KUOPION YLIOPISTON JULKAISUJA C. LUONNONTIETEET JA YMPÄRISTÖTIETEET 184
KUOPIO UNIVERSITY PUBLICATIONS C. NATURAL AND ENVIRONMENTAL SCIENCES 184

MOHAMED AHMED IBRAHIM

Plant Essential Oils as Plant Protectants and Growth Activators

Doctoral dissertation

To be presented by permission of the Faculty of Natural and Environmental Sciences
of the University of Kuopio for public examination in Auditorium L3, Canthia building,
University of Kuopio, on Thursday 30th June 2005, at 12 noon

Department of Ecology and Environmental Science
University of Kuopio

KUOPION YLIOPISTO

KUOPIO 2005

Distributor: Kuopio University Library
P.O. Box 1627
FIN-70211 KUOPIO
FINLAND
Tel. +358 17 163 430
Fax +358 17 163 410
http://www.uku.fi/kirjasto/julkaisutoiminta/julkmyyn.html

Series Editors: Professor Pertti Pasanen, Ph.D.
Department of Environmental Sciences

Professor Jari Kaipio, Ph.D.
Department of Applied Physics

Author´s address: Department of Ecology and Environmental Science
University of Kuopio
P.O. Box 1627
FIN-70211 Kuopio
FINLAND
Tel. +358 17 163 205
Fax +358 17 163 230
E-mail: Mohamed.Ibrahim@uku.fi

Supervisors: Docent Jarmo Holopainen, Ph.D.
University of Kuopio

Professor Elina Oksanen, Ph.D.
University of Joensuu

Reviewers: Professor Riitta JulkunenTiitto, Ph.D.
University of Joensuu

Professor Jan Pettersson, Ph.D.
SLU, Swedish University of Agricultural Sciences
Uppsala, Sweden

Opponent: Professor Heikki Hokkanen, Ph.D.
University of Helsinki

ISBN 951-27-0342-4
ISBN 951-27-0017-4 (PDF)
ISSN 1235-0486

Kopijyvä
Kuopio 2005
Finland

Ibrahim, Mohamed Ahmed. Plant essential oils as plant protectants and growth activators. Kuopio University Publications C. Natural and Environmental Sciences 184. 2005. 143p.
ISBN 951-27-0342-4
ISBN 951-27-0017-4 (PDF)
ISSN 1235-0486

ABSTRACT

Introduction: The use of synthetic chemical insecticides for plant protection has created many problems in conventional farming system in the form of health hazards, deterioration of environment quality, development of resistance from various insect species, as well as interference with natural enemies of the pests and other non-target organisms. Furthermore, the use of synthetic pesticides is not permissible in organic farming. Therefore, the aims of this study were to investigate the effects of natural plant essential oils (PEO) focusing on the phytotoxic effects of monoterpene limonene, the impacts of the PEO on physiology and growth of crop plant, its efficiency to control major pests of strawberry, cabbage and carrot considering also its effects on non-target organisms such as natural enemies.

Materials and methods: In this study, the phytotoxicity of various concentrations of (R)-(+)-limonene sprayed on cabbage (cv Rinda and Lennox) and carrot (cvs. Parano and Splendid) plants was investigated. The effects of limonene, limonene + carvone and MeJA on pest insect species (*Galerucella sagittariae*, *Plutella xylostella* and *Trioza apicalis*) were studied in the laboratory. Furthermore, the influence of exogenously sprayed limonene on plant chemistry and physiology of carrot cultivars (Parano and Splendid) was studied to explore the possible effects of limonene on the thermotolerance of these plants. To detect plant physiological responses; net photosynthesis, stomatal conductance, and chlorophyll fluorescence were measured. Gas chromatography-mass spectrometry (GC-MS) was used to analyse the volatile organic compounds (VOCs) and plant extractives.

Results: No significant visible damage was observed at concentrations lower than 60 ml l^{-1} of limonene although the rate of damage was species- and cultivar-specific. *Galerucella sagittariae* larval feeding on strawberry was deterred by limonene, and its mixture with carvone (75:25%) (cv Jonsok). Limonene attracted *Plutella xylostella* and its parasitoid *Cotesia plutellae*, but the mixture with carvone was significantly repellent. Limonene stimulated *Trioza apicalis* females for oviposition. In addition, limonene was not sufficient to protect the carrot plants from stresses caused by high temperatures (>30°C). MeJA induced the emission of *E*,*E*-α-farnesene and DMNT from cabbage plants.

Conclusion: The positive indications of the mixture of limonene with carvone against *P. xylostella* found in this study can act as a stimulus for further research to find efficient essential oils which could be used in plant protection as alternatives to synthetic chemical pesticides in agriculture. However, due to species-specificity and the variable effects of PEOs on insect-plant interactions, the promising results obtained in cruciferous pests can not be generalised to the other crop plants. Therefore, the effects of PEOs on insect-insect interactions, their allelopathic effects and their effects on plants and other organisms should be studied carefully in the laboratory before they are subjected to field trials.

Universal Decimal Classification: 581.19, 581.5, 632.7, 632.93
CAB Thesaurus: essential oils; monoterpenes; limonene; methyl jasmonate; carvone; phytotoxicity; photosynthesis; chlorophyll; fluorescence; *Plutella xylostellae*; *Cotesia plutellae*; cabbages; carrots; volatile compounds; olfactometers; plant protection; natural enemies; heat stress

ACKNOWLEDGEMENTS

This study was carried out at the Department of Ecology and Environmental Science at the University of Kuopio, Finland and at the Agrifood Research Finland (MTT). I wish to acknowledge the financial support by Jenni and Antti Wihuri Foundation, Finnish Cultural Foundation, Ministry of Agriculture and Forestry, and University of Kuopio.

My deepest gratitude belongs to my main supervisor Docent Jarmo Holopainen for his excellent guidance, ideas, discussions, and for having introduced me to the field of plant essential oils. His encouragement and never ending support throughout this work is gratefully acknowledged.

I am also most grateful to my second supervisor Professor Elina Oksanen. Her excellent comments and helpful advices during the plant physiological experiments, data analysis and writing the papers were fundamental to the completion of this thesis.

My sincere thanks are due to the reviewers of this thesis, Professor Jan Pettersson and Professor Riitta Julkunen-Tiitto for their valuable comments and advice.

I owe my warmest thanks to the co-authors in the publications included in the thesis. I also thank to all the people who helped me with their ideas, time and efforts when I was doing the experiments. My particular thanks belong to the staff and colleagues at the Department of Ecology and Environmental Science for creating such a pleasant working environment. I also wish to acknowledge Ewen MacDonald for revision of the language.

The most crucial help and greatest encouragement were from my wife Halima. My reasons for gratitude to her are endless. She succeeded to fill my place in taking care of our children during my absence for my studies and work. Her patience through the entire work has enabled me to successfully complete this thesis.

I express my warmest thanks to my parents in-law for their help to our family during all these years. Finally, I wish to thank all my old and new friends, where ever they are.

Kuopio, June 2005

Mohamed Ahmed Ibrahim

ABBREVIATIONS

ADP	adenosine diphosphate
AS	asparagine synthetase
CT	control temperature
DBM	diamondback moth
DMNT	(*E*)-4,8-dimethyl-3,7-nonatriene
EOs	essential oils
F_m	maximal fluorescence level
F_v	variable fluorescence (F_m-F_0)
F_v/F_m	maximum photochemical yield of PSII (F_m-F_0)/Fm)
$gaikg^{-1}$	gram active ingredient per kilogram
GC-MS	gas chromatography- mass spectrometry
GDP	geranyl diphosphate
GLM	general linear models
GLVs	green leaf volatiles
hrs	hours
HT	high temperature
LOX	lipoxygenase
MVA	mevalonate pathway
MeJA	methyl jasmonate
NADH	nicotinamide adenine dinucleotide
PEOs	plant essential oils
PSII	photosystem 2
R	absolute configuration of group around chiral carbon
RGR	relative growth rate
RH	relative humidity
RGR	relative growth rate
VOCs	volatile organic compounds

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text according to their chapter numbers.

Chapter 2	Ibrahim MA, Kainulainen P, Aflatuni A, Tiilikkala K and Holopainen JK. 2001. Insecticidal, repellent, antimicrobial activity and phytotoxicity of essential oils: With special reference to limonene and its suitability for control of insect pests. Agricultural and Food Science in Finland 10: 243-259.
Chapter 3	Ibrahim MA, Oksanen EJ and Holopainen JK. 2004. Effects of limonene on growth and physiology of cabbage (*Brassica oleracea* L.) and carrot (*Daucus carota* L.) plants. Journal of the Science of Food and Agriculture 84: 1319-1326.
Chapter 4	Ibrahim MA, Nissinen A and Holopainen JK. 2005. Response of *Plutella xylostella* and its parasitoid *Cotesia plutellae* to volatile compounds. Journal of Chemical Ecology. In press.
Chapter 5	Nissinen A, Ibrahim M, Kainulainen P, Tiilikkala K and Holopainen JK. Essential oil composition in the headspace and leaves of carrot (*Daucus carota*) cultivars damaged by the carrot psyllid (*Trioza apicalis*) or treated exogenously with limonene or methyl jasmonate. Submitted.
Chapter 6	Ibrahim MA, Nissinen A, Prozherina N, Oksanen E and Holopainen JK. 2005. The influence of exogenous monoterpene treatment and elevated temperature on growth, physiology, chemical content and headspace volatiles of carrot (*Daucus carota* L.). Environmental and Experimental Botany. In press

CONTENTS

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

CHAPTER 7

Chapter 1

General Introduction

GENERAL INTRODUCTION

1.1 The importance of pest control in agriculture

Since the dawn of civilization, the man has been able to improve his living conditions by practising agriculture (Gupta 2004). Therefore, the control of insects, weeds, fungi and other pests of cultivated crops for food and fodder has been always represented a challenge to protect essential food sources. One of the ways to control damage to crop by infestations of pests is to use insecticides either synthetic or natural. It is obviously important that these insecticides should not be toxic to man or be harmful to the environment.

The use of synthetic chemicals in plant protection against weed and pest control has improved the crop production quantitatively as well as qualitatively but has created undesirable side effects in the form of health hazards, deterioration of environmental diversity and quality, development of resistance from various insect species, and these compounds may also target the natural enemies of the pests as well as other non-target organisms (Batish *et al.* 2002, Duke *et al.* 2003). For these reasons major efforts have been exerted to create or find alternative pesticides (Isman 2000, Duke *et al.* 2003, Lacey & Shapiro-Ilan 2003).

However, in spite of widespread concern about the long term impacts of synthetic pesticides on health and negative effects on the environment particularly in the developed world, natural pesticides have yet to make any major in roads into the market. In other words, for over three decades, the insecticide market has been dominated by three chemical classes; organophosphates, carbamates and synthetic pyrethroids, while only about 1% of the global insecticide market is occupied by the two bio-insecticides (*Bacillus thuringiensis* based products) and plant-origin insecticides (such as pyretherum based products) (Isman 2000). Toxicity and development of insect resistance issues have put restrictions on the use of these chemical insecticides. Therefore, it is clearly desirable to develop alternative methods for pest control, including both biocontrol agents and biologically derived pesticides which are safe to both humans and the environment (Sakamoto *et al.* 2003, Duke *et al.* 2003). For this reason, researchers need to re-evaluate the toxicity of plant-origin pesticides to non-target organisms as there are some examples that certain neem oil formulations can have negative effects on natural enemies (Forget 1991, Bottrell 1996).

The thesis discusses how to discover new forms of insect control in agriculture, principally in organic farming system. It focuses on the control of vegetable pests using plant essential oils (PEOs), specifically monoterpene limonene investigating their various effects on the host plant, insect herbivore and the natural enemies.

1.2 The possible role of plant essential oils in plant protection

Plant extracts have been used as insecticides since before the ancient Romans, that civilization is renowned for having made extensive usage of these biologically active compounds (Franzios *et al.* 1997). These compounds include EOs which could be potential alternatives to the currently used chemical insecticides since there is now a desire for safer insecticides to the man, animals and plants (Franzios *et al.* 1997, Heldt 1998). The EOs have evolved as plants have designed to make themselves indigestible to animals feeding on them or conversely they may interact with other organisms for self defence. Furthermore, PEOs, could be a source of bioactive chemicals such as alkaloids, flavonoids and terpenoids against insects and pathogenic micro-organisms (Heldt 1998, Kim *et al.* 2003).

The vast majority of substances reported to have deterrent effects against herbivore insects have been isolated from plants and they can be found in all the major classes of secondary metabolites (Isman 2002). The PEOs are aroma components often responsible for characteristic plant odours (Bouwmeester *et al.* 1998) and they can be obtained by steam distillation of plant foliage or from the foliage itself of certain aromatic plants of Myrtacaeae and Lamiaceae family (Isman 2000, Hummelbrunner and Isman 2001). However, Pickett *et al.* (1997) suggested if the potential plant secondary metabolites are to be applied for crop protection, the trophic interactions of insect-insect and insect-plant mediated by plant secondary compounds (particularly EOs) need to be studied first.

1.3 The formation of monoterpenes in plants

Terpenoids represent the largest group of known plant chemicals which contains many volatile representatives. This large group of plant terpenoids includes isoprenes (C_5) which represent the basic and simplest terpenoid (hemiterpenes), monoterpenes (C_{10}), homoterpenes (C_{11}, C_{16}), sesquiterpenes (C_{15}) (Heldt 1998, Mahmoud and Croteau 2002, Dudareva *et al.* 2004). The mevalonate (MVA) pathway from acetyl-CoA which is localised in cytosol, and the methylerythritol phosphate (MEP) pathway from pyruvate which is localised in plastids are the two routes for the formation of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), the precursors of terpenoids (Dudareva *et al.* 2004). However, the MEP pathway is considered to be the principle pathway to provide hemiterpenes, monoterpenes and diterpenes while MVA is responsible for the biosynthesis of sesquiterpenes (Dudareva *et al.* 2004). Nevertheless, a cross-talk interaction between both pathways (cytosolic and plastidial) has been found (Laule *et al.* 2003). On the other hand, it was reported that a second type of cross-talk interaction between plant defence pathways (jasmonate and salicylate) which should not be confused with the previous one. This latter theory indicates that deploying defences against one attacker can positively or negatively affect other attackers (Thaler 2002). Jasmonate and salicylate pathways are two of the biochemical response mechanisms that can be activated by various attackers (McConn *et al.* 1997). Terpenoids in general are synthesized in various plant cellular organelles but are then stored in specialized secretory structures (such as leaf trichomes, resin ducts, laticifers) to protect the metabolic processes occurring in the plants from the toxic effects of these secondary metabolites (Langenheim 1994).

Monoterpenes are formed from two isoprene units having 10 carbon atoms (Gershenzon and Croteau 1993, Heldt 1998, Mahmoud and Croteau 2002). Monoterpenes are the main constituents of PEOs and they are all derived from the same precursor, the C_{10} acyclic intermediate geranyl diphosphate (GDP) (condensation of two C_5 units) (Tholl *et al.* 2004, Dudareva *et al.* 2004) converting this compound into the end monoterpene product (Dudareva *et al.* 2004). These reactions, the formation of basic C_5 units, their condensations to form GDP and the synthesis of the end products (monoterpenes) are mediated by a large group of enzymes called terpene synthases (Trap and Croteau 2001). Monoterpenes are produced in both angiosperm and gymnosperm plants (Trap and Croteau 2001) but with different degrees of occurrence (Langenheim 1994).

1.4 Insecticidal, repellent, and deterrent effects of monoterpenes

Terpenes in general can have various functions in the plant: they are toxic or make the plant indigestible to herbivores, antibiotics against pathogenic micro-organisms, and inhibiting germination and development of competing plants. Additionally, they are attractants to insect pollinators and some of them are used as natural insecticides (e.g. pyrethrin) while many of them are important components of food, beverages, cosmetics, and several vitamins (A, D, E) (Mahmoud and Croteau 2002, Dudareva *et al.* 2004).

Monoterpenes serve as plant defence against insect herbivores, plant pathogens or competing plant species (Regnault-Roger 1999, Grodnitzky and Coats 2002) and this means that they may be additionally useful as natural pesticides in organic farming systems (Isman 2002, Tworkoski 2002). They are very important for the plants, as they can attract and guide insect pollinators (Grodnitzky and Coats 2002, Reinhard *et al.* 2004) and help plants to defend themselves (Grodnitzky and Coats 2002, Kim *et al.* 2003, Miyazawa *et al.* 2004) because of their volatility (Grodnitzky and Coats 2002).

The activities and the role of the monoterpenes in plant protection have been studied by some researchers who have examined their insecticidal activities and a few of these insecticides are currently being used commercially as pesticides and repellents (d-limonene, menthol, citronellal and linalool) predominantly against household insects such as cockroaches and mosquitoes (Tsao and coats 1995). It has been reported that several monoterpenes (e.g. limonene, α-pinene, β-pinene, and myrcene) are toxic to many insects and insect herbivores and the monoterpene, menthol, has been claimed to be a potential insect repellent (Heldt 1998).

It was recently reported, that plant volatiles induced by herbivore damage serve as indirect plant defences (Dudareva *et al.* 2004) attracting predators or parasitoids of the insect herbivores (Dicke and Van Loon 2000). Interestingly, in some cases those herbivore-induced volatiles may also act as direct defences, exhibiting a repellent effect against the insect herbivores (De Moraes *et al.* 2001). In this respect De Moraes *et al.* (2001) found that plant volatiles induced by caterpillar attack are repellent to female moths of polyphagous tobacco budworm *Heliothis virescens* (Fabricius) (Lepidoptera: Noctuidae) and thus they guide conspecific females not to lay eggs on occupied plants. Christmas beetles (Anoplognathus spp., Coleoptera: Scarabaeidae) preferred feeding on non-host plants (*Schinus molle* (Anacardiaceae)) over their host plants (species of *Eucalyptus* (Myrtaceae)) due to the absence of monoterpene 1,8-cineole in *S. molle* (Steinbauer *et al.* 2002). Previously, monoterpenes which are thought to function principally in ecological roles, serving as inhibitors of herbivore feeding (Langenheim 1994) have been used as deterrents (Ntiamoah *et al.* 1996; Ntiamoah and Borden, 1996, Tiberi *et al.* 1999, Klepzig and Schlyter, 1999, Frank *et al.* 2002, Tripathi *et al.* 2003) or as repellents (Den Ouden *et al.* 1993, Dormont *et al.*1997, Luik *et al.* 1999, Choi *et al.* 2002). However, different activities of PEOs, especially, towards crop pests need to be investigated in detail to identify novel, potentially environmental friendly natural products for crop protection in agriculture.

1.5 Phytotoxic effects of monoterpenes

Monoterpenes (such as carvone, citronellol, fenchone, geraniol, and pulegone) have shown herbicidal activity exhibiting the inhibition or retarding of seed germination (Vaughn and Spencer 1996, Dudai *et al.* 1999, Singh *et al.* 2002, Vokou *et al.* 2003), inhibiting seedling growth (Singh *et al.* 2002, Vokou *et al.* 2003) or destroying the complete weed shoot development (Tworkoski 2002). Similarly, several other monoterpenes (camphor, pulegone, α- and β- pinene, carvone) have been shown to have phytotoxic effects on weeds and crop plants (Vaugn and Spencer 1993, Lee *et al.* 1997, Abrahim *et al.* 2000). The EOs of Kenaf (*Hibiscus cannabinus*) leaves have been found to exhibit phytotoxicity on lettuce (*Lactuca sativa*) and bentgrass (*Agrostis stolonifera*) (Kobaisy *et al.* 2001).

Although the mechanism by which PEOs affect plants is not well understood, several laboratory studies have suggested that PEOs (including monoterpenes such as 1,4-cineole and 1,8-cineole) inhibit certain phases of mitosis (Vaughn and Spencer 1993, Romagni *et al.* 2000a), and induce photosynthetic stress in the plants (Romagni *et al.* 2000a). It has been suggested that several monoterpenes (α- pinene, 1,4-cineole, 1,8-cineole, camphor, eucalyptol, limonene) may impair the mitochondrial respiration (Peñuelas *et al.* 1996, Abrahim *et al.* 2000, Abrahim *et al.* 2003, Weir *et al.* 2004). 1,4-cineole and 1,8-cineole have been found to be potent inhibitors of asparagine synthetase (AS) which is a key enzyme of asparagine biosynthesis in plants (Romagni *et al.* 2000b). However, to understand the effects and mode of action of monoterpenes in the plants further studies are required.

1.6 Allelopathic communication between plants

Monoterpenes are well known to play an important role in allelopathic communication between plants (Mahmoud and Croteau 2002, *Singh et al.* 2004). The allelopathic phenomenon could be explained the possible effect of compounds released by a plant on the growth of other plants. These compounds can be released into the surrounding environment in different ways such as volatilization, leaching by rain, decomposition of plant residues in the soil, or exudation from the roots (Ninkovic 2003, Weir *et al.* 2004). The germination and the growth of the roots and shoots of the receiving plants can be affected positively or negatively by the released compounds from the other neighbouring plants (Mizutani 1999). However, the release of allelopathic compounds by the plants into the surrounding environment is thought to represent one way for the plant to create around itself an environment detrimental to the development of other species and consequently this will be a benefit to the plant in the struggle for survival (Angelini *et al.* 2003). For example, the allelochemicals (monoterpenes, sesquiterpenes and flavones) of *Ageratum conyzoides* L. inhibited the germination and growth of associated plants, as well as affecting microbes and insects (Kong *et al.* 2004, Angelini *et al.* 2003). Recently, aerial communication between plants via volatilization has been verified by Ninkovic (2003) who described plant-plant communication between two barley cultivars which led to changes in the pattern of biomass allocation in plants of the second cultivar. However, it has also been reported that aerial allelopathy does not affect significantly the total biomass production (Ninkovic 2002). Previously, Pettersson *et al.* (1999) have shown that the volatiles from one barley cultivar affect the herbivore acceptance of another barley cultivar. Furthermore, the allelopathic effects can influence the behaviour of neighbouring plant herbivores (Glinwood *et al.* 2003). However, it was verified experimentally that the allelopathy phenomenon was present not only in laboratory under optimal conditions but also in field conditions (Pettersson 1999, Ninkovic *et al.* 2002) although the situation is more complex in the field than in the laboratory (Ninkovic 2002). The studies on the phytotoxic effects of PEOs mentioned above (section 1.5, Chapter 1) are also one kind of allelopathic communication between the plants (see also Chapter 3).

Table1: Summary of the experimental layout in the experiments with cabbage and carrot plants.

Study	Plant species	Tested compounds	Application method and way of analysis	Insects	Chapters to be referred
1.Phytotoxic effects: Visible symptoms, plant physiology parameters	Cabbage and carrot	Limonene	Spraying Photosynthesis, stomatal conductance, chlorophyll fluorescence	No	3
2. Pest responses: Ovipositioning, orientation behaviour, larval feeding , VOC collection	Cabbage	Limonene, limonene + carvone, and Methyl-jasmonate	Spraying, releasing from vermiculite, applied on filter paper Cage experiments Y-tube olfactometer Volatiles collected in adsorbent and analysed with GC-MS.	*Plutella xylostella* & *Cotesia plutellae (specialist* natural enemy of *plutella xylostella)*	4
3. Effects of essential oils (PEOs) on herbivore: Ovipositioning, VOC collection, chemical content	Carrot	Limonene & methyl jasmonate	Spraying Cage experiments Volatiles collected in adsorbent and analysed with GC-MS. Hexane extraction of leaves and analyses with GC-MS	Carrot psyllids (*Trioza apicalis*)	5
4. Thermotolerance: Influence of limonene and high temperature; Physiological parameters VOCs collections and their analysis Chemical content	Carrot	Limonene	Spraying Photosynthesis, stomatal conductance, chlorophyll fluorescence Volatiles collected in adsorbent and analysed with GC-MS Hexane extraction of leaves and roots and analyses with GC-MS	No	6

1.7 Aims of the study

PEOs are important plant secondary compounds and are one of the potential sources of safe and cheap plant origin pesticides to be used not only in organic farming but also in conventional farming system. The aim of the study was to investigate the effects of these oils, focusing on the phytotoxic effects of the monoterpene limonene, its impacts on physiology and growth of crop plant, its efficiency to control major pests of cabbage and carrot considering also its effects on non-target organisms such as natural enemies. Initially, I will review our current knowledge on the use of limonene as a plant protectant in agriculture and forestry, and will evaluate the potential control of crop pests with limonene using strawberry as an example crop (Chapter 2). The influence of prophylactic limonene sprayings on cabbage and carrot growth and physiology was evaluated in the first experimental study (Chapter 3). In the second experiment, responses of diamondback moth (DBM) (*Plutella xylostella*) - one of the most difficult pest to be controlled throughout the world - to limonene, and to a mixture of limonene and carvone were studied (Chapter 4). In addition, the effects of limonene on natural enemies of pest insects, the response of the DBM specialist parasitoid *Cotesia plutellae* were investigated (Chapter 4). In the third experiment, control of a serious carrot pest, carrot psyllid (*Trioza apicalis*) using PEOs, and the composition of carrot EOs after carrot psyllid damage, or after exogenous application of limonene or methyl jasmonate were studied (Chapter 5). The last experiment investigated whether limonene sprayed on plants exogenously could have any beneficial side effects by increasing the tolerance of the plants against higher temperatures (Chapter 6).

References

Abrahim D, Braguini WL, Kelmer-Bracht AM and Ishii-Iwamoto EL. 2000. Effects of four monoterpenes on germination, primary root growth, and mitochondrial respiration of maize. Journal of Chemical Ecology 26: 611-624.

Abrahim D, Francischini AC, Pergo EM, Kelmer-Bracht AM and Ishii-Iwamoto EL. 2003. Effects of α-pinene on the mitochondrial respiration of maize seedlings. Plant Physiology and Biochemistry 41: 985-991.

Angelini LG, Carpanese G, Cioni PL, Morelli I, Macchia M and Flamini G. 2003. Essential oils from Mediterranean lamiaceae as weed germination inhibitors. Journal of Agricultural and Food Chemistry 51: 6158-6164.

Batish DR, Singh HP, Saxena DB and Kohli RK. 2002. Weed suppressing ability of parthenin-A sesquiterpene lactone from partheium hysterophorus. New Zealand Plant Protection 55: 218-221.

Bottrell DG. 1996. The research challenge for integrated pest management in developing countries: A prospective for rice in Southeast Asia. Journal of Agricultural Entomology 13: 185-193.

Bouwmeester HJ, Gershenzon J, Konings MCJM and Croteau R. 1998. Biosynthesis of the monoterpenes limonene and carvone in the fruit of caraway. Plant Physiology 117: 901-912.

Choi WS, Park BS, Ku SK and Lee SE. 2002. Repellent activities of essential oils and monoterpenes against *Culex pipiens pallens*. Journal of the American Mosquito Control Association 18: 348-351.

De Moraes, CM, Mescher MC and Tumlinson JH. 2001. Caterpillar-induced nocturnal plant volatiles repel conspecific females. Nature 410:577-580.

Den Ouden H, Visser JH, Alkema DPW, De Vlieger JJ and Derks PSM. 1993. Experiments with volatile substances in slow release formulations causing repellency for oviposition of cabbage root fly, *Phorbia brassicae* Bché. (Dipt., Anthomyiidae). Journal of Applied Entomology 115, 307-312.

Dicke M and Van Loon JJA. 2000. Multitrophic effects of herbivore-induced plant volatiles in an evolutionary context. Entomologia Experimentalis et Applicata 97: 237-249.

Dormont L, Roques A and Malosse C. 1997. Efficiency of spraying mountain pine cones with oleoresin of Swiss stone pine cones to prevent insect attack. Journal of Chemical Ecology 23: 2261-2274.

Dudai NA, Poljakoff-Mayber A, Mayer M, Putievsky E and Lerner HR. 1999. Essential oils as allelochemicals and their potential use as bioherbicides. Journal of Chemical Ecology 25: 1079-1089.

Dudareva N, Pichersky E and Gershenzon J. 2004. Biochemistry of plant volatiles. Plant Physiology 135: 1893-1902.

Duke S, Baerson SR, Dayan FE, Rimando AM, Scheffler BE, Tellez MR, Wedge DE, Schrader KK, Akey DH, Arthur FH, De Leucca AJ, Gibson DM, Harrison, HF, Peterson JK, Gealy DR, Tworkoski T,Wilson CL and Morris JB. 2003. United States Department of Agriculture-Agricultural Research Service research on natural products for pest management. Pest Management Science 59: 708-717.

Forget G. 1991. Pesticides and the third world. Journal of Toxicology and Environmental Health 32: 11-31.

Frank T, Bieri K and Speiser B. 2002. Feeding deterrent effect of carvone, a compound from caraway seeds, on the slug *Arion lusitanicus*. Annals of Applied Biology 141: 93-100.

Franzios G, Mirotsou M, Hatziapostolou E, Kral J, Scouras ZG and Mavragani-Tsipidou P. 1997. Insecticidal and genotoxic activities of mint essential oils. Journal of Agricultural and Food Chemistry 45:2690-2694.

Gershenzon J and Croteau R. 1993. Terpenoid biosynthesis: the basic pathway and formation of monoterpenes, sesquiterpenes, and diterpenes. In: Moore T., Eds, Lipid metabolism in plants, CRC Press, Boca Raton, Florida, 333-338.

Glinwood R, Pettersson J, Ahmed E, Ninkovic V, Birkett M and Pickett J. 2003. Change in acceptability of Barley plants to aphids after exposure to allelochemicals from couch-grass (*Elytrigia repens*). Journal of Chemical Ecology 29: 261-274

Grodnitzky JA and Coats JR. 2002. QSAR evaluation of monoterpenoids' insecticidal activity. Journal of Agricultural and Food Chemistry 50: 4576-4580.

Gupta PK. 2004. Pesticide exposure-Indian scene. Toxicology 198: 83-90.

Heldt H-W.1998. Plant biochemistry and Molecular Biology. Chapter 16-17.Oxford University Press (Sd), pp 522.

Hummelbrunner LA and Isman MB. 2001. Acute, sublethal, antifeedant, and synergistic effects of monoterpenoid essential oil compounds on the tobacco cutworm, *Spodoptera litura* (Lep. Noctuidae). Journal of Agricultural and Food Chemistry 49: 715-720.

Isman MB. 2000. Plant essential oils for pest and disease management. Crop Protection 19: 603-608

Isman MB. 2002. Insect antifeedants. Pesticide Outlook 13: 152-157.

Jasicka-Misiak I., Lipok K and Kafarski P. 2002. Phytotoxic activity of the carrot (*Daucus carota* L.) seed oil and its major components. Journal of Essential Oil Bearing Plants 5: 132-134.

Kim SI, Roh JY, Kim DH, Lee HS and Ahn YJ. 2003. Insecticidal activities of aromatic plant extracts and essential oils against *Sitophilus oryzae* and *Callosobruchus chinensis*. Journal of Stored Products Research 39: 293-303.

Klepzig KD and Schlyter F. 1999. Laboratory evaluation of plant-derived antifeedants against the pine weevil *Hylobius abietis* (Coleoptera: Curculionidae). Journal of Economic Entomology 92: 644-650.

Kobaisy M, Tellez MR, Webber CL, Dayan FE, Schrader KK and Wedge DE. 2001. Phytotoxic and fungitoxic activities of essential oil of Kenaf (*Hibiscus cannabinus* L.) leaves and its composition. Journal of Agricultural and Food Chemistry 49: 3768-3771.

Kong CH, Hu F, Xu XH, Liang WJ and Zhang CX. 2004. Allelopathic plants XV. *Ageratum conyzoides* L. Allelopathy Journal 14: 1-12.

Lacey LA & Shapiro-Ilan DI. 2003. The potential role for microbial control of orchard insect pests in sustainable agriculture. Journal of Food, Agriculture and Environment 1: 326-331.

Langenheim JH. 1994. Higher plant terpenoids: A phytocentric overview of their ecological roles. Journal of Chemical Ecology 20: 1223-1280.

Laule O, Furholz, A, Chang, HS, Zhu T, Wang X, Heifetz PB, Gruissem W and Lange BM. 2003. Crosstalk between cytosolic and plastidial pathways of isoprenoid biosynthesis in *Arabidopsis thaliana*. Proceedings of the National Academy of Sciences of the USA 100: 6866-6871.

Lee S, Tsao R, Peterson C and Coats JR. 1997. Insecticidal activity of monoterpenoids to western corn rootworm (Coleoptera: Chrysomelidae), twospotted spider (ACari: Tetranichidae), and house fly (Diptera:Muscidae), Journal of Economic Entomology 90: 883-892.

Liuk A, Ochsner P and Jensen TS. 1999. Olfactory responses of seed wasps *Megastigmus pinus* Parfit and *Megastigmus rafni* Hoffmeyer (Hym., Torymidae) to host-tree odours and some monoterpenes. Journal of Applied Entomology 123: 561-567.

Mahmoud SS and Croteau RB. Strategies for transgenic manipulation of monoterpene biosynthesis in plants. Trends in Plant Science 7: 366-373.

McConn M, Creelman RA, Bell E, Mullet JE and Browse J. 1997. Jasmonate is essential for insect defense in Arabidopsis. Proceedings of the National Academy of Science of the USA 94: 5473-5477.

Miyazawa M, Tsukamoto T, Anzai J and Ishikawa Y. 2004. Insecticidal effect of phthalides and furanocoumarins from Angelica acutiloba against Drosophila melanogaster. Journal of Agricultural and Food Chemistry 52:4401-4405.

Mizutani J. 1999. Selected allelochemicals. Critical Reviews in Plant Sciences 18: 653-671.

Ninkovic V. 2002. PhD thesis: Plant volatiles mediate tritrophic interactions. Swedish University of Agricultural Sciences,Uppsala.

Ninkovic V. 2003. Volatile communication between plants affects biomass allocation. Journal of Experimental Botany 54: 931-1939.

Ninkovic V, Olsson U and Pettersson J. 2002. Mixing barley cultivars affects aphid host plant acceptance in field experiments. Entomologia Experimentalis et Applicata 102: 177-182.

Ntiamoah YA and Borden JH. 1996. Monoterpene oviposition deterrents for cabbage maggots, *Delia radicum* (L.) (Diptera:Anthomyiidae). Canadian Entomologist 128: 351-352.

Ntiamoah Ya, Borden JH and Pierce HD Jr. 1996. Identity and bioactivity of oviposition deterrents in pine oil for the onion maggot, *Delia antiqua.* Entomologia Experimentalis et Applicata 79: 219-226.

Pettersson J, Ninkovic V and Ahmed A. 1999. Volatiles from different barley cultivars affect aphid acceptance of neighbouring plants. Acta Agriculturae Scandinavica. Section B. Soil and Plant Science 49: 152-157.

Peñuelas J, Ribas-Carbo M and Giles L. 1996. Effects of allelochemicals on plant respiration and oxygen isotope fractionation by the alternative oxidase. Journal of Chemical Ecology 22: 801-805.

Pickett JA, Wadhams LJ and Woodcock CM. 1997. Developing sustainable pest control from chemical ecology. Agriculture, Ecosystems and Environment 64: 149-156.

Regnault-Roger C. 1999. Diversification of plants protection strategies: Interest of monoterpenes. Acta Botanica Gallica 146: 35-41.

Reinhard J, Srivivasan MV and Zhang S. 2004. Scent-triggered navigation in honeybees. Nature 427:411.

Romagni JG, Allen SN and Dayan FE. 2000a. Allelopathic effects of volatile cineoles on two weedy plant species. Journal of Chemical Ecology 26: 303-313.

Romagni JG, Duke SO and Dayan FE. 2000b. Inhibition of plant asparagine synthetase by monoterpene cineoles. Plant Physiology 123: 725-732.

Sakamoto N, Saito Sh, Taro H, Suzuki M, Matsuo S, Izumi K, Negatomi T, Ikegami H, Umeda K, Tsushima K and Matsuo N. 2003. The discovery of pyridalyl: a novel insecticidal agent for controlling lepidopterous pests. Pest Management Science 60: 25-34.

Singh HP, Batish DR and Kohli RK. 2002. Allelopathic effect of two volatile monoterpenes against bill goat weed (*Ageratum conyzoides* L.). Crop Protection 21: 347-350.

Singh HP, Batish DR, Kaur S, Vaid S and Kohli R. 2004. Weed suppressing ability of some monoterpenes. ZEITSCHRIFT FUR PFLANZENKRANKHEITIN UND PFLANENSCHUTZ- Journal of Plant Diseases and Protection 19: 821-828.

Steinbauer MJ and Wanjura WJ. 2002. Christmas beetles (*Anoplognathus* spp., Coleoptera: Scarabaeidae) mistake peppercorn trees for eucalypts. Journal of Natural History 36: 119-125.

Thaler JS, Karban R, Ullman DE, Boege K and Bostock RM.2002. Cross-talk between jasmonate and salicylate plant defense pathways: effects on several plant parasites. Oecologia 131: 227-235.

Tholl D, Kish CH, Orlova I, Sherman D, Gershenzon J, Picherschy E and Dudareva N. 2004. Formation of monoterpenes in *Antirrhinum majus* and *Clarkia breweri* flowers involves heterodimeric Geranyl Diphosphate synthases. The Plant Cell 16: 977-992.

Tiberi R, Niccoli A, Curini, M, Epifano F, Marcotullio MC and Rosati O. 1999. The role of the monoterpene composition in Pinus spp. needles, in host selection by the pine processionary caterpillar, *Thaumetopoea pityocampa*. Phytoparasitica 27: 263-272.

Trap SC and Croteau RB. 2001. Genomic organization of plant terpene synthases and molecular evolutionary implications. Genetics 158: 811-832.

Tripathi AK, Prajapati V and Kumar S. 2003. Bioactivities of l-carvone, d-carvone, and dihydrocarvone toward three stored product beetles. Journal of Economic Entomology 96: 1594-1601.

Tsao R and Coats JR. 1995. Starting from nature to make better insecticides. Chemtech 25: 23-28.

Tworkoski T. 2002. Herbicide effects of essential oils. Weed science 50: 425-431.

Vaughn SF and Spencer GF. 1993. Volatile monoterpenes as potential parent structure for new herbicides. Weed Science 41: 114-119.

Vaughn SF and Spencer GF. 1996. Synthesis and herbicidal activity of modified monoterpenes structurally similar to cinmethylin. Weed Science 44: 7-11.

Vokou D, Douvli P, Blionis G and Halley JM. 2003. Effects of monoterpenoids, acting alone or in pairs, on seed germination and subsequent seedling growth. Journal of Chemical Ecology 29: 2281-2301.

Weir TL, Park S-W and Vivanco JM. 2004. Biochemical and physiological mechanisms mediated by allelochemicals. Current Opinion in Plant Biology 7: 472-479.

Chapter 2

Insecticidal, repellent, antimicrobial activity and phytotoxicity of essential oils: With special reference to limonene and its suitability for control of insect pests

Ibrahim, M. A., Kainulainen, P., Aflatuni, A., Tiilikkala, K. and Holopainen, J. K.
Agricultural and Food Science in Finland, 10: 243-259.

AGRICULTURAL AND FOOD SCIENCE IN FINLAND

Vol. 10 (2001): 243–259.

Review

Insecticidal, repellent, antimicrobial activity and phytotoxicity of essential oils: With special reference to limonene and its suitability for control of insect pests

Mohamed A. Ibrahim
Department of Ecology and Environmental Science, University of Kuopio, PO Box 1627,
FIN-70211 Kuopio, Finland, e-mail: ibrahim@uku.fi
Pirjo Kainulainen
MTT Agrifood Research Finland, Plant Production Research, Plant Protection, FIN-31600 Jokioinen, Finland.
Current address: Department of Ecology and Environmental Science, University of Kuopio, PO Box 1627,
FIN-70211 Kuopio, Finland
Abbas Aflatuni
MTT Agrifood Research Finland, Regional Research, Tutkimusasemantie 15, FIN-92400 Ruukki, Finland
Kari Tiilikkala
MTT Agrifood Research Finland, Plant Production Research, Plant Protection, FIN-31600 Jokioinen, Finland
Jarmo K. Holopainen
MTT Agrifood Research Finland, Plant Production Research, Plant Protection, FIN-31600 Jokioinen, Finland.
Current address: Department of Ecology and Environmental Science, University of Kuopio, PO Box 1627,
FIN-70211 Kuopio, Finland

The interest in the use of monoterpenes for insect pest and pathogen control originates from the need for pesticide products with less negative environmental and health impacts than highly effective synthetic pesticides. The expanding literature on the possibility of the use of these monoterpenes is reviewed and focused on the effects of limonene on various bioorganisms. Limonene is used as insecticide to control ectoparasites of pet animals, but it has activity against many insects, mites, and microorganisms. Possible attractive effects of limonene to natural enemies of pests may offer novel applications to use natural compounds for manipulation of beneficial animals in organic agriculture. However, in few cases limonene-treated plants have become attractive to plant damaging insects and phytotoxic effects on cultivated plants have been observed. As a plant-based natural product limonene and other monoterpenes might have use in pest and weed control in organic agriculture after phytotoxicity on crop plants and, effects on non-target soil animals and natural enemies of pest have been investigated.

Key words: monoterpenes, limonene, essential oil, natural pesticides, plant protection, deterrent, insect control

Manuscript received April 2001

Introduction

Historical perspective

Essential oil-bearing plants have been collected from the forest and cultivated areas since pre-Christian time for their flavour and fragrance properties. Their volatility, which made them easy to discover in fragrant plant material and at the same time readily obtainable by simple distillation of plant parts, lent to them the term essential oil. The monoterpenes with to a lesser extent the sesquiterpenes, comprise the major components of essential oils. Monoterpenes operate as a chemical defence against herbivores and diseases, fragrances attractive to pollinators and allelopathic inhibition of seed germination and plant growth (Gershenzon and Croteau 1991, Langenheim 1994). Well-documented records show that before 1850, 20 plant species belonging to 16 different families were used for control of agricultural and horticultural pests in Western Europe and China (Needham 1986, Smith and Secoy 1981). The rich knowledge of plants with pesticide properties was not lost in China as evidenced by a recent report stating that in China different parts or extracts of 276 plant species are used as pesticides (Yang and Tang 1988).

Insecticidal properties have been recognized in the oil of many citrus fruits and in recent years, several products containing (+)-limonene, linalool, or a crude citrus oil extract have worked their way into the market place. Two of these oils, (+)-limonene and linalool are long-standing and widely used for food additives (Hooser 1990).

Chemical properties of monoterpenes

Terpenes are hydrocarbons classified by the number of five-carbon (isoprene) units that they contain. Monoterpenes contain a basic skeleton of 10-carbon atoms derived from of fusion of two C_5 isoprene units. The other classes of terpenoids are sesquiterpenes (C_{15}), diterpenes (C_{20}), triterpenes (C_{30}), tetraterpenes (C_{40}) and polyterpenes (C_n). The plastids of plants are regarded to be the site of monoterpene synthesis. All terpenoid compounds are biosynthesised from isopentenyl diphosphate (IPP), which may be derived by acetate/mevalonate or pyruvate/glyceraldehydes-3-phosphate pathway. The head-to-tail condensation of one molecule of IPP with one molecule of dimethylallyl diphosphate (DMAPP), itself derived from the reversible isomerization of IPP by IPP isomerase yields the C_{10} compound geranyl diphosphate (GPP) which is the immediate precursor of the monoterpenes (Lichtenthaler et al. 1997, Little and Croteau 1999).

Hydrocarbon variations that differ only in the arrangement of atoms are called isomers. Description of isomers and their chemical and biological functions are summarized in Table 1 according to Fessenden et al. (1998). Enantiomers are chiral molecules that have the same molecular formula but are mirror images of each other. Absolute configuration of chiral carbon (R or S) is not dependent on the direction of optical rotation (+ or –), but in each specific compound are always related, e.g. limonene has two enantiomers (R)–(+)-limonene and (S)–(–)-limonene, but the enantiomers of carvone are (R)–(–)-carvone and (S)–(+)-carvone. In this review we use (+ and –)-nomenclature, since optical isomers are related closely to the biological activity of monoterpenes. Even human sense of smell is able to discriminate the odours of the enantiomers of α-pinene, carvone and limonene. R-limonene is the smell of lemon or orange, while smell of S-limonene is close to that of pine turpentine (Laska and Teubner 1999).

Natural occurrence and functions of monoterpenes

More than 1000 naturally occurring monoterpenes have been isolated from higher plants (Gershenzon and Croteau 1991). Monoterpenes are volatile and responsible for the characteristic

Table 1. Description of isomers summarized from Fessenden et al. (1998) and chemical designation of some monoterpenes.

Type of the isomers	Nomenclature		Description
	Old	New	
A. Structural isomers			Different compounds that have the same molecular formula, but differ in order of attachment of atoms
B. Stereoisomers 1. Enantiomers (optical isomers)			Chiral molecules are mirror images of each other. Chemical properties are similar, but physiological properties differ.
	D L	R S	The prefixes R (clockwise) and S (counter clockwise) indicate absolute configuration of group around chiral carbon.
	D L	+ –	The prefixes (+)- and (–)- are used to designate the sign of optical rotation of plane-polarized light by the enantiomers
2. Diastereomers including achiral geometric isomers	*Cis* *Trans*	*Z* *E*	Molecules are not mirror images of each other. Geometric isomers result from groups being Z (same) and E (opposite) side.

odours of many plants. Most monoterpenes occur free in plant tissue, but some of them are found as glycosides. The monoterpenes occur in a variety of acyclic, monocyclic, bicyclic, and tricyclic structural types and derivates, representing one of the largest and most diverse families of natural compounds (Croteau 1987). They exist as hydrocarbons or as oxygenated moieties with aldehyde, alcohol, ketone, ester, and ether functionalities. Overall monoterpenes are insoluble in water, however, monoterpenes containing oxygen have greater solubility than hydrocarbons with comparable skeletons (Weidenhamer et al. 1993).

Because of lipophilic properties most of monoterpenes are stored in special structures as resin ducts, secretory cavities and epidermal glands (Dell and McComb 1981). Monoterpenes are most widely recognized constituents of conifers, mints (Lamiaceae), composites (Asteraceae), and citrus (Rutaceae). α-pinene and β-pinene are among the most widely distributed monoterpenes in the plant kingdom and are the major constituents of the various volatile oils (Schütte 1984). Overall, the variability in essential oil composition is determined both by genetic and epigenetic factors.

Generally, plants can produce a diverse range of secondary metabolites such as terpenoids, phenolic compounds and alkaloids (Benner 1993). Terpenoids are among the vast reservoir of secondry compounds produced by higher plants evolved in defence against herbivores and pathogens (Duke et al. 1991). Monoterpenes may interfere with basic behavioural functions of insects (Brattesten 1983). Some exhibit acute toxicity whereas others are repellents (Watanabe et al. 1993), antifeedants (Hough-Goldstein 1990), or disrupt on growth and development (Karr and Coats 1992) or reproduction (Sharma and Saxena 1974) and, interfere with physiological and biochemical processes (Gershenzon and Croteau 1991).

Since monoterpene composition of plant species is very distinctive, specialist herbivore insects that have only one plant species or one plant genus as host plant, use highly volatile monoterpenes as a cue to locate their specific host plant.

For many oligophagous and polyphagous insect herbivores monoterpenes have been demonstrated to act as toxins, feeding and oviposition deterrents. Thus, monoterpenes appear to play an important role in protecting plants from insect attack (Gershenzon and Croteau 1991, Langenheim 1994, Phillips and Croteau 1999). The best-known insect neurotoxins among monoterpenes are the pyrethroids, a group of monoterpene esters found in the leaves and flowers of certain *Chrysanthemum* species (Harborne 1993).

Some compounds in essential oils have shown promise as natural insect pest control agents because they naturally provide plants with chemical defences against phytophagous insects and plant pathogens. These advances are reviewed in this paper with a focus on the monoterpenes and especially limonene, a compound with low toxicity to humans and having even some antitumor activity (Crowell 1999).

Effects of monoterpenes on bio-organisms

Bacteria

Essential oil of plants has been shown to have activity against human, animal and plant pathogens, as well as food poisoning bacteria. The essential oils have effects on bacteria cells or their activity. The essential oils from *Melaleuca alternifolia* (tea tree oil) inhibit the respiration and increase the permeability of bacterial cytoplasmic membranes of Gram-negative bacterium *Escherichia coli* AG 100, the Gram-positive bacterium *Staphylococcus aureus* NCTC 8325. These essential oils also cause potassium leakage (Cox et al. 2000). The essential oil of *Cymbopogon densiflorus* showed a wide spectrum of activity against Gram positive and Gram negative bacteria in the range of 250–500 and 500–1000 ppm, respectively. The main essential oil components were limonene, cymenene, p-cymene, Z- and E-carveol, carvone, iso-piperitenone, p-mentha-1 (7), 8-dien-ol, and p-mentha-2, 8-dien-1-ol. (Takaisi-Kikuni et al. 2000).

The main constituents of the oil of *Calamintha nepeta* (limonene, menthone, pulegone, menthol) were tested against some bacteria species, and only pulegone showed antimicrobial activity, particularly against all *Salmonella* species (Flamini et al. 1999). The determination of the minimal bactericidal concentration of the essential oil from the leaves of *Peumus boldus* (main constituents: monoterpenes 90.5%, including 17% limonene) against several microorganisms showed antibacterial activities towards Gram-positive and Gram-negative bacteria. *Streptococcus pyogenes* and *Micrococcus* sp. were the more sensitive in the case of Gram-positive bacteria and *Shigella sonnei* in Gram-negative bacteria (Vila et al. 1999).

The antibacterial activity of the various oils (main constituents were (E)-anethole, limonene, fenchone, and methyl chavicol) hydrodistilled from the seeds of 3 varieties of *Foeniculum vulgare* (dulce or sweet, vulgare or bitter, and azoricum or Florence) against 25 microorganisms was evaluated. The essential oil from sweet fennel (at the early waxy seed stage) was the most effective antibacterial agent. Essential oils of *Rosmarinus officinalis* (from Giza, Egypt) showed a high antimicrobial activity against *Cryptococcus neoformans* and *Mycobacterium intracellularae* (Soliman et al. 1994).

In antibacterial assays, the essential oil of *Origanum onites*, *Thymus capitatus* and oregano were active against *Bacillus subtilis*, *E. coli*, *Hafnia alvei*, *Micrococcus luteus*, *Proteus vulgaris*, *S. aureus* and *Streptococcus faecalis* but not against *Pseudomonas aeruginosa*. *O. onites*, *T. capitatus* and oregano inhibited the growth of the 5 test fungi. It is suggested that the observed antimicrobial activities may be associated with the phenolic constituents in the essential oil of *O. onites*, *T. capitatus* and oregano (Biondi et al. 1993). Helander et al. (1998) tested the inhibitory activity of some essential oils, including (+)-carvone against *E. coli* 0157:H7 and *Salmonella typhymurium*, and determined that, (+)-

carvone was among less inhibitory compounds. Chanegriha et al. (1994) reported that, limonene and terpenyl acetate both inhibited the activity of *B. subtilis* and *E. coli.*

The essential oil of *Tagetes minuta* inhibited the multiplication of Gram-positive and Gram-negative bacteria showing 95–100% of inhibition (Hethelyi et al. 1987). Similarly, *Hyptis suaveolens*' essential oil including limonene inhibits the growth of both Gram-positive and Gram-negative bacteria (Iwu et al. 1990).

The bark essential oil of *Xylopia longifolia* exhibited antimicrobial properties against some microorganisms including *S. aureus* and *E. coli* (MIC values of 0.5 and 2 mg/l respectively) (Fuornier et al. 1993). The antimicrobial activity of the *Cotinus coggygria* oil was manifested by its strong inhibition of the multiplication of both Gram-positive and Gram-negative bacteria (Hethelyi et al. 1986). The main components of the essential oil of leaves and stems of *Ducrosia anethifolia* (α-pinene, myrcene, limonene, terpinolene and E-β-ocimene) were active against Gram-positive bacteria, yeast and fungi (Janssen et al. 1984).

Fungi

Cox et al. (2000) reported that fungal toxicity of *M. alternifolia* essential oils to the yeast *Candida albicans* is based on increased permeability of the plasma membranes. Essential oil of *H. suaveolens* including limonene has mild antifungal activity against *C. albicans* (Iwu et al. 1990). Monoterpenes (1R, 2S, 5R)-Isopulegol, (R)-carvone and Isolimonene showed good fungistatic activities against *C. albicans* (Naigre et al. 1996). All essential oils hydrodistilled from the seeds of 3 varieties of *Foeniculum vulgare* (dulce or sweet, vulgare or bitter, and azoricum or Florence) exhibited a marked antifungal activity against *Aspergillus niger* (Marotti et al. 1994).

Lippia alba essential oil was the most effective of essential oils extracted from various parts of 11 higher plants for their fungitoxicity against a range of fungal sugarcane pathogens. *L. alba* essential oil was fungistatic against *Colletotrichum falcatum* (*Glomerella Tucumanensis*) and *C. pallescens* at 700 ppm or less, and fungicidal at higher concentrations against all the other test pathogens such as: *Fusarium moniliforme*, *Ceratocystis paradoxa*, *Rhizoctonia solani*, *Curvularia lunata, Periconia atropurpuria* and *Epicoccum nigrum* (Singh et al. 1998).

A positive correlation between the monoterpene content of the oils (other than limonene and sesquiterpenes) and fungal inhibition was observed in an experiment testing the effect of volatile components of citrus fruit essential oils on *Penicillium digitatum* and *P. italicum. P. digitatum* was found to be more sensitive to the inhibitory action of the oils than *P. italicum* (Caccioni et al. 1998).

Essential oils extracted from leaves of *Ocimum canum* (*O. americanum*) and seeds of *Anethum graveolens* and *Pimpenella anisum* completely inhibited the growth of fungi at 3000 ppm. *P. anisum* oil showed fungicidal activity at 3000 ppm against *C. falcatum, C. paradoxa and P. solani* (Singh et al. 1998). (+)-limonene, cineole, β-myrcene, α-pinene, β-pinene and camphor showed high antifungal activity against *Botrytis cinerea* (Wilson et al. 1997). Volatiles from crushed tomato leaves inhibited hyphal growth of *Alternaria alternata* isolated from lesions of tobacco leaves and *Botrytis cinerea* isolated from infected strawberry fruit. Aldehydes, including C_6 and C_9 compounds, formed by lipoxygenase enzyme pathway upon wounding leaves, inhibited growth of both species. Terpene hydrocarbons, 2-carene, and limonene had no significant effect on hyphal growth (Hamilton-Kemp et al. 1992). Cardamom oil inhibited the growth of *A. flavus*, *A. parasiticus A. ochraceus*, *Penicillium sp*., *P. patulum*, *P. roquefortii* and *P. citrinum*, and of its components α-terpinyl acetate had the greatest antifungal spectrum, followed by linalool, limonene, and cineole (Badei 1992). In agar diffusion experiments the essential oil of *Tagetes minuta* inhibited the multiplication of fungi and showed 100% of inhibition (Hethelyi et al. 1986, 1987).

Nematodes

(+)-limonene at 100 ppm reduced the *Heterodera schachtii* population to less than 3% of the control after 3 months, when sugarbeet seedlings cv. SSY1 were inoculated with 2300 freshly hatched *H. schachtii* J2. However, root growth was also reduced by phytotoxic effects of (+)-limonene (Viglierchio and Wu 1989). Aqueous extracts of *H. suaveolens* leaves gave 100% mortality of *Meloidogyne incognita* larvae in 80 mins, whereas the whole oil gave 100% mortality in 30 mins. The nematicidal activity is therefore linked to the essential oil of *H. suaveolens*, of which (+)-limonene and menthol are two main constituents (Babu and Sukul 1990).

Mites

(–)-limonene, β-pinene, α-pinene and Δ_3-carene were toxic to adult females of the spruce spider mite *Oligonychus ununguis* when exposed for 24 hrs at concentrations below the calculated LC50s. All four compounds decreased oviposition in the mites while three of these compounds (limonene, β-pinene, and α-pinene) influenced movement (Cook 1992). Monoterpene vapours from peppermint have similar toxic effects on the spider mite *Tetranychus urticae* (Larson and Berry 1984). Acute toxicity of 34 naturally occurring monoterpenes were evaluated against *T. urticae* and most of the monoterpenes were lethal to the mite at high concentrations; carvomenthenol and terpinen-4-ol were especially effective (Lee et al. 1997).

Insects

Secondary plant metabolites play an important role in plant resistance to insects. Monoterpenes including limonene can be bioassayed to determine their possible fumigant, contact, and ingestion activity against insect pests and other pathogens. These substances can be toxic via penetration of the insect cuticle (contact effect), via the respiratory system (fumigant effect) and via the digestive apparatus (ingestion effect) (Prates et al. 1998).

Insecticidal use of limonene has been successfully applied for the control of insect parasitoids of pet animals. Weekly application of (+)-limonene reduced flea and tick infestations by 80% in 24 dogs and one cat, with no adverse effects on blood composition or liver and kidney function (Tonelli 1987). (+)-limonene was toxic to all life stages of the cat flea, *Ctenocephalides felis* (Hink and Fee 1986). (+)-limonene has shown to have efficacy against malathion-resistant fleas (Collart and Hink 1986), but dogs developed toxicity effects including extensive erythema, and therefore it should be used cautiously (Rosenbaum and Kerlin 1995). Limonene has shown insecticidal properties against human blood-sucking insects when tested against early 4th instar larvae of the mosquito *Culex quinquefasciatus*. The LC50 was 53.80 ppm after 24 h and 32.52 ppm after 48 h. Limonene-treated water was less favourable than untreated water for oviposition by females of the mosquito (Kassir et al. 1989). The oil of *Myrica gale* acts as a deterrent to biting midge *Culicoides impunctatus,* but limonene together with camphene and terpinene-4-ol were at the bottom of the scale of the activity (Stuart and Stuart 1998).

Some monoterpene showed efficacy against food and wood pests. Components (cymol and limonene) of the essential oils of *Eucalyptus camaldulensis*, *E. cameroni,* and of the peal of *Citrus aurantium* have significant insecticidal action, being lethal to the stored product pests *Rhyzopertha dominica* and *Tribolium castaneum.* (+)-limonene showed more effective control of *T. castaneum* than of *R. dominica* (Santos et al. 1997). A study carried out by Sharma and Raina (1998) indicated that linalool, citronellal, and carvone showed promising toxicity against the termite *Odontotermes brunneus*, while, eucalyptol, terpinene, and limonene did not show much activity. Application of high doses of (+)-limonene or linalool to oothecal (egg save) of gravid female of German cockroach (*Blatella* *germanica*) decreased significantly the probability of young emerging from them, but did not affect female mortality (Karr and Coats 1992). Untreated diet was significantly preferred compared with diet treated with high levels of (+)-limonene, linalool, and α-terpineol. However, (+)-limonene among other tested compounds reduced significantly the time required by cockroaches' nymphs to reach the adult stage (Karr and Coats 1992).

Limonene has been shown to be toxic to several bark beetles e.g. the Southern pine beetle, *Dendroctonus frontalis* (Coyne and Lott 1976), the Western pine beetle *D. brevicomis* (Smith 1975) and the mountain pine beetle *D. ponderosae* (Raffa and Berryman 1983). Laboratory bioassay indicated that myrcene, limonene and β-phellandrene applied topically at 20 ppm were toxic to 60% of adult spruce beetles, *D. rufipennis* (Werner and Illmann 1994). The vapours of the monoterpenes present in grand fir (*Abies grandis*) phloem caused a significant mortality of the fir engraver beetle (*Scolytus ventralis*). Toxicity was observed at doses normally found in the host tree, either in the attacked phloem or in the reaction tissue induced by the associated fungi (Raffa et al. 1985).

The substances dihydrocarvone and carvone were repelled the blow fly, *Protophormia terraenovae* in a net cage study. Other compounds like eucalyptol, limonene, p-cymene, gamma-terpinene, dihydrocarvyl-acetate, β-pinene, β-myrcene, eugenol, and α-humulene seemed to have a deterrent effect mainly by contact of the fly with the treated bait at concentrations of 17–25 μmolcm^{-2} (Thorsell et al. 1989). The essential oils of 0.5g citrus including (+)-limonene, α-pinene, and myrcene caused rapid knockdown (KT50) on *Musca domestica* in 10–20s, and also inhibition of the emergence rate of the pupae increased with the increased exposure time. The same dose of oils killed all treated flies within 24h (Liao and Liao 1999).

The internal concentration of limonene in plants or in artificial food of herbivorous insects has significant effects on the behaviour and food consumption of plant feeding insects (Table 2). Most of the studies indicate the attractive role of limonene for herbivorous insects of conifers, which have a high content of monoterpenes in their oleoresin. For specialised herbivores limonene can be a signal compound to detect the right host plant species of certain plant family as shown with *Trioza apicalis* by Valterova et al. (1997). The same carrot pest (*T. apicalis*) can even avoid carrot varieties with high limonene content (Kainulainen et al. 2001).

Phytotoxicity

Plant injuries from chemicals are called phytotoxicity, and are manifested in several ways. Leaf tips, margins, or the entire leaf surface can appear burned, growing tips and buds can be killed and roots can also be burned. Chlorosis or yellowing of leaves (in spots, along margins) or a general chlorosis of the entire leaf or leaf distortion may appear as curling, crinkling, or cupping of the leaf. Stunting of growth on all or parts of the plant is also one of the phytotoxic impacts. Phytotoxic chemicals can also stimulate abnormal either excessive growth (as aerial roots and suckering), or elimination and distortion of fruit or flowers. Symptoms of phytotoxicity can be confused with insect or mite damage, diseases, and other abiotic problems such as nutrient deficiencies, or environmental conditions (Fink 1999).

Phytotoxicity of some naturally occurring monoterpenes were tested on maize plant, and some of them have shown phytotoxicity to maize roots and leaves. On the other hand, D-Carvone was the most phytotoxic, whereas pulegone was the safest (Lee et al. 1997). But on the other hand, carvone inhibited sprouting of treated potato tubers during storage with low persistence. Carvone has to be reapplied about every 3 months during storage. The low persistence means that tubers could be consumed as soon as 15 days after treatment. Carvone also inhibited early sprouting of seed tubers (Reust 2000). In the Netherlands, carvone has been introduced as a commercial sprouting inhibitor for potatoes

Table 2. Summary of reports indicating negative or positive effects of internal limonene concentrations in plants or in artificial food on plant damaging insects.

Insect species	Host plant	Response	Reference
		Reduced activity of pest insect	
Pissodes strobi (Coleoptera: Curcullionidae) – Pine shoot feeder	Paired agar disc bioassay	Limonene inhibited feeding at high concentration.	Alfaro et al. 1980
Diaphania nitidalis (Lepidoptera, Pyralidae) – Pickleworm moth	Treated on artificial sites	A concentration of 20 µl of (–)-limonene 96% caused slight but significant reduction in oviposition.	Peterson et al. 1994
Trioza apicalis (Homoptera: Psyllidae) – carrot sucker	Various Apiacea species	Large amounts of either (–) or (+)-limonene were released by the Apiacea species of low preference of *T. apicalis*.	Valterova et al. 1997
Dioryctria zimmermanni, (Lepidoptera: Pyralidae) – pine trunk borer	Scots pine, *Pinus sylvestris*	Resistant proveniences emitted relatively high levels of limonene.	Sadof & Grant 1997
		Increased activity of pest insect	
Pissodes strobi (Coleoptera: Curcullionidae) – Pine shoot feeder	Paired agar disc bioassay	Limonene stimulated feeding at low concentration.	Alfaro et al. 1980
Pissodes strobi – pine shoot feeder	Eastern white pine, *Pinus strobus*	Limonene concentration was highest in trees attacked most frequently.	Wilkinson 1980
Dioryctria abietivorella (Lepidoptera: Pyralidae) – twig borer	Eastern white pine, *Pinus strobus*	Ten microliters of a test solution containing 10 µg (–)-limonene 95% elicited a significant oviposition response, but was the least stimulating monoterpene in EAG tests.	Shu et al. 1997
Cydia strobilella (Lepidoptera: Tortricidae) – spruce seed moth	Norway spruce, *Picea abies*	Limonene elicited the highest electroantennogram response, probably cue compound for ovipositing females.	Åhman et al. 1988
Dioryctria amatella (Lepidoptera: Pyralidae) – Southern pine coneworm	Loblolly pine, *Pinus taeda*	Combination of (+)-limonene, α-pinene, and myrcene was attractive to females.	Hanula et al .1985
Dioryctria sylvestrella (Lepidoptera: Pyralidae) – maritime stem borer	Maritime pine, *Pinus pinaster*	Attacked trees contained a significantly higher percentage of limonene together with longipinene and copaene.	Jactel et al. 1996
Thecodiplosis japonensis (Diptera: Cecidomyiidae) – Pine gall midge	Japanese pine, *Pinus thunbergii*	Higher limonene and β-pinene contents were associated to resistance.	Kim et al. 1976

(Bouwmeester et al. 1995). Limonene oxide and linalool both inhibited sprouting and fungal growth but tubers were soft after exposure. Limonene and α-pinene did not inhibit sprouting, and fungal growth was present on every tuber treated (Vaughn and Spencer 1991). Because of the phytotoxicity, monoterpenes may be a potential source of products used for haulm killing and weed control.

(+)-limonene is highly phytotoxic to sugarbeet seedlings (CV. SSY 1) at high concentrations (Viglierchio and Wu 1989). Carvone, (+)-limonene, and (–)-limonene were either less effective and/or more phytotoxic to wheat, barley and perennial ryegrass when seed dressing to control slugs were tested (Nijenstein and Ester 1998). Preliminary tests showed that limonene has phytotoxic activity at concentrations more than 3% to strawberry (cv. Jonsok and Honeoye) seedlings (Ibrahim 2000). Our preliminary observations suggest that cabbage and carrot seedlings are sensitive to limonene at the concentration of 9% (Ibrahim et al., unpublished results). In general, there is not much information available on the phytotoxicity threshold values of monoterpenes to different cultivated plants.

Suitability of limonene in control of plant-damaging insect pests

Insecticidal use

Experiments dealing with the use of monoterpenes extracted from plants like insecticides in plant protection are scarce. For insect pests that associated with plant roots, drenching with toxic monoterpene solution might increase larval mortality and reduce damage, but may also affect other soil animals (Karr et al. 1990). A wide range of monoterpenes has larvicidal effects on the western corn rootworm in the soil and effectively protects corn roots from attack by this larva under greenhouse conditions (Lee et al. 1997).

Deterrent

Internal limonene concentrations in plants have shown deterrent effects on only few insect pests (Table 2). Among these the carrot psyllid (*T. apicalis*) has shown a reduced oviposition rate on carrot varieties having a high concentration of limonene (Kainulainen et al. 2001). This observation suggests that, the selection of carrot varieties with high limonene contents can be used to reduce carrot psyllid damages in the areas, where the risk of damage is high. The selection of resistant varieties may be a suitable method for pest control in organic farming, but the factors determining pest deterrence should be known.

Exogenous treatment of cultivated plants with limonene extracted from other plant species have more often reduced insect attack than increased and attracted pest insects (Table 3). In leaf disk tests to estimate deterrent effects of (+)-limonene on larvae of *Galerucella sagittariae* (Coleoptera: Chrysomelidae) (Holopainen et al. 2000), plants from two strawberry varieties (Honeoye and Jonsok) were treated with 1% (+)-limonene, 1% mixture (75:25%) of (+)-limonene and (+)-carvone, and water in fumehood. The leaf discs (diam. 15 mm) were cut with a cylinder tube from strawberry leaves. For choice tests three leaf discs (one from each treatment) were put into Petri dish on filter paper, 2 hours and 24 hours after the treatment, one larva of *G. sagittariae* was immediately released into the middle of the Petri dish. After monitoring 24 hours the eaten area from the leaf discs by the larva was estimated visually.

Spraying of leaves with limonene or mixture of limonene and carvone did not significantly reduced the feeding ability of the larvae of *G. sagittariae* on leaf discs of the variety Honeoye if offered to the larvae 2 or 24 hours after spraying of leaves (Fig. 1a). In the test with strawberry variety Jonsok, larvae did not prefer feeding on leaf disk offered 2h after spraying, but limonene and, limonene and carvone in mixture significantly reduced feeding when leaf disks were offered to larvae 24 hrs after spraying (Fig. 1b). The result suggests that carvone in mixture with limonene can be as effective as pure limonene to reduce *G. sagittariae* larval feeding on certain strawberry varieties. For the

Table 3. Summary of reports of exogenous application of limonene against plant damaging insects.

Insect species	Host plant	Limonene application	Response	Reference
			Reduced activity of pest insect	
Thaumetopoea pityocampa (Lepidoptera: Notodontidae)	Pine *(Pinus nigra)*	Emulsified with water and sprayed on the foliage of pine seedlings	(+)-limonene reduced the number of egg cluster on plants sprayed with it	Tiberi et al. 1999
Hylobius abietis (Col., Curculionidae)	Pine (*Pinus sylvestris*), Spruce (*Picea abies*)	Exposure to limonene vapours	High limonene concentrations exhibited signs of poisoning within a few hours	Lindgren et al. 1996
Delia antiqua, (Diptera, Anthomyiidae)	Onion, Shallot, Leek	Volatile mixture 3-carene, limonene, and p-cymene releaser from capillary tubes near onion plants	Monoterpene mixtures deterred oviposition of adult flies	Ntiamoah et al. 1996
Trioza apicalis (Homoptera, psyllidae)	Carrot	Sprayed on the carrot in the field in 1–6% solutions (small amounts of carvone as contaminant)	Reduced damage and increased yield	Aaltonen et al. 2000
Megastigmus pinus and *M. rafini* (Hymenoptera: Torymidae) – Seed wasps	White fir (*Abies concolor*)	Olfactory responses to pure alpha-pinene and limonene	Limonene significantly acted as repellent	Liuk et al. 1999
Dendroctonus rufipennis, *D. simplex* (Col., Scolytidae) – Spruce beetle, Eastern larch beetle respectively	Spruce spp. (e.g. Sitka, white and Engelmann spruce)	Bioassayed for their toxicity	(+)-limonene at 60 ppm killed 100% of the pests after 24 hrs of exposure	Werner 1995
Delia radicum (Diptera, Anthomyiidae) – Cabbage maggot	Brassicaceae (e.g. Radish, turnip, cabbage, cauliflower, rape)	Olfactory stimuli for orientation behaviour	Limonene from the surface part of plant host was repellent	Kostal 1992
Acrolepiopsis assectella (Lepidoptera, plutellidae) – Leek moth	Leek & onion, but able develop on all Allium crop species	Olfactometer with two parallel air currents containing a Y-shaped nylon fibre	Limonene was repellent	Al-Rouz et al. 1988

continued on the next page

continued from the preceding page

Insect species	Host plant	Limonene application	Response	Reference
Costelytra zealandica (Col. Scarabaeidae) – Grass grub	Pasture grasses	Host selection test in glass chambers	Limonene had some repellent effect	Osborne & Boyd 1974
			Increased activity of pest insect	
Prays citri (Lepidoptera: Hyponomeutidae)	*Citrus limonum*, *Citrus decumana* & *Citrus aurantium*	Electroantennogram response to pure limonene	Limonene activated oviposition	El-Sayed et al. 1994
Helicoverpa armigera (Lepidoptera, Noctuidae) – Cotton bollworm	Polyphagous moth (e.g. cotton, maize cucurpitaceae, tomatoes, leguminous crops, conifers)	Electroantennography used to investigate electrophysiological responses	Attractive to 1–2 days old moths	Ding et al. 1997
Ips typographus (Col., Scolytidae) Spruce bark beetle	Spruce (e.g. Norway spruce)	Field bioassay using Pheromone baited traps with a mixture of limonene and α-pinene	(+)-limonene in mixture with α-pinene attracted adults	Reddemann & Schopf 1996
Papilio demoleus (Lepidoptera, Papilionidae) – Citrus Swallowtail	Fabaceae (e.g. *Cullen lenax, Psralea spp.*) and Rutaceae (e.g. *Murraya koenigii*, citrus)	Orientation responses to different odours in Olfactory	(–)-limonene showed maximum attaraction to the larvae of this pest	Saxena et al. 1975
			No effect	
Hylobius abietis (Col., Curculionidae)	Pine (*Pinus sylvestris*), Spruce (*Picea abies*)	Exposure to limonene vapours	Low limonene levels did not affect feeding activity	Lindgren et al. 1996
Hylobius abietis (Col., Curculionidae)	Pine (*Pinus sylvestris*), Spruce (*Picea abies*)	Scots pine (*Pinus sylvestris*) twigs were treated with limonene dissolved in ethyl acetate solvent	No antifeeding effects in 48h feeding trial with adult beetles	Klepzig & Schlyter 1999

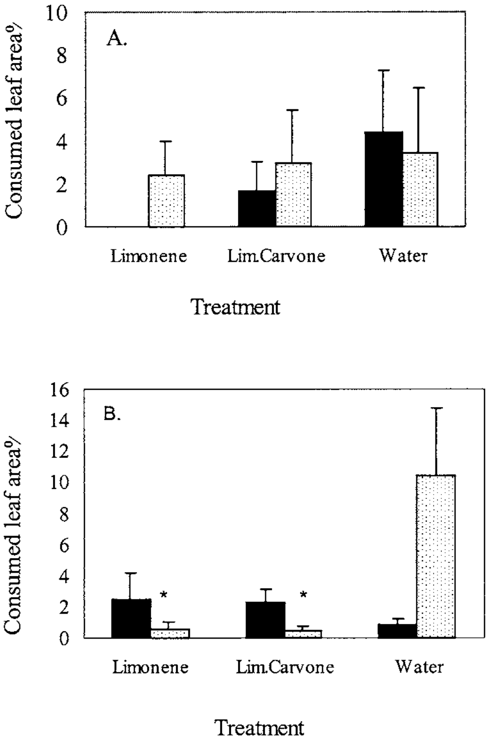


Fig. 1. The effect of 1% limonene treatment on the feeding of the larvae *Galerucella sagittariae* on cv. Honeoye (A) and cv. Jonsok (B) leaf discs when larvae were put into the Petri dishes for feeding 2 h (black bars) and 24 h after spraying (grey bars). Asterisk above bar indicate significant ($P < 0.05$) difference from the control (water) treatment according to oneway anova.

confirmation of results, field tests are needed, since high concentration of volatile compounds inside closed Petri dishes may reduce larval feeding also on control treatment as observed with the variety Jonsok when offered leaf discs sprayed only 2 h before feeding trial (Fig. 1b).

Field sprays of *Pinus uncinata* with oleoresin extracts of *P. cembra* cones significantly reduced the overall damage of specialized cone insects. None of the cones sprayed with oleoresin were attacked, whereas insects damaged 11% and 31% of the unsprayed control cones (Dormont et al. 1997). Several compounds including the major component of all citrus peel oils, (+)-limonene, were found to be bioactive, having a strong vapour insecticidal activity, against the cowpea weevil beetle *Callosobruchus maculatus* (Coleoptera: Bruchidae) (Don-Pedro 1996).

Limonene, mixed with the combination of α-pinene and ethanol on old clear cuttings, inhibited completely the catch of *Hylobius pinastri* (Coleoptera: Curculionidae) and that of *H. abietis* was reduced by two thirds. On fresh clear cuttings the inhibitory effect of limonene was small or absent (Nordlander 1990). Volatile oils of baladi orange and mandarin peels, which contain over 70% limonene, were highly toxic to 2nd larval instar stage of *Spodoptera littoralis*. Their toxicity consistently increased with increasing concentration of the volatile oils. The results from this experiment suggest that these volatile oils could be used as larval growth disruptors and also as repellent materials against moths for controlling programme of cotton leaf worm *S. littoralis* (Omer et al. 1997). Sesquiterpenes, in combination with tricyclene, camphene, myrcene, limonene, terpinolene, and the acetate fraction appear to be an effective mixture of defensive compounds against the western spruce budworm (Zou and Cates 1997).

Ntiamoah and Borden (1996) found that, a ternary mixture of carene, limonene and p-cymene in the choice bioassay significantly deterred the oviposition of cabbage maggots, but the deterrence was slight in the non-choice bioassay. Increasing the complexity of the blend to six monoterpenes increased the deterrent effect markedly. These results indicate that, as for onion maggots (Ntiamoah et al. 1996), monoterpenes are oviposition deterrents for cabbage maggots. The results also suggest that monoterpenes may be oviposition deterrents for other anthomyiids. Combining various deterrents used in different areas may develop a way of pest control more valuable than by using single deterrent.

Effects on non-target organisms and manipulation of natural enemies

Although the acute toxicity of monoterpenoids is low relative to that of conventional insecticides (Lee et al. 1997), the effects of field scale use on soil and water animals should be tested further. Potential drifts to aquatic ecosystem may have effects on food chains, since mortality of aquatic dipterans have increased after limonene treatment (Kassir et al. 1989). Toxic effects on earthworms (Karr et al. 1990) should be clarified more extensively, since potential pesticide use of limonene in organic farming may be harmful for whole agroecosystem if earthworm populations are reduced.

Also selectivity and harmlessness of limonene toward natural enemies and other biological agents needs testing. Interaction with entomopathogenes such as *Bacillus thuringiensis* should be understood. The potential use of limonene and other monoterpenes in integrated pest management can be achieved with the knowledge of all these interactions. For rapid attraction of large numbers of nestmates to newly discovered food sources, the polyphagous predator ant *Myrmicaria eumenoides* uses an efficient recruitment communication system based on the poison gland secretion that includes mainly (+)-limonene (Kaib and Dittebrand 1990). This observation suggests that a better understanding of the communication systems of predators and parasitoids may offer novel ways to increase the efficiency of natural enemies of pest insects with limonene and other monoterpenes.

Summary and future perspectives

Monoterpenes distilled from plants have effects on many bacterial, fungal, nematode and arthropod species and some compounds are effective sprouting inhibitors. The monoterpene limonene has shown deterrent and insecticide properties suggesting that as a plant-based natural product it might have use in pest control in organic agriculture. Possible attractive effects of limonene to natural enemies of pests may offer novel applications to use natural compounds for manipulation of beneficial animals in organic agriculture. However, since monoterpenes are phytotoxic to several cultivated plants, critical thresholds of limonene for plant physiology and limonene sensitivity should be determined. Also the potential harmful effects on natural enemies of pests and non-target soil animals should be investigated. After these evaluations, suitable recommendations for the use of limonene on specific crop plant species and against specific pest species can be given.

Acknowledgements. We thank Dr. G.V.P. Reddy for discussions and the comments on the manuscript and Dr. Simo Lötjönen for the information of limonene nomenclature. The work was funded by the Ministry of Agriculture and Forestry in Finland (MTT-project no: 130581) and Jenny and Antti Wihuri Foundation.

References

Aaltonen, M., Aflatuni, A. & Parikka, P. 2000. Limoneenilla kemppi kuriin. *Puutarha & Kauppa* 4, 16: 4–5.

Åhman, I., Wiersma, N. & Lindström, M. 1988. Electroantennogram responses in *Cydia strobilella* (L.) (Lepidoptera: Tortricidae) to flower and twig odours of its host *Picea abies* (L.) Karst. *Journal of Applied Entomology* 105: 314–316.

Alfaro, R.I., Pierce, H.D. Jr., Borden, J.H. & Oehlschlager, A.C. 1980. Role of volatile and nonvolatile components of Sitka spruce bark as feeding stimulants for *Pissodes strobi* Peck (Coleoptera: Curcullionidae). *Canadian Journal of Zoology* 58: 626–632.

Al-Rouz, H., Thibout, E. & Rouz, H.A.L. 1988. Olfactory responses to plant allelochemicals in *Acrolepiopsis assectella* larvae. *Entomologia Experimentalis et Applicata* 47: 231–237.

Babu, S.P. & Sukul, N.C. 1990. Essential oils as nematicidal principles. *Environment and Ecology* 8: 1118–1120.

Badei, A.Z.M. 1992. Antimycotic effect of cardamum essential oil components on toxigenic molds. *Journal of Egyptian Food Science* 20: 441–452.

Benner, J.P. 1993. Pesticidal compounds from higher plants. *Pesticide Science* 39: 95–102.

Biondi, D., Cianci, P., Geraci, C., Ruperto, G. & Piattelli, M. 1993. Antimicrobial activity and chemical composition of essential oils from Sicilian aromatic plants. *Flavour and Fragrance Journal* 8: 331–337.

Bouwmeester, J.H., Davies, J.A. & Toxopeus, H. 1995. Enantiomeric composition of carvone, limonene, and carveols in seeds of dill and annual and biennial caraway varieties. *Journal of Agricultural and Food Chemistry* 43: 3057–3064.

Brattesten, L.B. 1983. Cytochrome P-450 involvement in the interactions between plant terpenes and insect herbivores. In: Hedin, P.A. (ed.). *Plant resistance to insects.* American Chemical Society, Washington, DC. p. 173–195.

Caccioni, D.R., Guizzardi, M., Biondi, D.M., Renda, A. & Ruberto, G. 1998. Relationship between volatile components of citrus fruit essential oils and antimicrobial action of *Benicillium ditatum* and *Penicillium italicum. International Journal of Food Microbiology* 43: 73–79.

Chanegriha, N., Sabaou, N., Baaliiouamer, A. & Meklati, B.Y. 1994. Antibacterial and antifungal activity of the essential oil of Algerian cypress. *Rivista Italiana EPPOS* 12: 5–12.

Collart, M.G. & Hink, W.F. 1986. Development of resistance to malathion in cat flea (Siphonaptera: Pulicidae). *Journal of Economic Entomology* 79: 1570–1572.

Cook, S.P. 1992. Influence of monoterpene vapors on spruce spider mite *Oligonychus ununguis* adult females. *Journal of Chemical Ecology* 18: 1497–1504.

Coyne, J.F. & Lott, L.H. 1976.Toxicity of substances in pine oleoresin to southern pine beetles. *Journal of the Georgia Entomological Society* 11: 301–305.

Cox, S.D., Mann, C.M., Markham, J.L., Bell, H.C., Gustafson, J.E., Warmington, J.R. & Wyllie, S.G. 2000. The mode of antimicrobial action of the essential oil of *Melaleuca alternifolia* (tea tree oil). *Journal of Applied Microbiology* 88: 170–175.

Croteau, R. 1987. Biosynthesis and catabolism of monoterpenoids. *Chemical Reviews* 87: 929–954.

Crowell, P.L. 1999. Prevention and therapy of cancer by dietary monoterpenes. *Journal of Nutrition* 129: 775–778.

Dell, B. & McComb, A.J. 1981. Plant resins – Their formation, secretion and possible functions. *Advances in Botanical Research* 6: 278–316.

Ding, H.J., Guo, Y.Y. & Wu, C.H. 1997. Isolation and identification of semiochemicals from carrot flower and behavioural responses in cotton bollworm moths. *Acta Entomologica Sinica* 40: 73–78.

Don-Pedro, K.N. 1996. Investigation of single and joint fumigant insecticidal action of citrus peel oil components. *Pesticide Science* 46: 79–84.

Dormont, L., Roques, A. & Malosse, C. 1997. Efficiency of spraying mountain pine cones with oleoresin of Swiss stone pine cones to prevent insect attack. *Journal of Chemical Ecology* 23: 2261–2274.

Duke, S.O., Keeler, R.F. & Tu, A.T. (eds.). 1991. Plant terpenoids as pesticides. In: *Handbook of natural toxins.* Vol.6. Toxicology of plant and fungal compounds. Marcel Dekker, Inc., New York, USA. p. 269–296.

El-Sayed, E.M., Talaat, M.S., Desouki, S. & Sheashaa, M.A. 1994. Bioelectrical activity of citrus flower moth, *Prays citri* and host plant selection for oviposition. *Egyptian Journal of Biomedical Engineering* 10: 31–48.

Fessenden, R.J., Fessenden, J.S. & Logue, M. 1998. *Organic Chemistry.* 6th ed. Pacific Grove. Brooks/Cole. 1168 p.

Fink, S. 1999. *Pathological and regenerative plant anatomy.* Berlin. Gebrüder Borntraeger. 1095 p.

Flamini, G., Cioni, P.L., Puleo, R., Morelli, I. & Panizzi, L. 1999. Antimicrobial activity of the essential oil of Calamintha nepeta and its constituent pulegone against bacteria and fungi. *Phytotherapy Research* 13: 349–351.

Fournier, G., Hadjiakhoondi, A., Leboeuf, M., Cave, A., Fuorniat, J. & Charles, B. 1993. Chemical and biological studies of *Xylopia longifolia* A. DC. Essential oils. *Journal of Essential Oil Research* 5: 403–410.

Gershenzon, J. & Croteau, R. 1991. Terpenoids. In: Rosenthal, G.A. & Berenbaum, M.R. (eds.). *Herbivores. Their Interactions with secondary plant metabolites.* Academic Press, San Diego. p. 165–219.

Hamilton-Kemp, T.R., McCkracken, C.T. Jr., Loughrin, J.H., Andersen, R.A. & Hildebrand, D.F. 1992. Effects of some natural volatile compounds on the pathogenic fungi *Alternaria alternata* and *Botrytis cinerea. Journal of Chemical Ecology* 18: 1083–1091.

Hanula, J.L., Berisford, C.W. & DeBarr, G.L. 1985. Monoterpene oviposition stimulants of *Dioryctria amatella* in volatiles from fusiform rust galls and second-year loblolly pine cones. *Journal of Chemical Ecology* 11: 943–952.

Harborne, J.B. 1993. Introduction to ecological biochemistry. Fourth edition, Academic Press, Cambridge. 318 p.

Helander, I.M., Alakomi, H.-L., Kyösti, L.-K., Mattila-Sandholm, T., Pol, I., Smid, E.J., Gorris, L.G.M. & Wright, A.V. 1998. Characterization of the action of selected essential oil components on Gram-Negative bacteria. *Journal of Agricultural and Food Chemistry* 46: 3590–3595.

Hethelyi, E., Danos, B., Tetenyi, P. & Koczka, I. 1987. GC/MS analysis of the essential oils of four tagetes species and the microbial activity of *Tagetes minuta. Herba Hungarica* 26: 49–61.

–, Tetenyi, P., Kozka, I. & Domokos, J. 1986. Essential oil components and antimicrobial activity of *Cotinus coggygria. Herba Hungarica* 25: 73–85.

Hink, W.F. & Fee, B.J. 1986. Toxicity of D-limonene, the major component of citrus peel oil, to all life stages of the cat flea, *Ctenocephalides felis* (Siphonaptera: Pulicidae). *Journal of Medical Entomology* 23: 400–404.

Holopainen, J., Ibrahim, M.A., Aflatuni, A. & Tiilikkala, K. 2000. Limoneenin mahdollisuudet mansikan tuholaistorjunnassa. *Puutarha & Kauppa* 47/2000 plus: 16–17.

Hooser, S.B. 1990. Toxicology of selected pesticides, drugs, and chemicals. D-limonene, linalool, and crude citrus oil extracts. *Toxicology of selected pesticides, Drugs, and Chemicals* 20: 383–385.

Hough-Goldstein, J.A. 1990. Antifeedant effects of common herbs on the Colorado potato beetle (Coleoptera: Chrysomelidae). *Environmental Entomology* 19: 234–238.

Ibrahim, M.A. 2000. *Feeding of leaf beetle (Galerucella sagittariae) on Strawberry leaflets sprayed with plant volatiles.* M.Sc.Thesis. University of Kuopio, Department of Ecology and Environmental Science, 46 p.

Iwu, M.M., Ezeugwu, C.O., Okunji, C.O., Sanson, D.R. & Tempesta, M.S. 1990. Antimicrobial activity and terpenoids of the essential oil of *Hyptis suaveolens. International Journal of Crude Drug Research* 28: 73–76.

Jactel, H., Kleinhentz, M., Marpeau-Bezard, A., Marion, P. & Burban, C. 1996. Terpene variations in maritime pine constitutive oleoresin related to host tree selection by *Dioryctria sylvestrella* Ratz. (Lepidoptera: Pyralidae). *Journal of Chemical Ecology* 22: 1037–1050.

Janssen, A.M., Scheffer, J.J., Svendsen, A.B., Aynehchi, Y. & Baerheim-Svendsen, A. 1984. The essential oil of *Ducrosia Anethifolia* (DC.) Boiss. *Pharmaceuutisch weekblad, Scientific edition* 6: 157–160.

Kaib, M. & Dittebrand, H. 1990. The poison gland of the ant *Myrmicaria eumenoides* and its role in recruitment communication. *Chemoecology* 1: 3–11.

Kainulainen, P., Nissinen, A., Piirainen, A., Tiilikkala, K. & Holopainen, J.K. 2001.Essential oil composition in leaves of carrot varieties and preference of specialist and generalist sucking insect herbivores. (submitted manuscript).

Karr, L.L. & Coats, J.R. 1992. Effects of monoterpenoids on growth and reproduction of the German cockroach (Blattodae: Blattellidae). *Journal of Economic Entomology* 85: 425–429.

–, Drewes, C.D. & Coats, J.R. 1990. Toxic effects of d-limonene in the earthworm *Eisenia fetida* (Savigny). *Pesticide Biochemistry and Physiology* 36: 175–186.

Kassir, J.T., Mohsen, Z.H., & Mehdi, N.S. 1989. Toxic effects of limonene against *Culex quinquefasciatus* Say larvae and its interference with oviposition. *Anzeiger für Schädlingskunde, Pflanzenschutz, Umweltschutz* 62: 19–21.

Kim, C.S., Hong, S.H., Ryu, J.B, Choi, C., Kim, J.S. & Kim, D.S. 1976. Breeding of varieties of pines resistant to pine gall midge. II. Seasonal variation of needle monoterpene composition in resistant *Pinus thunbergii. Korean Journal of Breeding* 8: 137–142.

Klepzig, K.D. & Schlyter, F. 1999. Laboratory evaluation of plant-derived antifeedants against the pine weevil *Hylobius abietis* (Coleoptera: Curculionidae). *Journal of Economic Entomology* 92: 644–650.

Kostal, V. 1992. Orientation behaviour of newly hatched larvae of the cabbage maggot, *Delia radicum* (L.) (Diptera: Anthymiidae), to volatile plant metabolites. *Journal of Insect Behavior* 5: 61–70.

Langenheim, J.H. 1994. Higher plant terpenoids: A phytocentric overview of their ecological roles. *Journal of Chemical Ecology* 20: 1223–1280.

Larson, K.C. & Berry, R.E. 1984. Influence of Peppermint phenolics and monoterpenes on twospotted spidermite (Acari: Tetranychidae). *Environmental Entomology* 13: 282–285.

Laska, M. & Teubner, P. 1999. Olfactory discrimination ability of human subjects for ten pairs of enantiomers. *Chemical Senses* 24: 161–170.

Lee, S., Tsao, R., Peterson, C., Coates, J.R. & Lee, S.K. 1997. Insecticidal activity of monoterpenoids to western corn rootworm (Coleoptera: Chrysomelidae), twospotted spidermite (Acari: Tetranychidae), and housefly (Diptera: Muscidae*). Journal of Economic Entomology* 90: 883–892.

Liao, S. & Liao, S.C. 1999. Mortality and repellency effects of essential oils from citrus against the housefly and German cockroach. *Chinese Journal of Entomology* 19: 153–160.

Lichtenthaler, H.K., Schwender, J., Disch, A. & Rohmer, M. 1997. Biosynthesis of isoprenoids in higher plant chloroplasts proceeds via a mevalonate-independent pathway. *FEBS Letters* 400: 271–274.

Lindgren, B.S., Nordlander, G. & Birgersson, G. 1996. Feeding deterrence of verbenone to the pine weevil, *Hylobius abietis* (L.) (Coleoptera: Curculionidae). *Journal of Applied Entomology* 120: 397–403.

Little, D.B. & Croteau, R.B. 1999. Biochemistry of essential oil terpenes. A thirty year overview. In: Teranishi, L. & Wick, E.L. (eds). *Flavor Chemistry: 30 years of progress.* Kluwer Academic/Plenum Publishers, New York. p. 239–253.

Liuk, A., Ochsner, P. & Jensen, T. 1999. Olfactory responses of seed wasps *Megastigmus pinus* parfitt and *Megastigmus rafni* Hoffmeyer (Hymenoptera: Torymidae) to host tree odours and some monoterpenes. *Journal of Applied Entomology* 123: 561–567.

Marotti, M., Piccaglia, R., Giovanelli, E., Deans, S.G. & Eaglesham, E. 1994. Effects of variety and ontogenic stage on the essential oil composition and biological activity of fennel (*Foeniculum vulgare* Mill.). *Journal of Essential Oil Research* 6: 57–62.

Naigre, R., Kalck, P., Roques, C., Roux, I. & Michel, G. 1996. Comparison of antimicrobial properties of monoterpenes and their carbonylated products. *Planta medica* 62: 275–277.

Needham, J. 1986. *Science and Civilization in China.* vol. 6, 1. Cambridge University Press, Cambridge.

Nijenstein, J.H. & Ester, A. 1998. Phytotoxicity and control of the field slug *Deroceras reticulatum* by seed applied pesticides in wheat, barley and perennial ryegrass. *Seed Science and Technology* 26: 501–513.

Nordlander, G. 1990. Limonene inhibits attraction to a-pinene in the pine weevils *Hylobius abietis* and *H. pinastri. Journal of Chemical Ecology* 16: 1307–1320.

Ntiamoah, Y.A., Borden, J.H. & Pierce, H.D. Jr. 1996. Identity and bioactivity of oviposition deterrents in pine oil for the onion maggot (*Delia antiqua*). *Entomologia Experimentalis et Applicata* 79: 219–226.

– & Borden, J.H. 1996. Monoterpene oviposition deterrents for cabbage maggots, *Delia Radicum* (L.) (Diptera: Anthomyiidae). *Canadian Entomologist* 128: 351–352.

Omer, E.A., Youssef, A.A., Abo-Zeid, E.N. & Sharaby, A.M. 1997. Biochemical studies on the essential oils of Balady orange and mandarin. *Egyptian Journal of Horticulture* 24: 207–218.

Osborne, G.O. & Boyd, J.F. 1974. Chemical attractants for larvae of *Cotelytra zealandica* (Coleoptera: Scarabaeidae). *New Zealand Journal of Zoology* 1: 371–374.

Peterson, J.K., Horvat, R.J. & Elsey, K.D. 1994. Squash leaf glandular trichome volatiles: Identification and influence on behaviour of female pickleworm moth [*Diaphania nitidalis* (Stoll.)] (Lepidoptera: Pyralidae). *Journal of Chemical Ecology* 20: 2099–2109.

Phillips, M.A. & Croteau, R.B. 1999. Resin-based defenses in conifers. *Trends in Plant Science* 4: 184–190.

Prates, H.T., Santos, J.P., Waquil, J.M., Fabris, J.D., Oliveira, A.B. & Foster, J.E. 1998. Insecticidal activity of monoterpenes against *Ryzopertha dominica* (F.) and *Tribolium castaneum* (Herbst). *Journal of Stored Products Research* 34: 243–249.

Raffa, K.F. & Berryman, A.A. 1983. Physiological aspects of lodgepole pine wound responses to a fungal sympiont of mountain pine beetle *Dendroctonus ponderosae* (Coleoptera: Scolytidae). *Canadian Entomologist* 115: 723–734.

–, Berryman, A.A., Simasko, J., Teal, W. & Wong, B.L. 1985. Effects of grand fir monoterpenes on the fir engraver, *Scolytus ventralis* (Coleoptera: Scolytidae), and its symbiotic fungus. *Environmental Entomology* 14: 552–556.

Reddemann, J. & Schopf, R. 1996. The importance of monoterpenes in the aggregation of the spruce bark beetle *Ips typographus* (Coleoptera: Scolytidae). *Entomologia Generalis* 21: 69–80.

Reust, W. 2000. Carvone, a new natural sprouting inhibitor for potato storage. *Revue Suisse d'Agriculture* 32: 150–152.

Rosenbaum, M.R. & Kerlin, R.L. 1995. Erythema multiforme major and disseminated intravascular coagulation in a dog following application of a d-limonene based insecticidal dip. *Journal of the American Veterinary Medical Association* 207: 1315–1319.

Sadof, C.S. & Grant, G.G. 1997. Monoterpene composition of *Pinus sylvestris* varieties resistant and susceptible to *Dioryctria zimmermani*. *Journal of Chemical Ecology* 23: 1917–1927.

Santos, J.P., Prates, H.T., Waquil, J.M. & Oliveiria, A.B. 1997. Evaluation of plant origin substances on the control of stored product pests . *Pesquisa emm Andamento Centro Nacional de Pesquisa de Milho e sorgo* no. 19. 18 p.

Saxena, K.N., Prabha, S. & Shashi-Brabha 1975. Relationship between the olfactory sensilla of *Papilio demolius* L. Larvae and their orientation responses to different odours. *Journal of Applied Entomology* 50: 119–126.

Schütte, H.R. 1984. Secondary plant substances. Monoterpenes. *Progress in Botany* 46: 119–139.

Sharma, R.N. & Saxena, K.N. 1974. Orientation and developmental inhibition in the housefly by certain terpenoids. *Journal of Medical Entomology* 11: 617–621.

– & Raina, R.M. 1998. Evaluating chemicals for ecofriendly pest management. I: Terpenoids and fatty acids for building termites. *Journal of Scientific and Industrial Research* 57: 306–309.

Shu, S., Grant, G.G., Langevin, D., Lombardo, D.A. & MacDonald, L. 1997. Oviposition and electroantennogram responses of *Dioryctria abietivorella* (Lepidoptera: Pyralidae) elicited by monoterpenes and enantiomers from eastern white pine. *Journal of Chemical Ecology* 23: 35–50.

Singh, S.P., Rao, G.P. & Upadyaya, P.P. 1998. Fungitoxicity of essential oil of some aromatic plants against sugar cane pathogens. *Sugarcane* 2: 14–17.

Smith, A.E. & Secoy, D.M. 1981. Plants used for agricultural pest control in Western Europe before 1850. *Chemical Industry* 3: 12–17.

Smith, R.H. 1975. Formula for describing effect of insect and host tree factors on resistance to western pine beetle attack. *Journal of Economical Entomology* 68: 841–844.

Soliman, F.M., El-Kashoury, E.A., Dathy, M.M. & Gonaid, M.H. 1994. Analysis and biological activity of the essential oil of *Rosmarinus officinalis* L. *Egyptian Journal of Flavour and Fragrance* 9: 29–33.

Stuart, A.E. & Stuart, C.L.E. 1998. A microscope slide test for the evaluation of insect repellents as used with *Culicoides impunctatus*. *Entomologia Experimentalis et Applicata* 89: 277–280.

Takaisi-Kikuni, N.B., Tshilanda, D. & Babady, B. 2000. Antibacterial activity of the essential oil of *Cymbopogon Densiflorus*. *Fitoterapia* 71: 69–71.

Thorsell, W., Mikiver, A., Malm, E. & Mikiver, M. 1989. Fly repellents from extracts of *Mentha spicata* X crispata. Studies on *Protophormia terraenovae*. *Entomologisk Tidskrift* 110: 109–112.

Tiberi, R., Niccoli, A., Curini, M., Epifano, F., Marcotullio, M.C. & Rosati, O. 1999. The role of the monoterpene composition in *Pinus* spp. Needles, in host selection by the pine processionary caterpillar, *Thaumetopea pityocampa*. *Phytoparasitica* 27: 263–272.

Tonelli, E.A. 1987. Experimental use of d-limonene in dips for the treatment of flea and tick infestations in small animals. *Veterinaria Argentina* 4: 931–937.

Valterova, I., Nehlin, G. & Borg-Karlson, A.K. 1997. Host plant chemistry and preferences in egg-laying *Trioza apicalis* (Homoptera: Psylloidea). *Biochemical Systematics and Ecology* 25: 477–491.

Vaughn, S. & Spencer, G.F. 1991. Volatile monoterpenes inhibit potato tuber sprouting. *American Potato Journal* 68: 821–831.

Viglierchio, D.R. & Wu, F.F. 1989. Selected biological inhibitors for *Heterodera schachtii* control. *Nematropica* 19: 75–79.

Vila, R., Valenzuela, L., Bello, H., Canigueral, S., Montes, M. & Adzet, T. 1999. Composition and antimicrobial activity of the essential oil of *Peumus boldus* leaves. *Planta medica* 65: 178–179.

Watanabe, K., Shono, Y., Kakimizu, A., Okada, A., Matsuo, N., Satoh, A. & Nishimura, H. 1993. New mosquito repellent from *Eucalyptus camaldulensis*. *Journal of Agricultural and Food Chemistry* 41: 2164–2166.

Weidenhamer, J.D., Macias, F.A., Fischer, N.H. & Williamson, G.B. 1993. Just how insoluble are monoterpenes? *Journal of Chemical Ecology* 19: 1799–1807.

Werner, R.A. 1995. Toxicity and repellency of 4-allylanisole and monoterpenes from white spruce and tamarrac to the spruce beetle and eastern larch beetle (Coleoptera: Scolytidae). *Environmental Entomology* 24: 372–379.

– & Illmann, B.L. 1994. The role of stilbene-like compounds in host tree resistance of Sitka spruce to the spruce beetle, *Dendroctonus rufipennis*. In: *Proceedings, behavior, population dynamics and control of forest insect conference.* 6–11 February 1994, Mani, Hi. OARDC/ Ohio State University, Wooster, OH.

Wilkinson, R.C. 1980. Relationship between cortical monoterpenes and susceptibility of eastern white pine to white-pine weevil attack. *Forest Science* 26: 581–589.

Wilson, C.L., Solar, G.M., El-Ghaouth, A. & Wisniewski, M.E. 1997. Rapid evaluation of plant extracts and essential oils for antifungal activity against *Botrytis cinerea*. *Plant Disease* 81: 204–210.

Yang, R.Z. & Tang, C.S. 1988. Plants used for Pests'control in China, a literature review. *Economic Botany* 42: 376–406.

Zou, J. & Cates, R.G. 1997. Effects of terpenes and phenolic and flavonoid glycosodes from Douglas fir on Western spruce budworm larval growth, pupal weight, and adult weight. *Journal of Chemical Ecology* 23: 2313–2326.

SELOSTUS

Monoterpeenit kasvinsuojelussa: erityisesti limoneenin vaikutus eri eliöryhmiin

Mohamed A. Ibrahim, Pirjo Kainulainen, Abbas Aflatuni, Kari Tiilikkala ja Jarmo K. Holopainen

Kuopion yliopisto ja MTT (Maa- ja elintarviketalouden tutkimuskeskus)

Kiinnostus luonnonmukaisiin, kasveista peräisin oleviin ja vähemmän terveys- ja ympäristöhaittoja aiheuttaviin torjunta-aineisiin on lisääntynyt luomuviljelyn yleistyessä. Tässä katsauksessa selvitetään monoterpeenien käyttömahdollisuuksia kasvinsuojelussa ja arvioidaan erityisesti limoneenin vaikutuksia eri eliöryhmiin. Limoneenilla on torjuttu lemmikkieläinten ulkoloisia, mutta sen on todettu tehoavan myös moniin muihin hyönteisiin, punkkeihin ja mikrobeihin. Sekä karkottavaa että myrkkyvaikutusta on havaittu. Limoneenin houkuttavuus tuhohyönteisten luontaisille vihollisille voi tarjota mahdollisuuden käyttää sitä luomuviljelyyn sopivana biologisena torjuntamenetelmänä, jossa tuholaisten luontaisia vihollisia houkutellaan kasvustoon ennen tuholaisia. Joissain tapauksissa limoneenilla käsitellyt kasvit voivat kuitenkin altistua tuholaisille limoneenikäsittelyn seurauksena, ja korkeilla pitoisuuksilla limoneeni on kasveille myrkyllinen. Kasviperäisinä luonnontuotteina limoneenista ja muista monoterpeeneistä voi tulla luomuviljelyyn sopivia tuhoeläinten ja rikkakasvien torjunta-aineita. Tämä kuitenkin edellyttää, että aineiden mahdolliset haittavaikutukset viljelykasveihin, maaperäeliöstöön ja tuholaisten luontaisiin vihollisiin ensin selvitetään.

Chapter 3

Effects of limonene on growth and physiology of cabbage (*Brassica oleracea* L.) and carrot (*Daucus carota* L.) plants

Ibrahim, M.A., Oksanen, E. and Holopainen, J.K.
Journal of the Science of Food and Agriculture, 84: 1319-1326.

Journal of the Science of Food and Agriculture *J Sci Food Agric* **84**:1319–1326 (online: 2004)
DOI: 10.1002/jsfa.1819

Effects of limonene on the growth and physiology of cabbage (*Brassica oleracea* L) and carrot (*Daucus carota* L) plants

MA Ibrahim,* EJ Oksanen and JK Holopainen
Department of Ecology and Environmental Science, University of Kuopio, PO Box 1627, FIN-70211, Kuopio, Finland

Abstract: Monoterpenes are plant-based alternatives for pest control in sustainable agriculture. This study investigated the phytotoxic effects of the monoterpene limonene on white cabbage (cv Rinda and Lennox) and carrot (cv Parano and Splendid) plants. The effects of increasing concentrations (from 10 to 120 ml l^{-1}) of limonene on visible leaf injuries, growth, chlorophyll fluorescence, net photosynthesis and stomatal conductance were studied. Limonene concentrations of 90 and 120 ml l^{-1} were significantly phytotoxic in both cabbage and carrot, depending on the cultivar. However, there were major differences in limonene responses between the cultivars in both species. In carrot, lower shoot and root biomass was related to lower chlorophyll fluorescence (indicating lower efficiency of the photochemical processes) in cv Splendid, whereas in cv Parano it was related to lower stomatal conductance (indicating reduced gas exchange), suggesting that the mechanism behind the phytotoxicity may be different in different cultivars. In cabbage, better limonene tolerance and faster recovery processes in cv Lennox compared with cv Rinda were evident through increasing efficiency of photochemistry (chlorophyll fluorescence), and in the higher stomatal conductance and net photosynthesis rates, providing energy for defence and repair action. Since direct phytotoxic effects were detected on carrot and cabbage with limonene, we conclude that critical threshold concentrations for cultivated plants should be determined before monoterpenes are widely used as repellents and deterrents. These thresholds should, as far as possible, be species- and cultivar-specific in order to provide full protection of plants.

Keywords: phytotoxicity; photosynthesis; fluorescence; limonene; carrot; *Daucus carota* L; cabbage; *Brassica oleracea* L

INTRODUCTION

During the last decade, secondary plant metabolites have attracted considerable attention following their clear relationship with biotic factors.[1] The practical use of these plant-based products as pest control agents in particular is receiving attention, in part due to public fears concerning the potential human toxicity of synthetic pesticides and related residue problems in food products. Among these secondary plant metabolites are monoterpenes, many of which are phytotoxic in concentrations below 100 µl l^{-1}.[2]

Certain volatile monoterpenes appear to be involved in allelopathic interactions between higher plants, and have been shown to be potent growth inhibitors of plants and microorganisms.[2] However, they have low mammalian toxicity,[3] and are used commercially in large quantities in flavourings, natural medications and perfumes. Several monoterpenes have also been shown to have bactericidal and/or fungicidal action, repellence and deterrence effects in insects,[4,5] and to be attractive to pollinators.[6]

It has been shown that plants emit highly reactive volatiles which exert a protective effect against ozone phytotoxicity,[7] and that many classes of volatile monoterpenes are likely to inhibit plant growth[8] and induce phytotoxic reactions in other plants.[9,10] This phytotoxicity is manifested in several ways, such as stunting (delaying normal growth or plant development) or inhibiting the growth of the plant.[11,12] The phytotoxic chemicals can also stimulate excessive growth or elimination and distortion of fruit or flowers. However, symptoms of phytotoxicity can be confused with insect or mite damage, diseases and other abiotic problems such as nutrient deficiencies or environmental conditions.[13]

In general, the oxygenated monoterpenes [1,8-cineole, fenchone, limonene oxide and (−)-carvone] are more phytotoxic than the hydrocarbon monoterpenes (α-pinene, limonene and α-phellandrene).[12]

---

* Correspondence to: MA Ibrahim, Department of Ecology and Environmental Science, University of Kuopio, PO Box 1627, FIN-70211, Kuopio, Finland
E-mail: ibrahim@uku.fi

Contract/grant sponsor: Jenny and Antti Wihuri Foundation, Finland
(*Received 13 December 2002; revised version received 15 January 2004; accepted 6 April 2004*)

L-carvone was found to be the most phytotoxic of 16 monoterpenoids tested, in comparison with a control.[14] Similarly, the oxygenated monoterpenes (1,8-cineole, fenchone and limonene oxide) completely inhibited corn germination.[12] Some monoterpenoids were phytotoxic to both corn roots and leaves 3 and 10 days after the treatment.[14]

Carvone [derived from caraway seed (*Carum carvi* L)], which is an interesting compound in crop protection, especially in sprout inhibition for potatoes, appeared to be far more phytotoxic at 50 g active ingredient (gai) $\mathrm{kg^{-1}}$ seed, inhibiting 81% of seed germination of perennial ryegrass (*Lolium perenne*; cv Respect), than other compounds in caraway oil such as *S*- and *R*-limonene.[15] Seeds of winter wheat treated with carvone at 12 gai $\mathrm{kg^{-1}}$ seed also hardly germinated.[16] When (+)-limonene was applied to sugar beet seedlings (cv SSY 1), it was found to be highly phytotoxic at $\geq 200\,\mathrm{mg\,l^{-1}}$.[17] Carvone and (+)- and (−)-limonene were either less effective against *Deroceras reticulatum* and/or caused high phytotoxicity to wheat, barley and perennial ryegrass when seed was dressed to control slugs.[18]

However, phytotoxicity may be minimized by using coating agents (such as those that readily dissolve the chemical), and/or by developing analogues of the materials effective against the target pest.[15] The phytotoxic effect of some plant secondary metabolites depends on the plant species, method of application and pathogen or pest to be controlled.[13] Inhibition of certain key enzymes of photosynthesis and nutrient mobilization is the typical mechanism of action of phytotoxic monoterpenes,[19] but more information is needed on monoterpene phytotoxicity in plant biochemistry.

Replacement of synthetic pesticides with plant-based products that repel insect pests is increasingly demanded, and consequently more attention must be paid to the issue of the phytotoxicity of plant extracts. The aim of this study was to investigate the phytotoxic effects of spraying treatments with various limonene concentrations on the growth, net photosynthesis, stomatal conductance and fluorescence of cabbage and carrot cultivars. Cabbage and carrot were selected as test plants because both species commonly face pest problems in organic farming, and previous studies have already shown that a small amount of limonene is produced by the cabbage[20] and carrot[21] cultivars used in this study.

METHODS AND MATERIALS

Plant material

Cabbage (cv Rinda and Lennox) and carrot (cv Parano and Splendid) seeds were sown individually in 1 l plastic pots filled with a mixture (3:1) of *Sphagnum* peat (Finnpeat B2, Kekkilä Ltd, Tuusula, Finland) and sand (grain size 0.5–1.2 mm, Optiroc Ltd, Helsinki, Finland). The seedlings were watered daily with tap water and fertilized with $1\,\mathrm{g\,l^{-1}}$ 9-Superex (19:5:20 N:P:K, Kekkilä, Finland) at a rate of 0.5–1.0 dl per pot, starting 2 weeks after sowing at the time the seedlings developed their first leaf. The plants were grown under greenhouse conditions (temperature 24/18 °C (day/night), relative humidity (RH) 70/30% (day/night)) at the University of Kuopio in Central Finland. Seedlings of 4–5 and 4–6 leaves (cabbage and carrot, respectively) were used in the present study. The seedlings were 25 and 29 days old (cabbage and carrot, respectively) at the beginning of the experiment.

Limonene application

The monoterpene (*R*)-(+)-limonene ($970\,\mathrm{g\,kg^{-1}}$ purity) was obtained from Aldrich Chemical Co Ltd (Milwaukee, WI, USA). For each treatment, 30 seedlings from each cultivar were moved out of the greenhouse to a fume hood for spraying. For foliar damage and biomass experiments, limonene was tested at concentrations of 10, 30, 60, 90 and $120\,\mathrm{ml\,l^{-1}}$; ethanol at a concentrations of $50\,\mathrm{ml\,l^{-1}}$ was also tested. Pure water was used as control. For physiological response experiments (chlorophyll fluorescence and photosynthesis) the treatments were three sprays (3.5 ml) of 60, 90 and $120\,\mathrm{ml\,l^{-1}}$ of limonene (only at concentrations at which visible injuries developed), with pure water serving as control. The seedlings were returned to the greenhouse after 30 min in the fume hood for limonene evaporation.

Visible foliar damage

In order to determine the threshold level for the phytotoxic concentration of limonene, the visibly injured area as a proportion of the total leaf area was estimated for injured leaves 48 h after the spraying.

Chlorophyll fluorescence

Chlorophyll fluorescence was measured from 10 plants per treatment per cultivar. A field portable pulse modulated chlorophyll fluorometer (FMS 2-Field Fluorescence Monitoring System, Hansatech Instruments Ltd, Norfolk, UK) was used to measure the photochemical efficiency of PSII (F_v/F_m). The photochemical efficiency was measured by dark-adapting two leaves from each plant for 15 min. Fluorescence measurements were made using default parameters from each test mode (F_v/F_m). Repeated fluorescence measurements were conducted at 1, 5 and 7 days intervals after the spraying, hereafter referred as T1, T2 and T3, respectively, as shown in Fig 1.

Photosynthesis and stomatal conductance

Net photosynthesis and stomatal conductance were measured, in the same 10 plants used for fluorescence, with a CI-510 Portable Photosynthesis System (CID Inc, Vancouver, WA, USA) with a four-cell non-dispersive infrared gas analyser. For cabbage measurements, a rectangular cuvette (diameter 11 cm) was

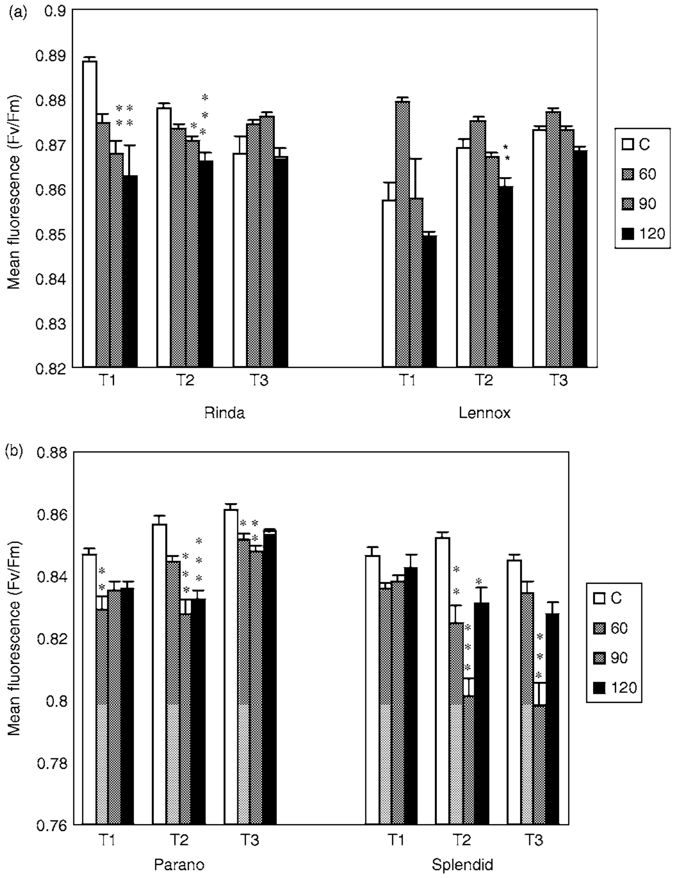


Figure 1. Effect of various concentrations (60, 90 and 120 ml l^{-1}) of limonene and water as control (C) on the chlorophyll fluorescence (F_v/F_m) of (a) cabbage and (b) carrot cultivars. T = time (1 (T1), 5 (T2) and 7 (T3) days after treatment). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

used, while for carrots a cylindrical cuvette (diameter 35 cm) was adopted. One leaf of similar developmental stage per plant was measured at a CO_2 concentration of 455 ± 32 and 390 ± 21 µl l^{-1}, corresponding to the prevailing greenhouse concentrations of CO_2 for cabbage and carrot, respectively. After the steady-state levels of photosynthesis had been determined, saturating light conditions with an average light intensity of 1504 ± 10 (additional LED lamp) were used for cabbage, while prevailing greenhouse light levels with an average light intensity of 191 ± 53 µmol m^{-2} s^{-1} were used for carrot measurements, because the additional light source was not technically adaptable for the cylindrical cuvette. Mean leaf temperature inside the cuvette was 24.2 ± 1.9 °C for cabbage and 25.4 ± 2 °C for carrot (mean outside temperature was 26 ± 2 and 26.7 ± 1.8 °C during the measurements, respectively). Measurements were made between 10.00 and 15.00 h. For cabbage, the same leaf was repeatedly measured 2, 5 and 7 days after spraying (T1, T2 and T3, respectively), as shown in Fig 2. For carrot, the net photosynthesis was measured only at 7 days after spraying, due to technical reasons.

Dry mass

For determination of the dry mass, the shoots and roots of 15 plants from the carrot cultivars for each treatment were cut into paper bags and dried at 60 °C for 3 days before weighing.

Statistical methods

The data were analysed using the SPSS for Windows, version 9.0 (SPSS Inc, Chicago, IL, USA). Analysis of multivariate repeated measures (general linear model procedure) with limonene treatment and cultivar as the between-subjects factor was carried out to determine the effect of limonene, time, cultivar and interactions between them on the fluorescence of both test species, and on photosynthesis and stomatal conductance of cabbage cultivars. Different sampling dates were represented on repeated trials. Thereafter, multiple comparisons were used for each time point using

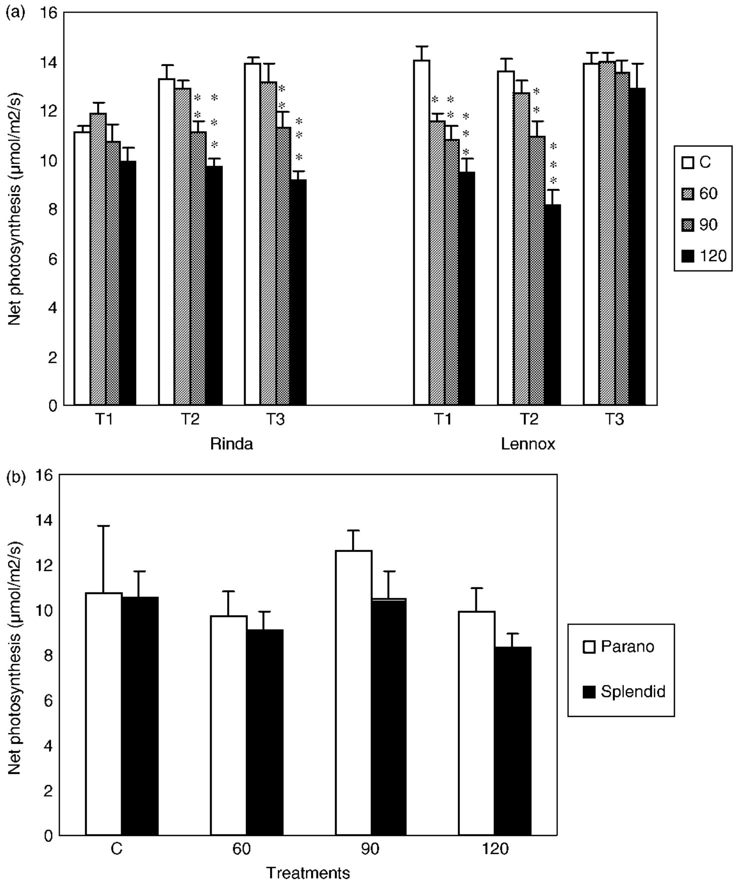


Figure 2. Effect of various limonene concentrations (60, 90 and 120 ml l^{-1}) and water as control (C) on the net photosynthesis (µmol m^2 s^{-1}) of (a) cabbage and (b) carrot cultivars. *T* = time (1 (T1), 5 (T2) and 7 (T3) days after treatment). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Tukey's multiple range tests. Analysis of variance (ANOVA) was used to analyse the leaf injuries of both plant species (the data were transformed with the ARSIN function), as well as biomass, photosynthesis and stomatal conductance of carrot. The experimental unit had 10 plants for fluorescence and photosynthesis, 15 plants for dry mass and 30 plants for visual symptoms. Differences were considered significant at $p \leq 0.05$.

RESULTS

Visible foliar damage

Phytotoxic symptoms of limonene application were characterized by wilting and curling of leaves followed by discoloration (yellowing), further necrotic symptoms in the upper leaf and, finally, leaf death. Judging from the leaf damage, limonene concentrations of 90 and 120 ml l^{-1} were significantly ($p < 0.05$) phytotoxic in cabbage (Table 1), while carrot showed significant injuries only at a limonene concentration of 120 ml l^{-1} (Table 1). There were no significant differences in limonene sensitivity between the cultivars in either species (Table 1).

Chlorophyll fluorescence

In cabbage, chlorophyll fluorescence was declining towards the end of the experiment in control plants of cv Rinda, while the opposite trend was evident in cv Lennox, indicating clear differences between the cultivars in performance of photochemical efficiency (Fig 1(a)). Cv Rinda showed a significant reduction of chlorophyll fluorescence at limonene concentration of 90 and 120 ml l^{-1} during the first two measuring days (1 and 5 days after treatment). In cv Lennox, limonene induced a significant reduction in chlorophyll fluorescence only at 120 ml l^{-1} during the second measurement (5 days after treatment; Fig 1(a)). However, complete recovery towards one week after treatment was evident in both cultivars (Fig 1(a)).

Table 1. Visible foliar damage (percentage of the total leaf area) in cabbage and carrot cultivars (mean ± SE) when treated with pure water, 50 ml l^{-1} of ethanol and limonene concentrations of 10, 30, 60, 90 and 120 ml l^{-1}

	Cabbage			Carrot		
Treatments	Rinda	Lennox	*p*-values	Parano	Splendid	*p*-values
Pure water	0 ± 0	0 ± 0	NS	0 ± 0	0 ± 0	NS
Ethanol	0 ± 0	0 ± 0	NS	0 ± 0	0 ± 0	NS
10	0 ± 0	0 ± 0	NS	0 ± 0	0 ± 0	NS
30	5.7 ± 2.1	7.7 ± 3.3	NS	0 ± 0	0 ± 0	NS
60	16.7 ± 4	0.5 ± 0.2	NS	0.8 ± 0.4	0.7 ± 0.2	NS
90	60.0 ± 6.4	55.0 ± 6.8	<0.001	1.5 ± 0.5	3.2 ± 0.7	NS
120	79.0 ± 5.0	83.2 ± 5	<0.001	17.5 ± 5.1	24.1 ± 4.9	<0.001

NS, not significant

In carrot, there were basic differences between the cultivars in chlorophyll fluorescence of the control plants: cv Parano showed increasing chlorophyll fluorescence, whereas it was almost stable in cv Splendid (Fig 1(b)). However, limonene concentrations from 60 to 120 ml l^{-1} caused a decline in chlorophyll fluorescence in both cultivars (Fig 1(b)). In cv Parano, there was a tendency to recover fluorescence, whilst in Splendid the opposite trend was noted (Fig 1(b)).

Net photosynthesis

In the control plants of cabbage cultivars, photosynthesis showed an increasing trend in cv Rinda, while in cv Lennox it was stable over the time of measurement (Fig 2(a)). In carrot, there were no differences between the cultivars in basic net photosynthesis (Fig 2(b)).

In cabbage, cv Rinda was not affected significantly by limonene 2 days after the treatment, whereas significant reduction in photosynthesis occurred at higher concentrations (90 and 120 ml l^{-1}) 5 and 7 days after spraying (Fig 2(a)). In cv Lennox, all limonene concentrations significantly reduced net photosynthesis 2 days after treatment, compared with the control (Fig 2(a)). Concentrations of 90 and 120 ml l^{-1} induced a significant reduction in net photosynthesis 5 days after the treatment, whereas all the plants recovered their photosynthesis activity at 7 days (Fig 2(a)). The effects of limonene on the photosynthesis of carrot were not significant (Fig 2(b)).

Stomatal conductance

In control plants of cabbage, rates of stomatal conductance decreased throughout the time of measurements in cv Rinda, while more stable values were observed in cv Lennox (Fig 3(a)). In carrot, control plants of cv Splendid showed about 30% lower stomatal conductance than those of cv Parano, indicating large physiological differences in water use (Fig 3(b)). The effects of different limonene concentrations on stomatal conductance were highly variable in cabbage over the time of the experiments, indicating disturbed stomatal control. In cv Rinda, concentrations of 90 and 120 ml l^{-1} caused significant decline in stomatal conductance 2 days after treatment, while a significant increase was found in plants treated with 60 ml l^{-1} limonene one week after treatment. In the Lennox cultivar, significant decreases were found at 60 and/or 120 ml l^{-1} limonene treatment on the first two measuring dates, whereas 90 ml l^{-1} limonene induced a significant increase one week after treatment (Fig 3(a)).

In carrot, only cv Parano showed a significant reduction at the highest concentration (120 ml l^{-1}) of limonene (Fig 3(b)).

Dry mass

In carrot, there was a significant reduction of shoot dry mass in cv Splendid at 90 ml l^{-1} (Table 2). On the other hand, in cv Parano limonene induced a significant dry mass increase at 60 ml l^{-1} (Table 2). There were no significant limonene effects on the root dry weight of carrot, although there was a slightly decreasing trend at higher limonene concentrations in both cultivars. In cv Parano, all treatments reduced the root dry mass, except at 60 ml l^{-1} (Table 2).

DISCUSSION

Limonene induced foliar injuries

In the present study we were able to demonstrate phytotoxic symptoms and minimal negative plant physiological responses to *R*-limonene on cabbage and carrot cultivars at concentrations exceeding 60 ml l^{-1} for cabbage and 90 ml l^{-1} for carrot. The higher limonene resistance of carrot is probably related to inherent resistance, since carrot is known to be a monoterpene-storing species which produces higher amounts of limonene (about 20 µg g^{-1} fresh weight)[21] in leaf tissues. However, cabbage emits small amounts of limonene (10 to 80 µg g^{-1} h^{-1}, dry weight basis),[20] while limonene is not normally detectable in cabbage leaf extracts (Vuorinen *et al*, unpublished).

The initial symptoms appearing in the plants treated with higher concentrations were wilting and curling of leaves, which developed into discoloration and necrotic symptoms, as previously reported by Vaughn and Spencer[22] in potato sprouts and more generally by Fink.[13] Limonene consistently caused darkening and necrotic injuries after 7 days on

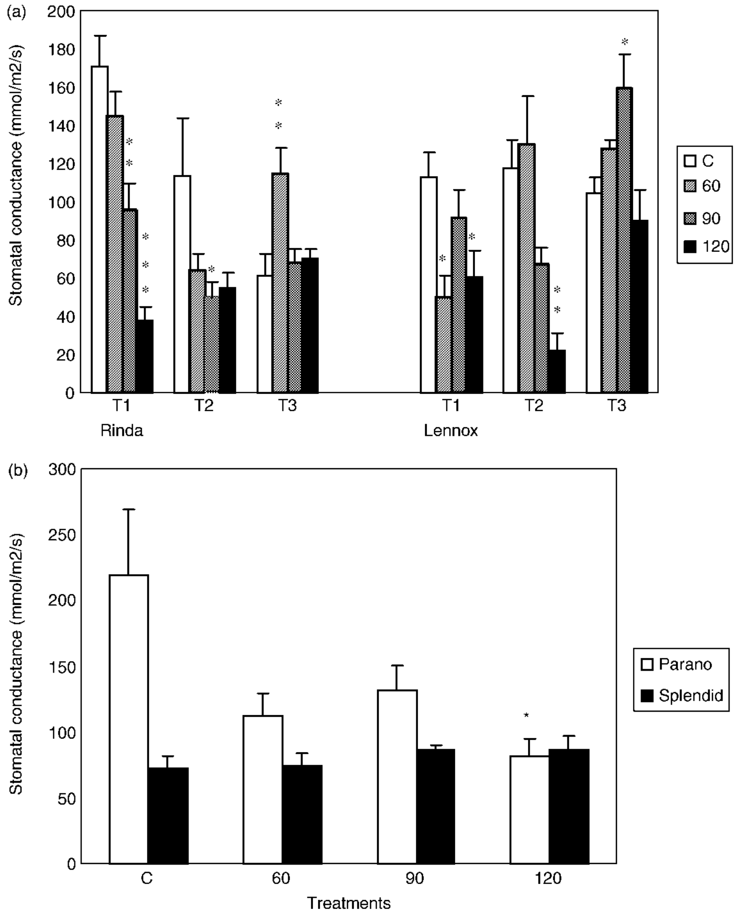


Figure 3. Effect of various concentrations of limonene (60, 90, and 120 ml l^{-1}) and water as control (C) on stomatal conductance of (a) cabbage and (b) carrot cultivars. T = time (1 (T1), 5 (T2) and 7 (T3) days after treatment). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Table 2. Effect of various concentrations of limonene, 50 ml l^{-1} of ethanol and pure water on the (mean ± SE) shoot and root dry mass (g) of the two carrot cultivars (Parano and Splendid)

	Parano			Splendid		
Treatment, ml l^{-1}	Shoot biomass	Root biomass	Shoot/root ratio	Shoot biomass	Root biomass	Shoot/root ratio
Pure water	0.78 ± 0.04	0.54 ± 0.06	1.46	0.83 ± 0.06	0.44 ± 0.05	1.88
Ethanol	0.80 ± 0.06	0.50 ± 0.04	1.58	0.72 ± 0.04	0.47 ± 0.07	1.53
10	0.82 ± 0.05	0.46 ± 0.04	1.78	0.75 ± 0.05	0.49 ± 0.05	1.53
30	0.70 ± 0.04	0.47 ± 0.03	1.52	0.81 ± 0.05	0.36 ± 0.05	2.25
60	1.01 ± 0.05*	0.57 ± 0.04	1.75	0.70 ± 0.03	0.37 ± 0.05	1.89
90	0.82 ± 0.05	0.44 ± 0.05	1.86	0.58 ± 0.03**	0.31 ± 0.03	1.87
120	0.70 ± 0.04	0.37 ± 0.04	1.86	0.70 ± 0.05	0.40 ± 0.03	1.76

Significant differences from the control, * $p < 0.05$.

potato tubers exposed in a continuous-flow system in which the compounds were released as volatiles into the headspace surrounding the tubers.[22] Carvone, which is a monoterpene closely related to limonene, significantly destroyed corn leaves at a concentration of 500 µl l^{-1}, and significantly reduced the dry weight of corn roots 10 days after treatment.[14] In comparison, limonene appeared to be much safer. Hence, the rather low phytotoxicity of limonene allows the use of high concentrations, which may have ecological significance for the control of insect pests on crop plants.

Impact of limonene on primary plant physiology

The present results constitute the first evidence of the negative impact of exogenous limonene sprayings on chlorophyll fluorescence, net photosynthesis and stomatal functioning of two cabbage and two carrot cultivars. In carrot, reduced chlorophyll fluorescence following spraying at a concentration of 90 ml l^{-1} in cv Splendid, indicating lower efficiency of photochemical processes (especially in Photosystem II), was followed by reduced shoot and root biomass. In cv Parano, on the other hand, the decline in shoot and root biomass was related to lower stomatal conductance at 120 ml l^{-1} treatment, indicating reduced gas exchange. Therefore, our results suggest that the mechanisms of phytotoxic limonene action may vary between the cultivars, and thereby cultivar-specific critical threshold levels are likely to be needed for full plant protection.

To our knowledge, only a few studies[19,23,24] have previously reported the impact of monoterpene compounds on plant physiology. For example, 1,4-cineole and 1,8-cineole have been reported to have a negative impact on growth[24] in two weedy plants, *Echinochloa crusgalli* L and *Cassia obtusifolia* L. There was a significant decrease in photosynthetic yield of 1,4-cineole treated plants, compared with control plants,[24] and our results are consistent with this. On the other hand, fumigation of the monoterpene emitter *Quercus ilex* with monoterpenes (α-pinene, sabinene, *cis*-β-ocimene, *trans*-β-ocimene and limonene) improved its tolerance to temperatures higher than 30 °C, due to increased photosynthesis at the higher temperatures.[25,26] Limonene also promoted the stimulation of oxygen uptake (dependent solely on substrates L-malate, succinate, L-glutamate and NADH) of mitochondrial respiration of maize in the concentration range 0.1–5.0 mM, and simultaneously caused inhibition of oxygen uptake in the presence of ADP (coupled respiration). At the same time, a concentration of 1.0 mM limonene increased basal respiration in the absence of exogenously added ADP.[27]

In the present study we found phytotoxic effects of limonene on the primary physiology and growth of carrot and cabbage, but Ester and Nijenstein[15] reported that neither *R*- nor *S*-limonene was phytotoxic at $\leq$400 gai kg^{-1} seed to the germination of the two cultivars (Respect and Carat) of perennial ryegrass (*Lolium perenne* L), while carvone appeared to be far more phytotoxic. It is suggested that the oil vapours of eucalyptus, including limonene, impair the respiratory as well as the photosynthesis of *Phaseolus aureus*, *Lens esculentum*, *Hordeum vulgare* and *Avena sativa*.[23] In addition to direct physiological phytotoxic effects, limonene may predispose the plants to other environmental stress factors, such as drought, through increased transpiration due to loss of stomatal control, as suggested by our results with cabbage.

Differences between the cultivars

This experiment revealed major differences both in physiological performance and in limonene responses between the cultivars, in both cabbage and carrot. In cabbage, the higher limonene tolerance and faster recovery processes in cv Lennox compared with cv Rinda could be explained by the increasing efficiency of photochemistry (chlorophyll fluorescence), and by higher stomatal conductance and net photosynthesis rates, which provide energy for defence and repair action.

In carrot, contrasting responses to limonene treatment between the cultivars became evident in the shoot dry mass. The significant increase of the shoot dry mass in cv Parano at 60 ml l^{-1} limonene may indicate some compensating growth of new foliage due to limonene stress, as supported by the increasing efficiency of Photosystem II during the experiment. The cultivar differences in phytotoxic and growth responses are difficult to explain by inherited limonene levels, which are significantly higher in cv Splendid than in cv Parano,[21] which showed slightly stronger symptoms.

In addition to the above-mentioned factors related to photosynthesis and water use, cultivar differences in phytotoxic responses might be related to different epidermal and morphological characteristics. For example, differences in the thickness and quality of the cultivar wax layer in cabbage may have a profound effect on limonene phytotoxicity. Furthermore, cv Lennox is capable of emitting significantly higher amounts of monoterpenes than cv Rinda (Vuorinen *et al*, unpublished). This trait might be related to the ability of this cultivar to cope better with exogenous limonene concentrations.

CONCLUSION

The results of our study reveal the primary phytotoxic effects of the limonene concentrations on carrot and cabbage. Since our results are consistent with those of numerous studies with several cultivated plants which indicate the high phytotoxicity of monoterpenes,[15,16,18,25,28] we suggest that critical thresholds of limonene for primary plant physiology (such as net photosynthesis, stomatal conductance and growth) and the variability in limonene sensitivity of cultivated plants should be determined urgently. Our study suggests that the critical threshold for carrot and cabbage is 60–90 ml l^{-1}, depending on the cultivar. If maximum non-phytotoxic concentrations could be determined, limonene would be a useful natural chemical for plant protection in organic farming systems. The exposure to non-phytotoxic concentrations of the exogenous monoterpenes may induce the internal production of monoterpenes[26] in crop plants, which may provide induced resistance against herbivores. In addition, the potential harmful effects of monoterpenes on the natural enemies of herbivores and non-target soil animals should be studied before application in integrated pest management programs.

ACKNOWLEDGEMENTS

We thank Dr GVP Reddy for his discussions and comments on the manuscript. This study was funded by the Jenny and Antti Wihuri Foundation, Finland. The English was revised by Mr Vivian Paganuzzi.

REFERENCES

1 Singh HP, Batish DR, Kaur S, Ramezani H, and Kohli RK, Comparative phytotoxicity of four monoterpenes against *Cassia occidentali*, *Ann Appl Biol* **141**:111–116 (2002).

2 Weidenhamer JD, Macias FA, Fischer NH and Williamson GB, Just how insoluble are monoterpenes?, *J Chem Ecol* **19**:1799–1807 (1993).

3 Vaughn SF and Spencer GF, Synthesis and herbicidal activity of modified monoterpenes structurally similar to Cinmethylin, *Weed Sci* **44**:7–11 (1996).

4 Dube S, Upadhyay PD and Tripathi SC, Antifungal, physiochemical, and insect-repelling activity of the essential oil of *Ocimum basilicum*, *Can J Bot* **67**:2085–2087 (1989).

5 Ibrahim MA, Kainulainen P, Aflatuni A, Tiilikkala K and Holopainen JK, Insecticidal, repellent, antimicrobial activity and phytotoxicity of essential oils: with special reference to limonene and its suitability for control of insect pests, *Agric Food Sci Finland* **10**:245–261 (2001).

6 Harborne JB, *Introduction to Ecological Biochemistry*, 4th edn. Academic Press, London (1992).

7 Lerdau M and Slobodkin K, Trace gas emissions and species-dependent ecosystem services, *Trends Ecol Evol* **17**:309–312 (2002).

8 Muller WH and Muller CH, Volatile growth inhibitors produced by Salvia species, *Bull Torrey Bot Club* **91**:327–330 (1964).

9 Muller CH and Chou CH, Phytoalexins. An ecological phase of phytochemistry, in *Phytochemical Ecology* Ed by Harborne JB. Academic Press, London, pp 201–216 (1972).

10 Gant RE and Clebsch EEC, The allelopathic influences of *Sassafras albidum* in old-field succession in Tennessee, *Ecology* **56**:604–615 (1975).

11 Williamson GB, Fischer NH, Richardson DR and De La Pena, A Chemical inhibition of fire-prone grasses by fire-sensitive shrub, *Conradina canescens*, *J Chem Ecol* **15**:1567–1577 (1989).

12 Vaughn SF and Spencer GF, Volatile monoterpenes as potential parent structure for new herbicides, *Weed Sci* **41**:114–119 (1993).

13 Fink S, *Pathological and Regenerative Plant Anatomy*, 1st edn. Bontraeger, Berlin (1999).

14 Lee S, Tsao R, Peterson CH and Coats JR, Insecticidal activity of monoterpenoids to western corn rootworm (Coleoptera: Chrysomelidae), twospotted spider mite (Acari: Tetranichidae), and house fly (Diptera: Muscidae), *J Econ Entomol* **90**:883–892 (1997).

15 Ester A and Nijenstein HJ, Control of field slug [*Deroceras reticulatum* (Müller)] by seed-applied pesticides in perennial ryegrass assessed by laboratory tests, *J Plant Dis Prot* **103**:42–49 (1996).

16 Ester A and Nijenstein HJ, Control of the field slug *Deroceras reticulatum* (Müller) (Pulmonata: Limacidae) by pesticides applied to winter wheat seed, *Crop Prot* **14**:409–413 (1995).

17 Viglierchio DR and Wu FF, Selected biological inhibitors for *Heterodera schachtii* control, *Nematropica* **19**:75–79 (1989).

18 Nijenstein JH and Ester A, Phytotoxicity and control of the field slug *Deroceras reticulatum* by seed applied pesticides in wheat, barley and perennial ryegrass, *Seed Sci Technol* **26**:501–513 (1998).

19 Romagni JG, Duke SO and Dayan FE, Inhibition of plant asparagine synthetase-by monoterpene cineoles, *Plant Physiol* **123**:725–732 (2000).

20 Vuorinen T, Reddy GVP, Nerg A-M and Holopainen JK, Monoterpene and herbivore-induced emissions from cabbage plants grown at elevated atmospheric CO_2 concentration, *Atmos Environ* **38**:675–682 (2004).

21 Kainulainen P, Nissinen A, Piirainen A, Tiilikkala K and Holopainen JK, Essential oil composition in leaves of carrot varieties and preference of specialist and generalist sucking insect herbivores, *Agric Forest Entomol* **4**:211–216 (2002).

22 Vaughn SF and Spencer GF, Volatile monoterpenes inhibit potato tuber sprouting, *Am J Potato Res* **68**:821–831 (1991).

23 Kohli RK and Singh D, Allelopathic impact of volatile compounds from *eucalyptus* on crop plants, *Biol Plant* **33**:475–483 (1991).

24 Romagni JG, Allen SN and Dayan FE, Allelopathic effects of volatile cineoles on two weedy plant species, *J Chem Ecol* **26**:303–313 (2000).

25 Loreto F, Förster A, Dürr M, Csiky O and Seufert G, On the monoterpene emission under heat stress and on the increased thermotolerance of leaves of *Quercus ilex* L fumigated with selected monoterpenes, *Plant Cell and Environ* **21**:101–107 (1998).

26 Delfine S, Csiky O, Seufert G and Loreto F, Fumigation with exogenous monoterpenes of a non-isoprenoid-emitting oak (*Quercus suber*): monoterpene acquisition, translocation, and effect on the photosynthetic properties at high temperature, *New Phytol* **146**:27–36 (2000).

27 Abrahim D, Braguini WL, Kelmer-Bracht AM and Ishii-Iwamoto EL, Effects of four monoterpenes on germination, primary root growth, and mitochondrial respiration of maize, *J Chem Ecol* **26**:611–624 (2000).

28 Reust W, Carvone, a new natural sprouting inhibitor for potato storage, *Rev Suisse Agric* **32**:150–152 (2000).

Chapter 4

Response of *Plutella xylostella* and its parasitoid *Cotesia plutellae* to volatile compounds.

Ibrahim MA., Nissinen A and Holopainen JK. Journal of Chemical Ecology.
In press

RESPONSE OF *PLUTELLA XYLOSTELLA* AND ITS PARASITOID *COTESIA PLUTELLAE* TO VOLATILE COMPOUNDS

Mohamed A. Ibrahim[1*], Anne Nissinen[1, 2] and Jarmo K. Holopainen[1]

1)Department of Ecology and Environmental Science, University of Kuopio, Kuopio, Finland
2) Agrifood Research Finland, Plant Protection, FIN-31600 Jokioinen, Finland.

Short title: Limonene attracts *Plutella xylostella* and its parasitoid

*Corresponding author. Email: Mohamed.Ibrahim@uku.fi

Abstract - The effects of limonene, a mixture of limonene + carvone (1:1, v/v), and methyl jasmonate (MeJA) on diamondback moth (DBM) (*Plutella xylostella* L.) oviposition, larval feeding and the behaviour of its larval parasitoid *Cotesia plutellae* (Kurdjumov) with cabbage (*Brassica oleracea* L. ssp. *capitata,* cvs. Rinda and Lennox) and broccoli (*B. oleracea* subsp. *Italica* cv Lucky) were tested. Limonene showed no deterrent effect on DBM when plants were sprayed with or exposed to limonene, although there was a cultivar difference. A mixture of limonene and carvone released from vermiculite showed a significant repellent effect, reducing the number of eggs laid on the cabbages. MeJA treatment reduced significantly the relative growth rate (RGR) of the larvae on cv Lennox leaves. In the Y-tube olfactometer tests C. *plutellae* preferred the odours of limonene and MeJA to filtered air. In cv Lennox, the parasitoid preferred significantly DBM-damaged plants with limonene to such plants without limonene. *C. plutellae* females were strongly repelled by the mixture of limonene + carvone. Exogenous MeJA induced the emission of the sesquiterpene (*E*,*E*)-α-farnesene, the homoterpene (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) and green leaf volatile (*Z*)-3-hexenyl acetate + octanal in both cultivars. The attraction effect of limonene and MeJA predicts that these two compounds can be used in sustainable plant protection strategies in organic farming.

Key Words-Limonene, methyl jasmonate, carvone, diamondback moth, *Cotesia plutellae,* cabbage, broccoli, vermiculite, olfactometer.

INTRODUCTION

The diamondback moth (DBM), *Plutella xylostella* (L.), (Lepidoptera: Yponometidae) is economically the most important pest of cruciferous crops throughout the world (Talekar and Shelton, 1993). DBM feed on all cruciferous crop plants, cole crops, and several green house plants (Reddy et al., 2004a). In this context, one of the most important biological control agents for DBM is the braconid *Cotesia plutellae* (Kurdjumov) (Hymenoptera: Braconidae). *C. plutellae* is a potential larval parasitoid which can parasitize in the first three instars of larval DBM (Reddy et al., 2002; Zu-hua et al., 2002).

Plants can defend themselves against herbivores by direct and indirect defence mechanisms. Direct defence directly affects the insect herbivore through toxic or anti-nutritional compounds, or through repellents or deterrents (Karban and Baldwin, 1997). In indirect defence, after herbivore damage, plants produce volatiles that can attract predators or parasitoids, as has been shown in many plants (Dickens, 1999; Shiojiri et al., 2001; Dicke et al., 2003; Vuorinen et al., 2004a,b). These volatile compounds permit insect parasitoids and predators to discriminate between intact and damaged plants (Reddy and Guerrero, 2004b; Vuorinen et al., 2004a). The parasitoid *C. plutellae* prefers the odour of damaged cabbage plants to that of intact cabbage in Y-tube olfactometer tests (Vuorinen et al., 2004a).

In previous studies, limonene alone or with other monoterpenes has been shown to have deterrent (Ntiamoah et al., 1996; Ntiamoah and Borden, 1996) or repellent (Peterson et al., 1994; Nehlin et al., 1994; Ibrahim et al., 2001) effects on insects. Chenier and Philogene (1989), for example, reported that monoterpenes, including limonene, attracted predators of conifer bark beetles. Carvone has been reported to inhibit the feeding of pales weevil (*Hylobius pales*) on *Pinus strobus* seedlings (Salom et al., 1996), of pine weevil (*Hylobius abietis,* Coleoptera: Curculionidae) on Scots pine (*Pinus sylvestris*) (Klepzig and Schlyter, 1999, Schlyter et al., 2004), and of the slug *Arion lusitanicus* on lettuce (Frank et al., 2002). Tripathi et al. (2003) reported feeding deterrence and contact and fumigant toxicity of carvone against stored product beetles, while Den Ouden et al. (1993) found a short-term oviposition repellence of carvone against cabbage root fly (*Delia radicum*).

Methyl jasmonate (MeJA) (a volatile derivative of jasmonic acid), which is involved in plant defence mechanisms against herbivores, has been found to increase the activities of defence-related proteins (Thaler et al., 1996). MeJA has been reported to protect genetically modified Arabidopsis plants (deficient in the jasmonate precursor linolenic acid) from attack by the larvae of *Bradysia impatiens* (Diptera: Sciaridae) (McConn et al., 1997). The relative growth rate (RGR) of *Spodoptera exigua* larvae fed on MeJA-treated leaflets has also been found to be lower than that of those reared on control leaflets (Thaler et al., 1996). However, Oka et al. (1999) found that MeJA did not show promising results as a nematocide to protect tomato from root-knot nematodes.

Natural compounds originating from plants might be potential sources of alternatives to conventional insecticides (Lee et al., 2001; Ibrahim et al., 2004). However, alternatives not persistent in the environment and are safe to natural enemies, non-target organisms and human beings are needed particularly in sustainable agriculture (Lacey and Shapiro-Ilan, 2003). Therefore, our aim was to investigate the role of limonene, a mixture of limonene + carvone, and MeJA in diamondback moth (DBM) control and their effects on the parasitoid *C. plutellae*.

METHODS AND MATERIALS

Plant Material and Insects. Cabbage (*Brassica oleracea* L. ssp. *capitata,* cvs. Rinda and Lennox) and broccoli (*B. oleracea* subsp. *Italica* cv Lucky) seedlings 4-5 weeks old grown at 24/18°C (day/night) and relative humidity (RH) of 60% were used. Diamondback moth *Plutella xylostella* L. and the parasitoid *Cotesia plutellae* (Kurdjumov) were from our own mass rearings (Vuorinen et al., 2004a). Second- and third-instar larvae of *P. xylostella* feeding on broccoli plants were offered to *C. plutellae* females for egg laying. The adults of *C. plutellae* that emerged were collected and released into a clean insect cage, and a honey-water solution (1:1) was provided for feeding. One- to three-day-old *C. plutellae* females were used in the behavioural assay.

Test compounds. (*S*)-(+)-carvone and (*R*)-(+)-limonene are the major compounds in the essential oil of caraway seeds (Bouwmeester et al., 1995; Bouwmeester et al., 1998; Hannukkala et al., 2002). Recently, there has been some interest in the use of caraway oil for plant protection purposes (Iacobellis et al., 2005). (*R*)-(+)-limonene has been found to be more effective than (*S*)-(-)-limonene against the pine processionary caterpillar, *Thaumetopoea pityocampa* (Tiberi et al., 1999). (*R*)-(+)-limonene (97% purity) and (*S*)-(+)-carvone (96%) obtained from Aldrich Chemical Co. Ltd. (Milwaukee, WI, USA), and methyl jasmonate (96%) provided by Bedoukian Research Inc. (Danbury, CT, USA) were used in this study.

DBM Egg-laying Experiments. The following three experiments were conducted for the egg-laying study.

Experiment 1: Cabbage plants sprayed with 3% limonene solution in a 5% ethanol (99.5% purity) solution in water were offered to DBMs for egg laying in a two-choice test where the moths were allowed to choose between two treatments (control and treated) 24 hrs after spraying in a acrylic polyester gauze cage (60 cm x 33 cm x 33cm, external dimensions). Plants sprayed with 5% ethanol in 95 ml of water were used as controls. The amount of solution reaching the plant surface was estimated to be 3-4ml. The test was replicated 15 times.

Experiment 2: Cabbage plants were exposed to limonene released from vermiculite (phyllosilicate mineral): 15ml of limonene was mixed in 300ml of vermiculite by vigorous stirring, and 25ml of the mixture was placed at the base of each plant. For controls, 25ml of vermiculite without limonene was placed at the base of the plants. Thereafter, the plants were introduced into the cages individually in a no-choice test where there was only one plant in the cage. The two tests were conducted simultaneously in two separate growth chambers and each test was replicated 10 times.

Experiment 3: As previously, 7.5ml of limonene and 7.5ml of carvone were mixed in 300ml of vermiculite, and thereafter 25ml of the mixture was placed at the base of each plant. For controls, 25ml of vermiculite without test compounds was placed at the base of each plant. Each test was replicated 10 times.

Ten moths (1:1 sex ratio) were released into the cage and allowed to lay eggs for 48 hrs for all three experiments. The moths were then removed from the cage and the eggs were counted.

Feeding Experiment. Cabbage (cv Lennox and Rinda) plants sprayed with 3% limonene (prepared as previously) or 4.5mM MeJA in 5% ethanol (99.5% purity) in water were used in this study. Plants sprayed with 5% ethanol in water were used as controls. Twenty four hours after spraying, the fully developed leaves of the sprayed plants were cut and the petiole was inserted into a 1.5ml Eppendorf tube filled with tap water. Thereafter, the leaf was placed in a plastic container (250ml) with a lid. Late second- or early third-instar larva of DBM was introduced onto the leaf and allowed to feed for 48 hours. The initial and final weights of the larvae were measured to calculate the RGR, using the following formula: $\mathrm{Ln}(W_f)-\mathrm{Ln}(W_i)$, where W_f is final weight and W_i initial weight. The experiment was replicated 20 times for the limonene and 15 times for the MeJA treatments.

Olfactometer Experiments. Cabbage plants from the above-mentioned cultivars and broccoli were used in this study. Ten µl of limonene, 10 µl of methyl jasmonate (MeJA), and 2 µl of a mixture of limonene + carvone (1:1 v/v) were applied by pipetting on Whatman filter paper (42.5mm^2) and left in a fume hood for 10 min to evaporate. The filter paper with the elicitor was introduced into the glass container for the test as the odour source with a plant or without, depending on the test. The filter paper was placed on a piece of aluminium foil in the glass container. This experiment was conducted in a Y-tube olfactometer (main arm 10.5 cm, other arms 10 cm, inner diameter 1.6 cm, angle between two arms ~ 90°).The plants were placed in 1-l glass containers closed with Teflon-sealed lids with two inlets. Pressurized air was activated-charcoal filtered and passed through the glass container holding the plant with the odour source (with or without limonene, with or without limonene + carvone, and with or without MeJa), and then to one of the Y-tube arms. The parasitoid *C. plutellae* females were released into the opening of the main arm-end of the Y-tube and observed for 5 min or until they made the final choice. This choice was recorded as the insect passed into the end of the Y-tube arm. The Y-tube was rotated 180° after each test run. The source of the odour, the glass container, the Y-tube device, and the lids of the containers were replaced after testing eight *C. plutellae* females in the limonene and MeJA experiments. Ten females were used for each test in the limonene + carvone assays, the filter papers with the mixture and the Y-tube being replaced after testing 5 females.

Collection of Volatile Compounds. Volatiles emitted from the foliage of the two cultivars of cabbage plants were collected using the headspace collection technique and analysed by GC-MS as described by Vuorinen et al. (2004a) one day after spraying with 3% limonene in 5% ethanol in water or 4.5mM MeJA in 5% ethanol in water. Five and six (Lennox and Rinda respectively) randomly selected seedlings per treatment were used for volatile collections. The roots of each sample plant were washed and pruned slightly before being inserted into a 15ml vial filled with water. Thereafter, the whole plant was enclosed in a 1.5-l (Lennox) or 1-l (Rinda) glass vessel. The glass vessels had two inlets, one for purified air and one for sampling. Charcoal-filtered air was led through Teflon tubing at a flow rate of 200 ml min^{-1} to the Tenax TA adsorbent tubes (150 mg), where the volatiles were collected for 30 min per sample. The samples were analysed by GC-MS (Hewlett Packard GC type 6890, MSD 5973). The compounds collected on the Tenax TA adsorbent tubes were released by thermodesorption at 250°C for 10 minutes. The compounds were cryofocused in a cold trap at -30 °C and subsequently injected onto a HP-5 capillary column (50.0 m * 0.2 mm i.d. * 0.50 µm film thickness). The column temperature was first held at 40°C for 1 minute. Thereafter, the temperature was programmed to rise from 40°C to 210°C at 5°C min^{-1} and finally to 250°C at 20°C min^{-1}.

An interval of 30 to 300 *m/z* range was considered by the MS runs. The compounds were identified using different external standards, one for terpenoids and one for green leaf volatiles (GLVs), by comparing the mass spectra of a single compound with those of pure standards and those in the Wiley library. The amount of α-thujene, (*E*)-4,8-Dimethyl-1,3,7-nonatriene (DMNT) and (*E*,*E*)-α-farnesene was calculated by assuming that the responses to them were the same as those to α-pinene, (*Z*)-ocimene and (*E*)-β-farnesene, respectively. The shoot biomass of the sample plants was determined to calculate the volatile emissions as ng g dry weight^{-1} h^{-1}. The volatiles were collected at a room temperature of 22°C and light intensity of 250 μmol m^{-2} s^{-1}. The light intensity was measured with Quantum Sensor LI-185B (LI-COR, inc. Lincoln, Nebraska, USA).

Statistics. Statistical analysis was performed using the SPSS 11.5 for Windows statistical package. One-way ANOVA and general linear models (GLM) procedures followed by Tukey's or Dunnett's T3 multiple comparison tests were used for RGR of larvae and for normally distributed volatile compounds. Other compounds which were not normally distributed were tested with the nonparametric Kruskal-Wallis test. The Mann-Whitney test with Bonferroni correction was used to analyse the differences between treatments. The data from the DBM egg-laying tests were analysed with the independent-samples *t*-test. The response of *C. plutellae* was analysed with the non-parametric binomial test.

RESULTS

Egg-laying Experiments. DBM females laid marginally significantly more eggs on cabbage (cv. Rinda) plants sprayed with limonene (Figure 1A) in the two-choice tests and when limonene was released from vermiculite (Figure 1B) in the no-choice test than on control plants. The number of eggs laid by the females on cabbage plants treated with limonene + carvone released from vermiculite was significantly lower in both cultivars in the no-choice tests (Figure 1C). On broccoli plants, the difference was not significant.

Feeding Experiment. Limonene had no deterrent effect on DBM larvae when detached leaves from plants sprayed with this compound were offered to them. The larval weight was not influenced by limonene (Figure 2A), but was significantly lower in cv Lennox sprayed with MeJA (Figure 2B).

Olfactometer Experiments. Females of *C. plutellae* significantly preferred the odours of pure compounds (limonene and MeJA) to filtered air without plants in the Y-tube olfactometer tests (Figure 3A), but were not able to discriminate between the limonene-carvone mixture and clean air. However, the number (n=12) of *C. plutellae* females responding to any of the odour sources was significantly (P <0.001) lower than that (n=38) of females which did not choose any of the odour source (Figure 3A). In another case, *C. plutellae* females showed a significant preference for the damaged plant with limonene over the damaged plant alone in cv Lennox (Figure 3B). In the tests with broccoli and with both cabbage cultivars, the females preferred significantly the damaged plant without the mixture of limonene + carvone to the damaged plant with the mixture of limonene + carvone (Figure 3C). The females were not able to differentiate between intact cabbage plants with limonene and those without it (Figure 3D). In the MeJA treatment, the females did not show significant preference for any of the odour sources when tested with intact or damaged plants (data not shown).

Collection of Volatile Compounds. In both cultivars, emission of homoterpene DMNT and the sesquiterpene *E,E*-α-farnesene was induced in the MeJA treatment (Tables 1 & 2). In cv Rinda, the concentration of total monoterpenes was higher in the limonene treatment than in the other two treatments (Table 2). In cv Lennox, the sabinene concentration was significantly lower in the MeJA treatment. In both cultivars, the GLV (*Z*)-3-hexenyl acetate + octanal was significantly higher in the MeJA treatment than in the other two treatments. In cv Rinda, the total GLVs were significantly higher in the MeJA treatment.

DISCUSSION

Limonene Attracts DBM and Its Parasitoid C. plutellae. We demonstrated for the first time that exogenous limonene alone attracts DBM females to cabbage cv Rinda for ovipositioning. Previously, Pivnick et al. (1994) have shown that DBMs are highly sensitive to an uncharacterised combination of volatiles released by intact plants and probably dominated by terpenes. In this regard, limonene has been found to be one of the main volatile compounds in the headspace of intact cabbages (Shiojiri et al., 2001; Vuorinen et al., 2004b). However, it has been found that DBM moths prefer the volatiles released from conspecific-damaged cabbage plants over undamaged plants (Shijiori and Takabayashi, 2003). Vuorinen et al. (2004a) found that the emission of total monoterpenes from cv Rinda was significantly induced by the 48h feeding damage of DBM larvae. We found that the total monoterpene emission from limonene-treated cv Rinda was significantly higher than that in other treatments. This may indicate that the volatile emission from exogenously limonene-treated cabbage cv Rinda resembles that of DBM damaged cv Rinda.

Our results show that limonene has no deterrent effect on DBM larval feeding. This is in contrast with the findings with other pest insects by Peterson et al. (1994), Nehlin et al. (1994) and Ntiamoah et al. (1996), who have shown that limonene deterred significantly the oviposition of pickleworm moths (*Diaphania nitidalis*), carrot psyllids (*Trioza apicalis*) and onion maggots (*Delia antiqua* M.), respectively. In Y-tube olfactometer tests, females of *C. plutellae* preferred infested plants with limonene to infested plants without limonene in cv Lennox. This observation is in agreement with results of our previous study, which indicated that limonene is an attractant to the generalist predator *Podisus maculiventris* (Hemiptera: Pentatomidae) (Ibrahim and Holopainen, 2002).

The Combination of Limonene with Carvone Improves its Repellent and Deterrent Effect. We found that a mixture of limonene and carvone reduces significantly the number of eggs laid by DBMs on cabbage. Our results are consistent with the findings of Ntiamoah et al. (1996) and those of Ntiamoah and Borden (1996), who have shown, respectively, that a mixture of limonene, 3-carene and p-cymene deters oviposition by the onion maggot (*D. antique* M.) and by the cabbage maggot (*D. radicum* L.). Similarly, the mixture of limonene and carvone has shown to repel females of *C. plutellae* in Y-tube olfactometer. Although the use of this mixture in Y-tube olfactometer has not been reported previously, our findings principally agree with those of Nehlin et al. (1994), Ntiamoah et al. (1996) and Ntiamoah and Borden (1996). This demonstrates that the deterrent effect of limonene increases when it is combined with other terpenoids. In previous studies, Salom et al. (1996), Klepzig and Schlyter (1999), Frank et al. (2002) and Schlyter et al. (2004) have reported a feeding deterrent effect of carvone on insects and slugs.

MeJA Induces the Emission of Volatiles. We found that MeJA treatment induces the emission of the sesquiterpene *E*,*E*-α-farnesene and the homoterpene DMNT, as previously reported by Rodriguez et al. (2001) for cotton plants treated with exogenous MeJA. Similarly, the exogenous application of MeJA on oilseed rape (*Brassica rapa subsp. oleifera*) has been reported to increase significantly the amount of these compounds (Loivamäki et al., 2004). In addition to constitutive monoterpenes (sabinene, limonene, β-pinene, myrcene, 1,8-cineole, α-thujene and α-pinene), infested cabbage plants emit induced compounds such as DMNT, and (*E,E*)-α-farnesene, which are known to attract herbivores and their natural enemies (Dicke et al., 1999; Rodriguez et al., 2001; Vuorinen et al., 2004a,b). Although the mechanism whereby MeJA induces DMNT and

(*E,E*)-α-farnesene is not fully understood, Mandujano-Chavez et al. (2000) found that MeJA can induce the expression of sesquiterpene cyclase genes in cell culture of tobacco. However, it has been reported that MeJA has limited ability to induce later steps in the sesquiterpene pathway (Mandujano-Chavez et al., 2000). On the other hand, MeJA has been found to induce the expression of lipoxygenase (LOX) in common bean (Porta et al., 1999) and in maize (Kim et al., 2003), which may indicate that MeJA affects the emission of DMNT and (*E,E*)-α-farnesene via lipoxygenase pathway by inducing the accumulation of endogenous jasmonic acid (JA).

As recently reported (Bogahawatte and van Emden, 1996; Potting et al., 1999; Vuorinen et al., 2004a), the volatile compounds emitted from plants damaged by DBM allow *C. plutellae* to discriminate between intact and damaged plants. In the present study, we demonstrated that the parasitoid *C. plutellae* is able to discriminate between limonene, MeJA and clean air. The response of *C. plutellae* varies with the plant species and probably with the cultivar (Liu and Jiang, 2003), and the volatiles induced from infested plants seem to be very important cues to *C. plutellae* (Shiojiri et al., 2001; Shiojiri and Takabayashi, 2003). Reddy et al. (2002) have also shown that the parasitoids of DBM, including *C. plutellae,* are attracted to a variety of chemical cues related to their host.

In conclusion, our results suggest that limonene and MeJA can be used as attractants for natural enemies of insect herbivores, particularly those of DBM, in organic agriculture. A mixture of limonene with carvone can improve the effectiveness of limonene as a deterrent on crop plants, but deterrents have negative effects on natural enemies of DBM. Limonene can perhaps be used as an attractant of DMB on trap crop plants.

Acknowledgments-This study was funded by the Jenny & Antti Wihuri Foundation and the Finnish Cultural Foundation. The English text was revised by V. Michael Paganuzzi, MA.

REFERENCES

BOGAHAWATTE, C. N. L. and VANEMDEN, H. F. 1996. The influence of the host plant of diamondback moth (*Plutella xylostella*) on the plant preferences of its parasitoid *Cotesia plutellae* in Sri Lanka. *Physiol. Entomol.* 21: 93-96.

BOUWMEESTER, H. J., DAVIES, J. A. R. and TOXOPEUS, H. 1995. Enantiomeric composition of carvone, lomonene and carveols in seeds of dill and annual and biennial caraway varieties. *J. Agric. Food Chem.* 43: 3057-3064.

BOUWMEESTER, H. J., GERSHENZON, J., KONINGS, M. C. J. M. and CROTEAU, R. 1998. Biosynthesis of the monoterpenes limonene and carvone in the fruit of caraway. *Plant Phsiol.* 117: 901-912.

CHENIER, J. V. R. and PHILOGENE, B. J. R. 1989. Field responses of certain forest coleopteran to conifer monoterpenes and ethanol. *J. Chem. Ecol.* 15: 1729-1745.

DEN OUDEN, H., VISSER, J. H., ALKEMA, D. P.W., DE VLIEGER, J. J., and DERKS, P. S. M.1993. Experiments with volatile substances in slow release formulations causing repellency for oviposition of cabbage root fly, *Phorbia brassicae* Bché. (Dipt., Anthomyiidae). *J. Appl. Ent.* 115, 307-312.

DICKE, M., GOLS, R., LUDEKING, D., and POSTHUMUS, M. A. 1999. Jasmonic acid and herbivory differentially induce carnivore-attracting plant volatiles in lima bean plants. *J. Chem. Ecol.* 25: 1907-1922.

DICKE, M., VAN POECKE, R. M. P., and DE BOER, J. G. 2003. Inducible indirect defence of plants: from mechanisms to ecological functions. *Basic Appl. Ecol.* 4: 27-42.

DICKENS, J. C. 1999. Predator-prey interactions: olfactory adaptations of generalist and specialist predators. *Agr. Forest Entomol.* 1: 47-54.

FRANK, T., BIERI, K., and SPEISER, B. 2002. Feeding deterrent effect of carvone, a compound from caraway seeds, on the slug *Arion lusitanicus*. *Ann. Appl. Biol.*141: 93-100.

HANNUKKALA, A., KESKITALO, M., LAAMANEN, J., and RASTAS, M. 2002. Control of potato late blight with Caraway and Dill extracts. In: H.T.A.M. Schepers and C.E.

Westerdijk, editors. Proceedings of the sixth workshop of a European network for development of an integrated control strategy of potato late blight. Edingburgh, Scotland, 26-30 September 2001. PPO-special report 8: 279-280.

IACOBELLIS, N. S., LO CANTORE, P. CAPASSO and SENATORE, F. 2005. Antibacterial activity of *Cuminum cyminum* L. and *Carum carvi* L. essential oils. *J. Agric. Food Chem.* 53: 57-61.

IBRAHIM, M. A., KAINULAINEN, P., AFLATUNI, A., TIILIKKALA, K., and HOLOPAINEN, J. K. 2001. Insecticidal, repellent, antimicrobial activity and phytotoxicity of essential oils: With special reference to limonene and its suitability for control of insect pests. *Agr. Food Sci. Finland* 10, 243-259.

IBRAHIM, M. A. and HOLOPAINEN, J. K. 2002. *Podisus maculiventris* (Hemiptera: Pentatomidae), a potential biocontrol agent of *Plutella xylostella* is not repelled with limonene treatments on cabbage. In: Kirk, A. A & Bordat, D (eds). Improving biocontrol of *Plutella xylostella, Proceed. Inter. Symp.* CIRAD 2004, pp 200-202.

IBRAHIM, M. A., OKSANEN, E. J., and HOLOPAINEN, J. K. 2004. Effects of limonene on growth and physiology of cabbage and carrot plants. *J. Sci. Food Agr.* 84: 1319–1326.

KARBAN, R. and BALDWIN, I. T. 1997. Induced responses to herbivory. University of Chicago Press, Chicago, Illinois, USA.

KIM, E. S., CHOI, E., KIM, Y., CHO, K. W., LEE, A., SHIM, J., RAKWAL, R. AGRAWAL, G. K. and HAN, O. S. 2003. Dual positional specificity and expression of non-traditional lipoxygenase induced by wounding and methyl jasmonate in maize seedlings. *Plant Mol. Biol.* 52: 1203-1213.

KLEPZIG, K. D. and SCHLYTER, F. 1999. Laboratory evaluation of plant-derived antifeedants against the pine weevil *Hylobius abietis* (Coleoptera: Curculionidae). *J. Econ. Entomol.* 92: 644-650

LACEY, L. A. and SHAPIRO-ILAN, D. I. 2003. The potential role for microbial control of orchard insect pests in sustainable agriculture. *J. Food Agr. Env.* 1: 326-331.

LEE, B. H., CHOI, W. S., LEE, S. E., and PARK, B. S. 2001. Fumigant toxicity of essential oils and their constituent compounds towards the rice weevil, *Sitophilus oryzae* (L.). *Crop Prot.* 20: 317-320.

LIU, S. S. and JIANG, L. H. 2003. Differential parasitism of *Plutella xylostella* (Lepidoptera : Plutellidae) larvae by the parasitoid *Cotesia plutellae* (Hymenoptera : Braconidae) on two host plant species. *B. Entomol. Res.* 93: 65-72.

LOIVAMÄKI, M., HOLOPAINEN, J. K., and NERG, A-M. 2004. Chemical changes induced by methyl jasmonate in oilseed rape grown in the laboratory and in the Field. *J. Agr. Food Chem.* 52: 7607-7613.

MANDUJANO-CHAVEZ, A., SCHOENBECK, M. A., RALSTON, L. F. LOZOYA-GLORIA, E. and CHAPPELL, J. 2000. Differential induction of sesquiterpene metabolism in tobacco cell suspension cultures by methyl jasmonate and fungal elicitor. *Arch Biochem Biophys.* 381: 285-294.

McCONN, M., CREELMAN, R. A., BELL, E., MULLET, J. E., and BROWSE, J.1997. Jasmonate is essential for insect defense Arabidopsis. *Proc. Natl. Acad. Sci. USA* 94: 5473-5477.

NEHLIN, G., VALTEROVA, I., and BORG-KARLSSON, A. K. 1994. Use of conifer volatiles to reduce injury caused by carrot psyllid, *Trioza apicalis*, Förster (Homoptera, Psylloidea). *J. Chem. Ecol.* 20: 771-783.

NTIAMOAH, Y. A. and BORDEN, J. H. 1996. Monoterpene oviposition deterrents for cabbage maggots, *Delia radicum* (L.) (Diptera:Anthomyiidae). *Can. Entomol.* 128: 351-352.

NTIAMOAH, Y. A., BORDEN, J. H., and PIERCE, H. D. Jr. 1996. Identity and bioactivity of oviposition deterrents in pine oil for the onion maggot, *Delia antiqua*. *Entomol. Exp. Appl.* 79: 219-226.

OKA, Y., COHEN, Y., and SPIEGEL, Y. 1999. Local and systemic induced resistance to the root-knot nematode in tomato by DL- beta -amino-n-butyric acid. *Phytopathology* 89: 1138-1143.

PETERSON, J. K, HORVAT, R. J., and ELSEY, K. D. 1994. Squash leaf glandular trichome Volatiles: Identification and influence on behavior of female pickleworm moth (*Diaphnia nitidalis* Stoll.) (Lepidoptera: Pyralidae). *J. Chem. Ecol.* 20: 2099-2109.

PEÑUELAS, J. and LLUSIÀ, J. 2002. Linking photorespiration, monoterpenes and termotolerance in *Quercus*. *New Phytol.* 155, 227-237.

PIVNICK, K. A., JARVIS, B. J., and SLATER, G. P. 1994. Identification of Olfactory cues used in host-plant finding by Diamondback moth, *Plutella xylostella* (Lepidoptera: Plutellidae). *J. Chem. Ecol.* 20: 1407-1427.

PORTA, H., RUEDA-BENITEZ, P., CAMPOS, F., COLMENERO-FLORES, J.M., CARMONA, M. J. COVARRUBIAS, A. A., and ROCHA-SOSA, M. 1999. Analysis of lipoxygenase mRNA accumulation in the common bean (*Phaseolus vulgaris* L.) during development and under stress conditions. *Plant Cell Physiol.* 40: 850-858.

POTTING, R. P. J., POPPY, G. M., and SCHULER, T. H. 1999. The role of volatiles from cruciferous plants and pre-flight experience in the foraging behaviour of the specialist parasitoid *Cotesia plutellae*. *Entomol. Exp. Appl.* 93: 87-95.

REDDY G. V. P., HOLOPAINEN, J. K., and GUERRERO, A. 2002. Olfactory responses of *Plutella xylostella* natural enemies to host pheromone, larval frass, and green leaf cabbage volatiles. *J. Chem. Ecol.* 28: 131-143.

REDDY, G. V. P., TABONE E., and SMITH M. T. 2004a. Mediation of host selection and oviposition behavior in the diamondback moth *Plutella xylostella* and its predator *Chrysoperla carnea* by chemical cues from cole crops. *Biol. Control* 29: 270-277.

REDDY, G. V. P. and GUERRERO, A. 2004b. Interactions of insect pheromones and plant semiochemicals. *Trends Plant Sci.* 9: 253-261.

RODRIGUEZ, S. C., CRAFTS, B. S. J., PARE, P. W., and HENNEBERRY, T. J. 2001. Exogenous methyl jasmonate induces volatile emissions in cotton plants. *J. Chem. Ecol.* 27: 679-695.

SALOM, S. M., GRAY, J. A., ALFORD, A. R., MULESKY, M., FETTIG, C. J., and WOODS, S. A. 1996. Evaluation of natural products as antifeedants for the pales weevil

(Coleoptera: Curculionide) and as fungitoxins for Leptographium procerum. *J. Entomol. Sci.* 31: 453-465.

SCHLYTER , F., SMITT, O., SJÖDIN, K., HÖGBERG, H.-E., and LÖFQVIST, J. 2004. Carvone and less volatile analogues as repellent and deterrent antifeedants against the pine weevil, *Hylobius abietis*. *J. Appl. Entomol.* 128: 610-619

SHIOJIRI, K., TAKABAYASHI, J., and YANO, S. H. 2001. Infochemically mediated tritrophic interaction webs on cabbage plants. *Popul. Ecol.* 43: 23-29.

SHIOJIRI, K. and TAKABAYASHI, J. 2003. Effects of specialist parasitoids on oviposition preference of phytophagous insects: encounter-dilution effects in a tritrophic interaction. *Ecol. Entomol.* 28: 573-578.

TALEKAR, N. S. and SHELTON, A. M. 1993. Biology, ecology and management of the diamondback moth. *Ann. Rev. Entomol.* 38: 275-301.

THALER, J. S., STOUT, M. J., KARBAN, R., and DUFFEY, S. S. 1996. Exogenous jasmonates simulate insect wounding in tomato plants (*Lycopersicon esculentum*) in the laboratory and field. *J. Chem. Ecol.* 22: 1767-1781.

TIBERI, R., NICCOLI, A., CURINI, M., EPIFANO, F., MARCOTULLIO, M. C., and ROSATI, O. 1999. The role of the monoterpene composition in Pinus spp. needles, in host selection by the pine processionary caterpillar, *Thaumetopoea pityocampa*. *Phytoparasitica* 27: 263-272.

TRIPATHI, A. K., PRAJAPATI, V., and KUMAR, S. 2003. Bioactivities of l-carvone, d-carvone, and dihydrocarvone toward three stored product beetles. *J. Econ. Entomol.* 96: 1594-1601.

VUORINEN, T., NERG, A-M., IBRAHIM, M. A., REDDY, G. V. P., and HOLOPAINEN, J. K. 2004a. Emission of *Plutella xylostella*-induced compounds from cabbages grown at elevated CO_2 and orientation behaviour of the natural enemies. *Plant Physiol.* 135: 1984-1992.

VUORINEN, T., REDDY, G. V. P., NERG, A-M., and HOLOPAINEN, J. K. 2004b. Monoterpene and herbivore-induced emissions from cabbage plants grown at elevated atmospheric CO_2 concentration. *Atmos. Env.* 38: 675-682.

ZU-HUA, S. H. I., SHU-SHENG, L. I. U., and YUAN-XI, L. I. 2002. *Cotesia plutellae* parasitizing *Plutella xylostella*: Host-age dependent parasitism and its effect on host development and food consumption. *Biocontrol* 47: 499-511.

Tables

TABLE 1. CONCENTRATION (ng g DW^{-1} h^{-1}). OF MONO- AND SESQUITERPENES AND SOME GREEN LEAF VOLATILES IN THE HEADSPACE EMISSIONS OF CABBAGE CULTIVAR LENNOX 24HRS AFTER TREATMENT

Compounds	Control	Limonene	MeJA
thujene	20.6 ± 1.7a	25.7 ± 4.6a	19.6 ± 5.5a
α-pinene	11.6 ± 0.8a	14.5 ± 2.5a	8.5 ± 0.5a
sabinene	61.6 ± 5.8a	77.1 ± 15.3ab	39.4 ± 2.1b
β-pinene + myrcene	23.6 ± 2.2a	29.3 ± 5.7a	16.7 ± 0.7a
limonene	40.3 ± 3.8a	254.9 ± 65.2a	35.5 ± 1.4a
1,8-cineole	30.1 ± 3.4a	35.4 ± 6.4a	23.2 ± 0.8a
Total monoterpenes	**187.9 ± 17.4 a**	**436.8 ± 96.6a**	**142.9 ± 7.4 a**
DMNT ((*E*)-4,8-dimethyl-1, 3,7-nonatriene)	0.0 ± 0.0 a	0.0 ± 0.0 a	10.9 ± 1.2 b
(*E,E*)-a-farnesene	4.6 ± 4.6 a	0.7 ± 0.6 a	49.3 ± 5.3 b
n-heptanal	0.1 ± 0.1a	0.0 ± 0.0a	0.0 ± 0.0a
(*Z*)-3-hexenyl acetate + octanal	0.2 ± 0.1 a	1.0 ± 0.7 a	29.8 ± 6.7 b

Control = 5% ethanol in water, Limonene = 3% of limonene in 5% ethanol in water, MeJA = 4.5 mM of methyl jasmonate in 5% ethanol in water. (N=5). Values are means ±se. Means followed by different letters are significantly (p<0.05) different.

TABLE 2. CONCENTRATION (ng g DW^{-1} h^{-1}) OF MONO- AND SESQUITERPENES AND SOME GREEN LEAF VOLATILES IN THE HEADSPACE EMISSIONS OF CABBAGE CULTIVAR RINDA 24HRS AFTER TREATMENT

Compounds	Control	Limonene	MeJA
thujene	38.29 ± 4.48a	27.05 ± 6.93a	27.71 ± 7.45a
α-pinene	23.78 ± 2.45a	22.88 ± 1.33a	20.38 ± 2.06a
sabinene	131.19 ± 16.63a	125.96 ± 5.49a	103.04 ± 13.59a
β-pinene + β-myrcene	48.29 ± 6.95a	47.34 ± 2.63a	43.27 ± 5.31a
limonene	77.11 ± 10.65a	528.07 ± 157.89b	99.97 ± 11.87a
1,8 cineole	52.23 ± 6.22a	58.13 ± 2.82a	53.58 ± 5.90a
γ-terpinene	0.00 ± 0.00a	0.00 ± 0.00a	0.60 ± 0.60a
Total monoterpenes	**370.89 ± 46.89a**	**809.422 ± 154.74b**	**348.55 ± 44.33a**
DMNT ((*E*)-4,8-dimethyl-1,3,7-nonatriene)	0.00 ±0.00a	6.46 ± 6.46ab	27.44 ± 3.86b
(*E,E*)- α-farnesene	18.72 ± 15.17a	27.64 ± 21.03a	202.22 ± 38.74b
3-hexen-1-ol	0.00 ± 0.00a	6.72 ± 4.35a	5.82 ± 2.47a
1-hexanol	0.00 ± 0.00a	7.29 ± 3.00a	0.00 ± 0.00a
1-octen-3-ol	0.00 ± 0.00a	17.09 ± 6.16a	0.00 ± 0.00a
(*Z*)-3-hexenyl acetate+octanal	34.02 ± 15.38a	35.73 ± 9.81a	108.81 ± 20.57b
nonanal	60.88 ± 3.90a	48.57 ± 12.75a	60.41 ± 3.22a
Total GLVs (green leaf volatiles)	**94.91 ± 14.80a**	**115.42 ± 17.02ab**	**175.03 ± 24.06b**

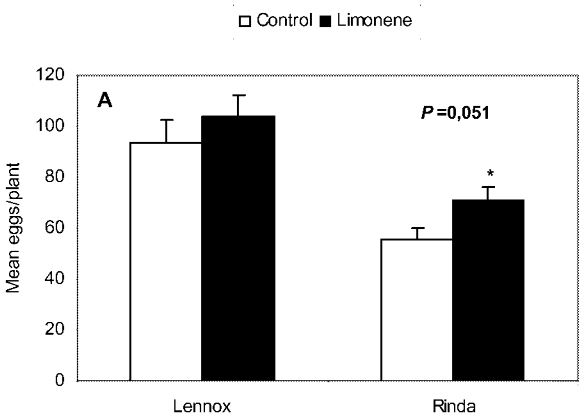
Control = 5% ethanol in water, Limonene = 3% of limonene in 5% ethanol in water, MeJA = 4.5 mM of methyl jasmonate in 5% ethanol in water. (N=6). Values are means ±se. Means followed by different letters are significantly (P<0.05) different.

Legend for Figures

FIG. 1. *P. xylostella* ovipositioning results on (A) cabbage plants sprayed with 3% of limonene (n=15) in 5% ethanol in water, (B) cabbage plants with limonene released from vermiculite (n=10), and (C) Cabbage (cv Lennox and Rinda) and broccoli plants exposed to the combination of limonene + carvone released from vermiculite (n=10). The data were analysed with independent samples T-test, and error bars are from SE values. * = $P<0.05$.

FIG. 2. Detached leaves from cabbage plants (cv Rinda and Lennox) sprayed with (A) 3% of limonene in 5% ethanol in water or (B) 4.5mM of MeJA in 5% ethanol (99.5%) in water were fed to the DBM larvae for 48 hrs and their RGR was calculated. Data were analysed with One-way ANOVA and error bars are from SE values. * = $P<0.05$.

FIG. 3. *C. plutellae* responses in Y-tube olfactometer tests to: (A) the odours of limonene, MeJA and limonene + carvone without plants (treatment) and clean air; (B) damaged plants by DBM larvae + limonene (treatment) or damaged plants without limonene as control; (C) damaged plants with limonene + carvone as treatment or damaged plants without limonene + carvone serving as controls; and (D) intact plants + limonene (treatment) or without limonene as control. B=Broccoli, L=Lennox, R=Rinda. Total = the total number of females used in the experiment. No choice= the number of females which did not choose any of the odour sources. The data were analysed using binomial tests: *= $P<0.05$, **=$P<0.01$, ***=$P<0.001$.


☐ Control ■ Limonene
Mean eggs/plant
0
20
40
60
80
100
120
A
P =0,051
*
Lennox
Rinda

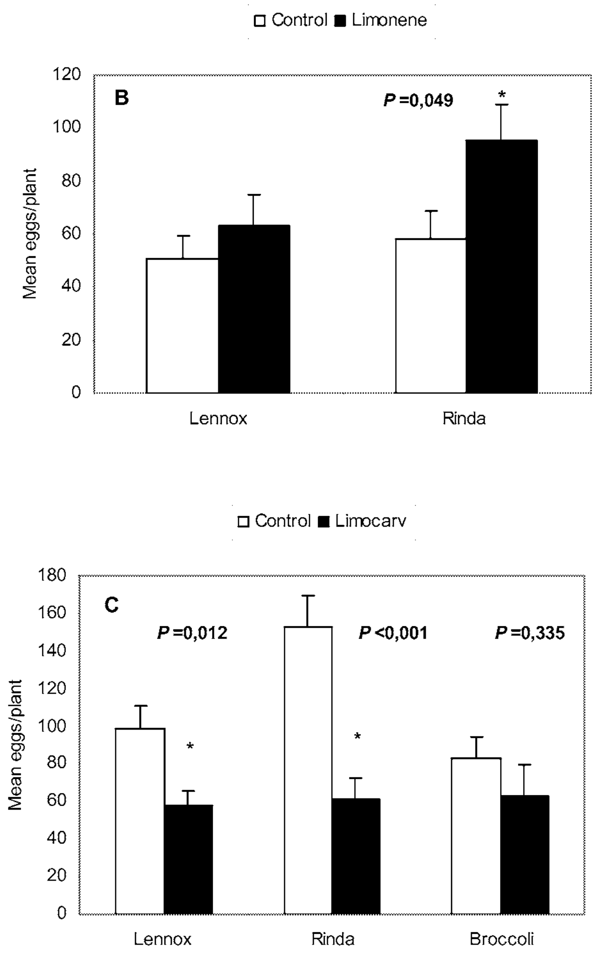

☐ Control ■ Limonene
B
P =0,049
*
Mean eggs/plant
0
20
40
60
80
100
120
Lennox
Rinda
☐ Control ■ Limocarv
C
P =0,012
P <0,001
P =0,335
*
*
Mean eggs/plant
0
20
40
60
80
100
120
140
160
180
Lennox
Rinda
Broccoli

FIG. 1.

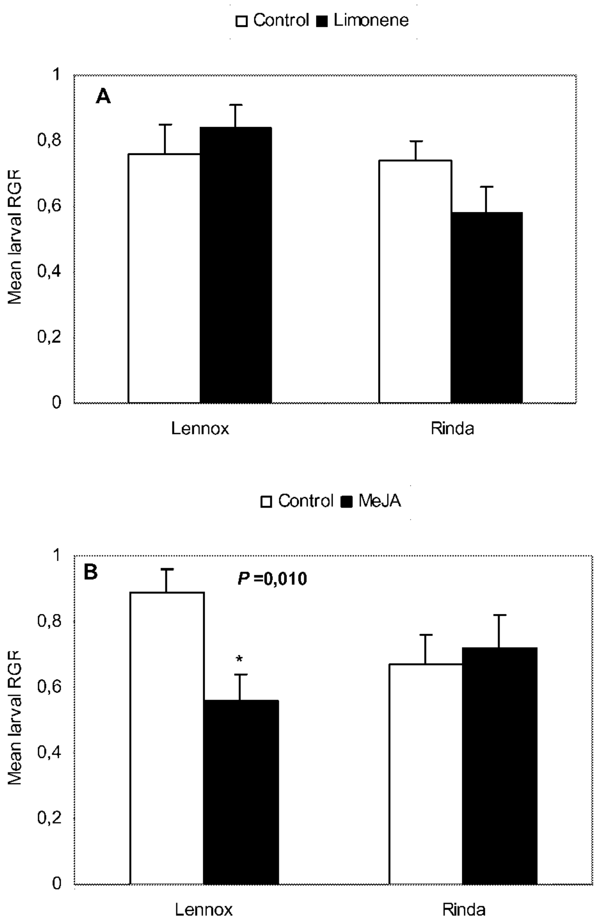
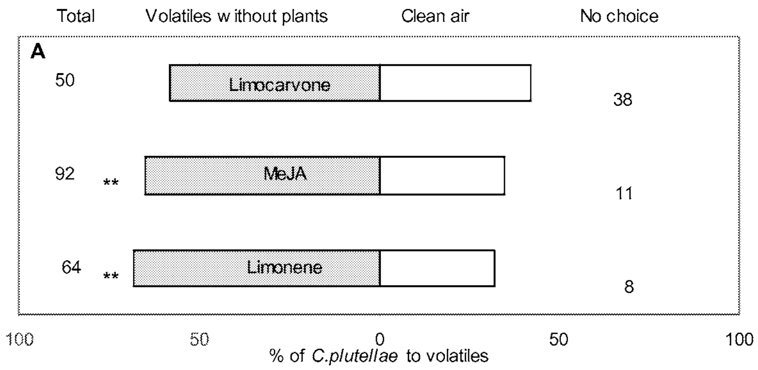
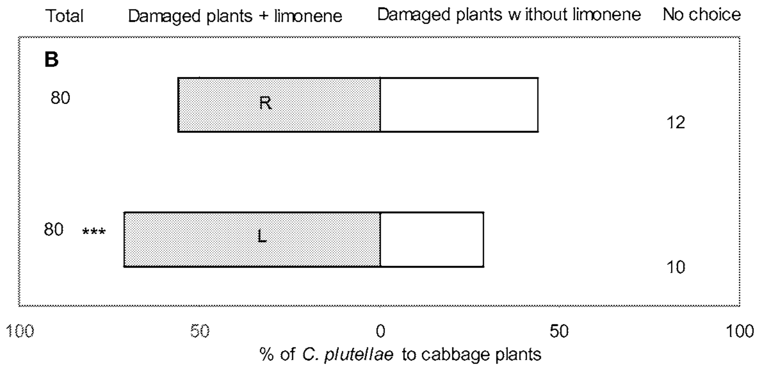

□ Control ■ Limonene
A
Mean larval RGR
1
0,8
0,6
0,4
0,2
0
Lennox
Rinda
□ Control ■ MeJA
B
P =0,010
*
Mean larval RGR
1
0,8
0,6
0,4
0,2
0
Lennox
Rinda

FIG. 2.



Total
Volatiles w ithout plants
Clean air
No choice
A
50
Limocarvone
38
92
**
MeJA
11
64
**
Limonene
8
100
50
0
50
100
% of *C.plutellae* to volatiles



Total
Damaged plants + limonene
Damaged plants w ithout limonene
No choice
B
80
R
12
80
***
L
10
100
50
0
50
100
% of *C. plutellae* to cabbage plants

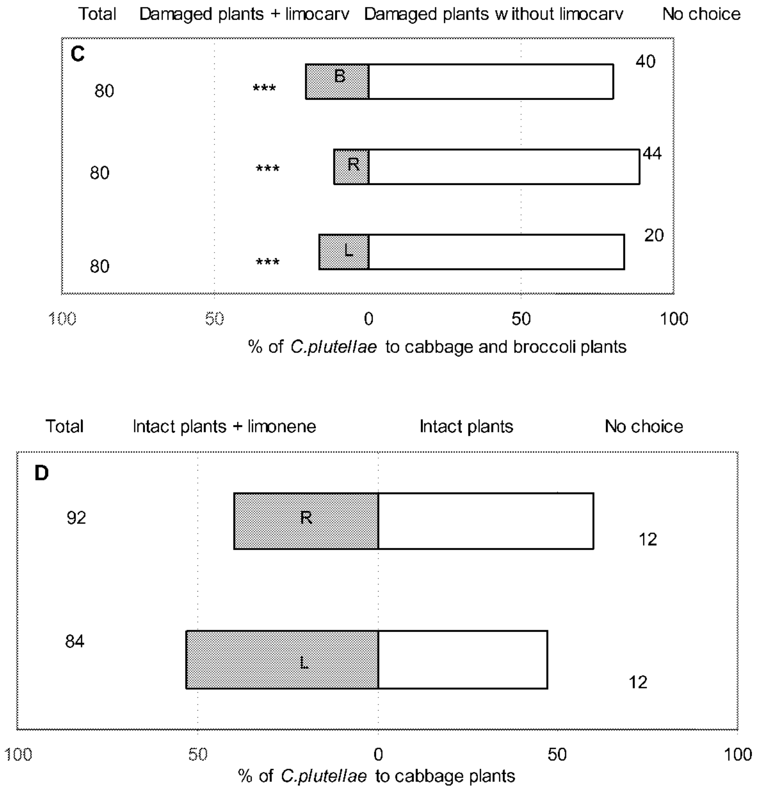

Total
Damaged plants + limocarv
Damaged plants w ithout limocarv
No choice
C
80
***
B
40
80
***
R
44
80
***
L
20
100
50
0
50
100
% of C.plutellae to cabbage and broccoli plants
Total
Intact plants + limonene
Intact plants
No choice
D
92
R
12
84
L
12
100
50
0
50
100
% of C.plutellae to cabbage plants

FIG. 3.

Chapter 5

Essential oil composition in the headspace and leaves of carrot (*Daucus carota*) cultivars damaged by the carrot psyllid (*Trioza apicalis*) or treated exogenously with limonene or methyl jasmonate

Nissinen A., Ibrahim, MA., Kainulainen, P., Tiilikkala, K. and Holopainen, J.K.
Submitted

Essential oil composition in the headspace and leaves of carrot (*Daucus carota*) cultivars damaged by the carrot psyllid (*Trioza apicalis*) or treated exogenously with limonene or methyl jasmonate

Anne Nissinen[1,2*], Mohamed Ibrahim[1], Pirjo Kainulainen[1], Kari Tiilikkala[2], and Jarmo K. Holopainen[1]

[1]*Department of Ecology and Environmental Science, University of Kuopio, P.O. Box 1627, FIN-70211 Kuopio, Finland*

[2]*Agricultural Research Centre of Finland, Plant Protection, FIN-31600 Jokioinen, Finland*

*Corresponding author:

Anne Nissinen
Department of Ecology and Environmental Science
University of Kuopio
P.O. Box 1627
FIN-70211 Kuopio
Finland

Telephone +358 17 163188
Telefax +358 17 163230
E-mail anne.nissinen@uku.fi

Abstract

The effect of carrot psyllid (*Trioza apicalis* Förster) feeding and limonene and methyl jasmonate (MeJA) treatments on the essential oil composition and headspace volatiles of carrot (*Daucus carota* ssp. *sativus*), cvs Parano and Splendid, leaves was studied. Carrot psyllid feeding significantly increased the concentration of sabinene, β-pinene, limonene, and longifolene and limonene treatment the concentration of (*Z*)-β-ocimene in the leaves of both cultivars. The limonene treatment significantly increased the concentration of total phenolics in the leaves of both cultivars and MeJA treatment in the leaves of cv Parano. Exogenous limonene spray did not decrease the number of carrot psyllid eggs laid either 2 or 24 h after treatment. The results suggest that carrot psyllid feeding induces endogenous mono- and sesquiterpene pool in the carrot leaves. Limonene and MeJA treatments affect some induced defenses of the carrot, but the exogenous limonene spray is not an effective oviposition deterrent against carrot psyllid.

Keywords *Trioza apicalis*, *Daucus carota*, damage, limonene, methyl jasmonate, monoterpenes, sesquiterpenes, biomass, total phenolics

Introduction

The carrot psyllid (*Trioza apicalis* Förster, Homoptera: Psylloidea) is an economically important carrot pest in the Nordic countries (*1*). The damage by overwintered females can cause 100% yield loss if control methods are not used (*2,3*). Pyrethroids have been used as chemical control methods against carrot psyllid (*1*) for two decades. Farmers have found the field monitoring of carrot psyllid with sticky traps difficult, because several *Trioza* and other psyllid species are found in the monitoring traps simultaneously (*4*). Therefore, the farmers in Finland tend to use calendar based spraying programs instead of supervised programs (*1*). However, recently there have been some doubts that the effectiveness of pyrethroid treatments has decreased in Nordic countries. Thus, the need for new control methods is increasing.

The symptoms of carrot psyllid feeding damage are leaf-curling and stunted growth of the carrot seedling (*2*). The systemic nature of psyllid damage, also demonstrated by Markkula et al. (*2*), indicates that the feeding damage may have an effect on the metabolism of the carrot. Accumulation of amino acids has been found in carrot leaves infested by carrot psyllid (*5*), however, there have been no attempts to analyze the secondary metabolites of damaged leaves. Herbivore induced defenses is a well-studied phenomenon of different herbivore-plant systems (e.g. *6,7*) In addition, some terpenoids detected in headspace volatiles of herbivore damaged plants have been found responsible for activation of defense genes in undamaged neighboring plants (e.g. *8*). However, these phenomena have not been studied on carrot.

Mono- and sesquiterpenes together with propenyl benzenes have been found to be the main substances in the essential oil composition of different carrot cultivars (*9*). The oil composition significantly differs between the leaves of different carrot cultivars (*9, 10, 11*). The proportion of propenyl benzenes decreases with the ageing of the seedling (*9*). Limonene, one of the monoterpenes in carrot essential oil, in high concentrations has previously been shown to have a repellent effect on carrot psyllids (*3, 12*). Previously, exogenous fumigation with monoterpenes has been shown to affect the emission of monoterpenes and the endogenous monoterpene pool in the leaves of *Quercus* leaves (*13, 14*). Similarly, exogenous applications of limonene have been found to confer a limited thermotolerance on carrot (*15*) or to *Quercus ilex* (*16*).

Based on the previous studies we hypothesize that limonene could be a repellent against carrot psyllid when exogenously sprayed on carrot leaves, but it may also affect the secondary chemistry of carrot. Therefore we studied the role of exogenous limonene spray in the control of carrot psyllid and its effects on the emission and the concentration of volatile compounds in the leaves. The specific aims of the present study were to investigate 1) if carrot psyllid feeding changes the concentrations of volatile compounds in carrot leaf tissue and plant headspace 2) if limonene can be used as a deterrent for carrot psyllids and 3) if limonene and methyl jasmonate can be used as elicitors that induce direct and indirect chemical defense in carrot plants. Methyl jasmonate (MeJA) treatment was included in the experiment, because it has been shown to have a similar kind of effect on plant volatiles as insect damage e.g. in cotton and in Sitka spruce (*17, 18*).

Materials and methods

Plant material and growing conditions. Three separate experiments were conducted using carrot cvs. Splendid and Parano, which are known to have different proportions of limonene (*11*). For the experiments 1 and 3, seeds were sown in plastic pots (vol. 0.75 L) and grown in a mixture of peat and sand (2:1 v/v). For experiments 1 and 3, one and three seedlings were allowed to grow in each pot, respectively. The seedlings were grown in growth chambers (Bioklim 2600T, Kryo-Service Oy, Finland) programmed to simulate the light and temperature conditions in June in Central Finland (19:12 °C, 50:80 RH and 22:2 h L:D photoperiod). For experiment 1, the seedlings were fertilized once with 0.1% Superex-9 solution (19:5:20 N:P:K, Kekkilä, Finland). For experiment 2 and for the biomass test of experiment 1, carrot seeds were sown in fertilized peat-sand mixture (Kekkilä TH1) in 1.1-L pots. For experiment 2, three seedlings were grown in each pot at similar conditions used for the carrot psyllid rearing. Extra light was given to plants from 04.00 to 24.00 with ten 400W metal halide lamps (Philips HP1-T, Philips, Netherlands) (one lamp/2 cages). For the biomass test of experiment 1, one seedling in each pot was grown in a greenhouse at a temperature of 19 °C:12 °C. The natural light period (14 h) was extended to 22 h with twelve 400W metal halide lamps (one lamp/2 cages).

Insect material. The carrot psyllids used in the experiment 1 were collected from an organic carrot field at Haukivuori (61°58'28" N, 27°11'00" E), central Finland, one day before the experiment and allowed to feed on carrot cv Nantura until the experiment was started.

In the biomass test of experiment 1 and experiment 2, we used carrot psyllids from our own continuous rearing which had been going on for several generations. The carrot psyllids were originally collected in June 2000 from the same farm as earlier mentioned. Thereafter, the carrot psyllids were reared in cages (33 * 33 * 60cm) on carrot cv Nantura in a greenhouse (20 °C: 17 °C, on average 50% RH, 20:4 h L:D). Each new carrot psyllid generation developed on average in 7-8 weeks. The adults of a new generation were allowed to copulate freely in the original cage for approximately two weeks. Thereafter, the gravid females were transferred to a new cage (50-80 individuals, 1:1 sex ratio).

Experiment 1. In experiment 1, the effect of carrot psyllid feeding on VOC emissions, leaf biomass and terpenoid concentration was studied. Two 3.5-week-old carrot seedlings were placed in one cylinder cage (volume 10 L) sealed at the top with a thin cloth. Four carrot psyllids (1:1 sex ratio) were released in each cage, except the control cages, and allowed to feed on the carrot seedlings for 7 days before they were removed. Each treatment (cages with and without psyllids) was replicated randomly four times in the growth chamber. The volatile organic compounds (VOCs) were collected with the equipment described in Loivamäki et al. (*19*) from 6 plants per treatment on the same day or one day after the removal of psyllids. The foliage of carrots growing in pots was sealed in 1.5-L glass containers with aluminium foil at the base of the container. The collection was performed in the laboratory under natural light (100-150 µmol/m/s) at room temperature. Charcoal filtered air was led into the container through 6-mm Teflon tubing at a flow rate of 100 mL/min and volatile samples were collected into Tenax TA adsorbent (150 mg/tube) for 2 h. Volatiles were analyzed with the method described below. The two youngest leaves (3rd and 4th leaf) of the same plant individuals as used in VOC collections were extracted with n-hexane 5 days after the collection of headspace volatiles. Due to the considerably higher fresh weight of undamaged leaves only half of the blades (cut along the petiole) were extracted, while from the damaged plants the whole blades were extracted. The leaf extracts analysis method is described below.

For determination of psyllid feeding effect on leaf biomass, four-week-old seedlings were transferred pairwise into insect cages (33 * 33 * 60 cm) and four carrot psyllids (1:1 sex ratio) were released into each cage except for control treatment. The insects were allowed to feed on the seedlings for 7 days before they were removed. There were 6 replicate seedlings of each treatment per cultivar. For biomass measurements, the blades and petioles were cut separately 24 h after the psyllids were removed and dried in an oven at 60 °C for two days and weighted.

Experiment 2. Egg-laying preference of the carrot psyllid was tested on limonene treated plants. Four-week-old carrot seedlings were treated with tap water, 5 % ethanol in aqueous solution, 3 % limonene in 5 % ethanol in aqueous solution or 6 % limonene in 5 % ethanol in aqueous solution. Treated plants were randomised into each of the corner of an insect cage. One gravid carrot psyllid female was released 2 h or 24 h after the treatments into the middle of the cage. The experiment was conducted two times with different randomization and each cultivar (with all the treatments) was replicated in 10 cages for each release time (2 h or 24 h), except the

2^{nd} randomization for 24 h with 9 cages/cultivar. Thus, there were all together 20 and 19 replicates per each cultivar for the release 2 h and 24 h after the treatment respectively. The choice of the female was recorded and it was allowed to lay eggs for 48 h. Thereafter, the female was removed and the eggs were counted under a microscope.

Experiment 3. The effect of limonene and MeJA treatments on emission of volatiles and concentrations of essential oil and total phenolics was studied. Four-weeks-old seedlings, with 3-4 true leaves, were treated with 5% of ethanol (control), 3% limonene in 5% of ethanol in aqueous solution or 13.4 mM (3 mg/mL) MeJA in 5% ethanol in aqueous solution (5mg/plant). Headspace volatiles were collected 24 h after the treatment from 5 seedlings per treatment. The roots of each seedling were washed and pruned slightly before being introduced into a small glass vial filled with water. Thereafter, the plant was sealed in a 1.5 L glass container. During the collection, additional light was given to the plants (one lamp/container) at light intensity of 250 µmol/m/s. The charcoal-filtered air was led through the container at a flow rate 200 mL/min and the headspace volatiles were collected for 1 h. The volatiles were analyzed using the method described below. Immediately after the collection of volatiles, the youngest fully developed (3^{rd}) leaf of each seedling was extracted with hexane. The rest of the plant was dried in the oven (40 °C, 5 days) to obtain the dry weight and the total phenolics of the plant. To determine the long term effects of the treatments the 2^{nd} extraction (9 seedlings/teatment) was performed six days after the treatment.

VOC analysis. Plant volatiles were analyzed by GC-MS (Hewlett Packard GC 6890, MSD 5973) equipped with a HP-5 capillary column (50.0 m * 0.2 mm i.d. * 0.50 µm film thickness). Compounds adsorbed to Tenax were desorbed using thermal desorption unit (ATD 400, Perkin-Elmer) at 250 °C for 10 min and cryofocused in a cold trap at -30 °C. First, the column temperature was maintained at 40 °C for 1 min, thereafter increased to 210 °C at 5 °C/min and finally to 250 °C at 20 °C/min. Mass numbers from 30 to 350 *m/z* were recorded. The absolute amounts of terpenoids were calculated based on the peak area of the pure compounds in external standard consisting of known amount of several mono- and sesquiterpenes, propenyl benzenes and green leaf volatiles.

Analysis of leaf extracts. Leaf samples were extracted with n-hexane for 2 h at room temperature and the plant residues were washed two times. In experiment 1, the plant extracts were analyzed by GC-MS (Hewlett Packard GC 6890, MSD 5973) and in experiment 3 by GC-MS (Hewlett Packard GC type 5890, MSD 5970). Both devices used similar 30-m-long HP-5MS (0.25 mm i.d., 0.25 µm film thickness) capillary column and helium as carrier gas. In experiment 1, the column temperature was held at 50 °C for 2 min, increased to 110 °C at 10 °C/min, increased to 150 °C at 5 °C/min and finally increased to 270 °C at 30 °C/min and then held at 270 °C for 5 min while in experiment 3 it was held at 50 °C for 2 min and then followed by increases of 10 °C/min to 110 °C and 5 °C/min to 200 °C. SCAN technique, recording mass numbers between 30 and 300 *m/z*, was used for the samples. In quantification single ions 69, 93, 95, 121, 133, 136, 154, 161, 196 and 204 were monitored for terpenes and 163, 178, 193 and 208 for propenyl benzenes. Quantification of the compounds was accomplished using known amounts of available pure terpenes and propenyl benzenes relative to known amount of internal standard (1-chloro-octane).

Total phenolics. In experiment 3, for total phenolic analysis, all the blades from each carrot seedling were dried at 40 °C and powdered. Approximately 40 mg of powdered leaf material was extracted with 5 mL of 80% aqueous acetone. The suspension was centrifuged at room temperature at 2200 *g* for 2 min and the supernatant was saved. The residue was re-extracted twice with 80% acetone and supernatants were pooled and made up to 10 mL with 80% acetone. One hundred microlitres of this extract was mixed with 1 mL of Folin-Ciocalteu reagent and 5 mL of 20% sodium carbonate, diluted to 10 mL with water, and mixed thoroughly. The absorbance was measured from two parallel samples after 20 min with a spectrophotometer (Shimadzu UV-1201) at wave length of 735 nm using tannic acid as standard. Results are expressed as tannic acid equivalents.

Statistical analyzes. In experiment 1, two control plants of cv Parano, one mechanically damaged and one showing abnormal growth, had to be omitted from the VOC data before statistical analyzes, because they emitted extremely high concentrations of terpenoids and propenyl benzenes. In cv Parano, slight aphid infestation was observed together with the psyllid damage on three seedlings. The differences in the volatile emissions and terpenoid concentration of carrot psyllid damaged and undamaged carrot leaves were tested by GLM-procedure using the lg (x+1) transformed terpenoid concentrations as dependent variables, cultivar and treatment as fixed factors and the sampling day and the aphid infestation as covariants. The fresh weight of insect damaged plants was tested with independent samples T-test, while the fresh and dry weight of elicitor-treated plant was tested with ANOVA. The biomass of the carrot psyllids damaged carrots was analyzed with GLM-procedure using fresh and dry weight of blades and petioles as dependent factors, cultivar and damage as fixed factors and number of true leaves as a covariant. In experiments 3, the differences in VOC emissions and the essential oil composition of elicitor treated carrots were tested by GLM-procedure using the lg (x+1) transformed terpenoid concentrations as dependent variables and cultivar, day of the extraction and treatment as fixed factors. In experiment 2, the choices of carrot psyllid females between control and limonene treatments and differences in egg-laying preference were tested with non parametric Kruskall-Wallis test followed by Dunnett T3 as post hoc.

Results

Exp. 1. Psyllid feeding effects

VOCs and foliar essential oils. Carrot psyllid feeding significantly increased the concentrations of sabinene, β-pinene, limonene, and longifolene (Table 1) in the leaves of both cultivars. The only significant volatile induction was of emission of (*Z*)-β-ocimene from cv Parano leaves (Table 2). A significant cultivar × feeding interactive effect was found both in foliar concentration and emission of (Z)-β-ocimene (P = 0.005, P = 0.013 respectively). Foliar concentrations of sabinene, myrcene, limonene, bornylacetate and methyl isoeugenol and emissions of sabinene, (*Z*)-β-ocimene, limonene, methyl salicylate, bornylacetate and β-caryophyllene differed significantly between cultivars.

Biomass. The visible carrot psyllid feeding damage, caused over 7 days, had no significant effect on the fresh or dry weight of the blades, but it significantly reduced the fresh and dry weight of petioles (Table 3). When the leaves were extracted (5 days after the psyllid removal) the fresh weight of undamaged seedlings was significantly higher than that of damaged seedlings in both cultivars (Parano and Splendid) (P = 0.031, P = 0.002 respectively).

Exp. 2: Egg-laying preference of carrot psyllid on limonene treated plants

The limonene treatment did not influence the number of eggs laid by psyllids released in cages 2 h after the limonene treatment. However, in cv Splendid, limonene treatment marginally significantly (P = 0.089) increased the numbers of eggs laid by psyllids, which were released in cages 24 h after the treatment (Fig.1).

Exp. 3. Effects of limonene and MeJA

VOCs. The emissions of several mono- and sesquiterpenes and methyl isoeugenol were significantly different between cultivars. In contrast, no significant differences in emission of terpenes after limonene and MeJA treatments were found. However, emission of (*Z*)-3-hexenyl-acetate was significantly different between the treatments (Table 4). There was a significant cultivar × treatment interaction on emission of (*Z*)-3-hexenyl-acetate (P = 0.012) and methyl salicylate (P = 0.040). No significant differences in the fresh or dry weight of the blades or petioles between limonene and MeJA treated carrots were found (data not shown).

Foliar essential oil. Elicitor treatment had a significant effect only on (*Z*)-β-ocimene concentration of the leaves 24 hours after the treatment. We found significant cultivar effect on the concentrations of sabinene, myrcene, limonene, bornylacetate, β-caryophyllene and methylisoeugenol (Table 5). None of the compounds was affected by 6 day after the treatments, thus no long-term effects of the treatment was detectable (data not shown). There was a significant effect of cultivar × treatment interaction on limonene ($P = 0.005$), while none of the other interactive effects was significant.

Total phenolics. The concentration of total phenolics in the leaves of Parano was significantly higher in limonene and MeJA treatment than in control treatment while in Splendid only the total phenolic concentration of limonene treated leaves was significantly higher than that of the control (Table 6).

Discussion

Psyllid effect on leaf chemistry and VOCs. The carrot psyllid feeding increased the concentration of sabinene, β-pinene, limonene, and longifolene in the leaves of both cultivars. The foliar concentration of (*Z*)-β-ocimene decreased with the feeding damage in the leaves of cv Splendid, while it increased in the leaves of cv Parano, which may indicate a cultivar difference in response to carrot psyllid feeding. However, the feeding did not markedly affect the emissions of carrot leaves: only the emission of (*Z*)-β-ocimene was increased from leaves of cv Parano. Similarly, no clear correlation has been found between the foliar concentration and emission of terpenes in some terpene-storing species, like conifers, while some non-storing species, like oaks, emit a large amount of terpenes (*20*). Previously, the feeding of pear psylla on pear trees increased the emission of (*E,E*)-α-farnesene and methyl salicylate (*21*), and quantitative and qualitative differences were found in the emission of pear cultivars infested with *Cacopsylla* spp. (*22*). However, it has been reported that some other phloem feeders, such as aphids or whiteflies, do not induce volatile emission (*6, 7, 23*) probably because they avoid damaging cells during stylet injection (*6*). Similarly, psyllids living on *Eucalyptus* have been shown to avoid oil glands during stylet penetration (*24*) which may explain the relatively low induction of volatiles caused by psyllid feeding.

There has been some evidence that Homoptera benefit from group feeding (*24*). Previously, a higher concentration of free amino acids has been found in carrot psyllid damaged leaves than undamaged (*5*), which may predict that carrot psyllids benefit from conspecific feeding. However, pear psylla females seemed to avoid pear branches with conspecific infestation (*25*). Thus, the ecological function of psyllid induced defenses and other chemical changes remains to be studied.

Psyllid effect on plant growth. Carrot psyllid feeding did not reduce foliar fresh weight at the end of a 7-day feeding period, but the difference was observed 5 to 6 days after the removal of the psyllids. This delayed reduction of blade fresh weight may be due to malformation of plant cells caused by carrot psyllid feeding. Nymphal feeding of another gall-forming species *Diaphorina truncata* (Homoptera: Psylloidea) caused e.g. hyperplasia, hypertrophy and nuclear gigantism in the mesophyll cells (*27*). When the psyllids are removed, the undamaged leaves will continue their growth, but the damaged leaves will remain stunted, which may explain the difference of the fresh weight observed later between the treatments.

Limonene treatment and egg-laying. Carrot psyllid females released into the cage 2 h after the treatment, showed no preference over the treatments which suggests that increased proportion of limonene does not affect oviposition behavior of carrot psyllids. In cv Parano, there was no difference in egg-laying preference of carrot psyllids 24 h after the treatment, while in cv Splendid there were marginally more eggs on plants treated with 3% of limonene than on control plants. Even though, the difference was only marginally significant it was interesting because limonene has previously been regarded as possible repellent against carrot psyllid (*3, 12*). Previously, Ibrahim et al. (*15*) found increased limonene emission from carrot leaves 24 h after limonene treatment. However, in this study the emission of limonene was not significantly increased 24 h hours after the treatment, but the concentration of (Z)-β-ocimene was increased in the carrot leaves, which may indicate that limonene spray may change the total volatile emission spectrum of carrot seedlings even more attractive to the pest in 24 h. Thus, the limonene spray on carrot leaves to repell carrot psyllids is not recommended.

Limonene and MeJA effect on leaf chemistry and VOC emissions. However, the leaf concentration of (*Z*)-β-ocimene was induced after the limonene treatment, it did not affect the emission of this compound. Emission of (*Z*)-3-hexenyl-acetate was higher in the control than in MeJA treatment and significant cultivar × treatment interaction on (*Z*)-3-hexenyl-acetate and methyl salicylate was observed indicating that these compounds reacted differently to the elicitor treatments in different cultivars. The period from MeJA treatment to VOC collection (24 h) may have been too short to detect MeJA induced volatiles. Recently, the emission of homoterpene (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) and the sesquiterpene (*E,E*)-α-farnesene and (*Z*)-3-hexenyl-acetate were found to be increased significantly in the headspace of oil seed rape (non-terpene-storing species) three days after MeJA treatment (*19*) and the emission of monoterpene linalool from Sitka spruce needles 60 to 66 h after MeJA treatment (*18*). However, terpene-storing species, like carrot, and non terpene-storing species, like oil seed rape, may respond differently to MeJA treatment. Previously, it has been found that MeJA did not induce the emission of stored terpenes in cotton (*17*). Similarly, linalool is de novo formed in Sitka spruce needles (*18*).

In contrast to carrot psyllid feeding, mono- and sesquiterpene concentrations were not significantly influenced by the MeJA either one or seven days after the treatment, suggesting that 13.4 mM concentration may be too low for carrot in the manipulation of indirect chemical defense which is based on inducible volatile compounds. The result suggests that MeJA does not influence foliar monoterpene concentrations of monoterpene-storing species like carrot. In conifers, this observation is supported by the results of Heijari et al. (*28*) who found that spraying with low (10 mM) or high (100 mM) concentrations of MeJA did not affect the concentrations of foliar monoterpenes in Scots pine, although resin acids were affected. Similarly, Miller et al. (*18*) found no increased accumulation of total mono- and sesquiterpenoids in mature needles of MeJA treated Sitka spruce.

We observed a higher level of volatile emission in experiment 3 with limonene and MeJA treatment than in the carrot psyllid feeding experiment. The difference may be due to the higher light intensity used during the volatile collection in experiment 3 or due to the different collection method. However, the emission quantity and quality of the main compounds in the VOC collections of eperiment 3 were on a quite similar level to that reported by Ibrahim et al. (*15*) where the collection method used was similar i.e. the whole plant was enclosed inside a cuvette and the same light intensity was used. However, these results are difficult to compare because the carrots used in Ibrahim et al. (*15*) were older (7 weeks) and grown in higher temperatures than in this study. The lower concentration of methyl isoeugenol in the leaf extractions of experiment 1 than in experiment 3 is likely due to the ageing of the carrots (*9*). Even though, the carrots were a few days younger in experimet 1, the average fresh weight of the carrot leaves was considerably higher (71-100%) than in experiment 3.

The emissions of sabinene, (*Z*)-β-ocimene, limonene, methyl salicylate, bornylacetate and β-caryophyllene differed between the cultivars Parano and Spendid, which is in agreement with the recent results by Ibrahim et al. (*15*). We found that the foliar concentration of mostly the same compounds (sabinene, myrcene, limonene, bornylacetate, β-caryophyllene) as reported by Kainulainen et al. (*11*) differed between the cvs. Parano and Splendid.

Limonene and MeJA effects on the leaf chemistry. Limonene treatment increased the concentration of (*Z*)-β-ocimene in the leaves which differs form the earlier observation of Ibrahim et al. (*15*). We found that limonene treatment increased the total phenolic concentration of both cultivars. These results may indicate that exogenous limonene treatment can affect the induced defense of carrot. Limonene treatment could have some value as a control method against carrot pathogens because high phenolic concentration has been shown to increase the resistance of carrot roots to fungal pathogens (*29, 30*). The phenolic content of carrot leaves should be more carefully studied with regard to carrot psyllid behavior, because high phenolic content of the olive buds was associated with a certain degree of resistance to olive psylla (*Euphyrulla olivina*) (*31*) and the feeding of pear psyllids increased the phenolic concentration of the pear leaves (*32*).

Our results indicate that (*Z*)-β-ocimene may be the most easily induced compound in these carrot cultivars, because its leaf concentration was affected by both insect feeding and limonene treatment. However, in most cases no significant treatment effects on volatile emissions were detectable because they may have been masked due to high variation between individual plants. Although limonene spraying was not an effective deterrent against carrot psyllid oviposition, it significantly increased phenolic concentration in the carrot leaves, which might have consequences for the performance of the psyllid nymphs. The use of methyl jasmonate as an elicitor to manipulate the direct and indirect chemical defense of carrot plants did not give very encouraging results. Total phenolics were increased by MeJA treatment only in cv Parano, however, the phenolic methyl isoeugenol in leaf tissue or in headspace was not affected. Similarly, the leaf concentration of mono- and sesquiterpenes was not affected by MeJA treatment. Obviously, volatile concentrations and emissions of monoterpene-stroring plant species, like carrot, are not as easily influenced as those of non-monoterpene-storing plant species.

Acknowledgements

We thank Outi Järvinen and Jaana Rissanen for assistance at laboratory work and Virgina Mattila for language revision. This work was funded by the Ministry of Agriculture and Forestry projects number 20121100 and number 310061, and the Jenny & Antti Wihuri Foundation.

Literature Cited

1. Tiilikkala, K.; Ketola, J.; Taivalmaa, S-L. Monitoring and threshold values for control of the carrot psyllid. *IOBC/WPRS Bulletin* **1995**, 19, 18-24.
2. Markkula, M.; Laurema, S.; Tiittanen, K. Systemic damage caused by *Trioza apicalis* on carrot. *Symp. Biol. Hung.* **1976**, 16, 153-155.
3. Nehlin, G.; Valterová, I.; Borg-Karlson, A.-K. Use of conifer volatiles to reduce injury caused by carrot psyllid, *Trioza apicalis*, Förster (Homoptera, Psylloidea). *J. Chem. Ecol.* **1994**, 20, 771-783.
4. Burckhardt, D.; Freuler, J. Jumping plant-lice (Hemiptera, Psylloidea) from sticky traps in carrot fields in Valais, Switzerland. *Mitt. Schweiz. Ent. Gesl.* **2000**, 73, 191-209.
5. Laurema, S. Free amino acids in the psyllid *Trioza apicalis* Först. (Homopt., Triozidae) and in carrot leaves. *Ann. Agr. Fenn.* **1989**, 28, 113-120.
6. Turlings, T. C. J.; Bernasconi, M.; Bertossa, R.; Bigler, F.; Caloz, G.; Dorn, S. The induction of volatile emissions in maize by three herbivore species with different feeding habits: possible consequences for their natural enemies. *Biol. Control.* **1998**, 11, 122-129.
7. van Poecke, R. M. P.; Roosjen, M.; Pumarino, L.; Dicke, M. Attraction of the specialist parasitoid *Cotesia rubecula* to *Arabidopsis thaliana* infested by host or non-host herbivore species. *Ent. Exp. Appl.* **2003**, 107, 229-236.
8. Arimura, G.; Ozawa, R.; Shimoda, T.; Nishioka, T.; Boland, W.; Takabayashi, J. Herbivory-induced volatiles elicit defence genes in lima bean leaves. *Nature.* **2000**, 406, 512-515.
9. Kainulainen, P.; Tarhanen, J.; Tiilikkala, K.; Holopainen, J. K. Foliar and emission composition of essential oil in two carrot varieties. *J. Agric. Food Chem.* **1998**, 46, 3780-3784.
10. Habegger, R.; Schnitzler, W.H. Aroma compounds in the essential oil of carrots (*Daucus carota* L. ssp. *sativus*). 2. Intra-leaf distribution. *J. Appl. Bot.* **2000**, 74, 229-232.
11. Kainulainen, P.; Nissinen A.; Piirainen, A.; Tiilikkala, K.; Holopainen, J. K. Essential oil composition in leaves of carrot varieties and preference of specialist and generalist sucking insect herbivores. *Agr. Forest Entomol.* **2002**, 4, 211-216.
12. Valterová, I.; Nehlin, G.; Borg-Karlson, A.-K. Host Plant chemistry and preferences in egg-laying *Trioza apicalis* (Homoptera, Psylloidea). *Biochem. Syst. Ecol.* **1997**, 25, 477-491.
13. Loreto, F.; Förster, A.; Dürr, M.; Csiky, O.; Seufert, G. On the monoterpene emission under heat stress and on the increased thermotolerance of leaves of *Quercus ilex* L. fumigated with selected monoterpenes. *Plant Cell Environ.* **1998**, 21, 101-107.
14. Delfine, S.; Csiky, O.; Seufert, G.; Loreto, F. Fumigation with exogenous monoterpenes of a non-isoprenoid-emitting oak (*Quercus suber*): monoterpene acquisition, translocation, and effect on the photosynthetic properties at high temperatures. *New Phytol.* **2000**, 146, 27-36.
15. Ibrahim, M. A.; Nissinen, A.; Prozherina, N.; Oksanen, E. J.; Holopainen, J. K. The influence of exogenous monoterpene teratment and elevated temperature on growth, physiology and headspace volatiles of two carrot cultivars (*Daucus carota* L.). *Environ. Exp. Bot.* **2005**, (in press).

16. Llusiă J., Peňuelas J., Asensio, D and Munnè-Bosch S. Airborne limonene confers limited thermotolerance to *Quercus ilex*. *Physiol. Plantarum*. **2005**,123, 40-48.
17. Rodriguez-Saona, C.; Crafts-Brandner, S. J.; Paré P. W.; Henneberry, T. J. Exogenous methyl jasmonate induces volatile emissions in cotton plants. *J. Chem. Ecol.* **2001**, 27, 679-695.
18. Miller, B.; Madilao, L. L.; Ralph, S.; Bohlmann, J. Insect-induced conifer defence. White pine weevil and methyl jasmonate induced traumatic resinosis, de novo formed volatile emissions, and accumulation of terpenoid synthase and putative octadecanoid pathway transcripts in Sitka spruce. *Plant Physiol.* **2005**, 137, 369-382.
19. Loivamäki, M.; Holopainen, J. K.; Nerg, A.-M. Chemical changes induced by methyl jasmonate in oilseed rape grown in the laboratory and in the field. *J. Agric. Food Chem.* **2004**, 52, 7607-7613.
20. Kesselmeier, J.; Staudt, M. Biogenic volatile organic compounds (VOC): an overview on emission, physiology and ecology. *J. Atmos. Chem.* **1999**, 33, 23-88.
21. Scutareanu, P.; Drukker, B.; Bruin, J.; Posthumus, M. A.; Sabelis, M.W. Volatiles from *Psylla*-infested pear trees and their possible involvement in attraction of anthocorid predators. *J. Chem. Ecol.* **1997**, 23, 2241-2260.
22. Scutareanu, P.; Bruin, J.; Posthumus, M. A.; Drukker, B., Constitutive and herbivore-induced volatiles in pear, alder and hawthorn trees. *Chemoecology*. **2003**, 13, 63-74.
23. Rodriguez-Saona, C.; Crafts-Brandner, S. J.; Cañas, L.A. Volatile emissions triggered by multiple herbivore damage: beet armyworm and whitefly feeding on cotton plants. *J. Chem. Ecol.* **2003**, 29, 2539-2550.
24. Brennan, E. B.; Weinbaum, S. A. Stylet penetration and survival of three psyllid species on adult leaves and 'waxy' and 'de-waxed' juvenile leaves of *Eucalyptus globulus*. *Ent. Exp. Appl.* **2001**, 100, 355-363.
25. Way, M. J.; Banks, C. J. Intra-specific mechanism in relation to the natural regulation of numbers of *Aphis fabae* Scop. *Ann. Appl. Biol.* **1967**, 59, 189-205.
26. Soroker, V.; Talebaev, S.; Harari, A. L.; Wesley, S. D. The role of chemical cues in host and mate location in the pear psylla *Cacopsylla bidens* (Homoptera: Psyllidae). *J. Insect Behav.* **2004**, 17, 613-626.
27. Balakrishna, P.; Raman, A. Cecidogenesis of leaf galls of *Strychnos nux-vomica* (Loganiaceae) induced by the jumping plant louse species *Diaphorina truncata* (Homoptera: Psylloidea: Psyllidae). *Entomol. Gen.* **1992**, 17, 285-292.
28. Heijari, J.; Nerg, A.-M.; Kainulainen, P.; Viiri, H.; Vuorinen, M.; Holopainen, J.K. Application of methyl jasmonate reduces growth but increases chemical defence and resistance against *Hylobius abietis* in Scots pine seedlings. *Ent. Exp. Appl.* **2005**, 115,117-124.
29. Mercier, J.; Arul, J.; Julien, C. Effect of food preparation on the isocoumarin, 6-methoxymellein, content of UV-treated carrots. *Food Research International.* **1994**, 27, 401-404.

30. Mercier, J.; Roussel, D.; Charles, M. T.; Arul, J. Systemic and local responses associated with UV- and pathogen-induced resistance to *Botrytis cinerea* in stored carrot. *Phytopathology*. **2000**, 90, 981-986.
31. Zouiten, N.; Ougass, Y.; Hilal, A.; Ferriere, N.; Macheix, J. J.; El Hadrami, I. Interaction of olive trees and Psylla: Characterization of phenol compounds from new shoots and flower buds and relation to the degree of cultivar resistance. *Agrochimica*. **2000**, 44, 1-12.
32. Scutareanu, P.; Ma, Y. L.; Claeys, M.; Dommisse, R.; Sabelis M. W. Induction of a p-coumaroyl trihydroxy triterpene acid in *Psylla*-infested and mechanically damaged pear trees *J. Chem. Ecol.* **1999**, 25, 2177-2191.

Table 1. Concentrations (mean ± SE, µg/g f.w.) of some mono- and sesquiterpenes, and propenyl benzenes in the leaves of undamaged and carrot psyllid (*Trioza apicalis*) damaged leaves of two carrot cultivars Parano and Splendid (n = 6)[a]

	Parano		Splendid		Main effects	
Compounds	Undamaged	Damaged	Undamaged	Damaged	Cultivar	Treatment
α-pinene	4.5 ± 1.0	20.3 ± 6.0	9.7 ± 4.9	16.4 ± 6.2	ns	ns
camphene	0.2 ± 0.1	0.6 ± 0.1	0.5 ± 0.3	0.9 ± 0.3	ns	ns
sabinene	66.9 ± 14.9	102.2 ± 10.1	0.9 ± 0.1	1.5 ± 0.2	0.000	0.027
β-pinene	nd	4.9 ± 2.4	0.6 ± 0.2	2.7 ± 1.3	ns	0.008
myrcene	61.6 ± 8.1	92.2 ± 13.0	47.9 ± 10.7	53.9 ± 10.0	0.045	ns
limonene	9.7 ± 0.8	14.3 ± 1.2	17.7 ± 4.2	28.7 ± 5.4	0.004	0.054
(*Z*)-β-ocimene	2.1 ± 0.4	5.4 ± 1.5	5.7 ± 1.8	2.3 ± 0.9	ns	ns
terpinolene	0.6 ± 0.4	4.8 ± 3.0	0.3 ± 0.2	0.8 ± 0.4	ns	ns
bornylacetate	0.5 ± 0.2	1.4 ± 0.5	0.9 ± 0.2	3.0 ± 0.8	0.012	ns
α-copaene	tr	0.1 ± 0.1	nd	nd	ns	ns
longifolene	0.1 ± 0.0	0.3 ± 0.1	0.0 ± 0.0	0.3 ± 0.1	ns	0.000
β-caryophyllene	9.3 ± 1.7	15.6 ± 5.6	19.8 ± 6.6	26.6 ± 5.9	ns	ns
α-humulene	nd	nd	nd	1.0 ± 1.0	ns	ns
methylisoeugenol	27.5 ± 18.2	42.3 ± 10.2	88.9 ± 18.7	99.4 ± 12.4	0.007	ns
α-asarone	0.4 ± 0.2	1.4 ± 0.6	2.3 ± 1.1	1.1±0.4	ns	ns

[a] Carrot psyllids were allowed to feed 7 days on the carrots before they were removed. Terpenoids were extracted 5 or 6 days after the removal of the carrot psyllids. Main effects were tested with GLM-procedure using lg (x+1) transformed data. Ns indicates non-significant main effect, nd indicates non-defined compound and tr a compound with concentration under 0.1 µg/g f.w..

Table 2. Concentrations (mean ± SE, ng/plant/h) of some mono- and sesquiterpenes and propenyl benzenes in the headspace emission of undamaged and carrot psyllid (*Trioza apicalis*) damaged leaves of two carrot cultivars Parano (control n = 4, damaged n = 6) and Splendid (n = 6)[a]

	Parano		Splendid		Main effects	
Compound	Undamaged	Damaged	Undamaged	Damaged	Cultivar	Treatment
α-pinene	1.3 ± 0.6	26.2 ± 21.4	7.6 ± 2.8	17.2 ± 14.2	ns	ns
camphene	nd	0.5 ± 0.3	0.6 ± 0.2	0.8 ± 0.7	ns	ns
sabinene	12.6 ± 4.0	100.6 ± 73.1	1.8 ± 0.7	2.2 ± 2.0	0.003	ns
β-pinene + myrcene	21.5 ± 9.7	82.1 ± 31.5	52.0 ± 25.2	98.5 ± 88.6	ns	ns
(Z)-3-hexenyl-acetate	nd	nd	0.0 ± 0.0	0.7 ± 0.7	ns	ns
(Z)-β-ocimene	0.4 ± 0.4	1.5 ± 0.5	nd	nd	0.000	0.013
limonene	2.5 ± 0.4	9.5 ± 4.6	105.4 ± 62.6	63.8 ± 47.6	0.008	ns
terpinolene	nd	10.0 ± 8.3	4.0 ± 4.0	1.7 ± 1.3	ns	ns
linalool	nd	nd	0.4 ± 0.4	0.0 ± 0.0	ns	ns
nonanal	nd	1.4 ± 1.4	0.0 ± 0.0	1.6 ± 1.6	ns	ns
methyl salicylate	0.4 ± 0.2	0.3 ± 0.2	1.7 ± 0.6	1.3 ± 0.6	0.038	ns
bornylacetate	nd	2.0 ± 1.3	0.5 ± 0.2	0.6 ± 0.2	0.007	ns
α-copaene	nd	0.1 ± 0.1	nd	nd	ns	ns
longifolene	nd	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.2	ns	ns
β-caryophyllene	0.1 ± 0.1	19.2 ± 15.9	6.3 ± 2.6	19.0 ± 15.4	0.008	ns
methylisoeugenol	0.0 ± 0.0	8.1 ± 6.7	17.8 ± 12.7	10.4 ± 5.0	0.051	ns

[a] Carrot psyllids were allowed to feed 7 days on the carrots before they were removed. Terpenes were collected within 32 h after the removal of the carrot psyllids. Main effects were tested with GLM-procedure using lg (x+1) transformed data. Ns indicates non-significant main effect and nd non-defined compound.

Table 3. Fresh and dry weights (mean ± SE, g) of undamaged and damaged leaves of two carrot cultivars (Parano and Splendid (n = 6)) after 7-day carrot psyllids feeding period[a].

	Parano		Splendid		Main effects	
	Undamaged	Damaged	Undamaged	Damaged	Cultivar	Treatment
Fresh weight, blades	0.570 ± 0.036	0.517 ± 0.029	0.349 ± 0.053	0.414 ± 0.046	0.002	ns
Dry weight, blades	0.088 ± 0.005	0.081 ± 0.005	0.047 ± 0.007	0.060 ± 0.007	0.000	ns
Fresh weight, petioles	0.585 ± 0.038	0.376 ± 0.051	0.352 ± 0.070	0.247 ± 0.028	0.009	0.000
Dry weight, petioles	0.052 ± 0.003	0.037 ± 0.005	0.028 ± 0.005	0.023 ± 0.002	0.000	0.001

[a] Main effects were tested with GLM-procedure, and ns indicates non-significant main effect.

Table 4. Concentrations (mean ± SE, ng/g d.w./h) of some mono- and sesquiterpenes, green leaf volatiles and propenyl benzenes in the headspace emissions of two carrot cultivars (Parano and Splendid) (n = 5) 24 h after the treatments[a]

	Parano			Splendid			Main effects	
Compound	Control	Limonene	MeJA	Control	Limonene	MeJA	Cultivar	Treatment
(*Z*)-3-hexen-ol	2.7 ± 2.7	nd	nd	12.6 ± 9.7	12.0 ± 12.0	nd	ns	ns
α-pinene	54.1 ± 19.6	116.1 ± 61.4	35.2 ± 11.9	72.9 ± 14.1	87.6 ± 29.9	53.6 ± 25.8	ns	ns
camphene	1.0 ± 1.0	1.4 ± 0.9	0.8 ± 0.8	6.1 ± 1.9	5.7 ± 1.8	2.6 ± 2.6	0.017	ns
sabinene	95.5 ± 42.3	62.0 ± 16.3	63.9 ± 9.3	10.3 ± 3.5	27.9 ± 14.4	8.1 ± 2.7	0.000	ns
myrcene	532.9 ± 131.0	308.0 ± 168.5	400.2 ± 94.2	671.4 ± 137.0	1669.6 ± 873.1	959.7 ± 285.2	0.013	ns
(*Z*)-3-hexenyl-acetate	105.9 ± 30.5	nd	nd	251.3 ± 242.9	167.1 ± 141.0	nd	ns	0.002
(*Z*)-β-ocimene	7.6 ± 3.6	3.5 ± 3.5	6.0 ± 4.7	37.6 ± 23.2	157.1 ± 105.2	76.8 ± 45.0	0.005	ns
limonene	94.6 ± 21.0	63.5 ± 19.5	50.0 ± 2.9	180.8 ± 52.3	522.5 ± 236.0	226.9 ± 61.2	0.001	ns
terpinolene	149.3 ± 131.2	90.0 ± 80.0	96.0 ± 15.3	119.7 ± 50.3	854.1 ± 462.3	123.8 ± 29.1	0.020	ns
nonanal	36.1 ± 4.3	30.7 ± 2.7	33.0 ± 2.8	34.0 ±6.5	36.3 ± 5.3	30.7 ± 7.4	ns	ns
methyl salicylate	2.0 ± 1.2	0.7 ± 0.7	nd	1.0 ± 1.0	11.0 ± 3.9	1.7 ± 1.7	ns	ns
bornylacetate	14.8 ± 4.6	16.5 ± 13.8	21.0 ± 5.8	25.6 ± 12.1	33.8 ± 10.0	30.1 ± 12.8	ns	ns
β-caryophyllene + (*E*)-β-farnesene	16.3 ± 8.0	106.1 ± 85.7	41.0 ± 26.3	211.7 ± 75.2	608.1 ± 307.4	126.7 ± 23.4	0.000	ns
α-humulene	nd	4.4 ± 4.4	1.6 ± 1.6	8.7 ± 4.3	31.0 ± 15.3	4.0 ± 1.7	0.004	ns
methylisoeugenol	nd	nd	nd	3.3 ± 2.1	16.0 ± 14.7	12.6 ± 6.8	0.005	ns

[a] Control = 5% ethanol in aqueous solution. Limonene = 3% of limonene in 5% ethanol in aqueous solution. MeJA = methyl jasmonate at concentration 13.4mM in 5% ethanol in aqueous solution. Main effects were tested with GLM-procedure using lg (x+1) transformed data, and ns indicates non-significant main effect and nd non-defined compound.

Table 5. Concentrations (mean ± SE, μg/g f.w.) of some mono- and sesquiterpenes, and propenyl benzenes in the leaves of carrot (cv Parano and Splendid) (n=5) 24 h after the treatment[a]

	Parano			Splendid			Main effects	
Compound	Control	Limonene	MeJA	Control	Limonene	MeJA	Cultivar	Treatment
α-pinene	4.4 ± 2.8	3.4 ± 2.5	2.3 ± 1.4	4.3 ± 2.7	5.7 ± 3.1	nd	ns	ns
sabinene	50.1 ± 25.4	70.8 ± 12.7	44.7 ± 11.7	nd	nd	nd	0.000	ns
myrcene	98.5 ± 14.6	71.7 ± 2.1	75.3 ± 10.8	42.4 ± 10.6	30.5 ± 3.8	37.8 ± 8.2	0.000	ns
limonene	18.9 ± 3.3	12.1 ± 0.6	12.2 ± 2.0	14.5 ± 1.1	20.3 ± 4.0	13.8 ± 2.1	0.033	ns
(Z)-β-ocimene	2.1 ± 2.1 a	9.2 ± 1.3 b	4.1 ± 2.8 ab	2.8 ± 1.8 a	11.3 ± 2.2 b	4.3 ± 2.7 ab	ns	0.002
linalool	0.9 ± 0.9	nd	nd	0.7 ± 0.7	nd	0.4 ± 0.4	ns	ns
bornylacetate	nd	nd	nd	3.0 ± 1.1	1.2 ± 0.8	0.4 ± 0.4	0.000	ns
β-caryophyllene	8.6 ± 2.7	17.8 ± 4.7	14.8 ± 8.5	19.4 ± 6.1	22.2 ± 6.2	18.2 ± 4.2	0.002	ns
methylisoeugenol	121.1 ± 39.3	98.9 ± 26.0	75.7 ± 19.5	244.0 ± 36.5	201.9 ± 27.0	135.6 ± 25.6	0.000	ns
α-asarone	3.5 ± 2.2	2.4 ± 1.7	2.5 ± 2.0	9.0 ± 5.6	36.3 ± 23.3	13.5 ± 8.4	ns	ns

[a] Control = 5% ethanol in aqueous solution. Limonene = 3% of limonene in 5% ethanol in aqueous solution. MeJA = 13.4mM methyl jasmonate in 5% ethanol in aqueous solution. Main effects were tested with GLM-procedure using lg (x+1) transformed data followed by Dunnett T3-test. Different letters within a row indicate significant differences between treatments. Ns indicates non-significant main effect and nd non-defined compound.

Table 6. Concentration (mean ± SE, mg/g d.w.) of total phenolics in the blades of two carrot cultivars (n=5) treated with limonene or methyl jasmonate[a]

	Parano	Splendid
Control	28.3± 1.7 a	28.2 ± 1,9 a
Limonene	37.2 ± 2.2 b	38.8 ± 1.9 b
MeJa	38.9 ± 1.8 b	29.6 ± 3.8 ab

[a]Differences between treatments tested with one way ANOVA followed by Dunnett T3-test. Different letters within a column indicate significant differences between treatments.

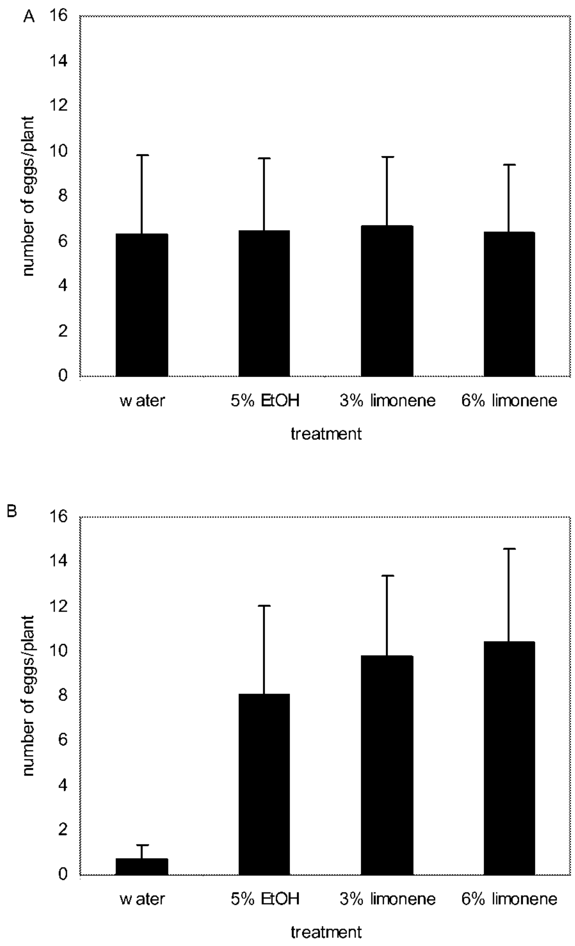


Figure 1. Mean number of eggs (+ SE) laid by carrot psyllids released 24 h after the limonene treatment. A = Parano (n = 20), B = Splendid (n = 19).

Chapter 6

The influence of exogenous monoterpene treatment and elevated temperature on growth, physiology, chemical content and headspace volatiles of carrot (*Daucus carota* L.)

Ibrahim MA., Nissinen A., Prozherina N., Oksanen E and Holopainen JK.

Environmental and Experimental Botany. In press

ELSEVIER

Available online at www.sciencedirect.com
SCIENCE DIRECT®

Environmental and Experimental Botany xxx (2005) xxx–xxx

Environmental
and Experimental
Botany

www.elsevier.com/locate/envexpbot

The influence of exogenous monoterpene treatment and elevated temperature on growth, physiology, chemical content and headspace volatiles of two carrot cultivars (*Daucus carota* L.)

M.A. Ibrahim[a,*], A. Nissinen[a,b], N. Prozherina[a],
E.J. Oksanen[a,c], J.K. Holopainen[a]

[a] *Department of Ecology and Environmental Science, University of Kuopio, P.O. Box 1627, FIN-70211 Kuopio, Finland*
[b] *Agrifood Research Finland, Plant Protection, FIN-31600 Jokioinen, Finland*
[c] *Department of Biology, University of Joensuu, P.O. Box 111, FI-80101 Joensuu, Finland*

Received 26 August 2004; accepted 18 January 2005

Abstract

We investigated the effects of application of aqueous solution (50 ml l^{-1}) of limonene spray (LS) on growth, physiology, foliar and root chemistry and volatile emissions of two carrot cultivars (cv Parano and Splendid) growing at control temperature (CT) of 25 °C and at high temperature (HT) of 35 °C. The reduction in net photosynthesis induced by HT tended to be counteracted by LS 2 h and 24 h after the first spraying in cv Parano, but the long-term effect was not significant. LS counteracted temperature-induced reductions seen in chlorophyll fluorescence after 21 days from the start of the experiment. A significant increase in the levels of monoterpenes α-pinene, camphene, sabinene, β-myrcene and limonene emission from foliage was observed at HT. HT elevated the concentrations of monoterpenes and sesquiterpenes (*E*)-β-farnesene and β-caryophyllene, in roots of both cultivars. The concentration of total phenolics in foliage was not affected by HT as expected by a protein competition model of phenolic allocation. However, the proportion of the single phenylpropanoid compound, methyl isoeugenol, was reduced by HT. HT increased the concentration of total soluble proteins in cv Parano, whereas in cv Splendid the concentrations of chlorophyll and carotenoids decreased. The dry mass of main root was significantly reduced at HT in both cultivars, probably reflecting reduced photosynthesis and carbon allocation costs to defence as a result of thermal stress. Limonene treatment was not sufficient to protect carrot root growth from thermal stress.

Keywords: Limonene; Carrot; Monoterpenes; Chlorophyll fluorescence; Temperature; Stomatal conductance

1. Introduction

Many plants produce and emit terpenoids, which constitute the major fraction of the biogenic volatile organic compounds (VOC) (Guenther et al., 1995). When

---

[*] Corresponding author. Tel.: +358 17 163 205; fax: +358 17 163 191.
E-mail address: Mohamed.Ibrahim@uku.fi (M.A. Ibrahim).

0098-8472/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.envexpbot.2005.01.006

monoterpenes are volatilized they can mediate multitrophic interactions (Dicke et al., 2003; Heil, 2004; Van Poecke and Dicke, 2004; Holopainen, 2004), attract pollinators (Caissard et al., 2004), or can act as a deterrent (Hummelbrunner and Isman, 2001; Wheeler and Isman, 2001; Ibrahim et al., 2001) to protect plants from insects and animals. It is well known that monoterpenes stored in reservoirs serve as constitutive or inducible defenses (Gershenzon and Croteau, 1993). The monoterpenes respond differently to temperature. The emission of cyclic monoterpenes such as α-pinene, sabinene, β-pinene, and limonene increases exponentially at temperatures from 5 to 35 °C but becomes reduced at temperatures higher than 35 °C, while acyclic monoterpenes such as (*Z*)-β-ocimene and (*E*)-β-ocimene are not or barely detected below 35 °C as they exhibit a sharp increase at temperatures from 35 to 45 °C (Loreto et al., 1998; Staudt and Bertin, 1998).

One intriguing hypothesis concerning the isoprenes and monoterpenes is that they are intended to protect the plant leaves against thermal stresses (Delfine et al., 2000) including bursts of heat (Delfine et al., 2000; Singsaas et al., 1997; Loreto et al., 1998) and increase the thermotolerance of intact leaves of isoprene emitting species (Sharkey and Singsaas, 1995) although these compounds do not exhibit this property in leaf discs (Logan and Monson, 1999). It was suggested also that monoterpene fumigation could be one way to improve the thermotolerance of non-isoprene emitting *Quercus* leaves (Loreto et al., 1998). Isoprenoid substances can help maintain photosynthesis from being damaged by high leaf temperatures and the recovery of photosynthesis can be increased by the emission of monoterpenes after brief high temperature episodes (Sharkey and Singsaas, 1995; Singsaas and Sharkey, 1998). Therefore, plant terpenoids in general could well be able to induce resistance to high temperature and possibly also to other environmental stresses such as drought (Sharkey and Loreto, 1993) and ozone (Loreto et al., 2004). However, exogenous monoterpene fumigation at low doses ($4–6\,\mu g\,l^{-1}$) does not result in any significantly protective effect on photosynthesis at high temperature as was observed with higher ($26–35\,\mu g\,l^{-1}$) (Delfine et al., 2000). On the other hand, fumigation of *Quercus ilex* L. (Fagaceae) leaves at high doses of monoterpenes resulted in a very high content of monoterpenes leading to an increase even in the concentration and emission of monoterpenes, which were not in the fumigation mixture (e.g. myrcene and limonene) (Delfine et al., 2000).

Although the role of essential oils including monoterpenes in carrots is poorly understood, previous studies have reported that cultivars of carrot differ in their essential oil contents (Senalik and Simoin, 1987; Kainulainen et al., 1998, 2002; Habegger and Schnitzler, 2000b) and that the leaves have higher amount of essential oils than the roots (Habegger and Schnitzler, 2000a), and that petioles have different amounts of essential oils from blades (Kainulainen et al., 1998; Habegger and Schnitzler, 2000b). The high terpene concentration (97–98%) of the total volatiles, present in carrot roots (Alasalvar et al., 1999; Kjeldsen et al., 2001) and foliage (Habegger and Schnitzler, 2000b) suggest that these essential oils have certain role in carrot defence. Most of the concentrations of terpenes detected in the essential oils of carrot became elevated as the temperature increased from 9 to 21 °C (Rosenfeld et al., 2002). However, the function of the isoprenoids inside the plants is still being debated, though the findings that endogenous and exogenous isoprene (Sharkey and Singsaas, 1995; Singsaas et al., 1997) and monoterpenes (Loreto et al., 1998) increase the thermotolerance of leaves suggested that isoprenoids are produced by the plants to help them cope with environmental stresses.

Limonene has potential repellent and deterrent effect against insects (Ibrahim et al., 2001), at least against carrot psyllid *Trioza apicalis* Först. (Homoptera: Triozidae) (Nehlin et al., 1994), which is one of the most serious carrot pests in Scandinavia. In several previous studies, it has been shown that when plants were fumigated with limonene together with other monoterpenes this could improve their thermotolerance (Delfine et al., 2000; Loreto et al., 1998; Peñuelas and Llusià, 2002). One possible field application technique for limonene could be spraying of an aqueous solution onto plants. There is also some evidence that terpenes, when applied in aqueous solutions, have similar effects on plant growth as fumigated terpenes (Jasicka-Misiak et al., 2002).

Phenolics are carbon-based secondary compounds synthesized from phenylalanine (Harborne, 1997). They are required for constitutive and induced protection and defence (Jones and Hartley, 1999). It has been suggested that a high temperature does not influence the content of total phenolics in plants (Jones and Hartley, 1998, 1999). However, different phenolic compound groups can have complex even opposite response to the environmental stresses (Lavola, 1998). One of the important groups of secondary compounds present in the leaves of several umbellifers, including carrots, is the polyacetylenes (Degen et al., 1999). Polyacetylenes have antifungal activity against carrot storage diseases (Olsson and Svensson, 1996) and they are known to be among oviposition stimulants of carrot fly (*Psila rosae*) (Degen et al., 1999), but to our knowledge there is no evidence of any link between the behaviour of carrot psyllid and polyacetylenes.

As monoterpenes are related to plant defence against high temperatures, we hypothesized that field application of limonene to carrots may influence carrot physiology, defence capabilities and secondary metabolites under thermal stress. Therefore, the specific aims of our study were to detect (1) how high temperature and exogenous limonene can influence carrot photosynthesis, (2) if there is a temperature-induced trade-off between carrot growth and chemical defence, (3) if high temperature can affect carbon allocation to terpenes and total phenolics, and (4) finally, if exogenous limonene treatment can compensate for these temperature-induced trade-offs.

2. Materials and methods

2.1. Plant material

Seeds of two carrot cultivars (Parano and Splendid) were sown individually in 1 l plastic pots filled with peat and sand (3:1). The seedlings were fertilized with 0.1% 9-Superex (19:5:20 N:P:K, Kekkilä, Finland) at the rate of 0.5–1.0 dl/pot, starting two weeks after sowing and watered daily with the tap water. The plants were grown under controlled growth chamber conditions, where day/night temperature was 23/18 °C, (10/6 h of transition time) relative humidity (RH%) ranged 40–60%, and maximum light intensity at canopy level was about 370 μmol m^{-2} s^{-1}. Four-week-old seedlings were transferred into four 2.6 m^{-3} controlled chambers (Holopainen and Kärenlampi, 1984) 3 days before the sprayings for acclimation to higher temperatures.

2.2. Limonene application and temperature levels

The treatments were: ambient conditions at 25 °C (CT), high temperature at 35 °C (HT), limonene spraying at 25 °C (CT + LS), and high temperature with limonene spraying (HT + LS). The maximum day-time (8 h) temperature level in (HT) treatment was raised from 25 to 35 °C. Each temperature level had two monoterpene treatment levels (one with limonene and the other without limonene spraying). The minimum temperature in all four chambers was set at 18 °C in the night (from 00:00 to 04:00 h.) (Holopainen and Kainulainen, 2004). The temperature level was raised from a minimum night temperature up to the maximum temperature (25 °C or 35 °C) reached at 10:00 h, and it was lowered linearly from 18:00 to 00:00 h. Similarly, the light was increased in the chambers gradually from 0 to 100% from 00:00 to 6:00 h. Day/night relative air humidity was 88/50%. The plants were rotated among and within the chambers weekly to avoid any systematic chamber effect.

Since limonene concentrations below 60 ml l^{-1} do not induce any visible phytotoxicity symptoms in carrot foliage (Ibrahim et al., 2004), we used a 50 ml l^{-1} of limonene solution in the present study. For spray treatment, 50 ml l^{-1} of monoterpene (*R*)-(+)-limonene of 97% purity (Aldrich Chemical Co. Ltd) was first dissolved in 50 ml l^{-1} of ethanol (99.5% purity) and then added into 900 ml of water. In the control treatment, 50 ml l^{-1} of ethanol was added into 950 ml of water. The seedlings were sprayed with the above-mentioned solutions once a week for 3 weeks (overall three times). The time of spraying was constant in each week and the spraying rate was 3 sprayings for each plant giving 3.5 ml of the solution.

2.3. Net photosynthesis and chlorophyll fluorescence

From each treatment, five seedlings per cultivar were used for the measurement of net photosynthesis, stomatal conductance and chlorophyll fluorescence. The measurements were conducted 2 and 24 h after each limonene spraying between 11:00 and 15:00 h to determine how exogenous limonene could affect plant physiological processes immediately after the spraying as well as one day after the spraying since limonene is a highly volatile monoterpene. A CI-510 Portable Photosynthesis System (CID, Inc., Vancouver, WA, USA) with a four-cell non-dispersive infrared gas analyzer was used to measure net photosynthesis and stomatal conductance at CO_2 concentration of 450 ± 20 ppm and light intensity of 700 ± 15 μmol m^{-2} s^{-1}. The mean outside temperature was 26 ± 1 °C during the measurements. For fluorescence, all measurements were conducted from the same leaves throughout the experiment with a portable pulse modulated chlorophyll fluorometer (FMS 2- Field Fluorescence Monitoring System, Hansatech Instruments Limited, Norfolk, England).

2.4. *Collections of headspace volatiles*

Volatiles emitted from the foliage of carrot plants were collected by the headspace collection technique as described by Vuorinen et al. (2004). Samples were collected 24 h after the first and the third spraying at 22 °C from four seedlings per treatment. Standard temperature during VOC sampling was used to detect the difference in emission due to volatile production capacity of test plant and to avoid any differences due to increased volatility of VOC at the higher temperature. One randomly selected seedling per treatment was enclosed into one of the four 1.5 l glass containers. The charcoal-filtered air was led to the container through teflon tubing at a flow rate 200 ml min^{-1} for 60 min. Volatiles were collected into Tenax TA adsorbent (150 mg/tube).

The plant volatiles were analyzed with GC-MS (Hewlett Packard GC 6890, MSD 5973) using HP-5 capillary column (50.0 m × 0.2 mm i.d. × 0.50 μm film thickness). Compounds absorbed to tenax were released by thermodesorption at 250 °C for 10 min and cryofocused in a cold trap at −30 °C. The column temperature was first held at 40 °C for 1 min, thereafter increased to 210 °C at 5 °C min^{-1} and finally raised to 250 °C at 20 °C min^{-1}. The mass numbers from 30 to 350 (*m*/*z*) were recorded. The absolute amounts of terpenes were calculated based on an external standard consisting of known amount of several mono and sesquiterpenes. The compounds were identified by comparing the mass spectrum of an individual compound to the spectra of compounds in external standard and in Wiley Library. The external standard consisted of known amount of (*Z*)-3-hexen-1-ol, α-pinene, camphene, sabinene, β-myrcene, (*Z*)-3-hexenyl acetate, limonene, (*Z*)-β-ocimene, terpinolene, linalool, nonanal, methyl salicylate, bornyl acetate, longifolelne, β-caryophyllene + (*E*)-β-farnesene, aromadendrene, α-humulene, methyl isoeugenol and α-asarone.

After the first VOC collection, the whole green mass of the seedling was dried at 40 °C (Julkunen-Titto 1985) for 5 days to obtain the dry mass. After the second VOC collection, one leaf per seedling was extracted. The rest of the plant, blades and petioles separately, were dried as described above. The estimate of the dry weight for each seedling was attained by calculating the fresh weight/dry weight ratio for the non-extracted leaves and using this for dry weight estimate of extracted leaves.

2.5. *Terpenoid extraction from shoots and roots*

Immediately after the second collection of volatiles, the youngest fully developed leaflet of the same plant individuals as used in VOC collections were extracted with 3 ml of *n*-hexane for 2 h at room temperature. After the filtration of the extract, the plant residues were washed twice with 1 ml of pure hexane. Plant extracts were analyzed with GC-MS (Hewlett Packard GC type 5890, MSD 5970) with a 30 m long HP-5MS (0.25 mm i.d., 0.25 μm film thickness) capillary column using helium as the carrier gas. The column temperature was maintained at 50 °C for 2 min. Then the temperature was elevated to 110 at 10 °C min^{-1} and finally to 200 °C at 5 °C min^{-1}. Mass numbers between 30 and 550 (*m*/*z*) were recorded. There was an error in the procedure of making the extraction solutions, thus the extraction solution used for the Splendid leaves contained an extremely high concentration of the internal standard (1-chloro-octane) and the extraction solution for Parano leaves contained no internal standard. Therefore, only the relative proportions of terpenoids and propenyl benzenes were calculated from the data. The compounds were identified by comparing the mass spectrum of an individual compound to the spectra of compounds in the external standard and in Wiley Library and retention indexes to Joulain and König (1998). The external standard consisted of known amounts of α-pinene, camphene, sabinene, β-pinene, β-myrcene, limonene, (*Z*)-β-ocimene, terpinolene, linalool, bornyl acetate, longifolene, β-caryophyllene, aromadendrene, (*E*)-β-farnesene, α-humulene, methyl isoeugenol, and α-asarone.

For the extraction from the roots, 10 pieces of roots, weighing 2–3 g, per treatment were cut from 8-week-old plants and stored at −80 °C until extraction. Two root pieces were combined to make one sample to obtain terpene concentrations detectable by GC/MS. The roots were cut into pieces and extracted for 2 h in 5 ml of *n*-hexane with 1-chloro-octane as the internal standard. The plant residues were not washed with pure hexane in order to keep the concentration as high as possible. The root extracts were analyzed with the same method as the leaf extracts.

2.6. *Total soluble proteins and pigment content of carrot leaves*

For each treatment of both cultivars, five blades (leaflets) used for the net photosynthesis measurements were cut after the second and the fourth measurement and placed into liquid nitrogen for total soluble protein and chlorophyll determinations. Frozen leaf samples were weighed (ca. 100 mg), and a crude extract was prepared using 2 ml of the extraction buffer, containing 50 mM MES, 20 mM $MgCl_2$, 50 mM β-mercaptoethanol, and 1% Tween-80. An aliquot for total soluble proteins was quantified by centrifuging the sample at 14,000 rpm for 5 min, re-suspending the pellet in 0.1 M NaOH and measuring the concentration according to the standard Bradford assay protocol (Sigma). An aliquot for chlorophyll and carotenoid concentrations was determined by centrifuging the sample at 10,000 rpm for 10 min and then the assays were performed according to Porra et al. (1989) and Wellburn and Lichtenthaler (1984), respectively.

2.7. *Total phenolics*

The total phenolics were extracted from five blades and five petioles from each cultivar of carrot plants dried at 40 °C. The highest amount of phenolics was achieved from willow leaves when dried at temperatures below 50 °C, but temperatures higher than 60 °C lowered the total phenolics (Julkunen-Tiitto, 1985). The dried leaf material was powdered with 80% (v/v) aqueous acetone, and analyzed with Folin-Ciocalteu reagent (Julkunen-Tiitto, 1985) to determine how the treatments would affect the total phenolic content of the two parts (blades and petioles) of the plant.

2.8. *Biomasss*

Ten of the 8-week-old plants from both cultivars were weighed for fresh weight (shoots and roots separately) and dried at 60 °C for over 3 days for the dry weight determination. Since a piece of the root was cut for terpenoid analysis, we determined the weight of these pieces for the fresh weight and then they were dried at 60 °C. The estimate of the dry weight for each main root was obtained by calculating fresh weight/dry weight ratio for the non-extracted pieces of a root and using this for the dry weight estimate of extracted piece of the root.

2.9. *Statistical analysis*

The data were analyzed using SPSS for Windows, version 11.5 (SPSS Inc., Chicago, IL, USA). For the fluorescence of test species analysis of multivariate repeated measures (General Linear Model procedures) with limonene treatment and cultivar as the between-subjects factors were carried out to determine the effect of limonene, temperature, time, cultivar, and the interactions between factors. Thereafter, multiple comparisons were used for each time point using Tukey's multiple range tests. For photosynthesis, biomass, total soluble proteins and pigments, and phenolics multivariate ANOVA were used, followed by Tukey's multiple range tests.

The data from the first VOC collection time were not analyzed statistically because the VOC emission of cv Parano was extraordinarily low. Effects of cultivar, temperature and limonene were tested with GLM when the distribution of a compound was possible to normalize with $\log x + 1$ transformation. The effect of cultivar was tested with Mann–Whitney test and effect of treatment with Kruskall–Wallis test with the rest of the compounds. The data from leaf extractions was first transformed with arcsin function and then tested with ANOVA. Differences between treatments were tested with Tukey or Dunnett T3 test (equality of variances assumed or not assumed, respectively). The effects of cultivar in the root extractions data were tested with non-parametric test (Mann–Whitney *U*). The effects of temperature and limonene were tested with GLM when the distribution of a compound was possible to normalize with $\log x + 1$ transformation, the rest of the compounds were tested with Mann–Whitney

U. Differences were considered significant at $P \leq 0.05$.

3. Results

3.1. Net photosynthesis, stomatal conductance and chlorophyll fluorescence

Net photosynthesis was not significantly affected by HT or LS in either cultivar (Fig. 1). In Parano cv, the stomatal conductance decreased significantly by HT, CT + LS and HT + LS treatments 2 h ($P = 0.008$, $P = 0.027$, and $P = 0.004$, respectively) and 24 h ($P = 0.001$, $P = 0.010$, and $P = 0.001$, respectively) after the first spraying while in Splendid, only HT and HT + LS decreased significantly ($P = 0.001$ and $P < 0.001$, respectively) the stomatal conductance immediately after the first limonene spraying (Fig. 2). The main effect of temperature in cv Parano was significant at the first three measurements ($P = 0.005$, $P = 0.001$, and $P = 0.011$, respectively), while limonene affected stomatal conductance significantly 2 and 24 h ($P = 0.026$ and $P = 0.016$, respectively) after the first limonene spraying treatment. In cv Splendid, the effects of temperature ($P < 0.001$ and $P = 0.002$) and limonene ($P = 0.018$ and $P = 0.002$) were evident 2 h after the first and the third spraying (Fig. 2).

The main effect of temperature appeared as reduced chlorophyll fluorescence 2 and 24 h after the first limonene spraying in both cultivars (Table 1). In cv Parano, HT reduced significantly the chlorophyll fluorescence 2 and 24 h after the first limonene spraying while in cv Splendid, chlorophyll fluorescence showed a significant reduction at 35 °C only 2 h after the first spraying. LS at control temperature 25 °C (CT + LS) did not cause any significant effect on the chlorophyll fluorescence in either cultivar. The decline in the fluorescence evoked by HT was not affected by simultaneous LS in either cultivar. A significant interactive effect between temperature and limonene treatment was noted in cv Parano in the last measurement day (Table 1), indicating that negative effect of limonene on fluorescence was ameliorated by HT.

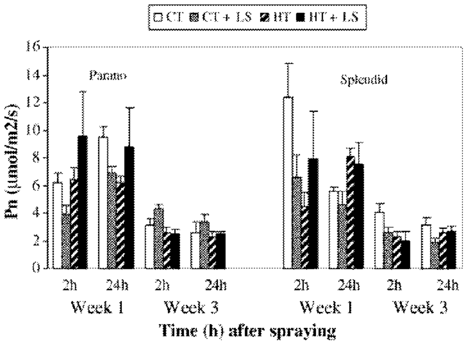


Fig. 1. Net photosynthesis (Pn) (µmol m^{-2} s^{-1}) of two carrot cultivars (cv Parano and Splendid), 2 h = 2 h after spraying; 24 h = 24 h after spraying; ($n = 5$), Bars show standard errors.

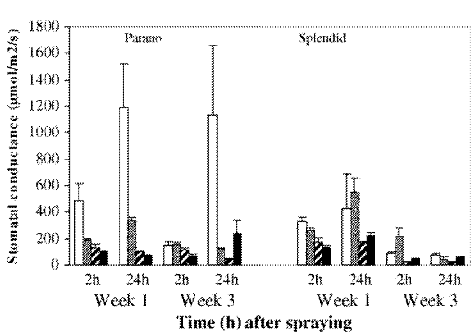


Fig. 2. The stomatal conductance (µmol m^{-2} s^{-1}) of two carrot cultivars (cv Parano and Splendid). For explanation of X-axis codes see legend of Fig. 1.

3.2. Headspace volatiles of carrot leaves

There was a significant difference between the concentrations of the monoterpenes sabinene, β-myrcene, limonene, an aldehyde nonanal and the combined concentration of sesquiterpenes β-caryophyllene and (*E*)-β-farnesene in the headspace volatiles of carrot cultivars Parano and Splendid (Table 2). HT had a significant effect on the emission of α-pinene, camphene, sabinene, β-myrcene, and limonene (Table 2). LS affected significantly only limonene emission. None of the interactive effects were significant.

Table 1
Chlorophyll fluorescence of two carrot cultivars (Parano and Splendid)

Measurement time	CT	CT + LS	HT	HT + LS	Main effects		Interaction
					Temperature	Limonene	
Parano							
T1 (week 1)	0.860 ± 0.006a	0.870 ± 0.024a	0.790 ± 0.005b	0.760 ± 0.016b	<0.001	0.452	0.154
T2 (week 1)	0.880 ± 0.004a	0.860 ± 0.017a	0.760 ± 0.007b	0.760 ± 0.024b	<0.001	0.687	0.488
T3 (week 3)	0.840 ± 0.016a	0.830 ± 0.012a	0.810 ± 0.030a	0.830 ± 0.012a	0.583	0.757	0.414
T4 (week 3)	0.850 ± 0.014a	0.820 ± 0.016a	0.790 ± 0.023a	0.840 ± 0.011a	0.261	0.685	0.019
Splendid							
T1 (week 1)	0.830 ± 0.018a	0.870 ± 0.022a	0.760 ± 0.006b	0.740 ± 0.017b	<0.001	0.684	0.130
T2 (week 1)	0.840 ± 0.020a	0.860 ± 0.01a	0.820 ± 0.014ab	0.790 ± 0.012b	0.003	0.458	0.144
T3 (week 3)	0.830 ± 0.019a	0.800 ± 0.025a	0.750 ± 0.028a	0.810 ± 0.015a	0.106	0.437	0.052
T4 (week 3)	0.760 ± 0.018a	0.810 ± 0.027a	0.730 ± 0.027a	0.760 ± 0.011a	0.070	0.115	0.664

Effects of control temperature (CT = 25 °C), high temperature (HT = 35 °C) and limonene spray (LS) on the chlorophyll fluorescence (Fv/Fm) of carrot plants (cv Parano and Splendid), ($n = 5$), T=time of measurement (T1 = 2 h after the spraying, T2 = 24 h after the spraying, T3 = 2 h after spraying, T4 = 24 h after spraying). Multivariate ANOVA followed by Tukey's multiple range test. Values are means ± S.E. Means (in columns) followed by different letters are significantly different $P < 0.05$.

3.3. Terpenoid content in leaves

The principal compounds in the leaves of cv Parano were β-myrcene, α-asarone, methyl isoeugenol, β-caryophyllene and sabinene, while in leaves of cv Splendid they were methyl isoeugenol, α-asarone, β-caryophyllene, β-myrcene and (*E*)-β-farnesene in descending order (Table 3). The β-myrcene content in the leaves of Splendid was significantly higher at HT with LS than in the CT or CT + LS (Table 3). In contrast, the content of methyl isoeugenol was significantly lower in HT + LS than in the CT or CT + LS. In the leaves of cv Parano, the contents of β-myrcene and limonene were increased by HT and LS, while the contents of other terpenes, (*E*)-β-ocimene, (*E*)-α-bergamotene, (*E*)-β-farnesene, and methyl isoeugenol were reduced by both HT and HT + LS (Table 3). Both cultivars showed a trend of increasing α-pinene content by HT + LS, although it was not significant.

3.4. Terpenoid concentration in roots

A significant difference between the concentrations of α-pinene, sabinene, β-pinene, limonene and β-caryophyllene ($P = 0.009$, $P = 0.005$, $P = 0.016$, $P = 0.047$, and $P = 0.047$, respectively) in roots of Parano and Splendid was observed (Fig. 3). The concentration of α-pinene, sabinene, β-pinene, β-myrcene, limonene, total monoterpenes and (*E*)-β-farnesene ($P = 0.035$, $P < 0.001$, $P = 0.001$, $P < 0.001$, $P < 0.001$, $P < 0.001$, and $P = 0.009$, respectively) in the roots of Parano was significantly increased by the elevated temperature. A significantly increased concentration of α-pinene, camphene, β-pinene, β-myrcene, limonene, total monotepenes and β-caryophyllene ($P < 0.001$, $P = 0.002$, $P = 0.026$, $P < 0.001$, $P < 0.001$, $P < 0.001$, and $P = 0.001$, respectively) with the higher temperature was found in the roots of Splendid (Fig. 3). The effect of LS was not significant for any of the compounds in either of the cultivars. However, there was a significant interactive effect between temperature and LS in β-caryophyllene concentration in the roots of Splendid (Fig. 3).

3.5. Concentrations of total soluble proteins and pigments

The main effect of HT appeared only at the first sampling day (31 or 32 days old plants) and differed between the cultivars in cv Splendid, lower contents of pigments and a lower chlorophyll to carotenoid ratio were found (Table 4), whereas in cv Parano HT increased the amount of soluble proteins and caused a higher chlorophyll a to b ratio (Table 5). Similarly, the main effects of limonene were found mainly in the first sampling day as a reduced amount of chlorophyll, carotenoids and total pigments in cv Splendid (Table 4),

Table 2
Concentration (ng gDW^{-1} h^{-1}) of mono and sesquiterpenes and some green leaf volatiles in the headspace emissions of two carrot cultivars (cv Parano and Splendid)

Compound	Parano				Splendid				Main effects		
	CT	CT + LS	HT	HT + LS	CT	CT + LS	HT	HT + LS	Cultivar	Temperature	Limonene
α-Pinene	37.0 ± 17.1	38.4 ± 20.5	52.7 ± 13.8	50.2 ± 21.8	16.4 ± 4.4	72.6 ± 48.2	207.2 ± 126.4	146.5 ± 77.4	ns	0.015	ns
Camphene	0.9 ± 0.4	1.0 ± 0.5	3.3 ± 1.2	2.8 ± 0.8	1.1 ± 1.1	7.7 ± 6.1	7.9 ± 2.6	9.4 ± 4.1	ns	0.005	ns
Sabinene	81.8 ± 57.0	32.1 ± 21.7	477.2 ± 197.6	556.3 ± 365.5	1.5 ± 1.5	10.7 ± 6.5	12.5 ± 6.2	11.1 ± 4.1	0.000	0.009	ns
β-Myrcene	107.4 ± 48.6	95.0 ± 39.5	673.9 ± 335.1	486.0 ± 153.3	191.2 ± 66.5	940.6 ± 519.5	1546.2 ± 1138.2	864.6 ± 260.0	0.044	0.004	ns
Limonene	14.4 ± 5.6	56.6 ± 13.4	67.6 ± 29.4	120.1 ± 18.3	53.8 ± 28.1	178.8 ± 75.4	199.9 ± 105.5	140.2 ± 53.0	0.024	0.010	0.009
Nonanal	0.8 ± 0.5	1.5 ± 1.2	5.5 ± 3.4	3.2 ± 2.1	22.7 ± 11.7	25.0 ± 9.0	14.1 ± 7.6	13.2 ± 4.3	0.000	ns	ns
β-Caryophyllene + (*E*)-β-farnesene	23.7 ± 12.9	28.5 ± 13.3	62.1 ± 34.6	35.7 ± 14.5	150.5 ± 98.6	617.3 ± 400.9	269.3 ± 179.4	226.4 ± 121.3	0.001	ns	ns
									Cultivar*	Treatment: P*	Treatment: S*
(*Z*)-β-Ocimene	0.0 ± 0.0	0.3 ± 0.3	4.6 ± 4.6	0.0 ± 0.0	18.5 ± 13.6	27.2 ± 27.2	5.2 ± 5.2	9.4 ± 9.4	ns	ns	ns
Terpinolene	13.8 ± 11.2	48.2 ± 24.5	14.4 ± 7.8	14.6 ± 7.4	56.0 ± 16.9	438.7 ± 261.0	111.4 ± 30.3	118.5 ± 70.8	ns	ns	ns
Bornyl acetate	3.9 ± 2.1	5.6 ± 1.4	5.9 ± 1.9	9.1 ± 7.8	15.1 ± 7.3	130.8 ± 73.9	16.9 ± 6.0	19.0 ± 12.6	ns	ns	ns
α-Humulene	1.0 ± 0.6	1.2 ± 0.7	2.4 ± 1.9	2.1 ± 1.0	7.9 ± 6.2	31.7 ± 21.9	15.2 ± 11.3	11.9 ± 6.8	ns	ns	ns
Methyl isoeugenol	0.4 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	23.5 ± 23.5	0.0 ± 0.0	ns	ns	ns
(*Z*)-3-Hexen-1-ol	2.1 ± 2.1	0.0 ± 0.0	28.3 ± 24.3	3.3 ± 1.4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	17.4 ± 17.4	ns	ns	ns
(*Z*)-3-Hexenyl-acetate	68.1 ± 27.9	23.8 ± 7.5	784.3 ± 561.5	244.0 ± 97.2	11.6 ± 11.6	2.4 ± 2.4	38.4 ± 30.1	344.6 ± 300.3	ns	ns	ns
Methyl salicylate	0.0 ± 0.0	0.7 ± 0.7	6.7 ± 6.7	1.9 ± 1.9	2.2 ± 2.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	ns	ns	ns

Plants ($n = 4$) were grown at CT or HT or with and without limonene treatments. Values are means ± S.E. Effects of cultivar, temperature and limonene are tested with GLM.
(*)Effect of cultivar tested with Mann–Whitney *U*-test and effect of treatment with Kruskall–Wallis test.

Table 3
Mean relative proportion (percentages) of mono and sesquiterpenes in leaves of two carrot cultivars (cv Parano and Splendid)

Compound	Parano				Splendid			
	CT	CT + LS	HT	HT + LS	CT	CT + LS	HT	HT + LS
α-Pinene	1.4	1.3	3.1	7.4	1.3	1.0	7.0	8.6
Camphene	0.0	0.0	0.0	0.2	0.0	0.0	0.2	0.2
Sabinene	8.5	8.9	19.3	17.8	0.0	0.0	0.2	0.6
β-Pinene	0.5	0.0	0.0	0.5	0.3	0.1	0.3	0.8
β-Myrcene	25.2a	17.4a	32.5ab	41.6b	12.6a	10.7a	23.3ab	38.2b
Limonene	2.6a	6.1ab	3.0a	8.7b	2.6	6.7	8.5	6.5
(*Z*)-β-ocimene	0.0	0.1	0.1	0.0	0.2	0.8	0.0	0.0
(*E*)-β-ocimene	3.2b	2.1ab	0.6a	0.5a	2.1	2.8	0.5	0.6
γ-Terpinene	0.5	0.1	0.5	0.4	0.0	0.0	0.0	0.0
Terpinolene	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0
Bornyl acetate	0.0	0.2	0.1	0.0	0.1	0.1	0.0	0.3
α-Terpinene	2.2	1.7	0.4	1.4	0.0	0.0	0.2	0.0
δ-Elemene	0.9	0.1	2.1	0.0	0.0	0.7	0.8	0.0
Methyleugenol	0.0	0.0	0.0	0.0	0.0	0.0	0.7	0.0
(*Z*)-α-bergamotene	2.2	1.2	0.7	0.8	1.2	2.3	2.4	2.1
β-Caryophyllene	9.3	7.3	11.5	8.6	10.7	13.8	10.9	15.4
(*E*)-α-bergamotene	2.3b	2.4b	1.6ab	0.4a	1.3	3.1	2.9	2.3
(*E*)-β-farnesene	3.3ab	3.9b	2.5ab	0.7a	3.7	6.2	5.5	4.9
(*Z*)-β-farnesene	2.4	2.5	2.1	0.5	2.8	4.5	3.9	3.1
Germacrene-d	4.0	6.6	7.3	4.1	3.3	2.5	3.4	3.7
β-Selinene	0.0	0.0	0.0	0.0	0.7	0.0	2.0	0.5
Methyl isoeugenol	10.6ab	16.9b	2.2a	0.0a	37.5b	38.6b	19.1ab	8.2a
(*E*,*E*)-α-farnesene	1.6	1.8	2.8	1.7	0.2	0.3	0.9	0.0
β-Bisabolene	0.0	0.3	0.3	0.0	0.7	0.6	0.4	0.0
(*E*)-γ-bisabolene	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0
(*E*)-α-bisabolene	2.8	3.0	3.2	1.8	0.0	0.0	0.0	0.0
β-Asarone	0.3	0.3	0.0	0.0	0.3	0.0	0.0	0.0
α-Asarone	14.7	15.2	0.0	0.1	17.2	3.6	4.7	2.6
α-Bisbolol	0.6	0.0	2.8	2.8	0.0	0.0	0.0	0.0
Unknown sesquiterpenes	1.0	1.2	0.8	0.1	0.8	1.7	2.2	1.4

Plants ($n = 5$) were grown at CT or HT or with and without limonene treatments. Different letters (in columns) indicate a significant difference in between treatments in Tukey or Dunnett T3-test.

but as a reduced amount of total soluble proteins in cv Parano (Table 5). In the second sampling (43 or 46 days old plants), the only significant main effect of limonene to be found was the reduced chlorophyll a to b ratio in cv Splendid (Table 4), while in cv Parano the total soluble proteins and the chlorophyll a to b ratio were significantly decreased by HT (Table 5). A significant temperature × limonene interaction was found in chlorophyll to carotenoid ratio in cv Splendid (Table 4), and in chlorophyll a, chlorophyll b, and total pigments in cv Parano (Table 5). In cv Splendid, the negative effect of HT on pigments was strengthened by LS, while in cv Parano, LS counteracted the negative effects of HT on chlorophyll (Tables 4 and 5, respectively).

3.6. Content of total phenolics in carrot leaves

There was no significant effect of HT or LS nor was there any interaction between them on total phenolic concentration in leaves. The total phenolic content in the blades of the two cultivars (cv Parano and Splendid) was significantly higher than that of petioles ($P = 0.026$ and 0.007, respectively). Cv Splendid exhibited a significantly ($P < 0.001$ and $P = 0.008$ for blades and petioles, respectively) higher phenolic content than cv Parano.

3.7. Biomass

In cv Parano, significant temperature and limonene main effects were found in the roots where their

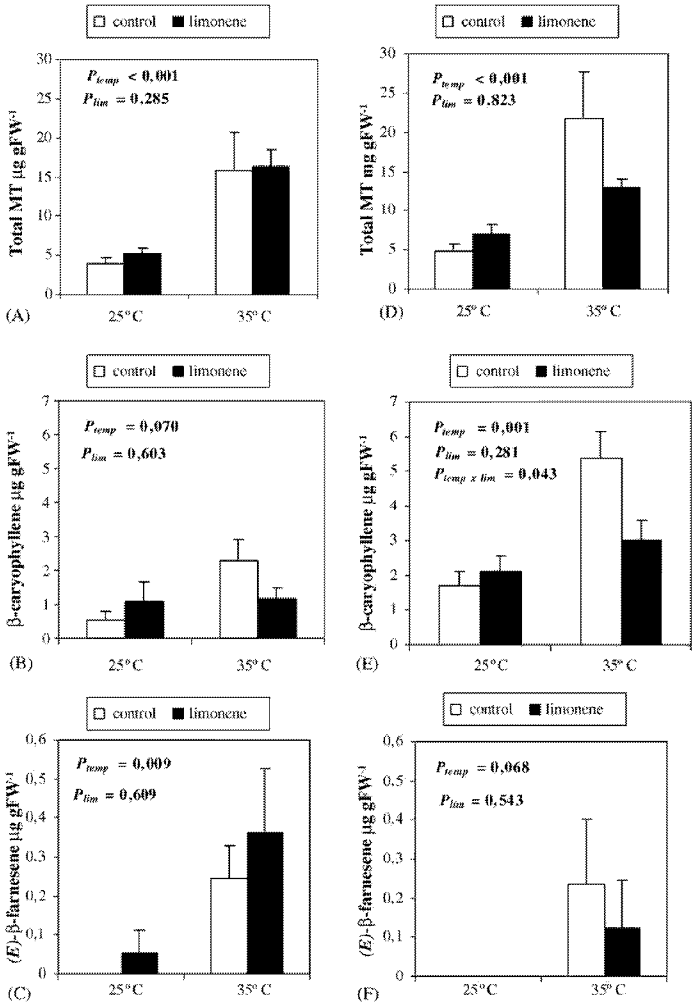


Fig. 3. Concentration (µg gFW^{-1}) of total monoterpenes (α-pinene, camphene, sabinene, β-pinene, β-myrcene, limonene, ocimene, terpinolene, bornyl acetate), β-caryophyllene and (*E*)-β-farnesene (mean + S.E.) in the roots ($n = 5$) of two carrot cultivars Parano (A–C) and Splendid (D and E) grown at control (25 °C) or elevated (35 °C) temperature or with or without limonene treatments. Bars show standard errors.

Table 4
Effects of elevated temperature and limonene treatment on total soluble proteins and pigment content of carrot leaves (cv Splendid) ($n = 5$)

	CT	CT + LS	HT	HT + LS	Main effects		Interaction
					Temperature	Limonene	
Splendid (32 days old)							
Total soluble proteins mg/g dry mass	94.71 ± 10.96a	64.87 ± 4.28a	81.58 ± 6.22a	81.52 ± 12.04a	0.115	0.847	0.117
Chlorophyll a mg/g dry mass	7.77 ± 0.12a	5.91 ± 0.24b	6.52 ± 0.24ab	4.37 ± 0.63c	<0.001	0.001	0.690
Chlorophyll b mg/g dry mass	2.71 ± 0.07a	2.09 ± 0.11b	2.28 ± 0.10a	1.57 ± 0.23b	<0.001	0.004	0.719
Carotenoids mg/g dry mass	2.28 ± 0.10a	1.70 ± 0.04b	1.77 ± 0.09b	1.41 ± 0.17b	0.001	0.003	0.319
Total pigments mg/g dry mass	12.76 ± 0.28a	9.70 ± 0.39b	10.57 ± 0.41ab	7.35 ± 1.03bc	<0.001	0.002	0.892
Ratio chlorophyll a/b	2.87 ± 0.04a	2.83 ± 0.04a	2.86 ± 0.03a	2.79 ± 0.03a	0.10	0.440	0.641
Ratio chlorophylls /carotenoids	4.61 ± 0.16ab	4.71 ± 0.11a	4.98 ± 0.15a	4.15 ± 0.10b	0.012	0.470	0.002
Splendid (46 days old)							
Total soluble proteins mg/g dry mass	56.75 ± 7.51a	58.41 ± 5.78a	53.47 ± 12.38a	50.51 ± 6.79a	0.940	0.520	0.790
Chlorophyll a mg/g dry mass	5.27 ± 0.44a	4.97 ± 0.47a	4.72 ± 0.95a	5.22 ± 0.52a	0.879	0.818	0.532
Chlorophyll b mg/g dry mass	1.91 ± 0.16a	1.81 ± 0.22a	1.71 ± 0.33a	2.07 ± 0.20a	0.589	0.917	0.349
Carotenoids mg/g dry mass	1.78 ± 0.10a	1.66 ± 0.15a	1.58 ± 0.21a	1.75 ± 0.13a	0.891	0.730	0.351
Total pigments mg/g dry mass	8.96 ± 0.69a	8.44 ± 0.83a	8.01 ± 1.49a	9.04 ± 0.84a	0.807	0.866	0.456
Ratio chlorophyll a/b	2.76 ± 0.02a	2.75 ± 0.09ab	2.76 ± 0.04a	2.52 ± 0.06ac	0.093	0.049	0.049
Ratio chlorophylls /carotenoids	4.01 ± 0.14a	4.06 ± 0.06a	3.94 ± 0.25a	4.14 ± 0.11a	0.427	0.990	0.639

Multivariate ANOVA followed by Tukey's multiple range test was used for data analyzes. Values are means ± S.E. Means followed by different letters between columns are significantly different, $P < 0.05$.

interaction was significant in the main roots and dry mass of the shoots indicating that the effect of limonene was dependent on temperature. HT induced a significant increase in the dry weight of the fine roots. The shoot dry mass was reduced significantly by HT and by CT + LS. The negative effect of HT on shoot dry mass was ameliorated by simultaneous treatment with limonene (Table 6).

In cv Splendid, a significant temperature main effect was observed on the roots. The growth of the main roots was only affected by HT while shoots were unaffected by any of the treatments. HT induced significant increase in the dry mass of fine roots. The shoot to root ratio was increased by HT and HT + LS in both cultivars (Table 6).

4. Discussion

4.1. Limonene helps carrot fluorescence to recover but not photosynthesis

The increase of day-time temperature from 25 to 35 °C did not significantly reduce carrot photosynthesis. Therefore, no significant compensating effect of exogenous monoterpene treatment was to be expected. However, the slight trend towards better photosynthesis at 35 °C immediately after the first limonene application does suggest that limonene may improve the thermotolerance of carrot photosynthesis, which is consistent with the findings by Loreto et al. (1998) and Delfine et al. (2000). The reduction of stomatal con-

Table 5
Effects of elevated temperature and limonene treatment on total soluble proteins and pigment content of carrot leaves (cv Parano) ($n = 5$)

	CT	CT + LS	HT	HT + LS	Main effects		Interactions
					Temperature	Limonene	
Parano (31 days old)							
Total soluble proteins mg/g dry mass	79.09 ± 4.24a	60.41 ± 3.91b	81.62 ± 3.36a	75.04 ± 3.03ab	0.003	0.032	0.118
Chlorophyll a mg/g dry mass	5.62 ± 0.08a	4.82 ± 0.22a	4.96 ± 0.31a	5.54 ± 0.38a	0.693	0.928	0.021
Chlorophyll b mg/g dry mass	1.81 ± 0.06a	1.68 ± 0.07a	1.52 ± 0.08a	1.92 ± 0.17a	0.201	0.834	0.021
Carotenoids mg/g dry mass	1.56 ± 0.03a	1.45 ± 0.05a	1.53 ± 0.07a	1.57 ± 0.13a	0.699	0.595	0.363
Total pigments mg/g dry mass	8.99 ± 0.16a	7.96 ± 0.34a	8.01 ± 0.46a	9.03 ± 0.67a	0.991	0.921	0.034
Ratio chlorophyll a/b	3.12 ± 0.07ac	2.87 ± 0.02b	3.26 ± 0.07a	2.90 ± 0.07bc	<0.001	0.178	0.404
Ratio chlorophylls /carotenoids	4.76 ± 0.02a	4.48 ± 0.12ab	4.23 ± 0.09b	4.77 ± 0.11a	0.179	0.213	<0.001
Parano (43 days old)							
Total soluble proteins mg/g dry mass	93.27 ± 2.89a	55.13 ± 7.84b	70.30 ± 5.27ab	68.08 ± 12.59ab	0.023	0.540	0.039
Chlorophyll a mg/g dry mass	6.38 ± 0.33a	4.43 ± 0.65b	5.21 ± 0.19ab	5.26 ± 0.61ab	0.066	0.731	0.054
Chlorophyll b mg/g dry mass	2.02 ± 0.15a	1.61 ± 0.26a	1.67 ± 0.06a	1.98 ± 0.21a	0.782	0.957	0.068
Carotenoids mg/g dry mass	1.89 ± 0.16a	1.47 ± 0.14a	1.75 ± 0.04a	1.78 ± 0.16a	0.163	0.530	0.111
Total pigments mg/g dry mass	10.30 ± 0.62a	7.51 ± 1.04a	8.63 ± 0.28a	9.02 ± 0.96a	0.147	0.925	0.061
Ratio chlorophyll a/b	3.17 ± 0.09a	2.78 ± 0.08b	3.12 ± 0.03a	2.64 ± 0.07b	<0.001	0.189	0.552
Ratio chlorophylls /carotenoids	4.49 ± 0.19a	4.04 ± 0.23a	3.92 ± 0.07a	4.03 ± 0.19a	0.353	0.129	0.142

Multivariate ANOVA followed by Tukey's multiple range test. Values are means ± S.E. Means followed by different letters between columns are significantly different, $P < 0.05$.

Table 6
Dry weight (g) of two carrot cultivars (cv Parano and Splendid) ($n = 10$) at HT and limonene treatments

	CT	CT + LS	HT	HT + LS	Main effects		Interaction
					Temperature	Limonene	
Parano							
Shoot	1.99 ± 0.10a	1.19 ± 0.07b	1.13 ± 0.06b	2.13 ± 0.16 a	0.760	0.366	<0.001
Fine root	0.41 ± 0.04b	0.32 ± 0.01b	0.58 ± 0.05a	0.43 ± 0.03ab	0.002	0.008	0.518
Main root	3.21 ± 0.17a	2.08 ± 0.14b	1.14 ± 0.70c	1.11 ± 0.13c	<0.001	<0.001	<0.001
Shoot/root	0.62	0.57	0.99	1.92			
Splendid							
Shoot	1.11 ± 0.18a	1.32 ± 0.26a	1.15 ± 0.14a	1.44 ± 0.14a	0.676	0.185	0.834
Fine root	0.45 ± 0.07b	0.43 ± 0.04b	0.84 ± 0.15a	0.60 ± 0.03ab	0.003	0.154	0.233
Main root	1.96 ± 0.33ab	2.21 ± 0.40a	0.95 ± 0.12b	1.13 ± 1.90ab	0.001	0.899	0.455
Shoot/root	0.57	0.59	1.21	1.27			

Multivariate ANOVA followed by Tukey's multiple range test was used for data analyzes. Values are means ± S.E. Means followed by different letters (between columns) are significantly different, $P \leq 0.05$.

ductance caused by the application of limonene in our study is consistent with the recent study of Rai et al. (2003) showing that the monoterpenes released from *Princepia utilis* L. could reduce the stomatal conductance of *Vicia faba* (Leguminosae) leaves.

The chlorophyll fluorescence of both carrot cultivars declined rapidly due to high temperature during the first and second measurements, irrespective of whether there was concomitant LS or not. The recovery of chlorophyll fluorescence, indicating the efficiency of Photosystem II to capture light energy, however was faster in plants receiving the limonene spray. Thus, our finding agrees with those reported previously (Delfine et al., 2000; Loreto et al., 1998; Sharkey and Singsaas, 1995; Rennenberg and Schnitzler, 2002), i.e. that the isoprenoids including monoterpenes can increase the thermotolerance of plants, particularly those plants emitting isoprenoids such as *Quercus* spp. However, the role of monoterpenes in the thermotolerance of the cultivated crops needs to be studied further since monoterpene spraying aimed to control pest problems may have substantial effects on crop plant physiology.

4.2. High temperature increases monoterpene emission from carrot leaves

As shown also in earlier studies (Senalik and Simoin, 1987; Kainulainen et al., 1998; Habegger and Schnitzler, 2000b; Kainulainen et al., 2002), the leaves of the two carrot cultivars exhibited a different essential oil composition. We found a significant increase in the emissions of α-pinene, camphene, sabinene, β-myrcene and limonene from carrot leaves grown at HT, though the emission of (*Z*)-β-ocimene was not significantly affected by the temperature. Loreto et al. (1998) reported increased emission of α-pinene, β-pinene, sabinene, myrcene and limonene from *Q. ilex* leaves with increasing temperature from 5 to 35 °C, while Staudt and Bertin (1998) found that emission of acyclic monoterpenes ((*Z*)- and (*E*)-β-ocimene) was not sensitive to this range of temperature, probably due to different enzymatic activities. Also several other authors (Loreto et al., 1996; Staudt et al., 2003) have reported increased emission or increased foliar concentrations (Sallas et al., 2003) of monoterpenes due to the elevated temperature. In our study, volatile sampling of plants from all the treatments was conducted at 22 °C. Despite this, the effect of high growing temperature was detectable, indicating that higher emissions were due to change in plant physiological properties and not caused merely by a higher volatility of monoterpenes at higher temperatures. This is in contrast with the observation of Loreto et al. (1996) who found that monoterpene emissions of *Q. ilex* were reduced within 30 min after reduction of the temperature. However, monoterpene-storing species like carrot may have a different response to the rapid temperature change compared to non-storing species.

Limonene treatment increased significantly only the limonene concentration in headspace volatiles of carrot. The detected limonene emission is likely due to the adsorption of the exogenous limonene solution onto the leaves of the carrots. In case of induced foliar synthesis of limonene, also the emission of the other monoterpenes would have increased (Delfine et al., 2000; Loreto et al., 1998; Peñuelas and Llusià, 2002).

4.3. Foliar secondary compounds are affected by high temperature and limonene

Simultaneously with the increased relative proportion of β-myrcene in the leaves, we measured a slightly reduced emission of β-myrcene from both cultivars at HT + LS compared to the emissions at HT alone. We also measured significantly reduced stomatal conductance two hours after the first limonene treatment in the leaves of both cultivars and 24 h after the treatment in the leaves of Parano. The stomatal closure driven by limonene treatment could explain the observed accumulation of β-myrcene in the carrot leaves. Previously, it has been shown that increased concentrations of external monoterpenes inhibit stomatal opening in *V. faba* (Rai et al., 2003). However, it has been reported that the emission rates of monoterpene compounds, which have high Henry's law coefficients (*H*-value), such as limonene or (*E*)-β-ocimene, are not related to stomatal conductance (Niinemets et al., 2002). Based on these results, the emission of β-myrcene should not be related to the stomatal conductance since β-myrcene has a low solubility to water, in other words, a high *H*-value. Thus, the reason for the observed high proportion of β-myrcene at the same time as we noted the lowered emission rate remains unclear. The proportion of phenylpropanoid, methyl isoeugenol, decreased at the elevated temperature and with limonene treatment in the leaves of both cultivars, which may be reflecting a change in plant carbon allocation towards the isoprenoid pathway at the expense of other metabolic pathways, although we did not observe any change in total phenolics.

4.4. High temperature increases levels of terpenoids in the roots

We found α-pinene, sabinene, β-pinene, β-myrcene, limonene, total monotepenes and (*E*)-β-farnesene had been induced in the roots of cv Parano and α-pinene, camphene, β-myrcene, limonene, total monotepenes and β-caryophyllene had been induced in the roots of Splendid, after HT treatment. (*E*)-β-farnesene was not detected in the roots of control plants indicating that the production of this compound is inducible by abiotic factors. Our findings were similar to the results of Rosenfeld et al. (2002) who have shown that the elevated growing temperature can increase the terpenoid content in the roots of carrot cv Panther. This is also in accordance with Habegger and Schnitzler (2000a) who have reported differences in the composition of essential oil in the roots of different carrot cultivars and changes in the composition of the essential oil content during plant growth. Significant temperature x limonene interaction was found in β-caryophyllene concentration in the roots of cv Splendid. This may indicate that limonene treatment at HT could affect the taste of the carrots.

It is known that volatile terpenes (α-terpinene, β-myrcene, β-caryophyllene, farnesene and α-humulene) are the compounds which are responsible for the bitter taste as demonstrated previously Rosenfeld et al. (2002). However, recently Czepa and Hofmann (2003) showed that polyacetylenes such as falcarinol and falcarindiol-3-acetate are the most important bitter taste indicators in carrots. β-caryophyllene was found to be responsible for terpene-like, spicy, woody aroma in carrots (Kjeldsen et al., 2003).

4.5. Foliar phenolics are not affected

Leaf phenolics of carrot did not show any significant response to elevated temperature. This is in agreement with the results of Holopainen and Kainulainen (2004) who found that a temperature increase from 2 to 8 °C did not have any significant effect on the concentration of needle phenolics of Scots pine. A 3–4 °C increase of growing temperature has resulted in somewhat conflicting results i.e. increase (Kuokkanen et al., 2003), no effect (Jones and Hartley, 1998, 1999; Sallas et al., 2003) or decrease (Veteli et al., 2002) of total foliar phenolics depending on the studied plant species. However, our results are in agreement with the protein competition model of phenolic allocation (Jones and Hartley, 1998, 1999), which predicts that there should be no effects on phenolic concentrations in most plant species in response to increasing temperature.

Our results revealed a significantly higher phenolic content in blades than in petioles, which to our knowledge have not been earlier reported. The foliar phenolic content was not affected by exogenous application of limonene, but exogenous methyl jasmonate (Francesch et al., 2002) or ethylene application (Tscharntke et al., 2001) has affected total phenolics in foliage of woody plants.

4.6. Differences between the cultivars in response to limonene

Growth analysis showed that cv Parano was more susceptible to high temperature stress than cv Splendid, and that the positive effect of limonene by diminishing the effects of heat stress was evident particularly in shoot mass and in pigment contents of cv Parano. This may be due to the fact that exogenous limonene can help cv Parano to overcome the effect of the heat stress since the leaves of cv Parano have lower endogenous concentrations of limonene compared with leaves of cv Splendid (Kainulainen et al., 2002). In cv Splendid, the effects of HT were seen as a decline in all pigment concentrations, whereas in cv Parano the chlorophyll a to b ratio and the amounts of total soluble proteins were increased, suggesting that these two cultivars differ in their sensitivity to HT.

The impact of limonene application on terpene content in the roots varied among the cultivars, and this was in accordance with earlier findings (Senalik and Simoin, 1987; Habegger and Schnitzler, 2000a) as LS at HT induced (*E*)-β-farnesene in the roots of both cultivars, but the effect of LS on the induction of this sesquiterpene was variable between the cultivars; an increasing concentration was found in cv Parano and a decreasing concentration in cv Splendid. Temperature induced a trade-off between root growth and the root concentration of terpenes in both varieties.

The negative effect of HT on the growth of main root, however, was not compensated by limonene spraying. Therefore, our results suggest that limonene application could not be able to improve the yield of the most heat-sensitive carrot cultivars.

In conclusion, our results showed that stomatal conductance of both carrot cultivars is affected by limonene treatment which may indicate that limonene treatment could improve the ability of carrots to tolerate the environmental stresses such as heat stress by inhibiting the stomatal opening. There was no clear evidence that limonene treatment was able to change the emission of VOCs from the carrot leaves. The increased emission of limonene could be due to the treatment and not to induction of the terpene. However, the reduced proportion of methyl isoeugenol may indicate a change in carbon allocation from phenolic pathways towards isoprenoid pathways. The dry mass of main root in both cultivars was significantly reduced by HT and the total monoterpenes in the roots significantly increased in response to HT which indicates that thermal stress creates metabolic costs for the carrots. On the other hand, LS at HT reduced the root concentrations of inducible sesquiterpenes and this may be due to a reduced thermal stress effect achieved by exogenous limonene treatment. However, root biomass analysis showed that exogenous application of limonene in aqueous solution did not provide sufficient protection against thermal stress. Limonene spraying influenced some physiological and chemical responses in the carrot, and these may be able to promote carrot resistance against biotic organisms. Therefore, before progressing to field applications of limonene and other monoterpene sprays for pest control purpose, the responses of major crop plants to limonene should be more thoroughly examined.

Acknowledgements

We thank Dr. Irene Vänninen, MTT Jokioinen, Finland, for her comments on the manuscript, Timo Oksanen for programming the climate chambers and Jaana Rissanen for assistance in the laboratory work. This work was financially supported by Jenny & Antti Wihuri Foundation, Finnish Cultural Foundation, and the Ministry of Agriculture and Forestry in Finland, Project number 310061. The language text was revised by Ewen Macdonald.

References

Alasalvar, C., Grigor, J.M., Quantic, P.C., 1999. Method for the static headspace analysis of carrot volatiles. Food Chem. 65, 391–397.

Caissard, J.C., Meekijjironenroj, A., Baudino, S., Anstett, M.C., 2004. Localization of production and emission of pollinator attractant on whole leaves of *Chamaerops humilis* (Arecaceae). Am. J. Bot. 91, 1190–1199.

Czepa, A., Hofmann, T., 2003. Structural and sensory characterization of compounds contributing to the bitter off-taste of carrots (*Daucus carota* L.) and carrot puree. Agric. Food Chem. 51, 3865–3873.

Degen, T., Buser, H.-R., Städler, E., 1999. Patterns of oviposition stimulants for carrot fly in leaves of various host plants. J. Chem. Ecol. 25, 67–87.

Delfine, S., Csiky, O., Seufert, G., Loreto, F., 2000. Fumigation with exogenous monoterpene of a non-isoprenoid- emitting oak (*Quercus suber*): monoterpene acquisition, translocation, and effect on the photosynthetic properties at high temperatures. New Phytol. 146, 27–36.

Dicke, M., Van Poecke, R.M.P., De Boer, J.G., 2003. Inducible indirect defence of plants: from mechanisms to ecological functions. Basic Appl. Ecol. 4, 27–42.

Franceschi, V.R., Krekhing, T., Christiansun, E., 2002. Application of methyljasmonate on *Picea abies* (Pinaceae) stems induces defense-related responses in phloem and xylem. Am. J. Bot. 89, 578–586.

Gershenzon, J., Croteau, R., 1993. Terpenoid biosynthesis: the basic pathway and formation of monoterpenes, sesquiterpenes, and diterpenes. In: Moore, T. (Ed.), Lipid Metabolism in Plants. CRC Press, Boca Raton, FL, pp. 333–338.

Guenther, A., Hewitt, N.C., Erickson, D., Fall, R., Geron, C., Graedel, T., Harley, P., Klinger, L., Lerdau, M., McKay, W.A., Pierce, T., Scholes, B., Steinbrecher, R., Tallamraju, R., Taylor, J., Zimmermann, P.A., 1995. Global model of natural volatile organic compound emissions. J. Geophys. Res. 100, 8873–8892.

Habegger, R., Schnitzler, W.H., 2000a. Aroma compounds in the essential oil of carrots (*Daucus carota* L. ssp. *sativus*). 1. Leaves in comparison with roots. J. Appl. Bot. 74, 220–223.

Habegger, R., Schnitzler, W.H., 2000b. Aroma compounds in the essential oil of carrot (*Daucus carota* L. ssp. *sativus*). 2. Intraleaf distribution. J. Appl. Bot. 74, 229–232.

Harborne, J.B., 1997. Plant secondary metabolism. In: Crawley, M.J. (Ed.), Plant Ecology, second ed. Blackwell Science, Oxford, UK, pp. 132–155.

Heil, M., 2004. Direct defense or ecological costs: responses of herbivorous beetles to volatiles released by wild lima bean (*Phaseolus lunatus*). J. Chem. Ecol. 30, 1289–1295.

Holopainen, J.K., Kainulainen, P., 2004. Reproductive capacity of the grey pine aphid and allocation response of Scots pine seedlings across temperature gradients: a test of hypotheses predicting outcomes of global warming. Can. J. Forest Res. 34, 94–102.

Holopainen, J.K., 2004. Multiple functions of inducible plant volatiles. Trends Plant Sci. 9, 529–533.

Holopainen, T., Kärenlampi, L., 1984. Injuries to lichen ultrastructure caused by sulphur dioxide fumigations. New Phytol. 98, 285–294.

Hummelbrunner, L.A., Isman, M.B., 2001. Acute, sublethal, antifeedant, and synergistic effects of monoterpenoid essential oil compounds on the tobacco cutworm, *Spodoptera litura* (Lep., Noctuidae). J. Food Chem. 49, 715–720.

Ibrahim, M.A., Kainulainen, P., Aflatuni, A., Tiilikkala, K., Holopainen, J.K., 2001. Insecticidal, repellent, antimicrobial activity and phytotoxicity of essential oils: With special reference to limonene and its suitability for control of insect pests. Agr. Sci. Finland 10, 243–259.

Ibrahim, M.A., Oksanen, E.J., Holopainen, J.K., 2004. Effects of limonene on growth and physiology of cabbage and carrot plants. J. Sci. Food Agric. 84, 1319–1326.

Jasicka-Misiak, I., Lipok, K., Kafarski, P., 2002. Phytotoxic activity of the carrot (*Daucus carota* L.) seed oil and its major components. J. Ess. Oil Bear. Plants 5, 132–134.

Jones, C.G., Hartley, S.E., 1999. A protein competition model of phenolic allocation. Oikos 86, 27–44.

Jones, C.G., Hartley, S.E., 1998. Global change and plant phenolic concentrations: species level predictions using the protein competition model. In: De Kok, L.J., Stulen, I. (Eds.), Responses of Plant Metabolism to Air Pollution and Global Change. Backhuys, Leiden, The Netherlands, pp. 23–50.

Joulain, D., König, W.A., 1998. The atlas of spectral data of sesquiterpene hydrocarbons. E.B.-Verlag, Hamburg.

Julkunen-Tiitto, R., 1985. Phenolics constituents in the leaves of northern willows: methods for the analysis of certain phenolics. J. Agr. Food Chem. 33, 213–217.

Kainulainen, P., Nissinen, A., Piirainen, A., Tiilikkala, K., Holopainen, J.K., 2002. Essential oil composition in leaves of carrot varieties and preference of specialist and generalist sucking insect herbivores. Agric. Forest Ent. 4, 211–216.

Kainulainen, P., Tarhanen, J., Tiilikkala, K., Holopainen, J.K., 1998. Foliar and emission composition of essential oil in two carrot varieties. J. Agric. Food Chem. 46, 3780–3784.

Kjeldsen, F., Christensen, L.P., Edelenbos, M., 2001. Quantitative analysis of aroma compounds in carrot (*Daucus carota* L.) cultivars by capillary gas chromatography using large volume injection technique. J. Agric. Food Chem. 49, 4342–4348.

Kjeldsen, F., Christensen, L.P., Edelenbos, M., 2003. Changes in volatile compounds of carrot (*Daucus carota* L.) during refrigerated and frozen storage. J. Agric. Food Chem. 51, 5400–5407.

Kuokkanen, K., Yan, S.C., Niemelä, P., 2003. Effects of elevated CO_2 and temperature on the leaf chemistry of birch *Betula pendula* (Roth) and the feeding behaviour of the weevil *Phyllobius maculicornis*. Agric. Forest Ent. 5, 209–217.

Lavola, A., 1998. Phytochemicals of deciduous trees in relation to environmental changes. Univ. Joensuu Publ. Sci. 46, 14.

Logan, B.A., Monson, R.K., 1999. Thermotolerance of leaf discs from four isoprene emitting species is not enhanced by exposure to exogenous isoprene. Plant Physiol. 120, 821–825.

Loreto, F., Pinelli, P., Manse, F., Kollist, H., 2004. Impact of ozone on monoterpene emissions and evidence for an isoprene-like antioxidant action of monoterpenes emitted by *Quercus ilex* leaves. Tree Physiol. 24, 361–367.

Loreto, F., Coccioli, P., Cecinato, A., Brancaleoni, E., Frattoni, M., Tricoli, D., 1996. Influence of environmental factors and air composition in the emission of α-pinene from *Quercus ilex* leaves. Plant Physiol. 110, 267–275.

Loreto, F., Förster, A., Dürr, M., Csiky, O., Seufert, G., 1998. On the monoterpene emission under heat stress and on the increased thermotolerance of *Quercus ilex* L. fumigated with selected monoterpenes. Plant Cell Environ. 21, 101–107.

Nehlin, G., Valterova, I., Borg-Karlson, A., 1994. Use of conifer volatiles to reduce injury caused by carrot psyllid, *Trioza apicalis*, Förster (Homoptera, psylloidae). J. Chem. Ecol. 20, 771–783.

Niinemets, Ü., Reichstein, M., Staudt, M., Seufert, G., Tenhunen, J.D., 2002. Stomatal constraints may affect emission of oxygenated monoterpenoids from the foliage of *Pinus pinea*. Plant Physiol. 130, 1371–1385.

Peñuelas, J., Llusià, J., 2002. Linking photorespiration, monoterpenes and thermotolerance in *Quercus*. New Phytol. 155, 227–237.

Olsson, K., Svensson, R., 1996. The influence of polyacetylenes on the susceptibility of carrots to storage diseases. J. Phytopathol. 144, 441–447.

Porra, R.J., Thompson, W.A., Kriedemann, P.E., 1989. Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochim. Biophys. Acta 975, 384–394.

Rai, V.K., Gupta, S.C., Singh, B., 2003. Volatile monoterpenes from *Prinsepia ulitis* L. leaves inhibit stomatal opening in *Vicia faba* L. Biol. Plantarum 46, 121–124.

Rennenberg, H., Schnitzler, J., 2002. VOCs-helping trees withstand global change? New Phytol. 155, 197–203.

Rosenfeld, H.J., Aaby, K., Lea, P., 2002. Influence of temperature and plant density on sensory quality and volatile terpenoids of carrot (*Daucus carota* L.) root. J. Sci. Food Agric. 82, 1384–1390.

Sallas, L., Luomala, E.-M., Utriainen, J., Kainulainen, P., Holopainen, J.K., 2003. Contrasting effects of elevated carbon dioxide concentration and temperature on Rubisco activity, chlorophyll fluorescence, needle ultrastructure and secondary metabolites in conifer seedlings. Tree Physiol. 23, 97–108.

Senalik, D., Simoin, P.W., 1987. Quantifying intra plant variation of volatile terpenes in carrot. Phytochemistry 26, 1975–1979.

Sharkey, T.D., Singsaas, E.L., 1995. Why plants emit isoperene. Nature 374, 769.

Sharkey, T.D., Loreto, F., 1993. Water stress, temperature, and light effects on the capacity for isoperene emission and photosynthesis of kudzu leaves. Oecologia 95, 328–333.

Singsaas, E.L., Lerdau, M., Winter, K., Sharkey, T.D., 1997. Isoprene increases thermotolerance of isoprene emitting species. Plant Physiol. 115, 1413–1420.

Singsaas, E.L., Sharkey, T.D., 1998. The regulation of isoprene emission responses to rapid leaf temperature fluctuations. Plant Cell Environ. 21, 1181–1188.

Staudt, M., Bertin, N., 1998. Light and temperature dependence of the emission of cyclic and acyclic monoterpenes from holm oak (*Quercus ilex* L.) leaves. Plant Cell Environ. 21, 385–395.

Staudt, M., Joffre, R., Rambal, S., 2003. How growth condition affect the capacity of *Quercus ilex* leaves to emit monoterpenes. New Phytol. 153, 61–73.

Tscharntke, T., Thiessen, S., Dolch, R., Boland, W., 2001. Herbivory induced resistance and interplant signal transfer in *Alnus glutinosa*. Biochem. Syst. Ecol. 29, 1025–1047.

Van Poecke, R.M.P., Dicke, M., 2004. Indirect defence of plants against herbivores: using *Arabidopsis thaliana* as a model plant. Plant Biol. 6, 387–401.

Veteli, T.O., Kuokkanen, K., Julkunen-Tiitto, R., Roininen, H., Tahvanainen, J., 2002. Effects of elevated CO_2 and temperature on plant growth and herbivore defensive chemistry. Glob. Change Biol. 8, 1240–1252.

Vuorinen, T., Reddy, G.V.P., Nerg, A.-M., Holopainen, J.K., 2004. Monoterpene and herbivore-induced emissions from cabbage plants grown at elevated atmospheric CO_2 concentration. Atmos. Environ. 38, 675–682.

Wheeler, D.A., Isman, M.B., 2001. Antifeedant and toxic activity of *Trichilia americana* extract against the larvae of *Spodoptera litura*. Entomol. Exp. Appl. 98, 9–16.

Wellburn, A.R., Lichtenthaler, H., 1984. Formulae and program to determine total carotenoids and chlorophylls a and b of leaf extracts in different solvents. In: Sybesma, C. (Ed.), Advances in Photosynthesis Research. Hague/Bosten/Lancaster, Netherlands, pp. 9–12.

Chapter 7

General Discussion

GENERAL DISCUSSION

7.1 The phytotoxic influence of prophylactic limonene sprayings on cabbage and carrot growth and physiology

In this work, concentrations of limonene higher than 60 ml l^{-1} exhibited phytotoxicity in cabbage and carrot. Some physiological processes such as photochemical efficiency of photosystem II and the stomatal conductance were disturbed although this was species- and cultivar-specific as described in Chapter 2 (see also section 4.1, Chapter 6). Carrot appeared to be more tolerant than cabbage due to the higher endogenous concentration of limonene in carrot leaves compared to cabbage leaves. The phytotoxicity of monoterpenes on plants has been extensively investigated but rather few researchers (such as Tellez *et al.* 2000, Kobaisy *et al.* 2001, Argandona *et al.* 2002, Singh *et al.* 2002, Abrahim *et al.* 2003) have studied the phytotoxic effects of essential oils on crop plants. The mechanisms by which monoterpenes act on plants are not yet clear but some recent reports have proposed that monoterpenes could have negative effects on mitochondrial respiration (Abrahim *et al.* 2003, Weir *et al.* 2004), cell division, photosynthesis (Romagni *et al.* 2000a), stomatal conductance (Rai *et al.* 2003), and evoke inhibition of the asparagine synthetase thereby disturbing amino acid metabolism (Romagni *et al.* 2000b). In general, hydrocarbon monoterpenes are less phytotoxic than the oxygenated monoterpenes (Vokou *et al.* 2003) but their activities individually or in combination against crop pests should be studied in parallel with their phytotoxicity.

7.2 Effects of monoterpenes on insect herbivores and natural enemies

In this study, the exogenous application of limonene on cabbage plants attracted both an insect herbivore (DBM) and its parasitoid *Cotesia plutellae.* Conversely, DBM and *C. plutellae* were repelled by the application of limonene combined with carvone in a cage and in a Y-tube olfactometer. Similarly, limonene alone and in a mixture with carvone (75:25%) have been found to have a deterrent effect on *Galerucella sagittariae* (Coleoptera: Chrysomelidae) larval feeding on strawberry (cv Jonsok) 24 hrs after spraying while no effect was observed on cv Honeoye (Chapter 2). This suggests that the mixture of limonene with carvone increases the efficiency of limonene. Limonene sprayed on carrot (cv Splendid) plants exhibited some stimulant activity to carrot psyllid (*Trioza apicalis*) females, whereas no effect was observed when it was sprayed on carrot cv Parano. This could be explained by the fact that the exogenous application of limonene on cv Splendid plants (which have a higher endogenous concentration of limonene than cv Parano) may induce the emission of other plant volatiles which consequently may take role in the attraction of carrot psyllid. These findings are in contrast with the results which have been previously reported by Nehlin *et al.* (1994), Valterova *et al.* (1997) and Kainulainen *et al.* (2002) showing that limonene is a repellent to the carrot psyllid. Although the effects of essential oils (e.g. limonene) on insects, especially on household and stored product insects have been under investigation for decades, research is still needed to reveal the action of mechanisms (herbicidal, fungicidal, bactericidal, insecticidal, repellence or deterrence) of these plant secondary compounds against crop pests. In addition to the cultivar-specific responses reported herein, the actions of individual monoterpenes seem to be also species-specific (Miller and Borden, 2000, Jasicka-Misiak 2002, Abrahim *et al.* 2003). Therefore, more laboratory and field studies into the effects of plant essential oils on plant-insect interactions are the only way to find the proper and safe way to use these compounds as alternatives for synthetic pesticides.

The total monoterpene emission from limonene treated cv Rinda plants was significantly increased (Chapter 4) suggesting that cabbage (cv Rinda) treated with limonene emits herbivore inducible compounds. This is supported by Vuorinen *et al.* (2004a), who found similar results with this cultivar after 48h feeding damage by DBM larvae. Limonene and methyl jasmonate (MeJA) treatment induced the concentration of (Z)-β-ocimene in the leaves of carrot cultivars (cv Parano and Splendid), while the herbivore feeding increased the concentration and the emission of this compound only in cv Parano (Chapter 5). In addition, limonene induced the emission of sesquiterpenes β-carophyllene + (*E*)-β-farnesene in cv Splendid at control temperature (25°C) (Chapter 6). Similarly MeJA treatment has been found to induce the emission of the sesquiterpene *E*,*E*-α-farnesene and the homoterpene DMNT from cabbage plants (Chapter 4). These compounds are known to be inducible compounds, attracting herbivores and their natural enemies (Dicke *et al.* 1999; Rodriguez *et al.* 2001; Vuorinen *et al.* 2004a,b). Moreover, limonene has been shown to be attractant to the DBM parasitoid *C. plutellae* in a Y-tube olfactometer set up (Chapter 4). This is in agreement with the previous findings that the plants have been shown to employ an indirect defence mechanism against herbivores by attracting the natural enemies of insect herbivores (De Moraes 2001, Vuorinen *et al.* 2004a,). It has been found that volicitin (N-(17-hydroxylinolenoyl)-L-glutamine), a compound identified in oral secretions of beet armyworm (*Spodoptera exigua*), stimulates volatile emission from corn (*Zea mays* L.) seedlings in a manner similar to the herbivory (Schmelz *et al.* 2001). Research on these indirect mechanisms have led to the intensification of efforts to find safe, effective and alternative natural products to synthetic pesticides (Hummelbrunner and Isman 2001, Frank *et al.* 2002, Singh *et al.* 2002, Isman 2002, Miyazawa *et al.* 2004, Tapondjou *et al.* 2005).

7.3 Ecological aspects of plant chemical defence

Plants are able to respond towards herbivore attack by direct and indirect defensive mechanisms. The direct defensive mechanisms have negative effect on the behaviour of the herbivore while the indirect defence affects the interaction between herbivore and its natural enemies. This latter mechanism is detrimental to the herbivore since it causes the arrival of carnivorous arthropods (Dicke and Hilker 2003) and this is likely to result in a plant fitness benefit i.e. the removal of the herbivore by the predator (Dicke and Van Loon 2000). It has been reported that these mechanisms involve in the induction of plant defensive traits when the plant is under attack by single or more attackers (Karban and Baldwin 1997, Agrawal *et al.* 1999). In the natural environment, plants are simultaneously attacked by number of herbivore species although many of the previous studies have been conducted in plant-insect interaction system with a plant infested by one single herbivore (Shiojiri *et al.* 2001). Infestation by both herbivore and carnivore species on the same plant may affect the production or function of prey-induced infochemicals in tritrophic interactions involving plants, herbivores, and parasitoids. If this is the case, the presence of non-prey herbivore species would either positively or negatively affect plant-parasitoid interactions mediated by infochemicals induced by the prey herbivores (Shiojiri *et al.* 2001). The literature contains many examples (Dicke *et al.* 1999, Shiojiri *et al.* 2001, De moraes 2001, Habollah *et al.* 2004, Vuorinen *et al.* 2004a, Heil 2004, Rasmann *et al.* 2005) about the ecological role of plant volatiles in plant-insect interactions. Similarly, our study pointed a role of limonene and MeJA in this interaction system (Chapter 4, 5). The recent publication by Rasmann *et al.* (2005) revealed the role of induced plant signals (volatiles) also in the belowground environment, where entomopathogenic nematode (*Heterorhabditis megidis*) of the Western corn root worm (WCR) is attracted by the insect-induced signal, (*E*)- β-carophyllene. This should lead to new studies on the ecological consequences of multitrophic interactions and the use of these kinds of belowground signals in crop protection (Rasmann *et al.* 2005).

Costs which are incurred by the direct plant defence can be categorized as allocation costs, resource-based tradeoffs between resistance and fitness, or as ecological costs, decreases in fitness resulting from interactions with other species (Strauss *et al.* 2002). These various costs can be derived directly from the plant resistance trait itself (such as auto-toxicity, allocation costs) or indirectly from plant-insect interactions (Karban and Baldwin 1997). Obviously, the resources available for growth and reproduction of the plant are reduced if the plant has to divert investment for defence (Strauss *et al.* 2002). However, type of defensive trait and the environmental conditions are the two factors which regulate the exact form of the plant defence cost against pathogens and herbivores and its transmission into an evolutionary fitness cost (Heil 2001). Recently, it was reported that as long as natural enemies are present in the environment, the cost of production of herbivore-induced signals is counterbalanced (Habollah *et al.* 2004). Finally, a better understanding of the evolutionary and ecological constraints will create the foundation so that these induced responses can be exploited in agricultural plant protection (Heil 2001).

7.4 Possible beneficial side effects of the exogenous use of limonene by increasing the thermotolerance of plant species

According to this work, limonene sprayings on carrot plants did not exhibit a clear protection of plants from thermal stress, although the stomatal conductance of both carrot cultivars was affected by limonene treatment. Previously, Delfine *et al.* (2000) proposed that exogenous monoterpene treatment could improve the ability of plants to tolerate environmental stresses such as heat stress by concurrent stomatal closure increasing internal concentration of monoterpenes. One of the terpenoid functions which has been proposed by Sharkey and Singsaas (1995), Singsaas *et al.* (1997), Loreto *et al.* (1998), Delfine *et al.* (2000), Rennerberg and Schnitzler (2002), and Peñuelas and Llusià (2002) is that isoprenoids (including monoterpenes) might have beneficial side effects to the plants by increasing the thermotolerance of isoprene emitting plant species, such as oak (*Quercus ilex*). However, individual monoterpenes such as limonene or limonene with α-pinene have been found to be rather inefficient at counteracting the negative effects of heat stresses on *Quercus ilex* (Peńuelas and Llusia 2002, Llusia *et al.* 2005). This is in concurrence with the previous indications of lower efficiency of monoterpenes compared to isoprene on the thermotolerance of plants (Peńuelas and Llusia 2002).

The real mechanism by which monoterpenes increase the thermotolerance is still unclear, but it has been proposed that the isoprenoids, including monoterpenes, increase thermotolerance by stabilizing thylakoid membranes at high temperatures (Sharkey and Singsaas 1995), serving as volatile mediators of bio-membrane fluidity (Sharkey and Yeh 2001), or by preventing membrane lipid denaturation following oxidative stress (Loreto and Velikova 2001, Loreto *et al.* 2001). It has been proposed that isoprenoids may stabilize the interactions between lipid compounds, lipid and proteins, or protein molecules (Singsaas *et al.* 1997). In this study, exogenous application of limonene assisted the recovery of chlorophyll fluorescence of carrot plants at high temperatures (35°C). Photosynthetic rates did not show positive responses to the limonene sprays in contrast to the results with Fv/Fm (Chapter 6). This may suggest that the photosynthetic electron transport chain has been damaged by the heat stress. It is possible that the photosynthetic rates have been affected by the high temperature which damaged the cytochrome complex or caused membrane leakiness as recently suggested by Llusia *et al.* (2005).

The concentration of monoterpenes (α-pinene, β-myrcene, limonene, and total monoterpenes) was increased by the elevated temperature in carrot roots (Chapter 6). Sabinene and β-farnesene induction was found particularly in carrot cv Parano roots while camphene and β-carophyllene were induced by the elevated temperature in the roots of cv Splendid. Accordingly, Rosenfeld *et al.* (2002) reported a similar increase of terpenoids in carrot roots at high temperatures. Furthermore, the identification of the belowground signal, (*E*)-β-carophyllene, a compound that attracts the root feeding herbivores (Rasmann *et al.* 2005) indicates that (*E*)-β-carophyllene is a compound the formation of which is induced by abiotic and biotic factors as indicated in our results in chapter 6 (Ibrahim *et al.* 2005). This plant-mediated belowground interaction may lead to the exploitation of herbivore induced compounds for crop protection (Rasmann *et al.* 2005). In our results (Chapter 6), one interesting finding was that (E)-β-farnesene was not detected in the roots of control plants, indicating that this sesquiterpene is an inducible compound in response to abiotic (Schmelz *et al.* 2001, Rosenfeld *et al.* 2002) and biotic factors (Williams *et al.* 2005). Consistently with the results in this study but in woody plants, monoterpene emissions have been found to be increasing in limonene fumigated *Q. ilex* plants at 50°C whereas the emissions from control plants started to decrease after 45°C (Llusia *et al.* 2005). A positive influence of exogenous limonene was found in carrot cv Parano, in that it diminished the negative effects of heat stress on the biomass of the shoots and pigment content by triggering limonene emission from the leaves at higher temperature more than that in cv Splendid. This may indicate that the exogenous application of limonene conferred slight thermotolerance to the cv Parano because of its lower internal concentration of limonene. Thus, the external application may activate other processes to protect the plants from the heat stress. In contrast, no improvement could be expected in cv Splendid after the external application of limonene due to its higher endogenous limonene biosynthesis rate (Kainulainen *et al.* 2002).

7.5 Methodological considerations

Exogenous PEO application reported in this thesis (Chapters 3, 4, and 5) was used to mimic the real spraying situation for plant protection in the field since our main goal was to evaluate the suitability of the tested compounds for plant protection. The spraying method as water or oil-based as this is the simplest way to apply any tested compound for crop protection in the same manner as used to apply an insecticide (Isman 2002). Previously, other workers have used similar application techniques for compounds of plant-origin for different purposes (Doughty *et al.* 1995, Rodriguez-Saona *et al.* 2001, Thaler *et al.* 2002). In earlier studies into the effects of monoterpenes on thermotolerance (Delfine *et al.* 2000, Peñuelas and Llusià 2002, Loreto *et al.* 1998) of plants, fumigation techniques have been used to simulate the process of vegetation-emitted PEOs and then to examine how other plants are affected. As far as I am aware, very few researchers (Dormont *et al.* 1997, Tiberi *et al.* 1999) have applied spraying method of limonene for the purpose of crop protection although the action of limonene against insects has been described previously (Mukherjee 2003, Bichao *et al.* 2003, Park *et al.* 2003, Lee *et al.* 2001). The use of limonene as slow release formulations has been investigated by Nehlin *et al.* (1994), Peterson *et al.* (1994), Ntiamoah *et al.* (1996), and Ntiamoah and Borden (1996) highlighting the potential crop protection of this monoterpene.

7.6 Conclusions

Natural compounds of plant origin including EOs are believed to have a role in direct and indirect plant defence and research on this interaction has led to intensification of efforts to find safe, effective and alternative natural products to the synthetic pesticides. Therefore, phytotoxicity, thermotolerance effects, the trophic interactions of insect-insect and insect-plant mediated by limonene, and limonene + carvone, and the possible role of limonene in plant protection were studied. The following conclusion can be drawn:

1. At high concentrations (>60ml l^{-1}), limonene is phytotoxic on cabbage and carrot cultivars, but the extent of damage is species- and cultivar-specific. Therefore, a threshold concentration of phytotoxicity should be determined for each plant species/cultivar before any experiments are conducted on repellence, deterrence or attraction activities of limonene and other monoterpenes to be used in plant protection.
2. On its own, limonene showed an attractant activity to crop pest *Plutella xylostella* and its parasitoid *Cotesia plutellae* which suggests that this monoterpene can possibly be used as an attractant of DBM and its parasitoid on trap crop plants.
3. Limonene sprayed on carrot (cv Splendid) was found to be oviposition stimulant to carrot psyllid females. Therefore, sprayed limonene is not a suitable essential oil for the protection of carrot from carrot psyllid unless it is used on trap crop plants for the attraction of natural enemies.
4. Concentrations of monoterpenes (sabinene, β-pinene, limonene, and longifolene) were elevated by the carrot psyllid feeding but (*Z*)-β-ocimene was found to be the most easily inducible compound in carrot after herbivore feeding, and limonene or MeJA treatment.
5. Limonene in a mixture with the monoterpene carvone (1:1 v/v) showed deterrent activity against DBM females. This suggests that carvone improves the effectiveness of limonene as a deterrent on crop plants. Both compounds are the main constituents of caraway oil (*Carum carvi* L.).
6. Limonene alone and in a mixture with carvone (75:25%) were shown to be deterrent against *Galerucella sagittariae* larval feeding on strawberry (cv Jonsok) 24 hrs after spraying while no effect was observed on cv Honeoye. This

demonstrates that the effect of limonene is cultivar-specific. Furthermore, this suggests that carvone improves the efficiency of limonene.

7. In concentrations which might be suitable for pest control, no significant advantage in the form of increased thermotolerance can be achieved.
8. Methyl jasmonate (MeJA) treatment induced the emission of the sesquiterpene *E*,*E*-α-farnesene and the homoterpene DMNT from cabbage plants. These compounds are known to be inducible compounds attracting herbivores and their natural enemies. The result suggests that MeJA can be used to activate indirect herbivore defence in cabbage crops.

References

Abrahim D, Takahashi L, Kelmer-Bracht AM and Ishii-Iwamoto EL. 2003. Effects of phenolic acids and monoterpenes on the mitochondrial respiration of soybean hypocotyl axes. Allelopathy Journal 11: 21-30.

Agrawal A, Tuzun S and Bent E. 1999. Induced plant defenses against pathogens and herbivores. Biochemistry, Ecology and Agriculture. APS Press, St Paul, MN, USA, 403 pp.

Argandona VH, Rovirosa J, San-Martin A, Riquelme A, Diaz-Marrero AR, Cueto M, Darias J, Santana O, Guadano A and Gonzalez-Coloma A. 2002. Antifeedant effects of marine halogenated monoterpenes. Journal of Agricultural and Food Chemistry 50: 7029-7033.

Bichao H, Borg-Karlson AK, Araujo J and Mustaparta H. 2003. Identification of plant odours activating receptor neurones in the weevil *Pissodes notatus* F. (Coleoptera, Curculionidae). Journal of Comparative Physiology A-Neuroethology Sensory Neural and Behavioral Physiology 189: 203-212.

Delfine, S, Csiky, O, Seufert, G and Loreto F. 2000. Fumigation with exogenous monoterpene of a non-isoprenoid- emitting oak (*Quercus suber*): Monoterpene acquisition, translocation, and effect on the photosynthetic properties at high temperatures. New Phytologist 146, 27-36.

De Moraes CM, Mescher MC and Tumlinson JH. 2001. Caterpillar-induced nocturnal plant volatiles repel conspecific females. Nature 410:577-580.

Dicke M, Gols R, Ludeking D and Posthumus MA. 1999. Jasmonic acid and herbivory differentially induce carnivore-attracting plant volatiles in lima bean plants. Journal of Chemical Ecology 25: 1907-1922.

Dicke M and Hilker M. 2003. Induced plant defences: from molecular biology to evolutionary ecology. Basic and Applied Ecology, 4: 3-14.

Dicke M and Van Loon JJA. 2000. Multitrophic effects of herbivore-induced plant volatiles in an evolutionary context. Entomologia Experimentalis et Applicata 97: 237-249.

Dormont L, Roques A and Malosse C. 1997. Efficiency of spraying mountain pine cones with oleoresin of Swiss stone pine cones to prevent insect attack. Journal of Chemical Ecology 23: 2261-2274.

Doughty KJ, Kiddle GA, Pye BJ, Wallsgrve RM and Pickett JA. 1995. Selective induction of glucosinoleates oilseed rape leaves by methyl jasmonate. Phytochemistry 38: 347-350.

Frank T, Bieri K and Speiser B. 2002. Feeding deterrent effect of carvone, a compound from caraway seeds, on the slug *Arion lusitanicus*. Annals of Applied Biology 141: 93-100.

Habollah ME, Köllner TG, Degenhardt J and Turlings TCJ. 2004. Costs of induced volatile production in Maize. Oikos 105: 168-180.

Heil M. 2001. The ecological concept of costs of induced systematic resistance (ISR). European Journal of Plant Pathology 107: 137-146.

Heil M. 2004. Direct defense or ecological costs: Responses of herbivorous beetles to volatiles released by wild lima bean (*Phaseolus lunatus*). Journal of Chemical Ecology 30: 1289-1295.

Hummelbrunner LA and Isman MB. 2001. Acute, sublethal, antifeedant, and synergistic effects of monoterpenoid essential oil compounds on the tobacco cutworm, *Spodoptera litura* (Lep. Noctuidae). Journal of Agricultural and Food Chemistry 49: 715-720.

Ibrahim MA, Nissinen A, Prozherina N, Oksanen E and Holopainen JK. 2005. The influence of exogenous monoterpene treatment and elevated temperature on growth, physiology, chemical content and headspace volatiles of carrot (*Daucus carota* L.). Environmental and Experimental Botany. In press

Isman MB. 2002. Insect antifeedants. Pesticide Outlook 13: 152-157.

Jasicka-Misiak I., Lipok K and Kafarski P. 2002. Phytotoxic activity of the carrot (*Daucus carota* L.) seed oil and its major components. Journal of Essential Oil Bearing Plants 5: 132-134.

Kainulainen P, Nissinen A, Piirainen A, Tiilikkala K and Holopainen JK. 2002. Essential oil composition in leaves of carrot varieties and preference of specialist and generalist sucking insect herbivores. Agricultural and Forest Entomology 4: 211-216.

Karban R and Baldwin IT. 1997. Induced responses to herbivorey. Chicago University Press, Chicago.

Kobaisy M, Tellez MR, Webber CL, Dayan FE, Schrader KK and Wedge DE. 2001. Phytotoxic and fungitoxic activities of essential oil of kenaf (*Hibiscus cannabinus* L.) leaves and its composition. Journal of Agricultural and Food Chemistry 49: 3768-3771.

Lee BH, Choi WS, Lee SE and Park BS. 2001. Fumigant toxicity of essential oils and their constituent compounds towards the rice weevil, *Sitophilus oryzae* (L.) Crop Protection 20: 317-320.

Loreto F, Förster A, Dürr M, Csiky O and Seufert G. 1998. On the monoterpene emission under heat stress and on the increased thermotolerance of *Quercus ilex* L. fumigated with selected monoterpenes. Plant, Cell and Environment 21, 101-107.

Loreto F and Velikova V. 2001. Isoprene produced by leaves protects the photosynthetic apparatus against ozone damage, quenches ozone products, and reduces lipid peroxidation of cellular membranes. Plant Physiology 127: 1781-1787.

Loreto F, Mannozzi M, Maris C, Nascetti P, Ferranti F and Pasqualini S. 2001. Ozone quenching properties of isoprene and its antioxidant role in leaves. Plant Physiology 126: 993-1000.

Llusiă J, Peňuelas J, Asensio D and Munnè-Bosch S. 2005. Airborne limonene confers limited thermotolerance to *Quercus ilex*. Physiologia Plantarum 123: 40-48.

Miller DR and Borden JH. 2000. Dose-dependent and species-specific responses of pine bark beetles (Coleoptera: Scolytidae) to monoterpenes in association with pheromones. Canadian Entomologist 132: 183-195.

Miyazawa M, Tsukamoto T, Anzai J and Ishikawa Y. 2004. Insecticidal effect of phthalides and Furanocoumarins from Angelica acutiloba against Drosophila melanogaster. Journal of Agricultural and Food Chemistry, 52:4401-4405.

Mukherjee S. 2003. Influence of plant allelochemicals on growth rate, nutritional physiology and mid-gut esterase activity in fifth instar larvae of *Spodoptera litura* (F.) (Lepidoptera: Noctuidae). Invertebrate Reproduction & Development 43: 125-132.

Nehlin G, Valterova I and Borg-Karlsson AK. 1994. Use of conifer volatiles to reduce injury caused by carrot psyllid, *Trioza apicalis*, Förster (Homoptera, Psylloidea). Journal of Chemical Ecology 20: 771-783.

Ntiamoah YA, Borden JH and Pierce HD Jr. 1996. Identity and bioactivity of oviposition deterrents in pine oil for the onion maggot, *Delia antiqua*. Entomologia Experimentalis et Applicata 79: 219-226.

Ntiamoah YA and Borden JH. 1996. Monoterpene oviposition deterrents for cabbage maggots, *Delia radicum* (L.) (Diptera:Anthomyiidae). Canadian Entomologist 128: 351-352.

Park IK, Lee SG, Choi DH, Park JD and Ahn YJ. 2003. Insecticidal activities of constituents identified in the essential oil from leaves of *Chamaecyparis obtusa* against *Callosobruchus chinensis* (L.) and *Sitophilus oryzae* (L.). Journal of Stored Products Research 39: 375-384.

Peñuelas J and Llusià J. 2002. Linking photorespiration, monoterpenes and thermotolerance in *Quercus*. New Phytologist 155: 227-237.

Peterson JK, Horvat RJ and Elsey, KD. 1994. Squash leaf glandular trichome Volatiles: Identification and influence on behavior of female pickleworm moth (*Diaphnia nitidalis* Stoll.) (Lepidoptera: Pyralidae). Journal of Chemical Ecology 20: 2099-2109.

Rai VK, Gupta SC and Singh B. 2003. Volatile monoterpenes from *Prinsepia ulitis* L. leaves inhibit stomatal opening in *Vicia faba* L. Biologia Plantarum 46: 121-124.

Rasmann S, Köllner T, Degenhardt J, Hiltpold I, Toepfer S, Kuhlmann U, Gershenzon J and Turlings TCJ. 2005. Recruitment of entomopathogenic nematodes by insect-damaged maize roots. Nature 732-737.

Rennenberg H and Schnitzler J. 2002. VOCs-helping trees withstand global change?. New Phytologist 155: 197-203.

Rodriguez SC, Crafts BSJ, Pare PW and Henneberry TJ. 2001. Exogenous methyl jasmonate induces volatile emissions in cotton plants. Journal of Chemical Ecology 27: 679-695.

Romagni JG, Allen SN and Dayan FE. 2000a. Allelopathic effects of volatile cineoles on two weedy plant species. Journal of Chemical Ecology 26: 303-313.

Romagni JG, Duke SO and Dayan FE. 2000b. Inhibition of plant asparagine synthetase by monoterpene cineoles. Plant Physiology 123: 725-732.

Rosenfeld HJ, Aaby K and Lea P. 2002. Influence of temperature and plant density on sensory quality and volatile terpenoids of carrot (*Daucus carota* L.) root. Journal of the Science of Food and Agriculture. 82: 1384-1390.

Schmelz EA, Alborn HT and Tumlinson JH. 2001. The influence of intact-plant and excised-leaf bioassay designs on volicitin- and jasmonic acid-induced sesquiterpene volatile release in *Zea mays*. Planta 214: 171-179.

Sharkey and Singsaas, 1995. Why plants emit isoprene. Nature 374:769.

Sharkey TD and Yeh S. 2001. Isoprene emission from plants. Annual Review of Plant Physiology and Plant Molecular Biology 52: 407-436.

Shiojiri K, Takabayashi J and Yano S. 2001. Infochemically mediated tritrophic interaction webs on cabbage plants. Population Ecology 43: 23-29.

Singsaas EL, Lerdau M, Winter K and Sharkey TD. 1997. Isoprene increases thermotolerance of isoprene-emitting species. Plant Physiology 115: 1413-1420.

Singh HP, Batish DR, Kaur S, Ramezani H and Kohli RK. 2002. Comparative phytotoxicity of four monoterpenes against *Cassia occidentalis* Annals of Applied Biology 141: 111-116.

Strauss SY, Rudgers JA, Lau JA and Irwin RE. 2002. Direct and ecological costs of resistance to herbivory. Trends in Ecology & Evolution 17: 278-285.

Tapondjou AL, Adler C, Fontem DA, Bouda H and Reichmuth C. 2005. Bioactivities of cymol and essential oils of *Cupressus sempervirens* and *Eucalyptus saligna* against *Sitophilus zeamais* Motschulsky and *Tribolium confusum* du Val. Journal of Stored Products Research 41: 91-102 2005.

Tellez MR, Dayan FE, Schrader KK, Wedge DE and Duke SO. 2000. Composition and some biological activities of the essential oil of *Callicarpa Americana* (L.). Journal of Agricultural and Food Chemistry 48: 3008-3012.

Thaler JS, Farag MA, Pare PW and Dicke M. 2002. Jasmonate-deficient plants have reduced direct and indirect defences against herbivores. Ecology Letters 5: 764-774.

Tiberi R, Niccoli A, Curini, M, Epifano F, Marcotullio MC and Rosati O. 1999. The role of the monoterpene composition in Pinus spp. needles, in host selection by the pine processionary caterpillar, *Thaumetopoea pityocampa*. Phytoparasitica 27: 263-272.

Vakou D, Douvli P, Blionis GJ and Halley JM. 2003. Effects of monoterpenoids, acting alone or in pairs, on seed germination and subsequent seedling growth. Journal of Chemical Ecology 29: 2281-2301.

Valterova I, Nehlin G and Borg-Karlson AK.1997. Host plant chemistry and preferences in egg-laying *Trioza apicalis* (Homoptera, Psylloidea) Biochemical Systematics and Ecology 25: 477-491.

Vuorinen T, Nerg A-M, Ibrahim MA, Reddy GV P and Holopainen, JK. 2004a. Emission of *Plutella xylostella*-induced compounds from cabbages grown at elevated CO_2 and orientation behaviour of the natural enemies. Plant Physiology 135: 1984-1992.

Vuorinen T, Reddy G VP, Nerg A-M and Holopainen JK. 2004b. Monoterpene and herbivore-induced emissions from cabbage plants grown at elevated atmospheric CO_2 concentration. Atmospheric Environment 38: 675-682.

Weir TL, Park S-W and Vivanco JM. 2004. Biochemical and physiological mechanisms mediated by allelochemicals. Current Opinion in Plant Biology 7: 472-479.

Williams L, Rodriguez-Saona, Pare PW and Crafts-Brandner SJ. 2005. The piercing-sucking herbivores *Lygus Hesperus* and *Nezera viridula* induce volatile emissions in plants. Archives of Insect Biochemistry and Physiology 58: 84-96.

Kuopio University Publications C. Natural and Environmental Sciences

C 165. Mäkinen, Milja. Dermal Exposure Assessment of Chemicals. An essential part of total exposure assessment at workplaces.
2003. 88 p. Acad. Diss.

C 166. Vauhkonen, Päivi J. Image reconstruction in three-dimensional electrical impedance tomography.
2003. 121 p. Acad. Diss.

C 167. Kolehmainen, Mikko T. Data exploration with self-organizing maps in environmental informatics and bioinformatics.
2004. 73 p. Acad. Diss.

C 168. Huttunen, Tomi. The ultra weak variational formulation for ultrasound transmission problems.
2004. 159 p. Acad. Diss.

C 169. Malinen, Matti. Computational methods for optimal control in ultrasound therapy.
2004. 126 p. Acad. Diss.

C 170. Kolmonen, Pekka. The Direct Control of the Multi-Leaf Collimator in the Inverse Problem of Radiotherapy Treatment Planning.
2004. 81 p. Acad. Diss.

C 171. Rautiala, Sirpa. Microbial exposure in remediation work.
2004. 74 p. Acad. Diss.

C 173. Tarvainen, Mika. Estimation Methods for Nonstationary Biosignals.
2004. 138 p. Acad. Diss.

C 174. Yppärilä, Heidi. Depth of sedation in intensive care patients. A neuropsysiological study.
2004. 104 p. Acad. Diss.

C 175. Sohlberg, Antti. Molecular small animal imaging with pinhole single-photon emission computed tomography.
2004. 104 p. Acad. Diss.

C 176. Kumlin, Timo. Studies on cancer-related effects of 50 Hz magnetic fields.
2004. 64 p. Acad. Diss.

C 177. Seppänen, Kari. Does mercury promote lipid peroxidation?: in vivo and in vitro studies concerning mercury and selenium in lipid peroxidation and coronary heart disease.
2004. 49 p. Acad. Diss.

C 178. Riikonen, Johanna. Modification of the growth, photosynthesis and leaf structure of silver birch by elevated CO2 and O3.
2004. 126 p. Acad. Diss.

C 179. Frank, Christian. Functional profiling of the xenobiotic nuclear receptors CAR and PXR.
2004. 87 p. Acad. Diss.