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Induced Volatile Emissions of Plants Under Elevated Carbon Dioxide and Ozone Concentrations, and Impacts on Indirect Antiherbivore Defence

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 Tuesday 21st June 2005, at 12 noon

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ABSTRACT

Introduction Tropospheric concentrations of ozone, [O₃], and carbon dioxide, [CO₂], are continuously rising due to human activities. O₃ is a phytotoxic gas which in high concentrations is able to cause severe damage to economically important crop species and wild plants, while elevated [CO₂] has variable effects on the physiology of plants. Effects of these gases on emissions of volatile organic compounds (VOCs) by plants have been studied to some extent. However, the effects on indirect defence recruiting natural enemies of herbivores which are attracted to the VOCs induced by their prey have been completely neglected. The aim of this research was to clarify the understanding of the emissions of crop plants and forest trees under future climate conditions, and to get primary information concerning the orientation behaviour of the natural enemies of herbivores when the host plants are grown at elevated [O₃] or [CO₂].

Experiments White cabbages (cvs. Lennox and Rinda) were grown under doubled ambient [CO₂] in growth chambers and damaged by larvae of Plutella xylostella and Spodoptera littoralis. Orientation behaviour of the generalist predator, Podisus maculiventris, and the specialist parasitoid, Cotesia plutellae, were studied. Two clones of silver birch trees were exposed to doubled ambient [CO₂] and [O₃] singly and in combination over three growing seasons in open-top chambers, and silver birch saplings were treated with 0, 50 and 100 ppb of O₃. Lima beans were exposed to acute 200 ppb of O₃ and damaged by Tetranychus urticae, and orientation behaviour of the specialist predator, Phytoseius persimilis, towards herbivore- and O₃-damaged plants was investigated.

Responses to elevated [CO₂] Growing under elevated [CO₂] decreased the constitutive monoterpenoid emission of herbivore-damaged cabbage plants, but did not significantly affect the P. xylostella-induced VOC emissions. P. maculiventris and C. plutellae showed higher preference to the odour of damaged cabbages grown at ambient [CO₂] than that of damaged cabbages grown at elevated [CO₂]. Emissions of silver birch trees were not significantly affected by the growth at doubled ambient [CO₂].

Responses to O₃ stress The emission from silver birch trees and saplings were not affected by the growth at moderately enhanced [O₃]. Lima beans treated with acetlyl-O₃ emitted some of the same compound that are also induced by T. urticae, but P. persimilis was not attracted to the O₃-induced blend of VOCs.

Conclusions Increasing atmospheric [CO₂] may have a decreasing effect on the constitutive monoterpane emission of plants, which may lead to reduced host-searching ability of the natural enemies of herbivores. The emissions from plants grown at elevated [CO₂] probably depend on whether the plant has storage structures for terpenes or if the carbon used in the formation of VOCs comes directly from photosynthesis. Extremely high [O₃] is capable of inducing the emission of VOCs, but moderately elevated [O₃] seems to have no effect on the emission by plants. It is likely that the natural enemies of the herbivores are not mislead by the O₃-induced emissions. To get a realistic view of the VOC emissions at elevated [CO₂] and [O₃] the experiments ought to be conducted in the field, since emission of VOCs is affected by several environmental factors. Behavioural tests also need to be conducted under elevated [CO₂] and [O₃], and more plant species and trophic systems should be studied to draw strong conclusions about the effects of elevated [CO₂] and [O₃] on VOC emissions and signalling between three trophic levels.

Universal Decimal Classification: 504.054, 546.264, 546.214, 632.151, 632.937
CAB Thesaurus: volatile compounds; organic compounds; carbon dioxide; ozone; monoterpenes; parasitism; predation; herbivores; defence; natural enemies; plants; cabbages; Betula pendula; Lima beans; Plutella xylostella; Spodoptera littoralis; Podisus maculiventris; Cotesia plutellae; Tetranychus urticae; Phytoseius persimilis
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Tampere, May 2005

Terhi Vuorinen
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>CO$_2$</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>[CO$_2$]</td>
<td>carbon dioxide concentration</td>
</tr>
<tr>
<td>DMAPP</td>
<td>dimethylallyl diphosphate</td>
</tr>
<tr>
<td>DMNT</td>
<td>(E)-4,8-dimethyl-1,3,7-nonatriene</td>
</tr>
<tr>
<td>GLV</td>
<td>green leaf volatile</td>
</tr>
<tr>
<td>JA</td>
<td>jasmonic acid</td>
</tr>
<tr>
<td>MeJA</td>
<td>methyl jasmonate</td>
</tr>
<tr>
<td>MeSA</td>
<td>methyl salicylate</td>
</tr>
<tr>
<td>NO$_x$</td>
<td>nitrogen oxides</td>
</tr>
<tr>
<td>O$_3$</td>
<td>ozone</td>
</tr>
<tr>
<td>[O$_3$]</td>
<td>ozone concentration</td>
</tr>
<tr>
<td>OTC</td>
<td>open-top chamber</td>
</tr>
<tr>
<td>PEP</td>
<td>phosphoenolpyruvate</td>
</tr>
<tr>
<td>SA</td>
<td>salicylic acid</td>
</tr>
<tr>
<td>TMTT</td>
<td>(E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene</td>
</tr>
<tr>
<td>VOC</td>
<td>volatile organic compound</td>
</tr>
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LIST OF ORIGINAL PAPERS

This thesis is based on the following papers which are referred to in the text by their chapter numbers.


Chapter 5  Vuorinen T, Nerg A-M, Syrjälä L, Peltonen P, Holopainen JK. VOC emissions induced by herbivory, pathogen infection and O₃ exposure from two silver birch clones and the host-searching behaviour of the generalist predator. Manuscript

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CHAPTER 1

GENERAL INTRODUCTION
GENERAL INTRODUCTION

1.1 Emission of volatile organic compounds (VOCs) from plants

1.1.1 Diversity of VOCs

Plants emit a great variety of VOCs depending on the family and species. It has been estimated that vegetation emits approximately $1.2 \times 10^{15}$ g of carbon annually (Guenther et al. 1995). VOCs are molecules that have a low boiling point, and therefore can change from liquid to gas phase at high rate at realistic temperatures (Lerdau & Slobodkin 2002).

Emissions from vegetation typically comprise of terpenes, C$_3$ isoprene, C$_{10}$ monoterpenes, C$_{15}$ sesquiterpenes, and several oxygenated compounds such as alcohols and aldehydes (Atkinson & Arey 2003). Isoprene and monoterpenes are the most prominent compounds emitted, followed by alcohols and carbonyls (Kesselmeier & Staudt 1999).

The release of VOCs can be divided into light-independent and light-dependent emissions. Light-independent emissions comprise e.g. the constitutive volatilisation of monoterpenes from storage pools which depends on their vapour pressure (Lerdau & Gray 2002). Monoterpenes are often stored in the specialised secretory organs of plants, e.g. resin ducts in conifers or glandular trichomes in angiosperms, and they have defensive and attractive functions (Gershenzon & Croteau 1991).

In contrast e.g. isoprene is rarely stored but immediately released in light-dependent manner (Lerdau & Gray 2002). Some plant species such as birches and oaks emit a variety of terpenes but they do not store them in specific structures (Hakola et al. 1998, Loreto et al. 1996, Loreto et al. 2001a). For example, monoterpenes α-pinene from holm oak leaves (Loreto et al. 1996) and sabinene from European beech (Kuhl et al. 1999) are immediately released into the atmosphere following production. Especially induced VOCs (see below) released after insect herbivory are synthesised de novo, at the site of the damage (Paré & Tumlinson 1997a,b). Although the storage structures for isoprenoids (isoprene and monoterpenes) may be absent in some species, the VOCs can be temporarily stored in the lipid or aqueous phase of the leaf depending on the chemical properties of the compound (Loreto et al. 1998, Niinemets et al. 2004).

Abiotic and biotic factors can modify the emission profile of plants by increasing or decreasing the level of constitutively emitted VOCs, or by triggering the emission of novel inducible VOCs (Takabayashi et al. 1994, Gouingué & Turlings 2002). Herbivore damage induces a special blend of VOCs which differs prominently from the odour of an intact plant (Karan & Baldwin 1997, Turlings & Wäcker 2004). Such a blend includes inducible compounds such as several mono-, homo- and sesquiterpenes, indole, C$_6$ compounds and aromatic methyl salicylate (MeSA) (Paré & Tumlinson 1999, Dicke & Hilker 2003).

Herbivore-induced compounds are derived mainly from the octadecanoid pathway with jasmonic acid (JA) as one of the main compounds (Dicke et al. 2003). The octadecanoid pathway has control over the lipoxygenase (LOX) pathway and two terpenoid pathways (Dicke et al. 2003): the acetate-mevalonate pathway in cytosol producing sesqui- and triterpenes (Phillips & Croteau 1999), and glyceroldehydes phosphate/pyruvate pathways in plastids producing precursors of mono-, di- and tetraterpenes (Lichtenhailer et al. 1997). So called green leaf volatiles (GLVs; C$_6$ compounds) indicating fresh leaf damage are formed from LOX-derived oxidative lipid breakage (Hatanoaka & Harada 1973).

1.1.2 Why plants emit VOCs?

Langenheim (1994) summarized the phytoecentric roles of terpene emissions as the attraction of pollinators and natural enemies of damaging herbivores, interactions in the troposphere, allelopathic effects on other plants, and influence on soil microbes. In addition, pathogen- and herbivory-induced
VOCs play a major role in plant-plant signalling by inducing defence responses in the neighbouring plants (Shulaev et al. 1997, Arimura et al. 2000, Karban et al. 2003). The herbivores may also exploit the induced VOC emissions by avoiding previously damaged plants (De Moraes et al. 2001, Heil 2004).

It has been shown that isoprene emission provides thermal protection to the plants (Sharkey & Singsaas 1995), and as an effective antioxidant protects plants from ozone (O₃) damage by direct quenching of O₃ (Loreto et al. 2001b). Actually a suggestion has been made that one of the original evolutionary functions of the VOCs might have been the reduction of phytotoxic O₃ by oxidation, and consequently by aerosol formation (Lerdau & Slobodkin 2002, Lerdau & Gray 2003). On the other hand, reactions with VOCs and O₃ can yield intermediates e.g. organic peroxides and hydroperoxides that might be more phytotoxic than O₃ itself (Salter & Hewitt 1992, Stokes et al. 1998).

1.2 Indirect defence of plants by VOCs

1.2.1 Tritrophic signalling

Plants defend themselves indirectly by producing and emitting VOCs that attract the natural enemies of their herbivores (e.g. Turlings et al. 1990, 1991, 1995, Dicke et al. 1991, 1994, 2003, De Moraes 1998). The plant utilizes the top-down control of the herbivore populations by the herbivore’s natural enemies, predators and parasitoids (Baldwin & Preston 1999). Thereby this indirect defence is often called tritrophic signalling.

The volatile blend induced by herbivore damage prominently differs from those of mechanically damaged and undamaged plants. Herbivory-induced VOCs emitted from damaged plants or plant parts comprise of monoterpeneoxides (e.g. (E)-β-ocimene, limonene), homoterpene oxides (DMNT, TMTT), sesquiterpenes (e.g. (E,E)-α-farnesene), indole, aromatics (MeSA) and GLVs ((Z)-3-hexenol and (E)-3-hexenyl acetate) which are mainly released due to the fresh mechanical damage (Paré & Tumlinson 1999, Dicke & Hilker 2003). In cotton the undamaged parts of the herbivore-damaged plant also emit induced VOCs in a systemic manner (Röse et al. 1996). This indicates an existence of a chemical messenger transporting a signal to the undamaged parts of the plant, and triggering the defence in advance (Karban & Baldwin 1997).

The induced emission is relatively specific so that some plants have the capability to respond differentially to different herbivore species (Turlings & Wäcker 2004). For example, damage caused by phytophagous mite, Tetranychus urticae, induces the emission of completely different set of compounds (Takabayashi et al. 1994) than leaf-chewing caterpillar, Spodoptera exigua, (Horiguchi et al. 2003) from Lima beans. The specialist predators may even avoid the odour of plants infested with a nonprofitable prey (Shimoda & Dicke 1999). Also the emission triggered by Heliothis virescens damage is clearly distinct from that of Helicoverpa zea damage in cotton plants, and the parasitic wasp can discriminate these odours to find its’ host (De Moraes et al. 1998).

The specialist predators and parasitoids of herbivores are not necessarily innately attracted to the induced plant-derived VOCs, but they learn to associate the induced volatiles to the feeding prey or host (Lewis & Tumlinson 1988, Lewis & Takasu 1990, Dukker et al. 2000).

1.2.2 Elicitors of indirect defence

This sophisticated signalling system between three trophic levels requires an elicitor which activates the plant to emit novel inducible VOCs of which the natural enemies are interested in. Thus far, only two compounds from the saliva of herbivores have been analyzed and identified. Volicitin (N-(17-hydroxylinolenoyl)-L-glutamine) was isolated from the regurgitant of Spodoptera exigua caterpillars. The compound induces the emission of VOCs that attract the natural enemies of the
caterpillars when applied to the damaged leaves of corn (Alborn et al. 1997).

A polysaccharide cleaving enzyme β-glucosidase is found in the saliva of many herbivores, including humans. Exogenous application of this enzyme causes the release of induced VOCs in a similar manner to feeding by Pieris brassicae larvae on brassica plants (Mattia et al. 1995), and Tetranychus urticae on Lima beans (Hopke et al. 1994). Also, human saliva together with mechanical damage induces the emissions of homoterpenes DMNT and TMTT from Lima bean plants (Boland et al. 1992). The blend induced by β-glucosidase from brassicas is highly attractive to the parasitic wasp Cotesia glomerata (Mattia et al. 1995).

As well as these herbivore-derived compounds phytohormones ethylene (Kahl et al. 2000) and JA (Hopke et al. 1994, Dicke et al. 1999, Thaler 1999, Ozawa et al. 2000) are elicitors of indirect defence. Exogenous application of methyl jasmonate (MeJA) and MeSA on plant leaves also induces the herbivore resistance (Ozawa et al. 2000, Rodriguez-Saona et al. 2001).

Plant pathogen inoculation also induces the emission of VOCs (Doughty et al. 1996, Piel et al. 1997, Shulaev 1997, Cardoza et al. 2002, Heiden et al. 2003). The enzyme cellulysin in plant parasitic fungi triggers the synthesis of VOCs by activating the octaadamoid signaling pathway (Piel et al. 1997). Also pathogen-derived peptides, glycoproteins, lipids and oligosaccharides have been characterised as to be elicitors inducing defence responses in plants (Nuemberger 1999).

1.3 Elevating CO2 concentration

1.3.1 Variation in the atmospheric CO2 concentration

For several thousand years prior to the industrial revolution the atmospheric CO2 concentration, [CO2], was stable at 280 ppm, but this has since risen to 367 ppm, and is predicted to double by the end of this century. Increase in [CO2] is predominantly due to the oxidation of organic carbon by fossil-fuel combustion and deforestation (Houghton et al. 2001).

Together with the other greenhouse gases, CO2 plays a major role in warming of the climate due to the greenhouse effect, and thus can indirectly affect emissions of VOCs, since climate warming is estimated to increase the emission of VOCs substantially (Constable et al. 1999a).

1.3.2 Effects of elevated [CO2] on plants and herbivores

Plants seem to benefit from the increasing atmospheric [CO2] because of its growth stimulating effect. Generally, the leaf growth and thickness increases, and plants grow faster when growing under elevated [CO2] (Saxe et al. 1998, Woodward 2002).

Elevated [CO2] stimulates higher rates of photosynthesis and increases the light-use efficiency. It also reduces stomatal density, stomatal conductance (Hetherington & Woodward 2003) and increases water use efficiency (Drake et al. 1996, Norby et al. 1999, Pritchard et al. 1999). Plants grown under elevated [CO2] also have increased carbon:nitrogen ratios (Bezemer & Jones 1998), and concentration of phenolic compounds (Bezemer & Jones 1998, Percy et al. 2002, Peltonen et al. 2005). Elevated [CO2] also alters other defence compounds. An increase in the amount of condensed tannins, flavonol glycosides (Kuokkanen et al. 2001), cuticular waxes and phenolic glucosides (Percy et al. 2002), and a decrease in amounts of antioxidants (Wustman et al. 2001) has been detected. The magnitude and direction of these changes in the foliar chemistry is species-specific (Lindroth et al. 1993).

Ecological interactions between plants and other organisms under increasing [CO2] have also been under investigation. Presumably herbivores as well as higher trophic levels will be affected by changes in the host plant quality. Leaf-chewers are able to cope with the diminished food quality (decreased nitrogen level in plant tissues) by compensatory feeding, although they seem to suffer higher larval mortality and prolonged development time (Lincoln & Couvet 1989,

1.3.3 Effects of elevated [CO₂] on emission of VOCs

Recent publications have clearly indicated that the elevated [CO₂] suppresses isoprene emission from plants (Rosenstiel et al. 2003, Rapparini et al. 2004, Scholefield et al. 2004), whereas elevated [CO₂] has lead to variable changes in the emission of monoterpenes from plants. A decrease (Loreto et al. 2001a, Rapparini et al. 2004), an increase (Staudt et al. 2001) and no effect (Pétilas and Llusá 1997, Constable et al. 1999b) in the emissions of monoterpenes have been observed. Also an increase in the emission of methyl ketones has been detected under elevated [CO₂] (Jasoni et al. 2004). It seems that enriched [CO₂] affects the constitutive emissions of plants, but elicitation of novel inducible compound under elevated [CO₂] has been neither detected nor expected.

The concentration of monoterpenes in the storage tissues of terpene-storing conifers may either decrease (Litvak et al. 2002, Sallas et al. 2003), increase (Sallas et al. 2001) or be unaltered (Kaimulainen et al. 1998) due to the increasing [CO₂]. Wounding has been shown to depress the monoterpene pool sizes at both ambient and elevated [CO₂] (Litvak et al. 2002).

1.4 Tropospheric O₃

1.4.1 Trends of tropospheric O₃ concentration

Background O₃ has several biogenic and anthropogenic sources: downward transport of stratospheric O₃, in situ O₃ production from methane emitted from swamps and wetlands reacting with natural NOₓ, in situ O₃ production from reactions of biogenic VOCs with natural NOₓ, and long-range transport of O₃ from distant pollutant sources (Vingarzan 2004).

O₃ is formed in complex photochemical reactions involving NOₓ and VOCs (Finlayson-Pitts & Pitts 1997). NOₓ are mainly produced by road traffic and industry while VOCs are mainly from biogenic sources. When NOₓ levels are low, oxidation of VOCs removes O₃ from the troposphere, while with high NOₓ concentration oxidation of VOCs increases the O₃ levels (Lerdau & Slobodkin 2002). In reactions with O₃ and VOCs aerosol particles are formed (Went 1960).

The mean tropospheric O₃ concentration, [O₃], in urban areas is continuously rising due to the human activities. The prevailing [O₃] have risen approx. two-fold since the early 20th century (Vingarzan 2004). The mean [O₃] can range from 20 to 60 ppb, but in the most polluted sites [O₃] can acutely reach a maximum of 120 to 500 nL L⁻¹ (Long & Naidu 2002). The transport of O₃ to rural areas is more hazardous to cultivated and natural crops than any other pollutant (Ashmore 2002).

1.4.2 Effects of tropospheric O₃ on plants

O₃ enters the plant cell mainly through stomata and decomposes in the apoplast to secondary products (Salter & Hewitt 1992, Sandermann 1996). When diffusing O₃ reacts with the constituents of the cell (cell wall, plasma membrane) forming reactive oxygen species such as hydroxyl (OH⁻) and superoxide (O₂⁻) radicals and hydrogen peroxide (H₂O₂) (Kangasjärvi et al. 1994, Pell et al. 1997). O₃ effects are usually biphasic starting with an initial phase of stress reaction and reduced photosynthesis, and followed by a second phase of visible symptom development (Sandermann 1996). Exposure to high [O₃] leads to lesion development, and potentially to plasmolysis and cell death (Pell et al. 1997).

O₃ stress can activate various defence responses in plants (Kangasjärvi et al. 1994) such as the expression of defence-related genes, the emission of ethylene and other VOCs, and the biosynthesis of signalling
molecules e.g. jasmonic acid (JA), salicylic acid (SA) and ethylene (Rao et al. 2000). O₃ is a phytotoxic gas and may lead to reduced crop yield, which makes O₃ an economically important issue. O₃ stress can also alter the leaf surface of the plant in such a way that pathogen infection becomes more likely (Karnosky et al. 2002).

An atmosphere enriched with O₃ increases the amount of cuticular waxes and decreases phenolic glycosides. It can also increase levels of rust infection and high aphid populations whilst decreasing the abundance of natural enemies (Percy et al. 2002). O₃ has also been shown to destroy insect pheromones (Arndt 1995).

1.4.3 Effects of O₃ stress on the emission of VOCs

It is plausible that changes in the VOC emissions by plants will occur in response to elevated [O₃], since O₃ exposure activates e.g. the JA-signalling cascade in plants (Kangasjärvi et al. 1994). Exposure of plants to elevated [O₃] may increase the constitutive emission of VOCs (Heiden et al. 1999, Peñuelas et al. 1999, Llusia et al 2002, Loreto et al. 2004), induce the emission of novel VOCs (Heiden et al. 1999, 2003) or leave the emission unaffected (Lindskog & Potter 1995).

The response of plants to O₃ stress depends on the [O₃] and the plant species. In open-top chamber (OTC) experiments the O₃ exposure has been continuous and realistic, approx. doubled ambient [O₃] (Lindskog & Potter 1995, Peñuelas et al. 1999, Llusia et al. 2002), while in the chamber experiments the acute O₃ exposure has reached concentrations of 170 ppb or more (Heiden et al. 1999).

The effects of elevated [O₃] on foliar terpene concentrations of conifers have varied. Exposure to realistically elevated [O₃] (max. 2-fold) may cause no changes (Kainulainen et al. 1998, Manninen et al. 2000) or increase (Kainulainen et al. 2000a) the concentrations of terpenes in needles. A decrease of foliar monoterpenes concentration was found when trees were exposed to high [O₃] (Kainulainen et al. 2000b).

1.5 The experiments

The thesis is composed of five independent studies. In three experiments effects of elevated [CO₂] (Chapters 2, 3 & 4) and in three experiments effects of elevated [O₃] (Chapters 4, 5 & 6) was studied (Table 1). Four experiments (Chapters 2, 3, 5 & 6) were conducted in the computer-controlled growth chambers (e.g. Sallas et al. 2003), and one experiment was a three-year field experiment (Chapter 4) at the field site of Suomenjoki Research Station (for details see Vapaavuori et al. 2002) from where we sampled VOCs in the last year of a running experiment. White cabbage was chosen for the CO₂ experiments because of its relatively slow growing time enabling 3-4 weeks growth and exposure at elevated [CO₂], and its capability to emit monoterpenes (Geervliet et al. 1997) (Chapters 2 & 3). The generalist herbivore Spodoptera littoralis and the specialist herbivore Plutella xylostella were chosen to investigate the specificity of induced emission of VOCs (Chapter 2) since the plant response might vary under pressure from differentially adapted herbivore species (De Moraes et al. 1998). To study the effects of O₃ on tritrophic signalling, we chose to study the signalling between Lima beans, spider mites (Tetranychus urticae) and predatory mites (Phytoseiulus persimilis) because that system is already well-established (Dicke 1994, Dicke et al. 1999, Arimura et al. 2000) (Chapter 6).

Silver birch was selected to gain information on the stress-induced VOCs (Chapters 4 & 5) of this tree species. Birches (B. pendula & B. pubescens) are estimated to dominate the forest tree species composition in Finland in 2100 (Kellomäki et al. 2001).

White cabbage (Brassica oleracea subsp. capitata) cvs. Lennox and Rinda and two silver birch (Betula pendula Roth) clones, and Lima bean (Phaseolus lunatus cv. Sieva) and two silver birch clones were used in CO₂ and O₃ experiments, respectively (Table 1). Silver birch clone 4 had been earlier classified as an O₃-tolerant genotype whereas clone 80 expressed an O₃-sensitive genotype (Pääkkönen et al. 1997).
Herbivores, predators and parasitoids used in the experiments are listed in Table 1. The orientation behaviour of the natural enemies was performed in a Y-tube olfactometer.

To see the emission profile of plants and induced compounds, VOCs were analysed by gas chromatography-masspectrometry (GC-MS) in all of the experiments, and calculated as ng g dry weight\(^{-1}\) h\(^{-1}\) (Chapter 2, 4, 5 & 6) and as ng cm leaf area\(^{-1}\) h\(^{-1}\) (Chapter 3). Because elevated [CO\(_2\)] affects plants' physiological properties, specific leaf area, leaf thickness and stomatal density was measured in one of the experiments (Chapter 3). The hypotheses of the experiment are summarised in Table 2, and the methods have been described in more details in the subsequent chapters.

<table>
<thead>
<tr>
<th>Table 1. Summary of the experiments and treatments</th>
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<tr>
<td>Chapter</td>
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<table>
<thead>
<tr>
<th>Table 2. Hypotheses tested in the original publications</th>
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<tr>
<td>Hypotheses</td>
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<tr>
<td>Doubled ambient [CO(_2)] alters the emission of constitutively emitted monoterpene.</td>
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<tr>
<td>Doubled ambient [CO(_2)] affects the emission of herbivore-induced VOCs.</td>
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<tr>
<td>Doubled ambient [CO(_2)] disturbs trophic signalling.</td>
</tr>
<tr>
<td>The specialist and the generalist herbivore will induce VOC emission in different manner.</td>
</tr>
<tr>
<td>Enhanced [O(_3)] elicits the emission of novel herbivore-inducible VOCs and enhances constitutively emitted VOCs.</td>
</tr>
<tr>
<td>Elevated [O(_3)] disturbs trophic signalling.</td>
</tr>
</tbody>
</table>

\(^a\)Loreto et al. 2001a, \(^b\)Staudt et al. 2001, \(^c\)Peñuelas & Llusia 1997, \(^d\)De Moraes 1998, \(^e\)Heiden et al. 1999, \(^f\)Lindskog & Potter 1995
1.6 Aims of the research

There have been a number of studies concerning the emission of VOCs from several crops and tree species, but the effects of climate change factors such as elevated \([O_3]\) and \([CO_2]\) on the emissions is quite rarely studied. In addition, the impacts of these greenhouse gases on tritrophic signalling have not been studied at all.

The aim of this research was to clarify the understanding of VOC emissions under future climate conditions, and to get primary information about the orientation behaviour of the natural enemies of herbivores to the plants grown at elevated \([O_3]\) or \([CO_2]\). Two crop species, Lima bean and white cabbage, and a tree species, silver birch, were selected in the study. The answers to the following questions were sought.

1) What compounds are emitted from CO\(_2\)-treated and O\(_3\)-exposed plants?

2) What impacts do elevated \([CO_2]\) have on the emission of herbivore-induced VOCs and constitutive VOCs?

3) Does elevated \([O_3]\) induce the emission of novel VOCs in a similar manner to herbivory?

4) How do the natural enemies of herbivores respond to the herbivore-induced VOCs and to the compounds induced or affected by elevated \([CO_2]\) or \([O_3]\)?

5) Do natural enemies of herbivores get false alarms from the plants grown at elevated \([CO_2]\) or \([O_3]\)?

References


Bezemer TM, Jones TH (1998) Plant-insect herbivore interactions in elevated atmospheric CO\(_2\): quantitative analyses and guild effects. Oikos 82, 212-222


Hakola H, Rinne J, Laurila T (1998) The hydrocarbon emission rates of tea-leafed willow (Salix phylicifolia), silver birch (Betula pendula) and European aspen (Populus tremula). Atmos Environ 32, 1825-1833
Hatanaka A, Harada T (1973) Formation of cis-3-hexenal, trans-2-hexenal and cis-3-hexenol in macerated Thea sinensis leaves. Phytochemistry 12, 2341-2346
Lewis WJ, Takasu K (1990) Use of learned odours by a parasitic wasp in accordance with host and food needs. Nature 348, 635-636
Litvak ME, Constable JH, Monson RK (2002) Supply and demand processes as controls over needle monoterpene synthesis and concentration in Douglas fir (Pseudotsuga
menziesii (mirb.) Franco). Oecologia 132, 382-391
Manning WJ, Tiedemann A v (1995) Climate change: potential effects of increased atmospheric carbon dioxide (CO2), ozone (O3), and ultraviolet-B (UV-B) radiation on plant diseases. Environ Pollut 88, 219-245
Paré PW, Tumlinson JH (1997a) Induced synthesis of plant volatiles. Nature 385, 30-31
Paré PW, Tumlinson JH (1997b) De novo biosynthesis of volatiles induced by insect herbivory in cotton plants. Plant Physiol 114, 1161-1167
Turlings TCJ, Tumlinson JH, Heath RR, Provenux AT, Doolittle RE (1991) Isolation and identification of allelochemicals that attract the larval parasitoid, Cotesia marginiventris (Cresson), to the microhabitat of one of its hosts. J Chem Ecol 17, 2235-2251
by host-seeking parasitic wasps. Science 25, 1251-1253


CHAPTER 2

MONOTERPENE AND HERBIVORE-INDUCED EMISSIONS FROM CABBAGE PLANT GROWN AT ELEVATED CO₂ CONCENTRATION


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Monoterpenes and herbivore-induced emissions from cabbage plants grown at elevated atmospheric CO₂ concentration

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Abstract

The warming of the lower atmosphere due to elevated CO₂ concentration may increase volatile organic compound (VOC) emissions from plants. Also, direct effects of elevated CO₂ on plant secondary metabolism are expected to lead to increased VOC emissions due to allocation of excess carbon on secondary metabolites, of which many are volatile. We investigated how growing at doubled ambient CO₂ concentration affects emissions from cabbage plants (Brassica oleracea subsp. capitata) damaged either by the leaf-chewing larvae of crucifer specialist diamondback moth (Plutella xylostella L.) or generalist Egyptian cotton leafworm (Spodoptera littoralis (Boisdruval)). The emission from cabbage cv. Lennox grown in both CO₂ concentrations, consisted mainly of monoterpenes (α-pinene, sabinene, β-pinene, myrcene, γ-terpinolene and γ-terpinene), (Z)-3-hexenyl acetate, sesquiterpenes (E, E)-α-farnesene and homoterpenes (E)-4,8-dimethyl-1,3,7-nonatriene (DMNT) were emitted mainly from herbivore-damaged plants. Plants grown at 720 μmol mol⁻¹ of CO₂ had significantly lower total monoterpenes emissions per shoot dry weight than plants grown at 360 μmol mol⁻¹ of CO₂, while damage by both herbivores significantly increased the total monoterpenes emissions compared to intact plants. (Z)-3-Hexenyl acetate, (E, E)-α-farnesene and DMNT emissions per shoot dry weight were not affected by the growth at elevated CO₂. The emission of DMNT was significantly enhanced from plants damaged by the specialist P. xylostella compared to the plants damaged by the generalist S. littoralis. The relative proportions of total monoterpenes and total herbivore-induced compounds of total VOCs did not change due to the growth at elevated CO₂, while insect damage increased significantly the proportion of induced compounds. The results suggest that VOC emissions that are induced by the leaf-chewing herbivores will not be influenced by elevated CO₂ concentration.

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Keywords: Herbivory; Induced defense; Monoterpenes; Plutella xylostella; Spodoptera littoralis; VOCs

1. Introduction

CO₂-induced warming of the lower atmosphere as well as elevated CO₂ concentration intrinsically, are predicted to increase the emissions of volatile organic compounds (VOCs) from plants (Constable et al., 1999a). Biogenic VOCs substantially contribute to the hydrocarbon load into the atmosphere, and significantly affect ozone and aerosol formation, methane oxidation and carbon monoxide budget (Andreae and Crutzen, 1997; Pehues and Llusia, 2001, 2003). Nevertheless, the number of experiments to analyse VOC emission from plants grown at elevated CO₂ is still limited.

Feeding damage of leaf-chewing insect larvae on plant tissue result in emission of wide variety of VOCs including monoterpenes, sesquiterpenes, ketones, aldehydes, esters, nitriles, sulfides, (iso)thiocyanates, carboxylic acids, and others (Geervliet et al.,...
1997). The ratios among herbivore-induced monoterpene (MTs) and sesquiterpenes may vary considerably between plant varieties (Gouinguené et al., 2001). The proportion of MTs attains nearly 50% of the total VOC emission spectrum in intact white cabbage plants, but remains below 25% in herbivore-damaged plants (Geervliet et al., 1997). Green leaf volatiles (GLVs), like (Z)-3-hexen-1-ol and (Z)-3-hexenyl acetate, are derived from the octadecanoid pathway and they are emitted rapidly after mechanical or herbivore damage (Turlings et al., 1998; Kessler and Baldwin, 2001), while the induced terpenes are emitted more slowly, starting to be released after 4 h and peaking within 10 h after the damage (Turlings et al., 1998).

In the few studies investigating the effects of elevated CO₂ concentration on plant emissions the results are controversial. Staudt et al. (2001) studied emissions of non-terpene storing Quercus ilex L. seedlings, and measured on average 1.8-fold higher MT emission capacities in plants grown at elevated CO₂ than in plants grown at ambient CO₂. They did not detect any short-term effect of assay CO₂ concentration (350 and 700 µl l⁻¹) on the emission capacities in both CO₂ treatments. On the other hand, elevated CO₂ inhibited the emission of certain MTs from Q. ilex seedlings due to concurrent down-regulation of corresponding MT synthase activities (Loreto et al., 2001). In the case of limonene, the down-regulation of corresponding enzyme activity did not occur, leading to the enhanced emission of limonene at elevated CO₂ (Loreto et al., 2001). Constable et al. (1999b) found that in Ponderosa pine and in Douglas fir, which both store terpenes in specialized secretory organs, there was no significant effect of elevated CO₂ (700 µmol mol⁻¹) on either needle MT concentration or emission rate per unit biomass. However, they concluded that the effect of elevated CO₂ on leaf area index and the effect of climate warming on MT biosynthesis and volatilization could increase MT emissions from the canopy.

The objective of our present study was to evaluate the effects of growth at elevated CO₂ concentration on VOC emission from cabbage plants damaged by either crucifer specialist or generalist insect herbivore. Cabbage is an important crop plant which is cultivated in both southern and northern hemispheres. Diamondback moth is the most serious pest of crucifers throughout the world (Talekar and Shelton, 1993). Also another lepidopteron species, polyphagous Egyptian cotton worm, was selected since plant response might be different to particular herbivore species (DeMeeus et al., 1998). Another aim was to provide information of the emission response of plants to the insect damage under stress factor of global change. This information is needed in the estimation of future biogenic VOC emissions under changing climatic conditions, as well as in the development of novel plant protection methods based on tritrophic signalling (Dicke et al., 1990; Lewis et al., 1997; Thaler, 1999; Cortesero et al., 2000; Hunter, 2001), repellents (Brahim et al., 2001) and attractants (Raddy and Guerrero, 2000). As far as we know, this is the first investigation to examine the capability of plants grown at elevated CO₂ to emit VOC after herbivore damage.

2. Materials and methods

2.1. Plant material and CO₂ exposure

Seedlings of white cabbage (Brassica oleracea subsp. capitata cv. Lento) were individually sown in 1–1 plastic pots filled with Sphagnum peat and sand (3:1 v/v). Seedlings were grown 24–26 days under ambient (360 µmol mol⁻¹) or elevated (720 µmol mol⁻¹) CO₂ concentration in growth chambers (Bioklim 2600T, Kryo-Service Oy, Helsinki, Finland) constructed for air pollutant exposures (e.g. Sallas et al., 2003) at 23±1°C, 70±10% RH and 22h light:2h dark photoperiod (250 µmol m⁻² s⁻¹ PAR during light period). The CO₂ exposure was maintained 24 h day⁻¹ for the whole experimental period. The seedlings were watered daily with tap water and fertilized weekly with 0.1% of 9– Superex (19:5:20 N:P:K, Kekkilä, Finland) at the rate of 0.05–0.11 plant⁻¹, starting 2 weeks after sowing. The CO₂ treatments and seedlings were rotated among the two chambers weekly to randomize any systematic chamber effect across the seedlings. The growth chamber conditions were constantly monitored.

2.2. Insect feeding

The larvae of leaf-chewing moths were used to cause herbivore damage on cabbage seedlings. Larvae of crucifer specialist Plutella xylostella L. (Lepidoptera: Yponomeutidae) were reared in acrylic polyester gauge cages (60 cm x 33 cm x 33 cm, external dimensions) at 25°C, 50% RH and 16 h light:8 h dark photoperiod. Each cage contained a 5–6-weeks-old broccoli (B. oleracea subsp. italica) seedling. Fresh plants were provided every 3–4 days for larval feeding. Larvae of generalist Spodoptera littoralis (Boisduval) (Lepidoptera: Noctuidae) were reared in the laboratory at 26±1°C, 75±10% RH and 16 h light:8 h dark photoperiod on slightly modified artificial diets from those previously reported (Poutout and Bues, 1974). Both insect cultures were maintained in the laboratory for several generations before the current experiments started.

Herbivore feeding damage was caused by 48-h feeding either by transferring eight third instar larvae of P. xylostella or four second or third instar larvae of S. littoralis on five randomly selected plants grown at
ambient or elevated CO₂. Five additional intact plants from both CO₂ levels were used as controls. To avoid any disturbance in control plants induced by VOC emissions from herbivore-damaged plants, plants with the feeding larvae were kept in a separate growth chamber in similar environmental conditions as described earlier.

2.3. Collection of volatile compounds

After 48-h larval feeding, five intact control plants from ambient air or elevated CO₂ concentration, as well as five P. xylostella or S. littoralis-damaged plants from both CO₂ concentrations were used in VOC collections. Whole plants with carefully rinsed and slightly pruned root system in a 15 ml vial filled with tap water were individually enclosed inside 1 l glass cuvettes, which were closed with Teflon-sealed lids with one inlet for purified air with ambient CO₂ concentration and one for sampling. The airflow was calibrated with the mini-Buck calibrator (Model M-5, A.P. Buck, Inc., Orlando, FL, USA) and flow rate was set to 0.110 l min⁻¹ for filtered and pressured air, and 0.190 l min⁻¹ for sampling. The collection was performed at 22°C and at light intensity of 250 µmol m⁻² s⁻¹ under ambient CO₂ concentration. VOCs were collected for 1 h on ca. 150 mg Tenax-TA adsorbent (Supelco, mesh 60/80) by pulling the sample through 6 mm diameter Teflon tubing with a vacuum pump (KNP, Neuherberg, Inc., Freiburg, Germany, Model N002AN.18). More details of the collection system are given by Turtola et al. (2002). Samples were analysed by GC-MS (Hewlett Packard GC type 6890, MSD 5973). Trapped compounds were desorbed (Perkin Elmer ATD400 Automatic Thermal Desorption System) at 250°C for 10 min, cryofocused at −30°C and injected onto HP-5 capillary column (50 m × 0.2 mm i.d. ×0.5 µm film thickness, Hewlett Packard). The carrier gas was helium. The temperature program began at 40°C for 1 min, followed by increases of 5°C min⁻¹ to 210°C and 20°C min⁻¹ to 250°C. Compounds were identified by comparison of the mass spectra with those in the Wiley library and pure standards. For quantification commercially available reference substances were used. Reference substances for α-thujene, DMNT and (E,E)-α-farnesene were not available; therefore, the concentration of these compounds were calculated by proposing the responses to be the same as the responses of α-pinene, (Z)-ocimene and (E)-β-farnesene, respectively. After VOC collection, the above-ground biomass of the plants was determined and emissions were calculated as ng g dry weight⁻¹ h⁻¹.

2.4. Statistical analyses

Statistical analyses were performed using SPSS 11.0 for Windows statistical package. VOC data were analysed mainly with multivariate general linear model (GLM) procedure using CO₂ as two-level fixed variable and herbivore damage as three-level fixed variable, and Tukey and Dunnett T3 were used as post hoc tests. In the case of (Z)-3-hexenyl acetate and DMNT, the main effect of herbivore damage was tested with Kruskal–Wallis test. Plant properties between two CO₂ levels in each herbivore damage treatment were tested with independent samples t-test.

3. Results

The dry weight of control cabbage shoots and S. littoralis-damaged shoots was higher in plants grown at elevated CO₂ concentration than at ambient CO₂ (Table 1). The number of leaves and the fresh/dry weight ratios of shoots were almost equal in all treatments at both ambient and elevated CO₂ (Table 1). Intensity of leaf damage did not differ between CO₂ treatments, but in plants grown at ambient air S. littoralis caused more (t = −2.54, df 8, p = 0.035) small-sized feeding holes than P. xylostella.

The emission rates of individual MTs from cabbage cv. Lennox grown at ambient or elevated CO₂ were in descending order: sabine, limonene, α-thujene, 1,8-cineole, β-pinene + myrcene (compounds not separated on HP-5 column), α-pinene and γ-terpinene. The emissions per shoot dry weight of all the MTs, except γ-terpinene, were significantly decreased when plants were grown at elevated CO₂, but increased by herbivore damage (Table 2). In general, total MT emission per shoot dry weight was approximately 27% reduced from plants grown at elevated CO₂ and this proportion was not affected by insect damage (Fig. 1a). On average, insect feeding increased total MT emission significantly (Fig. 1a), emission being significantly higher from P. xylostella-damaged plants than from control plants. On the other hand, total MT emission expressed per shoot fresh weight, was only slightly reduced in plants grown at elevated CO₂ (F = 3.899, p = 0.060), while herbivore damage again increased total MT emission significantly (F = 5.092, p = 0.014). Using shoot dry weight as a covariate, the total MT emission per plant did not respond to CO₂ treatment, but increased significantly due to herbivore damage (F = 8.294, p = 0.002).

The total VOC emission per shoot dry weight was also significantly increased by damage of both insect species compared to control; P. xylostella-damage causing higher increase in emission than S. littoralis-damage (Fig. 1b). When total VOC emission was expressed per shoot fresh weight, corresponding significant increase in emission by both herbivores was found (F = 16.923, p<0.001). Total VOC emission expressed per shoot dry weight or shoot fresh weight was not affected by the growth CO₂ concentration. Using plant dry weight as a
Table 1
Plant dry weight, the number of leaves per plant, fresh/dry weight ratio, number of small feeding holes (Ø < 2 mm) and number of big feeding holes (Ø > 2 mm) per leaf (mean ± SE) in intact control cabbage plants and in cabbage plants damaged by P. xylostella or S. littoralis grown under ambient (360 μmol mol⁻¹) or elevated (720 μmol mol⁻¹) CO₂ (n = 5)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ambient CO₂</th>
<th>Elevated CO₂</th>
<th>t</th>
<th>p-value</th>
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<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plant dry weight (g)</td>
<td>0.89 ± 0.03</td>
<td>1.33 ± 0.13</td>
<td>-3.038</td>
<td>0.016</td>
</tr>
<tr>
<td>Leaves per plant</td>
<td>5.80 ± 0.20</td>
<td>5.80 ± 0.20</td>
<td>0.000</td>
<td>1.000</td>
</tr>
<tr>
<td>Fresh/dry weight ratio</td>
<td>7.51 ± 0.57</td>
<td>6.21 ± 0.41</td>
<td>1.851</td>
<td>0.101</td>
</tr>
<tr>
<td>P. xylostella</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plant dry weight (g)</td>
<td>1.02 ± 0.09</td>
<td>1.15 ± 0.09</td>
<td>-0.984</td>
<td>0.354</td>
</tr>
<tr>
<td>Leaves per plant</td>
<td>5.40 ± 0.40</td>
<td>5.20 ± 0.20</td>
<td>0.447</td>
<td>0.667</td>
</tr>
<tr>
<td>Fresh/dry weight ratio</td>
<td>7.18 ± 0.28</td>
<td>6.53 ± 0.53</td>
<td>1.089</td>
<td>0.312</td>
</tr>
<tr>
<td>Ø &lt; 2 mm feeding holes per leaf</td>
<td>0.90 ± 0.48</td>
<td>2.09 ± 0.59</td>
<td>-1.569</td>
<td>0.155</td>
</tr>
<tr>
<td>Ø &gt; 2 mm feeding holes per leaf</td>
<td>2.40 ± 0.35</td>
<td>3.27 ± 0.54</td>
<td>-1.341</td>
<td>0.217</td>
</tr>
<tr>
<td>S. littoralis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plant dry weight (g)</td>
<td>0.84 ± 0.06</td>
<td>1.12 ± 0.10</td>
<td>-2.422</td>
<td>0.042</td>
</tr>
<tr>
<td>Leaves per plant</td>
<td>5.20 ± 0.20</td>
<td>5.40 ± 0.24</td>
<td>-0.632</td>
<td>0.545</td>
</tr>
<tr>
<td>Fresh/dry weight ratio</td>
<td>6.89 ± 0.16</td>
<td>6.84 ± 0.39</td>
<td>0.139</td>
<td>0.909</td>
</tr>
<tr>
<td>Ø &lt; 2 mm feeding holes per leaf</td>
<td>2.35 ± 0.36</td>
<td>3.60 ± 0.79</td>
<td>-1.442</td>
<td>0.187</td>
</tr>
<tr>
<td>Ø &gt; 2 mm feeding holes per leaf</td>
<td>2.95 ± 0.71</td>
<td>2.19 ± 0.66</td>
<td>0.781</td>
<td>0.457</td>
</tr>
</tbody>
</table>

CO₂ effect was tested with independent samples t-test.

Table 2
The significance (p-values from GLM analysis) of CO₂ and herbivore damage effects on the emission of individual monoterpenes

<table>
<thead>
<tr>
<th>Compound</th>
<th>CO₂</th>
<th>Insect damage</th>
<th>CO₂ x insect damage</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Thujene</td>
<td>0.042</td>
<td>0.019</td>
<td>0.997</td>
</tr>
<tr>
<td>α-Pinene</td>
<td>0.027</td>
<td>0.034</td>
<td>0.92</td>
</tr>
<tr>
<td>Sabinene</td>
<td>0.032</td>
<td>0.031</td>
<td>0.98</td>
</tr>
<tr>
<td>β-Pinene + myrcene</td>
<td>0.030</td>
<td>0.022</td>
<td>0.96</td>
</tr>
<tr>
<td>Limonene</td>
<td>0.047</td>
<td>0.003</td>
<td>0.961</td>
</tr>
<tr>
<td>1,8-Cineole</td>
<td>0.029</td>
<td>0.021</td>
<td>0.818</td>
</tr>
<tr>
<td>γ-Terpineene</td>
<td>0.060*</td>
<td>0.241</td>
<td>—</td>
</tr>
</tbody>
</table>

*Tested with independent samples t-test.

covariate for total VOC emission from whole plant, CO₂ treatment did not have any effect, while herbivore damage increased significantly the total VOC emission (F = 25.487, p < 0.001). Again, P. xylostella-damaged plants had higher emission than control or S. littoralis-damaged plants. Nonetheless, statistical analysis did not indicate significant interactive effects of CO₂ and herbivore treatments.

(Z)-3-Hexenyl acetate (Fig. 2a) and sesquiterpene (E,E)-α-farnesene (Fig. 2b) were emitted mainly from P. xylostella and S. littoralis-damaged plants, while homoterpene (E)-4,8-dimethyl-1,3,7-nonatriene (DMNT) (Fig. 2c) was emitted only from P. xylostella- and S. littoralis-damaged plants. The emission of DMNT was significantly higher from plants damaged by P. xylostella than from plants damaged by S. littoralis (Fig. 2c). In contrast to MTs, growth of cabbage at elevated CO₂ did not affect the emissions per shoot dry weight of these herbivore-inducible compounds (Fig. 2a-c). Furthermore, few herbivore-damaged plants emitted small amounts of linalyl acetate, (Z)-3-hexenol and (E)-2-hexenal.

Relative proportions of total MTs, total induced compounds (DMNT, (E,E)-α-farnesene and (Z)-3-hexenyl acetate) and total other compounds (nonanal, decanal and hexanal) of the total VOC emission did not change due to growth at elevated CO₂ concentration. However, insect damage altered the proportions of these compound groups by increasing the proportion of induced compounds, and thereby decreasing the proportions of MTs and other compounds (Table 3).

4. Discussion and conclusions

Our results indicate that intact cabbage plants and herbivore-damaged plants grown at elevated CO₂ concentration had reduced MT emissions per shoot dry weight, while such reduction did not take place in case of emission of herbivore-induced compounds per shoot dry weight. The dry mass of plants grown at elevated CO₂ was increased, which is in agreement with the earlier studies showing that thicker and heavier

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leaves are a typical response to elevated CO$_2$ (Pritchard et al., 1999; Staadt et al., 2001). Studies with excised leaves may exaggerate emission rates induced by mechanical damage and oral secretions of moth larvae showing even eight-fold greater emissions (Schmelz et al., 2001). Expressing emissions on dry weight basis might distort the CO$_2$ effect on emissions to some extent, but quantification of emissions per dry weight has been frequently used (e.g. Peñuelas and LLusia, 1997; Constable et al., 1999b; Staadt et al., 2001).

It is unclear whether *Brassica* species has specialized storage ducts or glands for terpenes, from where the emission could occur. In *Q. ilex*, which does not store terpenes, the incorporation of photosynthetic carbon to emitted MTs may take place within 20 min (Loreto et al., 1996). In the case of conifers, volatile terpenes are emitted to the surrounding environment from storage structures (Langeheim, 1994). Our results of reduced MT emissions from cabbage are consistent with the results of Loreto et al. (2001) who demonstrated reduced MT emission from *Q. ilex* leaves at elevated CO$_2$. However, their results were expressed as emission per leaf area, and sampling was conducted at elevated CO$_2$, whereas we presented VOC emission per shoot dry weight and sampled VOCs at ambient air. During early ontogeny stem tissues may significantly contribute to photosynthesis (Sack et al., 2002), and thereby probably to VOC emissions. Loreto et al. (2001) showed that decrease in total MT emission resulted from decrease in α-pinene, β-pinene, and sabine emissions, similarly as in our experiment. However, limonene emission was increased from *Q. ilex* (Loreto et al., 2001) while we detected significant reduction in limonene emission from cabbage plants grown at elevated CO$_2$. The explanation for reduced MT emissions from cabbage grown at elevated CO$_2$ could be the reduced MT synthesis due to depressed photosynthesis at ambient CO$_2$ and consequent lack of carbon for their synthesis as shown by Loreto et al. (2001). On the other hand, Staadt et al. (2001) found that *Q. ilex* leaves grown at elevated CO$_2$ have higher MT emission per protected leaf area and per leaf dry mass than leaves grown at ambient CO$_2$ regardless of the assay CO$_2$ concentration. However, we conducted VOC collections under ambient CO$_2$ concentration for both CO$_2$ treatments.

The studies with terpene storing plant species have indicated a non-significant effect of elevated CO$_2$ on MT emissions per dry weight (Peñuelas and LLusia, 1997; Constable et al., 1999b). However, MT concentration in storage tissues may either decrease (Litvak et al., 2002; Sallás et al., 2003), increase (Sallás et al., 2001) or be unaltered (Kainulainen et al., 1998) in conifers grown under elevated CO$_2$. Litvak et al. (2002) showed that wounded Douglas fir needles had lower total MT, α-pinene and β-pinene pool sizes than intact needles at both ambient and elevated CO$_2$. In our study the emission of MTs from insect-damaged plants was higher than the emission of MTs from intact plants regardless of the CO$_2$ concentration during growing. Also, insect-damage clearly triggered the emission of three inducible compounds indicating that cabbage learns on induced defence as suggested earlier by Geervliet et al. (1997). Litvak et al. (2002) concluded that elevated CO$_2$ might induce the reduction in the rate of MT accumulation in relation to other constituents, like starch, in needles. They also suggested that Douglas fir depends more on constitutive defence than on induced defence, since there was no notable induction of MT cyclase activity in wounded needles.
On the present study, excess carbon might be allocated to the formation of other secondary metabolite groups leading to reduced MT emissions on dry weight basis and possible down-regulation of MT synthesis. It is often demonstrated that plants grown at elevated CO₂ have higher concentrations of phenolic compounds (e.g. Agrell et al., 2000; Sallas et al., 2001; Coviella et al., 2002). In cotton plants elevated CO₂ may increase amounts of phenolic compounds, but terpenes are not affected as shown by Coviella et al. (2002). In cabbage seedlings (cv. Lemos) elevated CO₂ slightly increased the concentration of total phenolics, and one of the aromatic glucosinolates in foliage (Reddy et al., unpublished data). This trade-off between different biosynthetic pathways under high CO₂ could be species specific or dependent on the developmental stage of plants.

So far, majority of the publications suggest that elevated CO₂ concentration will not substantially increase MT emissions from most of the plant species, but indirectly CO₂-induced increase of temperature may increase MT emission from several plant species. We quantified VOC emissions from cabbages plants and detected that there was a reduction in MT emission but not in emission of induced compounds on dry weight basis in plants grown at elevated CO₂. On whole plant level there was CO₂ effect neither in MT nor in induced compounds emission, but herbivore-damage increased these emissions significantly. Based on this study, the effect of insect herbivores overcomes the effect of elevated CO₂ on the quantity and quality of the cabbage emission. However, further evaluation of direct effects of CO₂ on VOC emission rates is needed, to define the current models of vegetation-based VOC emissions expecting 12% increase in VOC emissions by doubled CO₂ concentration (Constable et al., 1999a).

Acknowledgements

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CHAPTER 3

EMISSION OF *PLUTELLA XYLOSTELLA*-INDUCED COMPOUNDS FROM CABBAGES GROWN AT ELEVATED CO₂ AND ORIENTATION BEHAVIOR OF THE NATURAL ENEMIES


*Plant Physiology* 135, 1984-1992

Emission of *Plutella xylostella*-Induced Compounds from Cabbages Grown at Elevated CO₂ and Orientation Behavior of the Natural Enemies

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Several plant species defend themselves indirectly from herbivores by producing herbivore-induced volatile compounds that attract the natural enemies of herbivores. Here we tested the effects of elevated atmospheric CO₂ (720 μmol mol⁻¹) concentration on this indirect defense, physiological properties, and constitutive and induced emissions of white cabbage (*Brassica oleracea* ssp. *capitata*, cv Lennox and Rinda). We monitored the orientation behavior of the generalist predator *Polistes maculiventris* (Heteroptera: Pentatomidae) and the specialist parasitoid *Cotesia plutellae* (Hymenoptera: Braconidae) to plants damaged by *Plutella xylostella* (Lepidoptera: Plutellidae) in the Y-tube olfactometer. Elevated CO₂ levels did not affect stomatal densities but reduced specific leaf area and increased leaf thickness in cv Lennox. In addition to enhanced constitutive monoterpane emission, *P. xylostella*-damaged cabbages emitted homoterpene (E)-4,8-dimethyl-1,3,7-nonatriene, sesquiterpene (E,E)-α-farnesene, and (Z)-3-hexenyl acetate. Growth at elevated CO₂ had no significant effect on the emissions expressed per leaf area, while minor reduction in the emission of homoterpene (E)-4,8-dimethyl-1,3,7-nonatriene and (E,E)-α-farnesene was observed at elevated CO₂ in one of two experiments. The generalist predator *P. maculiventris* discriminated only between the odors of intact and *P. xylostella*-damaged cv Rinda plants grown at ambient CO₂ concentration, preferring the odor of the damaged plants. The specialist parasitoid *C. plutellae* preferred the odor of damaged plants of both cultivars grown at ambient CO₂ but did not detect damaged cv Lennox plants grown at elevated CO₂. The results suggest that elevated atmospheric CO₂ concentration could weaken the plant response induced by insect herbivore feeding and thereby lead to a disturbance of signaling to the third trophic level.

Several plant species express traits which can indirectly participate in controlling the herbivore population. i.e. the release of volatile compounds after herbivore damage which participate in top-down control of herbivores by attracting predators and parasitoids (e.g. Dicke et al., 1990; Turlings et al., 1991). The emission of these herbivore-induced volatile compounds are strongly affected by abiotic factors such as soil and air humidity, temperature, light, and fertilization rate (Gunningham and Turlings, 2002) as well as biotic factors such as plant cultivar, growth stage of the leaf, and attacking herbivore species (Talabatashi et al., 1994).

The concentration of atmospheric CO₂ is expected to double by the end of this century (Houghton et al., 2001), and a simultaneous increase in the emissions from plants is predicted due to climate warming (Constable et al., 1999a, 1999b). This will affect the hydrocarbon load into the atmosphere and cause changes in ozone and aerosol formation (Andreae and Crutzen, 1997; Petruelas and Llusia, 2001, 2003). Monoterpenes (MTs) have an antioxidative role protecting plants probably in a wide range of stress situations (Loreto et al., 2004), and these changes in MT formation may well be an important issue to be considered in climate change studies. Changes in the atmospheric CO₂ concentration affect plant physiology: plants are larger, grow faster, have increased carbon:nitrogen ratios and a decreased specific leaf area (SLA) under elevated CO₂ (Bazzaz, 1990; Bezemer and Jones, 1998; Poorter and Navas, 2003; Sallas et al., 2003). Presumably higher trophic levels, predators, and parasitoids will also be affected through changes in the host plant quality. An elevated CO₂ concentration has evoked variable changes in the MT emission of plants: no effect (Petruelas and Llusia, 1997; Constable et al., 1999b). MT emission has been higher at elevated CO₂ than at ambient air (Staudt et al., 2001), or CO₂ exposure has decreased total MT emission (Loreto et al., 2001, Vuorinen et al., 2004). Changes in the emitted MTs and other compounds might be dependent on the availability of photosynthetic carbon which is needed in the production of volatiles (Loreto et al., 2001). Even though an elevated CO₂ level stimulates plant growth, the density of stomata might be decreased, as detected with Aribidopsis (Woodward et al., 2002), and this could

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potentially diminish the emission of volatile compounds.

A variety of induced compounds are released from herbivore-damaged plants (Geervliet et al., 1997; Paré and Tumlinson, 1999; Van Den Boom et al., 2004; Vuurinen et al., 2004). Feeding damage of both specialist Platella xylostella and generalist Spodoptera littoralis larvae induced the emission of (Z)-3-hexenyl acetate and (E,E)-4-methyl-3-penten-2-one (DMNT) emission was higher after specialist than generalist damage in cabbages (Brassica oleracea; Vuurinen et al., 2004). Also, larvae of Pieris rapae and Pieris brassicae induced the emission of DMNT and (Z)-3-hexenyl acetate and caused twice as high total emissions as produced by control cabbages (Geervliet et al., 1997). Potting et al. (1999) showed that P. xylostella-damaged Brassica napus plants were attractive to the parasitoid Cotesia plutellae. The induced compounds should contain the chemical information needed by predators and parasitoids to locate their hosts. Although, a number of studies has been conducted on volatiles that attract the natural enemies of herbivores, there has been no report yet on the volatiles emitted from plants grown at elevated CO₂ level and the subsequent consequences on the orientation behavior of the natural enemies.

The objective of this study was to evaluate the effects of an elevated CO₂ concentration on the emission of cabbage plants damaged by the crucifer specialist, the diamondback moth, P. xylostella, which is one of the most serious pests of cruciferous plants found throughout the world (Talekar and Shelton, 1993). The orientation behavior of the generalist predator Podisus maculiventris and the specialist parasitoid C. plutellae were determined, since the response of specialists would be expected to differ from that of generalists. These results are important for evaluation and modeling of the effects of climate change on multitrophic signalling by plant volatiles in food chains both in natural and man-made ecosystems.

**RESULTS**

**Plant Growth and Properties**

In the first experiment, the growth of cv Lennox was more responsive to elevated CO₂ than growth of cv Rinda, cv Lennox exhibited enhanced shoot dry weight and decreased fresh/dry weight ratios in both intact

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**Table 1. Physiological properties (mean ± se) of the intact control cabbages in experiments 1 and 2, and P. xylostella-damaged cabbages in experiment 2**

Plants were grown at ambient (340 μmol mol⁻¹) or elevated CO₂ (770 μmol mol⁻¹) concentration. The CO₂ effect was tested with independent samples t-test.

<table>
<thead>
<tr>
<th></th>
<th>Rinda</th>
<th>Lennox</th>
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<tbody>
<tr>
<td></td>
<td>Ambient CO₂</td>
<td>Elevated CO₂</td>
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<tr>
<td>SLA (cm² g dw⁻¹)</td>
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<td></td>
</tr>
<tr>
<td>5</td>
<td>273.7 ± 70.9</td>
<td>269.8 ± 27.8</td>
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<tr>
<td>SLA (cm² g dw⁻¹)</td>
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<tr>
<td>5</td>
<td>199.1 ± 49.3</td>
<td>143.1 ± 24.6</td>
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<tr>
<td>Shoot fresh weight (g)</td>
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<tr>
<td>5</td>
<td>15.8 ± 4.9</td>
<td>16.8 ± 2.4</td>
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<tr>
<td>Shoot dry weight (g)</td>
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<tr>
<td>5</td>
<td>1.7 ± 0.6</td>
<td>2.2 ± 0.4</td>
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<td>Fresh/dry weight ratio</td>
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<tr>
<td>5</td>
<td>9.6 ± 1.4</td>
<td>7.6 ± 1.0</td>
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<tr>
<td>P. xylostella</td>
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<tr>
<td>Leaf area (cm²)</td>
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<tr>
<td>5</td>
<td>254.8 ± 27.9</td>
<td>227.94 ± 29.2</td>
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<tr>
<td>SLA (cm² g⁻¹)</td>
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<tr>
<td>5</td>
<td>172.5 ± 10.4</td>
<td>158.2 ± 38.5</td>
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<tr>
<td>Shoot fresh weight (g)</td>
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<tr>
<td>5</td>
<td>15.4 ± 2.4</td>
<td>14.23 ± 2.5</td>
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<tr>
<td>Shoot dry weight (g)</td>
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<tr>
<td>5</td>
<td>1.8 ± 0.4</td>
<td>1.78 ± 0.4</td>
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<tr>
<td>Fresh/dry weight ratio</td>
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<tr>
<td>5</td>
<td>8.7 ± 1.1</td>
<td>8.17 ± 1.1</td>
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<tr>
<td>&lt; 2 mm holes per leaf</td>
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<tr>
<td>5</td>
<td>2.1 ± 1.6</td>
<td>2.6 ± 3.3</td>
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<tr>
<td>&gt; 2 mm holes per leaf</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>7.3 ± 4.2</td>
<td>5.7 ± 2.4</td>
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</tbody>
</table>

and P. xylostella-damaged plants and enhanced shoot fresh weight in intact plants (data not shown). Leaf thickness was increased and SLA was decreased in cv Lennox at elevated CO$_2$ but stomatal density was not affected in either of the cultivars (Table 1). In the second experiment, leaf area, SLA, shoot fresh weight, and shoot dry weight in intact Rinda and Lennox cvs did not differ between ambient and elevated CO$_2$ concentrations, although SLA of P. xylostella-damaged plants of cv Lennox was decreased. Even though P. xylostella caused more feeding holes in cv Lennox grown at elevated CO$_2$, the shoot dry weight of those plants remained higher at elevated CO$_2$ than at ambient CO$_2$ (Table 1).

**Figure 1.** Emission of intact control and P. xylostella-damaged cabbages ( cvs Rinda and Lennox) grown at ambient or elevated CO$_2$ concentration. A and B: Emissions of total MTFs (sabinene, limonene, 8-pinene + myrcene, 1,8-cineole, α-pinene, and α-phellandrene; mean ± SD). C and D: DMNT. E and F: (E,E)-farnesene. G and H, (Z)-3-hexenyl acetate (ng cm$^{-2}$ h$^{-1}$) from intact control and P. xylostella-damaged cabbage plants (n = 5) grown at ambient (360 μmol mol$^{-1}$) or elevated (720 μmol mol$^{-1}$) CO$_2$ concentration in experiment 1. P-values indicate the main effect tested by Kruskal-Wallis-test. Different letters above the bars indicate significant differences between the treatments by Tukey or Dunnett T3 post hoc tests.

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The Emission of Volatile Compounds

The composition of the cabbage emission profile in both experiments was identical. The emission of intact control cabbages consisted mainly of constitutive MTs (sabinene, limonene, β-pinene + myrcene [these two compounds were not separated on our HP-5 column], 1,8-cineole, α-thujene, and α-pinene), while P. xylostella-damaged cabbages emitted in addition to MTs also induced compounds, DMNT, sesquiterpenes (E,E)-α-

Figure 2. Emission of intact control and P. xylostella-damaged cabbages (cv. Rinda and Lennox) grown at ambient or elevated CO2 concentration. A and B, Emissions of total MTs (sabinene, limonene, β-pinene + myrcene, 1,8-cineole, α-thujene, and α-pinene; mean ± se). C and D, DMNT. E and F, (E,E)-α-farnesene. G and H, (Z)-3-hexenyl acetate (ng cm⁻² h⁻¹) from intact control and P. xylostella-damaged cabbage plants (n = 5) grown at ambient (360 μmol mol⁻¹) or elevated (720 μmol mol⁻¹) CO2 concentration in experiment 2. P-values indicate the main effect tested by Kruskal-Wallis test. Different letters above the bars indicate significant differences between the treatments by Dunnett T3 post hoc test.

farnesene, and (Z)-3-hexenyl acetate. The emission data expressed on a leaf area basis and collated from both experiments revealed that growing the plants at elevated CO$_2$ had no effect on the emission of total MTs (Figs. 1 and 2, A and B) or individual (data not shown) MTs of intact control and P. xyllostella-damaged cabbage cv Rinda and Lennox. Emissions of individual MTs of cv Rinda in experiment 1 were significantly higher from herbivore-damaged than intact control plants (main effect one-way ANOVA $P < 0.005$), while in experiment 2 only the emission of a-thujene was enhanced by P. xyllostella-damage (main effect K-W $P = 0.037$). On the other hand, a-pinene and b-pinene emissions in experiment 1 and the emissions of all individual MTs of cv Lennox in experiment 2 were significantly higher from herbivore-damaged plants than from intact plants.

In both cultivars and both experiments, the emissions of induced compounds, DMNT, (E,E)-a-farnesene, and (Z)-3-hexenyl acetate, were not particularly responsive to the CO$_2$ concentration (Figs. 1 and 2) even though elevated CO$_2$ seemed to diminish the emission of DMNT from cv Lennox and (E,E)-a-farnesene from cv Rinda in experiment 2. The emissions of induced compounds were naturally higher from herbivore-damaged plants than from intact plants (Figs. 1 and 2, C–H). P. xyllostella-damage tended to increase more clearly the emission of total MTs and induced compounds from plants grown at ambient CO$_2$ than at elevated CO$_2$.

The relative proportions of total MTs, total induced compounds, and total other compounds (hexanal, (Z)-3-hexenyl, heptanal, nonanal, and decanal) in relation to total volatiles were not affected by the elevated CO$_2$ concentration in either of the experiments. Again, P. xyllostella-damage increased significantly ($P < 0.001$) the proportion of total induced compounds and thereby decreased significantly the proportion of total MTs ($P < 0.001$) in both cultivars and in both experiments (Fig. 3). In experiment 1, the percentage of induced compounds tended to be larger than in experiment 2 (Fig. 3).

**Behavioral Response of** P. *maculiventris* **and C. *platellae***

In experiment 1, the generalist predator *P. maculiventris* discriminated only between the odors of intact and P. xyllostella-damaged cv Rinda plants, which were grown at ambient CO$_2$ concentration, preferring the odor of the damaged plants (Fig. 4A). In experiment 2, the specialist parasitoid *C. platellae* preferred the odor of damaged plants (both cultivars) when the plants had been grown at ambient CO$_2$ but did not detect damaged cv Lennox plants when they were grown at elevated CO$_2$ (Fig. 4B).

**Discussion**

**Elevated CO$_2$ and Plant Properties**

As indicated by the decreased SLA and the increased leaf thickness in experiment 1, cv Lennox was more responsive to elevated CO$_2$ than cv Rinda. The SLA of Lennox was reduced at elevated CO$_2$ in both experiments although in experiment 2 only in the plants fed by *P. xyllostella*. This intraspecific variation in the SLA response to elevated CO$_2$ is well known in the family Brassicaceae (Van Der Kooij et al., 2000). The analysis of more than 100 plant species has revealed that there is an average 11% reduction of stomatal density attributable to doubled atmospheric CO$_2$ (Hetherington and Woodward, 2003). We did not find significantly diminished stomatal density in cabbages grown at elevated CO$_2$, suggesting that the small

![Figure 3. Percentage of MTs and induced compounds in the total emission. A. Emission of total MTs (linalone, linalool, b-pinene + myrcene, 1,8-cineole, a-thujene, and a-pinene), total induced compounds (DMNT, (E,E)-a-farnesene, (Z)-3-hexenyl acetate, and (Z)-3-hexenyl), and total other compounds (hexanal, (Z)-3-hexenyl, nonanal, and decanal) in experiments 1 and 2 from intact control and P. xyllostella-damaged cabbage cv Rinda and Lennox (n = 10).](image-url)
Figure 4. The orientation behavior of P. maculiventris and C. plittellae. The percentage of A. P. maculiventris in experiment 1 and B. C. plittellae in experiment 2 choosing for P. xylostella damaged or intact control cabbage (cv. Rinda and Lennox) plants grown at ambient CO\textsubscript{2} (CC) or elevated CO\textsubscript{2} (EC) in the leaf-beat olfactometer. Asterisks indicate significant (\(P < 0.05\), **\(P < 0.01\), *binomial test preference toward other odor source. a indicates the total number of predators or parasitoids used in the assay including the individuals that did not show any preference for either odor source. DBM, diamondback moth. P. xylostella.

reduction in the emission of DMNT and \((E,E)-\alpha\)-farnesene from P. xylostella-damaged cabbages is not a consequence of a lower emission rate through stomata but probably is a reflection of restricted synthesis of volatile compounds. However, at the moment the importance of the density and behavior of stomata for controlling total emission is still unclear (Nitisse et al., 2003).

**Elevated CO\textsubscript{2} and Plant Emissions**

The elevated CO\textsubscript{2} concentration per se had no significant effect on constitutive MT emission from intact control plants, while it seemed to have overall reducing, although nonsignificant, impact on MT emission from herbivore-damaged plants. In an earlier study, we detected decreased MT emissions per shoot dry weight from intact and herbivore-damaged cv Lennox cabbages grown at elevated CO\textsubscript{2} (Vuorinen et al., 2004). At least in the case of intact cabbages, the observed decrease in the MT emission might have been a consequence of expressing emission results per shoot dry weight since the intact control plants grown at elevated CO\textsubscript{2} concentration had higher shoot dry weights than plants grown at ambient CO\textsubscript{2} (Vuorinen et al., 2004). In general, emissions from plants have been expressed on a dry weight basis (e.g., Pertuelas and Llusia, 1997; Constable et al., 1999b; Staudt et al., 2001) or on a leaf area basis (e.g., Loreto et al., 2001, 2004). If the MT emission in this study was expressed on a shoot dry weight basis (data not shown), then it was significantly decreased from herbivore-damaged cabbages grown at elevated CO\textsubscript{2}.

In earlier studies, the reduction of MT emission from species which do not store terpenes, such as Quercus ilex (Loreto et al., 2001) and cabbage (Vuorinen et al., 2004), has been detected at elevated CO\textsubscript{2} concentration. In contrast, Staudt et al. (2001) found that Q. ilex leaves grown at elevated CO\textsubscript{2} exhibited higher MT emission per projected leaf area and per leaf dry mass than leaves grown at ambient CO\textsubscript{2} regardless of the CO\textsubscript{2} concentration during sampling. We collected volatile compounds at ambient CO\textsubscript{2}. In a trial test, P. xylostella-damaged plants grown and sampled at elevated CO\textsubscript{2} had significantly lower MT emission than those plants grown at elevated CO\textsubscript{2} and sampled at ambient CO\textsubscript{2}, while CO\textsubscript{2} concentration during sampling of volatile compounds did not affect MT emissions from intact cabbages (data not shown). Assumingly the decrease in emission of MTs at elevated CO\textsubscript{2} would have been stronger if the sampling would have been conducted at elevated CO\textsubscript{2}. When one considers terpene storing plants such as conifers (Constable et al., 1999b) or Rosmarinus officinalis (Pertuelas and Llusia, 1997), elevated CO\textsubscript{2} concentration appeared to have no impact on MT emission. Litvak et al. (2002) detected a lower total MT pool size in wounded Douglas fir needles than in intact needles at ambient and elevated CO\textsubscript{2} concentrations, suggesting that the rate of MT accumulation decreases in relation to other compounds such as starch at elevated CO\textsubscript{2}. They also concluded that Douglas fir depends more on constitutive MTs than on induced compounds, which is not the case with cabbage. The Brassica species do not have MT pools in their leaves but are able to produce herbivore-induced volatile compounds.

In our earlier study we found no impact of CO\textsubscript{2} levels on the emission of induced compounds (Vuorinen et al., 2004), while in this study CO\textsubscript{2} concentration seemed to decrease the emissions of \(P. xylostella\)-induced compounds. The variation in the emission of induced compounds between individual plants was notable, and therefore no clear statistically significant CO\textsubscript{2} effect was observed. Also, feeding habits and the type of damage caused by \(P. xylostella\) larvae is not necessarily equal among the plants that might have great impact on the emission rates of induced compounds. Herbivore-induced compounds are derived.
mainly from the jasmonic acid signaling pathway, which modulates lipoygenase pathway producing green leaf volatiles, and two terpenoid pathways (Dicker et al., 2003), malononic acid pathway in the cytosol and the deoxyxylulose phosphate/methylerythritol pathway in plastids (Spurný, 2002). In parallel to our earlier results (Vuorinen et al., 2004), at least volatile compounds from the jasmonic acid signaling pathway were detectable from cabbage after *P. xylostella*-damage.

**Trophic Interactions**

*P. maculiventris* nymphs preferred the odor of damaged plants grown at ambient CO₂ but showed little preference between damaged and intact plants when the cabbages were grown at elevated CO₂. According to the opinions of Vet and Dickie (1992) herbivore-derived volatiles are the most reliable cues for generalist predators for prey locating. Therefore predators should not intramly react to herbivore-induced plant-derived semiochemicals, which are more abundant but less reliable and which can be used for prey locating after associative learning process. However, Reddy et al. (2012) observed that the generalist predator *Chrysopeola carnea* is attracted to the odor of (Z)-3-hexenyl acetate, which is released in large amounts from herbivore-damaged cabbages. Furthermore, Dickens (1999) detected that *P. maculiventris* has olfactory receptors for (Z)-3-hexenyl butrate which is induced by the Colorado potato beetle, and the predator was also attracted to a blend comprising (E)-2-hexenol, (Z)-3-hexenol, (Z)-3-hexenyl, (E)-2-hexenyl, and MeA. Sant’Ana et al. (1999) found that third- and fifth-instar nymphs reacted to common plant green leaf volatiles (E)-2-hexenal and (E)-2-hexenyl, which is also the dominant odor of the male-produced aggregated pheromone. The fifth-instar responded also to nonenal and (Z)-2-hexenal (Sant’Ana et al., 1999). Neither (E)-2-hexenol nor (Z)-2-hexenyl was emitted from the damaged cabbages in this study. The greatly reduced emission of (Z)-3-hexenyl acetate from *P. xylostella*-damaged cv Kinka plants grown at elevated CO₂ may be an explanation for the reduced host-searching efficiency of *P. maculiventris* in this study. On the other hand, there seemed to be elevated emission of (Z)-3-hexenyl acetate at elevated CO₂ from *P. xylostella*-damaged cv Lennox plants, but nonetheless the predator still did not show a preference for the odor of damaged plants.

Orientation of the parasitoid *C. plutellae* toward damaged plant was stronger than that of the generalist predator. The response of parasitoids to plant-herbivore complexes differs among the plant species and herbivores involved. For instance, *C. plutellae* shows a specific response toward the host-plant complex, unlike *C. gilgerata*, and the presence of the norbactin affects the specificity of the response of the wasps (Shoji et al., 2004, Liu and Jiang, 2003) showed that volatile compounds from Chinese cabbage were more attractive to female *C. plutellae* than those from white cabbage when both plant species were either intact or infested with *P. xylostella*. DMNT and other terpenes appeared to be important cues for orientation of *C. plutellae* to *P. xylostella*-damaged plants (Shoji et al., 2003). (E,E)-α-Farnesene is one of the *P. xylostella*-induced compounds in all cabbage varieties and in this respect differs from DMNT and (Z)-3-hexenyl acetate (Shoji et al., 2004). The decreasing impact of elevated CO₂ on DMNT and MT emissions from cv Lennox probably explain the reduction in host-searching efficiency of *C. plutellae*. As our results point to a reduced orientation efficiency of the specialist parasitoid toward herbivore-damaged plants grown at the forecasted elevated atmospheric CO₂ concentration, this finding deserves further and more extensive research on other plant species, *C. plutellae* and other Braconids at the top of food chains maintain important position in terms of global biodiversity (Dolphin and Quicke, 2001). In the future, these parasitoid species may become threatened also by elevated atmospheric CO₂ concentration if their host-searching efficiency is impaired.

**MATERIALS AND METHODS**

**The Experiments, Plant Material, and CO₂ Exposure**

We performed two separate experiments under similar growth conditions for the plants. produced similar *Plutella xylostella*-damaged, sampled volatiles, and used the Volite olfactometer in a similar manner in both experiments. In the first experiment, we studied the orientation behavior of the generalist *Pelliaus maculiventris*, and in addition to plant growth measurements we also measured leaf thickness and stomatal density from intact control plants. In the second experiment, we tested the orientation behavior of the specialist * Orius virens. Emission data from both experiments are presented.

White cabbage (Brassica oleracea var. capitata cv. Lennox and Rindal seedlings were grown in 14 plastic pots filled with sphagnum peat and sand (1:1, v/v) and grown for 24 to 28-d at ambient (360 µmol mol⁻¹) or elevated (720 µmol mol⁻¹) CO₂ concentrations in growth chambers (Biohim 2000, Koyo-Service Oy, Helsinki) at 25°C, 75% relative humidity, and 22.2° photoperiod (1500-3000 µmol m⁻² s⁻¹) photosynthetically active radiation during the light period). CO₂ enhancement was maintained for 24 d. The CO₂ treatments and seedlings were rotated among the two chambers weekly to randomize any systematic chamber effect across the seedlings. The seedlings were watered daily and fertilized weekly with 0.1% of N-(15); NPK, Rokkla, Finland) at a rate of 0.08 with 1 L plant⁻¹, starting 2 weeks after sowing.

**Leaf Properties**

The SLA was determined by scanning the leaf with a Logitech scanner and analyzing the leaf area using a Logitech Photo Touch Color-program (Logitech, Morges, Switzerland), and dividing the leaf area by the leaf dry weight. Leaf thickness was measured from the 3rd and 5th youngest leaves between the veins in the left top one-quarter of the leaf with a microscope (Mitutoyo Mod. ID-G1 BLE, Mitutoyo Kanagawa, Japan). Stomatal density was determined after collection of volatiles by pressing a small proportion of the adaxial and abaxial leaf surfaces against a glass microscope slide painted with instant glue (Locutte Super Glue +, Hanked Locutte, Cleveland). After drying for 1 to 2 min, the leaf was peeled off and replicas were examined at 200x magnification with a light microscope (Oikosan, 2003).

**Insects and *P. xylostella*-Damage**

Larvae of the crucifer specialist *P. xylostella* (aphidesiphon: Plutellidae) were reared at 25°C, 50% relative humidity, and 16:8 h photoperiod on broccoli (B. Plant Physiol. Vol. 135, 2004
ethylene gas, foliar infections. Feeding damage was caused by transferring eight third-instar larvae onto five randomly selected plants grown at ambient or elevated CO2 for 48 h. Five intact plants from both CO2 concentrations were used as controls. Plants with the feeding larvae were kept in a separate growth chamber in similar environmental conditions as described earlier.

Spined soldier bug P. maculicruris (Hemiptera: Pentatomidae) nymphs were obtained from Kappert Biological Systems, Netterl in Heino, The Netherlands. The nymphs were fed mainly with larvae of yellow meadowlark, Sturnella neglecta (Sturnellidae: Sturnellidae) at 20°C ± 1°C, 75 ± 5% relative humidity, and a 16:8-h photoperiod. First instars were used in the behavioral assay.

C. platycarpe Kudjurose (Hymenoptera: Braconidae) pupae were obtained from the Centre de Coopération Internationale en Recherche Agronomique pour le Développement (Montpellier, France). Second instar (larvae of P. zonatum) feeding on brood was offered to C. platycarpe females for egg laying at 25°C ± 1°C, 50% relative humidity, and a 16:8-h photoperiod. Pupae were placed in a clean cage until emergence. Adults were provided with 20% honey-water solution for feeding. From 3- to 5-d-old C. platycarpe females were used in the behavioral assay. Predators and parasitoids had not experienced the odor of P. zonatum-damaged white cabbage before the behavioral assay.

Collection of Volatile Compounds

Intact control plants and P. zonatum-damaged plants from both CO2 concentrations from both cultures (n = 10) were used for the collection of the volatile compounds. Cabbage with a clipped and a slightly pruned near system in a 15-L, vial filled with tap water were individually enclosed inside 1-L glass vessels, which were closed with a glass lid sealed with Teflon tape and parafilm. After 5 min adjustment, a sample was collected for 30 min after approximately 180 mg Texas-7A adenine (Sigma, mol wt 180) by pulling the sample through 6-mm diameter Teflon tubing with a vacuum pump (KNF Neuberger, Freiburg, Germany. Model 7022 22HL). An inlet for purified air and an outlet for sampling were on the top of the vessel. The airflow was calibrated with the mini-Stack calibrator (Model M-5, A.P. Beck, Ottershain, FL) and flow rate was set to 0.235 L·min⁻¹ for 20°C and 258 mm Hg. A 50 mL syringe was filled with 0.5 mL H₂O and air before sampling. The sampling was performed at 22°C and 50% RH at 0.5 mL·min⁻¹ by 20°C and 258 mm Hg. A 50 mL syringe was filled with 0.5 mL H₂O and air before sampling. The sampling was performed at 22°C and 50% RH

Behavioral Assay

The Vitrino olfactometer (main arm: 10 cm, other arm 10 cm, lid: 1 cm, and angle between two arms approximately 90°) was used in this test. Its size and volume corresponded to a P. maculicruris and C. platycarpe. The insects were given a choice between P. zonatum-damaged and intact cabbages grown at ambient or elevated CO2 concentrations. The plants were carefully placed in the 1-L vessels which were closed with Teflon sealed lids with a slit. The presentation area was purged with activated carbon and divided via Teflon tubing into two separate flows (0.20 L·min⁻¹) which both passed through a 1-L glass vessel containing cabbage as an odor source and leading towards one arm of the Vitrino. The airflow was adjusted with pressure and needle valves and calibrated daily with 0.1 mL·min⁻¹ of 5-mm min-5-01, 101, 0111, 1111, and 1011. The insects were introduced to the downwind end of the 1-lids and observed for 5 min or until they made their final choice. The choice was recorded when the insect passed two-thirds or the far end of the Vitro arm for P. maculicruris and C. platycarpe, respectively. Other sources, vessels, and lids were replaced after testing six P. maculicruris or eight C. platycarpe to avoid any errors caused by the olfactometer system itself.

Induced Compounds at Elevated CO2 and Trichothecene Signaling

Glucosinolate content was highest treated at 18°C before use, and the V tube was turned horizontally around after each test insect and reared with 10% ethanol at least after every third tested insect. In the experiments, approximately 60 P. zonatum nymphs and 60 C. platycarpe females were tested at 18 and 8 different odor source pairs, respectively, from both CO2 concentrations and cultivars.

Statistical Analyses

Statistical analyses were performed using SPSS 11.5 for Windows statistical package. The main effect of treatments was tested by nonparametric Kruskal-Wallis test and one-way ANOVA and multiple comparisons by Dunnett T3 and Tukey post hoc tests. ANOVA-transformed relative proportions of compound groups were tested by independent samples t test. The data from the behavioral assay were analyzed with the nonparametric binomial test to test whether there was a significant difference in attraction between the two odor sources from plants receiving different treatments (test proportion was set to 0.5).

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LITERATURE CITED


CHAPTER 4

EMISSION OF VOLATILE ORGANIC COMPOUNDS FROM TWO SILVER BIRCH (*BETULA PENDULA* ROTH) CLONES GROWN UNDER ELEVATED CO₂ AND O₃ CONCENTRATIONS


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Emission of volatile organic compounds from two silver birch 
(Betula pendula Roth) clones grown under ambient and 
elevated CO₂ and different O₃ concentrations

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Abstract

We analysed the emission of volatile organic compounds (VOCs) from two clones (4 and 80) of silver birch (Betula pendula Roth) trees exposed to doubled ambient CO₂ and O₃ singly and in combination, in open-top chambers. VOCs were collected in July and in August from detached twigs. The effect of twig detachment as such on emissions was separately studied, and it increased the emissions of green leaf volatiles. The emission in July from both clones was dominated by sesquiterpenes (SQTs) germacrene D, (E,E)-α-farnesene, α-copaene and β-bourbonene, while in August, the emission was dominated by monoterpenes (MTs) (E)-β-cocine and (Z)-ocimene. Elevated CO₂ concentration marginally decreased total MT emission in July, while in August the total MT emission was enhanced by elevated CO₂. O₃ or CO₂ + O₃-exposure did not have any effect on total MT or total SQT emissions. In general clones 4 and 80 emitted total quantified VOCs (19 compounds) 12520 and 8590 ng g⁻¹ fw h⁻¹ in July, and 4640 and 4990 ng g⁻¹ fw h⁻¹ in August, respectively. Clone 4 emitted more β-pinene + myrcene, (Z)-ocimene and (E)-β-cocine in July than clone 80, which emitted more linalool in July, and hexanal in August than clone 4. Elevated CO₂ tended to decrease the emissions of nonanal and (E)-β-cocine in July, while O₃ and CO₂ + O₃ had no effects on emissions. Our results indicate that elevated CO₂ and O₃ concentrations do not have considerable effect on silver birch emissions by increasing the carbon allocation to VOCs or by inducing the emission of novel compounds. Other factors, such as temperature, light and herbivores might conceal the effects of these atmospheric gases. High SQT proportion in emission profile suggests that B. pendula may have substantial role in biogenic aerosol formation in boreal forests.

Keywords: Green leaf volatiles; Induced defense; Monoterpene; Sesquiterpene; VOCs

1. Introduction

Atmospheric concentrations of greenhouse gases CO₂ and O₃ are continuously rising due to human activities (IPCC, 2001). The elevating atmospheric CO₂ concentration results in the warming of the lower atmosphere, which might lead to a higher emission of volatile organic compounds (VOCs) from plants (Constable et al., 2002).
Phytotoxic ozone itself can cause severe damage to cultivated and natural crops leading to foliar injury, and thereby to yield reduction (Ashmore, 2002). Ozone also activates several defence responses in plants which may cause changes in secondary metabolism (Kangasjärvi et al., 1994). There are few studies investigating the effects of singly elevated CO₂ (Constable et al., 1999b; Loreto et al., 2001a; Staude et al., 2001; Vuorinen et al., 2004a) or elevated O₃ (Heiden et al., 1999, 2003; Peláez et al., 1999; Lussi et al., 2002; Vuorinen et al., 2004a) on the emission of plants, but the effects of elevated CO₂ and O₃ concentrations in combination are not reported at all.

Guenther et al. (1995) estimated the annual global VOC emission from vegetation to be ~1.2 x 10¹¹ g of carbon. The concentration of carbon-based secondary and structural compounds in plants is expected to increase due to the enhanced atmospheric CO₂ concentration (Peláez and Estiarte, 1998), leading also to the enhanced emission of plants. VOCs can regulate the oxidative capacity of the troposphere, carbon monoxide, O₃ and aerosol budgets, and together with high concentration of nitrogen oxides in the sunlight they form more phytotoxic O₃. On the other hand, when levels of nitrogen oxides are low, oxidation of VOCs removes O₃ from the troposphere (Lerdau and Slobodkin, 2002).

Lately a question has risen whether the plant secondary metabolites were evolved as a defence against herbivores or as a protection against prevailing climate conditions. Close and McArthur (2002) suggested that phenolic compounds of plants function rather as a sunscreen than a protection against herbivores. Loreto et al. (2001b) showed that isoprene emission can actually protect plants from O₃ damage by direct quenching of O₃. It has also been suggested that isoprene emission protects the photosynthetic apparatus against heat stress (Sharkey and Singsaas, 1995).

Betula pendula has been reported to be a monoterpene (MT) emitter, releasing mainly α-pinene, β-pinene, sabine, 4-ocimene (Hakola et al., 1998, 2001; Lindfors et al., 2000), and in early summer also 3-carene (Hakola et al., 1998). Zhang et al. (1999) found also sesquiterpenes (SQTs), aliphatic and aromatic compounds from the emission of intact silver birch branches.

The objective of this study was to examine the quality and quantity of the emission from silver birch clones 4 and 80 grown under elevated CO₂ and O₃, singly and in combination. The information is needed when estimating the emission of plants and carbon load to the atmosphere under future climate conditions. We tested the following hypotheses: (1) increased carbon resource at elevated atmospheric CO₂ concentration may result in carbon allocation to secondary metabolites, and lead to enhanced emissions of constitutive VOCs, (2) elevated O₃ may induce the emission of herbivore-inducible VOCs, and thereby lead to enhanced emission of inducible plant volatiles, (3) combination of elevated CO₂ and O₃ may result in substantial increase of both constitutive and inducible VOC emission, and (4) the clones differ in the emission of VOCs depending on their tolerance to tropospheric ozone.

2. Materials and methods

2.1. Experimental site, plant material and CO₂ and O₃ exposure in open-top chambers

Two fast-growing clones of silver birch (B. pendula Roth) with different ozone-sensitivity were selected amongst 15 clones growing at the experimental site at Suonenjoki Research Station (Finland, 62°38'N, 27°03'E). Clone 4 has been earlier classified as an ozone-tolerant and clone 80 as an ozone-sensitive genotype (Pääkkönen et al., 1997). One-year old silver birch saplings were planted in the experimental site in spring 1993. The selected clones were exposed to elevated CO₂ and O₃ singly and in combination in an open-top chamber (OTC) system from 1999 to 2001 during growing seasons. Five treatments were established: outside control (OC), chamber control (CC), elevated CO₂ (2 x ambient, EC), elevated O₃ (2 x ambient, EO), and elevated CO₂ and elevated O₃ concentration (both 2 x ambient, EC + EO). The experiment was organised as a randomised incomplete blocks design. Both clones were replicated four times in each treatment, thus there were 40 trees in the experiment. Ozone was produced from pure oxygen with an ozone generator (FisherOG5, VTU Umwelt- und Verfahrenstechnik GmbH, D-53359 Rheinbach, Germany) and was monitored with an ozone analyser (Thermo Environmental 49C, Thermo Environmental Instruments Inc., Franklin, MA, USA). For CO₂ exposure, pure liquid CO₂ was supplied from tanks to vapouriser and further through the CO₂ dispensing system to the chambers. The CO₂ concentration in the chambers was monitored with CO₂-H₂O analyser (LiCor 6262, Li-Cor Inc., Lincoln, NE). The trees were fertilised annually and received additional fertilisation of 22, 33 and 41 kg N·ha⁻¹ in 1999–2001, respectively. For more details see Vapaavuo et al. (2002).

2.2. Collection of VOCs from CO₂ and O₃-exposed birches

In the midsummer 2001, VOCs were collected on 3rd and 5th of July from clones 80 and 4, respectively. In the autumn, sampling was conducted on 28th and 30th of August from clones 4 and 80, respectively. We took one sample from each tree (n = 4 clones/treatment/sampling time). The sample twig including 4-8 fully expanded
intact leaves was cut from the top of the branch (long shoots) and placed into 4-ml vial filled with tap water. Each twig was set individually in a 1-L glass chamber, closed with a lid and immediately transported to the laboratory in a cooler for VOC collections. Four samples from different trees were collected simultaneously. The rest of the samples were kept in the closed chambers in another laboratory. For those samples which were to be collected, the regular lid was replaced with Teflon sealed lid with an inlet for purified air and an outlet for sampling. Air purified with activated carbon led to the VOC collection chamber at the rate of 0.110 L min⁻¹ for 0-min prior to the sampling to flush the chamber air. After 0-min adjustment at room temperature and ~300–350 μmol m⁻² s⁻¹ PAR, a sample was pulled through a purified stainless steel tube (Perkin Elmer, ATD sample tubes) filled with approx. 150 mg Tenax-TA adsorbent (Supelco, mesh 60/80) with a vacuum pump (KNF Neuberger, Inc., Freiburg, Germany, Model N022AN.18) at rate 0.100 L min⁻¹ for 1 h. The airflow was calibrated daily with the mini-Buck calibrator (Model M-5, A.P. Buck, Inc., Orlando, FL, USA). The samples were collected under ambient CO₂ at 25°C in July and 22°C in August (room temperature). Samples were analysed by GC-MS (Hewlett Packard GC type 6890, MSD 5973). Trapped compounds were desorbed (Perkin Elmer ATD400 Automatic Thermal Desorption System) at 220°C for 10 min, cryofocused at ~30°C and injected onto HP-5 capillary column (50 μm x 250 μm x 0.25 μm, Hewlett Packard). The carrier gas was helium. The temperature program was 50°C for 1 min, followed by increases of 5°C min⁻¹ to 210°C and 20°C min⁻¹ to 250°C. Compounds were mainly identified by comparison of the mass spectra with those in the Wiley library and pure standards. For quantification of emissions, 19 commercially available reference substances were used. The reference substances for (E)-β-oicinone, (E)-4,6-dimethyl-1,3,7-nonatriene (DMNT) and (E,E)-x-farnesene were not available, therefore the concentration of these compounds were calculated by assuming that the responses would be the same as the responses of (Z)-oicinone for (E)-β-oicinone and DMNT, and (E)-caryophyllene for (E,E)-x-farnesene. After VOC collection, the total mass of the sample twig and leaves was determined and emissions were calculated as ng g⁻¹ total fresh weight h⁻¹. Since reference substances for all the emitted and identified terpenes were not available, the emission of total MTs and total SQTs were expressed as peak area g⁻¹ total fresh weight h⁻¹.

2.3. Testing the effects of twig detachment

Three-year-old tissue cultured silver birch saplings of clones 4 and 8 were used to study the effects of twig detachment on VOC emissions. The saplings were transferred from the experimental field of University of Kuopio to the computer-controlled growth chamber (19/12°C, 50–80% RH, 18 h light:6 h dark photoperiod and 500 μmol m⁻² s⁻¹ PAR) in the end of April before bud burst (5.5 weeks before the start of the experiment) to avoid natural herbivory in the field. The seedlings were watered when needed and fertilised weekly with 0.2% Superphos-9 (19:5:20 N:P:K, Kekkilä, Finland) at the rate of 0.1 L per plant. Herbivores (mainly aphids) were removed manually every day. VOC samples from twigs of intact saplings (n = 4 per clone) were collected by enclosing the twig bearing 5–15 leaves into a 1.5-L glass chamber. Care was taken not to cause damage to the plant. The chamber attached to the tripod, was clamped together with a Teflon plate with one hole for the twig. Air purified with activated carbon entered the chamber from the top via Teflon tubing at the rate of 0.210 L min⁻¹. Sample was pulled through a purified stainless steel tube filled with approx. 150 mg Tenax-TA adsorbent with a vacuum pump at rate 0.200 L min⁻¹ for 0.5 h. The airflow was calibrated daily with the mini-Buck calibrator. The samples were collected at ~300 μmol m⁻² s⁻¹ PAR at 22°C (room temperature). Samples were analysed by GC-MS as described above. Next day the same twig was detached, twig base was placed in a test tube with tap water, and whole twig was enclosed in the 1.5-L chamber, which was closed with a glass lid sealed with Teflon tape and parafilm. VOCs were collected and analysed as explained above. The emissions were calculated as ng g⁻¹ total fresh weight h⁻¹.

2.4. Statistical analyses

Statistical analyses were performed using SPSS 11.0 for Windows statistical package. Data were analysed and are represented as the means of four replicates per clone and treatment. Independent samples t-test was used to analyse the effects of time of the growing season, chamber (OC vs. CC) and clone on emission of total MTs, total SQTs, and individual quantified compounds from both silver birch clones. Main effects and interactions of CO₂, O₃ and clone on emissions at each time, and main effects and interactions of clone and detachment on emissions of individual quantified compounds was analysed by multivariate general linear model (GLM).

3. Results

3.1. Emission profiles

Both clones of silver birch emitted quite similar profile of VOCs. The emission in July was dominated by SQTs, relative proportion of total SQTs ranging from 39% to
### Table 1
Relative proportions of VOCs (% of the total) emitted by two clones of silver birch in July 2001

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Clone 4</th>
<th>Clone 80</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OC</td>
<td>CC</td>
</tr>
<tr>
<td>Monoterpenes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-pinene</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Sabinene</td>
<td>0.3</td>
<td>0.2</td>
</tr>
<tr>
<td>β-Pinene + myrcene</td>
<td>0.5</td>
<td>0.6</td>
</tr>
<tr>
<td>(Z)-ocimene</td>
<td>6.0</td>
<td>13.5</td>
</tr>
<tr>
<td>Limonene</td>
<td>0.3</td>
<td>0.1</td>
</tr>
<tr>
<td>1,8-cineole</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>(E)-β-ocimene</td>
<td>15.8</td>
<td>29.5</td>
</tr>
<tr>
<td>(Z)-farnesol oxide</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>(E)-farnesol oxide</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Terpinolene</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Linalool</td>
<td>1.1</td>
<td>1.7</td>
</tr>
<tr>
<td>Allo-ocimene</td>
<td>0.8</td>
<td>1.8</td>
</tr>
<tr>
<td>p-methoxy-1,5,8-triene</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>Sum of all monoterpenes</td>
<td>25.6</td>
<td>48.5</td>
</tr>
<tr>
<td>Homoterpenes (DMNT)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.9</td>
<td>1.1</td>
</tr>
<tr>
<td>Sesquiterpenes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-elemene</td>
<td>&lt;0.1</td>
<td>0</td>
</tr>
<tr>
<td>α-cedrene</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Acicyphylene</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>α-selinene</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>α-copaene</td>
<td>7.6</td>
<td>6.6</td>
</tr>
<tr>
<td>β-bourbonene (unidentified)</td>
<td>6.3</td>
<td>2.4</td>
</tr>
<tr>
<td>α-bourbonene</td>
<td>0.4</td>
<td>0.2</td>
</tr>
<tr>
<td>β-caryophyllene</td>
<td>3.8</td>
<td>3.4</td>
</tr>
<tr>
<td>β-selinene</td>
<td>1.5</td>
<td>0.9</td>
</tr>
<tr>
<td>3,7,guayacene</td>
<td>0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>α-humulene</td>
<td>3.1</td>
<td>1.9</td>
</tr>
<tr>
<td>α-amorphene</td>
<td>1.7</td>
<td>1.3</td>
</tr>
<tr>
<td>(E,E)-farnesene</td>
<td>10.6</td>
<td>3.8</td>
</tr>
<tr>
<td>Germacrene D</td>
<td>14.5</td>
<td>7.6</td>
</tr>
<tr>
<td>α-muurolene</td>
<td>1.6</td>
<td>1.1</td>
</tr>
<tr>
<td>s-cadinene</td>
<td>1.5</td>
<td>1.2</td>
</tr>
<tr>
<td>β-cadinene</td>
<td>3.6</td>
<td>3.3</td>
</tr>
<tr>
<td>(Z)-calamenene</td>
<td>0.5</td>
<td>0.4</td>
</tr>
<tr>
<td>Cadina-1,4-diene</td>
<td>0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>s-cadinene</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>α-cadinene</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>Caryophyllene oxide</td>
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<td>0.3</td>
</tr>
<tr>
<td>Unidentified sesquiterpenes</td>
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</tr>
<tr>
<td>Sum of all sesquiterpenes</td>
<td>65.9</td>
<td>38.9</td>
</tr>
</tbody>
</table>

Other Compounds

|                     | 8.5 | 11.5| 12.1| 12.1| 11.7 | 9.3 | 11.9| 7.2 | 7.5 | 12.9 |

Data are the means of four replicates in each clone and treatment. OC = outside control, CC = chamber control, EC = elevated CO₂, EO = elevated O₃, EC+EO = elevated CO₂ and elevated O₃.

71% of total VOCs (Table 1) depending on the treatment and the clone. The proportion of MTs varied from 15% to 49%. The main SQTs emitted from silver birch clones in July were germacrene D, (E,E)-farnesene, α-copaene, and β-bourbonene, and the main MTs emitted were (E)-β-ocimene and (Z)-ocimene (Table 1). The emission of silver birch in August was dominated by MTs ranging from 42% to 73% of total VOCs, while SQTs ranged from 16% to 36% (Table 2). The main MTs emitted from silver birch clones in August were the ocimenes, and the main SQTs were (E,E)-farnesene, α-copaene, β-bourbonene and β-caryophyllene (Table 2). Clone 4
Table 2
Relative proportions of VOCs (% of the total) emitted by two clones of silver birch in August 2001

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Clone 4</th>
<th></th>
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<th>Clone 80</th>
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<td>CC</td>
<td>EC</td>
<td>EO</td>
<td>EC+EO</td>
<td>OC</td>
<td>CC</td>
<td>EC</td>
<td>EO</td>
<td>EC+EO</td>
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<td>&lt;0.1</td>
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<td>47.9</td>
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<td>&lt;0.1</td>
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<tr>
<td>Caryophyllene oxide</td>
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<td>0.2</td>
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<td>3.2</td>
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<td>3.6</td>
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<td>5.5</td>
<td>18.8</td>
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</tr>
</tbody>
</table>

Data are the means of four replicates in each clone and treatment. OC = outside control, CC = chamber control, EC = elevated CO2, EO = elevated O3, EC+EO = elevated CO2 and elevated O3.

emitted small amounts of sabinene in July and in August, whereas sabinene emission was barely detected from clone 80 in August (Tables 1–2). Emission of terpinolene was detected only from clone 4 (Tables 1–2). Both silver birch clones emitted also a wide variety of alkenes, alcohols (e.g. (Z)-3-hexenol), ketones, aldehydes, esters (e.g. (Z)-3-hexenyl acetate) and aromatic compounds in July and in August, named as “other compounds” in Tables 1 and 2.

The emissions of total MTs and total SQTs from both silver birch clones were highly larger (independent samples t-test, P ≤ 0.002 in all cases) in July than in August (Fig. 1). MT emission from clone 4 in July was generally larger than from clone 80 (Fig. 1a), but there
was no notable clonal variation when only control trees (OC and CC; n = 8) were used in the test. A significant chamber effect was found in total MT emission in August (Fig. 1b) so that MT emission was higher from CC trees than from OC trees. Growth at elevated CO₂ treatment marginally decreased total MT emission in July (P = 0.082; Fig. 1a), and increased it in August (P = 0.085; Fig. 1b). Growing under O₃ or CO₂ + O₃ treatment had no effect on total MT and total SQT emissions (Fig. 1a–d).

3.2. Emission quantities

Emission of 19 individual compounds (2-butanol, hexanal, (Z)-3-hexenal, hexanol, α-pinene, β-pinene + myrcene (compounds were not separated on HP-5 column), (Z)-3-hexenyl acetate, (Z)-ocimene, limonene, linalool, nonanal, (Z)-3-hexenyl butyrate, decanal, α-copaene, (E)-caryophyllene, (E)-4,8-dimethyl-1,3,7-nonatriene (DMNT), methyl sulfoxide (MeSO₂), (E)-β-ocimene and (E,E)-α-farnesene) were quantified (Table 3). Regardless of the treatments, silver birch clones 4 and 80 emitted total quantified VOCs 12520 and 8590 ng g⁻¹ fwhm in July, and 4640 and 4990 ng g⁻¹ fwhm in August, respectively. The emission rates of all other quantified compounds, except MeSO₂ and (E)-β-ocimene, were affected by the time of the growing season, emissions being significantly higher in early July than in late August (independent samples t-test, P < 0.05). A significant chamber effect was found on the emission of (E)-β-ocimene causing higher emission from CC trees than from OC trees (t = -2.699; P = 0.014; n = 6). Also emissions of (Z)-3-hexanal (t = -2.825; P = 0.018; n = 8) in July and (Z)-ocimene (t = -2.625; P = 0.026; n = 8) in August were enhanced from the CC trees compared to the OC trees. When only control treatments (OC and CC) were considered, clone 4 emitted significantly more β-pinene + myrcene (t = 2.537; P = 0.034), (Z)-ocimene (t = 2.394; P = 0.04) and (E)-β-ocimene (t = 2.276; P = 0.039) in July than clone 80, which on the contrary emitted more linalool (t = -4.999; P < 0.001) in July, and more hexanal (t = -3.922; P = 0.002) in August than clone 4 (n = 8). Growth at elevated CO₂ concentration seemed to decrease emission of nonanal and (E)-β-ocimene in July (Table 3). A significant interactive effect of CO₂ × O₃ effect was found only in DMNT emission in August: clone 4 emitted diminished, while clone 80 emitted increased amounts of DMNT when grown at elevated O₃ (Table 3). Neither O₃ nor CO₂ + O₃ exposure had effects on the emissions.

3.3. Effects of twig detachment

As a consequence of the detachment of the twig, the emission of total GLVs (36 compounds) increased 27%
and 16% from clones 4 and 80, respectively, leading to the simultaneous decrease of the percentage of total MTs (saibanene, \( \alpha \)-pinene, \( \beta \)-pinene + myrcene, limonene, 1,8-cineole, (E)-\( \beta \)-ocimene, terpinolene and linalool) and SQTs (\( \alpha \)-copaene, \( \beta \)-bourbonene, (E)-caryophyllene, (E,E)-\( \alpha \)-farnesene and germacrene D) (data not shown).

The emissions of quantified MTs and SQTs did not respond to the twig detachment (Table 4). Nevertheless, clone 4 emitted significantly more \( \alpha \)-pinene, saibanene and \( \beta \)-pinene + myrcene than clone 80 which emitted only linalool more than clone 4. The emission of several GLVs (\( Z \)-3-hexenol, hexenal, \( Z \)-3-hexenyl acetate and \( Z \)-3-hexenyl butyrate) increased drastically in response to the twig detachment (Table 4), and emission of \( Z \)-3-hexenyl, \( Z \)-3-hexenal, \( Z \)-3-hexenyl butyrate, dodecane, tridecane and pentadecane was detected only from the detached twigs (data not shown).

4. Discussion and conclusions

4.1. VOC emissions are not affected by growth at elevated CO\(_2\) and O\(_3\)

According to our first hypothesis based on source-sink balance theory (e.g. Honkakari et al., 1999), growth at elevated CO\(_2\) did not have unequivocal direct enhancing effect on VOC emission of silver birch trees. Effects of elevated CO\(_2\) on secondary metabolism of plants which do not store terpenes have often been controversial. Loreto et al. (2001a) discovered that the emission of \( \alpha \)-pinene, saibanene and \( \beta \)-pinene from Quercus ilex L. expressed on a leaf area basis was inhibited under elevated CO\(_2\) concentration, and Vaarinen et al. (2004c) detected reduced \( \alpha \)-huenine, \( \alpha \)-pinene, saibanene, \( \beta \)-pinene + myrcene, limonene and 1,8-cineole emissions expressed on a dry weight basis from Brassica oleracea subsp. capitata grown at doubled ambient CO\(_2\) concentrations. Such reduction was not observed when emissions of MTs from intact cabbage plants were expressed on a leaf area basis (Vaarinen et al., 2004b). On the other hand, Stauble et al. (2001) found that Quercus leaves grown at elevated CO\(_2\) have higher MT emission per projected leaf area and per leaf dry weight than leaves grown at ambient CO\(_2\) regardless of the CO\(_2\) concentration during sampling. In contrast to the studies of Loreto et al. (2001a) and Stauble et al. (2001), we sampled VOCs from detached twigs in the laboratory at ambient CO\(_2\) and 0 ppb of O\(_3\) to gain better yield of reactive MTs and SQTs, and assuming that the change in CO\(_2\) concentration does not affect the emission rate expressed per leaf dry mass as previously suggested (Stauble et al., 2001).

Storage capacity of terpene-storing organs may restrict the incorporation of excess carbon into terpenoids at elevated CO\(_2\) concentrations, and leave the constitutive terpene emission unaffected. Constable et al. (1999b) found that MT emission rate of ponderosa pine and Douglas fir was not affected by elevated CO\(_2\), even though CO\(_2\) treatment increased significantly leaf area index. Peñuelas et al. (2002) reported that foliar concentration of phenolics and other carbon-based secondary metabolites were unaltered in two terpene-storing (Myrurus communis, Juniperus communis) and in one non-storing (Erica arborea) shrubs grown near natural CO\(_2\) spring (700 µmol mol\(^{-1}\)). Similarly, 3-year exposure of terpene-storing Pinus sylvestris growing in a forest site to doubled ambient CO\(_2\) or O\(_3\) did not substantially affect the foliar concentration of total phenolics or total MTs (Kainulainen et al., 1998). However, in actively growing young Picea abies and P. sylvestris seedlings reduced MT levels were detected at elevated CO\(_2\), while enhanced temperature increased MT concentrations in current-year needles (Sallas et al., 2003).

The question, whether B. pendula is a terpene-storing species, is open. Hakola et al. (2001) detected that MT emission declined gradually within sudden darkness, and emission of MTs almost vanished completely, indicating that MTs are not stored and photosynthetic carbon incorporated into these compounds is emitted quickly. However, SQT emission was not affected by darkness suggesting that SQTs could be stored in trichomes or resin glands of B. pendula leaves (Valkama et al., 2003) or in bark. In fact, Iridonov et al. (2004) detected at least 61 compounds from buds of B. pendula of which almost all were SQTs. In terpene-storing species carbon allocation to MTs show higher plasticity than allocation to phenolics (Sallas et al., 2003) under enhancing atmospheric CO\(_2\) concentrations. In B. pendula seedlings grown under elevated CO\(_2\), the concentration of condensed tannins and flavonoid glycosides were found to increase when compared to those seedlings grown under ambient CO\(_2\) (Koikkalanen et al., 2001). Likewise, in the leaves of birches analysed in this study, elevated CO\(_2\) increased the concentration of condensed tannins and the overall sum of phenolics (Peltonen et al., unpublished data) indicating enhanced partitioning of carbon in secondary compounds.

In the present study, growing under O\(_3\) or CO\(_2\)+O\(_3\) fumigation did not have any effect on emissions of total MTs, SQTs, or individual quantified VOCs of silver birch clones, although total foliar phenolics were significantly increased by elevated O\(_3\) (Peltonen et al., unpublished data). There was a significant interactive clone x O\(_3\) effect on emission of DMNT, clone 4 had decreased and clone 80 increased emission when exposed to ozone. DMNT is one of the compounds emitted from herbivore-damaged plants (Dícke, 1994). A pulse treatment of O\(_3\) induced the emission of DMNT, (Z)-3-hexenyl acetate and 1-penten-3-ol from Lima beans (Vaarinen et al., 2004a), MeSA, several SQTs and C\(_8\)
Table 2
Mean emission (ng g⁻¹ h⁻¹) of quantified VOCs from twigs of silver birch clones 4 and 80 grown under double ambient CO₂ (EC) and O₃ (EO) singly and in combination (EC × EO) in July and in August, and a summary of treatment effects on emissions tested by analysis of variance (ANOVA) and interactions tested by GLM procedure.

<table>
<thead>
<tr>
<th>July</th>
<th>CC</th>
<th>EC</th>
<th>EO</th>
<th>CC + EO</th>
<th>Clone 4</th>
<th>EC</th>
<th>EO</th>
<th>EC × Clone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green leaf volatiles</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-butanal</td>
<td>79 ±13</td>
<td>147 ±35</td>
<td>238 ±93</td>
<td>315 ±87</td>
<td>26 ±13</td>
<td>304 ±182</td>
<td>146 ±54</td>
<td>83 ±39</td>
</tr>
<tr>
<td>Hexanal</td>
<td>102 ±15</td>
<td>120 ±22</td>
<td>75 ±16</td>
<td>74 ±23</td>
<td>66 ±14</td>
<td>79 ±14</td>
<td>67 ±9</td>
<td>87 ±16</td>
</tr>
<tr>
<td>(Z)-3-hexenol</td>
<td>802 ±362</td>
<td>619 ±273</td>
<td>619 ±292</td>
<td>321 ±223</td>
<td>899 ±166</td>
<td>711 ±502</td>
<td>517 ±198</td>
<td>680 ±189</td>
</tr>
<tr>
<td>Hexanol</td>
<td>38 ±13</td>
<td>39 ±13</td>
<td>34 ±9</td>
<td>34 ±9</td>
<td>18 ±12</td>
<td>53 ±12</td>
<td>58 ±17</td>
<td>45 ±14</td>
</tr>
<tr>
<td>(Z)-3-hexenyl acetate</td>
<td>1751 ±815</td>
<td>1473 ±520</td>
<td>1708 ±658</td>
<td>912 ±518</td>
<td>930 ±144</td>
<td>914 ±508</td>
<td>742 ±237</td>
<td>1233 ±378</td>
</tr>
<tr>
<td>Neocanal</td>
<td>12 ±6</td>
<td>62 ±9</td>
<td>55 ±7</td>
<td>79 ±18</td>
<td>50 ±10</td>
<td>93 ±14</td>
<td>53 ±11</td>
<td>87 ±8</td>
</tr>
<tr>
<td>(Z)-3-hexenyl butyrate</td>
<td>38 ±30</td>
<td>39 ±30</td>
<td>30 ±16</td>
<td>30 ±16</td>
<td>13 ±11</td>
<td>23 ±12</td>
<td>16 ±16</td>
<td>14 ±10</td>
</tr>
<tr>
<td>Decanol</td>
<td>20 ±6</td>
<td>54 ±12</td>
<td>35 ±8</td>
<td>40 ±10</td>
<td>33 ±3</td>
<td>45 ±14</td>
<td>51 ±8</td>
<td>59 ±11</td>
</tr>
<tr>
<td>Aromatics (MeSA)</td>
<td>36 ±7</td>
<td>147 ±72</td>
<td>45 ±20</td>
<td>31 ±15</td>
<td>62 ±53</td>
<td>62 ±25</td>
<td>31 ±30</td>
<td>23 ±10</td>
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<tr>
<td>Terpenes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-pinene</td>
<td>29 ±7</td>
<td>13 ±4</td>
<td>12 ±2</td>
<td>16 ±4</td>
<td>17 ±4</td>
<td>18 ±4</td>
<td>12 ±3</td>
<td>16 ±1</td>
</tr>
<tr>
<td>β-pinene + myrcene</td>
<td>93 ±33</td>
<td>67 ±14</td>
<td>68 ±2</td>
<td>31 ±9</td>
<td>33 ±9</td>
<td>33 ±9</td>
<td>36 ±5</td>
<td>27 ±3</td>
</tr>
<tr>
<td>(Z)-ocimene</td>
<td>4366 ±1082</td>
<td>2879 ±551</td>
<td>2576 ±316</td>
<td>2297 ±659</td>
<td>1006 ±508</td>
<td>668 ±210</td>
<td>2114 ±823</td>
<td>438 ±98</td>
</tr>
<tr>
<td>Limonen</td>
<td>10 ±10</td>
<td>25 ±11</td>
<td>27 ±9</td>
<td>16 ±9</td>
<td>36 ±9</td>
<td>36 ±9</td>
<td>62 ±31</td>
<td>21 ±2</td>
</tr>
<tr>
<td>Linalool</td>
<td>387 ±147</td>
<td>281 ±79</td>
<td>217 ±77</td>
<td>146 ±34</td>
<td>1155 ±241</td>
<td>2314 ±572</td>
<td>1000 ±552</td>
<td>957 ±250</td>
</tr>
<tr>
<td>(E)-β-ocimene</td>
<td>8109 ±1848</td>
<td>5516 ±1402</td>
<td>5033 ±1444</td>
<td>4490 ±1198</td>
<td>2415 ±879</td>
<td>1787 ±474</td>
<td>4883 ±1724</td>
<td>1206 ±230</td>
</tr>
<tr>
<td>DMNT</td>
<td>267 ±54</td>
<td>346 ±67</td>
<td>359 ±43</td>
<td>337 ±10</td>
<td>314 ±103</td>
<td>312 ±34</td>
<td>340 ±66</td>
<td>245 ±63</td>
</tr>
<tr>
<td>Compound</td>
<td>August</td>
<td>August</td>
<td>August</td>
<td>August</td>
<td>August</td>
<td>August</td>
<td>August</td>
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<tr>
<td>alpha-copaene</td>
<td>707 ± 162</td>
<td>1187 ± 731</td>
<td>767 ± 135</td>
<td>428 ± 34</td>
<td>888 ± 610</td>
<td>779 ± 164</td>
<td>426 ± 116</td>
<td>505 ± 21</td>
</tr>
<tr>
<td>(E)-caryophyllene</td>
<td>446 ± 75</td>
<td>707 ± 421</td>
<td>417 ± 83</td>
<td>273 ± 22</td>
<td>704 ± 480</td>
<td>499 ± 102</td>
<td>352 ± 66</td>
<td>355 ± 14</td>
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<tr>
<td>(Z)-3-farnesene</td>
<td>385 ± 77</td>
<td>494 ± 208</td>
<td>413 ± 52</td>
<td>320 ± 88</td>
<td>541 ± 450</td>
<td>539 ± 125</td>
<td>372 ± 79</td>
<td>375 ± 149</td>
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<tr>
<td>Sum of all compounds</td>
<td>17776 ± 3473</td>
<td>14164 ± 2397</td>
<td>12708 ± 2988</td>
<td>10071 ± 1866</td>
<td>9141 ± 3355</td>
<td>9356 ± 745</td>
<td>11143 ± 2345</td>
<td>6461 ± 882</td>
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</tbody>
</table>

**Green leaf volatiles**

<table>
<thead>
<tr>
<th>Compound</th>
<th>August</th>
<th>August</th>
<th>August</th>
<th>August</th>
<th>August</th>
<th>August</th>
<th>August</th>
<th>August</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexanal</td>
<td>4 ± 4</td>
<td>17 ± 9</td>
<td>17 ± 3</td>
<td>17 ± 5</td>
<td>23 ± 3</td>
<td>17 ± 1</td>
<td>19 ± 6</td>
<td>15 ± 5</td>
</tr>
<tr>
<td>(Z)-3-hexenol</td>
<td>16 ± 16</td>
<td>0</td>
<td>332 ± 295</td>
<td>8 ± 0</td>
<td>0</td>
<td>0</td>
<td>195 ± 195</td>
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<tr>
<td>Hexanol</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3 ± 3</td>
<td>0</td>
</tr>
<tr>
<td>(Z)-2-hexenyl acetate</td>
<td>94 ± 65</td>
<td>123 ± 54</td>
<td>664 ± 474</td>
<td>62 ± 27</td>
<td>42 ± 9</td>
<td>23 ± 7</td>
<td>557 ± 523</td>
<td>30 ± 10</td>
</tr>
<tr>
<td>Nonenal</td>
<td>6 ± 4</td>
<td>17 ± 12</td>
<td>8 ± 5</td>
<td>3 ± 3</td>
<td>16 ± 5</td>
<td>8 ± 3</td>
<td>6 ± 4</td>
<td>17 ± 1</td>
</tr>
<tr>
<td>(Z)-2-hexenyl butyrate</td>
<td>0</td>
<td>0</td>
<td>25 ± 25</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5 ± 5</td>
<td>0</td>
</tr>
<tr>
<td>Decanal</td>
<td>0</td>
<td>12 ± 13</td>
<td>11 ± 0</td>
<td>5 ± 5</td>
<td>16 ± 16</td>
<td>7 ± 7</td>
<td>7 ± 7</td>
<td>43 ± 29</td>
</tr>
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</table>

**Aromatics (MeSA)**

<table>
<thead>
<tr>
<th>Compound</th>
<th>August</th>
<th>August</th>
<th>August</th>
<th>August</th>
<th>August</th>
<th>August</th>
<th>August</th>
<th>August</th>
</tr>
</thead>
<tbody>
<tr>
<td>alpha-pinene</td>
<td>3 ± 2</td>
<td>7 ± 4</td>
<td>8 ± 4</td>
<td>0 ± 4</td>
<td>1 ± 1</td>
<td>1 ± 1</td>
<td>4 ± 2</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>beta-pinene + myrcene</td>
<td>11 ± 3</td>
<td>23 ± 8</td>
<td>13 ± 7</td>
<td>13 ± 4</td>
<td>9 ± 4</td>
<td>10 ± 3</td>
<td>6 ± 2</td>
<td>16 ± 4</td>
</tr>
<tr>
<td>(Z)-4-ocimene</td>
<td>1073 ± 301</td>
<td>2230 ± 899</td>
<td>796 ± 360</td>
<td>981 ± 481</td>
<td>1308 ± 538</td>
<td>1545 ± 663</td>
<td>639 ± 285</td>
<td>1928 ± 779</td>
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<tr>
<td>Limonene</td>
<td>0</td>
<td>5 ± 5</td>
<td>16 ± 10</td>
<td>3 ± 3</td>
<td>0</td>
<td>0</td>
<td>8 ± 5</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>Linalool</td>
<td>3 ± 3</td>
<td>7 ± 7</td>
<td>9 ± 9</td>
<td>5 ± 3</td>
<td>11 ± 7</td>
<td>13 ± 8</td>
<td>57 ± 30</td>
<td>58 ± 44</td>
</tr>
<tr>
<td>(E)-beta-caryophyllene</td>
<td>2948 ± 763</td>
<td>5536 ± 1930</td>
<td>2153 ± 956</td>
<td>2522 ± 1176</td>
<td>3420 ± 1296</td>
<td>3956 ± 1673</td>
<td>1671 ± 739</td>
<td>4986 ± 1871</td>
</tr>
<tr>
<td>DMNT</td>
<td>79 ± 36</td>
<td>60 ± 15</td>
<td>78 ± 9</td>
<td>28 ± 3</td>
<td>33 ± 5</td>
<td>35 ± 3</td>
<td>57 ± 37</td>
<td>51 ± 11</td>
</tr>
<tr>
<td>alpha-copaene</td>
<td>45 ± 70</td>
<td>119 ± 62</td>
<td>89 ± 35</td>
<td>128 ± 70</td>
<td>37 ± 37</td>
<td>30 ± 13</td>
<td>71 ± 34</td>
<td>80 ± 42</td>
</tr>
<tr>
<td>(E)-caryophyllene</td>
<td>32 ± 13</td>
<td>49 ± 25</td>
<td>44 ± 13</td>
<td>50 ± 25</td>
<td>29 ± 25</td>
<td>27 ± 18</td>
<td>92 ± 35</td>
<td>61 ± 32</td>
</tr>
<tr>
<td>(Z,E)-farnesene</td>
<td>230 ± 96</td>
<td>163 ± 51</td>
<td>112 ± 37</td>
<td>109 ± 63</td>
<td>72 ± 9</td>
<td>48 ± 20</td>
<td>72 ± 16</td>
<td>67 ± 12</td>
</tr>
<tr>
<td>Sum of all compounds</td>
<td>4621 ± 1068</td>
<td>8499 ± 2716</td>
<td>4344 ± 1206</td>
<td>3927 ± 1582</td>
<td>4994 ± 1776</td>
<td>5732 ± 2318</td>
<td>3449 ± 809</td>
<td>7349 ± 2518</td>
</tr>
</tbody>
</table>

Data are the means (± SE) of four replicates per clone and treatment.

ns = not significant.
Table 4
Mean (± SE) emissions of quantified VOCs (ng g⁻¹ h⁻¹) from intact and detached (DET) twigs of 3-year old silver birch saplings of clones 4 and 80 (n = 4), and a summary of main effects of clone and detachment on emissions tested with GLM procedure.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Clone 4</th>
<th>Clone 80</th>
<th>P-values</th>
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<tbody>
<tr>
<td></td>
<td>Intact</td>
<td>DET</td>
<td>Intact</td>
</tr>
<tr>
<td><strong>Green leaf volatiles</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Z)-3-hexenol</td>
<td>80.4±18</td>
<td>2004±465</td>
<td>174±130</td>
</tr>
<tr>
<td>Hexanol</td>
<td>7.4±4</td>
<td>354±118</td>
<td>6±3</td>
</tr>
<tr>
<td>(Z)-3-hexenyl acetate</td>
<td>107.7±29</td>
<td>547±236</td>
<td>54±27</td>
</tr>
<tr>
<td>Theanone</td>
<td>54.2±28</td>
<td>61±20</td>
<td>72±13</td>
</tr>
<tr>
<td>(Z)-3-hexenyl butyrate</td>
<td>5.2±2</td>
<td>98±29</td>
<td>21±14</td>
</tr>
<tr>
<td>(Z)-3-hexenyl isovalerate</td>
<td>2.1±1</td>
<td>6±1</td>
<td>5±3</td>
</tr>
<tr>
<td>(Z)-3-hexenyl fagine</td>
<td>0</td>
<td>1±1</td>
<td>0</td>
</tr>
<tr>
<td><strong>Aromatics (MeSA)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Terepenes</td>
<td>1.2±1</td>
<td>3±2</td>
<td>3±2</td>
</tr>
<tr>
<td>Sabinene</td>
<td>19.4±5</td>
<td>15±3</td>
<td>7±4</td>
</tr>
<tr>
<td>α-pinene</td>
<td>15.2±4</td>
<td>11±2</td>
<td>7±3</td>
</tr>
<tr>
<td>β-pinene + myrcene</td>
<td>19.5±5</td>
<td>13±3</td>
<td>7±4</td>
</tr>
<tr>
<td>Limonene</td>
<td>11.4±3</td>
<td>11±2</td>
<td>7±3</td>
</tr>
<tr>
<td>1,8-cineole</td>
<td>12.4±4</td>
<td>12±3</td>
<td>5±3</td>
</tr>
<tr>
<td>Linalool</td>
<td>1±1</td>
<td>4±3</td>
<td>23±7</td>
</tr>
<tr>
<td>(E)-β-ocimene</td>
<td>16±3</td>
<td>32±3</td>
<td>42±25</td>
</tr>
<tr>
<td>DMNT</td>
<td>0</td>
<td>9±5</td>
<td>8±4</td>
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<tr>
<td>α-copaene</td>
<td>1±1</td>
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<td>1±1</td>
</tr>
<tr>
<td>(E)-caryophyllene</td>
<td>17±6</td>
<td>12±3</td>
<td>8±3</td>
</tr>
<tr>
<td>(E,E)-α-farnesene</td>
<td>14±10</td>
<td>60±39</td>
<td>22±3</td>
</tr>
</tbody>
</table>

ns = not significant.

Compounds from O₃-sensitive tobacco cultivar Bel W3 (Heiden et al., 1999, 2003), and C₆ compounds from corn and tomato (Heiden et al., 2003). Ozone exposure in OTCs has often led to increased emissions of total VOCs in several plant species (Peuhula et al., 1999; Llusia et al., 2002). Ozone activates defense responses in plants, and thereby may trigger the emission of inducible VOCs from several defense pathways (Heiden et al., 1999, 2003; Vuorinen et al., 2004a).

4.2. VOC emissions are affected by growth environment and genotype

In the field, several environmental factors such as temperature, light, humidity and fertilisation, affect the VOC emission of the plants (Gouinguené and Turling, 2002). In the present study, laboratory temperature during VOC collection was ~25°C in July and ~22°C in August. Zhang et al. (1999) revealed significant increases in the release of MTS, GLVs, and SQFs from silver birch when temperature was elevated from 16°C to 32°C. Herbivore-damage also triggers strongly the emission of certain herbivore-induced compounds e.g. (Z)-3-hexenyl acetate, (E)-β-ocimene, DMNT, and MeSA (Karban and Baldwin, 1997). In the summer the trees were fed by aphids, cicadas and moth larvae (Yapaavuo et al., 2002), and in the autumn some of the trees were infected by leaf pathogens. Feeding damage of larvae in the birch foliage induces the emission of (Z)-ocimene, linalool, β-ocimene, (E)-β-ocimene, DMNT, (E,E)-α-farnesene and MeSA (Vuorinen et al., unpublished data).

Anyhow, in this study visible herbivore-damage in the sampled twigs did not affect the emission of total MTS, total SQFs, or emission of individual VOCs (data not shown).

According to the data from this OTC experiment clone 4 was more sensitive to phytotoxic O₃ than clone 80 (Riikonen et al., 2004). We discovered that clone 4 emitted more MTS in general in July than clone 80. Also, the twig detachment experiment revealed higher MT emission from clone 4 than from clone 80. Lower growth rate (Riikonen et al., 2004) and higher investment to phenolic compounds (Pelkonen et al., unpublished data) suggest that clone 4 is investing in greater extend to chemical defence than clone 80.

Hakola et al. (2001) found that MT emission from silver birch was high soon after bud burst, thereafter emission declined for a month, and increased after the cumulative temperature sum 400 d·d was exceeded when leaves were green, harder, and expanded to their full size. In the present study, profile of VOCs changed in course of the season. Also the emissions of all other

60
compounds except MeSA and (E)-β-ionone were larger in July than in August which might partly be due to the higher temperature in July. Trees with fully expanded leaves emitted mostly SQTs in July, whereas the same trees emitted mainly MTs in August when the growth had ceased. Hakola et al. (2001) found that emission profile of B. pendula changed from quite evenly distributed MTs to ocimenes and sabine. Surprisingly, the sabine emission was minor in the present study. In accordance with the results by Zhang et al. (1999), our results indicate that B. pendula clones are capable of emitting also SQTs during warm weather in July. In previous field studies B. pendula has been considered mainly as a MT emitter (Hakola et al., 2001), which might be due to the high reactivity and particle forming capacity of SQTs (Bonn and Moortgat, 2003) in the atmosphere even during the sampling (Helmi et al., 2004). Chemical lifetime of SQTs is approximated to be less than 4 min during daytime and less than 2 min in the night (Kesselmeier and Staudt, 1999). Since SQTs contribute strongly to the aerosol formation in the troposphere, the role of B. pendula in aerosol formation in boreal zones might be more important than currently expected. In future the role of silver birch in aerosol formation could even increase as it has been estimated that the forest tree species composition in Finland in 2100 would mainly consist of birches (B. pendula and B. pubescens, 55% of total forest area) while in 1990 the proportion of birches from the total forest area was only 13%. (Kellomäki et al., 2001).

Schmelz et al. (2001) found that excised leaves may exaggerate the SQT emission rates induced by mechanical damage and volatilization (secretions of Spodoptera larvae) or jasmonic acid, while excision alone did not alter the emission from undamaged control plants. In the present study, we found significantly larger emission of GLVs from detached silver birch twigs than from intact twigs, while individual MTs and SQTs did not respond to the detachment. This is consistent with the results of Hakola et al. (2001) who demonstrated that rough handling of the birch leaves causes very high emission rates of (Z)-3-hexenol, (Z)-3-hexenyl acetate, 2-hexenal and 1-hexanol, while MT emission remains unaffected. Thus, in the present study the GLV emission may mostly be due to the detachments of twigs, but the emission of terpenes should not be over-exaggerated because of the detachment. Our data show that intact twigs emitted at least five SQTs indicating that the emission of SQTs does not result in the detachment. It is also possible that the emissions were enhanced due to the concentration of the sample air in the collection chambers prior to sampling, but there were no significant changes in course of the sampling time in the emissions of individual VOCs (data not shown).

In conclusion, our results indicate that growing under elevated CO₂ and O₃ do not have considerable effects on the emissions of silver birch, or other abiotic and biotic factors might conceal the effects of these gases. In the framework of source-sink balance hypothesis, we were not able to show that growing at elevated atmospheric CO₂ concentration increases the VOC emission from B. pendula. Neither the second hypothesis that ozone induces the emissions of herbivore-induced VOCs gained support. However, constant emission of DMNT suggests that either herbivore- or pathogen-induced defences were switched on in all of the experimental trees, which makes ozone-induced effects difficult to detect. Therefore joint effect of CO₂ and O₃ on VOC emission as expected in the third hypothesis could not be detected.

Acknowledgements

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References


CHAPTER 5

VOC EMISSIONS INDUCED BY HERBIVORY, PATHOGEN INFECTION AND O₃ EXPOSURE FROM TWO SILVER BIRCH CLONES AND THE HOST-SEARCHING BEHAVIOUR OF THE GENERALIST PREDATOR


Manuscript
VOC emissions induced by herbivory, pathogen infection and O$_3$ exposure from two silver birch clones and the host-searching behaviour of the generalist predator

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Summary. Induced volatile organic compounds (VOCs) emitted by various plant species play a major role in the indirect defence of plants against herbivores by attracting the natural enemies of herbivores. In laboratory experiments, we analysed the emission of VOCs from two clones (clone 4 and 80) of silver birch, Betula pendula Roth damaged by larvae of Epiprita autunnata (Lepidoptera: Geometridae), infected with pathogenic leaf spot causing fungus Marssonina betulae or exposed to 50 ppb and 100 ppb of ozone. Also the orientation behaviour of the generalist predator Podisus maculiventris (Heteroptera: Pentatomidae) to herbivore-damaged or pathogen-infected silver birch twigs was studied in the Y-tube olfactometer. Our results revealed that within 48 h, the herbivory did not induce systemic release of VOCs from intact leaves of damaged twigs (indirect herbivory), while pathogen-infected twigs had larger emission of (Z)-ocimene, (E)-β-ocimene, and (E,E)-α-farnesene than uninfected twigs of both clones. In the twigs suffering direct herbivore damage, the emissions of (Z)-ocimene, linalool, (E)-β-ocimene, DMNT, β-caryophyllene, (E,E)-α-farnesene and MeSA were induced. Exposure of silver birch clones to 50 ppb or 100 ppb of ozone had no effect on the emissions. P. maculiventris did not show significant orientation responses either towards herbivore-damaged or to pathogen-infected silver birch twigs. Our results suggest that a fungal pathogen is able to induce the emissions of several compounds from silver birch, but the blend differs from that of directly herbivore-damaged leaves.

Key words. Epiprita autunnata (Lepidoptera: Geometridae) – pathogenic fungus (Marssonina betulae) – Podisus maculiventris (Heteroptera: Pentatomidae) – tritrophic interaction – VOCs

Introduction

Plants are damaged by numerous attackers in the nature; herbivores, fungi and viruses. Therefore plants have been forced to evolve several defence methods to cope with these attackers. For example, after a plant is wounded it emits a wide range of volatile organic compounds (VOCs) which many attract carnivorous insects or parasitoids (Dicke et al. 2003). Alternatively, these compounds may function as signals activating disease resistance in the neighbouring plants (Shulaev et al. 1997). The composition of the emitted volatile blend depends on abiotic factors (e.g. humidity, temperature, light and fertilisation) (Gouinguéné & Turlings 2002) and biotic factors (e.g. plant cultivar, growth stage and attacking herbivore species) (Takabayashi et al. 1994). In general, silver birch, Betula pendula Roth (Fagales: Betulaceae), emits a variety of C$_{10}$ monoterpenes (König et al. 1995; Hakola et
al. 1998; Zhang et al. 1999; Lindfors et al. 2000; Hakola et al. 2001; Vuorinen et al. 2005), C_{15} sesquiterpenes, and also aliphatic (König et al. 1995; Zhang et al. 1999; Vuorinen et al. 2005) and aromatic compounds (Zhang et al. 1999). Two herbivory-inducible monoterpenes, (Z)-ocimene and (E)-β-ocimene, are two of the main compounds emitted from undamaged silver birch (Zhang et al. 1999; Hakola et al. 2001; Vuorinen et al. 2005).

Herbivore feeding has been shown to trigger the emission of novel induced VOCs from several tree species. Feeding by Agelastica alni increases the emission of mono-, homo and sesquiterpenes, green leaf volatiles (GLVs) and aromatic compounds from alder (Alnus glutinosa) leaves (Tschirnke et al. 2001), while Psylla-infested (P. pyricola) pear trees emit more (E)-α-farnesene, MeSA and four GLVs than uninfested trees (Scutareanu et al. 1997). Spider mite (Panonychus ulmi)-damaged apple trees also emit larger amounts of VOCs than uninfested trees, and therefore are attractive to the predatory mites (Lusià & Peñuelas 2001). Pathogen inoculation has been noted to induce the emission of terpenoids (Doughty et al. 1996; Piel et al. 1997; Cardoza et al. 2002), C_{6} GLVs (Heiden et al. 2003), aromatic compounds (Shulaev et al. 1997) and isothiocyanates (Doughty et al. 1996). Cardoza et al. (2003) showed that beet armyworms (Spodoptera exigua) preferred the white mold infected peanut plants for oviposition which might be due to the altered emissions released from infected plants (Cardoza et al. 2002).

Emissions of ozone-stressed plants are still poorly known. Higher total VOC emissions have been detected from Ceratonia siliqua, Olea europaea and Quercus ilex rotundifolia (Lusià et al. 2002) and Solarum lycopersicium L. (Peñuelas et al. 1999) in open-top chambers (OTCs) fumigated with 40 nL L\(^{-1}\) of O\(_{3}\) over ambient level in comparison with the non-fumigated OTCs. Loreto et al. (2004) detected increased monoterpenes (α-pinene, β-pinene and sabinene) emission from Q. ilex leaves exposed to either mild and repeated or 250 ppb acute ozone concentrations. An ozone-resistant variety of Nicotiana tabacum released sesquiterpenes shortly after O\(_{3}\) fumigation pulse, while an ozone-sensitive variety emitted sesquiterpenes, methyl salicylate (MeSA) and C\(_{6}\) compounds in much greater amounts at 24 h after the start of the O\(_{3}\)-fumigation when the visible symptoms appeared (Heiden et al. 1999). Recently it has been shown that acute ozone exposure can induce the emission of novel herbivore inducible compounds such as homoterpenes DMNT and TMTT, and (Z)-3-hexenyl acetate from lima beans (Vuorinen et al. 2004a).

Stress-induced VOCs and tritrophic interactions have not been studied in silver birch. The objective of this study was to analyse indirect (intact foliage of herbivore-damaged twigs) and direct herbivory-, pathogen- and ozone-induced VOCs from twigs of two clones of silver birch. Also, the orientation behaviour of the generalist predator, the spined soldier bug Podisus maculiventris (Hemiptera: Pentatomidae), to the odour of indirectly and directly herbivore-damaged and pathogen-infected twigs was tested in the Y-tube olfactometer. The hypotheses were: 1) herbivore damage on the branch triggers the release of novel VOCs systemically from the undamaged parts of the branch and locally from damaged parts of the branch, 2) pathogen-infection induces the emission of novel VOCs but in a different manner to that seen in leaves with herbivore-damage, 3) ozone-exposure enhances the constitutive emission and also induces the emission of novel VOCs, and finally 4) the generalist predator prefers those twigs emitting the induced novel VOCs.
Materials and methods

Experiments, plant material and growth conditions

We conducted two separate experiments using two silver birch clones. Clone 4 had been earlier classified as an ozone-tolerant genotype whereas clone 80 expressed an ozone-sensitive genotype (Pääkkönen et al. 1997). First, we studied the effects of herbivore infestation and pathogen infection on the VOC emission of silver birch clones, and the behavioural response of *P. maculiventris* to damaged twigs. Secondly, we studied the effects of ozone on VOCs of the same clones and saplings.

Cuttings of silver birch (*Betula pendula* Roth) saplings (clone 4 and 80) were tissue cultured in the Department of Applied Biology, University of Helsinki in the spring of 2000. Saplings were transferred to a greenhouse at Suomenjoki Research Station (Finland, 62°40′N, 27°03′E 120 m a.s.l.) in May 2000, and planted in pots in a mixture (1:3 v/v) of sand and fertilized peat (Finnpeat, Kekkilä). In September 2000 the plants were transferred outside and, in February 2001 taken to the greenhouse and planted into underground irrigation containers. The plants were inoculated with *Pyrenopeziza betulicola* 13.3.2001 for another experiment, and in May 2001 the saplings were cut at the stem-base. By the fall of 2001 new shoots had emerged, and the plants were placed outside over winter. On the 6th May 2002, the plants were placed in a controlled growth chamber (Bioklim 2600T), at 19/12°C, 50-80% RH, 18L/6D photoperiod and 500 μmol m⁻² s⁻¹ PAR two weeks prior to the first-year experiments with pathogenic fungus and *E. autumnata* to avoid natural herbivory in the field. The herbivores, aphids, cicadas and lepidopteran larvae, were removed daily with a fine paint brush. The saplings were divided into three treatments: controls, pathogen-infected and herbivore-damaged, which were all kept in separate growth chambers to avoid induction evoked by neighbouring plants receiving different treatments. After the first-year experiments, at the beginning of June 2002, the healthy control and herbivore-infested saplings were transferred to the field site of Kuopio University Garden (62°13′N, 27°13′E) to be used for the ozone experiment in 2003. At that time, before the opening of the buds, the saplings were transferred from the field site back to the growth chamber (19/12°C, 50-80% RH, 18L/6D photoperiod and 500 μmol m⁻² s⁻¹ PAR) 3.5 weeks before the ozone experiment. The saplings were fertilised weekly with 0.2% 9-Superex (19:5:20 N:P:K, Kekkilä, Finland). The saplings were divided into three different treatments, 0, 50 and 100 ppb of ozone, and again kept in separate growth chambers.

Insect feeding and predators

Four 3rd or 4th-instar larvae of the autumnal moth *Epirrita autumnata* (Lepidoptera: Geometridae) representing progenies of at least eight females were placed into a clip cage (hemispherical cage made of rubber foam and cloth) which then was attached around the branch of a sapling. Four leaves were enclosed into the cage with the feeding larvae and 6-8 leaves were left undamaged outside the cage above the clip cage system. This intact top of the branch (indirect herbivory; IH; *n* = 5 per clone), and the part of the branch where larvae were feeding (direct herbivory; DH; *n* = 3 per clone) were later on separately used in VOC collections and behavioural assay. The VOC samples from IH and DH twigs were collected 48 h and 72 h after the start of the larval feeding, respectively. Subsequently, the behavioural tests with *P. maculiventris* were done with IH twigs after 48 h or 72 h feeding damage and with DH twigs after 72 h damage. Control twigs for IH (*n* = 2 per clone) and DH (*n* = 2 per clone) treatment twigs had empty clip cage attached around the branch in a similar fashion to the herbivore-damaged twigs. Larvae of *E. autumnata* were reared in the laboratory at +22°C and occasionally at +4°C and +11°C in order to
synchronise nymphal development by slowing the growth of the first-hatched larvae. Nymphs of Podisus maculiventris (Hemiptera: Pentatomidae) were obtained from Schetelig (Vantaa, Finland) and approx. 50% of the nymphs were reared on larvae of E. autumnata. The others were naïve. Both adults and nymphs were used in the behavioural assay.

Pathogen inoculation

The pathogenic fungus Marssonina betulae was grown for production of conidia for 18 days on 3.9% PDA plates covered with a cellophane membrane (+15°C, continuous light). Spores were then collected by rinsing the membrane gently with sterilized water. The suspension for inoculation contained 102 500 spores per ml sterilized water. One spray with a spray bottle contained approximately 918 μl (approx. 94095 spores) of suspension. The suspension was also sprayed on 1.6% water agar plates to check the germination ability of the spores under a light microscope. After 27 h (+15°C, continuous light) 95.1% of the 616 studied spores had germinated. The pathogen was sprayed onto the top of the birch branch with 12-14 leaves, 5 sprays per branch underneath the leaves, and the sprayed top of the branch was enclosed in a 1.5-L plastic bag (one spray of water was sprayed into the bag) for incubation 72 h in advance of the VOC collections and behavioural assay. Control twigs (n = 2 per clone) were sprayed with water and enclosed in a plastic bag similar to the pathogen-infected twigs. The plastic bags were removed 18 h before the VOC collections and behavioural assay.

Ozone exposure

The saplings were divided into three different growth chambers and exposed to 0, 50 and 100 ppb of ozone for 12 h (9:00 am-9:00 pm) each day over two days. Untreated birches grown under filtered air with 0 ppb of ozone were used as controls (n = 6 per clone per treatment). Ozone was produced from pure oxygen by a Fisher OZ 500 ozone generator (Fisher, Bonn, Germany) and analyzed with a Dasibi 1008-RS ozone analyzer (Dasibi Environmental Corp., Glendale, CA, U.S.A.).

Collection of volatile compounds

VOCs were sampled from the top of the branch with 6-8 intact leaves (indirect herbivory) or pathogen-inoculated leaves, and herbivore-damaged parts of the branch (direct herbivory) with 4 leaves. The twig was detached and placed into a 4-ml vial filled with tap water, set in a 1-L glass vessel and closed with a Teflon sealed lid with an inlet for purified air and an outlet for sampling. Air purified with activated carbon entered the vessel at a rate of 0.210 L min⁻¹. After a 5-min adjustment period, the sample was pulled through a purified stainless steel tube (Perkin Elmer, ATD sample tubes) filled with approx. 150 mg Tenax-TA adsorbent (Supelco, mesh 60/80) with a vacuum pump (KNF Neuberger, Inc., Freiburg, Germany, Model N022AN.18) at a rate of 0.200 L min⁻¹ for 30 minutes. The samples were collected at ~300 μmol m⁻² s⁻¹ PAR at 22°C (room temperature). The airflow was calibrated daily with the mini-Buck calibrator (Model M-5, A.P. Buck, Inc., Orlando, FL, U.S.A.). Samples were analyzed by GC-MS (Hewlett Packard GC type 6890, MSD 5973). Trapped compounds were desorbed (Perkin Elmer ATD400 Automatic Thermal Desorption System) at 220°C for 10 minutes, cryofocused at -30°C and injected onto an HP-5 capillary column (50 m × 250 μm × 0.25 μm, Hewlett Packard). The carrier gas was helium, and the temperature program was 50°C for one min, followed by increases of 5°C min⁻¹ to 210°C and 20°C min⁻¹ to 250°C. Compounds were identified by comparison of the mass spectra with those in the Wiley library and pure standards. For quantification of the emissions, commercially available reference substances were used. Reference substances
for (E)-β-ocimene, (E)-4,8-dimethyl-1,3,7-
nonatriene (DMNT) and (E,E)-α-farnesene
were not available, therefore the
concentrations of these compounds were
calculated by assuming the responses to be
the same as the responses of (Z)-ocimene
for (E)-β-ocimene and DMNT, and (E)-β-
farnesene for (E,E)-α-farnesene.

Behavioural assay

The Y-tube olfactometer (main arm 10.5
cm, other arms 10 cm, inner diameter 1.6
cm) was used to test the orientation
behaviour of 3rd, 4th and 5th instar nympha
and adults of *Podisus maculiventris*
(Hemiptera: Pentatomidae). Nymphs were
used to test the attractiveness of the twigs
with indirect herbivory and pathogen-
infestation. Adults were used to test the
attractiveness of the twigs with direct
herbivore damage. The pressurized air was
purified with activated carbon and divided
via Teflon tubing into two separate flows
(0.500 L min⁻¹) which both passed through a
1-L glass vessel containing a silver birch
twig as an odour source leading to one of
the arms of the Y-tube. The airflow was
adjusted with pressure and needle valves
and calibrated daily with M-5 mini-Buck
calibrator. Nymphs or adults of *P.
maculiventris* were introduced to the
downwind end of the Y-tube and observed
for 5 minutes or until they made their final
choice. The choice was recorded when the
predator reached the far end of the other arm
of the Y-tube. Odour sources, glass vessels,
Y-tube, and lids were replaced after testing
six predators. All the glassware was heat-
treated at 120°C before use, and the Y-tube
was turned horizontally around after each
tested predator, and rinsed with ethanol after
every third insect.

Statistical analyses

Statistical analyses were performed using
SPSS 11.5 for Windows. VOC emission
data from herbivore and pathogen
experiments were analysed by independent
samples t-test, and the data from the ozone
exposure experiment were analysed by
General Linear Model procedure. Behavioural assay data were analysed with
non-parametric binomial test to analyse
whether there was a significant difference in
the attraction between the two odour sources
from plants undergoing different treatments
(test proportion was set to 0.50).

Results

Effects of herbivore damage and pathogen
infection on VOCs

Defoliation caused by four *E. autumnata*
larvae extended to 10-50% of the offered
leaves. All the pathogen-infected silver
birch leaves had brownish dots three days
after the inoculation indicating pathogen
infection or hypersensitive reaction caused
by the pathogen. Regardless of the
treatments, the total emission comprised
mainly of (Z)-3-hexenol and (Z)-3-hexenyl
acetate (Table 1), but these GLVs have been
found to be partly a consequence of the
detachment of the twig (Vuurinen *et al.*
2005). Therefore indirect herbivory, direct
herbivory or pathogen infection had no
effect on these emissions. The emission of
(Z)-3-hexenol accounted for 10-35%, (Z)-3-
hexenyl acetate 26-70%, mono- and
homoterpenes 2-10% and sesquiterpenes 2-
12% of the total emission, depending on the
clon and treatment (Table 1).

Indirect herbivory at the lower part of the
branch did not lead to any significant
systemic changes in the emission quality
(Table 1) or quantity (Table 2) from the
intact top of the branch in either of the
clones. Nevertheless indirect herbivory
seemed to induce the emission of DMNT
from both clones. Both clones of pathogen-
infected silver birch twigs exhibited greater
emission of (Z)-ocimene, (E)-β-ocimene,
and (E,E)-α-farnesene than uninfected twigs
(Table 3). The emission of DMNT was
induced in pathogen-infected clone 4, but
not in clone 80 (Table 3).
Table 1 Relative proportions (%) of VOCs (mean ± SD) emitted from detached intact control twigs (C; n = 7 including two twigs with a plastic bag, two with a clip cage and three without any plastic bag or clip cage), intact twigs with indirect herbivory (IH; n = 5), pathogen-infected twigs (PAT; n = 5), and damaged twigs with direct herbivory (DH; n = 3) of silver birch clones 4 and 80.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Clone 4</th>
<th>Clone 80</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>IH</td>
</tr>
<tr>
<td>Ketones</td>
<td>0.6 ± 0.5</td>
<td>0.3 ± 0.3</td>
</tr>
<tr>
<td>Alkanes</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Alcohols (Z)-3-hexenol</td>
<td>22.8 ± 6.3</td>
<td>31.9 ± 9.7</td>
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<tr>
<td>Aldehydes</td>
<td>0.2 ± 0.3</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>Esters (Z)-3-hexenyl acetate</td>
<td>69.8 ± 17.2</td>
<td>61.0 ± 24.8</td>
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<tr>
<td>Oximes</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Aromatic (MeSA)</td>
<td>0</td>
<td>&lt;0.1 ± 0.0</td>
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Mono- and Homoterprenes

<table>
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<tr>
<th>Compounds</th>
<th>Clone 4</th>
<th>Clone 80</th>
</tr>
</thead>
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<tr>
<td>α-pinene</td>
<td>0.3 ± 0.1</td>
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</tr>
<tr>
<td>Sabinene</td>
<td>0.3 ± 0.2</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>β-pinene + myrcene</td>
<td>0.4 ± 0.2</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>(Z)-ocimene</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Limonene</td>
<td>0.2 ± 0.2</td>
<td>0.1 ± 0.0</td>
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<tr>
<td>1,8-cineol</td>
<td>0.3 ± 0.1</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>(E)-β-cimene</td>
<td>0.3 ± 0.3</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>(Z)-linalool oxide</td>
<td>&lt;0.1 ± 0.0</td>
<td>&lt;0.1 ± 0.0</td>
</tr>
<tr>
<td>(E)-linalool oxide</td>
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<td>0.0</td>
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<tr>
<td>Terpinolene</td>
<td>0.2 ± 0.1</td>
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<tr>
<td>Linalool</td>
<td>0.5 ± 0.2</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>DMNT</td>
<td>0</td>
<td>&lt;0.1 ± 0.0</td>
</tr>
<tr>
<td>Total MTs and HTs</td>
<td>2.5 ± 1.3</td>
<td>2.5 ± 0.9</td>
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Sesquiterpenes

<table>
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<tr>
<th>Compounds</th>
<th>Clone 4</th>
<th>Clone 80</th>
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<tr>
<td>α-copaene</td>
<td>0.2 ± 0.2</td>
<td>0.4 ± 0.4</td>
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<tr>
<td>Unidentified SQT</td>
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<tr>
<td>β-bourbonene</td>
<td>0.1 ± 0.1</td>
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<tr>
<td>Unidentified SQT (bp 161)</td>
<td>&lt;0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>Compounds</td>
<td>C</td>
<td>IH</td>
</tr>
<tr>
<td>-------------------------</td>
<td>------------</td>
<td>------------</td>
</tr>
<tr>
<td>α-pinene</td>
<td>63.8 ± 22.9</td>
<td>77.2 ± 22.9</td>
</tr>
<tr>
<td>Sabinene</td>
<td>134.6 ± 46.9</td>
<td>119.7 ± 55.6</td>
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<tr>
<td>β-pinene + myrcene</td>
<td>115.8 ± 33.6</td>
<td>104.8 ± 30.5</td>
</tr>
<tr>
<td>(Z)-ocimene</td>
<td>0.0</td>
<td>4.2 ± 9.3</td>
</tr>
<tr>
<td>Limonene</td>
<td>64.4 ± 20.0</td>
<td>54.0 ± 21.8</td>
</tr>
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<td>1,8-cineole</td>
<td>65.9 ± 21.6</td>
<td>61.8 ± 20.7</td>
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<tr>
<td>Linalool</td>
<td>249.6 ± 152.6</td>
<td>520.4 ± 319.4</td>
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<tr>
<td>(E)-β-ocimene</td>
<td>128.3 ± 15.9</td>
<td>216.9 ± 97.7</td>
</tr>
<tr>
<td>DMNT</td>
<td>0.0</td>
<td>23.8 ± 32.9</td>
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<tr>
<td>β-caryophyllene</td>
<td>42.4 ± 6.6</td>
<td>59.7 ± 49.1</td>
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<td>δ-cadinene</td>
<td>45.6 ± 5.0</td>
<td>79.6 ± 26.2</td>
</tr>
<tr>
<td>(E, E)-α-farnesene</td>
<td>529.4 ± 185.7</td>
<td>1037.8 ± 790.4</td>
</tr>
</tbody>
</table>

Table 2. Emission data (ng g dw⁻¹ h⁻¹) of quantified terpenes (mean ± SD) from detached intact control twigs (C; n = 2) with a clip cage and from indirectly herbivore-damaged (IH; n = 5) twigs of silver birch clones 4 and 80. P-values are from independent samples t-test.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Clone 4 C</th>
<th>t</th>
<th>df</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-pinene</td>
<td>63.8 ± 22.9</td>
<td>77.2 ± 22.9</td>
<td>0.345</td>
<td>1.857</td>
</tr>
<tr>
<td>Sabinene</td>
<td>134.6 ± 46.9</td>
<td>119.7 ± 55.6</td>
<td>0.380</td>
<td>2.263</td>
</tr>
<tr>
<td>β-pinene + myrcene</td>
<td>115.8 ± 33.6</td>
<td>104.8 ± 30.5</td>
<td>0.366</td>
<td>2.243</td>
</tr>
<tr>
<td>(Z)-ocimene</td>
<td>0.0</td>
<td>4.2 ± 9.3</td>
<td>-1</td>
<td>4</td>
</tr>
<tr>
<td>Limonene</td>
<td>64.4 ± 20.0</td>
<td>54.0 ± 21.8</td>
<td>0.608</td>
<td>2.064</td>
</tr>
<tr>
<td>1,8-cineole</td>
<td>65.9 ± 21.6</td>
<td>61.8 ± 20.7</td>
<td>0.230</td>
<td>1.805</td>
</tr>
<tr>
<td>Linalool</td>
<td>249.6 ± 152.6</td>
<td>520.4 ± 319.4</td>
<td>-1.533</td>
<td>4.287</td>
</tr>
<tr>
<td>(E)-β-ocimene</td>
<td>128.3 ± 15.9</td>
<td>216.9 ± 97.7</td>
<td>-1.983</td>
<td>4.467</td>
</tr>
<tr>
<td>DMNT</td>
<td>0.0</td>
<td>23.8 ± 32.9</td>
<td>-1.616</td>
<td>4</td>
</tr>
<tr>
<td>β-caryophyllene</td>
<td>42.4 ± 6.6</td>
<td>59.7 ± 49.1</td>
<td>-0.788</td>
<td>4.003</td>
</tr>
<tr>
<td>δ-cadinene</td>
<td>45.6 ± 5.0</td>
<td>79.6 ± 26.2</td>
<td>-2.771</td>
<td>4.613</td>
</tr>
<tr>
<td>(E, E)-α-farnesene</td>
<td>529.4 ± 185.7</td>
<td>1037.8 ± 790.4</td>
<td>-1.348</td>
<td>4.813</td>
</tr>
</tbody>
</table>

ns = not significant
Table 3 Emission data (ng g⁻¹ h⁻¹) of quantified terpenes (mean ± SD) from detached uninfected control twigs in a plastic bag (C; n = 2), and from pathogen-infected (PAT; n = 5) twigs of silver birch clones 4 and 80. P-values are from independent samples t-test.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Clone 4</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>Clone 80</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Pathogen</td>
<td>t</td>
<td>df</td>
<td>P</td>
<td>Control</td>
<td>Pathogen</td>
<td>t</td>
<td>df</td>
<td>P</td>
</tr>
<tr>
<td>α-pinene</td>
<td>123.0 ± 59.4</td>
<td>69.5 ± 15.5</td>
<td>1.256</td>
<td>1.055</td>
<td>ns</td>
<td>101.3 ± 11.2</td>
<td>51.4 ± 30.8</td>
<td>3.140</td>
<td>4.931</td>
<td>ns</td>
</tr>
<tr>
<td>Sabinene</td>
<td>210.5 ± 117.8</td>
<td>95.8 ± 28.8</td>
<td>1.361</td>
<td>1.048</td>
<td>ns</td>
<td>180.3 ± 10.1</td>
<td>75.3 ± 65.6</td>
<td>3.478</td>
<td>4.423</td>
<td>ns</td>
</tr>
<tr>
<td>β-pinene + myrcene</td>
<td>179.5 ± 102.7</td>
<td>90.7 ± 21.2</td>
<td>1.213</td>
<td>1.034</td>
<td>ns</td>
<td>151.7 ± 17.1</td>
<td>72.2 ± 56.1</td>
<td>2.854</td>
<td>4.995</td>
<td>ns</td>
</tr>
<tr>
<td>(Z)-ocimene</td>
<td>0.0</td>
<td>51.4 ± 28.6</td>
<td>-4.016</td>
<td>4</td>
<td>0.016</td>
<td>0.0</td>
<td>52.9 ± 44.3</td>
<td>-2.673</td>
<td>4</td>
<td>0.056</td>
</tr>
<tr>
<td>Limonene</td>
<td>96.3 ± 89.9</td>
<td>52.8 ± 13.6</td>
<td>0.875</td>
<td>1.031</td>
<td>ns</td>
<td>82.3 ± 7.0</td>
<td>40.1 ± 27.4</td>
<td>3.190</td>
<td>4.893</td>
<td>ns</td>
</tr>
<tr>
<td>1,8-cineole</td>
<td>104.0 ± 58.6</td>
<td>47.6 ± 13.1</td>
<td>1.349</td>
<td>1.040</td>
<td>ns</td>
<td>91.9 ± 18.3</td>
<td>39.7 ± 37.0</td>
<td>2.481</td>
<td>4.165</td>
<td>ns</td>
</tr>
<tr>
<td>Linalool</td>
<td>247.9 ± 223.8</td>
<td>161.0 ± 82.6</td>
<td>0.535</td>
<td>1.111</td>
<td>ns</td>
<td>97.3 ± 15.0</td>
<td>215.5 ± 139.0</td>
<td>-1.874</td>
<td>4.222</td>
<td>ns</td>
</tr>
<tr>
<td>(E)-β-ocimene</td>
<td>206.3 ± 4.1</td>
<td>2042.5 ± 1488.8</td>
<td>-2.758</td>
<td>4</td>
<td>0.051</td>
<td>549.7 ± 280.9</td>
<td>2004.0 ± 1847.8</td>
<td>-1.711</td>
<td>4.417</td>
<td>ns</td>
</tr>
<tr>
<td>DMNT</td>
<td>0.0</td>
<td>144.5 ± 128.6</td>
<td>-2.512</td>
<td>4</td>
<td>0.066</td>
<td>56.2 ± 3.3</td>
<td>46.5 ± 53.9</td>
<td>0.399</td>
<td>4.072</td>
<td>ns</td>
</tr>
<tr>
<td>β-caryophyllene</td>
<td>61.3 ± 73.7</td>
<td>92.7 ± 51.3</td>
<td>-0.551</td>
<td>1.411</td>
<td>ns</td>
<td>79.0 ± 2.9</td>
<td>36.3 ± 18.7</td>
<td>5.498</td>
<td>4.534</td>
<td>0.004</td>
</tr>
<tr>
<td>δ-cadinene</td>
<td>56.5 ± 28.0</td>
<td>112.4 ± 51.5</td>
<td>-1.833</td>
<td>3.795</td>
<td>ns</td>
<td>45.2 ± 10.5</td>
<td>35.7 ± 19.1</td>
<td>0.846</td>
<td>3.734</td>
<td>ns</td>
</tr>
<tr>
<td>(E,E)-α-farnesene</td>
<td>1218.6 ± 946.2</td>
<td>2117.2 ± 855.8</td>
<td>-1.166</td>
<td>1.716</td>
<td>ns</td>
<td>1260.1 ± 46.2</td>
<td>2728.6 ± 2502.2</td>
<td>-1.261</td>
<td>4.066</td>
<td>ns</td>
</tr>
</tbody>
</table>

ns = not significant (P > 0.1)
Emissions of α-pinene, sabinene, β-pinene, myrcene, limonene, 1,8-cineole and β-caryophyllene were significantly larger from uninfected twigs than those from pathogen-infected twigs (Table 3). The plastic bags as such used in pathogen infection increased the emission of several VOCs compared to the intact twigs without the plastic bag (data not shown). Direct *E. autumnata*-damage in the foliage induced the emission of (Z)-ocimene, linalool, (E)-β-ocimene, DMNT, β-caryophyllene, (E,E)-α-farnesene and MeSA (Table 4).

**Effects of ozone exposure on VOCs**

Exposure of silver birch clones to 50 ppb and 100 ppb of ozone had no effect on the emission of volatile compounds (Table 5). Again, (Z)-3-hexenol and (Z)-3-hexenyl acetate were the main compounds emitted from the detached twigs. Despite the high O₃ concentration, no visible ozone symptoms were detectable in any of the O₃-exposed saplings. Clone 80 emitted significantly larger amounts of linalool and β-caryophyllene than clone 4 (Table 5).

Table 4 Pooled emission data (ng g dw⁻¹ h⁻¹) of quantified terpenes and MeSA (mean ± SD, n = 6) from detached intact control (C) and *E. autumnata*-damaged (DH) silver birch clones 4 and 80. P-values are from independent samples t-test.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>C</th>
<th>DH</th>
<th><em>t</em></th>
<th>df</th>
<th><em>P</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>α-pinene</td>
<td>18.9 ± 18.3</td>
<td>40.7 ± 29.2</td>
<td>1.555</td>
<td>10</td>
<td>ns</td>
</tr>
<tr>
<td>Sabinene</td>
<td>37.8 ± 35.5</td>
<td>43.2 ± 48.7</td>
<td>0.220</td>
<td>10</td>
<td>ns</td>
</tr>
<tr>
<td>β-pinene + myrcene</td>
<td>34.7 ± 28.4</td>
<td>49.9 ± 40.5</td>
<td>0.754</td>
<td>10</td>
<td>ns</td>
</tr>
<tr>
<td>(Z)-ocimene</td>
<td>0</td>
<td>30.4 ± 7.4</td>
<td>10.039</td>
<td>5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Limonene</td>
<td>34.5 ± 11.5</td>
<td>36.3 ± 20.6</td>
<td>0.193</td>
<td>10</td>
<td>ns</td>
</tr>
<tr>
<td>1,8-cineole</td>
<td>30.9 ± 23.2</td>
<td>14.2 ± 12.1</td>
<td>-1.561</td>
<td>10</td>
<td>ns</td>
</tr>
<tr>
<td>Linalool</td>
<td>39.3 ± 25.1</td>
<td>708.7 ± 406.5</td>
<td>4.026</td>
<td>5.038</td>
<td>0.010</td>
</tr>
<tr>
<td>(E)-β-ocimene</td>
<td>44.9 ± 27.0</td>
<td>1118.9 ± 390.7</td>
<td>6.717</td>
<td>5.048</td>
<td>0.001</td>
</tr>
<tr>
<td>DMNT</td>
<td>11.9 ± 18.5</td>
<td>714.2 ± 353.4</td>
<td>4.861</td>
<td>5.027</td>
<td>0.005</td>
</tr>
<tr>
<td>β-caryophyllene</td>
<td>35.4 ± 5.8</td>
<td>100.4 ± 40.1</td>
<td>3.939</td>
<td>5.209</td>
<td>0.010</td>
</tr>
<tr>
<td>δ-cadinene</td>
<td>22.2 ± 34.8</td>
<td>112.5 ± 161.4</td>
<td>1.399</td>
<td>10</td>
<td>ns</td>
</tr>
<tr>
<td>(E,E)-α-farnesene</td>
<td>473.3 ± 331.7</td>
<td>1833.2 ± 540.4</td>
<td>5.254</td>
<td>10</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MeSA</td>
<td>17.8 ± 21.3</td>
<td>170.2 ± 111.3</td>
<td>3.302</td>
<td>5.366</td>
<td>0.019</td>
</tr>
</tbody>
</table>

ns = not significant
Table 5 Emission data (ng g\textsuperscript{-1} dw\textsuperscript{-1} h\textsuperscript{-1}) of quantified terpenes and MeSA (mean ± SD, \(n = 6\)) from detached twigs of silver birch clones 4 and 80 exposed to 0, 50 or 100 ppb of ozone over two days for 12 h day\textsuperscript{-1}. P-values are from GLM procedure.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Clone 4</th>
<th></th>
<th>Clone 80</th>
<th></th>
<th>Clone</th>
<th>O3</th>
<th>O3 × O3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 ppb O3</td>
<td>50 ppb O3</td>
<td>100 ppb O3</td>
<td>0 ppb O3</td>
<td>50 ppb O3</td>
<td>100 ppb O3</td>
<td>n = 6</td>
</tr>
<tr>
<td>α-pinene</td>
<td>51 ± 3</td>
<td>48 ± 18</td>
<td>65 ± 21</td>
<td>74 ± 71</td>
<td>48 ± 26</td>
<td>74 ± 55</td>
<td>ns</td>
</tr>
<tr>
<td>Sabinene</td>
<td>80 ± 5</td>
<td>73 ± 33</td>
<td>113 ± 40</td>
<td>125 ± 146</td>
<td>79 ± 54</td>
<td>129 ± 109</td>
<td>ns</td>
</tr>
<tr>
<td>β-pinene + myrcene</td>
<td>72 ± 4</td>
<td>69 ± 27</td>
<td>95 ± 33</td>
<td>106 ± 113</td>
<td>68 ± 39</td>
<td>108 ± 87</td>
<td>ns</td>
</tr>
<tr>
<td>Limonene</td>
<td>49 ± 3</td>
<td>49 ± 17</td>
<td>65 ± 22</td>
<td>75 ± 67</td>
<td>53 ± 24</td>
<td>81 ± 62</td>
<td>ns</td>
</tr>
<tr>
<td>1,8-cineole</td>
<td>49 ± 3</td>
<td>46 ± 14</td>
<td>67 ± 24</td>
<td>72 ± 83</td>
<td>47 ± 32</td>
<td>73 ± 61</td>
<td>ns</td>
</tr>
<tr>
<td>Linalool</td>
<td>135 ± 73</td>
<td>80 ± 46</td>
<td>139 ± 74</td>
<td>393 ± 275</td>
<td>279 ± 317</td>
<td>312 ± 206</td>
<td>0.004</td>
</tr>
<tr>
<td>(E)-β-ocimene</td>
<td>56 ± 19</td>
<td>61 ± 26</td>
<td>70 ± 24</td>
<td>109 ± 83</td>
<td>125 ± 126</td>
<td>91 ± 68</td>
<td>ns</td>
</tr>
<tr>
<td>DMNT</td>
<td>17 ± 18</td>
<td>14 ± 13</td>
<td>9 ± 10</td>
<td>46 ± 52</td>
<td>18 ± 29</td>
<td>38 ± 27</td>
<td>0.046</td>
</tr>
<tr>
<td>α-copaene</td>
<td>42 ± 20</td>
<td>68 ± 76</td>
<td>82 ± 72</td>
<td>40 ± 21</td>
<td>28 ± 32</td>
<td>103 ± 119</td>
<td>ns</td>
</tr>
<tr>
<td>β-caryophyllene</td>
<td>32 ± 12</td>
<td>40 ± 27</td>
<td>50 ± 26</td>
<td>54 ± 14</td>
<td>53 ± 27</td>
<td>81 ± 56</td>
<td>0.044</td>
</tr>
<tr>
<td>α-humulene</td>
<td>5 ± 7</td>
<td>9 ± 18</td>
<td>14 ± 17</td>
<td>13 ± 8</td>
<td>3 ± 8</td>
<td>22 ± 26</td>
<td>ns</td>
</tr>
<tr>
<td>δ-cadinene</td>
<td>20 ± 10</td>
<td>37 ± 36</td>
<td>42 ± 31</td>
<td>27 ± 13</td>
<td>19 ± 15</td>
<td>46 ± 46</td>
<td>ns</td>
</tr>
<tr>
<td>(E, E)-α-farnesene</td>
<td>87 ± 28</td>
<td>137 ± 82</td>
<td>178 ± 37</td>
<td>147 ± 79</td>
<td>181 ± 108</td>
<td>173 ± 119</td>
<td>ns</td>
</tr>
<tr>
<td>MeSA</td>
<td>7 ± 7</td>
<td>9 ± 12</td>
<td>13 ± 21</td>
<td>7 ± 11</td>
<td>10 ± 17</td>
<td>4 ± 7</td>
<td>ns</td>
</tr>
</tbody>
</table>

ns = not significant
Host-searching behaviour of *P. maculiventris*

The nymphs of *P. maculiventris* did not significantly discriminate between the odours of intact twig with indirect herbivory and the intact twig in either of the clones (Fig. 1a). Furthermore the nymphs did not discriminate between pathogen-infected twigs and intact twigs in clone 4 which was more sensitive to the pathogen than clone 80 (Fig. 1b). However, *P. maculiventris* adults did exhibit a marginal preference (65%, *P* = 0.100) towards the twigs which had suffered direct herbivore feeding damage compared to the undamaged twigs in clone 4 (Fig. 1c).

![Figure 1](image-url)

**Figure 1** Percentage of *P. maculiventris* choosing between the odours of A, intact top of the branch where *E. autumnata* larvae had been feeding in the lower part (indirect herbivory; IH) or undamaged (C), B, pathogen-infected (PAT) or uninfected (C) twig, and C. *E. autumnata*-damaged (direct herbivory; DH) or undamaged (C) twig of silver birch in the Y-tube olfactometer. Clones 4 and 80 were used in experiment A, and only clone 4 in experiments B and C. 3rd, 4th and 5th instar nymphs of *P. maculiventris* were used in A and B, and adults in C. *n* indicates the total number of predators used in the assay including the individuals that did not choose between the two odour sources (no choice).

**Discussion**

Effects of herbivore damage and pathogen infection on VOCs

In disagreement with our starting hypothesis, the indirect herbivore damage did not lead to systemic release of novel VOCs from the undamaged upper part of the branch, while directly herbivore-damaged leaves emitted several inducible VOCs. Tomato plants damaged by *Manduca sexta* larvae systemically emit mono- and sesquiterpenes also from the undamaged parts of the plant, while GLVs are emitted only from the damaged leaves (Farag & Paré 2002). The systemic induction of VOCs has also been observed in cotton and maize (Rodriguez-Saona et al. 2002), and activation of defence genes has been observed in intact Lima beans exposed to the induced volatiles from spider mite damaged Lima beans (Arimura et al. 2000).

There are a few reports concerning systemic response in crop plants, but nonetheless the systemic induction of VOCs in trees has not received very much attention. However, Tscharntke et al. (2001) found that unattacked alder leaves had an increased proteinase inhibitor concentration when they were in contact with *A. alni*-damaged leaves indicating a systemic defence response against the feeding herbivore.

We were able to show that pathogen-infection can increase the emission of ocnemones, DMNT and (E,E)-a-farnesene, which were also triggered by *E. autumnata* damage. However, in agreement with our second hypothesis the emission profiles of pathogen-inoculated twigs and the twigs with direct herbivore were not identical. Corresponding results have been previously published by Cardoza et al. (2002) who showed that the infection of peanut plants with white mould fungus increased their emissions of (Z)-3-hexenyl acetate, linalool, DMNT and MeSA, and by Doughity et al. (1996) who reported a release of isothiocyanates and sesquiterpenes from *Brassica rapa* plants inoculated with *Alternaria brassicae*. We found MeSA emission only from *E. autumnata*-damaged silver birch twigs, while Shulav et al. (1997) reported it to be a major compound released from tobacco plants infected with tobacco mosaic virus. The similarities between pathogen- and herbivore-induced compounds indicate that the same defence pathways are activated by both pathogen and herbivore damage.
The phytohormones jasmonic acid (JA), salicylic acid (SA) and ethylene are involved in triggering the defence responses against herbivore damage (Rojo et al. 2003). JA modulates the octadecanoid pathway and SA the shikimic acid pathway (Dicke, 2003). Piel et al. (1997) found that cellulyisin from the plant parasitic fungus elicits the biosynthesis of VOCs in higher plants by activating the octadecanoid signalling pathway, leading to the induction of (E)-β-ocimene and DMNT emission, and this can be viewed as mimicking JA treatment. On the other hand, Heiden et al. (2003) showed with several plant species that pathogen attack, exposure to high ozone concentrations, and wounding induce the emission of (Z)-3-hexenol, C₆ aldehydes and alcohols derived from a JA-controlled lipoxygenase pathway.

Effects of ozone exposure on VOCs

In disagreement with our third hypothesis, ozone exposure did not enhance the constitutive emission or induce the emission of novel VOCs from silver birch twigs. This is in contrast with the previous studies where emissions have been reported to be enhanced (Loreto et al. 2004; Vuorinen et al. 2004a) by the ozone fumigation. Neither did we detect inducible terpenes (Heiden et al. 1999; Vuorinen et al. 2004a) or C₆ compounds (Heiden et al. 2003) which have been detectable from O₃-exposed plants. Heiden et al. (2003) also reported that when visible symptoms appeared, there was also emission of LOX products. In the present study the O₃ concentration was quite low compared to those studies where induction of VOCs has been observed. In addition, the exposure time was short, and no visible O₃ symptoms in the leaves were observed. We detected high emission of GLVs (e.g. (Z)-3-hexenyl acetate and (Z)-3-hexenol) from all of the treatments, but those emissions were considered most likely to be a consequence of our VOC analyzing method where the twig was cut (Vuorinen et al. 2005). The emission of GLVs can increase approx. 20% due to detachment. Also, Hakola et al. (2001) demonstrated that rough handling of the birch leaves causes high emissions of (Z)-3-hexenol, (Z)-3-hexenyl acetate, 2-hexenal and 1-hexanol, though MT emission was unaffected. Also, the saplings were used in the pathogen infection and herbivore damage experiments prior to the ozone experiment which may have launched systemic resistance against other stresses (Bostock 1999).

Host-searching behaviour of P. maculiventris

P. maculiventris nymphs were not attracted to the odours of pathogen-infected twigs or twigs with indirect herbivory, even though pathogen-infected twigs emitted novel VOCs. On the other hand, twigs with direct herbivore damage were slightly attractive to P. maculiventris adults. Apparently foliar damage caused by chewing herbivores plays an important role in the attractiveness, since systematic release of induced volatiles was not found from the twigs with indirect herbivore damage. P. maculiventris possesses olfactory receptors to Colorado potato beetle-induced (Z)-3-hexenyl butyrate (Dickens 1999). The predator is also attracted to the blend comprising (E)-2-hexenol, (Z)-3-hexenol, (±)-linalool, nonanal and MeSA (Dickens 1999), and 3rd and 5th instar nymphs are responsive to common plant GLVs (E)-2-hexenal and (E)-2-hexenal (Sant’Ana et al. 1999). The emission of GLVs in the present study was perhaps skewed compared to the natural situation because of the detachment of the twig. On the other hand, the emission of MeSa was higher from directly herbivore-damaged twigs compared to undamaged twigs. It has also been suggested that P. maculiventris uses vibration signals produced by the chewing of common prey species as cues for host-locating (Pfannenstiel et al. 1995). It is noteworthy that the number of predators in the behavioural assay was quite small, but our results show a trend in which the predator was attracted to the odour of herbivore-damaged foliage. Cardoza et al. (2003) found that the parasitoid Cotesia marginiventris was more responsive to the
odour of white mold-infected and S. exigua-damaged peanut plants compared to the plants with S. exigua-damage only. This finding still does not reveal whether pathogen-infection singly could attract the predator or the parasitoid. In our earlier study (Vuoris et al. 2004b), orientation of the specialist parasitoid Cotesia plutellae towards damaged cabbage plants was found to be stronger than that of the predator P. maculiventris. Vet & Dicke (1992) proposed that generalist predators should not innately react to herbivore-induced semiochemicals, instead herbivore-derived volatiles are the most reliable cues. After an associative learning process, the predators are able to use the volatiles produced by plants for prey-locating. In the present study, we used both nymphs which had not experienced the odors emitted by E. autumnata-damaged birch leaves, and adults and nymphs which had been reared on E. autumnata larvae feeding on birch leaves, but there was no difference in the orientation behavior of experienced and naïve predators.

Conclusions

Our results suggest that a fungal pathogen is able to induce the emission of several compounds known as herbivore-induced volatiles from silver birch, but the composition of the volatile blend differs from that of directly herbivore-damaged leaves, and most likely thereby does not attract the generalist predator. One question still remains: could pathogen-induced VOCs mislead the specialist parasitoid from its prey? Furthermore, to better understand the ecological and evolutionary significance of inducible VOCs, experiments combining both biotic and abiotic stresses on plants need to be conducted.

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References

Cardoza YJ, Teal PEA, Tumlinson JH (2003) Effect of peanut plant fungal infection on oviposition preference by Spodoptera exigua and on host-searching behaviour by Cotesia marginiventris. Environ Entomol 32: 970-976
Hakola H, Rinne J, Laurila T (1998) The hydrocarbon emission rates of tea-leaved willow (Salix phylicifolia), silver birch (Betula pendula) and European aspen (Populus tremula). Atm Environ 32: 1825-1833
compounds from ozone-exposed plants. Ecol Appl 9: 1160-1167
Vuorinen T, Nerg A-M, Holopainen J (2004a) Ozone exposure triggers the emission of herbivore-induced plant volatiles, but does not disturb tritrophic signalling. Environ Pollut 131: 305-311
CHAPTER 6

OZONE EXPOSURE TRIGGERS THE EMISSION OF HERBIVORE-INDUCED PLANT VOLATILES, BUT DOES NOT DISTURB TRITROPHIC SIGNALING


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Ozone exposure triggers the emission of herbivore-induced plant volatiles, but does not disturb tritrophic signalling

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“Capsule”: O3 induced emission of some of the same compounds as spider mites, but did not disturb signalling between lime beans, spider mites and predatory mites.

Abstract

We evaluated the similarities between ozone-induced and mite-induced emission of volatile organic compounds (VOCs) from lime beans, and tested the response of the natural enemies of herbivores to these emissions using trophic system of two-spotted spider mites and predatory mites. The acute ozone-exposure and spider mite-infestation induced the emission of two homoterpenes, (E)-4,8-dimethyl-1,3,7-nonatriene and (E,E)-4,8,12-trimethyl-1,3,7,11-trideatetraene, and (Z)-3-hexenyl acetate. Only plants with spider mite-infestation emitted the monoterpenes (E)-β-citralene. Predatory mites were equally attracted to ozone-exposed and unexposed plants, but discriminated between spider mite-infested and uninfested plants, when both were exposed to ozone. The similarities between ozone and herbivore-induced VOCs suggest that plant defence against phytotoxic ozone and the production of VOCs for attraction of the natural enemies of herbivores may have adaptive coevolution. However, the expected elevated ozone concentrations in future may not disturb tritrophic signalling, unless herbivore-induced VOCs are lost in the process of aerosol formation.

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I. Introduction

Plants are able to defend themselves indirectly against herbivores via herbivore-induced VOCs that attract the natural enemies of herbivores (e.g. Dicke et al., 1990a; De Moraes et al., 1999). These compounds, which function as foraging cues to the predators, are induced by herbivore feeding, but not usually by mechanical damage (Karban and Baldwin, 1997). It is also well known that predatory mites are attracted to the odour of spider mite-induced plant volatiles (Dicke et al., 1990b; Lussi and Perelschatz, 2001). Lately, it has been shown in natural population of Nicotiana attenuata plants that releasing VOCs due to herbivore attack can increase predation of the herbivore eggs and decrease oviposition rate, which strongly suggests that indirect defence operates not only in climate-controlled laboratories but in nature too (Kessler and Baldwin, 2001).

There are several environmental factors, such as light, humidity, temperature and fertilization (Gouinguené and Turlings, 2002) that can affect the emission of VOCs from plants in the field. Also, the effects of abiotic (light intensity, season and water stress) and biotic factors on tritrophic signalling have already been under investigation to some extent (Takahayashi et al., 1994). However, the effects of air pollutants such as ozone on this signalling system between three trophic levels have remained completely unexplored. The mean concentration of tropospheric ozone in urban areas is rising, and the transport of ozone to rural areas can cause more damage to cultivated and natural crops than any other pollutant (Ashmore, 2002). In the most polluted sites
plants are exposed to acute peak ozone concentrations of 120–500 nL L⁻¹ (Long and Naidu, 2002). As a phytoxic pollutant, ozone can activate various defence responses in plants (Kangasjärvi et al., 1994): the expression of defence-related genes, the emission of ethylene and other VOCs, and the biosynthesis of signalling molecules such as jasmonic acid (JA), salicylic acid (SA) and ethylene (Rao et al., 2000). Accordingly, it has been suggested that ambient ozone can act as a significant abiotic elicitor (Sanderman et al., 1998).

The ozone-induced emissions of plants are still poorly known. Llusia et al. (2002) showed that total VOC emissions of Ceratonia siliqua in summer, and that of Olea europaea and Quercus ilex rotundifolia in spring increased in open-top chambers (OTCs) fumigated with 40 nL L⁻¹ of O₃ over ambient level in comparison with the filtered air and non-fumigated OTCs. Persuad et al. (1999) also detected higher emissions of total VOCs from Solanum lycopersicum in OTCs fumigated with 40 nmmol mol⁻¹ of O₃ above ambient level. Ozone-resistant Bel B variety of Nicotiana tabacum released sesquiterpenes shortly after 5 h (70 nmmol mol⁻¹ O₃) fumigation pulse, while ozone-sensitive Bel W3 variety emitted sesquiterpenes, methyl salicylate (MeSA) and C₆ compounds in much greater amounts after 24 h of the start of the O₃-fumigation when the visible symptoms appeared (Heiden et al., 1999).

It has been suggested that one of the original evolutionary functions of the VOCs might have been the reduction of phytotoxic ozone by oxidation, and consequently by aerosol formation (Lerdau and Slobodkin, 2002; Lerdau and Gray, 2003). The aerosol forming capacity of VOCs is highest among sesquiterpenes, occasionally reaching nearly 100% of the emission (Andreae and Crutzen, 1997). Therefore, the ecological signals transmitted by reactive VOCs might suffer at high ozone concentrations. The ozone reactivity of herbivore-inducible homoterpenes is comparable to the reactivity of sesquiterpenes in the atmosphere (Roger Atkinson, personal communication). Jansen et al. (2002) suggested that it is unlikely that plants originally started to produce volatiles to attract the natural enemies of herbivores, as it would have required a simultaneous change in plants (to produce volatiles) and predators (to respond to these volatiles). Therefore, it is more likely that the pathways to produce plant volatiles may have evolved for some other reason, but once predators and parasitoids started responding to these volatiles, plants were able to use natural enemies as an indirect defence against herbivores.

The present study was conducted to test the hypothesis that acute ozone exposure may induce the formation and emission of the same plant volatiles in Lima bean plants that herbivorous mites induce. The second hypothesis was that due to similarities in ozone- and mite-induced emissions, ozone may mask the tritrophic signalling between herbivore-damaged plant and the natural enemies of the herbivore. To test these hypotheses we selected the most extensively studied tritrophic signalling system consisting of Lima beans, spider mites, and predatory mites (Dicke, 1994; Dicke et al., 1999; Arimura et al., 2000).

2. Materials and methods

2.1. Plant material and mites

Lima bean plants (Phaseolus lunatus CV. Siiva, Dormpe WHSE Co, Crows Landing, CA, U.S.A.) were grown in plastic pots (9 × 9 × 9 cm) filled with a mixture (3:1) of quartz sand and fertilized Sphagnum peat. The plants were maintained during rearing and experiments in computer controlled growth chamber (Bioclime 2600T), at 25/20 °C, 50/70% RH, 16L:8D and depending on the experiment at 250 or 400 μmol m⁻² s⁻¹ (PAR). Plants were used in experiments 5–7 days after germination when the primary leaves were unfolded.

Two-spotted spider mites (Tetranychus urticae) were originally obtained from a greenhouse population in the Research Greenhouse of University of Kuopio. Mites were reared on detached Lima bean leaves at 24–25 °C for better control of the population density. Adult female spider mites were used to infest Lima bean leaves in the experiments. Predator mites (Phytoseiulus persimilis) were obtained from a commercial source (OY Schetelig AB, Vantaa, Finland) and reared in our laboratory on T. urticae-infested detached Lima bean leaves at 24–25 °C. Adult female predatory mites were used in the behavioural assay.

2.2. Treatments

In all experiments the plants were first grown together in one filtered air chamber, and just before the experiment, the plants were distributed evenly into treatment and control chambers. To investigate the emission of O₃-induced VOCs from Lima bean plants together with the behaviour response of predatory mites, we conducted three separate experiments. VOC samples from plants were collected just before the behavioural assay. In the first experiment Lima bean leaves with just unfolded primary leaves were infested with 30 T. urticae females per leaf, and kept in the growth chamber at 250 μmol m⁻² s⁻¹. Spider mites were transferred on the leaves using fine paintbrush. The infested plants were used in the experiment 24 h after the infestation. Another set of uninfested plants was used as controls. In the second experiment the plants were first exposed to 150 nL L⁻¹ of O₃ for 4 h (1:00 pm to 5:00 pm) and thereafter to 200 nL L⁻¹ of O₃ for another 4 h (1:00 pm to 5:00 pm) in the growth chamber at 400 μmol m⁻² s⁻¹ during
the same day. Next day, plants were exposed to 150 mL L\(^{-1}\) of \(\text{O}_3\) from 8:00 am until they were used in the collection of plant volatiles and behavioural assays. Unexposed plants grown in filtered air were used as controls. Ozone was produced from pure oxygen with Fisher OZ 500 ozone generator (Fisher, Bonn, Germany) and analyzed with Dasibi 1008-BS ozone analyzer (Dasibi Environmental Corp., Glendale, CA, U.S.A.). In the third experiment the plants were infested with 30 \(T. \text{urticae}\) females per leaf and simultaneously exposed to 150 mL L\(^{-1}\) of \(\text{O}_3\) for 4 h and to 200 mL L\(^{-1}\) of \(\text{O}_3\) for 4 h as described previously. Control plants were exposed to ozone in another growth chamber but not infested with \(T. \text{urticae}\). Spider mites remained on the plants during VOC collections and behavioural assays. Every experiment lasted for three days.

2.3. Collection of volatile compounds

The VOCs from individual lima bean plants \((n = 5)\) were collected at 250 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) (PAR) using closed flow-through device, before the same plants were used for the behavioural assay described below. Whole plant with unfolded primary leaves was cut from stem-base, just above soil level, and placed into 4-mL vial filled with tap water. The whole plant was immediately set in a 1-L glass cuvette and closed with Teflon sealed lid with an inlet for purified air and an outlet for sampling. Air purified with activated carbon entered the cuvette at the rate of 310 mL min\(^{-1}\). After 5-min adjustment, the sample was pulled through a purified stainless steel tube (Perkin Elmer, ATD sample tubes) filled with ca. 150 mg Tenax\-TA adsorbent (Supelco, mesh 60/80) with a vacuum pump (KNF Neuberger, Inc., Freiburg, Germany, Model N922AN.16B) at a rate of 300 mL min\(^{-1}\) for 20 min. The airflow was calibrated daily with the mini-Buck calibrator (Model M-5, A.P. Buck, Inc., Orlando, FL, U.S.A.). Samples were analyzed by GC-MS (Hewlett Packard GC type 6890, MSD 5973). Trapped compounds were desorbed (Perkin Elmer ATD400 Automatic Thermal Desorption System) at 220 °C for 10 min, cryofocused at −30 °C and injected onto HP-5 capillary column \((30 \text{ m} \times 250 \mu \text{m} \times 0.25 \mu \text{m}, \text{Hewlett Packard})\). The carrier gas was helium. The temperature programme was 50 °C for 1 min, followed by increases of 5 °C min\(^{-1}\) to 210 °C and 20 °C min\(^{-1}\) to 250 °C. Compounds were identified by comparison of the mass spectra with those in the Wiley library and pure standards. The total VOC emission from plants was calculated by combining the peak areas of all identified compounds and dividing by dry weight (peak area g dw\(^{-1}\)).

2.4. Behavioural assay

The Y-tube olfactometer (main arm 10.5 cm, other arms 10 cm, inner diameter 1.6 cm, and angle between two arms ~90°) was used to test the behaviour of predatory mites. In the first experiment, predatory mites were given a choice between \(T. \text{urticae}\)-infested and uninfested plants. In the second experiment, predatory mites had to choose between \(\text{O}_3\)-exposed and unexposed plants, and in the third experiment, predatory mites made a choice between \(\text{O}_3\)-exposed plants and plants that had been infested with \(T. \text{urticae}\) and exposed to ozone simultaneously. The cuvettes and lids in the Y-tube olfactometer were the same that were used in the collection of VOCs. The pressurized air was purified with activated carbon and divided via Teflon tubing into two separate flows \((500 \text{ mL min}^{-1})\) which both passed through a 1-L glass cuvette containing one lima bean plant as an odour source, and leading to either arm of the Y-tube. The airflow in the Y-tube was checked with smoke, and no turbulence was detected between the two arms of the Y-tube. The airflow was adjusted with pressure and needle valves and calibrated daily with M-5 mini-Buck calibrator. Female predatory mites collected 24 h earlier from the mass rearing, were introduced to the downwind end of the Y-tube and observed for 5 min or until they made the final choice. The choice was recorded when the predator passed two-thirds of the arm of the Y-tube. Odour sources, cuvettes, Y-tube, and lids were replaced after testing five predators to avoid any errors caused by the olfactometer system itself. All the glassware was heat-treated at 120 °C before use, and the Y-tube was turned around after each tested predator. In each experiment, 100 predators were tested on 20 different sets of odour sources over three days.

2.5. Statistical analyses

For statistical analyses SPSS 10.0 for Windows was used. The treatment effects on inducible VOCs were analyzed by the non-parametric Mann–Whitney U-test, when variances were unequal, and parametric independent samples t-test, when variances were equal. The data from behavioural assay were analyzed with the non-parametric binomial test to test whether there was a significant difference in attraction between the two odour sources having plants from different treatments (test proportion was set to 0.50).

3. Results

3.1. Induced VOC emissions

Gas chromatographic-mass spectrometry analysis of the VOCs revealed similarities between the emissions of \(T. \text{urticae}\)-infested plants, \(\text{O}_3\)-exposed plants, and simultaneously \(T. \text{urticae}\)-infested and \(\text{O}_3\)-exposed lima bean plants (Fig. 1, A–D). The feeding of \(T. \text{urticae}\) induced clearly the emission of (Z)-3-hexenyl acetate.
7. *urticae* induced volatiles

8 10 12 14 16

9 11 13 15 17

10 12 14 16 18

3.2. Behavioural assay

In two-choice behavioural assay, predatory mites preferred the odour of lima bean plants infested with *T. urticae* to that of uninfested plants (Fig. 4). Predators were also able to discriminate between *T. urticae*-infested plants and uninfested plants when both were exposed to ozone. However, the predators did not distinguish between the odour of O₃-exposed plant and unexposed plant (Fig. 4).

4. Discussion

In this study, exposure of lima bean plants to ozone led to increased emission of two homoterpenes, DMNT and TMTT, which are the same compounds that are elicited by *T. urticae*-infection. Homoterpenes are derived from their C₁₅ and C₂₀ precursors, farnesyl- and geranylglycerol-pyrophosphate (Donnath and Boland, 1994). Ozone fumigation also increased the emission of (Z)-3-hexenyl acetate, which is produced via lipoygenase pathway (Paré and Turnbull, 1999). Contrary to earlier studies (Dícke et al. 1999b; Dícke et al., 1999) we did not detect the emission of (Z)-3-hexenol from *T. urticae*-infested plants even though trace amount of (Z)-3-hexenol was emitted from O₃-exposed lima beans in one of the experiments. Croft et al. (1993) found that (Z)-3-hexenol is induced by pathogen attack during hypersensitive response, but it also might be induced by mechanical damage (Takahayashi et al., 1994b). Rao et al. (2000) suggested that visible injury and responses of the plant to
acute ozone exposure are similar to the hypersensitive response occurring as a result of a pathogen attack.

Heiden et al. (1999) found that O₃-sensitive tobacco cultivar Bel W3 emitted MeSA, several sesquiterpenes, and C₆ compounds after a pulse treatment of O₃, but contrary to our results they did not detect typical herbivores-induced homoterpenes. Nevertheless, the emission of sesquiterpenes or MeSA was not detected from O₃-exposed lima beans in the present experiment. We did not detect the emission of MeSA from spider mite-infested lima beans either, which has been reported quite consistently in other studies (Dicke et al., 1999; Arimura et al., 2000). In the present study the spider mite-induced VOCs from whole plants were collected 24 h after the beginning of infestation, while in other studies infestation period has been longer, and experiments have been often done with excised leaves only. In agreement with our study Peñuelas et al. (1999) reported that tomato plants fumigated with 40 μL L⁻¹ of ozone over ambient level had significantly increased VOC emissions.

Fig. 3. The emission (mean ± SE) of total VOCs from three separate experiments on the basis of peak areas. FA = intact plants grown in filtered air, TU = T. urticae-infested, O₃ = O₃-exposed, and O₃ + TU = O₃-exposed and T. urticae-infested lima beans, n = 7 in exp. 1, and n = 6 in exp. 2 and 3. Asterisks indicate a significant (*P < 0.05; independent samples t-test) increase in emission.

Fig. 4. The behavioural response of predatory mite P. persimilis. The percentage of P. persimilis choosing for (A) T. urticae-infested (TU) or uninfested (FA), (B) O₃-exposed (O₃) or unexposed (FA), and (C) O₃-exposed and T. urticae-infested (O₃ + TU) or O₃-exposed (O₃) lima beans in the Y-tube olfactometer. In each experiment preference of 100 predatory mite females was tested. Asterisks indicate significant (***P < 0.001, **P < 0.01, *P < 0.05; binomial test) preference of predatory mite towards either odour source. No choice indicates the number of predatory mites that did not choose between two odour sources.
Since ozone induces the formation of both signalling molecules JA and SA, and triggers the emission of VOCs (Kangasjarvi et al., 1994), it is likely that plant response to ozone is similar to the plant response to the spider mite-infestation. In previous studies the composition of JA-induced VOCs has been found to be similar, but not completely identical, to that of VOCs emitted by lima bean leaves infested with T. urticae (Hopke et al., 1994; Dicke et al., 1999). Recently, the production of T. urticae-induced volatiles has found to be under control of both JA-related and SA-related signalling pathways, whilst JA-related signalling pathway alone is involved in the production of caterpillar-infected volatiles (Ozawa et al., 2000).

Schmelz et al. (2001) reported that excised leaves of corn plants (Zea mays L.) emitted 2.5 to 8.0-fold more JA- and volatilin (N-(1-hydroxyindol-3-yl)-L-glutamine)-induced sesquiterpenes than similarly treated intact plants. Our study was done with whole plants that were cut from stem-base, and used in the collection of VOCs and behavioural assays not more than 30 min after cutting. It is unclear whether the cutting of the whole plant affects the emission pattern or the odour profile of treated lima bean leaves, but detached leaves are often used when herbivore-induced VOCs of this plant species are measured (Arimura et al., 2000).

Although O3-exposed lima bean plants release some of the same volatile compounds as mite-infested plants, predatory mites can distinguish between odours from mite-stressed and ozone-stressed plants. The relative proportion of individual compounds, especially the proportion of (E)-β-oicinene in the odour plume, and the whole blend of volatiles, are probably the key factors for P. persimilis for location of T. urticae-damaged plants. The attractiveness of (E)-β-oicinene to the predator P. persimilis has already been demonstrated without a doubt by Dicke et al. (1999b). In addition, according to our findings, (E)-β-oicinene appears to be an even more effective foraging cue to the predators than DMN, which has previously been reported to act as an alarm signal luring predators (Dicke, 1994).

We were able to show that after ozone fumigation predatory mites still can detect the volatile cues from mite-infested plants. However, the laboratory conditions are not comparable to the natural conditions, where ozone-induced release of volatiles leads to atmospheric chemical reactions with the prevailing ozone that originally induced the emissions. In high ozone concentrations, the mite-induced volatiles in the atmosphere are probably in lower concentrations than in our olfactometer that is using filtered air. To test the response of predatory mites properly in simulated natural atmospheric conditions, an olfactometer that allows control of ozone concentration and UV-B radiation levels as well as measurements of aerosol particle yield from the system during behavioural test should be used. However, our experiment is the first one that tries to estimate the possible effects of ozone on signalling through three trophic levels, between plant and the natural enemies of its herbivores.

5. Conclusions

Predatory mites did not respond to the blend of volatiles from ozone-exposed plants, although ozone exposure triggered the emission of some of the same compounds as did spider mite-infestation. Our results suggest that elevated mean ozone concentrations in future might increase the total VOC emission and trigger the emission of herbivore-inducible compounds from plants, but this emission does not interfere with tritrophic signalling on lima bean. However, for more general conclusions the effects of ozone exposure on tritrophic cascades should be studied with several plant species using a wide variety of herbivores and their natural enemies.

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References


CHAPTER 7

GENERAL DISCUSSION
GENERAL DISCUSSION

7.1 Effects of doubled atmospheric [CO₂]

7.1.1 Growth at elevated [CO₂] reduces or leaves the emission of VOCs unaltered

One of the two separate experiments (Chapter 2) revealed that three-weeks growing at elevated [CO₂] in a growth chamber decreases the quantity of constitutively emitted monoterpenes from intact and herbivore-damaged white cabbage cv. Lennox, while emissions of herbivore-induced compounds were unaltered. In an other experiment (Chapter 3) with the same set-up, growing under doubled ambient [CO₂] had a reducing, although non-significant, effect on the emissions of monoterpenes and herbivore-induced compounds from cabbage cvs. Lennox and Rinda, when plants were damaged by P. xylostella.

However, the emission of VOCs from two clones of silver birch grown at doubled ambient [CO₂] in OTCs over three growing seasons (Chapter 4) did not respond clearly or significantly to CO₂ enhancement, although it did increase the growth, biomass and total leaf area of the experimental trees (Riikonen et al. 2004).

Traditionally, an increase of allocation of excess carbon to carbon based secondary metabolites under elevated atmospheric [CO₂] has been predicted based on carbon-nutrient balance (Bryant et al. 1983) and growth-differentiation balance (Herms & Mattson 1992) hypotheses. This may be true with the phenolic compounds as their concentration has been shown to increase several times (Bezemer & Jones 1998, Percy et al. 2002, Peltonen et al. 2005), but is not the case with secondary metabolites such as terpenes (Bezemer & Jones 1998, Koricheva et al. 1998). Lawler et al. (1997) suggested that this discrepancy is probably due to the different biochemical pathways of phenolics and terpenes, and the possibility that precursors of phenolics are more abundant at elevated [CO₂].

It has been found in several cases that elevated [CO₂] decreases the emission of isoprene and monoterpenes from various plant species (Loreto et al. 2001, Rosenstiel et al. 2003, Rapparini et al. 2004), Dimethylallyl diphosphate (DMAPP), the precursor of isoprene synthesis, decreases in parallel with the isoprene emission observed under elevated [CO₂], and Rosenstiel et al. (2003) suggested that intracellular metabolic competition for phosphoenoypyruvate (PEP) could be the reason for this suppression. Elevated [CO₂] also reduces the activity of monoterpen synthases (Loreto et al. 2001).

The CO₂ effect probably depends on whether the plant has storage-structures for the constitutively emitted terpenes or not. The capacity of terpene-storing organs may restrict the incorporation of excess carbon into terpenoids at elevated [CO₂], and leave the constitutive terpene emission unaffected. Constable et al. (1999) found that monoterpen emission rate of ponderosa pine and Douglas fir was not affected by elevated [CO₂], even though CO₂ treatment increased leaf area index significantly.

While P. xylostella (Chapters 2 & 3) and S. littoralis (Chapter 2) damage significantly enhanced constitutive VOC emissions and induced the emissions of novel VOCs from cabbages grown both at ambient and elevated [CO₂], Litvak et al. (2002) showed that wounded Douglas fir needles had lower total monoterpenes, α-pinene and β-pinene pool sizes than intact needles at both ambient and elevated [CO₂]. Litvak et al. (2002) concluded that elevated [CO₂] may cause the reduction in the rate of monoterpen accumulation in relation to other constituents, like starch, in needles. They also suggested that Douglas fir depends more on constitutive defence than on induced defence, since notable induction of monoterpen cyclase activity in wounded needles did not occur. Based on this assumption, white cabbage probably relies more on induced defence since herbivore-damage considerably increased the
emissions of VOCs and elicited the emission of novel compounds (Chapters 2 & 3).

Rapparini et al. (2004) found that the lifetime exposure of *Q. pubescens* and *Q. ilex* to high [CO₂] did not affect the emission of isoprene and monoterpenes, respectively. On the other hand, Rapparini et al. (2004) detected a decrease in the emissions when control plants were suddenly exposed to elevated [CO₂]. Perhaps the adaptation of the plants to the new growth conditions (e.g. increasing [CO₂]) has been underestimated. The two-fold increase in the atmospheric [CO₂] would not happen instantly even though the increase has been dramatic since the industrial revolution (Houghton et al. 2001).

White cabbage cv. Lennox had greater dry weight (Chapter 2), reduced specific leaf area (SLA) and increased leaf thickness (Chapter 3) when grown at elevated than at ambient [CO₂]. Elevated [CO₂] had no effect on stomatal density, while an average 11% reduction of stomatal density attributable to doubled atmospheric CO₂ had been revealed earlier (Hetherington & Woodward 2003). VOCs formed in the leaves must enter the atmosphere through hydrophobic cuticle, if they are unable to diffuse through the cuticle (Llusia & Peñuelas 1998). Still, the importance of the density and behavior of stomata for controlling total emission is unclear. Niinemets et al. (2004) suggested that the stomatal sensitivity depends on the Henry’s law constant of the particular compound, and they concluded that e.g. methanol and organic acids are stomatal sensitive while isoprene and non-oxygenated monoterpenes are stomatal insensitive.

Since growing under elevated [CO₂] affects the physiology of the plants, it is important to pay attention to the quantitation method used in the emission calculations.

Even though the emissions seem to decrease when plants are grown under elevated [CO₂], it may stimulate the growth of the plants, and thereby leave the emissions unaltered or even increase them on a canopy basis (Loreto et al. 2001). In addition, in future climate conditions elevated [CO₂] is followed by an increase in the temperature, which is expected to enhance the emission of VOCs (Constable et al. 1999).

7.1.2 Growth at elevated [CO₂] may reduce the host-searching efficiency of the natural enemies

The results from one of the experiments (Chapter 3) suggest that doubled atmospheric [CO₂] could reduce the plant response induced by insect herbivore feeding and thereby lead to a disturbance of signalling to the third trophic level. Both, the generalist predator *P. maculiventris* and the specialist parasitoid *C. plutellae* seemed to locate their prey, *P. xylostella* better in the Y-tube olfactometer, when cabbage plants had been grown under ambient [CO₂] than under elevated [CO₂] (Chapter 3).

The specialist parasitoid seemed to find the host more efficiently than the generalist predator. Vet & Dicke (1992) proposed that herbivore-derived VOCs are the most reliable cues for generalist predators for prey-locating. Therefore predators should not innate react to herbivore-induced plant-derived semiochemicals, which are more abundant but less reliable, and which can be used for prey-locating after an associative learning process.

To my knowledge, there have not yet been any other studies concerning tritrophic signalling via plant volatiles under elevated atmospheric [CO₂]. Roth & Lindroth (1995) found that the mortality of the parasitoid *C. melanoscela* was higher on the hosts fed foliage grown under elevated [CO₂] than under ambient [CO₂] indicating that parasitism may decrease under future [CO₂].

The worst case scenario would be that plants grown under increasing [CO₂] are fed on more by herbivores due to compensatory feeding (Bezemer & Jones 1998, Hunter 2001), increased mortality decreases the number of natural enemies parallel to the herbivores (Hunter 2001), the natural
enemies are misled from their prey or host (Chapter 3), and the populations of the natural enemies will become threatened because their host-searching efficiency is weakened.

I totally agree with Hunter (2001) who claims that the effects of elevated [CO₂] on the natural enemies under field conditions ought to be studied in future research since these issues have been barely addressed.

7.2 Effects of elevated [O₃]

7.2.1 Realistic [O₃] does not affect, while high [O₃] elicits the emission of VOCs

The VOC emission of two silver birch clones grown at doubled ambient [O₃] over three growing seasons in OTCs did not respond to elevated [O₃] (Chapter 4). The same non-responsive trend was observed with silver birch saplings exposed to 50 and 100 ppb of O₃ in growth chambers over two days (Chapter 5).

However, emission of novel inducible VOCs were elicited from Lima bean plants exposed to a minimum 4h of 200 ppb of O₃ (Chapter 6). Exposure to 150 ppb of O₃ did not cause qualitative changes on VOC emission profile. These O₃-induced compounds (DMNT, TMTT, and (Z)-3-hexenyl acetate) are also induced by feeding of T. urticae, but the profile of VOCs emitted from O₃- and T. urticae-injured plants were prominently different (Chapter 6).

Unexpected non-responsiveness in emissions from silver birch trees (Chapter 4) and saplings (Chapter 5) contrasts with the previous studies where enhancement of emissions have been reported (Peñuelas et al. 1999, Llusia et al. 2002, Loreto et al. 2004) or emission of novel inducible VOC have occurred (Heiden et al. 1999, 2003) as a consequence of O₃ fumigation. The response of plants to O₃ may be dependent on the type of exposure. Chronic long-term exposure seems to enhance the constitutive emissions, while acute exposure to high [O₃] elicits the emission of novel VOCs.

The Lima beans exposed to O₃ (Chapter 6), only emitted novel inducible compounds after the appearance of visible O₃ symptoms. The visible symptoms were not detectable in the leaves of silver birch saplings exposed to O₃. Heiden et al. (2003) also reported that when visible symptoms appeared, there was also emission of LOX products, C₆ GLVs. Either the visible symptoms and VOC emission are linked or the response to O₃ is postponed, and emission of novel VOCs starts parallel to the visible symptoms.

Homoterpenes are derived from their C₁₅ and C₂₀ precursors, farnesyl- and geranylgeranly-pyrophosphate (Donath & Boland 1994) and are produced from the terpenoid pathway, while GLV (Z)-3-hexenyl acetate is produced via the LOX pathway (Paré & Tumlinson 1999). The similarities between O₃ and herbivore-induced VOCs suggest that plant defence against phytotoxic O₃ and the production of VOCs for attraction of the natural enemies of herbivores may have adaptive coevolution.

7.2.2 O₃ does not affect the tritrophic signalling

Even though exposure to high [O₃] triggered the emission of some of the same compounds from Lima beans as T. urticae-damage, the specialist predator, P. persimilis, was not attracted to the O₃-induced VOCs (Chapter 6). Also, predators discriminated uninfested and T. urticae-infested plants when both were exposed to O₃. Monoterpene (E)-β-ocimene was not among the compounds induced by high [O₃], but was present in the blend from T. urticae-damaged plants. This compound has been proven to attract P. persimilis (Dicke et al. 1990). This indicates that although some of the same compounds are induced by O₃ and herbivory, the natural enemies distinguish the whole odour blend, not just a single compound.
Even though $O_3$ is capable of eliciting the emission of VOCs it also reacts with biogenic VOCs forming aerosols (Andreae & Crutzen 1997, Bonn & Moortgat 2003, Joutsensaari et al. 2005). For example, the chemical lifetime of sesquiterpenes is approximated to be less than 4 minutes during daytime and less than 2 minutes during the night (Kesselmeier and Staedt 1999). It is likely that the host-searching ability of the natural enemies will be disturbed, if those VOCs induced by herbivory and exploited by the natural enemies are lost in the aerosol forming processes.

7.3 Methods

Firstly, attention must be paid to the sampling and analysis of VOCs. What we know is that detaching the branches or twigs will trigger emissions of GLVs as will any other type of mechanical damage (Chapter 4). We do not know whether trimming the roots or cutting the stems will affect the emissions. In order to get as realistic results as possible VOCs should be sampled from intact plants.

Secondly, behavioural assays of the natural enemies should be conducted at elevated $[CO_2]$ and $[O_3]$ in order to get more reliable results of the host-searching behaviour of the natural enemies under projected future climate conditions. Even though high $[O_3]$ can elicit the emission of certain VOCs, it will react with these compounds leading to aerosol formation in the atmosphere (Joutsensaari et al. 2005), and therefore may eliminate the essential information used by the natural enemies.

Thirdly, with few exceptions (e.g. De Moraes 1998, Thaler 1999, Kessler & Baldwin 2001, Karban et al. 2003) the indirect defence studies have been conducted in controlled laboratory conditions. To see whether these trirophic interactions have any relevance in nature where the climate changes and plants are stressed by various attackers more field studies are needed (see opinions of Hunter 2002). Also it would be fascinating to know how far the natural enemies can detect the induced VOCs elicited by their prey. Karban et al. (2003) found that the plant-plant signalling via VOCs was effective when the VOC-producing plant and the receiver plant grew within 10 cm of each other.

7.4 Conclusions

The results of this research indicate that to achieve comprehensive understanding of the diverse impacts of global climate change on ecosystems, the environmental impact assessment of the effects of gaseous air pollutants related to climatic change should be extended to evaluation of food-chain responses.

This study reveals that doubled ambient $[CO_2]$ and high acute $[O_3]$ can alter the emission of VOCs from some plant species. The changes in VOC emission caused by elevated $[CO_2]$ may disturb the signalling to the third trophic level, and therefore reduce the defence of the plants. It is unlikely that high $[O_3]$ would mislead the natural enemies, but may affect the atmospheric chemistry by inducing novel VOCs from plants which react quickly after emission. The answers to the addressed questions:

1) What compounds are emitted from CO$_2$-treated and O$_3$-exposed plants?

   Elevated [CO$_2$] alters the constitutive emission of cabbage plants, but does not affect the emissions of silver birch. O$_3$-exposed Lima beans emit novel inducible VOCs, while O$_3$ exposure did not have an impact on the emissions of silver birch trees and saplings.

2) What impacts do elevated [CO$_2$] have on the emission of herbivore-induced VOCs and constitutive VOCs?

   Doubled ambient [CO$_2$] seems to diminish or leave unaltered the emission of monoterpenes from herbivore-damaged
cabbages. The same trend was found with the herbivore-inducible VOCs. Silver birch trees did not indicate any clear responses to elevated [CO₂].

3) Does elevated [O₃] induce the emission of novel VOCs in a similar manner to herbivory?
Exposure to high [O₃] (~200 ppb or more) can elicit the emission of some of the same compounds from Lima beans as herbivory does, but the emission profile is not the same. For example, O₃ does not induce the emission of (E)-β-octimene, which is one of the herbivory-induced monoterpenes. Realistic, doubled ambient [O₃] is unable to induce the emission of novel compounds from Lima bean and silver birch.

4) How do the natural enemies of herbivores respond to the herbivore-induced VOCs and to the compounds induced or affected by elevated [CO₂] or [O₃]?
The natural enemies of herbivores orientate better to host-plant complexes when plants have been grown under ambient than under elevated [CO₂]. O₃-exposure and O₂-induced VOCs do not disturb the host-locating of the predatory mite.

5) Do natural enemies of herbivores get false alarms from the plants grown at elevated [CO₂] or [O₃]?
On the basis of this study, no they do not get false alarms. However, elevated [CO₂] may reduce the host-searching ability of the natural enemies due to the possible changes in quality and quantity of the VOC emissions of damaged plants.

References

Bezemer TM, Jones TH (1998) Plant-insect herbivore interactions in elevated atmospheric CO₂: quantitative analyses and guild effects. Oikos 82, 212-222


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