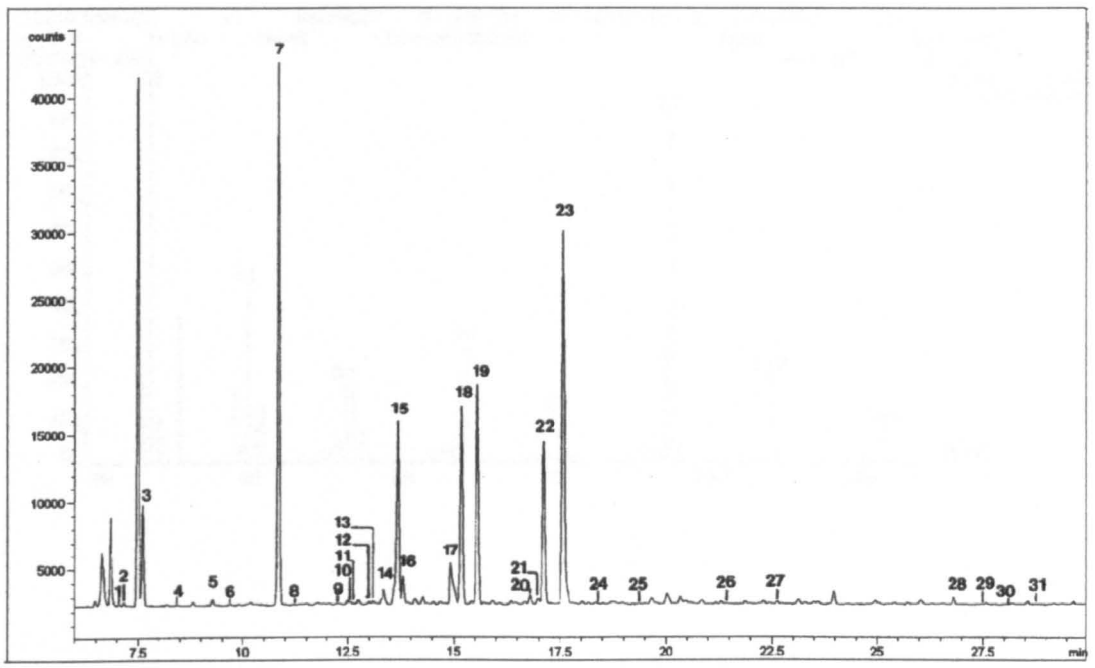


Figure 3.6: **Annotated GC trace (HP-1) of *C. maculatum* foliage air entrainment, showing electrophysiologically active compounds (and major peaks of interest) which were identified.**

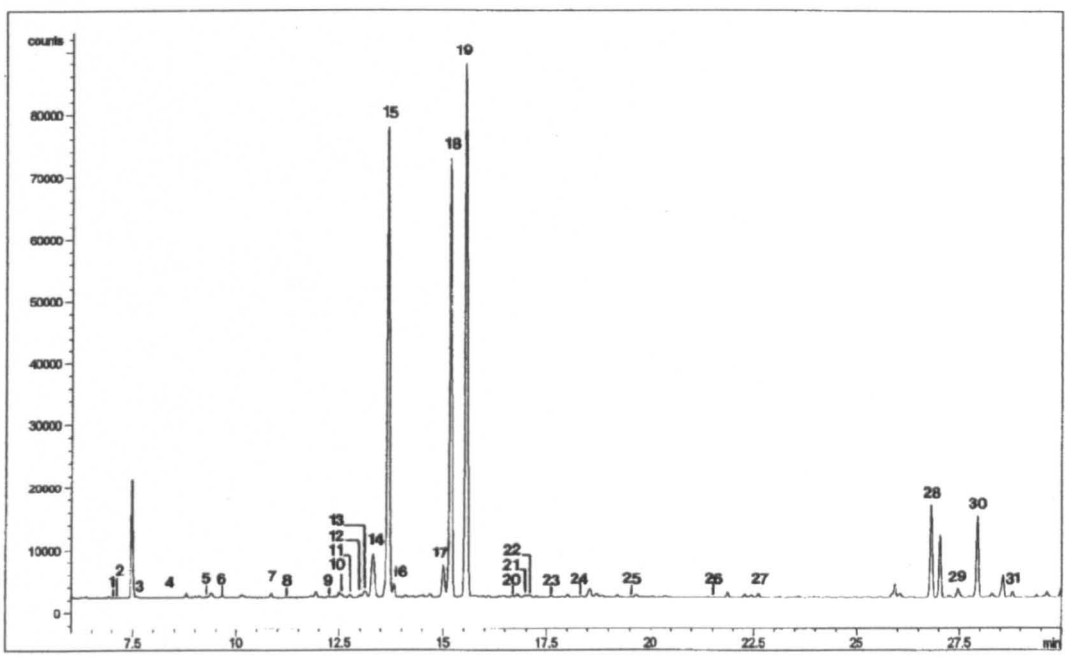


Figure 3.7: **Mass spectrum for EAG active peak number 17, tentatively identified as γ-coniceine.**

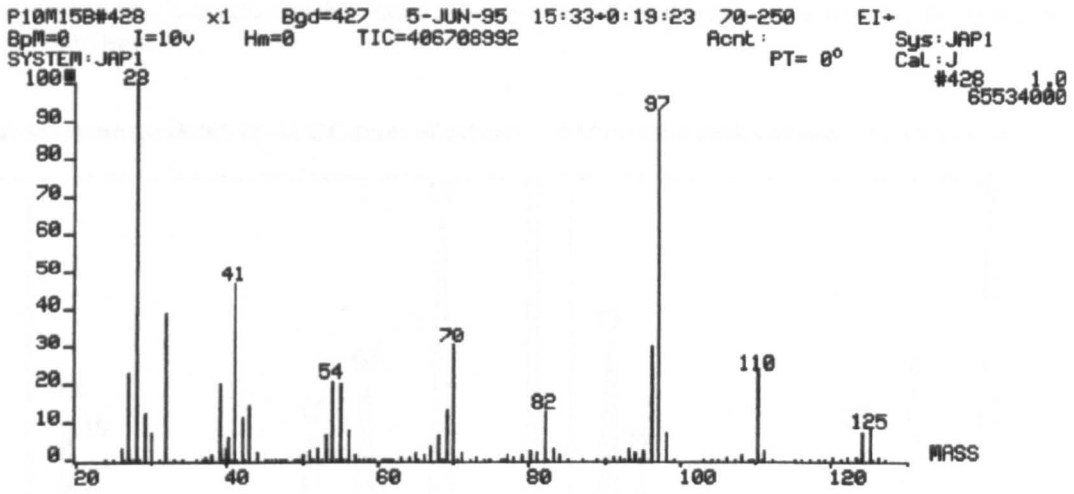
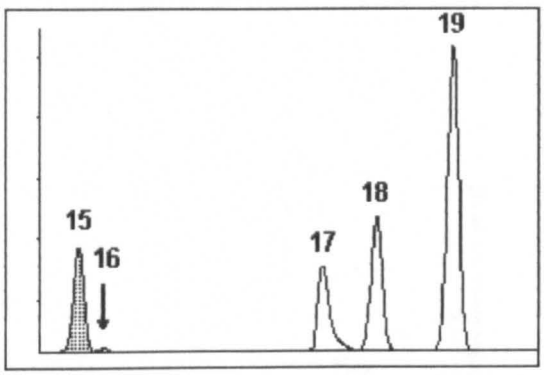


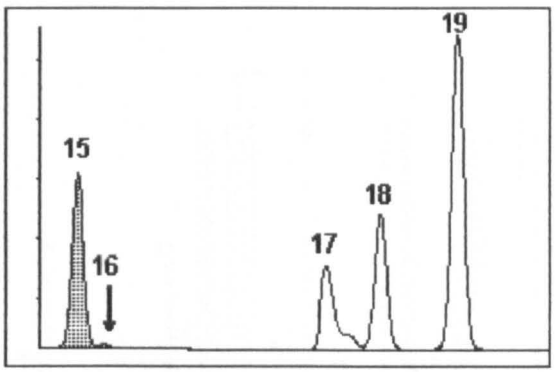
Figure 3.8: Sample confirmations of compound identities by GC peak enhancement.

Expansions of parts of the GC trace (HP-1) of *C. maculatum* MAD extract, showing confirmation of compound identity by peak enhancement. Annotated compound peaks of interest are shaded, and shown before and after coinjection with an equal quantity of authentic standard (for myrcene, γ-coniceine and β-caryophyllene).

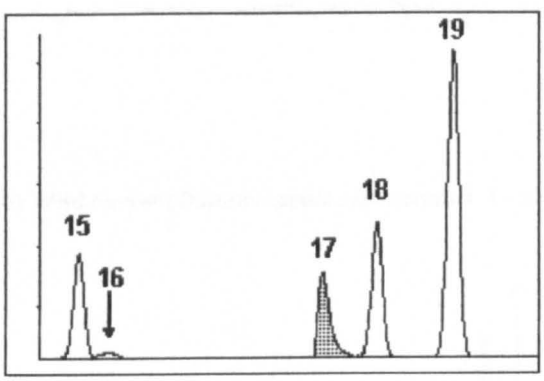
a) Myrcene (peak N°. 15) in GC trace of extract



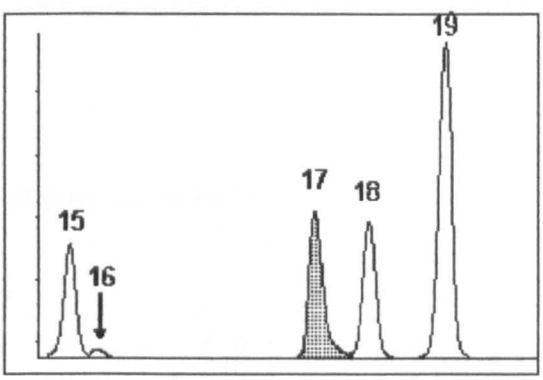
b) Myrcene peak enhanced by coinjection



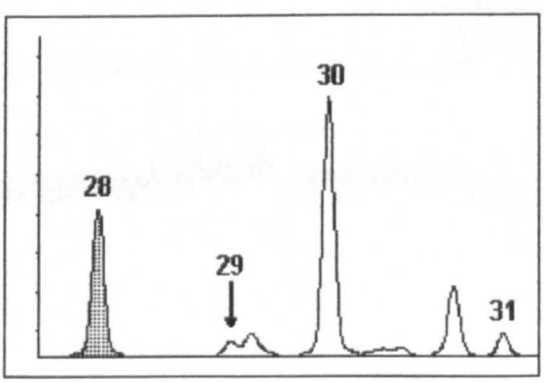
c) γ-Coniceine (peak 17) in GC trace of extract



d) γ-Coniceine peak enhanced by coinjection



e) β-Caryophyllene (Peak 28) in trace of extract



f) β-Caryophyllene peak enhanced by coinjection

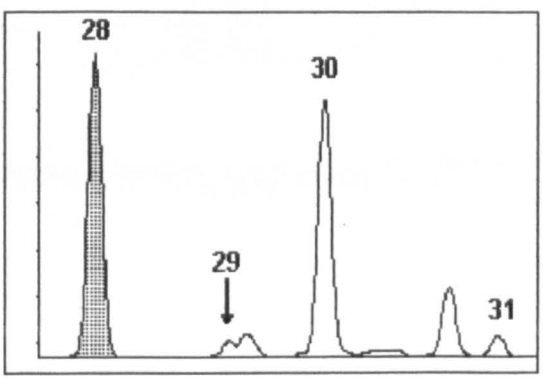
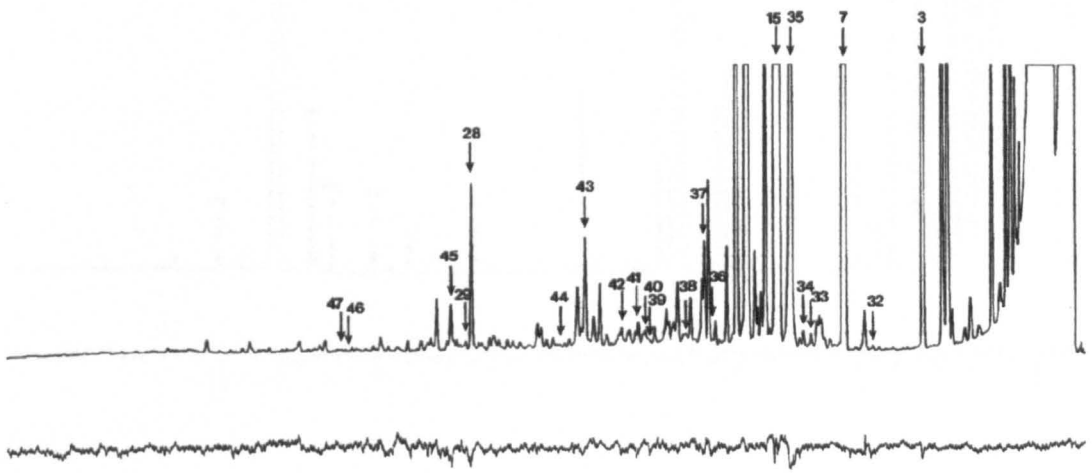


Figure 3.9: ***P. rosae*** **coupled GC-EAG traces for a range of host plant extracts**

Sample annotated GC-EAG traces: upper trace shows the gas chromatogram of the host-plant extract; lower trace shows corresponding EAG response of *P. rosae* antenna. Electrophysiologically active peaks have been ascribed peak reference numbers; the number of times each peak was found to be active over a number of GC-EAG runs is shown in Table 3.4.

a) Carrot (*Daucus carota* ssp.*carota* L.) - MAD foliage extract (n=5)



b) Wild carrot (*Daucus carota* ssp. *carota* L.) - MAD foliage extract (n=1)

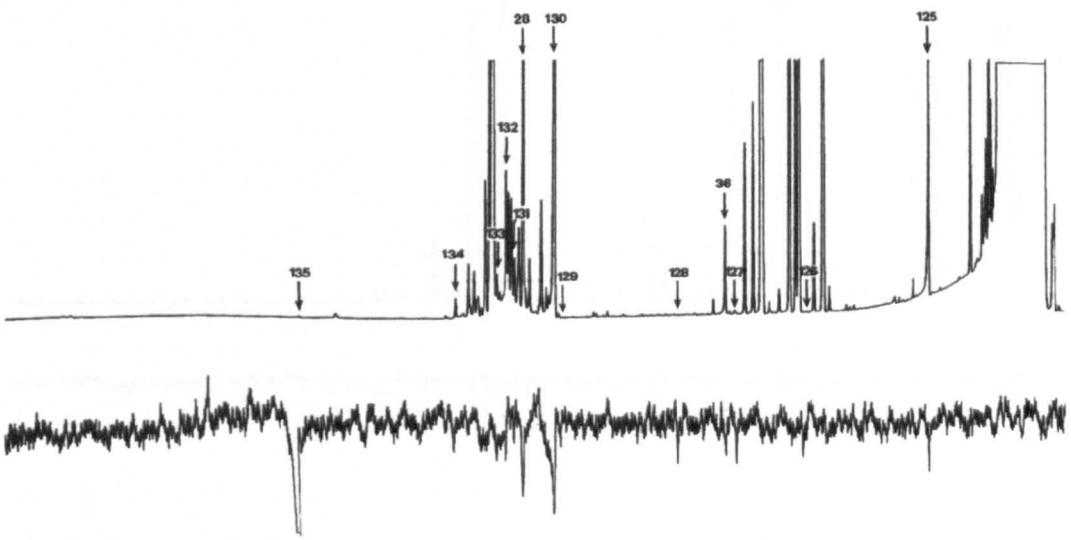
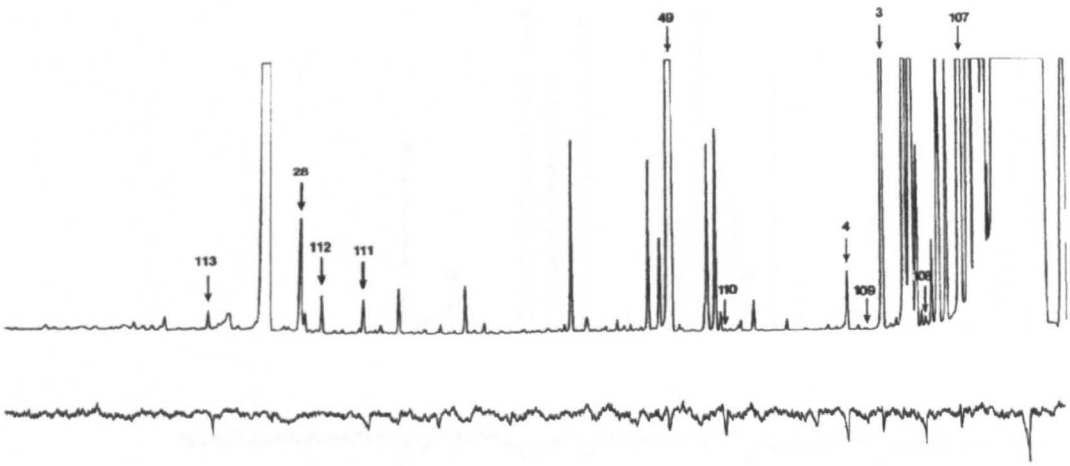


Figure 3.9: ***P. rosae*** **coupled GC-EAG traces for a range of host plant extracts (continued)**

c) Celery (*Apium graveolens* L.) - MAD foliage extract (n=2)



d) Longleaf (*Falcaria vulgaris* Bernh.) - MAD foliage extract (n=4)

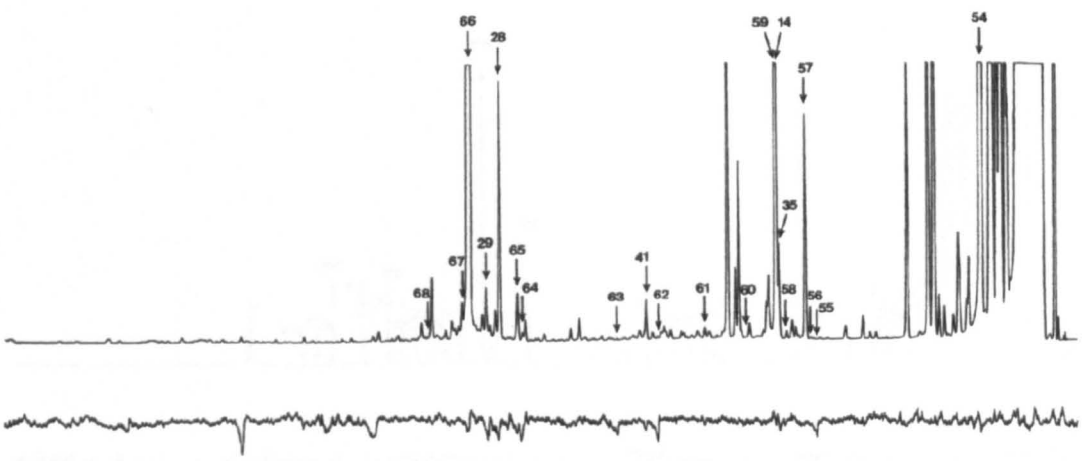
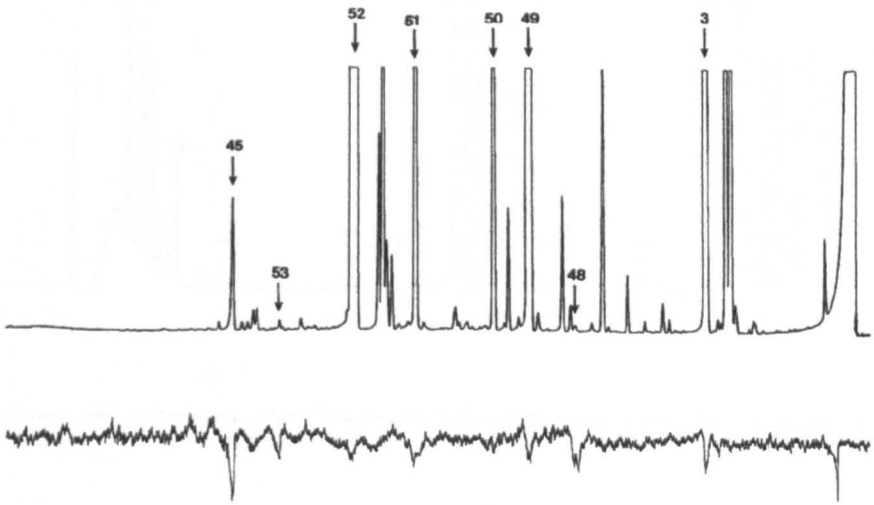


Figure 3.9: ***P. rosae*** **coupled GC-EAG traces for a range of host plant extracts (continued)**

e) Fennel (*Foeniculum vulgare* Mill.) - MAS extract re-extracted by air entrainment (n=2)



f) Giant hogweed (*Heracleum montegazzianum* Somm. & Lev.) - Seed extract (English Hops Ltd.) (n=5)

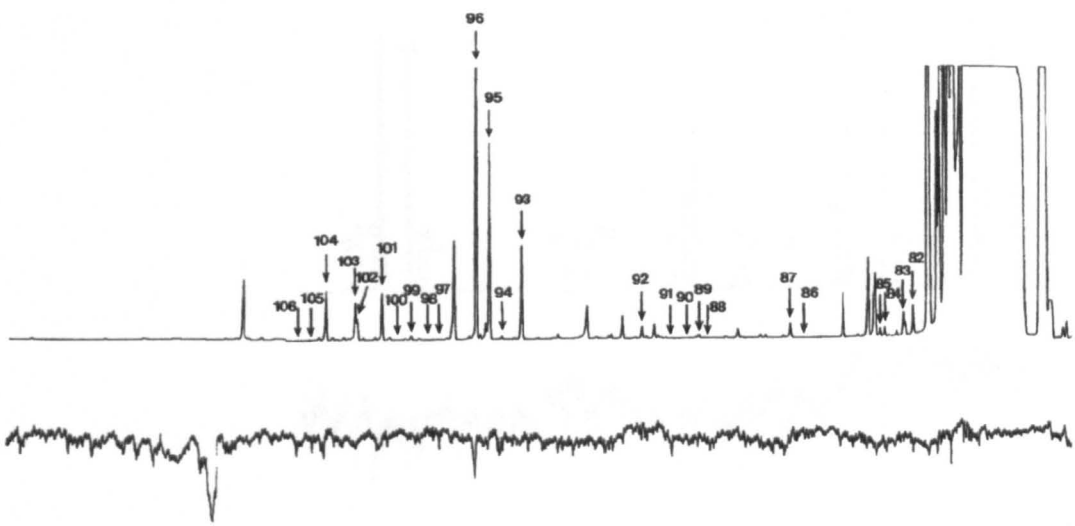
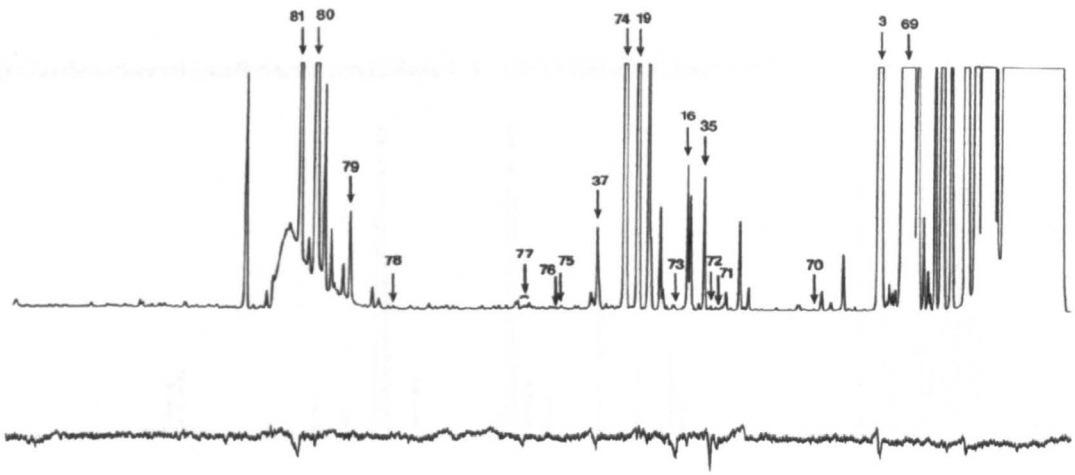


Figure 3.9: ***P. rosae*** **coupled GC-EAG traces for a range of host plant extracts (continued)**

g) Hogweed (*Heracleum sphondylium* L.) - MAD foliage extract (n=5)



h) Parsnip (*Pastinaca sativa* ssp. *sativa* L.) - MAD foliage extract (n=2)

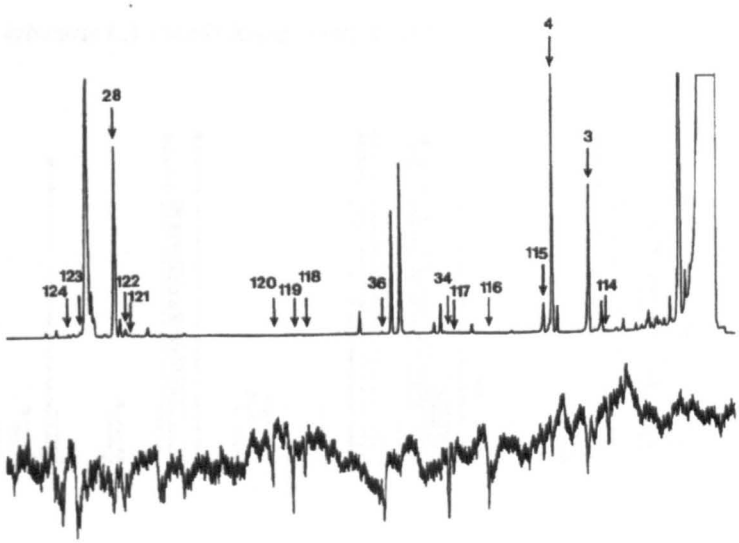
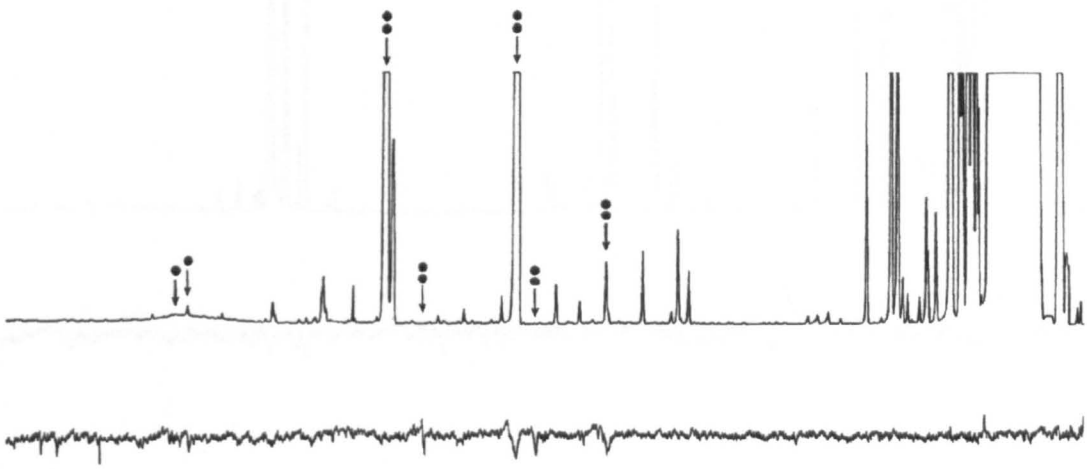


Figure 3.9: ***P. rosae*** **coupled GC-EAG traces for a range of host plant extracts (continued)**

Sample annotated GC-EAG traces: upper trace shows the gas chromatogram of the host-plant extract; lower trace shows corresponding EAG response of *P. rosae* antenna. Electrophysiologically active peaks have been ascribed dots to represent the number of times each peak was found to be active over a number of GC-EAG runs.

i) Garden chervil (*Anthriscus cerefolium* L.) - MAD foliage extract (n=2)



j) Cow parsley (*Anthriscus sylvestris* L.) - MAD foliage extract (n=3)

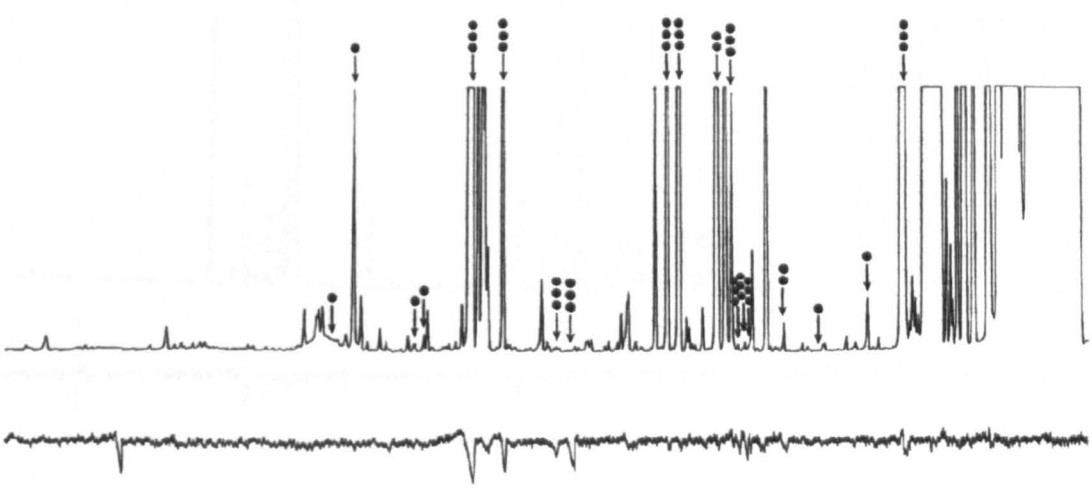
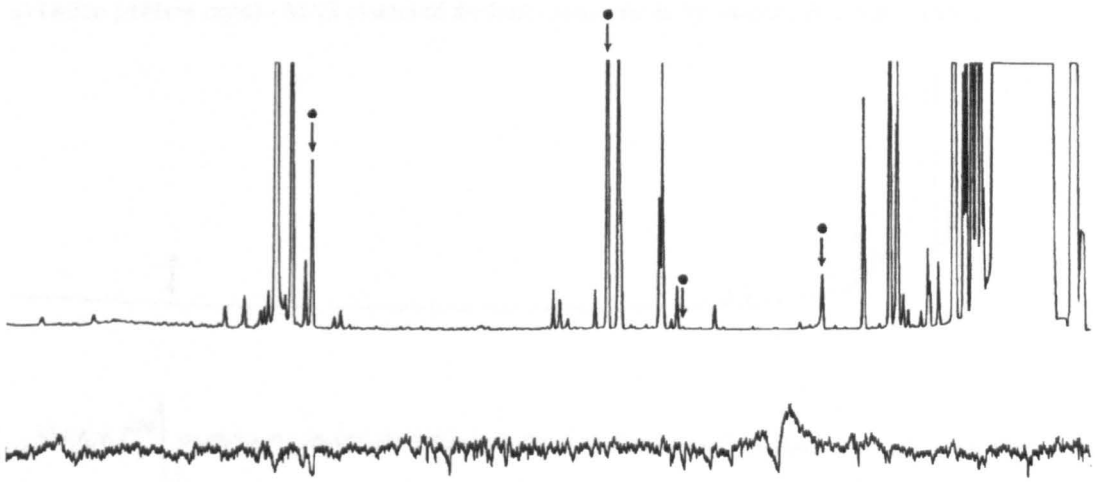


Figure 3.9: ***P. rosae*** **coupled GC-EAG traces for a range of host plant extracts (continued)**

k) Rough chervil (*Chaerophyllum temulentum* L.) - MAD foliage extract (n=1)



l) Parsley (*Petroselinum crispum* Mill.) - MAD foliage extract (n=3)

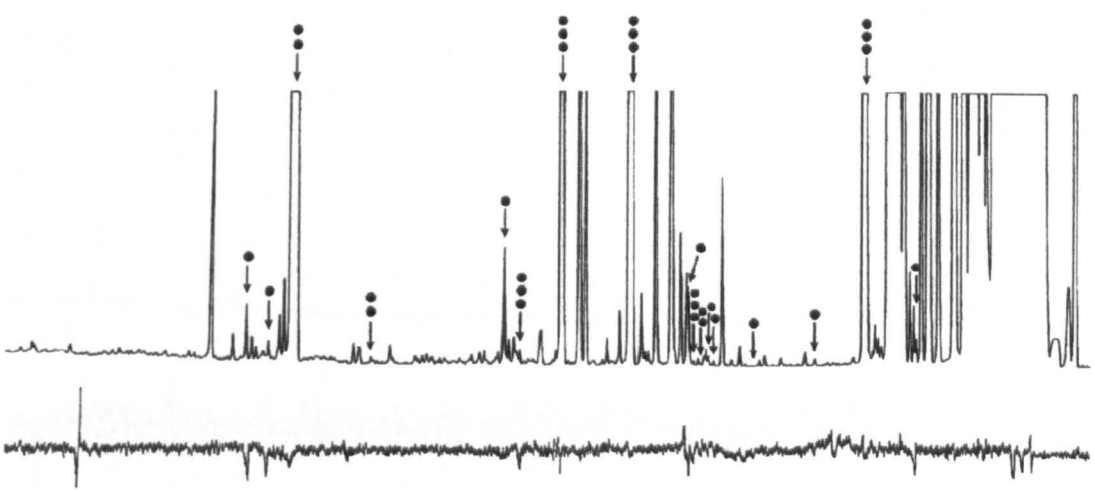
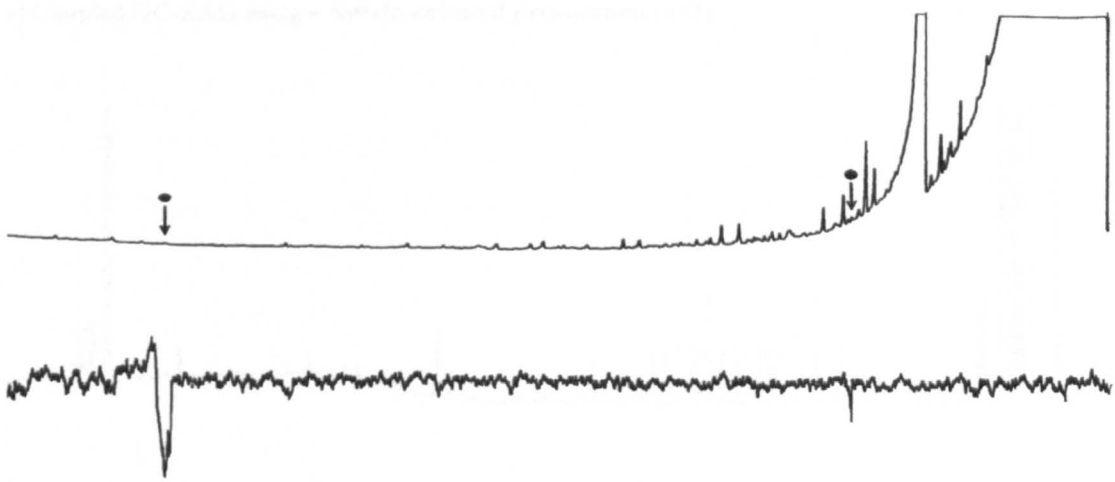


Figure 3.10: ***P. rosae*** **coupled GC-EAG traces for two non-host plant extracts**

Annotated GC-EAG traces: upper trace shows the gas chromatogram of the non-host plant extract; lower trace shows corresponding EAG response of *P. rosae* antenna. Electrophysiologically active peaks have been ascribed dots representing the electrophysiologically active peaks in these single GC-EAG runs.

a) Onion (*Allium cepa*) - MAS extract of the bulb, re-extracted by vacuum distillation (n=1)



b) *Medicago litoralis* - MAD foliage extract (n=1)

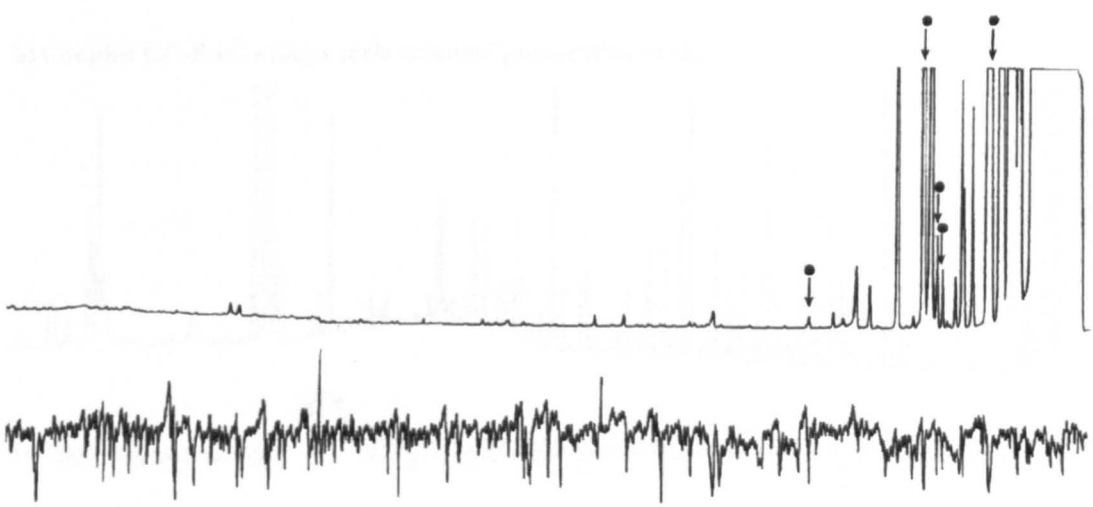
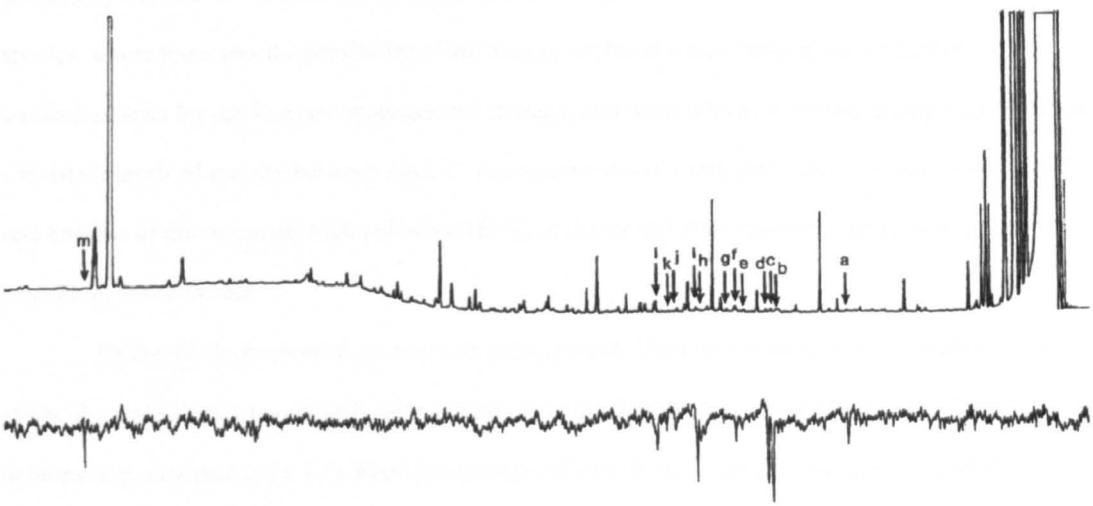


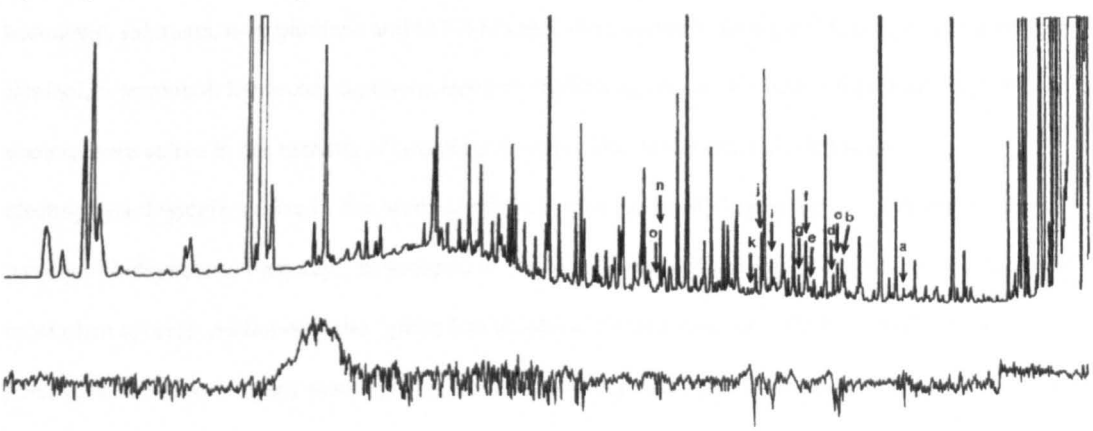
Figure 3.11: ***P. rosae*** **coupled GC-EAG traces for an air entrainment extract of mixed sex *P. rosae***

Sample annotated GC-EAG traces: upper trace shows the gas chromatogram of an air entrainment of 25 male and 25 female *P. rosae*; lower trace shows corresponding EAG response of *P. rosae* antenna. Electrophysiologically active peaks have been ascribed reference letters, and the number of times each peak was found to be active over a number of GC-EAG runs is shown in Table 3.6.

a) Coupled GC-EAG using a female antennal preparation (n=3)



b) Coupled GC-EAG using a male antennal preparation (n=2)



3.4 DISCUSSION

3.4.1 Biologically Active Compounds From Host Plant Species

The GC-EAG study of 13 apiaceous host plant species revealed a large number of electrophysiologically active peaks in the gas chromatograms of the extracts. Although some of this activity corresponded with responses to major components in the extracts, by far the majority of the activity was elicited by minor components. As so many areas of activity were evident, identification of the active components was necessarily limited. Consequently, GC-MS identification work was focussed upon the common crop species, where there was the possibility of utilising agricultural waste material for extraction of semiochemicals for use in a pest management strategy, and upon wild host species giving significant and consistent levels of activity between runs. *C. maculatum* was of particular interest as a preferred wild host, and because of the unusually high (off-scale) EAG response and slow recovery rate to peak 17 (γ-coniceine) in the extract.

Of the 42 electrophysiologically active compounds identified or tentatively identified in this study, the majority have previously been identified, in the literature, as present in the volatiles of apiaceous species (see table 3.7). Eight have not previously been reported; these were identified as 2-hexanol, 3-hexanol, 1-octen-3-ol, (*E*)-limonene-1,2-epoxide and 4-ethyltoluene, and tentatively identified as carvyl acetate, isoeugenol and 2,3,4-trimethylheptane. Of the nine species of Apiaceae where electrophysiologically active peaks were identified, β-caryophyllene and octane were active in six, α-humulene, sabinene, α-terpinolene and (*E*)-2-hexenal were active in three, and myrcene, (*E*)-β-ocimene, linalool, α-terpineol, limonene, β-pinene, *trans*-methylisoeugenol, (*Z*)-3-hexenyl acetate, 1-octen-3-ol and nonane were active in the extracts of two plant species. The compounds identified as electrophysiologically active in this study are from a wide range of chemical types, originating from a variety of biosynthetic pathways, as grouped in Table 3.7. Many of these compounds are ubiquitous to most plant species, particularly the 'green leaf volatiles' (Visser and Ave, 1978) and (*Z*)-3-hexenyl acetate, or common to many plant species, as is the case for many of the terpenoids (Schoonhoven *et al*, 1998). Others such as the methoxyphenyls are relatively specific to the Apiaceae, occurring in a number of its species, especially *trans*-asarone (although not exclusively as it also occurs in *Acorus calamus*) and

trans-methylisoeugenol. The piperidine alkaloids γ-coniceine, and the tentatively identified conhydrine and conhydrinone, are present only in one species of the Apiaceae, *C. maculatum*. As many of the compounds identified are generally occurring plant compounds, it seems likely that host location by *P. rosae* is accomplished by means of olfactory senses tuned to both specific qualities of the 'green odour' complex, as well as more host specific volatiles, as has previously been suggested in the literature (Guerin and Visser, 1980).

Two unlikely compounds, octane and nonane, were identified as electrophysiologically active in a number of extracts during this study. Both of these alkanes were initially thought to be of little consequence, as they were also found to be contaminants originating in the solvent (distol hexane, Fisher Scientific) and were concentrated during the excess solvent removal steps. However, they may in fact be implicated in the host location of *P. rosae*, as both these compounds, along with the other alkanes (hexane, decane, undecane and tridecane), have been identified as present in the extracts of a number of apiaceous species (Borg-Karlson, 1994; MacLeod, 1989; Potter and Fagerson, 1990; and Kasting *et al*, 1972). With these alkanes appearing so frequently in the volatiles of apiaceous species, despite differences in extraction methods, it seems unlikely that they could all be the result of accidental (or solvent) contaminations. Although γ-coniceine and the two other tentatively identified piperidine alkaloids (conhydrine and conhydrinone) were seen to elicit electrophysiological responses, it is possible that these responses could have resulted from a toxic physiological effect of these compounds on the olfactory receptors of the antenna. This could explain the extremely high electrophysiological response to γ-coniceine, which is the most potent toxin of the piperidine alkaloids identified in *C. maculatum*. This however may not be the case, as *P. rosae* frequently locate *C. maculatum* which is utilised as a preferred wild host species; consequently further investigations were performed to determine if γ-coniceine was biologically active for *P. rosae*. It was also noticed that, although both (*Z*) and (*E*) isomers of β-ocimene were present as major peaks in extracts of *C. maculatum* and *H. sphondylium*, only (*E*)-β-ocimene was identified as electrophysiologically active. Myrcene was also identified as a major peak in a number of extracts (i.e. *C. maculatum*), but was only rarely identified as electrophysiologically active (i.e. from *D. carota*).

One compound which was noticeable by its absence from the list of electrophysiologically active compounds was hexanal. This compound has previously been identified as highly active in EAG work by Guerin and Visser (1980). Hexanal may not have been identified as active in this study because of its low concentration in the extracts, or because of its earlier retention time than the other compounds identified. This would place it eluting further into the solvent front, where some electrophysiological activity was seen but no MS identifications were possible due to the large numbers of peaks co-eluting in this region of the GC trace. In order to increase the efficiency of identifying early retention time components such as hexanal, it would be advantageous to supplement the main extraction method (MAD) with a thermal desorption technique, which could obviate the use of solvent, and facilitate GC analyses. This could be either a solid phase microextraction (SPME) or an air entrainment technique, preferably with a short entrainment period to collect a higher proportion of the most volatile compounds. This method could also be useful in confirming the presence of alkane components in the volatiles of apiaceous plants, as solvent contaminants could be ruled out. The main disadvantages of using these supplementary thermal desorption techniques are the possibility of heat induced artefact production, the small quantity of material extracted, and the fact that each thermal desorption into the GC injector uses the entire sample. Numerous similar extracts would therefore be required in order to complete each step of the identification process.

Whilst undertaking electrophysiological studies of this kind, it should be kept in mind that the EAG response is a summation of potentials from a large number of chemoreceptors (Schneider, 1957; and De Kramer and Hemberger, 1987). It is normally useful for detecting responses to the most important behaviourally active compounds under investigation. However, if the antenna only possesses a limited number of receptors for a specific behaviourally important compound, a large EAG response will not be produced. Similarly, insects whose antennae possess relatively few receptors, such as aphids, are known to produce very weak EAG responses (Wadhams, 1990). The single cell recording (SCR) technique, although more difficult to establish, can be important in such instances because insect chemoreceptosr are often highly specialised for particular compounds, and even if a specialised chemoreceptor type for a given compound is uncommon, its response will be easily observed once the receptor has been located (Bjostad, 1998). Examples of this have been reported in a study of antennal perception of volatiles from oilseed rape, *Brassica napus*, by the cabbage seed weevil, *Ceutorhynchus assimilis*, where EAG and SCR

techniques were compared (Blight *et al*, 1995). Here, specific olfactory cells were located by SCR for five compounds that did not elicit significant EAG responses. Ideally, any future work on identification of biologically active compounds for *P. rosae* should include GC-SCR and SCR analysis.

3.4.2 Biologically active compounds from non-host plant species (repellent intercrops)

Although both non-host plant species, onions (*Allium cepa*) and *Medicago litoralis*, have been shown to reduce damage to carrots by *P. rosae* larvae in intercropping field experiments, very few electrophysiologically active components can be seen in their extracts. In both cases, this is probably a result of the low levels of volatiles actually present in the extracts. In the case of the onion extract, this may be attributed to poor extraction of volatiles during the vacuum distillation step of the extraction. For *Medicago litoralis*, it is probably a result of the low levels of volatiles present in the plant material, as the other species investigated are mainly aromatic plant species such as the Apiaceae, which produce relatively high quantities of volatile terpenoids.

In light of the low levels of volatiles released and the lack of electrophysiological activity in the extract, it would seem that any reduction in crop damage afforded by intercropping with *Medicago litoralis* (Rämert, 1993 and 1996) is likely to result from means other than olfactory repellence, deterrence or host odour masking. Consequently, any reduction in *P. rosae* attack is probably due to the behavioural barrier effect described by Finch and Collier (2000) in their theory of host plant selection based on 'appropriate/inappropriate landings'. However, onion intercropping has been shown to reduce the number of *P. rosae* actually entering a site, especially during the early stages of field trials where the onion plants were still young and had not yet begun to bulb (Uvah and Coaker, 1984). This reduction in flies entering the crop was seen to diminish as the onions developed. These observations suggest a repellent or masking effect of the onion volatiles, which reduce the host location ability of the flies, and requires further study. This would ideally involve GC-EAG analysis of air entrainment samples from young intact onion plants, up to the stage where they begin to bulb.

3.4.3 Biologically active compounds from air entrainments of *P. rosae*

Due to the small quantity of total material in the air entrainment of mixed sex *P. rosae*, there was insufficient to enable GC-MS identification and confirmation of peak identities by GC peak enhancement. However, a limited number of GC-EAG runs were performed using antennal detectors prepared using antennae from each sex of the fly. These show a number of electrophysiologically active peaks present in the air entrainment sample of *P. rosae*, that were not seen in the entrainment sample from the control chamber (containing sugar and water only), all of which seem to be elicited in response to minor components in the entrainment sample. It also appears that the female EAG detectors produced far greater electrophysiological responses to all the active components (with the exception of peaks 'n' and 'o') than the males. These greater responses seem to be above the expected two-fold higher response of females over males reported by Guerin and Visser (1980), and necessitated a ten-fold concentration of the extract in order to discern the male EAG responses above the baseline fluctuation. Although male and female EAG responses to individual active components are similar, minor differences can be seen, with peaks 'c', 'd' and 'i' eliciting particularly strong responses from the females, while males showed no response to peak 'h'. The males may also be responding to a pair of closely associated peaks, 'n' and 'o', not seen in the responses of the female, although this response was only seen in one male EAG run and may be an artefact. These differences between sexes in amplitudes and components to which responses were seen may suggest the presence of pheromone components in the extract. The much greater response of the female antennae to components 'c', 'd' and 'i' suggests that it may possess more olfactory receptors for these compounds, which may act as male-produced sex pheromones. In order to investigate further the possibility of *P. rosae* produced pheromones, it would be advantageous to repeat this experiment on a larger scale to provide sufficient material for identifications to be performed. It would also be preferable to entrain each sex separately, to determine whether the active components are produced by only one sex, where they would act as sex pheromones, or by both, in which case they may be acting as aggregation pheromones. SCR techniques may also be useful in identification of the relative proportions of receptors on the antennae of each sex to these putative pheromone components.

Finally, with regard to EAG studies, it should be borne in mind that not all compounds found to be electrophysiologically active are behaviourally active (or not in the context that an investigator may

expect). For example, with compounds that are EAG-active for tsetse flies (*Glossina morsitans*), some are attractants (1-octen-3-ol, 4methylphenol, and 3-propenylphenol), some are repellents (acetophenone, 2-methoxyphenol), and some have no apparent behavioural activity (2,6,10,10-tetramethyl-1-oxaspiro [4.5] dec-2-en-8-one) (Gough *et al*, 1987; Bursell *et al*, 1988). Also, not all behaviourally active compounds are picked up by EAG, particularly if the antennae have few receptors for the compound, or the receptors produce small amplitude responses.

Table 3.7: **Summary of compounds identified as electrophysiologically active by GC-EAG, with an example of where they have been previously identified in extracts of the Apiaceae.**

Peak Nº	Compound	Species and location of previous identifications in extracts of Apiaceae	Reference
		Isoprenoids	
	Monoterpenes		
Acyclic monoterpenes			
15	Myrcene	*Daucus carota* - leaf, petiole, seed, root	[3] and [4]
19	(*E*)-β-Ocimene	*Daucus carota* - leaf, petiole	[4]
37	Linalool	*Daucus carota* - leaf, petiole, seed, root	[3] and [4]
Cyclic monoterpenes			
36	Terpinolene	*Daucus carota* - leaf, petiole, root	[3] and [4]
38	α-Terpinene	*Daucus carota* - leaf, root	[3] and [4]
41	α-Terpineol	*Daucus carota* - seed, root	[3]
49	Limonene	*Daucus carota* - leaf, seed, root	[3] and [4]
111	Carvyl acetate	*Aegopodium podagraria* – flowering foliage	[1]
Bicyclic monoterpenes			
14	β-Pinene	*Daucus carota* - leaf, petiole, seed, root	[3] and [4]
24	(*E*)-Limonene-1,2-epoxide	*Apium graveolens* - foliage	[5]
35	Sabinene	*Daucus carota* - leaf, petiole	[4]
50	Fenchone	*Foeniculum vulgare* - leaf,seed	[6]
57	α-Pinene	*Daucus carota* - leaf, petiole, seed, root	[3] and [4]
	Sesquiterpenes		
28	β-Caryophyllene	*Daucus carota* - leaf, petiole, seed, root	[3] and [4]
29	α-Humulene	*Daucus carota* - leaf, petiole	[4]
46	(*E,E*)-α-Farnesene	*Heracleum sibiricus* - flowering foliage	[1]
66	Germacrene-D	*Daucus carota* - leaf, petiole	[4]
112	Copaene	*Carum carvi* – flowering foliage	[1]
		Amino Acid Metabolites	
	Methoxyphenyl compounds		
45	*trans*-Methyl isoeugenol	*Daucus carota* - leaf, petiole, root	[3] and [4]
51	4-Allylanisole (estragole)	*Foeniculum vulgare* - leaf, seed	[6]
52	*trans*-Anethole	*Foeniculum vulgare* - leaf, seed	[6]
113	*trans*-Asarone	*Daucus carota* - leaf, petiole	[4]
130	Isoeugenol		
	Piperidine alkaloids		
17	γ-Coniceine	*Conium maculatum* – leaf, seed, root	[2]
22	Conhydrine	*Conium maculatum* – leaf	[2]
23	Conhydrinone	*Conium maculatum* – minor alkaloid	[7]
	Others		
10	4-Ethyltoluene		
40	Methyl salicylate	*Heracleum sibiricus* - flowering foliage	[1]
56	Benzaldehyde	*Heracleum sibiricus* - flowering foliage	[1]

Table 4.7: **Summary of compounds identified as electrophysiologically active by GC-EAG (continued).**

		Lipoxygenase Products	
	Alcohols		
1	3-Hexanol		
2	2-Hexanol		
5	1-Hexanol	*Apium graveolens* – leaf	[3]
34	1-Octen-3-ol		
115	(*Z*)-3-Hexen-1-ol	*Apium graveolens* – leaf	[3]
	Aldehydes		
4	(*E*)-2-Hexenal	*Petroselinum crispum* – leaf	[3]
	Esters		
16	(Z)-3-Hexenyl acetate	*Apium graveolens* – leaf	[3]
95	Octyl acetate	*Heracleum sibiricus* - flowering foliage	[1]
96	Hexyl butanoate	*Pastinaca sativa* - flowering foliage	[1]
	Alkanes		
3	Octane	*Apium graveolens* - foliage	[5]
7	Nonane	*Aegopodium podagraria* - flowering foliage	[1]
33	2,3,4-Trimethyl heptane		

Key to references : [1] Borg-Karlson *et al* (1994), [2] Cromwell (1955), [3] Guerin and Visser (1980), [4] Kainulainen *et al* (1998), [5] MacLeod and Ames (1989), [6] Muckensturm *et al* (1997), [7] Panter and Keeler (1989).

4 ELECTROPHYSIOLOGICAL RESPONSES OF *P. rosae* TO IDENTIFIED COMPOUNDS

4.1 INTRODUCTION

Electrophysiological techniques have proven very useful in the isolation, identification and elucidation of the behavioural and physiological roles of semiochemicals, particularly for insects whose chemoreceptors are readily accessible (Bjostad, 1998). One of the most frequently used techniques is the electroantennogram, for recording from whole insect antennae, and this method has been successfully used on a wide range of insect species from most orders (Roelofs, 1984). The 'EAG is essentially the sum of many olfactory receptor potentials recorded more or less simultaneously by an electrode located in the sensory epithelium' (Schneider, 1963). An odour stimulus causes a negative deflection of the receptor potential, which rises relatively fast and declines more slowly after the end of the stimulus. The amplitude and shape of the EAG response depends on the chemical structure of the stimulus and stimulus concentration, until a saturation level is reached (Roelofs, 1984). For a general review of insect chemosensory electrophysiology, see De Kramer and Hemberger (1987).

Having identified compounds from extracts of the Apiaceae, by GC-EAG, GC-MS and comparative studies with authentic compounds, the electrophysiological activity was confirmed and investigated further by EAG using physiologically discriminating doses. Compounds previously identified from the literature as present in the Apiaceae were also included in this study, looking at differences in response to isomeric or enantiomeric forms (where available) including a range of chemically related polyacetylenic compounds, many of which commonly occur in the Apiaceae (Bohlmann, 1971). A number of organic isothiocyanates, typical of the Brassicaceae [(used to be Cruciferae) Blight *et al*, 1995], were also included in this study to assess responses to non-host volatiles.

Since EAG responses (in mV) can vary with time and between individual antennae, and can be affected by temperature, airflow rate, juxtaposition of the air tube to the antenna and previous responses (Roelofs, 1984), response amplitudes were normalised to a particular standard. In this study, 1-octen-3-ol was chosen as the standard because of its known activity in a number of dipterous species (Blackwell *et al*, 1996; van-Naters *et al*, 1996), as well as its common occurrence in volatile plant emissions (Buttery *et al*, 1984). In order to establish a suitable EAG protocol for testing the identified plant compounds, a range

of preliminary experiments were performed. These were aimed at determining a discriminating stimulus concentration at which to apply both standard and test compounds, the usable life span of an EAG preparation, and how closely in time consecutive test stimuli could be applied to obviate interference effects.

4.2 METHODS

4.2.1 Electrophysiology

Electroantennograms were recorded from recently emerged female flies (2-3 days old) using silver-silver chloride (Ag-AgCl) glass electrodes filled with saline (see method 3.2.3). The insect was momentarily anaesthetised by placing it in a specimen tube in an ice bucket; the head was excised and mounted on the indifferent electrode. This was performed by guiding the tip of the electrode through the occipital opening to the base of the antenna, and just inside the pedicellus. The tip of the recording electrode was placed over the excised tip of the arista on the same antenna (Fig. 3.2). This preparation reduced movement artefacts and desiccation to a minimum. The signals were passed through a high impedance amplifier (Syntech UN-06, Hilversum, Netherlands) and data storage and processing were carried out with a PC-based interface and software package (AutoSpike - Syntech). Room temperature was maintained at 20°C.

The stimulus delivery system used for application of test compounds utilised a disposable Pasteur pipette cartridge. The sample in 10µl of hexane was applied to a filter paper strip (4mm x 65mm), the solvent was allowed to evaporate (20 seconds) and the filter paper was inserted into a Pasteur pipette. After 40 seconds for equilibriation, the vapour from the cartridge was injected, by means of an air stream, into a second air stream (600ml/min), purified but not humidified, flowing continuously over the preparation. The former was controlled with a solenoid valve operated by an electronic timer. The stimulus duration was 1 second and the volume of air delivered through the pipette was 5 ml. Fresh cartridges were prepared for each new stimulation.

Preliminary dose-response study for 1-octen-3-ol

To determine a suitable concentration for standard stimuli to be applied, a preliminary dose-response study for 1-octen-3-ol was carried out, with the concentration eliciting a response of approximately 1mV chosen for all further experiments. This concentration was also chosen as the discriminating dose at which to test the whole range of compounds investigated in this study. Solutions of 1-octen-3-ol in hexane were presented to six individual EAG preparations in increasing concentrations, to reduce the effects of previous stimuli on those following. Concentrations were increased by a factor of ten each time, in the

range $1x10^{-11}$ to $1x10^{-4}$ g of 1-octen-3-ol per filter paper strip. A hexane control stimulus was presented to the preparation between every two stimuli of 1-octen-3-ol stimuli. To allow for complete recovery of the sensilla, stimuli were presented at intervals of 10 minutes until a significant response above the control level was evident, when the period between 1-octen-3-ol stimuli was increased to 15 min (estimated to be above that required for full recovery of the preparation). EAG responses evoked by 1-octen-3-ol were compared statistically to those of the control using Student's *t*-test and a dose-response curve was constructed.

Determination of the average life span of an EAG preparation

Preliminary tests with 1-octen-3-ol were also carried out to determine the average active life span of an antennal preparation. Five individual preparations were repeatedly subjected to standard stimuli, equivalent to $1x10^{-5}$ g of 1-octen-3-ol on a filter paper strip, at 10 min intervals for a duration of four hours. Once every hour, the preparation was also exposed to a blank (air only) and a hexane control stimulus for comparison.

Determination of the optimum recovery period necessary between consecutive test compounds

A further preliminary experiment was performed to determine how closely in time two similar test compounds could follow one another, without causing a reduction in the response to the latter stimulation. The time intervals between a series of consecutive paired stimulations were 30 sec, 1 min, 3 min, 5 min and 10 minutes. γ-Coniceine was chosen for the paired stimuli because it elicited the largest electrophysiological response, with the longest recovery from the deflection, in the previous GC-EAG study (Ch.3), which should therefore show with maximum effect any interference likely to occur. The paired stimuli were delivered at a concentration equivalent to $1x10^{-5}$ g per filter paper strip, which was to be used as the discriminating dose for all subsequent compounds. Seven replicates for each time period were delivered in a random order to seven EAG preparations, with each test preceded and followed by a hexane control and a 1-octen-3-ol standard ($1x10^{-5}$ g per filter paper strip). Each test was separated by fifteen minutes from the next test pair (estimated to exceed the necessary recovery period for the preparation). In order to accommodate changes in absolute antennal responses, response amplitudes were

calculated relative to the 1-octen-3-ol standards. The response to the second stimulation was then assessed relative to the response elicited by the first stimulation in each pair, with the null hypothesis being that they are both equal.

4.2.2 EAG Investigation of Compounds Identified in the Apiaceae: Closely Related Compounds and some Non-Host Compounds

Having determined a suitable concentration for the 1-octen-3-ol standard (Fig. 4.1), the average usable life span of a preparation (Fig. 4.2) and how closely in time consecutive test compounds could be applied to obviate interference effects (Fig. 4.3), the following protocol was established.

Compounds were separated into groups of twelve, with each compound being delivered to the EAG preparation at the electrophysiologically discriminating dose previously identified for the 1-octen-3-ol standard, equivalent to $1x10^{-5}$ g per filter paper strip. A hexane control and the 1-octen-3-ol standard stimulus was included between each three test compounds, and compounds were delivered consecutively to the EAG preparation with a six-minute recovery period allowed between each stimulus. This allowed all twelve compounds, and controls, in a group to be tested on each of six individual EAG preparations, within the predetermined usable life span of each preparation (approximately 2.5 hours). Where possible, compounds within similar chemical groupings were separated within their test group, to reduce further the possibility for interference. An element of randomness was added to the tests by delivering the compounds of each group in the reverse order for three EAG runs out of the six replicates carried out on each group. The only alteration to this protocol was in the case of the polyacetylenes, as all these compounds were grouped together and presented in a random order during testing, and some of the least important of this group received fewer than the normal six replicates (as detailed in table 4.1). This was due to their limited availability and highly unstable nature (Bohlman, 1971). To allow for variation among antennal preparations and for time dependent changes of a single preparation, responses were normalised with respect to the average response obtained from the two standards given before and after the test chemicals. Compounds were obtained commercially, with the exceptions of γ-coniceine and N-methylconiine, which were synthesised by Dr Mike Birkett (Biological and Ecological Chemistry Department, IACR-Rothamsted) and the polyacetylenes, which were provided as a kind gift by Dr Franz Hadacek. All compounds tested were greater or equal to 98% in purity, with the exceptions of (*Z*) and (*E*)-β-ocimene,

(-)-α and (-)-β-phellandrene, (*E,E*)-α and (*E*)-β-farnesene, which were 95-98% pure, myrcene, which was 90% pure; and *cis*-asarone, which was only 70% pure, the remaining 30% being *trans*-asarone.

4.3 RESULTS

4.3.1 Preliminary Studies

Preliminary dose-response study with 1-octen-3-ol

Preliminary results of the dose-response study with 1-octen-3-ol are given in Fig. 4.1. The concentration that produced a mean response of 1mV was chosen as a discriminating dose for application of the standard compound (1-octen-3-ol) and as the application concentration for all other test compounds. This concentration was equivalent to $1x10^{-5}$g of compound per filter paper strip.

Determination of the average life span of antennal preparations

Preliminary results of the determination of average life span of an antennal preparation are given in Fig. 4.2. It was found that the difference in response to the 1-octen-3-ol and the hexane controls decreased with time over the four-hour test period, with one preparation ceasing to respond to any stimuli after 190 minutes. To keep a reasonable difference in the responses between the test compounds and controls throughout experiments, the usable life span of a preparation was determined to be between two and a half to three hours. This was determined to be the period that provided a reasonable separation between the mean response lines for 1-octen-3-ol and the hexane control (with no overlap between the standard error bars for each stimuli) as plotted in Fig. 4.2.

Determination of optimum timing for consecutive stimulations

Results of the determination of optimum timing for consecutive stimulations are given in Fig. 4.3. It can be seen, in Fig. 4.3, that the mean response of an EAG preparation to a second identical stimulus of γ-coniceine was greatly reduced when the interval was less than three minutes. At longer intervals, the responses equalised (at approximately 4 min). This was followed by a short period during which the second response slightly exceeded the first (5-6 min), after which the second response returned to being hundred percent of the first response (at approximately 9-10 min). To minimise these distortions, a recovery period of ten minutes between consecutive stimuli would seem ideal for γ-coniceine. However, as such a long interval would greatly limit the number of compounds possible to test per EAG preparation,

and because the majority of compounds to be tested were much less stimulatory than γ-coniceine and therefore required a shorter recovery period, a compromise of six minutes between consecutive stimuli was allowed during all subsequent experiments.

4.3.2 Electroantennogram Investigation of Volatile Compounds for Electrophysiological Activity

Results from the electroantennogram investigation of volatile compounds are given in table 4.1, along with references as to which compounds have previously been found to be present in apiaceous extracts, and which compounds have previously been found to be electrophysiologically active for *P. rosae*. Compound structures are also provided in Appendix 1.

The following compounds, tentatively identified for the first time as electrophysiologically active by GC-EAG investigation of extracts from the Apiaceae, were confirmed as significantly active in this EAG investigation: (*E*)-β-ocimene, fenchone, α-humulene, (*E,E*)-α-farnesene, copaene, *trans*-limonene-1,2-epoxide, carvyl acetate, γ-coniceine, methyl salicylate, 3-hexanol and 1-octen-3-ol.

For fenchone and 1-octen-3-ol, enantiomeric forms were also investigated. Both (+)- and (-)-fenchone were significantly active, with the (-)-form eliciting a slightly higher response than the (+)-form; in the case of 1-octen-3-ol, both the (*R*)- and (*S*)-forms were found to be significantly active, both having similar responses to the (+/-)-1-octen-3-ol standard (taken as 100% response).

Germacrene-D, conhydrine, conhydrinone, octyl acetate, hexyl butanoate, benzaldehyde, 2,3,4-trimethylheptane and isoeugenol were unavailable as pure compounds, so their EAG activity could not be confirmed. Results from sabinene (52%, ±5; of response to standard), 4-ethyltoluene (39%, ±-3; of response to standard) and 2-hexanol (47%, ±3; of response to standard) were not significantly different from the hexane control.

Of the 106 compounds isomers and enantiomers tested, 69 showed significant activity at $1x10^{-5}$g per filter paper strip compared to the hexane control. Significant responses were seen to compounds in all the chemical groups tested, with the exception of the hydrocarbon ketones and alkanes. The chemical groups with the highest and most frequently significant responses were: the piperidine alkaloids, with γ-coniceine showing the largest relative response from any group (273%, ±33; of response to standard); the methoxyphenyl compounds, in which *trans*-asarone showed the largest relative response (153%, ±20;

of response to standard); the isoprenoids (especially the sequiterpenes, oxygenated sesquiterpenes and oxygenated monoterpenes). In the remaining groups, including the non-host isothiocyanates, approximately half of the compounds tested elicited significant responses.

Table 4.1: Summary of mean EAG responses of female *P. rosae*.

This table shows mean EAG responses of female *P. rosae* (± SE) to volatile compounds (10^{-5}g) identified in the Apiaceae, including closely related compounds (i.e. isomers, enantiomers) and some non-host compounds; expressed as a percentage of their response to a 1-octen-3-ol standard (10^{-5}g). References are also given for compounds, that have previously been identified in apiaceous extracts, and for those previously shown to elicit EAG responses in *P. rosae*.

Compound	Mean EAG response (%)	(±) SE (n = 6)	Statistical significance	Peak numbers from GC-EAG runs (Ch.3)	References to previous identifications in the extracts of Apiaceae	References to previous EAG responses with *P. rosae*
Isoprenoids						
Monoterpenes-Acyclic						
Linalool	**102**	3	***		1, 5, 14, 16, 21	11
Myrcene	**50**	3	ns	15	1, 5, 6, 7, 8, 9, 12, 13, 14, 15, 16, 21, 24	11
(*E*)-β -Ocimene	**66**	3	***	37	5, 14	
(*Z*)-β-Ocimene	**41**	3	ns	18	5, 14, 16	
Monoterpenes-Cyclic						
(-)-Carveol	**82**	6	***		16	
(+)-Carvone	**146**	7	***		8, 26	11
(-)-Carvone	**105**	8	***		8, 26	11
(-)-Carvyl acetate	**83**	4	***	111	#	
m-Cymene	**49**	4	ns		#	
o-Cymene	**42**	4	ns		#	
p-Cymene	**38**	3	ns		5, 16	
(+)-Limonene	**68**	6	**	49	1, 5, 6, 8, 9, 12, 13, 14, 16, 17, 21, 24, 27	11
(-)-Limonene	**71**	4	***	49	1, 5, 6, 8, 9, 12, 13, 14, 16, 17, 21, 24, 27	11
(-)-α-Phellandrene	**53**	6	ns		12, 13, 7, 15, 17	11
(-)-β-Phellandrene	**42**	4	*		5, 16, 17	
α-Terpinene	**38**	4	ns	38	6, 1, 9, 14, 16, 17	11
γ-Terpinene	**47**	4	ns		21, 6, 1, 9, 12 13, 15, 24, 5, 16, 17	11
α-Terpineol	**130**	6	***	41	1, 6, 16, 25	11
Terpinolene	**49**	3	**	36	21, 6, 1, 9, 12 7, 15, 14, 5, 16	11

Table 4.1: **Summary of mean EAG responses of female *P. rosae* (continued).**

Monoterpenes-Bicyclic						
Bornyl acetate	**64**	5	***		21, 6, 1, 9, 12, 14	11
(+)-Camphene	**50**	4	ns		21, 6, 1, 9, 12 13, 15	11
(+)-2-Carene	**37**	3	ns		#	
(+)-3-Carene	**46**	2	***		5, 13	11
(+)-Fenchone	**58**	4	**	50	17	
(-)-Fenchone	**89**	9	***	50	17	
(+)-*cis*-Limonene-1,2-epoxide	**79**	4	***	24	#	
(+)-*trans*-Limonene-1,2-epoxide	**94**	5	***	24	#	
(+)-α-Pinene	**68**	5	***	57	1, 5, 6, 7, 9, 12, 13, 15, 16, 21, 24, 27	11
(-)-α-Pinene	**50**	5	ns	57	1, 5, 6, 7, 9, 12, 13, 15, 16, 21, 24, 27	11
(+)-β-Pinene	**35**	3	ns	14	1, 5, 6, 7, 9, 12, 13, 15, 16, 21, 24, 27	11
(-)-β-Pinene	**35**	3	ns	14	1, 5, 6, 7, 9, 12, 13, 15, 16, 21, 24, 27	11
Sabinene	**52**	5	ns	35	3, 5, 16	
Sesquiterpenes						
γ-Bisabolene	**118**	11	***		1, 6, 9,14	11
β-Caryophyllene	**82**	6	***	28	1, 5, 6, 9,12,14, 15, 16, 21, 24, 27	11
(-)-α-Copaene	**64**	5	***	112	5, 14	
(*E*,*E*)-α-Farnesene	**81**	5	***	46	5	
(*E*)-β-Farnessene	**95**	7	***		5	
α-Humulene	**57**	2	***	29	14, 16	
Oxygenated Sesquiterpenes						
α-Ionone	**88**	**12**	**		#	
β-Ionone	**73**	**2**	***		1, 9	11
Amino Acid Metabolites						
Methoxy Phenyl compounds						
4-Allylanisole (estragole)	**77**	4	***	51	17, 22	11
trans-Anethole	**118**	15	***	52	17	10
p-Anisaldehyde	**91**	11	***		22	11
cis-Asarone	**145**	20	***		14	10
trans-Asarone	**153**	20	***	113	14	10
Eugenol	**71**	3	***		#	10, 11
Methyleugenol	**142**	14	***		14	10
trans-Methylisoeugenol	**125**	15	***	45	9, 14	10, 11

Table 4.1: **Summary of mean EAG responses of female *P. rosae* (continued).**

Piperidine Alkaloids						
G-Coniceine	**273**	33	***	17	18	
(+/-)-Coniine	**233**	23	***		18	
2-Ethylpiperidine	**127**	6	***		#	
N-Methylconiine	**170**	22	***		18	
2-Methylpiperidine	**102**	4	***		18	
Linear Furanocoumarins						
Bergapten	**48**	8	ns		2	23
Psoralen	**44**	3	*		2	
Xanthotoxin	**61**	5	***		2	
NON-HOST Isothiocyanates						
Allyl isothiocyanate	**51**	2	**		non-host	
Butyl isothiocyanate	**46**	4	ns		non-host	
2-Phenylethyl isothiocyanate	**67**	10	*		non-host	
Lipoxygenase Products						
Alcohols						
1-Heptanol	**76**	4	***		8	11
(+/-)-2-Heptanol	**76**	5	***		#	
1-Hexanol	**62**	4	***	3	8, 5	11
2-Hexanol	**47**	3	ns	2	#	
3-Hexanol	**49**	3	*	1	#	
1-Hexen-3-ol	**39**	5	ns		#	
(*E*)-2-Hexen-1-ol	**56**	5	*		25	11
(*Z*)-3-Hexen-1-ol	**57**	8	*	115	7, 25	11
1-Octanol	**95**	5	***		5	
1-Octen-3-ol (standard)	**100**	-	-	34	#	
(*R*)-1-Octen-3-ol	**97**	3	***	34	#	
(*S*)-1-Octen-3-ol	**89**	5	***	34	#	
1-Pentanol	**34**	2	ns		#	
Aldehydes						
1-Butanal	**30**	2	ns		#	
1-Heptanal	**44**	3	**		16	
Hexanal	**43**	3	ns		8, 16	11
(*E*)-2-Hexenal	**50**	5	ns	4	25	11
(*E*)-2-Nonenal	**76**	6	***		1, 6, 9, 19	11
1-Propanal	**31**	2	ns		#	
trans,trans-2,4-Decadienal	**88**	4	***		6	11
Ketones						
1-Butanone	**28**	2	ns		#	
4-Heptanone	**33**	2	ns		#	
3-Pentanone	**47**	4	ns		#	
Esters						
(*Z*)-3-Hexenyl acetate	**57**	4	ns	16	7, 26	11
Hexyl acetate	**78**	6	***		5	
Hydrocarbons						
Decane	**47**	2	ns		5	
Nonane	**42**	4	ns	7	5, 16,19	
Octane	**39**	5	ns	3	16	

Table 4.1: **Summary of mean EAG responses of female *P. rosae* (continued).**

Acetylenes and Polyacetylenes						
C13-Ene-diyne-diene (Aethusin) [6]	**67**	7	***		4	
C13-Diene-yne-diene [6]	**66**	10	**		4	
C13-Ene-yne-diene [6]	**62**	11	**		#	
C13-Ene-diyne-ene-10-ol (Aethusanol A) [5]	**46**	4	***		4	
C17-Ene-diyne-diene [5]	**59**	7	***		4	
C17-Ene-diyne-diene-14-ol [5]	**38**	4	*		4	
C17-Diyne-triene-1-ol (Cicutol) [4]	**31**	5	ns		4	
C17-Diyne-triene-1,14-diol (Cicutoxin) [5]	**38**	4	ns		4	
C17-Diyne-diene-1-ol [3]	**35**	5	ns		4	
C17-Diyne-diene-1,12-diol [3]	**35**	5	ns		4	
Falcarinol [6]	**49**	4	***		4	23
Falcarindiol [6]	**36**	3	ns		4	23
Dehydrofalcarinol [5]	**49**	5	***		#	
Dehydrofalcarinon 5]	**61**	6	***		#	
(*E*)-Spiroketalenolether [4]	**52**	3	***		#	
OTHERS						
Bi-phenyl	**81**	6	***		1, 6, 9	11
2,4-Dimethylstyrene	**39**	3	ns		9, 12	11
4-Ethyltoluene	**39**	3	ns	10	#	
Methyl salicylate	**114**	6	***	40	5	
(E/Z)-Artemidin [4]	**40**	4	*		#	
CONTROLS						
Hexane [234]	**37**	1	-			
Diethyl ether (solvent used for furocoumarins)	**41**	4	ns			
Air blank [$]	**32**	-	-			

NB. Statistical significance: ns= not significant; '*' $P = 0.1$; '**' $P = 0.01$; '***' $P = 0.001$.
All compounds were tested on six separate EAG preparations (n=6), with the exception of those with square brackets after their names, which contain the number of replicates tested.

'#' Compounds not known to be present in the Apiaceae but having structures closely related to known host plant compounds (i.e. isomers and enantiomers).

'$' Air blank estimate from a separate experiment, for comparison only.
The mean hexane control was calculated to be 114.8% of response to the estimated air blank control (calculated from responses normalised to the air blank, taken as 100% response; n=19).

Key to References (refers to compounds only, not specific enantiomers):
1 = Alabran *et al*, 1975; 2 = Beier and Oertli, 1983; 3 = Berüter and Städler, 1971; 4 = Bohlmann, 1971; 5 = Borg-Karslon *et al*, 1994; 6 = Buttery *et al*, 1968; 7 = Freeman *et al*, 1975; 8 = Gold and Wilson, 1963;9 = Guerin, 1978; 10 = Guerin *et al*, 1983; 11 = Guerin and Visser, 1980; 12= Heatherbell *et al*, 1971;13 = Ikeda *et al*, 1962; 14 = Kainulainen *et al*, 1998; 15 = Kasting *et al*, 1972; 16 = Mac Leod and Ames, 1989; 17 = Mukensturm *et al*, 1997; 18 = Panter and Keeler, 1989; 19 = Potter and Fagerson, 1990; 20 = Salveson and Baerheim Svendsen, 1976; 21 = Seifert *et al*, 1968; 22 = Städler, 1972; 23 = Städler and Roessingh, 1991; 24 = Wilson, 1969a; 25 = Wilson, 1969b; 26 = Wilson, 1970; 27 = Zalkow *et al*, 1963.

Figure 4.1: **Mean response in EAG preparations with increasing concentration of 1-octen-3-ol. Values are plotted as means ± SE (n=6 in each case). Lines fitted by eye.**

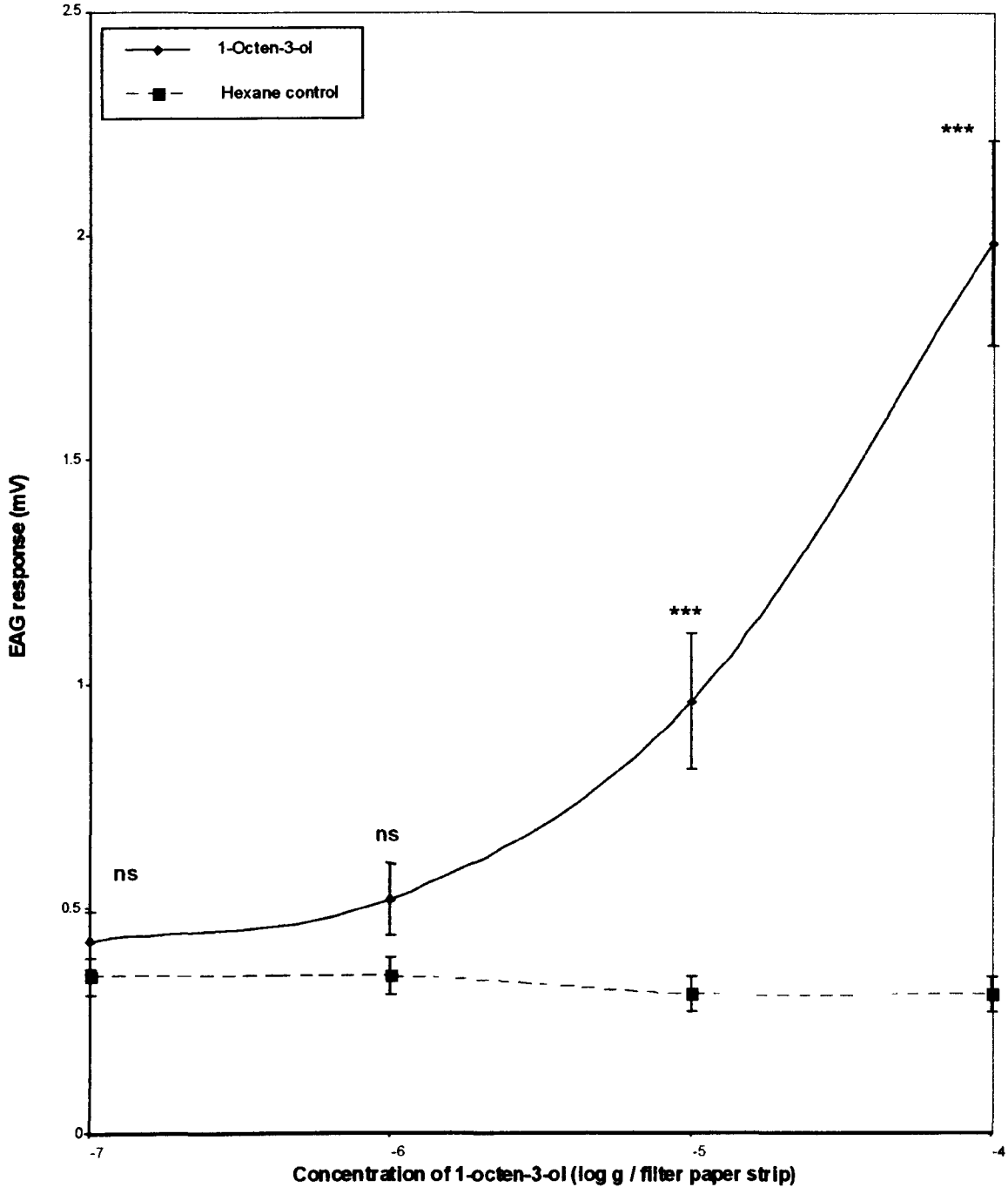


Figure 4.2: **Relationship between EAG response to 1-octen-3-ol ($1x10^{-5}$ g) and age of antennal preparation. Values plotted as means ± SE (n=5 in each case). Lines fitted by eye.**

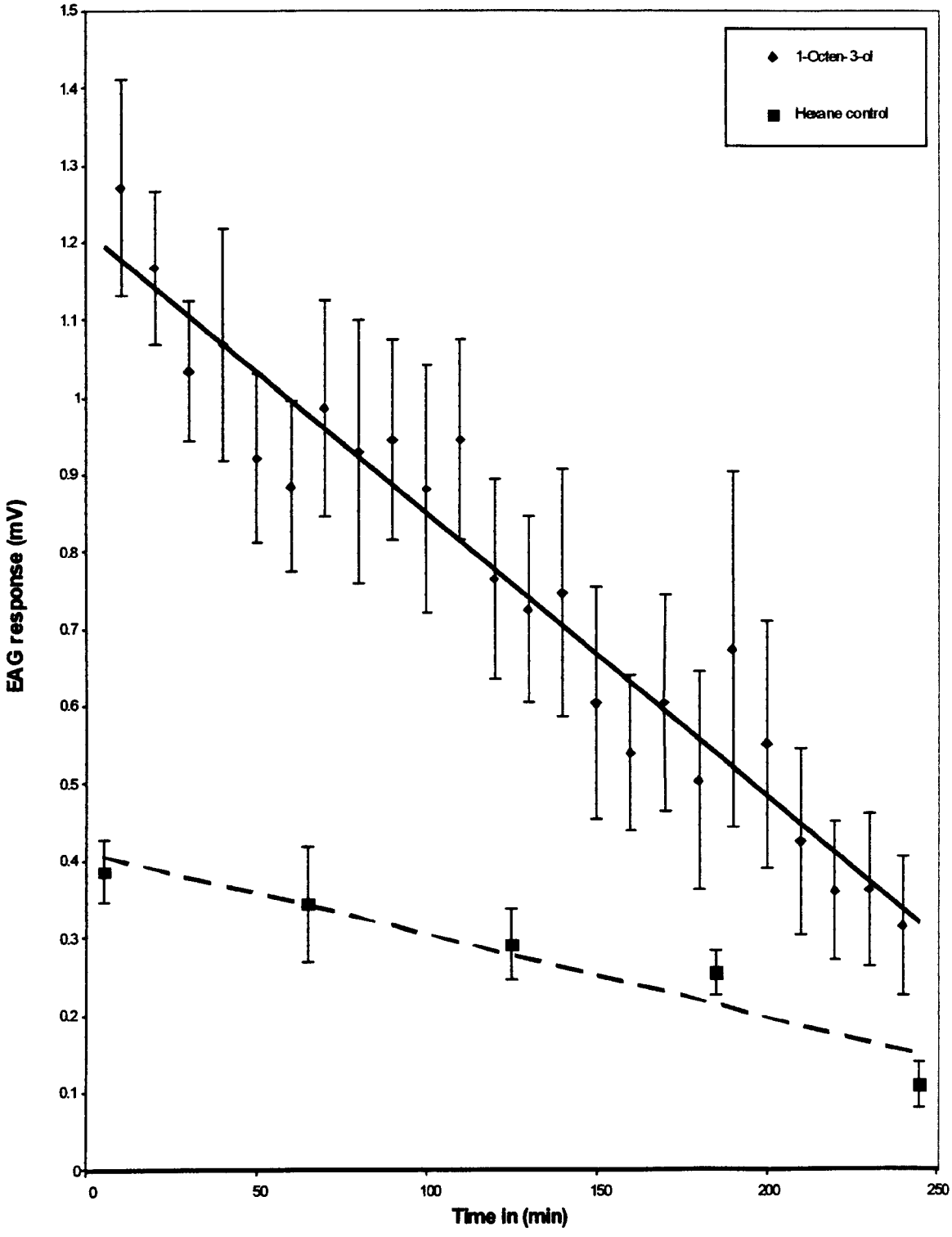
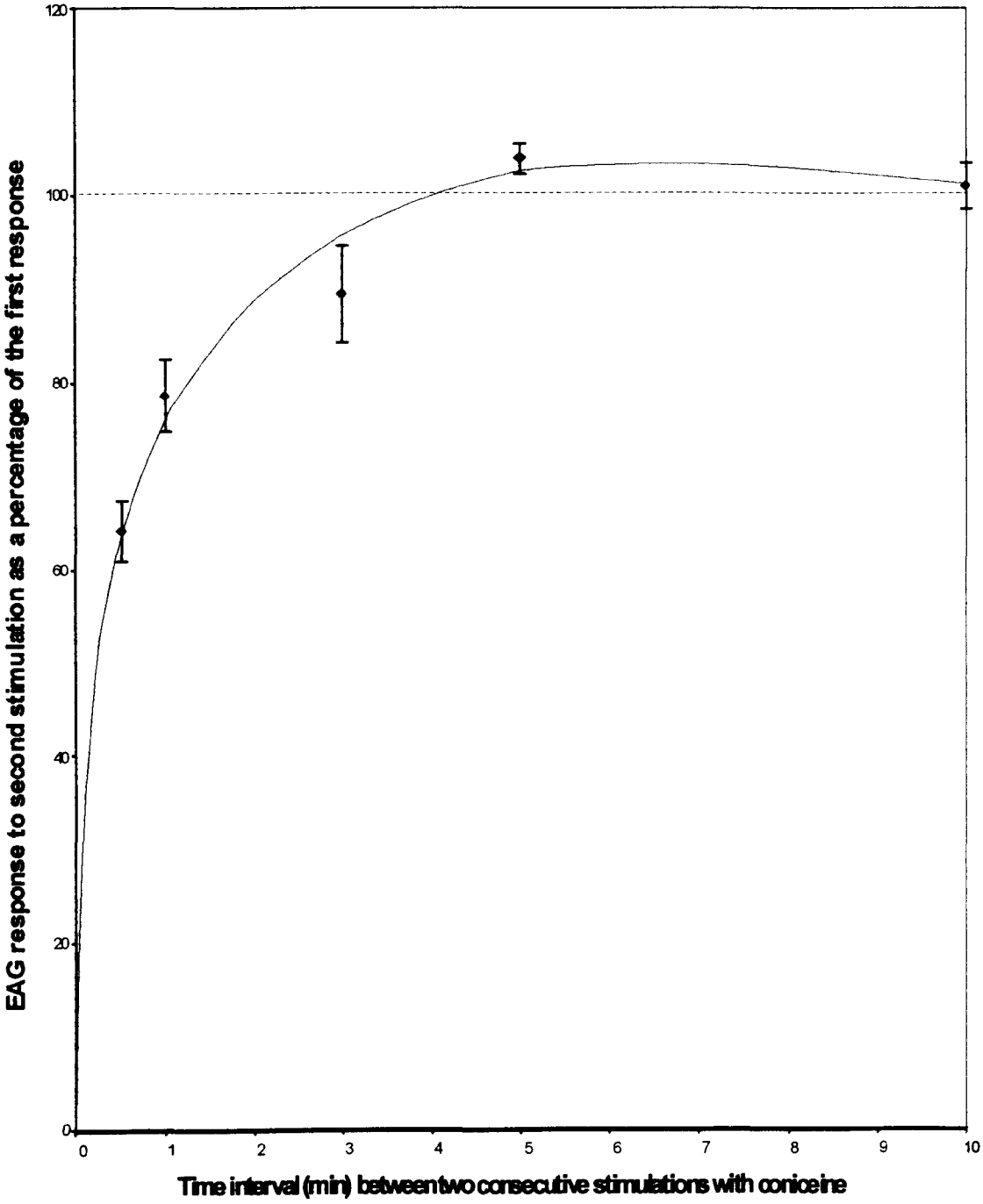


Figure 4.3: **Relationship between EAG response to γ-coniceine ($1x10^{-5}$ g) and time gap between consecutive stimulations. Values are plotted as means ± SE (n=7 in each case). Line fitted by eye.**



4.4 DISCUSSION

In this study, certain compounds previously identified in the GC-EAG investigation of plant extracts from the Apiaceae did not elicit responses significantly different from the hexane control at the level tested. These were octane, nonane, 2-hexanol, (*E*)-2-hexenal, (*Z*)-3-hexenylacetate, 4-ethyltoluene, sabinene, β-pinene, α-terpinene and myrcene. 3-Hexanol and (*Z*)-3-hexen-1-ol elicited only slightly higher responses than the hexane control. Some of these compounds have been reported to elicit moderate (β-pinene, α-terpinene and myrcene) or even high electrophysiological activity [(*E*)-2-hexenal, (*Z*)-3-hexenylacetate and (*Z*)-3-hexen-1-ol)] in previous studies on *P.rosae* (Guerin and Visser, 1980). However, this may have arisen because of differences in experimental protocol or the use of higher stimulus concentrations. There could also have been a residual level of hexane causing a response from the antenna in this experiment, which may have masked the activity of compounds eliciting a similar and especially a lower response. It is also possible that the residual hexane could interact with the same antennal receptor type as some of the test compounds. This would be most likely to occur with compounds having a similar size and/or structure to hexane and could suggest a reason for the lower than expected responses to some compounds, particularly the alkanes (octane and nonane) and compounds such as 2- and 3-hexanol. If it is the case that hexane (and diethylether, where used) elicits a significant electrophysiological response from the antenna, further investigations should be carried out using a less stimulatory solvent such as paraffin oil, as was utilised by Guerin and Visser (1980), which elicited no response relative to a control [(*Z*)-3-hexen-1-ol at 10^{-2} volume/volume]. Paraffin oil was not chosen as the solvent for this study, as it was thought that its sticky viscous properties might increase the likelihood of cross contamination occurring between test stimulations. To reduce the possibility of stimulation by residual hexane, the evaporation period allowed between application of the test solution and enclosing it within the pipette could be increased. However, this may not be advisable, as a corresponding loss of the more volatile test compounds would also occur.

4.4.1 Linear Furanocoumarins

The presence of linear, angular and substituted coumarins has previously been demonstrated in the Apiaceae (Beier and Oertli, 1983, Städler and Buser, 1984). The importance of the linear furanocoumarins

bergapten and xanthotoxin and the substituted coumarin osthol, as oviposition stimulants for *P. rosae*, was subsequently demonstrated in laboratory bioassays (Städler and Buser, 1984) and the electrophysiological activity of osthol and bergapten was later confirmed by EAG studies (Städler and Roessingh, 1991). This EAG study demonstrated electrophysiological activity of xanthotoxin and psoralen for the first time, which would be expected from their similarities in structure to bergapten, and from the previous oviposition results. However, these EAG results do not conform with the bioassay results of Städler and Buser (1984), as their estimated thresholds for oviposition stimulation suggested that *P. rosae* would be more sensitive to bergapten than to xanthotoxin, whereas in this study, the antennae showed greater responses to xanthotoxin than bergapten. The fact that bergapten did not elicit a response significantly different from the hexane control in this study, whereas it was seen to elicit a significant response by Städler and Roessingh (1991), further supports the possibility that some active compounds for *P. rosae* may have been overlooked in this experiment due to low level responses to hexane residues.

4.4.2 Acetylenes and Polyacetylenes

The presence of a wide range of polyacetylenes in many species of the Apiaceae has long been known, with falcarinol being first isolated from *Falcaria vulgaris* (Bohlmann *et al*, 1966). The polyacetylenes are found more regularly in the root tissues than the arial parts of plants (Harborne *et al*, 1998), although falcarinol and falcarindiol have been identified in the leaves of carrot (Städler and Buser, 1984), along with falcarindiol monoacetate (Maki *et al*, 1989). A number of the polyacetylenic compounds have also been shown to play a crucial role in the relationship between *P. rosae* and its host species, with falcarindiol and, to a lesser extent falcariol and falcarinone, acting as potent stimulants in pseudo-leaf oviposition bioassays (Städler and Buser, 1983). All three compounds were found to elicit significant electrophysiological activity in EAG studies (Städler and Roessingh, 1991). Falcarinol, falcarindiol and falcarindiol monoacetate were also shown to be potent electrophysiological stimuli for *P. rosae* larvae, with falcarinol significantly active as an attractant in behavioural assays (Maki *et al*, 1989). The combination of these three polyacetylenes was suggested to be a major contributing factor, in the superior root resistance of the carrot variety Vertou over the more susceptible variety Long Chantenay (Maki *et al*, 1989). It was proposed that, as the roots contained much higher concentrations of these compounds than

the aerial parts, they may contribute significantly to the overall volatile odour of the plant and may even surpass leaf odour as an oviposition stimulant (Maki *et al*, 1989). This suggestion is backed up by the results of Guerin and Städler (1982), who found that carrot foliage had to be supplemented with carrot root in order to elicit EAG responses for *P. rosae* that were significantly larger than those elicited by the non-host species Brussels sprouts (*Brassica oleracea*) and leek (*Allium porrum*). It is therefore not surprising that a large number of the acetylenes and polyacetyleneic compounds, many from the Apiaceae, investigated in this study elicited significant EAG responses. Although many of these responses were of medium to low intensity, this may be expected due to their relatively low volatilities, as with the furocoumarins. Comparison of the neurophysiological responses of larvae to falcarinol and falcarindiol with the responses of adults in this study showed slight differences. The larval responses were similar for both compounds, (approximately 5 times the response to a control, Maki *et al*, 1989), whereas with the adults, falcarinol elicited a significantly higher response than falcarindiol, which was not significantly different from the control. This difference in response is in accordance with a previous EAG study on adult *P. rosae* (Städler and Roessingh, 1991), which showed that response magnitudes decreased in the order falcarinol, falcarinone and falcarindiol, although unlike in this study, all three compounds were seen to be significantly active. The finding that falcarindiol is less electrophysiologically active than falcarinol is surprising, and appears to contradict oviposition bioassay results in which falcarindiol was a significantly stronger oviposition stimulant than either falcarinol or falcarinone (Städler and Buser, 1984).

As a range of 16 closely related acetylenes and polyacetyleneic compounds were investigated in this study, it was possible to compare structural features and relate these to the size and significance of the EAG responses they elicited. Such a comparison showed that the response was not totally dependent upon chain length, as 13 carbon and 17 carbon compounds both showed highly significant responses. The presence of straight chain structures rather than ring structures did not appear to be important, as compounds from both these groups elicited highly significant responses. It can also be seen that the presence of an oxygenated group is not essential. The results suggest that one important structural feature is the presence of at least one alkene (double bond) occurring in the early portion of the carbon chain, with no previous alkyne (triple bonds) occurring before it (as in the following compounds: aethusin, trideca-2,4,8,10-tetraen-6-yne, trideca-4,8,10-trien-6-yne, aethusanol A, heptadeca-2,8,10-trien-4,6-diyne,

falcarinol, falcarindiol, dehydrofalcarinol and dehydrofalcarinone); larger responses are seen with compounds containing the first alkene (double bond) between carbon numbers 2-3 or 4-5, rather than between carbons 1-2 (i.e. the falcarinol compounds). The presence of alkyne (triple bonds) earlier than an alkene (double bond) in the chain appears to reduce the antennal response substantially, as seen with the following compounds for which no significant responses were seen: 14-hydroxyheptadeca-2,8,10-trien-4,6-diyne, cicutol , cicutoxin, 1-hydroxyheptadeca-8,10-dien-4,6-diyne and 1,2-dihydroxyheptadeca-8,10-diene-4,6-diyne. Comparison of aethusin and trideca-2,4,8,10-tetraen-6-yne suggests that substitution of an alkene (double bond) for an alkyne (triple bond), after an initial alkene (double bond) in the chain, does not significantly alter the amplitude of the response. Comparison of heptadeca-2,8,10-trien-4,6-diyne with 14-hydroxyheptadeca-2,8,10-trien-4,6-diyne suggests that the addition of an alcohol (-OH) group to position 14 significantly reduces the EAG response, although from these results the effect of position of the alcohol group cannot be determined. Comparison of the responses to falcarinol and falcarindiol also shows a reduction in response as a result of addition of an alcohol group, this time to position 8, which may suggest that an alcohol group attached to a high carbon number in the chain is responsible for reduced antennal stimulation. Finally, comparison of dehydrofalcarinol and dehydrofalcarinone suggest that ester groups (possibly attached early in the carbon chain) elicit greater antennal responses than does an alcohol groups.

4.4.3 Piperidine Alkaloids

Investigation of the available piperidine alkaloids confirmed that γ-coniceine elicited the strongest EAG response of all compounds tested in this study, as seen originally in the GC-EAG analysis of *C. maculatum* extract (Ch.3). It also suggested that strong responses would be likely for the two tentatively identified but unavailable compounds conhydrine and conhydrinone, in keeping with similarities in their structures. The level of response to these compounds seems to depend upon the length of the carbonyl side chain (attached to carbon 2 of the ring), with the highest responses being seen to a side chain length of 3-carbons (propyl, i.e. coniine), with decreasing responses seen for 2-carbon 'ethyl' and 1-carbon 'methyl' side chains (i.e. 2-ethylpiperidine and 2-methylpiperidine), respectively. The magnitude of responses was highest when the secondary amine group of the ring became a double bond imine group, as seen in γ-

coniceine. A change in this region of the ring from a secondary amine to a tertiary amine, as seen in N-methylconiine, has the reverse effect and substantially reduces the observed EAG response. These trends seem to conform to the order of toxicity for this group of compounds, with the most toxic members such as γ-coniceine and coniine eliciting the largest responses.

This is the first report of host-derived nitrogen-containing compounds acting as electrophysiological stimuli for *P. rosae*. Because of the high toxicity of these alkaloids, behavioural evidence would be required before a role in host location or acceptance could be attributed to them, particularly as the observed electrophysiological activity could merely result from toxic effects of these compounds upon the antennal receptors. However, it should be borne in mind that *C. maculatum* is a preferred wild host of *P. rosae* (Petherbridge *et al*, 1942; Petherbridge and Wright, 1943; Wainhouse and Coaker, 1981) and that other proven biologically active compounds for *P. rosae* are also potent toxins (e.g. the polyacetylenes and furocoumarins). Nonetheless, it is surprising that this insect appears to have developed such a highly specialised response for this group of compounds, since *C. maculatum* is the only species in the Apiaceae known to possess any alkaloid compounds. However, eight other nitrogen containing compounds, including *o*-aminobenzaldehyde and benzyl cyanide, have recently been identified in the flower volatiles of a number of species from the Apiaceae (Borg-Karlson *et al*, 1994), some of which may also prove to be electrophysiologically active.

4.4.4 Methoxyphenyl Compounds

The highly significant EAG responses to the methoxyphenyl compounds tested was in agreement with previous studies by Guerin and Visser (1980) and Guerin *et al* (1983). The large EAG responses to *trans*-asarone and *trans*-methylisoeugenol are also in keeping with their known function as oviposition stimulants and field attractants (Guerin *et al*, 1983). The activity generally conformed to the structure-activity analysis of methoxyphenyl compounds previously performed by Guerin *et al* (1983), with a few notable exceptions. Firstly, the response to *trans*-asarone was less than expected, being only slightly higher than the response to *trans*-methylisoeugenol, whereas in the previous study it was ten times higher. The unusually large response to *cis*-asarone in this study was almost certainly due to the 30% impurity of *trans*-asarone in this sample, and can be discounted. The response to methyleugenol was also higher than

expected, being similar in response to that of *trans*-asarone in this study, whereas it was 23 times lower in the previous study. Whether these differences were due to differences in experimental design between these two studies, or was due to regional differences in the responses of *P. rosae*, was not determined.

4.4.5 Lipoxygenase Products

The EAG results for the lipoxygenase products show some interesting trends; of the alcohols tested, the largest responses were seen to the 8 carbon members, particularly 1-octen-3-ol, followed by the 7 carbon alcohol 1-heptanol. Lower activity was seen for the 6 carbon alcohols, where larger responses were elicited when the alcohol (-OH) group was terminal (i.e. 1-hexanol). The response to the 5-carbon alcohol, 1-pentanol, was not significant. These results are similar to the results of Guerin and Städler (1982), who found a similar trend in responses to the aliphatic aldehyde series pentanal to decanal, in which greatest EAG activity was seen with *P. rosae* for aldehydes of between 7 and 9 carbon atoms. In the aldehydes, only 1-heptanal and (*E*)-2-nonenal showed significant responses at the level tested, with hexanal and (*E*)-2-hexenal not significant. This result highlights the fact that not all biologically active compounds were picked up as significant in this investigation, as both hexanal and (*E*)-2-hexenal have previously been shown to elicit significant EAG activity, as well as acting as attractants in field studies (Guerin *et al*, 1983). The response to (*E*)-2-nonenal is not surprising as it has been shown to repel, and at higher levels to paralyse and kill the larvae of *P. rosae* (Guerin and Ryan, 1980), making it a likely repellent for ovipositing females.

4.4.6 Isoprenoids

The results of this EAG study correspond with some of the findings of the previous GC-EAG study (Ch.3). Myrcene, although present in many apiaceous host plant species of *P. rosae*, was confirmed as showing no significant EAG response for *P. rosae*. During the previous study, both *cis*- and *trans*- isomers of β-ocimene were found to be present as major peaks in *C. maculatum* extracts, but only the *trans*-isomer showed electrophysiological in the GC-EAG analysis. This observation was confirmed by EAG analysis, which showed that only the *trans*-isomer, (*E*)-β-ocimene, was significantly active.

Comparison of EAG responses to the isoprenoids showed some interesting trends. Members from all the groups elicited significant antennal responses, with the most frequent responses being to the sesquiterpenes, all of which were highly active, whilst the highest overall responses were seen to the monocyclic monoterpenes, (+)-carvone and α-terpineol. In the monoterpenes, approximately half of the compounds tested showed significant electrophysiological activity, with the majority of significant responses being seen to the oxygenated compounds in each sub-group.

Of the four acyclic monoterpenes tested, the highest response was seen to the oxygenated compound linalool, with the only other significant response in this group seen to the non-oxygenated compound (*E*)-β-ocimene. Of the fifteen monocyclic monoterpenes tested, the five oxygenated compounds elicited the highest antennal responses, with the most active being (+)-carvone, whilst of the non-oxygenated compounds in this group, only four showed significant activity [(-)- and (+)-limonene, terpinolene and (-)-β-phellandrene). Of the thirteen bicyclic monoterpenes tested, the five oxygenated compounds were among the six compounds eliciting the highest significant antennal responses, with the oxygenated compound, (+)-*trans*-limonene-1,2-epoxide, being the most active in this group. Of the non-oxygenated compounds, only two showed significant activity [(+)-α-pinene and (+)-3-carene]. Of the eight sesquiterpenes tested, the highest EAG responses were seen to the non-oxygenated compounds, γ-bisabolene and (*E*)-β-farnesene, although the two oxygenated compounds in this group still elicited highly significant antennal responses.

Comparison of the EAG responses elicited to different enantiomers of the chiral monoterpenes tested showed some significant differences. The (-)-forms of limonene and fenchone elicited slightly higher responses than the (+)-forms, with all four enantiomers showing significant EAG activity. The reverse was found for α-pinene and carvone, where the (+)-forms elicited higher responses than the (-)-forms; in the case of α-pinene, only the (+)-form was significantly active. No significant EAG activity was elicited by either optical isomer of β-pinene.

The significance of the differences in the carrot flies' perception of some chiral host plant compounds is unclear, as few data are available on the quantities of enantiomers present in different species of the Apiaceae. However, from the available data, large differences between the relative proportions of enantiomers of chiral compounds do occur, and the ratios between the two enantiomers

varies widely between different plant species in the Apiaceae. For example, the enantiomers of α-pinene occur in the ratio 21:1 (+)- to (-) in *A. sylvestris*, but in the opposite ratio of 2:1 (-)- to (+)-α-pinene in *Aegopodium podagraria* (Borg-Karlson *et al*, 1994). As no enantiomers appear to be particularly novel for the species of Apiaceae investigated here, they may play more of a role in host preference of *P. rosae* than in its identification of host versus non-host species.

4.4.7 Non-Host Isothiocyanates

Significant responses were elicited by two of the three organic isothiocyanates tested (allyl- and 2-phenylethyl-isothioyanate). These compounds are mainly confined to species within the Brassicaceae and are not found in the Apiaceae, indicating that *P. rosae* is able to perceive non-host compounds, as well as those from its host plants. As these compounds have relatively high volatilities, they may be perceived at some distance from non-host brassicaceous plants, and as such, may be utilised for avoidance of unsuitable plant species (Pickett *et al*, 1999).

5 LABORATORY CULTURE OF P. rosae AND LABORATORY BEHAVIOURAL BIOASSAYS

5.1 INTRODUCTION

A bioassay is defined as an assay in which the detector is a living organism or part of its sensory system (Finch, 1986). During this study attempts, were made to develop a bioassay suitable for testing the behavioural responses of adult *P. rosae* to volatile compounds involved in host plant location, and detection at some distance from their source (i.e. primarily to look at olfactory host plant attractants). The aim was to produce a simple technique, whereby the presentation of an olfactory stimulus would elicit behavioural responses that could be scored and quantified, and that were discriminating and reproducible. Once developed, this bioassay could be used to supplement behavioural studies in the field, aimed at identifying attractant host plant extracts and/or compounds for *P. rosae* that could be incorporated into monitoring and pathogen traps to improve their efficiency. Bioassay could also be employed for ascertaining the 'long range' attraction of *P. rosae* to a range of its host species, producing a preference hierarchy to supplement the oviposition preference (involving 'short range' olfactory and contact chemical cues) and larval survival hierarchies determined in studies by Degen, Städler and Ellis (1999a, b and c). Such information would be very useful in providing a more complete picture of the stimuli involved in the various stages of the complex host location and acceptance behaviour of *P. rosae*. Such information would also be helpful in focussing attempts to identify the most important host plant attractants on those plants attracting the most flies in the field. Bioassays would also be useful in assessing the behavioural significance of the numerous (new and previously reported) compounds identified as electrophysiologically active in GC-EAG and EAG investigations during this study (Ch.3 and 4).

Bioassays for behavioural responses to volatiles detected at some distance from their source have been developed for both inter- and intra-specific interactions of insects. However, in general these have shown that insects' responses to host odours are much more difficult to monitor, being less specific than their intraspecific responses to pheromones, which are more likely to produce clear observable responses (Finch, 1986). As such, the use of a known pheromone for initial development of a bioassay method would be more likely to succeed, and the 'working' bioassay could be optimised in relation to various factors before being applied to the less focussed plant finding behaviour. Unfortunately, no pheromones

have yet been discovered or shown to exist for *P rosae*, so development of the bioassay had to proceed using only responses to host plant odours. As these attempts were unsuccessful, an alternative oviposition bioassay method was used to investigate key plant extracts and compounds of importance to this study (using a small number of flies). The oviposition bioassays during this study were performed using the improved oviposition assay for the carrot fly described by Degen and Städler (1997).

5.2 METHODS

5.2.1 Laboratory Rearing of *P. rosae*

P. rosae used in bioassays, electrophysiological experiments and other work performed throughout this study were reared in the laboratory, using a method based upon that of Städler (1971). Flies were initially collected in the field (Norfolk, England) as pupae, and were supplemented with donations from Dr. T. Degen and Dr. E. Städler of the Swiss Federal Research Station for Arboriculture, Viticulture and Horticulture, (CH-8820, Wädenswil, Switzerland). After emergence, flies were introduced into the rearing programme. *P. rosae* were reared in cubic screen cages (0.6m x 0.6m x 0.8m) in a climate controlled room (L12:D12, 20°C, 80% r.h.) with overhead fluorescent lighting. Cultures of *P. rosae* were supplied with water (glass bottle reservoir, with a cotton wool wick) and food (4 parts cane sugar: 1 part yeast hydrolysate: 1.5 parts water), which was applied to a filter paper hung near the top of the rearing cage and replaced weekly. Suitable oviposition sites were provided in the form of large square polyethylene bowls (0.4m x 0.4m x 0.2cm) containing host plants grown in a glasshouse. Each bowl contained approximately fifty two-month old carrot seedlings (cultivar Danvers 126 half long), 5 hemlock seedlings and 2 fully grown carrots (which were pushed into the peat). The bowls of plants were watered daily and replaced twice weekly with fresh ones. Bowls removed from the rearing cages and containing *P. rosae* eggs were stored in fresh rearing cages until the adults emerged.

Theoretically, larval experience might have altered subsequent feeding or oviposition behaviour of the adult insects (i.e. larval memory). However, Degen (1998b; 1998c) found that adult *P. rosae* were not biased by the larval food plant (carrot) used during fly culture, and no problems were envisaged with the use of laboratory reared flies for subsequent bioassay studies in this investigation.

5.2.2 Olfactometer Studies

During this investigation, numerous variations of the two-arm 'Y'-tube olfactometer, and the Petterson 'four-armed' or 'star' olfactometer (Petterson, 1970, and Vet *et al.*, 1983) were tested, to see if it was possible to observe differentiation in behaviours with different treatments. These included real host plant leaves, host MAS extracts, EAG active compounds and the known synthetic attractants (*trans*-asarone and

hexanal). However, none of the designs tested showed significant behavioural discrimination between the treatments, so the details of these experiments have been omitted.

5.2.3 Oviposition Bioassay

'Three dimensional hogweed' leaf models were used as the basis for these experiments, as they had been shown to elicit the highest oviposition responses from *P. rosae* of a range of other, 2-D, and 3-D leaf shapes (Degen and Städler, 1997a and b). Leaf models were made from light green cardboard ($300g/m^2$), with an area of $100cm^2$ (leaf section of 12cm x 12cm, with cut-outs) and a stem 9cm long and 1cm wide. The stem was strengthened and kept upright with a metal wire running down its centre and held in place with light green insulation tape. At the base of the stem, the wire protruded at a right angle and was used to attach the leaf to the oviposition device. The leaf models were folded to 45° angles across each leaflet and centrally down the length of the leaf and stem, mimicking natural leaf patterns. The leaves were then coated with a thin layer of paraffin wax, by dipping it into a water bath containing melted paraffin wax (Aldrich, m.p. 52-58°C). This had the effect of darkening the shade of the green cardboard noticeably and giving it a glossy texture. Host plant extracts (0.25ml of 25g/ml extract, equivalent to 6.25g of leaf) and compounds (γ-coniceine, 1ml of 0.1mg/ml hexane solution) were applied to the leaf models by spraying both sides of the leaf area and stem. Control leaf models were made similarly, but only sprayed with hexane solvent. Real host leaves used in these experiments were grown in a glasshouse and replaced daily during the experiments. Leaves of a similar size to the leaf models, and cut to the same length, were introduced through a narrow central opening in the grid into a small water-filled plastic vessel incorporated into the oviposition device.

The oviposition experiments were performed in a large mesh cage (1.0m x 1.0m x 1.6m) with a Perspex front, and a hatch allowing access. The cage contained approximately 200 adult *P. rosae* (of mixed age and sex) transferred from the laboratory culture. The cage was situated in an environmentally controlled room (L13:D12, 20°C, 80% r.h.) with overhead fluorescent lighting. Whilst in the cage, *P. rosae* were fed on a sucrose and yeast hydrolysate solution applied to filter paper and hung near the top of the cage; water was also provided via soaked cotton wool in a glass dish placed in the centre of the cage. The oviposition devices used in this bioassay consisted of glass dishes (9cm in diameter and 4cm high)

with a wet foam rubber lining covered with a black cloth and a black polyethylene grid (mesh 1mm) (Städler, 1971). Leaf models were fixed to the dish with two rubber bands, and the dishes were topped by an inverted black plastic pot with a 5cm x 5cm wide opening that allowed the flies only restricted access to the oviposition substrate. These covers were used because they increased the resolution of differences between treatments by reducing 'stray' oviposition, e.g. from flies that landed on neighbouring dishes instead of on the dish accompanying the leaf which had stimulated them to oviposit (Degen and Städler, 1997a). Two or four different treatments (as indicated in the individual experimental details) were presented simultaneously to the flies in choice bioassays. Eight treatment positions were marked out equidistantly in a circle of 0.25m radius on the base of the cage. Treatments were allocated to these positions randomly, with identical replicate treatment pairs opposite each other in the circle, or placed in a regular alternating sequence for experiments with only two replicates. The oviposition devices containing test leaves were exposed to the flies for one-day periods, after which the oviposition dishes were removed, and the eggs laid onto the black cloths were counted and removed. The treatment leaves were then replaced in the cage, with their positions rotated by one place in each subsequent experimental period (block), so that at the end of the experiment, each treatment had been in each position in the circle once. Egg counts were log (n+1) transformed, to normalise the count data which contained some zero counts, and were analysed by ANOVA as a randomised block experiment. The number of replicates (n) is given by the number of leaf models with the same treatment, multiplied by the number of experimental periods (of one day each) during which the treatments were exposed to *P. rosae*. The number of eggs expressed in the graphs are percentages of the total eggs laid during the experiment (back transformed from ANOVA), with 95% confidence intervals.

The sources of synthetic compounds and plant materials extracted for use in these oviposition bioassays are the same as those given in Chapter 6. Plant extracts were produced by microwave-assisted solvent extraction using hexane as solvent (for further details see Method 2.2.2). Plant extracts were applied as treatments at a dose of 0.25ml of 25g/ml extract, equivalent to the extract of 6.5g of fresh leaf material; this was representative of the weight of a real leaf of the size used in these experiments.

The first experiment was performed to check that there was a good resolution between the levels of oviposition elicited by the control leaf model (green three dimensional hogweed model, lacking

chemical oviposition stimulants), and a real host plant leaf (*D. carota*), which would provide a strong source of chemical oviposition stimulants. This experiment was set up as described in Table 5.1 Exp.1.

The second experiment was performed to compare the oviposition stimulatory effects of three host plant leaf extracts (*D. carota*, *A. graveolens* and *C. maculatum*), which were the main plant extracts tested in field experiments (see the following chapter - Ch. 6). This experiment was set up as described in Table 5.1 Exp.2.

The third experiment was performed to determine the behavioural activity of a newly identified electrophysiologically active compound, γ-coniceine. In this experiment, the oviposition stimulatory effects of γ-coniceine alone, and in combination with *D. carota* extract, were compared. This experiment was set up as described in Table 5.1 Exp.3.

Table 5.1: Experimental Details of Oviposition Experiments

Experiment 1
Treatments:
A) Control leaf model (treated with solvent only)
B) Real carrot leaf
Each treatment was replicated four times in each experiment, and the experiment was performed for two days (n=8 for each treatment).

Experiment 2
Treatments:
A) Control leaf model (treated with solvent only)
B) Leaf model - treated with *D. carota* leaf extract
C) Leaf model - treated with *A. graveolens* leaf extract
D) Leaf model - treated with *C. maculatum* leaf extract
Each treatment was replicated twice in each experiment, and the experiment was performed for four days (n=8 for each treatment).

Experiment 3
Treatments:
A) Control leaf model (treated with solvent only)
B) Leaf model treated with *D. carota* leaf extract
C) Leaf model treated with γ-Coniceine (1ml, of 0.1mg/ml solution in hexane)
D) Leaf model treated with *D. carota* leaf extract, plus γ-coniceine
Each treatment was replicated twice in each experiment, and the experiment was performed for four days (n=8 for each treatment).

5.3 RESULTS

5.3.1 Laboratory Rearing of *P. rosae*

This method of rearing provided a constant supply of adult *P. rosae*, usually within the range of 50-300 per rearing cage, with a minimal time requirement for its upkeep.

5.3.2 Olfactometer Studies

None of the olfactometer designs tested produced significant behavioural discrimination between different treatments, including real host plant leaves, host MAS extracts, EAG active compounds and the known synthetic attractants (*trans*-asarone and hexanal), so the results of these experiments have been omitted for brevity.

5.3.3 Oviposition Bioassay

The results of the first experiment (Table 5.2 Exp.1, Fig.5.1) showed a highly significant difference between the numbers of eggs elicited by the two treatments. The figure shows that the real carrot leaf elicited significantly more oviposition than the control, a host-leaf model with no chemical oviposition stimulants applied.

The results of the second experiment (Table 5.2 Exp.2, Fig.5.2) showed a highly significant difference between the numbers of eggs elicited by the four treatments. The figure shows that *C. maculatum* leaf extract elicited the highest level of oviposition, receiving significantly more eggs than the other three treatments. The control, *D. carota* leaf extract and *A. graveolens* leaf extract all received similar numbers of eggs, and from the 95% c.i. were not significantly different from one another.

The results of the third experiment (Table 5.2 Exp.3, Fig.5.3) showed a highly significant difference between the numbers of eggs elicited by the four treatments. The figure shows that *D. carota* leaf extract plus γ-coniceine elicited the highest level of oviposition, receiving significantly more eggs than the other three treatments. The control, *D. carota* leaf extract alone and γ-coniceine alone all received similar numbers of eggs, and from the 95% c.i. these were not significantly different from one another.

Table 5.2: **Statistical Analyses of Oviposition Experiments: Analysis of Variance, Log_{10} (number of eggs +1).**

Experiment 1

Source of Variation	d.f.	s.s.	m.s.	*F*	*P*
Day	1	0.0132	0.0132	0.13	
Treatment:	1	6.9015	6.9015	67.75	<0.001
Error	13	1.3243	0.1019		
Total	15	8.2390			

Experiment 2

Source of Variation	d.f.	s.s.	m.s.	*F*	*P*
Day	3	0.24444	0.08148	1.78	
Treatment:	3	2.01572	0.67191	14.66	<0.001
Error	25	1.14607	0.04584		
Total	31	3.40623			

Experiment 3

Source of Variation	d.f.	s.s.	m.s.	*F*	*P*
Day	3	0.49185	0.16395	3.40	
Treatment:	3	1.148515	0.49505	10.27	<0.001
Error	25	1.20450	0.04818		
Total	31	3.18150			

Fig. 5.1: **Mean number of eggs laid during oviposition choice assay. Data are percentages of total eggs laid, ± 95% confidence intervals. Details of design are in Table 5.1 Exp1.**

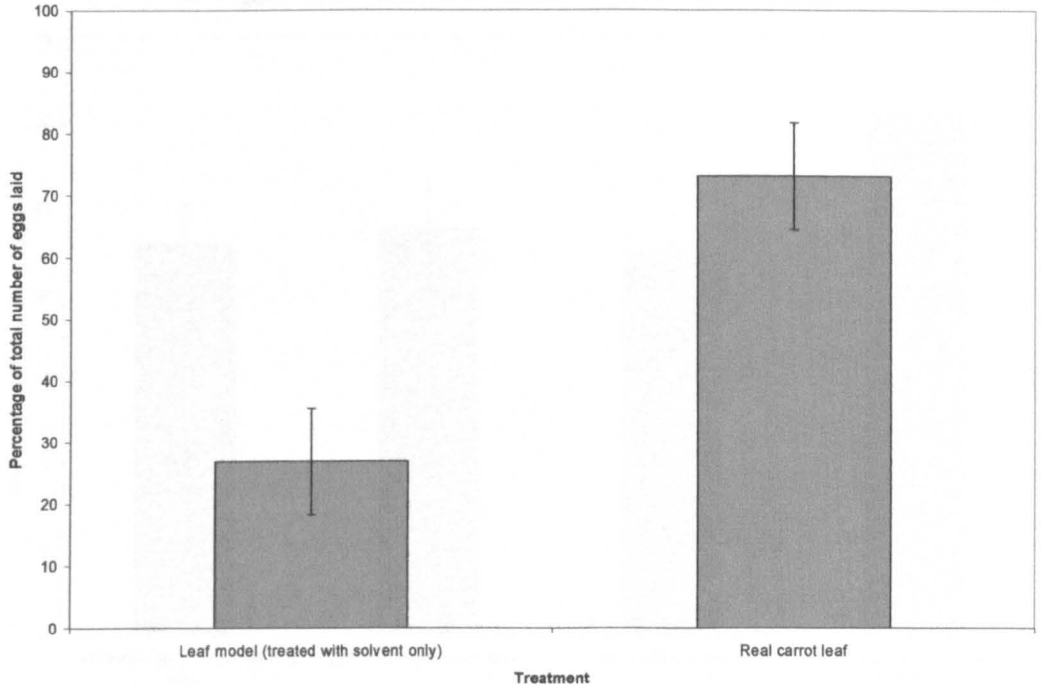


Fig.5.2 : **Mean number of eggs laid during oviposition choice assay. Data are percentages of total eggs laid, ± 95% confidence intervals. Details of design are in Table 5.1 Exp2.**

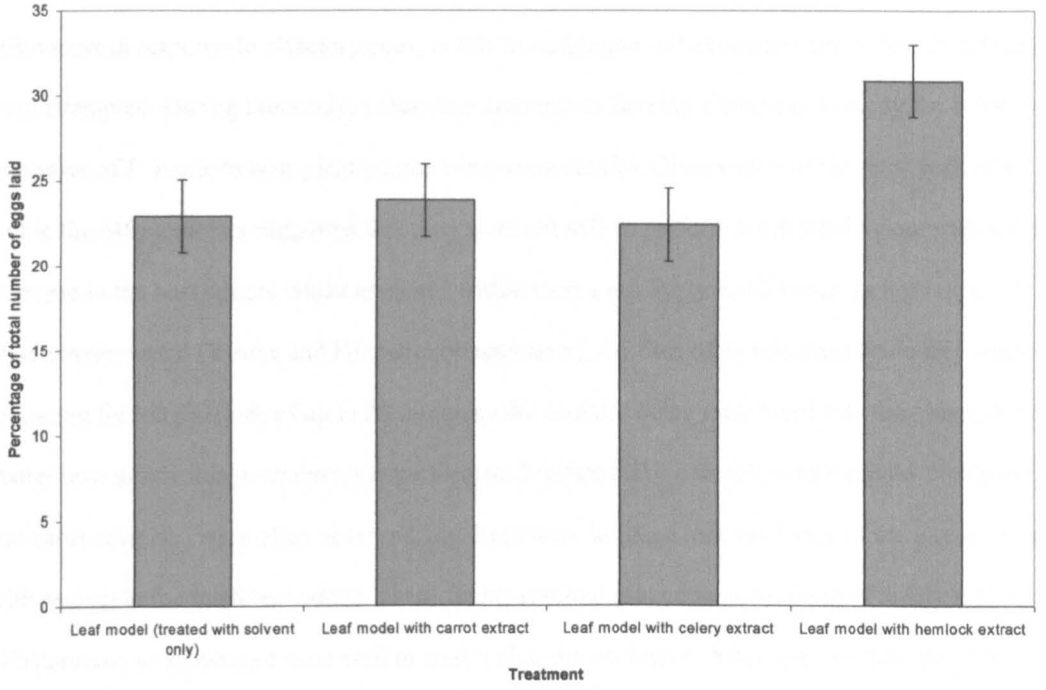
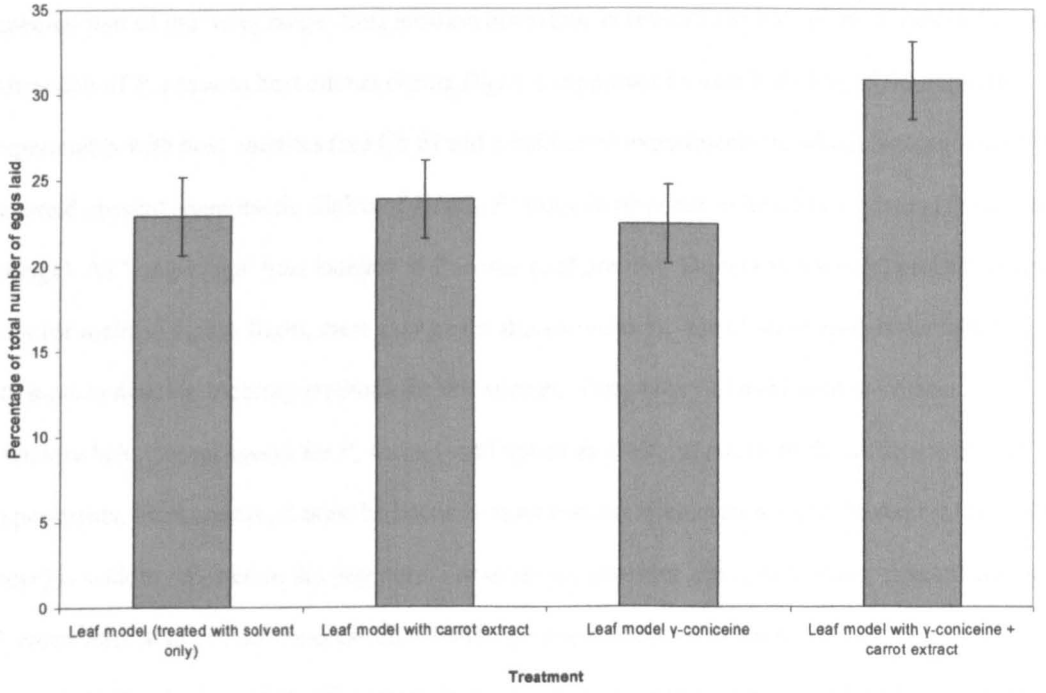


Fig. 5.3: **Mean number of eggs laid during oviposition choice assay. Data are percentages of total eggs laid, ± 95% confidence intervals. Details of design are in Table 5.1 Exp3.**



5.4 DISCUSSION

There are a number of different bioassay methods which might be used to differentiate between behaviours in response to olfactory cues; in this investigation, olfactometers and oviposition bioassays were attempted. During this study, exhaustive attempts to develop a bioassay to study the behavioural responses of *P. rosae* to host-plant odours were unsuccessful. Observation of the flies' behaviour within the olfactometers suggested that they were not able to perform normal behaviour patterns in response to the host odours whilst enclosed within such a relatively small space. In both types of olfactometer tested (Y-tube and Four-arm olfactometer), the flies often remained stationary while grooming for long periods of up to 20 minutes after initially being introduced into the olfactometer, no matter how gently this procedure was performed. Frequent take-offs followed by rapid flights (of long and short duration) were often observed, but these were initiated independently of the insect's position with respect to the treatment odour. These flights resulted in random movement of the fly around the olfactometer, as it bounced from wall to wall within the enclosure. After this exertion, the flies alighted and were possibly stunned or physically damaged, as they often remained stationary over long periods, after which extended grooming sessions occurred. These results suggest that flight is an essential part of the 'long range' host location behaviour in response to host plant odours in *P. rosae*. Attraction of *P. rosae* to host odours during flight is supported by data both from field trapping experiments with host volatiles (see Ch.6) and wind tunnel experiments, in which Nottingham (1987) reported upwind anemotactic flights of female *P. rosae* in response to host plant odours (*D. carota* foliage). As 'long-range' host location in *P. rosae* (and possibly Diptera as a whole) probably occurs only (or mainly) during flight, there may be no alternative to the use of wind tunnels for future attempts to develop bioassay methods for this species. The paucity of published references to olfactory behavioural assays for *P. rosae* (and Diptera as a whole) attests to the difficulty of such experiments. Furthermore, it must be borne in mind that negative evidence (i.e. bioassays that did not work) is seldom reported in the literature. For example, previous attempts at using olfactometers with *P. rosae* have also proven unsuccessful (Städler personal communication).

In the absence of an olfactory bioassay for *P. rosae*, supplementary oviposition experiments were performed in order to determine the behavioural activity of the newly identified electro-physiologically active compound γ-coniceine.

The first experiment confirmed that the leaf model without host oviposition stimulant, was significantly less stimulatory for oviposition than a real host leaf (*D. carota*) (Fig.5.1). As such, it was suitable for use as a non-stimulatory control, and as a base upon which to apply chemical treatments.

The second experiment was used to gauge the oviposition stimulatory effects of microwave-assisted solvent extracts of three host plant species of interest. These included hemlock (*C. maculatum*), from which γ-coniceine had originally been identified as electrophysiologically active during GC-EAG studies (see Ch.3). The results of this experiment showed that *C. maculatum* extract was significantly more stimulatory than *D. carota* or *A. graveolens* extracts, neither of which elicited significantly more eggs than the control (Fig.5.2). This higher oviposition stimulatory activity of *C.* maculatum extract, relative to *A. graveolens* and *D. carota* (cultivar 'Danvers') extracts, was in accordance with its position in the oviposition preference hierarchy as determined by Degen, Städler and Ellis (1997b). As hemlock is highly preferred for oviposition, and is the only apiaceous species containing γ-coniceine (which produced large 'off-scale' EAG responses during electrophysiological experiments), it was thought that this compound might have a pivotal role as an oviposition stimulant and/or attractant for *P. rosae*.

The third experiment showed that γ-coniceine plus *D. carota* extract elicited significantly more eggs than either γ-coniceine alone, or *D. carota* extract alone, neither of which elicited significantly more eggs than the control (Fig.5.3). This result provides evidence that γ-coniceine is a behaviorally active compound for *P. rosae*, as was initially suggested by the large EAG response it evoked during the previous electrophysiological studies (Ch.3 and 5). As γ-coniceine alone did not elicit significantly more oviposition than the control, and needed to be supplemented with other relatively weak stimulants in the *D. carota* extract (see previous experiment) before flies were induced to lay significantly more eggs than for the control, γ-coniceine only seems to act as a relatively weak oviposition stimulant. However, these findings do discount the possibility that γ-coniceine acted as a repellent or deterrent, as neither γ-coniceine alone, or in combination with *D. carota* extract, produced a significant reduction in the number of eggs laid as compared to the control, and *D. carota* extract alone, respectively.

A possible alternative to γ-coniceine acting as a weak oviposition stimulant could be that it acted as an attractant or arrestant in the locality of the leaves to which it was applied. If this was the case, more flies may have been attracted onto, or kept for longer, on the leaves with γ-coniceine. In

either of these situations, more flies may have built up sufficient stimulation (either though more frequent landing or extended visits) from the weakly stimulatory *D. carota* extract to trigger oviposition below this leaf. Flies concentrated on the leaf with γ-coniceine alone would not have received oviposition stimulation while on this leaf (without *D. carota* extract) and so would not have oviposited below this treatment. In any case, further behavioral studies would need to be performed before a valid behavioral role could be ascribed to this compound.

In the absence of an olfactory bioassay for *P. rosae*, all further work aimed at identifying host plant attractants, or determination of the behavioural activity of the newly identified electrophysiologically active compounds, had to be performed in the field.

6 FIELD EVALUATION OF PLANT VOLATILES FOR ATTRACTANCY TO *P. rosae*

6.1 INTRODUCTION

Carrot fly monitoring is recommended by ADAS, HRI and the Pesticide Safety Directorate (1995) to all growers of susceptible apiaceous crops. This aids in the optimum timing of first insecticide application and indicates the need and frequency of subsequent applications, reducing unnecessary use. However, trapping efficiency in monitoring programmes is so low that economic thresholds are essentially based upon the presence or absence of captured flies (Dufault and Coaker, 1987).

The main objective of these semiochemical field trials was to try to increase the efficiency of *P. rosae* population monitoring, primarily by increasing sticky trap catches at low population densities and ideally increasing catch numbers by 4-5 times. To do this, plant extracts or compounds would need to be incorporated into the monitoring traps, and to stand out as particularly strong points of attraction from the general background of attractive *D. carota* volatiles and the visually attractive stimuli associated with the large expanse of surrounding carrot foliage. It would also be advantageous to increase the selectivity of the monitoring traps by reducing the number of non-target insects caught, as this would increase the efficiency of the traps as well as making counting *P. rosae* more accurate. Further studies were also aimed at identifying possibly repellent extracts or compounds, as these might also be useful in future carrot fly control measures, such as incorporation into an integrated pest management (IPM) 'push/pull' strategy.

P. rosae is dispersed throughout the UK, with areas of high population infestations occurring in the main carrot growing regions such as the Isle of Ely, Norfolk (Hinton, 1971; Coppock, 1974). Within these areas of high background populations, field trial sites were chosen based upon previous high catch data from ADAS population monitoring and their close proximity to areas where preferred host crops had been grown during the previous year, thereby providing a nearby source of newly emerging adult flies. Within areas of high background populations, it is known that adult flies are not dispersed randomly but are contagiously dispersed, mainly aggregating around roosting sites, particularly hedgerows and trees surrounding host crops (Petherbridge *et al.*, 1942; Wainhouse, 1975), with their numbers shown to decline further into the crop (Petherbridge *et al.*, 1942; Wright and Ashby, 1946a). Even within these field

surrounds, their distribution is not random, but aggregated, with particularly high numbers often associated with trees (possibly linked to tall silhouettes against the sky line; Städler, 1972), hedgerow density and particular herbage species, such as the presence of *Urtica dioica* (common nettle) and *C. maculatum* (hemlock) (Wainhouse and Coaker, 1981; Petherbridge *et al.* 1942; Coppock, 1974). Consequently, these factors had to be taken into account during the allocation of field sites, placement of traps in the field and during experimental design. Trap placements were standardised as much as possible, with traps placed around the edges of fields avoiding, where possible, obvious differences in nearby roosting site quality, such as gaps or stark changes in vegetation occurring within a block of traps. The effects of inconsistencies in hedgerow quality and the presence of "hot spots" in population densities were also limited by allocating treatments randomly to traps within each block. The distribution of adults with time is also uneven, as the adults emerge as distinct generations throughout the year (Wright and Ashby, 1946b; Coppock, 1974). Populations reach a peak when the majority of adults have emerged from pupation in each generation, after which numbers gradually tail off. The number of adult flies available for capture on monitoring traps also varies on a daily basis depending upon the number of flies leaving the roosting sites in the hedgerows, which has been shown to depend heavily upon climatic conditions including wind speed, light intensity, temperature, rain and humidity (Wakerley, 1963 and 1964; Esbjerg *et al.*, 1983). In order to reduce the effects of uneven distribution of adults in time, and their patchy distribution in the hedgerows (i.e. due to hot spots), the treatment positions within blocks were re-randomised with each successive date they were put out in the experiment. This was done according to a Latin square design (Fowler and Cohen, 1994a), with treatments allocated to each position following the successive rows of a Latin square. This meant that each treatment had been in each trap position within a block at the end of a complete experiment. Where flies were still present at the end of an experiment, it was usually continued following the same Latin square order, to facilitate statistical analysis of a whole Latin square of results, even if the early part of the experiment had showed infrequent fly catches.

As the visually attractive characteristics of the monitoring system have already been optimised (Collier *et al.*, 1990), these field trials focused on investigating the effects of incorporating the release of biologically relevant extracts or compounds from the visually attractive REBELL ® orange carrot fly

sticky trap. This was achieved by the release of extracts of known apiacious host plant species, or electrophysiologically active compounds (singly or in combination) which had previously been identified in the extracts of host species by GC-EAG and GC-MS (Ch.3 and 4). To facilitate the release of host derived extracts or compounds from REBELL ® orange traps, suitable lures had to be devised. These had to facilitate release of the extract or compounds over the whole period that the trap was out in the field (i.e. between consecutive randomisations). Additionally, in the case of individual compounds, the aim was also to devise lures capable of giving approximately constant release rates of compounds over this period. In order to achieve this, various lure designs were tested under laboratory conditions of constant temperature and airflow, in order to estimate their release rates and how long they would release for, prior to their inclusion in field experiments. However, it should be borne in mind that, once out in the field, their release rates would obviously fluctuate in response to fluctuations in the environmental conditions.

In previous field trials by Guerin *et al.* (1983), a wide range of apiaceous essential oils and individual host plant derived compounds and mixtures were tested. The combination of hexanal and *trans*-asarone was found to be the most attractive to *P. rosae*. This combination was shown to produce an approximate doubling of catch on a visually attractive sticky trap, compared to similar unbaited traps. As the best known field attractant for *P. rosae*, this combination was included as one of the treatments in many of the field experiments, where it was used along with an unbaited control to help gauge the effectiveness of other treatments as attractants

During this field investigation, a wide range of treatments were tested in various combinations, including various plant extracts, a number of different slow release mechanisms and a range of individual host plant compounds. This resulted in a complex series of experiments, which for clarity have been divided into three main categories: those involving plant extracts in general; those concentrating on *C. maculatum* extract and its constituent compounds; a comparison between standard and baited (*trans*-asarone and hexanal) monitoring traps, in collaboration with ADAS. Within the first category involving plant extracts, the experiments are further sub-divided into three groups: A) investigation in which extracts were released form 'polyethylene vial' lures; B) an attempt to improve upon this release system, which included a field comparison of the best new release system, with the old 'polyethylene vial lure'; C) investigations in which extracts were released from the improved 'glass bottle with wick' lures.

The newly developed method of microwave assisted solvent (MAS) extraction was chosen for release in these experiments, as in a previous comparative study of extraction techniques it was found to rapidly extract large quantities of biologically relevant volatiles, suitable for release in the field (see Ch. 2). The main aim of the initial field experiments was to determine whether host plant extracts produced using the new MAS extraction method could be used as attractants for *P. rosae* in the field, as previous field experiments using host plant 'essential oils' failed to show attraction (Guerin *et al.*, 1983). It was hoped that, as extracts produced using the microwave-assisted methods had produced significant electrophysiological responses during previous GC-EAG work (see Ch.3), the host MAS extracts might prove attractive in the field.

For these first field experiments, a 'polyethylene vial' lure, originally used by Guerin *et al.* (1983) for the release of synthetic mixtures of green leaf volatiles, was chosen as the slow release mechanism for plant extracts. This release system was chosen as the green leaf volatiles would have comparable volatilities to the compounds of interest identified in the microwave assisted solvent extracts, as seen by the similarities in GC elution times of these compounds during GC-EAG studies (Ch.3). Various host and non-host extracts were included in these experiments, either alone, or in combination with the known synthetic attractants *trans*-asarone and hexanal. The combined release of extracts plus synthetic attractants was performed in an attempt to boost catches above the level possible with either treatment alone, and it was hoped that some of these interactions might prove synergistic in nature (as was previously found to be the case for *trans*-asarone and hexanal). Extracts of *D. carota* and *A. graveolens* foliage were of particular interest, and were included in these experiments because they were available as waste products from agricultural crops. This would give an economic advantage if they proved to be attractants in the field, as bulk material would be readily available for extraction at a minimal cost. *C. maculatum* was also of special interest, and was included because it was known to be a preferred wild host species for *P. rosae* (Petherbridge *et al.* 1942; Petherbridge and Wright, 1943; Wainhouse and Coaker, 1981). The other host plant extracts tested included *F. vulgare* and *D. carota* root. Bulb extract of the non-host species *A. cepa* was included in one experiment as having a possible repellent or masking odour. The possible repellent nature of *A. cepa* for *P. rosae* was suggested following intercropping experiments, in which carrots planted with *A. cepa* received less larval damage than stands of carrots alone. In one

experiment an alternative extract release system ('glass bottle with wick' lure) to the 'polyethylene vial' lure was also tested using *A. graveolens* extract. In addition, a small investigation was undertaken into the effect on *P. rosae* catches when traps were positioned in two different types of field surround, i.e. hedgerows versus tall grasses.

Due to the poor performance of the 'polyethylene vial' lures in the first field experiments improvements to the release system used for plant extracts were investigated. This involved a laboratory investigation into a number of new lure designs (using *C. maculatum*), which focussed on extending the period over which the lures released volatile compounds. The efficiency of the 'best' new release system ('glass vial with wick') was then compared with that of the old 'polyethylene vial' lure, in a second field investigation using *D. carota* and *A. graveolens* extracts.

In the third field investigation, plant extracts were released from the improved 'glass bottle with wick' lure. Further experiments were performed to investigate the effects of combining the release of *A. graveolens* extract and the attractant combination of *trans*-asarone and hexanal. Preliminary release rate studies were also undertaken to identify suitable methods for the release of *trans*-methylisoeugenol, followed by some minor experiments investigating the effects of combining plant extracts with the release of an alternative attractant combination, *trans*-methylisoeugenol and hexanal.

In a forth field investigation *C. maculatum* extract and its constituent compounds were chosen for further investigation, in view of the status of this species as a preferred wild host and its strong links to the number of flies caught in field surrounds. γ-Coniceine was of particular interest for field investigation, as it had never previously been reported as biologically active for *P. rosae* yet was seen to elicit the strongest (off-scale) responses of any compound during the GC-EAG experiments (Ch.4). β-Caryophyllene and β-ocimene were also included in this investigation, as they were major components in the *C. maculatum* extracts as well as producing significant electrophysiological responses with GC-EAG.

In the later series of field experiments, the effect was also investigated of combining these compounds and *C. maculatum* extract with the attractant compounds *trans*-asarone and hexanal or *trans*-methylisoeugenol and hexanal. In order to incorporate these individual compounds into the field experiments, suitable lures for their release had to be developed and tested in release rate experiments. As γ-coniceine is known to be

a relatively unstable compound, a further air entrainment experiment was performed to determine the actual rates of γ-coniceine, and any breakdown products, being released from its lure.

During the field experiments as a whole, the attractant combination of *trans*-asarone and hexanal was consistently shown to double (or more) the number of *P. rosae* caught, compared to unbaited REBELL ® control traps. This increased catch was significantly higher than that seen in response to any plant extract alone. As a result, extensive field experiments were undertaken in collaboration with ADAS to investigate the performance of *trans*-asarone and hexanal baited REBELL® traps for improving carrot fly monitoring. This required comparison of catches on paired traps, one baited with *trans*-asarone and hexanal lures and the other unbaited, over a wide range of *P. rosae* populations in the UK.

Towards the end of these field investigations, a number of possible problems with the *trans*-asarone and hexanal lures were identified. Firstly, it was noted that some of the *trans*-asarone lures became discoloured while out in the field, with the chemically treated areas turning slightly brown. This suggested that some sort of chemical change or decomposition may have been occurring, possibly as a result of oxidation or light (or UV) induced changes. Consequently, a further air entrainment experiment was performed to determine the actual release rates of *trans*-asarone, and any possible breakdown products, from this lure. Secondly, ADAS operatives found activating the hexanal 'amber glass vial' lures in the field difficult, as the small solid caps used during transport had to be changed for caps with a hole, to allow hexanal release. It was also found that significant losses of hexanal sometimes occurred during postal transit to the ADAS operatives, due to leakage between the vial and polyethylene cap. Consequently, further experiments were performed aimed at identifying a suitable replacement for the hexanal 'amber glass vial' lure, which could be used in any future studies using this attractant. The aim was to solve the problem of leakage by producing a completely sealed lure which did not require any activation procedure before it started to release hexanal.

6.2 METHODS

Sources of Synthetic Compounds, Plant Materials and Extraction Techniques

The following solvents and synthetic compounds were obtained from commercial sources: *trans*-asarone (α-asarone or *trans*-2,4,5-trimethoxypropenylbenzene, 99% - Aldrich); *trans*-methylisoeugenol (1,2-dimethoxy-4-propenylbenzene) [93% *trans*- isomer (the rest 7% *cis*- isomer), - Aldrich]; hexanal (99% +, - Avocado); β-caryophyllene (99% - Fluka); β-ocimene [70:30 mix of (*E*) and (*Z*)- isomers (99%+, Quest)]; BHT anti-oxidant (2,6-di-*tert*-butyl-4-methylphenol or butylated hydroxytoluene, 99% - Sigma); nonane (99%+, - Avocado); hexane (99%+, Distol hexane, Fisher Scientific); AR-acetone (99%+, Fisher Scientific). γ-Coniceine (98%) was synthesised by Dr Mike Birkett, at IACR-Rothamsted.

The sources of plant materials extracted for use in these field experiments are given in Ch. 3.2.1. All plant extracts for release during these investigations were produced in bulk just prior to the start of the field experiments and were stored in a freezer (25g/ml fresh weight equivalent) until required for lure production. Plant extracts were produced by microwave-assisted solvent extraction using hexane as solvent, for further details see Method 2.2.2. This method was chosen as, in a previous comparative study of extraction techniques, it was found rapidly to extract large quantities of biologically relevant volatiles suitable for release in the field (see Ch. 2).

General Methods Used in Lure Production and Calculation of Release Rates

As many of the extracts and compounds identified as electrophysiologically active for *P. rosae* are highly volatile in nature, methods had to be employed to slow and steady their release from the traps in the field. The first method, used for plant extracts and hexanal in this study, involved enclosing the volatile material within a sealed vial with only small holes in the cap to limit the evaporation rate (Guerin *et al.*, 1983). Another method, used for most of the synthetic compounds, involved applying undiluted liquid compound to a piece of cellulose sponge, which was then heat-sealed into a small bag made from polyethylene tubing. This slowed the release of the compound, as it first had to diffuse through the polyethylene barrier before it could evaporate from the outer surface. Different release rates were obtained by altering the type and surface area of the cellulose sponge, and the thickness (gauge) of polyethylene (Smart and Blight, 2000).

During this investigation, two types of spongy material were used as support media in the preparation of this type of lure, thin and thick 'bread-like' sponge. Both were prepared from larger sponge sheets purchased from Sainsbury's plc. Thin sponges were made from 'Sainsbury's Coloured Sponge Cloths' (20.5cm x 18.5cm x 0.25cm), Code 0032 6865 (J. Sainsbury plc). Thick 'bread like' sponges were produced from 'Sainsbury's Cellulose Sponge Wipes' (15cm x 11.5cm x 1cm Code 0013 4811 (J. Sainsbury plc). In order to remove colorants and fungicide treatments with which the sponges were impregnated, they were washed and continuously extracted in chloroform using a Soxhlet Extraction apparatus as follows. The sponges were washed in warm soapy water and thoroughly rinsed in hot water. The excess water was removed and they were slowly dried in an oven at 24°C over 4 days. Once dry, the sponge sheets were cut into 2.5cm x 2.0cm rectangles and loosely packed into the body of a large Soxhlet extraction apparatus, to which a 1 litre round bottomed flask containing 850ml of AR-chloroform was attached. A heating mantle was then used to heat the flask until chloroform started to reflux, after which the sponges were left to be extracted continuously for 24 hours. The sponge pieces were then removed from the Soxhlet Extraction apparatus and the chloroform allowed to evaporate for 12 hours in a fume cupboard, followed by a further 4 hours in an oven at 24°C. Following this procedure, 2.5cm x 2.0cm sponge pieces were referred to as whole sponges (½ sponge = 2.0cm x 1.25cm; ¼ sponge = 1.25cm x 1.0cm; $^1/_8$ sponge = 1.0cm x 0.6cm). The maximum load capacity was estimated for different sized pieces of thin sponge (before leakage occurred) using hexanal. These were found to be approximately: 800µl for a whole thin sponge, 400µl for ½ a thin sponge, 200µl for ¼ of a thin sponge and 100µl for $^1/_8$ of a thin sponge.

Polyethylene tubing for construction of the bags was available in a range of different gauges (abbreviated to 'G' in this thesis): 100G, 250G, 500G and 1000G (A1 Packaging Ltd., London). When polyethylene thicker than 1000G was required, bags were sealed within larger bags (i.e. to produce 2000G bag lures). Where the release of compounds from polyethylene bags was still too rapid, a thicker polyethylene barrier was achieved by enclosing the compound in a small polyethylene micro test tube (250µl, Cat. No. 223-9470, Bio-Rad Laboratories), a polyethylene vial (0.75ml, Just Plastics Ltd. or 3ml, WP/5 - Fisons), or a polyethylene vial within a vial [0.5ml vial (Just Plastics Ltd) inside a 3ml vial (WP/5

- Fisons)]. Further reductions in release rate were obtained using higher density polypropylene micro test tubes (250µl, Cat. No. 223-9471, Bio-Rad Laboratories).

Nominal release rates from lures were determined by cumulative weight loss during preliminary laboratory experiments. Generally, three replicates, of a range of lure designs, were tested for each compound required in the field experiments. The materials used in construction of the lures were weighed (Analytical plus, OHAUSE®) prior to application of the required liquid compound, and again immediately after preparation of the lure. The lures were then hung in a constant temperature room at 20°C with 0.5m/sec airflow, and re-weighed on a regular basis until weight loss ceased. The mean cumulative weight loss for each type of lure was then plotted with standard errors (±SE), and these are presented in Appendix 2 (Fig. A1 - A14). From these graphs, the release rate period over which steady rates could be maintained, and any lag period before the onset of release, were estimated for each type of lure. This information was then summarised, and is presented in Table 6.3. In general, lures providing steady release rates of between 5 and 10 mg of compound per day, for at least four days to a week, were selected for use in the field.

The release rates of volatiles emanating from lures comprising multi-component extracts (in solvent) were determined by air entrainment. This method was also used in situations where decomposition of an unstable compound was suspected. Here, it was used to determine the actual release rate of the original compound, and for isolating any released contaminants for further investigation. These air entrainment experiments were performed as previously described in Method 4.4.4a., and the apparatus was assembled in a similar manner to that described in Method 4.4.4b (see Fig. 4.2). The experiments were performed in a temperature controlled room at 20°C, with fluorescent strip lighting operating in a 12-hour light / 12-hour dark regime to simulate field conditions. The cleaned airflow was split and entered a number of glass entrainment chambers. The first chamber was always left empty as a blank control, and the lures under investigation were suspended in the remaining chambers using stainless steel wire hooks. Where the lure under investigation produced only a slow release of volatiles (i.e. *trans*-asarone lure), the continuous method of entrainment was performed as previously described (method 4.4.4b).

As release rates from the other lures investigated were relatively high, the large quantities of volatiles collected would rapidly swamp the Porapak trap, resulting in breakthrough and loss of

compound. To solve this problem, lures releasing large quantities of material were entrained in short bursts (typically three hours). These short entrainments were spread periodically throughout the release rate experiment to reduce the quantities of volatiles collected on each Porapak tube. During these periods, the volatiles released inside the chambers were carried into the traps with an airflow rate of 700ml/ min, where they were adsorbed onto Porapak Q (100mg of 50-80 mesh). After each collection period, the volatiles were extracted with 1ml of redistilled diethyl ether per Porapak tube. During the periods when volatiles were not being collected, blank pieces of glass tubing were substituted for the Porapak traps. The samples from each period of entrainment were analysed by gas chromatography (HP 5880 GC, fitted with a 50m HP1 column), allowing their separation into individual compounds. Identification and quantification of the compounds was achieved by comparison with GC retention times and peak area counts of authentic standards (100ng) analysed on the same GC. Where excessive numbers of compounds were isolated by entrainments (i.e. from lures containing extracts), the resulting data was simplified for graphical presentation by only presenting the results of a number of main (or key) compounds.

General Field Trapping Method and Experimental Block Protocol

Field evaluation of the responses of *P. rosae* to volatile plant extracts and compounds was achieved using commercially available carrot fly monitoring traps, which were baited with lures releasing the volatile material. The monitoring trap chosen was the double-sided carrot fly sticky trap 'REBELL ® orange' (Swiss Federal Research Station, CH-8820 Wädenswil). This was made from polystyrole with cadmium-free pigment coated with TANGLE-TRAP insect trapping adhesive, and is the standard trap used by the ADAS carrot fly monitoring service in the UK. Traps were generally mounted vertically, at an initial height of 10cm above ground level where seedlings were just emerging (to reduce dust and rain splash contamination), or with the base of the trap just above the carrot foliage height, whichever was the greater. This method is in accordance with the manufacturer's instructions and ADAS monitoring protocols. Positioning of the traps was accomplished by attachment to 1.5m long aluminium angle-iron stakes, using two large foldback clips. Trap heights were adjusted to accommodate foliage growth with each randomisation of the traps. Traps were positioned along the edge of the carrot crop, with the traps running parallel to the crop border. Individual traps within an experimental block were positioned 15m apart, with

different blocks separated by at least 20m, unless otherwise stated in the individual experimental methods (i.e. due to site constraints). These spacings were chosen in order to minimise any visual or treatment interactions, both within and between experimental blocks. Treatments applied to traps within experimental blocks were allocated according to the first row of a Latin square design, with the treatment positions re-allocated according to the following rows of the Latin square every time the traps were removed for counting. Counting of the number of carrot flies caught per trap was achieved by putting the traps (and used lures) into clear polyethylene bags, labelled with the trap position and treatment. These were taken back to the laboratory for identification under magnification, and positions of identified carrot fly were marked on the bags along with the totals for each trap. Traps were generally removed for counting on a weekly or twice weekly basis, based upon ADAS monitoring data, in an attempt to keep the number of flies caught per randomisation more constant. Lures which released volatiles shortly after production were produced the night before they were required in the field and were stored in a fridge at 5°C. Others, identified as having a period of lag between production and the onset of volatile release, were produced earlier and preconditioned in the laboratory until volatile release started. Lures in the field were generally replaced weekly, when the traps were changed and re-randomised; where more frequent lure changes were necessary, details are given in the individual experimental methods. In the field, *trans*-asarone 'Energy board' strip lures were attached along the vertical edge of the REBELL ® traps, clamped using the two foldback clips which attached the trap to the stake. Similarly, the 'polyethylene bag' lures, used for releasing synthetic compounds, were attached by clamping the edge of the bag under one of the fold back clips. Lures consisting of two small vials wired together (i.e. 'polyethylene vial' lure for extracts, and the 'amber glass vial' lure for hexanal) were hung through the central hole in the trap, via the connecting wire between them, with one vial either side. The 'glass bottle with wick' lures for releasing extracts were hung from the central hole in the trap, via a wire hook attached to the lure.

General Statistical Methods Used in Analysis of Field Trial Data

As statistical analysis of the field trial results involved the comparison of several means of catch data, the parametric technique of analysis of variance (ANOVA) was applied. Where whole Latin squares of catch data were available, the Latin square version of ANOVA was applied, which enabled the partitioning of

sums of squares due to observations of treatments (the main effect of interest) from the sums of squares attributed to trap position and date (Fowler and Cohen, 1994a). In practice, it was not always possible to obtain a complete Latin square of results, as catch numbers often deteriorated unexpectedly due to the end of a generation or more often as a result of changing environmental conditions, especially very hot dry spells. In such cases, the data obtained were analysed according to a randomised block version of the ANOVA. All analyses were performed using a computer statistical software package (Genstat). Where small numbers of missing values occurred in the trap catch data, Genstat estimated them during the analysis. Missing data were most frequently attributed to traps having blown down in strong winds or occasionally knocked over by farm machinery. ANOVA assumes that sample data have been drawn from populations that are normally distributed, and requires this property for its correct application. As it is well known that this is not the case for samples of count data (i.e. catches), an initial look at the catch data was made, which showed the distribution of *P. rosae* catches to be positively skewed. In order to normalise this catch data, which also contained zero catches, a log (x+1) transformation was applied (Fowler and Cohen, 1994b). The transformed log (x+1) data showed an approximately normal distribution, and the transformation also had the effect of stabilising the variance by removing its dependence upon the mean (i.e. larger mean catches having larger variances).

6.2.1 FIELD INVESTIGATION INTO PLANT EXTRACTS

A) INVESTIGATIONS USING 'POLYETHYLENE VIAL' LURES

Preliminary Studies on Release Rates

trans-Asarone

In previous field experiments with *P. rosae* (Guerin *et al.*, 1983), the attractant *trans*-asarone (250mg) was applied directly to the surfaces of sticky traps in solution, leaving crystals in the glue coating once the solvent (pentane) had evaporated. The actual release rate of *trans*-asarone using this method is not given, although the release rate from 100mg of *trans*-asarone in an open Petri dish is given as approximately 0.37mg / day (at a maximum daily temperature of 28°C). However, this method of release for *trans*-asarone was found to be unsuitable for use during these field experiments, as it required excessive quantities of relatively expensive *trans*-asarone to yield a relatively low release rate, making the approach

economically unfeasible. Application of the *trans*-asarone in solvent also resulted in the trapping glue becoming slightly opaque and cloudy, which could have adversely affected the visually attractive properties of the REBELL® orange traps. An alternative method of releasing *trans*-asarone from a lure was therefore investigated.

Three replicate *trans*-asarone (50mg) lures were made up as described in the treatments of the field experiments below (also see Table 6.1). The mean release rate of volatiles was then determined by weight loss as described in the general methods of this chapter, but with the following amendments. On completion of the lures, the acetone solvent was allowed to evaporate from the lures for a period of two hours in a fume cupboard. The three lures were laid on a clean surface in the constant temperature room to reduce compound release from one side of the lure, as would occur in the field due to one side of the lure being clamped against the trap. Due to the highly absorbent nature of the 'Energy board' used to construct these lures, steps had to be taken to stop moisture from being absorbed during movement of the lures between the constant temperature room and that containing the scientific balance (due to differences in air humidity). To solve this problem, the three lures were placed in an air-tight tarred glass vessel just before they were removed from the constant temperature room, and were weighed together still in the air-tight vessel. As a result, the mean cumulative weight loss of the lures was plotted without standard errors in this experiment.

Hexanal

In the previous field trial experiments of Guerin *et al.* (1983), hexanal was released at a rate of approximately 15mg/day in warm weather (midday temperatures of 24-28°C) from polyethylene vial lures with four 1mm holes in their caps. However, during this study, an alternative release system was investigated for hexanal, as it was thought that enclosing the hexanal within an 'amber glass vial' might reduce any deleterious effects of exposure to strong sunlight and its ultraviolet component, known to occur with such compounds.

Three replicate hexanal 'amber glass vial' lures were also made up as described in the treatments of the field experiments below (also see Table 6.1), but using three different quantities of the hexanal solution: 30, 50 and 100µl, along with a blank control containing no hexanal solution. The mean release

rate of volatiles from each lure was then determined by weight loss, as described in the general methods of this chapter.

Field Experiments Using 'Polyethylene Vial' Lures For Extracts

These field experiments compared catches of *P. rosae* on REBELL ® monitoring traps baited with 'polyethylene vial' lures containing a range of possibly attractive host plant extracts, and a possibly repellent non-host extract. In the first experiment, host extracts were tested alone, while in the second and third experiments they were combined with *trans*-asarone and hexanal, in an attempt to boost attraction. The third experiment compared catches on traps positioned in two different (but typical) types of field surround: a windbreak hedgerow comprising of trees and bushes, and a drainage ditch with a border consisting of tall grasses. In the fourth experiment, *D. carota* root extract was tested as an alternative to the leaf extract, which had performed poorly in the previous experiments. A possibly repellent non-host extract (*A. cepa* bulb) was also tested in combination with the attractants *trans*-asarone and hexanal. Here it was hoped that any repellent effects of the extract might show up as a reduction in catch compared to that of the standard *trans*-asarone and hexanal treatment. During this experiment, the 'polyethylene vial' lures were replaced twice weekly, as during the earlier experiments it was observed that the lures had often lost their characteristic plant odour by the time they were changed each week. An alternative system for releasing plant extracts ('glass bottle with wick' lure) was also tested in this experiment, using *A. graveolens*, as the initial results of the earlier experiments suggested that the 'polyethylene vial' lures performed poorly.

Field experiments were set up as described in Table 6.2 (Exp.1, 2, 3 and 4), and the treatments were made up as follows. The control consisted of an unbaited REBELL ® orange trap, with dummy lures simulating those on the other treatments traps [i.e. a white 'Energy Board' strip (21cm x 3cm) without *trans*-asarone, and two 'amber glass vials' with caps, but without hexanal]). The *trans*-asarone lures were constructed from a 21cm x 3cm rectangle of white 'Energy board' onto which *trans*-asarone (0.5ml of 100mg/ml solution in AR-acetone) was applied over the surface of one side. The acetone solvent was then allowed to evaporate off in a fume cupboard. This lure was estimated to release *trans*-asarone at a steady rate of 0.22mg/day for over three weeks (at 20°C). The enhanced *trans*-asarone lure was constructed in the same way, but was impregnated with 250mg of *trans*-asarone. The hexanal lures were constructed from

two amber glass vials (1ml, 08-PEP 1, Chromacol Ltd.), which were wired together in pairs. As hexanal is air sensitive (readily oxidising), a hexanal solution containing 10% BHT antioxidant [weight for weight (w:w)] was incorporated in these lures. Hexanal solution (100µl) was added to each vial, and they were capped with polyethylene stoppers [08-CPV (A) 742, Chromacol Ltd.] through each of which a 1mm diameter hole had previously been drilled. These lures were estimated to release hexanal at a steady rate of 9.6mg/day for twelve days (at 20°C), and were replaced weekly.

The 'polyethylene vial' lures for releasing plant extracts were produced using two polyethylene vials (3ml, WP/5 - Fisons) with four 1mm diameter holes drilled through each cap. These were wired together as a pair and MAS extract (2ml of 25g/ml fresh weight equivalent, in hexane) was added to each vial, equivalent of 100g of plant extract. In the first three experiments, the four host plant leaf extracts released from 'polyethylene vial' lures were *D. carota*, *C. maculatum*, *A. graveolens and F. vulgare*. These lures were replaced weekly. In the final experiment, *D. carota* and *A. cepa* root extracts were released from 'polyethylene vial' lures which were replaced twice weekly.

The 'glass bottle with wick' lures for releasing *A. graveolens* leaf extract were produced by adding *A. graveolens* MAS extract (6ml of 25g/ml, in hexane) and nonane (6ml) to a 16ml wide-necked clear glass bottle. The 16ml bottles were coated with black electrical insulation tape, which was used to attach a wire hook to facilitate hanging the lures from the traps, and to screen the contents from light and UV radiation in the field. The bottles were sealed with a solid 'Bakelite' screw cap prior to use. Once in the field, this was replaced with a 'Bakelite' screw cap through which a 5mm-diameter hole had previously been drilled. A lamp-wick was then inserted through the hole to the bottom of the bottle and trimmed until only 0.5cm protruded. These lures were replaced twice weekly.

B) INVESTIGATION INTO THE EFFECTIVENESS OF A NEW 'GLASS BOTTLE WITH WICK' LURE, COMPARED WITH THE 'POLYETHYLENE VIAL' LURE

Preliminary Studies on Delivery Systems

Due to the poor performance of the 'polyethylene vial' lures in the previous field experiments, improvements to the release system used for plant extracts were investigated (using *C. maculatum* extract), with the aim of extending the period over which the lures released volatile compounds.

Two new release systems were investigated in laboratory experiments. The first design was a sealed polyethylene bag containing sponge impregnated with extract, where it was hoped that diffusion through the bag might slow the release of the volatile components. This 'polyethylene bag' lure was constructed by applying *C. maculatum* MAS extract (1ml of 25g/ml, in hexane) to a piece of thick 'bread-like' sponge (2.5cm x 2.0cm). This was heat-sealed into 1000G polyethylene tubing, forming a bag just large enough to contain the sponge.

The second was the 'glass bottle with wick' design, which seemed to be an improvement over the 'polyethylene vial' lure, when tested in the previous field experiment. This lure consisted of a glass bottle with slightly more plant extract than in was used in the 'polyethylene vial' lures. It was also supplemented by the addition of an equal quantity of a less volatile solvent (nonane), in order that the plant extract material would remain in solution for up to a week, even when the original solvent, hexane, had evaporated. No polyethylene was used in the construction of this lure, to remove the possibility of releasing polyethylene derived volatiles, which may have acted as repellents during the previous field experiments. A wick was incorporated into this design, in an attempt to force the release of some of the less volatile compounds in the extract by drawing them up to the evaporation surface rather than allowing them to simply settle out in the bottom of the lure. This 'glass bottle with wick' lure was constructed as described in the treatments of the previous field experiments, but using *C. maculatum* MAS extract (6ml of 25g/ml, in hexane) and nonane (6ml). The bottle was sealed with its 'Bakelite' screw cap and lamp-wick, but the coating of electrical insulation tape, usually wrapped around this lure in the field, was omitted. As numerous plant volatiles (as well as nonane in the 'glass bottle with wick' lure) would be released simultaneously from these lures, air entrainment was used to estimate the release rates of compounds of interest. Determination of release rates by air entrainment was performed as described in the general Methods of this chapter. The cleaned airflow was split into three and entered three 250ml glass entrainment chambers. The first chamber was left empty as a blank control, the 'polyethylene bag' lure was added to chamber two, and the 'glass bottle with wick' lure was added to the remaining chamber. Samples from periodic air entrainments were then analysed by gas chromatography, allowing their separation into individual compounds. The resulting data was simplified by only plotting the release rates of four of the main compounds in this extract [myrcene, γ-coniceine and (*Z*) and (*E*)-β-ocimene].

Field Comparison of Lures

From the preliminary experiment above, the *C. maculatum* 'glass bottle with wick' lure was found to release significant levels of the four compounds investigated for at least a week, and seemed the most suitable new lure design for releasing plant extracts in the field. As such, the aim of this field experiment was to compare the effectiveness of this new lure with that of the previously used 'polyethylene vial' lure (using *A. graveolens* and *D. carota* leaf extracts). This field experiment was set up as described in Table 6.2 (Exp.5), and the treatments were made up as follows. The control, *trans*-asarone and hexanal lures, and the 'polyethylene vial' and 'glass bottle with wick' lures, for *D. carota* and *A. graveolens* leaf extracts, were all constructed as described in the previous field experiments. In this experiment, both types of plant extract lure were changed twice weekly.

C) INVESTIGATIONS USING 'GLASS BOTTLE WITH WICK' LURES

Preliminary Studies on Release Rates

During this investigation, release rate studies were undertaken to identify a suitable method for releasing the known attractant *trans*-methylisoeugenol (Guerin *et al.*, 1983). A number of different *trans*-methylisoeugenol (300µl) 'polyethylene bag' lures were produced as described in Table 6.1. The mean release rate of volatiles from each type of lure was then determined by weight loss, as described in the general Methods of this chapter.

Field Experiments Using the Improved Release System for Extracts

In the previous experiment comparing the 'old' and 'new' lure designs for releasing plant extracts, significant increases in catch were seen with the new 'glass bottle with wick' system. This increase was particularly clear in the case of the *A. graveolens* extract, which seemed a superior attractant to the *D. carota* leaf extract. In the light of the findings, these experiments focused on comparing many of the host extracts used during the first field experiments (*A. graveolens*, *C. maculatum* and *F. vulgare*), but this time releasing them from the improved 'glass bottle with wick' lure. Various combinations of extracts alone, and in combination with known attractants (i.e. *trans*-asarone, *trans*-methylisoeugenol and hexanal), were tested in attempts to boost levels of attraction for *P. rosae*.

The first (main) experiment compared catches when *A. graveolens* extract was released alone, and in combination with *trans*-asarone and hexanal. The second (minor) experiment compared catches when *A. graveolens* extract was released with the alternative attractant *trans*-methylisoeugenol, either alone or in combination with hexanal. The third (minor) experiment compared *C. maculatum* and *F. vulgare* extracts in combination with *trans*-asarone and hexanal.

These field experiments were set up as described in Table 6.2 (Exp. 6, 7 and 8), and the treatments were made up as follows. The control, *trans*-asarone and hexanal lures and the 'glass bottle with wick' lures, for *A. graveolens*, *F. vulgare* and *C. maculatum* leaf extracts, were constructed as described in the previous field experiments. The 'glass bottle with wick' lures were changed twice weekly during these experiments. The *trans*-methylisoeugenol lures consisted of a thin sponge impregnated with *trans*-methylisoeugenol (300µl), heat-sealed into a 100G polyethylene bag. These lures were estimated to release *trans*-methylisoeugenol at a steady rate of 6.7mg/day for a period of over two weeks (at 20°C).

6.2.2 FIELD INVESTIGATIONS INTO *C. MACULATUM* AND ITS CONSTITUENT COMPOUNDS

Preliminary Studies on Release Rates

Individual Synthetic C. maculatum Compounds

To determine suitable release systems for the *C. maculatum* compounds γ-coniceine, β-caryophyllene and β-ocimene, a wide variety of polymer lure designs (bags and vials) were produced and tested (see Table 6.1 for specific details). Three replicates of each type of β-caryophyllene and β-ocimene lure were tested (with the exception of only one replicate for the β-caryophyllene 2000G bag). Only one replicate of each γ-coniceine lure was tested, due to the difficulty of obtaining γ-coniceine, which had to be synthesised. The mean release rate of volatiles from each lure type was then determined by weight loss, as described in the general Methods of this chapter.

Air Entrainment of γ-Coniceine

As γ-coniceine is a relatively unstable compound, it was thought possible that it might decompose in the lure whist out in the field. To determine if decomposition occurred, and to determine the ratios of any breakdown products released, an air entrainment experiment was performed using a lure similar to those in

the experiment above. γ-Coniceine (10μl) was applied to a thin sponge (1/8th) and heat-sealed into a 1000G polyethylene bag. Determination of the volatile release rates was then performed by air entrainment as described in the general Methods of this chapter. The cleaned airflow was split into two and entered two 250ml glass entrainment chambers. The first chamber was left empty as a blank control, and the γ-coniceine lure was suspended in the remaining chamber. Samples from periodic entrainments were then analysed by gas chromatography, allowing their separation into individual compounds. Identification and quantification of γ-coniceine was achieved by comparison with GC retention times, and peak area counts, for an authentic standard (100ng) analysed on the same GC. Quantification of breakdown products was achieved by assuming them to have similar GC response factors to that of γ-coniceine.

Field Experiments Using *C. maculatum* Extract and Compounds

These field experiments compared catches for *C. maculatum* extract and its major electrophysiologically active component compounds (γ-coniceine, β-ocimene and β-caryophyllene), released alone or in combination. Various combinations were tested during these experiments, in an attempt to boost levels of attraction above that provided by the extract alone and to identify key compounds involved in the attraction of *P. rosae* to this preferred wild host species.

The first experiment compared *C. maculatum* extract alone, in combination with γ-coniceine, and in combination with γ-coniceine, β-caryophyllene and β-ocimene. The second experiment compared γ-coniceine, and β-caryophyllene plus β-ocimene, alone and in combination. The third experiment compared combinations of γ-coniceine, and β-caryophyllene plus β-ocimene, with and without the additional attractants *trans*-asarone and hexanal, or *trans*-methylisoeugenol and hexanal.

These field experiments were set up as described in Table 6.2 (Exp.9, 10 and 11), and the treatments were made up as follows. The control, *trans*-asarone, *trans*-methylisoeugenol, hexanal, and *C. maculatum* 'glass bottle with wick' lures were constructed as described in the previous field experiments. The γ-coniceine lure was produced from a thin sponge impregnated with γ-coniceine (100μl), heat-sealed into a 1000G polyethylene bag. This lure was estimated to release γ-coniceine at a steady rate of 7.7mg/day for four days (at 20°C), and was replaced twice weekly. The β-caryophyllene lure was

produced from a thin sponge impregnated with β-caryophyllene (300µl), heat-sealed in a 1000G polyethylene bag. This lure was estimated to release β-caryophyllene at a steady rate of 7.0 mg/day for two weeks (at 20°C), and was replaced weekly. The β-ocimene lure was produced from a polyethylene micro test tube containing β-ocimene [250µl, 70:30 mix of (*E*) and (*Z*)- isomers], sealed with its integral cap. This lure was estimated to release β-ocimene at a steady rate of 6.2 mg/day nine days (at 20°C), and was replaced weekly.

6.2.3 COMPARISON OF STANDARD, AND *trans*-ASARONE AND HEXANAL BAITED MONITORING TRAPS (IN COLLABORATION WITH ADAS)

These extensive field experiments were undertaken in collaboration with ADAS to compare the performance of *trans*-asarone and hexanal baited traps for improving carrot fly monitoring. Five to ten paired REBELL ® traps, one baited with *trans*-asarone and hexanal and the other left unbaited, were set up at a large number of sites across England, during the second generation of flies in 1995 and 1996. The sites were chosen to cover a range of carrot fly population densities from both Northern England and East Anglia. Lures were prepared in the laboratory at IACR-Rothamsted, and posted to ADAS field operatives on a weekly basis. The ADAS workers then dealt with changing the traps and lures, as well as counting *P. rosae* catches on a weekly basis. These field experiments were set up as described in Table 6.2 Exp.12a and 12b, with the paired traps positioned around field borders 5m into the crop, in accordance with standard ADAS monitoring practices.

For comparison, a further three blocks of paired traps (baited and unbaited) were set up as part of the field experiments in 1996. This experiment was performed as described above (see Table 6.2 Exp.12c) but the paired traps were positioned on the crop border, rather than 5m into the crop as in the ADAS experiments.

The treatments were made up as follows. The hexanal lures were made up as described in the treatments of the previous field experiments, but were capped with solid polyethylene plugs to seal them during transport. These lures were packed in larger airtight glass jars along with sufficient pre-drilled (a 1mm diameter hole) polyethylene stoppers for each vial. During the 1996 experiments, the quantity of hexanal was increased to 150µl per vial, to compensate for possible losses during postage. The *trans*-

asarone lures were also produced as described in the previous field experiments, but once the solvent had evaporated they were stacked together, tightly wrapped in aluminium foil, and heat-sealed into a flat polyethylene tube for transport. Both types of lures were then sent by first class post to the individual ADAS operatives, taking between one to three days in transit. On receipt, the lures were stored the correct way up in a freezer until required in the field. In order to activate the release of hexanal from the packaged lures, the solid polyethylene plugs of each vial were removed and replaced with one of the pre-drilled plugs supplied. During these experiments, the hexanal lures were replaced twice weekly, and the *trans*-asarone lures were replaced weekly.

Further Release Rate Studies Aimed at Solving Problems Encountered with the *trans*-Asarone and Hexanal Lures

Towards the end of these field investigations, a number of possible problems with the *trans*-asarone and hexanal lures were identified. These included a discolouration of some of the *trans*-asarone lures while out in the field, with the chemically treated areas on the card strips turning slightly brown (possibly as a result of some sort of chemical change or decomposition). Significant losses of hexanal were also observed during postal transit of the capped 'amber glass vial' lures to ADAS operatives. Following the final field trials, these problems were investigated further, in order to solve any problems for future studies.

Air Entrainment of trans-Asarone Lures

As decomposition of *trans*-asarone in the'Energy board' strip lure was suspected, an air entrainment experiment was performed to determine the actual release of *trans*-asarone, and to identify any degradation products also being released. It was suspected that breakdown of this compound might result from oxidation, or light (or UV) induced reactions. To determine if oxidation was occurring, lures incorporating different quantities of antioxidant (BHT) were produced and included in this experiment. If oxidation was a problem, reduced levels of contaminants would be expected from these lures which were protected from oxidation. As light (or UV) induced breakdown was also a possibility, fluorescent strip lighting was used during the entrainment in order to simulate natural lighting conditions and catalyze any light induced reactions that may have occurred in the field.

The lures for this experiment were made up as follows. A standard *trans*-asarone (50mg) lure was prepared as described in the treatments of the previous field experiments. A further two *trans*-asarone

(50mg) lures were prepared using the same method, but using a *trans*-asarone solution (in acetone) containing 1%, and 10% BHT antioxidant (w:w). Determination of volatile release rates was performed by air entrainment as described in the general Methods of this chapter, with the following amendments. The cleaned airflow was split into four and entered four 500ml glass entrainment chambers. The first chamber was left empty as a blank control, the standard *trans*-asarone lure was added to chamber two, and the *trans*-asarone lures containing 1% and 10% BHT antioxidant were added to chambers three and four respectively. The entrainment was performed in a temperature controlled room at 20°C, lit by fluorescent strip lights operating in a 12hr light / 12hr dark mode. As the release rate of volatiles from this type of lure was known to be slow, volatiles were collected continuously over a two week period. The Porapak traps were removed and replaced with fresh ones at the end of each week, and the volatiles were extracted from the used traps with 1ml of redistilled diethyl ether. These extracts were then analysed by gas chromatography.

At the end of the two week period, the chemicals remaining in lures and any which had been adsorbed onto the glassware were extracted with solvent. The entrainment vessels were washed out using 5ml of redistilled diethyl ether. This solvent was collected and used to extract the lures, which were chopped into 1cm squares and then added to the solvent for extraction over two days. The chopped lures were removed and further extracted for a day using 10ml of fresh diethyl ether. The resulting extracts from each lure were then combined and analysed by gas chromatography, to determine the quantity and composition of the material remaining in the lure. Compound identification and quantification was achieved by comparison with GC retention times and peak area counts for authentic standards (100ng) analysed on the same GC.

Alternative Lures for the Release of Hexanal

In order to identify an alternative sealed type of lure which could replace the 'amber glass vial' lures for releasing of hexanal, a wide range of polymer lures (bags and vials) were produced and tested (see table 6.1 for details). The release rate of volatiles from each lure type was then determined by weight loss, as described in the general Methods of this chapter.

Table 6.1 **Summary of Lure Designs Tested in Release Rate Experiments (Determined by Cumulative Weight Loss)**

a) ***trans*-Asarone**
50mg on a white 'Energy Board' strip (21cm x 3cm)

b) **Hexanal solution - containing 10% BHT (w:w)**
30µl in an 'amber glass vial' with a 1mm hole in the cap
50µl in an 'amber glass vial' with a 1mm hole in the cap
100µl in an 'amber glass vial' with a 1mm hole in the cap

c) ***trans*-Methylisoeugenol**
300µl on a thin sponge, heat-sealed into a 100G polyethylene bag
300µl on a thin sponge, heat-sealed into a 250G polyethylene bag
300µl on a thin sponge, heat-sealed into a 500G polyethylene bag
300µl on a thin sponge, heat-sealed into a 1000G polyethylene bag

d) **γ-Coniceine**
10µl on $^{1}/_{8}^{th}$ of a thin sponge, heat-sealed into a 1000G polyethylene bag
100µl on $^{1}/_{8}^{th}$ of a thin sponge, heat-sealed into a 1000G polyethylene bag
100µl on a thin sponge, heat-sealed into a 250G polyethylene bag
100µl on a thin sponge, heat-sealed into a 500G polyethylene bag
100µl in a polyethylene micro test tube sealed using its integral cap

e) **β-Caryophyllene**
300µl on a thin sponge, heat-sealed into a 1000G polyethylene bag
300µl on a thin sponge, heat-sealed into a 2000G polyethylene bag
250µl in a polyethylene micro test tube sealed using its integral cap
300µl on a thin sponge in a tall polyethylene vial sealed using its integral cap

f) **β-Ocimene**
300µl on a thin sponge, heat-sealed into a 1000G polyethylene bag
250µl in a polyethylene micro test tube sealed using its integral cap
300µl on a thin sponge inside a tall polyethylene vial sealed using its own integral cap

g) **Hexanal solution - containing 10% BHT (w:w)**
50µl on $^{1}/_{8}^{th}$ of a thin sponge, heat-sealed into a 1000G polyethylene bag
100µl on $^{1}/_{8}^{th}$ of a thin sponge, heat-sealed into a 1000G polyethylene bag
100µl on $^{1}/_{4}$ of a thin sponge, heat-sealed into a 1000G polyethylene bag
150µl on $^{1}/_{4}$ of a thin sponge, heat-sealed into a 1000G polyethylene bag
50µl on a thin sponge, heat-sealed into a 1000G polyethylene bag
50µl on $^{1}/_{8}^{th}$ of a thin sponge, heat-sealed into a 3000G polyethylene bag
50µl on a thin sponge, heat-sealed into a 3000G polyethylene bag
50µl in a polyethylene micro test tube sealed using its integral cap.
50µl in a polypropylene micro test tube sealed using its integral cap
50µl on a thin sponge inside a polyethylene vial (WP/5) sealed using its integral cap

Table 6.2 Experimental Details of Field Experiments

Investigations on Plant Extracts (A): 'Polyethylene Vial' Lures

Experiment 1
Treatments:
A) Control
B) *C. maculatum*
C) *A. graveolens*
D) *F. vulgare*
This experiment was performed at IACR-Rothamsted 'Garden Plots 1 and 2' and 'Long Hoos 6 and 7', and ran from 26/5/94 to 23/6/94 (1st generation flies). Two blocks were put out, with a spacing of 6m between traps. Data from the 2 blocks, over 3 randomisations (26/5/94-16/6/94), were included and analysed as a randomised block experiment. Data from the last week of this experiment were omitted from the analysis as no flies were caught. In this analysis, the total treatment sums of squares were separated into two specific treatment contrasts [one ("control vs. extracts") = A vs. B+C+D; two ("among extracts") = B vs. C vs. D].

Experiment 2
Treatments:
A) Control
B) *trans*-Asarone and hexanal
C) *C. maculatum*, *trans*-asarone and hexanal
D) *C. maculatum*
E) *D. carota*, *trans*-asarone and hexanal
This experiment was performed at ADAS Arthur Rickwood 'Big Ground South (plot ECT 647)', and ran from 25/5/94 to 22/6/94 (1st generation flies). Two blocks were put out, with a spacing of 10m between traps. Data from the 2 blocks, over 4 randomisations (25/5/94-22/6/94), were included and analysed as a randomised block experiment. The total treatment sums of squares were not separated into specific treatment contrasts, because the treatments were all dissimilar (i.e. a mixture of synthetic attractants, different species extracts, and combinations of the two). There were no missing values in this analysis.

Experiment 3
Treatments:
A) Control
B) *D. carota*, *trans*-asarone and hexanal
C) *C. maculatum*, *trans*-asarone and hexanal
This experiment was performed at ADAS Arthur Rickwood 'Big Ground South (plot ECT 647)', and ran from 25/5/94 to 22/6/94 (1st generation flies). Two blocks were put out in two contrasting types of field boarder, with a spacing of 10m between traps.
3a. One block of traps was put out along a hedgerow consisting of trees and bushes, the traps were attached to branches at a height of 1.75m using foldback clips. Data from 1 block, over 4 randomisations (25/5/94-22/6/94), were included and analysed as a randomised block experiment. In this analysis, the total treatment sums of squares were separated into two specific treatment contrasts [one ("control vs. extracts") = A vs. B+C; two ("among extracts") = B vs. C]. There were no missing values in this analysis.
3b. One block of traps was put out along the edge of a ditch with the traps level with the top of the herbage border at a height of 1m, this boarder mainly consisting of tall grasses. Data from 1 block, over 3 randomisations (25/5/94-15/6/94) were included and analysed as a complete Latin square experiment. Data from the last week of this experiment were omitted from the analysis as no flies were caught. In this analysis, the total treatment sums of squares were separated into two specific treatment contrasts [one ("control vs. extracts") = A vs. B+C; two ("among extracts") = B vs. C]. There were no missing values in this analysis.

Table 6.2 Experimental Details of Field Experiments (Continued)

Experiment 4

Treatments:

A) Control
B) *trans*-Asarone and hexanal
C) Enhanced *trans*-asarone
D) *A. graveolens* ('glass bottle with wick' lure), *trans*-asarone and hexanal
E) *A. cepa* bulb extract ('polyethylene vial' lure), *trans*-asarone and hexanal
F) *D. carota* root extract ('polyethylene vial' lure)

This experiment was performed at IACR-Rothamsted 'Garden Plots 1 and 2' and 'Long Hoos 6 and 7', and ran from 17/8/94 to 14/9/94 (2nd generation flies). Two blocks were put out, with a spacing of 6m between traps. Data for 1 block (Garden Plot), over 10 randomisations (17/8/94-9/9/94), were included and analysed as a randomised block experiment. The results of last two dates in this block and all those from the Long Hoos block were omitted, as so few flies were caught. In this analysis, the total treatment sums of squares were not separated into specific treatment contrasts, as the treatments were a mixture of synthetic attractants, host extracts, and a possibly repellent extract. There was one missing value in these data, estimated as 3.2 during the analysis.

Investigations on Plant Extracts (B): Comparison of Two Lure Types

Experiment 5

Treatments:

A) Control
B) *D. carota* in a 'polyethylene vial' lure
C) *D. carota* in a 'glass bottle with wick' lure
D) *A. graveolens* in a 'polyethylene vial' lure
E) *A. graveolens* in a 'glass bottle with wick' lure
F) *trans*-Asarone and hexanal

5a. This experiment was performed at Bruce Butcher's fields 'Feltwell Anchor', and ran from 8/8/94 to 2/9/94. Eight blocks were put out, with a spacing of 15m between traps. Data from 8 blocks, over 6 randomisations (12/8/94-2/9/94), were included and analysed as a complete Latin square experiment. The first two dates were omitted from this analysis, because they were mostly zero catches as this generation of flies had only just started to emerge. In this analysis, the total treatment sums of squares were separated into four specific treatment contrasts [contrast 1 ("control vs. AH vs. extracts") = A vs. F vs. B+C+D+E; and the two by two nested factorial for "species" and "lures", which is equivalent to saying: contrast 2 ("species") = B+C (*D. carota*) vs. D+E (*A. graveolens*); contrast 3 ("lures") = B+D (polyethylene vial lures) vs. C+E (glass bottle with wick lures); contrast 4 is the "species" by "lures" interactions]. There were two missing values in these data, estimated as 11.22 and -1.22 during the analysis.

5b. This experiment was performed at ADAS Arthur Rickwood 'Big Ground South, plot ECT 647', and ran form the 8/8/94 to 2/9/94 (2nd generation flies). Two blocks were put out, with a trap spacing of 10m. Data from 2 blocks, over 6 randomisations (12/8/94-2/9/94), were included and analysed as a complete Latin square experiment. The first two dates were omitted from this analysis because they were mostly zeros. In this analysis, the total treatment sums of squares were separated into four specific treatment contrasts [contrast 1 ("control vs. AH vs. extracts") = A vs. F vs. B+C+D+E; and the two by two nested factorial for "species" and "lures", which is equivalent to saying: contrast 2 ("species") = B+C (*D. carota*) vs. D+E (*A. graveolens*); contrast 3 ("lures") = B+D (polyethylene vial lures) vs. C+E (glass bottle with wick lures); contrast 4 is the "species" by "lures" interactions]. There were no missing values in these data.

Table 6.2 Experimental Details of Field Experiments (Continued)

Investigations on Plant Extracts (C): 'Glass Bottle with Wick' Lures

Experiment 6
Treatments:
A) Control
B) *trans*-Asarone and hexanal
C) *A. graveolens*
D) *A. graveolens*, *trans*-asarone and hexanal

6a. This experiment was performed at Bruce Butcher's fields 'Feltwell Anchor', and ran from 2/9/94 to16/9/94 (2nd generation flies). Eight blocks were put out, with a spacing of 15m between traps. Data from 8 blocks, over 4 randomisations (2/9/94-16/9/94), were included and analysed as a complete Latin square experiment. In this analysis, the total treatment sums of squares were separated into the two by two factorial for "extract" and "AH", which is equivalent to saying: contrast 1 ("extract") = (*A. graveolens* present) C+D vs. (*A. graveolens* absent) A+B; contrast 2 ("AH") = (*trans*-asarone and hexanal present) B+D vs. (*trans*-asarone and hexanal absent) A+C; contrast 3 is the "extract" by "AH" interactions]. There were no missing values in these data.

6b. This experiment was performed at ADAS Arthur Rickwood 'Big Ground South, plot ECT647'. Two blocks were put out, with a spacing of 10m between traps. No statistical analysis of these results was possible, as so few *P. rosae* were caught, and catches ceased before a full Latin square of results could be obtained.

6c. This experiment was performed at Bruce Butcher's fields 'Feltwell Anchor', and ran from 16/9/94 to 30/9/94. Two blocks were put out, with a spacing of 15m between traps. No statistical analysis of these results was possible, as the data contained lots of zero catches, and catches in the two blocks were too different from each other, showing no discernible pattern with regard to treatments.

6d. This experiment was performed at ADAS Arthur Rickwood 'Big Ground South, plot ECT 647'. Three blocks were put out, with a spacing of 10m between traps. No statistical analysis of these results was possible, as so few *P. rosae* were caught, and catches ceased before a full Latin square of results could be obtained.

Experiment 7
Treatments:
A) Control
B) *trans*-Asarone and hexanal
C) *A. graveolens*, *trans*-methylisoeugenol
D) *A. graveolens*, *trans*-methylisoeugenol and hexanal

This experiment was performed at Bruce Butcher's fields 'Feltwell Anchor', and ran from 16/9/94 to 30/9/94 (2nd generation flies). One block was put out, with a spacing of 15m between traps. Data for 1 block, over 4 randomisations (16/9/94-30/9/94), were included and analysed as a complete Latin square experiment. In this analysis, the total treatment sums of squares were separated into two specific treatment contrasts [contrast 1 ("control vs. AH vs. extracts" = A vs. B vs. C+D; contrast 2 ("among extracts") = C vs. D]. There were no missing values in these data.

Experiment 8
Treatments:
A) Control
B) *trans*-Asarone and hexanal
C) *C. maculatum*, *trans*-methylisoeugenol and hexanal
D) *F. vulgare*, *trans*-methylisoeugenol and hexanal

This experiment was performed at Bruce Butcher's fields 'Feltwell Anchor', and ran from 16/9/94 to 30/9/94 (2nd generation flies). One block was put out, with a spacing of 15m between traps. Data for 1 block, over 4 randomisations (16/9/94-30/9/94), were included and analysed as a complete Latin square experiment. In this analysis, the total treatment sums of squares were separated into two specific treatment contrasts [contrast 1 ("control vs. AH vs. extracts" = A vs. B vs. C+D; contrast 2 ("among extracts") = C vs. D]. There were no missing values in these data.

Table 6.2 Experimental Details of Field Experiments (Continued)

Investigations on *C. maculatum* and its Constituent Compounds

Experiment 9

Treatments:

A) Control
B) *trans*-Asarone and hexanal
C) *C. maculatum*
D) *C. maculatum* and γ-coniceine
E) *C. maculatum*, β-caryophyllene, β-ocimene and γ-coniceine

9a. This experiment was performed at Bruce Butcher's field - Decoy Farm, Methwold Fens, and ran from 21/7/95 to 23/8/95 (2nd generation flies). Five blocks were put out, with a spacing of 15m between traps. Data for blocks 5 blocks, over 20 randomisations (7/7/95-15/7/95), were included and analysed as a randomised block experiment. As catch numbers were very low with lots of zero catches, the total catches per treatment for each block were analysed, rather than individual trap catches. In this analysis, the total treatment sums of squares were not separated into specific treatment contrasts. There were no missing values in these data.

9b. This experiment was performed at Bruce Butcher's field - Broad Fen Farm, and ran from 29/6/96-1/9/96 (2nd generation flies). Two blocks of these treatments were put out, with a spacing of 15m between traps. No statistical analysis of these results was possible, as so few *P. rosae* were caught.

Experiment 10

Treatments:

A) Control
B) *trans*-Asarone and hexanal
C) γ-Coniceine
D) β-Caryophyllene and β-ocimene
E) γ-Coniceine, β-caryophyllene and β-ocimene

This experiment was performed at Bruce Butcher's field - Decoy Farm, Methwold Fens', and ran from 21/7/95 to 23/8/95 (2nd generation flies). Five blocks were put out, with a spacing of 15m between traps. Data for 5 blocks, over 20 randomisations (7/7/95-15/7/95), were included and analysed as a randomised block experiment. As catch numbers were very low with lots of zero catches, the total catches per treatment for each block were analysed, rather than individual trap catches. In this analysis, the total treatment sums of squares were separated into those due to four specific treatment contrasts [contrast 1 ("AH vs. others") = B vs. A+C+D+E and the two by two factorial for "γ-con" and "β-car and β-oci" which is equivalent to: contrast 2 ("γ-con") = C+E (γ-coniceine present) vs. A+D (absent); contrast 3 ("β-car + β-oci") = D+E (β-caryophyllene and β-ocimene present) vs. A+C (absent); contrast 4 the "γ-con" by "β-car and β-oci" interactions]. There were no missing values in these data.

Experiment 11

Treatments:

A) Control
B) *trans*-Asarone and hexanal
C) γ-coniceine
D) γ-coniceine, β-caryophyllene and β-ocimene
E) γ-coniceine, β-caryophyllene, β-ocimene, *trans*-asarone and hexanal
F) γ-coniceine, β-caryophyllene, β-ocimene, *trans*-methylisoeugenol and hexanal

This experiment was performed at Bruce Butcher's field - Broad Fen Farm, and ran from 29/6/96-1/9/96 (2nd generation flies). Two blocks were put out, with a spacing of 15m between traps. No statistical analysis of these results was possible, as no *P. rosae* were caught during this experiment.

Table 6.2 Experimental Details of Field Experiments (Continued)

Comparison of Standard and Baited Monitoring Traps (in Collaboration with ADAS) Experiment 12

Treatments:
A) Control
B) *trans*-Asarone and hexanal

12a. This extensive ADAS field experiment was performed at ten field sites dispersed throughout the main carrot growing regions of England (East Anglia and Northern England), and ran from 12/7/95 to 4/10/95 (2nd generation flies). Sixty-five blocks were put out (5m into the crop), with the following distribution: EAST ANGLIA SITES (10 blocks at each site) - Clifton Breck, TGB, Oxborough. NORTHERN ENGLAND SITES: SITES (5 blocks at each site) - Crockery Hill (Yorkshire), Kellington (Humberside), Blidworth (Nottinghamshire), Holbeach (Lincolnshire), Marston (Lincolnshire), Misterton (Nottinghamshire), Burscough (Lancashire). Data from all ten sites (65 blocks), between the dates 12/7/95 to 4/10/95, were included and analysed as randomised block experiments. As catch numbers were very low with lots of zero catches, the total catches per treatment from each block were analysed, rather than individual trap catches.

12b. This extensive ADAS field experiment was performed at eight field sites dispersed throughout the main carrot growing regions of England (East Anglia and Northern England), and ran from 24/7/96-17/9/96 (2nd generation flies). Sixty blocks were put out (5m into the crop), with the following distribution: EAST ANGLIA SITES (10 blocks at each site) - Browns, Well Piece, 110 Acres, Mussel Hill. NORTHERN ENGLAND SITES: SITES (5 blocks at each site) - Naish, Thomas, Templeton, Travis. Data for all eight sites (60 blocks), between the dates 24/7/96 to 17/9/96, were included and analysed as randomised block experiments. As catch numbers were very low with lots of zero catches, the total catches per treatment from each block were analysed, rather than individual trap catches. At the Travis site, sufficient *P. rosae* were caught to enable re-analysis of the individual trap catches. Data from the Travis site (5 blocks), over 6 randomisations (6/8/96-17/9/96), were included and analysed as a multiple Latin square experiment. The first week's catches were not included in this analysis as so few flies were caught at any site during this period. There were no missing values in these data.

12c. This experiment was performed at Bruce Butcher's field - Broad Fen Farm, and ran from 29/6/96-1/9/96 (2nd generation flies). Three blocks were put out on the crop edge, as opposed to 5m into the crop for the ADAS experiments, with a spacing of 15m between traps. No statistical analysis of these results was possible, as very few *P. rosae* were caught during this experiment.

6.3 RESULTS

6.3.1 FIELD INVESTIGATION INTO PLANT EXTRACTS

A) INVESTIGATIONS USING 'POLYETHYLENE VIAL' LURES

Preliminary Studies on Release Rates

trans-Asarone

The results of the cumulative weight loss experiment on *trans*-asarone (50mg) 'Energy board' lures are presented graphically in Figure A1 (Appendix 2). From this Figure, the release rate of *trans*-asarone was estimated to be 0.22mg/day (at 20°C) for a period of over three weeks (Table 6.3a). As this rate was comparable to that estimated for the Guerin experiment, but required five times less *trans*-asarone, this method of release was used in these experiments, with the lures being replaced weekly to reduce any effects of weathering on the lure. Further increases in the rate of *trans*-asarone release could have been made, but would have required a larger surface area of card, and may have proven counterproductive with regard to its appearance being more noticeable when attached to the sticky trap.

Hexanal

The results of the cumulative weight loss experiment on hexanal 'amber glass vial' lures are presented graphically in Figure A2 (Appendix 2), and the estimated release rates are given in Table 6.3b. These lures all provided a steady release rate of 4.8mg/day, with the length of time this rate was maintained being dependent upon the quantity of the hexanal solution applied to the vial. To approximate the release rate of hexanal used in the Guerin experiments, it was decided to use two of the amber glass vial lures wired together, each containing 100µl of the hexanal/BHT solution and giving an approximate release rate of 9.8mg/day (at 20°C). These lures would give a steady release for approximately twelve days, and so would need to be replaced weekly.

Field Experiments Using Host Plant Extracts

The results of the first experiment (Table 6.4 Exp.1, Fig 6.1) showed that, overall, the three plant extracts caught significantly more flies than the unbaited control (contrast 1 "control vs. extracts"); the Figure shows that *A. graveolens* had a greater effect than the other two (*C. maculatum* and *D. carota*), and this

was almost significant (contrast 2 "among extracts"). From the 95% c.i., *A. graveolens* is the only one clearly different from the control, catching approximately twice the number of flies.

The results of the second experiment (Table 6.4 Exp.2, Fig 6.2) showed a significant difference between the five treatments. The Figure shows that *trans*-asarone and hexanal caught the most flies, and from the 95% c.i., is the only treatment clearly different from the control (catching approximately twice as many flies). The Figure also shows that both extracts (with or without *trans*-asarone and hexanal) caught slightly more flies than the control, with *C. maculatum* having a greater effect than *D. carota*. Interestingly, extracts in combination with *trans*-asarone and hexanal caught fewer flies than *trans*-asarone and hexanal alone.

The third experiment was performed in two contrasting types of field surround, firstly a hedgerow, and secondly in the tall grassy border of a ditch. The results at the first site (Table 6.4 Exp.3a, Fig. 6.3) showed no significant differences between the catch on the control and the overall catch with plant extracts plus *trans*-asarone and hexanal (contrast 1 "control vs. extracts"), or between the two species extracts (contrast 2 "among extracts"). However, the Figure shows that *C. maculatum* plus *trans*-asarone and hexanal caught slightly more flies than the control, or *D. carota* extract plus *trans*-asarone and hexanal (which both caught very similar numbers). The results at the second site (Table.6.4 Exp.3b, Fig. 6.4) also showed no significant differences between the catch on the control and the overall catch with plant extracts plus *trans*-asarone and hexanal (contrast 1 "control vs. extracts"), or between the two species extracts (contrast 2 "among extracts"). However, the Figure shows once again that *C. maculatum* plus *trans*-asarone and hexanal caught slightly more flies than *D. carota* extract plus *trans*-asarone and hexanal.

The results of the fourth experiment (Table 6.4 Exp.4, Fig 6.5) showed a significant difference between the six treatments. The Figure shows that *A. graveolens* (in a 'glass bottle with wick' lure) plus *trans*-asarone and hexanal caught the most flies, and from the 95% c.i., is the only treatment clearly different from the control (catching almost three times as many flies). Notably, *trans*-asarone and hexanal combined with *A. graveolens* caught more flies than *trans*-asarone and hexanal alone. In contrast, *trans*-asarone and hexanal combined with *A. cepa* bulb extract (in a 'polyethylene vial' lure) caught fewer flies than *trans*-asarone and hexanal alone. The *D. carota* root extract and enhanced *trans*-asarone (at five

times the standard dosage) produced only a slight increase in catch, and the numbers were very similar to the control.

B) INVESTIGATION INTO THE EFFECTIVENESS OF A NEW 'GLASS BOTTLE WITH WICK' LURE, COMPARED WITH THE OLD 'POLYETHYLENE VIAL' LURE

Preliminary Studies on Delivery Systems

The results of the air entrainment of the two new *C. maculatum* lures ('polyethylene bag' lure and 'glass bottle with wick' lure), showing the release rates of four main compounds (myrcene, γ-coniceine, and (*Z*)- and (*E*)-β-ocimene), are presented graphically in Figures A3 and A4 respectively. Figure A3 shows that release of the four compounds from the 'polyethylene bag' lure peaked within the first day of the experiment, and that almost all of the extract had been released within two days. Figure A4 shows that the four compounds were released from the 'glass bottle with wick' lure at substantial rates (over 0.5μg/hour) for approximately four days. The release of γ-coniceine peaked during the first day of the experiment (at approximately 3μg/hour) and tailed off fairly rapidly, to approximately 0.5μg/hour by day four. A steadier release was seen for myrcene, which peaked on day two (1.7μg/hour) and gradually trailed off to approximately 1.0μg/hour by day four. The release rates of (*Z*)- and (*E*)-β-ocimene were very similar and relatively steady at approximately 0.5μg/hour, only dipping to slightly to approximately 0.4μg/hour by day four.

Of the two new plant extract release systems tested, only the 'glass bottle with wick' lure provided a relatively steady release of plant volatiles for over a week. This lure design also seemed to have performed well, when tested in a previous field experiment, for the release of *A. graveolens* extract (see Fig. 6.6). As such, this system was chosen for comparison with the previously used 'polyethylene vial' lure for the release of *D. carota* and *A. graveolens* extracts, in the following field experiment. In order to keep the release of all the volatiles as steady as possible throughout the experiments, it was decided to replace the lure twice weekly,

Field Comparison of Lures

This experiment was performed at two sites. The results from site one (Table 6.4 Exp.5a, Fig. 6.6) showed a significant difference between the control, *trans*-asarone and hexanal, and the plant extracts overall

(contrast 1 "control vs. AH vs. extracts"). The Figure shows that *trans*-asarone and hexanal caught the most flies (almost twice that of the control), and from the 95% c.i., is clearly different from the rest. The results also show that *A. graveolens* extract caught significantly more flies than *D. carota* extract (contrast 2 "species"), and that the new 'glass bottle with wick' lures caught significantly more flies than the old 'polyethylene vial' lures (contrast 3 "lures"). The type of lure used was also found to have a significantly greater effect when *A. graveolens* extract rather than *D. carota* extract was released (contrast 4 "species x lures").

The results from site two (Table 6.4 Exp.5b, Fig. 6.7) showed a significant difference between the control, trans-asarone and hexanal, and the plant extracts overall (contrast 1 "control vs. AH vs. extracts"). The Figure shows that *trans*-asarone and hexanal caught significantly the most flies (over twice that of the control), and from the 95% c.i., is clearly different from the rest. The Figure shows that *A. graveolens*, released from both lure types, caught more flies than *D. carota* extract, and this was almost significant (contrast 2 "species"). Catches for the two types of lure were not significantly different in this experiment (contrast 3 "lures"), although *A. graveolens* caught slightly more flies when released from the 'glass bottle with wick' lure than with the 'polyethylene vial' lure. This pattern was not seen with *D. carota* extract, as catches for both types of lure were very similar to those on the control.

C) INVESTIGATIONS USING 'GLASS BOTTLE WITH WICK' LURES

Preliminary Studies on Release Rates

The results of cumulative weight loss experiments on a range of *trans*-methylisoeugenol 'polyethylene bag' lures are presented graphically in Figure A5, and the estimated release rates are given in Table 6.3c. The lure providing a release rate of 6.7mg/day, for over two weeks, was chosen for the release of *trans*-methylisoeugenol in the following field experiments.

Field Experiments Using the Improved Release System for Extracts

The first experiment was performed at two sites, over two consecutive periods. The results from the earlier experiment at site one (Table 6.4 Exp.6a, Fig 6.8) showed that *A. graveolens* extract produced a slight increase in catch, and this was almost a significant difference (contrast 1 "extract"). From the Figure, it

can be seen that both treatments incorporating *trans*-asarone and hexanal caught more flies than those without, and the effect of *trans*-asarone and hexanal was highly significant (contrast 2 "AH"). The Figure also shows that *trans*-asarone and hexanal plus *A. graveolens* extract caught the most flies, which was slightly more than with *trans*-asarone and hexanal alone. As the interaction between the extract and synthetic attractants was not significant, it suggests that the increase in catch when the two treatments were combined is additive, rather than synergistic, in nature. The results of the later experiment at site one (Table 6.5 Exp.6c) and both experiments at site two (Table 6.5 Exp.6b and 6d) were all unsuitable for statistical analysis. However, the treatment totals in all three of these experiments showed a very similar pattern of catches to that found in Experiment 6a, with the control and *A. graveolens* catching the least flies, and *trans*-asarone and hexanal alone, or in combination with *A. graveolens* catching the most. Furthermore, the combination of *trans*-asarone and hexanal plus *A. graveolens*, caught more flies than *trans*-asarone and hexanal alone, in two of the three experiments.

The results of the second experiment (Table 6.4 Exp.7, Fig 6.9) showed no significant differences between the catches on the control, *trans*-asarone and hexanal, and extracts overall (contrast 1 "control vs. AH vs. extracts"), or between *A. graveolens* extract and *trans*-methylisoeugenol, alone or in combination with hexanal (contrast 2 "among extracts"). From the Figure, it can be seen that all four treatments caught very similar numbers of flies.

The results of the third experiment (Table 6.4 Exp.8, Fig 6.10) showed no significant differences between the catches on the control, *trans*-asarone and hexanal, and extracts overall (contrast 1 "control vs. AH vs. extracts"), or between the two different extracts combined with synthetic attractants (contrast 2 "among extracts"), although the Figure shows that both extracts with *trans*-asarone and hexanal, and *trans*-asarone and hexanal, caught more flies than the control. Of the two extracts combined with trans-methylisoeugenol and hexanal, *C. maculatum* seemed to have a greater effect than *F. vulgare*, as it caught the most flies in this experiment.

6.3.2 FIELD INVESTIGATIONS INTO *C. maculatum* AND ITS CONSTITUENT COMPOUNDS

Preliminary Studies on Release Rates

Individual synthetic C. maculatum compounds

The results of cumulative weight loss experiments on a range of lure designs for the release of *C. maculatum* compounds [γ-coniceine, β-caryophyllene, and (*Z*)- and (*E*)-β-ocimene] are presented graphically in Figures A6 to A10, and the estimated release rates are given in Table 6.3. From the previous release rate study of *C. maculatum* volatiles in a 'glass bottle with wick' lure, γ-coniceine was seen to be released at rates of between 3.0 and 0.5 μg/hour, during the first four days of the experiment. In order to enhance the release of individual *C. maculatum* compounds in the field, compared to the rates at which they were released from extracts in 'glass bottle with wick' lures, a higher arbitrary release rate of between 5 and 10 mg/day was chosen for each compound. The lure providing a release rate of 7.7mg/day, for over four days, was chosen for γ-coniceine (Table. 6.3d). The lure providing a release rate of 7.0mg/day, for over two weeks (with a one day lag), was chosen for β-caryophyllene (Table. 6.3e), and the lure providing a release rate of 6.3mg/day for over nine days (with a one day lag), was chosen for β-ocimene (Table. 6.3f).

Air entrainment of γ-Coniceine

The results of the air entrainment experiment on a γ-coniceine lure, showing the release rates of γ-coniceine and any contaminants produced, are presented graphically in Figure A11. The Figure shows that approximately 65% of the volatiles released from the lure were γ-coniceine; the other 35% was composed of four minor contaminants, which were released in approximately equal proportions (i.e. less than 9% each). The release of γ-coniceine increased rapidly to a peak of above 78μg/hour on the first day of the experiment. It then dropped sharply to 40μg/hour by the start of day two, after which it tailed off more gradually to approximately 10μg/hour on day four, and ceased to release further after about a week. The release profiles of the four contaminants followed the release profile for γ-coniceine very closely, but the release rates were much lower (all peaking at approximately 15μg/hour). As the majority of the material released was found to be γ-coniceine, and knowing that γ-coniceine was relatively unstable, no problems were foreseen with its inclusion in the following field experiments.

Field Experiments Using *C. maculatum* Extracts and Compounds

The first experiment was performed at two different sites during consecutive years. The results from the earlier experiment at site one (Table 6.4 Exp.9a, Fig 6.11) showed no significant differences between the catches on the five treatments, probably due to the low number of flies caught during the experiment. From the Figure, it can be seen that *trans*-asarone and hexanal caught the most flies, while the control and *C. maculatum* extract alone caught the least. The addition of γ-coniceine, and to a lesser extent γ-coniceine plus β-caryophyllene and β-ocimene, produced a slight increase in the catch to an intermediate level, between that of the control and *trans*-asarone and hexanal treatments. The results from site two (Table 6.5 Exp.9b) were unsuitable for statistical analysis, as catches were very low ands contained lots of zero catches.

The results of the second experiment (Table 6.4 Exp.10, Fig 6.12) showed that the difference in catch between *trans*-asarone and hexanal, and the other treatments overall, was almost significant (contrast 1 "AH vs. others"). The Figure shows that *trans*-asarone and hexanal caught the most flies, and from the 95% c.i., is the only treatment clearly different from the control (catching almost twice as many flies). The effects of γ-coniceine, and β-caryophyllene plus β-ocimene, were not statistically significant (contrasts 2 "γ-con" and 3 "β-car and β-oci"), and no significant interactions between these treatments were found (contrast 4 "γ-con" x "β-car and β-oci"). However, the Figure shows a clear trend, with catches increasing as more *C. maculatum* compounds were combined although, even with all three hemlock compounds the catch was only intermediate, between *trans*-asarone and hexanal and the control.

The results of the third experiment (Table 6.2 Exp.11) were not analysed, as no *P. rosae* were caught during this experiment.

6.3.3 COMPARISON OF STANDARD, AND *trans*-ASARONE AND HEXANAL, BAITED MONITORING TRAPS (IN COLLABORATION WITH ADAS)

This experiment compared the catch on a standard un-baited REBELL ® monitoring trap (the control), with a similar trap baited with *trans*-asarone and hexanal. It was performed over a wide range of sites in two consecutive years (1995 and 1996); however, very few *P. rosae* were actually caught during either year.

The results form 1995 (Table 6.4 Exp12a) showed no significant difference in catch on the two treatments at any of the ten sites. However, the catch was slightly higher on the *trans*-asarone and hexanal baited traps at seven of the ten sites. During this experiments some ADAS workers noted that there was little or no hexanal solution left in the 'amber glass' lures when they were collected from the field; consequently, the quantity of hexanal used in these lures was doubled to 100µl in the following year.

The results from 1996 (Table 6.4 Exp12b) showed a significant difference between the treatments at one Northern site (Travis), but no significant difference at the other seven sites. In this year, the baited traps only caught more than the control at four of the eight sites. As the experiment at the Travis site caught a relatively large number of flies, it was possible to re-analyse the data from this site as a Latin square experiment (Table 6.4 Exp.12b re-analysis of Travis site, Fig. 6.13). The results at this site showed a highly significant difference between the baited (which caught 424 flies in total) and unbaited traps (which only caught 143 flies).

In the small comparative experiment performed at my field site in 1996 (Table 6.4 Exp.12c), no statistical analysis was possible, as so few *P. rosae* were caught. Only one block caught any carrot flies, and of the five caught, all were on the *trans*-asarone and hexanal baited traps.

Further Release Rate Studies Aimed at Solving Problems Encountered with the *trans*-Asarone and Hexanal Lures

Air entrainment of trans-asarone lures

The results of the air entrainment of *trans*-asarone lures (alone or with added antioxidant), showing the release rates of *trans*-asarone and any contaminants produced plus the quantities of compounds remaining in the lures after two weeks of entrainment, are summarised in Table 6.6. The GC analysis of the entrainment samples from the standard *trans*-asarone lure showed two major peaks, one at 32.47 min, which corresponded to the release of *trans*-asarone, and a contaminant peak with a retention time of 31.51 min. The GC retention time of this contaminant peak corresponded with the retention time of the major contaminant peak (less than 1%) in GC traces of *trans*-asarone standards. This contaminant was subsequently identified by GC co-injection with authentic standards as the *cis*- isomer of asarone.

During the first week of entrainment, the standard lure released a total of 1.57mg of *cis*- and *trans*-asarone. This was equivalent to a relatively slow release rate of 0.224mg/day, and was in close

accordance with the earlier release rate determined by weight loss of 0.22mg/day (see Table 6.3a and Fig. A1). However, of this total, only 57.3% (0.9mg) was released as *trans*-asarone, with the other 42.7% (0.67mg) being released as *cis*-asarone. In its second week of entrainment, a similar total quantity of asarone was released (1.48mg), although the ratio of *trans*- to *cis*- asarone shifted slightly, with a higher proportion of the *cis*- isomer being released (58.1%). The material remaining in the lure at the end of the two week experiment was found to consist of mainly *trans*-asarone (92.3%), with only a little *cis*-asarone (7.7%). Calculations of the total quantity of asarone extracted during the air entrainment, plus that extracted form the lure and entrainment vessel at the end of the experiment, showed a high level of recovery (approximately 98%), suggesting that little or no breakthrough of asarone occurred from the Porapak traps during the experiment.

The GC analysis of entrainment samples from the lures containing additional BHT antioxidant (1% and 10% w:w) showed it had little effect on the quantities or ratios of *cis*- and *trans*-asarone released, which remained similar to those released from the standard lure. This would be expected as no oxidation products were discovered as contaminants, so the addition of BHT antioxidant to the lure had no effect.

As *trans*-asarone is classed as a slightly light sensitive compound, the production of the only contaminant found, *cis*-asarone, was probably the result of light sensitive isomerisation (see Fig. 6.14). This isomerisation was obviously not an artefact of GC analysis, as little *cis*-asarone was seen in the GC traces of *trans*-asarone standards. As very little *cis*-asarone was found in the material remaining in the lure at the end of the experiment, compared to the entrainment samples, the isomerisation to *cis*-asarone probably occurred either whilst *trans*-asarone was in the vapour phase, or whilst it was adsorbed onto the Porapak trap. As this experiment was performed under fluorescent tube lighting, which has a relatively high ultraviolet component compared to natural daylight, light sensitive isomerisation may have occurred more rapidly, resulting in the production of more *cis*-asarone than would occur under field conditions.

Alternative Lures for the Release of Hexanal

The results of cumulative weight loss experiments on a range of hexanal polyethylene 'bag' and 'vial' lures are presented graphically in Figures A12 to A14, and the estimated release rates are given in Table 6.3g. The 'amber glass vial' lure has a release rate of 9.6 mg/day (at 20°C), and a suitable replacement would need to release hexanal at a similar rate for at least a week. The table shows that the 1000G 'polyethylene bag' lure, containing a $^{1}/_{4}$ sized piece of thin sponge impregnated with 150µl of hexanal solution, was the closest to fulfilling these requirements. This lure released hexanal at a rate of 15.5mg/day (at 20°C), but only for five days (Table 6.3g); however, this period could easily be extended to over a week by the incorporation of 200µl of hexanal solution, instead of 150µl.

Table 6.3 **Summary of Results from Release Rate Experiments, Determined by Cumulative Weight Loss**

Compounds and Lure Designs Tested	Initial Steady Release Rate (in mg/day)	Period of Steady Release (Lag Time Prior to Steady Release)
a) *trans*-Asarone		
50mg on a white 'Energy Board' strip	0.22	3 weeks +
b) Hexanal solution - containing 10% BHT (w:w)		
30µl in an 'amber glass vial' with a 1mm hole in the cap	4.8	4 days
50µl in an 'amber glass vial' with a 1mm hole in the cap	4.8	7 days
100µl in an 'amber glass vial' with a 1mm hole in the cap	4.8	12 days
c) *trans*-Methylisoeugenol		
300µl on a thin sponge, in a 100G polyethylene bag	6.7	2 weeks +
300µl on a thin sponge, in a 250G polyethylene bag	4.0	2 weeks +
300µl on a thin sponge, in a 500G polyethylene bag	2.5	2 weeks +
300µl on a thin sponge, in a 1000G polyethylene bag	1.8	2 weeks +
d) γ-Coniceine		
100µl on a thin sponge, in a 250G polyethylene bag	20.2	2 days
100µl on a thin sponge, in a 500G polyethylene bag	11.5	3 days
100µl on $^1/_8^{th}$ of a thin sponge, in a 1000G polyethylene bag	10.1	4 days
100µl on a thin sponge in a 1000G polyethylene bag	7.7	4 days
100µl in a polyethylene micro test tube	2.1	2 weeks + (2 day lag)
10µl on $^1/_8^{th}$ of a thin sponge, in a 1000G polyethylene bag	0.25	2 weeks + (2 day lag)
e) β-Caryophyllene		
300µl on a thin sponge, in a 1000G polyethylene bag	7.0	2 weeks (1 day lag)
300µl on a thin sponge in a 2000G polyethylene bag	2.8	4 weeks + (4 days lag)
250µl in a polyethylene micro test tube	0.26	2 weeks + (2 days lag)
300µl on a thin sponge in a tall polyethylene vial	0.10	2 weeks + (3 days lag)
f) β-Ocimene		
300µl on a thin sponge, in a 1000G polyethylene bag	88.8	2 days
250µl in a polyethylene micro test tube	6.2	9 days (1day lag)
300µl in a tall polyethylene vial	4.1	1 week + (1day lag)
g) Hexanal solution - containing 10% BHT (w:w)		
50µl on a thin sponge, in a 1000G polyethylene bag	17.9	1 day
150µl on $^1/_4$ of a thin sponge, in a 1000G polyethylene bag	15.5	5 days
100µl on $^1/_4$ of a thin sponge, in a 1000G polyethylene bag	13.0	2 days
100µl on $^1/_8^{th}$ of a thin sponge, in a 1000G polyethylene bag	11.4	4 days
50µl on $^1/_8^{th}$ of a thin sponge, in a 1000G polyethylene bag	10.2	2 days
50µl on a thin sponge, in a 3000G polyethylene bag	3.3	5 days
50µl on $^1/_8^{th}$ of a thin sponge, in a 3000G polyethylene bag	2.3	7 days (1 day lag)
50µl in a polyethylene micro test tube	2.1	17 days (1 day lag)
50µl in a polypropylene micro test tube	0.8	15 days + (4 days lag)
50µl in a polyethylene vial (3ml, WP/5)	0.1	15 days + (4 days lag)

Table 6.4: **Statistical Analyses of Field Experiments: Analysis of Variance, Log_{10} (number of carrot flies +1)**

Investigations on Plant Extracts (A): 'Polyethylene Vial' Lures

Experiment 1

Source of Variation	d.f.	s.s.	m.s.	*F*	*P*
Block	1	0.050	0.050	0.08	
Date	4	2.354	0.588	13.00	
Treatment:	(3)	(0.481)			
control vs. extracts	1	0.230	0.230	5.09	0.039 *
among extracts	2	0.251	0.125	2.77	0.095
Error	15	0.679	0.045		
Total	23	3.563			

Experiment 2

Source of Variation	d.f.	s.s.	m.s.	*F*	*P*
Block	1	0.240	0.240	0.25	
Date	6	5.816	0.969	31.05	
Treatment	4	0.380	0.095	3.04	0.033 *
Error	28	0.874	0.031		
Total	39	7.309			

Experiment 3a

Source of Variation	d.f.	s.s.	m.s.	*F*	*P*
Date	3	1.540	0.513	4.38	
Treatment:	(2)	(0.048)			
control vs. extracts	1	0.002	0.002	0.02	0.897
among extracts	1	0.046	0.046	0.39	0.555
Error	6	0.703	0.117		
Total	11	2.290			

Experiment 3b

Source of Variation	d.f.	s.s.	m.s.	*F*	*P*
Date	2	0.550	0.27516	21.44	
Position	2	0.166	0.08288	6.46	
Treatment:	(2)	(0.014)			
control vs. extracts	1	0.003	0.003	0.20	0.700
among extracts	1	0.011	0.011	0.86	0.453
Error	2	0.026	0.013		
Total	8	0.755			

Experiment 4

Source of Variation	d.f.	s.s.	m.s.	*F*	*P*
Date	9	8.329	0.925	10.41	
Treatment	5	1.145	0.229	2.58	0.040 *
Error	44 (1)	3.910	0.089		
Total	58	13.384			

Table 6.4: **Statistical Analyses of Field Experiments: Analysis of Variance, Log_{10} (number of carrot flies +1), Continued**

Investigations on Plant Extracts (B): Comparison of Two Lure Types

Experiment 5a

Source of Variation	d.f.	s.s.	m.s.	*F*	*P*
Block	7	4.732	0.676		
Date	40	27.495	0.687	22.66	
Position	40	2.768	0.069	2.28	
Treatment:	(5)	(3.223)			
control vs. AH vs. extracts	2	2.352	1.176	38.78	<0.001 ***
species	1	0.140	0.140	4.61	0.033 *
lures	1	0.588	0.588	19.38	<0.001 ***
species x lures	1	0.143	0.143	4.71	0.031 *
Error	193 (2)	5.853	0.030		
Total	285 (2)	44.015			

Experiment 5b

Source of Variation	d.f.	s.s.	m.s.	*F*	*P*
Block	1	0.001	0.001		
Date	10	1.510	0.151	5.74	
Position	10	0.663	0.066	2.52	
Treatment:	(5)	(1.402)			
control vs. AH vs. extracts	2	1.259	0.630	23.92	<0.001 ***
species	1	0.091	0.091	3.46	0.069
lures	1	0.017	0.017	0.64	0.428
species x lures	1	0.035	0.035	1.33	0.256
Error	45	1.184	0.026		
Total	71	4.760			

Investigations on Plant Extracts (C): 'Glass Bottle with Wick' Lures

Experiment 6a

Source of Variation	d.f.	s.s.	m.s.	*F*	*P*
Block	7	4.682	0.669		
Date	24	8.8565	0.369	20.86	
Position	24	0.891	0.037	2.10	
Treatment:	(3)	(1.582)			
extract	1	0.052	0.052	2.91	0.092
AH	1	1.518	1.518	85.82	<0.001 ***
extract x AH	1	0.012	0.012	0.68	0.411
Error	69	1.220	0.018		
Total	127	17.230			

Experiment 7

Source of Variation	d.f.	s.s.	m.s.	*F*	*P*
Date	3	2.567	0.856	78.73	
Position	3	0.128	0.043	3.94	
Treatment:	(3)	(0.005)			
control vs. AH vs. extracts	2	0.004	0.002	0.19	0.831
among extracts	1	0.001	0.001	0.06	0.815
Error	6	0.065	0.011		
Total	15	2.765			

Table 6.4: Statistical Analyses of Field Experiments: Analysis of Variance, Log_{10} (number of carrot flies +1), Continued

Experiment 8

Source of Variation	d.f.	s.s.	m.s.	*F*	*P*
Date	3	1.247	0.416	12.31	
Position	3	0.435	0.145	4.29	
Treatment:	(3)	(0.185)			
control vs. AH vs. extracts	2	0.161	0.080	2.38	0.173
among extracts	1	0.024	0.024	0.70	0.436
Error	6	0.203	0.034		
Total	15	2.069			

Investigations on *C. maculatum* and its Constituent Compounds

Experiment 9a

Source of Variation	d.f.	s.s.	m.s.	*F*	*P*
Block	4	3.173	0.793	16.04	
Treatment	4	0.413	0.103	2.09	0.130
Error	16	0.791	0.049		
Total	24	4.377			

Experiment 10

Source of Variation	d.f.	s.s.	m.s.	*F*	*P*
Block	4	0.366	0.091	2.63	
Treatment:	(4)	(0.189)			
AH vs. others	1	0.122	0.122	3.51	0.079
γ-con	1	0.030	0.030	0.88	0.363
β-car and β-oci	1	0.036	0.036	1.04	0.323
γ-con x β-car and β-oci	1	0.001	0.001	0.03	0.860
Error	16	0.556	0.035		
Total	24	1.112			

Comparison of Standard and Baited Monitoring Traps (in Collaboration with ADAS)

Experiment 12a

Site No	Treatment means		Standard error of the mean (s.e.m)	Reps.	*F*	*P*	Sig.
	A) Control	B) Asarone + Hexanal					
1	0.433	0.533	0.113	10	0.39	0.547	ns.
2	0.637	0.661	0.049	10	0.12	0.733	ns.
3	0.496	0.402	0.058	10	1.34	0.277	ns.
4	0.511	0.594	0.110	5	0.28	0.623	ns.
5	0.181	0.156	0.101	5	0.03	0.869	ns.
6	0.260	0.235	0.086	5	0.04	0.847	ns.
7	0.396	0.464	0.131	5	0.14	0.731	ns.
8	0.095	0.276	0.083	5	2.36	0.199	ns.
9	0.771	0.891	0.141	5	0.36	0.581	ns.
10	0.556	0.620	0.052	5	0.78	0.428	ns.

Table 6.4: **Statistical Analyses of Field Experiments: Analysis of Variance, Log_{10} (number of carrot flies +1), Continued**

Experiment 12b

Site No	Treatment means		Standard error of the mean (s.e.m)	Reps.	*F*	*P*	Sig.
	A) Control	B) Asarone + Hexanal					
1	0.324	0.281	0.075	10	0.17	0.694	ns.
2	0.273	0.346	0.028	10	3.33	0.101	ns.
3	0.173	0.120	0.072	10	0.27	0.615	ns.
4	0.151	0.198	0.051	10	0.44	0.522	ns.
5	0.156	0.311	0.128	5	0.74	0.437	ns.
6	0.834	0.820	0.054	5	0.04	0.856	ns.
7	0.520	0.610	0.162	5	0.13	0.738	ns.
8	1.439	1.920	0.045	5	256.27	0.002	$P<0.01$ **

Experiment 12b, Re-analysis of Travis site

Source of Variation	d.f.	s.s.	m.s.	*F*	*P*
Block	4	0.356	0.089		
Date	25	10.771	0.431	13.21	
Position	5	0.138	0.028	0.85	
Treatment	1	1.654	1.654	50.69	<0.001 ***
Error	24	0.783	0.033		
Total	59	13.702			

Table 6.5 Treatment Totals of Experiments (per Block) Not Analysed Statistically

Exp. 6b

Treatment	Block 1	Block 2	Total
A) Control	6	4	**10**
B) *trans*-Asarone + hexanal	5	15	**20**
C) *A. graveolens*	9	6	**15**
D) *A. graveolens* + *trans*-asarone + hexanal	12	13	**28**

Exp. 6c

Treatment	Block 1	Block 2	Total
A) Control	35	114	**149**
B) *trans*-Asarone + hexanal	49	112	**161**
C) *A. graveolens*	22	122	**144**
D) *A. graveolens* + *trans*-asarone + hexanal	46	129	**175**

Exp. 6d

Treatment	Block 1	Block 2	Block 3	Total
A) Control	2	4	2	**8**
B) *trans*-Asarone + hexanal	7	11	2	**20**
C) *A. graveolens*	1	1	3	**5**
D) *A. graveolens* + *trans*-asarone + hexanal	2	5	9	**16**

Exp. 9b

Treatment	Block 1	Block 2	Total
A) Control	1	2	**3**
B) *trans*-Asarone + hexanal	4	1	**5**
C) *C. maculatum*	1	0	**1**
D) *C. maculatum* + γ-coniceine	0	1	**1**
E) *C. maculatum* + γ-coniceine + β-caryophyllene and β-ocimene	1	0	**1**

Exp. 12c

Treatment	Block 1	Block 2	Block 3	Total
A) Control	0	0	0	**0**
B) *trans*-Asarone + hexanal	5	0	0	**5**

Table 6.6 Summary of Results from the Air Entrainment of *trans*-Asarone Lures

Compounds identified by GC co-injection	Retention time on GC	Blank control	Standard asarone lure	Asarone lure plus 1% BHT	Asarone lure plus 10% BHT
Total quantity of each compound released (in mg) from *trans*-asarone lures during first week of air entrainment					
BHT (Butylated hydroxytoluene)	29.96	none	none	0.44	4.97
cis-Asarone	31.51	none	0.67	0.68	0.59
trans-Asarone	32.47	none	0.90	1.03	1.01
Total asarone (*cis* + *trans*)	-	none	1.57	1.71	1.60
Total quantity of each compound released (in mg) from *trans*-asarone lures during the second week of air entrainment					
BHT (Butylated hydroxytoluene)	29.96	none	none	none	0.21
cis-Asarone	31.51	none	0.86	0.84	0.79
trans-Asarone	32.47	none	0.62	0.75	0.91
Total asarone (*cis* + *trans*)	-	none	1.48	1.59	1.70
Total quantity of each compound remaining in the *trans-asarone* lures (in mg), at the end of the two-week air entrainment					
BHT (Butylated hydroxytoluene)	29.96	none	none	none	none
cis-Asarone	31.51	none	3.52	2.56	1.49
trans-Asarone	32.47	none	42.28	41.96	43.41
Total asarone (*cis* + *trans*)	-	none	45.80	44.52	44.90
Total quantity of each compound recovered from the *trans*-asarone lures (in mg) by air entrainment and by solvent extraction					
Compound		**Blank control**	**Standard asarone lure**	**Asarone lure plus 1% BHT**	**Asarone lure plus 10% BHT**
Original BHT (Butylated hydroxytoluene)		-	-	0.50	5.00
Recovered BHT (Butylated hydroxytoluene)		-	-	0.44	5.18
Original *trans*-asarone		-	50.00	50.00	50.00
Total recovered asarone (*cis* + *trans*)		-	48.85	47.82	48.20

Figure 6.1: **Mean catch of *P. rosae* per sampling interval. Data plotted are back transformed (geometric) means ± 95% confidence intervals. Details of design are in Table 6.2 Exp. 1.**

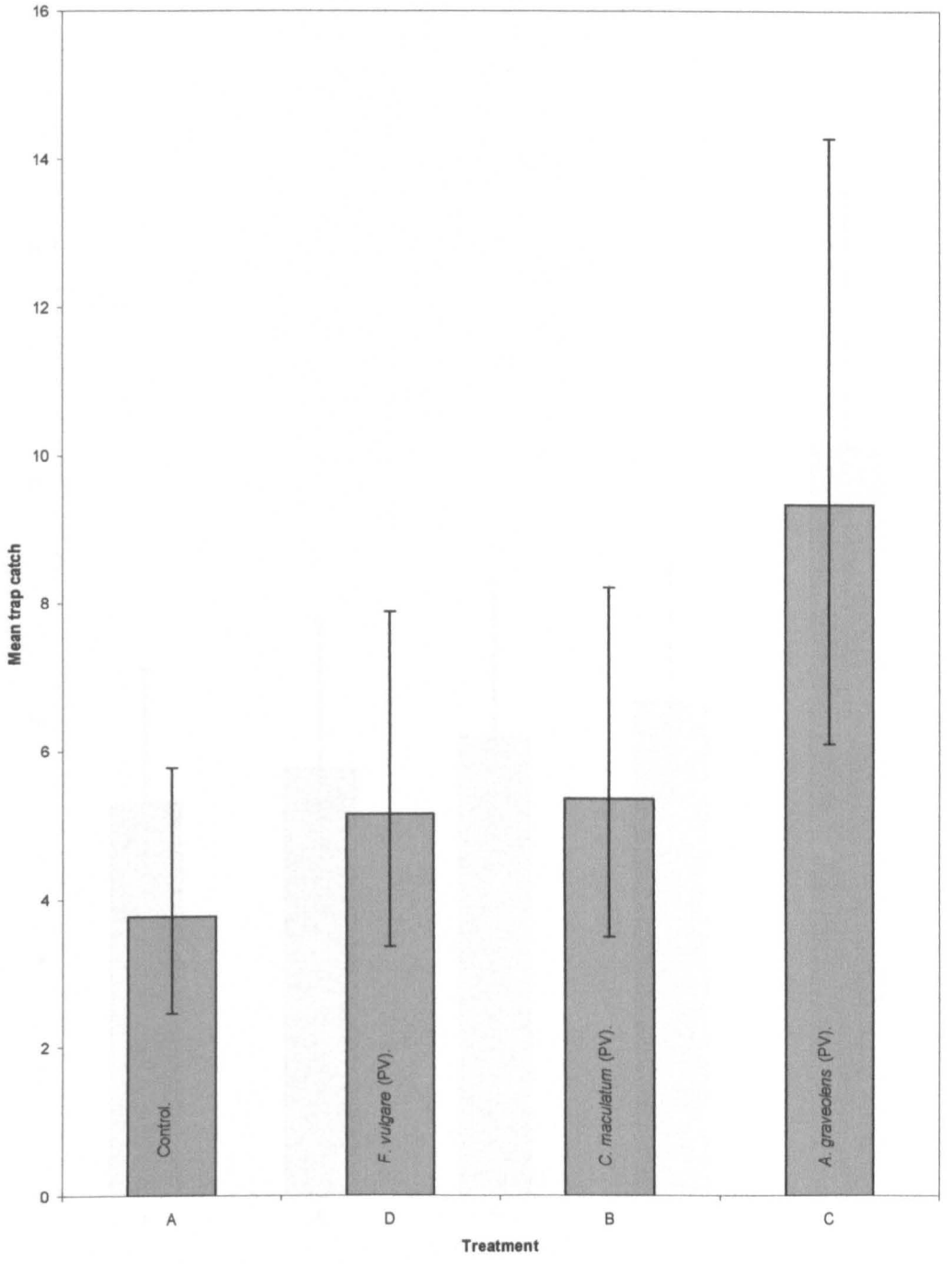


Figure 6.2: **Mean catch of *P. rosae* per sampling interval. Data plotted are back transformed (geometric) means ± 95% confidence intervals. Details of design are in Table 6.2 Exp. 2.**

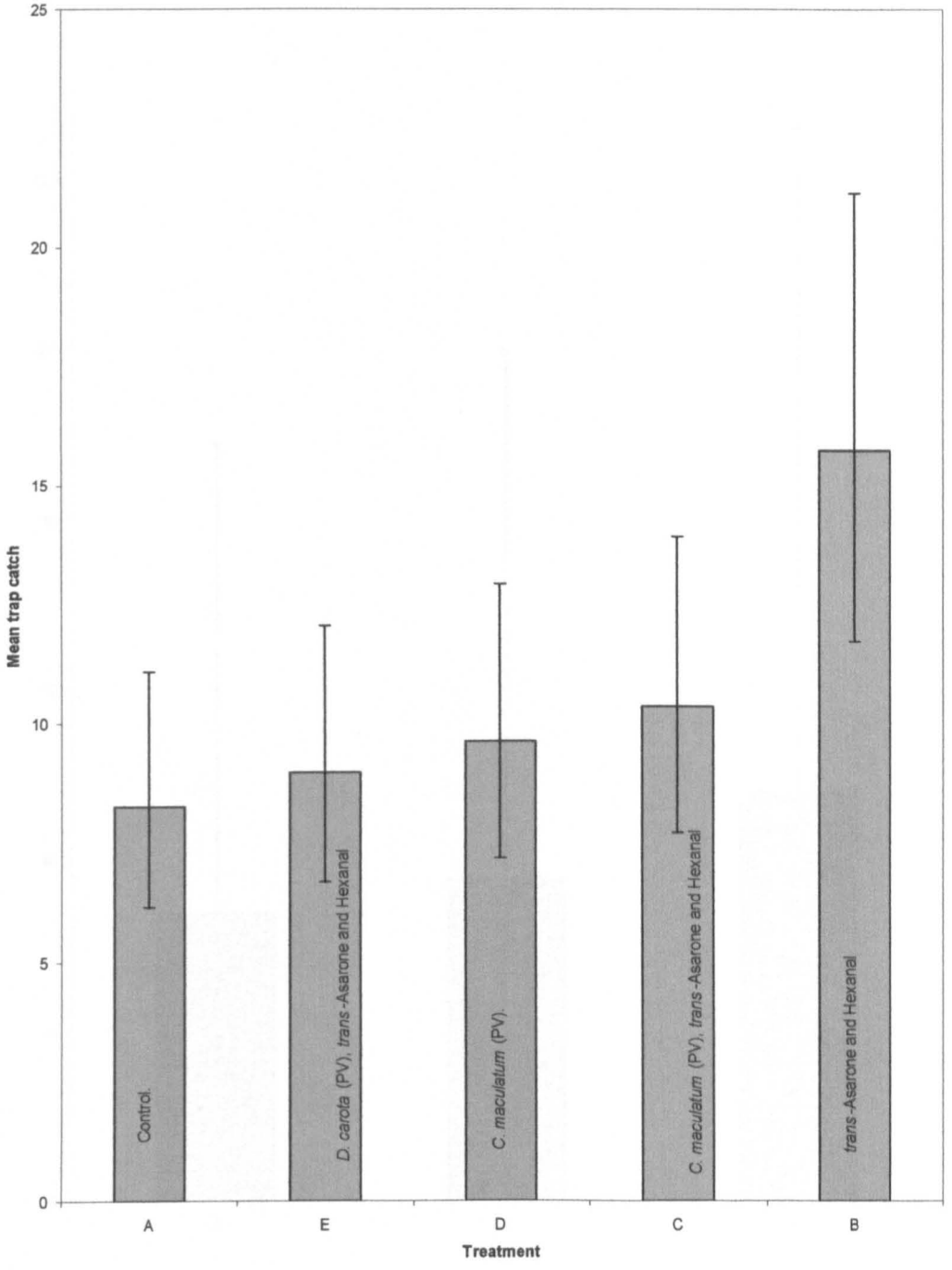


Figure 6.3: **Mean catch of *P. rosae* per sampling interval. Data plotted are back transformed (geometric) means ± 95% confidence intervals. Details of design are in Table 6.2 Exp. 3a.**

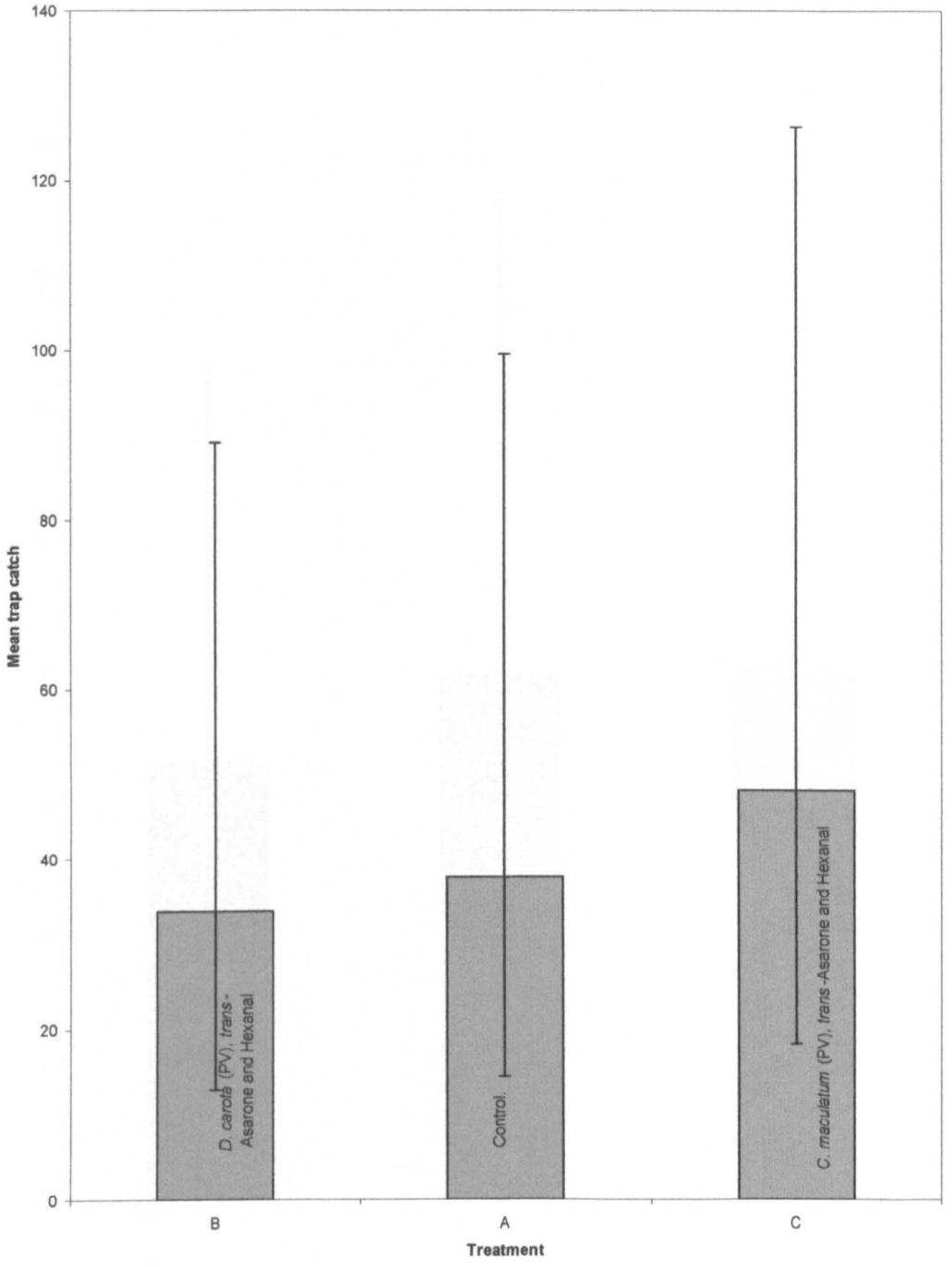


Figure 6.4: **Mean catch of *P. rosae* per sampling interval. Data plotted are back transformed (geometric) means ± 95% confidence intervals. Details of design are in Table 6.2 Exp. 3b.**

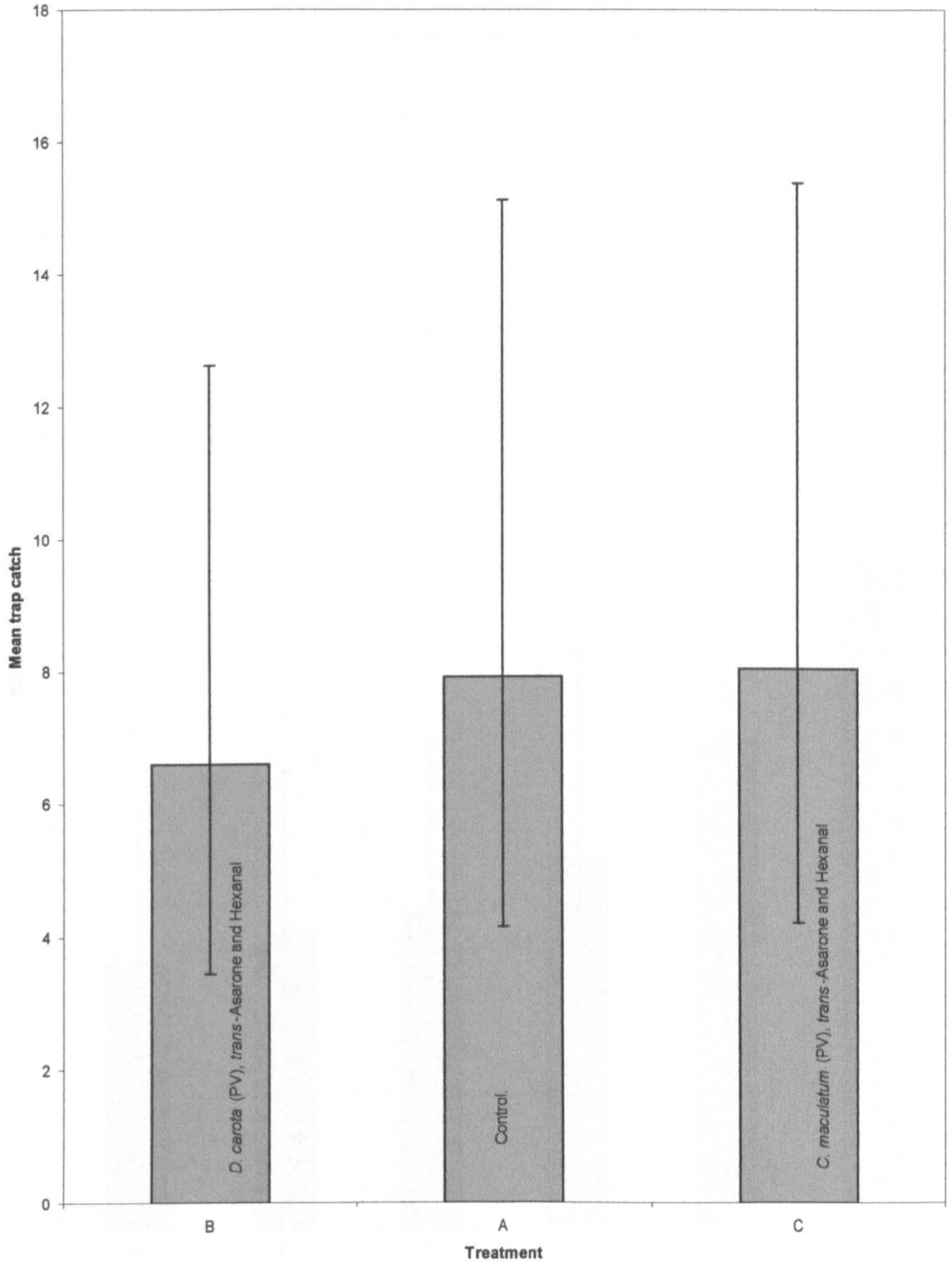


Figure 6.5: **Mean catch of *P. rosae* per sampling interval. Data plotted are back transformed (geometric) means ± 95% confidence intervals. Details of design are in Table 6.2 Exp. 4.**

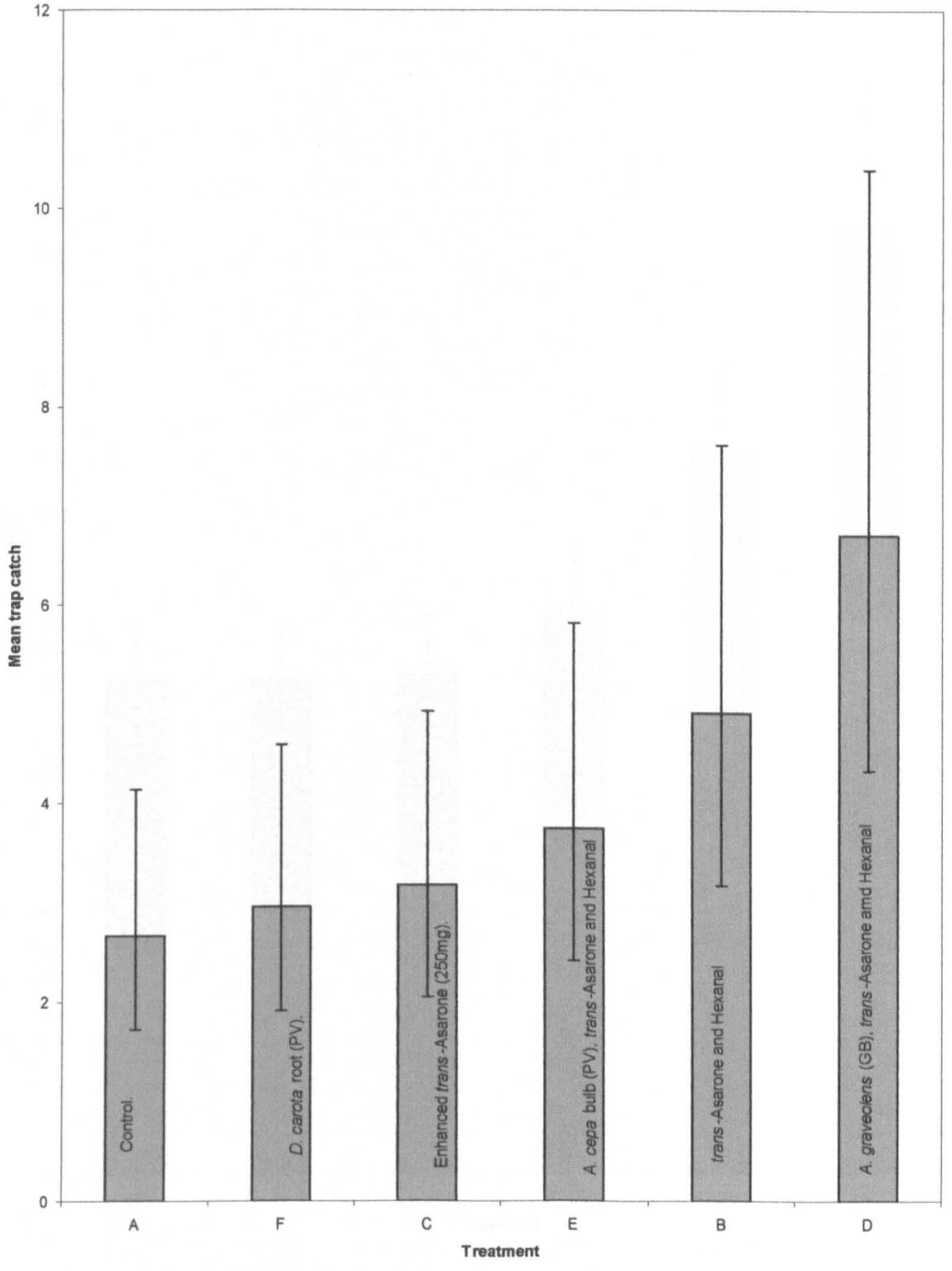


Figure 6.6: **Mean catch of *P. rosae* per sampling interval. Data plotted are back transformed (geometric) means ± 95% confidence intervals. Details of design are in Table 6.2 Exp. 5a.**

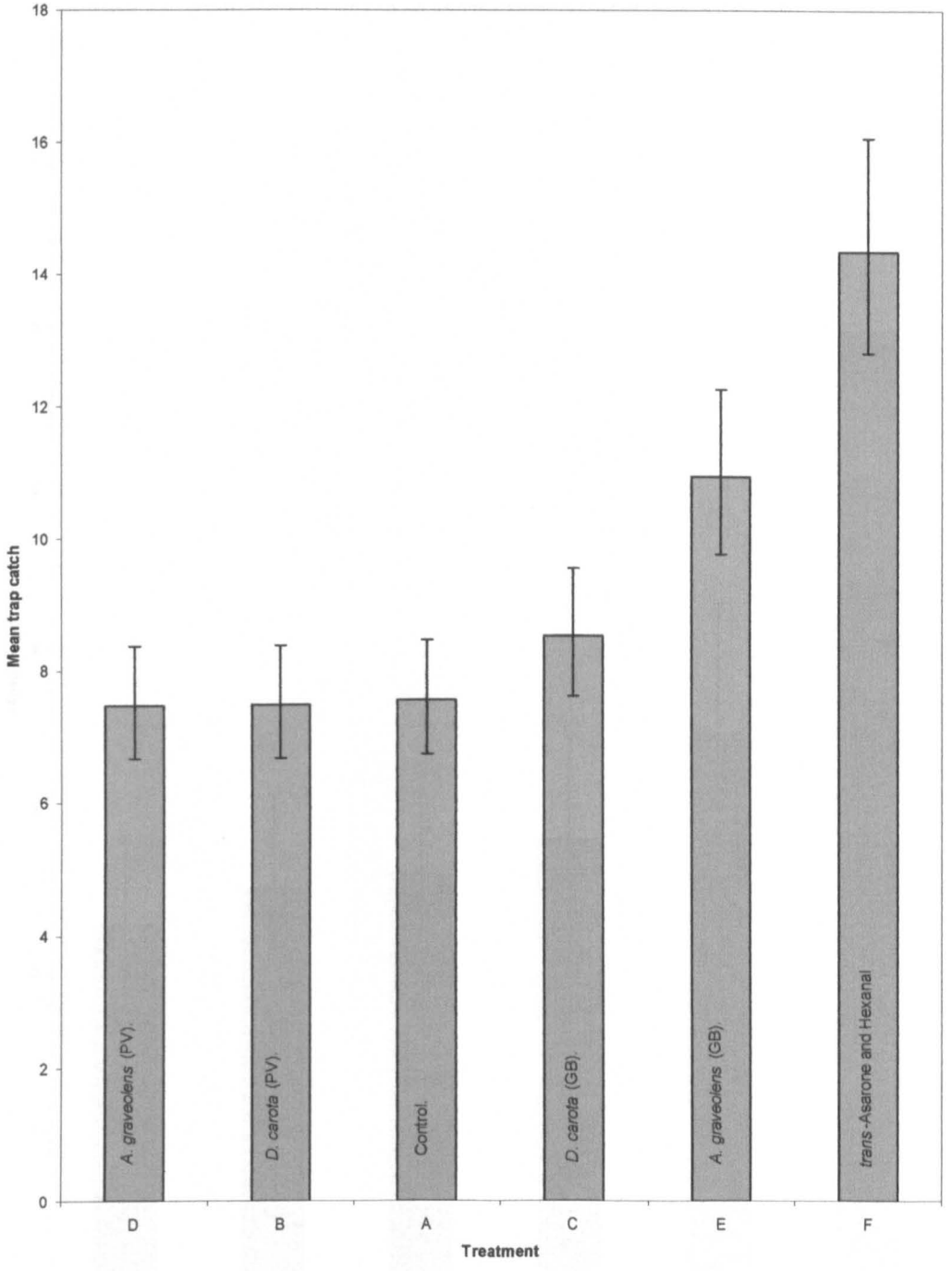


Figure 6.7: **Mean catch of *P. rosae* per sampling interval. Data plotted are back transformed (geometric) means ± 95% confidence intervals. Details of design are in Table 6.2 Exp. 5b.**

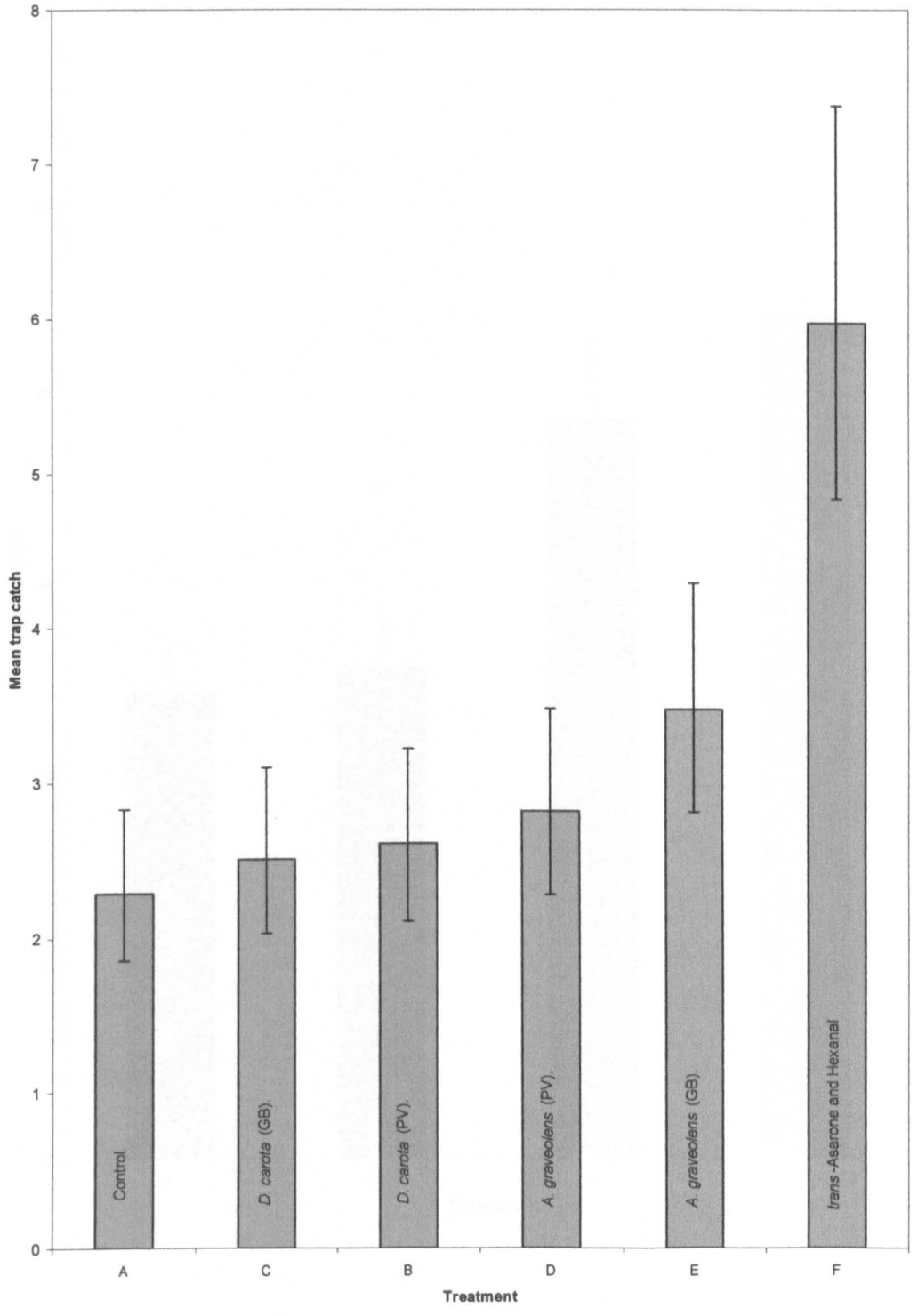


Figure 6.8: **Mean catch of *P. rosae* per sampling interval. Data plotted are back transformed (geometric) means ± 95% confidence intervals. Details of design are in Table 6.2 Exp. 6a.**

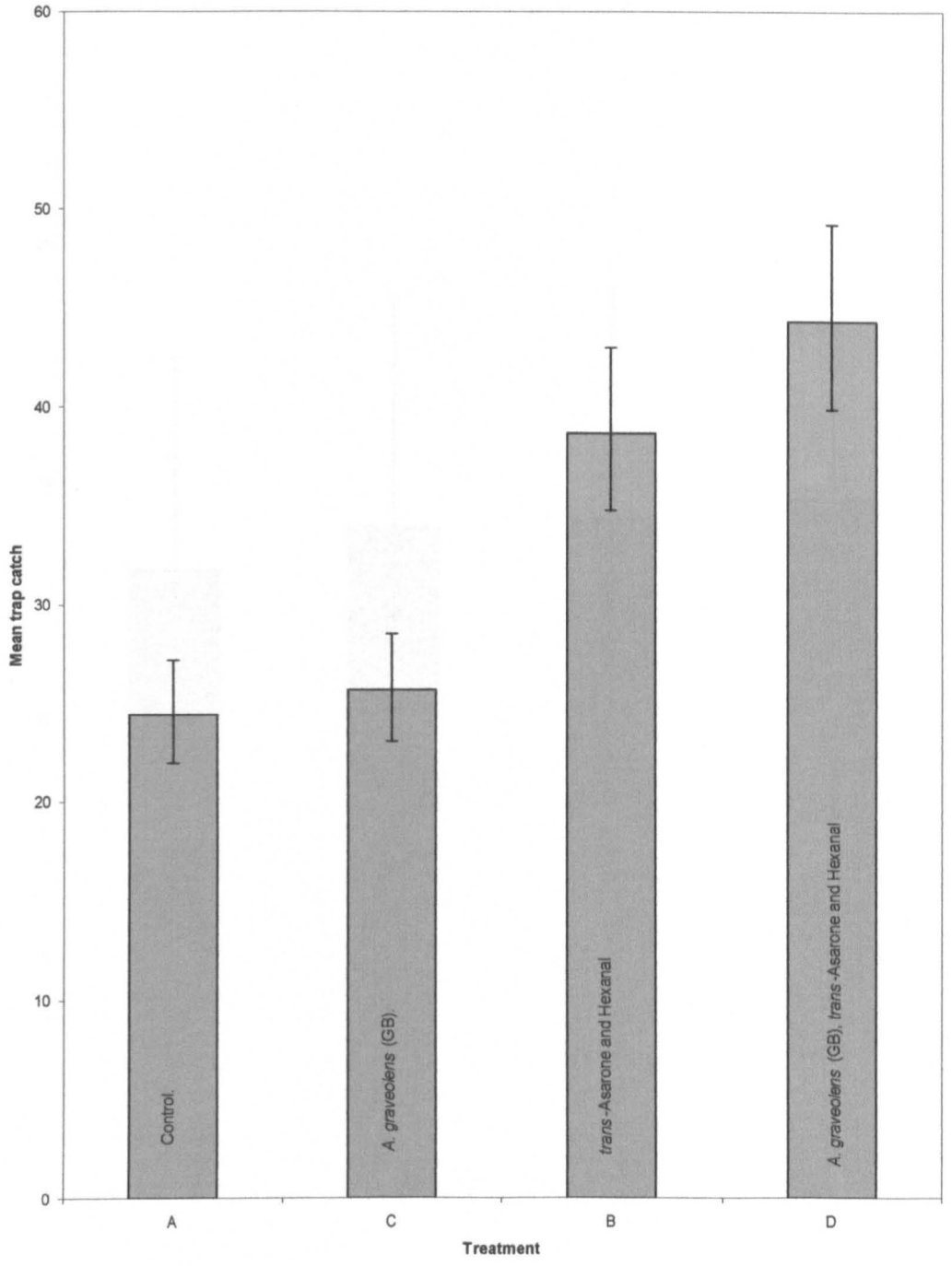


Figure 6.9: **Mean catch of *P. rosae* per sampling interval. Data plotted are back transformed (geometric) means ± 95% confidence intervals. Details of design are in Table 6.2 Exp. 7.**

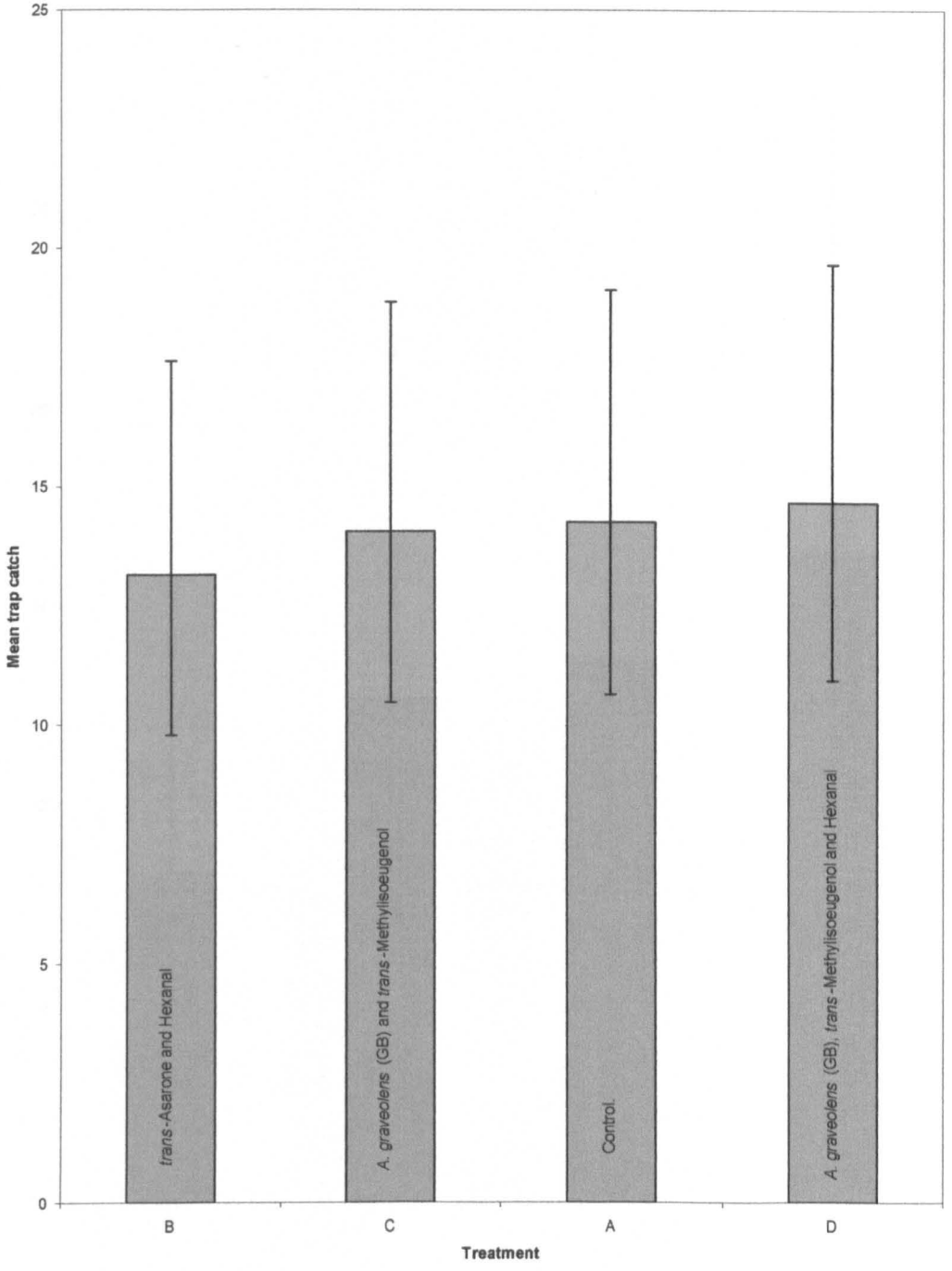


Figure 6.10: **Mean catch of *P. rosae* per sampling interval. Data plotted are back transformed (geometric) means ± 95% confidence intervals. Details of design are in Table 6.2 Exp. 8.**

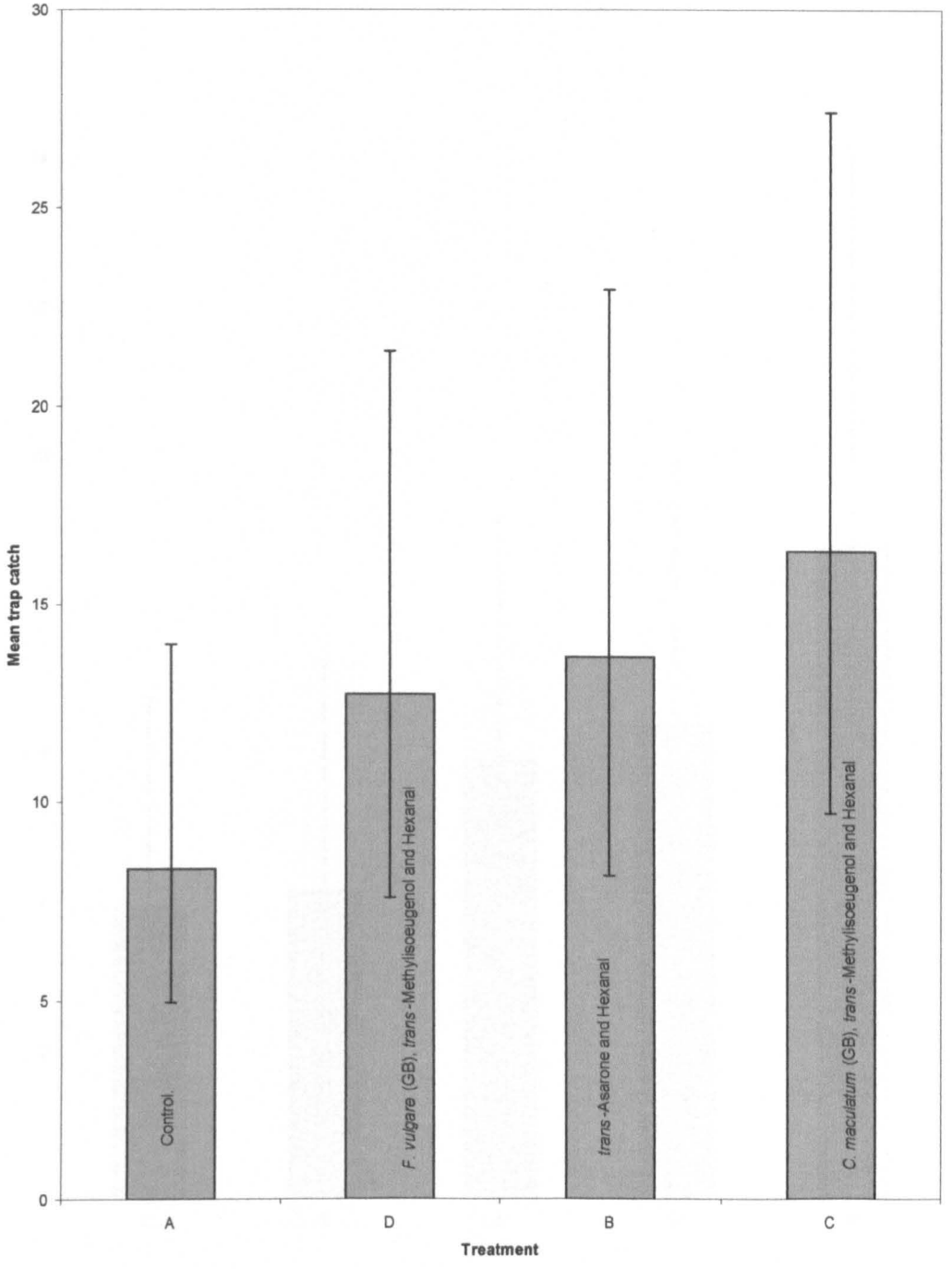


Figure 6.11: **Mean catch of *P. rosae* per sampling interval. Data plotted are back transformed (geometric) means ± 95% confidence intervals. Details of design are in Table 6.2 Exp. 9a.**

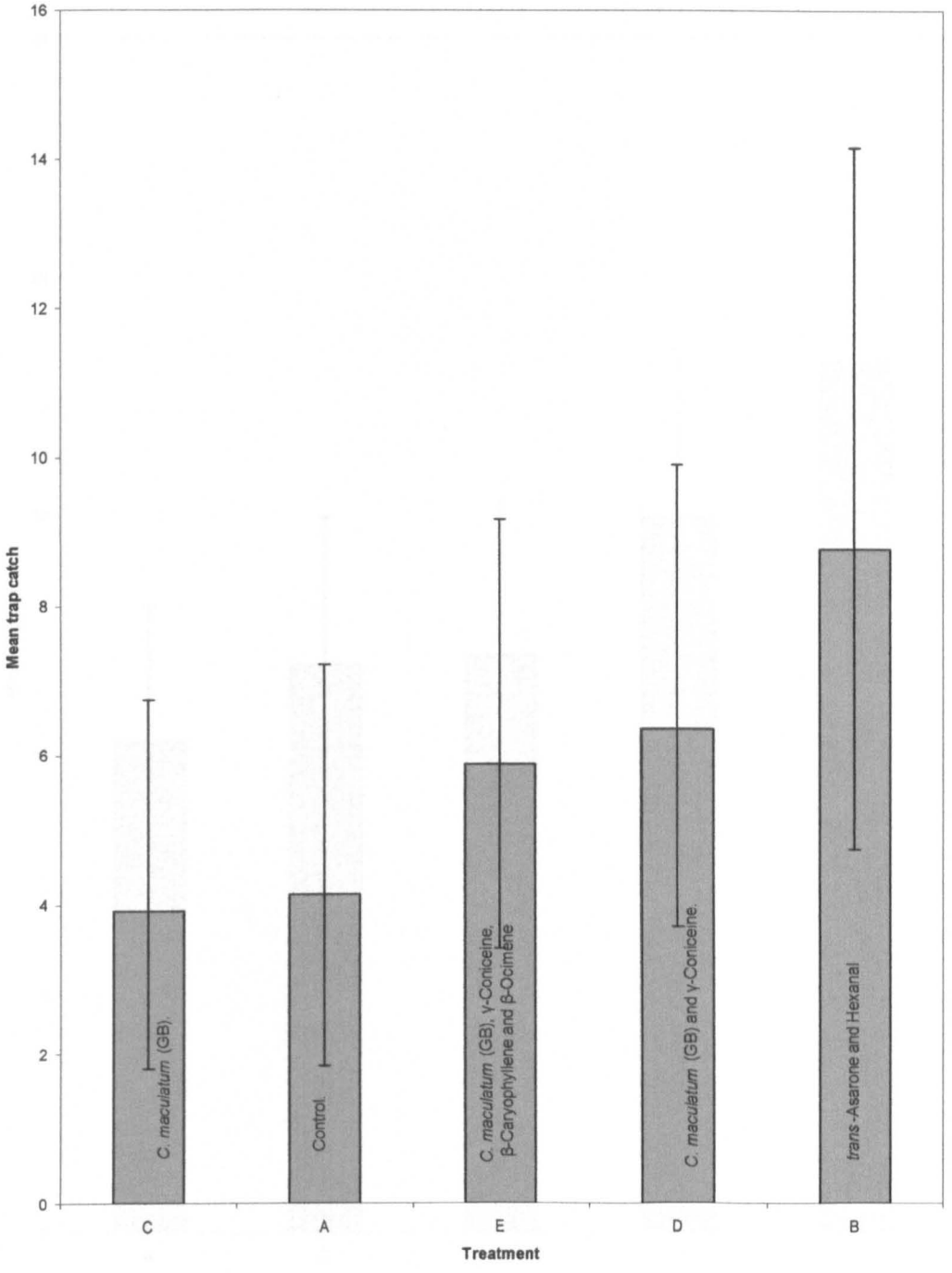


Figure 6.12: **Mean catch of *P. rosae* per sampling interval. Data plotted are back transformed (geometric) means ± 95% confidence intervals. Details of design are in Table 6.2 Exp. 10.**

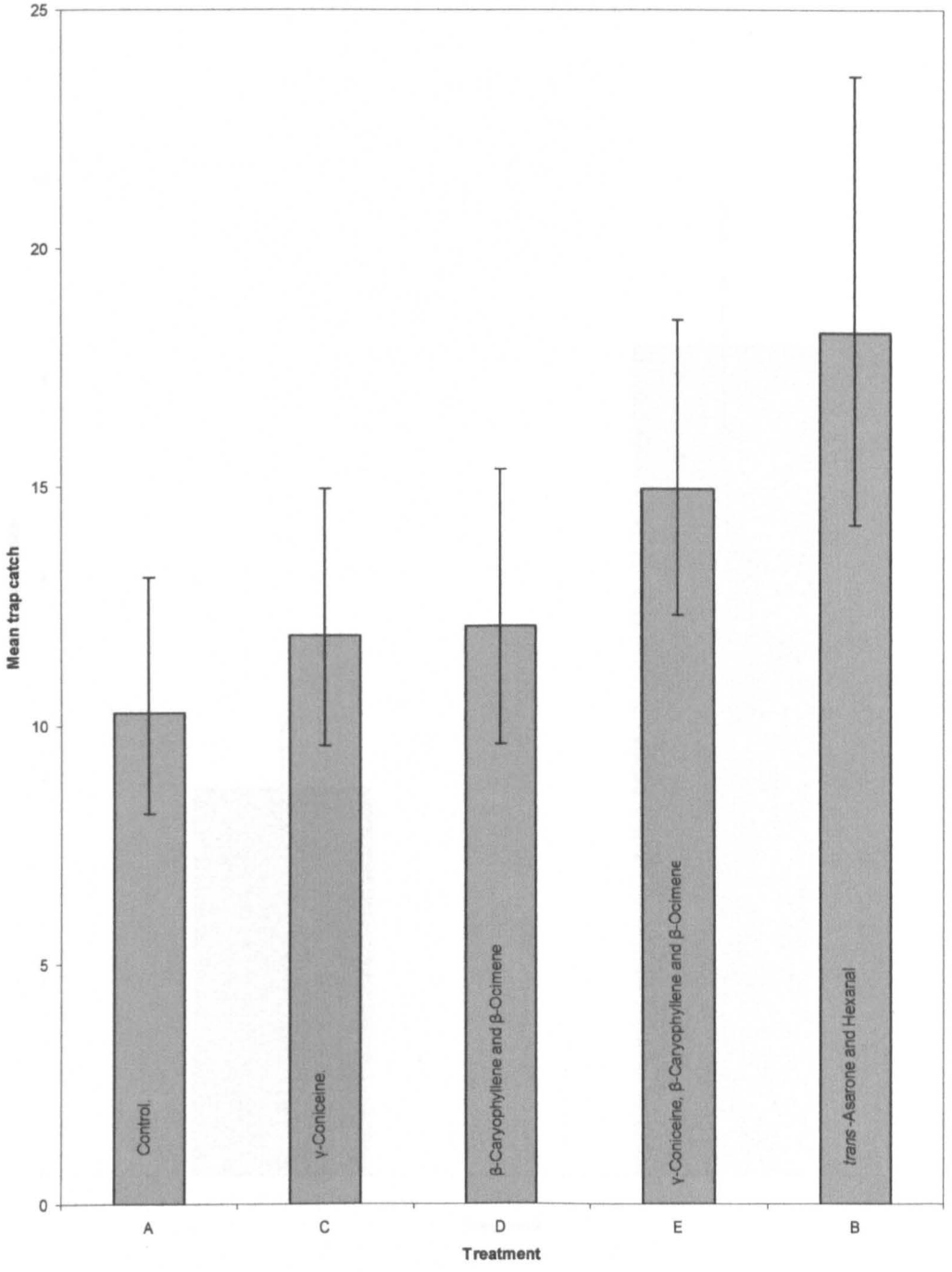
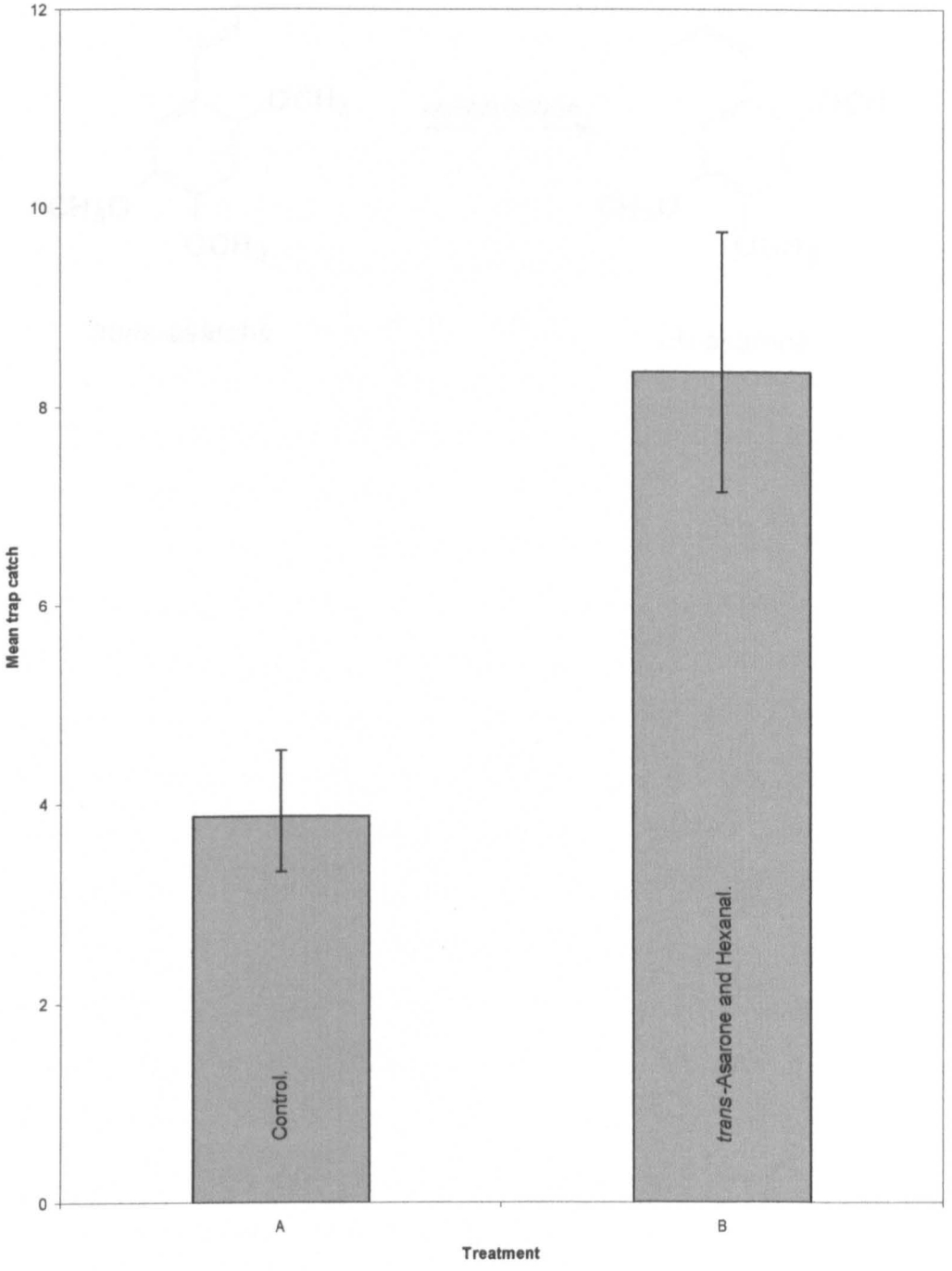
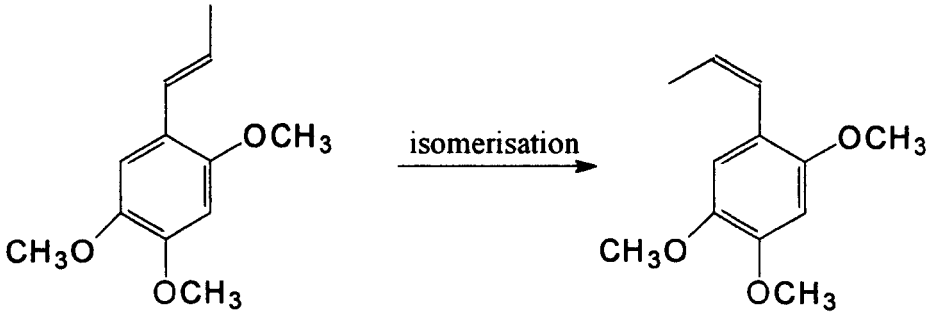


Figure 6.13: **Mean catch of *P. rosae* per sampling interval. Data plotted are back transformed (geometric) means ± 95% confidence intervals. Details of design are in Table 6.2 Exp. 12b (Re-analysis of Travis site).**



NB. In the figures above, (PV) = 'polyethylene vial' lure, and (GB) = 'glass bottle with wick' lure.

Figure 6.14: Isomerisation of *trans*-asarone to *cis*-asarone.



trans-asarone

cis-asarone

6.4 DISCUSSION

6.4.1 FIELD INVESTIGATION INTO PLANT EXTRACTS

A) INVESTIGATIONS USING 'POLYETHYLENE VIAL' LURES

The overall results from these experiments, using 'polyethylene vial' lures to release extracts, confirmed that host plant MAS extracts were attractive to *P. rosae* in the field, as well as producing significant electrophysiological responses in the laboratory (Ch.3). In all three experiments where host extracts were released alone (without *trans*-asarone and hexanal), some increase in catch was seen over the unbaited control (Fig.6.1, 6.2 and 6.5). This was particularly clear in the first experiment, where statistical analysis revealed that the host extracts caught significantly more flies than the control (Table 6.4 Exp.1, Fig.6.5). This finding suggests that the new microwave-assisted solvent extraction method is superior to steam- or hydro-distillation for producing biologically relevant samples, since previous experiments using similar monitoring traps and a range of host-derived essential oils showed no increase in *P. rosae* catches in the field (Guerin *et al.*, 1983).

In the first experiment, the difference in catch between the three host plant foliage extracts was almost significant; *F. vulgare* and *C. maculatum* extracts produced only a small increase in catch over the control, while *A. graveolens* extract had the greatest effect and, from the 95% c.i (Fig.6.1), was the only one clearly different from the control, catching approximately twice the number of flies. In the fourth experiment, *D. carota* root extract was also found to produce only a small increase in catch over the control (Fig.6.5), and this difference was not significant. Of all the plant extracts investigated alone, or in combination with *trans*-asarone and hexanal, *D. carota* extract appeared to be the least attractive. This might be expected, since the area close to these traps would be saturated with *D. carota* leaf volatiles from the carrot plot. Therefore, any attractant volatiles released from the *D. carota* lure might be swamped by the similar volatiles emanating from the crop. However, any dissimilar attractant volatiles being released from a lure containing an alternative host species (i.e. *A. graveolens*) might stand out above the overall attractant crop odour.

The known attractant combination of *trans*-asarone and hexanal was included in the second and fourth experiments, and in both cases resulted in a catch that was approximately twice that of the control (Fig.6.2 and 6.5); in the second experiment, this difference was also found to be statistically significant. This doubling of the catch with *trans*-asarone and hexanal was expected and was in agreement with results of the previous studies on *P. rosae* (Guerin *et al.*, 1983). In the second

experiment, foliage extracts of *D. carota* and *C. maculatum* were combined with the attractants *trans*-asarone and hexanal in an attempt to boost catches above those possible with either alone. However, the catches for both extracts, with *trans*-asarone and hexanal, were substantially lower than with *trans*-asarone and hexanal alone (Fig.6.2). The catch for *C. maculatum* plus *trans*-asarone and hexanal was only slightly higher than with *C. maculatum* alone (although none of these differences were statistically significant). This was an unexpected observation, as it was assumed that the addition of the extracts would enhance the catch due to the presence of additional attractant compounds, or at least make no difference to the catch compared to the attractant combination alone. This result seems to suggest some repellence, arising either from repellent compounds present in the extracts or in some way due to the function of the polyethylene lure used (possibly as a result of polyethylene-derived volatiles).

A comparison of the overall numbers of *P. rosae* caught in different block positions on the ADAS-Arthur Rickwood site suggested that trapping in the favourable roosting sites of the hedgerow resulted in substantial increases in catch. The control trap placed in the hedgerow (Exp.3a) caught a total of 173 flies in four weeks, which was approximately five times the number of flies caught on the controls of blocks placed around the edge of the carrot plot (Exp.2, mean total = 46.5 flies). Similar increases in catch were also seen on the traps baited with plant extracts plus *trans*-asarone and hexanal. Catch numbers on the controls for the block placed by the ditch, in tall grasses (Exp.3b, total = 26 flies), were approximately half those at the plot edge, suggesting that this was an unfavourable roosting site sheltering very few *P. rosae*. The large increase in catch at the favourable roosting site of the hedgerow is probably a result of a greater concentration of flies here, as well as reduced competition for the trap's visual and olfactory attractants, as the flies here were slightly removed from the large background stimulation of the crop's odour and its visually attractive foliage.

During the first three experiments, it was noted that the extracts were being released too rapidly from the 'polyethylene vial' lures, as by the end of the week only leaf waxes seemed to be left in the bottom of the vials and the lures had lost their characteristic "plant extract" odour. From daily checks, it was estimated that these lures only released appreciable quantities of plant volatiles for the first four days in the field. Because of these problems, the 'polyethylene vial' lures were replaced twice a week instead of once a week during the final experiment, and an alternative prototype extract release lure was also tested (using *A. graveolens*).

In the final smaller scale experiment, a number of treatments were tested as attractants and repellents to *P. rosae*. Of these, only the combination of *A. graveolens* extract (in the new release system) plus *trans*-asarone and hexanal caught significantly more flies than the control (Fig.6.5). Notably, this combination caught more flies (although not significantly more) than the *trans*-asarone and hexanal treatment (Fig.6.5), whereas in the earlier experiments, using 'polyethylene vial' lures, combinations of extract plus *trans*-asarone and hexanal always led to a reduction in catch compared to *trans*-asarone and hexanal alone. This suggests that the new release system ('glass bottle with wick' lure) may have overcome some of the problems initially encountered with the 'polyethylene vial' lures.

The release of *trans*-asarone (at five times its standard dosage) caught only slightly more flies than the control, suggesting that it was a relatively poor attractant when used alone. The treatment which combined the possibly repellent *A. cepa* bulb extract with the attractive *trans*-asarone and hexanal combination did show a small reduction in catch (Fig. 6.5), when compared with *trans*-asarone and hexanal alone (although this difference was not statistically significant). This result suggests that the *A. cepa* extract may indeed have had some repellent effect. However, a similar reduction in catch was also seen when host extracts were combined with *trans*-asarone and hexanal in the earlier experiments, and this effect may be attributed to some repellent aspect of the release system used. Even if the reduction in catch was attributable to repellence from the *A. cepa* extract, this effect does not seem strong enough to counteract the attractiveness of host plant volatiles drawing flies into a crop. In the light of this finding, any reduction in *P. rosae* attack afforded by intercropping with *A. cepa* probably results from some factor other than repellent olfactory cues. This is likely to result from reduced oviposition by the females, due to a higher proportion of inappropriate landings terminating the host acceptance before sufficient accumulation of oviposition stimulation has occurred (i.e. from non-volatile contact chemicals on the leaf surface), as suggested in the theory of host plant selection by Finch and Collier (2000).

B) INVESTIGATION INTO THE EFFECTIVENESS OF THE NEW 'GLASS BOTTLE WITH WICK' LURE COMPARED WITH THE OLD 'POLYETHYLENE VIAL' LURE

Due to the relatively poor performance, and problems, encountered with the 'polyethylene vial' lure during the previous experiments, a laboratory investigation was performed to investigate alternative methods for releasing extracts, with the aim of extending the life of the lure. The results of this

investigation, on two new lure designs, suggested that the new 'glass bottle with wick' system would be most suitable, as it released significant levels of plant volatiles for over a week. This lure also performed favourably, when tested in a previous field experiment using *A. graveolens* extract in combination with *trans*-asarone and hexanal, where it produced a significant increase in catch over the control, and for the first time in any field experiment, caught more flies than *trans*-asarone and hexanal alone (Fig.6.5). As these results seemed promising, a field comparison was made to see if the newly designed release system ('glass bottle with wick' lure) could improve the performance of host plant extracts, over their release from the previously used 'polyethylene vial' lures. The two extracts chosen for release in this comparison were those which had proved most (*A. graveolens*), and least (*D. carota*), attractive during the previous field experiments. This experiment was performed at two sites and the results at both showed that the new 'glass bottle with wick' lures caught significantly more flies than the old 'polyethylene vial' lures. As this new release system significantly improved the performance of plant extracts as attractants, it was incorporated into all further experiments, replacing the previously used lure.

The results of this experiment also confirmed that *A. graveolens* extract is a superior attractant to *D. carota*, as *A. graveolens* caught significantly more flies than *D. carota* in the first experiment, and almost significantly more in the second experiment. At both sites, *A. graveolens* extract, in the new release system, caught more flies than the control, both extracts in the 'polyethylene vial' lure, or *A. graveolens* in the 'polyethylene vial' lure (Fig.6.6 and 6.7). However, at both sites, *trans*-asarone and hexanal caught significantly more flies than any other treatment, and at each site this catch was approximately twice that of the control.

The relatively poor performance of the *D. carota* extract, even in the improved release system, was probably due to its lack of "apparence" to the flies, as a result of the high background levels of *D. carota* volatiles emanating from the carrot plots (as previously discussed for the earlier results).

C) INVESTIGATIONS USING 'GLASS BOTTLE WITH WICK' LURES

The overall results from these experiments, using the improved release system 'glass bottle with wick' to release extracts, confirmed many of the results found during the earlier experiments. These included

an approximate doubling of catch on *trans*-asarone and hexanal baited traps compared to the unbaited control, which was seen in four out of the six experiments.

The first and larger experiment was performed at two sites over two different dates, and investigated the effects of combining *A. graveolens* extract with *trans*-asarone and hexanal. The results showed that *A. graveolens* extract produced a small increase in catch (Fig.6.8), and this increase was almost statistically significant, whilst *trans*-asarone and hexanal resulted in a larger increase in catch, which was highly significant. In this experiment, the highest catch was with *A. graveolens* plus *trans*-asarone and hexanal. This confirmed the earlier finding that the combination of this extract, in the new lure type, with *trans*-asarone and hexanal did in fact produce an increase in catch over *trans*-asarone and hexanal alone, although this difference was still not statistically significant. Statistical analysis of the results found that the interaction between the extract and *trans*-asarone and hexanal was not significant. This suggests that the increase in catch seen when both treatments are combined is additive rather than a synergistic process, unlike the synergistic increase in catch suggested when *trans*-asarone and hexanal are combined (Guerin *et al.*, 1983). In the three replicates of this experiment at different sites or dates, insufficient flies were caught for statistical analysis; however, the treatment totals from these experiments showed a very similar pattern of catches (Table 6.5 Exp.6b, 6c, and 6d).

The smaller second and third experiments investigated the effect of adding the alternative attractants *trans*-methylisoeugenol and hexanal to plant extracts. Few conclusions could be drawn from these experiments, as no statistically significant differences were seen between any of the treatments due to the small sizes of these single block experiments (Fig. 6.9 and 6.10). However, in the third experiment, both the *F. vulgare* and *C. maculatum* extracts plus *trans*-methylisoeugenol and hexanal caught more flies than the control (Fig. 6.10). Of these two extracts, *C. maculatum* stood out as being most worthy of further investigation as it also caught more *P. rosae* than the *trans*-asarone and hexanal treatment.

6.4.2 FIELD INVESTIGATIONS INTO *C. maculatum* AND ITS CONSTITUENT COMPOUNDS

These experiments focused on *C. maculatum* extract, and a number of its main electrophysiologically active compounds (γ-coniceine, β-caryophyllene and β-ocimene), which were released either alone or in various combinations. However, during these investigations, the catches of *P. rosae* were extremely

low, and the catches on the chemically baited traps seemed anomalously low compared with the unbaited controls. As a result, no statistically significant differences were seen between any of the treatments in these experiments.

The first experiment was performed in two consecutive years (1995 and 1996), and investigated the effects of releasing *C. maculatum* extract alone and in combination with its synthetic component compounds. The results of the experiment performed in 1995 showed that *trans*-asarone and hexanal caught the most flies, and this catch was again approximately twice that of the control (Fig.6.11). In this experiment, the *C. maculatum* extract performed poorly, and this extract alone and the control caught the least flies. The addition of the synthetic *C. maculatum* compounds (γ-coniceine alone, or γ-coniceine plus β-caryophyllene and β-ocimene) to the extract resulted in an increase in catch, to a level between that of the control and the *trans*-asarone and hexanal treatments. During the replicate experiment in 1996, very few flies were caught and only *trans*-asarone and hexanal caught more then the control.

The second experiment was performed in 1995, and investigated the effects of releasing synthetic *C. maculatum* compounds without the extract. In this experiment, *trans*-asarone and hexanal again caught more flies than any other treatment (almost twice that of the control), and this difference was almost significant. Catches with the *C. maculatum* compounds were intermediate between those on the control and *trans*-asarone and hexanal treatments (Fig.6.12). However, the results showed a clear trend, with the catches increasing as more compounds were combined. The highest catch with *C. maculatum* compounds was achieved when γ-coniceine, β-caryophyllene and β-ocimene were combined, while similar and slightly lower catches were obtained for γ-coniceine alone, and for β-caryophyllene plus β-ocimene. A similar experiment was performed in 1996, but also included a combination of all three *C. maculatum* compounds with the attractants, *trans*-asarone plus hexanal, and *trans*-methylisoeugenol plus hexanal; however, no flies were caught on any of the treatments during this experiment.

The overall results of these experiments seem to suggest that the synthetic *C. maculatum* compounds were more attractive than the extract, and that γ-coniceine was probably slightly more attractive than the combination of β-caryophyllene plus β-ocimene.

6.4.3 COMPARISON OF STANDARD AND *trans*-ASARONE AND HEXANAL BAITED MONITORING TRAPS (IN COLLABORATION WITH ADAS)

This experiment was performed in collaboration with ADAS, to investigate the performance of *trans*-asarone and hexanal baited traps for improving carrot fly monitoring, by comparing baited and unbaited REBELL ® monitoring traps. The experiments were performed during two consecutive years (1995 and 1996), on a large number of field sites widely distributed across England. During these experiments, catch numbers were extremely low at all but one of the sites, although enough *P. rosae* were caught to perform statistical analyses of the block totals from each site. The overall results from these experiments showed no significant difference between the catches on the baited traps and unbaited control, at seventeen of the eighteen sites. The only exception was the Travis site in the North of England, which was also the only site to catch a relatively large number of flies. As more carrot flies were caught at this site, it was possible to re-analyse the individual catch data, which showed a highly significant difference between the two treatments. This difference was an approximately three-fold increase in catch on the *trans*-asarone and hexanal baited traps (424 flies), compared to the unbaited control (143 flies) (Fig. 6.13). The treatment totals from the other sites showed that baited traps only caught slightly more flies than the controls, at ten of the other seventeen sites. The similar small experiment performed at my field site caught so few flies that statistical analysis was not possible, although of the five flies caught, all were on the baited traps.

If the overall results from this experiment were representative of the general responses of *P. rosae* to *trans*-asarone and hexanal, it would suggest that these compounds were unsuitable for improving the efficiency of the standard REBELL ® monitoring trap. However, this does not seem to be the case, as a significant doubling of the catch over that on the control was found in most of the earlier experiments and at one site in this investigation. Unfortunately, in the absence of supporting evidence from this investigation, the incorporation of *trans*-asarone and hexanal into the wider commercial ADAS monitoring programme for *P. rosae* had to be abandoned.

Following the first experiments in 1995, meetings with ADAS operatives led to the suggestion that hexanal may have been released from its lure too quickly, possibly as a result of the very hot weather, and may have all gone before the end of the week it was out in the field. Consequently, the quantity of hexanal per lure was increased to 150µl per vial during the subsequent experiment. Further meetings following the 1996 experiments again suggested that the hexanal lures had failed to release hexanal for the whole week they were out in the field, even with the increased

hexanal content. It was also noticed that some of the *trans*-asarone 'Energy board' lures were becoming discoloured whilst out in the field, possibly as a result of the high temperatures.

Subsequent investigations into the cause of this problem concluded that the poor performance of the hexanal lures resulted from substantial leakage of up to 70% of the hexanal from some of the lures during postal transit. As this problem had not occurred before, during the postage of a trial package of lures sent prior to the start of these experiments, or in other experiments at IACR-Rothamsted (where such lures have often been sent by post), the materials used in the construction of the lures were scrutinised. This resulted in the discovery that the latest batches of polyethylene caps (for the amber glass vials) were not of the same standard as those previously supplied and were found to be somewhat harder than in previous batches. This was seen to affect the tightness of their seal with the amber glass vials, and resulted in substantial leakage when the vials of hexanal were left on their sides or inverted, as was likely to have occurred during transit. This finding suggests that many of the hexanal lures used in the ADAS experiments would have gone out into the field with only around 30µl of hexanal per vial during 1995, and 50µl per vial during 1996. Lures containing these levels of hexanal solution would release hexanal at the required rate for approximately four and seven days, respectively, at 20°C. This period of release would probably have been much shorter whist out in the field during 1995 and 1996, because of increased evaporation rates of the hexanal resulting from the high temperatures experienced during both seasons. However, the possible loss or reduction in hexanal release from a proportion of the hexanal lures may not have caused any major problem, as high catches of *P. rosae* were still seen on the *trans*-asarone and hexanal baited traps at the Travis site, to which the same lures were also sent by post.

Following these experiments, a further investigation was performed to identify an alternative method of releasing hexanal to overcome the problems of hexanal leakage during transport, and the cumbersome requirements of having to replace the solid polyethylene plugs used during transport with pre-drilled caps to activate hexanal release in the field. The 'amber glass vial' lure has a release rate of 9.6 mg/day (at 20°C), and a suitable replacement would need to release hexanal at a similar rate for at least a week. The results of experiments using sealed polyethylene bags and polymer vials suggested that the 1000G 'polyethylene bag' lure, containing a 1.25cm x 1.0cm piece of thin sponge impregnated with 150µl of hexanal solution, was the closest to fulfilling these requirements. This lure released hexanal at a rate of 15.5mg/day (at 20°C), but only for five days (Table 6.3g); however this

period could easily be extended to over a week by the incorporation of 200µl of hexanal solution, instead of 150µl. An alternative method of release could be the use of a precursor capable of releasing hexanal, such as sodium bisulphite adduct, which has been used to release aldehydes during work on control of the striped rice borer (*Chilo suppressalis*) (Beevor *et al.*, 1977). Use of sodium bisulphite also has the advantage that it is a stable, inert compound and is already accepted as a safe food additive (where it is used as a preservative).

In response to the observations that some of the 'Energy board' lures were becoming discoloured whilst out in the field, possibly as a result of decomposition of the *trans*-asarone, an investigation was also performed to determine the actual release rates of *trans*-asarone, and any contaminants formed, from this lure. Air entrainment of the *trans*-asarone lure showed a total release rate of volatiles equivalent to 0.22µg/day (Table 6.6), which was in close accordance with an earlier release rate estimated by weight loss (0.22µg/day). However, the air entrainment showed that only 53% of the volatiles released were *trans*-asarone, with the rest (43%) being a contaminant, identified as *cis*-asarone. As *trans*-asarone is classed as a slightly light sensitive compound, the production of the only contaminant found, *cis*-asarone, was probably the result of light sensitive isomerisation. As very little *cis*-asarone was found in the material remaining in the lure at the end of the experiment, compared to the air entrainment samples, the isomerisation to *cis*-asarone probably occurred either whilst *trans*-asarone was in the vapour phase or whilst it was adsorbed onto the Porapak trap. As this experiment was performed under fluorescent tube lighting, which has a relatively high ultraviolet component compared to natural daylight, light sensitive isomerisation may have occurred more rapidly, resulting in the production of more *cis*-asarone than would be likely to occur under field conditions. As the majority of the volatiles released from this lure still comprised of *trans*-asarone, it remained suitable for continued use during these field trials. Further investigations aimed at producing higher releases rates of *trans*-asarone, whilst reducing the production of its *cis*-isomer, might result in significant improvements in attraction for *P. rosae*. These investigations could include the addition of a UV-protectant, such as Congo red (Baskaran *et al.*, 1998; Shapiro, 1989), or fluorescent brighteners (Hamm, 1999; Dougherty *et al.*, 1996) that absorb energy from UV-light and emit it as visible light. Alternatively, *trans*-asarone could be applied to an 'Energy Board' disk, housed within an upturned Petri dish (painted black or lined with aluminium foil), and attached to the top edge of the REBELL ® trap. This would offer the *trans*-asarone lure protection from direct UV-light, rain, and might increase

the release of *trans*-asarone by heating the lure as the housing absorbed solar radiation. However, as much of the isomerisation seems to have occurred while asarone is in the vapour phase, and away from the lure, the addition of UV-protection to the lure may have very little effect.

6.4.4 GENERAL COMMENTS

During the field experiments of 1995 and 1996, very low numbers of *P. rosae* were actually caught, and treatments including extracts or synthetic compounds performed exceptionally poorly, generally only catching similar numbers of flies as the unbaited controls. This was especially obvious in the experiments performed in collaboration with ADAS, comparing catches on *trans*-asarone and hexanal baited, and unbaited, REBELL ® monitoring traps. Even taking into account the problems discovered with the *trans*-asarone and hexanal lures, as discussed above, the very poor performance of these known attractants seems anomalous, and was not in keeping with the significant increases in catch seen for these attractants during earlier experiments.

During this period, the summer weather was extremely hot and dry (often above 25°C) for extensive periods. Such climatic conditions are known to affect adversely the larval development of *P. rosae*, as well as initiating aestivation (or diapause) of the pupal stage. Consequently, population numbers were very low, leading to corresponding reductions in trap catches during these field trial experiments. In addition, the anomalous performance of the chemically baited traps may also be explained by the unusual climatic conditions during this period. The hot dry weather may have lead to the adults becoming dehydrated, which has been shown in electrophysiological experiments to affect adversely the olfactory (antennal) responses, resulting in the fly's inability to detect or orientate towards otherwise attractant volatiles emanating from the chemically baited traps. This dehydration would probably not have affected the fly's attraction to the visually stimulating trap coloration, and may therefore have led to a dilution of the effects that should have been observed on the chemically baited traps.

This hypothesis is backed up by the observation that, during the ADAS experiments, at the sites where the baited and unbaited traps caught similar numbers of *P. rosae*, the overall catches of flies were very low. This suggests that these sites experienced unfavourable hot dry conditions, capable of causing dehydration in the adults and leading to their loss of ability to respond to (or distinguish between) the baited and unbaited traps. Further evidence for this suggestion comes from the observation that the only site to show a highly significant, three-fold, increase in catch due to

trans-asarone and hexanal was also the site which caught substantial total numbers of *P. rosae*. It is likely that more favourable conditions (i.e. cooler and wetter) prevailed at this site. Thus, the antennal responses of these flies would remain unimpaired, allowing them to detect and orientate towards the attractive volatile cues emanating from the baited traps

During 1996, even fewer *P. rosae* were caught than during the previous year, with many experimental blocks failing to catch any carrot flies at all. This situation was quite understandable, considering that similar unfavourable hot dry weather conditions, which had already caused a decline in populations during 1995, were experienced again in 1996. Furthermore, if dehydration during 1995 had resulted in impairment of the antennal responses of adult flies, further larval mortality may have arisen from the reduction (or inability) of ovipositing females to locate suitable host plants around which to oviposit. Newly emerging larvae, from these sub-optimally positioned egg batches, may then have experienced high mortalities due to the lack of an acceptable food source close by.

7 INVESTIGATION INTO THE DEVELOPMENT OF A TRAP FOR THE AUTODISSEMINATION OF THE FUNGAL PATHOGEN *E. schizophorae* INTO *P. rosae* POPULATIONS

7.1 INTRODUCTION

During a five year study on the occurrence of fungal pathogens in populations of *P. rosae* in carrot fields (Denmark), Eilenberg (1988) concluded that *Entomophthora muscae* should be regarded as the most promising agent for biological control. This pathogen was by far the most common species found to infect *P. rosae*, causing up to three epizootics per year, and was highly virulent towards this pest. Eilenberg suggested that the development of a biological control product, possibly using *in vitro* produced spores of one or several strains of *E. muscae*, could be used for control of *P. rosae*. During his studies, similar patterns in the development of *E. muscae* infection of *P. rosae* populations were observed each year. Infections were found to be low at the beginning of the first generation of flies (late May to early June). The first epizootic generally developed just after the peak in fly numbers, with infection usually reaching its highest levels during early July. In the second generation of flies, infections generally developed very rapidly, producing a second epizootic in August. A third epizootic was observed in some years, generally developing in October, around one month after the peak in second generation flies (resulting in infected flies still being found in the field until early November). A similar pattern of infection was observed in England, where *E. muscae* infected *P. rosae* were usually found in the field during late summer and early autumn (Van't Sant, 1963), with little infection being observed during the first generation of flies. Eilenberg (1983) reported that levels of infection could reach up to 60% during some epizootics. He also found that epizootics developed during very different weather conditions, including warm dry conditions in August and cool wet conditions in October. Although high levels of infection in *P. rosae* populations did not result in protection of crops not protected with insecticides, this seemed to result from the initial lag in transfer of the fungi from the overwintering stage into the newly emerging first generation of flies.

The development of *E. muscae* epizootics in *P. rosae* populations is believed to be enhanced by the behaviour of the flies, which prefer high humidity (Wakerly, 1964) and usually stay in the hedges during the night and hot periods of the day. The flies are most active during the late afternoon and evening when females move into the crop to locate hosts for oviposition (Städler, 1975). As they tend to aggregate

within favourable roosting sites in the hedge, *E. muscae* infected individuals die within restricted areas of high fly density, which is also favourable for pathogen infection. Transmission of conidia within these sites is also enhanced by the stereotyped sequence of behaviours exhibited by *E. muscae* infected individuals just prior to death. This sequence starts with infected flies becoming sluggish and moving into elevated positions in the hedge (often 0.5m below the canopy), which is above the normal roosting height preferred by uninfected individuals. Here they become attached to the underside of leaves via fungal rhizoids emerging from their proboscis

Although *E. muscae* only infects adult *P. rosae*, its suitability as a biological control agent against this pest is enhanced because of its ability to reduce the number of eggs laid by infected females, as well as reducing the survival of any eggs that are laid. In a study by Eilenberg (1987a), *E. muscae* infection was found to influence both the oviposition behaviour and oviposition abilities of female *P. rosae*, with infected females laying fewer eggs than uninfected females due to their reduced life span. Uninfected females in the laboratory generally start laying eggs about three days after emergence, and are capable of laying numerous batches of eggs at three day intervals throughout their 45 day life span. However, infected flies only tend to live for about a week after exposure to conidia, and cease egg-laying about two days prior to death. Females infected on the day that they emerged are often unable to lay eggs, even though the eggs remain viable. This study also found that females infected within four to five days of emergence exhibit abnormal oviposition behaviour, and are unable to lay their eggs near suitable host plants. This led to eggs being laid in random locations, resulting in high egg and larval mortality as the eggs are sensitive to desiccation, whilst larvae would die from the lack of a suitable food plant nearby. This combination of factors led Eilenberg (1987a) to conclude that female *P. rosae* infected with *E. muscae* would not contribute progeny to subsequent populations of *P. rosae* in the field. This is of particular interest, as life-table studies on *P. rosae* in England showed that loss in potential natality (k_0) was one of the key factors in determining the population size in this species (Burn, 1984).

Although *E. muscae* has been shown to cause epizootics in *P. rosae* populations in the field, with large reductions in the adult population, effective control of larval damage on unprotected crops has never been achieved. The main reason for this seems to be the late development of fungal infection, particularly during the start of the first generation of flies. This lag in the spread of infection during the first generation

probably arises as a result of the pathogen having to switch from its overwintering dormant phase to becoming re-established in the new host population. The reasons for this lag, and the overwintering strategy employed by *E. muscae* on *P. rosae* in the field, are still unknown. However, it seems likely that it involves the production of resting spores, either in *P. rosae* or an unknown alternative host species. If resting spores are its means of overwintering, the initial lag in the infection process may result from the poor survival of resting spores during the winter, their slow germination in May, or from a poor transmission rate for germ conidia to the emerging flies. Because of this initial low level of infection, *E. muscae* may have to pass through a number of conidial cycles before enough infective material builds up in the environment to trigger a population-wide epizootic. In this situation, release of pathogen earlier than naturally occurs may allow more rapid establishment of the pathogen and result in an epizootic early in the first generation. If this could be brought about, and if sufficiently high egg mortality occurred due to the abnormal oviposition behaviour of *E. muscae* infected females, control of crop damage due to the first generation larval might be achieved. A high mortality in the first generation of flies would also have a knock-on effect, resulting in fewer adults emerging into the second generation and reducing the usual rapid build up of *P. rosae* during the year.

The most promising method for introducing *E. muscae* into field populations of *P. rosae* is probably an inoculative approach, as this requires a minimum of infective material. Primary and supernumerary conidia would have to be used as the infective agents in any release system, as although resting spores can be produced *in vitro*, not enough is known about their dormancy or germination requirements for production of germ conidia to be achieved. An inundative approach to the release of *E. muscae* would be both economically and technically unfeasible at present, primarily because suitable techniques for the mass production of conidia are not yet available. Application of conidia as a mycoinsecticide would also face a number of obstacles as the conidia are fragile, short-lived, and die rapidly in aqueous solution. The mucus coat surrounding conidia, which aids in host attachment and possibly protects them against desiccation, also presents problems for formulation and application. This sticky coat makes it difficult to harvest conidia from culture and also makes conidia difficult to suspend uniformly in a solution. As this mucus is also water-soluble it can easily be lost, resulting in a loss of conidial adhesive properties and a reduction in its protection from desiccation.

All previous attempts to use *E. muscae* as a biological control agent have focussed on its release into *M. domestica* populations in indoor livestock systems (including poultry and dairy farms: Kramer and Steinkraus, 1987; Geden *et al.* 1993; Six and Mullens, 1996; Shimazu and Kuramoto, 1994; Kuramoto and Shimazu, 1997). These studies were performed either in controlled cage situations or on actual farms. Two methods for introducing the pathogen were tested; these involved either the placement of sporulating *E. muscae*-killed cadavers at sites of high fly activity, or the release of live pathogen infected flies into the population. The results of these experiments showed that both release methods were equally capable of transferring infection into the general population, and often resulted in infection levels of up to 90%. However, even at these high levels of prevalence, little suppression of fly populations occurred on the farms. This was attributed to flies completing mating and oviposition before being killed by the pathogen, and it was also suggested that infected flies might have prolonged their longevity by resting or basking in warm locations in order to eliminate (or slow) pathogen development ('behavioural fever' response). Of the two release methods, the distribution of cadavers was found to be too time consuming and impractical for large-scale application, while the release of infected flies was comparatively simple and provided equally effective transmission of the pathogen (Geden *et al*, 1993).

Ignoffo (1978) urged the exploration of novel approaches to targeting pest species with emtomopathogens, and defined the relatively new technique of autodissemination as the use of insects to introduce and spread entomopathogens in the ecosystem. The hypothesis behind this technique is that pest behaviour can be manipulated using semiochemicals to encourage the spread of pathogens to susceptible populations earlier in the season than would normally occur. In this way, disease epizootics could establish and decimate small early-season pest populations before the crop was damaged (Pell *et al*, 2001). This strategy has been applied to the biological control of the diamondback moth (*Plutella xylostella*) using *Zoophthora radicans* (Pell *et al*, 1993; Pell and Wilding, 1994; Furlong and Pell, 1995), and an adaptation of this strategy would seem ideal for the control of *P. rosae* with *E. muscae*. This strategy involved male moths being attracted into specially designed fast-entry, slow-exit traps in response to synthetic female pheromone. While inside the trap, moths were exposed to a sporulating source of *Z. radicans* and became infected with conidia. After becoming habituated to the pheromone, the infected moths left the trap and returned to the crop, spreading the pathogen into the field population. The benefits

of this strategy are that the trap can be designed specifically to attract the target pest, thereby reducing the exposure of non-target organisms to the inoculum. As only small quantities of fungal inoculum are required for this method, some of the problems associated with mass production, formulation and storage can be overcome. As the inoculum is housed within the trap, it can be protected from the damaging effects of UV radiation and be provided with a microenvironment favouring conidial production and transmission of infection to the host (Pell *et al.*, 2001).

Recently, the fungal pathogen *E. muscae* has been found to consist of a complex of different species, which are referred to as *E. muscae* sensu lato (s.l.) (meaning "in a broad sense"). Forms within this complex differ considerably in characteristics such as size of conidia and number and size of nuclei, as well as in their host range (MacLeod et al., 1976; Keller, 1984a and 1987). This discovery went some way to explaining the large host range previously attributed to this pathogen, and led to the description of the new species *E. schizophorae* (Keller, 1987a) and the redescription of *E. muscae* sensu stricto (s.s.) (meaning "in a narrow sense") (Keller *et al.*, 1999). *E. muscae* s.l. is now believed to include at least six species, based primarily upon their host range, the size of the primary conidia, and the number and size of nuclei per conidia.

a) *E. schizophorae* is the species usually responsible for *P. rosae* infections in the field. This species is described as a pathogen of *Psila*, *Delia* and *Pollenia* species in Europe (Diptera: Schizophora), and has 3-6 nuclei per conidia (Keller, 1987a and 1999).

b) The redescribed *E. muscae* s.s. species is a pathogen of *Delia*, *Musca*, *Scopeuma*, *Melanostoma* and other species (Diptera: Cyclorrhapha), and has 10-27 nuclei per conidia (Keller, 1999).

c) *E. syrphi* is a pathogen of syrphids (Diptera: Sirphidae) and has 19-22 nuclei per conidia.

d) *E. scatophagae* often infects dung flies (Diptera: Scatophaga) and has 15-18 nuclei per conidia.

e) An as yet undescribed *E. muscae* 'group B' species, and one infecting *Delia kullensis* with 10-11 nuclei per conidia, are also included within this complex (Keller, 1984).

The separation of *E. muscae* s.l. into the species *E. muscae* s.s., *E. schizophorae* and *E. syrphi* has recently been supported by molecular genetics studies of this group (Jensen and Eilenberg, 2000). As *E. muscae* has only recently been recognised to be a complex of species, many early studies on *E. muscae* may actually have included studies of *E. schizophorae*. Because of the lack of clarity as to which species

was studied in many publications (as morphological features used to identify the pathogen were not given), the two species have been discussed together in this chapter. However, it is relatively safe to assume that any references to *E. muscae* infecting *P. rosae* populations in the wild are actually references to *E. schizophorae.*

A number of steps would be involved in the development of an autodissemination trap for the biological control of *P. rosae* using *E. schizophorae*, the first of which would be to design a trap capable of drawing flies into an enclosed area suitable for infection to occur. This could be achieved for *P. rosae* using a combination of visual and semiochemicals attractants. The rest of this chapter concerns the development and field evaluation of prototype autodissemination traps intended for the dispersal of *E. schizophorae* into *P. rosae* populations at agricultural sites, allowing the pathogen to be utilised as a biological control agent and ultimately to be incorporated into an IPM strategy for this pest. This work followed preliminary studies performed at IACR-Rothamsted by Dr. Guy Poppy (unpublished data), which showed that the host finding behaviour of *P. rosae* could be manipulated using visual and host-plant chemical attractants, causing *P. rosae* to enter relatively large box-type traps, similar in design to a Stephenson screen (25 x 25 x 20cm with walls made from overlapping horizontal strips, each angled at 45°). However, trapping efficiencies within this design were very low in comparison with the number of flies caught on standard unbaited REBELL® monitoring traps. The internal volume of this trap was also too large to allow efficient inoculation of flies by an enclosed source of inoculum, and the relatively open design was too exposed (due to wind passing through it) to allow adequate manipulation of the internal microclimate in favour of pathogen survival and high infection levels.

The first field experiments in this new study involved the evaluation of a range of prototype traps for their ability to direct flies into a relatively small enclosed space (an inoculation chamber); here, flies would be in close proximity to a source of inoculum in the fully functional trap, and environmental conditions could be optimised for pathogen survival and infection of target insects (high humidity and low UV). The trap designs tested attempted to manipulate the fly's host-finding behaviour using visually attractive cues (orange and yellow coloured materials) combined with semiochemical attractants (host-plant extracts and compounds), to draw the flies into the vicinity of the trap and hopefully towards the enclosed inoculation chamber. Both male and female *P. rosae* are attracted to materials with a high

reflectance in the yellow part of the visual spectrum, thought to be a general plant discrimination cue used by most foliage seeking insects (Prokopy and Owens, 1983), and particularly to traps in the field with a high ratio in reflectance at 560nm (yellow): 460nm (blue) wavelengths of the visible spectrum (Brunel and Langouet, 1970). Accordingly, REBELL® orange trap material (Polystyrol) and an equivalent orange Perspex material (both optimised for visual attraction by their colour and slight translucency), or bright canary-yellow paint where necessary, were used as visual attractants during trap design. Attempts to direct flies into an enclosed region were made in two different ways. In the 'space station' trap design, structural means (i.e. unidirectional baffles) along with attractant visual and chemical cues were employed. A more complex method, utilising the female's oviposition behaviour in response to host plant oviposition stimulants, was attempted with the 'funnel' and 'V'-board traps.

The oviposition behaviour of *P. rosae* involves females ready to oviposit landing on host plant leaves and performing exploratory runs, during which they gradually adopt a positive geotactic behaviour in response to oviposition stimulants on the leaf surface. The flies then proceed down the leaf stem axis to the soil (Bohlen, 1967), where they search for moist dark crevices suitable for egg laying (Overbeck, 1978). In oviposition assays, Degen and Städler (1997b) showed that green, yellow and orange artificial leaves with a thin coating of paraffin wax, sprayed with microwave-assisted hexane extracts of host leaves (or other stimulatory compounds), were highly efficient at stimulating this oviposition behaviour. Furthermore, Degen and Stadler (1997a) showed, during attempts to improve the resolution in their oviposition assays, that flies stimulated for oviposition on artificial host leaves would walk down the stem and through a relatively small gap in a cover surrounding the leaf stem (5cm diameter) on their journey to the oviposition substrate. The 'funnel' and ' V'-board traps were designed to take advantage of these behaviours by attracting flies and inducing them to land on the large surface area of the trap (via volatile semiochemical attractants and colouration), while the coating of paraffin wax sprayed with host plant extracts (containing natural leaf surface waxes and oviposition stimulants) and *trans*-asarone (an oviposition stimulant) were used to stimulate females to land and perform an oviposition run down the trap. It was hoped that such behaviour would funnel the flies into a black Petri dish below via a small aperture (simulating an oviposition site). Subsequent field experiments were aimed at optimising various factors in the design of the most successful of the prototype trap designs, with regards to size, visual and

chemical attractants, as well as finding the optimum trapping location for such traps in the field (hedgerows versus within the crop, and the optimum height for the traps).

7.2 METHODS

The sources of synthetic compounds and plant materials extracted for use in these field experiments are the same as those given in Ch.6. Plant extracts were produced by microwave-assisted solvent extraction using hexane as solvent, for further details see Method 2.2.2. Where traps were baited with synthetic attractants, hexanal was released from an 'amber glass vial' lure [constructed from two vials each containing 100µl of hexanal/BHT (10% w:w) solution,], and *trans*-asarone was released from an 'Energy board' lure (impregnated with 50mg of *trans*-asarone), unless stated otherwise in the individual experimental methods; these lures were produced as described in the methods of Ch.6, and were replaced weekly during all these experiments.

The design for these experiments was generally the same as that used for the field evaluation of plant volatiles for attractancy to *P. rosae* (Ch.6). Pathogen traps were positioned in blocks running parallel to the edge of the crop and the nearby hedgerow, at a height of 1m above the ground. Individual traps were spaced 15m apart, with at least 20m between different experimental blocks. The different pathogen trap designs (treatments) were positioned according to the first row of a Latin square design, with treatment positions re-allocated according to the following rows of the Latin square every time the insect trapping devices (or traps) were removed for counting (once or twice weekly, see individual experimental details). Flies entering the inoculation chamber of traps were generally caught using insect trapping adhesive (Oecotak A-5, Oecos Ltd., Kimpton, UK), although the actual trapping region within the inoculation chamber varied slightly depending on trap designs.

7.2.1 FIELD INVESTIGATION OF PROTOTYPE TRAP DESIGNS

These field experiments were performed to evaluate the effectiveness of a range of prototype trap designs, for their ability to direct *P. rosae* into a small enclosed space (an inoculation chamber). Three basic types of trap were tested, and these are referred to in the text as 'space station' traps, 'yellow funnel' traps and 'REBELL ® 'V'-board' traps. These basic designs were tested with different combinations of visually and chemically attractive cues, in an attempt to draw the flies into the inoculation chamber. The first experiment compared catches in the inoculation chambers of 'space station' and 'yellow funnel' traps while the second experiment compared catches in 'space station' and 'REBELL ® 'V' board' traps. An

externally sticky version of the 'REBELL ® 'V'-board trap' was also included in this experiment as a control, to estimate the numbers of *P. rosae* attracted to the exterior of the 'REBELL ® 'V' board' trap. This allowed a comparison to be made between the number contacting the trap's surface, and the number induced to perform an oviposition run down into the inoculation chamber of the operational version of the trap.

Field experiments were set up as described in Table 7.1 (Exp.1 and 2), and the prototype traps (treatments) were made up follows.

Space Station Trap: This trap (Fig.7.1) was based upon a pheromone trap for dispersal of *Zoophthora radicans* into populations of the diamondback moth (*Plutella xylostella* L.), developed by Pell, Macaulay and Wilding (1993). The body of the trap comprised two clear Perspex plates fixed horizontally one above the other, 5.5cm apart, by a series of four outer 'V'-shaped baffles and four inner right-angled baffles. These baffles were held in position by a number of small (3mm high Perspex) pegs, glued to the inner surfaces of the top and base plates with acrylic cement. The two plates were clamped together against the baffles by means of four 6.5cm lengths of stainless steel stud bar, which passed through each corner of the trap, secured with butterfly nuts. The inner baffles formed a 12.5cm^2 arena in the centre of the trap (inoculation chamber), which was relatively simple for the flies to enter but required a more protracted escape. Directly above the central arena was an 8cm-diameter hole, allowing access for placement and removal of the insect trapping device, and lures. This hole was sealed with an up-turned Petri dish base (9cm diameter) to stop flies exiting through the top of the arena and to protect it from rain and UV-radiation. This Petri dish base sat in a raised collar, made from an upturned Petri dish lid (9cm diameter) with an 8cm hole in its centre that was aligned with the hole in the top plate, and glued into position. The cover was secured in position by two lengths of elastic cord stretched diagonally between the butterfly nuts in the corners of the top plate.

The trap was set at a height of 1m above ground level, by means of a wooden post (5cm x 5cm cross section) in a metal post holder (Metpost). A polyethylene screw cap was bolted centrally to the underside of the trap's base plate, into which a polyethylene jar was screwed. This jar slotted into a cup, formed from a length of polyethylene pipe, screwed to the top of the post. The trap was secured to the post

by two lengths of nylon cord, each tied to holes in adjacent corners of the base plate, stretching to hooks screwed into the wooden post (Fig.7.3).

Entrapment of carrot fly was achieved by incorporation of a sticky cross (8cm x 8cm x 5cm) in the central arena of the trap. This was made from either clear Perspex or orange REBELL ® material (which gave an added visual cue), coated with insect trapping adhesive (Oecotak A-5) (Fig. 7.2). In the chemically baited traps, a hexanal lure was hung from the sticky cross, and a *trans*-asarone lure was cut in half and both parts were placed in the base of the central arena.

Yellow Funnel Trap: This trap (Fig.7.4) comprised a clear polyethylene funnel (30cm dia.), painted 'canary yellow' and coated with paraffin wax (by dipping in molten wax over a heated water bath). This wax coating had two functions: firstly, to enhance the flies' ability to walk on the funnel surface, by adding a leaf surface texture; secondly, to enable impregnation of the surface by plant extracts and *trans*-asarone, allowing the wax to act as a slow release matrix for the applied chemicals. The inoculation chamber of this trap was made from a modified Petri dish, which was painted black and had a entry hole in the top (4cm in diameter) to allow flies access as they walked down the outside of the trap. Entrapment of was achieved by coating the interior of the Petri dish with insect trapping adhesive. Where the trap was baited, this was achieved by hanging a hexanal lure from the rim of the funnel, whilst *trans*-asarone (100mg in acetone), and *D. carota* and *A. graveolens* foliage extracts (2ml of 25g/ml in hexane), were added by spraying them onto the exterior wax coated surface of the funnel.

REBELL ® 'V'-Board Trap: This trap (Fig.7.5) was similar in design to the 'yellow funnel' trap above, and comprised a modified REBELL ® orange sticky trap, from which the insect trapping adhesive had been removed by cleaning in hexane solvent. The REBELL ® trap was then cut into a 'V' shape and coated with paraffin wax. The inoculation chamber was made from a modified Petri dish, with an interior coating of insect trapping adhesive (as described above). Where the trap was baited, this was achieved by hanging a hexanal lure from the rim of the funnel, whilst *trans*-asarone (100mg in acetone), and *D. carota* and *A. graveolens* foliage extracts (2ml of 25g/ml in hexane), were added by spraying them onto the exterior wax coated surface of the funnel.

The externally sticky version of this trap (Fig.7.6) acted as a control to estimate the numbers of

P. rosae attracted to the 'REBELL ® 'V' board' trap. It was identical in design to the non-sticky trap, but retained its insect adhesive coating. This trap was baited, with a hexanal lure hung through its central hole and a *trans*-asarone lure attached to its top edge with paperclips.

7.2.2 INVESTIGATIONS USING 'SPACE STATION' TRAPS, AND THE OPTIMISATION OF THEIR USE

A) DETERMINATION OF OPTIMUM TRAPPING HEIGHT IN THE HEDGEROW

This field experiment was performed to determine the optimum trapping height for *P. rosae* in a hedgerow surrounding a carrot field, and compared catches on REBELL ® monitoring traps at three different heights. The experiment was set up as described in Table 7.1 (Exp.3), and the traps were made up as follows. A pole of approximately 5m in length was constructed of steel interconnecting sections (Ecos). The first 1m of this pole was driven into the ground, and the structure was stabilised by attaching three nylon cords to the top of the pole and pegging these cords into the ground. Three unbaited REBELL ® sticky traps were attached to the pole, using foldback clips, at the following heights: 1.5m (approximately level with top of herbage border), 2.5m (half way between tops of trees/bushes and top of herbage border) and 3.5m (approximately level with tops of trees/bushes). These poles of traps were positioned against the hedgerow, in the herbage boarder around the carrot field, with the three REBELL ® traps running parallel to the direction of the hedgerow (Fig 7.7).

B) OPTIMISATION OF SIZE, VISUAL CHARACTERISTICS AND CHEMICAL ATTRACTANTS

In the previous experiments, a range of prototype traps were tested for their ability to direct *P. rosae* into a small inoculation chamber. Of the designs tested, the 'space station' design was found to be the most successful. This trap caught the most flies when a visually attractive cue (a small cross made from REBELL ® orange material) and chemically attractive cues (*trans*-asarone and hexanal lures) were placed in its central arena. As such, this design was chosen for further investigation, and the following field experiments were aimed at optimising various factors in the design of this prototype trap, with regards to size, visual and chemical attractants, as well as finding the optimum trapping location for such traps in the field (hedgerow vs. within the crop). The results of the previous experiments also suggested that the

chemically attractive cues were of primary importance in determining the number of flies entering the trap, while the visual cue provided only a small increase in catch (possibly as a result of its very small size, and lack of apparence). As such, many of the trap designs in the following experiments incorporated larger visually attractive cues; this was achieved by constructing various parts of the trap from visually attractive orange materials (i.e. the baffles, and the top and base plates). As large sheets of the REBELL ® orange material were unavailable, an alternative yellow/orange Perspex material (with a similar spectral reflectance and translucent properties) was used as the visually attractive material in the construction of the traps. In these experiments, entrapment of flies was achieved using a clear acetate sheet coated on one side with insect trapping adhesive (Oecotak A-5), which was coiled into a cylinder (8cm dia.) with its tacky surface facing outwards. This trapping device was placed in the central arena of the trap, via the circular access hole in the top plate. Where traps were chemically baited, a hexanal lure was hung from the outside of the trapping device and a *trans*-asarone lure was cut in half and both parts were placed in the base of the central arena.

The first experiment investigated the effects of incorporating attractive chemical cues in the absence of visually attractive cues. It compared catches in traps with and without the release of *trans*-asarone and hexanal from traps with no visually attractive orange Perspex in their construction. This experiment was set up as described in Table 7.1 (Exp. 4).

The second experiment investigated the effects of increasing the quantity of visually attractive orange material used in the trap design, in the absence of chemical attractants. The trap designs included traps with no orange Perspex used in their construction, traps with only orange Perspex baffles (i.e. which presented a visually attractive profile), and traps constructed totally from orange Perspex (i.e. which presented a visually attractive profile, with attractive upper and lower surfaces) (see Fig.7.8). This experiment was set up as described in Table 7.1 (Exp. 5).

The third and fourth experiments investigated the effects of increasing the vertical height of traps which had orange Perspex baffles (i.e. increasing the height of the visually attractive profile), using traps constructed without (see Fig.7.9) and with (see Fig.7.10) visually attractive top and base plates. This experiment was set up as described in Table 7.1 (Exp.6 and Exp.7, respectively).

In the experiments above, 'space station' designs with visually attractive baffles 10cm and 15cm high were more effective than those with a smaller baffle height of 5.5.cm, whilst the catches for traps with the two higher baffle heights were very similar. As such, all the trap designs tested in the following experiments were constructed with baffle heights of 10cm. The higher baffle height of 15cm was not used, because it would probably only provide a slightly higher catch than with the 10cm high baffles but would also make the traps more unstable in wind, greatly increase the internal volume of the inoculation chamber (which was undesirable), and would require more of the relatively expensive orange Perspex material during construction of the traps.

The fifth experiment investigated the effects of increasing the quantity of visually attractive orange material used in the trap design, and the effect of incorporating attractive chemical cues, using traps with an optimised baffle height of 10cm. This experiment was performed in two consecutive years, with a slight modification made to the experimental block design in the second year. In this year, a REBELL ® monitoring trap was positioned at each end of the block of 'space station' traps; these acted as controls to estimate the number of flies available for capture in the vicinity of the experiment. This experiment was set up as described in Table 7.1 (Exp.8).

In the experiments above, the optimum 'space station' trap design was found to be one constructed entirely from visually attractive orange Perspex, with 10cm high baffles, which also included the chemical attractants *trans*-asarone and hexanal in its inoculation chamber. As such, this design was chosen for further investigation in the final experiment. The sixth experiment was performed to identify the optimum trapping location, for the optimised 'space station' design, in the field. It compared the effects of placing traps against a hedgerow surrounding a crop (at a height of 1m), compared to five metres into the crop (at a height of 20cm) and in these two positions, the effects adding *trans*-asarone and hexanal were also investigated.

C) LABORATORY ESTIMATION OF RESIDENCE TIME

This experiment was performed to determine the average length of time *P. rosae* would spend in the inoculation chamber of an optimised 'space station' trap design (as determined in the field experiments above), once they had entered the trap in response to its visual and chemical attractants.

Approximately 150 adult *P. rosae* (mixed sex) were transferred from rearing cages into a large mesh cage (1.0 x 1.0 x 1.6m), with a Perspex front and an access hatch. The cage was situated in an environmentally controlled room at 20°C and 80% relative humidity, with overhead fluorescent lighting (operating in 12:12 hour on:off mode). Food was provided on a sponge hung from the top of the cage, to which a sucrose and yeast hydrolysate solution was applied, and water was provided via soaked cotton wool in a glass dish at the bottom of the cage. During periods of high fly activity (i.e. in the latter half of the day), an optimised 'space station' trap (10cm high and constructed completely from orange Perspex, with *trans*-asarone and hexanal lures), was introduced into the cage (at a height of 0.6m). During this period, flies were observed entering the pathogen trap and their residence times within the central inoculation chamber were recorded (including periods when the flies journeyed back into the baffles but returned to the inoculation chamber), for up to a maximum of 15 minutes. Over a 3 day period, the residence times of 25 individual flies were recorded.

Table 7.1 Experimental Details of Field Experiments

FIELD INVESTIGATION OF PROTOTYPE TRAP DESIGNS

Experiment 1
Treatments:
A) Space station trap: 5.5cm high, with sticky clear Perspex cross in the central arena (unbaited)
B) Space station trap: 5.5cm high, with a sticky orange (REBELL ® board) cross in the central arena (unbaited)
C) Space station trap: 5.5cm high, with sticky orange (REBELL ® board) cross in the central arena; baited with *trans*- asarone and hexanal
D) Yellow funnel trap: (unbaited)
E) Yellow funnel trap: baited with *trans*-asarone and hexanal
F) Yellow funnel trap: baited with *trans*-asarone, hexanal and *D. carota* foliage extract
G) Yellow funnel trap: baited with *trans*-asarone, hexanal and *A. graveolens* foliage extract
This experiment was performed at Bruce Butcher's field - Feltwell Anchor, and ran from 12/8/94-2/9/94 (2nd generation of flies). One block was put out, and the traps were randomised twice weekly.

Experiment 2
Treatments:
A) Space station: 5.5cm high, with sticky clear Perspex cross in central arena (unbaited)
B) Space station: 5.5cm high, with sticky orange (REBELL ® board) cross in central arena (unbaited)
C) Space station: 5.5cm high, with sticky orange (REBELL ® board) cross in central arena; baited with *trans*-asarone and hexanal
D) Sticky REBELL ® 'V' board trap: baited with *trans*-asarone and hexanal
E) REBELL ® 'V' board trap: baited with *trans*-asarone, hexanal and *D. carota* foliage extract
F) Sticky REBELL ® 'V' board trap: baited with *trans*-asarone and hexanal
G) REBELL ® 'V' board trap: baited with *trans*-asarone, hexanal and *A. graveolens* foliage extract
This experiment was performed at Bruce Butcher's field - Feltwell Anchor, and ran from 2/9/94-30/9/94 (2nd generation of flies). One block was put out, and the traps were randomised twice weekly.

INVESTIGATIONS USING 'SPACE STATION' TRAPS, AND THE OPTIMIZATION OF THEIR USE

Experiment 3
Treatments:
A) REBELL ® trap at a height of 3.5m (approximately level with tops of trees/bushes)
B) REBELL ® trap at a height of 2.5m (half way between tops of trees/bushes and top of the herbage border)
C) REBELL ® trap at a height of 1.5m (approximately level with top of herbage border)
This experiment was performed at Bruce Butcher's field, Graves and Graves Ltd. - Decoy Farm, and ran from 21/7/95-23/8/95 (2nd generation of flies). Ten blocks were put out, and the traps were changed weekly.

Table 7.1 Experimental Details of Field Experiments (Continued)

Experiment 4
Treatments:
A_1) Space station trap: 5.5cm high, with clear top and base plates, and orange baffles (unbaited)
B_1) Space station trap: 5.5cm high, with clear top and base plates, and orange baffles; baited with *trans*-asarone and hexanal
A_2) Space station trap: 5.5cm high, with clear top and base plates, and orange baffles (unbaited)
B_2) Space station trap: 5.5cm high, with clear top and base plates, and orange baffles; baited with *trans*-asarone and hexanal
This experiment was performed at Bruce Butcher's field Graves and Graves Ltd. - Decoy Farm, and ran from 21/7/95-23/8/95 (2^{nd} generation of flies). In this experiment, one block of four traps was put out, and the two treatments (presence and absence of *trans*-asarone and hexanal) were allocated to the traps in an alternating pattern, which was randomised weekly.

Experiment 5
Treatments:
A) Space station trap: constructed totally from clear Perspex
B) Space station trap: 5.5cm high, with a clear top and base plate, and orange baffles (unbaited)
C) Space station trap: 5.5cm high, with a clear top and base plate, and orange baffles (unbaited)
This experiment was performed at Bruce Butcher's field Graves and Graves Ltd. - Decoy Farm, and ran from 21/7/95-23/8/95 (2^{nd} generation of flies). One block was put out, and the traps were randomised weekly.

Experiment 6
Treatments:
A) Space station trap: 5.5cm high, with a clear top and base plate, and orange baffles (unbaited)
B) Space station trap: 10cm high, with a clear top and base plate, and orange baffles (unbaited)
C) Space station trap: 15cm high, with a clear top and base plate, and orange baffles (unbaited)
This experiment was performed at Bruce Butcher's field Graves and Graves Ltd. - Decoy Farm, and ran from 21/7/95-23/8/95 (2^{nd} generation of flies). One block was put out, and the traps were randomised weekly.

Experiment 7
Treatments:
A) Space station trap: 5.5cm high, with an orange top and base plate, and orange baffles (unbaited)
B) Space station trap: 10cm high, with an orange top and base plate, and orange baffles (unbaited)
C) Space station trap: 15cm high, with an orange top and base plate, and orange baffles (unbaited)
This experiment was performed at Bruce Butcher's field Graves and Graves Ltd. - Decoy Farm, and ran from 21/7/95-23/8/95 (2^{nd} generation of flies). One block was put out, and the traps were randomised weekly.

Table 7.1 Experimental Details of Field Experiments (Continued)

Experiment 8
Treatments:
A) Space station trap: 10cm high, with a clear top and base plate, and clear baffles (unbaited)
B) Space station trap: 10cm high, with a clear top and base plate, and clear baffles; baited with *trans* asarone and hexanal
C) Space station trap: 10cm high, with a clear top and base plate, and orange baffles (unbaited)
D) Space station trap: 10cm high, with a clear top and base plate, and orange baffles; baited with *trans*-asarone and hexanal
E) Space station trap: 10cm high, with an orange top and base plate, and orange baffles (unbaited)
F) Space station trap: 10cm high, with an orange top and base plate, and orange baffles; baited with *trans*-asarone and hexanal
8a. This experiment was performed at Bruce Butcher's field Graves and Graves Ltd.-Lords Ground Farm, and ran from 23/4/96-4/6/96 (1st generation of flies). Four blocks were put out, and the traps were randomised twice weekly.
8b. This experiment was performed at Bruce Butcher's field Graves and Graves Ltd. - Broad Fen Farm, and ran from 30/6/96-1/9/96 (2nd generation of flies). Five blocks were put out, and the traps were randomised weekly. In this year, a REBELL ® trap was placed at the end of each block of 'space station' traps as a control to estimate the number of flies available for capture in the vicinity of the experiment. These REBELL traps were referred to as R^1 and R^2, respectively and were not randomised within the block structure, but remained static.

In this experiment, the number of other insects (excluding *P. rosae*, and over 2.5mm in length) was also counted. These data were analysed as a complete Latin square experiment (+1 extra randomisation). In this analysis, the total treatment sums of squares were separated into the 3 by 2 factorial for 'chemical' (absent = A, C and E vs. present = B, D and F) and 'visual' (no orange = A and B, part orange = C and D, and totally orange = E and F), with the 'chemical' by 'treatment' interactions. There were no missing values in these data.

Experiment 9
Treatments:
R^1) REBELL ® trap control: always next to the unbaited pathogen trap (unbaited)
A) Space station trap: 10cm high, with an orange top and base plate, and orange baffles (unbaited)
B) Space station trap: 10cm high, with an orange top and base plate, and orange baffles; baited with *trans*-asarone and hexanal
R^2) REBELL ® trap control: always next to the baited pathogen trap (unbaited)
This experiment was performed at W.H.Knights Ltd - Broom Bits East, and ran from 30/6/96-1/9/96 (2nd generation of flies).
Ten blocks of traps were positioned around the edge of a parsnip field (*Pastinaca sativa*), running parallel to the crop. The position of blocks, either 5m into the crop (**Exp.9a**) at a height of 20cm above ground level, so as to be visible above the crop foliage; or in the field surround against a hedgerow (**Exp.9b**), at a height of 1.5m so as to be visible above the level of the rough herbage, were allocated in an alternating pattern around the field. The two treatments for the 'space station' traps (baited and unbaited) were randomised weekly, while the two REBELL ® traps were changed weekly but their possitons remained static.

In this experiment, the number of other insects (excluding *P. rosae*, and over 2.5mm in length) caught in the traps was also counted. These data were analysed as three complete Latin square experiments. There were no missing values in these data.

Figure 7.1: Exploded diagram of space station trap (Pell *et al.*, 1993).

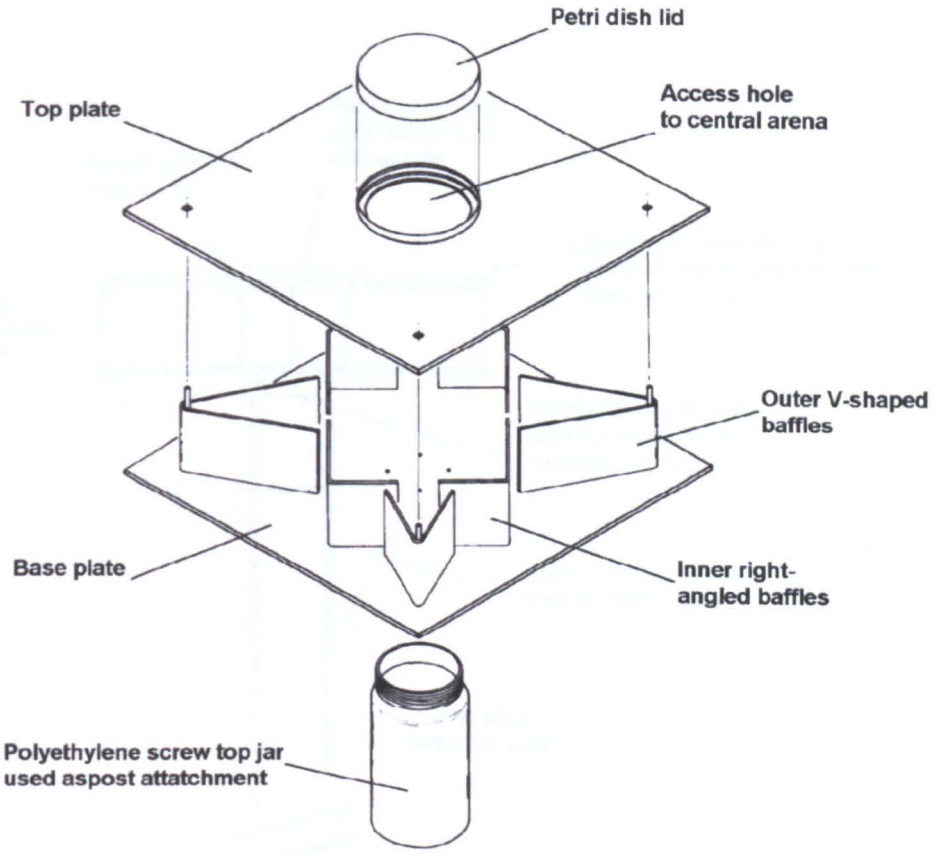


Figure 7.2: Insect entrapment device and lures as used in central arena of space station trap.

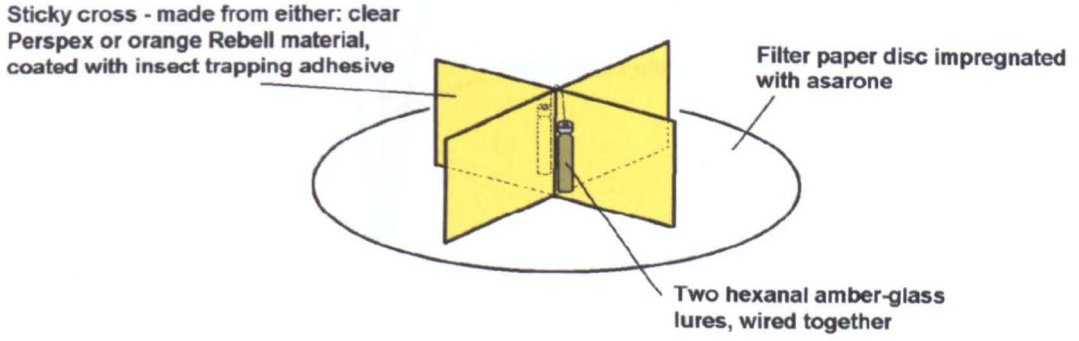


Figure 7.3: Side view of space station trap as deployed in the field.

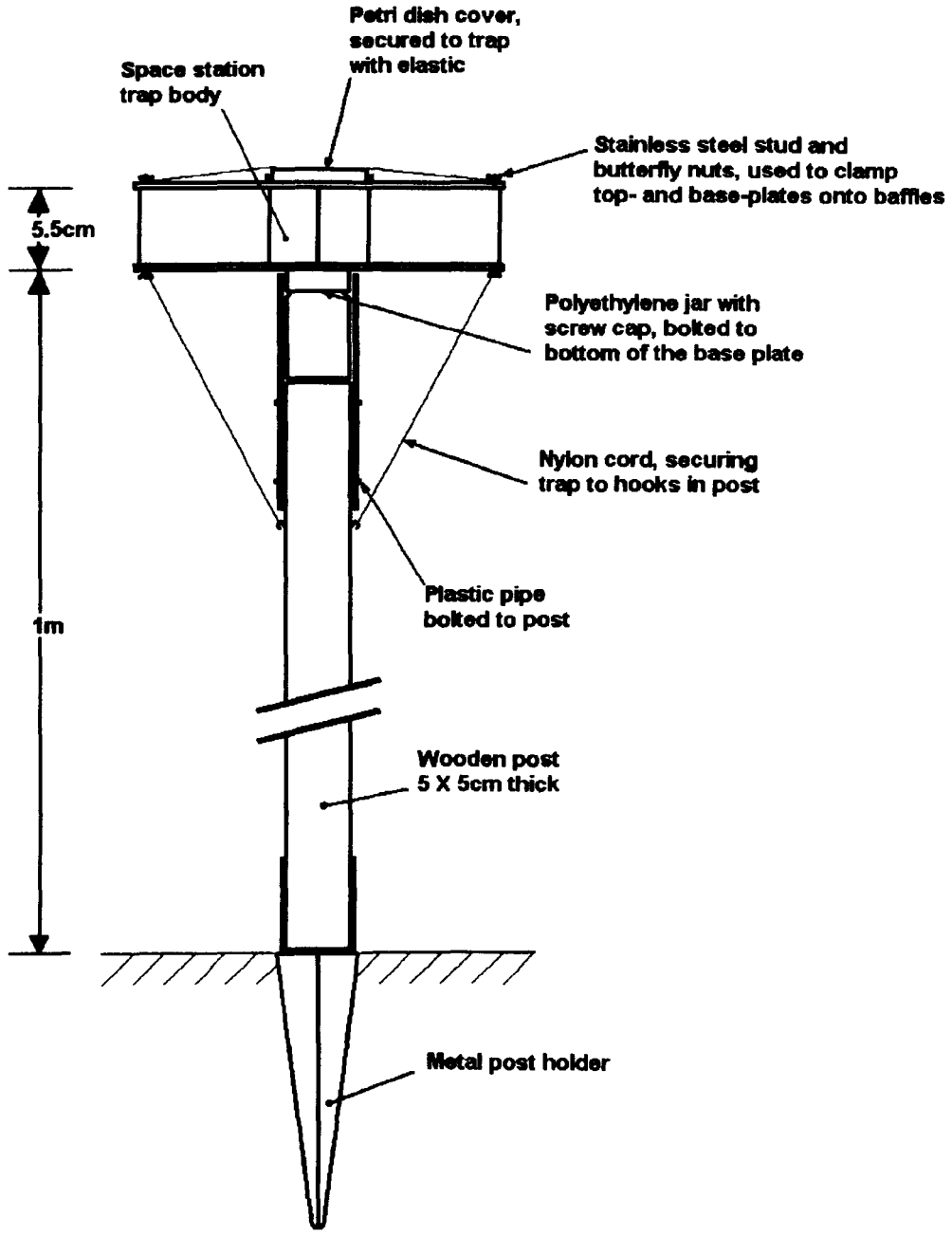


Figure 7.4: Yellow funnel trap

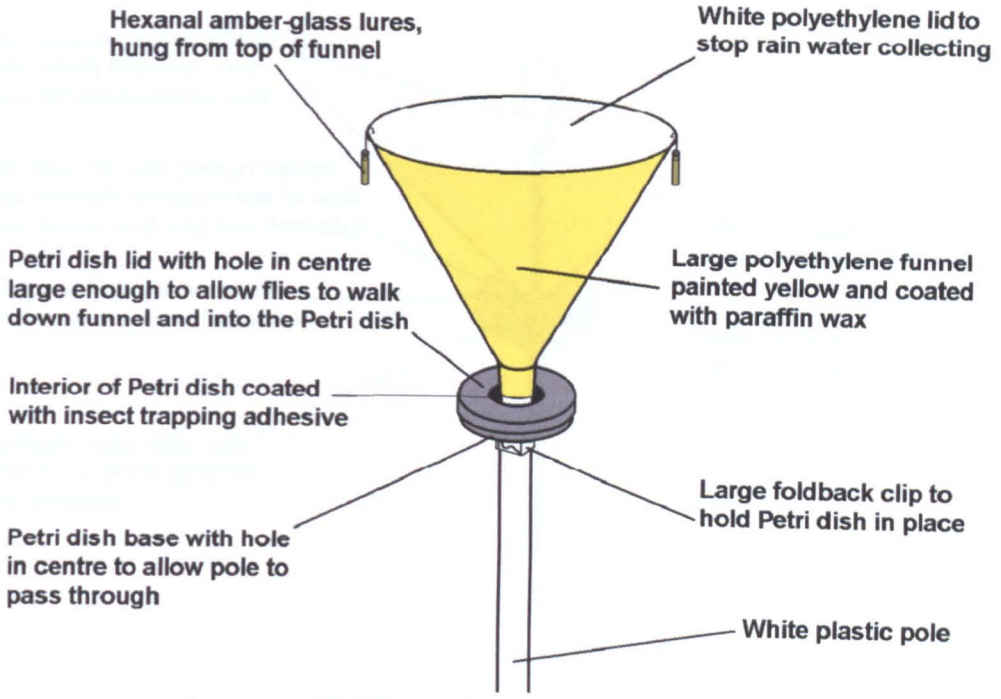


Figure 7.5: Sticky REBELL ® 'V'-board trap

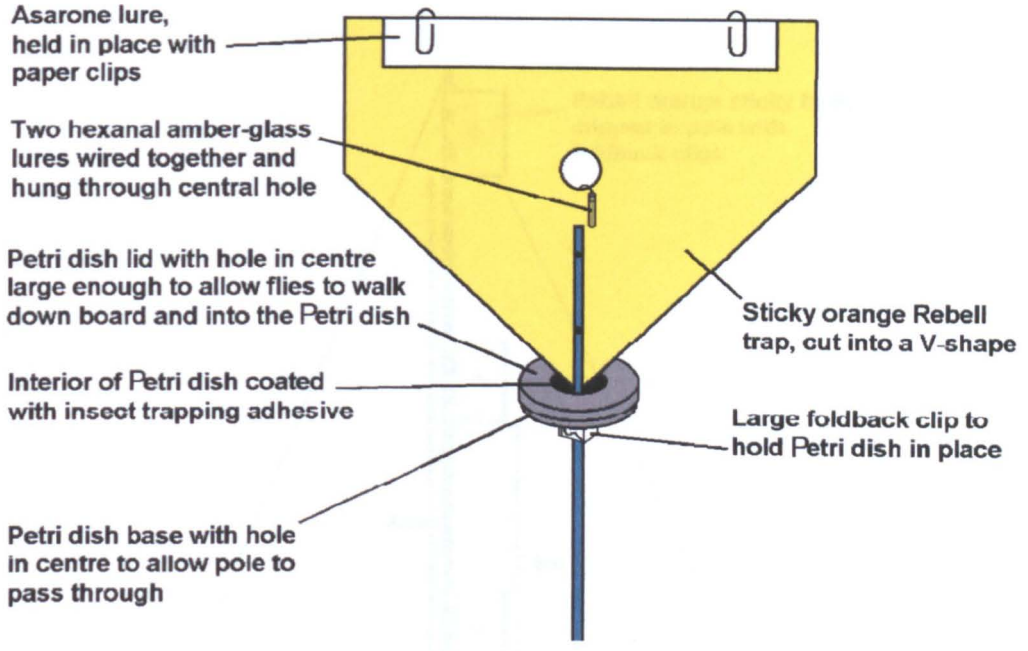


Figure7.6 : Non-sticky REBELL ® 'V'-board trap

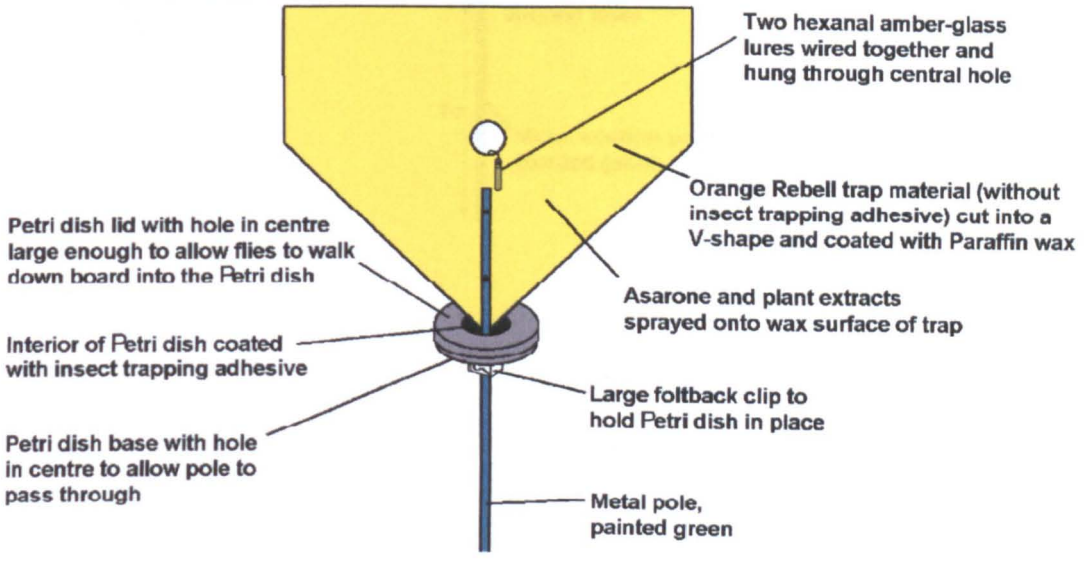


Figure 7.7: **Height trap design.**

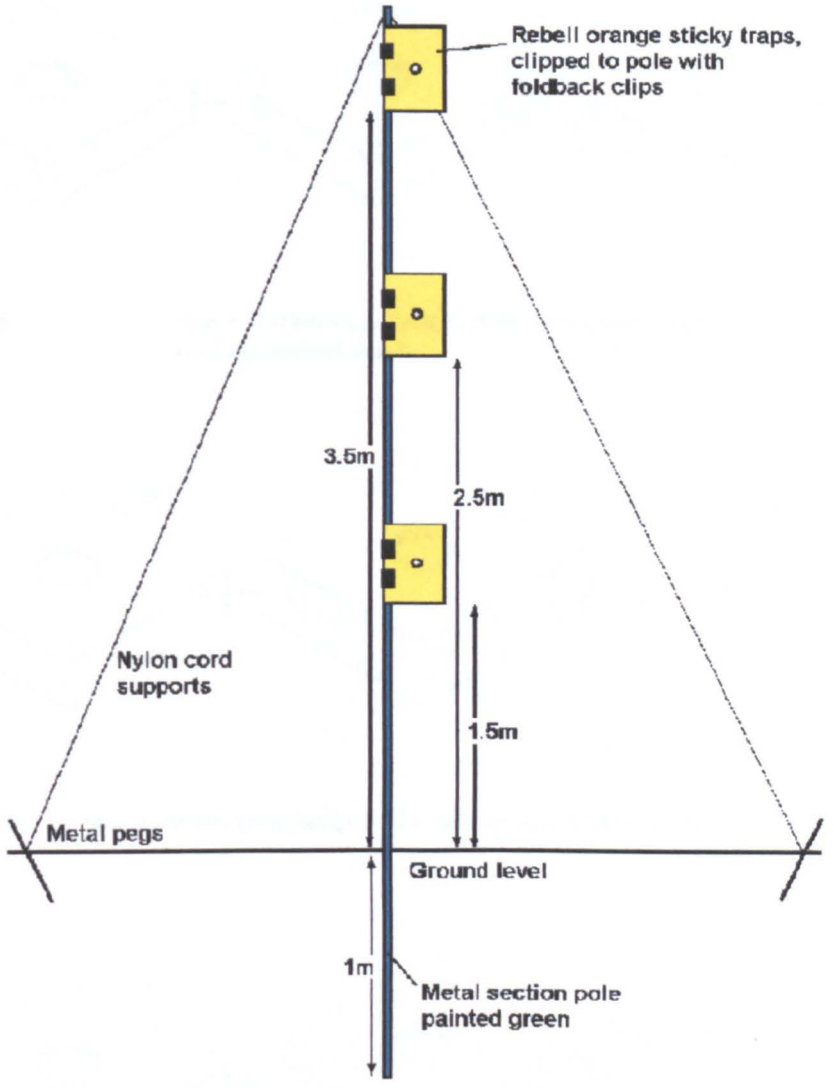


Figure 7.8: **Space station trap designs with increasing use of visually attractive orange Perspex.**

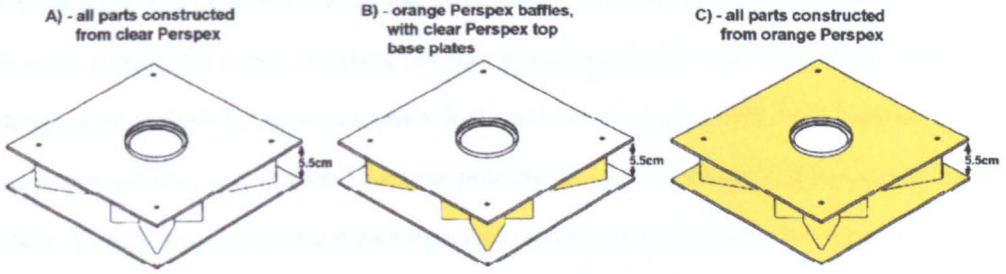


Figure 7.9: **Space station trap designs with increasing baffle height (orange baffles only).**

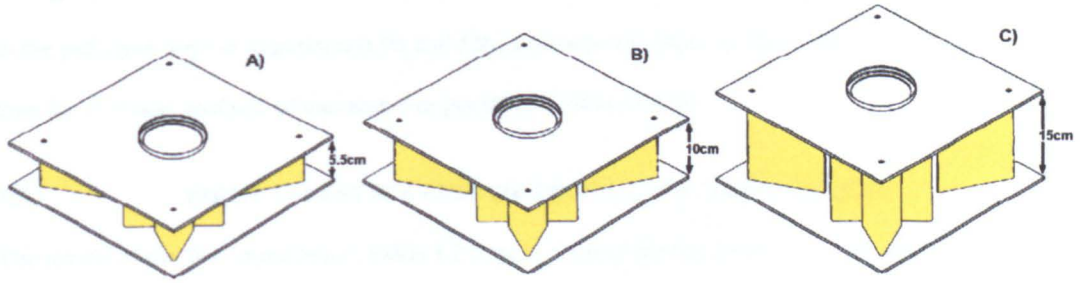
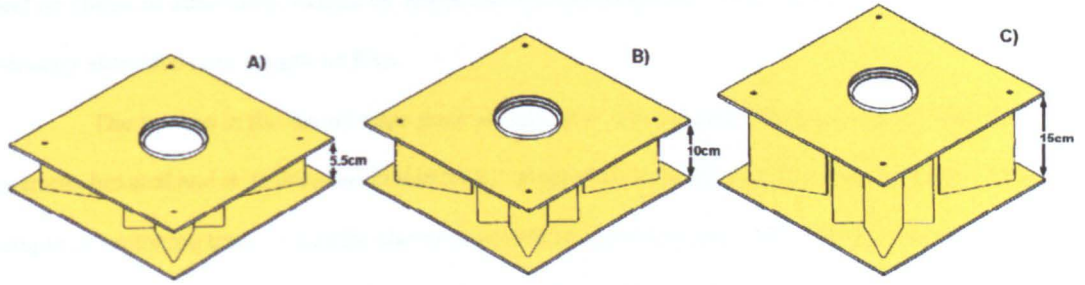


Figure 7.10: **Space station trap designs with increasing baffle height (totally orange).**



7.3 RESULTS

During this study, work on pathogen trap development was hampered to a large extent by a crash in *P. rosae* populations in the UK, hence few flies were caught in the field. These field experiments were designed for analysis by parametric analysis of variance; however, counts of *P. rosae* were too low to allow this analysis, so the results have been presented as tables of means (catches per sampling interval, ±SE). As such, any conclusions drawn from these results can only be tentative suggestions. For calculation of the means, only blocks catching some *P. rosae*, and trapping periods (randomisations) during which at least one *P. rosae* was caught on a block, were included. This was necessary as *P. rosae* were particularly scarce and unevenly distributed in the field, and over time, with flies often disappearing for days or weeks during experiments. The numbers of other insects (i.e. excluding *P. rosae*, and >2.5mm in length) caught in the pathogen traps in experiments 9b and 10b, were also counted. As these catches were much higher than for *P. rosae*, analysis of variance was possible on these results.

7.3.1 FIELD INVESTIGATION OF PROTOTYPE TRAP DESIGNS

The results of the first experiment (Table 7.2 Exp.1) showed that the most successful trap design, for directing *P. rosae* into an enclosed inoculation chamber, was the clear Perspex space station trap with a visually attractive orange target and *trans*-asarone and hexanal lures, placed centrally. This trap caught 76% of the total number of flies caught in prototype traps as a whole. A similar trap with an orange target, but no chemical attractants, caught far fewer flies (3% of the total), whilst the trap without chemical or visually attractive cues caught no flies.

The catches in the funnel traps were all very low. The most effective was baited with *trans*-asarone, hexanal and *A. graveolens* leaf extract, but this trap only caught 9% of total number of flies caught in prototype traps. A similar control trap with no chemical attractants, and two others baited with *trans*-asarone and hexanal alone, or *trans*-asarone and hexanal plus *D. carota* leaf extract, caught very few flies.

The results of the second experiment (Table 7.2 Exp.1) again showed that the most successful trap design was the clear Perspex space station trap with a visually attractive orange target and *trans*-asarone and hexanal lures, placed centrally. This trap caught 71% of the total number of flies caught in

prototype traps (excluding the catch on the stick 'V'-board controls). A similar trap with an orange target, but no chemical attractants, caught fewer flies (13% of the total), whilst the trap without chemical or visually attractive cues caught no flies.

The catches in the non-sticky 'V'-board traps were all very low. The most effective was baited with *trans*-asarone, hexanal and *A. graveolens* leaf extract, but this trap only caught 10% of the total number of flies caught in prototype traps. A similar trap baited with *trans*-asarone and hexanal plus *D. carota* extract caught slightly fewer flies (6% of the total).

Comparison of the catches in the inoculation chambers of prototype traps with the number of flies available for capture, as estimated from catches on the sticky 'V'-board traps, showed that the most effective prototype trap ('space station' with chemical and visually attractive cues) only caught approximately 7% of the flies available for capture. Only about 1% of the flies attracted to the surface of the non-sticky 'V'-board traps were actually directed into the inoculation chamber.

The slightly lower catch of *P. rosae* in the most effective 'space station' traps in the second experiment (mean 4.6), compared to the first experiment (mean 9.9), may have resulted from the inclusion of sticky 'V'-board traps, which caught large number of flies and possibly reduced the number available for capture in the other traps.

7.3.2 INVESTIGATIONS USING 'SPACE STATION' TRAPS, AND OPTIMISATION OF THEIR USE

A) DETERMINATION OF OPTIMUM TRAPPING HEIGHT IN THE HEDGEROW

The results of this experiment (Table 7.2 Exp.3) showed little differences between the total catches at the 3 different heights (3.5m = 35% of total catch, 2.5m = 36% and 1.5m = 29%). However, a large proportion of the flies caught at 3.5m were in one anomalously high catch of 142 flies, which occurred on a single trap during one week. This high catch was particularly notable, as the overall population of *P. rosae* in the field was low, and flies were not caught in this position either before or after this event.

After exclusion of this anomalous result, the largest numbers of flies were caught at a height of 2.5m (48%), which was about half way between the top of the hedgerow and the herbage boarder. Only slightly fewer (38%) flies were caught at a height of 1.5m, which was approximately level with the top of

the herbage boarder, whilst the fewest flies were caught at 3.5m (14%), which was about level with the top of the hedgerow and trees.

The anomalously high catch of *P. rosae* occurred during the first week that flies were caught in the field. The trap which caught these flies was situated near the top of a tall willow tree, in an otherwise relatively uniform hedgerow, and flies were not caught in this location in any other trapping period during this experiment. This suggests that some sort of specialised behaviour may have occurred in the locality, during the emergence of the second generation of flies.

B) OPTIMISATION OF SIZE, VISUAL CHARACTERISTICS AND CHEMICAL ATTRACTANTS

The results of the first experiment (Table 7.2 Exp.4) showed that the *trans*-asarone and hexanal baited 'space station' traps without visually attractive cues failed to catch more *P. rosae* than similar unbaited control traps, catching (37.5%, and 62.5% of total catch, respectively). However, these results have little significance as only eight *P. rosae* were caught in total during this experiment.

The results of the second experiment (Table 7.2 Exp.5) showed that both 'space station' traps constructed partially or wholly from the visually attractive orange Perspex, caught equal proportions of the total number of flies caught during this experiment (i.e. 50% each), whilst the trap without visually attractive orange Perspex failed to catch any flies. However, these results have little significance as only two *P. rosae* were caught in total during this experiment.

The results of the third and fourth experiments (Table 7.2 Exp.6, and Exp.7) showed that for 'space station' traps with visually attractive orange Perspex baffles, designs with baffle heights of more than 5.5cm were the most effective, whether or not the trap also had visually attractive orange Perspex top and base plates. In the experiment using traps with clear top and base plates, a similar proportion of the total catch was caught in traps with 10cm and 15cm high orange baffles (42% and 50% of the total, respectively), whilst very few flies were caught in traps with 5.5cm high orange baffles (8%). In the experiment using traps with orange top and base plates, the majority of flies were caught in the trap with the higher (15cm) baffle height (62.5% of the total), fewer were caught in the trap with 10cm high baffles (25%) and about half as many again were caught in the trap with the smallest baffle height (12.5% of the total).

The fifth experiment investigated the effects of increasing the quantity of visually attractive orange Perspex used in the construction of 'space station' traps, and the effects of incorporating the chemical attractants *trans*-asarone and hexanal. This experiment was performed at two sites (on different dates). The results at the first site (Table 7.2 Exp.8a) showed that the effectiveness of the traps increased as more visually attractive orange Perspex was used in their construction. This trend was particularly clear when comparing the catches in the chemically baited traps, where traps with no orange Perspex, those with orange baffles, and those constructed wholly from orange Perspex, caught 8%, 17%, and 50% of the total number of flies caught in this experiment, respectively. The importance of both visual and chemical attractants for increasing the number of flies entering the central inoculation chamber can be clearly seen. Traps lacking both of these attractive cues failed to catch any flies. Traps with only chemical attractants caught 8% of the total, and traps with only visual attractant cues (i.e. partly or wholly from orange Perspex) caught 8% and 17% of the total, respectively. In general, adding chemical attractants to a trap increased its catch over a similar unbaited trap, and this effect was strongest for traps constructed wholly from orange Perspex, where a baited trap caught 50% of the total number of flies caught during the experiment, but a trap without chemical attractants only caught only 8% of the total.

The results at the second site (Table 7.2 Exp.8b) reflected those from the first site, and showed similar trends. In general, catches increased as more visually attractive material was used in the construction of the 'space station' traps, and this was again particularly obvious with the chemically baited traps: traps with no orange Perspex, those with orange baffles, and those constructed wholly from orange Perspex, caught 0%, 14% and 70% of the total number of flies in this experiment, respectively. The inclusion of the chemical attractants (*trans*-asarone and hexanal) in traps with some visually attractive orange material led to an increase in catch; this was particularly obvious for the totally orange traps, where catches rose from 8% to 70% of the total catch, when chemical lures were added. The number of flies caught on the most effective pathogen trap in this experiment (10cm high, constructed wholly from orange Perspex and including *trans*-asarone and hexanal lures) was higher (mean 0.87) than the catch on the REBELL® monitoring traps, placed at either end of the blocks of 'space station' traps (mean 0.55). This showed a surprisingly high efficiency for the 'space station' traps, considering that flies had first to

negotiate their way into the trap through a series of baffles and into the inoculation chamber before being captured, whilst those caught on the REBELL® traps had only to come into contact with it.

In this experiment, the numbers of other insects (excluding *P. rosae*, and over 2.5mm in length) caught in the traps were also counted; the catches of these 'other insects' were much higher than the catches of *P. rosae* (Table 7.3 Exp 8b), and were suitable for statistical analysis (Table 7.4 Exp.8b, and Fig.7.11 and 7.12). The results showed no significant interactions between the two factors (contrast 3 'chemical' by 'visual'), and they can therefore be considered separately. The difference between catches in traps, with and without chemical lures, was found to be significant (contrast 1 'chemical). The Figure (Fig.7.11) shows that the presence of *trans*-asarone and hexanal lures within the inoculation chamber of traps reduced their catch of 'other insects' by approximately 13%. The difference between catches in traps with three different quantities of orange Perspex used in their construction (none, only orange baffles and wholly orange) was also highly significant (contrast 2 'visual' and from the 95% c.i. (Fig.12), the catches on all three types were clearly different. The Figure shows a clear trend, with numbers of 'other insects' caught in the inoculation chamber of 'space station' traps dropping significantly as more orange Perspex was used in their construction. The highest catch was in the wholly clear Perspex trap. The part orange trap (orange baffles only) caught approximately three times fewer insects, and the wholly orange trap caught approximately six times fewer insects, than the clear Perspex trap. Catches of 'other insects' on the REBELL ® traps, located at either end of the blocks of 'space station' traps, were too numerous to count. However, they were estimated to catch over 500 insects, per trap, per week, although the criterion of only counting insects over 2.5mm length excluded carrot psyllids (*Trioza apicalis*) and pollen beetles (*Meligethes* species). These were often too numerous to count on the REBELL ® traps, but were almost absent in the pathogen traps. Comparison of the estimated catch on the REBELL ® traps with catches in the 'space station' traps showed that the REBBELL ® traps caught far more (at least 6 times more) 'other insects' than any of the 'space station' trap designs.

The overall results from the previous experiments suggest that the optimum design for a 'space station' trap is one with 10cm high baffles, constructed entirely from visually attractive orange Perspex, and baited with *trans*-asarone and hexanal.

The sixth experiment investigated the effects of placing optimally designed 'space station' traps, with and without *trans*-asarone and hexanal, in two different locations in the field (5m in to the crop vs. in the hedgerow). The results from the traps placed 5m into the field (Table 7.2 Exp.9a) showed that four times as many *P. rosae* were caught in the inoculation chambers of traps baited with *trans*-asarone and hexanal than in unbaited traps. At this location, catches in the chemically baited 'space station' traps were approximately 8.5 times lower than those on the REBELL ® control traps (placed either end of the blocks of 'space station' traps). The results from the traps placed in the hedgerow (Table 7.2 Exp.9b) showed that twice as many *P. rosae* were caught in the inoculation chambers of traps baited with *trans*-asarone and hexanal than in unbaited traps. At this location, catches in the chemically baited 'space station' traps were approximately three lower than those on the REBELL ® control traps.

The overall results showed that 'space station' traps baited with *trans*-asarone and hexanal were more effective than unbaited traps in both locations. The chemical attractants may have had a slightly greater effect in traps located 5m into the crop (i.e. a 4-fold increase) than when they were located in the hedgerow (i.e. a two-fold increase). However, the 'space station' traps performed particularly poorly in this experiment, catching far fewer *P. rosae* than the REBELL ® control traps. The results also showed that the 'space station' traps caught similar numbers of *P. rosae* in both the crop and in the hedgerow, while the REBELL ® controls caught more flies when placed 5m into the crop than when placed in the hedgerow. This result was unusual, as catches of *P. rosae* are usually much higher (up to ten-fold higher) in the hedgerow than at the crop edge, and numbers usually decline rapidly further into the crop (ADAS unpublished data). As a result, this experiment gave no clear indication as to the best position in the field for the 'space station' traps to be placed for greatest effectiveness. The reason for the unusual distribution of flies at this site probably stemmed from the exposed nature of the field, which had very sparse hedgerows providing little shelter for the flies. This probably led to them taking shelter in the crop, rather than returning to their normal roosting site in the hedgerow and field surrounds.

In this experiment, the number of 'other insects' (excluding *P. rosae*, and >2.5mm in length) were also counted; these catches were much higher than for *P. rosae* and were suitable for statistical analysis (Table7.4 Exp.9a, Fig.7.13, and Table7.4 Exp.9b, Fig.7.14). The results from both trapping positions (5m into the crop, and in the hedgerow) showed a significant difference between catches in

'space station' traps, with and without chemical lures. The Figures (Fig. 7.13 and 7.14) show that at both locations, the presence of *trans*-asarone and hexanal lures within the inoculation chamber of the traps, reduced their catch of 'other insects' by approximately 30-40%. In general, more 'other insects' were caught in the hedgerow than 5m into the crop. This might be expected, as the hedgerow provides shelter and acts as a reservoir for many diverse insect species. Comparison of the catches in the 'space station' traps with those on the REBELL® control traps (in both locations) showed that the 'space station' traps caught approximately 85% fewer 'other insects' when in the crop, and about 92% fewer 'other insect' when in the hedgerow. The criterion of only counting insects over 2.5mm length excluded carrot psyllids (*Trioza apicalis*) and pollen beetles (*Meligethes* species), which were often numerous on the REBELL® traps, but were almost absent in the pathogen traps.

C) LABORATORY ESTIMATION OF RESIDENCE TIME

Observations of twenty-five *P. rosae* which entered an optimised 'space station' trap (10cm high and constructed wholly from orange Perspex, and baited with *trans*-asarone and hexanal) found that fifteen of the flies remained in the inoculation chamber for more than fifteen minutes (at which point they were removed). The other ten flies (40%) remained within the inoculation chamber for periods ranging from 2 to11 minutes, with a mean residence time of 5minutes 18seconds (±0.9).

Table 7.2: Results of Field Experiments: Mean Catch of *P. rosae* per Sampling Interval (±SE)

FIELD INVESTIGATION OF PROTOTYPE TRAP DESIGNS

Experiment 1 (n=5)

Treatments	Total catch	Mean (± SE)
A) Space station trap - with sticky clear Perspex cross in central arena, unbaited	0	0
B) Space station trap - with sticky orange (REBELL ® board) cross in central arena, unbaited	2	0.40 (± 0.25)
C) Space station trap - with sticky orange (REBELL ® board) cross in central arena, baited with *trans*- asarone and hexanal	50	9.90 (± 4.42)
D)Yellow funnel trap - unbaited	2	0.40 (± 0.25)
E) Yellow funnel trap - baited with *trans*-asarone and hexanal	2	0.40 (± 0.25)
F) Yellow funnel trap - baited with *trans*-asarone, hexanal and carrot MASE	4	0.80 (± 0.49)
G) Yellow funnel trap - baited with *trans*-asarone, hexanal and celery MASE	6	1.20 (± 0.80)

Experiment 2 (n=8)

Treatments	Total catch	Mean (± SE)
A) Space station trap - with sticky clear Perspex cross in central arena, unbaited	0	0
B) Space station trap - with sticky orange (REBELL ® board) cross in central arena, unbaited	7	0.88 (± 0.30)
C) Space station trap - with sticky orange (REBELL ® board) cross in central arena, baited with *trans*- asarone and hexanal	37	4.63 (± 1.24)
D) Sticky REBELL ® 'V' board trap – baited with *trans*-asarone and hexanal	513*	64.13 (± 17.01)
E) REBELL ® 'V' board trap – baited with *trans*-asarone, hexanal and carrot MASE	3	0.38 (± 0.18)
F) Sticky REBELL ® 'V' board trap – baited with *trans*-asarone and hexanal	490*	61.25 (± 14.30)
G) REBELL ® 'V' board trap – baited with *trans*-asarone, hexanal and celery MASE	5	0.63 (± 0.26)

N.B. combined mean and SE for the two identical sticky 'V'-board traps (n=16) was 62.69 (± 10.74).

Experiment 3 (n=68)

Treatments	Total	Mean (+/- SE) (n=68)	Mean (± SE) (excluding anomalous catch) (n=67*)
A) REBELL trap at a height of 3.5m	203	2.99 (+/- 2.09)	0.91 (± 0.28)*
B) REBELL trap at a height of 2.5m	214	3.15 (+/- 0.96)	3.15 (± 0.96)
C) REBELL trap at a height of 1.5m	170	2.50 (+/-0.62)	2.50 (±0.62)

NB. 142 of the 203 carrot flies caught on the trap at 3.5m were caught on a single trap during one week. This anomalous catch was omitted from the second calculation of means*, as it was not representative of the overall pattern of *P. rosae* distribution, and no other flies were caught in this position during the rest of the experiment. There were 2 missing sets of data, from poles which collapsed.

Table 7.2: Results of Field Experiments: Mean Catch of *P. rosae* per Sampling Interval (±SE) (Continued)

Experiment 4 (n=4)

Treatments	Total	Mean (± SE)
A_1) Space station trap - unbaited	1	0.25 (± 0.25)
B_1) Space station trap – baited with *trans*-asarone and hexanal	2	0.50 (± 0.50)
A_2) Space station trap - unbaited	4	1.00 (± 0.41)
B_2) Space station trap – baited with *trans*-asarone and hexanal	1	0.25 (± 0.25)

Experiment 5 (n=1)

Treatments	Total	Mean (± SE)
A) Space station trap - constructed totally from clear Perspex (unbaited)	0	0
B) Space station trap - with a clear top and base plate, and orange baffles (unbaited)	1	1
C) Space station trap - constructed totally from orange Perspex (unbaited)	1	1

Experiment 6 (n=2)

Treatments	Total	Mean (± SE)
A) 5.5cm high Space station trap (clear top and base plates, orange baffles, unbaited)	1	0.50 (± 0.50)
B) 10cm high Space station trap (clear top and base plates, orange baffles, unbaited)	5	2.50 (± 0.50)
C) 15cm high Space station trap (clear top and base plates, orange baffles, unbaited)	2	1.00 (± 0.00)

Experiment 7 (n=3)

Treatments	Total	Mean (± SE)
A) 5.5cm high space station trap (constructed totally from orange Perspex, unbaited)	1	0.33 (± 0.33)
B) 10cm high space station trap (constructed totally from orange Perspex, unbaited)	6	2.00 (± 0.58)
C) 15cm high space station trap (constructed totally from orange Perspex, unbaited)	5	1.67 (± 0.67)

Experiment 8a (n=6)

Treatments	Total	Mean (± SE)
A) Totally clear space station - unbaited	0	0
B) Totally clear space station - baited with *trans*-asarone and hexanal	1	0.17 (± 0.17)
C) Orange centred, clear top/base space station - unbaited	2	0.33 (± 0.21)
D) Orange centred, clear top/base space station baited with *trans*-asarone and hexanal	2	0.33 (± 0.21)
E) Totally orange space station - unbaited	1	0.17 (± 0.17)
F) Totally orange space station - baited with *trans*-asarone and hexanal	6	1.00 (± 0.00)

Table 7.2: **Results of Field Experiments: Mean Catch of *P. rosae* per Sampling Interval (±SE) (Continued)**

INVESTIGATIONS USING 'SPACE STATION' TRAPS, AND THE OPTIMISATION OF THEIR USE

Experiment 8b (n=30)

Treatments	Total	Mean (± SE)
R^1) Control unbaited REBELL ® trap	15	0.50 (± 0.17)
A) Totally clear space station – unbaited	0	0
B) Totally clear space station - baited with *trans*-asarone and hexanal	0	0
C) Orange centre, clear top/bottom space station – unbaited	3	0.10 (± 0.06)
D) Orange centre, clear top/bottom space station - baited with *trans*-asarone and hexanal	5	0.17 (± 0.11)
E) Totally orange space station – unbaited	3	0.10 (± 0.06)
F) Totally orange space station - baited with *trans*-asarone and hexanal	26	0.87 (± 0.25)
R^2) Control unbaited REBELL ® trap	18	0.60 (± 0.20)

N.B. Combined mean and SE for the two identical REBELL® traps (n=60) was 0.55 (± 0.12).

Experiment 9 - 5m into the crop at a height of 20cm (n=30)

Treatments	Total	Mean (± SE)
R^1) Control unbaited REBELL ® trap	65	2.17 (± 0.45)
A) Totally orange space station - unbaited	3	0.10 (± 0.06)
B) Totally orange space station - baited with *trans*-asarone and hexanal	12	0.40 (± 0.13)
R^2) Control unbaited REBELL ® trap	140	4.67 (± 0.82)

N.B. combined mean and SE for the two identical REBELL® traps (n=60) was 3.42 (± 0.49).

Experiment 9 - traps in the hedgerow at a height of 1.5m (n=30)

Treatments	Total	Mean (± SE)
R^1) Control unbaited REBELL ® trap	24	0.80 (± 0.18)
A) Totally orange space station - unbaited	5	0.17 (± 0.08)
B) Totally orange space station - baited with *trans*-asarone and hexanal	10	0.33 (± 0.10)
R^2) Control unbaited REBELL ® trap	38	1.27 (± 0.28)

N.B. Combined mean and SE for the two identical REBELL® traps (n=60) was 1.03 (± 0.17).

Table 7.3: Results of Field Experiments: Mean Catch of 'Other Insects' per Sampling Interval (±SE)

Experiment 8b (n=35)

Treatments	Total	Mean (± SE)
R[1]) Control unbaited REBELL ® trap	>17500	>500
A) Totally clear space station – unbaited	2543	72.66 (± 11.91)
B) Totally clear space station - baited with *trans*-asarone and hexanal	2309	65.97 (± 9.24)
C) Orange centre, clear top/bottom space station – unbaited	658	18.80 (± 2.39)
D) Orange centre, clear top/bottom space station - baited with *trans*-asarone and hexanal	767	21.91 (± 3.33)
E) Totally orange space station – unbaited	365	10.43 (± 1.25)
F) Totally orange space station - baited with *trans*-asarone and hexanal	445	12.71 (± 1.75)
R[2]) Control unbaited REBELL ® trap	>17500	>500

Experiment 9a (n=30)

Treatments	Total	Mean (± SE)
R[1]) Control unbaited REBELL ® trap	1753	58.43 (± 5.84)
A) Totally orange space station - unbaited	302	10.07 (± 1.28)
B) Totally orange space station - baited with *trans*-asarone and hexanal	207	6.90 (± 0.92)
R[2]) Control unbaited REBELL ® trap	1749	58.30(± 6.06)

N.B. Combined mean and SE for the two identical REBELL® traps (n=60) was 58.37 (± 4.17).

Experiment 9b (n=30)

Treatments	Total	Mean (± SE)
R[1]) Control unbaited REBELL ® trap	3812	127.07 (± 9.50)
A) Totally orange space station - unbaited	249	8.30 (± 1.27)
B) Totally orange space station - baited with *trans*-asarone and hexanal	156	5.20 (± 0.82)
R[2]) Control unbaited REBELL ® trap	3548	118.27 (± 9.10)

N.B. Combined mean and SE for the two identical REBELL® traps (n=60) was 122.67 (± 6.55).

Table 7.4: **Statistical Analyses of Field Experiments: Analysis of Variance, Log_{10} (number of 'other insects' +1)**

Experiment 8b

Source of Variation	d.f.	s.s.	m.s.	*F*	*P*
Block	4	4.089	1.022		
Date	30	13.517	0.451	14.54	
Position	25	1.828	0.073	2.36	
Treatment:					
chemical	1	0.187	0.187	6.03	0.015 *
visual	2	19.911	9.955	321.27	<0.001 ***
chemical x visual	2	0.122	0.061	1.97	0.143 ns
Error	145	4.493	0.031		
Total	209	44.147			

Experiment 9a

Source of Variation	d.f.	s.s.	m.s.	*F*	*P*
Block	4	1.539	0.385		
Date	25	2.245	0.090	3.06	
Position	5	0.187	0.037	1.28	
Treatment:	1	0.288	0.288	9.82	0.005 **
Error	24	0.704	0.029		
Total	59	4.963			

Experiment 9b

Source of Variation	d.f.	s.s.	m.s.	*F*	*P*
Block	4	1.146	0.286		
Date	25	3.643	0.146	3.77	
Position	5	0.124	0.025	0.64	
Treatment:	1	0.260	0.260	6.74	0.016 *
Error	24	0.928	0.039		
Total	59	6.101			

Figure 7.11: **Mean catch of 'other insects' per sampling interval, in traps with and without *trans*-asarone and hexanal lures. Data plotted are back transformed (geometric) means ± 95% confidence intervals. Details are in Table 7.1 Exp. 8a.**

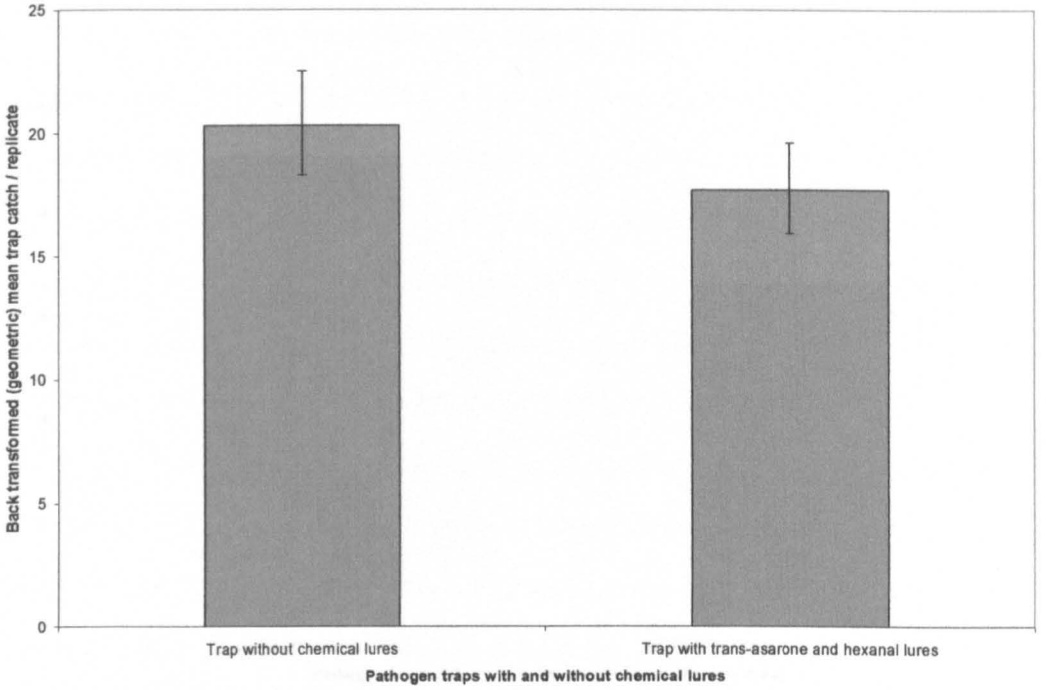


Figure 7.12: **Mean catch of 'other insects' per sampling interval, in traps with different quantities of orange Perspex used in their construction. Data plotted are back transformed (geometric) means ± 95% c.i. Details are in Table 7.1 Exp. 8b.**

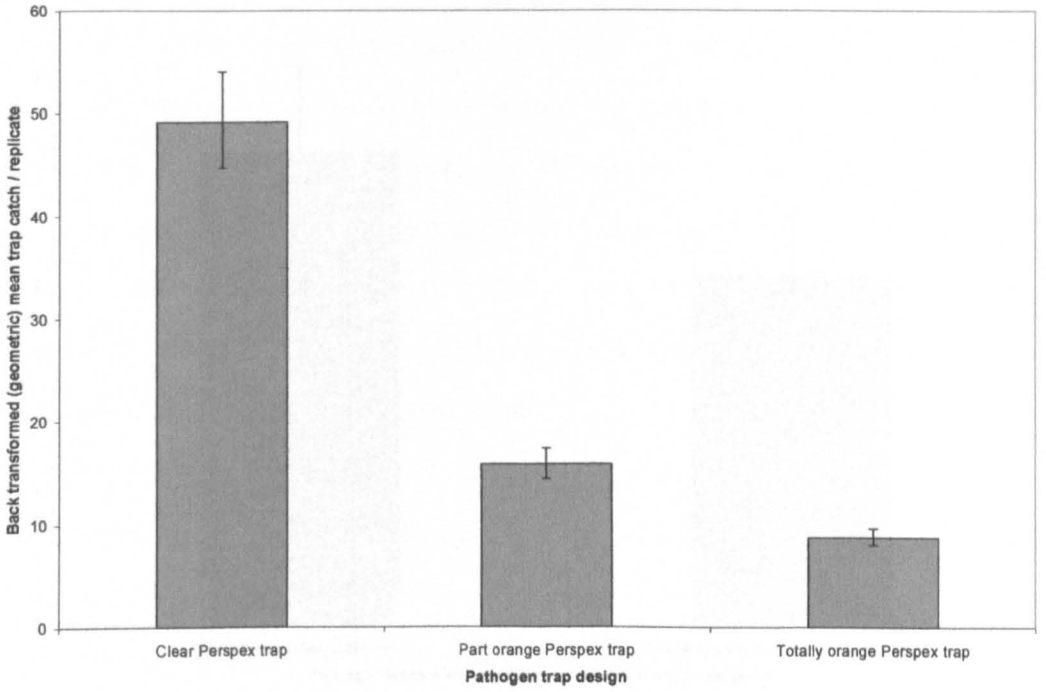


Figure 7.13: **Mean catch of 'other insects' per sampling interval, in traps placed 5m into the crop. Data plotted are back transformed (geometric) means ± 95% confidence intervals. Details are in Table 7.1 Exp. 9a.**

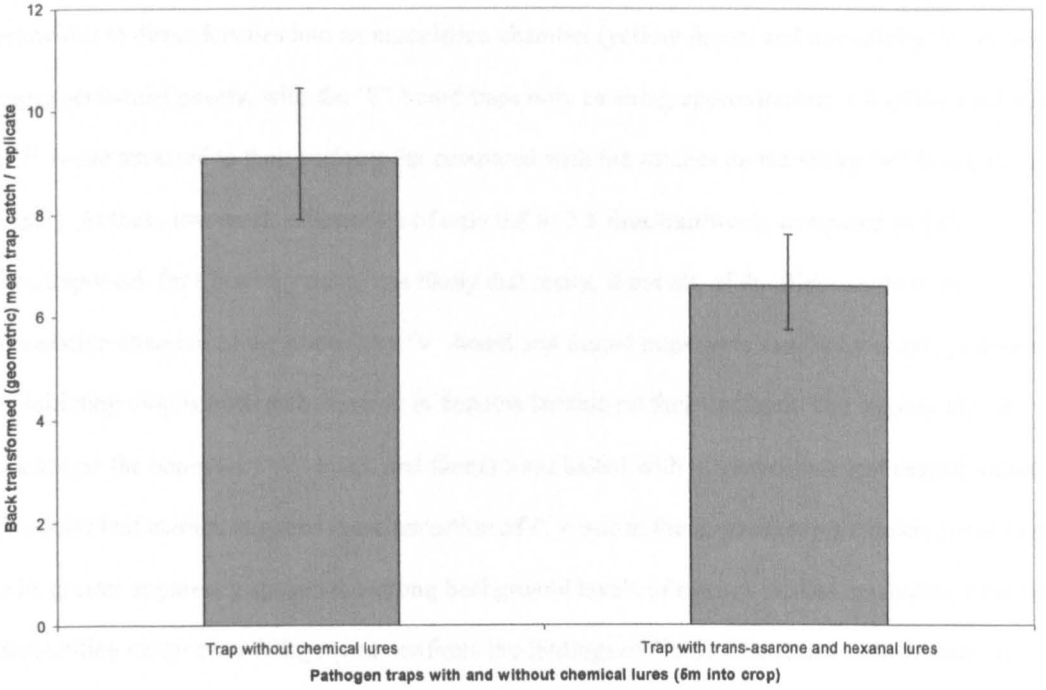
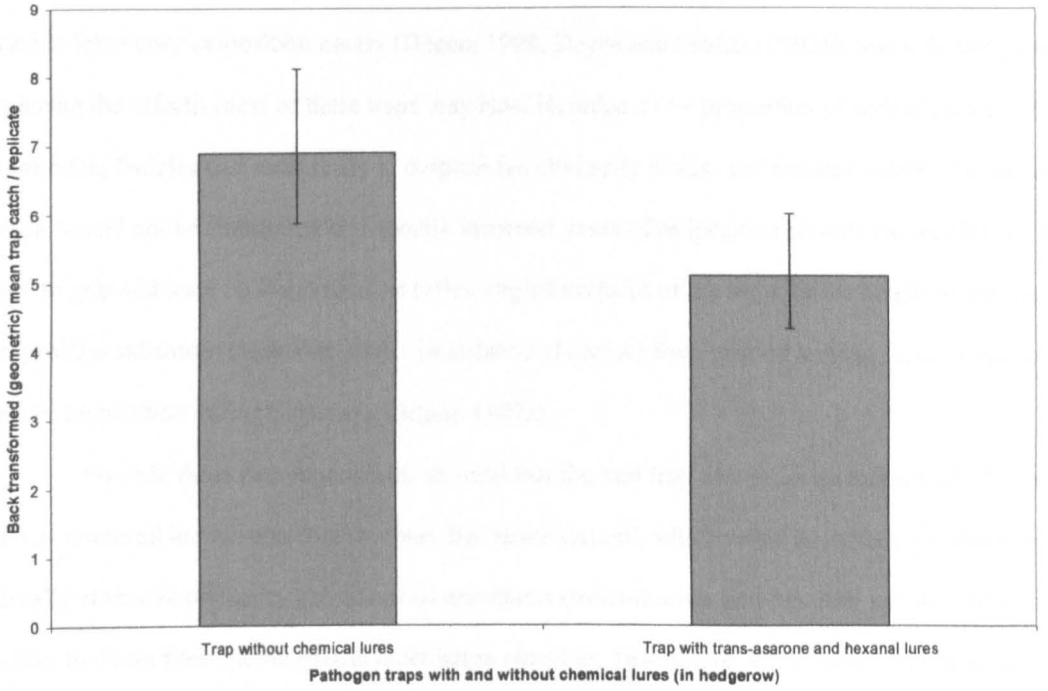


Figure 7.14: **Mean catch of 'other insects' in per sampling interval, in traps placed in the hedgerow. Data plotted are back transformed (geometric) means ± 95% confidence intervals. Details are in Table 7.1 Exp. 9b.**



7.4 DISCUSSION

7.4.1 FIELD INVESTIGATION OF PROTOTYPE TRAP DESIGNS

In the first and second experiments, the two trap designs which relied on inducing oviposition behaviour to direct females into an inoculation chamber (yellow funnel and non-sticky 'V'-board traps) performed poorly, with the 'V'-board traps only catching approximately 1% of the total number of *P. rosae* attracted to their surfaces (as compared with the catches on the sticky 'V'-board traps in Exp.2). At these low catch efficiencies of only 0.8 to 2.5 flies/trap/week, compared to 125 flies/trap/week for the sticky traps, it is likely that many, if not all, of the flies caught in the inoculation chamber of the non-sticky 'V'-board and funnel traps were caught by chance, rather than by initiating oviposition run behaviour in females landing on their surfaces. The slightly higher catches on the non-sticky 'V'-board and funnel traps baited with *A. graveolens* leaf extract, rather than *D. carota* leaf extract, suggests more attraction of *P. rosae* to the *A. graveolens* extract, possibly due to its greater apparency against the strong background levels of carrot volatiles emanating from the surrounding carrot crop foliage (and confirms the findings of Ch.6). The factors which caused the poor performance of these two trap designs (funnel and 'V'-board traps) are not known, as they included many of the main factors required for oviposition stimulation (colour, surface texture, chemical attractants and oviposition stimulants), and in many respects simulated the pseudo-leaves used in laboratory oviposition assays (Degen, 1998; Degen and Städler, 1997a). Some factors possibly reducing the effectiveness of these traps may have included a low proportion of flies attracted to the traps being females that were ready to oviposit (as obviously males, and females without mature egg loads, would not be stimulated to oviposit), incorrect doses of oviposition stimulants, inability of the flies to grip and walk on the vertical or reflex-angled surfaces of the traps, or the height of the trap or oviposition substrate (black Petri dish - inoculation chamber) from ground level (a factor found to reduce oviposition during bioassays; Degen, 1997a).

Overall, these two experiments showed that the best trap design for directing adult *P. rosae* into an enclosed inoculation chamber was the 'space station', which relied upon the combination of a visually attractive orange target, chemical attractants (*trans*-asarone and hexanal) and unidirectional baffles to direct flies into its central inoculation chamber. This design was at least seven times as effective as the best trap design relying on oviposition run behaviour to direct flies into an enclosed space. As such, the space station design was chosen for further work aimed at increasing its catch

efficiency for *P. rosae* by optimising various factors in its construction, as well as trying to determine the most appropriate field location for maximum catches of *P. rosae*. The importance of both the visual cue (although very small in these experiments) and chemical attractants for directing *P. rosae* into the inoculation chambers of space station traps were obvious in both experiments, as flies were not caught in traps without either cue, while small numbers were caught in traps with only the visual target (0.8 and 1.8 flies/trap/week respectively), and between 5 and 26 times more flies were caught in traps with both visual and chemical attractants (20.6 and 9.3 flies/trap/week respectively).

7.4.2 INVESTIGATIONS USING 'SPACE STATION' TRAPS, AND OPTIMISATION OF THEIR USE

A) DETERMINATION OF OPTIMUM TRAPPING HEIGHT IN THE HEDGEROW

After exclusion of one anomalous catch, the results of this experiment showed that the optimum height to place traps in the hedgerow was 2.5m (half way between the top of the herbage boarder and the top of the hedge), as the highest proportion of flies were caught at this height (48% of the total for the experiment), with catches at 1.5m (38%) and 3.5m (14%) being lower. However, there was only a small reduction in catch (10% of the total) between traps placed at a height of 2.5m and those at 1.5m. As 1.5m was a more practical height for mounting and servicing the traps, and reduced the risk of the bulky traps been blown down in high winds, this small reduction in catch is probably an acceptable trade-off in the light of its advantages; as such it was chosen as the most suitable height for locating functional pathogen traps in the hedgerow.

One anomalously high *P. rosae* catch occurred during the first week that *P. rosae* were caught in the field, on a trap situated near the top of a tall willow tree in an otherwise relatively uniform hedgerow, with no other flies being caught at this site during the rest of the experiment. This suggests that something unusual may have occurred at this site during the emergence of the second generation of flies. This massive catch in an area of low fly density may be further evidence that *P. rosae* utilise tall silhouettes during dispersive flights (as suggested by Städler, 1972), or possibly evidence that *P. rosae* use such features as aggregation cues during mating (i.e. the mating lecs of other dipteran species).

B) OPTIMISATION OF SIZE, VISUAL CHARACTERISTICS AND CHEMICAL ATTRACTANTS

During the 1995 second generation experiments on different space station trap designs (Exp. 4-7), few conclusions could be drawn due to the low population densities of *P. rosae*, exacerbated by the placement of pathogen traps on the crop edge rather than in the hedgerows where fly numbers were found to be higher (see Exp.3). However, the overall results from experiments 6 and 7 (part-orange and totally-orange space station traps, with vertical heights of 5cm, 10cm and 15cm) showed that catches of *P. rosae* increased with the vertical height of the traps. This was expected, as it increased the visually attractive surface area of the trap's profile, and increasing surface the area of visually attractive sticky board traps has previously been found to increase catches of *P. rosae* in the field. A large increase in catch was achieved by increasing the trap height from 5.5cm to 10cm in both experiments, but only a small increase was achieved, in one experiment, by increasing the height from 10cm to 15cm (and a slight reduction was seen in the other, Exp. 7). From these results, and the fact that the 10cm high traps were more economical to produce (using less expensive orange Perspex), and would be more resistant to damage in strong winds (lower profile), than the 15cm high traps; the 10cm high trap size was chosen for subsequent studies. Using this intermediate sized trap would also have significant advantages in an operational pathogen trap, as the inoculation chamber would be smaller, bringing flies which entered the inoculation chamber into closer proximity with the inoculum source and increasing their exposure to higher conidal densities. It would also make it easier to maintain a favourable microclimate for conidial production, survival, and germination within the chamber (i.e. high humidity).

Further investigations into the effects of increasing the quantity of visually attractive orange Perspex used in trap construction, both with and without chemical attractants (*trans*-asarone and hexanal), were performed in experiments 8a and 8b. In both these experiments, the importance of including both visual and chemical attractants to increase the number of *P. rosae* attracted to, and directed into, the inoculation chamber, was clear. In both experiments, clear Perspex traps without chemical lures failed to catch any *P. rosae*, while clear traps with chemical attractants only caught a small proportion (8.3%) of the total flies caught in (Exp 8b). For the part-orange traps (orange baffles only), a small number of flies were caught in traps without chemical lures (16.7% and 8.1% of the totals for experiments 8a and 8b respectively), while slightly more were caught in traps with chemical attractants (16.7% and 13.5% of the total catch respectively). For the totally orange traps (orange

baffles and orange top and base plates), similar numbers of *P. rosae* were caught in traps lacking chemical lures (8.3% and 8.1% of total catch, in Exp. 8a and 8b respectively) as in the unbaited part-orange traps, in these two experiments. However, large increases in catch were seen for the totally orange traps baited with chemical attractants (a 6 and 9-fold increase, in Exp. 8a and 8b respectively), over those without lures. These results showed that visual attractants had a significant effect in increasing catches of *P. rosae*, even in the absence of chemical attractants. However, increasing the quantity of orange material in the traps, from part-orange to totally orange, had little effect on increasing catch, unless chemical attractants were also included. In both of these experiments, the totally orange Perspex traps with chemical attractants (*trans*-asarone and hexanal) were by far the most effective pathogen traps tested, catching 50% and 70% of the total number of *P. rosae* caught in experiments 8a and 8b respectively. The mean catch of *P. rosae* in this most effective pathogen trap design (1.04 flies/trap/week) was also higher than the mean catch on the REBELL® control traps (0.66 flies/trap/week), which were positioned at each end of the pathogen trap blocks, in experiment 8b. This suggested a very high efficiency for this pathogen trap design, considering that flies caught on the (unbaited) REBELL® traps only had to make contact with its surface, while those caught in pathogen trap had first to actively negotiate the system of baffles, and home in on the chemical attractants within the inoculation chamber.

During experiment 8b, the numbers of insects other than *P. rosae* and over 2.5mm in length were also counted; the criterion of 2.5mm length excluded carrot psyllids (*Trioza apicalis*) and pollen beetles (*Meligethes* species), which were often numerous on the REBELL ®traps, but were almost absent in the pathogen traps. The results of this experiment showed a highly statistically significant effect of the quantity of orange Perspex used in the construction of the traps, with numbers of non-target insects dropping significantly as more orange Perspex was included. The totally orange Perspex traps caught 82% fewer non-target insects than the totally clear Perspex traps, while the traps with orange baffles and clear Perspex top and base plates caught 68% less (Fig. 7.12). The presence of *trans*-asarone and hexanal lures in pathogen traps also had a statistically significant effect of reducing the catch of non-target insects by approximately 13% (Fig. 7.11). Analysis of the results also showed that these two effects, amount of orange Perspex and chemical lures, acted independently of each other. All the pathogen trap designs tested caught far fewer (at least 6 times less) non-target insects, than the REBELL® control traps which caught at least 500 other insects/trap/week. The low catches

of other non-target insects in the pathogen traps probably reflected the difficulty of entering the inoculation chamber of the trap, without the aid of visual and chemical attractants, specific to these diverse insect species. It is unclear, from this experiment, whether the reduced catches of non-target insects in traps made partly or wholly from orange Perspex resulted from increased repellence as the quantity of orange Perspex increased, or visa versa, from a reduced attraction to the trap as less clear Perspex was used in its construction. As many non-target insects were caught on the similarly coloured REBELL® traps, and as this material had a high reflectance in the yellow region of the visible spectrum, which is known to act as a plant discrimination cue for most foliar insects (Prokopy and Owen, 1983), it seems unlikely that the orange Perspex acted as a repellent to many insect species. It may therefore be possible that the clear Perspex had some attractive properties perceptible to the non-target insect's vision, possibly stress patterns which would be visible in the UV spectrum, but would not be visible to the human eye. The significant effect of *trans*-asarone and hexanal in reducing the numbers of non-target insects caught in the pathogen traps suggests that it had a repellent effect on some of the non-target insect species caught in traps without chemical lures. The low number of non-target insects entering the most efficient pathogen trap design for catching *P. rosae* (totally orange Perspex with *trans*-asarone and hexanal lures) would be advantageous in an operational pathogen trap, as it would reduce the exposure of non-target insects to high levels of fungal inoculum found in the inoculation chamber. This would reduce losses of fungal inoculum, as less would be carried out on non-target insects, whilst also minimising the possibility of cross-infection of less susceptible non-target species in the field.

In the final field experiment, the most effective pathogen trap developed during this study (10cm high 'space station' trap, constructed wholly from orange Perspex) was tested, both with and without chemical attractants (*trans*-asarone and hexanal), at two different localities within the field: 5m into the crop at an optimum height of 20cm, and in the hedgerow at an optimum height of 1.5m (see Exp. 3). As expected, the traps with chemical attractants caught more *P. rosae* than those without, in both positions in the field, although the doubling of catch in the hedgerow, and four-fold increase in the field, were not as high as expected from previous experiments. Unexpectedly, pathogen traps placed in the hedgerow, and in the field, caught similar numbers of *P. rosae*, while catches on the REBELL® control traps were approximately four times as high in the crop as in the hedgerow. This finding was opposed to the findings in all other field trials, where distributions of flies were higher in

the hedgerows surrounding crops than at the crop edge, and tended to drop dramatically further into the crop (Dr. J. Blood-Smyth, ADAS, personal communication). Comparison of catches in the chemically baited pathogen traps with those on the REBELL® controls traps also showed that the pathogen traps had performed particularly poorly in this experiment, as the REBELL® traps caught approximately 8.5 times more *P. rosae* in the crop, and 3 times more in the hedgerow, than the most effective pathogen trap. This anomalous finding may have resulted from the exposed nature of this particular field site, which had sparse, thin hedges that would have provided little shelter for roosting *P. rosae*, and may have caused the flies to become more evenly distributed throughout the crop as they searched for shelter and higher humidity. Although unlikely, this unusual distribution of flies may also reflect different behaviours of the flies due to the different crop species, parsnip (*Pastinaca sativa*), grown at this site, as all other field experiments were performed in carrot fields (*D. carota*).

The number of non-target insects (>2.5mm in length) were also counted during this experiment (Exp.9), and statistically significant reductions in catch were again seen in response to the presence of *trans*-asarone and hexanal. This effect was higher than that seen in experiment 8b, and was similar at both locations in the field, being a 37% reduction for traps in the crop (Fig. 7.13) and a 32% reduction for traps in the hedgerow (Fig. 7.14), as compared to the catches of non-target insects caught in pathogen traps without chemical lures. Again, in this experiment, all the pathogen traps caught far fewer non-target insects than the REBELL® companion traps, with 88% fewer non-target insects being caught in the totally orange chemically baited trap in the crop, and 96% less in those in the hedgerow.

The overall results of this study into the development of an autodissemination trap for release of *E. schizophorae* into *P. rosae* populations in the field showed that the most suitable trap design was one based upon a trap designed by Pell *et al.* (1993) for the autodissemination of *Zoophthora radicans* into field populations of *Plutella xylostella*. During this study, the design of this trap was refined for use with *P. rosae*, by optimisation of the visually attractive cues used in its construction and by the incorporation of semiochemical attractants. This process led to an optimum design for an autodissemination pathogen trap, suitable for *P. rosae*, constructed completely from visually attractive orange Perspex, with a vertical height of 10cm, which was baited with chemical attractant lures (*trans*-asarone and hexanal) in its central inoculation chamber. The best location for this pathogen trap

was shown to be at a height of 1.5m against the hedgerow surrounding the crop, as highest densities of adult *P. rosae* were caught in this position.

The efficiency of this optimum trap design, as determined by its catches of *P. rosae* relative to the catches on REBELL® control traps nearby, varied widely between experiments in different years and generations of flies. The highest efficiency for this trap was seen during experiment 8b (1996, 2nd generation of flies), where it caught 58% more flies than the REBELL® traps (0.87 flies/trap/week vs. 0.55 flies/trap/week), when positioned on the crop edge, near the hedgerow. Much lower efficiencies were seen during experiment 9 (1997, 1st generation of flies), when it caught 68% (0.33 flies/trap/week vs. 1.03 flies/trap/week) and 88% (0.40 flies/trap/week vs. 3.42 flies/trap/week) fewer flies, than the REBELL® traps, when positioned in the hedgerow, and 5m into the crop, respectively. Much of the variability in the efficiency of this optimised trap design may be attributed to the very low numbers of *P. rosae* in the field during these experiments, combined with poor responses to the chemical attractants incorporated into the trap; with both factors resulting from the hot dry weather experienced by the flies during field experiments from 1995 onwards (see Ch. 6). Variability in the effectiveness of the chemical attractants can be seen in the differences in catch in 10cm high orange pathogen traps with chemical lures, relative to the catches on similar unbaited traps, during different years and generations of flies. During experiment 8b, 8 times more flies were caught in the chemically baited traps than in unbaited traps; during experiment 9, only 3 times, and double, the catches were seen for chemically baited traps positioned in the crop, and in the hedgerow, respectively. Similar variability in the performance of chemical attractants, including *trans*-asarone and hexanal, was also found during field experiments at a wide range of locations to investigate the effectiveness of various semiochemicals for their attractancy to *P. rosae* in the field (see Ch.6). The poor performance of the chemical attractants during these experiments can probably be attributed to reduced antennal responses to olfactory chemicals as a result of dehydration in the flies (which is known to affect EAG responses to olfactory stimulants in the laboratory), resulting from the hot dry conditions they experienced in the field. The suggestion that low catch numbers of *P. rosae* during the 1995 and 1996 field trials was a result of high mortality in general carrot fly populations, due to extremely unfavourable hot dry weather conditions, was supported by the findings of pest threshold experiments performed at HRI-Wellesbourne. These found that the high temperatures experienced

during this period, especially during June and July, resulted in the death of most first instar larvae shortly after they emerged from the eggs (J. Vincent, and S. Finch. personal communication).

C) ESTIMATION OF RESIDENCE TIME

In study by Furlong and Pell (1995), male moths (*P. xylostella*) were found to spend a mean time of 88 seconds in the inoculation chamber of a similar pathogen dispensing pheromone trap in the field. Their laboratory experiments showed that this was less than the lower estimate of the time they needed to spend under a shower of conidia of primary spores of *Z. radicans* to have a 50% chance of picking up a lethal infection (LD_{50} 306 sec, 95% c.i. 126-630 sec). However, by increasing the amount of sporulating mycelia in the trap from one 9cm plate above the moths, to two 9cm plates (one above and one below), they dramatically reduced the time needed to pick up a lethal infection to less than 60-seconds. Under these conditions, they found that a male remaining in the trap for the mean duration measured in the field was almost certain to acquire a lethal dose of fungal infection.

In this investigation, the time required within a conidial shower of *E. schizophorae* for *P. rosae* to acquire a lethal infection was not estimated. However, a laboratory investigation of the time spent inside the inoculation chamber of an optimised 'space station' trap showed that the majority of *P. rosae* entering, remained inside for more than 15minutes. With regards to an operational trap, and assuming that the time required within a conidial shower of *E. schizophorae* for lethal infection of *P. rosae* is similar to that required for infection of *P. xylostella* by *Z. radicans*, *P. rosae* remaining in the trap for the duration measured in the laboratory (over 15min) would almost certainly acquire a lethal dose of fungal infection.

7.4.3 FUTURE PROSPECTS

During this study, a trap was designed and optimised in many respects, with regard to the number of *P. rosae* it could draw into a small enclosed area, suitable for its infection with a fungal pathogen. This is the first step in the process of developing an autodissemination trap for the biological control of *P. rosae* using the fungal pathogen *E. schizophorae*. A great deal of further work would be required to complete this objective; the major steps involved in this process, along with how they might be achieved, are discussed below.

A suitable strain of pathogen would need to be isolated; this could be achieved by the collection of a number of isolates of the pathogen from a range of locations. Pathogen-killed cadavers could be collected from hedges at infection sites, or live *P. rosae* could be collected (i.e. using sweep nets) and then incubated to check for infection. The different isolates would then need to be kept in *in vivo* culture. The most virulent and host specific isolates could be determined using laboratory assays to compare the number of conidia necessary for host infection and the host range of each isolate. The environmental fitness of the most virulent strains could also be evaluated in a similar manner, comparing fungal development and conidial persistence under a range of environmental conditions. Once the most suitable isolate was determined, it could be kept *in vivo* on *P. rosae* or *M. domestica*. The use of *M. domestica* for *in vivo* culture has the advantages that these flies are very simple to mass rear and each cadaver produces more conidia than with *P. rosae*, due to its larger size. The drawback with using this method is that numerous passages of the pathogen through this alternative host could lead to changes in its pathogenicity towards *P. rosae*. Laboratory assays could be used to determine if this was happening, and occasionally passing the pathogen back through its original host (*P. rosae*) may help to reduce the problem. Alternatively, it could be kept as an *in vitro* culture, as both *E. muscae* and *E. schizophorae* have been successfully cultured *in vitro* (Carruthers *et al.*, 1985, Latgé *et al.*, 1988; Eilenberg and Bresciani, 1990). A method for the small-scale production of actively discharged primary conidia has also been developed by Eilenberg and Bresciani (1990). This involves the initial growth of protoplasts in liquid culture (GLEN medium containing - glucose, yeast extract, lactalbumin hydrolysate, NaCl and foetal calf serum; Beauvais and Latgé 1988; Latgé *et al.*, 1988), followed by transferral of protoplasts onto solid medium [Sabouraud Dextrose Agar (SADEY) supplemented with egg-yolk and milk; Eilenberg and Bresciani, 1990]. After 16 days incubation at 20°C, primary conidia are discharged from the surface of the medium and continue for up to 16 days, with an average of 4.79×10^6 conidia produced per Petri dish.

The selected pathogen could be incorporated into the autodissemination trap, either as recently pathogen-killed cadavers (of *P. rosae* or *M. domestica*), or as pieces of *in vitro* produced culture material capable of producing conidia. This material would ideally be placed in the top of the trap, so that actively discharged primary conidia rained down onto flies attracted into the inoculation chamber. Infection would be transmitted as a result of primary conidia, and supernumerary conidia produced by conidia that did not land on a host. The humidity within the trap could be elevated to

favour conidial production and disease transmission by incorporating a reservoir of water and wick system. The fungal inoculum could also be kept moist by embedding it in water agar. Once inside the trap, the flies would need to be exposed to sufficient conidia for infection to occur. This would depend upon the rate of conidial discharge from the infection source, the proportion landing on the fly, and the fly's residence time within the trap. Laboratory investigations of the residence times of *P. rosae* entering the inoculation chamber of this pathogen trap design showed that the majority of flies remained inside for more than 15minutes, which seems ample for transfer of conidia from a sporulating source to the flies. However, all of these factors would need to be manipulated, in order to maximise the number of flies which became infected after entering the trap.

Once a fully functioning trap, incorporating a sporulating source of conidia and capable of infecting the majority of *P. rosae* entering the trap in the laboratory, had been developed, field experiments would be required to prove the autodissemination trap was capable of spreading infection into field populations of *P. rosae* and to determine its efficiency in causing high levels of infection and epizootics. The infection levels in the field population of *P. rosae* would also need to be related to the levels of larval damage occurring in the crop. Simply comparing the effects of *E. schizophorae* release on *P. rosae* populations at release sites with the effects in nearby control sites (without pathogen release) would not be adequate for this pathogen, as it occurs naturally throughout the UK. This would make it necessary to distinguish between *P. rosae* infected with the introduced fungal isolate and infections due to resident strains of the pathogen. A number of molecular techniques are now available, making this possible (Bidochka, 2001). One of the most promising methods would be to use the RAPD (random amplification of polymorphic DNA) technique, as this is relatively easy and quick to perform, and has been used successfully in several studies on entomophthoralean fungi (i.e. Hodge *et al.*, 1995). Even if this biological control strategy was shown to control adult *P. rosae* populations and larval damage in the field effectively, a number of major obstacles with regard to mass production, formulation and storage of fungal inoculum for the traps would still have to be overcome. In conclusion, this study has provided a basis for future work on the biological control of *P. rosae* using a suitable pathogen such as *E. schizophorae*. However, a great deal of work would still be required to validate the effectiveness of this biological control strategy for *P. rosae* in the field.

8 GENERAL SUMMARY

During this investigation into the chemical ecology of *P .rosae*, a wide range of techniques were employed to study electrophysiological and behavioural responses to semiochemicals, particularly host plant volatiles. In a comparison of extraction techniques for host plant semiochemicals, the relatively novel microwave-assisted hexane extraction system was found to be a very rapid method of extracting large quantities of foliage, and provided high yields of plant volatiles. These extracts of host species, and particularly *A. graveolens*, were found to be attractive to *P. rosae* in the field during this study. This is the first time a host plant extract has been found to increase significantly catches of *P. rosae* in the field, and suggests that this extraction method is superior to hydro-distillation for producing biologically relevant extracts, since a range of host plant essential oils showed no activity in the field (Guerin *et al.* 1983). Extracts produced using this MAS extraction method were also found to be almost as acceptable as real host leaves in oviposition bioassays (Degen, Poppy and Städler, 1999). However, as this direct solvent extraction method produced extracts containing large quantities of non-volatile compounds, including leaf-surface waxes, it was not suitable for analysis by GC.

Air entrainment of head space volatiles was found to produce very 'clean' extracts ideally suited to GC analysis, but the extracts were very time consuming to perform and gave very low yields of volatiles. A relatively novel microwave-assisted distillation method was also found to produce very 'clean' extracts from foliage, suitable for GC analysis. This extraction procedure was very rapid and gave much higher yields of volatiles than were obtained using air entrainment; this made it an ideal method for the production of extracts from a wide range of host species, which were subsequently investigated for their biological activity using GC-EAG techniques.

GC-EAG studies of 13 apiaceous host plant species revealed a large number of electrophysiologically active peaks in the gas chromatograms of their extracts, the majority of this activity being elicited by minor components in the extracts (as seen in the GC-profiles of the extracts). The majority of the 42 electrophysiologically active compounds, identified in this study by GC-MS, have previously been reported as present in the volatiles of apiaceous plants. The eight not previously reported

in the Apiaceae were 2-hexanol, 3-hexanol, 1-octan-3-ol, (*E*)-limonene-1, 2-epoxide and 4-ethyl toluene, and tentatively identified, carvyl acetate, isoeugenol and 2,3,4-trimethylheptane. The compounds most frequently found to elicit EAG responses during GC-EAG analysis, of the range of host extracts tested, were β-caryophyllene, octane, α-humulene, sabinene, α-terpinolene and (*E*)-2-hexenal.

C. maculatum was of particular interest during this study as it is a preferred wild host of *P. rosae,* and has been shown to contribute significantly to the local concentration of adults in the boundaries surrounding host crops (Wainhouse and Coaker, 1981). During the GC-EAG investigations of host extracts, an unusually high (off-scale) EAG response and slow recovery rate was associated with one of the peaks in the GC profile of a *C. maculatum* extract. The compound eliciting this response was subsequently identified as γ-coniceine, the main alkaloid toxin of this plant. Two related piperidine alkaloids tentatively identified as conhydrine and conhydrinone also elicited large electrophysiological responses during GC-EAG analysis of this extract.

The host plant compounds identified as electrophysiologically active, over the range of plant extracts investigated, were from a wide range of chemical types, originating from a variety of biosynthetic pathways which can be grouped as: lipoxygenase products (alcohols, aldehydes, esters and alkanes), isoprenoids (monoterpenes and sesquiterpenes) and amino acid metabolites (methoxyphenyl compounds, piperidine alkaloids and others).

GC-EAG analysis of the non-host plant species *M. litoralis,* which has been shown to reduce larval damage to carrots by *P. rosae* in intercropping field experiments (Rämert, 1993 and 1996), found little electrophysiological activity and only low levels of volatiles could be extracted from this plant. These findings suggest that any reduction in crop damage afforded by *M. litoralis* is likely to result from an effect other than olfactory repellence or host odour masking, possibly involving leaf surface contact chemicals and/or the behavioural barrier effects described by Finch and Collier (2000) in their host selection theory based on 'appropriate/inappropriate landings' by pest insects.

GC-EAG analysis of an extract produced by the air entrainment of adult *P. rosae* (mixed sex) revealed a number of electrophysiologically active peaks elicited by minor components in the extract which were not seen in control entrainments. However, due to the small quantity of total material extracted, there was insufficient to enable GC-MS identification of the compounds responsible. Although

only a limited number of GC-EAG runs were performed on each sex of fly, it appears that the female EAG detector produced far greater electrophysiological responses to all the active components (except two) than the males. This greater response seemed at least ten times higher than that of the males, which is much higher than the expected two-fold higher responses of females over males, found for most host plant volatiles and reported by Guerin and Visser (1980). Although male and female EAG responses to the individual active components are generally similar, some differences were observed. The much greater response of the female antenna to three of the active components suggests that it may possess more olfactory receptors for these compounds, which may act as male-produced sex pheromones. Further investigations are required to determine the validity of these results, and it would be advantageous to repeat this experiment on a larger scale to provide sufficient material for identifications to be performed. It would also be preferable to entrain male and female flies separately, to determine whether the active components are produced by only one sex (perhaps to act as sex pheromones), or by both sexes, in which case they may be acting as aggregation pheromones. Single-cell recording techniques may also be useful in identifying the relative proportions of receptors on the antenna of each sex to these putative pheromone components.

Having identified host plant volatiles by GC-EAG and GC-MS, their electrophysiological activity was confirmed and investigated further by EAG analysis with authentic compounds, using a physiologically discriminating dose. Certain compounds previously identified in the GC-EAG investigation of apiaceous plant extracts did not elicit EAG responses significantly different from the hexane control, at the level tested. These were octane, nonane, 2-hexanol, (*E*)-2-hexenal, (*Z*)-3-hexenylacetate, 4-ethyltoluene, sabinene, β-pinene, α-terpinene and myrcene; 3-hexanol and (*Z*)-3-hexen-1-ol elicited only slightly higher responses than the hexane control.

As a range of 16 closely related acetylenes and polyacetylenic compounds were investigated in this study, it was possible to compare their structural features and relate these to the size and significance of the EAG responses they elicited. The results suggest that one important structural feature in these compounds is the presence of at least one alkene (double bond) occurring in the early portion of the carbon chain, with no previous alkyne (triple bonds) occurring before it (as in the following compounds:

aethusin, trideca-2,4,8,10-tetraen-6-yne, trideca-4,8,10-trien-6-yne, aethusanol A, heptadeca-2,8,10-trien-4,6-diyne, falcarinol, falcarindiol, dehydrofalcarinol and dehydrofalcarinone); larger responses were also seen for compounds containing the first alkene (double bond) between carbon numbers 2-3 or 4-5, rather than between carbons 1-2 (i.e. the falcarinol compounds).

Investigation of the available piperidine alkaloids confirmed that γ-coniceine elicited the strongest EAG response of all the compounds tested during this study, as was originally found in the GC-EAG analysis of *C. maculatum* extract. This EAG study also suggested that strong responses would be likely for the two tentatively identified, but unavailable, compounds conhydrine and conhydrinone, in keeping with their similarity in structure to γ-coniceine. The EAG responses elicited by piperidine alkaloids seem to depend upon the length of the carbonyl side chain (attached to carbon 2 of the ring), with the highest responses being seen to compounds with a side chain length of 3 carbons (propyl, i.e. coniine); the amplitude of the responses decreased for compounds with 2-carbon ethyl (i.e. 2-ethylpiperidine) and 1-carbon methyl side chains (i.e. 2-methylpiperidine). The highest responses were seen to compounds in which the ring contained a double-bonded imine group (i.e. γ-coniceine), rather than a secondary amine group (i.e. coniine). A change in this region of the ring structure, from a secondary amine to a tertiary amine (as seen in N-methylconiine), substantially reduced the observed EAG response. These trends seem to conform to the order of toxicity for this group of compounds, with the most toxic members such as γ-coniceine and coniine eliciting the largest EAG responses. This is the first time a host-derived nitrogen-containing compound has been reported to act as an electrophysiological stimulus for *P. rosae*. However, because of the high toxicity of these compounds, behavioural evidence would be required to discount the possibility that the EAG responses observed were not simply the result of toxic effects on the antennal receptors. This would seem unlikely since *P. rosae* utilise *C. maculatum* as a host plant, and in light of the fact that many other proven biologically active compounds for *P. rosae* are also potent toxins (e.g. the polyacetylenes and furanocoumarins). Nonetheless, it seems surprising that *P. rosae* appears to have developed such a highly specialised response for this group of compounds, since *C. maculatum* is the only apiaceous species known to possess any alkaloid compounds. Eight other nitrogen-containing compounds, including *o*-aminobenzaldehyde and benzyl cyanide, have recently been identified in the flower volatiles of a number of other apiaceaeous plant species (Borg-Karlson *et al*, 1994), and the

antennal receptors responsible for the response to the piperidine alkaloids may also respond to a number of these and possibly other unknown nitrogen-containing compounds in various species of the Apiaceae.

The results of this EAG study correspond with those of the previous GC-EAG studies, showing no significant response to myrcene (even though it is present in many species of the Apiaceae), and that only the *trans*-isomer of β-ocimene was significantly active. This confirmed the observation during the GC-EAG investigation, in which both *cis*- and *trans*-isomers of β-ocimene were seen to be present as major peaks in GC profiles of *C. maculatum* extracts, yet only the *trans*-isomer showed EAG activity. The EAG responses observed during this EAG investigation showed some interesting trends in activity. Members from all the isoprenoid groups tested elicited significant antennal responses, with the most frequent responses being to the sesquiterpenes (all of which were highly active), whilst the highest overall responses were seen to the monocyclic monoterpenes, (+)-carvone and α-terpineol.

All the methoxy-phenyl compounds tested elicited highly significant responses, with the largest response observed for *trans*-asarone. The pattern of responses observed in this study agreed closely with the findings of previous studies on the EAG responses of *P. rosae* to these compounds (Guerin and Visser, 1980; Guerin *et al.*, 1983).

The most significant difference between the antennal responses to enantiomers of host plant monoterpenes was seen for α-pinene, for which the (+) form was electrophysiologically active, while its (-) form showed no significant antennal response. For limonene and fenchone the (-) forms elicited slightly higher responses than the (+) forms, while for β-pinene, no significant EAG activity was elicited by either optical isomer. The role, if any, that these differences in perception of some chiral compounds play in the host selection process of *P. rosae* is unclear, since few data are available on the quantities of enantiomers present in different species of the Apiaceae. However, the existing data suggest that large differences in the enantiomeric composition of host compounds occur between different host plant species. The ratios of enantiomers often switch between different species; for example, enantiomers of α-pinene occur in the ratio 21:1 (+)- to (-) in cow parsley (*Anthriscus sylvestris*), but in the ratio 2:1 (-)- to (+)-α-pinene in ground elder (*Aegopodium podagraria*) (Borg-Karlson *et al*, 1994). As no enantiomers appear to be particularly novel for the apiaceaeous species investigated here, they probably play more of a role in differentiation between host species than in the identification of host versus non-host species.

The significant responses to two of the organic isothiocyanates tested (allyl- and 2-phenylethyl-isothiocyanate), which are typical of brassicaceous species and do not occur in species of the Apiaceae, show that *P. rosae* is able to perceive non-host compounds, which it may utilise for avoidance of unsuitable plant species (Pickett *et al*, 1999).

With regard to EAG studies, it should be borne in mind that not all compounds found to be electrophysiologically active are behaviourally active (or not in the context that an investigator may expect). For example, with compounds that are EAG-active for tsetse flies (*Glossina morsitans*), some are attractants (1-octen-3-ol, 4methylphenol and 3-propenylphenol), some are repellents (acetophenone, 2-methoxyphenol), and some have no apparent behavioural activity (2,6,10,10-tetramethyl-1-oxaspiro[4.5]dec-2-en-8-one) (Gough *et al*, 1987; Bursell *et al*, 1988).

In order to determine the behavioural activity of host plant extracts and the electrophysiologically active compounds identified during this study, field experiments were performed, and attempts were made to develop an olfactometer bioassay for *P. rosae*. Exhaustive attempts to develop a bioassay which was simple, discriminating and replicable, to study the behavioural responses of *P. rosae* to host plant odours, were unsuccessful. None of the olfactometers tested (Y-tube and Four-arm olfactometers) showed significant behaviour discrimination between the treatments tested, which included real host plant foliage, host plant MAS extracts, EAG-active compounds, and the best known synthetic attractant combination of *trans*-asarone and hexanal. During bioassays, the flies often remained stationary while grooming for long periods, and performed frequent take-offs followed by rapid flights (of long and short duration), which were initiated independently of the insect's position with respect to the treatment. From these observations it seems likely that flight is an essential part of the 'long-range' host location process for *P. rosae*. Attraction of *P. rosae* to host odours during flight is supported by data from field trapping with host volatiles (see Ch.6 this study, and field experiments by Guerin *et al.*, 1983), and wind tunnel experiments in which Nottingham (1987) reported upwind anemotactic flights of 6 to 9-day old gravid females in response to host plant odours (*D. carota* plants). As 'long-range' host location in *P. rosae* (and possibly Diptera as a whole) probably occurs mainly during flight, there may be no alternative to using wind tunnels for future attempts to develop an olfactory bioassay for this species. In the absence of an olfactory

bioassay for *P. rosae*, supplementary oviposition experiments were performed to determine the behavioural activity of the newly identified electrophysiologically active compound γ-coniceine. The results from this investigation confirmed that γ-coniceine was behaviourally active in *P. rosae*, as γ-coniceine plus *D. carota* extract elicited significantly more eggs than either γ-coniceine or *D. carota* extract alone (neither of which elicited significantly more eggs than the control). This showed for the first time, that γ-coniceine acts as an oviposition stimulant for this species, although the fact that it had to be applied in conjunction with a host plant extract before a significant oviposition was observed suggests that it is only weakly stimulating. This finding does, however, discount the possibility that γ-coniceine acts as a repellent or deterrent for *P. rosae*.

Further problems were also encountered during field investigations into the behavioural responses of *P. rosae* to host plant volatiles, either released as extracts or individual host-derived compounds. During these investigations very, few *P. rosae* were caught in the field, resulting in little statistical significance between treatments in the majority of experiments, even for the proven field attractant combination *trans*-asarone and hexanal. The low catches of *P. rosae* were a result of high mortality in general carrot fly populations, due to extremely unfavourable hot dry weather conditions, supported by the results of pest-threshold experiments performed at HRI-Wellesbourne. These found that the high temperatures experienced during 1995 and 1996, especially during June and July, resulted in the deaths of most first-instar larvae shortly after they emerged from the eggs (Vincent. J. and Finch. S., personal communication to ADAS). Furthermore, responses observed to the known host plant attractants *trans*-asarone and hexanal were unusually low and often erratic between different field locations and experiments. This was thought to result from the poor condition of adults which survived the unfavourable conditions, which may have seriously impaired their ability to detect olfactory cues since antennal responses are known to diminish as a result of dehydration during EAG experiments.

During these field experiments, a newly designed release system (glass bottle with wick) was found to improve significantly the performance of host plant extracts as attractants over their release from the previously-used polyethylene-vial lures. This resulted from a steadier and more sustained release of volatiles over experimental periods of one week.

A comparison of the overall numbers of *P. rosae* caught in different positions in the field suggested that trapping in the favourable roosting sites of the hedgerow resulted in substantial increases in catch. The control trap placed in the hedgerow caught approximately five times the number of flies caught on the controls placed around the edge of the carrot plot, with similar increases in catch also seen on the traps baited with plant extracts plus *trans*-asarone and hexanal. The large increase in catch at the favourable roosting site of the hedgerow is probably a result of a greater concentration of flies here, as well as reduced competition for the trap's visual and olfactory attractants, since the flies here were slightly removed from the large background stimulation of the crop's odour and its visually attractive foliage. The overall results of the field experiments found that microwave-assisted hexane extracts of host plant foliage were attractive to *P. rosae* in the field. As in most cases where these extracts were added as lures (i.e. *D. carota*, *F. vulgare*, *C. maculatum* or *A. graveolens*, without *trans*-asarone and hexanal), some increase in catch was seen over the unbaited REBELL ® control traps. This was particularly clear for *A. graveolens* extracts, which produced a statistically significant doubling of the catch seen on the control traps. These findings suggest that the MAS extraction method is superior to steam or water distillation for producing biologically relevant samples, since previous experiments using similar monitoring traps and a range of host-derived essential oils showed no increases in *P. rosae* catches in the field (Guerin *et al.*, 1983). The combination of *trans*-asarone and hexanal with *A. graveolens* extract in the new lure type produced a higher catch than that did *trans*-asarone and hexanal alone, although this difference was not statistically significant. However, analysis of the results suggested that this increase was an additive rather than a synergistic effect, unlike the synergistic increase in catch suggested when *trans*-asarone and hexanal are combined (Guerin *et al.*, 1983). The general trend in attraction of *P. rosae* to the host plant extracts tested was as follows, in order of increasing trap catch: *D. carota*, *F. vulgare*, *C. maculatum* and *A. graveolens* (although only the catch with *A. graveolens* extract was significantly different from the control). The order of activity of these extracts, observed for trap catches in the field, differs significantly from the order of these compounds in the oviposition preference hierarchy of *P. rosae*, as determined by Degen, Städler and Ellis (1999a). *D. carota* extract was consistently the least attractive MAS extract (even when combined with *trans*-asarone and hexanal). This might be expected, since the area close to these traps would be saturated with *D. carota* leaf volatiles from the carrot plot. Therefore, any attractant

volatiles released from the *D. carota* lure might be swamped by the similar volatiles emanating from the crop.

Guerin *et al.* (1983) found that *trans*-asarone and hexanal baited traps caught significantly fewer flies when placed in fallow ground than in a stand of host plants. This was attributed either to the interaction between the trap-released and crop-released volatiles, since mixtures of functionally different types of volatiles prove most attractive to *P. rosae*, or as a result of the crop-released volatiles having an arresting effect on the flies, thus increasing the number of possible respondents to traps within the crop. The larger catches seen for all the 'non-carrot' host-plant extracts released in this study probably also resulted from the interaction between the trap-released and crop-released (*D. carota*) volatiles, with the 'non-carrot' extracts 'standing out' against the background of carrot volatiles, and providing greater attraction due to the mixture of functionally different types of volatiles in the vicinity of these lures.

Of the individual host-derived compounds tested, the known attractant combination of *trans*-asarone and hexanal generally showed the expected statistically significant doubling of catch over the control trap. The release of individual *C. maculatum* compounds was seen to increase trap catches to a level intermediate between the lowest catches (on the unbaited control and those baited with *C. maculatum* extract alone) and the highest catches (on the *trans*-asarone and hexanal treatment). Of the compounds tested, γ-coniceine was slightly more attractive than the combination of β-caryophyllene plus β-ocimene, and combining all three compounds did generally produce a slight increase in trap catch, although none of these differences were statistically significant.

The failure of attempts to develop a working bioassay for the study of olfactory responses to host plant volatiles, combined with low fly catches in the field and their poor responses to host plant volatiles due to unusually high summer temperatures, meant that many of the behavioural experiments performed had to be repeated in subsequent seasons or years in an attempt to obtain statistically significant results. Because of this, the number of different treatments tested and the scope of the behavioural investigations performed had to be limited to those directly aimed at increasing trap catches, rather than including investigations into the specific behavioural mechanisms involved in these increases. Consequently, the results from these experiments do not give any clear indication of the mechanisms involved in host plant location in *P. rosae*. As all the treatments involved the release of volatiles from visually attractive

REBELL ® orange traps, it was impossible to separate the effects of the visual attractants from those of the chemical attractants in these experiments.

The experiments aimed at increasing trapping efficiencies of *P. rosae* in prototype 'space station' traps do shed some light on the relative importance of the visual and chemical factors involved in this process. Because only very small numbers of *P. rosae* were caught during these experiments, statistical analysis of the results was not possible and none of the findings discussed are statistically significant. In experiments 8a and 8b, a range of 'space station' trap designs were compared. These included traps which did not incorporate either visual or chemical attractants in their design, traps with only a visually attractive component, traps with only chemical attractants, and traps which combined both. As expected, the traps which contained neither visually attractive materials (constructed from clear Perspex), nor chemical attractants, caught no *P. rosae*. Traps which had only chemical attractants (*trans*-asarone and hexanal) located in their central arena caught a small proportion of the flies caught overall in these experiments (zero and 8% of the totals, in Exp. 8a and 8b, respectively). Although only consisting of one fly in one of the two experiments, this catch does suggest (however weakly) that one or both of the volatiles acted as a true attractant operating in the absence of any colour stimulus, as was shown to be the case for *trans*-methylisoeugenol in experiments by Guerin *et al.* (1983) using colourless sticky traps. Traps with only a visually attractive component (either part or wholly constructed from orange Perspex) caught a slightly higher proportion of the flies caught overall in these experiments, generally about 8% (one fly), but ranging from 8% to 17%. Traps which combined both visually attractive materials (part or wholly constructed from orange Perspex) and the use of chemical baits (*trans*-asarone and hexanal) caught the highest number of flies, which ranged from 13.5% to 70% of the total for these experiments. These results generally confirmed the results found in earlier experiments on prototype pathogen traps in this study. In experiments 8a and b, the increase in catch when visual and chemical attractants were combined was particularly high for the entirely orange Perspex traps (rather than the part-orange traps, with only orange baffles). The catches in these traps were 50% and 70% of the flies caught overall in experiments 8a and 8b, respectively. The very high catches in these traps suggested that combining the two types of attractant (visual and chemical) had a synergistic effect on the number of *P. rosae* entering the traps, as catches were

far higher than would be suggested by addition. The findings of these experiments clearly show that both visual and chemical cues play a significant role in the effectiveness of these traps for directing *P. rosae* into the enclosed region of the central arena, where in an operational pathogen dispensing trap, the flies would be showered with infective fungal conidia. The effects of the two factors seem similar, although the visual cues only seem of slight importance compared to the incorporation of a chemical attractant.

One reason for the possibly greater importance of the visual cues could be that the traps were positioned on the edges of large plots of host crops (generally carrots), and flies in the vicinity of the traps would also be stimulated by the high levels host volatiles released by from crop. If, as suggested by Finch and Collier (2000), these acted as arrestants and induced flies to land on nearby visually attractive surfaces (i.e. normally the green foliage), they would preferentially land on the traps constructed from highly visually attractive material (i.e. the orange Perspex - designed to act as super-optimal visual stimulus), rather than on traps lacking these visual cues, or on the less visually attractive surrounding foliage. The presence of the host volatiles emanating from the crop probably also initiated a change from linear locomotion (ranging) to a more convoluted 'local search' pattern (as described by Visser, 1988), this would have increased the likelihood that flies attracted to the trap would manage to negotiate the baffle system and enter the central arena of trap.

On the other hand, traps with only chemical baits and no visually attractive cues should only catch *P. rosae* if the volatiles released from within the trap actually acted as attractants, and then only if the flies were able to home-in on the odour source relatively precisely, allowing them to negotiate the system of baffles before being caught in the central arena of the trap. If the host volatiles released from the trap only acted as arrestants and induced landing on visually attractive objects, no flies would have entered these traps as they would preferentially have landed on the visually attractive surrounding foliage. Consequently this finding does suggest, however weakly, that the trap-released host volatiles did act as true attractants.

Considerable doubts have been expressed by Finch and Collier (2000) about whether host plant volatile chemicals are truly attractants or simply arrestants for receptive insects, and similar doubts were expressed by Degen (1998a), who questioned whether *P. rosae* was able to locate its hosts precisely using anemotactic flight from a distance. In both cases, it was pointed out that the quantities of host plant

volatiles required to increase trap catches were generally several orders of magnitude higher than the levels emitted by undamaged host plants, as was determined for *trans*-asarone and *trans*-methylisoeugenol released as attractants for *P. rosae* in the field (Städler, 1992). Similarly, during my field investigations, significant increases in trap catch were only obtained by releasing relatively high levels of host plant extracts or synthetic host compounds from lures.

Finch and Collier (2000) suggested that volatile chemicals emanating from plants indicate to receptive flying insects that they are passing over suitable host plants, and that the primary action of these volatiles is to stimulate the insects to land. They also suggested that, under suitable weather conditions, these chemicals may also provide some directional information, but this is of secondary importance. In general, they suggested that the amounts of volatile chemicals released from plants which impinge upon the insect's receptors are sufficient to arrest, but rarely provide accurate directional information to flying insects. Once stimulated to land, the insects would preferentially land on visually attractive green objects, and avoid landing on brown surfaces such as bare soil (Kostal and Finch, 1994). The Kostal-and-Finch theory of host plant selection then revolves around the number of 'appropriate/inappropriate' landings made by the insects, on host plants and non-host plants respectively, and the level of oviposition stimulation the insects receives on each landing.

My field investigations into prototype pathogen traps showed that relatively large catches of flies only occurred when host plant volatiles were released from traps containing a visually attractive cue, and the results suggested that the combination of the two factors operated in a synergistic, rather than additive, manner. The release of host volatiles without the presence of a visual cue, and vice versa, resulted in a considerable reduction in trap catches. These findings support the theory that the host plant volatiles acted primarily as arrestants/landing stimuli; however, the small catch in traps baited with host volatiles, but lacking visually attractive cues, also suggested a level of attraction to the volatiles. These findings seem in keeping with the behavioural roles suggested by Finch and Collier. The possibility that the primary role of host plant volatiles is to stimulate the insects to land on visually attractive objects, and only secondarily to provide directional cues, could explain the failure to develop an olfactory bioassay for *P. rosae* during this study, because attractive visual cues were not included in the arms of the olfactometers tested. This was a major oversight on my part, and in subsequent unrelated olfactometer studies on aphids (*Aphis fabae* and

Myzus persicae), the inclusion of a small yellow bead at the end of each arm of a four-arm olfactometer was found to bring about significant responses to host volatiles, which prior to this modification had failed to occur (Selby, unpublished data).

Two groups of compounds have been shown to act as olfactory attractants/arrestants increasing trap catches of *P. rosae* in the field, with the propenyl benzenes (trans-asarone and trans-methylisoeugenol) and the 'green leaf' aldehydes (hexanal, heptanal and trans-3-hexenal) increasing catches in the field. These two classes of compounds almost certainly interact with two different classes of receptor on the antennae of *P. rosae*, as both the EAG responses and trap catch (synergistically) increase when combinations of there two groups of compounds are presented as stimuli; only a small additive increase in EAG was observed when the antennae were presented with combinations of compounds from within the same compound class (Guerin *et al.*, 1983). During this study, the relatively optimal combination of *trans*-asarone and hexanal was again generally found to provide the largest increase in trap catches in the field. For the first time, a host plant extract (*A. graveolens* foliage) was also shown to significantly increase catches of *P. rosae* on visually attractive traps. As the combination of this extract with *trans*-asarone and hexanal only produced a small additive type increase in trap catch compared to the two treatments alone, rather than a synergistic increase, it seems very likely that the compounds responsible for the attractant/arrestant effects in the extract are acting on the same groups of antennal receptors that respond to the propenyl benzenes and 'green leaf' aldehydes, and are probably similar compounds. The very large EAG response observed to γ-coniceine during this study suggests that this highly volatile alkaloid may also act as a host plant attractant/arrestant in the field, as consistent relatively small increases in trap catch were observed to this compound, which produced catches that were intermediate between those on *trans*-asarone and hexanal baited traps and those of the unbaited control. However, this was not a statistically significant increase, possibly because of the poor condition of the flies during this study; they were presumably suffering from dehydration due to high temperatures at the time, which possibly reduced their ability to detect olfactory cues in the field. As γ-coniceine shares an aromatic ring structure with the propenyl benzenes, it is likely that it may also interact with the same class of antennal receptors as this class of compounds. However, the inclusion of nitrogen in the ring structure of the piperidine alkaloids is a significant difference between these two classes of compound, and as such

the response elicited by this compound may come about by interactions with a separate (as yet unknown) class receptors on the antennae of *P. rosae*.

Although Degen's studies (1998a) did not directly investigate host plant finding in *P. rosae*, he suggested that characteristic volatiles possibly enhanced the likelihood of encounters with hosts by attracting the flies towards vegetation that comprised patches of host plants, while the landings on hosts interspersed within non-hosts probably occurred more or less at random. He corroborated this theory with evidence from the results of his observations of caged flies, in which flies were not found to alight more frequently on leaf mimics treated with host plant extracts than on those without (Degen and Städler, 1997b). His studies into the non-chemical traits involved in host acceptance (Degen and Städler, 1996; 1997b) showed that *P. rosae* were able to discriminate between different leaf shapes before landing, and showed a preference for landing on compound leaf shapes. This leaf shape is typical but not unique to apiaceous host plants, and may allow *P. rosae* to distinguish roughly between broad plant categories, increasing the probability of 'correct' host plant recognition. Degen suggested that this may enhance their efficiency in host plant finding by allowing them to avoid alighting on plants with non-pinnate leaves, or those with narrow blades (e.g. grasses). However, Degen found no evidence that *P. rosae* was able to distinguish between the more subtle differences among apiaceous host plants.

These suggestions as to how host location may occur in *P. rosae*, and phytophagous insects in general, seem to fit well with the results obtained during this study. The relatively high EAG responses recorded from *P. rosae* to non-host isothiocyanates during this study also lend some support to the suggestion that these volatiles may be perceived at some distance from non-host brassicaceous plants and, as such, may be utilised for the avoidance of these unsuitable species (Pickett *et al.* 1999).

The mechanisms involved in host plant acceptance by *P.rosae* have been extensively studied by Degen (Degen, 1998a; Degen, Buser and Städler, 1999; Degen, Städler and Ellis, 1999a, b, and c), who suggested that a mixture of the identified oviposition stimulants act as a 'chemical search image' (Atema *et al.*, 1980) and enable *P. rosae* to distinguish between host and non-host species, while as yet unidentified polar stimulants and deterrents mediate the oviposition preferences between particular species within the Apiaceae. Städler (1986) suggested that the combination of propenylbenzens, furanocoumarins and the C17-polyacetylenes is characteristic of the Apiaceae. However, Degen's study found that, in all

the host species analysed, at least one of these groups of stimulants was present in very low amounts, and probably imperceptible to the fly. This led him to suggest that unless, the unidentified stimulatory compounds were also typical of the Apiaceae, host recognition must rely primarily on the C17-polyacetylenes for positive host-specific stimuli (Degen, Buser and Städler, 1999).

The overall results of the study into the development of an autodissemination trap for release of *E. schizophorae* into *P. rosae* populations in the field showed that the most suitable trap design was one based upon a trap designed by Pell *et al.* (1993) for the autodissemination of *Zoophthora radicans* into field populations of *Plutella xylostella*. During this study, the design of the trap was refined for use with *P. rosae* by optimisation of the visually attractive cues used in its construction and by the incorporation of semiochemical attractants (as described above). This process led to an optimum design for an autodissemination pathogen trap suitable for *P. rosae*, constructed completely from visually attractive orange Perspex, with a vertical height of 10cm, and baited with chemical attractant lures (*trans*-asarone and hexanal) in its central inoculation chamber. The best location for this pathogen trap was shown to be at a height of 1.5m against the hedgerow surrounding the crop, because the highest densities of adult *P. rosae* were caught in this position. The efficiency of this optimum trap design, as determined by its catches of *P. rosae* relative to the catches on REBELL® companion traps nearby, varied widely between experiments in different years and generations of flies. The highest efficiency for this trap was seen during experiments on the second generation of flies in 1996, when it caught 58% more flies than the REBELL® traps (0.87 flies/trap/week vs. 0.55 flies/trap/week). Much lower efficiencies were seen during experiments on the first generation of flies in 1997, when it caught 68% (0.33 flies/trap/week vs. 1.03 flies/trap/week) and 88% (0.40 flies/trap/week vs. 3.42 flies/trap/week) fewer flies than were caught on the REBELL® traps, when positioned in the hedgerow and 5m into the crop respectively. Much of this variability in the efficiency of this optimised trap design may be attributed to the very low numbers of *P. rosae* in the field during these experiments, combined with poor responses to the chemical attractants incorporated into the trap, as mentioned previously.

All pathogen trap designs tested caught far fewer non-target insects than REBELL® traps (which often caught more than 500 non-target insects per week); this reduction was generally over 90% less for

the optimum pathogen trap design. In this experiment, only insects over 2.5mm in length were counted, and this criterion excluded carrot psyllids (*T. apicalis*) and pollen beetles (*Meligethes* species), which were often numerous on the REBELL® traps, but were almost absent in the pathogen traps. The prototype pathogen trap experiments showed a statistically significant effect of the quantity of orange Perspex used in the construction of the traps on the number of non-target insects caught in the traps. The number of non-target insects caught dropped significantly as more orange Perspex was included in the trap design, with totally orange traps catching 82% fewer non-target insects than totally clear traps in one experiment. The presence of *trans*-asarone and hexanal lures in pathogen traps also had a statistically significant effect of reducing the catch of non-target insects, by up to 37% in one experiment. Analysis of the results showed that these two factors (i.e. trap colour and chemical lures) acted independently of each other. The low number of non-target insects entering the most efficient pathogen trap design for catching *P. rosae* (totally orange Perspex with *trans*-asarone and hexanal lures) would be advantageous in an operational pathogen trap, as it would reduce the exposure of non-target insects to high levels of fungal inoculum found in the inoculation chamber. This would reduce losses of fungal inoculum as less would be carried out on non-target insects, while it would also minimise the possibility of cross-infection to less susceptible non-target species in the field.

In terms of the initial aims of this investigation, we were unable to identify attractive host plant volatiles (extracts or compounds) capable of increasing trapping efficiencies in the field sufficiently to warrant their incorporation into the *P. rosae* monitoring programme. However, only a small proportion of the electrophysiologically active compounds identified in host plant extracts during this study were actually tested for behavioural activity. This was due to difficulties in development of a 'working' bioassay method, as well as the low population and poor responses to attractants of *P. rosae* during field experiments. One solution to the poor responses of *P. rosae* to its host plant volatiles could be the use of either sex alone, or aggregation pheromones as attractants, since tentative evidence for their existence was found in GC-EAG investigations during this study. If the electrophysiologically active compounds found in air entrainments of adult *P. rosae* are identified and confirmed as pheromones, they could have significant implications for IPM strategies for *P. rosae* in the future, providing a simple way of increasing both monitoring and pathogen trap efficiencies, and their specificity for this pest species.

With regards to the development of an autodissemination trap for the release of the pathogenic fungus *E. schizophorae* into *P. rosae* populations, some progress was made in spite of the low numbers of *P. rosae* during field experiments. The optimum pathogen trap design determined during this study showed a relatively high trapping efficiency during some experiments (although this varied), considering that the flies behaviour had to be manipulated in order to direct them through a baffle system and into an enclosed inoculation chamber suitable for their infection with a pathogen. Once inside the inoculation chamber, relatively long residence times of over 15min were observed for most *P. rosae* in the laboratory. This would seem ample for infection with a pathogen to occur, since adult male moths (*P. xylostella*) attracted to traps baited with synthetic pheromone only spent a mean time of 88 seconds within the inoculation chambers of similar traps, and this was found to be comparable with the period the moths had to spend within a shower of *Z. radicans* conidia in order to become infected (Furlong and Pell, 1995).

During experiments aimed at identifying the optimum position to locate pathogen traps in the hedgerow surrounding a host crop, an unexpected and surprisingly high catch of *P. rosae* was found on a trap placed near the top of a tall willow tree in an otherwise relatively uniform hedgerow, during the start of emergence of the second generation of flies. This massive localised catch near the top of a tree supports the suggestion of Städler (1972) that *P. rosae* utilise tall silhouettes during dispersive flights; alternatively, these sites may be utilised as visual cues during aggregations flies during mating. The massive catch of *P. rosae* (142 flies in one week) in an area of low fly density might be of further interest with regard to future *P. rosae* control. Positioning operational pathogen traps in such a locality (if practical) would optimise the number of flies entering the traps, as well as exposing flies to the pathogen immediately after their emergence; both these factors would contribute greatly to the establishment of an early season epizootic in the *P. rosae* population, which in turn would optimise the chances of successfully controlling this pest by the early season inoculative release of *E. schizophorae*.

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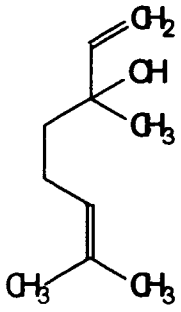
APPENDIX 1 - COMPOUND STRUCTURES

ISOPRENOIDS

MONOTERPENES – ACYCLIC



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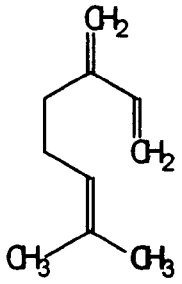

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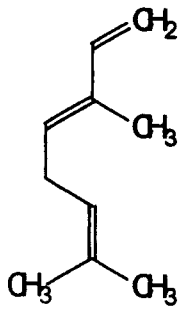

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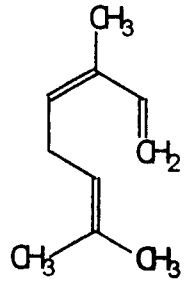

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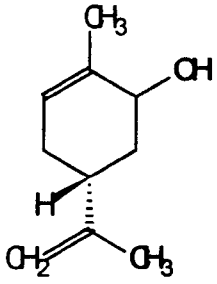
MONOTERPENES – CYCLIC



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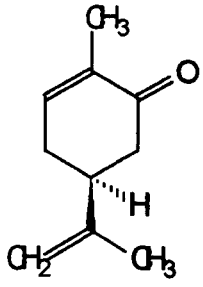

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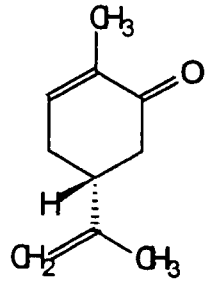

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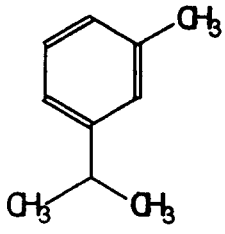

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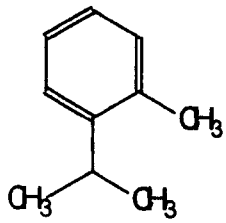

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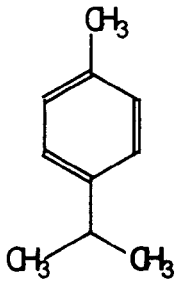

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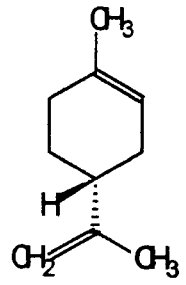

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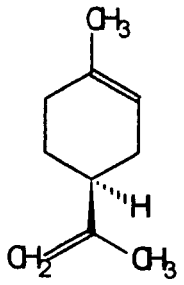

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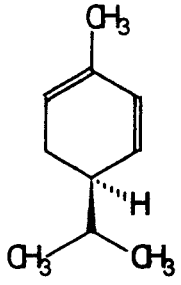

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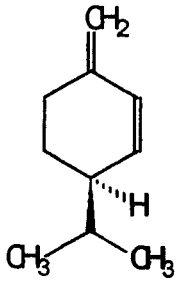

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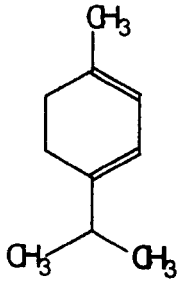

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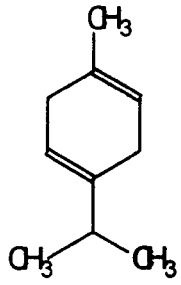

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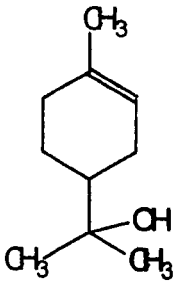

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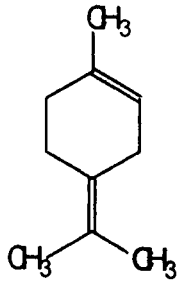

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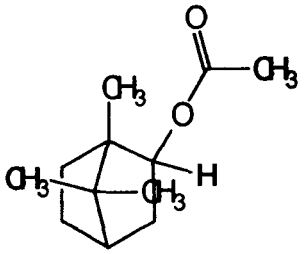

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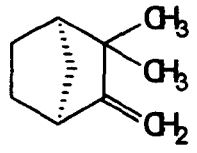

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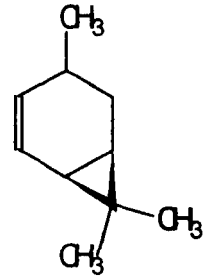

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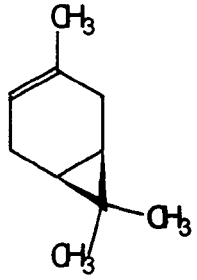

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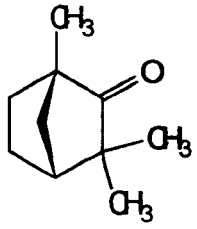

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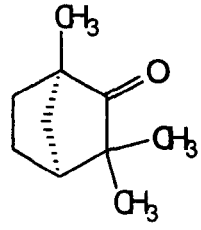

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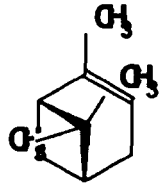

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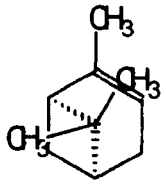

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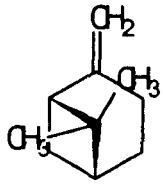

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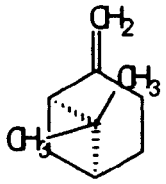

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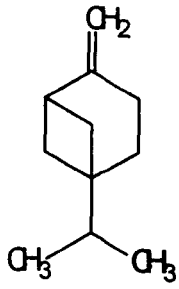
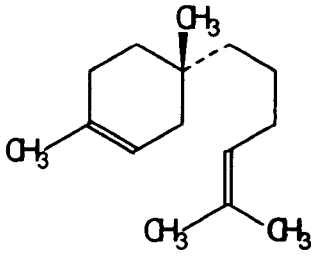


Sabinene

SESQUITERPENES



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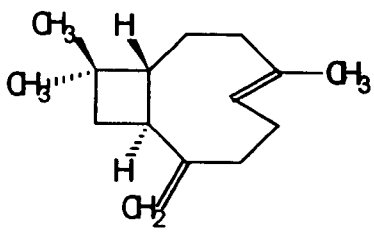

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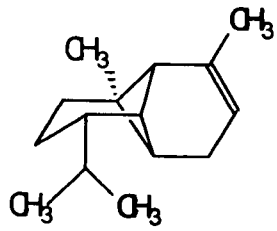

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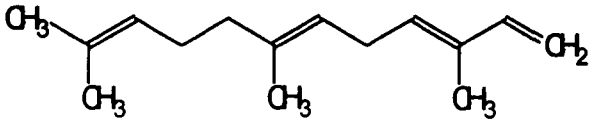
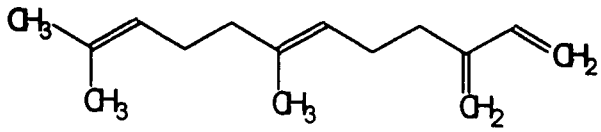


(*E,E*)-α-Farnesene



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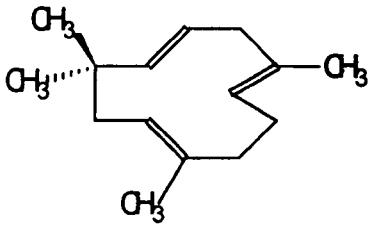
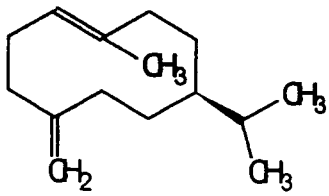
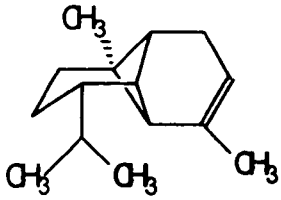


α-Humulene

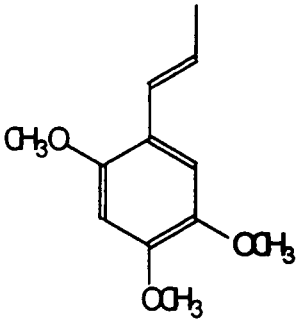


Germacrene D

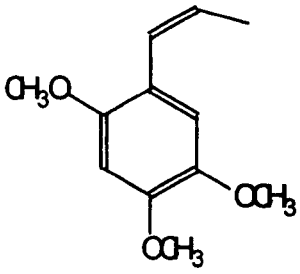


Ylangene

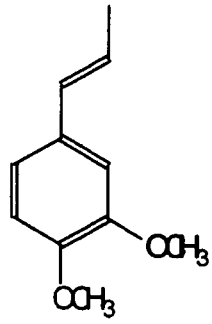
METHOXYPHENYL COMPOUNDS



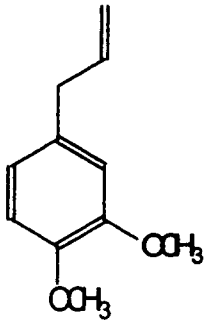
trans-Asarone



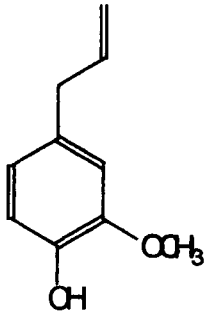
cis-Asarone



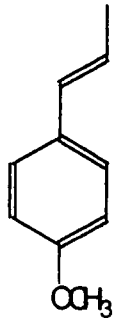
trans-Methylisoeugenol



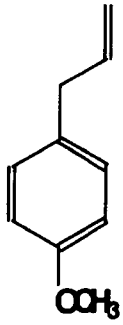
Methyleugenol



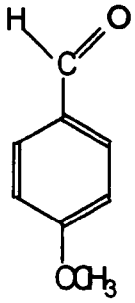
Eugenol



trans-Anethole

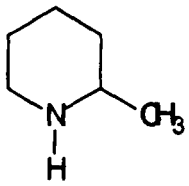


4-Allylanisole
(Estragole)

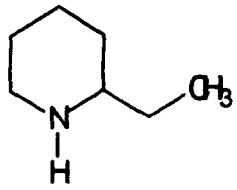


p-Anisaldehyde

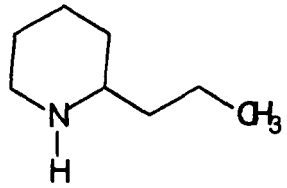
PIPERIDINE ALKALOIDS



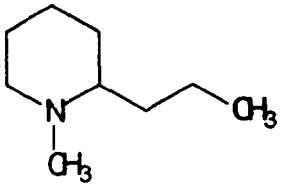
2-methyl piperidine



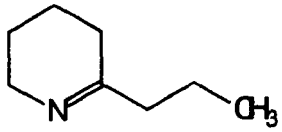
2-Ethyl piperidine



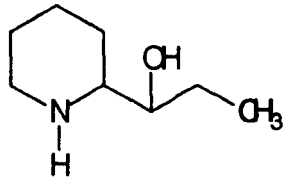
Coniine



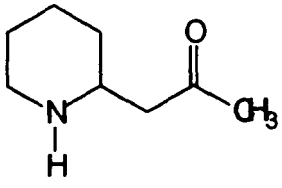
N- methyl coniine



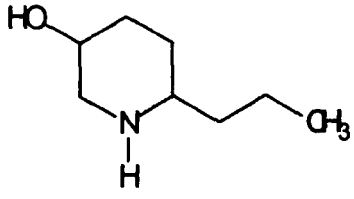
γ - coniceine



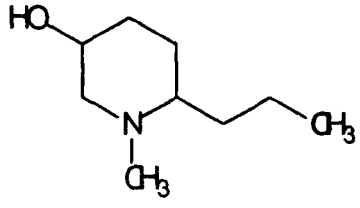
Conhydrine



Conhydrinone

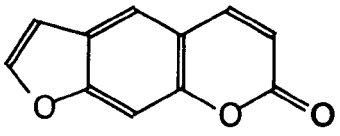


Pseudoconhydrine

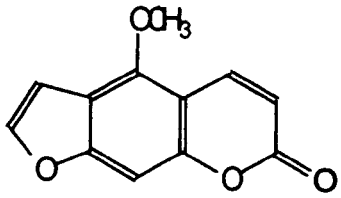


N-methylpseudoconhydrine

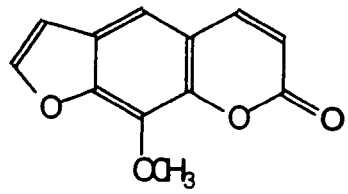
LINEAR FURANOCOUMARINS



Psoralen

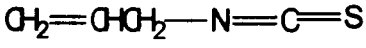


Bergapten
(5-Methoxypsoralen)

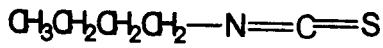


Xanthotoxin
(8-Methoxypsoralen)

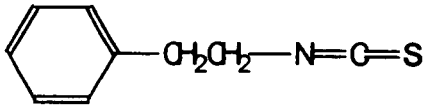
ISOTHIOCYANATES – (Non-Host)



Allyl isothiocyanate



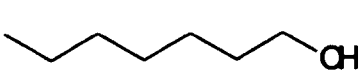
Butyl isothiocyanate



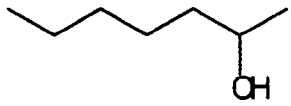
2-Phenylethyl isothiocyanate

LIPOXYGENASE PRODUCTS

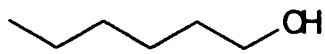
ALCOHOLS



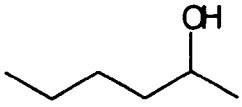
1-Heptanol



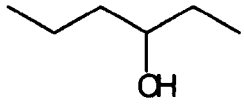
2-Heptanol



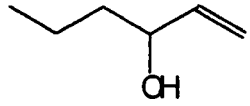
1-Hexanol



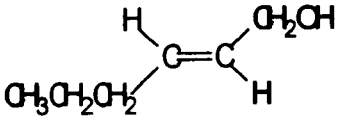
2-Hexanol



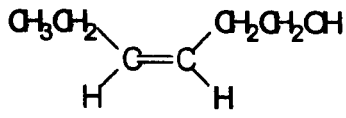
3-Hexanol



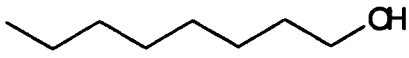
1-Hexen-3-ol



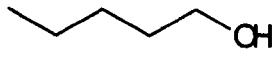
(*E*)-2-Hexen-1-ol



(*Z*)-3-Hexen-1-ol

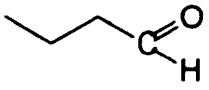


1-Octanol

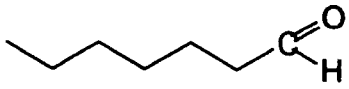


1-Pentanol

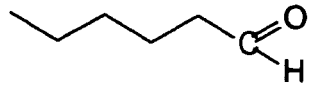
ALDEHYDES



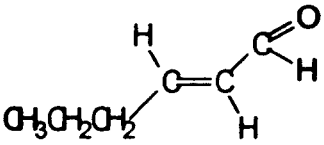
1-Butanal



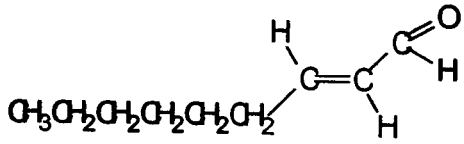
1-Heptanal



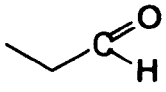
1-Hexanal



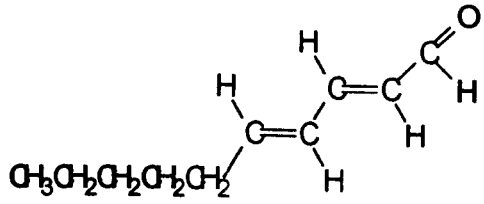
(*E*)-2-Hexenal



(*E*)-2-Nonenal

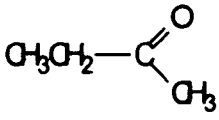


1-Propanal

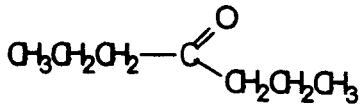


trans,trans-2,4-Decadienal

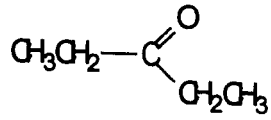
KETONES



1-Butanone

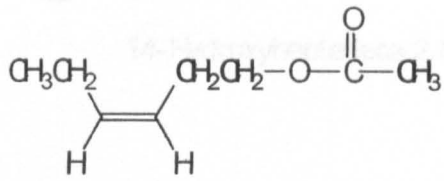


4-Heptanone

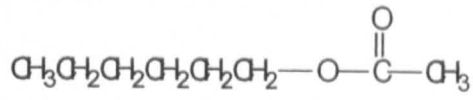


3-Pentanone

ESTERS

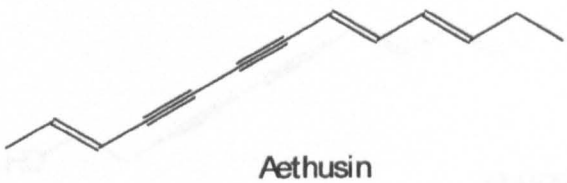


(*Z*)-3-Hexenyl acetate



Hexyl acetate

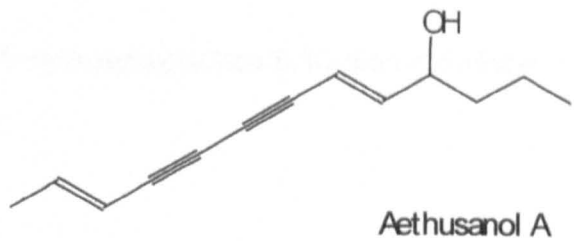
ACETYLENES AND POLYACETYLENES



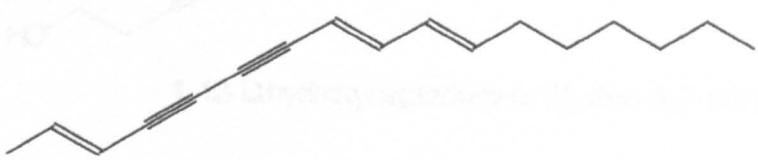
Aethusin

Trideca-2,4,8,10-tetraen-6-yne

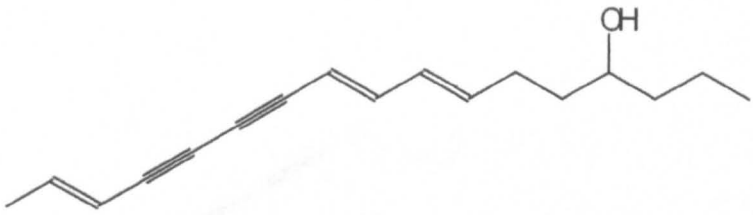
Trideca-4,8,10-trien-6-yne



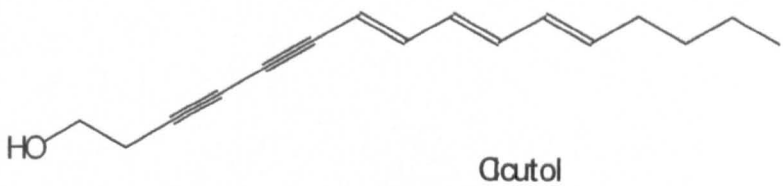
Aethusanol A



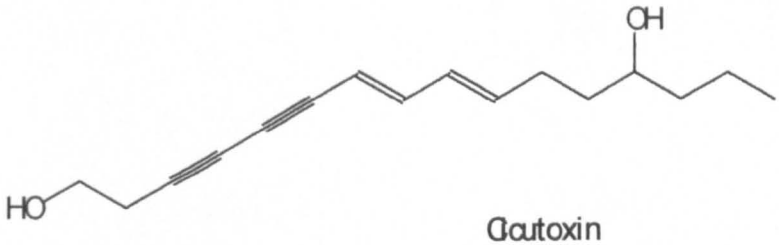
Heptadeca-2,8,10-trien-4,6-diyne



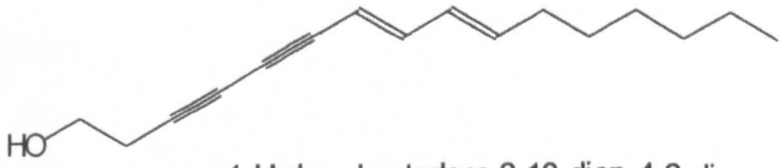
14-Hydroxyheptadeca-2,8,10-trien-4,6-diyne



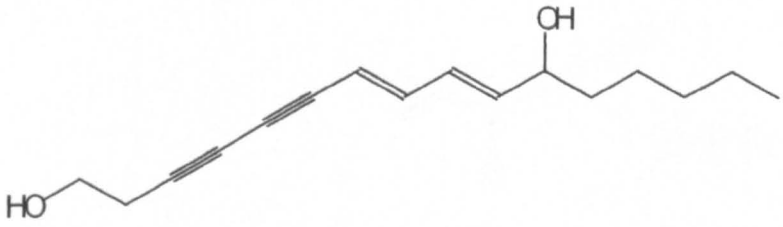
Cicutol



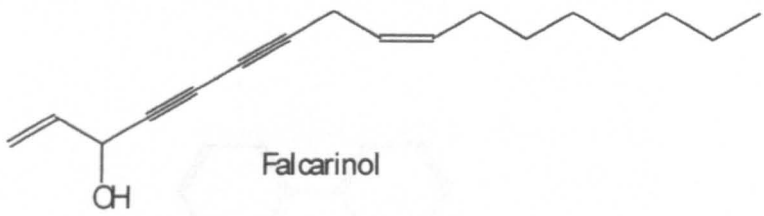
Cicutoxin



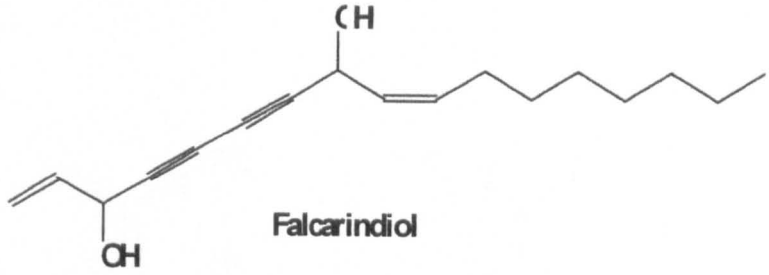
1-Hydroxyheptadeca-8,10-dien-4,6-diyne



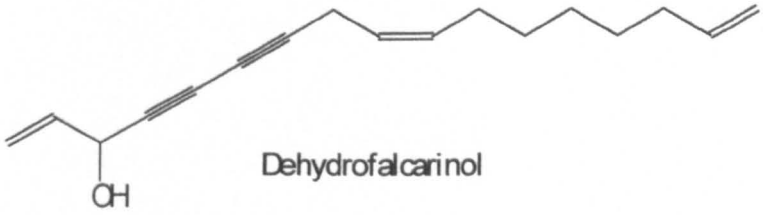
1,12-Dihydroxyheptadeca-8,10-dien-4,6-diyne



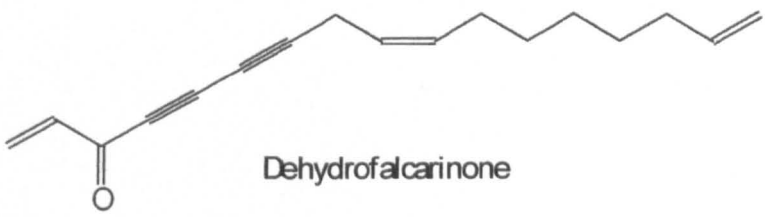
Falcarinol



Falcarindiol

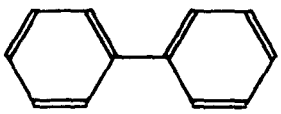


Dehydrofalcarinol

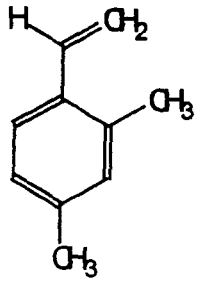


Dehydrofalcarinone

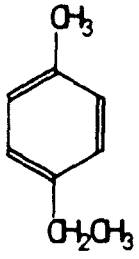
OTHERS



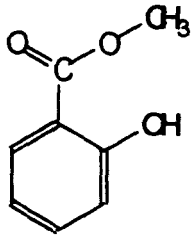
Biphenyl



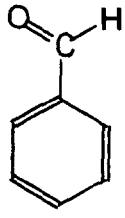
2,4-Dimethylstyrene



4-Ethyltoluene



Methyl salicylate



Benzaldehyde

APPENDIX 2 - GRAPHS SHOWING RESULTS OF RELEASE RATE STUDIES OF VARIOUS LURE DESIGNS

Figure A1: **Mean cumulative weight loss from a *trans*-asarone 'Energy Board' lure (n=3)**

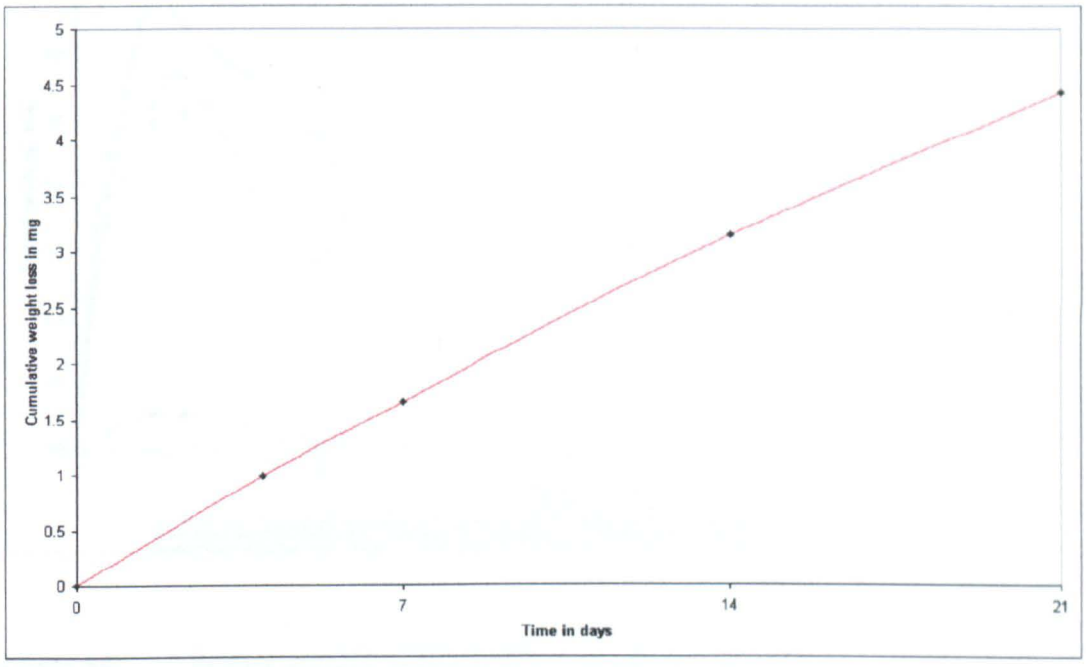
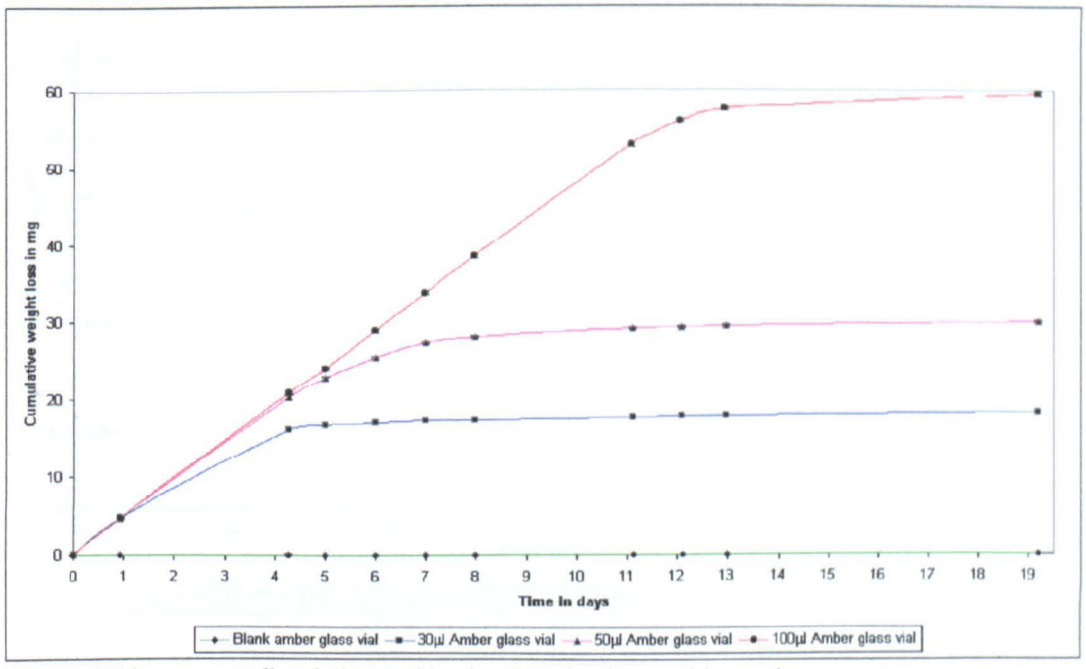


Figure A2: **Mean cumulative weight loss from hexanal 'amber glass vial' lures (n=3, ±SE).**



NB. SE bars are small and obscured by the plotted points on this graph

Figure A3: **Release rates of volatiles from a *C. maculatum* 'polyethylene bag' lure, by air entrainment (line fitted by eye, n=1).**

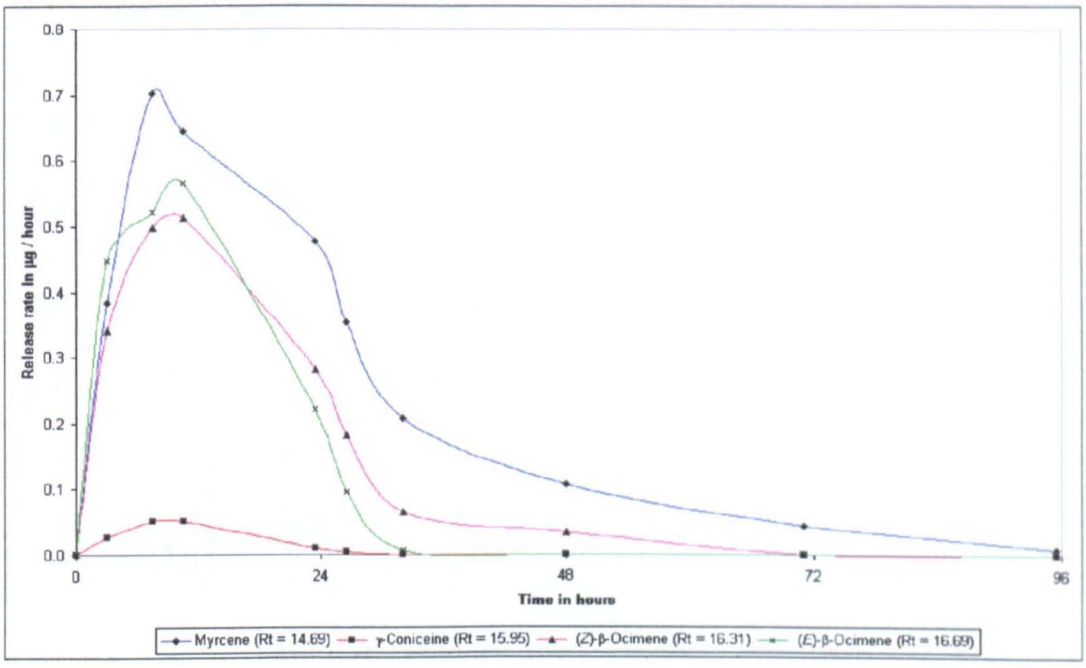


Figure A4: **Release rates of volatiles from a *C. maculatum* 'glass bottle with wick' lure, by air entrainment (line fitted by eye, n=1).**

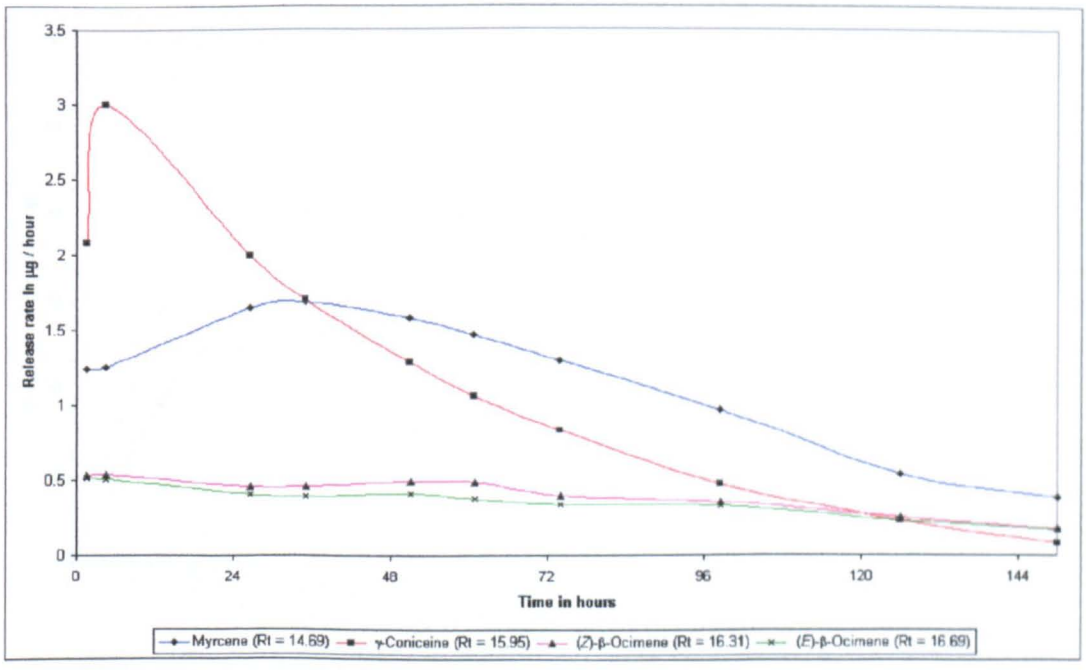


Figure A5: **Mean cumulative weight loss from *trans*-methylisoeugenol 'polyethylene bag' lures (n=3, ±SE).**

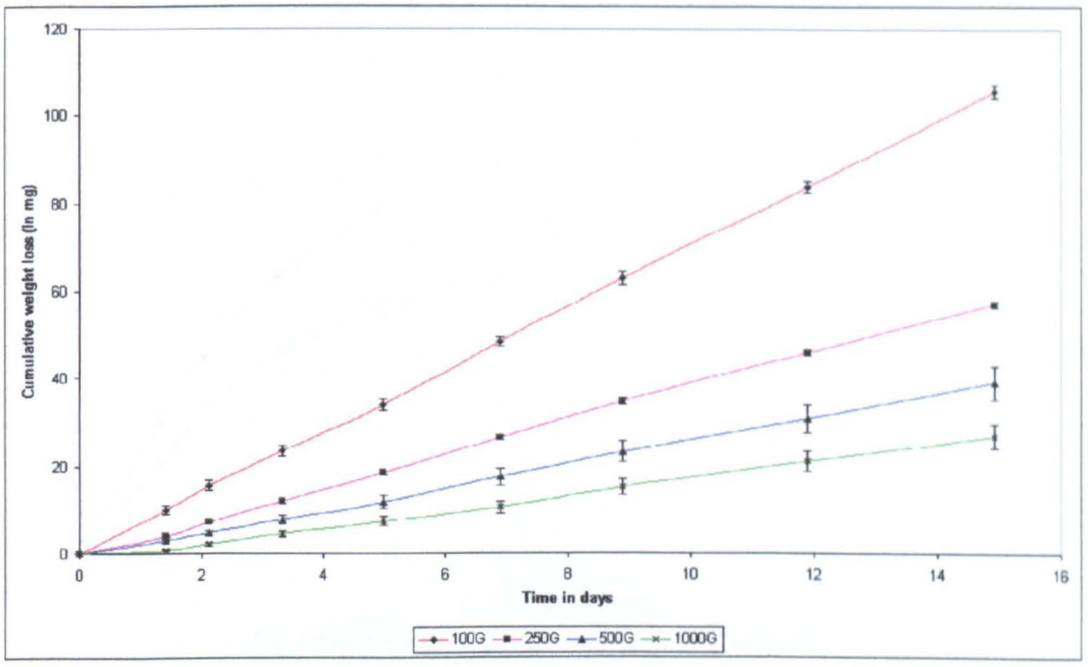


Figure A6: **Mean cumulative weight loss from γ-coniceine 'polyethylene bag' lures [a] (n=1).**

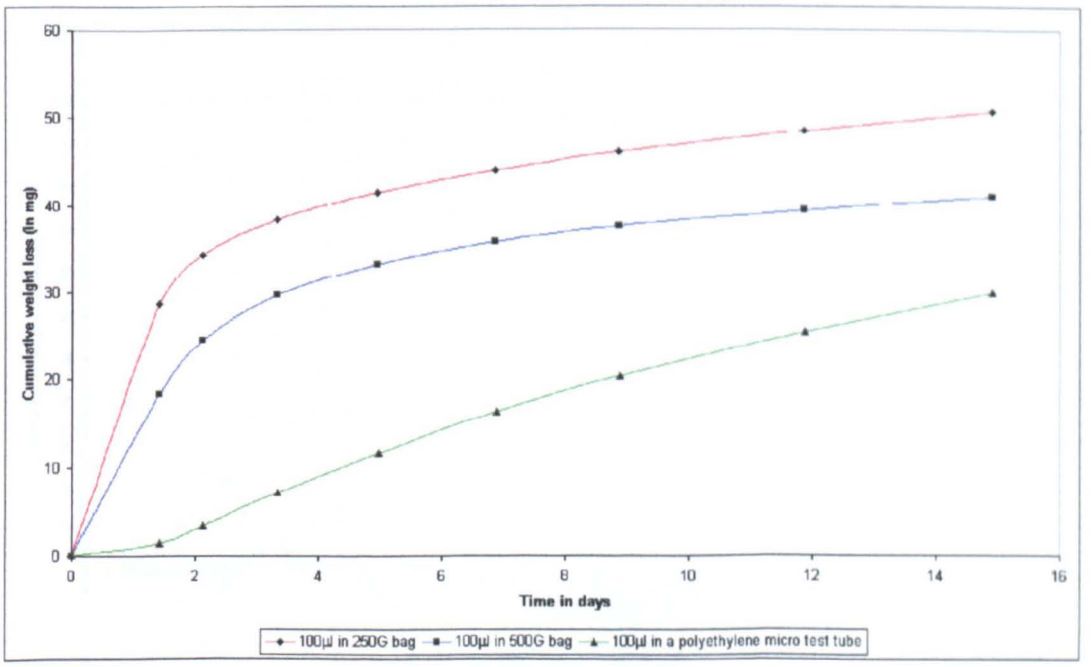


Figure A7: **Mean cumulative weight loss of γ-coniceine from 'polyethylene bag' lures [b] (n=1).**

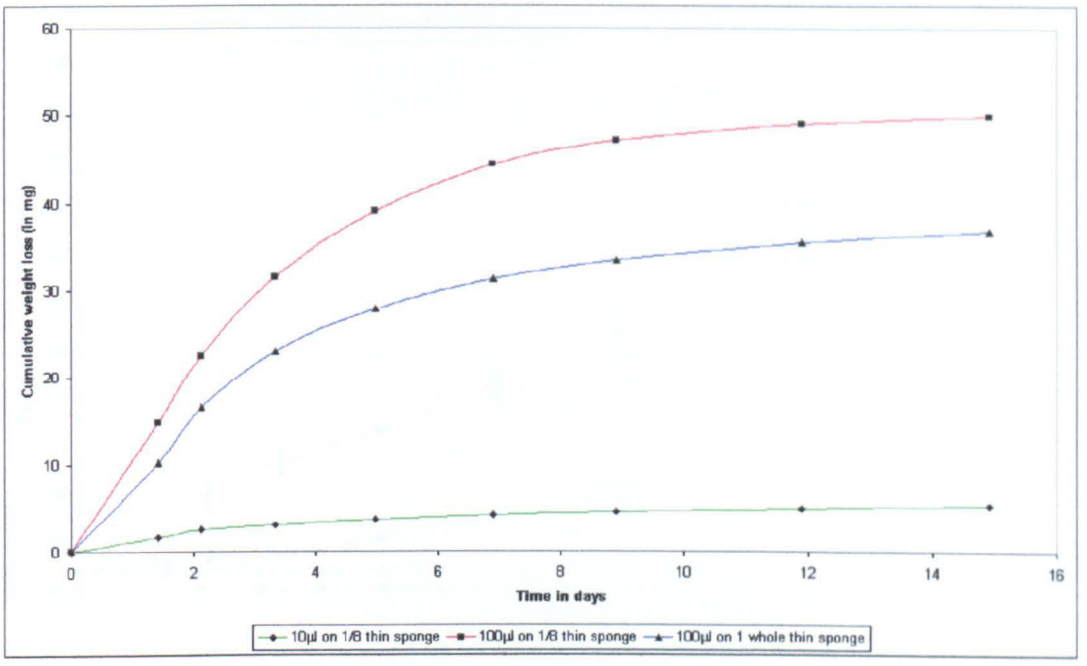
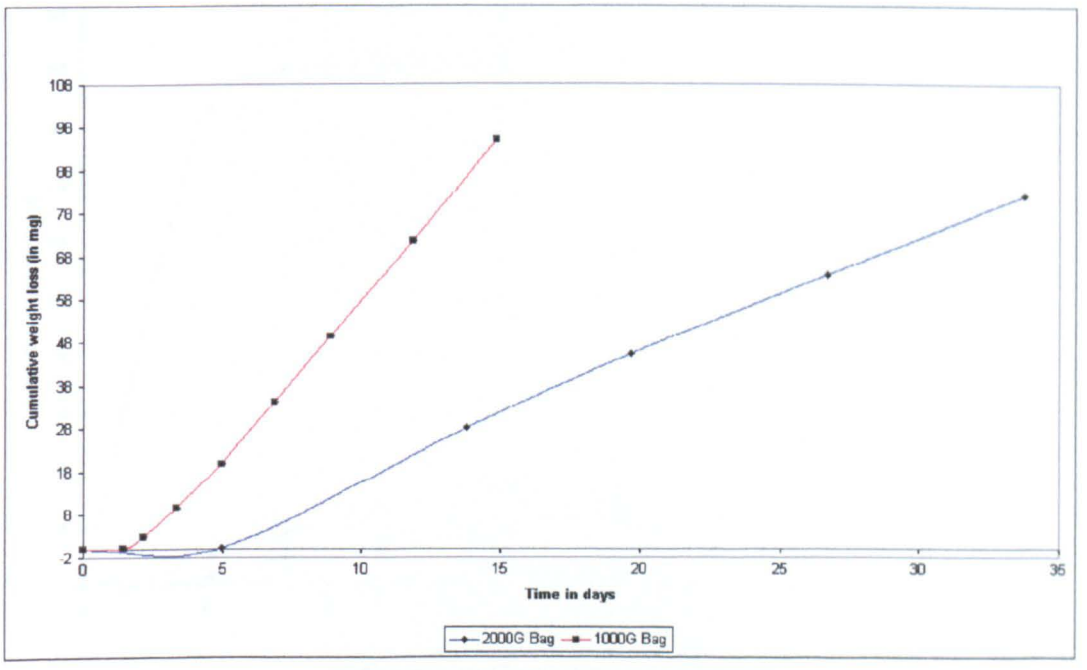


Figure A8: **Mean cumulative weight loss from β-caryophyllene 'polyethylene bag' lures [a] (n=3, ±SE).**



N.B. SE bars are small and obscured by the plotted points on this graph.

Figure A9: **Mean cumulative weight loss from β-caryophyllene 'polyethylene vial' lures [b] (n=3, ±SE).**

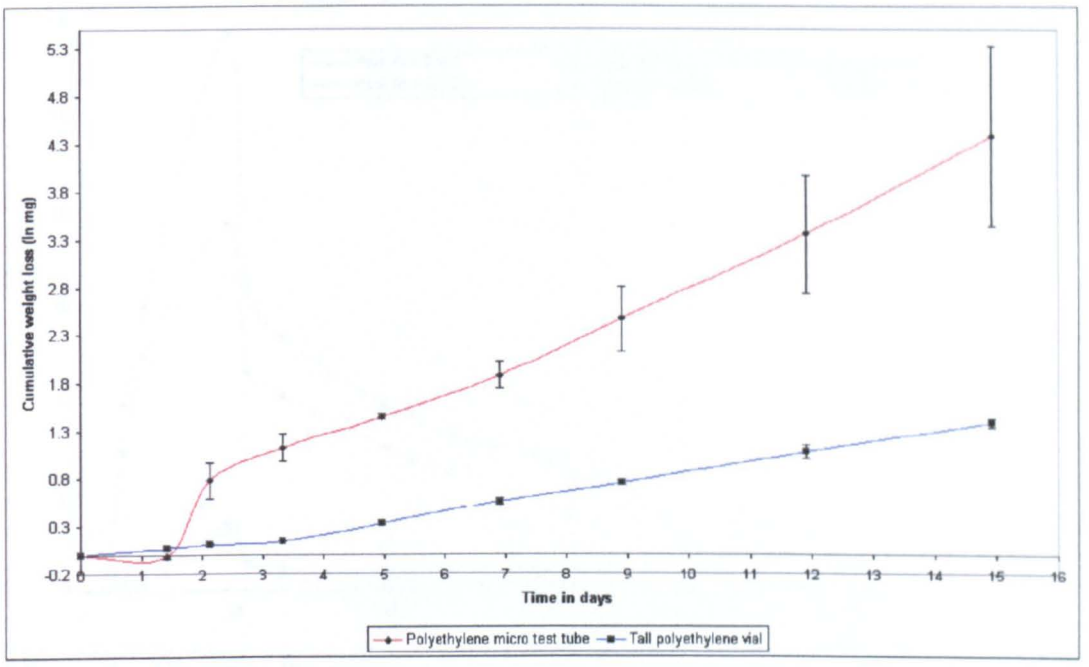


Figure A10: **Mean cumulative weight loss from β-ocimene polyethylene lures (n=3, ±SE).**

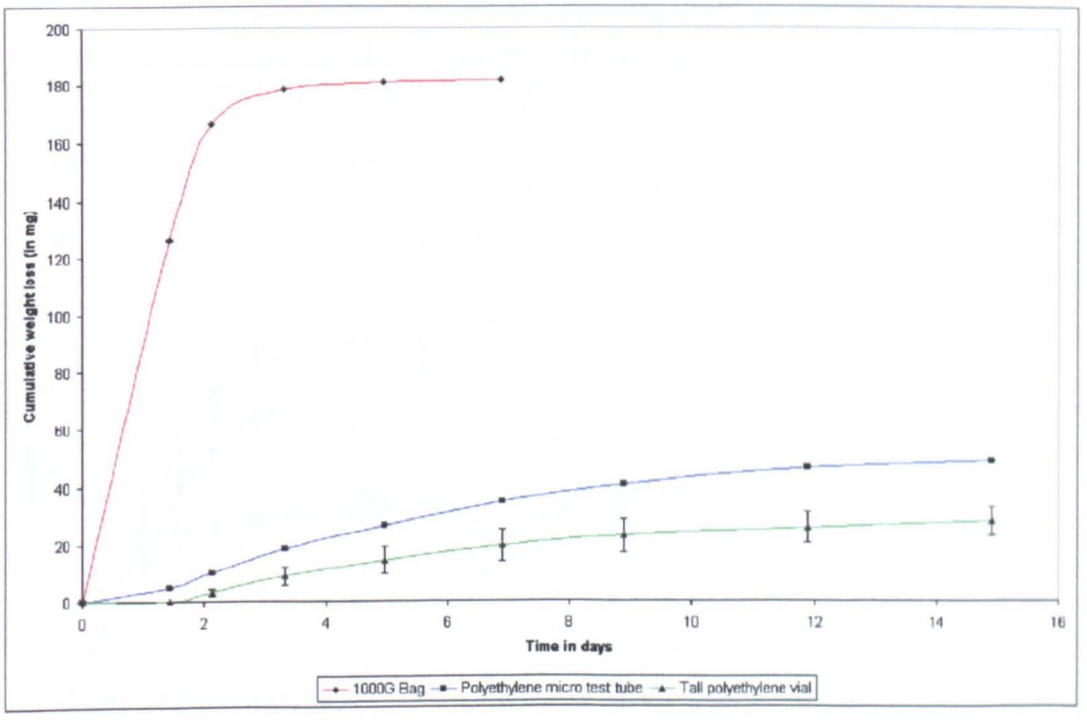
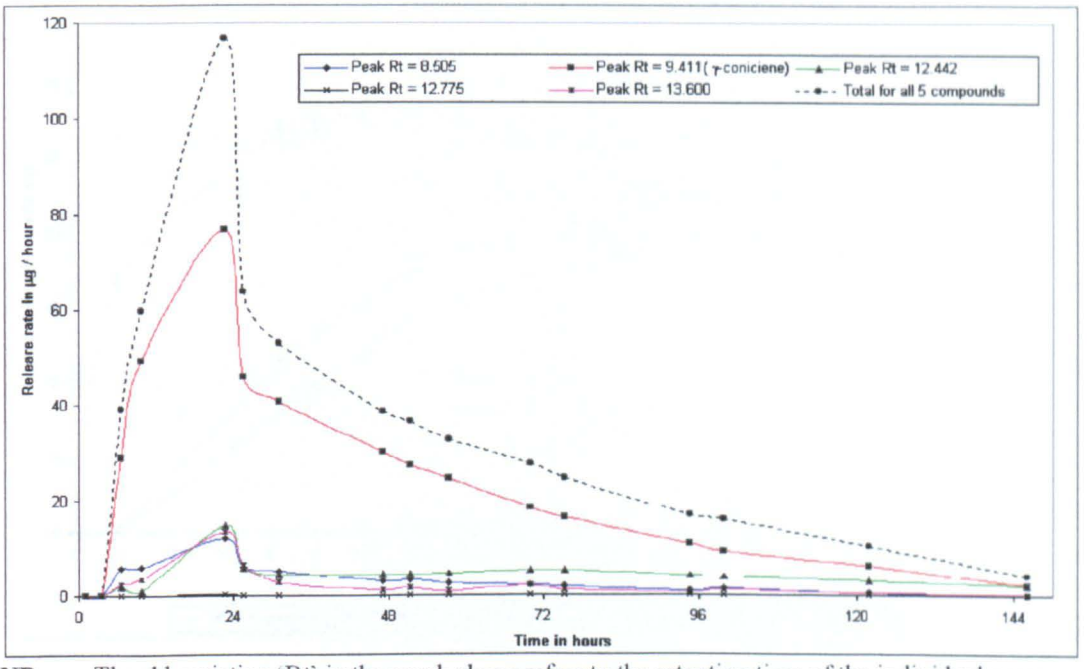


Figure A11: **Release rates of volatiles from a γ-coniceine 1000G 'polyethylene bag' lure, determined by air entrainment (line fitted by eye, n=1).**



NB. The abbreviation 'Rt' in the graph above refers to the retention time of the individual compound peaks during GC analysis.

Figure A12: **Mean cumulative weight loss from hexanal 'polyethylene bag' lures [a] (n=3, ±SE).**

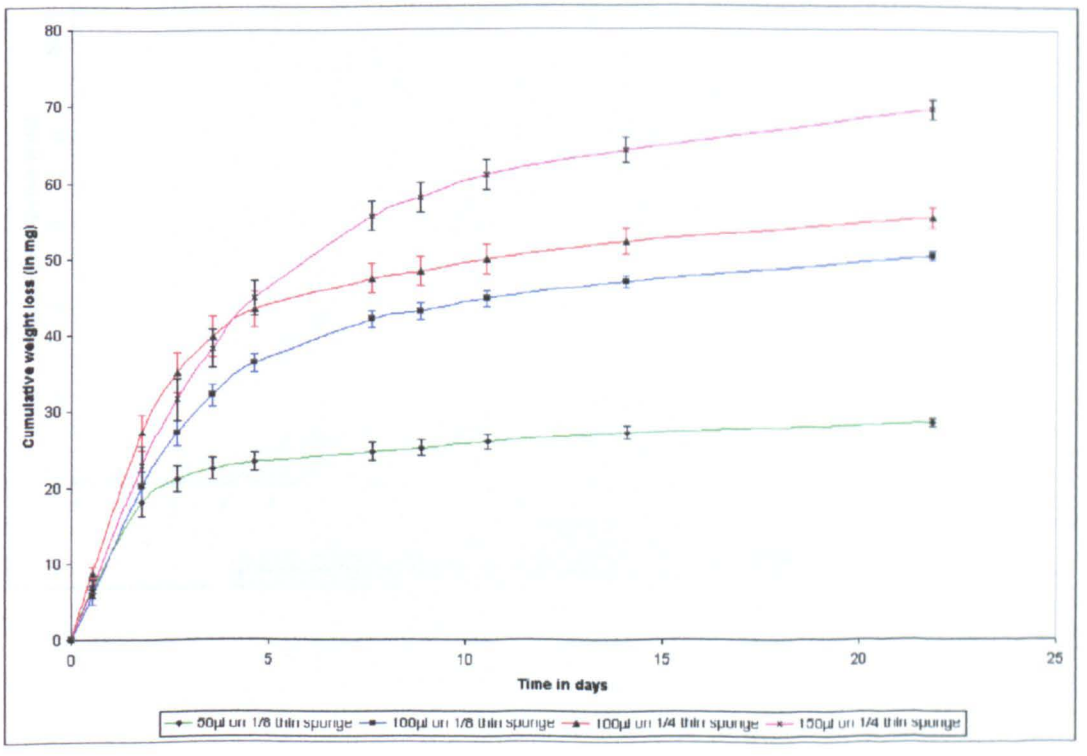


Figure A13: **Mean cumulative weight loss from hexanal 'polyethylene bag' lures [b] (n=3, ±SE).**

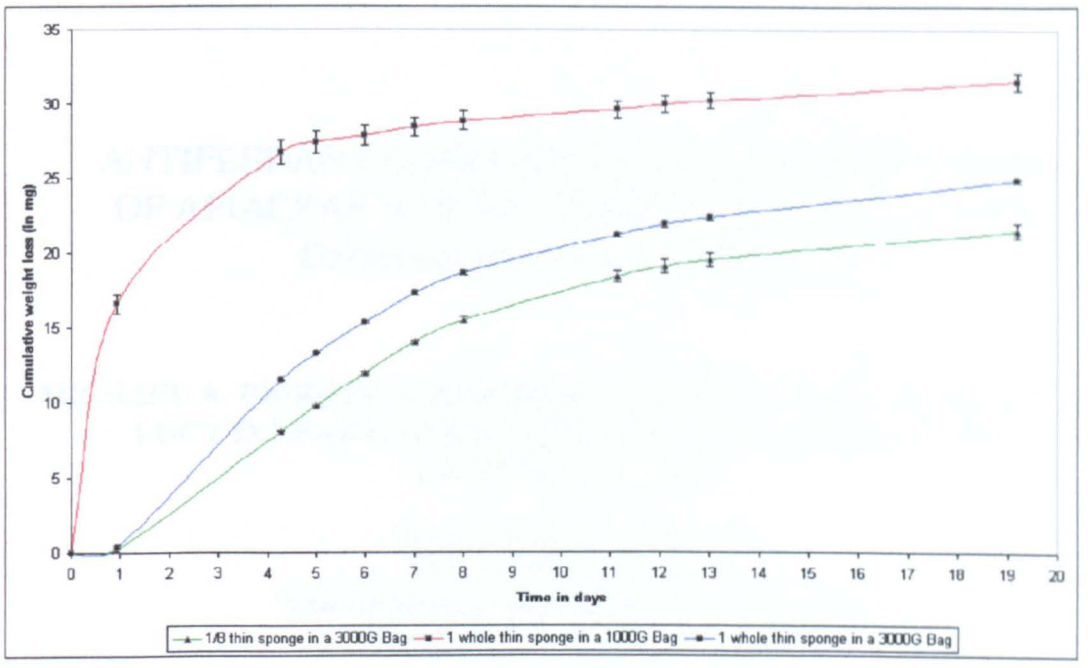


Figure A14: **Mean cumulative weight loss from hexanal 'polyethylene bag' lures [c] (n=3, ±SE).**