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Olfactory Mechanisms Underlying Host-Finding by the *Eucalyptus* Woodborer, *Phoracantha semipunctata* Fab. (Coleoptera: Cerambycidae).

A Behavioural and Electrophysiological Approach

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Ph.D. thesis submitted to the University of Évora, Portugal

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To my parents;

Maria Teresa & Eduardo

for

their ever-present love and support

Contents

PART II - EXPERIMENTAL WORK

Sumário

A selecção da planta hospedeira por insectos fitófagos resulta de uma sequência de respostas comportamentais a uma série de estímulos associados a plantas hospedeiras e não hospedeiras. As características visuais e olfactivas das plantas detectadas pelo insecto a uma certa distância, podem influenciar o seu comportamento de orientação durante a "fase de localização" da planta hospedeira. Na planta, o insecto efectua a sua identificação em resultado da detecção das suas características mecânicas e gustativas. Nesta "fase de identificação", o insecto aceita a planta para alimentação e/ou oviposição ou rejeita a planta e reinicia o processo de selecção. Para algumas espécies de fitófagos, a fase de identificação da planta é determinante no processo de seleção da planta hospedeira, sendo ainda matéria de discussão se os odores vegetais influenciam o comportamento de orientação na fase localização. No entanto, para outras espécies de fitófagos tem sido demonstrado que o seu comportamento de orientação é influenciado por odores vegetais, que tendo efeitos repelentes ou atractivos, permitem ao insecto discriminar, a uma certa distância, aquelas espécies ou indivíduos que são mais adequados para alimentação e/ou oviposição.

O xilófago do eucalipto, *Phoracantha semipuncatata* Fabricius, é um cerambicídeo de origem Australiana cujas principais plantas hospedeiras são espécies do género *Eucalyptus*. Este insecto foi introduzido em várias regiões do mundo após a introdução de eucaliptos como plantas ornamentais ou para exploração económica de madeira e óleos essenciais. Na sua região de origem, *P. semipunctata* é uma espécie sem importância económica, enquanto que nas regiões em que foi introduzida, incluíndo Portugal e practicamente todos os países mediterrânicos (e.g. Espanha, Itália, Marrocos, Tunísia), tornou-se uma importante praga do eucalipto. Em Portugal, o insecto foi detectado em 1980, na península de Setúbal. Desde então, as populações de *P. semipunctata* cresceram rápidamente e a sua presença estendeu-se a todo o país, com particular incidência nas regiões do interior sul onde têm provocado perdas económicas significativas aos produtores e à indústria de celulose e pasta de papel.

Os adultos de *P. semipunctata* voam durante o crepúsculo e noite. As fêmeas depositam os ovos nas fendas do ritidoma e, após a eclosão, as larvas penetram o tronco e irradiam em todas as direcções, alimentando-se do câmbio vascular e lenho jovem. Os métodos actuais para controlo de *P. semipunctata* aproveitam o facto de as fêmeas depositarem preferencialmente os seus ovos em eucaliptos enfraquecidos ou abatidos recentemente, nos quais há maior

probabilidade sucesso do desenvolvimento larvar. Assim, armadilhas de toros para os ovos têm sido utilizados com o objectivo de reduzir os efectivos populacionais na geração seguinte.

Tem sido sugerido que as fêmeas de *P. semipunctata* utilizam o sentido olfactivo para localizar as espécies de eucalipto, ou os indivíduos de uma espécie, mais adequados para o desenvolvimento larvar, no entanto isto nunca foi investigado. A possibilidade de utilização de compostos voláteis de origem vegetal em estratégias de controlo de *P. semipunctata*, foi a motivação para a realização desta tese, tendo sido investigado: (1) se o comportamento de localização da planta hospedeira é influenciado pelo odor do eucalipto; (2) quais os compostos produzidos por espécies de eucalipto e por espécies não hospedeiras que são detectados pelos neurónios receptores olfactivos nas antenas; (3) como são codificados os odores de espécies hospedeiras e não hospedeiras pelos neurónios olfactivos.

A tese está dividida em quatro partes: a parte I, contém dois capítulos (1 e 2) que apresentam a revisão da literatura sobre o conhecimento científico de base para o desenvolvimento do trabalho experimental; a parte II, contém quatro capítulos (3 a 6) que apresentam o trabalho experimental desenvolvido; a parte III, contém um só capítulo (7) que é uma discussão dos resultados experimentais como um todo, e onde são apontadas direcções de investigação com o objectivo de estabelecer estratégias de "protecção integrada" do eucalipto contra *P. semipunctata*; a parte IV, contém as referências bibliográficas.

Na parte I, o capítulo 1 apresenta uma revisão dos mecanismos olfactivos subjacentes ao comportamento de localização da planta hospedeira e dos aspectos neurofisiológicos do sentido olfactivo dos insectos; o capítulo 2 apresenta uma sinopse da biologia de *P. semipunctata*, englobando a posição taxonómica, os aspectos morfológicos e outras características biológicas mais marcantes, as espécies de plantas hospedeiras conhecidas, o ciclo de vida, a distribuição geográfica, os métodos de controlo actualmente practicados e as estratégias de controlo a desenvolver no futuro.

Na parte II, os quatro capítulos (3 a 6) estão dividido em quatro secções: introdução, materiais e métodos, resultados e discussão. De seguida é apresentado um sumário de cada um destes capítulos.

O capítulo 3 investigou se machos e fêmeas de *P. semipunctata* são atraídos pelo odor de *E. globulus* (atracção primária). Para esse objectivo, foi realizada uma experiência na plantação de *E. globulus* da Serra d'Ossa (Redondo, sul de Portugal), durante as primeiras 3 semanas de

Julho de 1991, utilizando armadilhas para insectos adultos em vôo. Foram usadas armadillhas vazias e armadilhas com um dos seguintes iscos: um toro sêco descascado de *E. globulus*, um toro de um espécimen saudável de *E. globulus* abatido recentemente, ou folhas do mesmo espécimen. As armadilhas foram dispostas na plantação segundo uma distribuição de 6 blocos casualisados. Os iscos foram renovados semanalmente e a sua posição em cada bloco casualisada de novo. A análise dos resultados mostrou que, as armadilhas iscadas com folhas capturaram o maior número médio de machos e de fêmeas, seguidas das armadilhas iscadas com um toro cortado recentemente. As armadilhas iscadas com um toro sêco e as armadilhas vazias capturaram número baixo de adultos.

Em laboratório, foram registados electroantenogramas (EAG) em machos (n=5) e fêmeas (n=4) em resposta à estimulação da antena com odor emanado por amostras (1,5 g) de casca do tronco, rebento do tronco, folha e fruto de *E. globulus*, assim como em resposta à estimulação com alguns compostos voláteis constituintes do seu odor, i.e. α-pineno, α-terpineno, canfeno, 1,8-cineol e etanol. O estímulo consistiu num sopro de ar (3 ml) de uma seringa ligada a uma pipeta de Pasteur contendo uma amostra de material de *E. globulus* ou uma porção de papel de filtro impregnado com 25 µl de uma diluição em óleo de parafina (10^{-4} a 1 v/v) de cada composto. O sopro de ar foi injectado num fluxo contínuo de ar puro (80 ml/s) sobre uma das antenas do insecto vivo. As amplitudes de EAG registadas para machos e fêmeas não mostraram diferenças significativas. De entre as várias amostras de *E. globulus*, a amostra de fruto provocou as maiores amplitudes de EAG relativas ao estímulo padrão (1,8-cineol, 10^{-1} v/v), seguida das amostras de folha, rebento do tronco e por último casca do tronco. O α-pineno provocou as maiores amplitudes relativas de EAG nas concentrações de 10^{-4} a 10^{-2} v/v. O canfeno na concentração de 10^{-1} v/v provocou amplitudes relativas de EAG semelhantes às provocadas pelo α-pineno na mesma concentração, enquanto que na concentração de 1 v/v, foi aquele que provocou EAG de maior amplitude relativa. As curvas de dose-resposta obtidas para α-terpineno e 1,8-cineole foram semelhantes, enquanto o etanol provocou as menores amplitudes relativas de EAG em todas as concentrações testadas.

Os resultados mostraram que, folhas e toros de *E. globulus* são atractivos para machos e fêmeas de *P. semipunctata*. As folhas foram mais atractivas do que os toros, possivelmente porque emanaram odor mais intenso do que o odor emanado pela casca dos toros recentemente cortados.

O capítulo 4 investigou o comportamento de orientação de *P. semipunctata* para um toro de *E. globulus* em túnel de vento (2,5 m x 1 m x 1 m). Um toro recentemente cortado e posicionado verticalmente na entrada do ar no túnel (velocidade = 50 cm/s) produziu uma pluma de odor, à qual machos (n=92) e fêmeas (n=76) foram submetidos, após terem sido introduzidos individualmente na zona central do túnel. Em experiências controlo, machos (n=52) e fêmeas (n=46) foram submetidos à presença de um tubo de PVC de dimensões semelhantes ao toro e posicionado de igual modo no túnel, constituíndo um estímulo visual sem odor de *E. globulus*. A forma e a largura da pluma odorífera foram determinados por simulações registadas em vídeo com fumo de acetato de amónia formado numa esponja que revestiu a superfície do tubo de PVC. O comportamento de locomoção e de vôo foi observado sob iluminação de lâmpadas incandescentes vermelhas e registado em vídeo. O trajecto de cada insecto foi desenhado em folha de acetato justaposta ao monitor vídeo, tendo sido a sua posição marcada em cada 10 imagens (0,4s) quando em locomoção, e em cada 3 imagens (0,12 s) quando em vôo.

Não foram encontradas diferenças estatísticas entre a respostas comportamentais de machos e fêmeas. Nenhum insecto aterrou no tubo de PVC, enquanto 48% dos insectos submetidos à pluma odorífera (n=168) aterraram no toro de *E. globulus*. A análise dos resultados revelou que a pluma odorífera influenciou o comportamento de locomoção do seguinte modo: (1) aumentou a frequência de insectos com locomoção de sentido contrário ao do vento, diminuíu a frequência de insectos com locomoção no sentido do vento e a de insectos com locomoção sem orientação preferencial; (2) aumentou a frequência de locomoção (tempo em locomoção/tempo de observação); (3) diminuíu o tempo médio de paragem; (4) diminuíu o tempo médio até levantar vôo. Estes resultados mostraram que o odor de *E. globulus* influencia parâmetros direcionais e nâo direcionais do comportamento de locomoção de *P. semipunctata*, sugerindo que as respostas comportamentais ao estímulo odorífero envolveram uma combinação de "anemotaxis" positiva e "ortokinesis".

Em relação ao comportamento de vôo, a análise dos resultados revelou que a pluma odorífera aumentou a frequência de insectos com vôo de sentido contrário ao do vento e, diminuíu a frequência de insectos com vôo de sentido perpendicular ao do vento, com vôo no sentido do vento e com vôo sem orientação preferencial. Verificou-se ainda que, de entre os insectos que aterraram no toro (n=80): (1) em 40% dos casos, o insecto voou dentro dos limites da pluma odorífera, exibindo uma progressão rápida contra o sentido do vento, frequentemente em ziguezague; (2) em 48% dos casos, ocorreu perda de contacto do insecto com a pluma odorífera, após o qual o insecto mudou rápidamente o curso de vôo, exibindo por vezes uma sequência de curvas e contra-curvas, voando de seguida em sentido perpendicular ao do vento ou

no sentido do vento, restabelecendo o contacto com a pluma odorífera, após o qual reiniciou o vôo em ziguezague com sentido contrário ao do vento; (3) em 12% dos casos, o insecto voôu em sentido contrário ao do vento, progredindo de modo errático em consequência de contactos frequentes com o tecto do túnel de vento. Na presença do tubo de PVC, os trajectos em vôo foram mais lineares (sem ziguezague), ocorrendo com igual frequência em todas os sentidos, tornando-se erráticos apenas quando ocorreram contactos frequentes com o tecto do túnel de vento.

Estes resultados mostraram que o odor de *E. globulus* influencia parâmetros direcionais do comportamento de vôo de *P. semipunctata*, sugerindo que as respostas comportamentais ao estímulo odorífero envolveram uma integração de "anemotaxis-optomotora" positiva com um programa interno de "ziguezague auto-dirigido".

O capítulo 5 identificou os components voláteis produzidos por plantas hospedeiras e não hospedeiras que são detectados pelos neurónios receptores olfactivos de *P. semipunctata*. Compostos voláteis, emanados por 3 espécies de *Eucalyptus* com diferente grau de atracção para *P. semipunctata* (*E. globulus* e *E. camaldulensis* - atractivas; *E. tereticornis* - menos atractiva) e por 2 espécies não hospedeiras, *P. pinaster* (pinheiro) e *O. europeae* (oliveira), foram adsorvidos em filtros de porapak Q por uma técnica de "headspace". Os solventes, hexano ou éter dietílico, foram utilizados para eluir dos filtros de porapak Q, os compostos voláteis adsorvidos e obter soluções de voláteis de cada uma das espécies. A técnica de cromatografia de fase gasosa ligada ao registo simultâneo de EAG (CG-EAG) foi utilizada para identificar os components voláteis de espécies hospedeiras e não hospedeiras que provocaram respostas electrofisiológicas nos neurónios receptores de fêmeas (n=6). A identificação química dos componentes activos foi efectuada por espectrometria de massa e por métodos indirectos com compostos padrão. Amostras autenticadas dos compostos identificados, foram testadas quanto ao seu efeito electrofisiológico (1µl, 10^3 ppm em hexano) em machos (n=6) e fêmeas (n=6), utilizando a técnica de CG-EAG. Foram também testadas amostras autenticadas de compostos voláteis produzidos pelo metabolismo secundário de várias plantas (incluíndo alcoois, aldeídos, esteres derivados de ácidos gordos vegetais, e monoterpenos e sesquiterpenos derivados do isopreno) que não foram detectados nos cromatogramas dos voláteis das espécies estudadas. No total, foram testados 42 compostos voláteis.

Foram localizados nos cromatogramas das soluções de voláteis obtidas das várias espécies vegetais, 43 picos com efeito electrofisiológico. Destes, 22 (incluíndo picos de pequena

e grande dimensão) foram correlacionados com EAG de amplitude superior a 300 μV (resposta forte), aos quais foi dada maior importância na análise de resultados.

Espécies hospedeiras. Na solução de voláteis de *E. globulus*, os compostos correlacionados com respostas electrofisiológicas fortes foram: 3-hidroxi-2-butanona; 3-metil-1-butanol; 2,3-epoxi-4,4-dimetilpentano; etil-3-metilbutanoato; (Z)-3-hexen-1-ol; α-pineno; β-pineno; *p*-cimeno; 1,8-cineol; limoneno; guaine e um composto não identificado. Na solução de voláteis de *E. camaldulensis*, excepto guaine, todos os outros compostos mencionados acima provocaram respostas electrofisiológicas fortes. Além destes, foram também correlacionados com fortes respostas electrofisiológicas o α-terpineno e o linalol (em maior concentração do que nas soluções das outras espécies de eucalipto), e um composto não identificado. Na solução de voláteis de *E. tereticornis*, excepto etil-3-metilbutanoato, (Z)-3-hexen-1-ol, 1,8-cineol e limoneno (correlacionados com EAG de amplitude ≤300 μV), os mesmos compostos produzidos por *E. globulus* foram também, neste caso, correlacionados com respostas electrofisiológicas fortes.

Espécies não hospedeiras. Na solução de voláteis de *P. pinaster*, os compostos correlacionados com respostas electrofisiológicas fortes foram: etil-3-metilbutanoato; (Z)-3-hexen-1-ol; α-pineno; β-pineno; mirceno; 1,8-cineol; limoneno; trans-β-ocimeno; linalool e α-cubebeno. O sesquiterpeno, α-cubebeno, foi identificado apenas nesta espécie. Na solução de voláteis de *O. europeae*, os compostos correlacionados com respostas electrofisiológicas fortes foram: 3-hidroxi-2-butanona; 3-metil-1-butanol; 2,3-epoxi-4,4-dimetilpentano; etilpropanoato; (Z)-3-hexen-1-ol; 4,8-dimetilnona-1,3,7-trieno; α-pineno e três compostos não identificados. O hidrocarboneto não terpenoidíco, 4,8-dimetilnona-1,3,7-trieno, e dois dos compostos não identificados, foram detectados apenas nesta espécie.

Os resultados do teste de respostas electrofisiológicas a 42 compostos voláteis mostraram não haver diferenças sexuais nas amplitudes de EAG relativas ao estímulo padrão [(E)-2-hexenal]. Os compostos que provocaram amplitudes relativas de EAG mais elevadas (>60%) foram os seguintes: (E)-2-hexenal, 3-hidroxi-2-butanona, 3-metil-1-butanol, (+)fenchona, 3-careno, (Z)-3-hexen-1-ol e 1-hexenol.

Os resultados mostraram que espécies de *Eucalyptus* com diferente grau de atracção para *P. semipunctata* produzem diferentes quantidades relativas de metabolitos secundários (hidrocarbonetos não terpenoidícos, monoterpenos e sesquiterpenos) detectáveis pelos neurónios receptores nas antenas. Muitos destes compostos são produzidos também por espécies não hospedeiras. Estes resultados sugerem que a localização da planta hospedeira depende da

detecção de vários tipos de metabolitos secundários, sendo importante a sua concentração relativa no odor para a ocorrência de respostas comportamentais conducentes ao contacto com a planta. Verificou-se ainda que, metabolitos secundários produzidos em pequenas quantidades apenas por espécies não hospedeiras provocaram fortes respostas electrofisiológicas. Estes compostos poderão ser importantes para identificar, de modo inequívoco, odores de espécies não hospedeiras, podendo actuar como repelentes.

O capítulo 6 investigou a codificação de odores de *E. globulus*, *P. pinaster* e *O. europeae* pelos neurónios receptores de *P. semipunctata*. Para esse objectivo, foi utilizada a técnica de cromatografia de fase gasosa ligada ao registo extracelular de potenciais de acção de neurónios receptores associados a sensilas olfactivas nas antenas. Neste estudo, foram utilizadas as mesmas soluções de voláteis obtidas para a realização do estudo anterior (capítulo 5).

No total, foi estudada a actividade de 41 neurónios receptores em resposta à estímulação com os componentes voláteis de *E. globulus*, *P. pinaster* e *O. europeae*. Todos os neurónios receptores exibiram actividade espontânea e todas as respostas ao estímulo olfactivo consistiram no aumento de frequência de potenciais de acção, correlacionado com a concentração dos compostos activos na solução de voláteis. Os compostos voláteis que activaram os neurónios receptores foram a maioria dos que no estudo anterior (capítulo 5) provocaram fortes amplitudes de EAG e ainda 16 componentes voláteis de *E. globulus* e *P. pinaster* que não tinham provocado evidentes respostas electrofisiológicas durante CG-EAG. Estes resultados mostraram que, a técnica de CG-EAG não permite identificar todos os componentes do perfil de voláteis de uma planta que são detectados pelos neurónios receptores de *P. semipunctata*.

Os neurónios foram classificados em 23 tipos de acordo com os compostos a que responderam. Neurónios de 10 tipos responderam a apenas um composto (especialistas). Outros tipos de neurónios responderam a dois, três ou 4 compostos. Cada um destes tipos de neurónios respondeu a compostos com estrutura química relacionada (i.e. hidrocarbonetos C4-C6 não terpenoidícos, monoterpenos ou sesquiterpenos). Estes resultados sugerem que, os neurónios receptores de odores vegetais têm espectros estreitos de resposta, sendo cada neurónio especializado na recepção de informação sobre um ou alguns compostos relacionados químicamente.

A maioria dos neurónios foram activados por compostos produzidos em diferentes quantidades pelas várias plantas. Isto sugere que, a actividade relativa provocada em neurónios de diferentes tipos é o código para o cérebro sobre a qualidade de um odor vegetal. A informação sobre a presença de alguns compostos no odor é preservada pelos neurónios especialistas,

ocorrendo alguma integração da qualidade do odor pelos neurónios que respondem a mais do que um composto.

A informação sobre α-cubebeno, produzido apenas por *P. pinaster*, e sobre um composto não iedentificado, produzido apenas por *O. europeae*, é transportada para o cérebro apenas por dois tipos de neurónios especialistas. Isto sugere a existência de neurónios especializados na detecção de compostos odoríferos de plantas não hospedeiras. A sua actividade pode estar relacionada com eventuais respostas comportamentais de *P. semipunctata* a estes odores, que resultem no evitar o contacto com espécies não hospedeiras.

Na parte III, o capítulo 7 apresenta uma discussão geral dos resultados experimentais, da qual resultaram as seguintes conclusões:

1. A localização da planta hospedeira por *P. semipunctata* resulta de respostas comportamentais iniciadas e mantidas por odores emanados de plantas hospedeiras e transportados pelo vento. O insecto adulto afastado da planta hospedeira responde ao estímulo olfactivo voando em sentido contrário ao do vento, sendo sugerido que mantém este curso de vôo por controle de "feedback" com o padrão do movimento aparente das imagens nos olhos - "anemotaxis optomotora" induzida pelo odor. Os resultados do estudo do comportamento de orientação em túnel de vento sugerem ainda que, existe um programa interno de vôo em ziguezague que produz desvios alternados à resultante do curso de vôo. Este programa interno de orientação pode aumentar a probabilidade de contacto frequente com a pluma odorífera, especialmente quando ocorrem mudanças bruscas na direcção do vento. Assim, a "anemotaxis optomotora" polariza o vôo em ziguezague auto-dirigido que de outro modo seria errático, resultando desta integração de mecanismos comportamentais, um deslocamento resultante de sentido contrário ao do vento, que aumenta a probabilidade de encontro com a planta hospedeira.

2. Espécies de *Eucalyptus* com diferente grau de atracção para *P. semipunctata* produzem diferentes quantidades relativas dos mesmos metabolitos secundários que são detectáveis pelos neurónios receptores nas antenas. Muitos destes compostos são produzidos também por espécies não hospedeiras. Consequentemente, sugere-se que a ocorrência e manutenção dos comportamentos que aumentam a probabilidade do contacto com a planta hospedeira, depende da detecção de misturas de compostos odoríferos em determinados quocientes de concentração.

3. O grau de especialização relativamente elevado dos neurónios receptores sugere que a qualidade de odores vegetais é codificada através da actividade relativa promovida em diferentes tipos de neurónios receptores nas antenas.

4. A actividade promovida em neurónios especialistas que detectam componentes dos odores de plantas não hospedeiras, pode estar relacionada com eventuais respostas comportamentais que permitem ao adulto de *P. semipunctata* evitar o contacto com essas espécies vegetais.

5. Existe evidência experimental sugerindo que, os adultos de *P. semipunctata* não possuem mecanismos de comunicação intraespecífica eficientes a distâncias longas. Assim, a capacidade para discriminar olfactivamente odores vegetais, subjacente a comportamentos de orientação que levam ambos os sexos ao encontro com plantas hospedeiras adequadas ao desenvolvimento larvar, parece ter sido uma importante adaptação que minimiza a energia dispendida na procura de parceiro sexual e de plantas hospedeiras, e tem a vantagem de reduzir os riscos de predação e aumentar o investimento na reprodução.

Summary

The host selection in phytophagous insects is a sequence of behavioural responses to an array of stimuli associated with host and non-host plants, and it can be divided arbitrarily as host finding and host recognition (acceptance or rejection), including several behavioural steps: orientation, landing, probing, feeding and/or oviposition. The stimuli involved include visual, mechanical, olfactory, and gustatory characteristics of the plants. The visual and olfactory cues are usually associated with long-range host finding, whilst host plant recognition is based mostly on the other two types of stimuli after the insect has made contact with the plant. For some species, the role of host plant odours in host-finding behaviour is unclear and is a matter of debate, whereas for other species, it has been shown that both attractive and repellent odours are used for discriminating between plants during the process of selecting a suitable host.

The eucalyptus woodborer, *Phoracantha semipunctata* Fabricius, is a cerambycid of Australian origin that has host plant range restricted to species mainly of *Eucalyptus*. It has been introduced to several regions of the world following the introduction of *Eucalyptus* for ornamental purposes or economic exploitation of its wood and essential oils. Whereas this insect species is of no economic importance in Australia, it has become a severe pest of eucalyptus plantations in the introduced regions, including Portugal and several other Mediterranean countries (e.g. Spain, Italy, Marrocco and Tunisia). In Portugal, *P. semipunctata* was detected for the first time in the peninsula of Setúbal in 1980. Since then, the populations have increased dramatically and have spread throughout nearly all eucalyptus plantations of the country, causing significant economic losses for the growers and the pulp paper industry, mainly in the central and southern regions of Portugal.

Adults of *P. semipunctata* fly at dusk and during the night in search for mates and oviposition sites. The females oviposit in bark cracks or under loose bark, and preferentially on recently felled or weakened trees where larval development is more successful. Tree damage is produced by the larvae feeding on the bark, resulting in the destruction of the cambium and phloem tissues along the trunk. Heavy infestations may result in the death of the tree. Current methods of *P. semipunctata* control exploit the extraordinary ability of females to colonise recently felled trees, and log traps for the eggs have been used by *Eucalyptus* growers in order to reduce the beetles' population size of the next generation.

It has been suggested that *P. semipunctata* females use olfaction to find suitable hosts for larval development, but this has never been thoroughly investigated. Motivated by the potential

use of plant volatile compounds in strategies to control *P. semipunctata* populations, the present thesis investigated: (1) whether host-finding behaviour of *P. semipunctata* beetles is influenced by *Eucalyptus* odour; (2) which volatile compounds of host and non-host plant species are detected by olfactory receptor neurons in the antennae; (3) how host and non-host plant odours are encoded by receptor neurons.

The thesis is divided in four parts. Part I comprises two chapters (Ch. 1 and 2) providing the scientific background knowledge relevant to the experimental work carried out. Part II comprises four chapters (Ch. 3 to 6) describing the experimental work. Part III is comprised of a single chapter (Ch. 7) where the integrated results are discussed as well as their implications for further research aiming at the establishment of integrated pest management strategies of *P. semipunctata*. Part IV comprises the bibliographic references in the thesis.

In part I, chapter 1 is a review on olfactory mechanisms underlying host-finding behaviour in phytophagous insects, and on neurophysiological aspects of olfaction in insects. Chapter 2 is a synopsis on the biology of *P. semipunctata*, including the taxonomic position of the species, its distinguishing morphological and other biological characteristics, range of host plant species, life cycle, geographical distribution, and current and future methods of pest control.

In part II, each chapter is divided in four sections: introduction, materials and methods, results, and discussion. A summary of each of these chapters is presented below.

Chapter 3 investigated whether *P. semipunctata* males and females are attracted to host plant odour. A field experiment was conducted during the first three weeks of July 1991 in a plantation of *E. globulus* in the south of Portugal, using traps for flying beetles. Empty traps and traps baited with a dry debarked log, a freshly cut log or with leaves of a common host species, *E. globulus*, were placed in plantation according to a six randomised block design. The baits were renewed weekly, and their place within each block randomised. The traps baited with leaves caught the highest mean number of males and females, followed by the traps baited with a freshly cut log. Empty traps and traps baited with a dry log caught the fewest insects.

Recording of electroantennograms (EAGs) was carried out to test whether the beetles' olfactory receptor neurons detect volatiles emanating from *E. globulus*. EAGs were recorded from 5 males and 4 females in response to stimulation with odour emanating from samples (1.5g) of bark, trunk node, leaf, and fruit of *E. globulus*, as well as to stimulation with volatile

compounds of *Eucalyptus* (α-pinene, α-terpinene, camphene, 1,8-cineole, and ethanol) The stimulus was a "puff" of air (3 ml) blown by a syringe attached to a Pasteur pipette containing the plant material or a piece of filter paper impregnated with 25 µl of a dilution of each volatile compound in paraffin oil (10^{-4} to 1 v/v). The stimuli were injected in a tube with a continuous airflow (80 ml/s) over the antenna. There were no sexual differences between the EAG amplitudes elicited by the various stimuli. Among the samples of *E. globulus* material, the fruit elicited the highest EAG amplitudes relative to the standard stimulus (1,8-cineole, 10^{-1} v/v), followed by leaf, trunk node, and bark. α-Pinene was the volatile compound that elicited the highest relative EAG amplitudes at concentrations of 10^{-4} to 10^{-2} v/v. Camphene elicited relative EAG amplitudes similar to those elicited by α-pinene at 10^{-1} v/v, and elicited the highest relative amplitudes at 1 v/v. The dose-response relationships obtained for α-terpinene and 1,8-cineole were similar, whereas ethanol elicited the lowest relative EAG amplitudes at every concentration tested.

The results showed that freshly cut *E. globulus* material was clearly attractive to *P. semipunctata* males and females. The leaves showed higher attractiveness than the logs, which may have resulted from higher concentration of volatile compounds in the odour blend evaporated from the leaves than in the one evaporated from the logs.

Chapter 4 investigated the orientation behaviour *P. semipunctata* males (n=92) and females (n=76) to logs of *E. globulus*. The beetles were subjected to an odour plume originating from a freshly cut log placed vertically at the upwind end of a wind tunnel. In control experiments, males (n=52) and females (n=46) were subjected to a PVC pipe in the same position as the log, providing a visual stimulus without host plant odour. The shape and width of the odour plume emanating from the log was determined from simulations recorded on video using ammonium acetate smoke originated from a cloth covering the PVC pipe. The beetles' behaviour was recorded on video, and detailed observations of behavioural responses were made by playback of video recordings. Analysis was done from acetate sheet tracings of the walk and flight made on the video monitor screen, the position of the beetle being marked every 10 frames (0.4s) when walking and every 3 frames (0.12s) when flying.

No statistical differences were found between the behavioural responses of males and females. No beetles landed on the PVC pipe, whereas 48% of the beetles subjected to host odour plume landed on the log. Behavioural analysis revealed that the odour plume affected the walking behaviour as follows: (1) increased the frequency of beetles walking upwind, and

decreased the frequency of those walking downwind or with no predominant direction; (2) increased the walking frequency (time walking/time of observation); (3) decreased mean stop time; (4) decreased mean take-off time. These results showed that host plant odour affected directional and non-directional parameters of the walking behaviour, suggesting that walking responses involved a combination of positive odour induced-anemotaxis and chemo-orthokinesis.

As concerns flight behaviour, the behavioural analysis revealed that the odour plume increased the frequency of beetles flying upwind, and decreased the frequency of those flying crosswind, downwind, or with no predominant direction. In addition, the traces of flight tracks of beetles that had landed on the log (n=80) superimposed on the odour plume trace as obtained from simulations showed that: (1) in 40% of the cases, the beetle flew quickly upwind within the boundaries of the odour plume, often with narrow zigzag movements; (2) in 48% of the cases, the beetle eventually lost contact with the odour plume, and exhibited a sharp turn or quick counterturns followed by crosswind or downwind excursions that led it to regain contact with the odour plume, and then resumed upwind flight with narrow zigzag movements provided it flew within the boundaries of the plume; (3) in 12% of the cases, the beetle flew erratically upwind as result of frequent contact with the ceiling of the wind tunnel. The type of movements described were never observed in beetles flying in the presence of the PVC pipe, whose flight tracks were more linear, occurring with equal in all direction categories (upwind, crosswind, downwind, no predominant direction), except when the beetle bounced off the ceiling of the wind tunnel. These results showed that host plant odour affected the direction and pattern of flight tracks, suggesting that flight responses involved both odour induced-anemotaxis and self-steered zigzag counterturns.

Chapter 5 identified volatile compounds of host and non-host plant species that are detected by *P. semipunctata* receptor neurons. Headspace volatiles of leaves from three *Eucalyptus* species with different attractiveness to the beetles (*E. globulus*, *E. camaldulensis*, and *E. tereticornis*) and from two non-hosts (*Pinus pinaster* and *Olea europeae*) were trapped on porapak Q filters. Hexane and diethyl ether were used to wash out the trapped compounds providing solutions of volatiles from each plant species. The technique of gas chromatography linked with simultaneous recording of EAGs (GC-EAD) was used to identify components in each plant species solution of volatiles that elicit electrophysiological responses. Active components were chemically identified by GC linked with mass spectrometry (GC-MS) and by indirect identification methods. Authentic samples of compounds chemically identified in the

headspace volatiles of the various plant species were tested for their relative electrophysiological effect in males (n = 6) and females (n = 6) at 10^3 ppm in hexane, using the GC-EAD technique. In addition, authentic samples of various secondary metabolites including ubiquitous plant fatty acid ("green leaf" volatiles) and isoprene derivatives (terpenoids) not detected in the headspace volatiles were also tested for their relative electrophysiological effect. In total, 42 synthetic compounds were tested.

Altogether, 43 stimulatory compounds, were located in the gas chromatograms of the various volatile blends by GC-EAD. Out of these, 22 elicited strong EAG responses (amplitude > 300 μV), including major and minor components of each volatile blend.

Host species. In the blend of *E. globulus*, the compounds correlated with strong EAG responses were 3-hydroxy-2-butanone, 3-methyl-1-butanol, 2,3-epoxy-4,4-dimethylpentane, ethyl-3-methylbutanoate, (Z)-3-hexen-1-ol, α-pinene, β-pinene, *p*-cymene, 1,8-cineole, limonene, guaine, and one unidentified compound. In the blend of *E. camaldulensis*, except for guaine that was absent in this species, all the compounds mentioned above were correlated also with strong EAG responses. In addition, α-terpinene and linalool (produced by this species in higher amount than by the other two host species), and one unidentified compound were also correlated with strong EAG responses. In the blend of *E. tereticornis*, except ethyl-3-methylbutanoate, (Z)-3-hexen-1-ol, 1,8-cineole, and limonene (correlated with EAG amplitudes ≤ 300 μV), the same compounds produced by *E. globulus* were also correlated with strong EAG responses.

Non-host species. In the blend of *P. pinaster*, the compounds that elicited strong EAG amplitudes were ethyl-3-methylbutanoate, (Z)-3-hexen-1-ol, α-pinene, β-pinene, myrcene, 1,8-cineole, limonene, trans-β-ocimene, linalool, and α-cubebene. The sesquiterpene α-cubebene was exclusively detected in this blend of volatiles. In the blend of *O. europeae*, the compounds that elicited strong EAG responses were 3-hydroxy-2-butanone, 3-methyl-1-butanol, 2,3-epoxy-4,4-dimethylpentane, ethyl propanoate, (Z)-3-hexen-1-ol, 4,8-dimethylnona-1,3,7-triene, α-pinene, and three unidentified compounds. The non-terpenoid 4,8-dimethylnona-1,3,7-triene and two of the unidentified compounds were exclusively detected in this blend of volatiles.

There were no sexual differences between the EAG amplitudes relative to the standard [(E)-2-hexenal] elicited by each of the 42 synthetic volatile compounds tested. Among these, (E)-2-hexenal, 3-hydroxy-2-butanone, 3-methyl-1-butanol, (+)fenchone, 3-carene, (Z)-3-hexen-1-ol, and 1-hexenol elicited the strongest EAG responses (>60%). The two "green leaf" volatiles, (E)-2-hexenal and 1-hexenol, and the monoterpenes, (+)fenchone and 3-carene, were not

identified as stimulatory compounds in the headspace volatiles of the various plant species during GC-EAD.

The results showed that *Eucalyptus* species with different attractiveness to *P. semipunctata* beetles produce different ratios of secondary metabolites (non-terpenoid hydrocarbons, monoterpenes and sesquiterpenes) detected by the receptor neurons. Most of these compounds are produced also by non-host species. This suggests that *P. semipunctata* beetles use certain ratios of several host related compounds instead of relying on single types of compounds for host-finding. In addition, compounds exclusively produced in small amount by *P. pinaster* or *O. europeae* species elicited strong EAG responses. These compounds may be important for identifying the odour of these non-host plants, and may act as repellents or may mask attractive odour blends.

Chapter 6 investigated the responses of single receptor neurons of *P. semipunctata* to plant volatile compounds, using coupled gas chromatography-single cell recordings (GC-SCR). In this study, it was used the same hexane solutions of headspace volatiles from leaves of *E. globulus* and *O. europeae* and needles of *P. pinaster*, as used in the previous study of GC-EAD recordings (Ch. 5).

Forty-one receptor neurons were tested for the volatiles of the host, *E. globulus*, and the two non-hosts, *P. pinaster* and *O. europeae*, via the GC using a non-polar capillary column. All responses appeared as increased firing rates which followed the concentration profiles of the stimulatory compounds eluting from the GC-column. The compounds that activated the receptor neurons included most of the compounds that had elicited strong EAG responses during GC-EAD in the previous study (Ch. 5), and 16 unidentified components of *E. globulus* and *P. pinaster* that had not elicited significant EAG responses during GC-EAD. These results showed that GC-EAD alone is not sufficient for identifying all the components in a blend of plant volatiles that are detected by the receptor neurons of *P. semipunctata*.

The neurons were classified in 23 neuron types according to the compounds they responded to. Ten neuron types responded to only one compound (specialist neurons). Other neuron types responded to two, three, or four compounds. Each of these neurons responded to chemically related compounds (i.e. non-terpenoid hydrocarbons, monoterpenes, or sesquiterpenes). It suggests that the plant odour receptor neurons are rather narrowly than broadly tuned, and that each neuron is specialised for receiving information about one or a few related compounds.

Most neurons responded to compounds present in both host and non-host plants but in different amounts, whereas others are activated by compounds present only in one plant species. This suggests that the relative activity elicited in neurons of different type is the code to the brain about the quality of a plant odour. Information about the presence of some compounds in the blend is preserved by specialist neurons. In addition, there is some integration of blend quality by neurons responding to more than one compound of the blend.

The information about α-cubebene detected only in *P. pinaster* and about one chemically unidentified compound detected only in *O. europeae* is conveyed only by two specialist neurons. This suggests that some specialist neurons convey information to the brain about non-host plant odours, and their activity may be related with possible avoidance reactions of *P. semipunctata* to non-host plant species.

In part III, chapter 7 presents a discussion of the integrated results conducting to the following conclusions:

1. Host-finding by *P. semipunctata* beetles results from behavioural responses initiated and sustained by odour blends emanating from host plants, and transported by the wind. Downwind of the source, the beetle responds to olfactory stimulation flying upwind. This flight course is maintained by visual feedback from the apparent movement of images over the eyes, i.e. odour-induced optomotor anemotaxis. In addition, it is suggested that self-steered counterturning is integrated with optomotor anemotaxis.

2. *Eucalyptus* species with different attractiveness to *P. semipunctata* beetles produce different ratios of the same volatile compounds that are detected by receptor neurons. In addition, most of these compounds are also produced by non-host plant species, but in different ratios. Therefore, it is suggested that the ratio of host related compounds in the odour plume is important for initiating and sustain behavioural responses taking the beetles upwind to the host plant.

3. The narrow response spectra of plant odours receptor neurons suggest that odour blend quality is encoded by the relative activity in receptor neurons of different type.

4. The activity elicited in specialist neurons by compounds exclusively produced by non-host plants may be related with avoidance reactions that prevent *P. semipunctata* beetles of make contact with non-host plant species.

5. There is experimental evidence suggesting that *P. semipunctata* has not evolved long-range intraspecific communication systems. Thus, olfactory discrimination of plant odours

underlying behavioural responses taking males and females to suitable host plants for larval development, can be viewed as a primary foraging adaptation that minimises the energy spent on searching for mates and suitable host plants, and has the advantage of reducing predation risk and increases the investment in reproduction.

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General Introduction

Introduction

Ecological relationships of plants and animals are universal, of fundamental importance, and paradoxical. Plants use solar energy to fix carbon, and all animal life ultimately depends on use of energy stored by plants. How can an Earth teeming with herbivores, ranging in size from aphids to elephants, be so green ? Animals do not eat all plants. Somehow most plants, most of the time, manage to avoid being eaten.

Charles Darwin (1859) provided the key to questions borne of this paradox in the "On the Origin of Species" (Darwin, 1987). He proposed the theory of evolutionary adaptation by natural selection. Darwin argued that plants and animals best fitted to their environments leave more offspring than other members of their species and, consequently, pass their inherited advantages on to future generations. Darwin even provided insight into the nature of plant defences and animal ability to cope with those defences. He noted that sheep of different breeds have different susceptibilities to plant poisons. The fittest herbivores have an inherited ability to cope with those plant defences, and therefore to prosper on plants that their competitors cannot eat. Plants have evolved an enormous array of mechanical and chemical defences against herbivores. Herbivores, on the other hand, evolved an array of adaptations to overcome the plant defences. As pointed out by Ehrlich and Raven (1964), "the plant-herbivore interface may be the major zone of interactions responsible for generating terrestrial organic diversity".

The phytophagous insects make up a more than a quarter of all macroscopic organisms, and the plants upon which they feed make up another quarter (Bernays and Chapman, 1994). Every green plant has a set of insect herbivores feeding on its roots, steams, buds, leaves, flowers and fruits. Although there is a continuous spectrum of phytophagous insects species, from those feeding on one plant species to those feeding on a wide range of plants of different families, it is common to classify them as follows: 1) monophagous include species that feed on plants within a single genus; 2) oligophagous includes species that feed on various plants in different genera within one plant family; and 3) polyphagous refers to insects feeding on a relatively large number of plant species of different families (Bernays and Chapman, 1994). Most insects have relatively narrow range of host plant species. Out of approximately 310,000 known phytophagous insect species, about 75% are monophagous or oligophagous (Bernays and Chapman, 1994) and these will be faced with the necessity of selecting the appropriate host plant at some stage of their life

cycle. The study of the behavioural mechanisms underlying the selection of a suitable host plant by phytophagous insects is a challenging endeavour, and is central to understand how the present insect-plant associations have evolved, and to know what are the proximate causes of the narrow host ranges of most phytophagous insect species.

The evolutionary questions have provided motivation for the study of insect-plant interactions. At the ultimate level, insect-plant associations can be explained in terms of relative fitness of insects on their host plants and, presumably, natural selection reinforces behavioural preference for suitable plants and avoidance of unsuitable ones (Feeny, 1992). Phytophagous insects may have had a role to play in the evolution of plants, by selecting for diverse chemical and physical defences. Many ecologists believe that the current diversity of both plants and insects is, in part, a result of their co-evolution (refs. in Jermy, 1993). Through an evolutionary "arms race" between plants and insects, the plants have evolved defences to reduce herbivore pressure whilst the insects evolved ways for overcoming some of the defences. This again had consequences in a multitude of animals at higher trophic levels. Others believe that the adaptation and diversity of the insects has tended to follow that of the plants, in this case being less important the influence of insects on the evolution of plants. In other words, it is proposed that the evolution of insect-plant associations results primarily from autonomous evolutionary events, i.e. heritable functional changes within the insects' behavioural and sensorial mechanisms would mediate changes in insect-plant associations, and the ecological factors would play a secondary role by either supporting or preventing the establishment of the new genotype with the novel plant preferences (Jermy, 1993). The study of phytophagous insect behaviour tends to have been neglected, yet the scientific knowledge of the proximate causes of behaviour and their variation is important to answer the evolutionary questions. The behavioural changes are important adaptations to be selected when an insect shifts or widens its host range. Any morphological or physiological changes, potentially adaptive to exploit a new host plant, are inconsequent if they do not occur together with behavioural changes. Thus, knowledge about behaviour and its variation is essential to the major evolutionary questions of insect-plant relationships.

Another important motivation for studying insect-plant interactions is that specialists as well as generalist phytophagous insect species have become economically important as pests on agricultural crops. Increased concern for the environment, the continued need to minimise possible hazards from crop protection agents, and the rapid development of pesticide resistance have placed tremendous demands on research to provide new methods of pest control. These can

only be successfully implemented on the basis of a detailed knowledge about the behavioural mechanisms by which phytophagous insects select host plants.

The host plant selection process consists of a sequence of behavioural responses to an array of stimuli associated with host and non-host plants (Visser, 1986). Plant secondary metabolites are important stimuli in this process. These include a wide variety of organic compounds not directly involved in the primary metabolism of plants (photosynthesis, respiration, biosynthesis of proteins), and some of them have been implicated in regulating chemical mediated interactions with other organisms acting as semiochemicals[1]. Several plant secondary compounds contribute to defense against various organisms, including fungal pathogens, other plants, and herbivorous animals ranging from insects to man, acting as general toxicants or biocides, or may have a specific toxic action directed at a particular target. They may also act as chemical messengers or signals with purely behavioural effect, and in many cases plant chemical defenses have become signals to herbivores for selection of suitable host plants. In phytophagous insects, volatile secondary compounds have been implicated in the process of host plant selection. These are detected at some distance from the plant, affecting insects' orientation during host-finding behaviour. They act as chemical signals that attract the insect to suitable host plants or induce avoidance reactions to unsuitable plants for feeding and/or oviposition.

A large part of insects' olfactory system is thought to be specifically involved in conveying and processing information about plant volatile compounds (Mustaparta, 1992). However, it is scarce the knowledge concerning which volatile compounds or blends of compounds affect the behaviour of phytophagous insects. There is a need to focus research on what are the plant odours (host and non-host) important in host selection of different insect species with different ranges of host plants. Attraction and avoidance are important characteristics of olfaction, and it is therefore important that chemical signals from both host and non-host plants are identified and studied, as regard their behavioural effect on insects and how such chemo-sensorial input is detected and processed.

Semiochemicals that regulate insect-plant interactions have long been seen to have potential for developing new methods of pest management, e.g. through the exploitation of plant

[1] The term "semiochemicals" is used for a variety of compounds that are involved in various chemically mediated interactions: "pheromones" were defined by Karlson and Lücher (1959) to classify the signals used within one species; the term "allelochemicals" is used to classify signals acting between species (Whitaker and Feeny, 1971), and these are further subdivided as "allomones" - chemicals involved in interactions of advantage for the producing species, "kairomones" - those of advantage for the receiving species, and "synomones" - those that mediate interaction that are advantageous for both the emitting and the receiving species (Brown *et al.*, 1970; Nordlund, 1981).

secondary metabolism for modification of insect behaviour, or to develop resistant crop cultivars (Pickett *et al.*, 1991; Hallahan *et al.*, 1992). The resistance of some agricultural crops varieties results in many cases from plant chemical features that have an effect on the insect behaviour, however the resistant plants have often been developed without knowledge about what modifies the behaviour. With a greater knowledge about the mechanisms by which the insects select host plants, many more resistant varieties can be developed.

The eucalyptus woodborer, *Phoracantha semipunctata* Fabricius, is a cerambycid of Australian origin that has a range of host plants restricted to species mainly of *Eucalyptus* (Myrtaceae). It has been introduced to several regions of the world following the introduction of *Eucalyptus* for ornamental purposes or economic exploitation of its wood and essential oils. Whereas this insect species is of no economic importance in Australia, it has become a severe pest of eucalyptus plantations in the introduced regions, including Portugal and several other Mediterranean countries (e.g. Spain, Italy, Marrocco and Tunisia). In Portugal, *P. semipunctata* was detected for the first time in the peninsula of Setúbal in 1980 (Figo, 1981). Since then, the populations have increased dramatically and have spread throughout nearly all eucalyptus plantations of the country, causing significant economic losses for the growers and the pulp paper industry, mainly in the central and southern regions of Portugal.

P. semipunctata males and females fly at dusk and during the night in search for mates and oviposition sites. The females oviposit in bark cracks or under loose bark, preferentially on recently felled or weakened trees. Tree damage is caused by the larvae feeding on the bark. Heavy infestations result in the destruction of cambium and phloem tissues along the trunk leading to the death of the tree (Chararas, 1969; Drinkwater, 1975; Scriven *et al.*, 1986). Current methods of *P. semipunctata* control exploit the extraordinary ability of females to colonise recently felled trees. Log traps for the eggs have been used by *Eucalyptus* growers in order to reduce the beetles' population size of the next generation (e.g. Egea, 1982; Tirado, 1984, 1990). Some authors have suggested a role for the odour of *Eucalyptus* in host plant selection by *P. semipunctata* (Powell, 1978; Hanks *et al.*, 1993); however this has never been thoroughly investigated.

The characteristic odour of *Eucalyptus* results from volatilisation of essential oils[2] that are accumulated in glands distributed more or less abundantly throughout the foliar parenchyma and bark of many *Eucalyptus* species (Chattaway, 1954; Carr and Carr, 1969, 1970, 1976). These

[2] Essential oils are volatile isolates normally obtained from plants by steam distillation or by mechanical processes and, being largely insoluble in water, separate spontaneously from the aqueous phase; many plants synthesise and store such essential oils in specialised secretory glands and ducts (Doran, 1991).

essential oils are composed predominantly terpenes[3], i.e. monoterpenes and sesquiterpenes, produced by a secondary metabolic pathway (Doran, 1991). Traditionally, eucalyptus oils have been regarded as relatively toxic waste products of metabolic processes with limited adaptive value to the plant (Penfold and Willis, 1955), but now it is thought that essential oil constituents are involved in chemically mediated interactions (Doran, 1991), including interaction with phytophagous insects (Ohmart and Edwards, 1991). For example, variation in terpene composition between *Eucalyptus* species and between individual trees has been related to their resistance/susceptibility to the attack of defoliator Christmas beetles, *Anoplognathus* spp. (Edwards *et al.*, 1990, 1993).

Volatile compounds of *Eucalyptus*, as well as those of non-host plant species may influence host-finding behaviour of *P. semipunctata*. Motivated by the potential use of plant volatile compounds in strategies to control *P. semipunctata* populations, this thesis presents research work undertaken to enlighten olfactory mechanisms underlying host-finding by *P. semipunctata* beetles.

Objectives and Thesis Contents

Experimental research was carried out to investigate (1) whether host-finding behaviour of *P. semipunctata* beetles is influenced by eucalyptus odour; (2) which volatile compounds of host and non-host plant species are detected by olfactory receptor neurons on the antennae; and (3) how host and non-host plant odours are encoded by the receptor neurons.

The thesis is divided in four Parts. Part I comprises two chapters providing a synthesis of the background of knowledge relevant to the experimental work. Chapter 1 is a review on mechanisms underlying host finding behaviour of phytophagous insects, and on neurophysiological aspects of olfaction in insects. Chapter 2 is a synopsis on the biology of *P. semipunctata*. This chapter includes the taxonomic position of the species, its distinguishing biological characteristics, the geographical distribution, the range of host plants, and current and future methods of pest control. Part II comprises four chapters, describing the experimental work carried out. Each chapter is divided into four sections: introduction, materials and methods, results, and discussion. Chapter 3 (the first chapter of Part II) describes a field experiment

[3] This is a generic term that designates one of the largest and biologically most important classes of natural products, which exhibit a remarkable structural and functional diversity, especially in view of their common origin from the same C5 isoprene units (Mabry and Gill, 1979).

conducted to test whether *P. semipunctata* males and females are attracted to the host plant. Recording of electroantennograms was carried out to test whether the beetles' olfactory receptor neurons detect volatiles emanating from eucalyptus. Chapter 4 describes a wind tunnel assay to study the beetles' orientation behaviour (locomotion and flight) to plant odour sources. Chapter 5 describes the use of gas chromatography linked with electroantennogram recordings for identifying components of volatile blends of host and non-host plant species that are detected by *P. semipunctata* receptor neurons. Analytical chemistry techniques, including mass spectrometry, were used to identify compounds that elicit electrophysiological responses. Chapter 6 investigated the encoding of plant odours by receptor neurons in the antennae of *P. semipunctata* using gas chromatography linked with single-cell recordings. Part III is comprised of a single chapter (chapter 7) providing a genearl discussion of the integrated results, and points out future research targets aiming at the development of pest control strategies of *P. semipunctata* based on plant semiochemicals. Part IV lists the bibliographic references in the thesis. At the end, an appendix shows mass spectra of compounds in volatile blends of host and non-host plants that elicited electrophysiological responses.

PART I

SCIENTIFIC BACKGROUND

1.

Host-Finding Behaviour in Phytophagous Insects - A Review of Proximate Causes and Neurophysiological Aspects of Olfaction

> "...the plant world is not coloured green; it is coloured morphine, caffeine, tannin, phenol, terpene, canavanine, latex, phytohemaglutin, oxalic acid, saponin, and L-dopa (Janzen, 1978). To a slug, beetle, or hare, a forest or meadow is a many-textured landscape of scents and tastes, the distinction of which may be a life and death matter (Howe and Westley, 1988)..."

1.1. Introduction

The host selection of phytophagous insects depends on their behavioural responses to plant features. During the host plant selection process in a certain habitat, the insect will first be able to detect and locate its host from a distance and then, having arrived at the plant, it must confirm the appropriateness of that plant for feeding and/or oviposition. This process consists of a sequence of behavioural responses to an array of stimuli associated with host and non-host plants (Visser, 1986), and it can be divided arbitrarily as **host finding** and **host recognition** (acceptance or rejection) (Miller and Strickler, 1984), including several behavioural steps: orientation, landing, probing, feeding and/or oviposition. The stimuli involved include visual, mechanical, olfactory, and gustatory characteristics of the plants (Städler, 1976, 1992). The visual and olfactory cues are usually associated with long-range host finding, whilst host plant recognition is based mostly on the other two types of stimuli once the insect has contact with the plant. Figure 1.1 represents a simplified sequence of behavioural events leading a generalised insect to feed or oviposit on a host plant. It is presented for convenience of the reader, and does not pretend to cover all examples of phytophagous insects nor every behavioural mechanisms that may be involved in the response to host or non-host plant stimuli. The scheme is restricted to flying insects in a physiological state of readiness to respond to the host plant stimuli. An insect can progress through the steps, remain for an extended time at a step, or regress to any prior step in the sequence if conditions do not stimulate progression. Rather than emphasising the

uniqueness of a stimulus eliciting a given response, figure 1.1 suggests overlap of the stimuli eliciting the range of behaviours. From left to the right in the scheme, there is an increase in: 1) number of sensory modalities stimulated (e.g. vision, olfaction, gustation), and 2) stimulus strength. The term "find" has been used to describe the phenomenon of insects arriving near or on a resource (Miller and Strickler, 1984). Here, the term is used in the sense described by Miller and Strickler (1984), meaning to behave and maintain proximity to something, sensed by the finder's nervous system, that was previously apart and of undetermined location.

An insect, flying around the host plant habitat, eventually detects non-contact host related features such as olfactory and/or visual cues. An oriented movement is initiated leading the insect to the host plant. When approaching, the higher intensity of the external stimulation (chemical and physical) increases the probability of the insect maintaining an oriented movement that ends with landing on the host plant. Once on the plant other contact sensory modalities take part in the process of host recognition. The olfactory stimulation reaches its highest intensity, and probing takes place by detection of gustatory and mechanical characteristics of the plant which may lead to full acceptance as suitable site for feeding and/or oviposition. Non-host plants may also provide a multitude of sensory cues for the insect, which may act at any step, e.g. olfactory cues may induce avoidance at some distance from the source, or rejection may occur upon reception of contact sensory stimulation. A phytophagous insect may reject as well a unsuitable specimen of the host species, providing that it does not receive enough stimulation to continue the process of host selection or it receives unambiguous information reflecting the inappropriateness of the plant specimen. Host plant selection involves not only choosing the right species, but also selecting an individual plant within that species that is suitable for feeding and/or oviposition.

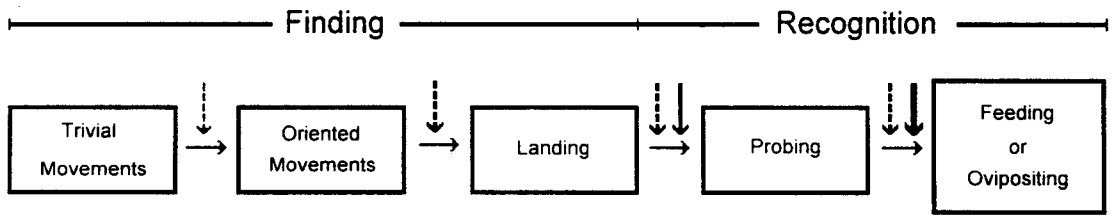


Figure 1.1. Schematic overview of behavioural events leading a generalised flying insect to feed or oviposit on a host plant - host selection. Broken and solid downward arrows indicate non-contact and contact sensory cues, respectively (modified from Miller and Strickler, 1984).

In phytophagous insects, the narrow host ranges observed is thought to depend on the plants chemical profile (Bernays and Chapman, 1994). Plant volatile compounds have been shown to be of primary importance in the host selection of many phytophagous insects, and large part of their olfactory system is thought to have evolved to detect and process information about plant volatiles (Mustaparta, 1992).

Olfaction plays a major role in the control of important behaviours in insects. Orientation and movement toward mating partners, appropriate sites for oviposition and sources of food involve in many cases olfactory signals that initiate, sustain and guide the behaviour. These olfactory signals are pheromones and plant odours transported by the wind. Both pheromones and plant odours are chemical messages comprised of a blend of volatiles. Insect pheromones consist of mixtures of several compounds of specific isomers in a species-specific ratio. The blend composition of plant odours is more complex, to a large extent comprised of unspecific chemically diverse compounds present in varied concentrations across plant species and across individuals of the same species (cf. Visser, 1986; Bernays and Chapman, 1994). Downwind of the odour source insects of one species respond to these chemical messages by walking or flying upwind towards the source. Other insect species do not respond in this way to the same chemical message.

The olfactory system of insects is well adapted to recognise and discriminate with sensitivity and accuracy a wide range of different odours. The detection of odours is largely made by numerous receptor neurons with various degrees of specialisation associated with olfactory sensilla located on the antennae. These receptor neurons encode the intensity and quality of odourants into patterns of afferent neural signals conveyed to the brain. Here, the olfactory information is further processed and integrated with other sensory modalities (e.g. vision) by complicated networks of interneurons which are connected with the motor system controlling the insect's behaviour (cf. Boeckh and Ernst, 1983; Boeckh, 1984; Visser and De Jong, 1988; Homberg *et al.*, 1989; Masson and Mustaparta, 1990; Christensen *et al.*, 1996; Hildebrand, 1996a,b).

Three major question in olfaction are: (1) Which are the odourants of biological significance for the animal, (2) how is the odour molecule able to elicit reactions in a receptor neuron, and (3) how do the receptor neurons and central neurons encode the odour information? These questions have been better answered with respect to insect pheromones. These are the odourants that have been predominantly studied, and the research on behavioural and neurophysiological mechanisms of insects' response to pheromones is therefore ahead of research on plant odours. This research on insect pheromones has been partially encouraged by

the potential of its use in insect pest control (Ridgeway *et al.*, 1990). Whereas insect pheromones are identified in numerous species (Arn *et al.*, 1992) and their use in pest control has been established, the identification of plant derived products as insect odourants has only just started.

Below, focus will be put mainly on the importance of olfaction in host finding behaviour of flying insects and what are the neurophysiological mechanisms underlying insects' olfaction.

1.2. Plant Volatiles Implicated in the Host Finding Behaviour

Plants produce a wide variety of secondary compounds that have been implicated in regulating chemical mediated interactions with other organisms (cf. Rosenthal and Janzen, 1979; Bell, 1981; Harborne, 1988; Howe and Westley, 1988; Rosenthal and Berenbaum, 1992). Some secondary compounds are classified by chemical function, e.g. alkaloids which are heterocyclic molecules containing nitrogen or the plant phenolics and glucosinolate derivatives like the sulphur-containing compounds termed isothiocyanates. Other compounds, with widely varied functionality, are classified by terms that relate to their biosynthesis, e.g. isoprenoid derivatives (terpenes) and non-lipid plant fatty acid derivatives (so called "green leaf" volatiles).

Although many plant secondary compounds are known to be toxic to some insects, often blocking specific biochemical reactions beyond a low threshold of concentration[1], many phytophagous insects have evolved ways to overcome some of the plant chemical defences. Generalised enzyme systems[2] provide protection against an enormous variety of plant toxins, and some insects seem to have evolved specific detoxification mechanisms for toxins of their host plants (Howe and Westley, 1988), and in many instances volatile secondary compounds became important chemical cues in host finding behaviour.

Leaves and other plant parts release complex mixtures of volatiles from the wax, stomata, and other structures, such as glands and trichomes, and the olfactory systems of insects detect some of these plant volatiles (Visser, 1986; Städler, 1992). According to their distribution in the

[1] For example, the toxic effects of alkaloids include inhibition of DNA and RNA synthesis (caffeine), inhibition of mitosis (colchicine), stimulation of ribosome breakdown (mescaline), and breakdown of membrane compartmentation (tomatine). Another important class of compounds that includes chemicals with toxic effects are the terpenoids. For example, the pyrethroid monoterpenes found in species of *Chrysanthemum* (Asteraceae) are effective insecticides, and other monoterpenes have been implicated in the resistance of cedar and conifers to insect attack. The toxicity of some monoterpenes is due to an inhibition of respiration in mitochondria, whereas for others it is unknown the toxicity mechanism.

[2] Mixed-function oxidases (MFO) are membrane-bound enzymes that detoxify a wide variety of plant and synthetic poisons (Brattsten, 1979). The capacity for insects to develop MFO resistance to novel pesticides is of tremendous concern in agriculture and public health. The MFO systems that have evolved for neutralizing natural plant products preadapt many crop pests for developing resistance to synthetic pesticides (Brattsten *et al.*, 1986).

plant kingdom, these secondary compounds can be broadly divided in two groups (Visser, 1986, Hallahan *et al.*, 1991): a) chemicals that occur commonly and are derived from biosynthetic pathways present throughout the plant kingdom; and b) more restricted chemicals that are found in only a few related plant species. Examples of odourants included in the first group are the "green leaf" volatiles that comprise a group of six-carbon alcohols, aldehydes and ester derivatives produced from unsaturated fatty acids[3], fermentation products like ethanol and ethyl acetate, and terpenoid compounds derived from polymerisation of isoprene units. Some plant specific odourants are the isothiocyanates (e.g. allyl-isothiocyanate) which are volatile catabolites of the non-volatile glucosides characteristic of the Cruciferae (Finch, 1978, 1980; Visser, 1986; Blight *et al.*, 1995), the disulphides (e.g. n-propyl disulphide) produced from dipropyl sulfinate by the Liliaceae (e.g. onions) (Städler, 1992), and the phenylpropanoids derivatives (methyleugenol and asarone) in carrots (Guerin *et al.*, 1983). Lists of referenced examples of general and specific plant odour components that elicit behavioural responses in phytophagous insects are found in Visser (1986), Städler (1992), and Bernays and Chapman (1994).

1.3. Finding the Host Plant from a Distance

Due to their importance in host finding and host recognition, the chemicals produced by plants were classified according to their behavioural effect on insects as defined by Dethier, Barton Browne and Smith in 1960:

- **attractant** - a chemical that causes an insect to make oriented movements towards the source of the stimulus
- **repellent** - a chemical that causes an insect to make oriented movements away from the source
- **feeding or oviposition stimulant** - a chemical that elicits feeding or oviposition
- **deterrent** - a chemical that inhibits feeding or oviposition.

Notice that attractants and repellents have an influence on the orientation of the insect, and they can be effective at some distance from the plant, whereas the other two functional definitions

[3] Examples - alcohols: 1-hexanol, cis-3-hexenol, trans-2-hexenol, 1-octanol, 2-heptanol; aldehydes: hexanal, propanal, butanal, trans-2-hexenal, cis-3-hexenal; ketones and esters: 3-pentanone, butanone, 4-heptanone, cis-3-hexenyl acetate.

apply to compounds that act when an insect is on the plant, probing or touching; a deterrent compound may cause an insect to stop feeding, but it does not cause the insect to move away from the plant. This is a functional classification based on each insect's biology. Not all known secondary plant metabolites present in the host plants of an insect *a priori* have the expected effect of attracting or stimulating feeding in adapted specialists (monophagous and oligophagous) or repelling or deterring most generalists (Sätdler, 1992). A compound that is attractant or stimulates feeding on one insect species, maybe a repellent or a deterrent to another species. In order to understand the effects of particular compounds it is necessary to evaluate them individually.

Our knowledge on the chemistry of plant odours is still very incomplete, nevertheless the research work undertaken so far on host plant selection behaviour and its sensory aspects, have shown that insect responses to classical sign-stimuli came to be viewed as relatively extreme situations, where one compound or groups of compounds constitute "key" stimuli. More typically, effective behavioural stimulation that leads to host finding and host recognition requires mixtures of several compounds, i.e. a multicomponent chemical message where the individual components may not elicit significant behavioural activity alone (Feeny, 1992). The individual plant odourants often interact with each other to produce a behavioural response, and these effects within the insects are not necessarily additive. Synergistic effects among the components of plant odour blends have been shown in several studies. Phenylethyl propanoate, eugenol and geraniol show synergism in trap catches of the Japanese beetle, *Popillia japonica* (Ladd, 1980). The mixture of fatty acid esters released by apple has a weak attraction on *R. pomonella* flies, and the acetate components have no effect; however the mixture of both fractions is as highly attractive as the crude apple extract (Fein *et al.*, 1982). The "green leaf" volatiles have also been shown to synergize the effect of aggregation pheromones in several beetles (Blight *et al.*, 1984; Dickens, 1989; Dickens *et al.*, 1990).

Specific host odour blends of ubiquitous plant volatiles. Several examples in the literature suggest that monophagous or oligophagous insects are attracted to their host plants by an odour blend comprised of specific ratios of generally distributed plant odourants. The green leaf volatiles (i.e. (E)-2-hexenal, (Z)-3-hexenyl acetate, (Z)-3-hexenol and (E)-2-hexenol) are important components of the odour that attracts the Colorado potato beetle, *Leptinotarsa decemlineata*, to its host plant (Visser and Avé, 1978). However, wind tunnel studies showed that none of these odourants have an attraction effect alone, and when added to potato leaf odour they prevented the beetles from locating the host plant. This suggested that the attraction of the Colorado beetle is dependent on specific ratios of certain green leaf volatiles in the host plant

odour. Another example, where the attraction of the insect is dependent on the ratio of certain non-specific compounds in the host plant is the case of the red sunflower seed weevil, *Smicronyx fulvus*. As shown by Roseland *et al.* (1992), effective attractiveness of baited field traps to the beetle is only achieved by a mixture of five ubiquitous terpenoids (α-pinene, β-pinene, camphene, limonene, bornyl acetate) in the ratio they occur in the air above susceptible sunflower cultivars. In the case of the damson-hop aphid, *Phorodon humuli*, a mixture of (E)-2-hexenal and β-caryophyllene in the natural ratio was more attractive than the aldehyde alone (Campbell *et al.*, 1993).

Specific host odour blends of specific plant volatiles. For some phytophagous insects it has been shown that attraction is to plant specific compounds. Host-specific phenylpropanoids (e.g. trans-asarone) are involved in the attraction of the carrot fly, *Psila rosae*, to its host plant (Guerin *et al.*, 1983). Crucifer specialists such as the mealy cabbage aphid, *Brevicoryne brassicae*, and the turnip aphid, *Lipaphis erysimi,* are attracted by isothiocyanate catabolites of glucosinolates (Nottingham *et al.*, 1991). Onion flies, *Delia antiqua*, are attracted to sulphur-containing volatiles (disulphides) characteristic of the Liliaceae (Ishikawa *et al.*, 1978; Pierce *et al.*, 1978). Another specialist of some Liliaceae, the leek moth (*Acrolepiopsis assectella*), reacts to the precursor of the disulphides (dipropyl sulfinate) which exists in the air around the leek plants (*Allium porrum*) (Städler, 1992). Generally distributed plant volatiles may synergize the effect of specific odourants as has been suggested to explain the higher attraction of *P. rosae* to traps containing a host specific compound (trans-asarone) and hexanal (Guerin *et al.*, 1983).

Most examples in the literature report monophagous and oligophagous insect species being attracted by the odour of their host plants, but polyphagous species are also attracted by host plants odour. In two polyphagous noctuid moths, *Heliothis virescens* and *Trichoplusia ni*, it was shown that host related volatiles[4] are involved in host finding behaviour (Mitchell *et al.*, 1991; Heath *et al.*, 1992; Tingle and Mitchell, 1992; Landolt, 1993).

In sum, plant odours providing cues for host location of phytophagous insects can be divided into odour blends comprised of 1) compounds specific of certain groups of plants and not found in unrelated plant species, 2) compounds that are generally distributed among the plant kingdom, but present at certain ratios in the host plants, and 3) combinations of general and more specific plant volatiles. In most of the cases where plant odours were implicated in the host finding behaviour of phytophagous insects it is not known the entire set of odourants responsible for the attraction.

[4] Host related odourants means volatile compounds produced by the host plant, but also by other plants that are non-hosts.

1.3.1. How are flying insects able to orient and find a distant odour source ?

Städler (1992), reviewed the literature on whether insects are attracted over distance to their host plant and if so, from how far". Although this question was asked long before by Thorsteinson (1960), conclusion was reached that, in contrast to sex pheromones, we have little idea how widespread distant olfactory responses to plant odours are in nature, and their occurrence cannot be taken by granted. He states, "from the evidence available, it can be concluded that odour-modulated optomotor anemotactic flight to host plants is in the order of 10 m at most".

The dispersion of odour molecules in the air has been studied, and this was reviewed as concerns its implication in the orientation of flying insects to pheromone sources (Cardé, 1984; Elkinton and Cardé, 1984; Murlis, 1986; Murlis *et al.*, 1992). Because of air turbulence, concentration gradients of plant odours are unlikely to exist more than a few centimetres away from a plant. Instead, the air coming from a plant contains pockets of odour carried downwind in a mass of "clean" air, so that an insect at some distance from the plant will perceive a series of bursts of odour separated by periods without odour. The concentration of odour within a pocket, or burst, is very variable and, even some distance downwind from the source, some pockets will still carry high concentrations. The frequency of bursts tend to decrease with the distance from the source. Consequently, although on average the odour concentration measured over a period will be lower at greater distances from the plant, there is no odour gradient which the flying insect could follow to reach the plant.

The present knowledge on orientation mechanisms of flying insects to odour sources is mostly based on the study of male moths orienting to sex pheromone sources (e.g. Kennedy *et al.*, 1981, 1983; Kuenen and Baker, 1982, 1983; Willis and Baker, 1984; Baker, 1986; Vickers and Baker, 1994). These studies have shown that flight behaviour towards the odour source involves a complex integration of visual and olfactory cues, an odour-modulated optomotor anemotaxis mechanism that was first demonstrated to occur in insects by Kennedy (1940). Once airborne, there are no mechanosensory means by which the insect can tell the direction of movement of the airmass which it is in. It will use vision. An insect flying forward in still air will perceive images moving over the eye from front to back at moderate speed. If it is flying into wind, it will continue to make headway provided its airspeed exceeds the speed of the wind. The insects examined increase their airspeed so as to maintain a more or less constant speed over the ground (groundspeed), as determined by the rate of image movement across the eye. This type optomotor reaction enables the insects to compensate also for directional drift, and they can

maintain orientation to the wind at any angle, not just directly up- or downwind (Cardé, 1984; Murlis *et al.*, 1992; Vickers and Baker, 1994). Ultimately, if the windspeed is too high, and the insect is unable to compensate, it will be blown backwards. In this case, the movement of images across the eye will be from back to front, and the insects usually land or turn to fly with the wind. In night flying insects the eyes are adapted to maximise the use of the available light at the expense of acuity (Bernays and Chapman, 1994). Wind tunnel observations showed that male moths following a sex pheromone plume fly upwind in a zigzag pattern (Kennedy *et al.*, 1981; Kennedy, 1983). This pattern of movement is programmed into the insect's nervous system and continues as long as the insect is stimulated by the odour. If it looses contact with the odour, the insect directs its flight more across the wind direction, and if it fails to remake contact with the odour, it ceases to make progress upwind and may turn to fly downwind.

The use of optomotor responses during host finding is probably widespread amongst phytophagous insects. Kennedy (1977) concluded that "odour-induced optomotor anemotaxis" was the only behavioural mechanism involved in distant orientation of flying insects to the host plant, and so far, all the evidence collected by different investigators supports this conclusion (Städler, 1992). Direct evidence was provided by wind tunnel observations on females of *Manduca sexta* showing that they use an optomotor response as they zigzag upwind towards an host plant (Willis and Arbas, 1991), in the same way as when male moths are following a female sex pheromone plume.

1.3.2. What is the role of visual features of the plant ?

Synergism between chemical and visual/physical cues have been recognised for several decades (refs. in Kennedy, 1977). Visual attraction might result from responding to the colour or the form of the host plant, but because these features vary so greatly within a plant species, visual responses often only synergize with the appropriate olfactory stimulus, and do not constitute by itself a reliable cue for host finding (Bernays and Chapman, 1994). Vision may be of importance previous to the landing of the flying insect on the plant. Harris and Miller (1984), showed that oviposition by females of the onion maggot, *Delia antiqua*, is markedly greater in the presence of onion chemicals and a yellow glass model "onion stem" than in the presence of either the chemical or visual/physical stimuli alone. In the black swallowtail butterfly, *Papilio polyxenes*, the number of landings made by females and the number of eggs laid on artificial leaves was greatly enhanced by the presence of carrot volatiles (Feeny *et al.*, 1989). This occurred even

when the odour pervaded the whole cage in which the experiments were carried out, suggesting that the landing responses were probably also associated with the visual stimulus. Shape of objects may interact with odour in the attraction of insects as demonstrated in the apple maggot fly, *Rhagoletis pomonella* (Bernays and Chapman, 1994).

In sum, attraction to a host plant from a distance involves both olfaction and vision. The olfactory signal indicates the presence of the appropriate host plant, and the subsequent orientation behaviour that leads to host finding is the result of integration of both plant odour and visual stimuli.

1.4. Discrimination Between Host and Non-host Plants from a Distance

Investigators observing the orientation of flying phytophagous insects, both in the field and in the laboratory, report that many species are unspecific in their landing on plants, landing on both hosts and non-hosts. Non-host plants are often approached and often rejected only after contact, and it has been suggested that there is a major role for the presence/absence of deterrents on the discrimination between suitable and unsuitable host plants (Chapman and Bernays, 1989; Bernays and Chapman, 1994). The large number of plant compounds that have been shown to be deterrents, together with many reports identifying receptor cells sensitive to deterrents, question the general role of discriminative distant olfactory responses to host and non-host plant volatiles in phytophagous insects (Städler, 1992). A strong random element has been suggested in the host finding behaviour of several phytophagous insect species (Feeny, 1992; Bernays and Chapman, 1994). For example, there are two theories on how bark beetles[5] find the suitable host trees (Byers 1995): 1) they locate such trees by oriented movements from a distance mediated by volatiles released from damaged or diseased host tree species (primary attraction); 2) they encounter the suitable host tree at random, and host selection occurs after landing on a tree where host recognition takes place by short-range olfaction and/or gustation. The two theories are not mutually exclusive, and one or the other may operate primarily in a particular species (Byers, 1995). For example, in *Dendroctonus brevicomis* and *Ips paraconfusus*, two important pests of *Pinus ponderosa*, it has been shown that adults' mass attack (aggregation), attack density (intraspecific competition) and termination of aggregation is mediated by a complex intraspecific

[5] The bark beetles (order Coleoptera: family Scolytidae) comprise a taxonomic group of dendrophagous species that look similar although they differ widely in their ecology and biochemical adaptations (Byers, 1995).

chemical communication system, involving pheromones synthesised from ingested host plant secondary compounds (e.g. α-pinene and myrcene) (Byers, 1989).

Females of *D. brevicomis* and males of *I. paraconfusus* are pioneer beetles, initially choosing the host tree on which the mass attack might take place, without being attracted by any aggregation pheromone. These pioneer beetles are unspecific in their landing responses, landing on both host and non-hosts (Moeck *et al.*, 1981; Wood, 1982). Thus, host finding is suggested to be a random process, and host selection occurs after landing on the bark, where the probability of remaining on the tree is thought to be influenced by the presence of non- or low-volatile compounds. Species of bark beetles that attack and kill living trees (termed aggressive) have been shown invariably to make use of aggregation pheromones, but are weakly if at all attracted by host volatiles alone (Byers, 1995).

However, the so-called secondary bark beetle species[6], including species in the genera *Scolytus*, *Dendroctonus*, *Hylurgops*, *Trypodendron* and *Tomicus*, may not use aggregation pheromones, and are generally attracted to either host monoterpenes, ethanol, or a combination of both (Byers, 1995). For example, *T. piniperda*, which seems to produce no aggregation pheromone, was shown to be strongly attracted by a mixture of monoterpenes (terpinolene, α-pinene and 3-carene), volatilizing from wound oleoresin of Scots pine (Byers, 1989), and it was shown that this bark beetle is able to detect the unsuitability of host trees in flight (Byers *et al.*, 1989).

To discriminate between plants from a distance an insect may be attracted to odours from the suitable host plants, it may not respond to odours from non-hosts or it may be repelled by them. In contrast with the relative large amount of experimental work showing host plant odour blends attracting insects, there are very few reports on the identification of repellents or repellent odour blends associated with non-host plants. Since repellents are "chemicals which cause the insect to make oriented movements away from its source", their action is probably restricted to close range, and it is difficult to demonstrate their presence in field experiments. It has been shown that odours released from non-host plants may interfere with the orientation of insects to the host plants, inhibiting their attraction (refs. in Pickett *et al.*, 1991; Hallahan *et al.*, 1992; Städler, 1992). It is inherently more difficult to isolate repellents and inhibitors used in avoidance behaviour of phytophagous insects than to isolate attractants, since tests of avoidance require one to first isolate the attractive host odours and then present these with and without the potentially inhibitory non-host odours.

[6] Secondary species is a term used to group bark beetles that arrive later after the tree has already been killed by the aggressive species or that feed as saprophytes in decaying trees (Byers, 1995).

Several authors report that the population of an insect pest is reduced in vegetationally diverse agricultural crops, and this is particularly noted to affect specialised phytophagous insects. A common suggestion from such observations is that the chemical complexity of plant odours in mixed crop systems interferes with the insects' chemo-orientation behaviour to the host plant (Perrin, 1980; Cromartie, 1981; Stanton, 1983; Price, 1984). A good example of this is the case of the flea beetle, *Phyllotreta cruciferae*, which is more abundant on collards grown in monocultures, than on stands of collards interplanted with tomatoes and tobacco (Tahvanainen and Root, 1972). The mechanisms of the masking effect of host plant odours have been most thoroughly studied in the laboratory for the Colorado potato beetle, where the odour-conditioned anemotaxis towards the potato odour is distorted by artificially changing the ratios of some individual "green leaf" volatiles (Visser and Avé, 1978) and by mixing the host plant odour with odour released from non-host plants (e.g. tomatoes or cabbage) (Thiery and Visser, 1986, 1987).

These mechanisms have been investigated also in aphids, and evidence is accumulating for the role of non-host plant volatiles as agents capable of masking host kairomones and repellents (Pickett *et al.*, 1992). For example, in the damson-hop aphid, a slow chemical release of isothiocyanates in the hop crop (*Humulus lupulus*) decreased colonisation by spring migrants of *P. humuli* (Pickett *et al.*, 1991).

In other cases, laboratory bioassays have shown the existence of non-host plants repellent odour. The black bean aphid, *Aphis fabae*, is repelled by the odour of the summer savory (*Satureja hortensis*) which also inhibits the attraction to the host plant (beans - *Vicia faba*), and the cabbage aphid (*Brevicoryne brassicae*) is repelled by the odour of tansy (*Tanacetum vulgare*) which, according to the concentration, also has a repellent effect in combination with the odour of the host plant (brussel sprouts - *Brassica oleracea*) or shows an inhibitory effect on the attraction to the host (Nottingham *et al.*, 1991). In the same study, it was reported that single isothiocynates (4-pentenyl-, 3-butenyl- and allyl- isothiocyanates) induce repellence in *A. fabae* or inhibited its attraction to the host plant (beans). Also more generally distributed plant volatiles, i.e. methyl salycilate and myrtenal[7], are repellents for the black bean aphid and inhibit the attraction to the host plant (Hardie *et al.*, 1994).

The existence of repellents associated with unsuitable plants is not confined to phytophagous insects with a narrow range of host plants. Also in polyphagous moths, it was shown that repellents may be involved in the discrimination between host and non-host plants.

[7] Methyl salicylate is the methylated metabolite of salicylic acid, a systemic plant component inducing a range of defence mechanisms (Pettersson *et. al.* 1994), and it is present in several groups of plant species, e.g. Poaceae, Rosaceae and Salicaceae (Pettersson *et al.* 1994; Hardie *et al.*, 1994); and myrtenal is a component of the resins produced by gymnosperms (Hardie *et al.*, 1994).

The cabbage looper, *T. ni*, which is attracted by the steam distillates of susceptible soybean plants, whereas those from a resistant variety repelled them (Khan *et al.*, 1987).

1.5. Detection and Coding of Odours

1.5.1. Detection

Most olfactory receptor neurons (RNs) are located on the antennae of insects, associated with sensilla, the walls of which possess many pores (Zacharuk, 1980, 1985). According to the outer structure, many types of olfactory sensilla have been described (refs. in Masson and Mustaparta, 1990), including the hair-like sensilla trichodea (long hairs) and sensilla basiconica (short hairs). One or more RNs are associated with each olfactory sensillum, the dendrites of which project into the hair lumen filled with the sensillum lymph. After adsorption on the cuticular surface, the odour molecules diffuse through the pores and reach the receptor/acceptor sites in dendritic membranes of RNs.

The conduction system of the odour molecule has been most thoroughly studied in the sensilla trichodea of silk moths, *Antheraea* spp., with associated RNs specialised in the reception of sex pheromones (Kaissling, 1987; Masson and Mustaparta, 1990). The odour molecules reach the dendrite branches by diffusion through pore tubules found to make connections between the pores on the sensillum wall and the dendritic membranes or these may be reached directly through the lymph. In the lymph of sensilla trichodea and sensilla basiconica, pheromone binding proteins (PBPs) and general odourant binding proteins (GOBPs), have been shown to be involved in the transport of odourants to the membrane receptors in the dendrites (Vogt and Riddiford, 1981; Laue *et al.*, 1994), as suggested for the olfactory systems of several vertebrates (Pelosi, 1990, 1994). Kaissling (1996) describes the present state of theory about the perireceptor events[8] in a moth olfactory sensilla, where the PBPs are thought to have an important role on the delivery of the pheromone molecules to the dendritic membrane-bound receptor proteins[9], and on the subsequent inactivation of the pheromone molecule, which involves enzymatic degradation to form an inactive metabolite.

[8] Perireceptor events are processes that influence the entry, exit or residence time of odourant molecules in the vicinity of membrane receptors (Getchell *et al.*, 1984).

[9] Prestwich *et al.* (1995) reported that two PBPs, in the moth *A. perneyi*, have diffrent affinities for two pheromone components, and a model was proposed where PBPs encode ligand specificity and present the pheromone ligand to a G-protein coupled receptor where the interactions from both the bound ligand and ligated PBP would be required for receptor activation. A similar model was envisaged for vertebrate OBPs (Pelosi, 1994).

1.5.2. Specificity of receptor neurons

The specificity of the RNs is thought to be determined by dendritic membrane-bound proteins as in other systems involving the reception of chemicals (Satelle *et al.*, 1980), where the binding between the odour molecule and the membrane receptor should lead to a conformational change of the receptor into an active state, and subsequent opening of ion channels (Masson and Mustaparta, 1990). The more generalist RNs, sensitive to many chemically diverse odourants, may have different types of membrane receptors, whilst more specialised RNs, tuned to a specific compound or to a narrow range of stimulatory compounds similar in chemical structure, may have only one type of membrane receptor (Masson and Mustaparta, 1990). Breer (1994) describes two alternative principles for odour recognition by analogy with colour vision and with the large antibody repertoire of the immune system: the specificity of odour detection may be based on only a few receptor types, each reacting with a wide range of odourants; or, there might be thousands of distinct receptors, each specialised for one or a small number of odourants. In the latter case much of the discrimination between odours may occur in the periphery and thus alleviate input processing in the brain. Unravelling the nature as well as the diversity and specificity of receptors for odourants is therefore crucial to the understanding of olfaction.

1.5.3. Transduction

Transduction includes processes leading from the recognition of the stimulus to the generation of electrical responses (Kaissling, 1987). The biochemistry of the transduction process of odourants by the RNs is not yet fully understood. In attempt to detect receptor proteins, the dendritic membranes have been isolated and membrane structural units (3 nm) with a density of 30,000/μm^2 have been found, which were suggested to represent protein molecules having a receptor function (Kaissling *et al.*, 1985). Membrane receptors of olfactory RNs have not yet been isolated and identified biochemically in insects. Kaissling (1996) provides a summary review of the research results on the transduction mechanisms in pheromone-sensitive RNs of moths. It has been shown that the activation of the membrane receptor(s) initiates a sequence of production of different second messengers (e.g. IP_3, cAMP, cGMP) acting on several types of ion channels, which leads to increased ion conductance of the dendritic membrane. Ion channels can also be opened directly via G-protein coupled receptors (Brown and Birnbaumer, 1990).

In a review paper on the recognition and transduction of olfactory stimuli in vertebrates, Breer *et al.* (1994) present the notion that two alternative pathways of second messenger cascades are implicated in the chemo-electrical transduction and may co-exist within the same RN. Both pathways are initiated by the activation of G-protein coupled receptors. In one case, receptor-ligand interaction increases the production of cAMP, which in turn activates cyclic nucleotide gated cation channels. In the other case, the activation of the membrane receptor enhances the hydrolysis of phosphatidylinositol producing IP_3, which is supposed to target IP_3-gated cation channels. The existence of two alternative transduction cascades might be related to excitatory or inhibitory responses of RNs, as was demonstrated for the olfactory cells of lobsters, where a cAMP-pathway mediates hyperpolarisation of the cell membrane and inhibitory responses, whereas the IP_3-pathway leads to depolarisation and excitatory responses (Fadool and Ache, 1992).

1.5.4. Intensity coding

The RN message relevant for the insect is a sequence of nerve impulses with frequency dependent on the stimulus intensity. In general, olfactory RNs exhibit spontaneous activity (i.e. a spontaneous firing of action potentials), the frequency of which can be increased or decreased by an odour stimulus[10] (Kaissling, 1987). Activation of the receptor molecules leads to a change in the ion conductance of the dendritic cell membrane, producing local depolarisation or hyperpolarisation (receptor potential) which spreads passively (electrotonically) towards an electrically-sensitive region of the cell membrane where impulses are elicited or, conversely in the case of hyperpolarisation, the resting impulse activity is suppressed (Kaissling, 1987).

At low stimulus intensities the rate of nerve impulses remains constant throughout the stimulus duration (tonic responses). At high stimulus concentration, an initial burst of impulses lasting a fraction of a second is followed by a lower level of firing that may remain constant for minutes depending on the stimulus duration (phasic-tonic). The dynamics of impulse response as well as of receptor potential vary among different RN types even within the same sensillum (Kaissling, 1987). Within the natural environment insects encounter frequent and extensive changes of odour concentration, and it has been found that repetitive odour pulses elicit more upwind orientation to pheromone sources than constant odour concentrations (Kramer, 1986).

[10] Without odour stimulation, a spontaneous activity of 0.086 nerve impulses per s was observed in a total of 1070 measurements from RNs in the moth, *Bombyx mori* (Kaissling, 1987).

The frequency of odour pulses decreases with the distance to the odour source (Murlis and Jones, 1981), therefore the function of the phasic peak of nerve impulses at higher stimulus concentration may be to improve the resolution of frequent odour pulses closer to the source (Kaissling, 1987).

1.5.5. Quality coding

In early studies on the responses of single RNs, the conclusion was reached that receptor neurons could be classified as either specialists or generalists (Boeckh *et al.*, 1965). Specialists are narrowly tuned to the reception of one or a few compounds similar in chemical structure, whereas generalists are broadly tuned, responding to many, chemically diverse compounds. The research on the specificities of pheromone-sensitive RNs, as determined by the dose-response relationships to pheromone components, chemical analogues or other relevant compounds, has revealed that pheromonal information is, in general, received by RNs narrowly tuned to one component - specialists - (e.g. Visser and De Jong, 1988; Kaissling, 1996; Mustaparta, 1995, 1996a; Hildebrand, 1996b).

It was common to classify RNs sensitive to plant/food odours as generalists on the basis of electrophysiological studies of responses to synthetic compounds. However, a RN may respond to many compounds at high concentrations that may not necessarily be of biological significance (Mustaparta, 1996b). Nevertheless, interesting results came out of investigations with a wide variety of synthetic compounds some of which are biologically relevant (e.g. Mustaparta, 1975; Sass, 1978; Selzer, 1981; Kafka, 1987), suggesting that despite the considerable overlap between the response spectra the plant/food odour RNs may be highly selective. This led to a modification in the traditional concept of a dichotomy between generalist (plant/food odour RNs with low sensitivity and broad overlapping response spectra, highly variable between individual neurons) and specialists (characterised by high sensitivity to individual components of pheromone blends). The new knowledge led to the classification of plant/food odours RNs into reaction groups with different degrees of specialisation, falling in between two extremes (Visser, 1986): (1) groups that respond solely to one class of chemicals (specialists); and (2) groups with wider response profiles, showing considerable overlap in their response spectra (generalists).

Related with the specialist-generalist classification of RNs, two mechanisms of peripheral codification of olfactory information were suggested, labelled-line and across-fibre code patterns

(cf. Kaissling, 1971; Boeckh, 1984; Visser and De Jong, 1988; Masson and Mustaparta, 1990). The labelled-line coding mechanism involves specialist RNs, each responding to one compound of the odour blend. The discrimination of blend composition in the central nervous system would then be a comparison between neural activities in separate channels The labelled-line mechanism is well established for the encoding of pheromone blends in moths. (see section 1.6). The across-fibre mechanism involves broadly tuned RNs, i.e. the information about one compound is mediated by different types of neurons. The discrimination of blend composition in the central nervous system would require an evaluation of patterns of neural activity across an array of RNs with overlapping response profiles.

The across-fibre mechanism has been suggested for the coding of food/plant odour blends on the basis of the broad overlapping response spectra exhibited by RNs stimulated with various synthetic compounds at relatively high concentrations (Schneider, 1987). However, even pheromone-sensitive RNs may respond to many different compounds when stimulated with high enough concentrations which are likely to be out of the natural range. Thus, there is need to identify the compounds for which RNs conveying non-pheromonal odour information have evolved. A major problem in this investigation is that food/plant odours have a complex composition, and it is not clear which of their components have biological significance for the insect.

In order to identify these compounds in complex volatile profiles of plants, gas chromatography (GC) has been linked to electrophysiological recordings of single RNs (GC-SCR). This technique was introduced by Wadhams (1982) for identification of pheromone components, and is now being used for the identification of plant odour components that are detected by RNs in a few species of beetles (Tømmerås and Mustaparta, 1987; Blight *et al.*, 1995; Wibe and Mustaparta, 1996). The results have shown that RNs often respond specifically to one or a few structurally similar compounds present in the plant odour blends (Mustaparta, 1996b). Furthermore, RNs frequently respond to compounds that are present in a very low amount (minor components), sometimes hardly detectable by the GC (Wibe and Mustaparta, 1996).

This underlines the importance of knowing the compounds for which olfactory RNs have evolved for studying their specificities, and that RNs sensitive to food/plant odours can be rather specialised. Both labelled-line and across-fibre coding mechanism may be used for the encoding of plant odours, as they are not mutually exclusive, and may represent the two extremes of a continuum, which may also operate within the same individual (Masson and Mustaparta, 1990; Frazier, 1992). In some cases, specific neurons may convey critical information, specifying a

particular plant compound by a labelled-line pattern. For example, Hardie *et al.* (1994) showed that the black bean aphid detects each of two non-host plants derived compounds, methyl salycilate and myrtenal, by specialised RNs, and the authors suggest that this could lead to a direct inhibitory input to the central nervous system as a possible mechanism for the masking effects observed on the attraction to host plant volatiles (see section 1.4). In other cases a unique across-fibre pattern code, matching some template present in the central nervous system, may provide the insect with the capacity to recognise a plant odour, without any particular neuron or set of neurons playing a dominant role. In other words, each plant volatile blend elicits a defined pattern of neural activity in the RNs (the peripheral odour image), and this forms the basis of the code to the brain about odour quality.

Most research on olfactory coding mechanisms of plant odours has involved the assessment of response profiles of RNs to single compounds. One important question in olfaction is how volatile compounds interact on the same RN (i.e. by addition, synergistic or suppression effects), and whether patterns of neural activities determined upon stimulation with single compounds resemble olfactory codes produced on stimulation with complete plant odours blends. To answer this question, Visser and De Jong studied the olfactory code in the adult Colorado potato beetle (De Jong and Visser, 1988a; Visser and De Jong, 1988). The attraction of this oligophagous insect to its solanaceous host plants species depends on the ratios between some "green leaf" volatiles released (see section 1.3). The study of single RN responses to "green leaf" volatiles showed a gradual differentiation of individual RNs that ranged from generalist to specialist. When the effect of binary mixtures (1:1) of such compounds was studied, they found additive effects on some neurons, but the vast majority of responses to mixtures showed various degrees of suppression. Furthermore, the suppression effects were more accentuated in the specialised RNs. The authors suggest two channels for coding the plant odour blend. The neural activities in the first channel, consisting of generalist RNs, arise from addition of the neurons' responses to individual components, and this channel is mainly affected by the quantities of components of the odour blend. The second channel, consisting of specialised RNs, responds to the odour blend by suppression, i.e. the change in neural activities depends on ratios between the components of the blend. This two channels concept was suggested to occur also for AL neurons (De Jong and Visser, 1988b) (see section 1.6).

1.6. Central Processing of Olfactory Information

The RNs in the antennae send their axons directly to the brain via the antennal nerve. Central processing of odour information begins in the deutocerebrum (cf. Homberg *et al.*, 1989; Masson and Mustaparta, 1990; Christensen *et al.*, 1996). The deutocerebrum is a bilateral structure receiving the two antennal nerves. Each side consists of two distinct regions called the antennal lobe (AL) and the antennal mechanosensory and motor centre (AMMC), also called the dorsal lobe. Both areas of the deutocerebrum receive the axons from sensory neurons in the antennae. Most and possibly all axons of olfactory RNs in the flagellum have terminal arborisations in the AL, whereas the AMMC receives axons from mechanoreceptors in the two basal segments of the antennae, the scape and pedicel (Homberg *et al.*, 1989).

1.6.1. Antennal lobe

The AL is the first relay station to process and integrate the olfactory input by a complex network of interneurons. The AL has been studied in a few species of insects including the moths *M. sexta*, *B. mori*, and *A. polyphemus*; cockroaches, mostly *Periplaneta americana*; some Hymenoptera, including honey bees and ants; and a few species of flies (refs. in Homberg *et al.*; 1989, Masson and Mustaparta, 1990; Christensen *et al.*, 1996).

Besides primary afferents that project, via the antennal nerve, strictly ipsilaterally, the neuropil of the AL comprises neurites of three classes of central neurons: local interneurons (LNs), projection neurons (PNs), and centrifugal neurons. The LNs are amacrine cells with arborisations confined to the AL. The PNs (also called output or principal neurons) have dendrites in the AL and axons that project into the protocerebrum. Centrifugal neurons have extensive dendritic arborisations in the protocerebrum and send axons into one or both ALs.

Synapses in the AL are largely or entirely restricted to the glomeruli. The glomeruli are small discrete units of dense neuropile with spheroid or ovoid shape where the endings of RN axons establish chemical synapses with central neurons (Homberg *et al.*, 1989; Masson and Mustaparta, 1990). A number of neuroanatomical studies have shown that the glomeruli are discretely identifiable, and their number is constant within each insect species (e.g. Rospars, 1983). In *Manduca*, the glomeruli have a glial investment and a characteristic size and position in a shell-like glomerular array around a central region of coarse neuropil, and computer-assisted neuroanatomical methods have been used to demonstrate unique identities, and provide a

detailed map of the glomeruli in the AL of males (Rospars and Hildebrand, 1992). The corresponding array of glomeruli in female AL closely resembles its male counterpart, but quantitative mapping of glomeruli in female AL is still under way (Hildebrand, 1996b). Each RN axon terminates within a single glomerulus in the ispsilateral AL (refs. in Hildebrand, 1996b). In *Locusta migratoria*, up to six terminals per axon were identified by the Golgi technique, with each terminal linked to up to three different glomeruli (Ernst *et al.*, 1977). Mobbs (1982), also using the Golgi staining technique, showed that in the honeybee (*Apis mellifera*) most RN axons terminate in a single glomerulus, and the same was found in the ant *Formica pratensis* (Goll, 1967 in Masson and Mustaparta, 1990). Uniglomerular projections are also found in *P. americana* (Ernst and Boeckh, 1983).

Each glomerulus includes terminals of RNs and arborisations of LNs and PNs. The LNs are restricted to the AL, and may in principle have a function analogue with the amacrine cells of the visual system by integrating the input information from various RNs (Masson and Mustaparta, 1990). Each LN innervates groups of glomeruli[11] where receives input directly from RNs, and also interacts synaptically with the PNs. The PNs have arborisations in one or several glomeruli, where receive little direct input from RNs, but instead are involved with synaptic connections with LNs, and send an axon to one or more higher-order olfactory centres in the protocerebrum (cf. Homberg *et al.*, 1989; Masson and Mustaparta, 1990; Hildebrand, 1996a,b).

The glomeruli in the AL constitute an "interface" between the axons of the RNs and the dendritic arborisations of central neurons which is characterised by an enormous neuronal convergence between the RNs and central neurons (Masson and Mustaparta, 1990). For instance, the 180,000 olfactory RNs an antenna of a male *P. americana* are connected to about 250 PNs (Boeckh, 1984). For the sexual pheromone pathway in this insect, the convergence is even greater. There are about 36,000 RNs for each of the two components of the female pheromone (Sass, 1983), and all the 72,000 RNs are connected to about 20 PNs in the macroglomerular complex[12], giving a ratio of about 4000:1 (Ernst and Boeckh, 1983). This input convergence results in amplification of the original signal as well as in improvement of signal-to-noise ratio level[13].

[11] One LN may establish synapses with as many as 70-80 glomeruli (Boeckh and Ernst, 1983).

[12] The macroglomerular complex (MGC) is a sexually dimorphic glomerular structure in the AL that appears in a number of insect species (cf. Masson and Mustaparta, 1990). In moths, the MGC is present near the entrance of the antennal nerve in the ipsilateral AL of males, and it is the site of first order synaptic processing of olfactory information about at least some of the components of the female's sex pheromone (cf. Hildebrand, 1996a,b).

[13] There is a 10- to 100-fold difference between the "average" RN threshold (the concentration to which more than 50% of the RNs respond) and the central neurons threshold (the concentration to which every neuron responds). The convergence of many RNs to a single central neuron enables the latter to detect excitation of even a few RNs and to sum signals from enough RNs to overcome the critical signal-to-noise level (Boeckh, 1984).

The features of olfactory information processing in the AL are better understood for pheromones, especially in moths. For instance, in *Manduca*, male specific sensilla trichodea (type I) are innervated by two RNs, of which one RN is highly sensitive and specific to one of the two components (E10,Z12-hexadecadienal or component A) of the female's sex pheromone, while the second RN is tuned to the second essential component (E10,E12,Z14-hexadecatrienal or component B) (refs. in Hildebrand, 1996b). These pheromone-specific RNs have extremely narrow molecular receptive ranges, and each type represents a narrowly tuned input channel that conveys information about the presence, concentration, and temporal patterning of one key pheromone component to the brain (Hildebrand, 1996b). In *Manduca*, as well as in other male moth species, the pheromone-sensitive RNs project their axons to one large macroglomerular complex (MGC) at the entrance of the antennal lobe (AL), and this is well separated from the "ordinary" glomeruli, processing non-pheromonal odour information (Boeckh and Boeckh, 1979; Ernst and Boeckh, 1983; Christensen and Hildebrand, 1987; Homberg *et al.*, 1989). Furthermore, RNs tuned to the detection of each pheromone component project their axons to different glomerular locations in the MGC of *M. sexta* and *Agrotis segetum* (Hansson *et al.*, 1991, 1992).

In *Manduca*, the MGC is made up of three distinct glomeruli: the "cumulus", "toroid", and "horseshoe" (Hildebrand, 1996a; Christensen *et al.*, 1996). AL neurons with arborisations in the MGC have been studied in detail (refs. in Hildebrand, 1996b), and of particular interest are PNs projecting their axons to higher olfactory centres in the protocerebrum. PNs that respond preferentially to stimulation of the antenna with component A of the female's sex pheromone have arborisations restricted to the "toroid", whereas PNs that respond to component B have arborisations confined to the "cumulus". PNs that respond to both components have arborisations in both "toroid" and "cumulus". Thus anatomically distinct MGC glomeruli are also functionally distinct with respect to their roles in processing of sex-pheromonal information received from different input channels with narrow and specific molecular receptive ranges, i.e. a labelled-line mechanism.

On the basis of their responses to antennal stimulation with pheromone components PNs have been classified in two broad categories: pheromone generalists and specialists (Christensen and Hildebrand, 1987; Christensen *et al.*, 1989; Christensen and Hildebrand, 1990). Pheromone generalists respond similarly to stimulation with either component A or component B, and do not respond differently when the antenna is stimulated with the complete pheromone blend. In contrast, pheromone specialists discriminate between antennal stimulation with component A and stimulation with component B. These PNs preserve information about individual key components of the species-specific pheromone blend. Another subset of pheromone-specialist

PNs respond to both pheromone components, but the physiological effects of the two components are opposite (inhibition or excitation). When the antenna is stimulated with mixtures of components A and B the response of these PNs depends on the ratio of the components in the mixture. Thus, different groups of PNs from the MGC convey information about different aspects of the pheromonal stimulus (Hildebrand, 1996b).

A labelled-line mechanism is also mediating interspecific chemical information in heliothine moths, i.e. the inhibition of attraction of males of one species to females of sympatric species (Mustaparta, 1996a). All heliothine species for which sex pheromones are identified (e.g. *H. virescens*, *Helicoverpa zea*, *H. assulta*) produce the same major compound, Z11-hexadecanal or component A. The species-specificity of the pheromone blend results from the production of a second essential component B, C, or D, of which B and D are also interspecific signals (refs. in Mustaparta, 1996a). As for *Manduca*, the heliothine species have RNs tuned to the detection of each pheromone component.

In *H. virescens*, it was shown that the RNs tuned to each of the two essential pheromone components (components A and B) (Almaas and Mustaparta, 1991; Berg *et al.*, 1995) project to the same glomerulus which is the largest of the four glomeruli in the MGC of this species (Hansson *et al.*, 1995). A third type of RN tuned to the pheromone component D of a sympatric moth species project to another glomerulus of the MGC, in which no termination of the pheromone RNs were found (Mustaparta, 1996a). Among the PNs that arborise in the glomerulus that receive input from RNs tuned to component A and RNs tuned to component B, a subset responds selectively to antennal stimulation with component A, and a second subset of PNs responds to both essential components A and B (Christensen *et al.*, 1995; Mustaparta, 1996a). Other PNs respond selectively to the interspecific signal (component D), and these are likely to receive the input of RNs tuned to component D of the interspecific signal that project to another glomerulus in the MGC (Christensen *et al.*, 1995; Mustaparta, 1996a). Thus, different levels of activities in the two pathways may result in different behavioural reactions. When *H. virescens* males detect the pheromone blend of conspecific females, the neural pathway for the pheromone components will exhibit activity according with the qualitative and quantitative composition of the species-specific pheromone blend, whereas the neural pathway for the interspecific signal is not activated. This results in attraction. In contrast, when *H. virescens* males detect the pheromone blend of a sympatric species, the activity in the pheromone pathway will be different from the one elicited by the conspecific female pheromone blend, and the neural pathway for the component D of the interspecific signal is activated. Thus, *H. virescens* males are not attracted to the sympatric species.

In *H. zea*, the RNs tuned to each pheromone component (A or C) project to different glomeruli of the MGC and convey information to subsets of PNs that respond selectively either to component A or C (Christensen *et al.*, 1991; Mustaparta, 1996a). However, in this species the PNs that respond to component C are also excited by component B of *H. virescens* pheromone blend, but antennal stimulation with the interspecific signal evokes higher frequency firing in these PNs than stimulation with component C. It is suggested that different levels of activity in the two subsets of PNs may result in different behavioural reactions (Mustaparta, 1996a). When the antennae are stimulated with the pheromone blend (A+C) there is high level in the subset of PNs receiving input from RNs tuned to component A and low level in the subset of PNs receiving input from RNs tuned to component B and RNs tuned to component C. This results in attraction to conspecific females. When the antennae are stimulated with the pheromone blend of the sympatric species (A+B) there is high level in the subset of PNs receiving input from RNs tuned to component A and high level in the subset of PNs receiving input from RNs tuned to component B and RNs tuned to component C. This results in interruption of pheromone attraction to sympatric *H. virescens* females.

The results from the studies carried out in moths species show that the MGC is subdivided into functional units. In *Manduca* and other moths (e.g. *A. segetum* and *H. zea*), different units receive information from each major component of the sex-pheromone blend, and in *H. virescens* the pheromone and interspecific signal information is also processed in different MGC units. The information from different pheromone components and from interspecific signals is integrated by some PNs, whereas others receive information about single components.

Is there a functional specialisation also in the "ordinary" glomeruli that process non-pheromonal information? The literature about electrophysiological studies of central AL neurons responses to antennal stimulation with single plant/food odour compounds and mixtures is scarce and do not permit an answer to this question.

In *P. americana*, central AL neurons were tested for their responses to antennal stimulation with various food odours (apple, banana, lemon, orange) and synthetic compounds (Boeckh *et al.*, 1977; Selzer, 1979). These electrophysiological studies have revealed PNs with broad response spectra responding to different aromas (e.g. orange and lemon odours), and PNs with less broad response spectra responding exclusively to one such stimulus. No response spectrum of a central neuron simply reflects the activity of one RN type. Different RNs all respond to a series of different odours but to a different degree (Sass, 1978; Selzer, 1981). In addition, it was found that single components of food odour blends (e.g. pentanol, octanol, terpineol) are less effective stimuli than their mixture and than a fruit odour blend. As suggested

by Ernst *et al.* (1977), this implies that the processes involved are not simply convergence or divergence of RNs into central AL neurons, but a rather more complicated network of integration. Boeckh and Ernst (1983), suggested that a convergence of different inputs at a central neuron elicits higher responses than an equally strong activity at only one input, implying the following different functions between the RNs and the central neurons: (1) the RNs analyse which type of compounds, which concentrations and which ratio of concentrations are present in a given odour; (2) each central neuron receives a special set of receptor inputs and puts together those stimulus constituents which are characteristic for a given odour. The authors noticed, however, that this interpretation should be regarded as preliminary because of lack of evidence on summation effects at the inputs of the central neurons, as well as on the receptor types which converge at a single PN. In addition, it is not known what is the projection pattern of different RN types to the glomeruli in the AL.

In the Colorado potato beetle (*L. decemlineata*), AL neurons were tested for responses to antennal stimulation with five "green leaf" volatiles present in the host plant odour (potato), i.e. (Z)-3-hexen-1-ol, (E)-2-hexenal, (Z)-3-hexenyl acetate, (E)-2-hexen-1-ol and 1-hexanol (De Jong and Visser, 1988b). The response spectra of AL neurons could be roughly divided in two classes: one class comprising neurons which are not very specific for the tested compounds (broadly tuned), and another class with neurons responding selectively to one compound (specialist neurons). When the antenna was stimulated with potato odour, the broadly tuned neurons showed excitatory responses, whereas the specialist neurons showed no response. Based on these results, the authors suggest a simplified mechanism for processing plant odour information in the AL based on two separate channels: channel A for processing information about stimulus quality (broadly tuned neurons that are excited by single "green leaf" odour components as well as by their mixture), channel B for processing information about stimulus quantity (specialist neurons that evaluate component ratios).

According with the two channels concept, a mechanism of host plant odour recognition in the Colorado potato beetle was illustrated by three possible situations: (1) Both channels do not respond to antennal stimulation. In this case important leaf odour components are not present and there is no detection of plant odour (absence of attraction). (II) Only channel A responds to stimulation. In this situation there is a stimulation with "green" leaf odour components, and these components are in the correct ratio since neurons of channel B do not respond. In this case the beetles would be attracted to the host plant by positive anemotaxis. (III) Both channels respond to stimulation. Thus, the stimulus contain "green leaf" odour components in a ratio that differs from

the one in potato leaf odour (absence of attraction).[14] However, since it was not tested whether antennal stimulation with mixtures of "green leaf" components in ratios different from the one present in potato leaf odour elicits responses in neurons of channel B, it is necessary to provide more experimental evidence to support these hypothesis.

Modality convergence. Although the primary function of the AL is to process olfactory information, some AL neurons including LNs and PNs receive multimodal sensory information. Some neurons respond not only to antennal olfactory stimulation (plant/food odours or pheromones), but also to stimulation with a puff of air[15] (mechanosensory stimulus), and to temperature, humidity and acoustic stimuli (cf. Homberg *et al.*, 1989). As pointed out by Boeckh and Ernst (1983), this is not surprising, since in addition to the 190,000 olfactory RNs on an antenna of a male cockroach, some 70,000 taste cells, 19,000 mechanoreceptors, 2,100 hygroreceptors and 90 thermoreceptors terminate in the glomeruli. In addition, some of these responses may be mediated through centrifugal neurons that have various connections to other areas of sensory convergence in the protocerebrum (Homberg *et al.*, 1989). Furthermore, although the pathways for processing pheromonal and non-pheromonal information are separated, some AL neurons respond to both pheromones and plant/food odours. These responses are thought to result from lateral interactions mediated through multiglomerular LNs (Matsumoto and Hildebrand, 1981)[16], since the MGC gets input only from pheromone receptors. The possibility that plant/food odours directly stimulate pheromone RNs has also been ruled out in *P. americana* through single-cell recordings (Sass, 1983). Thus, in the AL, there is integration of information about pheromones and plant/food odours, and integration of olfactory information with other sensory modalities.

1.6.2. Higher brain centres

After synaptic processing in the AL, information about odours (pheromones and plan/food odours) is relayed to the protocerebrum and the subesophageal ganglion by way of the axons of AL PNs (Homberg *et al.*, 1989; Masson and Mustaparta, 1990; Hildebrand, 1996b).

[14] See section 1.3 for the behavioural studies on the orientation of *L. decemlineata* to host plant volatiles, and section 1.4 for the masking effect of non-host volatiles in the attraction of the insect to the host plant.

[15] Multimodal LNs found in *P. americana* do not respond when the mechanoreceptors at the base of the antenna are stimulated (Boeckh *et al.*, 1984), which is in accordance with the fact that these mechanoreceptors project their axons to the dorsal lobe of the deutocerebrum.

[16] Local interneurons were found in *M. sexta* that have arborisations both to the macroglomerulus and to "ordinary" glomeruli (Matsumoto and Hildebrand, 1981).

Olfactory foci in the protocerebrum of *Manduca* include the calyces of the mushroom body[17], the lateral horn of the protocerebrum, and an olfactory focus and a sex-pheromone focus in the inferior lateral protocerebrum (Homberg *et al.*, 1989). In these olfactory foci the olfactory information is further processed and integrated with other sensory modalities, and output neurons are connected with the motor system controlling the insect's motor patterns (Boeckh and Ernst, 1987; Visser and De Jong, 1988). Multimodal neurons that respond to pheromone, light, and mechanical stimuli have been found in the lateral protocerebrum of moths, and some of these cells project out of the brain and may mediate pheromonal arousal or motor responses (Light, 1986).

In order for an airborne male moth to perform pheromone-induced optomotor anemotaxis, stimulation from pheromone must be integrated at some level with feedback about motion from the visual system (Kennedy, 1986). Such integration is in evidence in the multimodal neurons descending from the protocerebrum of *B. mori* and heading toward the pterothoracic ganglia (Olberg, 1983). Cells were found which changed their firing frequency from either a high to low state (or vice-versa) in response to successive pulses of pheromone. Some of these "flip-flopping" interneurons also changed states when a light was switched on or off. Some cells responded only to pheromone fluctuations, while others responded only to changes in the visual field. Others were multimodal, or stimulated by different modalities such as odour and visual stimuli. Interestingly, some of the "flip-flopping" interneurons responded to the transverse movement of images across the eyes. The close association of cells responsive to such movement - precisely the kind which be used by insects to steer in compensation for wind-induced drift (see section 1.3.1) - and fluctuating pheromone stimulation, appears to provide solid evidence for the neuronal activity needed in order for moths to sustain their upwind flight toward conspecific females (Baker, 1989).

In general, the amount of research work on processing of olfactory information and its integration by neurons beyond the deutocerebrum is very limited. The exploration of their morphological and physiological properties has only just started (Hildebrand, 1996b).

[17] The mushroom bodies, in addition to participating in a wide variety of behaviour patterns in insects (cf. Howse, 1974), have long been thought to be involved in olfactory learning and memory (cf. Homberg *et al.*, 1989; Masson and Mustaparta, 1990).

2.

Synopsis of the Biology of *Phoracantha semipunctata*

2.1. Taxonomic Position

The insect species was described for the first time in 1775 by Juan Christian Fabricius as *Stenocorus semipunctatus*. In 1840, Newman suggested that it should be considered under the new genus *Phoracantha*, and the species designation changes to *Phoracantha synonyma* (Santis, 1945). The actual description as *Phoracantha semipunctata* is due to Aurivillius in 1912 (Santis, 1945), and its full taxonomic position is (Santis, 1945; Duffy, 1963; Henriques, 1986):

Order Coleoptera
Superfamily Cerambycoidea
Family Cerambycidae
Subfamily Cerambycinae
Tribe Phoracanthini
Genus *Phoracantha*
Species *Phoracantha semipunctata*

2.2. Distinguishing Morphological and Other Biological Features

The morphological features of the species have been described in detail by several authors (e.g. Santis, 1945; Duffy, 1963; Henriques, 1986). Here, it is provided a general description of some distinct morphological and other general biological features of the species.

Eggs. The eggs are pale yellow, 2-3 mm long, elongated and spindle-shaped, deposited on the trunk of the host tree species, beneath dead bark stripes or in bark crevices. The females lay the eggs in batches which varies between 10 and 110, as reported by several authors (e.g. Chararas, 1969a; Bytinski-Salz and Neumark, 1952).

Larvae. The larvae are flattened and with a yellowish colour, and can achieve 38 mm length and 8,5 mm width at the prothorax level. For a complete morphological description see Santis (1945) or Duffy (1963). Neonate larvae penetrate the bark and feed along the cambium

and phloem until mature. Mature larvae bore into the sapwood to construct the nymph chambers, packing the opening to the surface with frass. The adults emerge by chewing their way out from the nymph chambers.

Nymph. Whitish coloured, and the entire body is covered with a fine delicate cuticle. For a complete detailed description see Santis (1945) or Duffy (1963).

Adult (figure 3.1). The adult insect is 13-30 mm long, flattened, glistening longhorn beetles. It has a dark brown coloration with a yellow transverse band and apical spots on the elytra. Elytra very coarsely punctured for basal half; apical half smooth; apices with out and sutural angles strongly spined. A pair of well developed metaesternal scent-glands and associated reservoirs open to the surface through small pores near the outer distal borders of the metasternum. When molested, the beetles exude drops of a secretion that contains 5 significant volatile components[1] which are presumed to play a defensive role and/or to have a more subtle function, regulating interspecific competition (Moore and Brown, 1972).

The antennae have 9 flagelar segments, and the first 5 are strong spined apically on inner angle. In the female, the antennae extend to at least as far as elytral apices, and well beyond apices in male. The antennae of both sexes have large number of different sensilla types, including sensilla chaetica (mechanoreceptor organs), and sensilla basiconica and trichodea (chemoreceptor organs) (Lopes, 1990). The receptor neurons associated with the olfactory sensilla project their axons to numerous glomeruli in the antennal lobe where no macroglomerulus structure have been found in males or females (Lopes, pers. comm.).

The ovipositor can extend to a length of 2 cm, and it is specialised to detect tigmotactically suitable sites for oviposition on the tree trunk (Chararas, 1969a,b; Drinkwater, 1975). The females do not prepare the substrate with the mandibles for oviposition. The eggs are placed under the trunk loose bark or in bark crevices, and this is achieved exclusively with the aid of the ovipositor.

Both sexes are able to produce sounds by friction between the inner edge of the posterior margin of the prothorax and a specialised striated area on a median anterior prolongation of the mesonotum. Within the Cerambycidae, this stridulatory plate varies from group to group and can be used in the classification of genera and tribes, but less commonly at the species level (Linsley, 1959). Since both sexes of cerambycid species stridulate when captured, the function is commonly regarded as defensive, but it may be also involved in mate location (Linsley, 1959). In

[1] Volatile components of the metasternal gland secretion (Moore and Brown, 1972): the most abundant is 2-hydroxy-6-methyl-benzaldehyde (1); the second most abundant was named phoracanthol and shown by synthesis to be (5-ethylcyclopent-1-enylmethanol) (2); the three other components are aldehydes derivable from phoracanthol and cis- and trans-dihydrophoracanthols, respectively.

P. semipunctata, preliminary recordings of sound emitted by stridulation of hand disturbed beetles showed power spectra where most of the signal is in the ultrasound range (Fonseca and Barata, unpublished). This suggests that, if involved in mate location, sound emitted is most likely effective only at close-range.

Evidence collected so far suggests that there is no long-range intraspecific chemical communication, e.g. sexual or aggregation pheromones, involved in mate location (Marques, 1992, 1996).

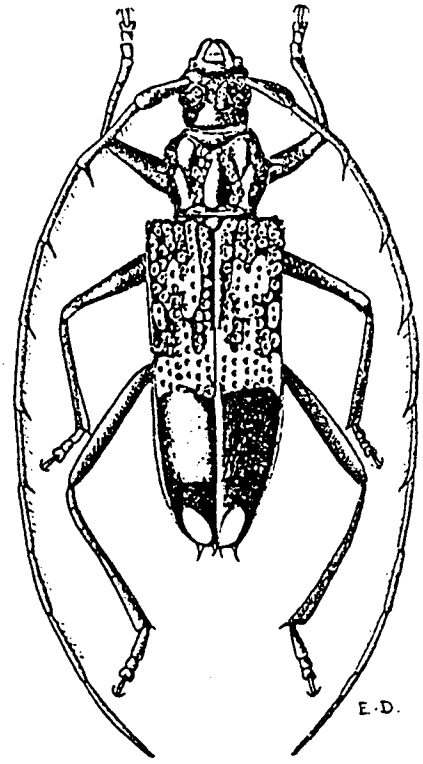


Figure 2.1. *Phoracantha semipunctata* adult male (from Duffy, 1963).

2.3. Range of Host Plant Species

Most authors describe *P. semipunctata* as woodborer specific of *Eucalyptus*, but the taxonomically related genera *Angophora* and *Syncarpia* are also attacked, i.e. *A. floribunda* (Moore, 1963), *A. intermedia* (Duffy, 1963), and *S. laurifolia* (Duffy, 1963). Drinkwater (1975) reports a case where a specimen of one unrelated species, *Cupressus lindleyi* (Cupressaceae), was attacked in Malawi. It has been shown that *P. semipunctata* prefer some *Eucalyptus* species to

others for oviposition (Powell, 1978), and within a species the preference goes to recently felled trees or standing specimens under physiological stress, e.g. due to water deficit (Chararas, 1969a; Hanks *et al.*, 1993). The preference for drought stressed specimens may be related with the fact that such trees produce less kino[2], which has been implicated in the resistance to larval progression (Chararas, 1969a,b), and have lower bark moisture. The bark moisture content plays a critical role in preventing penetration of neonate larvae into the bark (Hanks *et al.*, 1991).

2.4. Life Cycle

The life cycle of *P. semipunctata* has been studied in Tunisia (Chararas, 1969a,b); Zambia (Loyttyniemi, 1983), Israel (Mendel, 1985), California (Hanks *et al.*, 1990), and Portugal (Lima *et al.*, 1988; Lima, 1989; Araujo *et al.*, 1990, 1991). The adult insects fly actively during dusk and night in search for mates and suitable host trees for oviposition when the temperature is above 15° C (Chararas, 1969a). Eggs and larvae development time, the emergence pattern of the adults, the flight and oviposition period, and the number of generations per year depends on the regions and seems to be related with the annual temperature profile. In Portugal, one or two overlapped generations can be completed in a year (Lima *et al.*, 1988; Araujo *et al.*, 1990, 1991). In Tunisia, two generations per year are observed (Chararas, 1969b), and in Zambia and Israel a maximum of three overlapped generations can be completed in a year (Löyttyniemi, 1983; Mendel, 1985).

2.5. Geographical Distribution and Pest Status

This cerambycid species of Australian origin became widely distributed, following in general the geographical distribution of host plant species introduced to several regions of the world. The importation of infested eucalyptus wood was suggested as the main cause for the appearance of *P. semipunctata* in far regions (Tooke, 1929), and the species has become a severe pest of eucalyptus in introduced regions. Table 2.1 indicates regions of the world where *P. semipunctata* has been introduced and whether the insect achieved pest status.

[2] Kino, a sticky, gum is an aqueous solution of polyphenolic compounds retained in veins or pockets under the bark (Tippett, 1986).

Table 2.1. Geographical distribution of *Phoracantha semipunctata* and pest status.

Region	Reference	Pest Status	Reference
Australia	Duffy (1963)	No	Duffy (1963)
New Zealand	Duffy (1963)	no report	--------
New Guinea	Duffy (1963)	no report	--------
South Africa	Tooke (1929)	Yes	Drinkwater (1975)
Malawi	Majawa (1981)	Yes	Majawa (1981)
Zambia	Löyttyniemi (1980)	Yes	Löyttyniemi (1983); Selander & Bubala (1983)
Mozambic	Cadahia (1986)	no report	--------
Maurice and Ruperez Islands	Duffy (1963)	no report	--------
Egypt	Lepesme (1950)	no report	--------
Marrocco	El-Yousfi (1989)	Yes	El-Yousfi (1989)
Algeria	Cadahia (1986)	no report	--------
Tunisia	Billioti & Schoenberger (1963)	Yes	Chararas (1969)
Israel	Bytinski-Salz & Neumark (1952)	Yes	Mendel (1984)
Palestine	Bytinski-Salz & Neumark (1952)	no report	--------
Turkey	Acatay (1959)	Yes	Acatay (1981)
Cyprus	Duffy (1963)	no report	--------
Corse	Orousset (1984)	no report	--------
Italy	Parenzan (1976)	Yes	Cavalcaselle (1986)
Spain	Sotres & Vasquez (1981)	Yes	Tirado (1986)
Portugal	Figo (1981)	Yes	Paiva & Araújo (1985)
Canarian Islands	Estevez (1988)	no report	--------
Argentina	Santis (1945)	Yes	Santis (1945)
Uruguay	Santis (1945)	no report	--------
Chile	Duffy (1963)	no report	--------
Brazil	Santis (1945)	no report	--------
California (USA)	Scriven *et al.* (1986)	Yes	Hanks *et al.* (1993)

In natural populations of *Eucalyptus* in Australia, two species of *Phoracantha* are known (Penfold and Willis, 1961): *P. semipunctata* and *P. recurva*. These do not constitute a pest problem where predators (clerid beetles) and parasitoids control the eucalyptus woodborer populations to such an extent that the damage on the host species populations is limited (Penfold and Willis, 1961; Moore, 1963, 1972). Furthermore, the natural multispecific forests of *Eucalyptus* in Australia are well adapted to the edapho-climatic conditions, and noticeable attacks of *Phoracantha* spp. are restricted to sick or felled trees (Chararas, 1969). Outside

Australia, the outbreaks of *P. semipunctata* closely represent a gradient type[3] (Selander, 1985), and are generally associated with sites where eucalyptus trees are growing under edapho-climatic conditions leading to physiological stress (Chararas, 1969a,b; Ivory, 1977).

Economic losses in 300,000 ha of *Eucalyptus* planted in the entire south-west of Spain due to *P. semipunctata* activity during 1983 were estimated as 529 millions ptas (Tirado, 1986). In Portugal there are over 300,000 ha of planted *Eucalyptus* (Henriques, 1986), mostly used for production of paper pulp. The eucalyptus woodborer is responsible for important economic losses for growers and paper pulp companies, mainly in plantations in central and southern regions of the country, however no estimates of economic losses were available to present here.

2.6. Current Methods of Pest Control

Control of *P. semipunctata* by insecticides has proven prohibitively costly both economically and environmentally, as well as being of limited effectiveness (Scriven *et al.*, 1986; Ali and Garcia, 1988). Adults are nocturnal fliers and spend daylight hours under loose bark and may be located high in the canopy. In addition, females lay their eggs under loose bark, and larvae and nymphs are protected inside the bole. This limits exposure to sprayed insecticides. Use of systemic insecticides is not effective also, since larvae destroy conductive tissues of the trunk, thus preventing the movement of these chemicals. In addition, the adults do not feed on foliage nor on the bark.

Current methods of pest control exploit the extraordinary ability of *P. smipunctata* females to locate and colonise recently felled trees. A number of freshly cut logs distributed in *Eucalyptus* plantations have been used by growers to attract females and trap the eggs in order to reduce the population size of the next generation (e.g. Chararas, 1969b; Egea, 1982; Löyttyniemi, 1980, 1983; Tirado, 1984, 1990). In the south of Spain, log traps of 15 freshly cut logs treated with lindane were placed in various *Eucalyptus* plantations at a density of 1 trap per 25 ha (Tirado, 1984). Female beetles laid eggs on the sprayed logs and then died from contact with the insecticide. The logs were replaced every two weeks with fresh ones, and the old logs were destroyed within two months to prevent beetle emergence. These measures apparently limited the impact of the beetle in some areas, and it was estimated that economic losses decreased from 592

[3] Berryman and Stark (1985) have provided frameworks for risk assessment models by classifying destructive insect pest outbreaks as gradient, cyclical, or eruptive. The gradient and cyclical outbreaks are generated by stand and site conditions conducive to the reproduction and survival of the pest or stressful for the host plant, whereas eruptive outbreaks refer to situations in which pest density remains low for long periods of time, but eruptions of very high density may occur at irregular intervals, triggered by site conditions and/or environmental disturbances .

millions ptas in 1983 to 524 millions in 1984 (Tirado, 1986). It is not possible to provide any information on whether these measures have been continued and if so what was their impact on further reduction of *P. semipunctata* impact in Spain. In Portugal, log traps (without insecticide) have been used more or less consistently, however it was not possible to obtain data as regard their efficiency in controlling the populations of *P. semipunctata*.

2.7. Future Methods of Pest Control

Long-term control of *P. semipunctata* populations is probably achieved only through integrated pest management strategies (Scriven *et al.*, 1986; Ali and Garcia, 1988; Araújo, 1985; Paiva and Araújo, 1985; Hanks *et al.*, 1990), a conclusion reached also during the international workshop on "*Phoracantha semipunctata* Integrated Pest Management" organised by the association of paper pulp companies (ACEL) in Lisbon, Portugal (1991).

Integrated Pest Management (IPM) is an interdisciplinary approach to the solution of agricultural problems, aiming at the reduction of the present tremendous use of insecticides with negative impact on public health and environment (Wysoki, 1996). The IPM concept is based on the recognition that no single approach to pest control offers a universal solution, and that long-term pest control can be provided only by an integration of various tactics into practices based on sound ecological principles. The objective of the concept is to integrate all suitable techniques, and biological and ecological information available into the most economical and ecologically acceptable control system, either to reduce and maintain pest populations below economic damaging levels or to manipulate them in such a manner as to prevent their causing damage. These control systems include, adequate cultural practices, use of plant resistant varieties, use of plants better adapted to environmental conditions, use of biological control agents (e.g. bacterial insecticides, viruses, protozoan, fungal insecticides, nematodes, parasitoids, predators), use of naturally occurring agents to interfere with insect behaviour (semiochemicals), their growth and development (growth regulators and juvenile hormones), and use of selective naturally occurring insecticides. A fuller incorporation of these biologically based ingredients into the IPM systems is essential to develop the concept to its full potential as a long-term approach to pest control, and to limit the use of wide spectrum insecticides so many times overused.

The efficiency of IPM strategies to control *P. semipunctata* is dependent on the biological knowledge that can be achieved about the insect and about its host plants. The host plant selection mechanisms of *P. semipunctata* are poorly understood. Host plant volatiles may be

stimuli involved in the host finding behaviour of this insect, but this has never been demonstrated. Motivated by the potential use of semiochemicals in IPM strategies to control *P. semipunctata*, research work was carried out to enlighten mechanisms by which the beetles find the host tree, and this is presented in the next chapters of the thesis.

PART II

EXPERIMENTAL WORK

3.

Primary Attraction of *Phoracantha semipunctata* to *Eucalyptus globulus* and Electroantennogram Responses to Host Plant Odours

3.1. Introduction

In a review of the ecology of the Cerambycidae, published almost 40 years ago, Linsley (1959) suggested that olfaction is the most important sense used by long-horned beetles to locate suitable host plants. However, the cerambycids have not been subjected to intense research on host selection mechanisms, and since 1959 only a few reports have been published, in which the existence of primary attraction is demonstrated. In the Japanese pine sawyer, *Monochamus alternatus*, males and sexually mature females are attracted to recently felled pine trees (*Pinus densiflora* and *P. thunbergii*) and to a mixture of host tree volatiles comprised of monoterpenes and ethanol (Ikeda *et al.*, 1980; Ikeda, 1981). In the case of *Plagithmysus bilineatus*, which specifically attacks Ohia trees (*Metrosideros polymorpha*) in Hawaii, males and females are more attracted to stressed trees than to healthy ones (Stein and Nagata, 1986).

In contrast to the scarce research work on cerambycids, host selection mechanisms in Scolytidae which mass attack living coniferous trees using pheromones have been studied extensively (e.g. Byers, 1989, 1995). For several species in the western United States, the pioneer bark beetles do not use plant volatiles to find host trees. Ponderosa pines that were killed by freezing and screened to prohibit beetle attack, did not exhibit higher landing rates for *Ips paraconfusus*, *Dendroctonus brevicomis* and *D. ponderosae*, than did healthy trees (Moeck *et al.*, 1981). Thus, host selection in these species is thought to be a random process as regard the landing of pioneer beetles on host and non-host trees (Moeck *et al.*, 1981; Wood, 1982; Byers, 1989, 1995). The host plant volatiles do not provide any long range cue for host finding, and host selection appears to occur through host recognition after landing on the bark and on the basis of contact sensory modalities. The mass attack of individual trees is mediated by the release of pheromones synthesised from ingested host plant monoterpenes that attract large numbers of individuals of both sexes to mate and oviposit on the host tree (Byers, 1995).

In other species that do not use aggregation pheromones, there is a long-range influence of host tree volatiles in the host plant selection process. This is the case of male and female

Tomicus piniperda, which are attracted by monoterpenes from Scots pine evaporated from wound oleoresin (Byers, 1989), and the case of *Hylurgopinus rufipes* males and females that are more attracted to diseased elms than to healthy elms (Millar *et al.*, 1986).

The eucalyptus wood borer of Australian origin, *P. semipunctata*, became a severe pest of eucalyptus plantations in several regions of the world (chapter 2, this thesis). The economic importance of this cerambycid species has been the source of motivation for studies concerning its general biology and ecology (Tooke, 1935; Santis, 1945; Chararas, 1969a, 1969b; Drinkwater, 1975; Löyttyniemi, 1980; Hanks *et al.*, 1991, 1993a), life cycle (Löyttyniemi, 1983; Mendel, 1985; Lima *et al.*, 1988; Araújo *et al.*, 1991), life tables (Powell, 1982; Tirado, 1987), host species preference (Powell, 1978; Hanks *et al.*, 1993b), and control methods (Löyttyniemi, 1980; Mendel, 1984; Tirado, 1984, 1990; El-Yousfi, 1989).

The life cycle of *P. semipunctata* has been studied in the south of Portugal (Lima *et al.*, 1988; Lima, 1989; Araújo *et al.*, 1991). In general, the emergence of adults starts in June, peaks in July, and an oviposition peak may be observed 2 to 3 weeks later. Neonate larvae penetrate the bark and feed on the phloem and cambium during Summer months, and the mature larvae bore into the sapwood to construct chambers where the nymphs spend the winter. In years exceptionally dry and warm in the south of Portugal, a second generation of adults may emerge in September, resulting from eggs laid in early June. However, the rule is that nymphs overwinter in hardwood chambers and emerge as adults in summer the following year. During the flight period, the females readily attack trees under physiological stress, or recently felled trees (Chararas, 1969a,b). However, no direct evidence has been provided to show that *P. semipunctata* females are able to make oriented movements towards the host tree mediated by host chemical cues, i.e. a "primary attraction" mechanism in their host-finding behaviour. It is also not known whether the males are attracted by host tree odour.

In this study, a field experiment was designed to test the existence of such a primary attraction mechanism in males and females to a common host species, *E. globulus*. The experiment was undertaken in a monospecific plantation of *E. globulus* in the south of Portugal during a peak of adult emergence detected by survey. The ability of the adults to detect the odour emanating from *E. globulus* material, and also host related volatile chemicals was further tested by recording electroantennogram (EAG) responses. The EAG, assumed to summate receptor potentials of olfactory neurons, is measured as a slow potential change between the base and the tip of the antenna in response to olfactory stimulation (Schneider, 1957, 1963), and it has been extensively used in the identification of insect pheromones as well as of host plant odours (Masson and Mustaparta, 1990).

3.2. Materials & Methods

3.2.1. Field Experiments

Study area. An area of the *E. globulus* plantation of Serra d'Ossa, in the vicinity of Redondo (southern Portugal) was chosen to survey the emergence of *P. semipunctata* adults from infested trees, and to undertake a field experiment to test the attractiveness of logs and foliage of *E. globulus* to flying males and females.

Surveillance of adult emergence. In early June 1991 (week 24), 53 infested trees were randomly chosen, and an area of their trunks was debarked to expose the wood chambers containing nymphs of *P. semipunctata*. In total, 288 chambers were trapped (1-6 per tree) as described by Araújo (1988), and the emergence of *P. semipunctata* adults was surveyed from June until early September.

Field experiment. A trap designed to catch flying adults of *P. semipunctata* is shown in figure 3.1. It consisted of a 1 m long and 25 cm diameter wire net cylinder (ca. 2 cm mesh size) covered with tanglefoot, and suspended ca. 1m above ground level on a nylon cable attached to two trees from adjacent rows. Placed under the net cylinder, a plastic tray with water collected the insects that fell from the net. At the onset of the week 27 (July), empty traps and traps baited with a debarked log, a freshly cut log, or leaves of *E. globulus*, were placed in the plantation according to a six complete randomised block design. The experiment lasted for 3 weeks. Both debarked and freshly cut logs were 1 m long and ca. 14 cm diameter, and were suspended vertically in the middle of the net cylinder. The cut leaves filled up wire net cylinders of dimensions similar to the logs, and were also suspended vertically inside the net cylinder of the trap. The trap plus baits appeared to the human eye as vertical silhouettes at dusk or under moonlight. The baits were renewed weekly, their position within each block was determined at random. The insects trapped were collected daily, and the females were taken to the laboratory for observation of the ovaries.

Statistics. The total number of males and females captured per trap within each block at the end of the experimental period was transformed by $\log_{10}(X+1.5)$. Analysis of variance was made separately for each sex on the transformed data, and significant differences between means of insects caught per bait were tested by the Student-Newman-Keuls' test (S-N-K test) (Zar, 1984).

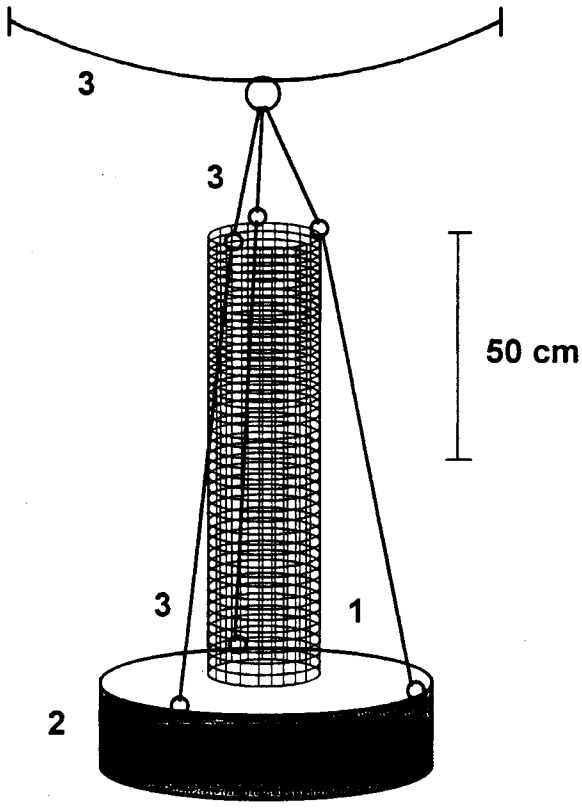


Figure 3.1. Field trap for flying adults of *Phoracantha semipunctata* suspended between two trees: 1 - Wire net cylinder with tanglefoot; 2 - plastic tray; 3 - nylon cables.

3.2.3. Electroantennograms (EAGs)

Insects. Adult females and males *P. semipunctata* were obtained by individual trapping upon emergence from naturally infested *E. globulus* logs, as described by Araújo (1988). After emergence, the insects were kept in individual plastic boxes in a controlled environment room at 25° ± 3° C, 60 ± 10% RH, and 16 hr photoperiod provided by fluorescent bulbs. The insects were fed weekly with a honey solution (7 g/100 ml) poured on cotton pads. With this rearing procedure, the longevity of males was 89 ± 26 days and that of females was 96 ± 20 days (mean ± SD), and females achieved full maturation of the ovaries after 21 days (Marques, 1992). The males and females used for electrophysiological recordings were 20-25 days old.

EAG Recordings. The EAG recordings were obtained from alive adult specimens of *P. semipunctata* mounted on a Plexiglas block and secured with wax (Utility Wax Rods, Kerr®),

using tungsten electrodes. The recording electrode was inserted in the cut tip of the antenna and the reference electrode was placed into the ipsilateral eye. The signal was delivered to a differential amplifier (x100 amplification) and monitored with an oscilloscope. Samples of recordings were digitised with a data translation computer board (DT2821F-8DI), and analysed by a software designed by P. Fonseca (University of Lisbon).

Olfactory stimuli and stimulation procedure. The antennae of 5 males and 4 females of *P. semipunctata* were stimulated with the odour from a cut portion of bark, a trunk node, a leaf, or a fruit of *E. globulus*, and with dilution series in paraffin oil (10^{-4} to 1 v/v) of α-pinene, camphene, α-terpinene, 1,8-cineole and ethanol, obtained from commercial sources. A constant stream of air (80 ml/s) was directed over the antenna by a glass tube (1 cm i.d.) connected to a bottle with purified air by a flexible PVC tube. The olfactory stimulus consisted of a 3 ml odour puff injected into the air stream 1.5 m away from the antenna. This was made with a syringe attached to a Pasteur pipette containing the odour source: 1.5 g of each plant material or a piece of filter paper impregnated with 25 µl of a chemical dilution. Each insect was tested for all stimuli in a random order, and each chemical was tested at successively higher concentrations. Between each stimulation, the insects were allowed to recover for 5-10 min, depending on the intensity of the preceding response. A standard stimulus (1,8-cineole at 10^{-1} v/v) was applied at the end of each stimulation chemical series, and the EAG amplitude values were expressed relative to this standard.

3.3. Results

3.3.1. Emergence of Adult Beetles and Field Experiment

In total, 85 males and 95 females were caught as they emerged, corresponding to 62.5% of the individually trapped wood chambers. Figure 3.2 shows the cumulative percentage of beetles emerged. The emergence pattern of both sexes was identical until week 31. The emergence began in week 26, peaked occurred during weeks 28 to 31, and then virtually ceased.

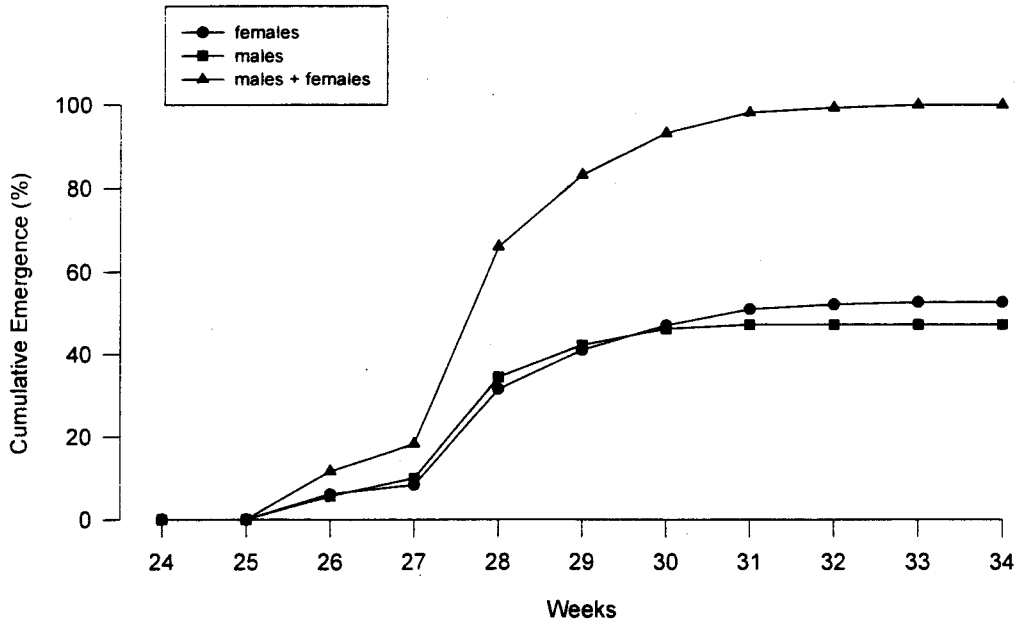


Figure 3.2. Emergence pattern of males and females *Phoracantha semipunctata* from infested *Eucalyptus globulus* specimens at the plantation of Serra d'Ossa from week 24 until week 34 of 1991. In total, 85 males and 95 females had emerged at the end of the survey period.

Tables 3.1 and 3.2, show the results of the analysis of variance made on the total number of insects caught per baited trap over weeks 27, 28 and 29, and in figure 3.3 is shown the mean number of males and females caught per trap. For both sexes, the number of specimens caught was significantly dependent of the bait in the trap; the leaves of *E. globulus* caught the highest mean number of males and females, followed by the freshly cut log (figure 3.3). The empty traps and traps baited with dry logs caught the fewest insects, and the traps with a dry log caught significantly less males than the empty traps (figure 3.3, A). Each type of trap caught more males than females.

Table 3.1. Analysis of variance of the number of flying *Phoracantha semipunctata* males caught in the four types distributed in the plantation of *Eucalyptus globulus* according to a six complete randomised block. Significant differences ($p < 0.05$) are marked with *.

Effect	df	MS	F values	p
Type of Trap	**3**	**1.123**	**80.690**	**0.000 ***
Blocks	**5**	**0.058**	**4.139**	**0.015 ***
error	15	0.014		

Table 3.2. Analysis of variance of the number of flying *Phoracantha semipunctata* females caught in the four types distributed in the plantation of *Eucalyptus globulus* according to a six complete randomised block. Significant differences ($p < 0.05$) are marked with *.

Effect	df	MS	F values	p
Type of Trap	**3**	**0.784**	**25.018**	**0.000 ***
Blocks	**5**	**0.137**	**4.366**	**0.012 ***
error	15	0.031		

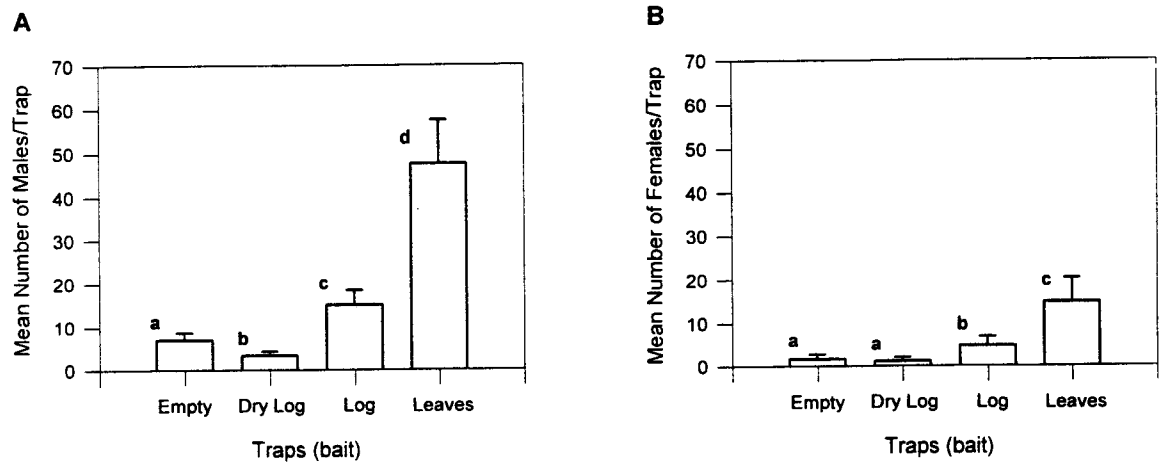


Figure 3.3. Mean number (± 95% confidence interval) of *Phoracantha semipunctata* males (A) and females (B) caught in empty traps, and traps baited with a dry log, a freshly cut log or leaves from *Eucalyptus globulus*. Different letters indicate significant differences between the means (S-N-K test, $p<0.05$)

The analysis of the total number of males and females (figure 3.4) captured at the end of each of the three experimental weeks shows that the proportion of males caught in each type of trap was independent of the weeks ($\chi^2_6 = 5.55$; $p > 0.05$), but that there was a significant difference in the total number of males caught in each week ($\chi^2_2 = 132.9$; $p < 0.05$). The total number of males increased from week 27 to week 29, following the increase in male emergence over the same period. For the females, it is shown that their number in each type of trap was also independent of the experimental week ($\chi^2_6 = 10.26$; $p > 0.05$), but for them, unlike the males, the the total numbers caught each week were not significantly different from a 1:1:1 ratio ($\chi^2_2 = 2.49$; $p > 0.05$), and did not follow the emergence pattern observed over the same period.

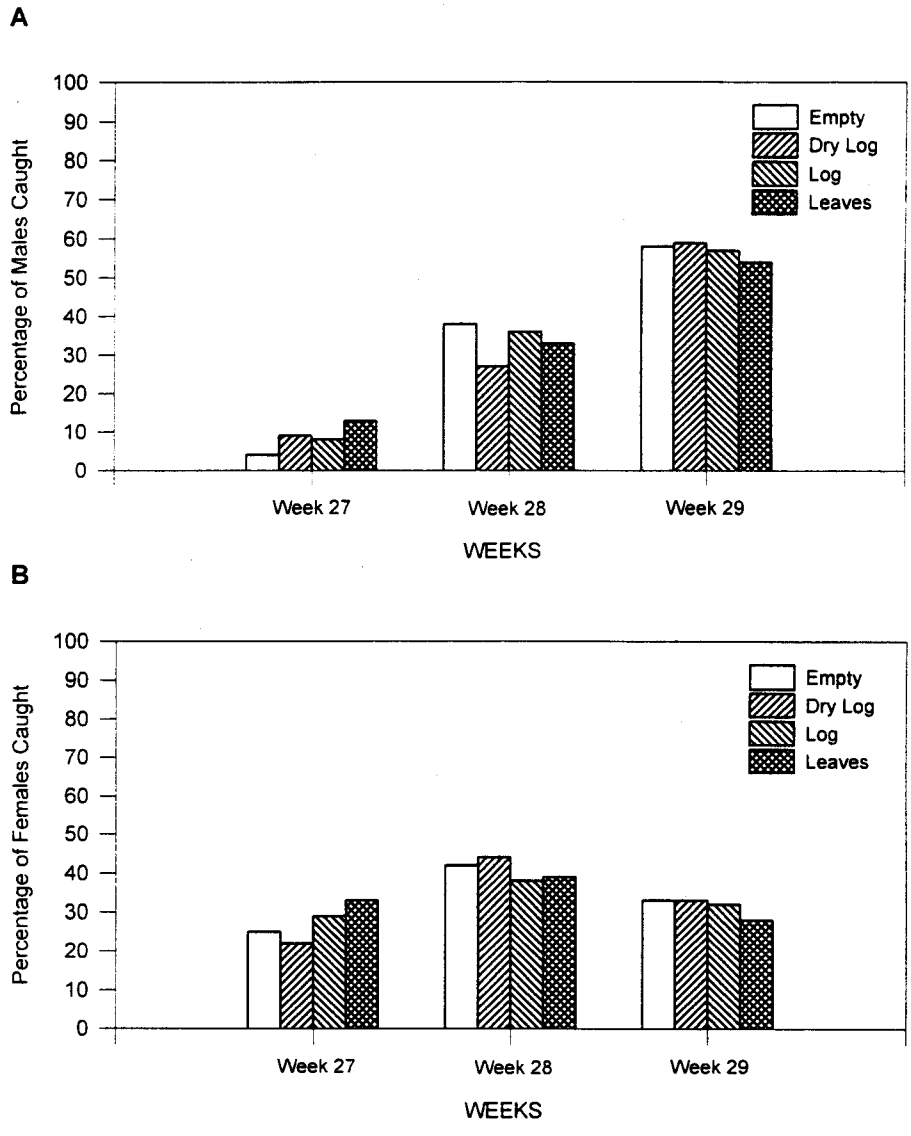


Figure 3.4. Percentage of *Phoracantha semipunctata* males (A) and females (B) caught in empty traps, and traps baited with a dry log, a freshly cut log or with leaves from *Eucalyptus globulus*, at the end of each experimental week. Total number of males caught : 51 in week 27; 153 in week 28; 252 in week 29. Total number of females caught: 45 in week 27; 58 in week 28; 44 in week 29.

From a total of 147 females caught in the traps, 90% had mature ovaries. The remain females had immature ovaries, and were distributed across the different types of traps.

3.3.2. Electroantennograms

In figure 3.5, are shown typical EAGs from a *P. semipunctata* female, elicited upon stimulation with a puff of air from Pasteur pipettes containing a filter paper with paraffin oil, or with increased amount of camphene in paraffin oil (10^{-3}, 10^{-2}, and 10^{-1} v/v). Stimulation with paraffin oil did not elicit any measurable response. The onset of the EAG was delayed in relation to the time of stimulation due to the long distance from the antenna at which the stimulus was injected, in addition to the relatively low airstream flow used for stimulus delivery to the antenna.

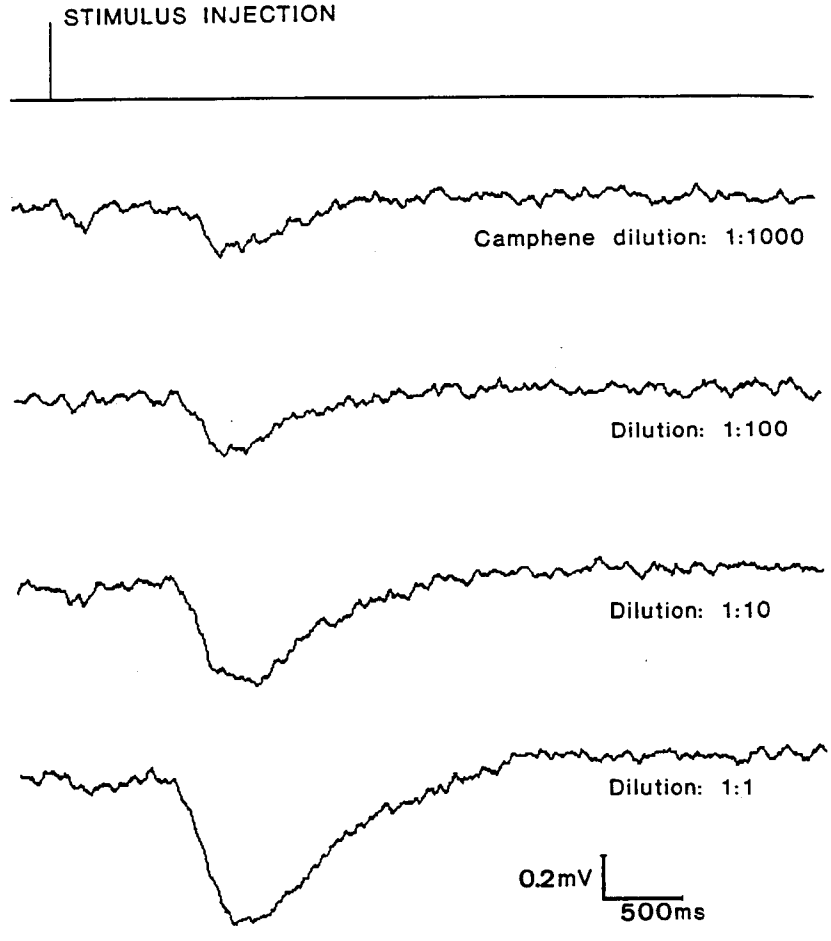


Figure 3.5. Typical EAG responses of a *Phoracantha semipunctata* female stimulated with increased amounts of camphene in paraffin oil (v/v).

For each preparation, the EAG amplitudes elicited by the standard stimulus (1,8-cineole at 10^{-1} v/v) did not decrease with time, and the mean EAG amplitude was 279 ± 181 μV (± SD). No differences were found between males and females in the EAGs elicited by each stimulus, therefore the data from both sexes were pooled.

The samples of fruit of *E. globulus* elicited the highest relative EAG amplitudes, followed by the leaf, trunk node and bark (table 3.3).

Table 3.3. Mean relative EAG amplitudes (± 95% confidence interval) from *Phoracantha semipunctata* beetles elicited by 1.5 g samples of a fruit, a leaf, a trunk node and bark from *Eucalyptus globulus*.

Samples of *Eucalyptus globulus* Material			
Fruit	Leaf	Trunk Node	Bark
193 ± 30	106 ± 20	62 ± 10	29 ± 6
(n = 9)	(n = 9)	(n = 9)	(n = 9)

Figure 3.6 shows the dose-response curves obtained for stimulation with α-pinene, camphene, α-terpinene, 1,8-cineole and ethanol at five concentrations in paraffin oil (v/v). Except for camphene and α-pinene, the remaining three compounds produced rather smooth dose-response relationships, and those of α-terpinene and 1,8-cineole were similar. At concentrations, from 10^{-4} to 10^{-2} (v/v), α-pinene elicited the largest relative EAG amplitudes, whereas camphene, α-terpinene and 1,8-cineole showed similar dose-response relationships. At concentrations above 10^{-2}, camphene was the compound showing the steepest increase in EAG amplitude with dose, and elicited the largest relative EAG at the highest concentration. α-Pinene elicited smaller EAG amplitudes than camphene only at the highest concentration. The other two monoterpenes were clearly less effective at concentrations above 10^{-2} , and ethanol was clearly the less effective odourant at each concentration tested.

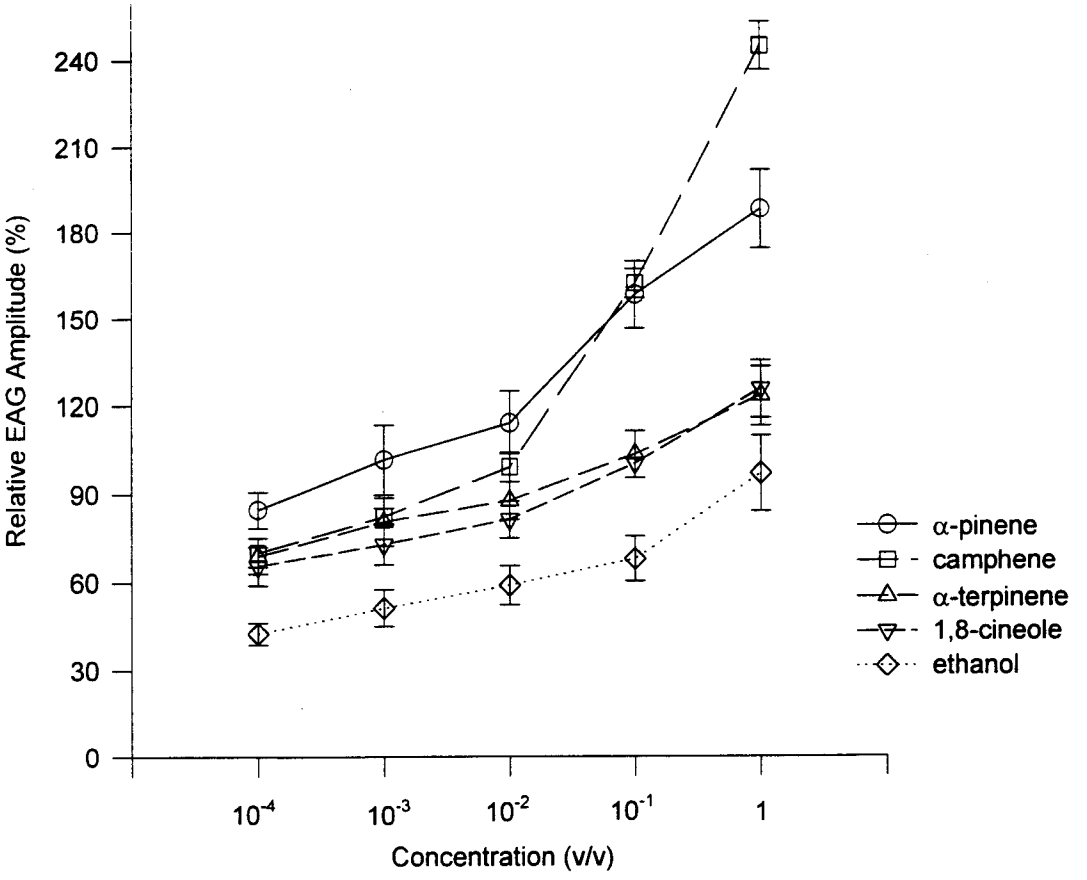


Figure 3.6. Relative EAG amplitudes (mean ± 95% confidence limits, n = 9) from *Phoracantha semipunctata* beetles elicited by α-pinene, camphene, α-terpinene, 1,8-cineole, and ethanol. Standard stimulus: 1,8-cineole; 10^{-1} v/v.

3.4. Discussion

3.4.1. Field Data

The freshly cut *E. globulus* material was clearly attractive to both male and female *P. semipunctata*, the leaves showing higher attractiveness than the logs, which may have resulted from higher concentration of volatiles evaporated from the leaves. This hypothesis is supported by the larger EAG amplitudes elicited by a leaf sample of *E. globulus* than those elicited by a similar amount of bark. The field experiment carried out showed that landing of *P. semipunctata* beetles on the host tree does not occur at random, and a primary attraction mechanism (i.e. orientation to host odour at a distance) mediates the host finding by both males and females.

However, one should not underestimate the role of vision in the landing decisions of *P. semipunctata*. The various baits in the traps provided visual as well as olfactory cues for the flying beetles, whereas the empty traps presumably provided much weaker visual cues and may have caught at random the beetles flying to nearby host trees. The lower number of beetles caught in the traps baited with a dry debarked log of *E. globulus* than in the empty traps (although the difference was not statistically significant for the females), suggest that the flying beetles avoided this type of trap. One cannot exclude the possibility of a repellent odour emanating from the dry logs, but it is also possible that the beetles flying around saw the vertical silhouette and avoided landing on it due to the absence of host odour. On the other hand, an appropriate visual stimulus, mimicking the vertical silhouettes provided by standing trees, may interact with the tree odour in the attraction and landing of *P. semipunctata*. In a field study with the ambrosia beetle, it was demonstrated that the vertical visual stimulus provided by perforated cylinders ("cone traps") synergised with host plant related compounds and pheromone, as higher landing rates were observed on this type of trap than in flight barriers ("window traps") of similar trapping surface and odour bait (Vité and Bakke, 1979).

At present, there is no evidence to support the existence of an aggregation pheromone or a sexual attractant acting over long distances in *P. semipunctata*. Observations on mating behaviour in the laboratory did not reveal any oriented movement from a distance of one sex towards the other, and sexual recognition seems to take place only at close range after contact of the male antenna with the female antenna or with its body parts, suggesting the existence of a contact pheromone for mate recognition (Marques, 1992, 1996; pers.obs.). The existence of epicuticular pheromones for mate recognition has been shown for another cerambycid, *Semanotus japonicus* (Kim *et al.*, 1993). The absence of long-range aggregation pheromone or sexual attractants implies that host odour is of paramount importance as the attractants for both male and female *P. semipunctata*, promoting encounters between sexes on the host tree where copulation is frequently observed (pers.obs.). The close dependence of mate-finding on host-finding has been suggested for other cerambycid species, e.g. *M. sutor* and *M. urussovi* (Grodnitski, 1987), and *Paraglenea fortunei* (Wang *et al.*, 1990), and also for the bark beetle *H. rufipes* (Gardiner, 1979; Lanier, 1983). Once on the host plant, the encounter of males and females may be promoted by close-range sexual pheromones as suggested for *P. fortunei* (Wang *et al.*, 1990) and *M. alternatus* (Kim *et al.*, 1992) or by a contact pheromone laid on the surface of the host tree trunk by *M. sutor* and *M. urussovi* females, which induces an intense local search in males (Grodnitski, 1987).

All types of traps caught more males than females. In a field experiment conducted at the same plantation area in week 28 of the previous year, more males than females (4:1) were caught in similar empty traps or traps baited either with a log or with foliage of *E. globulus* (Barata *et al.*, 1991). In the present study, the unbalanced sex ratio (3:1) observed at the end of the experimental period resulted from an increase of the male population, which was not observed for the females, as would have been expected from the emergence pattern. One must assume that a proportion of the female population was not searching for oviposition sites.

\At the time of emergence from the nymphal chambers, the males have mobile sperm and are able to inseminate the females, whereas the females exhibit immature ovaries (Marques, 1992). For many insects, feeding on protein and carbohydrates such as nectar and honeydew is necessary to provide nutrients for the development of eggs (Städler, 1992), and for most cerambycid species in the adult stage, feeding appears to be an essential prerequisite to egg maturation and oviposition (Linsley, 1959). The feeding requirements of *P. semipunctata* adults are poorly known. Some authors report feeding on the nectar of eucalyptus and other plant species (Chararas, 1969b; Luck and Scriven, 1987; Hanks *et al.*, 1993; pers.obs.) and also on eucalyptus sap (Drinkwater, 1975; pers.obs.). In the laboratory, both males and females fed exclusively on water have a considerably shorter longevity than those fed daily on a honey solution, and only the females fed on honey complete the vitellogenesis to achieve full maturation of the ovaries ca. 21 days after emergence (Marques, 1992). The females caught in the different types of traps were all sexually mature, and a similar phenomenon was observed by Ikeda (1981) for *M. alternatus* females attracted to recently felled pine trees. Immediately after emergence, *P. semipunctata* males are ready to mate, and may allocate their metabolic energy into flight activity, searching for suitable host trees where sexually mature females may be found. The allocation of metabolic energy in females has to be divided between flight activity and vitellogenesis. Thus, it is possible that recently emerged females do not actively search for a suitable host on which to oviposit, but spend more time near appropriate food sources feeding in order to mature their eggs, food sources which may be diverse and not necessarily associated with the host tree suitable for larval development.

A dispersal period is often observed in dendrophagous insects (c.f. Barbosa and Wagner, 1989; Byers, 1995). The insects may respond to different stimuli when foraging for food from when foraging for oviposition sites, and periods of feeding may interrupt oviposition for the development of additional egg batches (Städler, 1992). Ikeda (1981) reported the dependence of EAG amplitudes elicited by α-pinene on the age of *M. alternatus* females: females up to 25 days

after emergence were less sensitive than males, whereas females over 30 days were more sensitive. Thus, it is possible that sexually immature *P. semipunctata* females do not respond to host plant odour as strongly as females ready to oviposit do. The hypothesis of the dependence of female *P. semipunctata* active search for suitable oviposition sites on its physiological condition would be better supported by the data if the females had started emerging earlier than the males, so that early emerged males would have the possibility to mate with already sexually mature females searching for suitable host trees and mates. However, previous surveys of adult emergence from attacked trees in the same eucalyptus plantation showed that females started emerging one week earlier than the males (Lima *et al.*, 1988), and an earlier female emergence from infested logs has been consistently observed over the years (pers. obs.).

3.4.2. Electroantennograms

The electrophysiological recordings showed that *P. semipunctata* detects the odour evaporated from various *E. globulus* material. *Eucalyptus* odour results from the volatilisation of various terpenoid compounds (Doran, 1991) that are accumulated in glands distributed more or less abundantly throughout the foliar parenchyma and bark (Chattaway, 1954; Carr and Carr, 1969, 1970, 1976). Monoterpenes which have been chemically identified in *E. globulus* (Zrira, 1988; Barton, 1989) and ethanol elicited EAG responses suggesting their role as chemical cues in *P. semipunctata* host-finding behaviour. Ethanol elicited lower EAG amplitudes than those elicited by the monoterpenes. This cerambycid readily attacks eucalyptus under physiological stress or recently downed trees (Chararas, 1969a,b), so one should not minimise the possible role of ethanol in the host finding behaviour of *P. semipunctata* on the basis of the low EAG amplitudes elicited. Ikeda (1981), reported that ethanol alone had no attractiveness to *M. alternatus*, but acted as a synergist to the attraction to a mixture of host associated monoterpenes, and to α-pinene. A similar effect was described by Vité and Bakke (1979) for the ambrosia beetle, *Trypodendron lineatum*, where ethanol synergised the attraction of α-pinene. Ethanol, which is a natural product of anaerobic fermentation emitted from the phloem, sapwood, and leaves of stressed, dying or dead trees (Moeck, 1970; Kimmerer and Kozlowski, 1982), may provide an additional chemical cue for the insect, as concerns the physiological condition of the plant.

4.

Orientation of *Phoracantha semipunctata* to *Eucalyptus globulus* in Wind Tunnel

4.1. Introduction

In the previous chapter, it was shown that eucalyptus odour mediates landing of both males and females *P. semipunctata* on host trees. A primary attraction mechanism was suggested, in the form of the flying insects making odour-modulated oriented movements towards the host tree. However, the flight orientation of the beetles was not observed and the establishment of such mechanism in host-finding behaviour requires more direct evidence.

The effective odour blend used by the insect to locate its host plant is unknown. Electrophysiological recordings showed that *P. semipunctata* beetles are sensitive to the odour emanating from *E. globulus*, and to some of its monoterpenoid components (chapter 3, this thesis), but the eucalyptus produces a wide variety of unspecific mono- and sesquiterpenoid compounds (Brophy *et al.*, 1991), and only a small fraction of these was screened. The host finding behaviour of *P. semipunctata* may rely on a complex blend of those compounds in critical ratios, as suggested for other phytophagous species responding to host related compounds (e.g. Visser, 1986; Roseland *et al.*, 1992; Campbell *et al.*, 1993). Field tests designed to investigate the odour blend that is attractive to *P. semipunctata* were not feasible, due to the absence of any information about how many compounds within the complex blend of volatiles produced by *Eucalyptus* spp. can be detected by *P. semipunctata* and might be involved in the host-finding behaviour.

Laboratory bioassays allow an easier direct behavioural observation of the flight orientation of *P. semipunctata* and may be a useful tool that have advantages over field tests for investigating the behavioural effect of synthetic blends of host related chemicals. Wind tunnels have been used to study the manoeuvres of flying insects subjected to odour plumes derived from sources of pheromone (e.g. Kennedy, 1983; Kuenen and Baker, 1983; Baker and Linn, 1984; Vickers and Baker, 1994), or plant odour (e.g. Kennedy, 1977; Tingle *et al.*, 1990; Willis and Arbas, 1991; Kaiser and Cardé, 1992; Tingle and Mitchell, 1992; Aluja *et al.*, 1993). Such studies have been quite useful in elucidating which behavioural mechanisms are used by flying insects to find odour sources. As pointed out by Städler (1992), the evidence collected so far by different investigators supports the conclusion of Kennedy (1977) that "odour-modulated optomotor anemotaxis" is the only behavioural mechanism involved in distant orientation of

flying insects to the host plant. Besides their utility for investigating the orientation mechanisms of flying insects to odour sources, wind tunnels provide bioassays which have some definite advantages over field bioassays involving capture of wild insects. A major advantage is that a wind tunnel is a physical model of the environment that allows the experimental manipulation of one variable at a time (Baker and Linn, 1984). Temperature, humidity, wind speed, and odour stimulation conditions (intensity, composition, shape, etc.) can be reproduced day after day, and the experimenter does not have to face the influence of environmental variation on the results, as is common to field tests (and that must be factored out by replication and experimental design). Thus, cause-effect relationships from these variables can be inferred more easily from wind tunnel observations than from field tests. Wind tunnel studies may also provide relevant information such as optimal odour blend composition, optimal odour releasing rates, optimal design of field traps. These studies can be performed throughout the year in preparation for the time when field tests can ultimately be performed with synthetic compounds (Baker and Linn, 1984).

In the present study, a wind tunnel bioassay was established to study the locomotion and flight behaviour of *P. semipunctata* beetles subjected to an odour plume originating from freshly cut logs of *E. globulus*.

4.2. Materials & Methods

Wind tunnel and experimental environment. The wind tunnel designed and constructed for this study is represented in figure 4.1. The work section (WS), where the behavioural observations took place, was 2.5 m long and 1 m high and wide, and made out of clear PVC plates connected to each other by an aluminium frame. On the side, two doors allowed access to the entire WS. To provide a visual ground pattern for the flying beetles, white cardboard with randomly placed black dots (20 cm diam) covered the entire floor of the WS and part of the WS side walls. At the centre of the WS, there was a white circular platform (50 cm diam) placed 20 cm above the floor. Each beetle tested was introduced in the wind tunnel through a small door on the side of the WS and released on the platform where the locomotion behaviour before take-off flight was observed. The other sections of the wind tunnel had opaque grey PVC walls. The connection between the fan and the wind tunnel was via a pyramidal section made out of zinc-plated steel. All fixed joints were air-sealed with silicone and the access doors with foam.

The air was drawn from both outside and inside the room housing the wind tunnel by a power-controlled centripetal fan (Woods, max. rpm = 1440 rpm), and blown into the WS after

passing through two cloth screens. No activated charcoal filter was used to clean the air before entering the wind tunnel, but dust was removed by a fine mesh cloth screen, before passing through the fan. The beetles were prevented from leaving the WS by a nylon net screen placed at the downwind end. The windspeed was measured in the middle of the WS by a thermo-anemometer (Alnor®) and set at 50 cm/s, air temperature was 25° ± 2° C and humidity was 55 ± 5%. The locomotor and flight behaviour of the beetles was filmed by a Stemmer CCD-video camera (light sensitivity of 0.02 lux) equipped with a 9mm TV lens (Fujinon) fitted with a 0.5x wide angle attachment (Hamma®), and videotaped with an 8mm recorder (Sony, EV-S550E). The video camera was suspended on the room ceiling, ca. 1.4 m above the top of the wind tunnel, providing a wide angle image that covered ca. 3/5 of the WS at the ceiling level and almost its entire length at the floor level, as illustrated in figure 4.1. Red light, emitted by darkroom red lamps (Osram, 15E, E27), was reflected from the white walls and ceiling of the room, providing a fairly uniform light intensity of 0.4 lux along the WS.

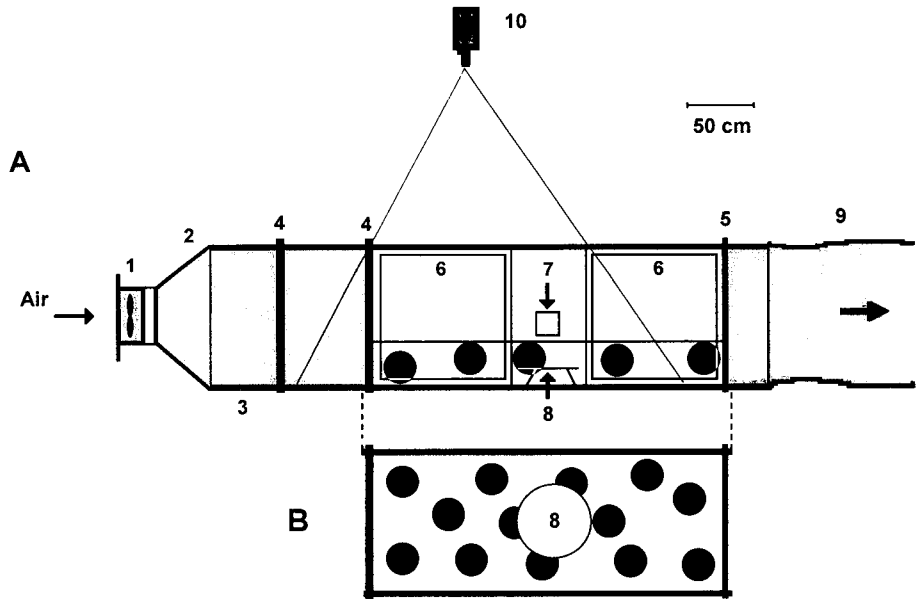


Figure 4.1. Diagram of the wind tunnel. A - side view of the full length of the wind tunnel; B - top view of the working section (WS). 1 - fan; 2 - pyramidal zinc connection; 3 - opaque PVC walls; 4 -cloth screens; 5 - nylon net screen; 6 - access doors to the WS; 7 - door for introduction of the beetles; 8 - release platform; 9 - way out through a flexible plastic connection; 10 - suspended video camera and limits of wide angle image obtained.

Insects. Adult females and males were obtained from naturally infested logs of *E. globulus* and reared in the laboratory as described in chapter 3. The beetles tested in the wind tunnel were unmated and their age was between 21 and 45 days. In order to prepare the beetles for the light conditions of the wind tunnel observations, they were subjected to a light regime of 16 hours of white light provided by fluorescent bulbs, followed by 8 hours of red light (2.5 lux) provided by darkroom red lamps (Osram, 15E, E27). The red light period started at 22:00 hrs. A study on the circadian locomotion activity of *P. semipunctata* males and females under a similar light regime showed that the beetles synchronise their activity with the red light period (Barata, unpublished).

Stimuli. Logs of *E. globulus* 1 m long and ca. 14 cm diameter were obtained from 6-year old trees that had not been attacked at the plantation of Fonte Santa, in the vicinity of Redondo, southern Portugal. The logs were kept in a cooled room (5° C) until use up to three days after being cut. In the morning of the experimental day, a log was moved to ambient temperature. Immediately before the onset of the experiments, the log was placed vertically at the upwind end of the WS, providing visual and olfactory stimuli. In control experiments, a grey PVC drainpipe (1 m long and 14 cm diam) was placed in the WS in the same position as the log, providing a visual stimulus without eucalyptus odour. The odour emitted from the log of *E. globulus* was simulated using ammonium acetate smoke generated by mixing the vapours from ammonium hydroxide (NH_4OH) and acetic acid (CH_3CO_2H). Vapours of CH_3CO_2H were blown over the PVC pipe covered with a cloth soaked with NH_4OH, and the smoke emitted from the entire surface of the pipe, formed a filamentous plume that spread out from the source and covered the width of the release platform in the middle of the WS (figure 4.2). Just in front of the pipe there was a higher concentration of smoke, due to low air speed and turbulence.

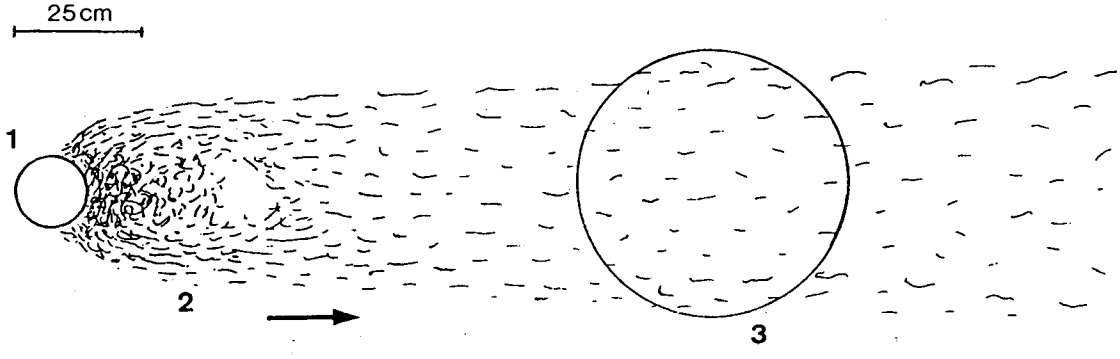


Figure 4.2. Representation of the plume of ammonium acetate smoke (2) originated on the PVC pipe (1) based on video image recorded on tape with the camera suspended over the wind tunnel WS (see figure 4.1). The filamentous plume spread out from the pipe surface and covered the width of the release platform (3) at the middle of the WS.

Experimental procedure. The behavioural observations took place over a total of 14 experimental days between 7 July and 3 August 1994. In each experimental day, 25 specimens of the same sex were submitted either to the log or to the PVC pipe. For each sex, a different log of *E. globulus* was used in each of 4 experimental days, and the same PVC pipe was used in 3 days. The sequential order of different experimental days was determined at random. In total, 100 specimens of each sex were tested with the log, and 75 with the PVC pipe. After each experimental day with a log, the wind tunnel walls and release platform were cleaned with alcohol. During the red light period of the day previous to each experiment, the beetles to be tested were given a chance to fly spontaneously in the wind tunnel. On the morning of the experimental day, they were removed from the wind tunnel and placed in individual plastic boxes with an easily removable lid. Fifteen minutes after the onset of the red light, a box containing a single insect was placed on the centre of the release platform as shown in figure 4.3. One minute later the lid was removed, allowing the beetle to leave the box and walk on the platform until it took off flight. After its first landing (as observed on a video monitor) the beetle was removed from the wind tunnel, and the same procedure was repeated for the other beetles. If a beetle did not walk out of the box within 5 minutes, or failed to fly after a first take-off attempt from the release platform (bad flyers), it was removed and not considered in the analysis. The observations on each experimental day lasted up to 4 hours after the onset of the red light. Each beetle was tested only once.

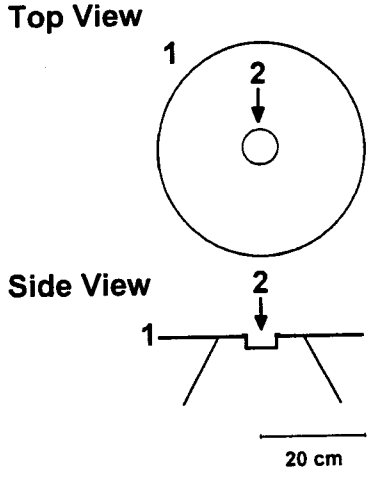


Figure 4.3. Release platform (1) in the wind tunnel and positioning of the plastic box (2) with the insect.

Behavioural data. The detailed observations of behavioural responses were made by playback of the video recordings, and on the platform included rate of walking, frequency of walking, stop time, overall direction of walk, time to take-off flight; in flight, overall flight direction and landing place was recorded. The rate of walking was measured between the moment the beetle left the box and initiated flight or reached the edge of the release platform. The frequency of walking was measured as the number of stops per distance walked until the beetle initiated flight or reached the edge of the release platform. The stop time was measured as the mean stop time per beetle during the walking period on the release platform. Sometimes, the beetle went under the release platform, came back to the top and then initiated flight from the edge. The time to take-off was measured as the total time spent on the platform after the beetle had left the box, including the time the beetle may have spent under the platform.

Analysis was done from acetate sheet tracings of the walk and flight made on the video monitor screen, the position of the beetle being marked every 10 frames (0.4 s) when walking and every 3 frames (0.12 s) when flying (until it landed or left the video view). The direction of walking or flight was scored as upwind, crosswind, downwind or non-directional, on the basis of the predominant direction of the track. Landing place was scored as landing on the log, on the PVC pipe or elsewhere in the tunnel.

Data analysis. Categorical data were summarised as frequencies and analysed separately for each behavioural category, as multi-way frequency tables, using a log-linear analysis procedure. This procedure uses a linear model approach to the analysis, applying general weighted least-square regression techniques to estimate cell proportions in multidimensional categorical layouts (Bishop *et al.*, 1974; Everitt, 1977; Kleinbaum and Kupper, 1978). A "model" in statistical analysis is an expression of how observed data are affected by variables and combination of variables, and "log-linear" refers to a procedure whereby a multiplicative relationship is transformed to a linear relationship by the use of logarithms (Zar, 1984). In the terminology of log-linear models, one tests interactions of variables (instead of independence). This approach allowed the testing for significant interactions and main effects of variables and to contrast for levels of main effects for a specific response, using the chi-square (χ^2) statistic.

Specifically tested was whether there were interactions between the experimental day, the sex and a specific behavioural response either to the log of *E. globulus* or to the PVC pipe. Interactions between the stimuli, the sex and a specific behavioural response were also tested. A log-linear model was accepted to fit the data if the probability of the χ^2 was higher than 0.1, and the effects were eliminated if their significance was higher than 0.05. Response time data

collected in different experimental days were pooled for each sex and stimulus. The rate of locomotion and the time to take-off data were analysed using two-way analysis of variance after transformation by $Log_{10}(X+1.5)$ to reduce heterogeneity of variances and the proportional relationship between mean and variance values. Subsequent mean separation between groups was carried out with the Tukey's Honest Significant Difference (HSD) test for unequal replications (significant probability level for differences, $p < 0.05$). The data on frequency of locomotion and mean stop time per beetle data were analysed using the non-parametric Kruskal-Wallis Anova test, and the median differences were subsequently tested by the Median Test (significant probability level for differences, $p < 0.05$).

4.3. Results

Table 4.1 shows the total number of beetles of each sex subjected to the *E. globulus* log or to the PVC pipe, and indicates the number of flyers, bad flyers and inactives. All beetles exhibited flight take-off attempts within 1 min of leaving the box, so the occurrence of flight was independent of the stimulus in the wind tunnel; all the beetles considered for analysis were therefore flyers (n = 266).

Table 4.1. Total number of males and females *Phoracantha semipunctata* tested either with log of *Eucalyptus globulus* or with a PVC pipe, discriminating the number flyers, bad flyers and inactive insects.

Stimulus	Sex	Flyers	Bad Flyers	Inactive	Total
Log	Male	92	5	3	100
Log	Females	76	10	14	100
PVC Pipe	Males	52	10	12	75
PVC Pipe	Females	46	12	17	75
	Total	266	37	46	340

4.3.1. Walking Behaviour

Most beetles exhibited a period of walking before take-off flight, and also for most of them take-off occurred at the edge of the release platform. The predominant direction of locomotion was independent of the experimental day for each stimulus. For the *E. globulus* logs, the data fit a log-linear model that considers only significant differences in the beetles' direction

of locomotion (Pearson $\chi^2_{28} = 23.4$; $p = 0.71$), and a similar model fits the data collected in the presence of the PVC pipe (Pearson $\chi^2_{20} = 20.3$; $p = 0.44$). There were no significant two-way interactions between sex, the experimental day and the overall direction of locomotion (tables 4.2 and 4.3), but in the presence of a log 81.5% of the beetles walked in the upwind direction, whereas in the presence of the PVC pipe only 51.6% did so. The data from each experimental day were pooled and the subsequent log-linear analysis revealed that the data fit a model that considers a single two-way interaction between the direction of locomotion and the stimulus (Pearson $\chi^2_8 = 10.4$; $p = 0.24$). The direction of locomotion was significantely influenced by the type of stimulus ($\chi^2_3 = 23.6$; $p < 0.05$; table 4.4), and no differences were observed between the responses of males and females, thus the data from both sexes were pooled (figure 4.5). In the presence of the log, there was a significantly higher frequency of beetles walking upwind, and a significantly lower frequency of insects walking downwind or with no predominant direction.

Table 4.2. Specimens of *Phoracantha semipunctata* in the presence a *Eucalyptus globulus* log: tests of partial and marginal association of all effects in the three-way contingency table of (I) direction of locomotion (4 levels) X (II) experimental days (4 levels) X (III) sex (2 levels). Significant differences ($p<0.05$) are marked with *.

Effect	df	Part. Ass. χ^2	p	Mrg. Ass. χ^2	p
I	3	181.120	**0.00000 ***	181.120	**0.00000 ***
II	3	6.598	0.08588	6.598	0.08588
III	1	0.589	0.44298	0.589	0.44298
I x II	9	5.662	0.77321	5.661	0.77333
I x III	3	6.339	0.09625	6.338	0.09630
II x III	3	0.640	0.88733	0.638	0.88760

Table 4.3. Specimens of *Phoracantha semipunctata* in the presence of a PVC pipe: tests of partial and marginal association of all effects in the three-way contingency table of (I) direction of locomotion (4 levels) X (II) experimental days (4 levels) X (III) sex (2 levels). Significant differences ($p < 0.05$) are marked with *.

Effect	df	Part. Ass. χ^2	p	Mrg. Ass. χ^2	p
I	3	32.804	**0.00000 ***	32.804	**0.00000 ***
II	2	2.561	0.27788	2.561	0.27788
III	1	0.340	0.56000	0.340	0.56000
I x II	6	11.870	0.06452	11.745	0.06795
I x III	3	1.161	0.76237	1.0165	0.79726
II x III	2	0.235	0.88936	0.0900	0.95601

Table 4.4. Tests of partial and marginal association of all effects in the three-way contingency table of (I) direction of locomotion (4 levels) X (II) sex (2 levels) X (III) stimulus (2 levels). Significant differences ($p<0.05$) are marked with *.

Effect	df	Part. Ass. χ^2	p	Mrg. Ass. χ^2	p
I	3	206.717	**0.00000 ***	206.717	**0.00000 ***
II	1	1.125	0.28879	1.125	0.28879
III	1	13.666	**0.00022***	13.666	**0.00022 ***
I x II	3	7.192	0.06604	6.536	0.08830
I x III	3	24.271	**0.00022 ***	23.614	**0.00003 ***
II x III	1	0.660	0.41658	0.003	0.95380

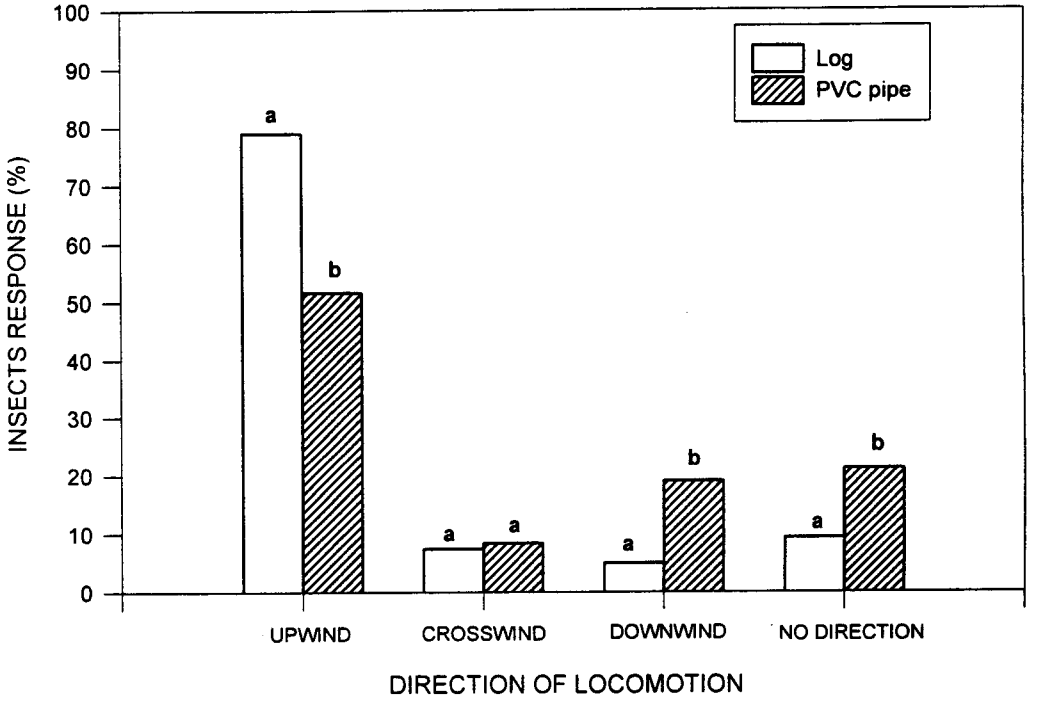


Figure 4.5. Overall direction of walking of *Phoracantha semipunctata* in the presence of a *Eucalyptus globulus* log or a PVC pipe (male and female data pooled). For each direction, different letters indicate significant difference in response (χ^2; $p < 0.05$) for individual comparisons. The numbers of observations for each stimulus were: log, 163; PVC pipe, 95.

There were no significant differences in the distance walked by the two sexes in the presence of a log or the PVC pipe (table 4.5). Most beetles reached the edge of the release platform before take-off, regardless of their sex and stimulus, and walked on average 27.1 ± 17.9

cm (mean ± SD). Also, the walking speed was not significantely different between sexes, and was not influenced by the stimulus (table 4.6). On average, the walking speed on the platform was 6.2 ± 1.1 cm/s (mean ± SD). However, the time to take-off of males and females was significantely influenced by the stimulus (table 4.7). In the presence of a log, both sexes took a shorter time to initiate flight than they did in the presence of the PVC pipe (figure 4.6). The frequency of walking did not differ between the sexes ($H = 1.71$; $p > 0.05$), and was significantely influenced by the stimulus ($H = 4.13$; $p < 0.05$). In the presence of the log a significantely higher proportion of beetles stopped more frequentely than in the presence of the pipe ($\chi^2 = 5.10$; $p < 0.05$). The mean stop time per beetle did not differ between the sexes ($H = 2.61$; $p > 0.05$), and it was significantely influenced by the stimulus ($H = 7.15$; $p < 0.05$). In the presence of the log a significantely higher proportion of beetles had a shorter mean stop time than in the presence of the pipe ($\chi^2 = 5.10$; $p < 0.05$).

Table 4.5. Two-way analysis of variance of the distance walked by males and females *Phoracantha semipunctata* on the release platform in the presence of a *Eucalyptus globulus* log or a PVC pipe. Significant differences ($p < 0.05$) are marked with *.

Effect	df	MS	F values	p
Sex	1	0.096	1.047	0.307
Stimuli	1	0.334	3.645	0.057
interaction	1	0.042	0.456	0.500
error	262	0.092		

Table 4.6. Two-way analysis of variance[1] of the locomotory rate of males and females *Phoracantha semipunctata* on the release platform in the presence of a *Eucalyptus globulus* log or a PVC pipe. Significant differences ($p < 0.05$) are marked with *.

Effect	df	MS	F values	p
Sex	1	0.003	0.444	0.506
Stimuli	1	0.009	1.536	0.216
interaction	1	0.006	0.989	0.321
error	254	0.006		

[1] Eight beetles that initiated flight at the edge of the release box were not considered in this analysis.

Table 4.7. Two-way analysis of variance of the time of flight take-off in males and females *Phoracantha semipunctata* on the release platform in the presence of a *Eucalyptus globulus* log or a PVC pipe. Significant differences ($p < 0.05$) are marked with *.

Effect	df	MS	F values	p
Sex	1	0.000	0.000	0.991
Stimuli	**1**	**1.933**	**24.183**	**0.000 ***
interaction	1	0.159	1.992	0.159
error	262	0.080		

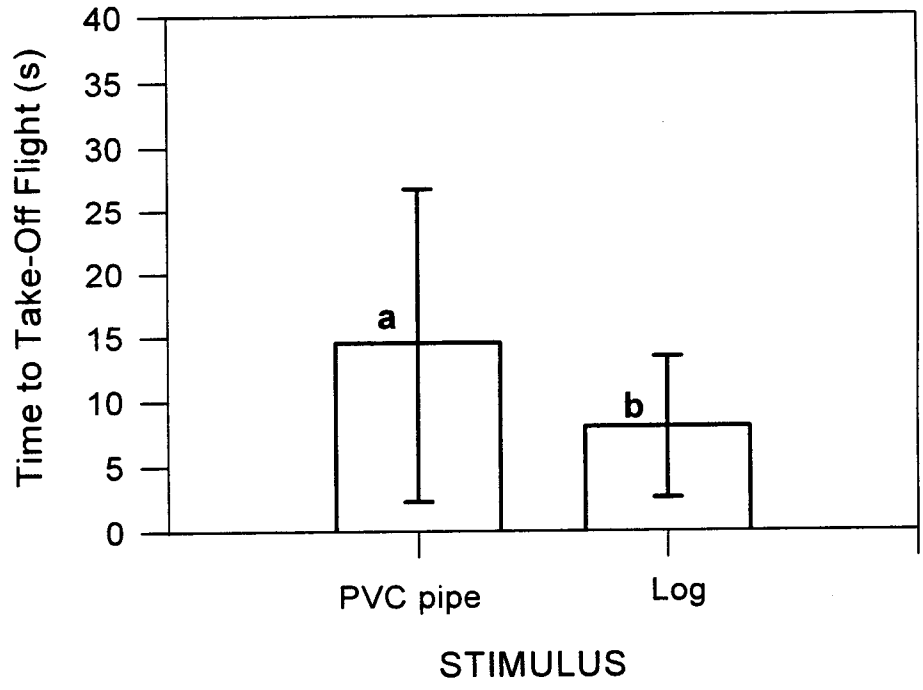


Figure 4.6. Mean time ± SD (indicated by vertical bars running through the top of each bar) to take-off flight of *Phoracantha semipunctata*, in the presence of the PVC pipe or a *Eucalyptus globulus* log. Data for males and females were pooled. Different letters indicate significant differences between the means of the data transformed by $\log_{10}$ (X+1.5) (HSD test; $p < 0.05$). The number of observations in the presence of each stimulus were: PVC pipe, 98; log, 168.

4.3.1. Flight Behaviour

The overall flight direction was found to be independent of the experimental day for both log and PVC pipe. For logs, the data fit a log-linear model that considers the absence of any significant two-way interactions between sex, the experimental day and the flight direction (Pearson $\chi^2_{28} = 29.3$; $p = 0.40$) (table 4.8), and a similar model fit the data collected in the presence of the PVC pipe (Pearson $\chi^2_{20} = 10.9$; $p = 0.95$) (table 4.9). However, in the presence of the logs, there were significant differences between the frequencies of each flight direction, whilst in the presence of the PVC pipe, there were no significant differences between the four directions (table 4.8 and 4.9). The data from each experimental day were pooled and the subsequent log-linear analysis revealed that the data fit a model that considers a single two-way interaction between the flight direction the stimulus (Pearson $\chi^2_8 = 9.7$; $p = 0.29$). The overall flight direction was significantely influenced by the type of stimulus ($\chi^2_3 = 45.7$; $p < 0.05$; table 4.10), and no significant differences were observed between the sexes, so the data from both were pooled (figure 4.7). In the presence of a log, there was a significantly higher frequency of beetles flying upwind, and a significantly lower frequency flying crosswind, downwind or with no predominant direction.

Table 4.8. Specimens of *Phoracantha semipunctata* in the presence of a *Eucalyptus globulus* log: tests of partial and marginal association of all effects in the three-way contingency table of (I) flight direction (4 levels) X (II) experimental days (4 levels) X (III) sex (2 levels). Significant differences ($p < 0.05$) are marked with *.

Effect	df	Part. Ass. χ^2	p	Mrg. Ass. χ^2	p
I	3	120.256	**0.00000 ***	120.286	**0.00000 ***
II	3	2.651	0.44860	2.651	0.44860
III	1	1.998	0.15750	1.998	0.15750
I x II	9	10.075	0.34446	10.163	0.33752
I x III	3	5.103	0.16441	5.191	0.15836
II x III	3	0.915	0.82192	1.002	0.80080

Table 4.9. Specimens of *Phoracantha semipunctata* in the presence of a PVC pipe: tests of partial and marginal association of all effects in the three-way contingency table of (I) flight direction (4 levels) X (II) experimental days (4 levels) X (III) sex (2 levels). Significant differences ($p < 0.05$) are marked with *.

Effect	df	Part. Ass. χ^2	p	Mrg. Ass. χ^2	p
I	3	4.077	0.25334	4.077	0.25334
II	2	0.561	0.75545	0.561	0.75545
III	1	0.230	0.63194	0.230	0.63494
I x II	6	2.163	0.90416	2.101	0.91017
I x III	3	4.750	0.19108	4.689	0.19611
II x III	2	0.332	0.84695	0.271	0.87336

Table 4.10. Tests of partial and marginal association of all effects in the three-way contingency table of (I) flight direction (4 levels) X (II) sex (2 levels) X (III) stimulus (2 levels). Significant differences ($p < 0.05$) are marked with *.

Effect	df	Part. Ass. χ^2	p	Mrg. Ass. χ^2	p
I	3	94.428	**0.00000** *	94.428	**0.00000** *
II	1	1.768	0.18361	1.768	0.18361
III	1	18.083	**0.00002** *	18.083	**0.00002** *
I x II	3	6.935	0.06604	6.741	0.08066
I x III	3	45.763	0.07401	45.569	**0.00000** *
II x III	1	0.270	0.60342	0.075	0.78377

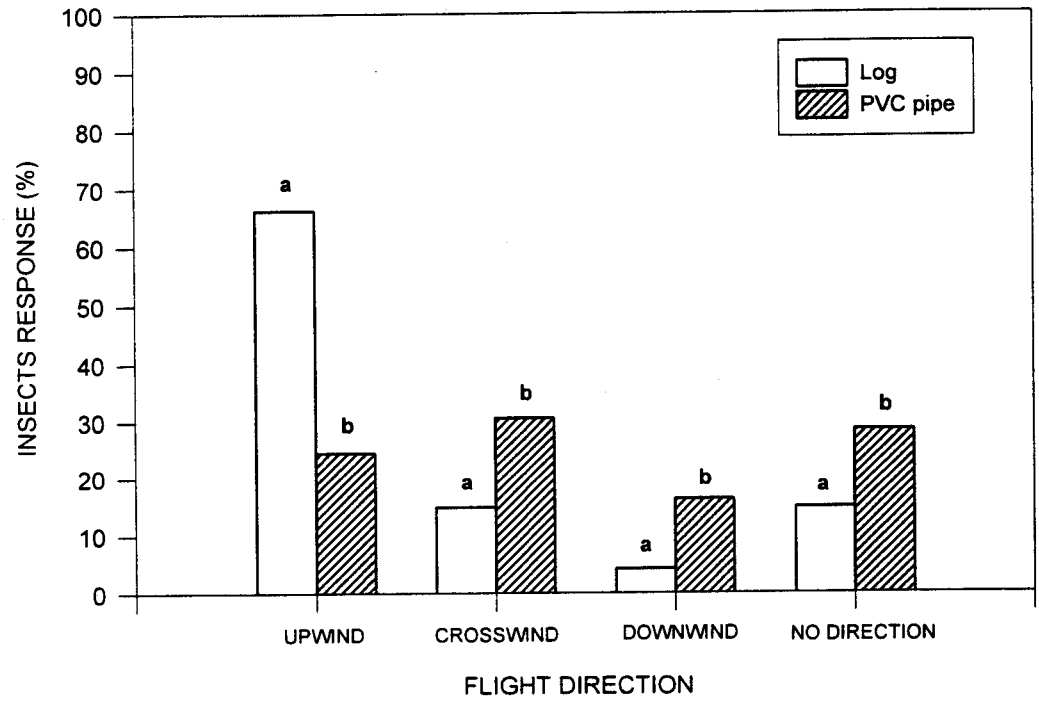


Figure 4.7. Overall flight direction of *Phoracantha semipunctata* in the presence of a *Eucalyptus globulus* log or a PVC pipe (male and female data pooled). For each direction, different letters indicate significant difference in response (χ^2; $p < 0.05$) for individual comparisons. The numbers of observations for each stimulus were: log, 168; PVC pipe, 98.

There were no beetles that landed on the PVC pipe (n = 98), and no significant differences were found between the landing frequency of males and females on different logs of *E. globulus* over the 4 experimental days (table 4.11), therefore these data were pooled and showed that 48% of the beetles tested landed on the log (n = 168). In a total of 80 beetles that

landed on the *E. globulus* log, 89% walked upwind on the release platform, whereas 5% walked crosswind, 5% showed no predominant direction of locomotion, and 1% initiated flight from the edge of the release box.

Table 4.11. The best log-linear model found to fit the data does not account for any interactions between the landing frequency on the log, the sex and the experimental day (Pearson $\chi^2_{12} = 5.8$; $p = 0.92$): tests of partial and marginal association of all effects in the three-way contingency table of (I) landing place (2 levels) X (II) experimental days (4 levels) X (III) sex (2 levels). Significant differences ($p < 0.05$) are marked with *.

Effect	df	Part. Ass. χ^2	p	Mrg. Ass. χ^2	p
I	1	0.364	0.546	0.364	0.546
II	3	3.023	0.388	3.023	0.388
III	1	1.457	0.228	1.457	0.228
I x II	3	0.951	0.813	1.024	0.795
I x III	1	0.364	0.546	0.438	0.508
II x III	3	0.688	0.876	0.762	0.859

Figures 4.8, 4.9 and 4.10, show the ground projections of some individual flight tracks of the *P. semipunctata* beetles that landed on an *E. globulus* log, superimposed on the odour plume edges as obtained from simulation with ammonium acetate smoke. In 32 cases (40%), the upwind flight path progressed within the boundaries of the odour plume as in figure 4.8. These beetles exhibited a quick upwind progression, often with narrow zigzag movements. In 38 cases (48%), the flying beetle eventually lost contact with the odour plume, and exhibited a sharp turns or quick counterturns followed by crosswind or downwind excursions that led it to regain contact with the odour plume, and resumed zigzagging upwind progression provided it flew within the boundaries of the plume (figure 4.9. In 10 cases (12%), the beetle flew erratically upwind as result of frequent contact with the ceiling of the wind tunnel (figure 4.10). The type of movements described were never observed in beetles flying in the presence of the PVC pipe, where the flight tracks were more linear, occurring with equal frequency in all direction categories (figure 4.11), except when the beetle bounced off the ceiling of the wind tunnel.

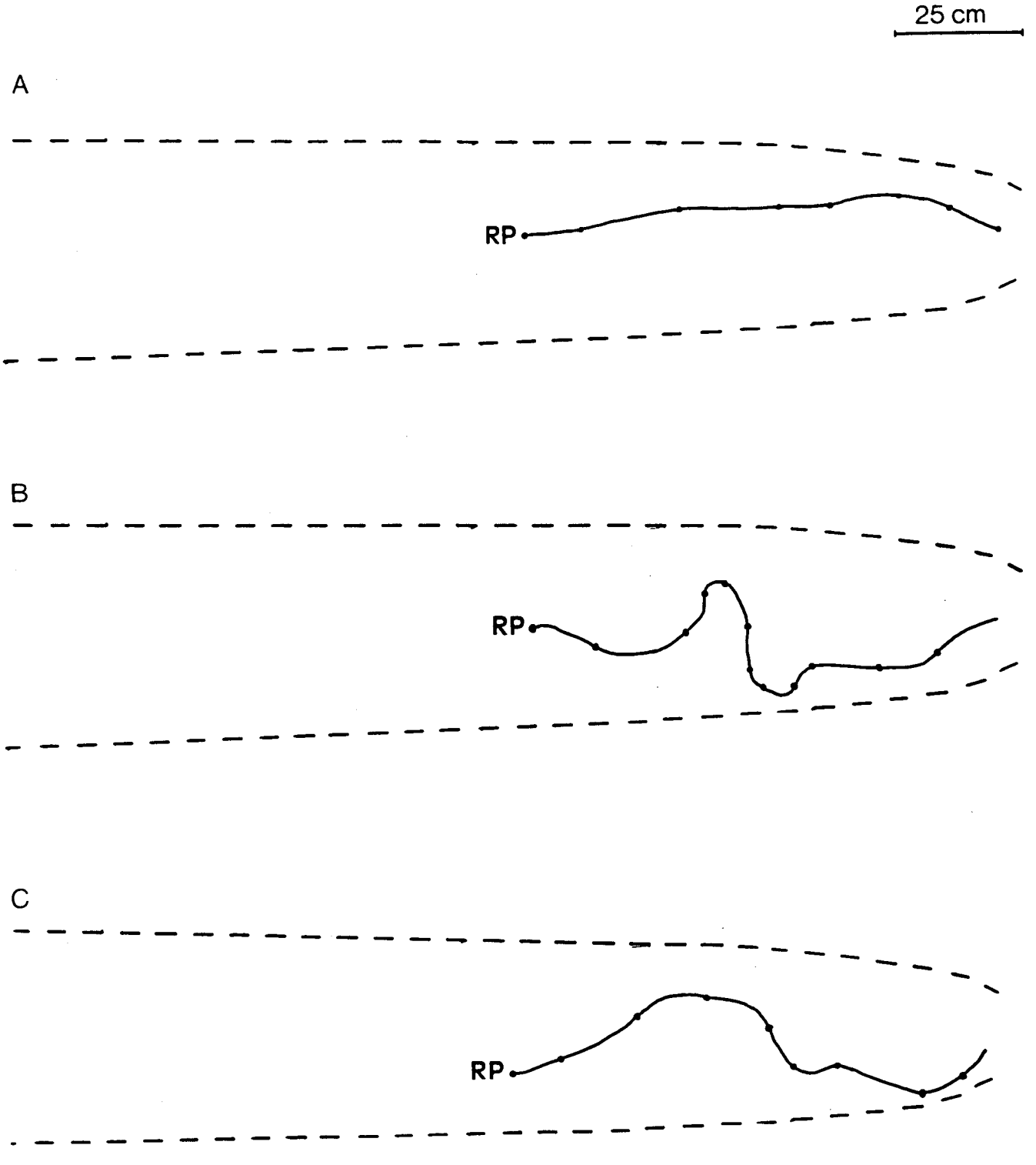


Figure 4.8. Within plume flight tracks *Phoracantha semipunctata* beetles videotaped from above. The broken lines indicate the edges of the odour plume from a *Eucalyptus globulus* log placed vertically at the upwind end of the wind tunnel. These three beetles (A-C) landed on the log after upwind progression within the boundaries of the odour plume. The dots represent the beetle's locations at consecutive 0.24 s intervals. The wind (50 cm/s) was blowing from right to left. The beetles initiated flight from the release platform (RP) and flew from left to right.

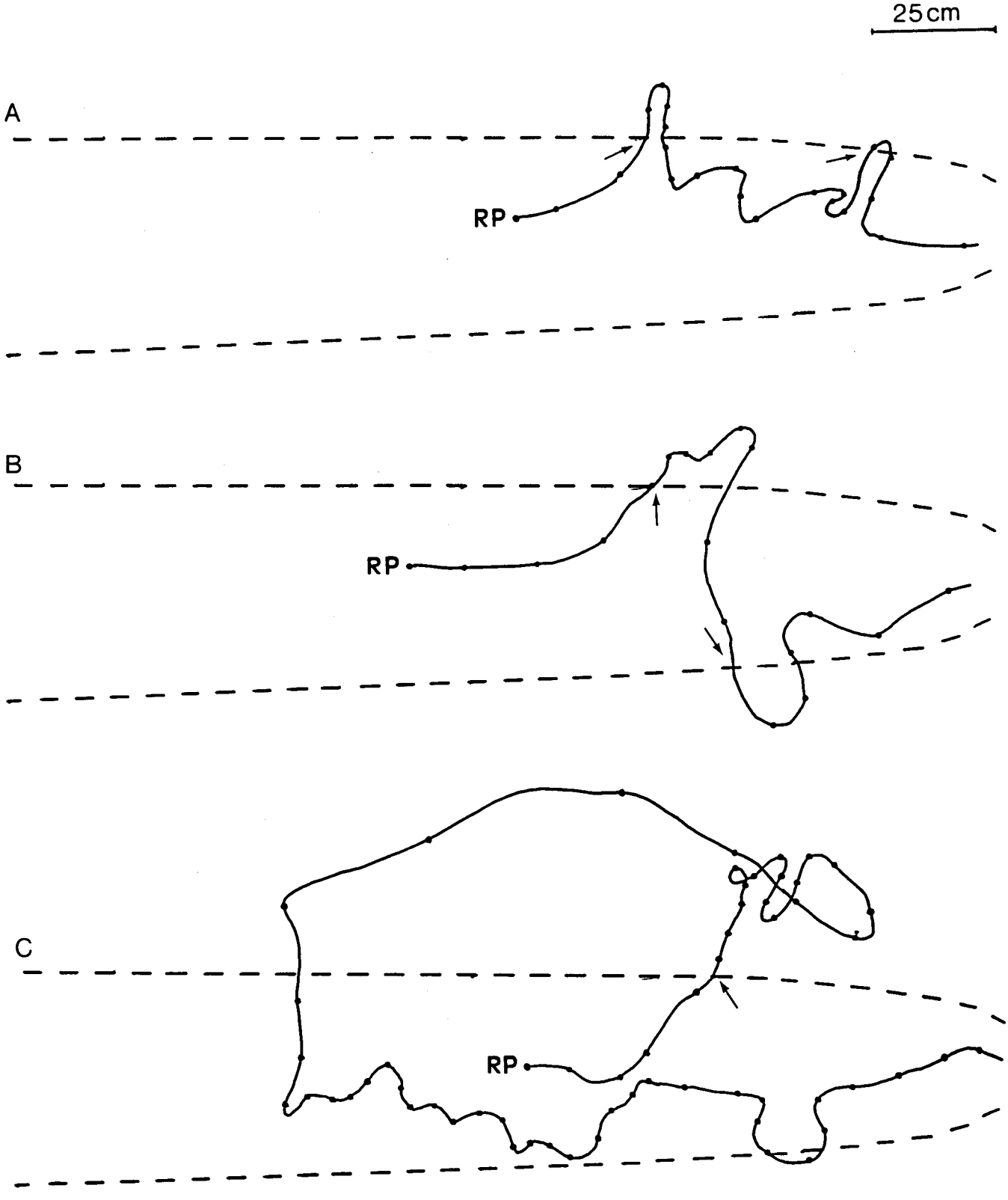


Figure 4.9. Plume-loss flight tracks *Phoracantha semipunctata* beetles videotaped from above. The broken lines indicate the edges of the odour plume from a *Eucalyptus globulus* log placed vertically at the upwind end of the wind tunnel. These three beetles (A-C) landed on the log. During flight, the beetle lost contact with the odour plume as indicated by the arrows. Upwind progression was resumed after crosswind (A-B) or downwind (C) flight that led to regain contact with the odour plume. The dots represent the beetle's locations at consecutive 0.24 s intervals. The wind (50 cm/s) was blowing from right to left. The beetles initiated flight from the release platform (RP) and flew from left to right.

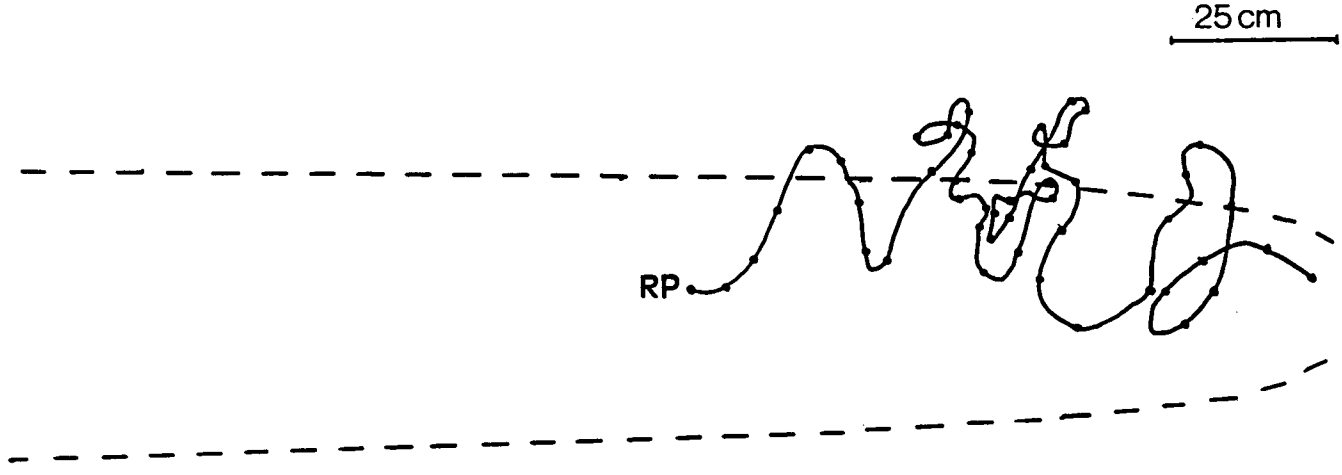


Figure 4.10. Erratic flight track of a *Phoracantha semipunctata* beetle videotaped from above. The broken lines indicate the edges of the odour plume from a *Eucalyptus globulus* log placed vertically at the upwind end of the wind tunnel. The erratic upwind progression of the beetle was influenced by bouncing off the wind tunnel ceiling as indicated by the arrows. The dots represent the beetle's locations at consecutive 0.24 s intervals. The wind (50 cm/s) was blowing from right to left. The beetle initiated flight from the release platform (RP) and flew from left to right.

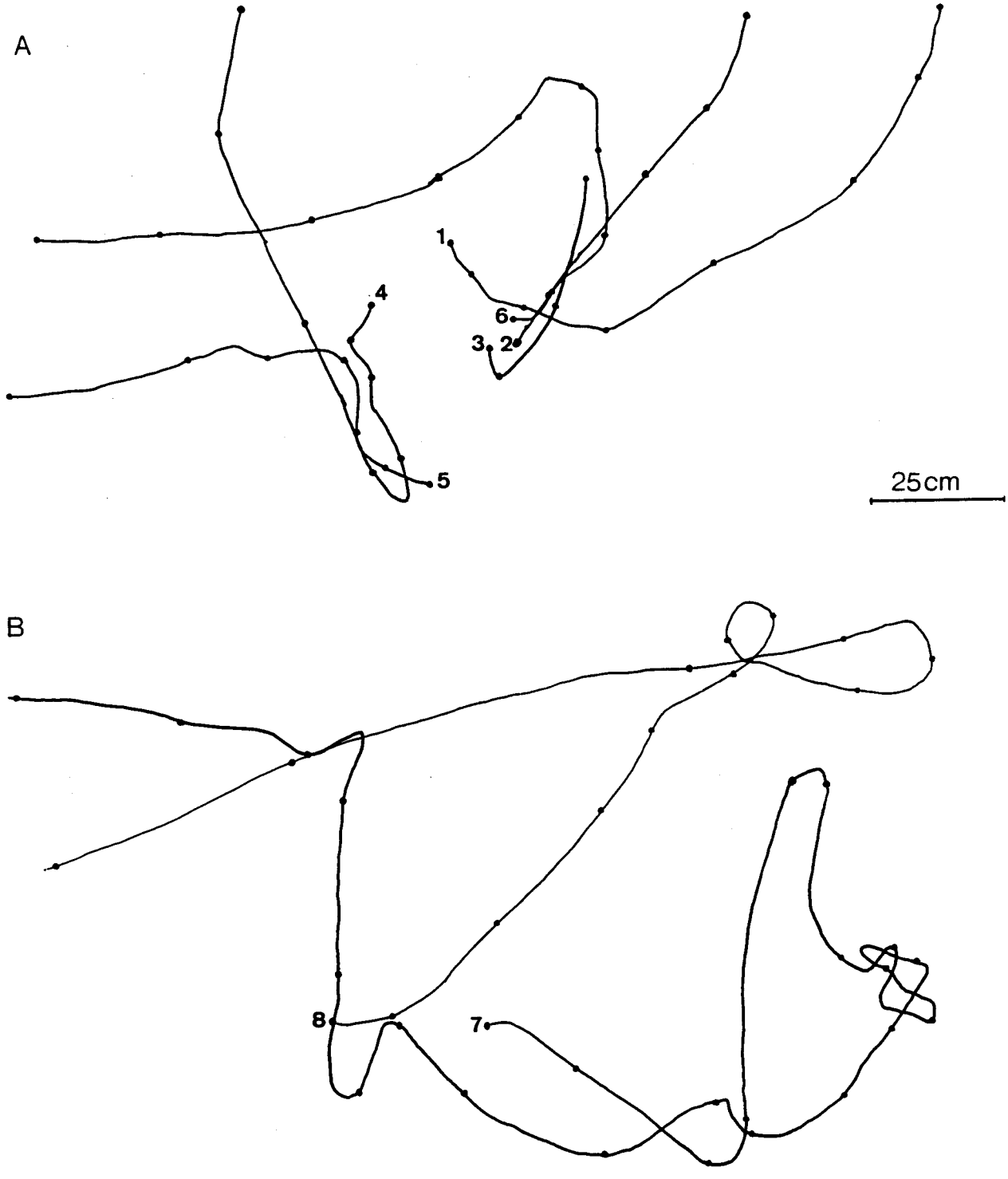


Figure 4.11. Random flight tracks of *Phoracantha semipunctata* beetles videotaped from above in the presence of the PVC pipe placed vertically at the upwind end of the wind tunnel. In the absence of host plant odour, the beetles flew without zizagging upwind (A: 1 and 2), crosswind (A: 3 and 4), downwind (A: 5 and 6), or without any predominant direction (B: 7 and 8). The dots represent the beetle's locations at consecutive 0.24 s intervals. The wind (50 cm/s) was blowing from right to left. The beetle initiated flight from the release platform (numbers position).

4.4. Discussion

The locomotory and flight orientation responses of the *P. semipunctata* beetles to each stimulus (i.e. the PVC pipe and the *E. globulus* logs), as well as the landing frequency on the logs, did not differ between the several experimental days. Thus, experimental days with the PVC pipe were reliable control replications, despite the absence of a charcoal filter at the air entrance of the wind tunnel to control of volatile chemical "contaminants" with a potential influence on the insects' behaviour. It indicates also that the different *E. globulus* logs produced similar odour stimulation over the several experimental days.

Kennedy (1977), provided a classification of insect locomotory responses to odour sources based on terms defined by Fraenkel and Gunn (1961). Two major groups of chemo-orientation responses were distinguished: 1) nondirected responses termed chemo-kineses (singular, kinesis); and 2) responses directed with reference to the odour source termed chemo-taxes (singular, taxis). Chemo-kinesis was subdivided into chemo-orthokinesis, where the response is a change in the amount or speed of linear movement (starting, stopping, slowing down or speeding up); and chemo-klinokinesis, where the response is a change in the rate or frequency of turning. Chemo-taxis was subdivided into chemo-klinotaxis, where the directed response results from detecting the left-right difference of stimulus intensity at successive moments in time; and chemo-tropotaxis, where the directed response results from detecting the difference of stimulus intensity in the two antennae at one moment in time. Another important non-chemical directed response is anemotaxis (positive or negative), where the walking insect responds to the wind direction as detected by mechanoreceptors.

The present results showed that the odour plume originated in the *E. globulus* logs had an effect downwind on the walking behaviour of *P. semipunctata* on the release platform. In the presence of a log, a higher proportion of beetles walked upwind and a lower proportion walked downwind or with no direction than did so in the presence of the PVC pipe. The beetles' response cannot be explained by any type of chemotaxis response to odour gradient because the odour plume simulation with smoke showed a uniform dispersion of smoke filaments over the release platform. Furthermore, the insects walking upwind in the presence of the odour plume showed a behaviour similar to that of the beetles walking upwind in the control experiment, exhibiting a fairly linear path without successive left-right turns typical of chemotactic responses in the presence of a chemical gradient. This suggests that an odour-induced positive anemotactic response occurred to the host odour plume. There was no influence of the odour plume on walking speed, but a higher proportion of beetles stopped less frequently and for less time in the

presence of host odour than in its absence. This suggests chemo-orthokinetic response in addition to the odour-induced anemotaxis.

That host odour induced flight is indicated by the shorter time to take-off in beetles exposed to *E. globulus* logs than in those exposed to the PVC pipe, although the ultimate occurrence of flight might have been independent of the presence of host odour. Once airborne, the flight direction of the beetles was clearly influenced by the odour plume. More beetles flew upwind and fewer crosswind, downwind or with no predominant direction in host odour than in its absence (when flights were randomly distributed). Host odour also induced the landing on the log, whereas no landings occurred on the PVC pipe, even when the beetles flew upwind in its direction (figure 6.9, D). This supports the hypothesis that flying beetles can visualise vertical silhouettes and avoid landing on them when there is no host odour present, as suggested from the results of the field experiment presented in the previous chapter.

The beetles that landed on the *E. globulus* logs were evidently strongly responsive to the host odour: nearly all of them walked upwind on the release platform, and their flight orientation was clearly influenced by the odour plume (figures 6.7 and 6.8).When in permanent contact with the odour plume (locked onto the plume), they flew quickly upwind with narrow zigzag movements. Upon loss of contact with the odour, they exhibited casting flight crosswind without upwind progression, or else flew downwind. Both responses normally led to their regaining contact with the plume and resuming upwind zigzag movements.

The zigzag and casting movements have been observed in wind tunnel studies with flying male moths orienting to female pheromone sources (e.g. Kennedy, 1981; Willis and Baker, 1984) and plant odour (Willis and Arbas, 1991), as well as in field studies (David *et al.*, 1982, 1983). Sex pheromone apparently releases two programmes which are thought to be widespread in flying insects: odour-induced optomotor anemotaxis (upwind orientation), and a self-steered programme of zigzag counterturns (Kennedy, 1986). The first programme requires that the airborne insect determine the wind's direction by optomotor reactions to visual features (e.g. Kennedy, 1977; David, 1986; Vickers and Baker, 1994), whereas the second is initiated and modulated by the olfactory stimulus, but not steered by it. Instead, the manoeuvre is controlled by an internal programme utilising current and stored information about the insect's own steering activity (Kennedy, 1986; Bell *et al.*, 1995). The idea that the self-steered programme is modulated by the odour is based on the fact that this behaviour is influenced by two major factors: 1) changes in pheromone concentration, and 2) loss of contact with the pheromone plume (Bell *et al.*, 1995). When the concentration is low, the zigzags are wide, and when the concentration is high they are narrow, adding to the efficiency of locking onto the plume when

the male is closer to the odour source. Loss of contact with the odour decreases the frequency of zigzagging, and increasing the angle of each turn so that the insect flies back and forth across the wind (casting) until the odour plume is regained and the upwind zigzag is resumed. The data presented here suggest that both odour-induced optomotor anemotaxis and self-steered zigzag counterturns are involved in *P. semipunctata* host location behaviour.

It is likely that orientation behaviour of *P. semipunctata* to the host plant is influenced by a complex blend of *Eucalyptus* related volatiles. The volatile compounds that are detected by *P. semipunctata* receptor neurons have to be identified, and wind tunnel assays can be designed further in time to investigate the behavioural effect of synthetic odour blends.

5.

Coupled Gas Chromatography - Electroantennogram Responses of *Phoracantha semipunctata* to Plant Volatiles

5.1. Introduction

Phoracantha semipunctata is a woodborer that attacks *Eucalyptus* and a few species in closely related genera (Duffy, 1963; chapter 2, this thesis). In its region of origin, Australia, *P. semipunctata* occurs wherever *Eucalyptus* grows (Fox and Curry, 1980), but its attacks are restricted to dead or sickly trees and downed limbs or logs (Tooke, 1935; Chararas, 1969a,b). In other parts of the world, standing trees are also attacked and *P. semipunctata* has become a pest of *Eucalyptus* plantations (chapter 2, this thesis). In the introduced regions, *P. semipunctata* attacks preferentially trees under physiological stress, e.g. due to poor adaptation to growing conditions (Chararas, 1969a). In addition, variation between *Eucalyptus* species in their susceptibility to attack has been observed in several countries (Chararas, 1969a,b; Drinkwater, 1975; Powell 1978; Löyttyniemi, 1983; Mendel *et al.*, 1984; Tirado, 1986). In Portugal, this is evident in Mata do Escaropim, an arboretum area with 400 ha situated at the south margin of the Tejo river in the vicinity of Lisbon. Here 110 species of *Eucalyptus*, planted in side by side plots in the period 1953-1958 (Goes, 1985), showed large species differences concerning colonisation by *P. semipunctata*. Lencart reported in 1988 that *E. bicostata*, *E. gigantea*, *E. globulus*, *E. ovata* and *E. viminalis* were heavily attacked in this arboretum. In 1994, some plots of *E. globulus* had been totally devastated by *P. semipunctata* larvae, whereas plots with other species including *E. tereticornis* did not show any signs of colonisation attempts (pers.obs.).

The importance of olfaction in *P. semipunctata* host finding behaviour is now well established on the basis of experimental evidence provided previously in this thesis. Logs and leaves of *E. globulus* are attractive to *P. semipunctata* males and females (chapter 3, this thesis), and wind tunnel observations suggest that odour-induced optomotor anemotaxis is involved in host-finding behaviour (chapter 4, this thesis). Furthermore, survival of *P. semipunctata* larvae is significantly different in logs of different *Eucalyptus* species and adult beetles are more attracted to the tree species that give highest survival for their progeny (Hanks *et al.*, 1993). When presented as an array of logs in a natural setting, more suitable hosts (*E. camaldulensis* and the

hybrid *E. trabutii*) attracted two or three times more beetles than did logs of less suitable species (*E. cladocalyx*, *E. grandis* and *E. tereticornis*).

Eucalyptus produce large numbers and large amounts of volatile terpenes that are accumulated in glands abundantly distributed throughout the foliar parenchyma and bark (Chattaway, 1954, Carr and Carr, 1969, 1970, 1976). The terpenes are plant secondary metabolites that commonly derive from the acetate-mevalonate biosynthetic pathway ubiquitous throughout the plant kingdom. Terpene biosynthesis has been extensively revised by several authors (e.g. Francis, 1971; Herout, 1971; Banthorpe *et al.*, 1972; Mabry and Gill, 1979; Seigler, 1981; Beale, 1990), and is summarised in figure 5.1. Through stepwise phosphorilation and decarboxylative elimination, mevalonic acid yields isopentenyl pyrophosphate. Isomerisation of the latter to dimethylallyl pyrophosphate provides the two basic C5 isoprene units which are the precursors to all other intermediates of the pathway. Condensation of isopentenyl pyrophosphate and dimethylallyl pyrophosphate catalysed by prenyl transferase leads to geranyl pyrophosphate, the immediate percursor of monoterpenes (C10), while further elongation provides farnesyl pyrophosphate (C15) and geranylgeranyl pyrophosphate (C20), the immediate precursors of the sesquiterpenes and diterpenes, respectively (Gijzen *et al.*, 1993). Although the details of the biosynthetic pathways involved in the synthesis of particular compounds are still to be elucidated, the levels of precursors and activity of enzymes appear to be the governing factors (Beale, 1990). The monoterpenes and sesquiterpenes are the major constituents of *Eucalyptus* essential oils, and their yield and composition is to a large extent under genetic control (Doran, 1991). Variation between *Eucalyptus* species in their content of such compounds has been related to host species preference by paropsine chrysomelid defoliators (Li, 1993), and has been useful in the chemotaxonomy of the genus (Li *et al.*, 1995).

As concerns the plant odour blends mediating host finding behaviour in phytophagous insects, two hypothesis have been discussed (Visser, 1986). (1) Insects use highly specific compounds of the host that are not present in unrelated plants. (2) Insects use a particular ratio of host related compounds which have ubiquitous distribution in the plant kingdom. Host finding by *P. semipunctata* may depend on the detection of a complex blend of terpenes produced by *Eucalyptus* in critical ratios, as suggested for other phytophagous species responding to blends of host related compounds (e.g. Visser, 1986; Roseland *et al.*, 1992; Campbell *et al.*, 1993). Specific variation in the production of host related volatiles may provide a chemical cue for *P. semipunctata* to find the most suitable *Eucalyptus* species for larval development.

In addition to host odours, the insects may also be influenced by odours of non-host plants. For instance, in aphids, avoidance reactions to non-host plants have been shown to be

induced by specific plant compounds present in non-hosts such as isothiocyanates characteristic of the cruciferaceae (Nottingham *et al.*, 1991) and by more generally distributed compounds, methyl salicylate and myrtenal (Hardie *et al.*, 1994). The restrict host range of *P. semipunctata* may be due to host species lacking compounds that induce avoidance behaviour, whereas non-host plants may release such compounds. It is interesting to note that although *P. semipunctata* has been introduced to several regions of the world following the introduction of *Eucalyptus*, it has never adapted to new host trees within the wide range of species present in the new environments. For example, *Pinus pinaster* and *Olea europeae*, which are adapted to the Portuguese edapho-climatic conditions where *Eucalyptus* have been planted, have never suffered colonisation attempts by *P. semipunctata*.

In order to investigate the mechanisms underlying host attraction and/or non-host avoidance, it is important to identify compounds that are detected by the beetles' receptor neurons. Previous electroantennograms (EAG) showed that receptor neurons of *P. semipunctata* males and females detect *Eucalyptus* odour, including some monoterpenes, i.e. α-pinene, camphene, α-terpinene, and 1,8-cineole (chapter 3, this thesis). The compounds tested for EAG responses are likely to be only a small fraction of the spectrum of plant odours that *P. semipunctata* beetles are able to detect.

Moorhouse *et al.* (1969) introduced the recording of EAG linked with the simultaneous gas chromatographic separation (GC) of insect semiochemicals. The GC effluent was accumulated over short periods at the end of the GC-column and flushed in nitrogen through a glass tube directed over the insect antenna. Later, Arn *et al.* (1975) introduced a more direct coupling by letting the GC effluent pass continuously over the insect antenna, a technique named as GC-electroantenographic detector (GC-EAD). This technique was then extended to the identification of compounds in host plant volatile blends detectable by insects (e.g. Guerin *et al.*, 1983). These studies make use of the high resolution capillary GC columns where the peak widths are only a few seconds, and the compounds reach the antenna with a sharp rise in concentration, can thus test sequentially the stimulatory ability of every component of a blend of plant volatiles on the insect olfactory receptors.

The present study aimed at identifying volatile compounds of host and non-host species that are detected by receptor neurons of *P. semipunctata*. The GC-EAD technique was employed to identify volatile components of the complex plant volatile blends that elicit EAG. Their chemical identification was achieved by gas chromatography linked with mass spectrometry (GC-MS) and by indirect identification methods. The compounds that elicited EAGs is compared

(1) for volatile blends of host species that vary in their attractiveness to *P. semipunctata* (i.e. *E. globulus*, *E. camaldulensis* and *E. tereticornis*), and (2) for blends of host and non-host species (i.e. *P. pinaster* and *O. europeae*). Authentic samples of various plant secondary metabolites, including the ubiquitous fatty acid and isoprene derivatives, were also tested for their relative electrophysiological effect on *P. semipunctata* receptor neurons at one concentration.

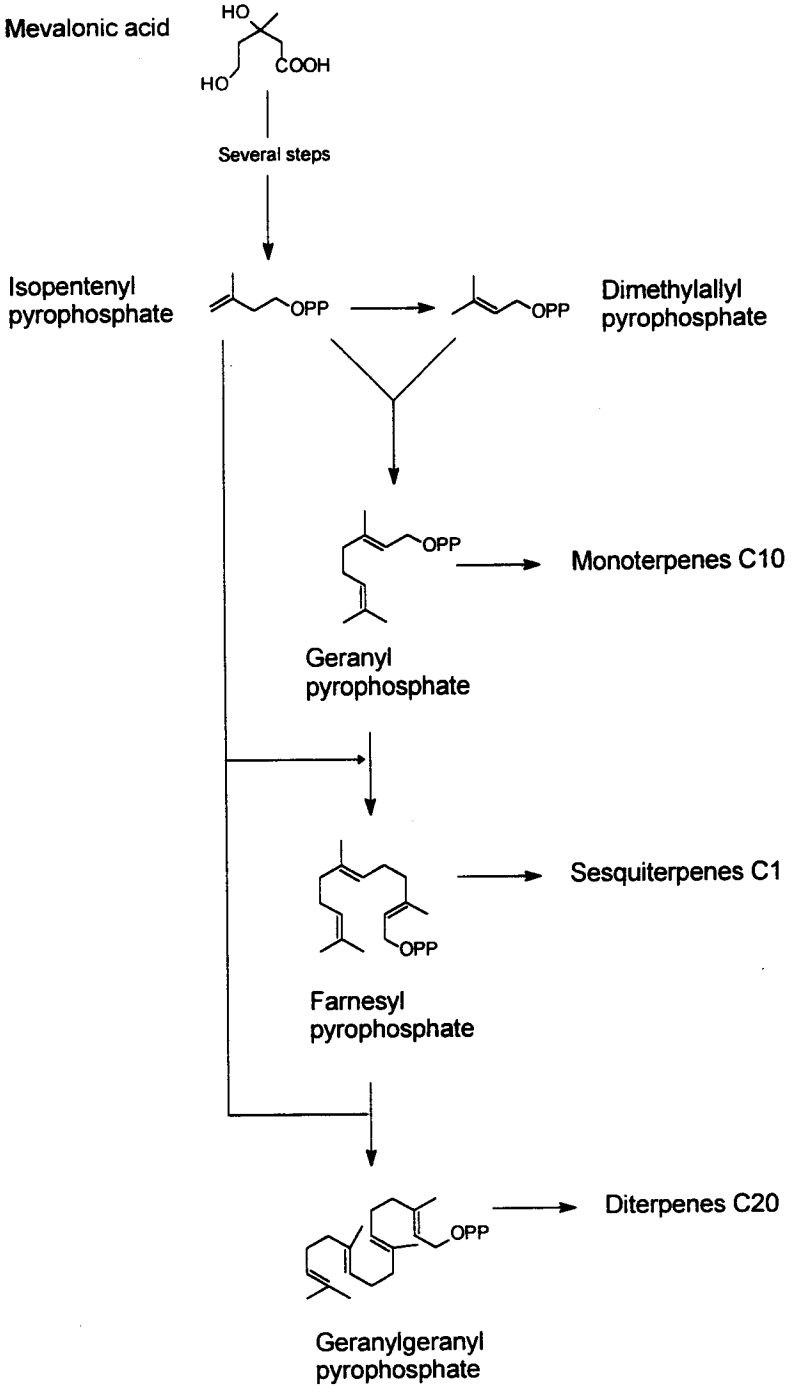


Figure 5.1. Overview of the isoprenoid pathway leading to monoterpenes, sesquiterpenes and diterpenes (derived from Gijzen *et al.*, 1993).

5.2. Materials & Methods

The GC-EAD technique was carried out at the University of Évora and at the Biological Ecological Chemistry Department of the Rothamsted Experimental Station (RES). The GC-peaks correlated with EAG responses were subsequently identified by GC-MS carried out at the RES.

Collection of plant material. Branches with mature foliage were collected from three specimens of *E. globulus*, *E. camaldulensis* and *E. tereticornis* in Mata do Escaropim, at the south margin of Tejo river, in the vicinity of Lisbon, Portugal. Branches with needles of *P. pinaster* and branches with leaves of *O. europeae*, were collected from three specimens of each species in Herdade da Mitra, in the vicinity of Évora, Portugal. Collection of plant material took place during June-July 1994, between 1200 and 1800 hrs, and was stored in a cooled room (5° C) until the next day for collection of headspace volatiles.

Collection of headspace volatiles. For each plant species, equal amounts of foliage from each of three individuals were pooled, and a 200 g sample was obtained for adsorption of headspace volatile compounds on Porapak Q (80-100 mesh). Glass tubes (6.6 cm x 0.5 cm i.d.) were filled with 400 mg of Porapak Q. Before use, the Porapak Q was cleaned in the glass tubes with dichloromethane and hexane, and left overnight perfused by a nitrogen (N_2) flow (60 ml/min) in a heating chamber at 180° C. A sample of 200 g of leaves was placed in a 1 l glass container, and the evaporated volatile compounds were drawn from the container with a N_2 flow onto paired glass tubes containing the Porapak Q. The N_2 flow was passed through the glass container, subsequently half devided with PTFE tubing and forced through the two Porapak Q filters placed in parallel. The N_2 flow was ca. 60 ml/min at the outlet of the tubes. The sorption period lasted for 24 h at 30° C. At the end of each sorption period, the Porapak Q tubes were sealed and stored in the dark at -3° C. The glassware, PTFE tubing and metal fittings used in the N_2 flow system were washed in hot water and soap, and then left in an ultrasound cleaning container filled with methanol for half an hour. The materials were then rinsed with distilled water and left overnight in an oven at 200° C for being used the next morning.

The trapped compounds on each Porapak Q filter were washed out with 1.5 ml of solvent dripping into a 1.8 ml glass vial. As solvents were used n-hexane (Merck, pro-analysis grade) for experiments carried out at the University of Évora and distilled diethyl ether for experiments carried out at RES. These solutions of plant volatiles were used in the GC-EAD recordings. When not used, the solutions were stored in the dark at -3° C.

Insects. The beetles used for the GC-EAD recordings were obtained from infested logs of *E. globulus* and reared in the laboratory as described in chapter 3. Successful GC-EAD recordings were obtained from 6 females at the University of Évora and 3 females at the RES. The females were 22-30 days old. The solutions of each plant species headspace volatiles (in hexane or diethyl ether) were tested on each female beetle in a randomised sequential order.

Electrophysiological preparation. The EAG recordings were obtained from live adults of *P. semipunctata*, mounted on a Plexiglas block and secured with wax (Utility Wax Rods, Kerr®). The recording of EAGs was made with glass electrodes filled with receptor lymph Ringer solution (Kaissling and Thorson, 1980 in Roelofs, 1984). Stable baselines were obtained by placing the recording electrode into the cut tip of the last segment of the antenna with the reference electrode inserted into the intersegmental membrane between the scape and the pedicel. With this positioning of the electrodes it was possible to minimise baseline drift and record EAGs for up to 6 h without detectable loss of sensitivity. The positioning of the reference electrode in various areas of the abdomen resulted in highly drifting baselines, often with irregular oscillations superimposed.

GC-EAD set-up. Figure 5.2 shows a diagram of the GC-EAD set-up at the University of Évora. A sample (1 µl) of a solution of plant volatiles or of a dilution of a synthetic compound in hexane, was injected on column in the gas chromatograph (Konik high resolution gas chromatograph) where the compounds were separated as they passed with the carrier gas (H_2) through the HP-1 capillary column (0.52 µm cross-linked methyl silicone, 50 m x 0.32 mm i.d.). The GC-oven temperature was maintained at 40° C for 1 min and then programmed at 5°/min to 120° C, then 15°/min to 150° C, and then at 10°/min to 200° C for 10 min. At the end of the column, a glass splitter (CRS Inc.) led one half of the effluent to the GC flame ionisation detector (FID) through a deactivated silica column, and the other half went through a similar length of silica column to a glass tube (1 cm i.d.) with a continuous flow of purified air (600 ml/min). The silica column that led the GC effluent to the air flow was permanently heated at 200° C. The air flow through the glass transfer tube was directed over the three most distal segments of the antenna where there is the highest density of olfactory sensilla (Lopes, 1990). The EAG signal was amplified x100 by a differential amplifier (SG1-93) supplied by S. Gabraek (University of Trondheim, Norway), and monitored on the screen of a Kikusui oscilloscope (COS5020 - 20 MHz). The FID-electrometer signal and the electrophysiological signal were delivered to an IBM compatible PC, and was analysed with specially designed commercial software (Konikrom™ Chromatography Data System). In this way, the EAGs and the FID-electrometer detection of GC

peaks were recorded simultaneously. The synchrony between the FID detection of a stimulatory compound and the occurrence of the EAG elicited was ascertained by on-column injection of 1 µl of (-)α-pinene diluted in hexane (10^3 ppm).

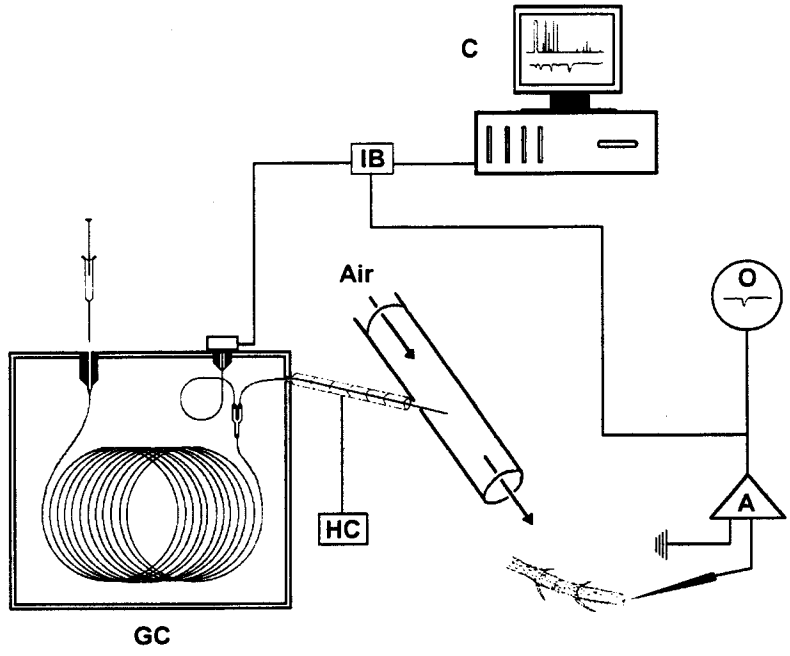


Figure 5.2. Diagram of GC-EAD set-up (not to scale). The EAGs were recorded using glass electrodes. Recording equipment comprises differential amplifier (x 100) (A), oscilloscope (O), computer (C) with a 2 channel signal acquisition interface board (IB) and software. Both the FID-electrometer and amplifier are connected to the interface board. The capillary column inside the GC is illustrated with a glass splitter for leading the effluent equally to the FID and out the GC through a metal tube to an air stream (600 ml/min) directed onto the antenna. A heat controller (HC) was set to heat the metal tube at a constant temperature of 200° C.

The GC-EAD technique employed at the RES followed the same general principle described above, but with some technical differences. An AI 93 gas chromatograph was used with the following temperature programme after injection: 40° C for 1 min, a rise of 5°/min to 100° C followed by 10°/min to 250° C. The electrophysiological signal was amplified 100 times by the Syntech UN-03b amplifier that was able to compensate for baseline drift. The FID-electrometer and electrophysiological signals were analysed with a computer, using the Syntech Computer GC-EAD System. Both electric signals were also delivered to a two-channel pen recorder.

Chemical identification of compounds eliciting EAGs. The chemical identification of some compounds in the blend of volatiles that elicited EAG responses was achieved by GC-MS

Chemical identification of compounds eliciting EAGs. The chemical identification of some compounds in the blend of volatiles that elicited EAG responses was achieved by GC-MS at the RES. A capillary column similar to the one used in GC-EAD was fitted in a Hewlett Packard 5890 gas chromatograph directly coupled to a mass spectrometer and integrated data system (70-250 VG Analytical). Ionisation was by electron impact at 70 eV, 230° C. The GC oven was maintained at 30° C for 5 min and then programmed at 5°/min to 180° C. At the University of Évora, indirect methods, i.e. comparison of calculated Kováts indices with published indices (e.g. Davies, 1990) and peak enhancement when the solutions of bark volatiles were coinjected with authentic samples of compounds, were used to identify EAG stimulatory compounds and to confirm the tentative GC-MS identifications. The indirect identification was achieved with two capillary columns of different polarity, 1) the non-polar HP-1 column used for the GC-EAD and GC-MS techniques, and 2) the polar Stabilwax® column (0.5 µm crossbonded Carbowax-PEG, 30 m x 0.32 mm i.d.).

Quantitation of EAG stimulatory compounds. The concentration of EAG stimulatory compound in the various solutions of plant volatiles was evaluated with the external standard p-cymene using both HP-1 and Stabilwax capillary columns. The linear equation, $Y = 91{,}191.X$, was used to calculate the concentration values. The equation was obtained by linear regression ($R^2 = 0{,}9992$) to determine the best calibration line for a series of 3 concentrations of the standard in hexane (10^1, 10^2, and 10^3 ppm) with 3 replications each.

Electrophysiological test of synthetic chemicals. At the University of Évora, GC-EAD was used to test 42 synthetic chemicals diluted in hexane (10^3 ppm) for EAG responses from 6 males and 6 females of *P. semipunctata.* A sample of 1 µl of each compound or mixtures of compounds with non-overlapping retention time was injected on the HP-1 capillary column. This method was preferred over the so-called "puff" method (e.g. Visser, 1979; Guerin and Visser, 1980; chapter 3, this thesis) because it excludes the contribution of impurities to the EAG. The set of chemicals comprised identified stimulatory compounds in the blends of plant volatiles and other plant derived compounds included in the green leaf volatile complex (Visser *et al.*, 1979) or identified by others as components of *Eucalyptus* essential oils (Lassak *et al.*, 1991). Chemically related compounds and isomeric forms were also included. Except 4,8-dimethylnona-1,3,7-triene which was synthesised at the RES, all the other chemicals were obtained from various commercial sources (table 5.1). The amplitude of the EAGs elicited by each compound was normalised with respect to the EAG amplitudes elicited by the standard, (E)-2-hexenal.

Table 5.1. Commercial sources of synthetic chemicals tested for EAG responses in *Phoracantha semipunctata* beetles.

Source	Compound	Purity (GC)
Aldrich Chemical	3-carene	95%
	(-)myrtenal	98%
	(-)myrtenol	96%
	(Z)-2-hexen-1-ol	92%
	3-hydroxy-2-butanone	80%
Extrasynthése	sabinene	98%
Fluka Chemie	(-)alloaromadendrene	98%
	aromadendrene	97%
	(-)α-cubebene	98%
	(-)α-gurjunene	97%
K+K	piperitone	96%
Merck	3-methyl-1-butanol	99%
Sigma	1,8-cineole	96%
	(dl)citronellol	95%
	(±)linalool	95-97%
	α-terpinene	89%
	γ-terpinene	95%
Tokyo Kasei Kogyo Co.	camphene	80%
	cuminaldehyde	95%
	p-cymene	96%
	(+)fenchone	98%
	(E)-2-hexenal	95%
	1-hexenol	98%
	(Z)-3-hexen-1-ol	97%
	(E)-2-hexenyl acetate	95%
	(Z)-3-hexenyl acetate	98%
	(-)limonene	95%
	linalool oxide	mixture of E and Z isomers
	linalyl acetate	95%
	4-methyl-2-pentanone	99%
	myrcene	96%
	3-octanone	97%
	α-phellandrene	95%
	(-)α-pinene	98%
	β-pinene	95%
	α-terpineol	98%
	terpinolene	95%
	terpinyl acetate	95%
	(α,β)thujone	95%
unknown	β-phellandrene	90%

5.3. Results

5.3.1. Host and Non-host Plant Volatiles that Elicited EAGs

Gas chromatographic analysis revealed a large number of compounds in a wide range of concentrations that contributed to the volatile blends emanating from foliage of host and non-host tree species. Many of these compounds as well as the solvents elicited EAGs that were consistently observed during replication of GC-EAD recordings in *P. semipunctata* females. Altogether, 43 stimulatory compounds were located in the gas chromatograms by GC-EAD. These included some of the major components of plant volatile blends as well as some of their minor components.

Figures 5.3 to 5.7 show typical GC-EAD recordings (obtained at the University of Évora) testing the volatiles of each plant species. The numbers in the gas chromatograms (1-43) indicate the compounds that elicit EAGs. The same compound is marked with the same number in all the chromatograms. The electrophysiological baseline shows numerous deflections, sometimes with amplitudes up to 350 μV which cannot be assigned to any GC-peak and were not reproducible. Such random deflections were considered as "noise". Baseline deflections consistently recorded in replications with 6 females and correlated to the elution of a GC-peak were considered EAGs and are marked with arrows. The EAG amplitudes were arbitrarily grouped in small (≤ 300 μV), medium (300-500 μV) and large (≥ 500 μV) as indicated in figures 5.8-5.12.

Out of the 43 stimulatory compounds, 26 were chemically identified as indicated in table 5.2. Unidentified compounds with the same number in different chromatograms are assumed to be the same compounds on the basis of similar relative retention time (rRt) and consistent EAGs elicited by each of them in the various volatile blends.

In the headspace volatiles of *E. globulus*, 30 compounds elicited EAGs (figures 5.3 and 5.8). Large EAG amplitudes were elicited by five minor components, 3-hydroxy-2-butanone (4), 3-methyl-1-butanol (5), 2,3-epoxy-4,4-dimethylpentane (6), ethyl-3-methylbutanoate (12), (Z)-3-hexen-1-ol (13), and by three major components, α-pinene (15), 1,8-cineole (24) and limonene (25). Due to high concentration, extensive overlap occurred in the GC-peaks of *p*-cymene, 1,8-cineole and limonene, which resulted in three superimposed EAG deflections. Ethyl-3-methylbutanoate and (Z)-3-hexen-1-ol co-eluted and elicited a single EAG deflection. Medium EAG amplitudes were elicited by five compounds in various concentrations, β-pinene (18),

linalool (31), guaine (40) and the unidentified compounds 1 and 38. Small EAG amplitudes were elicited by sixteen compounds corresponding to GC-peaks of different size: ethyl propanoate (10), (Z)-2-hexen-1-ol (14), myrcene (19), (Z)-3-hexenyl acetate (20), α-terpinene (22), trans-β-ocimene (26), γ-terpinene (27), 1-isopropenyl-3-methylbenzene (29), isopinocarveol (35), and the unidentified compounds 3, 7, 28, 33, 37, 42, and 43.

In the headspace volatiles of *E. camaldulensis*, 26 compounds elicited EAGs (figures 5.4 and 5.9). Large EAG amplitudes were elicited by two minor components, 3-hydroxy-2-butanone (4) and 3-methyl-1-butanol (5), and by four major components, α-terpinene (22), *p*-cymene (23), 1,8-cineole (24), and limonene (25). Due to high concentration, the GC-peaks of these four major components overlaped extensively, eliciting a large EAG outlasting the GC-peaks. Medium EAG amplitudes were elicited by eight compounds corresponding to GC-peaks of different size: 2,3-epoxy-4,4-dimethylpentane (6), ethyl-3-methylbutanoate (12), (Z)-3-hexen-1-ol (13), α-pinene (15), β-pinene (18), linalool (31), and the unidentified compounds 36 and 38. As observed in the chromatogram of *E. globulus* volatiles blend, ethyl-3-methylbutanoate and (Z)-3-hexen-1-ol co-eluted, eliciting a single EAG. Small EAG amplitudes were elicited by the remaining twelve compounds corresponding also to GC-peaks of a wide size range: (Z)-2-hexen-1-ol (14), sabinene (16), myrcene (19), (Z)-3-hexenyl acetate (20), α-phellandrene (21), trans-β-ocimene (26), γ-terpinene (27), 1-isopropenyl-3-methylbenzene (29), and the unidentified compounds 1, 3, 7, and 28.

In the headspace volatiles of *E. tereticornis*, 24 compounds elicited EAGs (figures 5.5 and 5.10). Large EAG amplitudes were elicited by a minor component 3-hydroxy-2-butanone (4) and by two major components, α-pinene (15) and *p*-cymene (23). Medium EAG amplitudes were elicited by five minor components: 3-methyl-1-butanol (5), 2,3-epoxy-4,4-dimethylpentane (6), β-pinene (18), guaine (40) and the unidentified compound 38. Small EAG amplitudes were elicited by sixteen compounds which are GC-peaks of much different sizes: ethyl-3-methylbutanoate (12), (Z)-3-hexen-1-ol (13), myrcene (19), α-phellandrene (21), α-terpinene (22), 1,8-cineole (24), limonene (25), trans-β-ocimene (26), γ-terpinene (27), 1-isopropenyl-3-methylbenzene (29), linalool (31), 1,2-dimethyl-3-isopropenylcyclopentane (34), and the unidentified compounds 1, 3, 28, and 30. As observed in the chromatograms of volatiles of the previous two host species, ethyl-3-methylbutanoate and (Z)-3-hexen-1-ol co-eluted as did 1,8-cineole and limonene, and a single EAG is correlated with each pair of compounds.

In the headspace volatiles of *P. pinaster*, 20 compounds elicited EAGs (figures 5.6 and 5.11). Large EAG amplitudes were elicited by two minor compounds, ethyl-3-methylbutanoate

(12) and (Z)-3-hexen-1-ol (13), and by four major compounds, α-pinene (15), β-pinene (18), myrcene (19), and (Z)-3-hexenyl acetate (20). As observed in the chromatograms of volatiles of *Eucalyptus* spp., ethyl-3-methylbutanoate and (Z)-3-hexen-1-ol co-eluted and a single EAG was correlated with the GC-peak. Due to high concentration of myrcene and (Z)-3-hexenyl acetate, their GC-peaks overlapped extensively and elicited a single EAG. Medium EAG amplitudes were elicited by five compounds in various concentrations: 1,8-cineole (24), limonene (25), trans-β-ocimene (26), linalool (31), and α-cubebene (39). Again, 1,8-cineole and limonene overlapped extensively in their elution from the GC-column, eliciting a single EAG. Small EAG amplitudes were elicited by nine compounds in various concentrations: 3-hydroxy-2-butanone (4), sabinene (16), α-phellandrene (21), α-terpinene (22), *p*-cymene (23), γ-terpinene (27), 1-isopropenyl-3-methylbenzene (29), and the unidentified compounds 2 and 28.

In the headspace volatiles of *O. europeae*, 16 compounds elicited EAGs (figures 5.7 and 5.12). Large EAG amplitudes were elicited by two minor compounds, 3-hydroxy-2-butanone (4) and the unidentified 17, and by the major compound, (Z)-3-hexen-1-ol (13). Medium EAG amplitudes were elicited by eight compounds in various concentrations: 3-methyl-1-butanol (5), 2,3-epoxy-4,4-dimethylpentane (6), ethyl propanoate (10), (Z)-2-hexen-1-ol (14), α-pinene (15), 4,8-dimethylnona-1,3,7-triene (32), and the unidentified compounds 8 and 38. Small EAG amplitudes were elicited by five minor compounds: trans-β-ocimene (26), *D*-germacrene (41) and the unidentified compounds 3, 9, and 11.

The mass spectra of compounds listed in table 5.2 are shown in the appendix of the thesis. In two cases, the tentative GC-MS identifications were not confirmed by indirect identification methods. The mass spectrum of GC-peak 5 in the chromatogram of *E. globulus* volatiles suggested the chemical structure of 4-methyl-2-pentanone (Appendix, figure A.2), but co-injection of the authentic compound with the solution of volatiles on the polar capillary column did not confirm the tentative GC-MS identification. GC-peak 5 was identified as 3-methyl-1-butanol in all plant species by co-injection on both non-polar and polar capillary columns. The mass spectrum of GC-peak 17 in the chormatogram of *O. europeae* volatiles suggested the chemical structure of 3-octanone (Appendix, figure A.7). Co-injection of the authentic compound with the solution of volatiles and simultaneous recording of electrophysiological responses showed an EAG elicited by the co-injected compound followed immediately by a second EAG elicited by GC-peak 17 eluting just after (figure 5.13). The mass spectrum of GC-peak 17 fitts also the chemical structures of 5-methyl-3-heptanone, 2,7-dimethyl-octane, 2-methyl-3-octanone, 2,3-octanedione, 3-nonanone, trans-4,5-epoxynonane,

and 1-nonen-3-ol, but none of these tentative identifications were tested by indirect methods due to the absence of authentic samples.

The mass spectrum of GC-peak 38 suggested the chemical structure of α-terpineol (Appendix, figure A.23). This tentative GC-MS identification was confirmed by the indirect methods, but authentic α-terpineol did not elicit EAG response from *P. semipunctata* (see below).

Ethyl-3-methylbutanoate and (Z)-3-hexen-1-ol co-eluted in the chromatograms of volatiles of *Eucalyptus* spp. and *P. pinaster*. The mass spectra of their GC-peak in the chromatograms of *E. globulus* and *E. tereticornis* suggested ethyl-3-methylbutanoate (Appendix, figure A.5). The co-injection of (Z)-3-hexen-1-ol in both polar and non-polar capillary columns provided evidence for its co-elution. Due to absence of authentic sample, the tentative GC-MS identification of ethyl-3-methylbutanoate was not confirmed by indirect methods. (Z)-3-Hexen-1-ol was also identified in *O. europeae* (Appendix, figure A.6).

Due to the absence of authentic samples, the tentative GC-MS identifications of 2,3-epoxy-4,4-dimethylpentane (Appendix, figure A.3), ethyl propanoate (Appendix, figure A.4), ethyl-3-methylbutanoate (Appendix, figure A.5), 1,2-dimethyl-3-isopropenylcyclopentane (Appendix, figure A.10), 1-isopropenyl-3-methylbenzene (Appendix, figure A.18), isopinocarveol (Appendix, figure A.21), guaine (Appendix, figure A.25), and *D*-germacrene (Appendix, figure A.26) were not confirmed by indirect methods, and test of their electrophysiological effect on *P. semipunctata* receptor neurons was not possible.

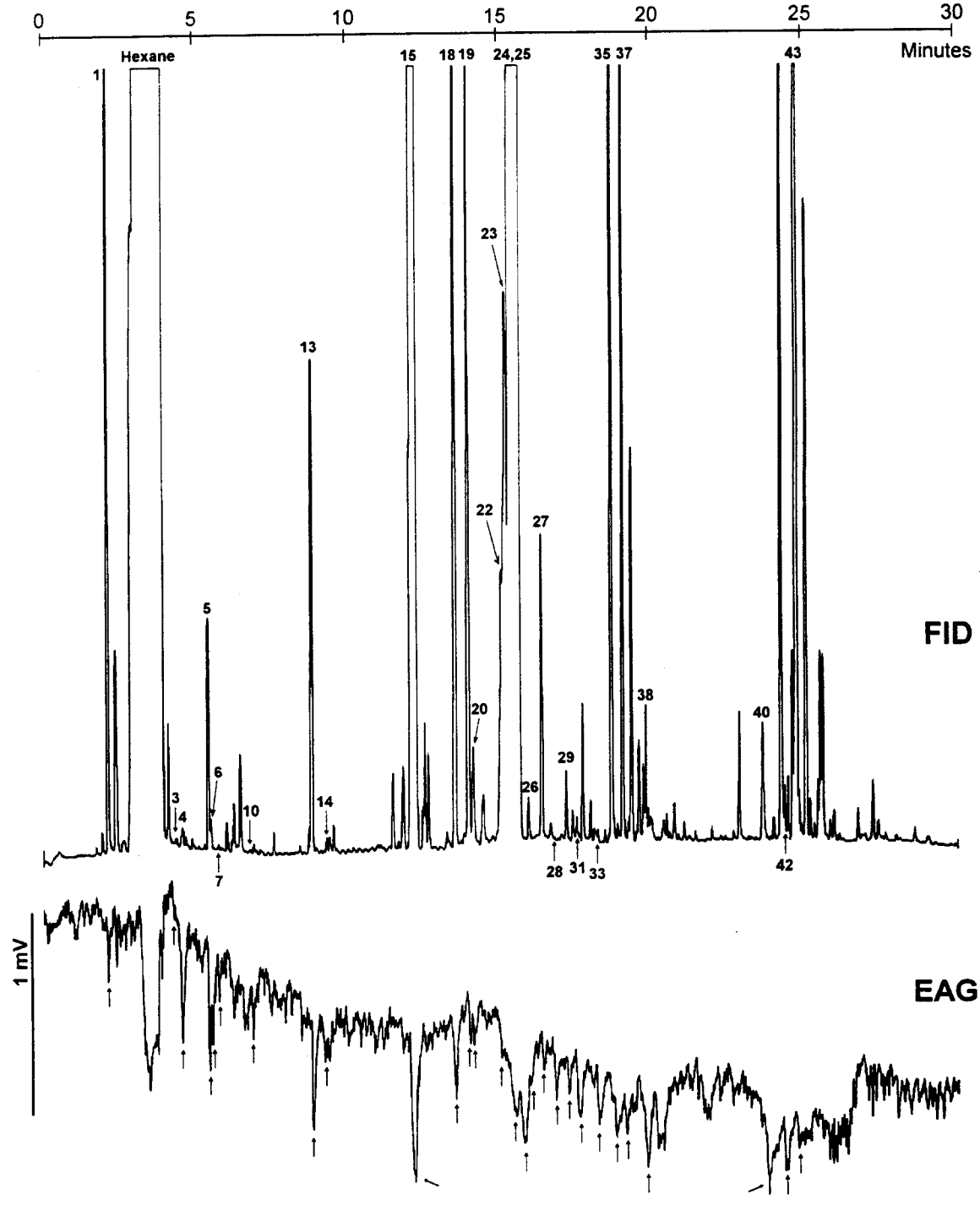


Figure 5.3. Gas chromatogram (FID) of headspace volatiles from *Eucalyptus globulus* and simultaneously recorded EAG responses from a *Phoracantha semipunctata* female. The numbers indicate GC-peaks that elicited EAG responses, and the arrows indicate the EAG responses. On column injection of 1 µl of the hexane solution of volatiles. Column effluent splitt at 1:1 ratio (FID:EAD). Temperature program: 40° C for 1 min, 5°/min to 120° C, 15°/min to 150° C, 10°/min to 200° C, and 200° C for 10 min.

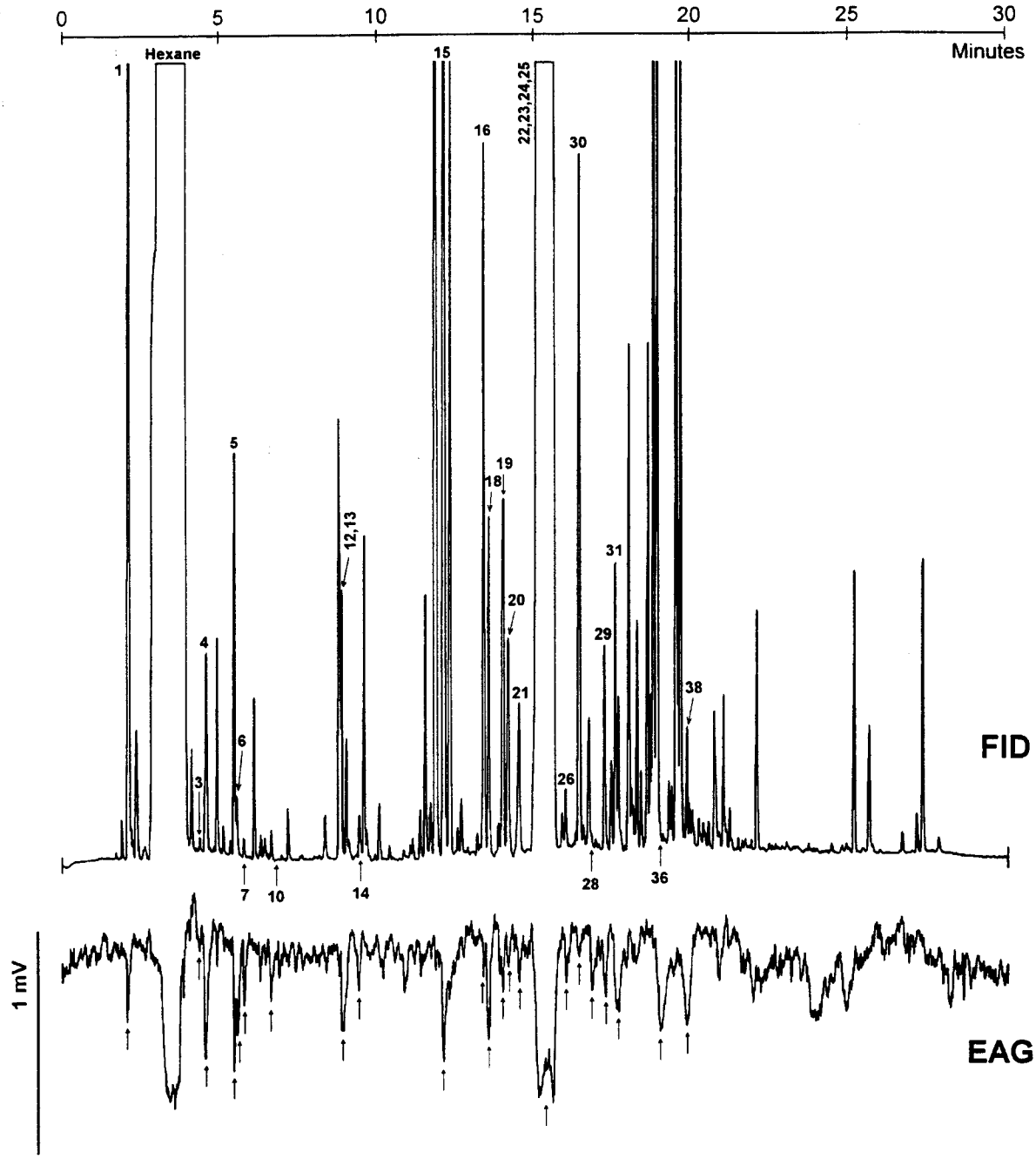


Figure 5.4. Gas chromatogram (FID) of headspace volatiles from *Eucalyptus camaldulensis* and simultaneously recorded EAG responses from a *Phoracantha semipunctata* female. The numbers indicate GC-peaks that elicited EAG responses, and the arrows indicate the EAG responses. On column injection of 1 µl of the hexane solution of volatiles. Column effluent splitt at 1:1 ratio (FID:EAD). Temperature program: 40° C for 1 min, 5°/min to 120° C, 15°/min to 150° C, 10°/min to 200° C, and 200° C for 10 min.

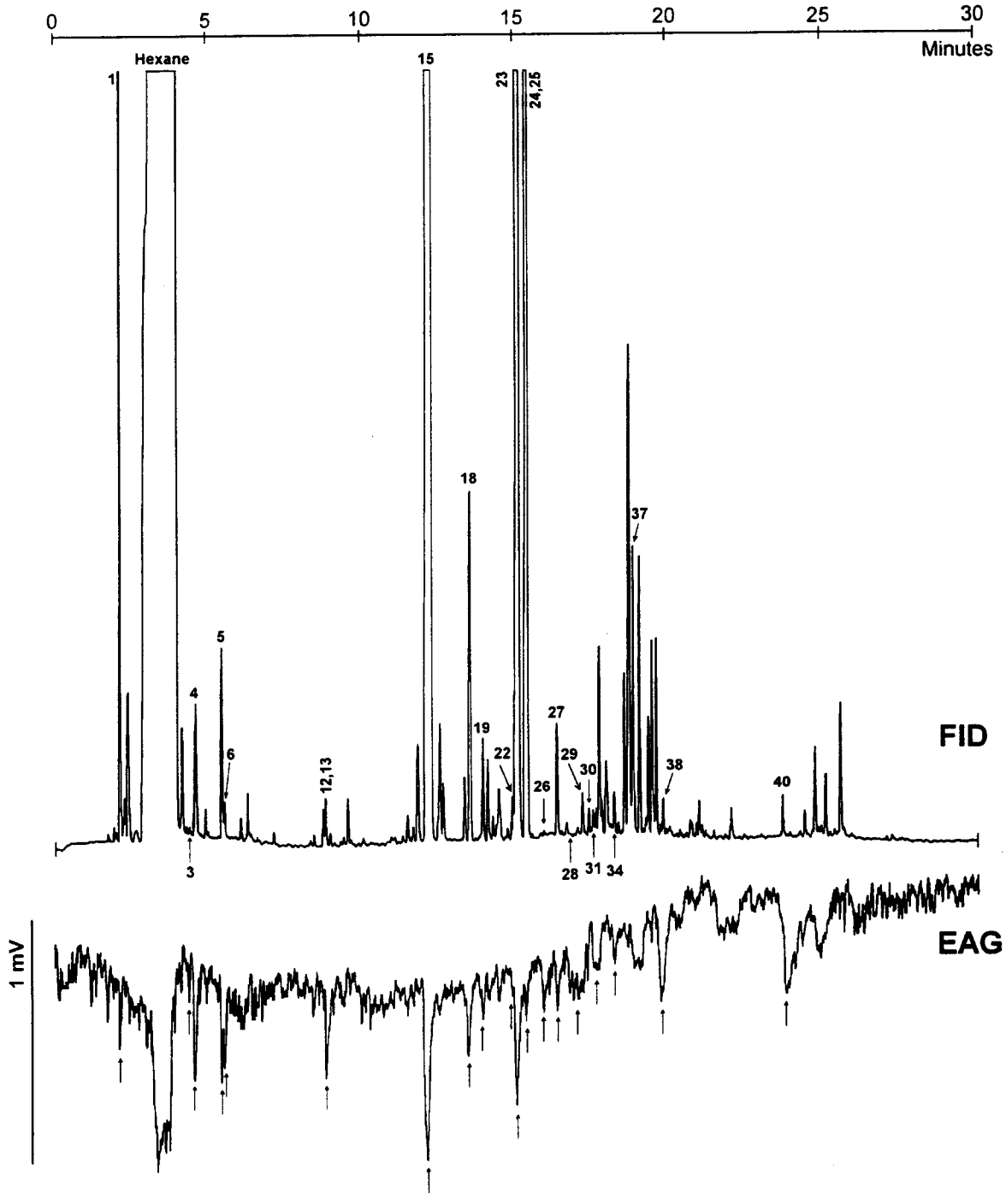


Figure 5.5. Gas chromatogram (FID) of headspace volatiles from *Eucalyptus tereticornis* and simultaneously recorded EAG responses from a *Phoracantha semipunctata* female. The numbers indicate GC-peaks that elicited EAG responses, and the arrows indicate the EAG responses. On column injection of 1 µl of the hexane solution of volatiles. Column effluent splitt at 1:1 ratio (FID:EAD). Temperature program: 40° C for 1 min, 5°/min to 120° C, 15°/min to 150° C, 10°/min to 200° C, and 200° C for 10 min.

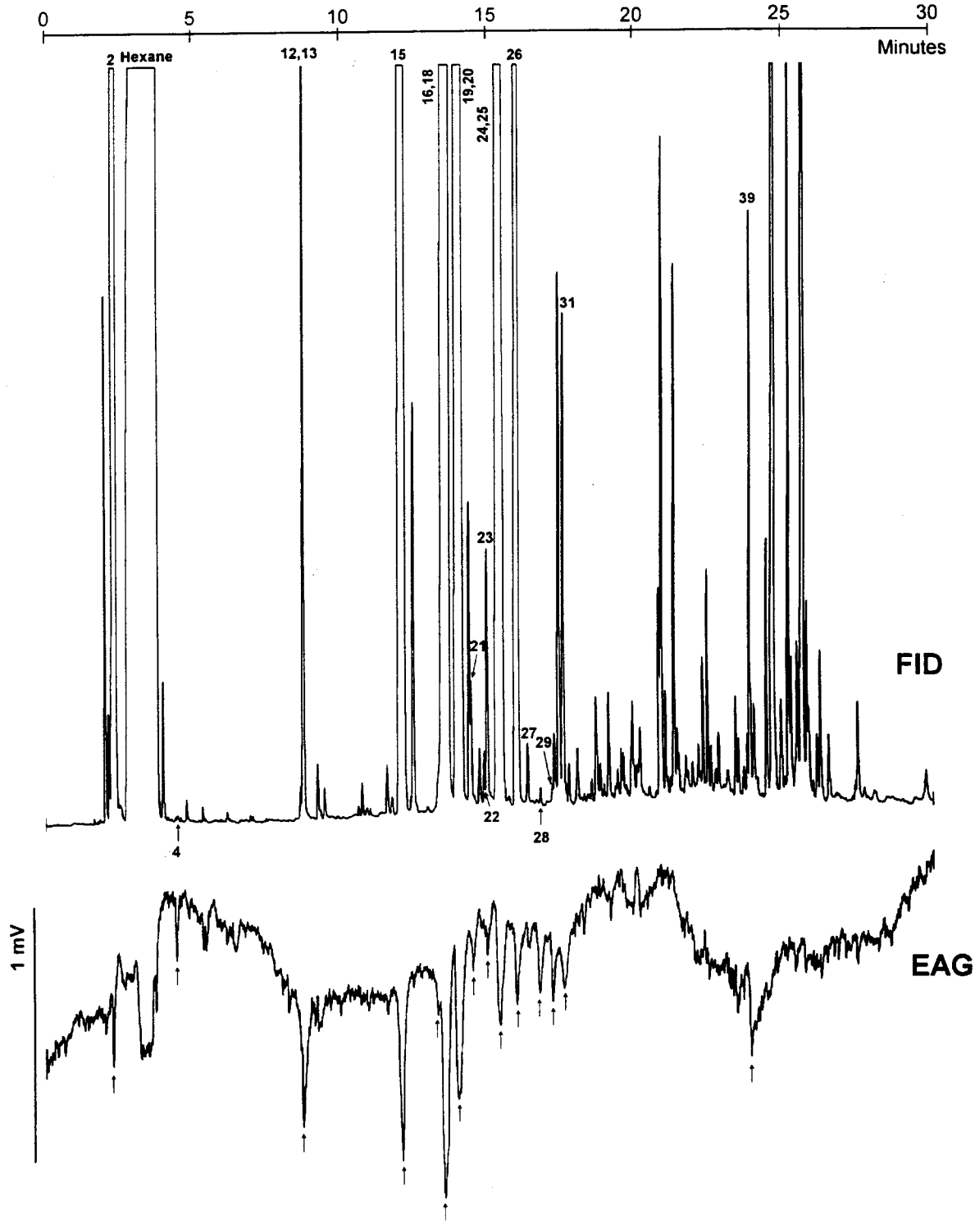


Figure 5.6. Gas chromatogram (FID) of headspace volatiles from *Pinus pinaster* and simultaneously recorded EAG responses from a *Phoracantha semipunctata* female. The numbers indicate GC-peaks that elicited EAG responses, and the arrows indicate the EAG responses. On column injection of 1 µl of the hexane solution of volatiles. Column effluent splitt at 1:1 ratio (FID:EAD). Temperature program: 40° C for 1 min, 5°/min to 120° C, 15°/min to 150° C, 10°/min to 200° C, and 200° C for 10 min.

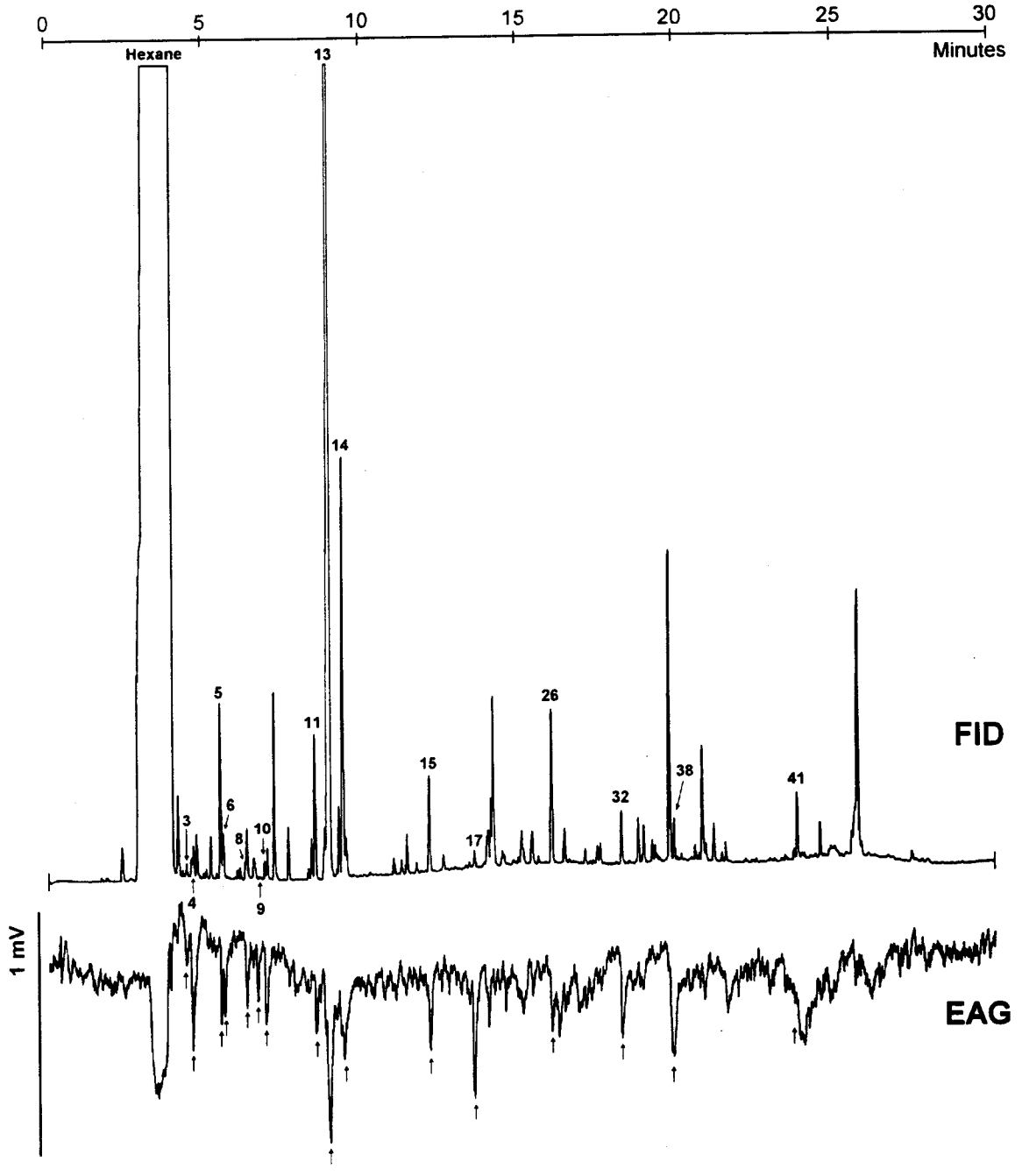


Figure 5.7. Gas chromatogram (FID) of headspace volatiles from *Olea europeae* and simultaneously recorded EAG responses from a *Phoracantha semipunctata* female. The numbers indicate GC-peaks that elicited EAG responses, and the arrows indicate the EAG responses. On column injection of 1 µl of the hexane solution of volatiles. Column effluent splitt at 1:1 ratio (FID:EAD). Temperature program: 40° C for 1 min, 5°/min to 120° C, 15°/min to 150° C, 10°/min to 200° C, and 200° C for 10 min.

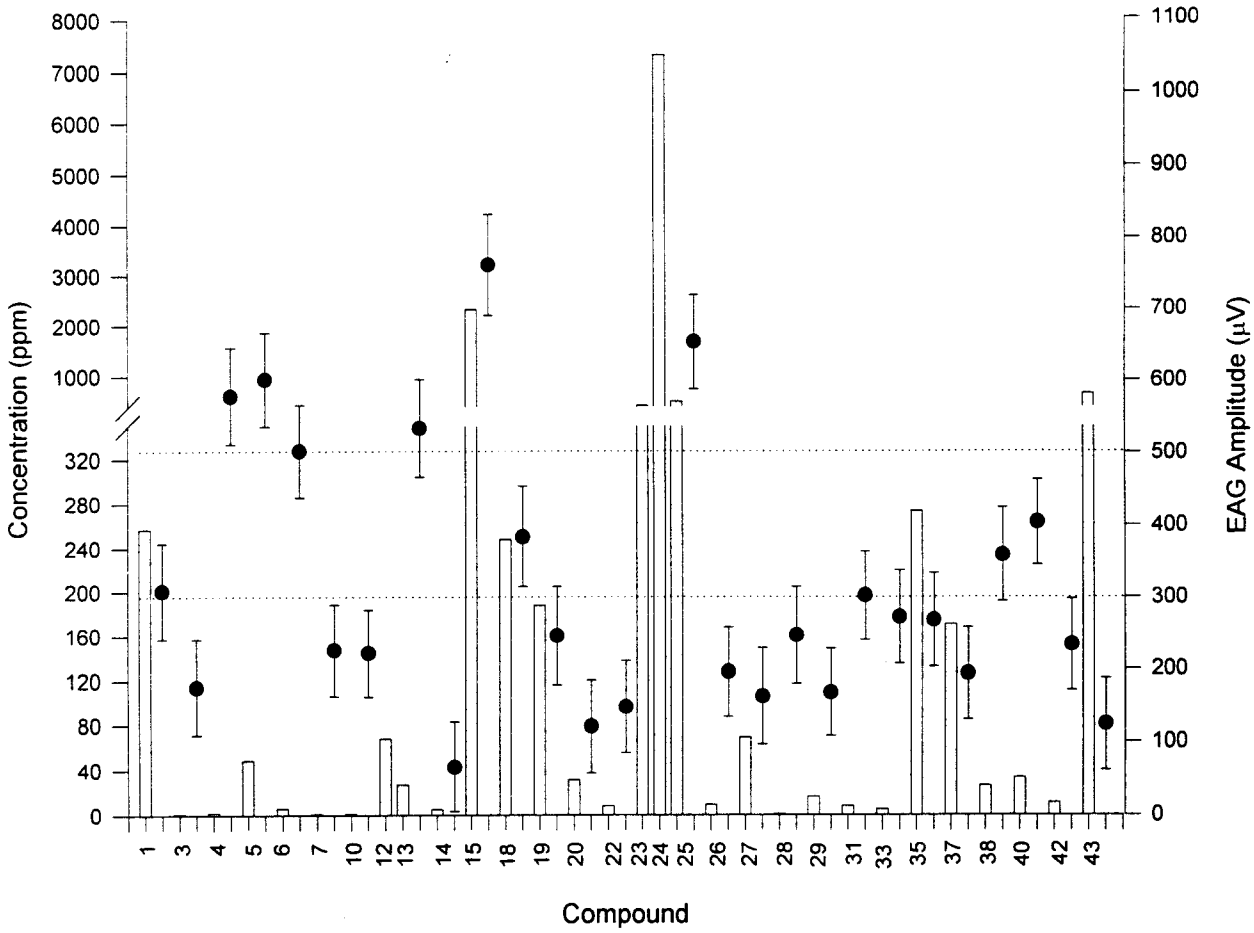


Figure 5.8. Concentration of stimulatory compounds in the solution of headspace volatiles of *Eucalyptus globulus* (bars) and correlated EAG amplitudes (mean ± SD) elicited from 6 *Phoracantha semipunctata* females during GC-EAD. The compound numbers correspond to the numbers in the chromatogram (figure 5.2). The mean EAG amplitudes were arbitrarily grouped in small (≤ 300 µV), medium (300-500 µV) and large (≥ 500 µV) as indicated by the horizontal dotted lines.

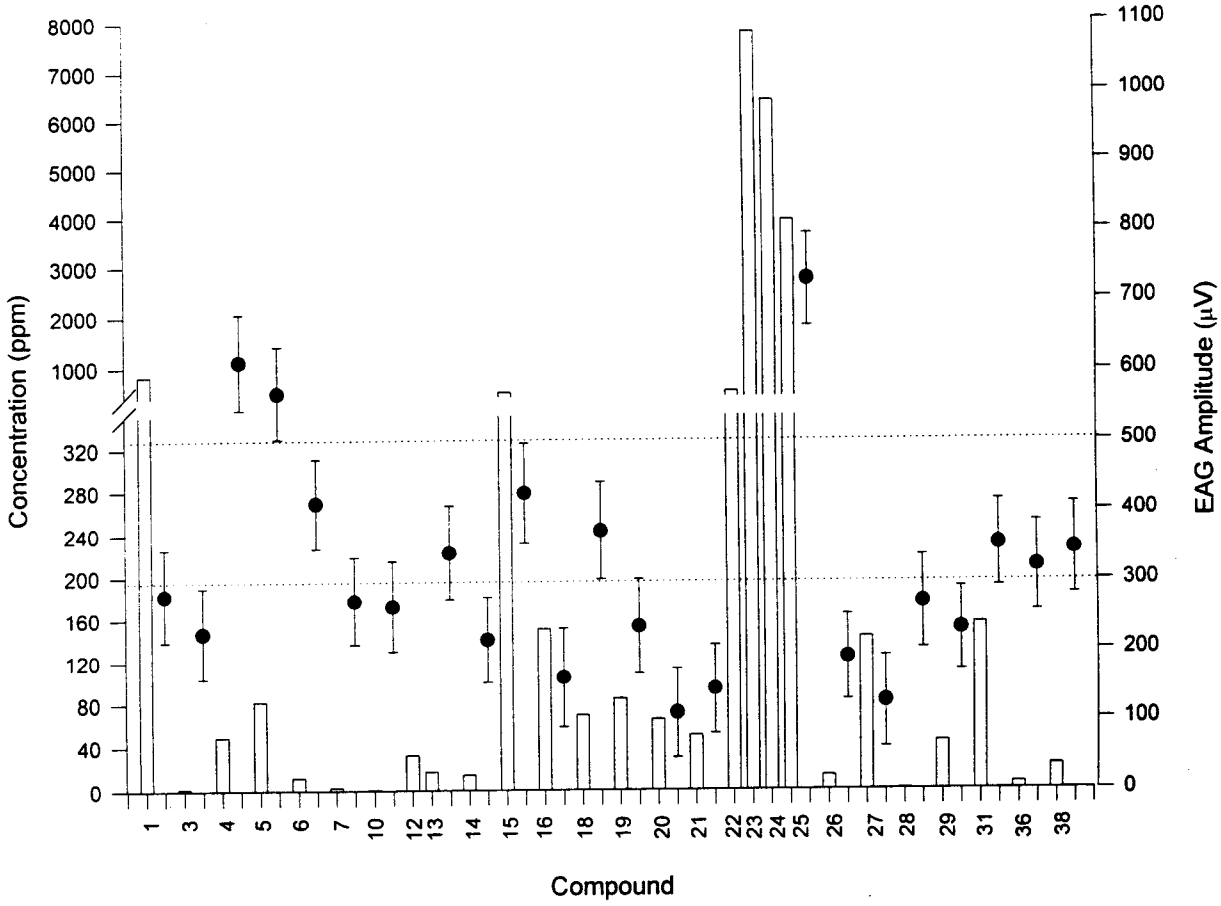


Figure 5.9. Concentration of stimulatory compounds in the solution of headspace volatiles of *Eucalyptus camaldulensis* (bars) and correlated EAG amplitudes (mean ± SD) elicited from 6 *Phoracantha semipunctata* females during GC-EAD. The compound numbers correspond to the numbers in the chromatogram (figure 5.3). The mean EAG amplitudes were arbitrarily grouped in small (≤ 300 µV), medium (300-500 µV) and large (≥ 500 µV) as indicated by the horizontal dotted lines.

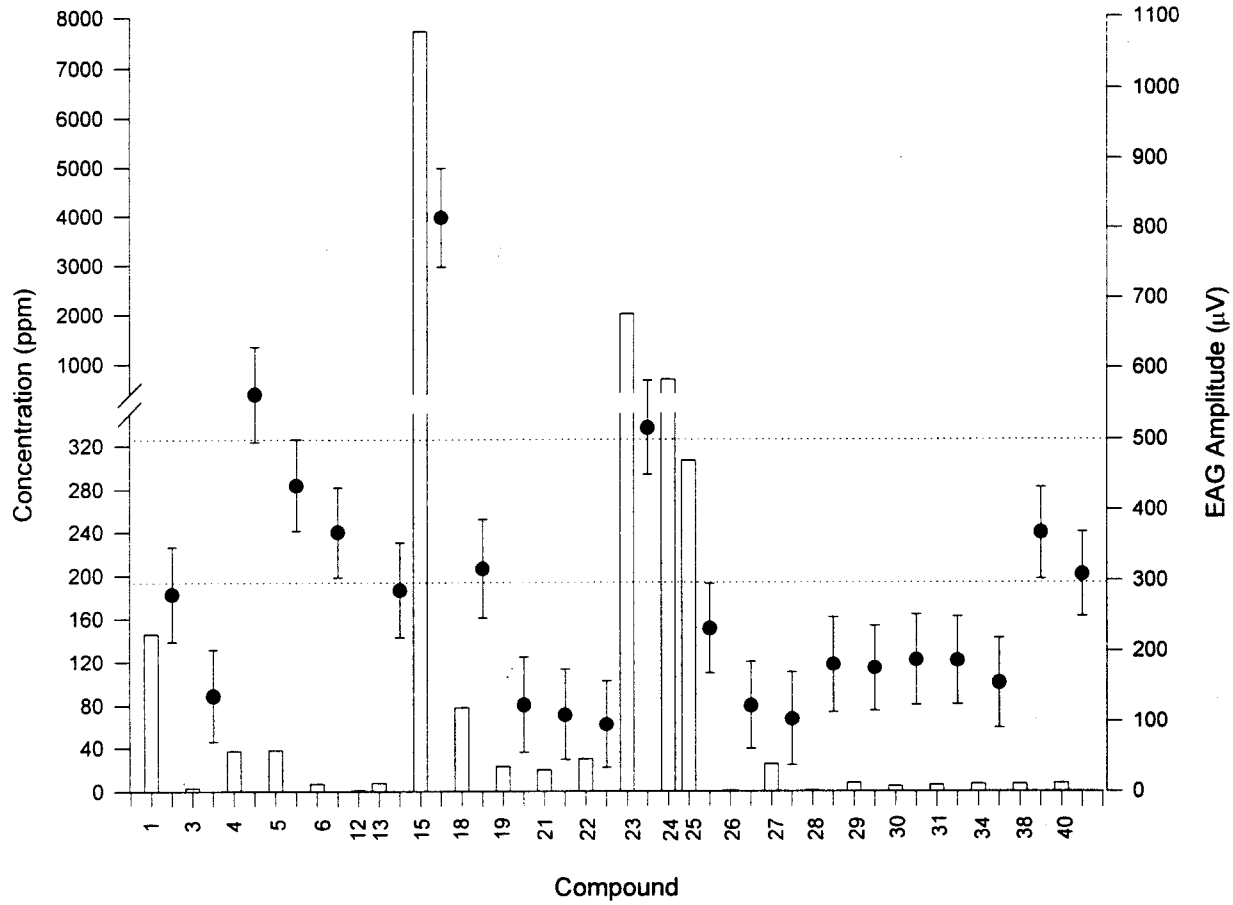


Figure 5.10. Concentration of stimulatory compounds in the solution of headspace volatiles of *Eucalyptus tereticornis* (bars) and correlated EAG amplitudes (mean ± SD) elicited from 6 *Phoracantha semipunctata* females during GC-EAD. The compound numbers correspond to the numbers in the chromatogram (figure 5.4). The mean EAG amplitudes were arbitrarily grouped in small (≤ 300 µV), medium (300-500 µV) and large (≥ 500 µV) as indicated by the horizontal dotted lines.

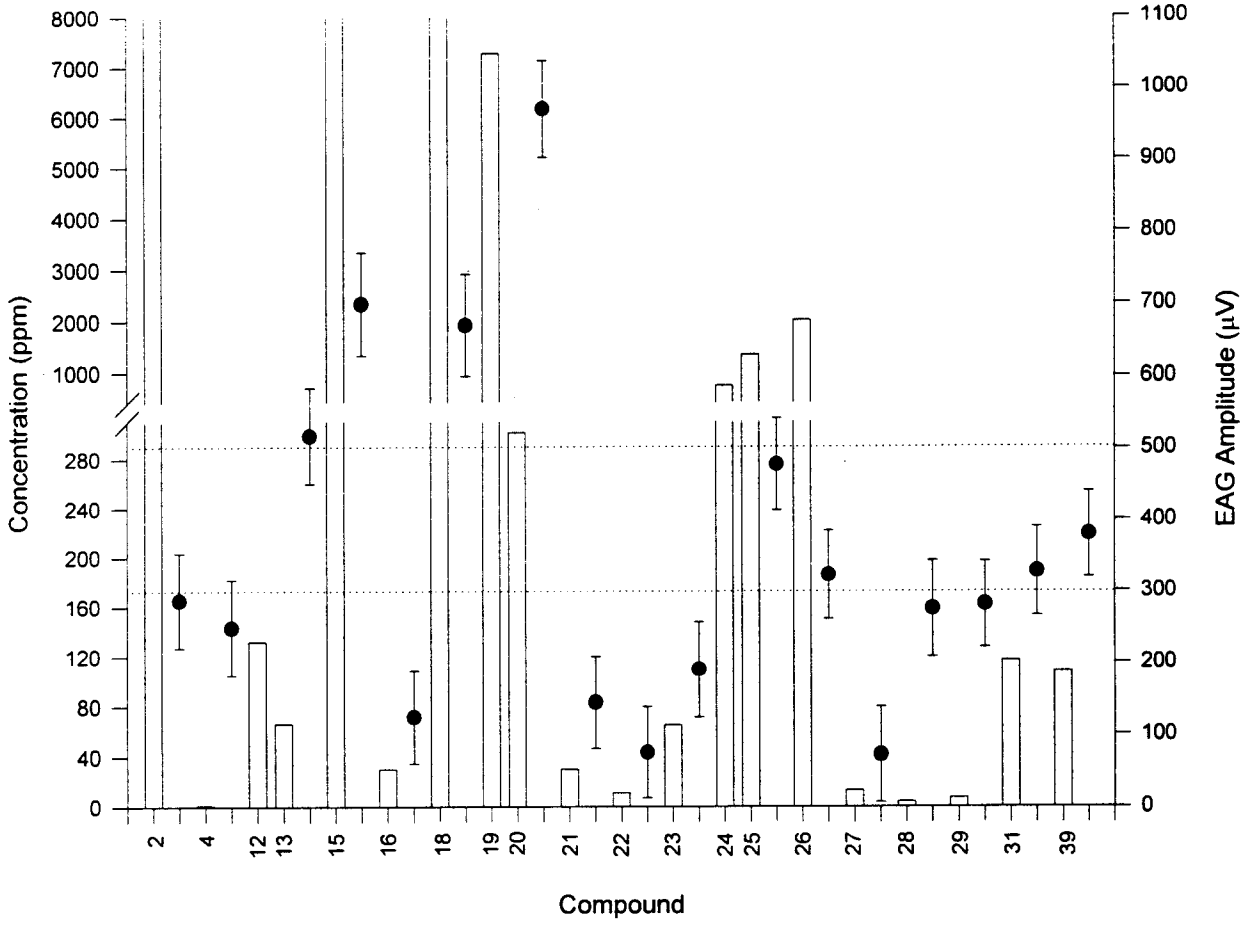


Figure 5.11. Concentration of stimulatory compounds in the solution of headspace volatiles of *Pinus pinaster* (bars) and correlated EAG amplitudes (mean ± SD) elicited from 6 *Phoracantha semipunctata* females during GC-EAD. The compound numbers correspond to the numbers in the chromatogram (figure 5.5). The mean EAG amplitudes were arbitrarily grouped in small (≤ 300 µV), medium (300-500 µV) and large (≥ 500 µV) as indicated by the horizontal dotted lines.

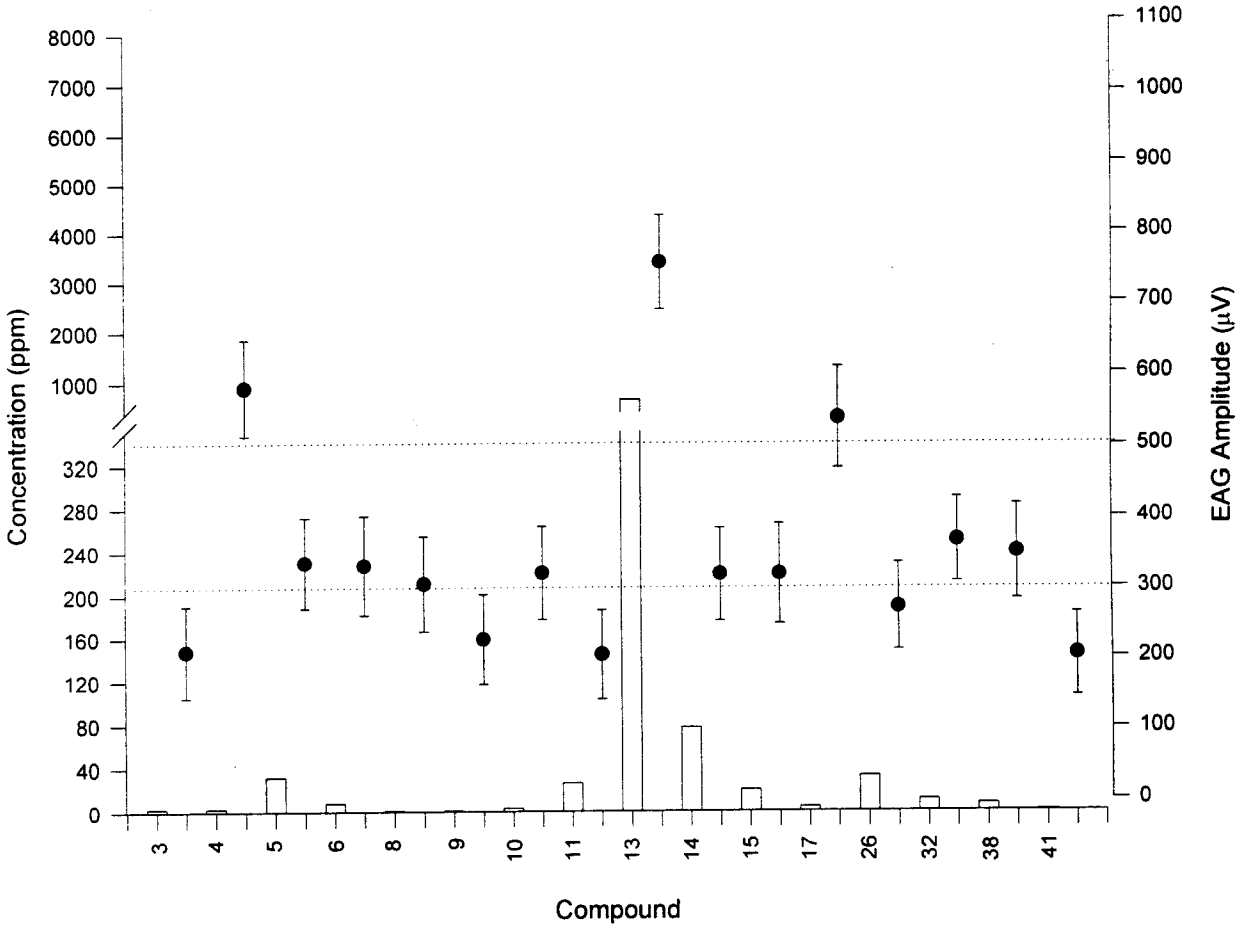


Figure 5.12. Concentration of stimulatory compounds in the solution of headspace volatiles of *Olea europeae* (bars) and correlated EAG amplitudes (mean ± SD) elicited from 6 *Phoracantha semipunctata* females during GC-EAD. The compound numbers correspond to the numbers in the chromatogram (figure 5.6). The mean EAG amplitudes were arbitrarily grouped in small (≤ 300 µV), medium (300-500 µV) and large (≥ 500 µV) as indicated by the horizontal dotted lines.

Table 5.2. Chemical identification method and calculated Kováts' indices in the HP-1 and Stabilwax (CWAX) capillary columns of EAG stimulatory compounds in the volatile blends of I - *Eucalyptus globulus*, II - *Eucalyptus camaldulensis*, III - *Eucalyptus tereticornis*, IV - *Pinus pinaster*, and VI - *Olea europeae*. The compounds are listed in order of their retention time in the HP-1 capillary column, and the numbers correspond to the GC-peaks in figures 5.2 to 5.6.

Compound [1]	Identification Method [2]					Kováts' Indices	
	Host Species			Non-host Species			
	I	II	III	IV	V	HP-1	CWAX
1 unidentified	rRt	rRt	rRt	ND	ND	-----	-----
2 unidentified	ND	ND	ND	unident.	ND	-----	-----
3 unidentified	rRt	rRt	rRt	ND	rRt	-----	-----
4 3-hydroxy-2-butanone	rRt	MS	MS	rRt	rRt	-----	-----
5 3-methyl-1-butanol *	ci	ci	ci	ci	ci	-----	1212
6 2,3-epoxy-4,4-dimethylpentane	MS	rRt	MS	rRt	rRt	-----	-----
7 unidentified	rRt	rRt	ND	ND	ND	-----	-----
8 unidentified	ND	ND	ND	ND	unident.	-----	-----
9 unidentified	ND	ND	ND	ND	unident.	-----	-----
10 ethyl propanoate	rRt	MS	ND	ND	rRt	-----	-----
11 unidentified	ND	ND	ND	ND	unident.	828	-----
12 ethyl-3-methylbutanoate	MS	rRt	MS	rRt	ND	841	-----
13 (Z)-3-hexen-1-ol	ci	ci	ci	ci	MS, ci	841	1394-95
14 (Z)-2-hexen-1-ol	ci	ci	ND	ND	ci	856-58	1365-67
15 α-pinene	MS, ci, Ki	ci, Ki	ci, Ki	ci, Ki	ci, Ki	933-37	1027-32
16 sabinene	ND	MS, ci, Ki	ND	ci, Ki	ND	967-69	1123-28
17 unidentified *	ND	ND	ND	ND	unident.	972	-----
18 β-pinene	MS, ci, Ki	ci, Ki	ci, Ki	MS, ci, Ki	ND	972-74	1109-13
19 myrcene	MS, ci, Ki	MS, ci, Ki	ci, Ki	ci, Ki	ND	984-86	1170-76
20 (Z)-3-hexenyl acetate	ci	MS, ci	ND	ci, Ki	ND	988	1331
21 α-phellandrene	ND	MS, ci, Ki	MS, ci, Ki	ci, Ki	ND	996-97	1170-76
22 α-terpinene	ci, Ki	ci, Ki	ci, Ki	ci, Ki	ND	1009-10	1182-86
23 *p*-cymene	MS, ci, Ki	ci, Ki	ci, Ki	ci, Ki	ND	1013-15	1280-83
24 1,8-cineole	MS, ci, Ki	ci, Ki	ci, Ki	ci, Ki	ND	1022-24	1214-19
25 limonene	MS, ci, Ki	ci, Ki	ci, Ki	ci, Ki	ND	1022-24	1205-10
26 trans-β-ocimene	Ki	Ki	Ki	MS, Ki	Ki	1039-41	1264-68
27 γ-terpinene	MS, ci, Ki	ci, Ki	ci, Ki	ci, Ki	ND	1050-52	1254-58
28 unidentified	rRt	rRt	rRt	rRt	ND	1064-65	-----
29 1-isopropenyl-3-methylbenzene	MS	rRt	MS	rRt	ND	1075	-----
30 unidentified	ND	ND	unident.	ND	ND	1081	-----
31 linalool	ci, Ki	MS	ci, Ki	ci, Ki	ND	1084-86	1563-64
32 4,8-dimethylnona-1,3,7-triene	ND	ND	ND	ND	MS	1098	-----
33 unidentified	unident.	ND	ND	ND	ND	1104	-----
34 1,2-dimethyl-3-isopropenylcyclopentane	ND	ND	MS	ND	ND	1108	-----
35 isopinocarveol	MS	ND	ND	ND	ND	1128	-----
36 unidentified	ND	unident.	ND	ND	ND	1141	-----
37 unidentified	unident.	ND	ND	ND	ND	1144	-----
38 unidentified	rRt	rRt	rRt	rRt	rRt	1177-78	-----
39 α-cubebene	ND	ND	ND	MS, ci	ND	1383	1497
40 guaine	MS	ND	MS	ND	ND	1381-83	-----
41 *D*-germacrene	ND	ND	ND	ND	MS	-----	-----
42 unidentified	unident.	ND	ND	ND	ND	1418	-----
43 unidentified	unident.	ND	ND	ND	ND	1448	-----

[1] Compounds which first tentative MS identification was not confirmed by indirect methods are marked with *(see text).

[2] Identification method: rRt - relative retention time; MS - mass spectrometry; Ki - comparison of calculated Kováts' indices with published indices (Davies, 1990); ci - co-injection on HP-1 and Stabilwax capillary columns; ND - not detected; unident. - unidentified.

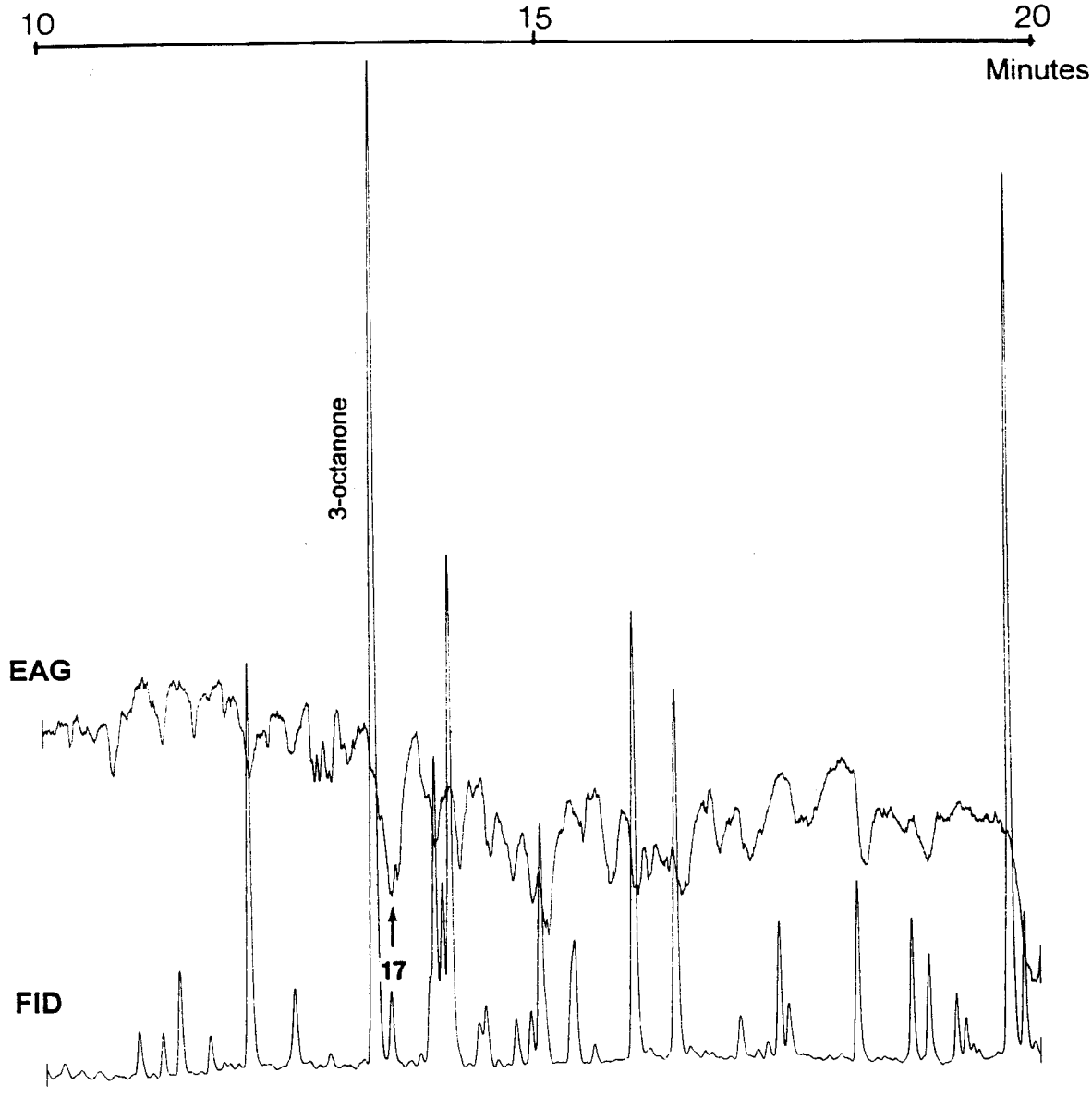


Figure 5.13. Section of the chromatogram of *Olea europeae* (10-20 min) solution of volatiles co-injected in the GC with 3-octanone (FID) and simultaneously recorded EAG responses from a *Phoracantha semipunctata* female. The GC-peak of 3-octanone eluted from the GC-column just before the elution of GC-peak 17. The onset of the EAG response is associated with the elution of 3-octanone, but a second superimposed EAG response is associated with the elution of GC-peak 17.

5.3.2. Comparison of EAG Profiles Elicited by Volatiles of Host and Non-host Plants

Out of the 43 EAG stimulatory compounds located in the volatile blends of the various plant species, 22 elicited large or medium EAG amplitudes during GC-EAD. The profile of large and medium EAG amplitudes elicited by the various plant species volatile blends is compared in table 5.3. Differences in the profile of EAG responses are related to differences in yield and composition between the various blends of volatiles shown in table 5.4. The compounds were grouped in non-terpenoids, monoterpenes, sesquiterpenes, and unidentified compounds.

Non-terpenoids. This group comprises: a ketone, 3-hydroxy-2-butanone; two alcohols, 3-methyl-1-butanol and (Z)-3-hexen-1-ol; two esters, ethyl propanoate and ethyl-3-methylbutanoate; an epoxy compound, 2,3-epoxy-dimethylpentane; and 4,8-dimethylnona-1,3,7-triene. Ethyl-3-methylbutanoate and (Z)-3-hexen-1-ol co-eluted in the chromatograms of volatiles of *Eucalyptus* species and *P. pinaster* (figures 5.3-5.6). Possibly, both compounds contributed to the single EAG response correlated with the GC-peak, but the ester is produced in higher amounts than the alcohol by *E. globulus*, *E. camaldulensis* and *P. pinaster*. Except 4,8-dimethylnona-1,3,7-triene (detected exclusively in *O. europeae*) and ethyl propanoate, the remaining non-terpenoids are minor components of the volatile blends of *E. globulus* and *E. camaldulensis* that elicited large or medium EAG amplitudes. In *E. tereticornis*, only three compounds are correlated with large or medium EAG amplitudes, i.e. 3-hydroxy-2-butanone, 3-methyl-1-butanol, and 2,3-epoxy-dimethylpentane. In *P. pinaster*, only ethyl-3-methylbutanoate and (Z)-3-hexen-1-ol are correlated to large EAG amplitudes, whereas the other non-terpenoids were absent. In *O. europeae*, ethyl-3-methylbutanoate is absent. (Z)-3-Hexen-1-ol is the major compound in this non-host species and it is correlated with large EAG amplitudes. 3-Hydroxy-2-butanone is also correlated with large EAG amplitudes, whereas the remaining non-terpenoids elicited medium EAG amplitudes.

Monoterpenes. This group of compounds dominated quantitatively the volatile blends of *Eucalyptus* spp. and *P. pinaster*, and all of them are common to the four plant species, but produced in markedly different amounts, resulting in different EAG profiles between the GC-EAD recordings. Although *P. semipunctata* beetles have lower olfactory sensitivity to the identified monoterpenes than to the non-terpenoids (figure 5.14), high amounts of these compounds are produced and elicited large or medium EAG amplitudes. In *E. globulus*, large EAG amplitudes are correlated with α-pinene, 1,8-cineole, and limonene, whereas medium EAG amplitudes are correlated with β-pinene and *p*-cymene. 1,8-Cineole is by far the most abundant

compound, followed by α-pinene, limonene, *p*-cymene and β-pinene. In *E. camaldulensis*, large EAG amplitudes were correlated to *p*-cymene, 1,8-cineole, limonene and α-terpinene which are the four compounds produced in higher amounts, whereas α-pinene, β-pinene and linalool are correlated with medium EAG amplitudes. In *E. tereticornis*, the large EAG amplitudes are correlated with α-pinene and p-cymene which are the compounds produced in higher amounts, whereas β-pinene is correlated with medium EAG amplitudes. In *P. pinaster*, the large EAG amplitudes are correlated with myrcene, β-pinene, and α-pinene which are the most abundant compounds. The medium EAG amplitudes are correlated with 1,8-cineole, limonene, trans-β-ocimene, and linalool. In *O. europeae*, only α-pinene is correlated with medium EAG amplitudes, whereas trans-β-ocimene elicited only small EAG amplitudes, and the remaining monoterpenes are absent.

Sesquiterpenes. Only two minor sesquiterpenes, α-cubebene and guaine, were correlated with medium EAG amplitudes. α-Cubebene was exclusively detected in *P. pinaster*, whereas guaine is present in *E. globulus* and *E. tereticornis* and absent in *E. camaldulensis*.

Unidentified Compounds. Four minor unidentified compounds are correlated with large and medium EAG amplitudes. Unidentified compounds 8 and 17 were exclusively detected in the non-host, *O. europeae*, and are correlated with medium and large EAG amplitudes, respectively. Unidentified compound 36 is only present in *E. camaldulensis*, and compound 38 is present in *Eucalyptus* spp. and *O. europeae*. These two compounds in the four plant species are correlated with medium EAG amplitudes.

Table 5.3. Contribution of electrophysiologically most effective compounds in I - *Eucalyptus globulus*, II - *Eucalyptus camaldulensis*, III - *Eucalyptus tereticornis*, IV - *Pinus pinaster*, and V - *Olea europeae* to the profile of EAG amplitudes elicited on *Phoracantha semipunctata* females during GC-EAD. The EAG amplitudes were classified as large (●), medium (⊙), and small (○). Absence of EAG is indicated by "----".

Compound [1]	EAG Amplitudes[2]				
	Hosts			Non-hosts	
	I	II	III	IV	V
Non-terpenoids:					
3-hydroxy-2-butanone (4)	●	●	●	○	●
3-methyl-1-butanol (5)	●	●	⊙	----	⊙
2,3-epoxy-4,4-dimethylpentane (6)	⊙	⊙	⊙	----	⊙
ethyl propanoate (10)	○	○	----	----	⊙
ethyl-3-methylbutanoate (12)	● ?	⊙ ?	○ ?	● ?	----
(Z)-3-hexen-1-ol (13)	● ?	⊙ ?	○?	● ?	●
→ 4,8-dimethylnona-1,3,7-triene (32)	----	----	----	----	⊙
Monoterpenes:					
α-pinene (15)	●	⊙	●	●	⊙
β-pinene (18)	⊙	⊙	⊙	●	----
myrcene (19)	○	○	○	●	----
α-terpinene (22)	○	●	○	○	----
p-cymene (23)	⊙	●	●	○	----
1,8-cineole (24)	●	●	○	⊙	----
limonene (25)	●	●	○	⊙	----
trans-β-ocimene (26)	○	○	○	⊙	○
linalool (31)	○	⊙	○	⊙	----
Sesquiterpenes:					
→ α-cubebene (39)	----	----	----	⊙	----
guaine (40)	⊙	----	⊙	----	----
Unidentified Compounds:					
→ (8)	----	----	----	----	⊙
→ (17)	----	----	----	----	●
(36)	----	⊙	----	----	----
(38)	⊙	⊙	⊙	----	⊙

[1] Arrows indicate compounds exclusively detected in non-host species. The numbers in parenthesis indicate the GC peak in the chromatograms.

[2] ? - indicates uncertainty in the correlation between the EAG amplitude and the compound.

Table 5.4. Concentration of compounds in volatile blends of I - *Eucalyptus globulus*, II - *Eucalyptus camaldulensis*, III - *Eucalyptus tereticornis*, IV - *Pinus pinaster*, and V - *Olea europeae* that elicited large or medium EAG amplitudes.

Compound [1]	Concentration (ppm)				
	Hosts			Non-hosts	
	I	II	III	IV	V
Non-terpenoids:					
3-hydroxy-2-butanone (4)	2	49	37	<1	3
3-methyl-1-butanol (5)	49	82	38	----	32
2,3-epoxy-4,4-dimethylpentane (6)	6	12	7	----	8
ethyl propanoate (10)	<1	<1	----	----	3
ethyl-3-methylbutanoate (12)	68 [a]	32 [a]	<1 [a]	132 [a]	----
(Z)-3-hexen-1-ol (13)	27 [a]	17 [a]	8 [a]	66 [a]	647
→ 4,8-dimethylnona-1,3,7-triene (32)	----	----	----	----	11
Monoterpenes:					
α-pinene (15)	2313	490	7712	9449	20
β-pinene (18)	248	69	78	14150	----
myrcene (19)	188	84	23	7256	----
α-terpinene (22)	8	500	30	11	----
p-cymene (23)	420	7776	2001	65	----
1,8-cineole (24)	7297	6378	681	742	----
limonene (25)	499	3917	306	1334	----
trans-β-ocimene (26)	9	13	<1	2011	32
linalool (31)	8	155	6	117	----
Sesquiterpenes:					
→ α-cubebene (39)	----	----	----	108	----
guaine (40)	33	----	8	----	----
Unidentified Compounds:					
→ (8)	----	----	----	----	1
→ (17)	----	----	----	----	4
(36)	----	6	----	----	----
(38)	26	22	7	----	7

[1] Arrows indicate compounds exclusively detected in non-host species. The numbers in parenthsis indicate the GC peak in the chromatograms.

[a] The concentration of (Z)-3-hexen-1-ol was calculated on Stabilwax capillary column; the concentration of ethyl-3-methylbutanoate was calculated by subtraction of the area of (Z)-3-hexen-1-ol on the Stabilwax column to the area of GC peak 12 on HP-1 capillary column.

5.3.3. Electrophysiological Test of Synthetic Plant Compounds

Figure 5.14 shows EAG responses of *P. semipunctata* to 42 synthetic compounds in hexane, 10^3 ppm. The tested compounds comprised: 1) ten non-terpenoid hydrocarbons, including six-carbon plant fatty acid derivatives: 1-hexanol, (Z)-3-hexen-1-ol, (Z)-2-hexen-1-ol, (E)-2-hexenal, (E)-2-hexenyl acetate, and (Z)-3-hexenyl acetate; 2) twenty seven bicyclic, cyclic and acyclic monoterpenes; and 3) four sesquiterpenes. Eighteen of the tested volatiles were chemically identified in host or non-host tree species and are marked with numbers corresponding to the numbers in the chromatograms (figures 5.3 to 5.7). From left to right in the horizontal axis of the plot, the compounds are grouped according to similarities in chemical structure. There were no significant differences between the relative mean EAG amplitudes elicited by each compound in males (n = 6) and females (n = 6), thus the data were pooled.

The standard, (E)-2-hexenal, was clearly the most effective stimulus. This compound was not detected in the gas chromatograms. After the standard, the compounds that elicited large EAG amplitudes (i.e. ≥ 60%) are ranked as follow: 3-hydroxy-2-butanone > (+)fenchone ≅ 3-methyl-1-butanol ≥ 3-carene ≅ (Z)-3-hexen-1-ol ≅ 1-hexanol ≥ β-pinene ≅ (-)α-pinene ≅ (-)limonene ≅ 1,8-cineole ≥ α-phellandrene ≥ myrcene. The compounds that elicited EAGs of moderate amplitudes (i.e., 40-60%) were: 4,8-dimethylnona-1,3,7-triene ≥ β-phellandrene ≅ α-terpinene ≅ camphene ≅ (α,β)thujone ≅ (E)-2-hexenyl acetate ≥ (±)linalool ≅ (E)-linalool oxide ≅ (Z)-2-hexen-1-ol ≅ 3-octanone ≥ *p*-cymene ≥ γ-terpinene ≅ (Z)-linalool oxide ≅ sabinene ≅ (-) myrtenal. The less effective stimulus (EAG amplitudes < 40%) were: 4-methyl-2-pentanone ≅ (Z)-3-hexenyl acetate > terpinolene > (-)α-cubebene > piperitone.

Significant differences were detected in the relative effect of some structural analogues. 1) Position isomers: (Z)-3-hexen-1-ol was more effective than (Z)-2-hexen-1-ol; (E)-2-hexenyl acetate was more effective than (Z)-3-hexenyl acetate; and the ranked relative effect of several monoterpene position isomers was, (-)limonene ≥ α-phellandrene > β-phellandrene ≅ α-terpinene > γ-terpinene > terpinolene. 2) Diastereomers: (E)-linalool oxide was more effective than (Z)-linalool oxide. 3) Functional-group analogues: among the C6 compounds, (E)-2-hexenal was the most potent stimulus followed by (Z)-3-hexen-1-ol and 1-hexanol, (Z)-2-hexen-1-ol and (E)-2-hexenyl acetate were moderate stimuli, and (Z)-3-hexenyl acetate was among the less effective stimuli; myrcene was more effective than (±)linalool, and linalyl acetate had no stimulatory effect; α-terpinene was a moderate stimulus and its ketone analogue, piperitone, was the least effective stimulus; (-)myrtenol is a moderate stimulus and the aldehyde, (-)myrtenal, has

no stimulatory effect; cuminaldehyde, α-terpineol and terpinyl acetate have no stimulatory effect, and these are aldehyde, alcohol and ester analogues of *p*-cymene, limonene and β-phellandrene, respectively.

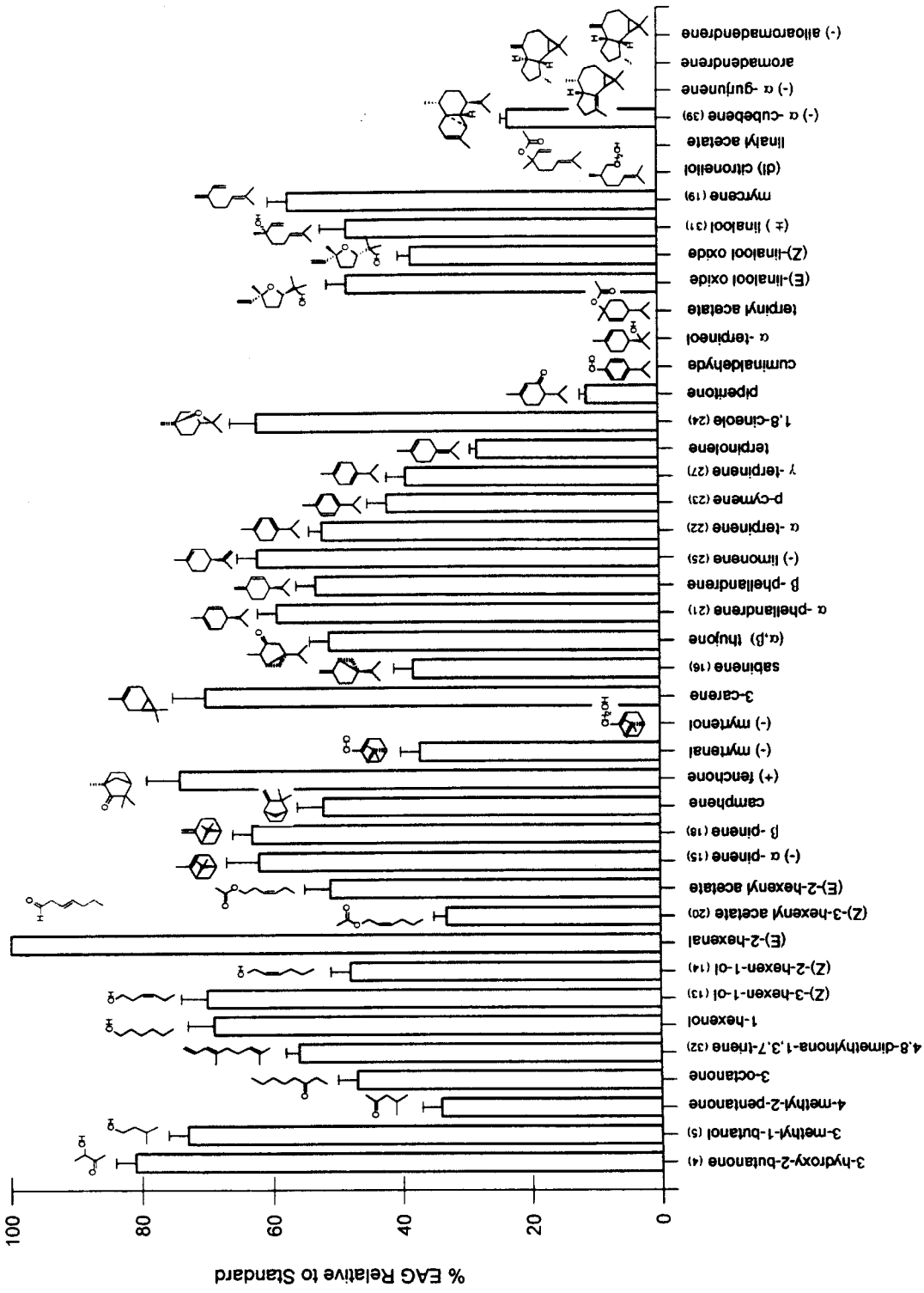


Figure 5.14. Mean EAG amplitudes (± SE; n = 12) relative to the standard, (E)-2-hexenal, elicited by 42 volatile compounds, stimulating the olfactory receptors of *Phoracantha semipunctata* via the GC at 10^3 ppm. It is shown the chemical structure of each compound. Eighteen of the tested volatiles were identified in host or non-host tree species, and are marked with numbers correspondig to the numbers in the chromatograms of figures 5.3 to 5.7.

5.4. Discussion

Collection of plant volatiles. The headspace technique for collecting volatiles on porapak Q was used in this study because it allows the trapping of airborne volatiles which the insect antennae normally are exposed to. This technique has been successfully used in the identification of insect pheromones (Silverstein and Rodin, 1966; Byrne *et al.*, 1975; Peacock *et al.*, 1975) and plant volatiles (Millar *et al.*, 1986; Pham-Delegue *et al.*, 1989; Blight *et al.*, 1995). Other techniques such as steam distillation (Schultz *et al.*, 1977) and solvent extraction (Etievant *et al.*, 1984) have been used, but these are more crude techniques yielding samples containing both volatile and non-volatile, making the identification of important volatiles more difficult.

Accumulative trapping of headspace volatiles over a time period onto porapak Q used in this study, yielded complex volatile blends of numerous major and minor components. This procedure may not have provided the precise natural ratio of volatiles released from plant material, because different compounds may have different affinity to the binding site of the adsorbant (Dobson, 1991). Cold-trapping of headspace volatiles has also been used in the identification of plant volatiles important to phytophagous insects (e.g. Guerin *et al.*, 1983; Baur *et al.*, 1993), and may give more precisely the ratio of the natural blend. In the present study, it was not critical to obtain the natural ratio of released volatiles, since the purpose was to identify which components of the blends elicit EAGs and to find out major differences between the various plant species in their production of these volatiles. Thus, it was used a relatively large adsorption period to collect large numbers and large quantities of compounds from host and non-host species, facilitating the identification of major as well as minor components. In addition, collection of headspace volatiles released from a pool of leaves of different individuals of the same plant species eliminated to some extent individual differences between trees which is reported in several studies (Squillace, 1976; Doran, 1991).

Identification of compounds eliciting EAGs. The GC-EAD proved to be a useful technique to locate in the gas chromatogram of complex volatile blends the peaks that elicited receptor neuron responses. Altogether, 43 components of the plants volatile profiles elicited EAG responses from *P. semipunctata*. From these, 26 compounds were identified by GC-MS and/or by indirect identification methods. GC-MS is a unique technique for the separation, detection and characterisation of the components of complex organic mixtures, allowing complete structural assignments of compounds based on their mass spectra. However, many compounds have identical or very similar mass spectra due to similarity of chemical structures, or due to various

fragmentation and rearrangements after ionisation (Davies, 1990). Hence, the GC-MS provides a tentative chemical identification of GC peaks that must be complemented with indirect identification methods. The two indirect methods used in this study were (1) the comparison of calculated Kováts' indices with similar indices published by Davies (1990) for terpenoid compounds and (2) the enhancement of GC-peaks by co-injection of authentic samples of compounds and solutions of plant volatiles on two capillary columns of different polarity. The co-injection method provided far better accuracy to confirm the tentative GC-MS identification of terpenes than the comparison of calculated Kováts' indices with published indices. The calculated indices of most terpenoid compounds deviated significantly from those reported by Davies (1990) on similar stationary phases (i.e. methyl silicone and Carbowax). These deviations are known and may occur due to the influence of temperature in the retention parameters (Karlssen & Siwon, 1975; Davies 1990). On Carbowax stationary phases, differences over 50 units may occur between indices determined under widely different conditions (Davies, 1990). As regard non-terpenoid compounds for which no published Kováts' indices were available, the co-injection of authentic samples did not confirm the GC-MS tentative identification of 4-methyl-2-pentanone in *E. globulus* and 3-octanone in *O. europeae* as GC-peaks correlated with EAG responses.

Due to the complexity of the volatile blends, co-elution and extensive overlap of compounds occurred in the chromatograms, and in some cases it was not possible to establish accurate correlation between EAG and GC-peaks. In one case, EAG responses were correlated to GC-peak 38 in the volatiles of *E. globulus*, *E. camaldulensis*, *E. tereticornis* and *O. europeae* (figures 5.2-5.4, 5.6). This GC-peak was identified as α-terpineol, but the authentic compound did not elicit any EAG. The active compound may have co-eluted with α-terpineol or correspond to a GC-peak that eluted just before, but this was not investigated. In another case, the GC-peak corresponding to the co-elution of (Z)-3-hexen-1-ol and ethyl-3-methylbutanoate in the volatile blends of *Eucalyptus* species and *P. pinaster*, was correlated with a single EAG response (figures 5.2-5.5). Both compounds may have contributed to the EAG, but due to the absence of authentic ethyl-3-methylbutanoate it cannot be conclusively established that this compound is also detected by *P. semipunctata* receptor neurons. Due to high concentration, some compounds overlapped extensively during elution from the GC-column, thus it was not possible to correlate reliably each of them with the recorded EAG responses. This was the case of α-terpinene, *p*-cymene, 1,8-cineole and limonene in *E. globulus* and *E. camaldulensis*, of 1,8-cineole and limonene in *E. tereticornis* and *P. pinaster*, and of myrcene and (Z)-3-hexenyl acetate in *P. pinaster* (figures 5.2-

5.5). The test with authentic samples showed that each of these compounds are detected by *P. semipunctata* receptor neurons, eliciting different relative EAG amplitudes (figure 5.14).

The chemical identification of ethyl propanoate, 2,3-epoxy-4,4-dimethylpentane, ethyl-3-methylbutanoate, 1-isopropenyl-3-methylbenzene, 1,2-dimethyl-3-isopropenylcyclopentane, isopinocarveol, guaine and *D*-germacrene, is based only on mass spectra. For the time being, this identification is just tentative, requiring further confirmation and test of the electrophysiological activity of authentic samples on *P. semipunctata* receptor neurons.

Profile of odourants in host and non-host plants. GC-EAD recordings showed that major and minor components of the various volatile blends elicited large or medium EAG amplitudes. Strong EAG responses to minor components of plant volatile blends have been demonstrated in other insect species using GC-EAD (Guerin *et al.*, 1983; Baur *et al.*, 1993). The EAG is considered to be the summation of receptor potentials of receptor neurons responding simultaneously (Schneider, 1957, 1969), and the EAG amplitude reflects the number of responding receptor neurons to an odourant as well as the response strengths of each neuron. Thus, it indicates the sensitivity of all olfactory receptor neurons to an odour. Major and minor components which elicited large or medium EAG amplitudes are more likely to be detected by *P. semipunctata* beetles at lower concentration and thus at longer distances, affecting the flight orientation. Major and minor components eliciting small EAG amplitudes may play a role in close range orientation and host recognition. However, since small EAG amplitudes may also result from secondary effects of chemical analogues in receptor neurons tuned to other compounds, more attention is given to the volatiles that elicited strong EAG responses.

The profile of large and medium EAG amplitudes elicited by the various plant species volatile blends was compared (table 5.3). Differences in the profile of EAG responses are related to differences in yield and composition between the various blends of volatiles (table 5.4). In the host species, large or medium EAG amplitudes were correlated with a group of sixteen compounds comprised of six minor non-terpenoids [3-hydroxy-2-butanone, 3-methyl-1-butanol, 2,3-epoxy-4,4-dimethylpentane, and possibly ethyl-3-methylbutanoate and (Z)-3-hexen-1-ol], seven major monoterpenes (α-pinene, β-pinene, α-terpinene, *p*-cymene, 1,8-cineole, limonene and linalool), one minor sesquiterpene (guaine), and two minor unidentified compounds (36 and 38). Except for guaine (not detected in *E. tereticornis*) and the unidentified compound 36 (exclusively detected in *E. camaldulensis*), all the other volatiles are present in the three *Eucalyptus*, eliciting EAG amplitudes that relate to their concentration. It is possible that variation in the attractiveness of *E. globulus*, *E. camaldulensis*, and *E. tereticornis* to *P.*

semipunctata beetles is related to specific quantitative differences in the production of these volatile compounds.

The females oviposition choice is critical for this wood-boring insect, because their larvae cannot compensate for poor host quality by moving away to new hosts. *P. semipunctata* beetles are attracted to the *Eucalyptus* species that represent the highest quality hosts for their progeny (Hanks *et al.*, 1993). Host quality is also evaluated among the individuals of an host species, since drought stressed specimens, which cannot prevent bark penetration of neonate larvae, are preferred for oviposition (Chararas, 1969; Hanks *et al.*, 1991). Although the profile of plant volatile compounds is under genetic control, it can be altered to some extent by environmental factors leading to physiological stress, such as variation in the amount of water available. Plants under physiological stress produce several volatile compounds by oxidation of fatty acids and fermentation products resulting from anaerobiosis (Kimmerer and Kozlowski, 1982). Prolonged water deficit situations may induce changes in the quantitative composition of volatiles in *Eucalyptus*, and these may produce a blend of volatiles that is more attractive to adult beetles. The beetles exhibited strong EAG response to (E)-2-hexenal, 1-hexenol, and (Z)-3-hexen-1-ol (figure 5.14). However, only (Z)-3-hexen-1-ol is related to strong EAG responses in the experiments with the volatile blends of *E. globulus*. These are compounds of the "green leaf" volatiles complex, a group of chemicals well known for being detected by many species of phytophagous insects (Visser, 1986). Since tissue damage increases the amount of "green leaf" volatiles released (Wallbank and Wheatley, 1976; Tollsten and Bergström, 1988; Whitman and Eller, 1990), some of these compounds could permit *P. semipunctata* beetles to find stressed or downed *Eucalyptus*.

Only very limited work have been done to investigate the influence of environmental factors on variation in the composition of *Eucalyptus* volatile compounds (Doran, 1991). Mateus *et al.* (1995) suggested a relationship between the physiological condition of *E. globulus*, their profile of major monoterpenes in the headspace volatiles, and attraction to *P. semipunctata*. Logs of *E. globulus* obtained from trees attacked by *P. semipunctata* were observed to be more attractive to the beetles than logs obtained from unattacked trees. The profile of α-pinene, α-terpinene, and α-phellandrene emitted by attacked logs was similar to the one obtained from logs of non-vigorous trees attacked by pathogens, but significantly different from that emitted by logs of apparently healthy trees. The EAG recordings in the present study show that in addition to these monoterpenes, *P. semipunctata* receptor neurons are able to detect other monoterpenes as well as non-terpenoids in *Eucalyptus*.

Qualitative and quantitative differences between the volatile blends of host and non-host species explain differences obtained in the profiles of large and medium EAG amplitudes (table 5.2 and 5.3). In the non-host, *P. pinaster*, nine compounds elicited large or medium EAG amplitudes, including: two non-terpenoids, ethyl-3-methylbutanoate and (Z)-3-hexen-1-ol; six monoterpenes, α-pinene, β-pinene, myrcene, 1,8-cineole, limonene, and trans-β-ocimene; and the sesquiterpene α-cubebene. Except for α-cubebene detected only in this blend of volatiles, all the other stimulatory compounds are also present in the Eucalyptus species but in much different concentration ratios. For instance, myrcene and trans-β-ocimene are present in host species volatile blends in evidently smaller concentration than in the blend of *P. pinaster*. In the other non-host, *O. europeae*, ten compounds elicited large or medium EAG amplitudes, including: six non-terpenoids, 3-hydroxy-2-butanone, 3-methyl-1-butanol, 2,3-epoxy-4,4-dimethylpentane, ethyl propanoate, (Z)-3-hexen-1-ol, and 4,8-dimethylnona-1,3,7-triene; one monoterpene, α-pinene; and three unidentified compounds, 8, 17 and 38. Among these, 4,8-dimethylnona-1,3,7-triene and the unidentified compounds 8 and 17 were exclusively detected in this species. Except for α-pinene, the terpenoids that give important contribution to the profile of EAG amplitudes elicited by the host volatile blends are absent in *O. europeae*. Other volatiles produced by *O. europeae* are also found in *Eucalyptus* but in different ratios.

Some important host related volatiles are absent in the blends of *P. pinaster* and *O. europeae*, whereas other important odourants of the host plants are also produced by these non-hosts, but in different ratios. In addition, *P. pinaster* and *O. europeae* produce volatile compounds which elicit strong EAG responses and were not detected in *Eucalyptus*. These compounds may induce avoidance reactions in *P. semipunctata* beetles. The identification of compounds in non-host plants eliciting strong EAG responses provides a basis for further experiments on the influence of individual chemicals on the flight orientation of *P. semipunctata*, and on their effect when mixed with attractive host blends of volatiles. Repellent effects of non-hosts odour has been shown in polyphagous moths and aphids. The cabbage looper moth, *T. ni*, is repelled by steam distillates of resistant varieties of soybean plants (Khan *et al.*, 1987). The black bean aphid, *A. fabae*, is repelled by the odour of summer savory, *Satureja hortensis*, and the cabbage aphid, *B. brassicae*, is repelled by the odour of tansy, *Tanacetum vulgare* (Nottingham *et al.*, 1991). In aphids, the behavioural importance of single non-host volatile compounds has been also demonstrated. Black bean aphids are repelled by single isothiocyanates (i.e. 4-pentenyl-, 3-butenyl-, and allyl-isothiocyanates) produced specifically by non-host crucifereae (Nottingham *et al.*, 1991), and by methyl salycilate and myrtenal that are more generally

distributed plant volatiles (Hardie *et al.*, 1994). When mixed with host plant blends of volatiles, these compounds inhibit the attraction of *A. fabae* to the host plant (beans - *Vicia faba*).

EAG responses elicited by synthetic plant compounds. The test of EAG responses to authentic samples of 3-hydroxy-2-butanone, 3-methyl-1-butanol, 4,8-dimethylnona-1,3,7-triene, (Z)-3-hexen-1-ol, (-)α-pinene, β-pinene, (-)limonene, α-terpinene, *p*-cymene, 1,8-cineole, linalool, myrcene, and (-)α-cubebene (figure 5.14) confirmed the chemical identification of components in headspace volatiles of the various plant species that were correlated with medium/large EAG amplitudes during GC-EAD. However, the relative EAG amplitudes elicited by (-)α-cubebene are of lower amplitude than those expected on the basis of its concentration in the headspace volatiles of *P. pinaster* and correlated EAG amplitudes (figure 5.6). It is possible that *P. pinaster* produce the two enantiomers of which (+)α-cubebene or a mixture of both forms might be electrophysiologically more effective than the enantiomer (-)α-cubebene.

Authentic samples of chemically identified components in the headspace volatiles of the various plant species that elicited small EAG amplitudes were also tested, i.e. (Z)-2-hexen-1-ol, (Z)-3-hexenyl acetate, sabinene, α-phellandrene, and γ-terpinene. Among these, α-phellandrene elicited relative EAG amplitudes not significantly different from those elicited by (-)limonene or β-pinene. In addition, (E)-2-hexenal, 1-hexenol, (+)fenchone, and 3-carene, which were not detected as stimulatory components in the various blends of plant volatiles, are among the compounds tested that elicited largest relative EAG amplitudes. These compounds have widespread occurrence among plants, and the monoterpenes α-phellandrene, (+)fenchone, and 3-carene have been identified as essential oil components of various *Eucalyptus* species (Brophy *et al.*, 1991). Thus, it is possible that they contribute for discrimination between odour blends of different *Eucalyptus* species as well as of other plants.

Authentic samples of several compounds which did not contribute to the profile of medium/large EAG amplitudes during GC-EAD with the various plant species volatile blends elicited moderate (60-40%) or small (<40%) relative EAG amplitudes (i.e. 4-methyl-2-pentanone, 3-octanone, (Z)-2-hexen-1-ol, (Z)-3-hexenyl acetate, (E)-2-hexenyl acetate, camphene, (-)myrtenal, sabinene, (α,β)thujone, β-phellandrene, γ-terpinene, terpinolene, piperitone, (E)-linalool oxide, and (Z)-linalool oxide) (figure 5.14). The EAG responses elicited by these compounds have limited value as indication of their biological importance for the insect, since they may have resulted from secondary effects on receptor neurons tuned to the detection of other compounds with related chemical structures. Information about how plant compounds are

encoded by the receptor neurons can only be obtained by electrophysiological recordings from single receptor neurons.

Correlation of EAG responses with behaviour. Finding the host plant is a first behavioural step in the process of host selection in phytophagous insects, followed by host recognition when the insect at close range responds to chemical and physical features that may lead to feeding and/or oviposition on the plant (Miller and Strickler, 1984; chapter 1, this thesis). Odour of *E. globulus* induces upwind flight in *P. semipunctata* beetles at a distance from the plant (chapter 4, this thesis). In this study, more importance was given to compounds in *Eucalyptus* volatile blends that elicit strong EAG responses, and quantitative variation in the production of such compounds was suggested to be related with the variation in the attractiveness of *E. globulus*, *E. camaldulensis*, and *E. tereticornis* to *P. semipunctata* beetles. It was assumed that compounds that elicited small EAG amplitudes during GC-EAD, regardless of their concentration in the volatile blends, are not behaviourally important at long distances from the plant. However, the magnitude of the EAG elicited by individual plant compounds do not always correlate with the behavioural responses (Averill *et al.*, 1988; Light *et al.*, 1992). It is conceivable that compounds producing a sensory response in only a limited number of receptor neurons, thus producing a weak EAG, may nevertheless have influence on the behaviour at long range as shown for some pheromone components (cf. Baker, 1989). Thus, volatiles in hosts and non-hosts eliciting small EAG amplitudes may also affect the behaviour of *P. semipunctata* beetles, and contribute to discrimination between plant odours.

In conclusion, the integrated results suggest that *P. semipunctata* beetles use certain ratios of several host related compounds (terpenoid and non-terpenoid hydrocarbons) instead of relying on single types of compounds for host-finding. Only behavioural studies may show conclusively what changes in the ratio of which compounds reduce or increase the attractiveness of natural volatile blends of preferred *Eucalyptus* species (e.g. *E. globulus*). Changes in the natural ratio of some host related odourants, i.e. ubiquitous "green leaf" volatiles, prevent the Colorado potato beetles, *L. decemlineata*, of finding the host plant in wind tunnel (Thiery and Visser, 1986, 1987). Also, in some Scolytidae, preference for species of conifers is not related to a single terpene in the profile of volatiles, instead the attraction of suitable host species is thought to result from synergistic effects of various terpenes (Chararas *et al.*, 1982). Other oligophagous insects rely on a few chemically related compounds specifically produced by their host plants. Examples are phytophagous insects attacking Liliaceae, which respond to certain organic disulphides, and many crucifer-associated insects that respond to isothiocyanates (refs. in Städler,

1992). In addition, compounds exclusively detected in non-host plants may induce avoidance reactions in *P. semipunctata* beetles.

The results of this study provide valuable information on the olfactory capabilities of *P. semipunctata* beetles for orienting further research to elucidate the mechanisms underlying host and non-host plants discrimination. The restrictions on the interpretation of the EAG results underline the importance of further investigation on behavioural reactions to single odourants and manipulated plant odour blends, as well as investigation on how plant odours are encoded by receptor neurons of *P. semipunctata*.

6.

Responses of *Phoracantha semipunctata* Receptor Neurons to Volatiles of Host and Non-host Plants Using Coupled Gas Chromatography-Single Cell Recordings

6.1. Introduction

The detection of plant volatiles by insects is largely made by receptor neurons of olfactory sensilla located on the antennae (cf. Masson and Mustaparta, 1990). The odour information is then conveyed as nerve impulses to the brain, where it is processed, and further conveyed to motor neurons, resulting in behavioural reactions. An important question in olfactory research is how information from odour mixtures is encoded by receptor neurons. This question is to a large extent answered for pheromone mixtures in many insect species, whereas the knowledge about the olfactory coding mechanisms for plants odour information is limited (cf. Mustaparta, 1996b). Early electrophysiological investigations of insects olfactory receptor neuron responses led to the conclusion that plant/food odours were detected by broadly tuned cells of low sensitivity, responding to many chemically diverse compounds, whereas the pheromone receptor neurons were of high sensitivity and specialised for receiving information about one or a few chemically related compounds (cf. Schneider, 1987). The studies on plant/food odour reception were conducted with synthetic compounds, randomly selected from a set of known volatile compounds present in host plants, and with wide distribution among plant species. A receptor neuron may respond at high concentrations to many compounds which may not necessarily be of biological importance. Therefore, it is possible that receptor neurons showing slight responses to a range of different chemicals may in fact be specialised to a particular compound not present among the volatiles tested (cf. Masson and Mustaparta, 1990).

Plant volatiles are of primary importance in host finding behaviour of many phytophagous insects, but little is known about which chemical signals of the complex plant volatile blends are used. In order to understand the mechanisms involved in host plant selection, it is important to know for which plant compounds the receptor neurons of phytophagous insects have evolved and how olfactory information is encoded at the peripheral level. The first study aimed at testing all compounds naturally released by a host plant was carried out on the carrot fly (Guerin *et al.*, 1983), using gas chromatography (GC) linked with the recording of

electroantennograms (EAG), a technique which was introduced to identify pheromones and was termed GC-electroantennographic detector (GC-EAD), described in chapter 5 (Arn *et al.*, 1975; Struble and Arn, 1984). The GC linked with the electrophysiological recording from single receptor neurons (GC-SCR) was later introduced for identifying insect pheromones (Wadhams, 1982), and has also been employed for studies on the reception of plant volatiles (Tømmerås and Mustaparta, 1987; Wibe and Mustaparta, 1992, 1996; Blight *et al.*, 1995). These studies have shown selective responses of the receptor neurons to major as well as minor components of the plants volatile blends. This is in contrast to the early idea that plant volatiles are detected by broadly tuned receptor neurons, mediating the odour information to the brain via an "across-fibre" mechanism, i.e. information about one compound is mediated by different types of neurons (Kaissling, 1971; Sass, 1978; Visser and De Jong, 1988; Masson and Mustaparta, 1990). However, since evidence is growing for the existence of receptor neurons specialised to receive information about a particular plant compound, it is possible that certain plant odours are encoded by specialised neural connections, i.e. a "labelled line" mechanism which is thought to be the mechanism for coding of pheromone information (cf. Mustaparta, 1995, 1996a,b; Hildebrand 1996). Both "labelled-line" and "across-fibre" codes may be used in plant odour recognition, since one does not exclude the other. They may represent the two extremes of a continuum and may both operate in one species or individual (Masson and Mustaparta, 1990; Frazier, 1992).

In chapter 5, it was suggested that for oviposition the eucalyptus wood borer, *P. semipunctata*, makes use of many chemically diverse host and non-host plant volatile compounds (including major and minor components) to discriminate between plant species. The compounds identified, using GC-EAD and GC-MS techniques, which might be of behavioural importance, included terpenoid and non-terpenoid volatiles with a wide distribution in the plant kingdom. However, the EAG data has limited value, since biologically important compounds may be overlooked, and others may elicit EAGs due to secondary effects on many neurons. Furthermore, the EAG data do not provide information about how single receptor neurons encode the odour information. Therefore, it is important to complement the results of EAG recordings with further studies using GC-SCR. The aim of the present study was to investigate which components of the volatile profiles of host (*E. globulus*) and non-host (*P. pinaster* and *O. europeae*) plant species are detected by single receptor neurons of *P. semipunctata*, using the GC-SCR technique.

6.2. Materials & Methods

Insects. The adult insects were collected from *E. globulus* infested logs, and reared in the laboratory as described in chapter 3. The electrophysiological recordings were obtained from 14-21 days old females.

Plant volatiles. In this study, it was used the same solutions of headspace volatiles from leaves of *E. globulus* and *O. europeae* and needles of *P. pinaster*, as used in the previous study of GC-EAD recordings (chapter 5). When not in use, these hexane solutions were stored in the dark at -3° C.

Preparation and electrophysiological recordings. The electrophysiological recordings were carried out at the Zoology Department of the University of Trondheim (Norway). The insect was mounted on a Plexiglas block and secured with wax (Utility Wax Rods, Kerr®). The preparation was placed under a Leitz stereoscope. Nerve impulses (spikes) from olfactory receptor neurons were recorded extracellularly using electrolitically sharpened tungsten electrodes. These were inserted into the base of an olfactory sensillum with a Leitz micromanipulator. the aid of a Leica. Most recordings were obtained from sensilla basiconica on the lateral (anterior) region of the 8th flagellar segment and a few were obtained on the corresponding region of the 7th and 9th (apical) segments. At these regions of these most distal flagellar segments of *P. semipunctata* the density of sensilla basiconica is highest (Lopes, 1990). The indifferent electrode was inserted into the base of the antenna through the inter-segment membrane between the scape and the pedicel. The treatment did not seriously injure the specimen. After one experiment, the beetles resumed normal walking and feeding activity, and lived for long periods, even when the same specimen had been used for electrophysiological recordings during three consecutive days.

When bioelectrical contact was achieved with a receptor neuron, it was screened for sensitivity to the volatile blends of *E. globulus*, *P. pinaster* and *O. europeae*. These tests were carried out with "puff"-stimulation from Pasteur pipettes containing a piece of filter paper impregnated with 3 µl of a solution of plant volatiles after hexane was evaporated. The "puff"-stimulus (5 ml of air for 0.5 s) was directed into a glass tube with a permanent flow of purified air (ca. 600 ml/min) blowing onto the three most distal segments of the antenna. The neurons that responded were classified as plant odour receptor neurons and further examined by stimulation via the gas chromatograph (GC) - Fisons Mega 2 Series (figure 6.1). A sample of 1 µl of the hexane solutions was injected onto the GC-column (HP-1, 50 m x 0.32 mm i.d.). The GC-

separation conditions were: helium as carrier gas (26 cm/s linear velocity; 1.55 ml/min gas flow at 100° C), on column injection at 40° C and temperature program rising to 200° C at 7°/min with a final isothermal period of 5 min. The column effluent was divided by a glass splitter with pressfit connectors at a 1:1 ratio to the flame ionization detector (FID) and to the antennal preparation. The deactivated silica capillary column leading the effluent to the insect antenna was inserted into a stainless steel tube (2 mm i.d.) secured to the GC oven wall. This metal tube was permanently heated at 200° C by a thermostatically controlled heating tape. This tube with the deactivated column was inserted into a glass tube (5 mm i.d.) through which a 600 ml/min purified airflow was directed onto the antenna.

The FID-electrometer and receptor neuron electric signals were simultaneously recorded with the Syntech Computer System for recording and analysis of electrophysiological signals, using the GC-EAD program (v. 1.4). The FID-electrometer signal was connected to a DC channel in the interface board, designated as the GC channel. A spike counter, connected between the amplifier output and a second DC channel in the Syntech interface board (designed to acquire EAG signals), was set to count spikes every 2s. In this way, both FID-electrometer and receptor neuron activity were displayed simultaneously on the computer monitor screen allowing the easy detection of GC-peaks that elicited a response from the receptor neuron. The neural signal was also recorded on tape for subsequent analysis with the Syntech Computer System using the Autospike program (v. 1.2a) to measure spike frequency and to distinguish spikes of different amplitudes. The FID-electrometer signal was also connected to a LDC Milton-Roy integrator and printer.

Chemical identification. The chemical identification of some compounds that elicited responses from plant odour receptor neurons in the various solutions of plant volatiles was achieved by direct (GC-MS) and indirect methods (co-injection, Kováts indices) as described in chapter 5.

Dose-response relationships. Dose-response curves were obtained for some plant odour receptor neurons. The stimulation procedure was the one described above to screen for sensitivity of receptor neurons to the plant volatiles. The Pasteur pipettes contained a piece of filter paper impregnated with 200 µl of a dilution in hexane (10^{-7} to 10^{-1} v/v) of a synthetic compound, i.e. 3-methyl-1-butanol, (Z)-3-hexen-1-ol, 1-hexenol, α-terpinene, (-)limonene, 1,8-cineole and linalool. When not in use, the Pasteur pipettes were stored in the dark at -3° C.

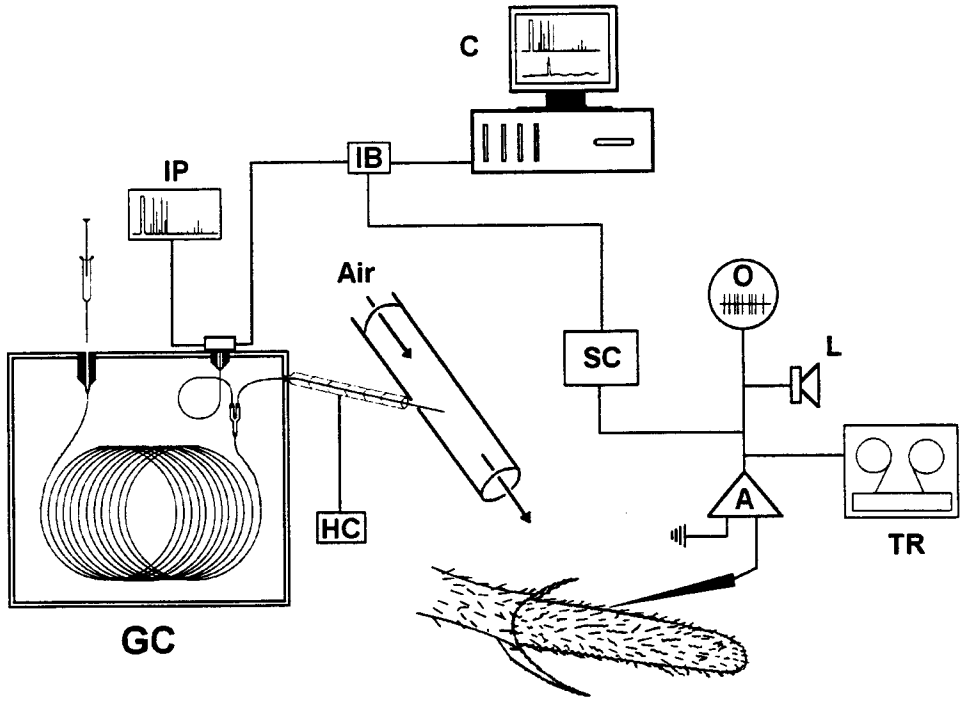


Figure 6.1. Diagram of the GC-SCR set-up (not in scale). Recording equipment comprises amplifier (x1000) **(A)**, audio-amplifier with loudspeaker **(L)**,tape recorder **(TR)**, oscilloscope **(O)**, spike counter **(SC)** with DC output to the interface box **(IB)** of Syntech Computer System, computer **(C)** with a signal acquisition interface board and software (Syntech). The FID-electrometer is connected to the interface box and to the integrator-printer **(IP)**. The capillary column inside the GC is illustrated with a glass splitter for leading the effluent equally to the FID and out the GC, through a metal tube, to an air stream directed onto the antenna. A heat controller **(HC)** was set to heat the metal tube at a constant temperature of 200° C.

6.3. Results

6.3.1. Volatiles of Host and Non-host Plants that Elicited Responses in Receptor Neurons

Several components of each plant species volatile blend elicited responses in RNs of *P. semipunctata*. Figures 6.2, 6.3 and 6.4, show gas chromatograms of the volatiles from the host (*E. globulus*) and from the non-host species (*P. pinaster* and *O. europeae*). The GC-peaks that elicited responses in RNs during the GC-SCR recordings are indicated with numbers in the chromatograms. The same numbers in the three chromatograms indicate the same compound. Altogether 32 compounds, being major as well as minor components of each volatile blend, elicited responses of the RNs. Fifteen of these compounds had been chemically identified by combined GC-EAD and GC-MS (chapter 5), the structures of which are shown in figure 6.5. These included the non-terpenoid hydrocarbons, 3-hydroxy-2-butanone (1), 3-methyl-1-butanol (2), 2,3-epoxy-4,4-dimethylpentane (3), ethyl-3-methylbutanoate (7), (Z)-3-hexen-1-ol (8) and (Z)-2-hexen-1-ol (9); the monoterpenes, α-pinene (10), β-pinene (12), myrcene (13), 1,8-cineole (14), limonene (15), trans-β-ocimene (16) and linalool (17); and the sesquiterpenes α-cubebene (27) and guaine (28). Some of the stimulatory compounds were exclusively detected in one plant species. In the host plant, *E. globulus*, these were guaine and the unidentified compounds 4, 19 to 24, 26 29, 30, and 32. A few compounds were exclusively detected in the non-host species. These were α-cubebene and the unidentified compounds 25 and 31, which were only present in *P. pinaster*, and the unidentified compound 11 which was only present in *O. europeae*. The other stimulatory compounds were present in *E. globulus* and in one of the two non-hosts. Ten of the compounds did not elicit significant EAG responses during the GC-EAD recordings described in chapter 5. These compounds were not chemically identified and are indicated in the chromatograms of *E. globulus* and *P. pinaster* volatiles (figures 6.2 and 6.3).

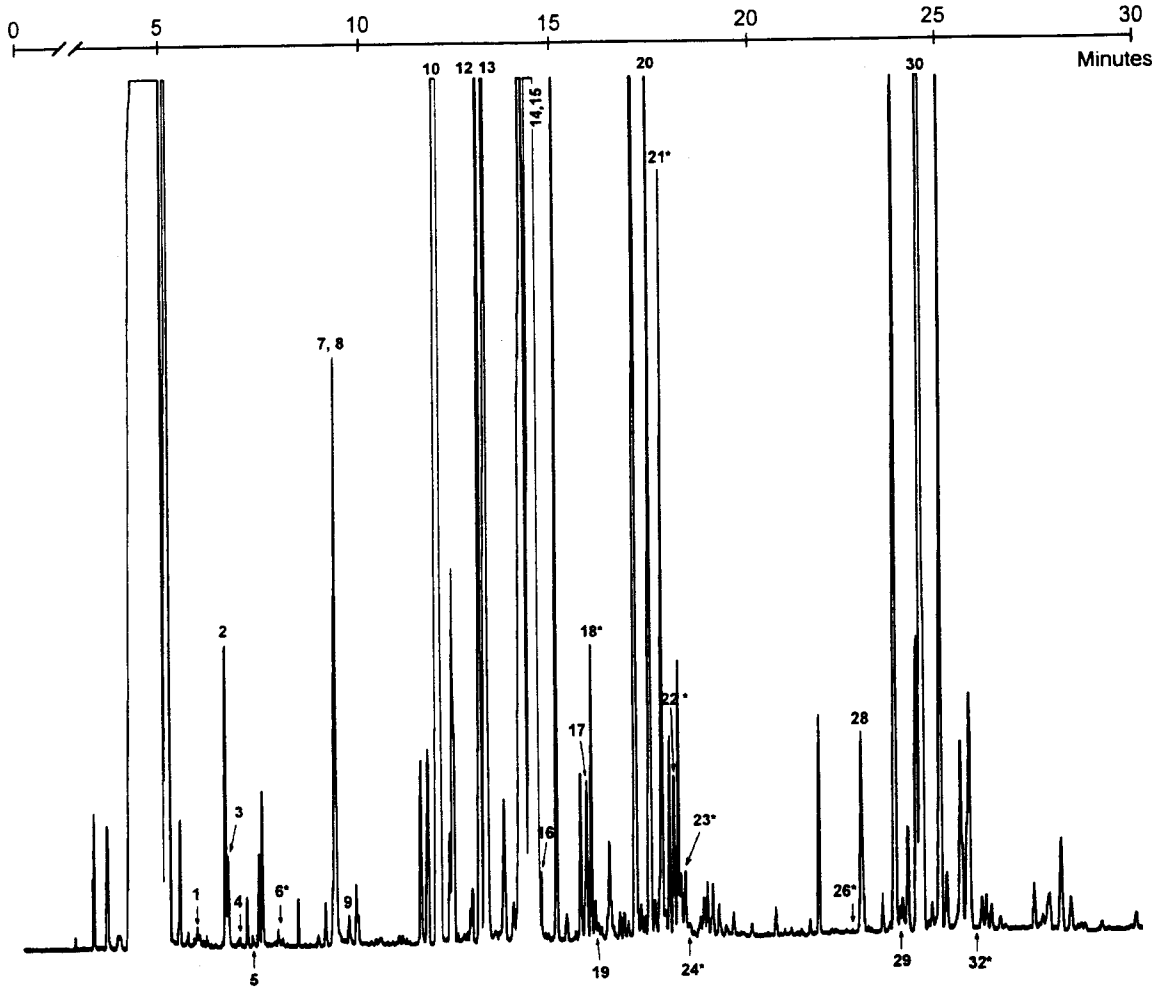


Figure 6.2. Gas chromatogram of headspace volatiles of *Eucalyptus globulus*. Numbered peaks elicited responses from *Phoracantha semipunctata* olfactory receptor neurons: (1) 3-hydroxy-2-butanone, (2) 4-methyl-1-butanol, (3) 2,3-epoxy-4,4-dimethylpentane, (4) unidentified, (5) unidentified, (6*) unidentified (7) ethyl-3-methylbutanoate, (8) (Z)-3-hexen-1-ol, (9) (Z)-2-hexen-1-ol, (10) α-pinene, (12) β-pinene, (13) myrcene, (14) 1,8-cineole, (15) limonene, (16) trans-β-ocimene, (17) linalool, (18*) unidentified, (19) unidentified, (20) unidentified, (21*) unidentified, (22*) unidentified, (23*) unidentified, (24*) unidentified, (26*) unidentified, (28) guaine, (29) unidentified, (30) unidentified, (32*) unidentified. The numbers marked with * indicate compounds that did not elicit significant EAG responses (see text).

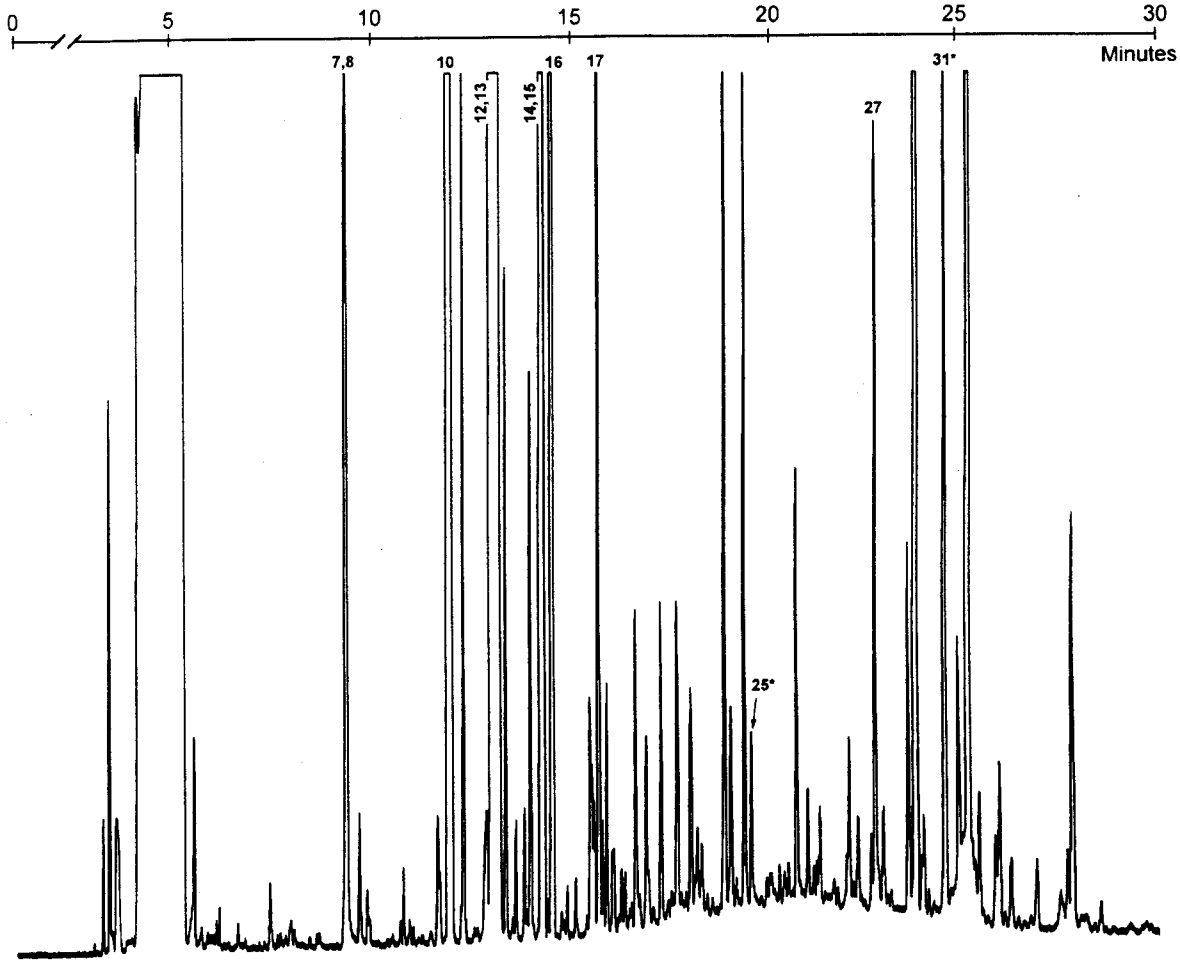


Figure 6.3. Gas chromatogram of headspace volatiles of *Pinus pinaster*. Numbered peaks elicited responses from *Phoracantha semipunctata* olfactory receptor neurons: (7) ethyl-3-methylbutanoate, (8) (Z)-3-hexen-1-ol, (10) α-pinene, (12) β-pinene, (13) myrcene, (14) 1,8-cineole, (15) limonene, (16) trans-β-ocimene, (17) linalool, (25*) unidentified, (27) α-cubebene, (31*) unidentified. The numbers marked with * indicate compounds that did not elicit significant EAG responses (see text).

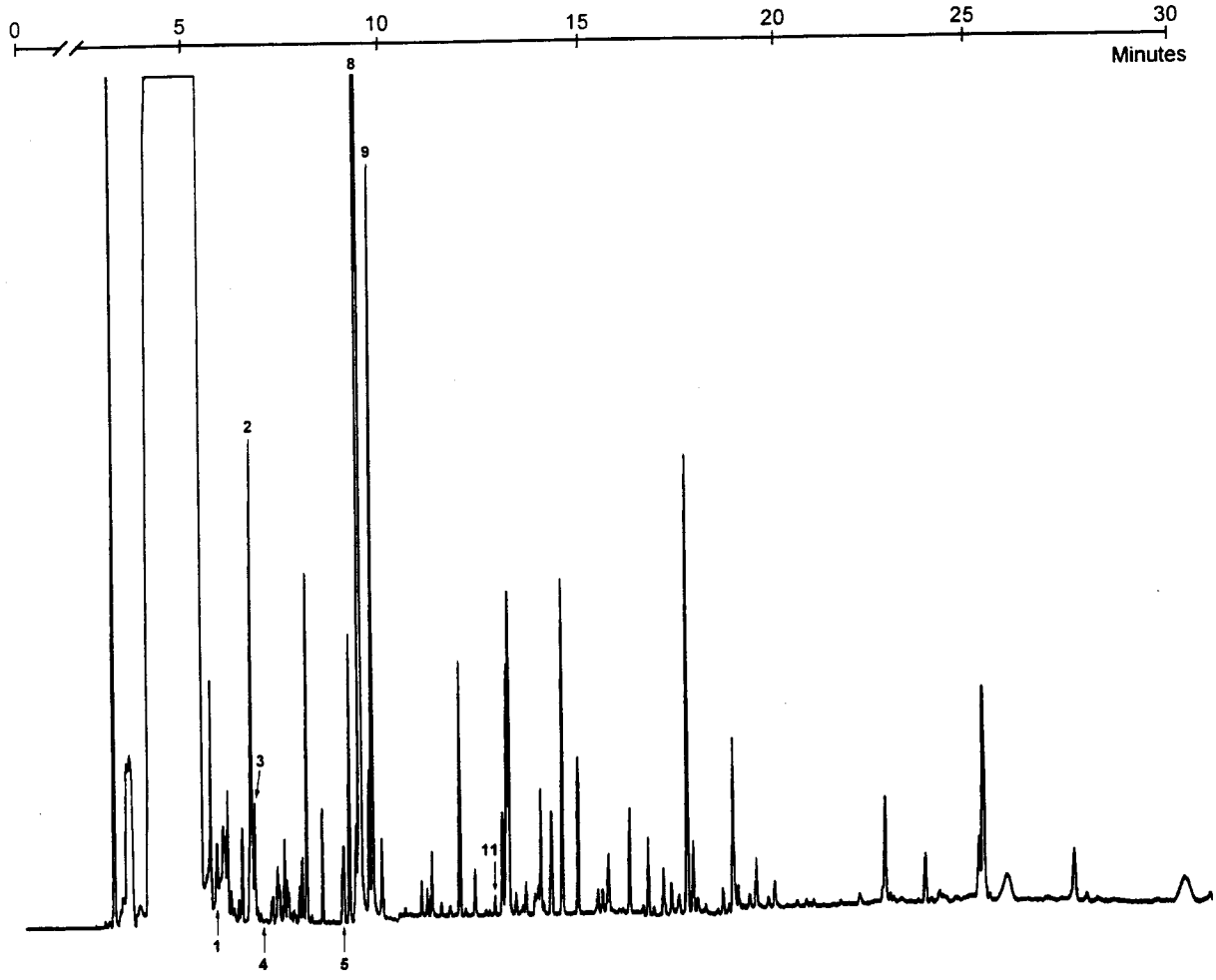
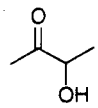


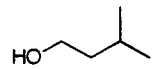
Figure 6.4. Gas chromatogram of headspace volatiles of *Olea europeae*. Numbered peaks elicited responses from *Phoracantha semipunctata* olfactory receptor neurons: (1) 3-hydroxy-2-butanone, (2) 4-methyl-1-butanol, (3) 2,3-epoxy-4,4-dimethylpentane, (4) unidentified, (5) unidentified, (8) (Z)-3-hexen-1-ol, (9) (Z)-2-hexen-1-ol, (11) unidentified.

Non-terpenoids

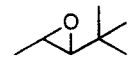
3-hydroxy-2-butanone (1)



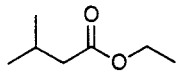
3-methyl-1-butanol (2)



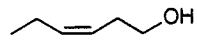
2,3-epoxy-4,4-dimethylpentane (3)



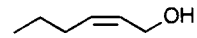
ethyl-3-methylbutanoate (7)



(Z)-3-hexen-1-ol (8)



(Z)-2-hexen-1-ol (9)

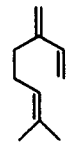


Monoterpenes

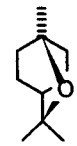
α-pinene (10)

β-pinene (12)

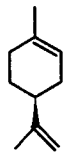
myrcene (13)



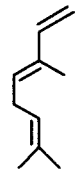
1,8-cineole (14)



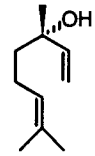
limonene (15)



trans-β-ocimene (16)

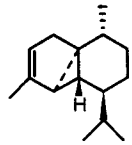


linalool (17)



Sesquiterpenes

α-cubebene (27)



guaine (28)

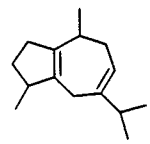


Figure 6.5. Chemical structures of compounds in volatile blends of host and non-host plants of *Phoracantha semipunctata* that elicited electrophysiological responses during GC-SCR recordings. The numbers in parenthesis correspond to GC-peaks in the chromatograms of figures 6.2-6.4.

6.3.2. Recording of Receptor Neuron Responses

The results are based on recordings from olfactory receptor neurons which displayed only one or two spike amplitudes and were thus possible to analyse. The recordings originated from 56 receptors neurons (RNs) associated with 45 olfactory sensilla of the 3 most distal flagellar segments (7^{th}, 8^{th} and 9^{th}). The recordings were obtained from 16 preparations of *P. semipunctata* females of which 3 preparations were used for three days, 5 preparations for two days, and the remaining 8 preparations for one day. The duration of each recording varied from some minutes up to five hours. The spike duration was 2 ms and the spontaneous activity varied from 0 to 6 spikes/s. Out of the 56 RNs, 9 responded to the volatiles of the host , *E. globulus*, and of the non-hosts, *P. pinaster* and *O. europeae*, 23 to the volatiles of *E. globulus* and of *P. pinaster*, 8 to the volatiles of *E. globulus* and *O. europeae*, 8 to the volatiles of only *E. globulus*, 4 to the volatiles of only *P. pinaster*, and 4 to the volatiles of only *O. europeae*. The duration of 33 recordings, including 41 RNs, was sufficient to complete at least one GC-SCR recording with one of the solutions of plant volatiles to locate components in the chromatogram that stimulated the RN.

The figures 6.6 to 6.10 illustrate the GC-SCR recordings obtained. Figure 6.6 shows a recording from a RN that responded selectively to the unidentified compound, 11, exclusively detected in the non-host *O. europeae*. This neuron did not respond to "puff "stimulation with the volatiles of *E. globulus* and *P. pinaster*. Figure 6.7 shows another GC-SCR recording where two spike amplitudes were clearly distinguished, indicating two RNs in the same sensillum. The neuron with large spikes responded to 3-methyl-1-butanol and to 2,3-epoxy-4,4-dimethylpentane, which were also detected in *O. europeae* (figure 6.8). The neuron with small spikes responded selectively to the unidentified compound 26 which eluted later in the chromatogram of *E. globulus* volatiles and was detected only in this plant species. The response of this neuron showed a slow decay, outlasting the GC-peak. This long lasting response applies to other RNs that responded to other compounds eluting in this part of the chromatograms of *E. globulus* (i.e. guaine and compounds 29, 30 and 32) and *P. pinaster* (i.e. α-cubebene and compound 31) volatiles. This region of the gas chromatogram corresponds in general to the area of sesquiterpenes. Figures 6.9 and 6.10 illustrate two GC-SCR recordings from two RNs tested with the volatiles of *E. globulus* and *P. pinaster*, respectively. Also in this recording two spike amplitudes were observed. Both neurons responded to the solvent peak of hexane. The neuron with large spikes (neuron A) responded to α-pinene, β-pinene and 1,8-cineole, and the response strengths correlated with the concentration of these three monoterpenes in *E. globulus* and *P.*

pinaster. The neuron with smaller spikes (neuron B) responded to the unidentified compounds 20 and 21 which were only detected in *E. globulus*.

Most of the 41 RNs tested for the volatile blends of host and non-host plants via the GC responded selectively to one or two compounds (78% of the RNs) whereas a smaller number responded to three or four. On the basis of the response spectra, the 41 RNs could be grouped in 23 different RN types. In table 6.1, the RN types were grouped according to their selective responses to non-terpenoids, monoterpenes, and sesquiterpenes identified in the gas chromatograms of the volatile blends from host and non-host plant species. RN types that responded to only chemically unidentified components in the volatile blends are grouped as "Chemical Group Unknown". For neurons responding to more than one compound, it is indicated which compound elicited the strongest response (best response) and which elicited less strong or weak responses (other responses).

The multiporous sensilla basiconica are the most abundant olfactory sensillum type in *P. semipunctata* antennae, and they are present at higher density in the anterior regions of the three most apical flagelar segments (Lopes, 1990). Thus, it is likely that the 41 RNs were associated with 33 sensilla basiconica distributed along the anterior regions of the 7^{th}, 8^{th} and 9^{th} flagellar segments, as indicated in table 6.2. Most recordings were made from sensilla basiconica on the 8^{th} segment. In 12 out of 33 recordings, two spike amplitudes could be distinguished suggesting two types of RNs associated with the same sensillum. Among the sensilla with two neurons, pairs of RN types were found between types 1 and 5, types 2 and 20, types 3 and 14, types 9 and 16, types 10 and 15, types 12 and 21, types 13 and 23. Due to the low number of recordings, it is not possible to ascertain the existence of consistent neuron-type pairs defining sensillum types, nor any pattern of inter- or intrasegmental spatial segregation of RN types in the antennae of *P. semipunctata* beetles.

The responses of each RN are shown in figure 6.11 where the response strengths to the plant volatile components are shown as histograms, expressing the response spectra of the neurons.

RN Type 1. One RN responded exclusively to 3-methyl-1-butanol when tested via the GC with the volatiles of *E. globulus* and of the non-host *O. europeae*. This neuron did not respond to the "puff"-stimulus of *P. pinaster*, and was not tested for this solution via the GC. This RN was also tested with an authentic sample of 3-methyl-1-butanol at different concentrations, resulting in the dose-response curve shown in figure 6.12.

RN Type 2. Two neurons (A and B) responded to 3-methyl-1-butanol and to 2,3-epoxy-4,4-dimethylpentane present in *E. globulus* and *O. europeae*. The RN A is the same shown in

figures 6.7 and 6.8 with large spike amplitudes. Considering the different size of the two GC-peaks and the magnitude of the electrophysiological responses, the RN A seems to be more sensitive to 2,3-epoxy-4,4-dimethylpentane than to 3-methyl-1-butanol. The dose-response curve obtained for 3-methyl-1-butanol indicates a higher sensitivity of this RN than RN type 1 (figure 6.12). Like RN type 1, the two RNs of type 2 did not respond to the "puff"-stimulus with *P. pinaster* volatiles.

RN Type 3. One neuron responded to 3-hydroxy-2-butanone, 2,3-epoxy-4,4-dimethylpentane, and to the unidentified compounds 4 and 6. Although compound 4 corresponds to a very small GC-peak in the chromatograms of *E. globulus* and *O. europeae* volatile blends, it elicited the strongest responses to that compound. This RN did not respond to the "puff"-stimulus of *P. pinaster* volatiles.

RN Type 4. Two RNs (A and B) responded to the unidentified compound 5 and to (Z)-3-hexen-1-ol. Although present at smaller amount than (Z)-3-hexen-1-ol in both *E. globulus* and *O. europeae*, compound 5 elicited a stronger response. The dose-response curve for (Z)-3-hexen-1-ol and 1-hexanol shows similar sensitivity of RN A to both compounds (figure 6.12). The two RNs, A and B, did not respond to the "puff"-stimulus of *P. pinaster* volatiles.

RN Type 5. One RN which responded to the "puff-stimulus" of the three plant species was tested via the GC for *E. globulus* volatile blend. This RN responded to only one GC-peak, which corresponds to the co-elution of ethyl-3-methylbutanoate and (Z)-3-hexen-1-ol.

RN Type 6. One RN responded to (Z)-3-hexen-1-ol present in *O. europeae*. This compound is also present in *E. globulus* and *P. pinaster*, but at lower concentrations which may explain why the neuron did not respond to the "puff"-stimulus of the volatiles from these two species. The dose-response curve to (Z)-3-hexen-1-ol in figure 6.12 shows that this neuron is less sensitive to (Z)-3-hexen-1-ol than neuron A of type 4.

RN Type 7. One RN responded to (Z)-3-hexen-1-ol and (Z)-2-hexen-1-ol present in *O. europeae*. This RN also responded to the "puff"-stimulus of *E. globulus* and *P. pinaster*, but due to the loss of bioelectrical contact it was not possible to perform GC-SCR recordings with the volatile blends of these species.

RN Type 8. Two RNs (A and B) responded exclusively to α-pinene when tested for *E. globulus* volatiles, neuron A with a stronger response than neuron B. Both neurons responded strongly to "puff"-stimulus of *P. pinaster* and weakly to the one of *O. europeae*. However, due to loss of bioelectrical contact, the neurons were not further tested via the GC with the blends of these two species.

RN Type 9. Three RNs (A, B and C) responded to α-pinene, β-pinene and 1,8-cineole in *E. globulus* and *P. pinaster*. The elution of 1,8-cineole and limonene overlapped extensively in the chromatograms of both species due to high concentration of these two compounds (figures 6.2 and 6.3). It was assumed that these RNs responded to 1,8-cineole and not to limonene since a weaker response was recorded when testing the volatile blend of *P. pinaster*, where 1,8-cineole is present at lower concentration and limonene at higher concentration than in *E. globulus* (see chapter 5). Considering the RN response strengths and the concentration of the stimulatory compounds, these RNs exhibited a highest sensitivity to α-pinene followed by β-pinene and 1,8-cineole. When stimulated with the "puff"-stimulus of O. europeae blend of volatiles, neurons A and B showed weak responses and neuron C did not respond. The neurons were not tested for these volatiles via the GC due to loss of bioelectrical contact.

RN Type 10. Two RNs (A and B) responded to 1,8-cineole and/or to limonene present in *E. globulus*. Both RNs responded to the "puff"-stimulation with *P. pinaster* volatiles, but were not tested for these volatiles via the GC. The "puff"-stimulation with *O. europeae* volatiles did not elicit any response. Since the GC peaks of 1,8-cineole and limonene overlapped extensively, it is not possible to know whether the neurons responded to both compounds or to only one of them.

RN Type 11. One RN responded selectively to the unidentified compound 11 exclusively detected in *O. europeae*. The "puff"-stimulation with *E. globulus* and *P. pinaster* volatiles did not elicit any response. The GC-SCR recordings carried out with the solutions of volatiles of these two plant species confirmed the absence of stimulatory compounds.

RN Type 12. Three RNs (A, B and C) responded to myrcene and limonene present in both *E. globulus* and *P. pinaster*. The "puff"-stimulation with *O. europeae* volatiles did not elicit responses in these RNs. Dose-response curves determined for neuron C (figure 6.12) showed no response to 1,8-cineole. It indicates that the responses of these neurons were elicited by limonene and not by 1,8-cineole in the volatile blends of the two plant species. Furthermore, the dose-response curves show that neuron C has higher sensitivity to myrcene and α-terpinene than to limonene. As shown in the previous chapter, α-terpinene is present in both *E. globulus* and *P. pinaster* volatile profiles, but at low concentrations (8 and 11 ppm, respectively). The low concentrations may be the reason why these neurons did not respond to α-terpinene when tested via the GC with the plant volatile blends.

RN Type 13. Three RNs (A, B and C) responded to α-pinene, myrcene, limonene, and trans-β-ocimene when stimulated with the volatile blends of *E. globulus* and/or *P. pinaster*.

Considering the concentration of the four compounds in the two species and the corresponding response strengths of the neurons, it is suggested that they have higher sensitivity to myrcene and trans-β-ocimene than to the other two compounds. Although 1,8-cineole and limonene overlapped extensively in the chromatograms of the two volatile blends, it is assumed that these neurons responded to limonene and not to 1,8-cineole. This is supported by the fact that neuron A had a lower response strength to the GC-peak of the two compounds in the chromatogram of *E. globulus* than to the corresponding GC-peak in the chromatogram of *P. pinaster*, and this relates with differences between the two volatile blends in the concentration of the two compounds. The concentration of limonene is higher in *P. pinaster* than in *E. globulus*, whereas the concentration of 1,8-cineole is higher in *E. globulus* than in *P. pinaster*.

RN Type 14. One RN responded to myrcene present in both *E. globulus* and *P. pinaster* and to the unidentified compound 19 only present in *E. globulus*. The response strengths to myrcene were related with its higher concentration in *P. pinaster* than in *E. globulus*. However, since compound 19 is a smaller GC-peak in the chromatogram of *E. globulus* than the GC-peak of myrcene in the chromatograms of *E. globulus* and *P. pinaster* volatiles, and elicited a stronger response than did myrcene, this neuron responds better to compound 19 than to myrcene. The "puff"-stimulation with *O. europeae* volatiles did not elicit responses.

RN Type 15. Three RNs (A, B and C) responded only to linalool in the volatile blends of *E. globulus* and *P. pinaster*. The response strengths of neurons A and B is correlated with the concentration of linalool in both species. Neuron C was not tested for the volatiles of *P. pinaster* via the GC. The "puff"-stimulation with *O. europeae* volatiles did not elicit any responses in these three RNs.

RN Type 16. One RN responded to the unidentified compounds 20, 21 and 23 present only in *E. globulus*. The "puff"-stimulation with the volatiles of *P. pinaster* and *O. europeae* did not elicit responses.

RN Type 17. One RN responded to the unidentified compounds 18 and 22 present only in *E. globulus*. Compound 22, a smaller GC-peak than compound 18, elicited the strongest response. The "puff"-stimulation with the volatiles of *P. pinaster* and *O. europeae* did not elicit responses.

RN Type 18. Three RNs (A, B and C) responded to linalool present in both *E. globulus* and *P. pinaster* volatile blends and to the unidentified compound 24 present only in *E. globulus*. Compound 24, a smaller GC-peak than linalool, elicited stronger responses in these neurons. The "puff"-stimulation with the volatiles of *P. pinaster* and *O. europeae* did not elicit responses.

RN Type 19. Three RNs (A, B and C) were selective to the unidentified compound 24 present only in *E. globulus*. The "puff"-stimulation with the volatiles of *P. pinaster* and *O. europeae* did not elicit responses.

RN Type 20. One RN responded selectively to the unidentified compound 26 present at very low concentration only in *E. globulus*. The "puff"-stimulation with the volatiles of *P. pinaster* and *O. europeae* did not elicit responses.

RN Type 21. Two RNs (A and B) responded selectively to α-cubebene present only in *P. pinaster*. The "puff"-stimulation with the volatiles of *P. pinaster* and *O. europeae* did not elicit responses.

RN Type 22. One RN responded to guaine and to the unidentified compounds 29, 30 and 32 in the blend of *E. globulus*. Compound 29, a GC-peak of smaller size than those of guaine and compound 30, elicited the strongest response. Compound 32 was hardly detected by the GC-detector, but elicited responses as strong as those elicited by guaine and compound 30. The "puff"-stimulation with the volatiles of *P. pinaster* elicited only a weak response, but it was not possible to obtain a GC-SCR recording. This neuron did not respond to "puff"-stimulus of *O. europeae* volatiles.

RN Type 23. Two RNs (A and B) responded to the unidentified compounds 25 and 31 present only in *P. pinaster*. The "puff"-stimulation with the volatiles of *E. globulus* and *O. europeae* did not elicit responses in this RN.

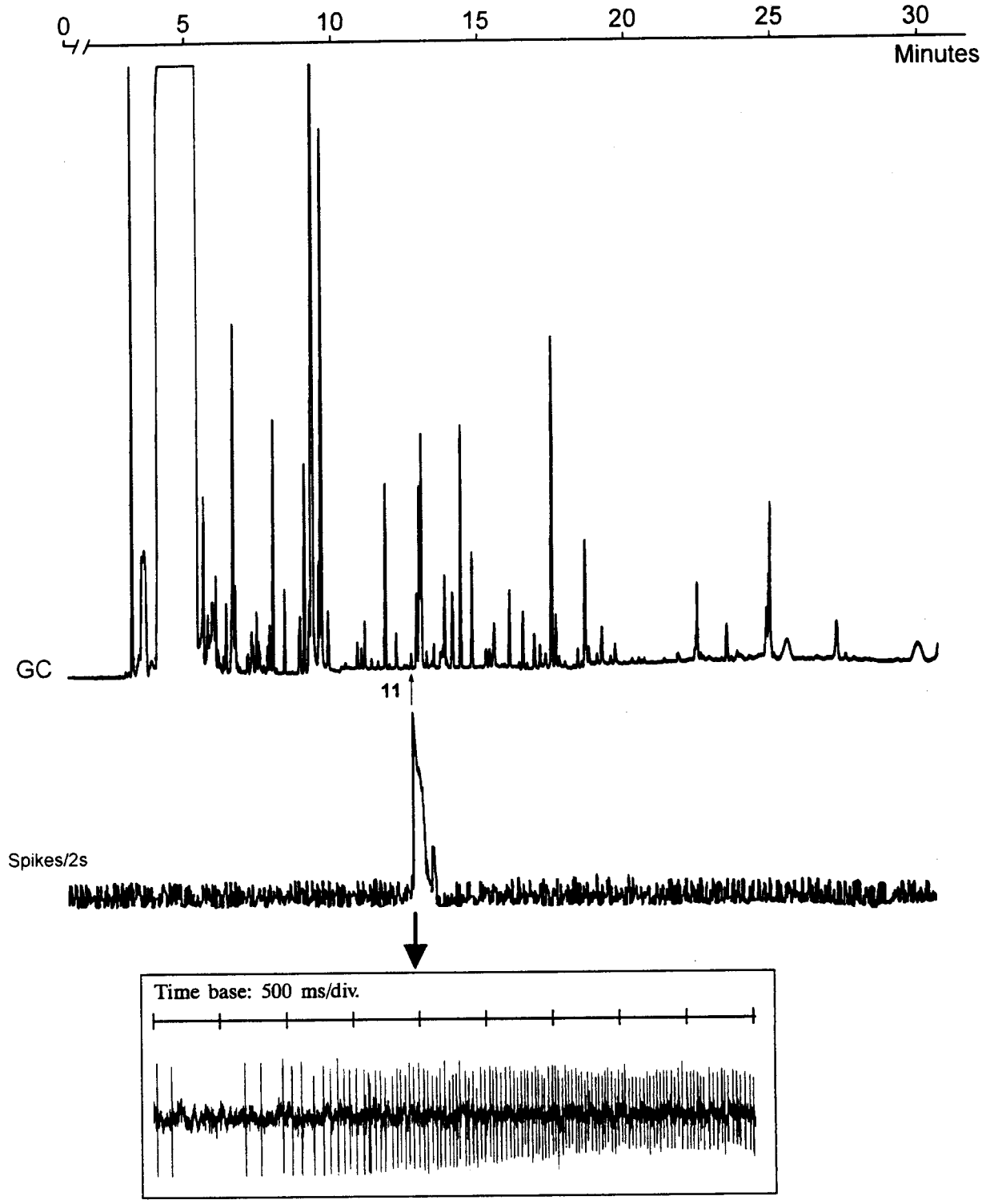


Figure 6.6. Gas chromatogram of the volatiles of *Olea europeae* and simultaneously recorded responses of one single receptor neuron associated with an olfactory sensillum of *Phoracantha semipunctata*. This receptor neuron responded selectively to the unidentified compound 11.

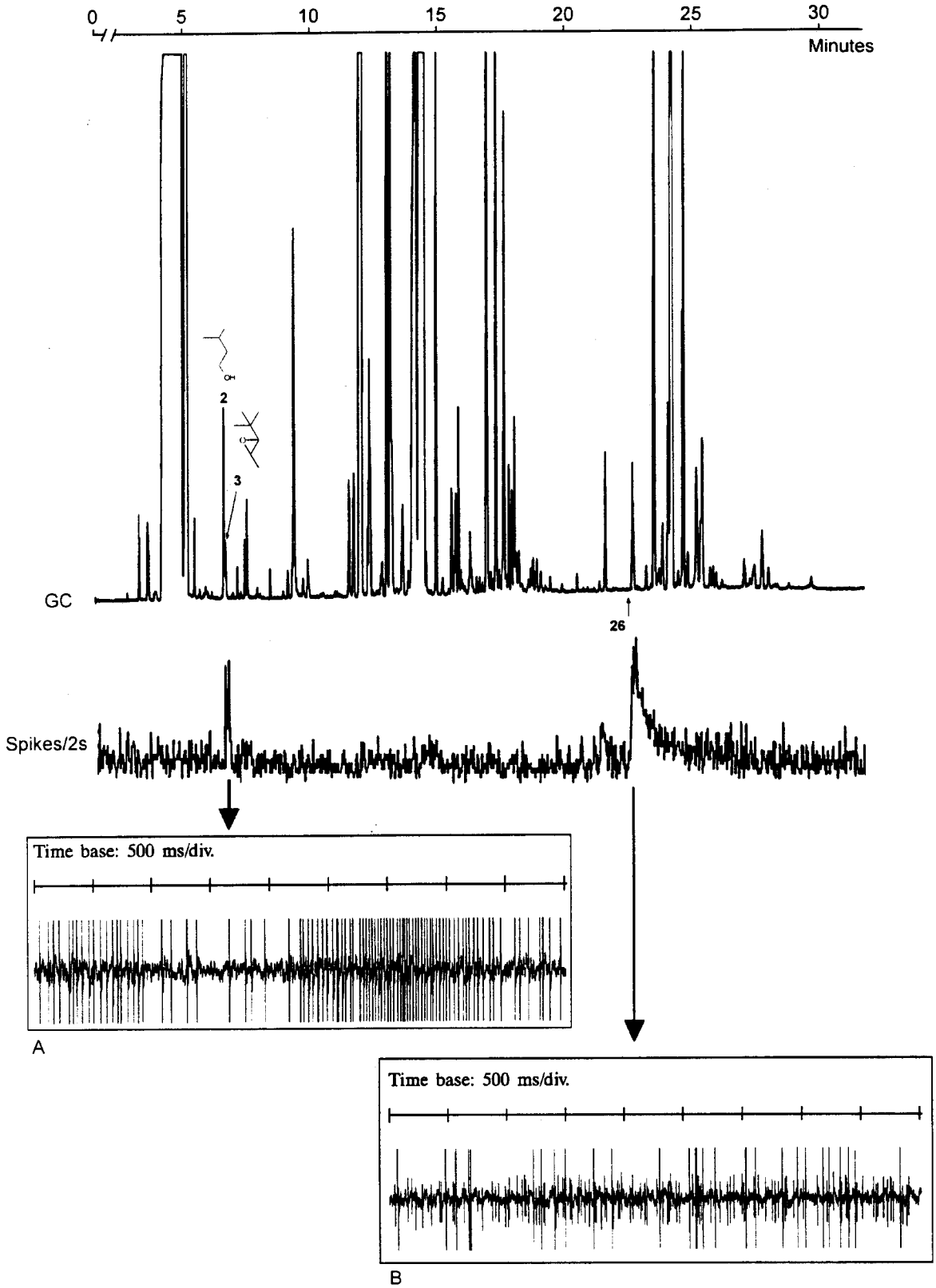


Figure 6.7. Gas chromatogram of the volatiles of *Eucalyptus globulus* and simultaneously recorded responses of two receptor neurons (A and B) in the same olfactory sensillum of *Phoracantha semipunctata*. The receptor neuron A (large spike amplitudes) responded to 3-methyl-1-butanol (2) and to 2,3-epoxy-4,4-dimethylpentane (3). The receptor neuron B (small spike amplitudes) responded selectively to the unidentified minor compound 26.

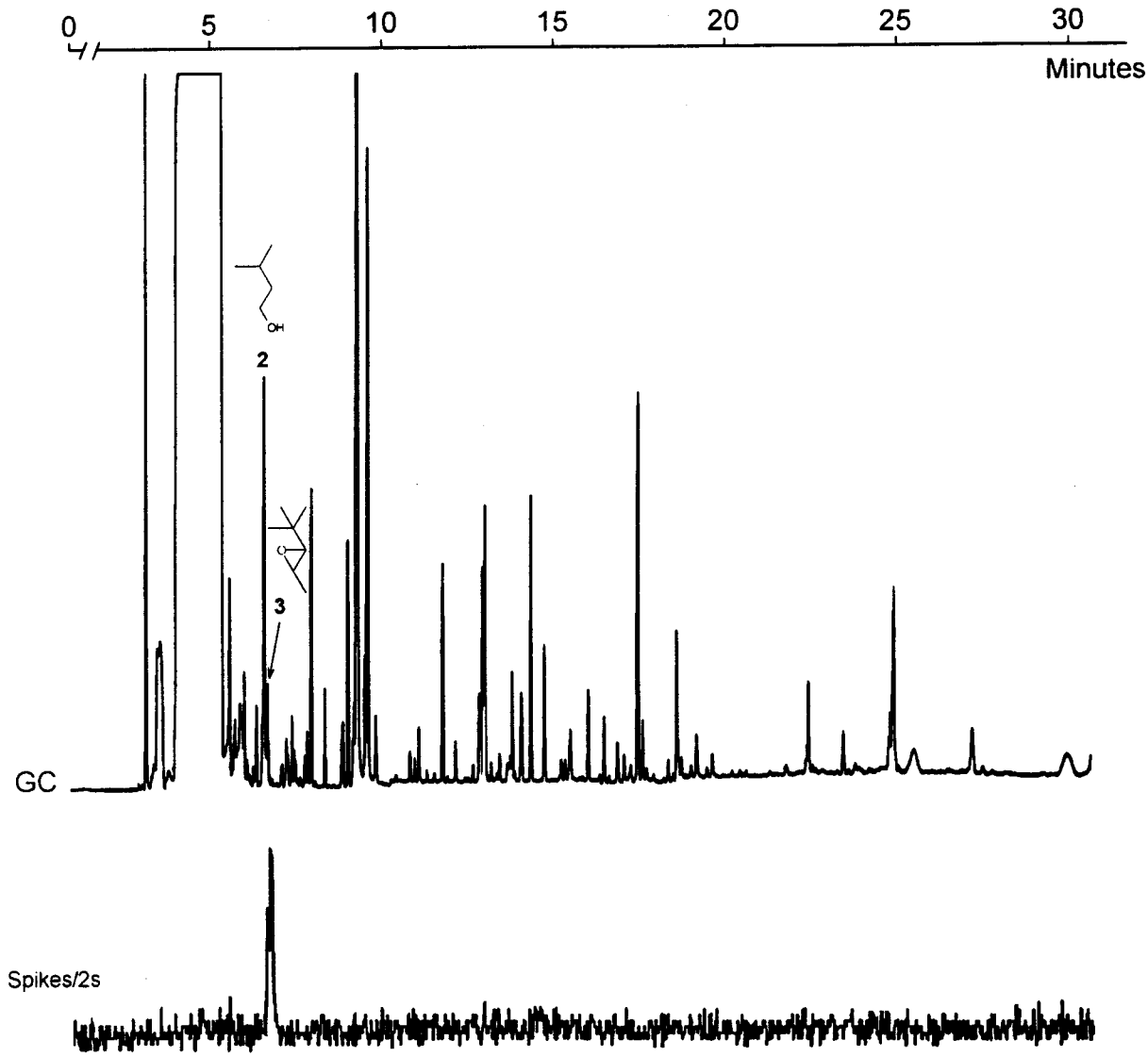


Figure 6.8. Gas chromatogram of the volatiles of *Olea europeae* and simultaneously recorded responses of a receptor neuron associated with an olfactory sensillum of *Phoracantha semipunctata*. This GC-SCR recording was obtained from the two receptor neurons shown in figure 6.7. Here, the neuron A responded to the same two compounds present in *Eucalyptus globulus*, 3-methyl-1-butanol (2) and 2,3-epoxy-4,4-dimethylpentane (3), and neuron B did not respond to any these plant volatiles.

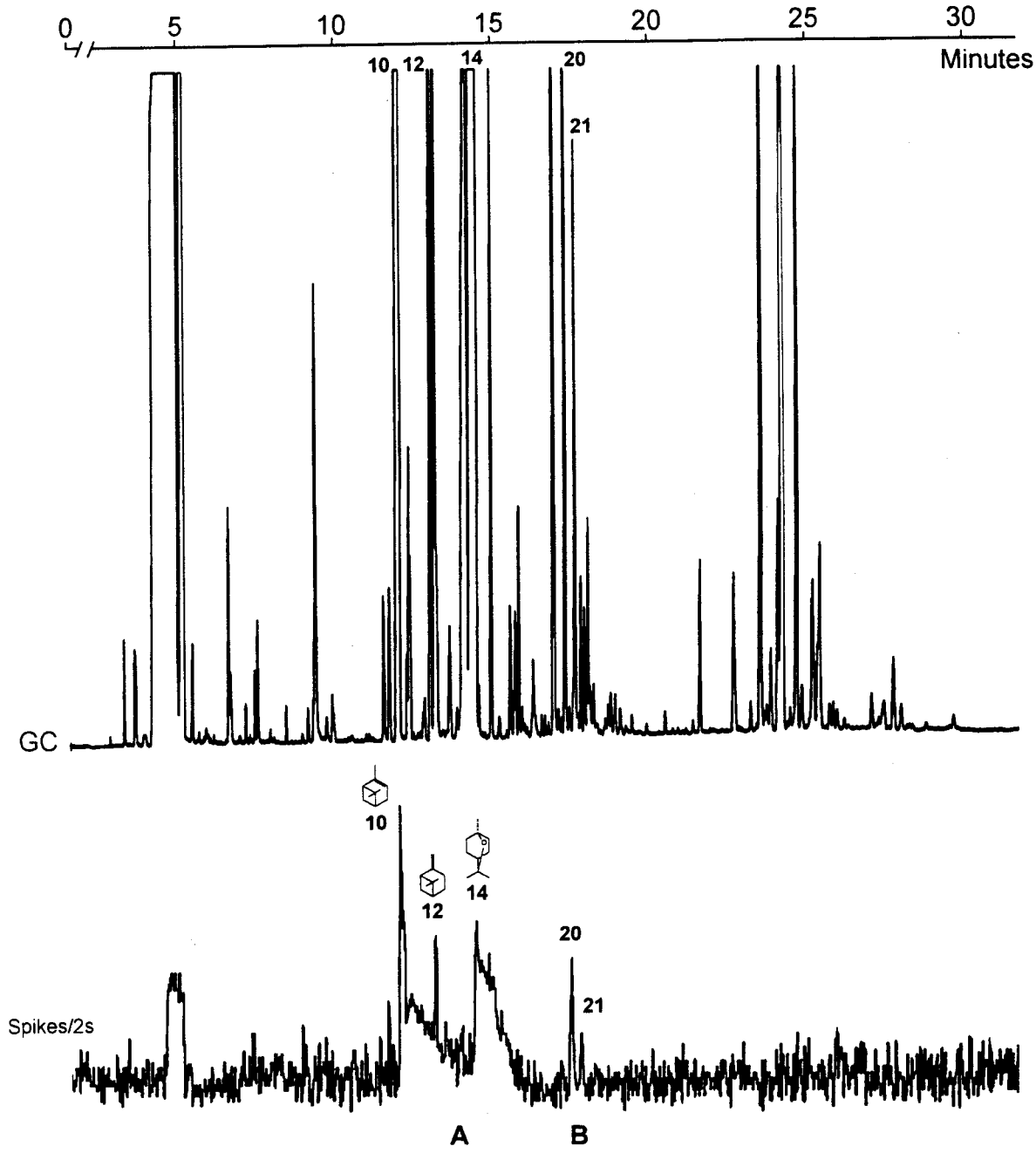


Figure 6.9. Gas chromatogram of the volatiles of *Eucalyptus globulus* and simultaneously recorded responses of two receptor neurons (A and B) in the same olfactory sensillum of *Phoracantha semipunctata*. Both receptor neurons responded to the solvent, hexane. The neuron A responded to α-pinene (10), β-pinene (12) and 1,8-cineole (14). The neuron B responded to the unidentified compounds 20 and 21.

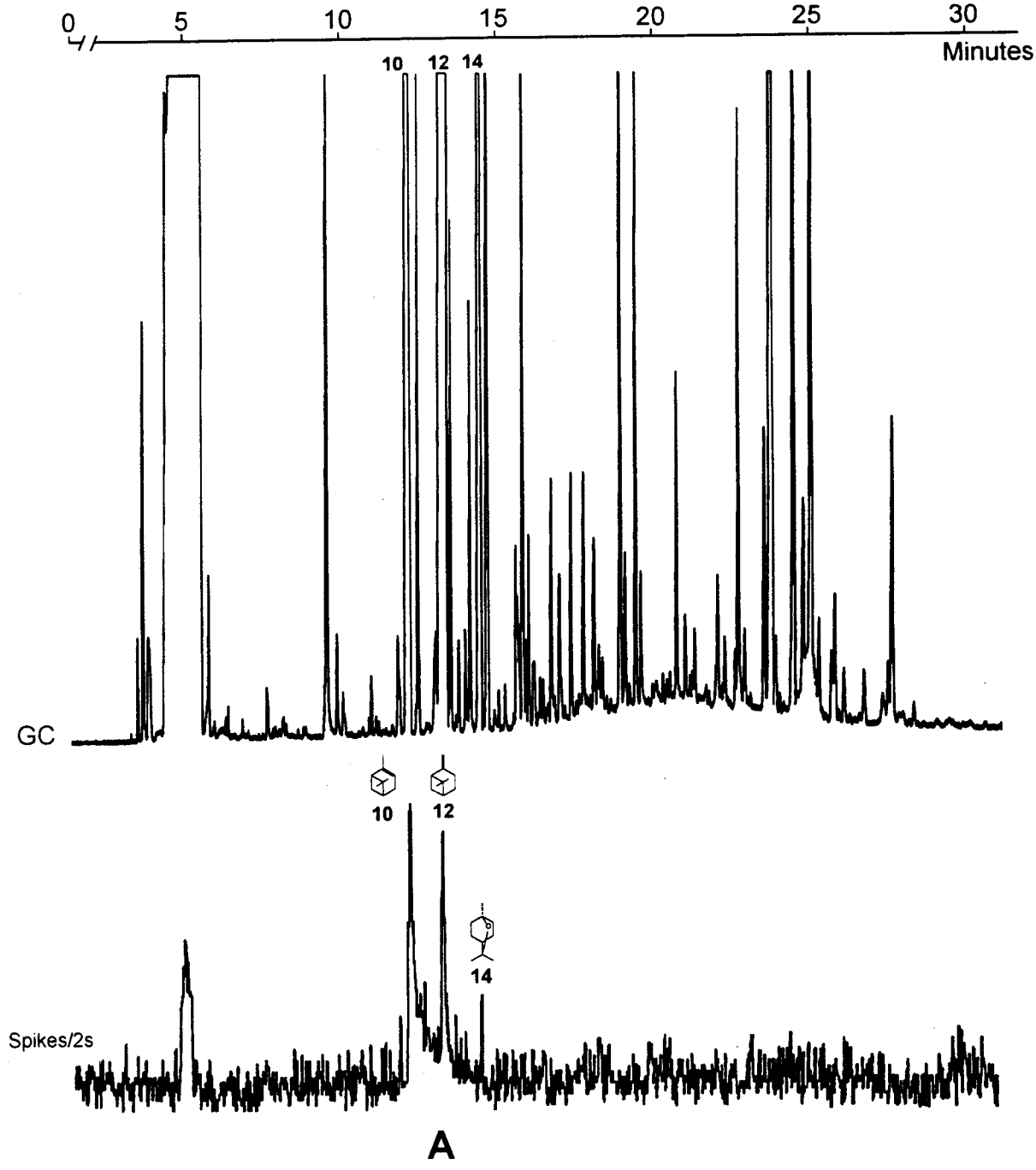


Figure 6.10. Gas chromatogram of the volatiles of *Pinus pinaster* and simultaneously recorded responses of a receptor neurons associated with an olfactory sensillum of *Phoracantha semipunctata*. This GC-SCR recording was obatined from neuron A shown in figure 6.9. Here, neuron A responded to the same three compounds present in *Eucalyptus globulus*, α-pinene (10), β-pinene (12), and 1,8-cineole (14). Neuron B did not respond to any of these plant volatiles.

Table 6.1. Groups of receptor neuron types (RN type) in *Phoracantha semipunctata* antennae that showed selective responses to non-terpenoids, monoterpenes, and sesquiterpenes identified in the gas chromatograms of volatile blends of host (*Eucalyptus globulus*) and non-host (*Pinus pinaster* and *Olea europeae*) plant species. For neurons responding to more than one compound, it is indicated which compounds elicited stronger responses (best response).

Chemical Group	RN Type	No. of neurons	Stimulatory Compounds[1]	
			Best Response	Other responses
Non-terpenoids				
	1	1	(2) 3-methyl-1-butanol	
	2	2	(3) 2,3-epoxy-4,4-dimethylpentane	(2) 3-methyl-1-butanol
	3	1	(4) unidentified	(1) 3-hydroxy-2-butanone (3) 2,3-epoxy-4,4-dimethylpentane (6) unidentified
	4	2	(5) unidentified	(8) (Z)-3-hexen-1-ol 1-hexenol [a]
	5	1	(7) ethyl-3-methylbutanoate or (8) (Z)-3-hexen-1-ol	
	6	1	(8) (Z)-3-hexen-1-ol	
	7	1	(9) (Z)-2-hexen-1-ol	(8) (Z)-3-hexen-1-ol
Monoterpenes				
	8	2	(10) α-pinene	
	9	3	(10) α-pinene	(12) β-pinene (14) 1,8-cineole
	10	2	(14) 1,8-cineole or (15) limonene	
	13	3	(13) myrcene (16) trans-β-ocimene	(10) α-pinene (15) limonene
	15	3	(17) linalool	
	12	3	(13) myrcene α-terpinene [a]	(15) limonene
	14	1	(19) unidentified	(13) myrcene
	18	3	(24) unidentified	(17) linalool
Sesquiterpenes				
	21	2	(27) α-cubebene	
	22	1	(29) unidentified (32) unidentified	(28) guaine (30) unidentified
Unknown				
	11	1	(11) unidentified	
	16	1	(20) unidentified (23) unidentified	(21) unidentified
	17	1	(22) unidentified	(18) unidentified
	19	3	(24) unidentified	
	20	1	(26) unidentified	
	23	2	(25) unidentified (31) unidentified	

[1] Numbers in parentheses indicate the GC-peaks in the gas chromatograms of figures 6.2 - 6.4.

[a] 1-Hexenol and α-terpinene are synthetic compounds that elicited responses when the neurons were subjected to "puff"-stimulation for obtention of dose-response curves (figure 6.12, RN type 4A and RN type 12C), they were not identified as stimulatory compounds when the neurons were tested with the volatile blends via GC.

Table 6.2. Olfactory sensilla location on the surface of the antennal flagellum of *Phoracantha semipunctata* females and associated RN types.

Sensilla #	Segment	Location	RN Type[1]
1	7	medial	8
2	7	medial	22
3	8	medial	1, 5
4	8	medial	2
5	8	medial	2, 20
6	8	medial	3, 14
7	8	medial	4
8	8	medial	7
9	8	medial	9
10	8	medial	9
11	8	medial	9, 16
12	8	medial	10
13	8	medial	10,15
14	8	medial	12
15	8	medial	12
16	8	medial	12, 21
17	8	medial	13, 23
18	8	medial	13, 23
19	8	medial	15
20	8	medial	17, ?
21	8	medial	18
22	8	medial	21, ?
23	8	distal	4
24	8	distal	6
25	8	distal	8
26	8	distal	11, ?
27	8	distal	15
28	8	distal	18
29	8	distal	18
30	8	distal	19
31	8	distal	19
32	8	distal	19
33	9	proximal	13, ?

[1] ? - means that there was a second RN, but the it was not possible to define its type due to the loss of bioelectrical contact before occurrence of any response.

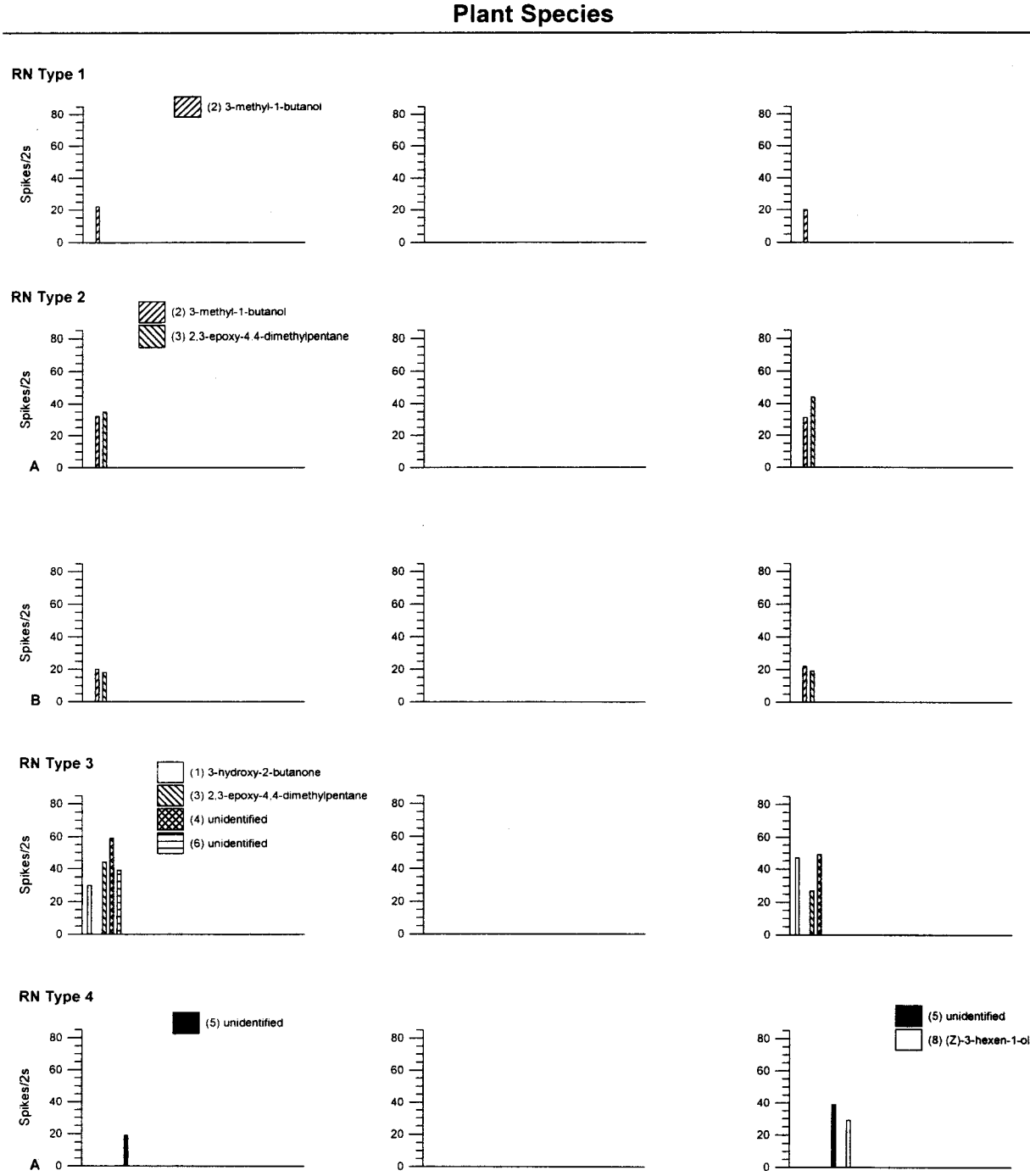


Figure 6.11. Olfactory receptor neuron (RN) types in *Phoracantha semipunctata* antennae. The histograms in the same row represent the response magnitude of a single neuron to volatiles of *Eucalyptus globulus* (host), *Pinus pinaster* and *Olea europeae* (non-hosts) during GC-SCR recordings. The position of the bars along the horizontal axis of each histogram reflects the retention time of the compound, and the numbers in parenthesis correspond to the GC-peaks in the gas chromatograms (figures 6.2 to 6.4). Absence of histogram in a row means that the neuron responded to "puff"-stimulation with the volatile blend of a plant species but no GC-SCR recordings were made.

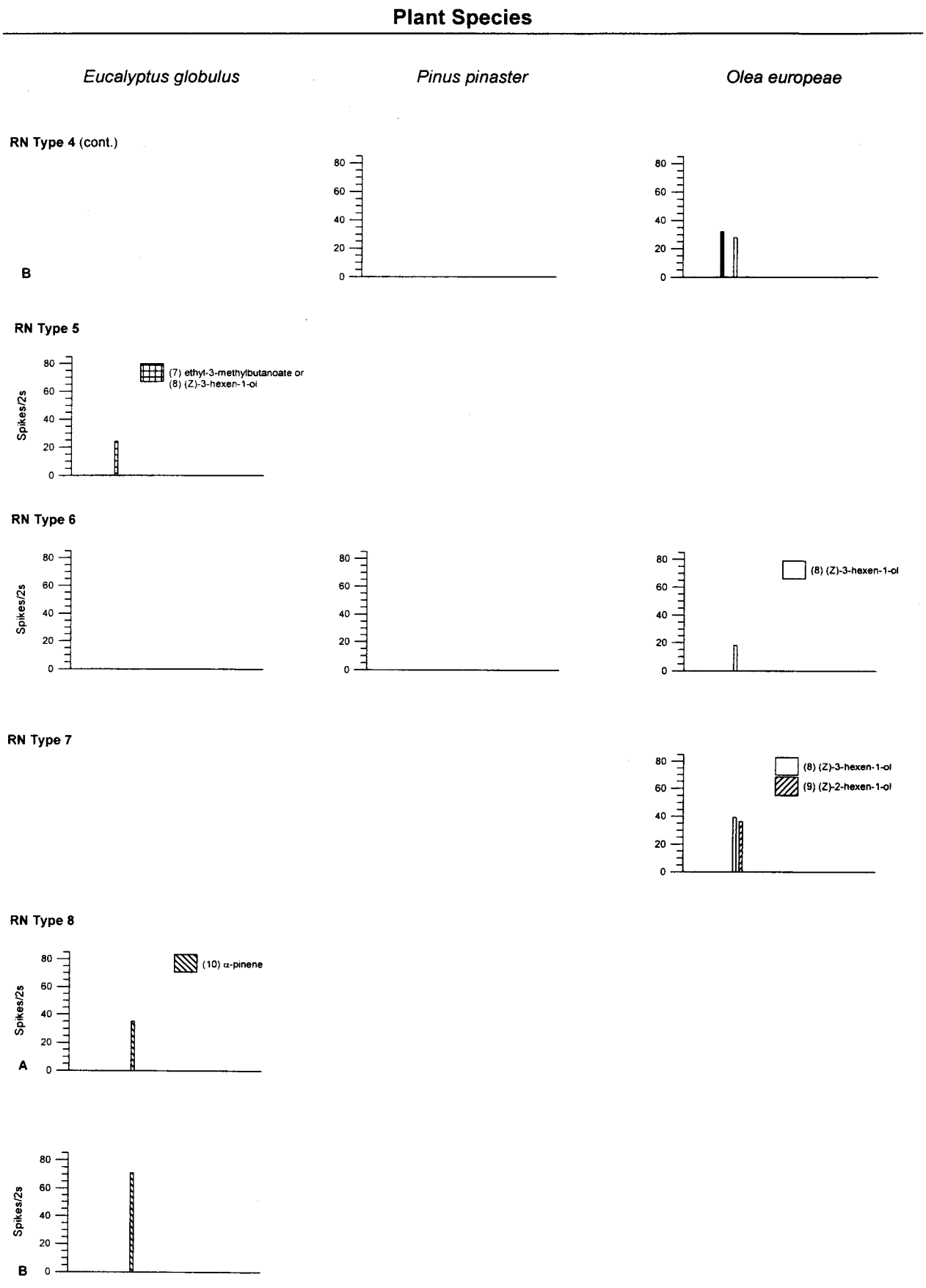
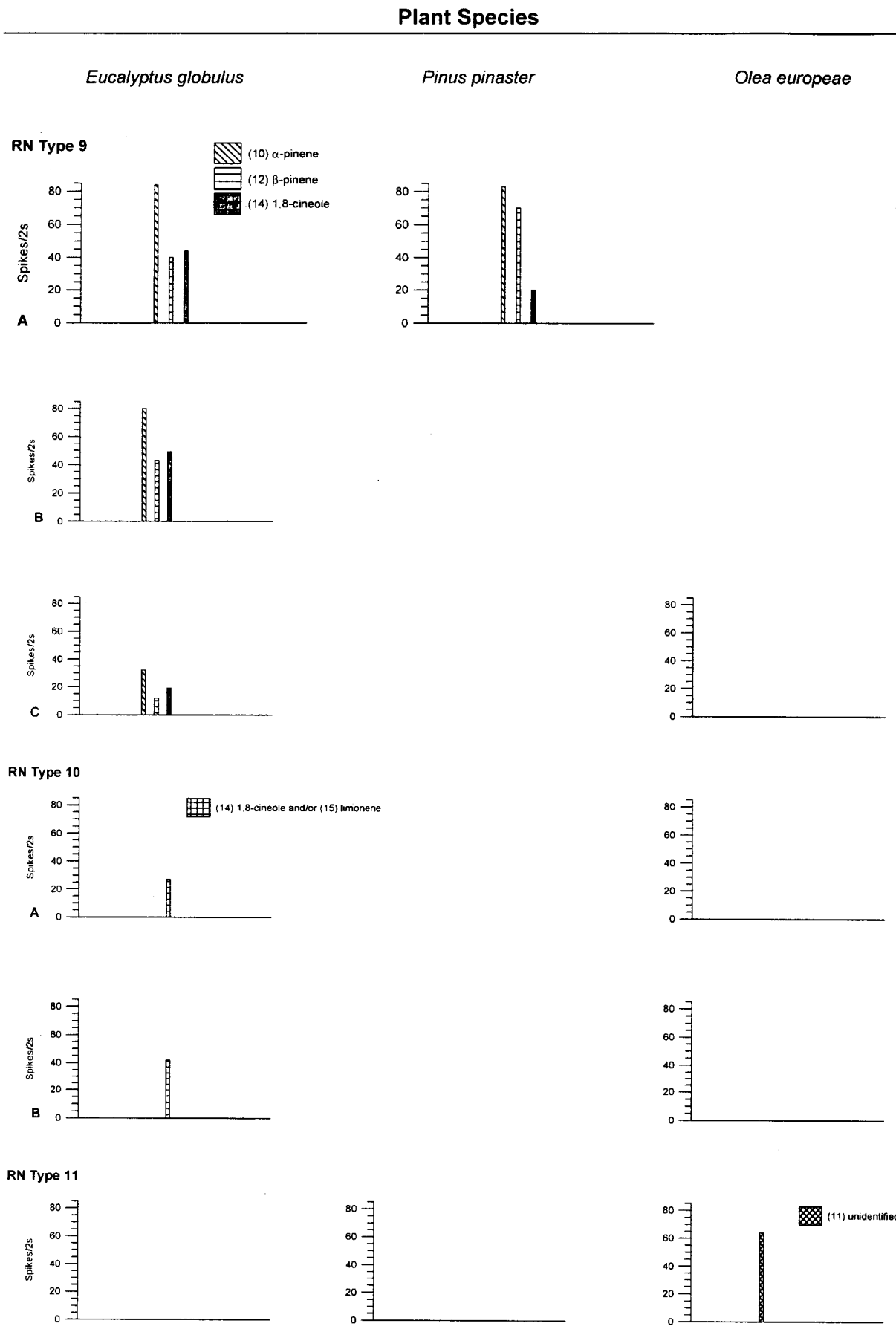


Figure 6.11. Continued



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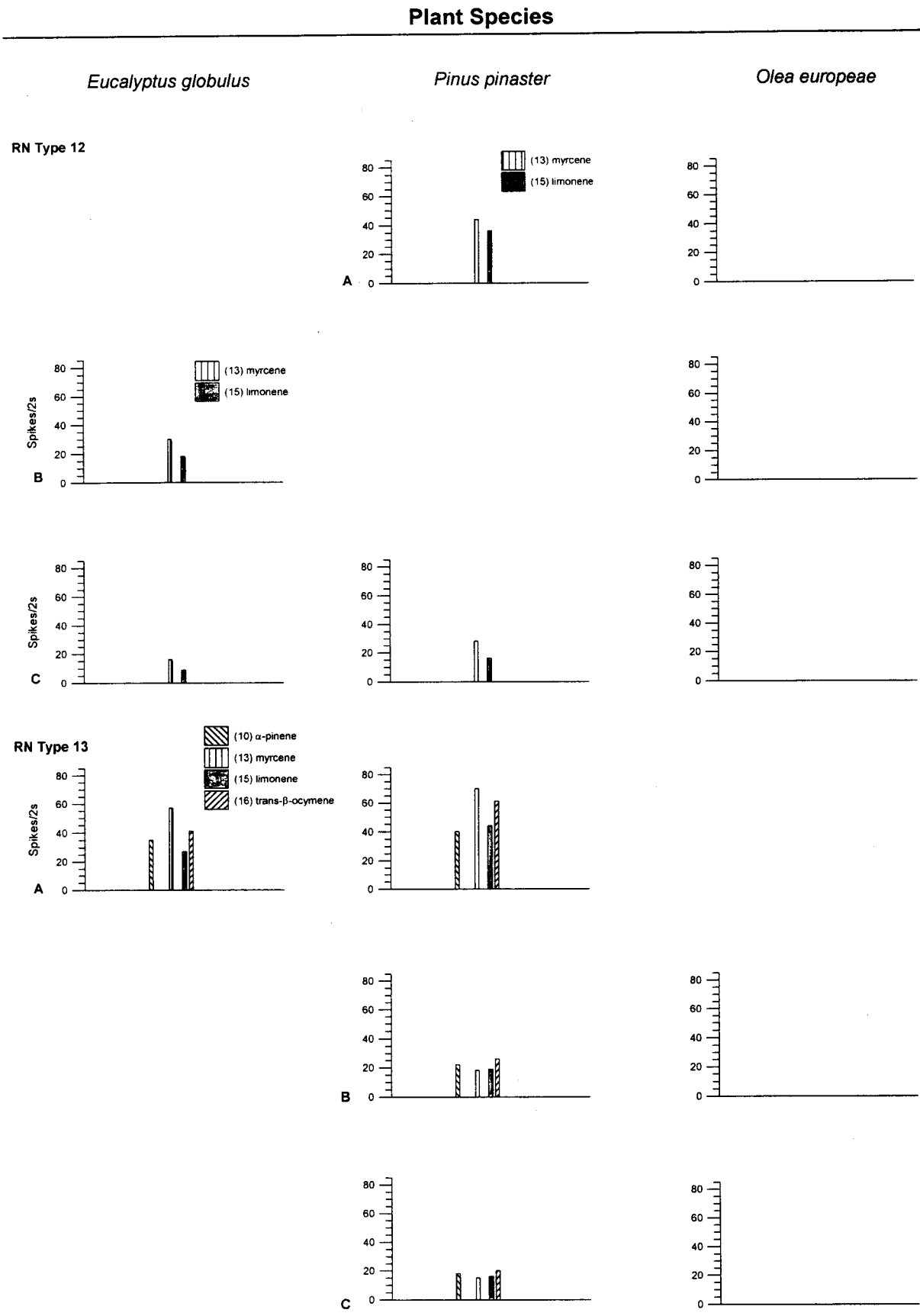

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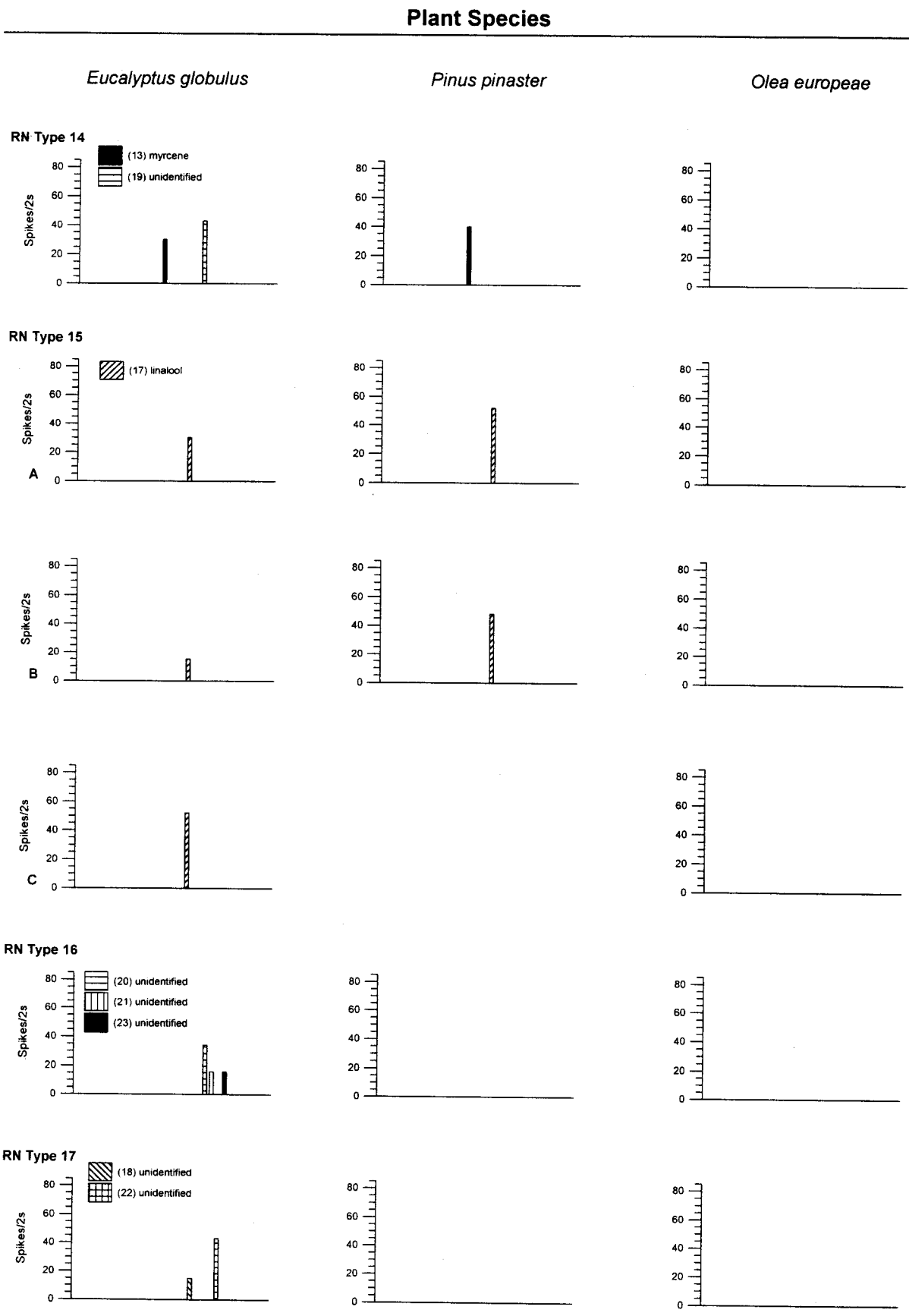
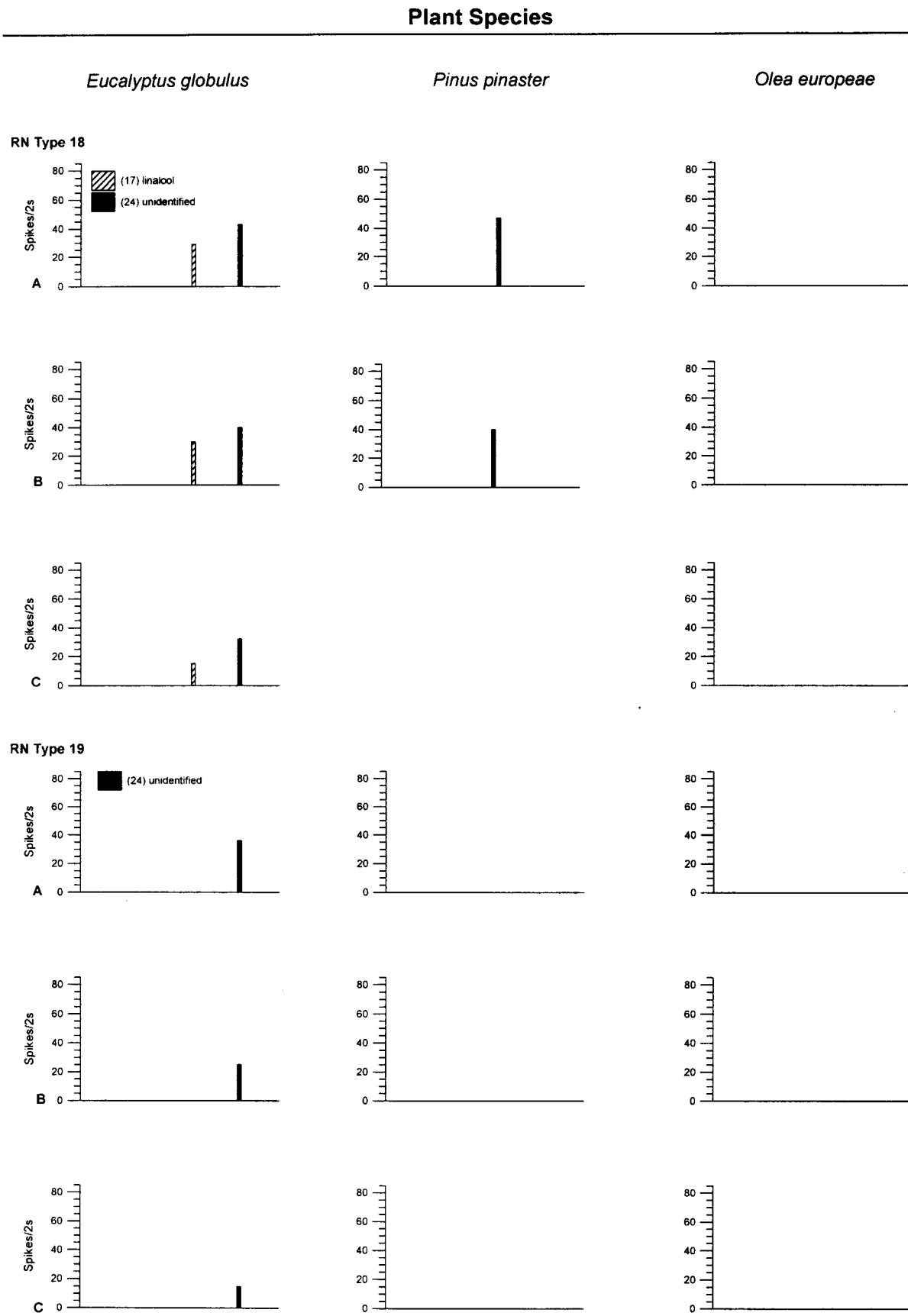


Figure 6.11. Continued



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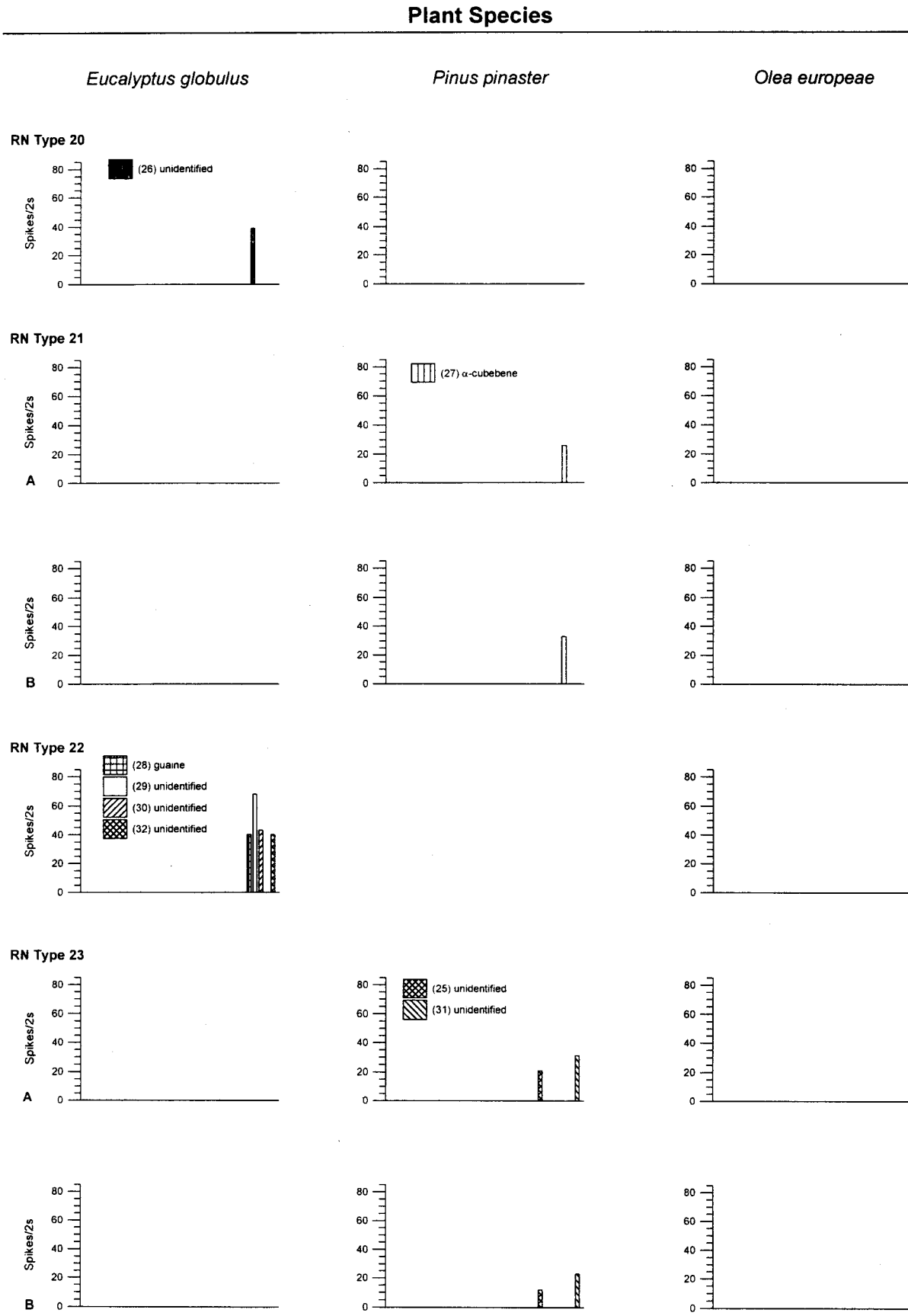

Figure 6.11. Continued

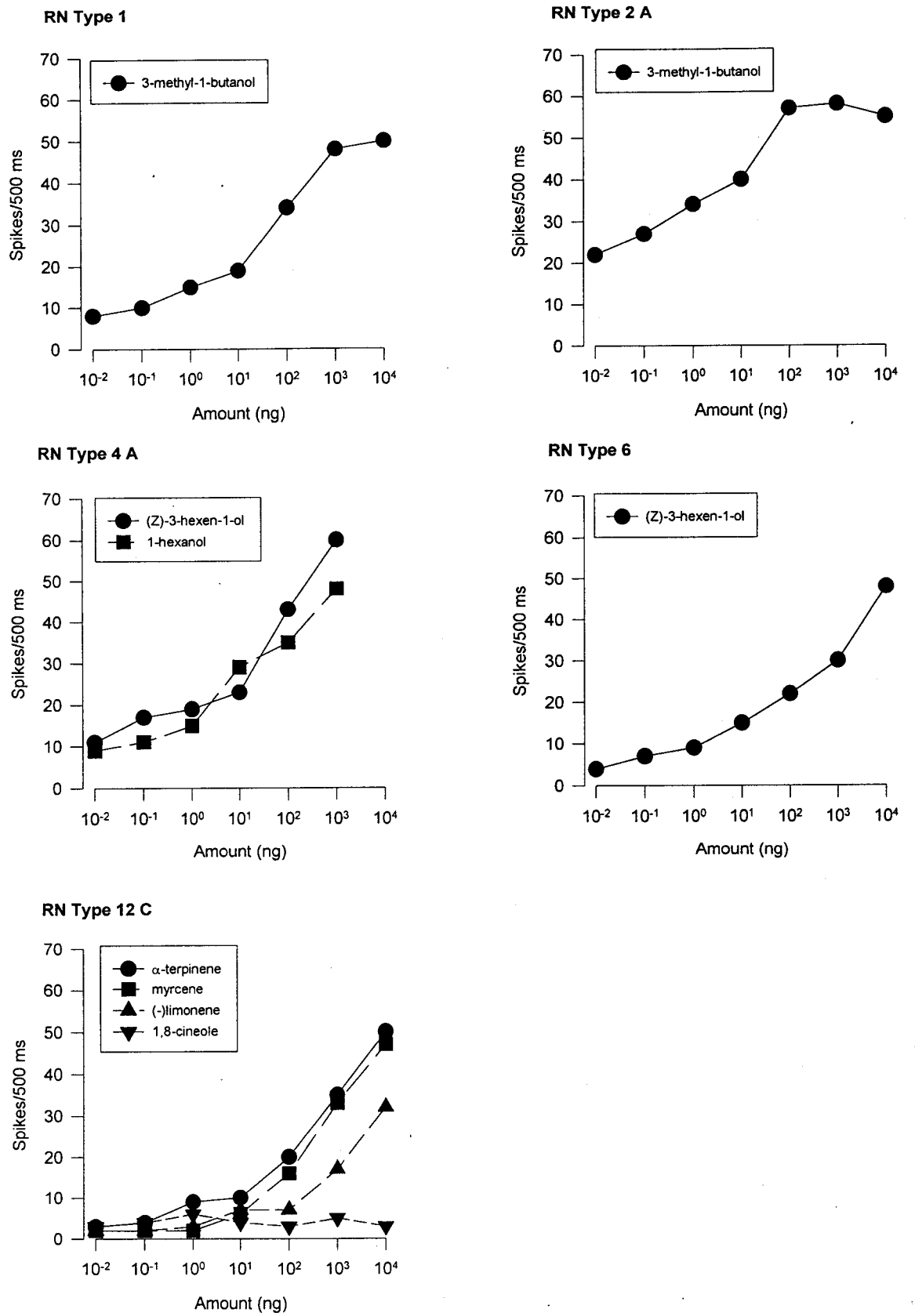

Figure 6.12. Dose-response curves obtained from five receptor neurons of different type in female *Phoracantha semipunctata* stimulated with synthetic plant compounds.

6.4. Discussion

The results obtained by stimulation of *P. semipunctata* RNs with host and non-host plant species volatile blends after separation in the GC-column were highly reproducible. This is demonstrated by GC-SCR recordings where the same neuron responded selectively to the same compounds present in different plant materials (e.g. figure 6.11, RNs type 4 and 9). Furthermore, the response strengths to a compound of different materials could be correlated with the size of the GC-peak in the chromatograms (figures 6.2 to 6.5).

Since GC-peaks 6, 21, 22, 23, 24, 26, 32 in the chromatogram of *E. globulus* (figure 6.2) and GC-peaks 25 and 31 in the chromatogram of *P. pinaster* (figure 6.3) did not elicit EAG responses during GC-EAD recordings made previously with the volatile blends of these species (chapter 5), it is demonstrated that GC-EAD alone is not sufficient for identifying all the components in a blend that are detected by the RNs of *P. semipunctata* beetles. The importance of using both the GC-EAD and the GC-SCR techniques was previously demonstrated in a study on the electrophysiological responses of the cabbage seed weevil (*Ceutorhynchus assimilis*) to blends of volatiles from oilseed rape (*Brassica napus*), where specific receptor neurons were found for five compounds that did not elicit significant EAG responses (Blight *et al.*, 1995).

The results show that RNs responded to one or a few compounds in the different plant volatile blends. This suggests that plant odour RNs in *P. semipunctata* are rather specialist types of olfactory neurons than neurons broadly tuned to many compounds. Some neuron types responded to only one compound. This was the case for RN type 1 to 3-methyl-1-butanol, RN type 5 to ethyl-3-methylbutanoate or to (Z)-3-hexen-1-ol, RN type 6 to (Z)-3-hexen-1-ol, RN type 8 to α-pinene, RN type 10 to 1,8-cineole or limonene, RN type 15 to linalool, RN type 21 to α-cubebene, RN type 11 to unidentified compound 11, RN type 19 to unidentified compound 24, and RN type 20 to unidentified compound 26 (table 6.1 and figure 6.11). It suggests that these neurons mediate information to the brain about only one compound, thus acting as "labelled-lines".

Other neurons responded to two, three or four compounds. In these cases the compounds, when chemically identified, were structurally related, so that the RN types could be grouped according to the chemical class of the stimulatory compounds, i.e. non-terpenoids [e.g. 3-methyl-1-butanol, 2,3-epoxy-4,4-dimethylpentane, (Z)-3-hexen-1-ol, (Z)-2-hexen-1-ol], monoterpenes (e.g. α-pinene, β-pinene, 1,8-cineole, limonene, myrcene, trans-β-ocimene, linalool), and

sesquiterpenes (e.g. guaine, α-cubebene) (table 6.1). RN types in different chemical classes did not show overlap of response spectra. Within each chemical class, the RN response spectra showed some overlap, but one or two compounds seemed to have the best stimulatory effect. This is exemplified by RN type 9 which responded best to α-pinene and showed less strong responses to β-pinene and 1,8-cineole; RN type 13 which responded best to myrcene and trans-β-ocimene, and showed weaker responses to α-pinene and limonene; and RN type 12 which responded best to myrcene and α-terpinene, and showed weaker responses to limonene. This suggests that some RN types may detect a narrow set of structurally related compounds, where some chemical structures are more efficient stimuli than others. Each of these neurons may convey information to the brain about different compounds, depending on their ratios in the blend released by a particular plant. For example, RNs type 9 are strongly activated by the volatile blend of *E. globulus*, caused by α-pinene and high concentration of 1,8-cineole (figure 6.9). They are also strongly activated by the blend of *P. pinaster*, but in this case caused by α-pinene and high concentration of β-pinene (figure 6.10). The blend of *O. europeae* elicited only weak or no response in this type of RNs probably because α-pinene is present at a very low concentration as shown in chapter 5. Thus, the information about some plant compounds is conveyed by an "across-fibre" pattern.

Figure 6.11 show some RN types responding to compounds present only in the host plant, many RN types responding to compounds present in both host and non-hosts but in different amounts, and some RN types responding to compounds present only in non-hosts. This suggests a chemical "fingerprint" mechanism (Schneider, 1987) for discrimination between odour blends of different plants, i.e. the relative activities elicited by many compounds in different neuron types mediate the code to the brain about the odour blend quality of a plant species or an individual plant. Information about the presence of some compounds is preserved by specialist neurons acting as "labelled-lines". In addition, there is some integration of odour quality by neurons responding to more than one compound in the odour blend. Thus, the results suggest that the code to the brain about an odour blend quality combines "labelled-line" and across-fibre" code patterns.

It is interesting that the information about some compounds exclusively present in non-hosts is conveyed only by specialist neurons (RN types 11 and 21). These may act as "labelled-lines" that convey important information about odour blends of non-host plants. The existence of specialised neurons for the detection of non-host plants related compounds was demonstrated in the black bean aphid, where neurons responding to methyl salycilate and myrtenal was suggested

to mediate a direct inhibitory input to the brain as a possible mechanism for masking the attraction to the host plant (Hardie *et al.*, 1994). This is similar to the mechanism for interruption of pheromone attraction in many insect species, e.g. heliothine males possess RNs specifically responding to sex pheromone components of sympatric species (Mustaparta, 1995, 1996a,b).

The RNs which responded selectively to one compound may possess one type of membrane receptor (Masson and Mustaparta, 1990). Thus, specific receptors sites may exist for 3-methyl-1-butanol, α-pinene, linalool, α-cubebene, and for a number of other chemically unidentified compounds in the various plant species tested. However, on the basis of the present electrophysiological results it is not possible to know whether each RN possess one type or several related types of membrane receptors. Some neurons responding best to the same compounds, but showing different sensitivities to other compounds may in fact possess e.g. different numbers of one receptor type or different composition of related receptor types. For instance, RNs of type 8, responding selectively to α-pinene, and RNs of type 9, responding best to α-pinene but also to β-pinene and 1,8-cineole, may both have one type of α-pinene receptors or different types α-pinene receptors. In other insect species it was suggested the presence of different types of membrane receptors (acceptors) in one RN. In the bark beetle, *D. frontalis*, differential adaptation of RNs (Payne and Dickens, 1976) suggested the existence of different membrane receptors specific for (+) and (-) enantiomers of the attractant pheromone frontalin (Payne *et al.*, 1982). In a cerambycid species (*M. notatus*) attacking *Pinus* spp., although not fully demonstrated, it was suggested that the same neuron may possess different membrane receptors for α-pinene and limonene (Dyer and Seabrook, 1978).

Another characteristic of RN responses is the ability to follow the concentration profile of the stimulatory components. Whereas the response frequency of neurons that responded to compounds eluting early from the GC-column (e.g. compounds with retention time corresponding to the region of monoterpenes in the chromatograms) followed the GC-peaks profile, characteristic long-lasting responses with slow decays were observed for all less volatile compounds with retention time in the region of the sesquiterpenes (e.g. figure 6.7, neuron B). No tailing was observed in these minor GC-peaks as detected by the FID-electrometer. Thus, the RN responses may have resulted by a slow inactivation of the stimulation for this type of neurons as suggested for the pine weevil, *Hylobius abietis*, that have RNs showing similar temporal patterns of response to some plant volatile components (Wibe and Mustaparta, 1996). Different temporal response patterns has also been recorded for pheromone neurons, even for neurons tuned to the same compound (Rumbo and Kaissling, 1989; Almaas and Mustaparta, 1991). Variation of

temporal response patterns of neurons may be important for the flight orientation mechanism towards an odour source where the different components may cause varying response duration of the neurons (cf. Kaissling, 1987).

In conclusion, the sensilla basiconica of *P. semipunctata* beetles possess many types of RNs for receiving plant odour information. Most RN types are narrowly tuned and convey information about non-terpenoid compounds, monoterpenes, and sesquiterpenes. Moreover, most neurons are activated by compounds present in volatile blends of both host and non-host plant species, whereas some are activated by compounds present only in one volatile blend. Thus, the relative activity elicited in neurons of different type is likely to be the code to the brain about the blend quality. This is in principle similar to the "labelled-line" system described for encoding pheromone information (cf. Mustaparta, 1995). However, an important difference is that neurons responding to two, three, or four compounds of related chemical structures convey information about different components depending on their concentration in the blend. This implies the involvement of an "across-fibre" code pattern. Thus, the results suggest that plant odour information in *P. semipunctata* beetles is mediated to the brain by a mechanism combining "labelled-line" and "across-fibre" code patterns. A combination of these two code patterns for plant odours was recently suggested for *H. abietis* (Wibe and Mustaparta, 1996).

PART III

GENERAL DISCUSSION

7.

General Discussion

7.1. Plant Odours are Involved in Host-Finding Behaviour

The first question asked in this thesis was whether eucalyptus odour plays a role in host-finding behaviour of *P. semipunctata* beetles. This was investigated in chapters 3 and 4, and the results support clearly a positive answer. The field experiment described in chapter 3 showed that primary attraction (i.e. attraction to host plant odour) mediates host-finding behaviour by *P. semipunctata* beetles. The behavioural meaning of attraction was discussed in the important paper of Kennedy (1978). The attraction that is induced by an odour is the end-result of actual behavioural reactions. As written by Baker (1989), "attraction is a displacement through space, and not a behaviour". The behaviour is the manoeuvres used by insects which allow them to control their position in space. The behaviour of *P. semipunctata* beetles in the presence of host odour was studied in wind tunnel, and the results suggest that attraction to the host plant results from odour-induced optomotor anemotaxis (steering with respect to the wind) and self-steered counterturning (chapter 4).

Experimental evidence for these two behavioural mechanisms has been obtained from studies of moths flying to pheromone sources (refs. in chapter 1, section 1.3.1). In order to control its direction and speed of displacement in the horizontal plane, a flying insect has only two reactions available at any instant: change its course angle (the direction towards which it is thrusting relative to the wind line) and change its airspeed (its speed through the air mass next to its body). Both optomotor anemotaxis and self-steered counterturning rely on these two behavioural reactions (Baker, 1989). In optomotor anemotaxis, feedback for the control of flight manoeuvres is derived visually from the apparent movement of images over the eyes. The control of course angle is a steering reaction and control of airspeed is a reaction related to the force of thrust created by the wing movement. In self-steered counterturning it is not known what external feedback is employed. The regularity in tempo of counterturns exhibited by moths both in and out of contact with pheromone indicate that there is a motor program underlying these reversals (Baker, 1989). This implies that the direction and force of the thrust (course angle and airspeed) may need no external feedback whatsoever, and the tempo at which the program runs appears to be set by the pheromone concentration. It has been suggested that optomotor anemotaxis

polarises the otherwise meandering zigzags into an orderly upwind resultant, taking the moth upwind the pheromone plume to the source (Baker *et al.*, 1984).

Why do moths zigzag? Answer to this question has been investigated by several researchers and there has been substantial disagreement as to why the tracks of male moths flying upwind in a plume have side-to-side deviations back and forth across the wind line (cf. Baker, 1989; Bell *et al.*, 1995). Not all moths necessarily integrate counterturning with optomotor anemotaxis while flying upwind in the plume, but it appears that all moths do so during casting flight moments after losing the plume (Baker, 1989). According to Baker (1989), a system which uses counterturning during both upwind flight and during casting may have advantages over straight-line upwind flight. It involves a continuum from narrow to wide zigzagging that depends on pheromone concentration, and may help facilitate contact with pheromone filaments in the plume, especially during rapid shift in wind direction.

The genus *Eucalyptus* comprises more than 600 species (Chippendale, 1988). Among these, some are better quality hosts (e.g. *E. camaldulensis*) than others (e.g. *E. tereticornis*) for larval development, and the best quality hosts are more attractive to the beetles than those of low quality (Hanks *et al.*, 1993). Host quality is also evaluated among individuals of one species, since specimens under physiological stress, e.g. due to water deficit and therefore unable to prevent bark penetration of neonate larvae (Hanks *et al.*, 1991), are more attractive than healthy ones. Perhaps, the best quality host species or specimens within a species emanate odour blends that sustain better upwind flight than blends emanating from low quality hosts. In general, both the concentration and composition of the odour blend of an host plant are important to perform sustained upwind flight in phytophagous insects (see chapter 1, section 1.3). In some insect species, field trials and wind tunnel assays have shown that potent attraction is only achieved to blends of several host specific or host related volatile compounds in the ratio they occur in the air above suitable host plants. Thus, it is possible that sustained upwind flight of *P. semipunctata* beetles depends on the quantitative and qualitative composition of odour blends emanating from *Eucalyptus* species.

In addition, flight orientation of *P. semipunctata* beetles may be affected also by volatile compounds of non-host species which may induce avoidance reactions (e.g. acting as repellents or inhibitors of attraction). In general, the study of behavioural reactions of phytophagous insects to volatile compounds or blends of non-host plants has been overlooked. However, attraction and avoidance are important aspects of olfaction, and it has been shown for some insect species that both attractants and repellents are used for discriminating between plants during the process of host selection (see chapter 1, section 1.4).

Since eucalyptus and many unrelated plants produce a wide range of ubiquitous volatile compounds, investigating the composition of odour blends that effectively affect the behaviour of *P. semipunctata* beetles is quite complex. This is certainly a less complicated task in terms of number of compounds for priority investigation by investigating first which volatile compounds are detected by olfactory receptor neurons, and how these encode plant odour blends. Answer to these two questions was provided in chapters 5 and 6, which presented results obtained by coupling gas chromatographic separation of headspace volatiles of host and non-host species with recording of electroantenograms (GC-EAD; chapter 5) and with single-cell recordings (GC-SCR; chapter 6). The results obtained are summarised in table 7.1 showing components of the volatile blends that are detected by receptor neurons of *P. semipunctata*, and the neuron types that convey information about each of the components.

The receptor neurons of *P. semipunctata* detect several major as well as minor components of complex volatile blends of host and non-host plants. The components chemically identified comprise non-terpenoid hydrocarbons, monoterpenes, and sesquiterpenes, many of which are present in both host and non-host plants, but in different amounts. Some components which were located in the chromatograms by activating certain type of neurons during GC-SCR, did not elicit significant EAG responses during GC-EAD recordings. This demonstrates that GC-SCR is a more accurate technique for identifying plant volatiles of biological importance to *P. semipunctata* beetles than GC-EAD. In addition, since some compounds that elicited strong EAG responses did not activate any of the neuron types tested during GC-SCR, it is conceivable the existence of more neuron types than those presented in this thesis.

The GC-SCR showed different types of receptor neurons, including specialist neurons responding to only one compound and neurons responding to two, three or four compounds of related chemical structures. Some neurons responded to compounds present only in the host plant (*E. globulus*), many neurons responded to compounds present in both host and non-host plants (*P. pinaster* and *O. europeae*), and some neurons responded to compounds present only in non-host plants. This suggests a chemical "fingerprint" for discrimination between odour blends of different plants, i.e. the relative activities elicited by many compounds in different neuron types is the code to the brain about the odour blend quality of a plant species or an individual plant. Information about the presence of some compounds in the blend is preserved by specialist neurons, acting as "labelled-lines". This is in priciple similar to the "labelled-line" system for encoding pheromone blends in male moths. However, an important difference is that neurons responding to more than one compound in the plant odour blend are responsible for some

integration of blend quality. Thus, the integrated results suggest that each plant odour blend is encoded by a mechanism combining "labelled-line" and "across-fibre" code patterns.

Patterns of neural activity elicited in receptor neurons by plant odour blends are conveyed to the central nervous system for further processing and integration with other sensory modalities (e.g. vision) by complex networks of interneurons which are connected with the motor system controlling the behaviour (see chapter 1, section 1.6). The integrated results strongly suggest that upwind flight by *P. semipunctata* beetles toward the host plants depends on the detection of odour blends comprised of several host related volatile compounds in certain ratios.

Major qualitative and quantitative differences were found between volatile blends of host and non-host species, *P. pinaster* and *O. europeae*. The behavioural reactions of *P. semipunctata* beetles to odour blends of non-host plants were not investigated. Nevertheless, it is conceivable that they fail to elicit sustained upwind flight or that induce obvious avoidance reactions by eliciting markedly different patterns of relative activities in the same and in different receptor neurons from those elicited by odour blends of suitable host species. It is interesting, however, that the information about some compounds exclusively produced by non-host plants is conveyed only by specialist neurons (neuron types 11 and 21). The activity elicited in these neurons may be important for generating behavioural responses of *P. semipunctata* beetles to the odour of non-host plants.

Whether the insects in general are using both attractive and repellent olfactory cues for finding and selecting a proper host plant is still unclear. At least for aphids and moths, it has been shown that some non-host plant odours are repellent (refs. in chapter 1, section 1.4). A relatively well studied example is the black bean aphid, *A. fabae* (Hardie *et al.*, 1994). This aphid is repelled by each of two compounds, methyl salycilate and myrtenal, not present in the hosts, but with wide distribution among plants. When each of the two compounds is added to the odour blends of suitable host plants, the attraction of the aphids is inhibited. Methyl salycilate and myrtenal are detected by specialised receptor neurons in the primary rhinaria of the antennae, and it was suggested that they could send a direct inhibitory input to the central nervous system, being in this way responsible for masking attractive host plant odours.

A "labelled-line" mechanism for encoding information about pheromone blends of sympatric species has been thoroughly studied in heliothine moths, which is related to inhibition of attraction of males of one species to females of a sympatric species (refs. in chapter 1, section 1.6). The pheromone blends of different heliothine species have a common compound as their major pheromone component (component A). The species specificity is provided by adding a second compound (B, C, D, etc.). In *H. virescens*, males have specialist receptor neurons for

detecting each of the two essential pheromone components (A and B), which project to the same glomerulus of the macroglomerular complex (MGC). A third type of receptor neurons is tuned to the detection of a pheromone component (D) of a sympatric species, and project their axons to another glomerulus of the MGC. These neurons convey an interspecific signal to the brain that inhibits the attraction of *H. virescens* males to females of a sympatric species. In the antennal lobe of *H. virescens*, two groups of projection neurons (interneurons that send axons to the mushroom bodies and the lateral lobe of the protocerebrum) receive input from the two major pheromone components, one receives input from component A and the other integrates the information from both components (A and B), whereas a third group receives input from the interspecific signal (D) that inhibits the attraction to the sympatric species (Mustaparta, 1996). It is unknown whether this type of mechanism is also acting in phytophagous insects repelled by odour of non-host plants or inhibited in their attraction to host plants by non-host odours.

In conclusion:

1. Host-finding by *P. semipunctata* beetles results from behavioural responses initiated and sustained by odour blends emanating from host plants, and transported by the wind. Downwind of the source, the beetle responds to olfactory stimulation flying upwind. This flight course is maintained by visual feedback from the apparent movement of images over the eyes, i.e. odour-induced optomotor anemotaxis. In addition, it is suggested that self-steered counterturning is integrated with optomotor anemotaxis.

2. *Eucalyptus* species with different attractiveness to *P. semipunctata* beetles produce different ratios of the same volatile compounds that are detected by receptor neurons. In addition, most of these compounds are also produced by non-host plant species, but in different ratios. Therefore, it is suggested that the ratio of host related compounds in the odour plume is important for initiating and sustain behavioural responses taking the beetles upwind to the host plant.

3. The narrow response spectra of plant odours receptor neurons suggest that odour blend quality is encoded by the relative activity in receptor neurons of different type.

4. The activity elicited in specialist neurons by compounds exclusively produced by non-host plants may be related with avoidance reactions that prevent *P. semipunctata* beetles of make contact with non-host plant species.

Figure 7.1. Compounds present (+) and absent (-) in the volatile blends of *Eucalyptus* spp., *Pinus pinaster*, and *Olea europeae* that elicited electrophysiological responses from receptor neurons of *Phoracantha semipunctata* during gas chromatography coupled with recording of electroantennograms (GC-EAD) or with single-cell recordings (GC-SCR).

Chemical Class	Compound[1]	Host Species	Non-host Species[2]		Neuron Types[3]
		Eucalyptus spp.	*Pinus pinaster*	*Olea europeae*	
Non-terpenoids					
	3-hydroxy-2-butanone	+	+	+	3
	3-methyl-1-butanol	+	-	+	1(s), 2
	2,3-epoxy-4,4-dimethylpentane	+	-	+	2, 3
	ethyl propanoate[a]	+	-	+	unknown
	ethyl-3-methylbutanoate	+	+	-	5
	(Z)-3-hexen-1-ol	+	+	+	4, 6(s), 7
	(Z)-2-hexen-1-ol	+	-	+	7
	4,8-dimethylnona-1,37-triene[a]	-	-	+	unknown
Monoterpenes					
	α-pinene	+	+	+	8(s), 9, 13
	β-pinene	+	+	-	9
	1,8-cineole	+	+	-	9, 10(s)?
	limonene	+	+	-	12, 10(s)?
	α-terpinene	+	+	-	12
	p--cymene[a]	+	+	-	unknown
	myrcene	+	+	-	12, 13, 14
	trans-β-ocimene	+	+	+	13
	linalool	+	+	-	15(s), 18
Sesquiterpenes					
	guaine	+	-	-	22
	α-cubebene	-	+	-	21(s)
Unknown					
	unidentified (4)[b]	+	-	+	3
	unidentified (5)[b]	+	-	+	4
	unidentified (8)[a]	-	-	+	unknown
	unidentified (6)[b]	+	-	-	3
	unidentified (11)	-	-	+	11(s)
	unidentified (18)[b]	+	-	-	17
	unidentified (19)[b]	+	-	-	14
	unidentified (20)[b]	+	-	-	16
	unidentified (21)[b]	+	-	-	16
	unidentified (22)[b]	+	-	-	17
	unidentified (23)[b]	+	-	-	16
	unidentified (38)[a]	+	+	+	unknown
	unidentified (24)[b]	+	-	-	18, 19(s)
	unidentified (25)[b]	-	+	-	23
	unidentified (26)[b]	+	-	-	20(s)
	unidentified (29)[b]	+	?	-	22
	unidentified (30)[b]	+	?	-	22
	unidentified (31)[b]	-	+	-	23
	unidentified (32)[b]	+	?	-	22

[1] Numbers in parenthesis indicate GC-peaks in the chromatograms of plant volatile blends that activated responses single receptor neuron types (chapter 6, figures 6.2-6.4); [a] these compounds elicited strong EAG responses during GC-EAD recordings (chapter 5, figures 5.3-5.7) but did not activate any type of neurons during GC-SCR (Neuron Type unknown); [b] these compounds were located in the gas chromatograms of *Eucalyptus globulus*, *Pinus pinaster*, or *Olea europeae* during GC-SCR, and did not elicit significant EAG responses during GC-EAD recordings

[2] Compounds whose presence in a plant volatile blend is uncertain are indicated by ? (chapter 6, figure 6.11).

[3] Neuron response spectra are shown in figure 6.11 (chapter 6). Specialist type of neurons, responding to only one compound are indicated by (s).

7.2. Host Plant Promotes Sexual Encounters

The results presented in this thesis strongly suggest that both males and females use olfactory input from plants to guide their flight behaviour and take landing decisions. Since there is no evidence supporting the existence of sexual or aggregation pheromones effective at long range or any non-chemical long-range intraspecific communication mechanism (i.e. sound) (chapter 2, section 2.2), it is not surprising that males are as effective as females in locating the host plant (chapters 3 and 4), and possess similar olfactory capabilities to detect plant volatile compounds (chapter 5). Thus, suitable host plants do not only provide a substrate for larval development, but also promote encounters between sexes, aggregating large numbers of beetles. At dusk and during the night, sexual interactions as well as aggressive interactions between males can be observed after large number of beetles have landed on stands of recently cut logs and leaves of *E. globulus* (pers. obs.). Once on the host plant, sexual pheromones effective at close range may further promote sexual encounters.

The close dependence of mate-finding on host-finding has been suggested for other oligophagous cerambycid species which attack conifers (*Pinus* spp.) under physiological stress or recently felled, e.g. *M. scutellatus* and *M. notatus* (Dyer and Seabrook, 1978), *M. alternatus* (Yamane and Asada, 1977 in Iwabuchi, 1982), *M. sutor* and *M. urussovi* (Grodnitski, 1987), and *P. fortunei* (Wang *et al.*, 1990). In some of these species, the existence of close-range sexual pheromones has been shown. In *P. fortunei*, an airborne pheromone released by females attracts the males within a range of about 3 cm (Wang *et al.*, 1991). In *M. alternatus*, males release a pheromone that attracts the males at close range (Kim *et al.*, 1992). A contact pheromone on females' body surface elicits copulation behaviour in males, but this is not directly related with sexual recognition since both sexes produce the contact pheromone that elicit copulatory behaviour (Kim *et al.*, 1992). In *M. sutor* and *M. urussovi*, females lay a contact pheromone on the surface of an host tree trunk which induces intense local search in males (Grodnitski, 1987). In *P. semipunctata*, observations of the sexual behaviour in the laboratory have shown that sexual interactions are initiated by females approaching immobile males, but no experiments were conducted to demonstrate the existence of a male pheromone that attracts females at close range (Marques, 1996), as shown for *M. alternatus* (Kim *et al.*, 1992). However, experimental evidence has been provided for the presence of a contact pheromone on the body surface of both sexes that elicits copulatory behaviour in males (Marques, 1996).

Apparently, these cerambycid species have not evolved intraspecific communication systems effective at long distances. Instead, sexual encounters are promoted by flying upwind to suitable host plants where is high the probability of finding a mate. Accepting the premise that evolution moulds organisms to high levels of efficiency their environment requires, then the long-range detection system of *P. semipunctata* beetles for odour blends emanating from host and non-host plants is a logical adaptation. This helps the decision process of takeoff and flying in a particular direction. Even near potential hosts, tree species or individuals fitting a particular set of form factors certainly look more alike than they smell for this nocturnal insect. It seems to be more efficient to discriminate by odour to avoid the expense of landing to test every seemingly suitable substrate than to rely only on contact chemical and/or physical features of plants for host selection. Thus, olfactory discrimination of plant odours underlying behavioural responses taking males and females to suitable host plants for larval development, can be viewed as a primary foraging adaptation. This permits *P. semipunctata* beetles to minimise the energy spent on searching for mates and suitable oviposition places, and has the advantage of reducing predation risk and increases the investment in the production of offspring.

7.3. Future Research Targets for Integrated Pest Management

The experimental results in this thesis provide a strong motivation for future research on *P. semipunctata* chemical ecology planned to find out the answer for two important questions: (1) which are the odour blends that most effectively attract *P. semipunctata* beetles to suitable host plants, and (2) which volatile compounds or blends (if any) are repellent or inhibit the attraction to host plants.

The results in the previous chapters are clearly pointing out ways to conduct further research on the behavioural effect of plant chemicals in *P. semipunctata* beetles. Through wind tunnel experiments, synthetic blends of chemically identified host related volatiles detected by the beetles' receptor neurons can be tested for their attractiveness. Likewise, repellent effects of single non-host compounds can also be tested, as well as their possible inhibitory effect on the attraction to host plants. The results of such investigation may be of usefulness in integrated pest management (IPM) strategies which are probably the only way to achieve a long-term control of *P. semipunctata* populations (chapter 2, section 2.7).

Decisions concerning how much effort should be put on pest control strategies and which tactics should be implement require accurate knowledge about the population level of the insect.

Thus, traps baited with attractive blends may be used to monitor the population of *P. semipunctata* beetles, and even used in mass-trapping programmes replacing the current use of log traps if chemically baited traps prove to be less costly and have at least the same control efficiency.

The attractive power of a trap is likely to be dependent not only on the correct formulation of the chemical blend but also on the presence of non-chemical cues acting synergistically (e.g. visual cues) (chapter 1, section 1.3.2). Although, *P. semipunctata* beetles are active at dusk and during the night, vision is likely to add olfaction in host-finding behaviour (chapter 3 and 4). For instance, moths of the African Armyworm (*Spodoptera exempta*) possess a four-colour visual system which reaches from the UV to the deep red and even functions in moonlight (Langer *et al.*, 1979). This sensorial capacity may provide additional non-chemical information adding the flying moths decision to land on ground vegetation for mating and egg-laying.

Besides vision, other non-chemical sensory modalities may add orientation towards the host plant and landing. These may include infrared radiation. Buprestid beetles detect infrared radiation by specialised thermoreceptors in metathoracic pit organs next to the coxae of the mesothoracic legs, enabling them to locate recently killed trees by fire where larval development takes place (Schmitz, 1996). Although, no organs with similar function have so far been described in *P. semipunctata* beetles, it is interesting to note that the beetles show strong oriented responses towards darkroom red light lamps (pers. obs.). These lamps emit also energy in the infrared wavelength. This does not prove that the beetles were responding to infrared energy, but the hypothesis is exciting and deserves further research.

The interest of such possibility lies on the fact that freshly cut logs and leaves of *Eucalyptus*, as well as the trunk of standing trees, emit infrared energy during the night (pers. obs.). Biological stress in agricultural crops such as sugar beet, cotton, and beans has been detected with infrared thermovision (Pinter *et al.*, 1979; Tu and Tan, 1985). The emission of infrared energy by conifer seedlings and its correlation with the physiological condition was demonstrated by Egnell and Örlander (1992). Increased temperature emission by needles indicates reduced transpiration rate, which could reflect the physiological condition of a seedling under certain circumstances. For instance, water stress caused by lack of soil water or inability to transport or absorb water induce a decrease in transpiration rate and this affects needle temperature due to decreased dissipation of incident energy (e.g. Gates, 1964; Hashimoto, 1980). In addition to odour blends of host plants, infrared emission by *Eucalyptus* might be a non-chemical stimulus that add host location by *P. semipunctata* beetles. This speculative idea

deserves some attention when testing the attraction efficiency of traps baited with synthetic blends of volatile compounds.

Repellents or inhibitors of attraction can be released in *Eucalyptus* plantations, and by interfering with the beetles' orientation they may induce disruption of both mating and oviposition. A volatile compound in minute amount in the chromatogram of *O. europeae* blend of volatiles, which is detected specialist receptor neurons of *P. semipunctata*, has promise in this respect (for mass spectrum see Appendix, figure A.7). After correct chemical identification has been achieved, and if is shown that this compound is repellent or mask attractive host plant odours, it could be slowly released from dispensers placed in selected areas of the plantations. Dispersion of the molecules by the wind would increase largely the active space generated from even point sources (Murlis, 1986) within the plantation. Furthermore, the high degree of turbulence within the canopy (Shaw, 1982), and some extent of mixing downwind from the planted area (Stanton, 1983) would combine to disrupt orientation of both resident and immigrant beetles.

Altering the genome of *Eucalyptus* to alter the profile of some key volatiles or to promote production of inhibitory agent(s) is also possible due to considerable advances in molecular biology research and techniques. With knowledge of the enzymatic steps leading to the production of secondary metabolites with known function in *P. semipunctata* behaviour, it is conceivable genetic manipulation to induce changes in the biosynthetic pathways to alter the profile of components of an attractive blend and/or promote the biosynthesis of inhibitory compound(s). The same research approach used when searching for *Eucalyptus* strains better adapted to environmental conditions, yielding higher production in result of increased growth rates and biomass, may also be used to find out genomes that yield non-attractive profiles of secondary metabolites. Due to the large number of species and chemical forms[1] within a single species, the genus *Eucalyptus* provide a diverse genetic pool which can be manipulated to control the biosynthesis of secondary compounds. Even different branches of one specimen have genetic variation responsible for variation in the composition of volatile oils, and this confers differential resistance among branches to defoliating Christmas beetles (*Anoplognathus montanus*) (Edwards *et al.*, 1990).

[1] Chemical forms are plants in naturally occurring population which cannnot be separated on morphological evidence, but which are readily distinguished by marked differences in the chemical composition of their essential oils (Penfold and Willis, 1953). Chemical forms are generally well defined and readily distinguishable from each other, and they do not appear to be a result of site differences, seasonal variation, leaf ageing effects or hybridisation (Doran, 1991). The occurrence of chemical forms appear to be relatively common in Eucalyptus and other genera of the Myrtaceae (Whiffin, 1982, 1992).

Plant secondary metabolites presents a difficult target for plant genetic engineering, since their biosynthesis involves a number of enzymatic steps and thereby a series of associated genes (Hallahan et al., 1992; Gijzen et al., 1993). Nonetheless, genetic engineering strategies may be devised that only require the limited modification of existing pathways, for example, providing that the genes for particular enzymes are appropriately expressed, they could be employed to augment existing pathways so as to enable biosynthesis of metabolites useful in crop plant protection (Hallahan *et al.*, 1992).

Successful IPM strategies can only be achieved through integration of various tactics. Integrative approaches involving semiochemicals and pathogens have been termed push-pull or stimulo-deterrent diversionary strategies (SDDS) (Miller & Cowles, 1990; Pickett et al., 1991; Pickett and Woodcock, 1992). The population, possibly by use of plant attractants and aggregation stimuli, would be aggregated on to a part of the crop where a selective insecticide or a biological agent could be used. On a larger scale, the harvestable crop is again protected by repellents or inhibitors but in addition, a reduction of host plant acceptability would be provided by genetic manipulation of the secondary pathways that produce attractants. An SDDS has been established for reducing the insecticide use in oilseed, *Brassica napus*, and such integrative approaches may be applied to other crop pests as more knowledge is achieved on their chemical ecology (Pickett and Woodcock, 1992).

To implement SDDS approaches is necessary to have a detailed knowledge on what the insects detect and how they respond to plant chemical stimuli. This is greatly facilitated by investigating the detection capabilities of receptor neurons as shown in this thesis. The plants produce a great range of volatiles within which those that are detected by the insects can be located and chemically identified by coupling electrophysiological recordings with high resolution gas chromatography. The study of insect behaviour is then a less complicated task in terms of numbers of compounds for priority investigation, and thereafter research targets can be defined aiming the implementation of SDDS for plant protection.

PART IV

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APPENDIX

Appendix

In this appendix is shown mass spectra of components of plant volatile blends of *E. globulus*, *E. camaldulensis*, *E. tereticornis*, *P. pinaster*, and *O. europeae*) that are detected by olfactory receptor neurons.

Mass Spectra of Non-terpenoid Hydrocarbons

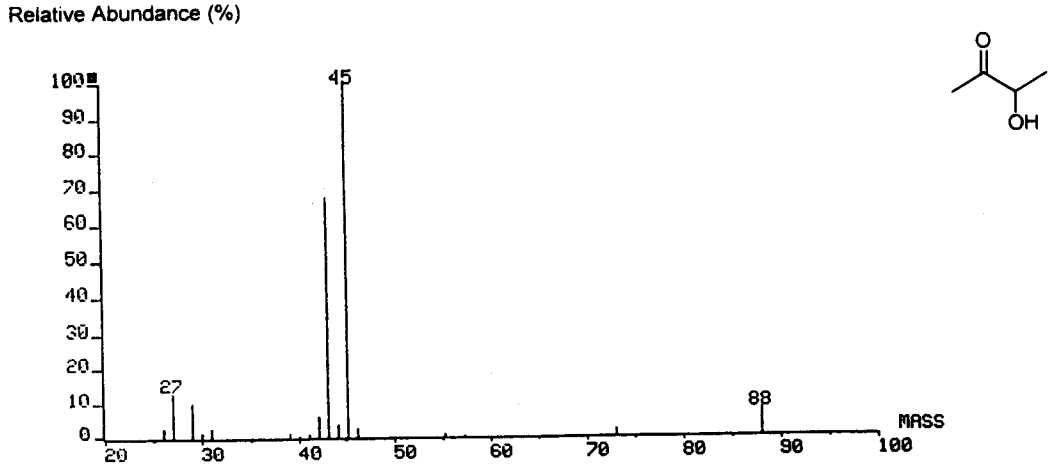

Figure A.1. Mass spectrum of GC-peak 4 in the gas chromatogram of *Eucalyptus camaldulensis* (chapter 5, figure 5.4), identified as 3-hydroxy-2-butanone.

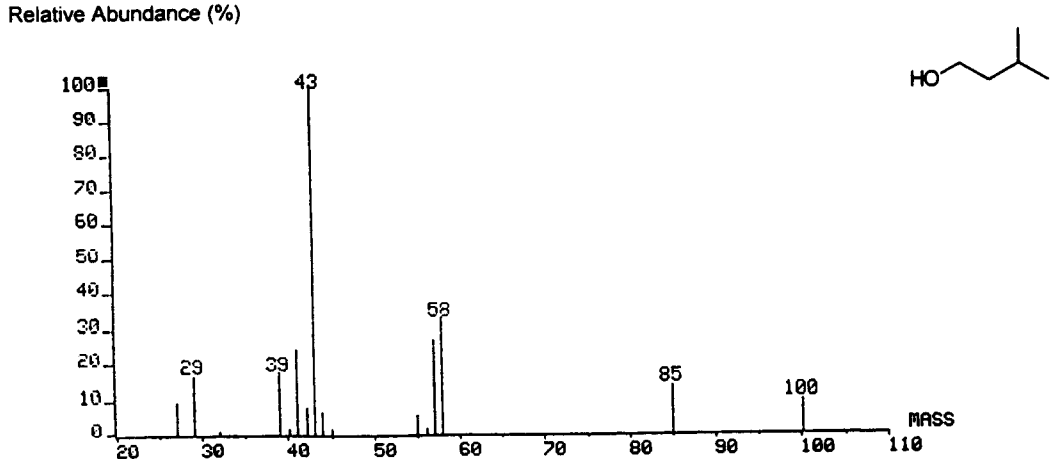

Figure A.2. Mass spectrum of GC-peak 5 in the gas chromatogram of *Eucalyptus globulus* (chapter 5, figure 5.3), identified as 3-methyl-1-butanol.

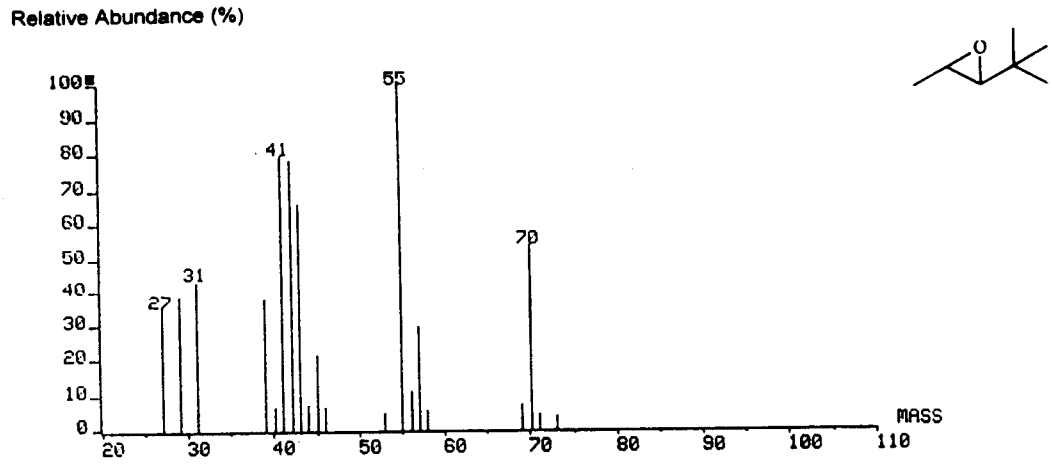

Figure A.3. Mass spectrum of GC-peak 6 in the gas chromatogram of *Eucalyptus globulus* (chapter 5, figure 5.3), tentatively identified as 2,3-epoxy-4,4-dimethylpentane.

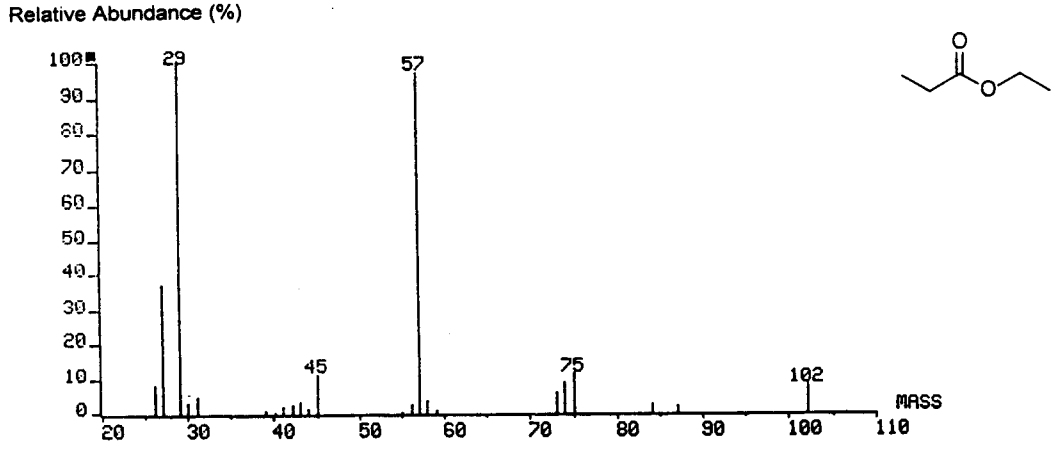

Figure A.4. Mass spectrum of GC-peak 10 in the gas chromatogram of *Eucalyptus camaldulensis* (chapter 5, figure 5.4), tentatively identified as ethyl propanoate.

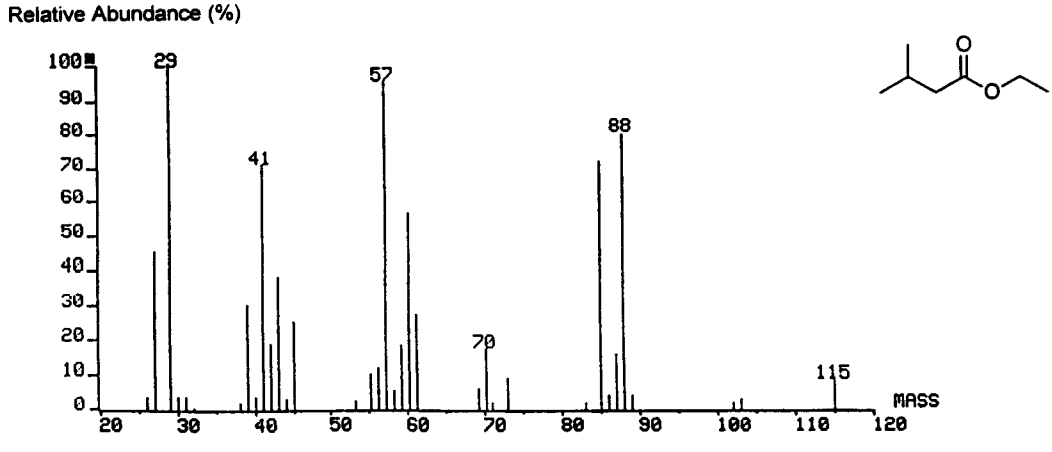

Figure A.5. Mass spectrum of GC-peak 12 in the gas chromatogram of *Eucalyptus globulus* (chapter 5, figure 5.3), tentatively identified as ethyl-3-methylbutanoate.

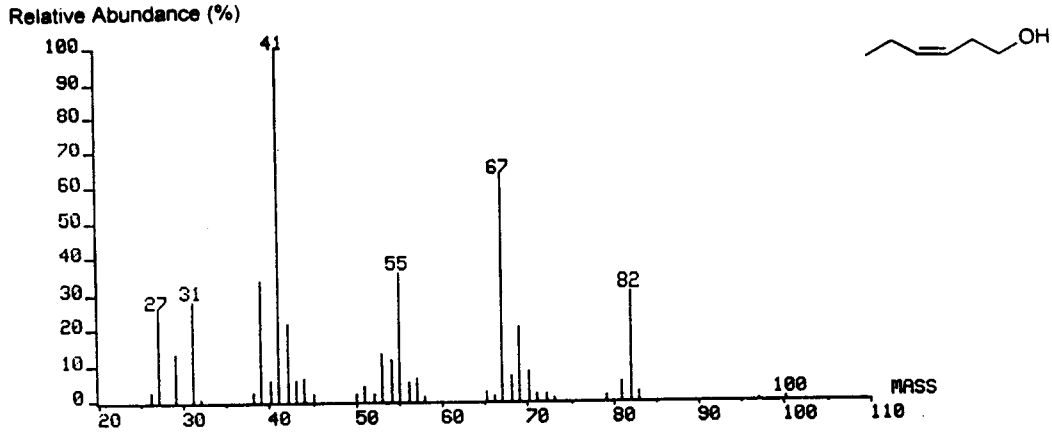

Figure A.6. Mass spectrum of GC-peak 13 in the gas chromatogram of *Olea europeae* (chapter 5, figure 5.7), identified as (Z)-3-hexen-1-ol.

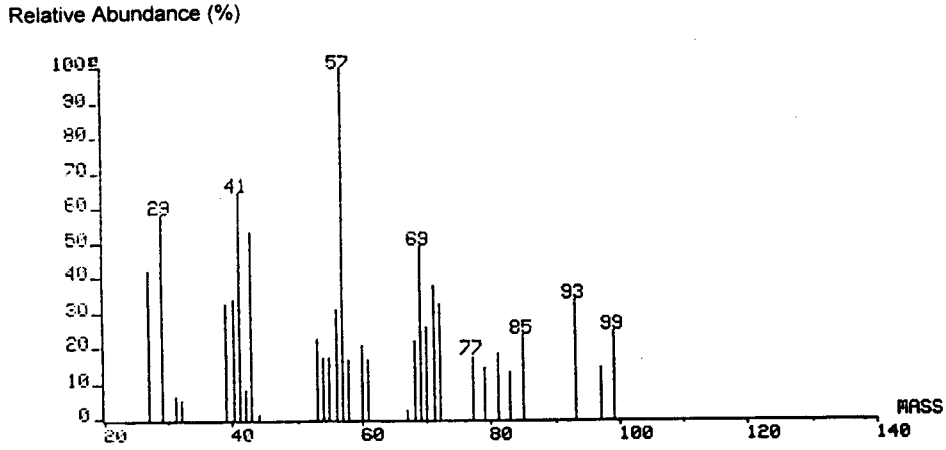

Figure A.7. Mass spectrum of GC-peak 17 in the gas chromatogram of *Olea europeae* (chapter 5, figure 5.7), unidentified compound.

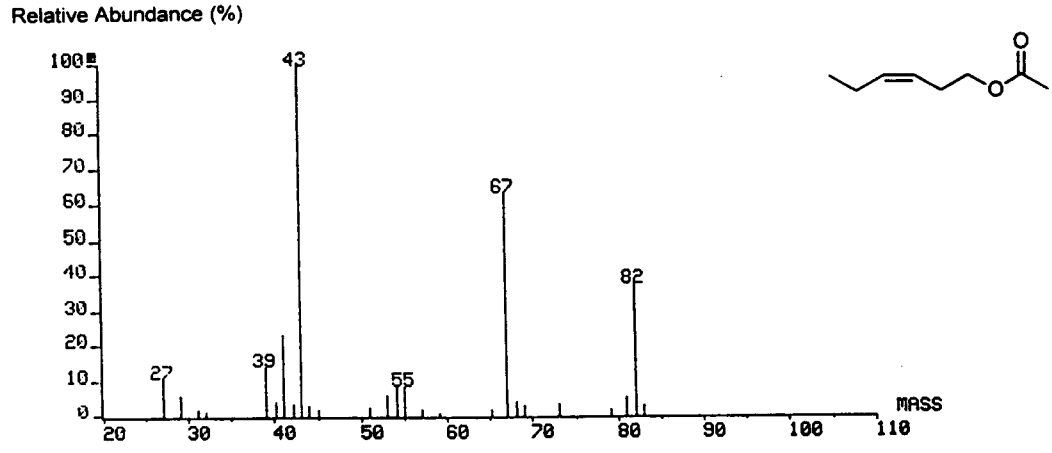

Figure A.8. Mass spectrum of GC-peak 20 in the gas chromatogram of *Eucalyptus camaldulensis* (chapter 5, figure 5.4), identified as (Z)-3-hexenyl acetate.

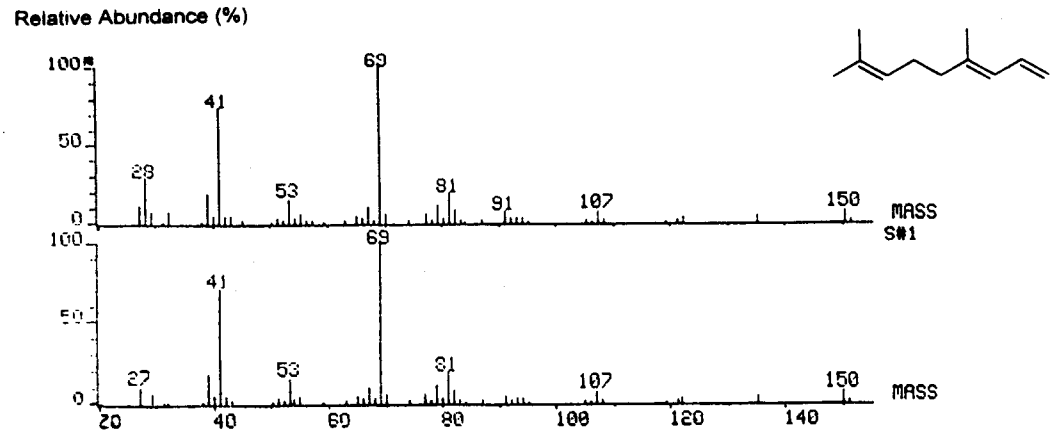

Figure A.9. Mass spectrum of GC-peak 32 in the gas chromatogram of *Olea europeae* (chapter 5, figure 5.7), identified as 4,8-dimethylnona-1,3,7-triene.

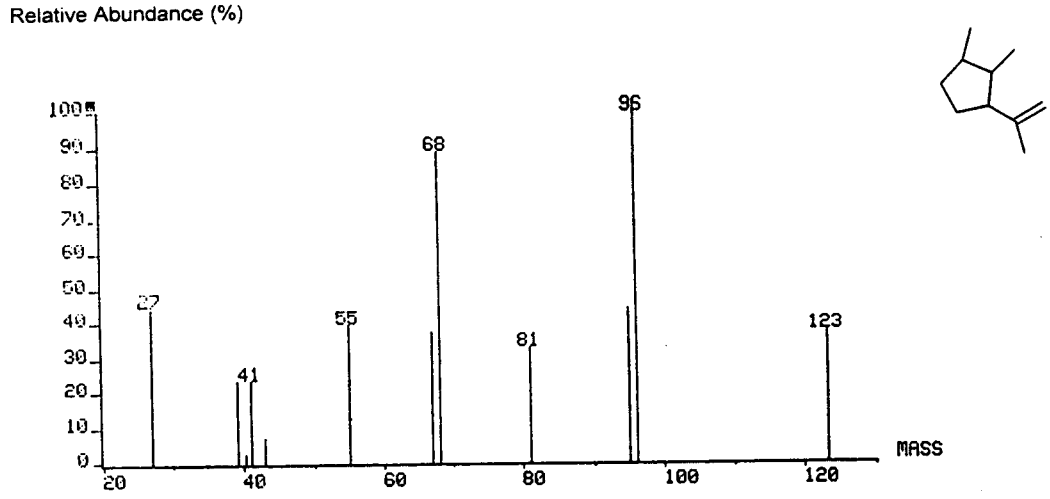

Figure A.10. Mass spectrum of GC-peak 34 in the gas chromatogram of *Eucalyptus tereticornis* (chapter 5, figure 5.5), tentatively identified as 1,2-dimethyl-3-isopropenylcyclopentane.

Mass Spectra of Monoterpenes

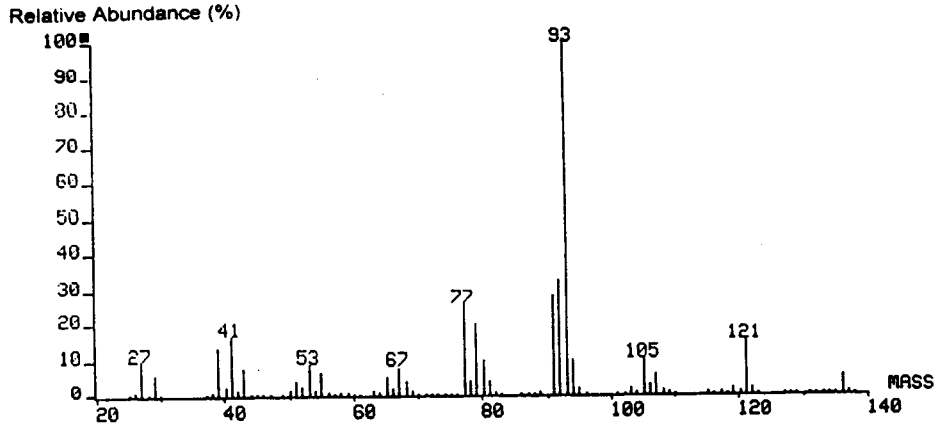

Figure A.11. Mass spectrum of GC-peak 15 in the gas chromatogram of *Eucalyptus globulus* (chapter 5, figure 5.3), identified as α-pinene.

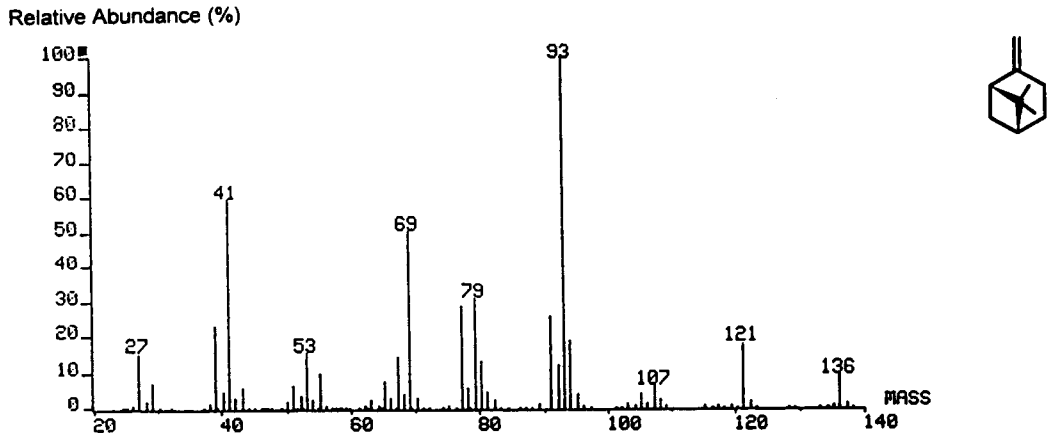

Figure A.12. Mass spectrum of GC-peak 18 in the gas chromatogram of *Pinus pinaster* (chapter 5, figure 5.6), identified as β-pinene.

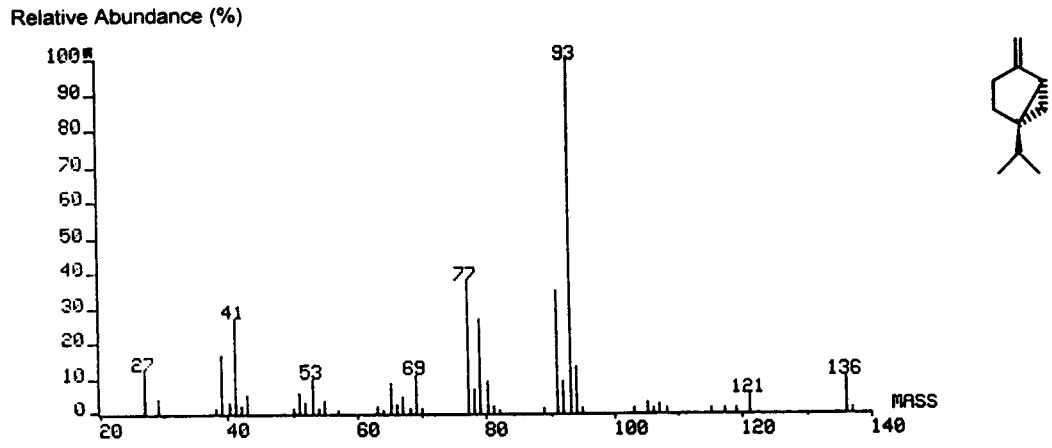

Figure A.13. Mass spectrum of GC-peak 16 in the gas chromatogram of *Eucalyptus camaldulensis* (chapter 5, figure 5.4), identified as sabinene.

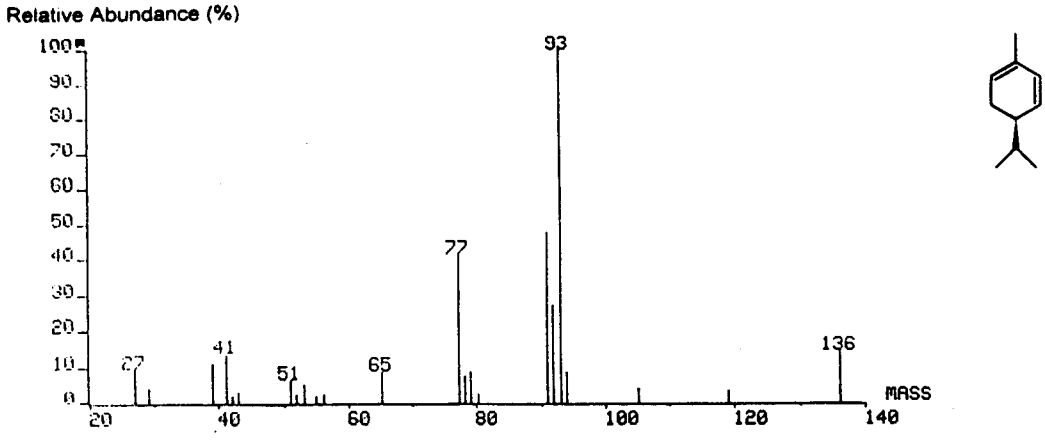

Figure A.14. Mass spectrum of GC-peak 21 in the gas chromatogram of *Eucalyptus camaldulensis* (chapter 5, figure 5.4), identified as α-phellandrene.

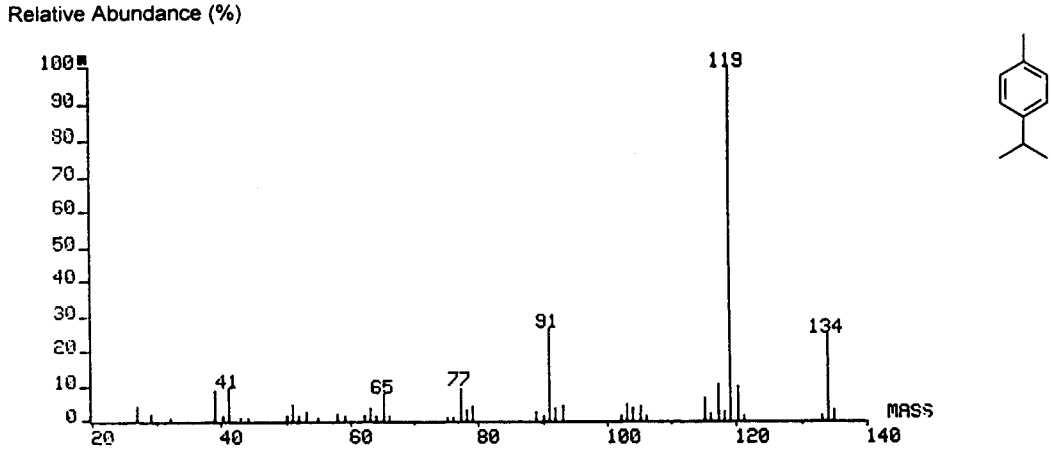

Figure A.15. Mass spectrum of GC-peak 23 in the gas chromatogram of *Eucalyptus globulus* (chapter 5, figure 5.3), identified as *p*-cymene.

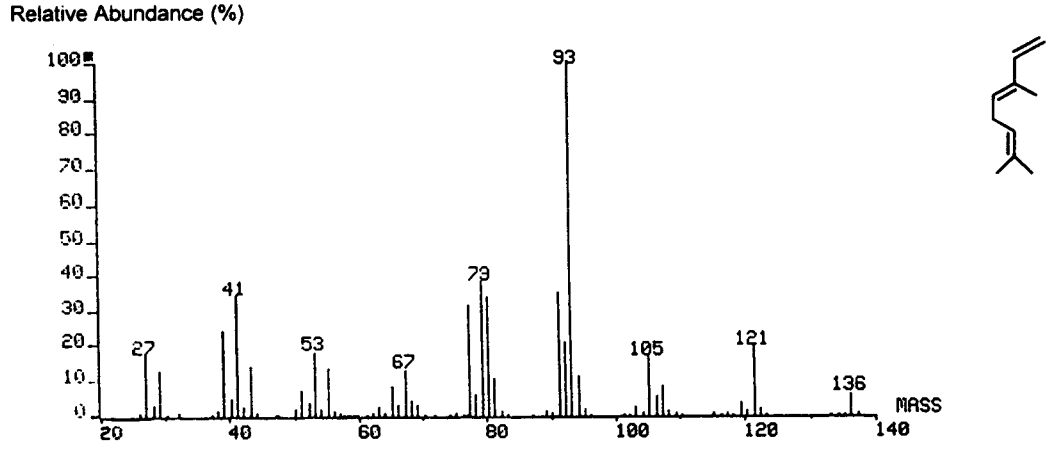

Figure A.16. Mass spectrum of GC-peak 26 in the gas chromatogram of *Pinus pinaster* (chapter 5, figure 5.6), identified as trans-β-ocimene.

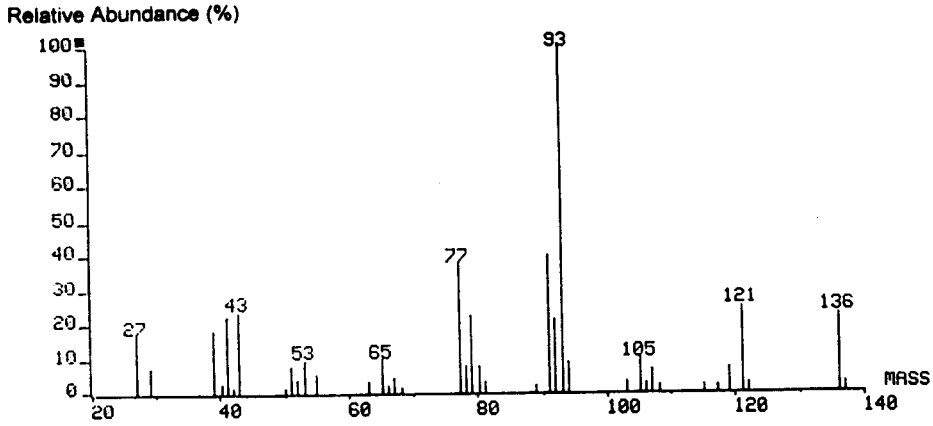

Figure A.17. Mass spectrum of GC-peak 27 in the gas chromatogram of *Eucalyptus globulus* (chapter 5, figure 5.3), identified as γ-terpinene.

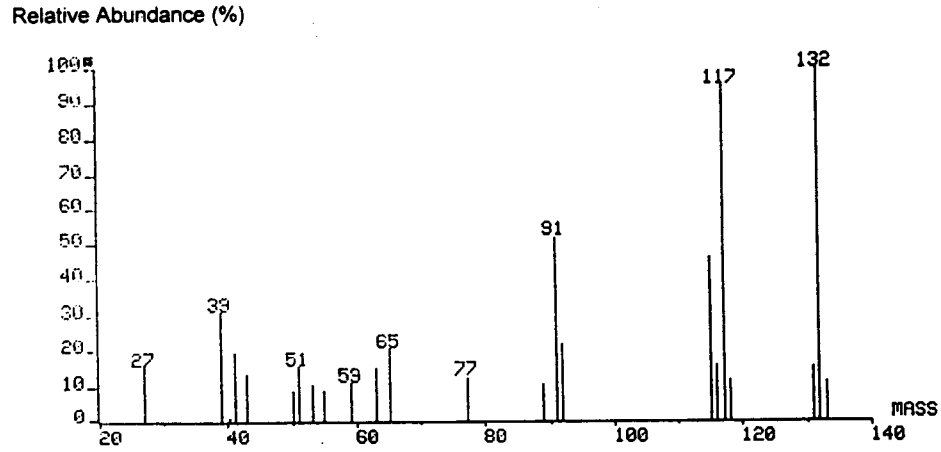

Figure A.18. Mass spectrum of GC-peak 29 in the gas chromatogram of *Eucalyptus tereticornis* (chapter 5, figure 5.4), tentatively identified as 1-isopropenyl-3-methylbenzene.

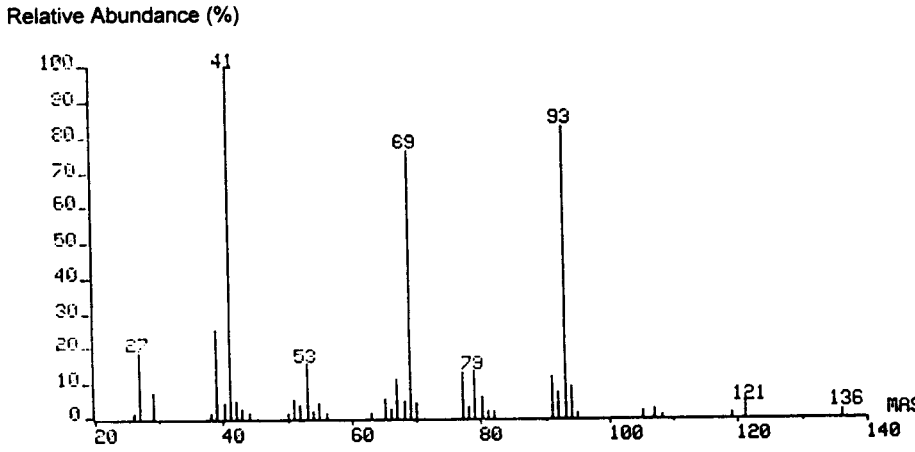

Figure A.19. Mass spectrum of GC-peak 19 in the gas chromatogram of *Eucalyptus globulus* (chapter 5, figure 5.3), identified as myrcene.

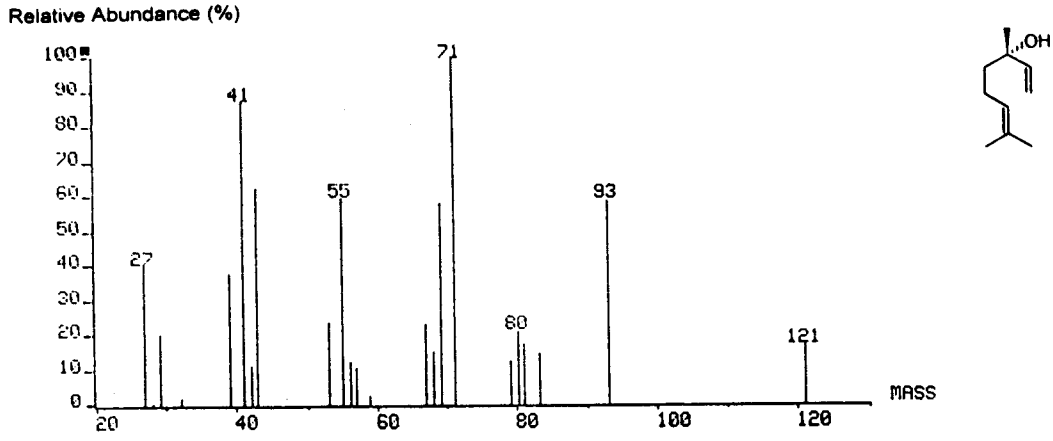

Figure A.20. Mass spectrum of GC-peak 31 in the gas chromatogram of *Eucalyptus camaldulensis* (chapter 5, figure 5.4), identified as linalool.

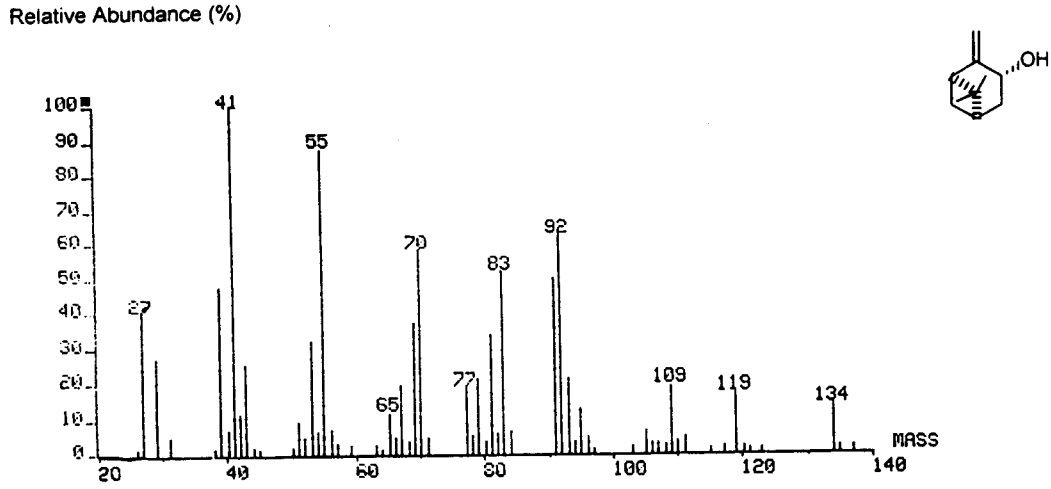

Figure A.21. Mass spectrum of GC-peak 35 in the gas chromatogram of *Eucalyptus globulus* (chapter 5, figure 5.3), tentatively identified as isopinocarveol.

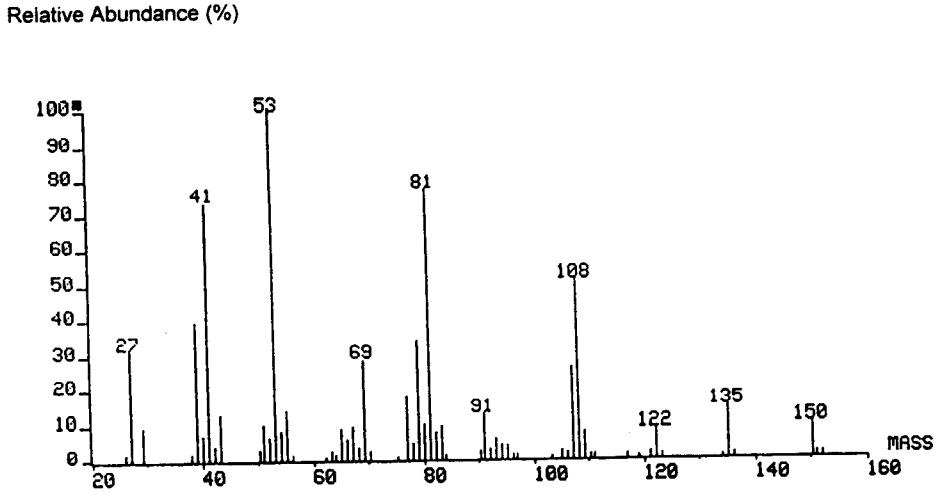

Figure A.22. Mass spectrum of GC-peak 37 in the gas chromatogram of *Eucalyptus globulus* (chapter 5, figure 5.3), unidentified.

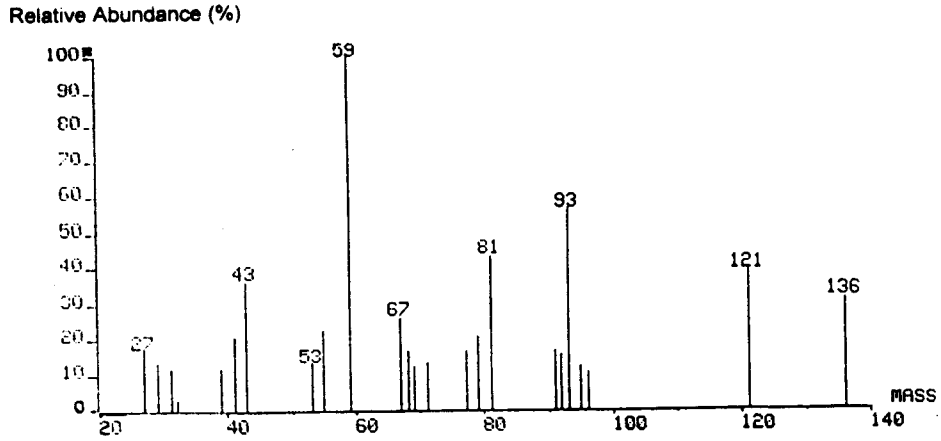

Figure A.23. Mass spectrum of GC-peak 38 in the gas chromatogram of *Eucalyptus tereticornis* (chapter 5, figure 5.5), unidentified.

Mass Spectra of Sesquiterpenes

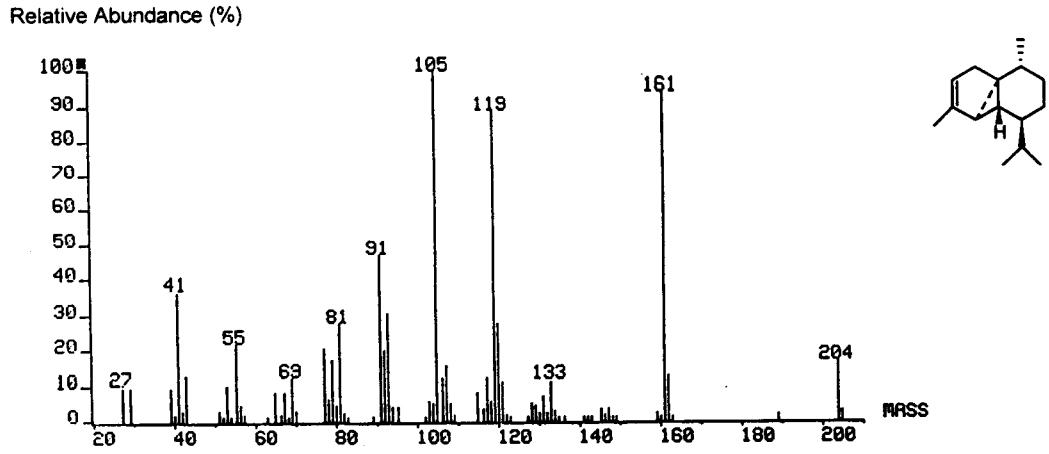

Figure A.24. Mass spectrum of GC-peak 39 in the gas chromatogram of *Pinus pinaster* (chapter 5, figure 5.6), identified as α-cubebene.

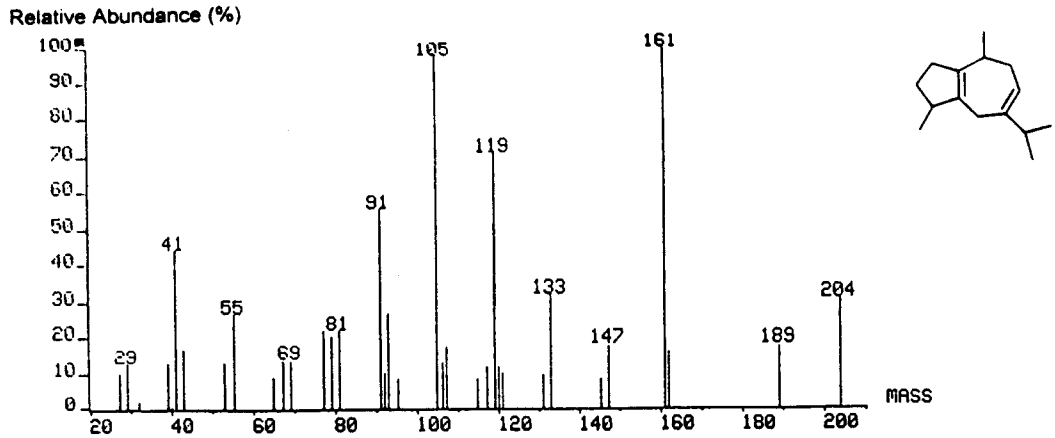

Figure A.25. Mass spectrum of GC-peak 40 in the gas chromatogram of *Eucalyptus globulus* (chapter 5, figure 5.3), tentatively identified as guaine.

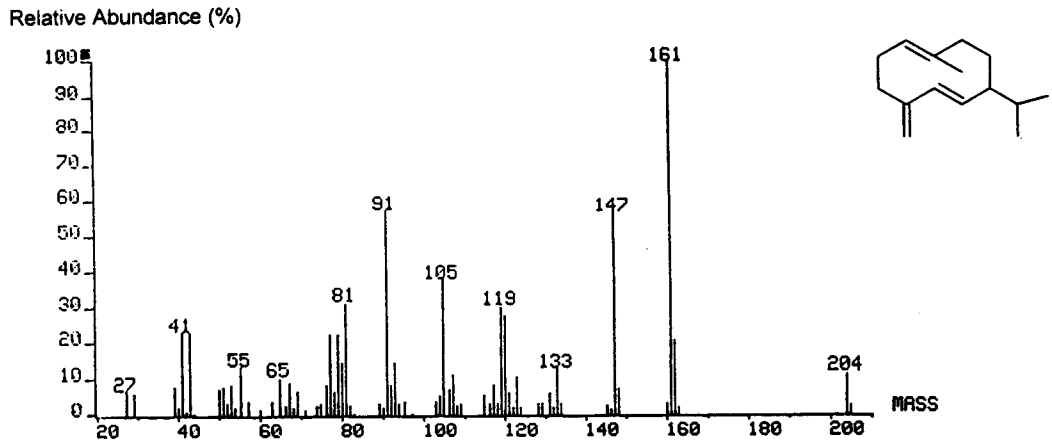

Figure A.26. Mass spectrum of GC-peak 41 in the gas chromatogram of *Olea europeae* (chapter 5, figure 5.7), tentatively identified as *D*-germacrene.