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The role of terpenes in the defensive responses of conifers against herbivores and pathogens

PhD Thesis

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to be eligible for the doctor degree

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Universitat Autònoma de Barcelona, September 2015

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Li faig un tall a l'arbre i surt un broll de lletra

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Abstract

Plants are organisms under constant biotic stress. Due to their sessile condition, they have developed a plethora of physical and chemical defences to make front to herbivores and pathogens. Chemical defences are very versatile: many plants have permanent reserves of chemical compounds (known as constitutive defences) to face aggressions immediately, but these defences can also increase, change and *de novo* compounds can be produced when biotic aggressions are detected (induced defences). We can also distinguish between direct defences, which fight the aggressions by intoxicating, deterring or slowing growth of the insects and pathogens, and indirect defences, which attract antagonists of the biotic agents attacking the plant, usually by volatile cues. Moreover, plants not only defend the attacked tissues (local defences), but also can rise the defences of still undamaged tissues (systemic defences). More than 100,000 chemical compounds are considered to play defensive roles in plants. About 20-30,000 of these compounds are terpenes, a type of hydrocarbons that has a very wide range of structures and functions. Terpenes are known to be very important defences in conifers, a small yet very important division of plants spread throughout the world.

This thesis aimed to describe and deepen in the role that terpenes play in the defence of two conifers, Scots pine (*Pinus sylvestris*) and Italian cypress (*Cupressus sempervirens*). We chose two of the most important pests attacking conifers in the Mediterranean basin: the pine processionary moth (PPM, *Thaumetopoea pityocampa*), the most important insect defoliatior of Mediterranean pines, and cypress bark canker (*Seiridium cardinale*), a fungal pathogen that embodies the main threat for cypresses worldwide. We have analyzed the terpenes from plant and fungal tissues with Gas Cromatography – Mass Spectrometry (GC-MS), and calculated their concentrations and emission rates of terpenes. Besides GC-MS, we also used techniques of molecular biology (real-time polymerase chain reaction, qPCR) to quantify the gene expression of terpene synthases. Regarding the effects of terpenes on the biotic agents, we conducted laboratory bioassays that determined the impact that these defences have on survival and growth of insects and pathogens.

The role of terpenes in interactions between Scots pine and the PPM had been studied before, but nothing was known about the responses of pines regarding terpene emission rates, systemic reactions or terpene biosynthesis. In the first chapter we observed that two Scots pine subspecies coexisting in natural stands decrease their terpene concentrations in needles when PPM feeds on them, which coincides with recent reports. A substantial increase of terpene emissions was coupled with the decrease of terpene

concentrations, but we could not ascertain whether the lower concentrations were due to a decrease in terpene biosynthesis or to high terpene emission losses masking a possible induction of terpene synthesis. In chapter 2, using qPCR, we observed that PPM feeding tended to reduce the gene expression of terpene synthases of local and systemic needles. This was unexpected, as synthases usually up-regulate in front of herbivory. Accordingly, needle terpene concentrations also decreased, suggesting a weak terpene response of Scots pine needles to PPM infestations. We also studied the terpene and nutritional responses of new Scots pine needles to previous defoliation by PPM in chapter 3. One Scots pine subspecies grew new needles with increased terpene concentrations, changes in terpene emission rates and decreased concentrations of nutrients, in local and systemic needles. The other subspecies did not show any clear response, suggesting that the two subspecies had clearly contrasting reactions to PPM herbivory. Surprisingly, these contrasting reactions did not produce any difference in the survival of young PPM larvae that fed on the needles of both pine subspecies, suggesting that PPM, a specialist herbivore, might be adapted to the defensive responses of pines.

The role of terpenes in the Italian cypress – *Seiridium cardinale* system was mostly unknown. In the 4th chapter we monitored during 90 days the reactions in the phloem and the leaves of cypresses artificially infected by two strains of this fungal pathogen. Results showed strong increases of terpene concentrations in infected phloem, including the formation of *de novo* terpenes and substantial changes in the proportions of terpene profiles. Terpene concentrations of leaves next to the infected tissues showed no clear changes, but terpene emissions of these leaves significantly increased 10 days after infection. Results suggested terpenes play an important role in the direct and likely the indirect defence of Italian cypress. The 5th and final chapter compared the terpene phloem responses of resistant and non-resistant cypresses, finding a more intense response in resistant trees, and corroborating the results of Chapter 4. We also tested the growth inhibition caused by Italian cypress defences, finding that many of the induced terpenes were very inhibitory and had more concentration-dependent inhibitions than the major, constitutive terpenes. We finally investigated the biotransformation capacities of *S. cardinale*, and observed that this fungus can biotransform and detoxify three cypress terpenes and can alter the chemical composition of its immediate environment.

This thesis provides thus new and valuable information about two important Mediterranean systems, but also deepens in some understudied features of terpene conifer defence, such as variety resistance, systemic reactions of conifers or terpene biotransformation capacities of conifer pathogens.

General introduction

General introduction

Plants: organisms under stress

In terrestrial ecosystems, plants are the autotroph organisms responsible for almost all the primary production (Begon et al. 2006; Woodwell and Whittaker 1968). On one hand, the nourishment of heterotrophs absolutely depends, directly or indirectly, on the organic compounds resulting of that primary production, and on the other hand, plants are sessile, and cannot escape from their antagonists. This combination has made plants subject to an enormous biotic stress. To face these constant and multiple aggressions (De Vos et al. 2007; Dicke et al. 2009), plants have developed a very wide range of defences.

Defences against biotic agents

Plant defences can be physical and can be chemical. Physical defences begin with the cell wall, a thick layer that protects plants from pathogenical infections (Hématy et al. 2009; Underwood 2012). At a major scale, physical defences include lignified cells, spines, thorns, hairs and tickles (Milewski et al. 1991; Myers 1991), that can prevent insect and mammal herbivory. Leaf toughness, for example, is a determining factor against herbivory (Cooke and Leishman 2011; Zovi et al. 2008). Hypersensitive responses, such as needle desiccation (Codella and Raffa 2002) or leaf abscission (Williams and Whitham 1986) also can prevent or defend plants from current or future biotic attacks. Another example would be resin, that despite it is commonly considered a chemical defence, is also useful to trap and drown insect antagonists such as bark beetles (Phillips and Croteau 1999).

Chemical defences make use of chemical compounds to fight herbivory and pathogenicity (Gershenzon and Dudareva 2007; Phillips and Croteau 1999). Plants produce a very wide range of compounds that can be used as chemical defences (Levin 1976), usually known as "secondary metabolites", due to their non-fundamental role in growth or reproduction (Howe and Jander 2008). These compounds include terpenes (mainly mono-, sesqui- and diterpenes), glucosinolates, phenolics (which include tannins and flavonoids), alkaloids or fatty acid derivates, among others (Dudareva et al. 2004; Kliebenstein 2012). Several works consider there are at least one hundred thousand plant compounds that can play defensive roles (Dixon 2001; Kliebenstein 2012; Wink 1988). Many plants have developed permanent chemical defences (known as constitutive defences), which have the purpose of mantaining the plant always defended, in order to immediately fight any aggression (Phillips and Croteau 1999; Wittstock and Gershenzon 2002). But once the aggression begins, plants can produce more defences, (known as induced defences). These induced defenses include increases in the concentration of certain compounds, changes in their proportions or even generation of *de novo* compounds, i.e., defences especially created for the occasion, also known as phytoalexins (Ahuja et al. 2012; Darvill and Albersheim 1984). The preference for constitutive or induced defenses depends on the level and type of attack that the plant suffers, and on cost/benefit strategies to optimize resource allocation (Karban 2011; Sampedro 2014). Two other kinds of defences are usually considered: i) direct defences, repelling substances that directly defend from antagonists when released (Hardie et al. 1994; Ngoh et al. 1998), reducers of the palatability of tissues (Rice et al. 1978), toxic compounds to herbivores (Hopkins et al. 2009; Isman 2000) or inhibitors of the growth of pathogens (Bridges 1987; Ejechi et al. 1999; Kusumoto et al. 2014) among others and ii) indirect defences, that attract antagonists of herbivores and pathogens. The volatile fraction of chemical defences (semiochemicals, the compounds of low molecular weight, such as mono- and sesquiterpenes, benzenoids or derivatives of fatty acids and aminoacids) play communicative roles among organisms, and can be attractive to predators and parasitoids of herbivores (Dicke 1994; Hilker et al. 2002; Mattiacci et al. 1995), and fungivores (Guevara et al. 2000; Holighaus et al. 2014). Semiochemicals that benefit the emitter (in our case, the plant) are known as allomones, but plant volatiles can also be a disadvantage for plants (known as kairomones), as some insects exploit plant volatile emissions to locate their hosts (Ruther et al. 2002; Wood 1982). Moreover, plants do not only emit volatile messages, they are also able to receive them. Plant-plant communication for defensive purposes has been reported since the 80s, and

described how attacked plants can emit blends that alert other plants of imminent danger (Baldwin and Schultz 1983; Heil and Karban 2010). Also, recent discoveries suggest that plants can even perceive insect emissions (such as sexual pheromones) too (Helms et al. 2013). Plants usually react to the biotic attacks in the tissues where the aggression has been produced (local reactions) (Lombardero et al. 2013; Viiri et al. 2001). But reactions in other unattacked plant parts, known as systemic reactions, have also been observed (Fäldt et al. 2006; Hilker et al. 2002; Viiri et al. 2001). The goal of systemic reactions is still quite unknown, but it could be a strategy to protect tissues in imminent danger (Heil 2014) and to boost the signal to a receptor that could help reducing biotic stress (i.e. a message is much more likely to reach a parasitoid if all the leaves of a plant emit an attractive blend than if only the attacked leaves emit that blend) (Dicke 1994; Hilker et al. 2002).

Terpenes

Terpenes are very widespread in nature, and occur virtually in all living organisms (Gershenzon and Dudareva 2007). Among the chemical classes of organic compounds described above, terpenes outstand as the group with highest number of different molecules. Estimations of their number range from 20.000 to 30.000 different products (Gershenzon and Dudareva 2007; Tholl 2006) and they also have the highest number of different structural types (Degenhardt et al. 2009). Terpenes were initially thought to be just the metabolic waste of organisms (Gershenzon and Dudareva 2007), but this perspective changed in the 1960s, when reports began to observe terpenes could be toxic to herbivores and pathogens, as well as attractive or deterrent to insects (Muller 1965; Perttunen 1957; Shrimpton and Whitney 1968). These hydrocarbons are formed by assembled pieces of isoprene, the C_5H_8 structural unit that forms terpenes, but it is common that these compounds also include oxygen. Terpenes are organized in acyclic and cyclic distributions, with cis- and trans- configurations and chiral carbons that allow the existence of several enantiomers (Fig. 1).

This impressive diversity of terpene compounds and structures has its base in just a few C_5 molecules: isopentenyl diphosphate (IPP) and its isomer dimethylallyl

diphosphate (DMPP) (Degenhardt et al. 2009; Tholl 2006). These two molecules were initially thought to be formed exclusively by the mevalonate pathway, but in the 90s the non-mevalonate pathway (essential in plants but not in archaea and animals) was unveiled (Rohmer et al. 1993). Geranyl pyrophosphate (GPP), formed by the union of one unit of DMPP and one unit of IPP, is the precursor of monoterpenes ($C_{10}H_{16}$), farneslyl pyrophosphate (FPP), formed by one DMPP and two IPPs, is the precursor of sesquiterpenes ($C_{15}H_{24}$) and finally, geranylgeranyl pyrophosphate (GGPP), formed by one DMPP and three IPPs, is the precursor of diterpenes ($C_{20}H_{32}$). These three molecules (GPP, FPP and GGPP) are the substrates used by one type of enzymes known as terpene synthases, which catalize the formation of all terpenes known. Terpene synthases are capable of producing just one terpene (e.g. *PaTPS-Myr*, isolated from *Picea abies* by Martin et al. (2004), produces the monoterpene myrcene) or various terpenes (e.g. *PsTPS2*, isolated from *Pinus sylvestris* by Köpke et al. (2008), produces four different sesquiterpenes).
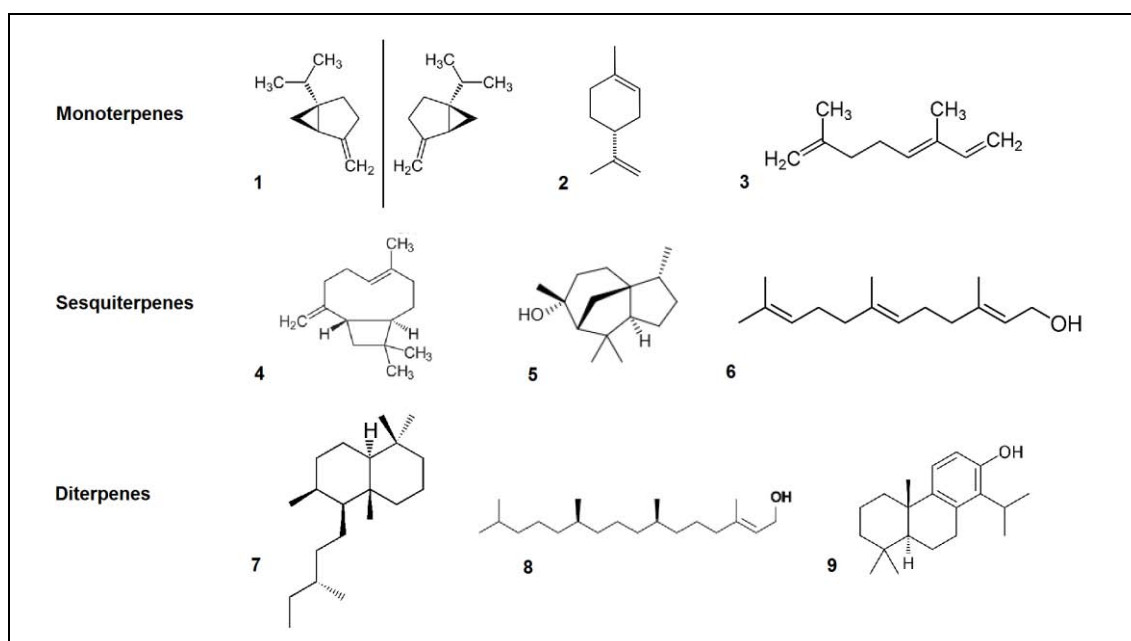
Figure 1. Examples of terpenes. **1** Two enantiomers of sabinene: (-)-sabinene and (+)-sabinene (bicyclic structure). **2** limonene (monocyclic). **3** α-ocimene (acyclic). **4** β-caryophyllene (tricyclic). **5** cedrol (tricyclic, oxygenated). **6** farnesol (acyclic, oxygenated). **7** labdane (bicyclic). **8** phytol (acyclic, oxygenated). **9** totarol (tricyclic, oxygenated). Photo credit: wikipedia, assembled by Ander Achotegui-Castells

The transcript levels of several terpene synthases usually increase when plants are wounded (Byun-McKay et al. 2006; Litvak and Monson 1998), or suffer herbivory (Arimura et al. 2004; Beyaert et al. 2012; Köpke et al. 2008; Miller et al. 2005) or

pathogenic infection (De Alwis et al. 2009), and authors have related these increases with defensive inductions. However, several mis-matches have been reported between the gene expression of synthases and the concentration and emission of their terpene products (Beyaert et al. 2012; De Alwis et al. 2009; Köpke et al. 2010). These discorrespondences have been attributed to the existence of products formed by more than one terpene synthase (Bohlmann 2000, Fäldt 2003) or post-transcriptional controls and factors influencing the activity of enzymes (Garms et al. 2008).

Monoterpenes and sesquiterpenes, the terpenes with lowest molecular weight, are volatile at ambient conditions, due to their high vapor pressures (Dudareva et al. 2004). Terpene emissions depend on multiple factors (Peñuelas and Llusià 2001), such as temperature (Guenther et al. 1993; Penuelas and Llusià 1999) or light (Yatagai et al. 1995), and their communicative role can be altered by contaminants such as ozone (Farré-Armengol et al. 2015). These properties, together with the high diversity of compounds explained above, make terpenes very useful for delivering specific signals (Gershenzon and Dudareva 2007). Nowadays there is a compelling amount of studies showing how volatile terpene signals mediate many different interactions between plants and other organisms (Hilker et al. 2002; Van Poecke et al. 2001; van Schie et al. 2006; Wegener et al. 2001)

Conifers and terpenes

Conifers, one of the 12 divisions of extant plants, are known to contain high quantities of terpenes in their tissues (Phillips and Croteau 1999). Despite conifers only include about 600 sp. (almost all of them trees), they are a group of enormous ecological importance and with remarkable evolutive success (Mumm and Hilker 2006; Nystedt et al. 2013). Spread throughout the world, they are especially dominant in the boreal forests of the northern hemisphere (Begon et al. 2006) (Fig. 2).

In conifers, terpenes occur in a mixture called oleoresin, formed half by the turpentine fraction (volatile, formed by mono- and sesquiterpenes) and half by the rosin fraction (non-volatile, formed by diterpenes). The turpentine fraction, which can be

insecticidal and fungicidal (Phillips and Croteau 1999), has the function of fluidifying oleoresin, and volatilizes in contact with atmosphere. The rosin fraction hardens when the turpentine fraction evaporates and, in case of attack or damage, covers the wound and traps insects within the oleoresin (Phillips and Croteau 1999; Trapp and Croteau 2001). Conifers have specialized structures to accumulate oleoresin called resin ducts, which can be found in the stems and needles of conifers (Wu and Hu 1997). Their creation can be induced by insects and pathogens (then called traumatic resin ducts) and has been associated to resistance against pests (Martin et al. 2002; Moreira et al. 2015). Most studies on the role of terpenes in conifer defences against herbivores and pathogens have been conducted in conifers of North America or Central-Northern Europe (Beyaert et al. 2012; Björkman and Larsson 1991; Hilker et al. 2002; Raffa and Berryman 1982; Raffa et al. 1998; Wang et al. 2013), while relatively few is known about conifers inhabiting the Mediterranean basin (Hodar and Zamora 2004; Madar et al. 1995; Moreira et al. 2013). It should also be noted that the vast majority of the studies cited here were conducted in interactions involving the pinaceae family, especially in *Pinus* sp., but also *Abies sp.* and *Picea sp.* The defensive role of terpenes in other taxa within the coniferales remain largely unstudied, especially in Podocarpaceae and Cupressaceae, which represent half of the coniferous species described.
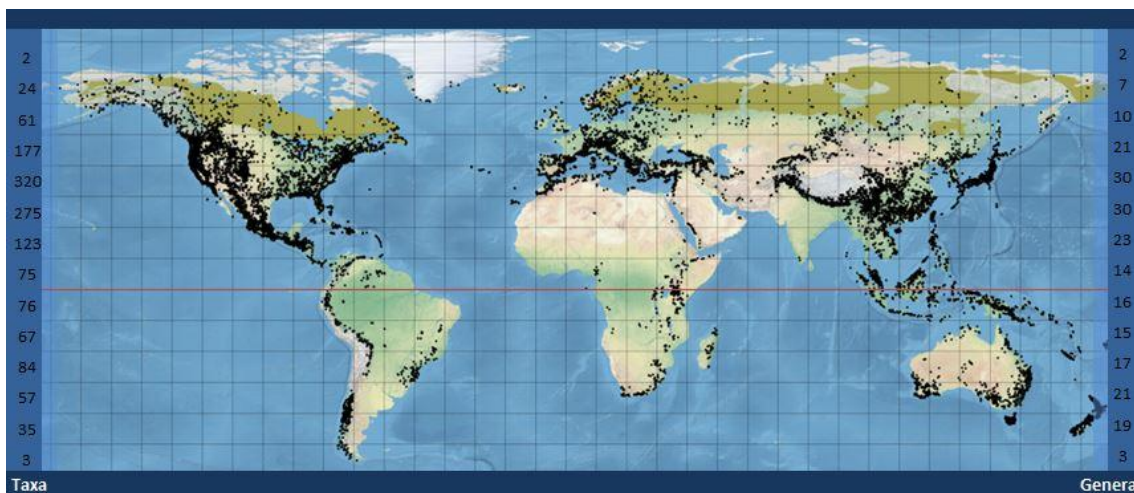

Figure 2. Distribution of conifers around the world. The Y axis of the left side, indicates the number of species at different latitudinal heights, while the Y axis of the right side indicates the number of genera at different latitudinal heights. Photo credit: http://herbaria.plants.ox.ac.uk/bol/conifers

Herbivore-conifer interactions

The role of terpenes in insect-conifer interactions has been extensively studied since the 60s-70s (Mumm and Hilker 2006; Nykanen and Koricheva 2004; Paine et al. 1997). A topic that has centered a lot of attention has been the bark beetle – conifer interactions, due to the importance of terpenes in these relations, and the great economical and ecological impacts of these pests, especially in the American forests (Paine et al. 1997; Trapp and Croteau 2001). Folivorous insects (insects feeding on conifer needles, such as lepidoptera) also have important impacts on conifer forests (Jacquet et al. 2012) (Fig. 3). The role of terpenes in these interactions also has been intensively studied (Mumm and Hilker 2006), and we will discuss their state of the art in the next paragraphs.

State of the art of folivorous insects and conifer terpenes

The role of terpenes in plant direct defence against folivorous insects is, after many decades of study, still controversial (Agrawal and Weber 2015; Mumm and Hilker 2006; Nykanen and Koricheva 2004). Different tree responses have been observed, but tipically, terpene concentrations of needles attacked by folivores tend to have unaltered or reduced terpene concentrations during and after attack (Beyaert et al. 2012; Hodar and Zamora 2004; Litvak and Monson 1998; Moreira et al. 2013; Nykanen and Koricheva 2004). Few is known about terpene systemic reactions to herbivory in conifers (Mumm and Hilker 2006). The recent characterization of enzymes and genes involved in the production of terpenes have been applied to the study of insect-plant interactions, allowing a better comprehension of the mechanisms underlying conifer defences. In contrast with concentrations reported, folivory usually up-regulates gene expression of terpene synthases and enzyme activity in conifers (Beyaert et al. 2012, Litvak and Monson 1998, Köpke et al. 2008, Köpke et al. 2010). Further research is thus needed to understand the links between gene expression, enzyme activity and terpene concentrations and emissions to understand the nature of folivore - conifer interactions (Köpke et al. 2010).

Regarding the effect of terpenes on larval performance and survival, laboratory tests have observed quite consistent toxic and deterrent effects of terpenes against insect folivores (Kanat and Alma 2004; Mumm and Hilker 2006; Zou and Cates 1997)whereas *in vivo* experiments have usually not been able to link foliar terpene concentrations to changes in the performance or survival of folivores (Chen et al. 2002; Hódar et al. 2015; Hodar and Zamora 2004; Nykanen and Koricheva 2004). Some authors have suggested that the specialization degree of insects could be related to the different tree responses observed and to the effects caused on insect performance and survival (Cornell and Hawkins 2003; Mumm and Hilker 2006). Terpene concentrations do not only depend on herbivory but on many other factors, such as droughts (Kainulainen et al. 1992; Llusia and Peñuelas 1998) or nutrient availability (Björkman et al. 1991; Sampedro et al. 2012). Apart from abiotic factors, many confounding factors, such as needle nutrients or physical defences seem to hinder the real contribution of terpenes to defensive tasks (Hódar et al. 2015; Watt et al. 1991). Hence, terpenes, despite being regarded as the most important chemical defences of conifers (together with phenols), are apparently not capable of explaining insect survival and performance by themselves (Nykanen and Koricheva 2004). As it has been recently suggested, to make front to these controversies, a more integrated view is needed to assess the effect of terpenes and other secondary metabolites in plant direct defences (Agrawal and Weber 2015).

The indirect defences of conifers against folivores are still poorly studied. Up to date, very few interactions have been well-described. To cite one example, when *Diprion pini* (Hilker et al. 2002) and *Neodiprion sertifer* (Mumm et al. 2005) lay their eggs on needles of *Pinus sylvestris,* this tree changes its terpene cues (Mumm et al. 2003), which become attractive for the egg parasitoid *Closterocerus rufforum.* Apart from that, several studies have reported changes in terpene emissions of conifers under real (Litvak and Monson 1998; Priemé et al. 2000) or simulated folivory (Faiola et al. 2015; Miller et al. 2005; Sampedro et al. 2010), describing cues that could potentially attract antagonists of herbivores. Further studies should test this cues with herbivore antagonists to ascertain if they are indirect defence signals or not. Several of these interactions have been characterized in laboratory conditions or simulating herbivory herbivory, and description and replication in nature with real herbivory is necessary to determine the real importance of indirect defence in conifers.

Pathogen-conifer interactions

The role of terpenes in pathogen-conifer interactions has been deeply studied, but not to the extent of insect-conifer interactions. In fact, a very significant part of this research field is also linked to bark beetles, who are often associated to pathogenic fungi (Paine et al. 1997; Six 2003).

Fungal spores can be dispersed by wind, raindrops, or vectors (Ingold 1971). Wind can disperse plant pathogens up to hundreds or thousands of kilometers (Brown and Hovmoller 2002). Regarding vector dispersal, fungi are so commonly associated with bark beetles that some of these coleopterans have developed specific structures to carry fungal spores (mycangia). Fungi can play two main roles that benefit bark beetles. The first, and main one, is to infect and weaken the conifer that bark beetles colonize, by releasing toxins, enzymes and invading host tissues with their hyphae (Paine et al. 1997). The second benefit is related to the nutrition of beetles, as some of these coleopterans can feed upon the fungus that they carry. Once the fungus enters in contact with the plant tissues, it begins to develop and becomes an important nutrition source for the beetle (Villari et al. 2001).

Figure 3. First instar larvae of the pine processionary moth (*Thaumetopoea pityocampa*) defoliating one twig of a Scots pine. Photo credit: Ander Achotegui

State of the art in the role of terpenes in conifer-fungal pathogens interactions

A remarkable number of the studies regarding the role of terpenes in the conifer-fungal pathogen interactions have studied the conifer terpene responses against fungi artificially inoculated in the phloem of the tree. Nowadays it is well established that conifers usually react to fungal infections increasing terpene concentrations in the infected tissues (Fig. 4) (Blodgett and Stanosz 1998; Faldt et al. 2006). These increases are substantial and can be dramatic, with several studies describing over 100-fold inductions of terpene concentrations (Raffa and Smalley 1995; Viiri et al. 2001). In the last decade, some studies have also described changes in terpene chirality after infection (Bonello et al. 2008; Faldt et al. 2006; Zamponi et al. 2007), but they are still minoritary. A few works have also studied tree systemic responses to infection, usually analyzing the reactions of stem a few decimeters away from the infected zone (Fäldt et al. 2006; Viiri et al. 2001). However, the systemic reactions in needles, such as changes in terpene concentrations or emissions (Lusebrink et al. 2011; Schiller and Madar 1991) that could be relevant cues for mutualist and antagonist insects, are still very understudied.

Figure 4. a) Resinosis in *Cupressus sempervirens* caused by the infection of the cypress pathogen *Seiridium cardinale.* b) When the bark is removed, it can be appreciated the cankered tissues of the phloem and xylem (reddish color).

The effect of terpenes in fungal growth has been extensively studied since the 1950s, mostly in *in vitro* conditions. Conifer terpenes can be very inhibitive to fungal

growth and germination (Bridges 1987; Kusumoto et al. 2014; Mason et al. 2015; Paine and Hanlon 1994). However, there are also studies reporting how the most common terpenes (pinenes, limonene) can stimulate growth, thus providing evidence that some specialist fungi are capable of making use of terpenes for their own benefit (Bridges 1987; Kotan et al. 2007; Mason et al. 2015). In contrast with the studies assessing conifer defence, terpene chirality is usually considered in these *in vitro* studies. Those studies have demonstrated that different enantiomers can have distinct effects on fungal growth (Bridges 1987; Kusumoto et al. 2014). However, the terpene concentrations tested *in vitro* are almost always arbitrary and thus may not represent accurately the reality. Efforts to mimic the concentrations found *in planta* are necessary for a proper interpretation of *in vitro* results (Andrews et al. 1980; Sherwood and Bonello 2013). Studies also need to assess the effects of exposition of several terpenes at the same time (Espinosa-garcia and Langenheim 1991), as combinations of several compounds could have additive, synergistic or antagonistic effects. Other interesting but understudied fields include the effects of phytotoxic terpenes produced by fungal pathogens attacking conifers (Evidente et al. 1993; Evidente et al. 2002; Oku 1988), the fungal biotransformation of defensive terpenes of conifers (Kusumoto et al. 2014; Yano et al. 1994) and the role of terpenes in multi-trophic communication among conifers, insect vectors, fungal pathogens and antagonists of the fungi or the insect vectors (Adams and Six 2008; Boone et al. 2008; Sullivan and Berisford 2004).

Thesis objectives

The goal of this PhD thesis was to study the terpene defensive reactions of Mediterranean conifers under biotic stress. We organised the thesis in two blocks to investigate a herbivore-conifer interaction between *Thaumetopoea pityocampa* and *Pinus sylvestris* and a pathogen-conifer interaction between *Seiridium cardinale* and *Cupressus sempervirens*. The defensive role of terpenes was poorly investigated or controversial in these interactions.

The first block addresses the defensive reactions of Scots pine (*Pinus sylvestris*) terpenes against pine processionary moth (*Thaumetopoea pityocampa)* herbivory.

Objective one: To describe and compare the changes in total and specific needle terpene concentrations and emissions of two Scots pine subspecies attacked by the most defoliating larval stage of the pine processionary moth, in natural pine stands. To study local and systemic reactions to attack.

Objective two: To monitor the changes in gene expression of two terpene synthases and the needle terpene concentrations of their products in young Scots pines during the whole larval cycle of the pine processionary moth. To describe local and systemic reactions in front of attack.

Objective three: To determine and compare the changes in total and specific terpene concentrations and emissions, as well as nutrients, in new needles of two Scots pine subspecies previously attacked by the pine processionary moth, in natural pine stands. To study local and systemic reactions to previous attack, linking nutrient and terpene concentrations.

The second block addresses the role of terpenes in the defence of Italian cypress (*Cupressus sempervirens*) against the pathogenical infection of the fungus *Seiridium cardinale.*

Objective four: To monitor the changes in phloem terpene concentrations and foliar terpene concentrations and emissions of canker-resistant Italian cypresses infected by *Seiridium cardinale* during the first 90 days after artificial inoculation.

Objective five: To compare the terpene phloem reactions of non-resistant and canker-resistant Italian cypresses 30 days after artificial inoculation with *Seiridium cardinale*. To perform *in vitro* experiments able to determine the fungal growth inhibition caused by concentrations of both cypress variety reactions, and to study the terpene biotransformation and detoxification capacity of *S.cardinale.*

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Chapter 1. Needle terpene concentrations and emissions of two coexisting subspecies of Scots pine attacked by the pine processionary moth (*Thaumetopoea pityocampa*)

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Abstract

Mediterranean pine forests are often attacked by caterpillars of *Thaumetopoea pityocampa* (Lep., Thaumetopoidae), one of the most important defoliators in the Mediterranean region causing large economic losses and ecological effects. The needle terpene concentrations and emissions may play a key role in the defense of pines. We studied two subspecies of *Pinus sylvestris*, *nevadensis* (an endemic and relict subspecies) and *iberica*, with different levels of caterpillar attack in Sierra Nevada mountains (Spain). GC–MS analyses showed large total concentrations of terpenes (6 to 39 mg g^{-1} of dry weight) in the needles of both subspecies under field conditions. Concentrations were 25 % higher in ''Non-Attacked Trees'' (NATs) of the *iberica* than in the *nevadensis* subspecies. The branches of NATs had terpene concentrations 20 % higher than those of ''Attacked Branches of attacked trees'' (ABs). Within attacked trees, the ''Non-Attacked Branches'' (NABs) also had terpene concentrations 20 % higher than those of ABs. Mainly α-pinene and germacrene D had higher concentrations in NATs and NABs than in ABs. Some terpenes had higher concentrations in NABs than in NATs, indicating possible systemic reactions. In subsp. *nevadensis*, the percentage of monoterpenes relative to total terpenes was higher in ABs than in other attack states. The rates of emission in *nevadensis* (standardized to 30 ºC) were ca. three times higher in ABs than in NABs and NATs. These results suggest that the lower terpene concentrations and high percentages of monoterpenes in ABs were produced by a combination of emission losses and terpene induction in response to herbivorous attack.

Introduction

Biogenic volatile organic compounds (BVOCs) stored in different plant tissues and released as emissions contribute to a broad array of mechanisms for protection, defense, allelopathy and communication in the face of abiotic and biotic stresses, such as severe droughts or herbivory (Llusia and Penuelas 2001; Penuelas and Staudt 2010). In conifers, the main direct defensive mechanism against folivorous insects is the production of phenolic compounds and oleoresins (Mumm and Hilker 2006). Oleoresins are complex mixtures of volatile (mono- and sesquiterpenes) and nonvolatile (diterpenes) terpenoids that act as toxins against insects and microbes and as biologically active agents that discourage predation by insects (Phillips and Croteau 1999). Needle-leaved conifers have resin ducts, specialized tissues for storing volatile isoprenoids (Blanch et al. 2011), that release oleoresins from the phloem, xylem and needles when these are damaged or injured (Sampedro et al. 2010).

Chemical defensive mechanisms can be constitutive (preformed) or induced by a specific stress. The synthesis and accumulation of resinous terpenoids in conifers are under genetic control, with remarkable variation occurring among species and even within populations (Trapp and Croteau 2001). Plant defences, although also respond plastically, and herbivorous attacks can raise (Sampedro et al. 2011) or decrease (Litvak and Monson 1998) the concentration of terpenoids and induce changes in the composition and production of resin and in the emission of volatiles, including terpenes (Staudt and Lhoutellier 2007). The emission of volatile terpenes by conifers also has important functions in indirect resistance, because these compounds act as airborne molecular messengers that deter herbivores, attract parasitoids of herbivores (Hilker et al. 2002) or warn other plants of attack, but may also be used as an olfactory cue by herbivores for their host selection (Penuelas and Llusia 2004). This fascinating complexity makes the role of terpenes in the defense of plants against defoliators controversial, needing further study to understand the functioning of these interactions.

Pinus sylvestris is one of the most widespread conifers in the world, having a southern limit of distribution in Spain in Sierra Nevada and Sierra de Baza (Boratynski

1991). In these areas, *P. sylvestris* subsp. *nevadensis*, an endemic and relict subspecies exclusive to this region of high altitude, coexists with reforested plantations of *P. sylvestris* subsp. *iberica*, one of the most common pines in Spain. The relict pine has recently been threatened by the uphill spread of the pine processionary moth (PPM), *Thaumetopoea pityocampa* (Hodar et al. 2003; Hodar and Zamora 2004), which has become a serious menace.

Thaumetopoea pityocampa (Lep., Thaumetopoeidae) is one of the most important defoliators of pine trees in the Mediterranean region, and its eruptive dynamics inflict serious economic and ecological losses, as well as severe allergic reactions in humans and other mammals (Lamy 1990). This insect is limited by the low winter temperatures, and climate warming is consequently expanding the limits of its distribution to more northerly territories and to higher altitudes, where it was not previously common (Battisti et al. 2005). This expansion of the PPMs range is thus raising the interest in understanding its relationship with *P. sylvestris*, a pine that had rarely encountered PPMs.

Several attempts have been made to understand the relationship between PPMs and their hosts (*Pinus* spp., *Cedrus atlantica* and *Larix decidua*). Several mechanisms and factors have been postulated to determine this insect's selection of host: unselective host colonization (Hodar et al. 2002), low needle concentrations of terpenes (Petrakis et al. 2005) such as limonene (Tiberi et al. 1999), needle toughness (Zovi et al. 2008) or visual and olfactory cues (Paiva et al. 2011) such as terpene emissions (Zhang et al. 2003). Which mechanisms determine host selection and which chemical, defensive and communication changes control this herbivore-plant interaction are thus still unclear.

To shed light on the chemical interactions of the PPM with *P. sylvestris* subsp. *nevadensis* and *P. sylvestris* subsp. *iberica*, we aimed to, (*i*) determine if, previous to any attack, the two coexisting subspecies of pine present differences in terpene composition and concentration, (*ii*) to determine if the total and specific terpene concentrations and emissions of the pine needles show any relationship with PPM attack and (*iii*) to determine whether terpene concentrations and emissions differ between attacked branches and non-attacked branches of attacked trees.

Materials and methods

Study site

The study was conducted in Collado de Matasverdes (37.05°N, 3.27°W, 1,900 m a.s.l.) in the Sierra Nevada National Park (Granada, SE Spain) at the beginning of March 2011. The climate is continental Mediterranean with hot summers, cold winters and usually with severe summer drought (July–August). The mean minimum temperature is in January (-0.1°C), and the mean maximum temperature is in July (30.1°C). Rainfall is concentrated mainly in autumn and spring, with an annual precipitation of 803 mm (average for 1998–2008 at the Jardín Botánico de La Cortijuela, 1.5 km distant and 300 m lower in altitude). The area was mainly covered with snow during the sampling, with unstable weather ranging from sunshine to snowfall.

Experimental design

The study site was located in an area with pure stands of *P. sylvestris* subsp. *nevadensis* and *P. sylvestris* subsp. *iberica* and had a notable presence of PPM colonies, which were scarce that year on the lower mountain slopes. Three different attack states were studied in the branches of the two subspecies. Branches of healthy, non-attacked trees (NATs) were used as controls, while the attacked trees were used to study their healthy, non-attacked branches (NABs) and their attacked branches (ABs).

Sampling of pine needles

Twenty-four trees of each subspecies were sampled. For each branch attack state (3) and each subspecies (2), 12 twigs were sampled (72 pine twigs in total) and stored in liquid nitrogen for subsequent treatment and analysis. All needles were lyophilized and stored at -80°C. Previous studies have shown that lyophilization does not cause losses of monoterpenes nor sesquiterpenes (Ormeno et al. 2007).

Sampling of BVOC emissions

Samplings of BVOC emissions were conducted at the study site over a period of 5 days, from 09:00 to 15:00 h (solar time) in the endemic pine subspecies, *nevadensis*. The selected branches were carefully wrapped with Teflon tape and then placed in a 10 l tedlar sampling bag (Saint Gobain, Akron, USA), avoiding damage to any needles. The open end of the bag was attached to the branch over the Teflon tape with a bridle, with a thermometer's probe (Velleman, Gavere, Belgium) and a tedlar tube (Saint Gobain, Akron, USA) inside the bag. The bag was connected by a Teflon tube to a modified air pump (Robert Bosch Tool Corporation, Mt Prospect, USA) that introduced an air flow of 2.67 ± 0.19 l min^{-1} ($\pm$SE). All measures of flow were performed with a Bios Defender 510 fluxometer (Bios International Corporation, Butler, USA). The photosynthetically active radiance (PAR) was also measured with a CI-340 Hand-Held Photosynthesis System (CID Bio-science, Camas, USA).

The system remained stabilized for at least 30 min before BVOC sampling. The tedlar bag had a valve connected to a metallic trap filled with 115 mg of Tenax and 230 mg of Unicarb (Markes International Inc. Wilmington, USA), where the BVOCs in the exhausted air coming from the bag were retained. At the other end of the metallic trap, a QMAX pump (Supelco, Bellefonte, Pennsylvania) pulled air from the sampling bag. The sampling time was 5 min, with a flow of 0.69 ± 0.01 l min^{-1} ($\pm$SE). The BVOC-filled tubes were stored in a portable refrigerator at 4ºC until stored in a -20ºC freezer at the end of the field work. Blank air from bags without branches was sampled in tubes for 5 min once a day. All metallic tubes used had been previously conditioned for 15 min at 300ºC with a purified stream of helium at a flow of 20 ml min^{-1}. Sample analyses Biomass terpene concentration and terpene identification The pine needles were ground manually with a mortar inside 50 ml Teflon tubes filled with liquid nitrogen to avoid evaporation of the BVOCs. After the needles were pulverized, 1 ml of pentane containing 0.5 nl of dodecane (used as an internal standard) was added. The Teflon tubes were rested for at least 12 h at -20ºC, and 300 µl of the supernatant was taken for gas chromatography–mass spectrometry analysis. The Teflon tubes with the remaining solution were dried to a constant weight and then weighed with a precision balance. The Teflon tubes were later exhaustively cleaned, dried and reweighed to determine the dry weight (dw) of the biomass analyzed.

Two µl of the needle extract were injected into a capillary column (HP 5MS, 30 m x 0.25 µm x 0.25 mm) in a Gas Cromatograph (GC) (7890A, Agilent Technologies, Santa Clara, USA) with a mass spectrometric (MS) detector (5975C inert MSD with Triple-Axis Detector, Agilent Technologies). The temperature of the column was 35ºC for the first 2 min, then was increased stepwise at 15ºC min^{-1} to 150ºC and maintained for 5 min, at 30ºC min^{-1} to 250ºC and maintained for 5 min and finally at 30ºC min^{-1} to 280ºC and maintained for 5 min. The flow of helium was 1 ml min^{-1}, and the total run time was 29 min. During the analyses, one blank every six analyses was also tested.

The identification of terpenes was performed by a comparison of the mass spectra with published spectra (Wiley 7n library) and known standards, while quantification of the peaks was conducted using the fractionation product with mass 93. The MS detection system was operating in SIM mode (Blanch et al. 2012; Llusia et al. 2012). Calibration curves for quantification were prepared with commercial standards of some of the most abundant compounds in the samples: four monoterpenes (α-pinene, sabinene, β-pinene and limonene), one sesquiterpene (α-caryophyllene) and one non-terpene internal standard (dodecane), all from Fluka Chemie AG, Buchs, Switzerland. Terpene calibration curves were always highly significant ($r^2 < 0.99$) in the relationship between signal and terpene concentration. The most abundant terpenes had very similar sensitivities (differences were < 5 %).

Rates of terpene emission

The terpene emissions trapped in the metallic tubes were released with an automatic sample processor (TD Autosampler, Series 2 Ultra, Markes International Inc. Wilmington, USA) and desorbed using an injector (Unity, Series 2, Markes International Inc. Wilmington, USA) in a GC (noted above) with an MS detector (noted above). A full-scan method was used in the chromatographic analyses. The desorbed sample was retained in a cryo-trap at -20ºC. The split was 1:20. The sample was desorbed again at 250ºC for 10 min and injected into the column with a transfer line at 250ºC. Following sample injection at 35ºC (initial time 1 min), the column temperature was increased stepwise at 15ºC min^{-1} to 150ºC and maintained for 5 min, at 50ºC min^{-1} to 250ºC and maintained for 5 min and finally at 30ºC min^{-1} to 280ºC and maintained for 5 min. Total run time was 26.7 min, and the helium flow was 1 ml min^{-1}.

The identification of terpenes was performed as above for the analysis of terpene concentrations. The rates of terpene emission were expressed in µg g^{-1} (dw) h^{-1}. Owing to the unstable weather, the rates of terpene emission for *P. sylvestris* subsp. *nevadensis* were standardized at 30ºC using an algorithm for terpene-storing species (Guenther et al. 1993),

E = Es {exp[βT-Ts)]}

where E is the emission rate in µg g^{-1} (dw) h^{-1} of monoterpenes at temperature T (in degrees Kelvin, K), Es is the emission factor in µg g^{-1} (dw) h^{-1} at standard temperature Ts (303 K) and b is an empirically determined coefficient, 0.09 (in degrees Kelvin, K).

Statistical analyses

The data obtained were analyzed using t-tests, one-way ANOVAs with least significant difference (LSD) post hoc tests, principal component analysis (PCA) and simple regressions to analyze the terpene concentrations and emissions of the different attack states. Non-parametric analyses such as the Mann–Whitney U test and the Kruskal–Wallis test (with Conover-Inman post hoc tests) were conducted in other cases. Statistica v. 6.0. for Windows (Statsoft Inc. Tulsa, USA) was used for the statistical analyses, and SigmaPlot v. 11.0 for Windows (Systat Software, Chicago, USA) was run for graphics and nonparametric statistical analyses.

Results

Needle terpene concentrations

The analysis identified 42 compounds as terpenes: 16 monoterpenes, 22 sesquiterpenes, two diterpenes and two unidentified terpenes (see Table 1; Fig. 3). Of these, 39 were found in both subspecies and for all attack states, while three monoterpenes were present only in ABs. These three monoterpenes could not be clearly identified due to their very low concentrations. The nine most abundant terpenes found in the needles of both subspecies accounted for more than 90 % of the

total terpene concentration. They were, in order of retention time: tricyclene, α-pinene, camphene β-pinene, β-myrcene (monoterpenes), β-caryophyllene, α-caryophyllene, germacrene D and bicyclogermacrene (sesquiterpenes). α-Pinene was the most abundant monoterpene, and germacrene D the most abundant sesquiterpene in both pines (Fig. 1).

Compound name	R.T.	Type of terpene	P. sylvestris subsp. nevadensis			P. sylvestris subsp. iberica		
			ABs	NABs	NATs	ABs	NABs	NATs
1 Tricyclene	7.69	Cyclic monoterpene	643.3+97.8	625.6 ± 54.2	760.6 ± 75.7	857.5 ± 122.0	1,157 ± 93	912.5 ± 104.0
2 α-pinene*	7.85	Cyclic monoterpene	8176 ± 612 a	9590 ± 714 b	9601 ± 410 b	10,176 ± 1,436 b	13,936 ± 482 a	14,323 ± 933 a
3 Camphene	8.05	Cyclic monoterpene	1392 ± 241	1306 ± 135	1632 ± 164	2039 ± 301	2645 ± 233	2066 ± 251
4 Sabinene*	8.34	Cyclic monoterpene	110.7 ± 29.7	106.4 ± 17.5	125.2 ± 15.7	201.3 ± 32.8	227.1 ± 29.2	177.1 ± 3.5
5 β-pinene*	8.40	Cyclic monoterpene	816.5 ± 104.4	921.0 ± 149.8	1315 ± 466	2097 ± 686 ab	2966 ± 600 a	1229 ± 134 b
6 β-myrcene	8.48	Acyclic monoterpene	518.7 ± 96.6	537.4 ± 53.6	646.1 ± 72.4	739.5 ± 110.9 ab	1016 ± 84 a	712.3 ± 103.3 b
7 α-phellandrene	8.71	Cyclic monoterpene	11.0 ± 1.7	11.5 ± 1.7	12.8 ± 1.6	25.3 ± 6.7	26.9 ± 8.9	15.5 ± 1.6
8 α-terpinene	8.85	Cyclic monoterpene	5.3 ± 1.0	5.9 ± 1.1	6.4 ± 1.1	10.0 ± 1.5	8.9 ± 0.9	9.2 ± 0.1
9 Limonene*	8.99	Cyclic monoterpene	84.0 ± 23.7	56.2 ± 12.9	67.2 ± 13.7	127.0 ± 24.1	117.0 ± 22.9	114.1 ± 2.5
10 Cis-β-ocimene	9.16	Acyclic monoterpene	156.3 ± 25.5 a	212.2 ± 19.3 ab	259.2 ± 33.5 b	424.6 ± 71.8	534.9 ± 61.1	427.7 ± 10.3
11 γ-terpinene	9.33	Cyclic monoterpene	11.6 ± 2.9	11.2 ± 1.4	13.8 ± 1.7	19.8 ± 2.8	23.2 ± 2.0	18.6 ± 0.3
12 α-terpinolene	9.68	Cyclic monoterpene	7.9 ± 1.7	10.0 ± 2.8	13.0 ± 3.0	77.1 ± 25.7	64.2 ± 32.5	72.7 ± 6.6
13 Unidentified terpene 1	9.85	Monoterpene	3.6 ± 0.8	Not found	Not found	2.8 ± 0.9	Not found	Not found
14 Unidentified terpene 2	9.94	Monoterpene	3.7 ± 1.2	Not found	Not found	2.6 ± 0.7	Not found	Not found
15 Unidentified terpene 3	10.24	Monoterpene	1.3 ± 0.6	Not found	Not found	5.1 ± 2.0	Not found	Not found
16 Borneol	10.66	Monoterpene alcohol	9.5 ± 2.5 a	9.8 ± 1.4 ab	16.2 ± 3.8 b	10.8 ± 2.3	13.3 ± 1.8	16.6 ± 3.0
17 γ-elemene	13.28	Cyclic sesquiterpene	21.2 ± 3.8 ab	15.8 ± 2.6 b	36.5 ± 11.3 a	30.3 ± 4.7	34.4 ± 5.3	24.8 ± 1.5
18 Copaene	14.17	Cyclic sesquiterpene	7.7 ± 0.8	9.2 ± 1.4	9.6 ± 0.8	11.5 ± 2.0	16.3 ± 2.6	11.5 ± 0.4
19 Unidentified terpene 4	14.31	Sesquiterpene	5.6 ± 0.9	7.4 ± 1.4	7.1 ± 1.5	6.4 ± 0.8	7.3 ± 0.9	7.1 ± 1.3
20 β-elemene	14.48	Cyclic sesquiterpene	111.6 ± 16.8	100.0 ± 23.6	135.7 ± 29.3	134.5 ± 20.5 ab	187.7 ± 26.9 a	115.2 ± 2.2 b
21 β-caryophyllene	15.23	Cyclic sesquiterpene	579.4 ± 75.3 a	1065 ± 122 b	838.9 ± 56.8 ab	729.3 ± 111.2 a	1170 ± 149 b	842.7 ± 91.0 ab
23 β-cubebene	15.37	Cyclic sesquiterpene	9.7 ± 2.9	14.7 ± 1.0	13.5 ± 2.1	15.3 ± 3.6	18.3 ± 3.0	17.6 ± 0.4
22 Epi-bicyclosesquiphellandrene	15.38	Cyclic sesquiterpene	5.8 ± 1.4 b	7.9 ± 3.1 ab	12.8 ± 2.2 a	20.7 ± 6.0 a	14.3 ± 1.9 ab	11.3 ± 0.8 b
24 α-gurjenene	15.59	Cyclic sesquiterpene	4.0 + 0.5 b	6.2 ± 1.0 a	4.6 ± 0.7 ab	5.9 ± 0.5	5.8 ± 0.4	4.2 ± 0.7
25 β-gurjunene	15.65	Cyclic sesquiterpene	2.8 ± 0.3 b	4.6 ± 0.8 a	4.1 ± 0.4 ab	4.8 ± 0.3 a	3.7 ± 0.3 b	4.2 ± 0.6 ab
26 α-caryophyllene*	15.80	Cyclic sesquiterpene	269.2 ± 38.0 a	517.5 ± 60.5 b	406.7 ± 29.0 ab	360.4 ± 55.1 a	563.2 ± 70.7 b	412.7 ± 46.2 ab
27 γ-muurolene	16.06	Cyclic sesquiterpene	23.1 ± 3.2 b	31.6 ± 5.5 ab	42.4 ± 4.6 a	43.9 ± 7.9	55.5 ± 9.4	36.4 ± 1.1
28 Germacrene-D	16.18	Cyclic sesquiterpene	649.1 ± 104.4 a	1580 ± 247 b	1862 ± 198 b	1056 ± 206 a	1866 ± 233 b	1752 ± 251 b
29 α-guaiene	16.26	Cyclic sesquiterpene	8.9 ± 1.5	14.2 ± 3.3 a	6.4 ± 0.9 b	9.1 ± 2.1 a	17.6 ± 3.6 b	11.5 ± 0.5 ab
30 Bicyclogermacrene	16.36	Cyclic sesquiterpene	279.2 ± 50.9 a	699.5 ± 204.0 b	405.8 ± 83.7 ab	286.6 ± 43.6 b	511.0 ± 65.7 a	361.8 ± 51.2 ab
31 γ-cadinene	16.55	Cyclic sesquiterpene	26.3 ± 3.5 a	104.0 ± 39.8 b	42.5 ± 6.1 ab	49.4 ± 15.1	72.0 ± 17.0	49.8 ± 6.4
32 δ-cadinene	16.62	Cyclic sesquiterpene	22.5 ± 3.0 b	26.8 ± 3.8 b	40.7 ± 4.9 a	35.7 ± 9.7	56.7 ± 11.3	45.8 ± 1.7
33 Cadinadiene 1-4	16.74	Cyclic sesquiterpene	3.8 ± 0.6	4.4 ± 0.8	4.5 ± 0.5	4.1 ± 0.6	4.9 ± 0.5	4.5 ± 1.1
34 α-cadinene	16.79	Cyclic sesquiterpene	2.5 ± 0.2	3.7 ± 0.6	3.3 ± 0.3	4.0 ± 0.9	4.5 ± 0.6	4.1 ± 0.7
35 Germacren-4-ol	17.23	Sesquiterpene alcohol	204.4 ± 41.4	174.5 ± 45.8	281.5 ± 64.9	322.7 ± 80.6	386.8 ± 93.7	466.7 ± 10.0
36 Caryophyllene oxide	17.33	Sesquiterpene oxide	16.3 ± 5.0 a	4.6 ± 0.9 b	7.2 ± 2.7 ab	9.5 ± 1.9	5.4 ± 0.7	7.6 ± 1.5
37 τ-cadinol	17.78	Sesquiterpene alcohol	19.4 ± 3.6 a	31.5 ± 3.6 ab	38.7 ± 6.6 b	42.3 ± 12.1	79.0 ± 17.2	45.7 ± 2.6
38 τ-muurol	17.87	Sesquiterpene alcohol	18.8 ± 3.9 a	61.4 ± 28.0 b	37.5 ± 5.8 ab	32.3 ± 13.3	63.6 ± 18.0	43.5 ± 4.4
39 Unidentified terpene 5	18.13	Unknown	5.0 ± 0.9	7.6 ± 1.6	8.9 ± 1.8	9.1 ± 1.3	6.0 ± 0.6	6.6 ± 0.9
40 β-springene	19.53	Acyclic diterpene	10.2 ± 1.4	9.2 ± 1.3	13.3 ± 1.9	15.2 ± 1.9	14.7 ± 3.3	14.2 ± 1.2
41 Phytol	20.92	Diterpene alcohol	9.3 ± 2.0	18.2 ± 5.0	6.4 ± 1.0	2.5 ± 2.0 b	4.5 ± 0.6 b	12.8 ± 2.8 a
42 Unidentified terpene 6	21.69	Unknown	4.3 ± 0.8 a	6.8 ± 1.0 b	5.1 ± 0.6 ab	9.5 ± 0.8	8.7 ± 0.9	7.1 ± 1.0

Table 1. Terpene concentrations (Mean ± SE, in µg g^{-1} of dry weight) in the needles of *Pinus sylvestris* subsp. *nevadensis* and *Pinus sylvestris* subsp. *iberica*. The main terpenes are shaded, also shown in Figs. 1, 2 and 4. The compounds marked with an asterisk belong to terpenes that were verified with standards. Different letters indicate statistically significant differences. (one-way ANOVA, LSD post hoc test $P < 0.05$) ABs attacked branches of attacked trees, NABs non-attacked branches of attacked trees, NATs non-attacked trees. R.T. retention time (expressed in minutes).

Non-Attacked Trees of *P. sylvestris* subsp. *iberica* presented higher needle concentrations of total terpenes than NATs of *P. sylvestris* subsp. *nevadensis* (24.44 ± 1.93 and 18.73 ± 1.37 mg g^{-1} (±SE) dw, respectively; t = -2.44, df = 22, *P* < 0.05) (Fig. 1). The concentration of α-pinene, the principal compound in the needles, was clearly higher in *iberica* than in *nevadensis* (14.32 ± 0.91 and 9.60 ± 0.41 mg g^{-1} (±SE) dw, respectively; t = -4.63, df = 22, *P* < 0.001). Limonene, α-terpinolene and phytol were also higher in NATs of *iberica* than in NATs of *nevadensis* (t-tests and Mann–Whitney tests, *P* < 0.05) (Table 1). The percentage of monoterpenes relative to total terpenes was higher in NATs of *iberica* than those of *nevadensis* (83.16 ± 1.17 % and 77.79 ± 1.23, respectively; t = -3.15, df = 22, *P* < 0.01).
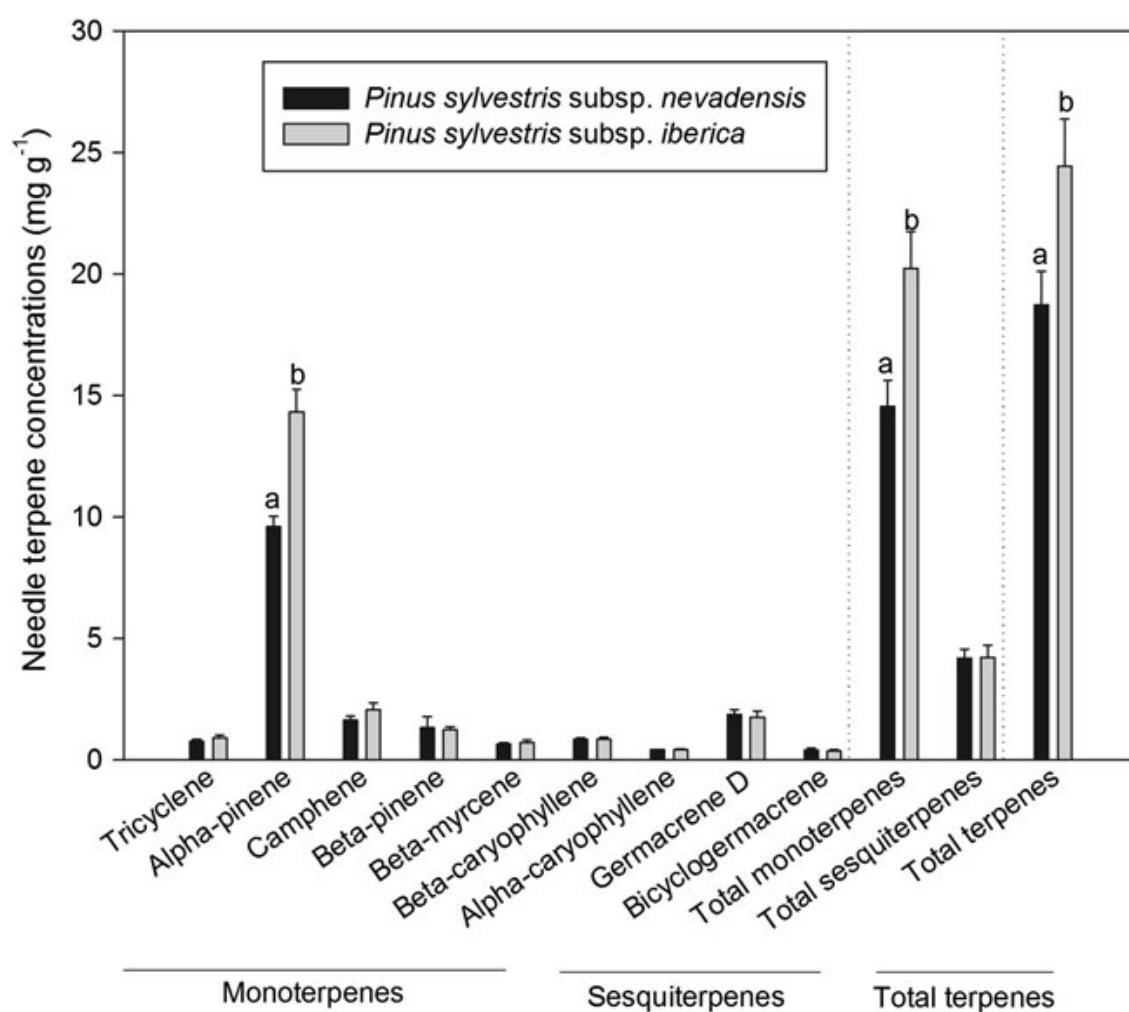

Figure 1. Mean concentrations (±SE) in mg g^{-1} of dry weight of the most abundant needle terpenes (five monoterpenes and four sesquiterpenes) and the total terpenes for non-attacked trees (NATs) of each subspecies. Different letters indicate statistically significant differences (t-tests, *P* < 0.05) for the two subspecies.

The needle terpene concentrations of several compounds were higher in NATs than in ABs for both subspecies, but especially in *nevadensis* (Fig. 2; Table 1). NATs of *nevadensis* presented higher total monoterpenes, sesquiterpenes and total terpenes than ABs (14.53 ± 1.08 and 11.17 ± 1.44 mg total monoterpenes g^{-1} dw (±SE), 4.19 ± 0.36 and 2.73 ± 0.38 mg total sesquiterpenes g^{-1} dw (±SE) and 18.73 ± 1.37 and 13.90 ± 1.69 mg total terpenes g^{-1} dw (±SE), respectively; one-way ANOVA, LSD post hoc test $P < 0.05$). Among the individual compounds, the concentrations of α-pinene and specially germacrene D were also higher in NATs than in ABs (oneway ANOVA, LSD post hoc test $P < 0.05$). The concentrations of camphene, β-caryophyllene and α-caryophyllene were also slightly higher in NATs (ANOVA on Ranks, Conover-Inman test $P < 0.10$ (marginally significant)). Other terpenes, such as β-ocimene, borneol, epi-bicyclosesquiphellandrene, γ-muurolene, δ-cadinene and τ-cadinol were also higher in NATs (one-way ANOVA, LSD post hoc test and ANOVA on Ranks Conover-Inman post hoc test, $P < 0.05$) (Table 1). Terpene concentrations in *iberica* did not differ between NATs and ABs, except for α-pinene, germacrene D and phytol (one-way ANOVA, LSD post hoc test $P < 0.05$) (Table 1).

Within the attacked trees of both subspecies, the concentrations of sesquiterpenes differed most between NABs and ABs, with the concentrations of β-caryophyllene, α-caryophyllene, germacrene D and bicyclogermacrene higher in NABs (one-way ANOVA, LSD post hoc test, $P < 0.05$) (Fig. 2; Table 1). In *nevadensis*, the difference between NABs and ABs was especially important for germacrene D (1.58 ± 0.25 and 0.65 ± 0.11 mg g^{-1} dw (±SE), respectively; $P < 0.01$). Other sesquiterpenes, such as β-gurjunene, γ-cadinene, τ-muurol and one unidentified terpene, also presented higher concentrations in NABs, except for caryophyllene oxide, whose concentration was the highest in the AB attack state (one-way ANOVA LSD post hoc test and ANOVA on Ranks Conover-Inman post hoc test, $P < 0.05$). In *iberica*, the concentrations of total terpenes and α-pinene were also higher in NABs than in ABs (one-way ANOVA LSD post hoc test, $P < 0.05$). ABs had a higher percentage of monoterpenes relative to total terpenes than did NABs for both subspecies, though the difference was significant only in *nevadensis* (81.29 ± 1.57 and 76.34 ± 1.76 % (±SE), respectively; one-way ANOVA LSD post hoc test, $P < 0.05$).

Differences between NABs and NATs were found in both subspecies but were most apparent in *iberica*, which had the highest concentrations in NABs (Fig. 2; Table

1). The concentrations of γ-elemene (*nevadensis*) and phytol (*iberica*) were higher in NATs than in NABs, while the concentrations of α-guaiene (*nevadensis*) and β-pinene, β-myrcene and β-elemene (*iberica*) were higher in NABs than in NATs (one-way ANOVA LSD post hoc test and ANOVA on Ranks Conover-Inman post hoc test, *P* < 0.05). Notably, both subspecies had marginally significant higher concentrations of β-caryophyllene and α-caryophyllene in NABs than in NATs (one-way ANOVA LSD post hoc test, *P* < 0.10).
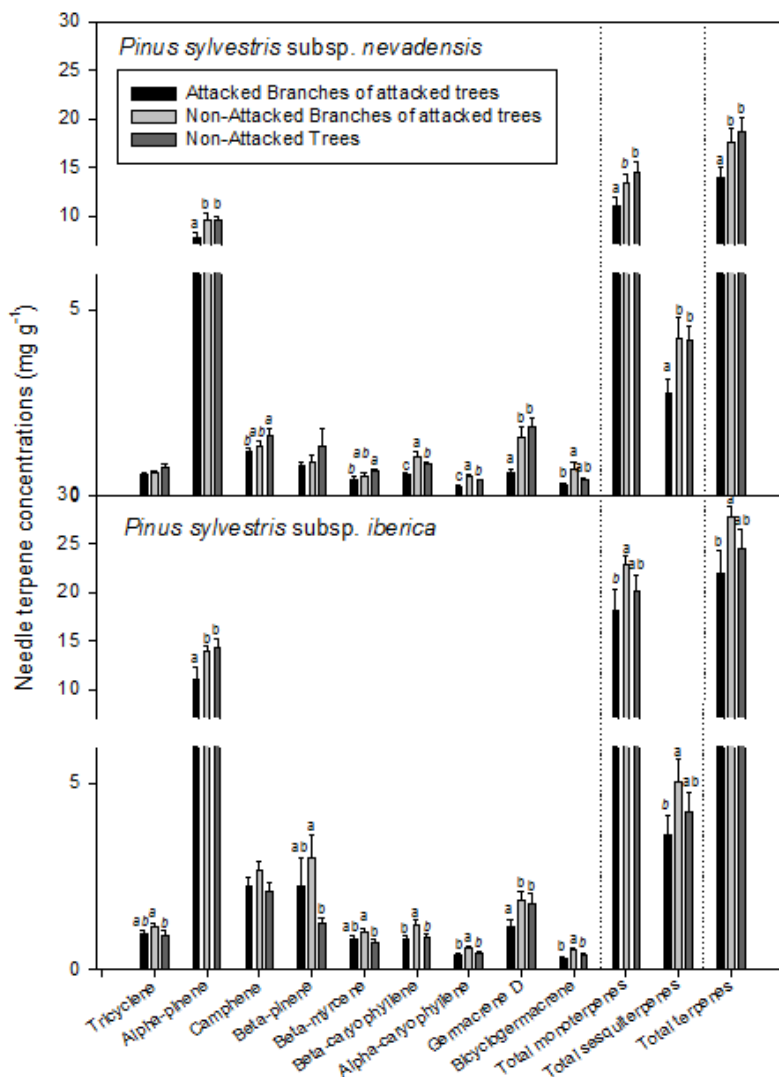

Figure 2. Mean concentrations (±SE) in mg g^{-1} of dry weight of the most abundant needle terpenes and total terpenes for the three attack states in the two subspecies. Different letters indicate statistically significant differences (one-way ANOVA, LSD post hoc test, *P* < 0.05), and *italicized* letters indicate marginally significant differences (*P* < 0.1).

A general overview of the differences in the needle concentrations and proportions of mono- and sesquiterpenes was achieved using a PCA that included all the concentrations and proportional variables of the main terpenes (Fig. 4). The first two PCs accounted for 57.8 and 20.6 % of the total variance, respectively. PC1 separated the cases by terpene concentration, and PC2 separated sesquiterpenes from monoterpenes. The PC1 and PC2 scores were different for *nevadensis* and *iberica* (PC1: 1.18 ± 0.35 and 21 ± 0.48 (±SE), respectively, of PCA scores, t = 4.00, df = 67, P < 0.001; PC2: 0.81 ± 0.23 and -0.83 ± 0.25 (±SE), respectively, of PCA scores, t = 4.79, df = 67, P < 0.0001), placing *nevadensis* close to low monoterpene percentages and low total terpene concentrations and *iberica* more displaced toward higher total terpene concentrations and higher monoterpene percentages. ABs were located toward lower terpene concentrations and higher monoterpene percentages than were NATs. In the main axis (57.8 %), *nevadensis* ABs differed from all other attack states for both subspecies, while in *iberica*, NABs differed from all the others (one-way ANOVAs LSD post hoc test, P < 0.05). In the second axis (20.6 %), *nevadensis* had higher values and had differences within its subspecies and with *iberica* attack states (one-way ANOVA LSD post hoc test, P < 0.05), while *iberica* showed no intrasubspecific differences (Fig. 4).
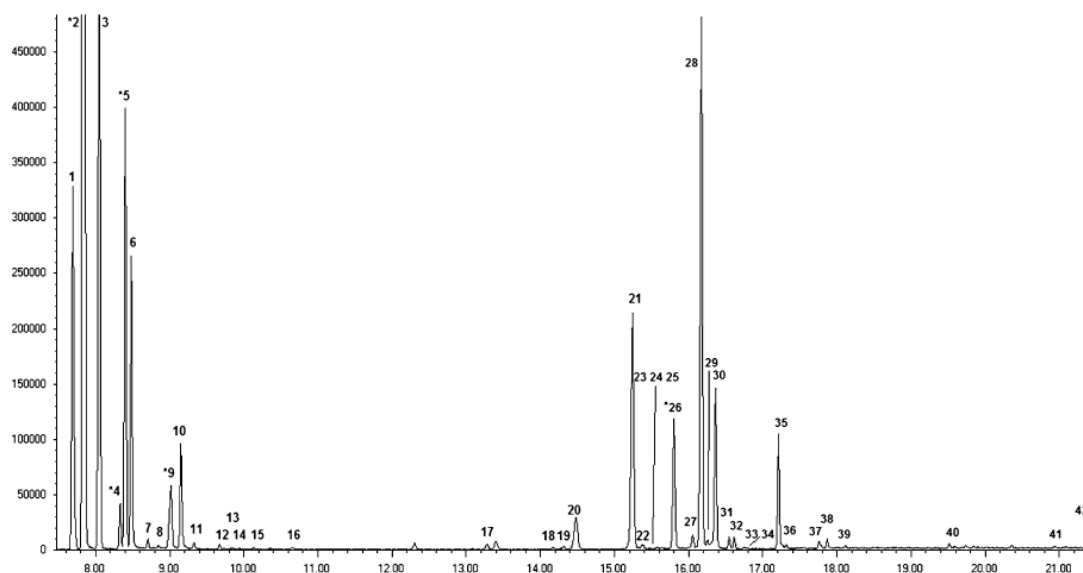

Figure 3. Gas chromatographic trace of an attacked branch (AB) of *Pinus sylvestris* subsp. *iberica*, seen as a fractionation product with mass 93. Peak numbers correspond to the terpenes listed in Table 1. Numbers marked with an asterisk belong to terpenes that were verified with standards

Rates of terpene emission

Sixteen terpenes were identified in the emissions. Eleven were monoterpenes, which was the main class of emitted terpenes, and five were sesquiterpenes. Sesquiterpenes had very low emission rates and only appeared in some samples (data not shown). ABs presented higher emissions of total terpenes, α-pinene and camphene than NATs, and higher emissions of total terpenes, tricyclene, α-pinene and β-myrcene than did NABs (one-way ANOVA LSD post hoc test, $P < 0.05$). The most notable differences were found in total terpenes (30.62 ± 3.11, 11.31 ± 2.52 and 6.34 ± 2.45 μg g^{-1} dw h^{-1} ($\pm$SE) for ABs, NABs and NATs, respectively; one-way ANOVA, $P < 0.001$) and α-pinene (19.28 ± 8.72, 5.57 ± 1.68 and 2.40 ± 1.60 µg g^{-1} dw h^{-1} ($\pm$SE); one-way ANOVA, $P < 0.01$). NABs tended to have higher emission rates than NATs, although the differences were not statistically significant for any emission (Fig. 5). α-Pinene had the highest rates of emission in all attack states, but implied a significantly higher percentage of total terpene emission in ABs than in NATs (61.07 ± 5.76 and 27.24 ± 9.79 % ($\pm$SE), respectively; one-way ANOVA LSD post hoc test, $P < 0.05$) (Fig. 6).

Discussion

Needle terpene concentrations

The terpene concentrations found in the needles of the two subspecies of *P. sylvestris* were within the range reported for other pines (Beyaert et al. 2012; Blanch et al. 2011; Sampedro et al. 2011). The main terpenes and their concentrations and proportions were similar to those described by Hodar et al. (2004) in a study conducted on needles of *P. sylvestris* subsp. *nevadensis* in a nearby area (200 m lower in altitude). The three terpenes that were only found in ABs could not be properly identified. For a better understanding of the system, the enantiomers and their proportional changes in the terpene concentrations (Faldt et al. 2006) of the different attack states should be analyzed in future studies.

In the comparison of NATs of both subspecies, the higher concentrations of total terpenes and total monoterpenes found in *P. sylvestris* subsp. *iberica* were basically due to the higher α-pinene concentrations, the only main compound that

differed between both subspecies. α-Pinene, despite being the most abundant component in needles, is not detected by the antennae of PPM females (Zhang et al. 2003) and seems not to act as an antifeedant (Petrakis et al. 2005). Among the other terpenes (Table 1), limonene, thought to have a deterrent effect on PPMs (Tiberi et al. 1999), was also higher in *iberica* than in *nevadensis*. These differences in terpene concentrations between NATs suggest constitutive differences between these two subspecies, because the study area was small, and the conditions of the habitat were not very heterogeneous. No apparent differences were seen in the variables known to affect terpene concentrations in plants, such as pathogens (Gershenzon and Dudareva 2007), PPM attacks in previous years, herbivores other than PPMs, droughts (Llusia et al. 2010) or availability of nutrients (Sampedro et al. 2011), which were not controlled in this study. Theoretically, lower concentrations of terpenes (understood as lower defensive ability) should render *nevadensis* more prone to PPM attack than *iberica*.

When comparing ABs with NATs, only α-pinene and germacrene D were clearly higher in NATs for both subspecies (Figs. 1, 2). Hodar et al. (2004) also found α-pinene and germacrene D to present higher concentrations in undefoliated pines than in pines defoliated by PPM the previous year. Germacrene D played a significant role in the feeding model of Petrakis et al. (2005) as a ''weak suppressor of feeding''. In Zhang et al. (2003), germacrene D was detected by the antennae of PPMs. In *nevadensis*, caryophyllene oxide, which is a well-known insect repellent (Gunasena et al. 1988) and plays a significant role in the feeding model of Petrakis et al. (2005), was the only compound that presented the highest concentration in the AB state, although its concentration was not statistically different from that in NATs (Table 1). This oxygenated sesquiterpene could have increased its concentrations when PPM bites exposed the resin ducts to the air and may act as a deterrent once the needle had been attacked. The *nevadensis* subspecies had larger differences of concentration between the ABs and NATs than *iberica* had (Fig. 2). Since NATs of both subspecies were very similar in individual terpene content (Fig. 1), the larger differences found in *nevadensis* should be generated by the lower concentrations found in their ABs. This difference could be due to larger constitutive differences between attacked trees and non-attacked trees of *nevadensis* or to a larger loss related to emissions.

Within the attacked trees, NABs had higher concentrations of sesquiterpenes than did ABs (Fig. 2) and lower percentages of monoterpene concentrations in the case of

nevadensis. Monoterpenes are more volatile than sesquiterpenes, so the losses due to emission in ABs should be larger for monoterpenes (and generate larger differences
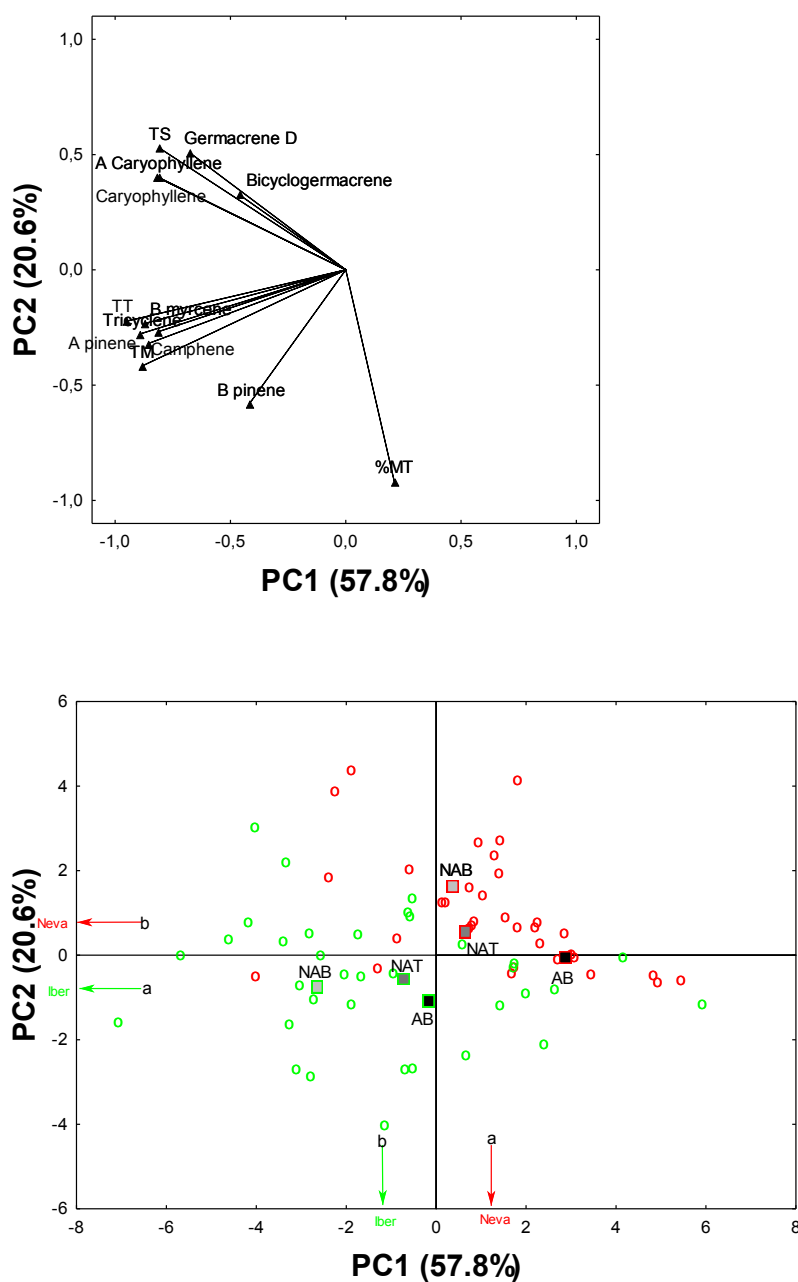
Figure 4. Principal component analysis conducted using 13 variables, including the needle concentrations of the most abundant terpenes (9) and total terpenes (3) (mg g^{-1} of dry weight) and the percentage of monoterpenes of total terpenes (%). The two biplots depict PCA variable loadings (above) and PCA case scores (below). Red circles indicate the *nevadensis* subsp., and green circles indicate the *iberica* subsp. The squares indicate the means of the different attack states. AB attacked branches of attacked trees, NAB non-attacked branches of attacked trees, NAT non-attacked trees, MT monoterpenes, ST sesquiterpenes and TT total terpenes. Arrows indicate the means of the axes of PCA scores for each subspecies, and the different letters indicate statistically significant differences between subspecies (oneway ANOVA, LSD test *P* < 0.05) (color figure online)
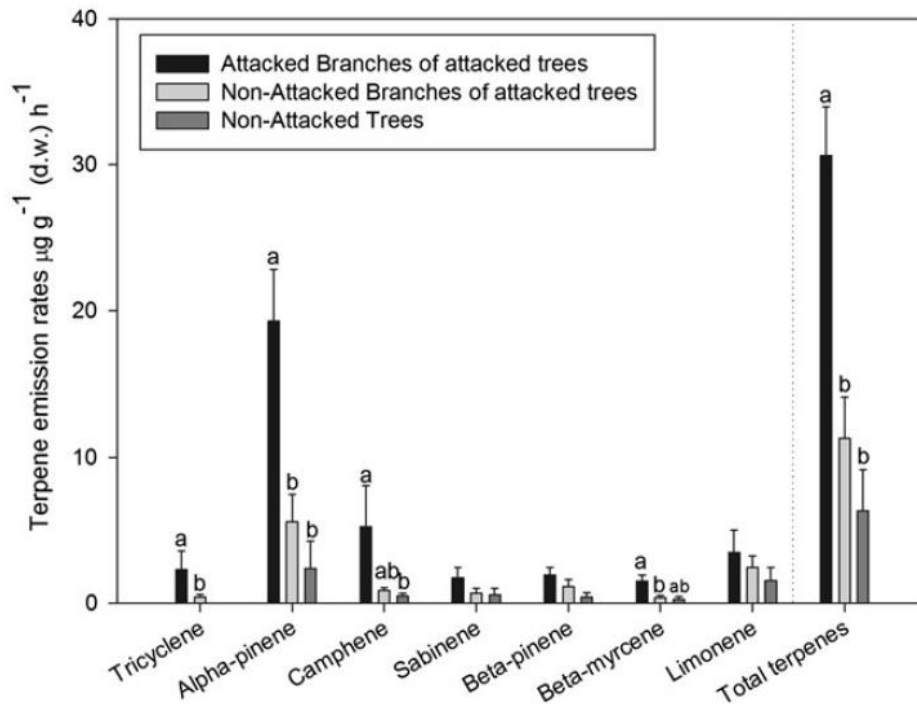
Figure 5. Mean rates of emission (±SE) in µg g^{-1} of dry weight h^{-1} of the most abundant and total terpenes in the three attack states for *Pinus sylvestris* subsp. *nevadensis*. Different letters indicate statistically significant differences (one-way ANOVA, LSD post hoc test $P < 0.05$)

among attack states) than for sesquiterpenes. Similarly, the percentages of monoterpenes should decrease in ABs. One plausible explanation for this contradictory situation is an induction of monoterpenes, as observed in several pines in response to attacks from the Tiger moth (Litvak and Monson 1998). Another possibility is a higher and/or earlier induction of monoterpenes than of sesquiterpenes, as seen in the stems of grand firs (Steele et al. 1998). Although presenting a similar pattern, the differences in percentage of monoterpene concentrations were not significant in *iberica*, perhaps because this subspecies seems to have a higher level of constitutive monoterpenes. The supposed induction of monoterpenes to generate differences between these two attack states would thus be more difficult. Other factors involved might be internal constitutive differences of the trees, unequal numbers of previous PPM attacks among branches or a larger loss of emissions of sesquiterpenes than of monoterpenes, as seen in Martin et al. (2003), although in their study, the conifers sustained no wounds and thus avoided exposing terpenes to the atmosphere.

When comparing NABs and NATs in both subspecies, our results suggest that β- and α-caryophyllene could have increased slightly their concentrations in NABs (marginally significant) as a systemic response to attack. β-caryophyllene was the most significant deterrent in the study by Petrakis et al. (2005). In *iberica*, additional major compounds like β-pinene and β-myrcene increased significantly in NABs, indicating that this subspecies may be reacting systemically to attack. The smaller number of possible systemic responses found in *nevadensis* may be due to its limited exposure to PPM attacks (Hodar and Zamora 2004). Prior to the current climatic warming, the isolation of *nevadensis* in high mountains too cold for the PPM has protected this pine from attack, but it may now be more vulnerable to the PPM.
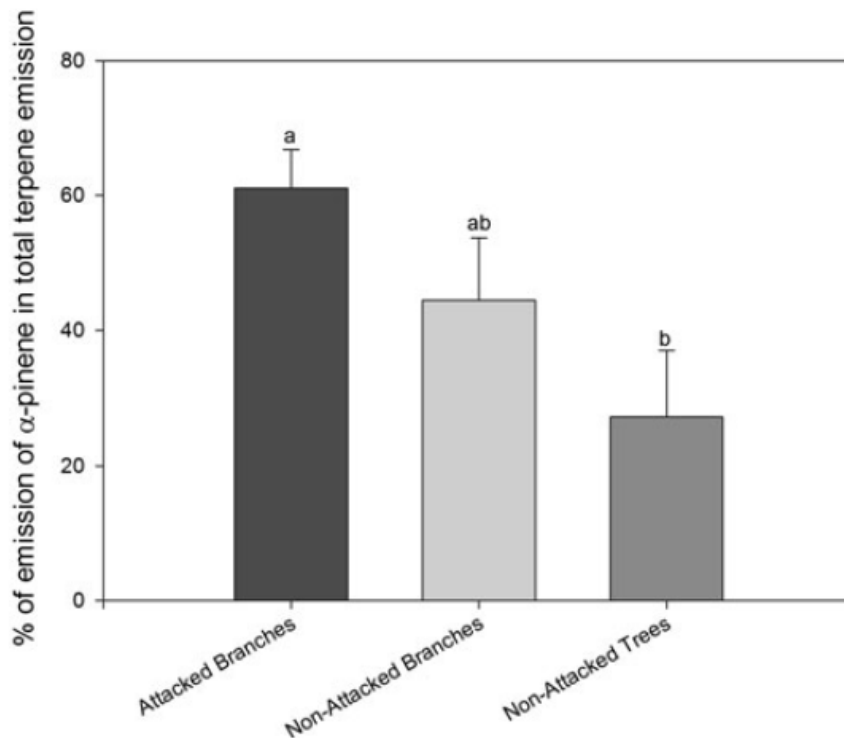
Figure 6. Mean (±SE) percentage of emission of α-pinene relative to total terpene emission in the three attack states for *Pinus sylvestris* subsp. *nevadensis*. Different letters indicate statistically significant differences (one-way ANOVA, LSD post hoc test $P < 0.05$)

Rates of terpene emission

The rates of terpene emission in *nevadensis* were also in the range reported in the literature for pines (Blanch et al. 2007; Llusia and Penuelas 2000). In *nevadensis*, ABs, which had the lowest needle terpene concentrations (Fig. 2), were the highest emitters in all cases (Fig. 5), followed by NABs and NATs (NATs had the highest needle

concentrations). The volatiles induced by insect damage depend on the feeding habits and on the level of herbivorous attack (Delphia et al. 2007). Attacked leaves usually emit more BVOCs than do non-attacked leaves (Llusia and Penuelas 2001; Paris et al. 2010). Further studies would be necessary to determine if these altered emissions could become a "cry for help" that would attract parasitoids or larval predators (Mumm and Hilker 2006; Vet and Dicke 1992) or if the differences in emissions could be an olfactory cue for the PPM to select the best trees for oviposition (Paiva et al. 2011). Although NABs usually presented higher emission rates than did NATs, the differences were not significant, indicating that NABs produced no clear systemic reactions.

The increased percentage of α-pinene in the emissions compared to total emissions in attacked states may be due to the high volatility of α-pinene. Although physical processes are plausible causes, a specific response of the plants to attack should not be discarded, e.g. Norway spruce can double its emissions of terpenes without wounding, but only by the application of methyl jasmonate (Martin et al. 2003). The proportions of the blends emitted by the attacked states studied were different for several terpenes, indicating that the emissions may be a possible olfactory cue. Enantiomers and their proportional changes in emissions in the different attack states should also be analyzed for obtaining a better understanding of this system (Yassaa and Williams 2007).

Our results cannot discriminate between the low terpene concentrations as the cause of the higher PPM attack (moths choose needles with lower terpene concentrations for oviposition, or the larvae feed on needles with lower terpene concentrations) or as the consequence of PPM attack (herbivory by caterpillars changes terpene concentrations). The role of terpenes in the defense of pine and other conifers against defoliators is still controversial, with reports of increased (Bauce et al. 1994; Wainhouse et al. 2009) and decreased concentrations (Litvak and Monson 1998; Nykanen and Koricheva 2004) when attacked. The first possibility (PPMs attack the trees and branches with lower terpene concentrations) agrees with the wellestablished fact that terpenes are one of the main direct defenses used by conifers against folivorous insects (Mumm and Hilker 2006). The lower concentrations of terpenes in ABs might be detected and chosen by the PPM in an effort to avoid toxins in their food as much as possible (Hesbacher et al. 1995). Some authors consider terpenes as determinants in the preferences in PPM feeding (Petrakis et al. 2005) or oviposition

(Tiberi et al. 1999). Inter- and intra-individual differences could be explained by constitutive variability, or by terpene induction differences caused in current or previous attacks by the PPM. Differences in previous PPM attacks, although taken into account, may have been overlooked because old colonies can fall from branches in less than a year, which would hinder an exact count.

The second possibility (PPM attack decreases terpene concentrations) is consistent with increased emissions of terpenes induced by feeding by caterpillars, provoking losses in the terpene content of needles. Our measurements of concentrations and emissions (Figs. 2, 4) allowed us to determine that the difference in total terpene concentrations between NATs and ABs could be completely explained by the loss from the higher emission rates of ABs during only 8 days. In Litvak and Monson (1998), the needles of attacked conifers significantly increased their activity of monoterpene cyclase, but the attacked needles had lower monoterpene concentrations than the controls rather than higher concentrations. The high emissions provoked by feeding by caterpillars were apparently more important than the induction of monoterpene synthesis by cyclase. After damage, these additional emissions should be stopped by the accumulation of oxidative products of the same emitted isoprenoids, but the time required to stop the emissions is not clear; 24 h (Pasqua et al. 2002), 96 h (Su et al. 2009) and 12 days (Litvak and Monson 1998), depending on the pine species, have been reported. This scenario may explain the reduction in needle terpene concentrations through emission. The most likely explanation for our results would be a combination of the two processes: the ABs had lower concentrations due to higher rates of emission, and the higher percentage of monoterpenes was due to an induction of monoterpenes (or at least a higher induction of monoterpenes than of sesquiterpenes).

In summary, the results of this study highlight a relationship between trees and branches with lower needle terpene concentrations and attacks by *T. pityocampa*. The Sierra Nevada endemic pine, *P. sylvestris* subsp. *nevadensis*, presented lower terpene concentrations than did *P. sylvestris* subsp. *iberica* in NATs, probably due to constitutive differences. ABs had lower terpene concentrations than did NABs and NATs. Some terpenes had higher concentrations in NABs than NATs, indicating possible systemic reactions to attack. In contrast with concentrations, ABs showed the highest rates of emission, as expected after herbivorous attacks. These results will

hopefully help in understanding and managing this dangerous and spreading pest of pines. Future studies should clarify the influence of evaporation and the induction of synthesis on the concentrations and emissions of terpenes in the needles of *P. sylvestris* by studying how terpene synthases react to attack and how atmospheric exposure affects the terpene content of needles damaged by herbivory.

Acknowledgments

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Chapter 2. Down-regulation of the expression of two sesquiterpene synthase genes after severe infestation of Scots pine by the pine processionary moth (*Thaumetopoea pityocampa*)

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Abstract

Mediterranean pines are commonly defoliated by the larvae of the pine processionary moth (PPM), *Thaumetopoea pityocampa,* a very voracious lepidopteran. Previous studies have reported unaltered or lower terpene concentrations in pine needles attacked by PPM larvae, coupled with high terpene emissions. Slightly higher terpene concentrations in unattacked branches of attacked trees (systemic reactions), however, have also been reported. We thus investigated if the needle terpene concentrations of Scots pine attacked by PPM larvae were the result of *i*) an induction of terpene biosynthesis masked by high terpene emissions or *ii*) reduced terpene biosynthesis due to the damage-induced down-regulation of the expression of genes encoding enzymes involved in terpene biosynthesis. We monitored the expression of two genes encoding sesquiterpene synthases of Scots pine (*PsTPS1* and *PsTPS2*) in the needles attacked by PPM larvae. *PsTPS1* encodes a synthase producing β-caryophyllene and α-humulene, and *PsTPS2* encodes a synthase producing β-elemene, bicyclogermacrene, α-amorphene and germacren-4-ol. Expression levels were monitored in locally attacked and systemic needles. Terpene concentrations in these needles were determined in parallel. Both the expression and chemical analyses were conducted at three time points after the onset of defoliation by the PPM. The expression of the two genes decreased as the defoliation progressed. The expression levels of both genes were significantly lower when the needles were heavily damaged by late instar larvae (L5). Needle terpene concentrations followed similar trends and were significantly correlated with the expression levels of the synthase genes. The *PsTPS2* products in the systemic needles, however, had higher concentrations at the intermediate time point, thus partially matching some previous results. The possible reasons for this response of Scots pine to PPM herbivory are discussed.

Keywords: *Pinus sylvestris – PsTPS1 – PsTPS2* – defoliation - herbivory

Introduction

Terpenes are important chemical defenses of conifers against pathogens and herbivorous insects (Keeling and Bohlmann 2006; Mumm and Hilker 2006; Phillips and Croteau 1999). Conifers maintain high levels of constitutive terpenes that act as a first line of defense ready against any aggression, but the biosynthesis of these compounds can also be induced by biotic stress (Pasquier-Barre et al. 2001; Raffa and Smalley 1995; Zou and Cates 1997). Induction has been demonstrated in attacked tissues (local defense) (Achotegui-Castells et al. 2015; Moreira et al. 2013) but also in undamaged tissues of an attacked plant (systemic defense) (Hilker et al. 2002; Viiri et al. 2001). Terpenes directly defend against herbivores by their toxicity (Kesdek et al. 2014; Petrakis et al. 2005; Zou and Cates 1997), but their volatile fraction (mono- and sesquiterpenes) can also be an indirect defense by attracting antagonists of the attacking herbivores, such as predators or parasitoids (Hilker et al. 2002; Paré and Tumlinson 1999). Both constitutive and induced terpenes are produced by a wide range of terpene synthases, a group of enzymes responsible for catalyzing the formation of thousands of different terpenes (Degenhardt et al. 2009; Tholl 2006).

Four genes encoding terpene synthases in the Scots pine (*Pinus sylvestris*) have been sequenced: *PsTPS1*, *2*, *3* and *5* (Köpke et al. 2010; Köpke et al. 2008). All catalyze the formation of several sesquiterpenes, and the expression of *PsTPS1*, *2* and *5* can be up- or down-regulated in response to oviposition by *Diprion pini* (Köpke et al. 2008) and *Neodiprion sertifer* on Scots pine needles (Köpke et al. 2010). The larvae of these two species of hymenopteran sawflies are folivorous, whereas the adults do not feed on pine needles. Beyaert et al. (2012) recently observed that the expression levels of *PsTPS1* and *2* increased strongly in response to larval feeding by *D. pini* (with and without previous oviposition) compared to an undamaged control.

The pine processionary moth (PPM), *Thaumetopoea pityocampa*, is a very important pine defoliator in the Mediterranean Basin (Battisti et al. 2005; Hódar et al. 2002). Scots pine is not the main host of the PPM, but the larvae successfully feed on this pine species (Hódar et al. 2002; Jactel et al. 2015). Several studies have reported

the terpene reactions of pines infested with this voracious caterpillar (Achotegui-Castells et al. 2013; Lombardero et al. 2013; Moreira et al. 2013), suggesting unaltered or decreased needle terpene concentrations. Achotegui-Castells et al. (2013) reported that needles damaged by the PPM generally had lower terpene concentrations but a 5-fold increase in the rates of terpene emissions. In contrast, the concentrations of several sesquiterpenes in systemic branches (unattacked branches of an attacked tree) slightly increased. These results, however, did not determine whether the lower terpene concentrations were due to feeding-induced volatile emissions or to reduced terpene biosynthesis. Information for the expression of pine terpene synthase genes in response to PPM feeding damage would help us to understand the dynamics that could account for the reported terpene concentrations.

The goals of this study were thus to *i*) monitor the expression of the terpene synthase genes *PsTPS1* and *PsTPS2* in Scots pines attacked by the PPM at three time points during larval development and *ii*) correlate the needle terpene concentrations at these time points with the levels of gene expression.

Materials and Methods

Study site and plant and insect material

The study was conducted in the experimental fields of the Universitat Autònoma de Barcelona (UAB) (Barcelona, Spain) (41°29'38"N, 2°05'54"E; 100 m a.s.l.), from the end of July 2013 to the end of January 2014. The experimental field had homogeneous sunlight and wind exposure. Forty-eight 1.5-2.5 m Scots pines in pots (acquired from Forestal Catalana SA, Barcelona, Spain) were randomly assigned to two treatment groups in the experimental field: 24 control pines (no PPM infestation) were placed at one end of the field, and 24 pines (assigned to future attack) were placed at the opposite end of the field to avoid or minimize any possible interplant communication (Karban et al. 2006; Kost and Heil 2006). The potted trees were fertilized with NPK at the beginning of the experiment and were watered three times a week.

We collected 500 PPM pupae from Venosta Valley (Bolzano, Italy) and stored them in Petri dishes. We buried the pupae at UAB in trays filled with sand. We allowed the emerged adults to mate and encouraged the females to oviposit on the pines to mimic the natural process of infestation. Scots pines can react to oviposition by herbivorous insects (Köpke et al. 2010; Köpke et al. 2008) but unfortunately we did not succed to make the moths oviposit. We thus collected PPM egg batches from natural stands of pines and attached them to the trees assigned to larval attack (Köpke et al. 2010; Köpke et al. 2008). All egg batches were collected from Scots pines during July and August at a forest in Tona (Northern Barcelona province: 41°50'27"N, 2°11'42"E; 400-700 m a.s.l.). The egg batches were attached to the needles, and the larvae could begin feeding from the moment of hatching. Each attacked pine received one egg batch consisting of about 150 eggs. The pines were monitored daily to record the time of hatching for each tree.

PPM larval development usually lasts from four to six months, typically from August to February, and larvae go through five larval stages (Hódar et al. 2002). In the first larval stage (L1), which lasts about 10 days, the gregarious larvae build a small silk nest in which they take refuge from predators. L1 has the highest mortality rates of the larval cycle (up to 60%), which strongly determines later survival (Hódar et al. 2002). The L1 larvae measure 2-6 mm and usually eat only the superficial parts of needles close to the nest. The second larval stage (L2) lasts 12-18 days, and the larvae grow to 10-12 mm. In the third larval stage (L3), which lasts about a month, the larvae develop the urticant hairs that deter mammals (Lamy 1990) and build a new and definitive silk nest. The fourth stage (L4) lasts about 40 days, and the fifth and last stage (L5), in which the larvae can reach 4 cm and are the most destructive, lasts another 40 days (Montoya and Hernández 1991). Once the larval cycle is completed, the caterpillars descend from the tree forming a *procession*, bury in the nearby soil or litter and pupate until July to August, when the adults emerge, mate and locate a pine for laying their eggs (Hódar et al. 2002).

We studied the effects of feeding damage by L1, L3 and L5 larvae on the chemistry of Scots pine needles and the expression of terpene synthase genes by sampling needles 4, 45 and 100 days after hatching. The samples were collected from different trees to avoid any alteration of terpene chemistry due to sampling wounds (i.e. picking a twig from a tree). Sixteen trees were sampled for each larval stage, eight

control pines and eight attacked pines (eight tree replicates * two treatments * three larval stages = 48 trees).

Experimental design and needle sampling

Four types of needles were collected. We collected Control needles from the control pines and Systemic needles (unattacked needles as far as possible from the PPM nest), Local-intact needles (unattacked needles close to the nest) and Local-bitten needles (freshly attacked needles close to the nest) from the attacked pines. Recently attacked needles (within approximately 1-24 h) have a watery green color, whereas those attacked more than one day previously are more yellowish and appear drier. Each sample was divided into two, one for terpene analysis and the other for molecular analysis. Sampled needles were immediately frozen in the field in liquid nitrogen and were then stored at -25 °C in the laboratory.

Needle terpene identification and analysis

Ten milligrams of needles were ground to a powder in a Teflon® tube filled with liquid nitrogen. The needle powder was covered with 1 ml of pentane containing 0.5 ng of dodecane (internal standard) and was then stored overnight at -25 °C for maximal extraction. One blank was included for every five needle extracts. The next morning, 300 µl of the supernatant were transferred to a vial for analysis by gas chromatography (GC)/mass spectrometry (MS). The Teflon® tubes were thoroughly cleaned, dried and reweighted to obtain the dry weight (dw) of the analyzed needles. Two microliters of the extract were injected into an HP SM5 capillary column (30 m × 0.25 mm × 0.25 µm) in a GC (7890A, Agilent Technologies, Santa Clara, USA) coupled to an MS detector (5975C inert MSD with triple axis detector, Agilent Technologies) (see details in Achotegui-Castells et al. (2013)). Terpenes were identified by comparison with published mass spectra (Wiley 07 and NIST 05 libraries) and known standards (the sesquiterpenes α-humulene, caryophyllene oxide and cedrol) that were also used to construct calibration curves for terpene quantification. The terpenes were quantified using Total Ion Chromatogram (TIC) mode.

PsTPS1 and *PsTPS2* gene expression analyses

Sampled needles were ground to a powder in liquid nitrogen with a mortar and pestle that had been baked at 200 °C for 4 h to inactivate any interfering RNases. RNA was extracted from 100 mg of the powder using the Invitrap® Spin Plant RNA Mini Kit (Stratec, Berlin, Germany) for plants containing phenolic compounds. The RNA was eluted in 50 µl of nuclease-free water. Residual DNA was digested following the protocol of the DNA-free™ kit (Ambion, Darmstadt, Germany). RNA concentration was determined spectrophotometrically with 2 µl of RNA extract on a µDrop™ plate (Thermo Scientific, Schwerte, Germany) with a Multiskan GO spectrophotometer (Thermo Scientific, Schwerte, Germany). Concentrations were calculated from the absorbance at 260 nm.

RNA integrity was visually assessed on a 1.1% TAE-agarose gel stained with 4 µl of 1% ethidium bromide (45 min at 120V). Five microliters of RNA extract were mixed with 2X loading dye of the HR-Ribo Ruler ladder (Thermo Fisher, Darmstadt, Germany), heated for 10 min at 70 °C and immediately cooled to 4 °C for 5 min prior to loading on the gel. Four microliters of the HR-Ribo Ruler ladder were identically treated prior to loading. Disintegrated samples due to transport, cooling at -25 °C or extraction were not used for further analysis.

c-DNA was synthesized from 1 µg of each RNA extract using native AMV reverse transcriptase (Roboklon, Berlin, Germany) following the manufacturer's protocol. *PsTPS1* and *PsTPS2* expression was analyzed by qPCR as described by (Köpke et al. 2008), with minor modifications. Thermal cycling used a Stratagene Mx3005P™ system (Stratagene, Santa Clara, USA), and cycle number was reduced to 45 cycles. Each reaction contained 25 ng of template cDNA. Ct values and efficiencies were determined with LinRegPCR (Ruijter 2014). Relative expression of the transcripts was calculated with the ΔCt method, including efficiencies.

$$\Delta Ct = \frac{E_{ref}^{(Ctref)}}{E_{target}^{(Cttarget)}}$$

where ΔCt is the expression relative to a reference gene (ubiquitin), E_{ref} is the efficiency of expression of the reference sample, Ct_{ref} is the Ct value of the reference sample, E_{target} is the efficiency of expression of the target sample (*PsTPS1* or *PsTPS2*) and Ct_{target} is the Ct value of the target sample.

Statistical analyses

We first eliminated the outliers using absolute deviation around the median with the *very conservative* method described by Leys et al. (2013). The data were then assessed for normality and homogeneity of the variances. Data not fitting the requirements were transformed or were analyzed with non-parametric tests. We performed one-way analyses of variance (ANOVAs) with Tukey's post-hoc tests (or Kruskal-Wallis ANOVAs for data that could not be normalized) to compare needle types and used Pearson's correlation coefficients to assess the statistical significance of linear correlations, transforming the non-normal variables.

Results

Expression levels of terpene synthase genes

Visual quantification of tree defoliation (mean±SE) indicated that L1-attacked pines suffered <5% defoliation, L3-attacked pines 15±6% defoliation and L5-attacked pines 91±3% defoliation. Expression levels of both *PsTPS1* and *PsTPS2* in the four types of needles did not differ significantly at the early time points of feeding damage (L1 and L3). Expression levels, however, were lower in the three types of needles of the attacked trees relative to the Control needles at the latest time point of larval feeding (L5), i.e. when the trees were heavily damaged (Fig. 1). The levels of *PsTPS1* and *PsTPS2* expression in the Control needles did not differ significantly ($P = 0.34$, $P = 0.20$, respectively) throughout the experiment (Figs. 1 and 2). *PsTPS1* expression was marginally significantly lower in the Systemic needles ($P < 0.10$) after the attack by L5 larvae (Fig. 1). *PsTPS2* expression was marginally lower in the Systemic needles after attack by L5 larvae than after attack by L3 larvae ($P < 0.10$) (Fig. 2). *PsTPS1* expression of Local-intact needles was marginally significantly lower ($P < 0.10$) (Fig. 1),

and *PsTPS2* expression was significantly lower ($P < 0.01$) (Fig. 2) in the Local-intact needles after attack by L5 larvae than after attack by L1 larvae. Gene expression in the Local-bitten needles did not differ significantly over time.

Concentrations of terpene products

The products of *PsTPS1* (β-caryophyllene and α-humulene) and *PsTPS2* (β-elemene, bicyclogermacrene, α-amorphene and germacren-4-ol, also known as 1(10),5-germacradiene-4-ol) were quantified. The concentrations of these terpenes did not differ between the needle types after attack by L1 larvae (Figs. 1 and 2). The Systemic needles had higher concentrations of terpene products of both synthase genes after attack by L3 larvae than the Control needles, but the difference was only significant for the *PsTPS2* products ($P < 0.05$). The concentrations of the *PsTPS1* and *PsTPS2* products were significantly lower in the Local-bitten needles ($P < 0.05$) and marginally significantly lower in the Systemic needles ($P < 0.10$) after attack by L5 larvae than in the Control needles. The concentrations of the *PsTPS2* products in the Control needles were marginally higher at the latest time point than at the first time point ($P < 0.10$) (Figs. 1 and 2). The concentrations of the *PsTPS1* and *PsTPS2* products in the Systemic needles were highest at the intermediate time point (L3), which were significantly higher than those at the late time point (L5) for *PsTPS1* products ($P < 0.05$) and higher than at the early (L1) and late (L5) time points for *PsPTS2* products ($P < 0.01$). The concentrations of the products of both synthase genes in the Local-intact needles decreased during defoliation, but the concentrations of the *PsTPS2* products differed only marginally significantly ($P < 0.10$) between the early (L1) and late (L5) time points. The terpene concentrations of the Local-bitten needles also tended to be lower over time, with significantly higher concentrations of *PsTPS1* products ($P < 0.05$) and marginally significantly higher concentrations of *PsTPS2* products ($P < 0.10$) at L1 than at L5. Details of the changes in concentrations of the specific terpenes due to needle damage at the three time points are shown in Table 1.

Relationships between the terpene synthase genes and their products

The differences in the concentrations of the terpenes in the four types of needles at the three time points were generally similar to the differences in gene expression. The expression levels of both terpene synthase genes were positively correlated with the

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concentrations of their terpene products in the needles (Fig. 3). The expression levels of *PsTPS1* were correlated with the concentrations of its products (R^2 = 0.25, P < 0.001), with a very strong correlation between the concentrations of β-caryophyllene and α-humulene (R^2 = 0.994, P < 0.001). The expression levels of *PsTPS2* were correlated with the concentrations of its products (R^2 = 0.167, P < 0.01), but the concentrations varied. The concentrations of β-elemene and bicyclogermacrene (R^2 = 0.903, P < 0.001) were very strongly correlated, whereas the concentration of germacren-4-ol was strongly correlated with those of β-elemene (R^2 = 0.724, P < 0.001) and bicyclogermacrene (R^2 = 0.684, P < 0.001). The concentration of α-amorphene correlated less well with the concentrations of the other *PsTPS2* products (β-elemene, R^2 = 0.380, P < 0.001; bicyclogermacrene, R^2 = 0.429 P < 0.001, germacren-4-ol, R^2 = 0.342, P < 0.001).

Discussion

Terpene synthase gene expression

The expression levels of *PsTPS1* and *PsTPS2* were not clearly affected by PPM herbivory during L1 and L3 but were markedly lower at L5 in the three needle types of the attacked pines. The unaffected or lower gene expression in this study differs from that in most other studies, which have reported significant increases in the expression of terpene synthase genes or cyclase activity in conifers facing herbivory (Beyaert et al. 2012; De Alwis et al. 2009; Litvak and Monson 1998; Miller et al. 2005) or even just woundings (Litvak and Monson 1998; Steele et al. 1998). For example, Beyaert et al. (2012) reported a strong induction of *PsTPS1* and *PsTPS2* expression for Scots pines attacked by *D. pini* caterpillars for two days. The expression of terpene synthase genes, though, can vary greatly over short time periods (De Alwis et al. 2009; Miller et al. 2005). *PsTPS1* and *2* expression can change abruptly depending on the day of measurement (Köpke et al. 2010; Köpke et al. 2008). Egg deposition by *N. sertifer* elicited a large increase in gene expression three days after sawfly oviposition, followed by a reduction after four days (relative to slightly wounded needles that mimicked needles wounded by oviposition) (Köpke et al. 2010).
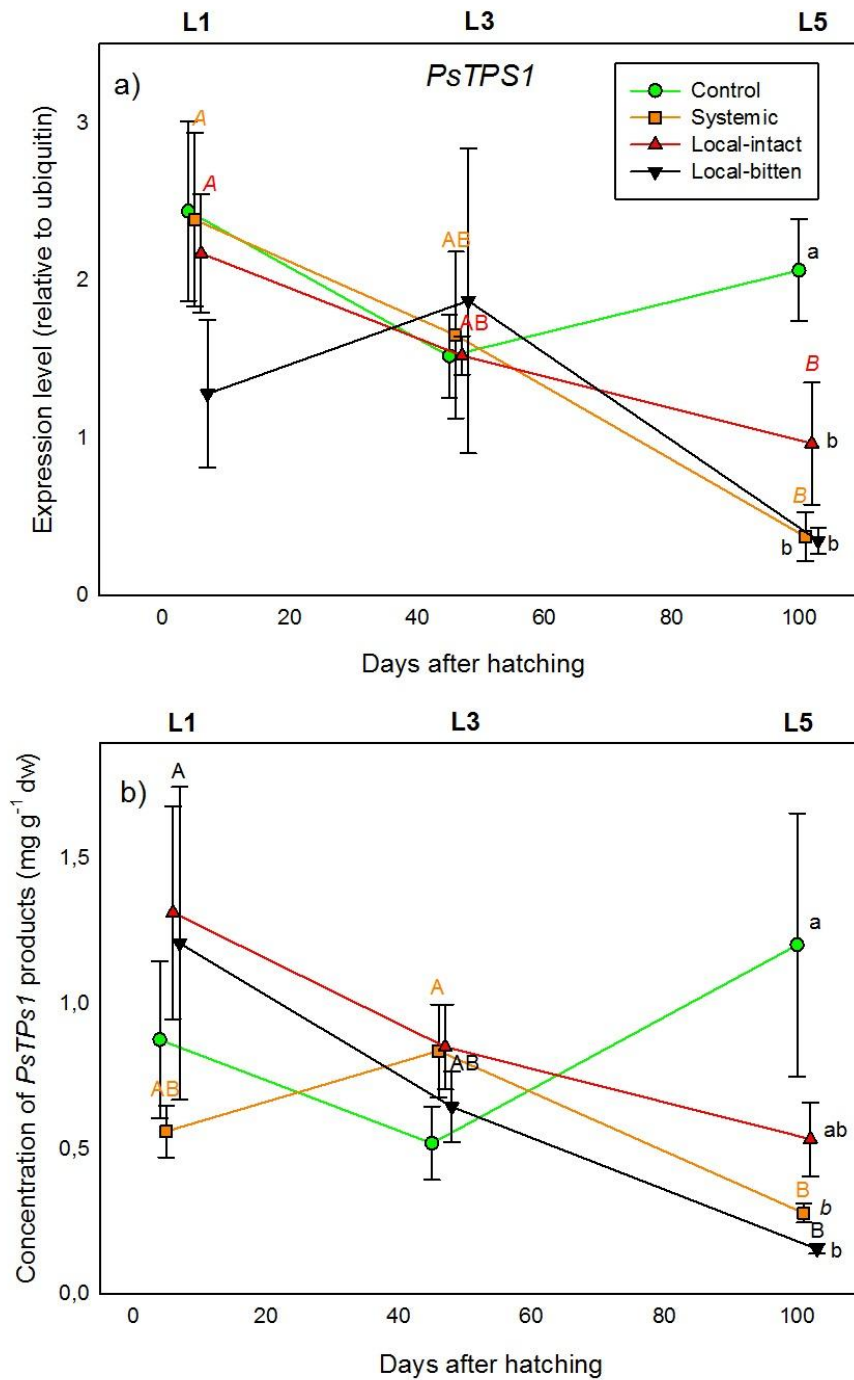
Figure 1. Comparison of a) the gene-expression level (relative to ubiquitin) of *PsTPs1* (mean ± SE) and b) the sum of the concentrations of the *PsTPS1* products (β-caryophyllene and α-humulene) at the three time points (L1, L3 and L5) of the larval cycle of *Thaumetopoea pityocampa*. Different lowercase letters indicate significant differences between the types of needles at the same time point (one-way ANOVA, Tukey's HSD post hoc test, $P < 0.05$), and different uppercase letters indicate significant differences between the time points within the same needle type. *Italicized* letters (lower- and uppercase) indicate marginally significant differences ($P < 0.10$). dw, dry weight.
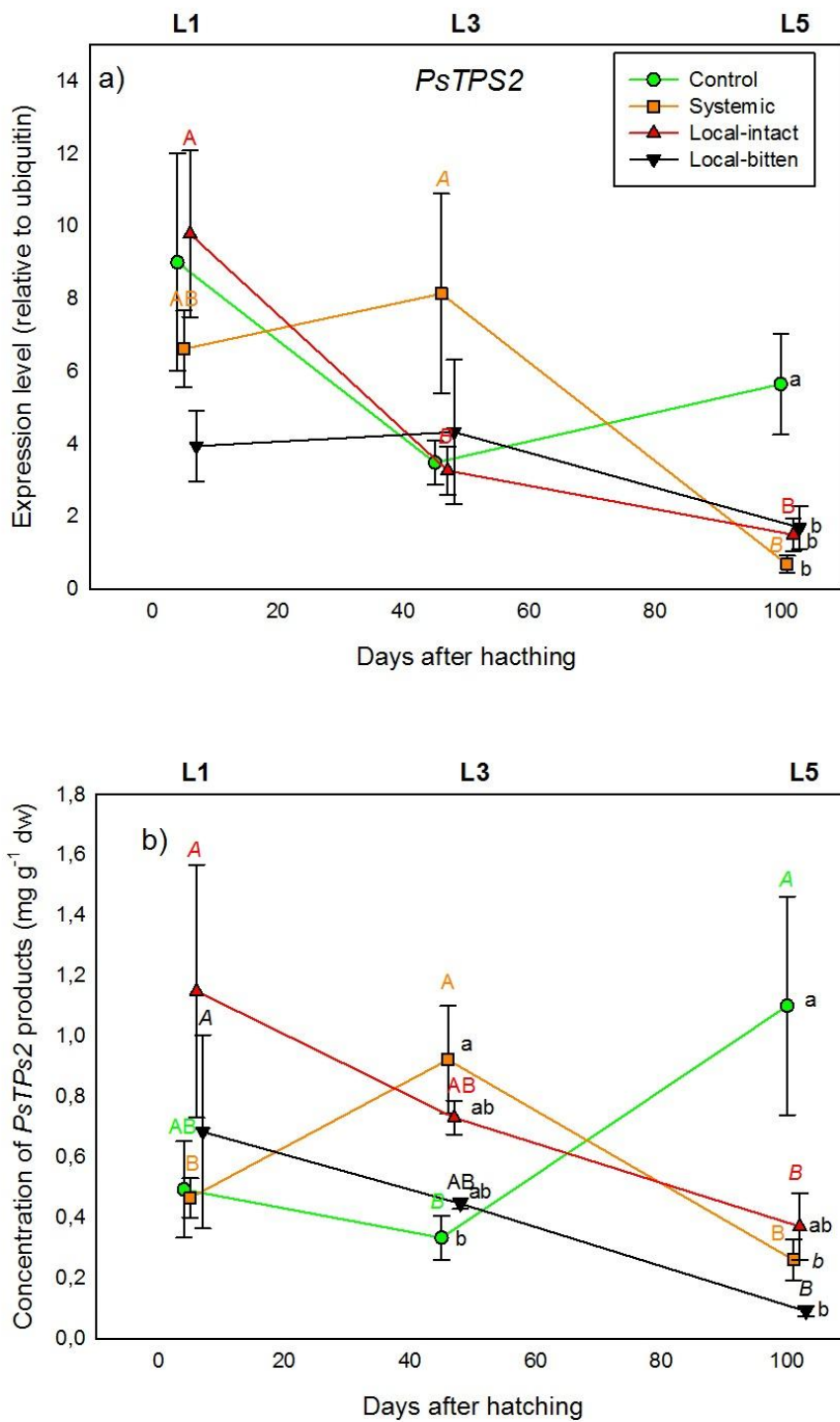
Figure 2. Comparison of a) the gene-expression level (relative to ubiquitin) of *PsTPS2* (mean ± SE) and b) the sum of the concentrations of the *PsTPS2* products, (β-elemene, bicyclogermacrene, α-amorphene and germacren-4-ol) at the three time points (L1, L3 and L5) of the larval cycle of *Thaumetopoea pityocampa*. Different lowercase letters indicate significant differences between the types of needles at the same time point (one-way ANOVA, Tukey's HSD post hoc test, $P < 0.05$), and different uppercase letters indicate significant differences between the time points within the same needle type. *Italicized* letters (lower- and uppercase) indicate marginally significant differences ($P < 0.10$). dw, dry weight.

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Larval stage	Needle types	β-elemene (*PsTPS2*)	β-caryophyllene (*PsTPS1*)	α-humulene (*PsTPS1*)	bicyclogermacrene (*PsTPS2*)
L1 (4 days)	Control	0.20±0.07	0.75±0.23	0.13±0.04	0.17±0.04
	Systemic	0.11±0.02 B	0.48±0.08 AB	0.080±0.014 AB	0.15±0.03 B
	Local-intact	0.17±0.06	1.1±0.3	0.19±0.057	0.26±0.09
	Local-bitten	0.16±0.07 A	1.0±0.5 A	0.18±0.08 A	0.21±0.07 A
L3 (45 days)	Control	0.10±0.02b	0.44±0.11	0.075±0.018	0.14±0.04b
	Systemic	0.19±0.04aA	0.72±0.14 A	0.12±0.02 A	0.29±0.06aA
	Local-intact	0.14±0.01ab	0.73±0.13	0.12±0.02	0.27±0.03ab
	Local-bitten	0.087±0.011abAB	0.55±0.10	0.093±0.018 A	0.18±0.02abA
L5(100 days)	Control	0.25±0.08a	1.0±0.4a	0.17±0.06a	0.39±0.14a
	Systemic	0.064±0.004bB	0.24±0.03bB	0.040±0.005b B	0.10±0.02bB
	Local-intact	0.088±0.023ab	0.46±0.11ab	0.094±0.009ab	0.12±0.04ab
	Local-bitten	0.031±0.004 B	0.13±0.01bB	0.020±0.002bB	0.035±0.011bB

| Larval stage	Needle types	α-amorphene (*PsTPS2*)	germacren-4-ol (*PsTPS2*)	Σ products *PsTPS1*	Σ products *PsTPS2*
L1 (4 days)	Control	0.044±0.011	0.16±0.08AB	0.88±0.27	0.49±0.16AB
	Systemic	0.030±0.007AB	0.12±0.03B	0.56±0.09AB	0.47±0.06B
	Local-intact	0.046±0.008B	0.31±0.11	1.3±0.4	1.1±0.4A
	Local-bitten	0.036±0.015	0.10±0.04	1.2±0.5A	0.68±0.32A
L3 (45 days)	Control	0.019±0.005b	0.061±0.015aB	0.52±0.13	0.33±0.07aB
	Systemic	0.050±0.010bA	0.36±0.09bA	0.84±0.16A	0.92±0.18bA
	Local-intact	0.13±0.04aA	0.22±0.04ab	0.85±0.15	0.73±0.06abAB
	Local-bitten	0.061±0.01ab	0.13±0.03ab	0.64±0.12A	0.45±0.02abAB
L5(100 days)	Control	0.045±0.02	0.30±0.10aa	1.2±0.5a	1.1±0.4aA
	Systemic	0.018±0.002 B	0.054±0.023bB	0.28±0.03bB	0.26±0.07bB
	Local-intact	0.036±0.012 B	0.12±0.06ab	0.53±0.13ab	0.37±0.11abB
	Local-bitten	0.015±0.001	0.008±0.003b	0.15±0.02bB	0.090±0.016bB

Table 1. Mean (±SE) of the needle terpene concentrations of the *PsTPS1* and *PsTPS2* products (mg g^{-1} dry weight) at three time points (L1, L3 and L5) of the larval cycle of *Thaumetopoea pityocampa*, presented by retention time order. Different lowercase letters indicate significant differences between the types of needles at the same time point (one-way ANOVA, Tukey's HSD post hoc test, $P < 0.05$), and different uppercase letters indicate significant differences between the time points within the same needle type. *Italicized* letters (lower- and uppercase) indicate marginally significant differences ($P < 0.10$)

Concentrations of terpene products and their correlations with synthase gene expression

The concentrations of sesquiterpenes tended to increase in the Control needles from L1 to L5 (Hanover 1992), similar to the Control levels in our previous reports prior to egg hatching (Achotegui-Castells et al. unpublished (chapter 3)) and before L5 (Achotegui-Castells et al. 2013). The concentrations of β-elemene and bicyclogermacrene in the present study were marginally higher in the Systemic needles than the Control needles at L3 (Table 1), in agreement with the observations by Achotegui-Castells et al. (2013) at L5.

The expression of the terpene synthase genes were moderately well correlated with the needle concentrations of their products, indicated by the significant correlations between these variables and the similar tendencies observed in the same needle types. The correlations nevertheless explained only 15-25% of the variability (Fig. 3), which may have been partially due to the needle terpene emissions that have also been correlated with the expression of terpene synthase genes (De Alwis et al. 2009; Köpke et al. 2008) but could not be included here. Post-transcriptional controls or factors influencing the activity of the enzymes may have also have altered the correlation between synthase gene expression and the needle concentrations of their products (Köpke et al. 2010; Köpke et al. 2008).

The most interesting trend was the higher gene expression and terpene concentrations at L5 in the Control needles than the needles of the attacked pines. The L5 larvae had defoliated the Scots pines a 90%, so the low level of gene expression may have represented a "deactivation" of these costly tree defenses (Gershenzon 1994) under such a critical situation, in which the trees had lost most of their photosynthetic capacity. The low terpene concentrations at L5 could be due to a combination of low synthase gene expression and high emission rates. Increased terpene emissions are common in attacked conifers, especially in bitten needles (Litvak and Monson 1998; Priemé et al. 2000) and this observation has also been reported in Scots pines attacked by the PPM (Achotegui-Castells et al. 2013).

The correlation between the concentrations of the *PsTPS1* products was very strong (R^2 = 0.99), suggesting that β-caryophyllene and α-humulene were exclusively produced by this synthase gene. The concentrations of some of the *PsTPS2* products were also strongly correlated, such as β-elemene, bicyclogermacrene and germacren-4-ol (with R^2 ranging from 0.68 to 0.90). The correlations with α-amorphene concentrations curiously had lower R^2 values (between 0.34 and 0.43). These weaker correlations, together with differences in concentration changes over time and in needle type compared with other *PsTPS2* products (Table 1), suggest that the biosynthesis of α-amorphene may not be regulated by *PsTPS2* alone or may be regulated post-transcriptionally.
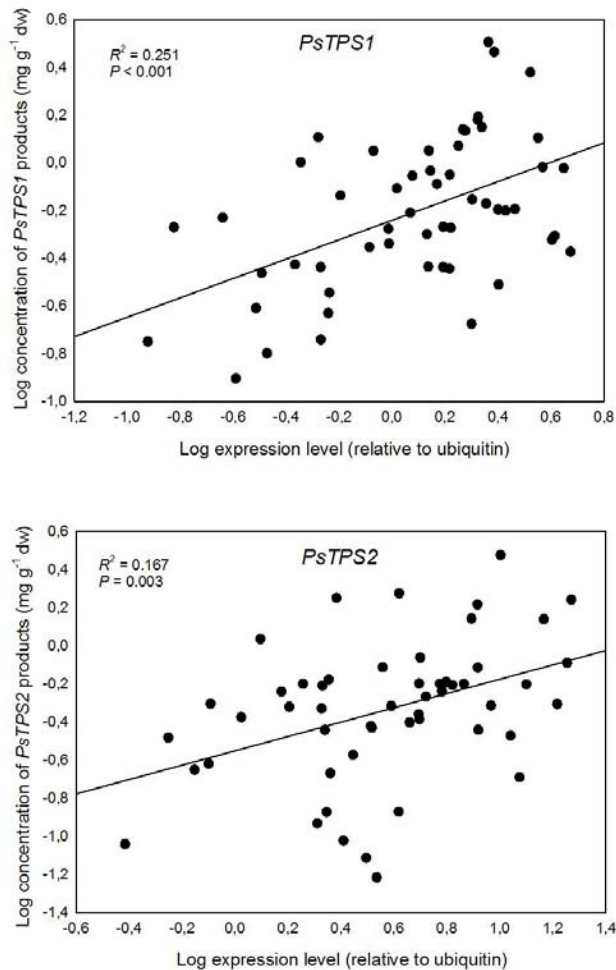
Figure 3. Correlations between terpene synthase gene expression and concentration of the *PsTPS1* and *PsTPS2* products. Correlations were calculated with Pearson's correlation coefficients. Data was log transformed. dw, dry weight.

Why is the pine reacting so weakly?

In contrast to the majority of studies of terpene synthase genes and herbivory, including those with the Scots pine, we found no clear sign of gene up-regulation or terpene induction in the pines attacked by the PPM. The Systemic needles at L3 nevertheless had higher terpene concentrations, corresponding to non-significantly higher levels of terpene synthase gene expression.

A number of possibilities may account for this situation. *i*) The expression of terpene synthase genes can vary greatly over time, so we may not have collected our samples at the best time to detect an induction. Needle terpene concentrations depend

on previous terpene concentrations, synthase gene expression and terpene emissions, so a mismatch between terpene synthase gene expression and needle terpene concentrations at the same time point is quite possible. *ii)* The "artificial feeding" in our study, not preceded by natural oviposition, may have altered the tree response, as suggested by Beyaert et al. (2012) with *D. pini*. *iii)* The sesquiterpenes produced by these two synthase genes may not contribute to the defensive reactions of pines against the PPM, even though systemic inductions have been reported (partially matching the results by Achotegui-Castells et al. (2013)) and the suggestion that β-caryophyllene is an antifeedant against the PPM (Petrakis et al. 2005). *iv)* Pines may not actively defended themselves from the PPM due to their limited and episodic defoliation, suggesting that constitutive defenses are the optimal response to the multiple stresses suffered by pines (Hódar et al. 2015). *v)* Several reports have indicated that the salivary enzymes of herbivores can limit plant defensive reactions, including terpene biosynthesis (Musser et al. 2002, Bede et al. 2006). The PPM is a herbivore specialized on *Pinus* sp. and thus may have evolved the ability to deter the defenses of their hosts. The reported systemic inductions, though, suggest that this possibility may occur only in local needles and not systemically throughout the entire plant.

Conclusions

The aims of this study were to monitor the expression of terpene synthase genes and the needle terpene concentrations of Scots pines throughout PPM larval development and to compare them to the findings from a previous study conducted under fully natural conditions to ascertain whether the lower needle terpene concentrations after PPM defoliation was due to increased terpene emission or reduced terpene biosynthesis. PPM herbivory did not clearly induce the expression of *PsTPS1* or *PsTPS2* in the Scots pines, even though a non-significantly higher level of *PsTPS2* expression in the Systemic needles coincided with significantly higher concentrations of *PsTPS2* products during L3, partially matching the results of previous reports. The expression of terpene synthase genes and the needle terpene concentrations were interestingly lower in the last larval stage (L5) of the PPM, at a time at which the pines had been defoliated by up to 90%, suggesting that heavily defoliated pines may stop investing resources in terpene defenses. Gene expression of terpene synthases was positively correlated with the concentrations of their products, but the relative weakness

of these correlations, explaining only 15-25% of the variance, suggests that emissions and unknown mechanisms could produce discrepancies between gene expression and terpene concentrations. Further studies should verify the synthase inductions we measured by analyzing enzymatic activity and collecting samples over several consecutive days of larval feeding. Any differences in the expression of terpene synthase genes in pines naturally (preceded by oviposition) and artificially infested by the PPM should be explored, including the analysis of terpene emission rates and the effect of PPM saliva on wounded needles.

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Chapter 3. Contrasting terpene and nutritional responses to previous defoliation by the pine processionary moth (*Thaumetopoea pityocampa*) in two Scots pine subspecies

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Abstract

The pine processionary moth (*Thaumetopoea pityocampa*, PPM) is amongst the most voracious defoliators of pine trees in the Mediterranean region. The mechanisms by which this insect selects its hosts and the factors that determine its larval survival are still poorly understood, despite some evidence of reduced larval survival in previously defoliated trees. We determined the terpene concentrations and emissions and the nutritional and physiological parameters of new needles in Control (uninfested) trees, and in trees previously defoliated by the PPM in Local (damaged) and Systemic (undamaged) branches of two coexisting *Pinus sylvestris* subspecies, *nevadensis* (endemic and relict) and *iberica* (reforested plantation), in the Sierra Nevada mountains in southern Spain. No clear responses were observed in *nevadensis*, but *iberica* responded strongly to previous defoliation at the Local and Systemic levels, with substantial increases of terpene concentrations and several changes in terpene emissions. The Local and Systemic needles of *iberica* had the highest carbon (C) and lowest nitrogen (N) and phosphorus (P) concentrations (including the highest C:N and C:P ratios). Nutritional changes were correlated with several terpene concentrations for both subspecies. The increases in terpene concentrations and the decreases in N and P concentrations in the needles of *iberica* were expected to lower PPM larval survival, but laboratory bioassay failed to found differences, suggesting changes in terpenes and nutrients may have little effect on the early larval stages of this herbivore. Interestingly, emission changes in *iberica* were observed for terpenes previously reported to have electrophysiological and behavioural effects on PPM adults, opening the possibility of host selection amongst defoliated and undefoliated trees. The contrasting responses between the Scots pine subspecies could be due to several reasons, such as different coevolutionary histories of PPM herbivory in *nevadensis* and *iberica* and/or different strategies of resource allocation under abiotic stress. The results suggest that Scots pines can react to previous PPM defoliation under certain selective pressures by inducing direct and indirect defensive mechanisms, despite the effect of these changes in PPM survival seems to be scarce.

Key words: *Pinus sylvestris – nevadensis - iberica -* monoterpenes –sesquiterpenes - carbon - nitrogen – phosphorus - needles – VOCs - emission – herbivory

Introduction

Plants cannot escape herbivorous attack and have consequently developed a very wide range of physical and chemical defences (Hanley et al. 2007; Mumm and Hilker 2006). Physical defences, such as spines (Myers 1991) or needle toughness (Zovi et al. 2008), confer mechanical protection, whereas chemical defences provide direct defence by inducing the production and changing the proportions of toxic compounds (Mithöfer and Boland 2012; Mumm and Hilker 2006) and provide indirect defence by attracting the antagonists of herbivores via emissions of volatile organic compounds (VOCs) (Hilker et al. 2002; Mattiacci et al. 1995). Complementary to these defensive mechanisms, the concentrations and proportions of essential nutrients in plant tissues have a strong influence on the host selection, performance, and survival of insects (Awmack and Leather 2002; Elser et al. 2000). The relationships between herbivory and plant concentrations of nitrogen (N) and phosphorus (P) and their proportions relative to carbon (C) (C:N, C:P, and N:P ratios) have been extensively studied in recent decades (Awmack and Leather 2002; Daufresne and Loreau 2001; Elser et al. 2000; Sardans et al. 2012). Herbivores, though, are able to cope with plant defensive mechanisms and can select poorly defended or decaying hosts (Byers and Zhang 2011; Covassi et al. 1975), associate with other organisms to attack plants (e.g. scolytids and fungal pathogens (Paine et al. 1997; Villari et al. 2012)), inhibit plant defensive responses (Alba et al. 2011; Musser et al. 2002), or even exploit theoretically toxic compounds for their own benefit (Nishida 2002).

Constitutive or rapidly induced plant defences may thus not always be capable of preventing defoliation. After an episode of defoliation, plants could then change some physical, chemical, or nutritional traits in their new leaves, not only in the damaged tissues, but also in other plant parts (systemic defences), to prepare for new attacks. Interestingly, a meta-analysis of herbivory and plant defences suggested that plant responses to previous defoliation are more effective against the performance and survival of herbivores than plant responses to current defoliation (Nykanen and Koricheva 2004). Several studies have observed significant relationships between reduced larval performance and survival in defoliated plants with changes in the chemical defences and nutrient concentrations in new leaves (Battisti 1988; Roitto et al. 2009; Traw and Dawson 2002), despite another important share of studies have failed to do so (Chen et al. 2002; Hódar et al. 2015; Hodar et al. 2004; Schuldt et al.

2012). Furthermore, some studies have found that host selection by folivores was influenced by previous tree defoliation (Leather et al. 1987; Sbabdji and Kadik 2011), but other studies found no host discrimination by the insects (Battisti 1988; Tammaru et al. 1995). Conclusive evidence of herbivore host selection and survival after an episode of defoliation is thus lacking, probably because host selection and larval survival after defoliation may be influenced by multiple factors often overlooked, such as environmental conditions (e.g. nutrient availability, drought, or temperature) or ecological factors (e.g. plant-herbivore coevolutionary history, pathogens, or plant competition) (Jactel et al. 2012; Karban 2011; Sampedro et al. 2011).

The caterpillar of the pine processionary moth (PPM) is the main defoliator of pine trees in the Mediterranean region (Hódar et al. 2003, Battisti 1988). It feeds on all pine species but can also attack cedars and larches. Apart from the ecological and economic problems derived from substantially reducing the growth and reproduction of pine stands (Hódar et al. 2003; Jacquet et al. 2012), the PPM has urticant hairs that are a serious public-health issue (Lamy 1990; Rodriguez-Mahillo et al. 2012). The adult females of this univoltine insect, which usually lives only for one night, oviposit batches containing between 70 and 300 eggs around a pair of pine needles (Tsankov et al. 1996). The gregarious larvae of a batch will live in colonies on tree branches during five larval stages, and feed on needles from the end of summer to early spring, when the caterpillars burrow into the soil to pupate. In the worst cases, pines can be completely defoliated (Hódar et al. 2002). PPM fitness is limited mainly by low winter temperatures, but current climatic change is favouring the colonisation of tree stands at higher latitudes and altitudes (Battisti et al. 2005).

More than a dozen studies have tried to identify the factors that determine PPM larval performance and survival, but conclusive evidence is still lacking. These studies have focused on terpenes and phenols, usually regarded as two of the main chemical defences of conifers against herbivores and pathogens (Mumm and Hilker 2006; Phillips and Croteau 1999). The large number of different pine species used in these studies and the variety of experimental approaches probably account for the contrasting results (Jactel et al. 2015). Three studies have found indices of reduced survival in PPM larvae growing in conifers previously defoliated by the PPM (Battisti 1988; Hodar et al. 2004; Sbabdji and Kadik 2011), despite Hódar et al. (2015) did not. Battisti (1988) associated a decrease in larval survival to lower N contents in *Pinus*

nigra and indirectly estimated, by energetic values, an increase in chemical defences. Hódar et al. (2004) analysed most of the nutritional and defensive parameters previously used in other PPM studies in *P. sylvestris nevadensis* but found no changes in needle traits that could explain the lower survival observed. Finally, Sbabdji and Kadik (2011) observed a large decrease in colony survival in previously defoliated trees of *Cedrus atlantica*, but the nutritive and defensive parameters of the needles were unfortunately not studied. Contrarily, Hódar et al. (2015) found neither clear tree response to previous defoliation nor differences in larval survival in three different pine species.

Host selection by the PPM has also attracted considerable attention. The PPM has been proposed to be unselective (Hódar et al. 2002) or to select hosts based on tree shape, VOC emissions, and thigmotactic recognition (Jactel et al. 2015). Five studies have directly investigated the role of pine VOC emissions in the preferences for moth oviposition. Zhang et al. (2003) found significant responses in the antennae of female moths to several minor terpenes emitted from *P. sylvestris*. Tiberi et al. (1999) observed that both limonene enantiomers sprayed on the foliage of several pine species were repellent to the PPM, thereby providing protection against oviposition. These results were supported by Mateus (2009), who also observed that (-)-β-pinene could attract PPMs. Niccoli et al. (2008), though, contradicted both of these studies by finding no protective roles of limonene enantiomers but suggested a deterrent effect of β-pinene (Jactel et al. 2015). Paiva et al. (2010) found further evidence of the roles of VOCs in host selection by observing ovipositional preferences in an experiment with identically shaped plastic trees sprayed with extracts of different pine species. Two other studies tested the effect of VOC emissions on host selection by the PPM, but using VOCs of broadleaved plant species (Dulaurent et al. 2011; Jactel et al. 2011). These two experiments succeeded in lowering PPM incidence by introducing non-host volatiles in *P. pinaster* stands, supporting the hypothesis that PPMs use volatile cues to select their hosts and that associational resistance could help to mitigate PPM outbreaks. Most of these studies have unfortunately used VOC extracts of needles instead of actual VOC emissions from the needles, which usually differ from the needle concentrations (Achotegui-Castells et al. 2013, Llusià and Peñuelas 2000). Furthermore, no PPM olfactometric tests have yet been published, nor have the terpene-emission profiles of pines previously defoliated by the PPM been described.

The main goals of this study were to determine *i*) if the concentrations and emissions of terpenes, and the C, N and P concentrations, of pine needles shift relative to a previous defoliation by the PPM, and *ii*) if pines attacked by PPM have different terpene and nutrient responses in local and systemic branches, and *iii*) how these chemical and nutritional changes affect PPM larval survival. To answer these questions, we measured terpenes and nutrients in the needles of two subspecies of *P. sylvestris* in Sierra Nevada Natural Park in southern Spain, where pine populations are naturally exposed to PPM attack, and used those needles to perform larval survival bioassays in the laboratory.

Materials and methods

Study site

The experiment was carried out between 18 and 22 July 2011 in Collado de Matasverdes in the Sierra Nevada National Park (Granada, SE Spain; 37.05°N, 3.27°W, 1900 m a.s.l.) (see Achotegui-Castells et al. (2013) for more details). The climate in the study area is continental Mediterranean with cold winters, hot summers, and severe summer drought during July and August. Rainfall is concentrated mainly in autumn and spring, with an annual rainfall of 803 mm (average for 1998–2008 at the Jardín Botánico de La Cortijuela, 1.5 km from and 300 m lower than the study site). The weather was stable during the sampling days, which were sunny, cloudless, and warm. This area contains two subspecies of *P. sylvestris*: *P. sylvestris* subsp. *nevadensis* (hereafter *nevadensis*) that is endemic to Sierra Nevada and is a relict tree (Hódar et al. 2003) that coexists with forest plantations of the other subspecies, *P. sylvestris* subsp. *iberica* (hereafter *iberica*), the most common subspecies of Scots pine in Spain. The *iberica* trees planted in the study zone are endemic to Navacerrada, central Spain.

Experimental design

The experiment was conducted in an area with pure stands of both pine subspecies that had hosted a moderate number of PPM colonies the previous winter (2010-2011).

We studied three attack states in the branches of *nevadensis* and *iberica* following the experimental design of our previous study (Achotegui-Castells et al. 2013): Local, branches near PPM colonies; Systemic, branches as far as possible from any colony on attacked trees; and Control, branches of healthy, non-attacked trees. A total of 48 trees were sampled, 24 of each subspecies. For each attack state (3; Local, Systemic and Control) and each subspecies (2; *nevadensis* and *iberica*), 12 replicate twigs were randomly sampled from 12 trees (3 × 2 × 12 = 72 pine twigs in total).

Field sampling

Pine needles

A small sunlit branch was removed from each tree with a pole, and the needles were quickly packed and frozen in liquid nitrogen. All samples were lyophilised in the laboratory and stored at -20 °C until the processing and analysis of their terpene concentrations. Lyophilisation does not cause the loss of mono- or sesquiterpenes from needles (Ormeno et al. 2007).

VOC emissions

The VOC emissions were sampled on five consecutive days from 09:00 to 15:00 (solar time). A portion of each branch was carefully wrapped with Teflon® tape and placed in the sampling chamber of a CI-340 Hand-Held Photosynthesis System (CID Bioscience Inc., Canmas, USA) or an LC-Pro+ Photosynthesis Measuring System (ADC BioScientific Ltd., Great Amwell, UK). The system was allowed to stabilise for 10 minutes prior to measuring various environmental and physiological parameters, such as temperature, photosynthetic active radiation (PAR), photosynthesis, and stomatal conductance (measured also during the VOC sampling).

Once the system was stabilised, a Tedlar® tube was placed in the chamber, by which the air was exhausted to a metallic trap filled with 115 mg of Tenax® and 230 mg of UniCarb™ (Markes International Inc., Wilmington, USA), where the VOCs were retained. A QMAX pump (Supelco, Bellefonte, USA) at the other end of the metallic trap pulled the air from the chamber at an internal rate of air flow of 0.5 L min^{-1}. The

VOCs were sampled for 5 min at a flow rate of 0.28 ± 0.04 L min^{-1} (±SE). The sampled VOC-filled tubes were then placed in a portable refrigerator at 4 °C and transported to the laboratory, where they were stored at -25 °C until analysis. One blank air sample was collected for 5 min after sampling three trees for subtracting the background volatiles from the pine samples. The blanks were collected near the sampled pines, but the chamber contained no pine twigs. All metallic tubes used in the samplings had been cleaned twice for 15 min at 300 °C with a purified helium stream at a flow rate of 100 mL min^{-1}.

Sample analyses

Needle terpene concentrations

Three or four randomly chosen lyophilised pine needles from each sample were ground inside a 50 mL Teflon® tube filled with liquid nitrogen to prevent VOC evaporation and to facilitate the crushing. One millilitre of pentane containing 0.5 nL of an internal standard (dodecane) was added to each of the tubes and samples. The samples were then stored at -20 °C for at least 12 h, and 300 µL of each supernatant was collected in vials for analysis by gas chromatography-mass spectrometry (GC-MS). The tubes containing the grinded biomass were then dried to constant weight and weighed on a precision scale. The Teflon® tubes were exhaustively cleaned, dried, and reweighed to calculate the dry weight (dw) of each analysed sample. Needle length did not differ amongst the attack states.

For each sample, two µL of the needle extract were injected into a capillary column (HP 5MS, 30 m × 0.25 µm × 0.25 mm) in a GC (7890A, Agilent Technologies, Santa Clara, USA) coupled to an MS detector (5975C inert MSD with Triple-Axis Detector, Agilent Technologies). The temperature was 35 °C for 2 min after the injection and increased at 15 °C min^{-1} to 150 °C and maintained for 5 min, increased at 30 °C min^{-1} to 250 °C and maintained for 5 min, and finally increased at 30 °C min^{-1} to 280 °C and maintained for 5 min. The helium flow was set to 1 mL min^{-1}, and total run time was 29 min. A blank was also injected after every six samples during the analysis.

The terpenes were identified by comparison to published mass spectra (NIST 05 and Wiley 7n libraries) and known standards and were quantified using the fractionation product with mass 93 while the system was operating in SIM mode.

Calibration curves for quantification were prepared with commercial standards of the most abundant compounds in the samples. Four monoterpenes (α-pinene, β-pinene, limonene, and sabinene), one sesquiterpene (α-caryophyllene), and one non-terpene internal standard (dodecane) were purchased from Fluka Chemie AG (Buchs, Switzerland). The terpene calibration curves were always highly significant ($r^2 \geq 0.99$) for the relationship between signal strength and terpene concentration. The most abundant terpenes responded very similarly in the GC-MS (differences were <5%).

Terpene emission rates

The emitted terpenes retained in the metallic tubes were desorbed with an automatic sample processor (TD Autosampler, Series 2 Ultra, Markes International Inc. Wilmington, USA) and readsorbed in a cryotrap at -25 °C placed in the injector. The redesorbed terpenes from the cryotrap were injected at 250 °C (Unity, Series 2, Markes International Inc. Wilmington, USA) in a GC (7890A, Agilent Technologies, Santa Clara, USA) with an MS detector (5975C inert MSD with Triple-Axis Detector, Agilent Technologies). The chromatographic analyses were performed in full-scan mode with a split of 1:20, and the samples were injected into the column with a transfer line at 250 °C. After sample injection at 35 °C (initial time 1 min), the column temperature was increased at 15 °C min^{-1} to 150 °C and maintained for 5 min, increased at 50 °C min^{-1} to 250 °C and maintained for 5 min, and increased at 30 °C min^{-1} to 280 °C and maintained for 5 min. Total run time was 26.7 min, and the helium flow was set to 1 ml min^{-1}.

The terpenes were identified as described above for the analysis of needle terpene concentrations. The terpene emission rates were expressed in µg g^{-1} dw h^{-1}. Even though the weather conditions during sampling were stable, the terpene emission rates for *nevadensis* were standardised at 30 °C using an algorithm for terpene-storing species (Guenther et al. 1993):

E = Es {exp[βT-Ts)]}

where *E* is the emission rate in µg g^{-1} dw h^{-1} of monoterpenes at temperature *T* (in degrees Kelvin, K), *E*s is the emission factor in µg g^{-1} dw h^{-1} at standard temperature *T*s (303 K), and β is an empirically determined coefficient, 0.09 (in degrees Kelvin, K).

Elemental analyses

Foliar processing for elemental analyses is described in detail by Rivas-Ubach et al. (2013). Briefly, an aliquot of the lyophilised pine needles were ground with a ball mill at 1600 rpm for 8 min (Mikrodismembrator-U, B. Braun Biotech International, Melsungen, Germany) and stored in plastic cans until analyses. For C and N determination, 1.4 mg of the powdered needles from each sample were inserted into a tin capsule, and concentrations were determined by combustion coupled to gas chromatography with a CHNS-O Elemental Analyser (EuroVector, Milan, Italy). P was extracted by acid digestion in a microwave under high temperature and pressure (Sardans et al. 2010). Briefly, 250 mg of powder for each sample were placed in a Teflon$^®$ tube, and 5 mL of HNO_3 and 2 mL of H_2O_2 were added. The acid digestions were performed with a MARSXpress microwave reaction system (CEM, Mattheus, USA). The digestions were transferred to 50-mL flasks and resuspended in Milli-Q water to a final volume of 50 mL. P concentrations were determined from each digestion by ICP-OES (Optic Emission Spectrometry with Inductively Coupled Plasma) (Perkin-Elmer Corporation, Norwalk, USA).

Larval survival bioassay

Needles contiguous to those sampled for terpene and nutrient analyses were used to conduct a larval survival bioassay in the laboratory. For every attack state (3) and subspecies (2) seven bioessays were performed (3 × 2 × 7 = 42 replicates). Each bioassay consisted of three technical replicates, which contained 20 larvae per replicate. Thus, the survival of 2520 larvae (3 × 2 × 7 × 3 × 20) was analyzed. The experimental unit of the bioassay consisted in a Petri dish where 20 newly-hatched 1st instar PPM larvae with a humidified filter paper (to prevent needle drying) were fed with current-year pine needles. First instar larvae were obtained from egg batches sampled from the study zone. Larvae were read on lab at 20º C and 14:10 photoperiod. The date of hatching was recorded, as well as the date of detection of moulting to 2nd instar. When half of the larvae (10 individuals) had moulted to 2nd instar, the experiment concluded and the number of living larvae was recorded. The percentage of survival of each technical replicate was calculated dividing the number of living larvae by the total number of larvae (20), and the final percentage of survival (%) of each replicate was the mean of the three technical replicates. The mean duration of the bioassays was 14.3 ± 0.2 days (mean ± SE).

Terpene		*Pinus sylvestris* subsp. *nevadensis*			*Pinus sylvestris* subsp.*iberica*		
		Control	Systemic	Local	Control	Systemic	Local
Tricyclene	[ ]	0.43±0.05a	0.30±0.03b	0.37±0.03ab	0.27±0.04b	0.53±0.07a*	0.57±0.06a*
	%	3.5±0.2^	3.4±0.2	3.5±0.2	2.8±0.2	2.9±0.2	3.2±0.2
α-pinene	[ ]	8.9±0.8	7.5±0.9	8.1±0.8	7.5±0.8b	13±1a*	13±1a*
	%	73±1	76±1	74±1	78±1	76±2	75±2
Camphene	[ ]	1.0±0.1a^	0.67±0.07b	0.87±0.07ab	0.64±0.12b	1.0±0.1ab	1.2±0.1a
	%	9.1±0.4*a* *	7.6±0.5*b*	8.3±0.4ab	6.2±0.6	6.5±0.6	7.6±0.4
Sabinene	[ ]	0.094±0.011*	0.066±0.009	0.085±0.009	0.044±0.011b	0.076±0.008*b*	0.11±0.01a
	%	0.79±0.07	0.70±0.07	0.82±0.09	0.60±0.10	0.61±0.07	0.64±0.06
ß-pinene	[ ]	0.72±0.08a	0.46±0.04b	0.65±0.06ab	0.42±0.07b	0.87±0.14a*	1.1±0.2a*
	%	5.8±0.1	5.5±0.2	5.8±0.2	5.1±0.3	7.0±1.5	7.7±1.3
ß-myrcene	[ ]	0.41±0.04^	0.31±0.04	0.35±0.04	0.23±0.04b	0.50±0.06a*	0.53±0.05a^
	%	3.2±0.1*	3.1±0.2	3.3±0.3	2.3±0.2	2.8±0.2	2.8±0.2
Limonene	[ ]	0.14±0.01	0.10±0.01	0.14±0.02	0.10±0.02b	0.21±0.03a*	0.22±0.02a*
	%	1.0±0.0	1.0±0.1	1.1±0.1	1.1±0.1	1.1±0.1	1.2±0
ß-ocimene	[ ]	0.30±0.03	0.24±0.03	0.23±0.02	0.27±0.05b	0.50±0.08a*	0.38±0.04ab
	%	2.3±0.2	2.4±0.2	1.9±0.1	2.5±0.3	2.7±0.2	2.2±0.2
γ-terpinene	[ ]	0.012±0.001a	0.008±0.001b	0.009±0.001*b*	0.011±0.001	0.015±0.002*	0.015±0.001*
	%	0.1±0.01a	0.083±0.005*b*	0.079±0.004b	0.12±0.02a	0.084±0.006b	0.085±0.007b
ß-elemene	[ ]	0.052±0.007	0.069±0.015	0.060±0.012	0.051±0.007*b*	0.059±0.01*ab*	0.090±0.016*a*
	%	1.9±0.3	1.9±0.3	2.1±0.3	2.2±0.2	1.8±0.2	2.4±0.3
Caryophyllene	[ ]	0.71±0.06	0.90±0.12	0.77±0.08	0.66±0.05b	0.96±0.05a	0.90±0.05a
	%	25±1	27±1	29±2	27±1	30±2	25±3
α-caryophyllene	[ ]	0.35±0.03	0.45±0.06	0.38±0.04	0.31±0.03b	0.47±0.03a	0.43±0.02a
	%	12±1	13±1	13±1	13±1	15±1	12±1
ST1	[ ]	0.022±0.002	0.030±0.005	0.020±0.002	0.018±0.002b	0.026±0.003ab	0.034±0.005a
	%	0.81±0.03	0.94±0.11	0.80±0.07	0.75±0.06	0.76±0.06	0.96±0.09
Germacrene D	[ ]	1.4±0.1	1.4±0.2	1.2±0.1	1.1±0.1	1.4±0.1	1.5±0.2
	%	51±2*a*	47±2ab	45±2*b*	45±2	42±2	44±4
Bicyclogermacrene	[ ]	0.11±0.02	0.15±0.03	0.13±0.03	0.11±0.02b	0.14±0.03*b*	0.24±0.04^
	%	4.2±0.6	4.3±0.7	4.4±0.7	4.7±0.5ab	4.3±0.7*b*	6.4±0.9*a*
γ-cadinene	[ ]	0.027±0.003	0.035±0.005	0.026±0.003	0.024±0.003b	0.037±0.005ab	0.048±0.007a*
	%	1.1±0.1	1.2±0.1	0.98±0.08	1.0±0.1	1.2±0.2	1.1±0.1
δ-cadinene	[ ]	0.020±0.002	0.026±0.004	0.019±0.002	0.018±0.002b	0.028±0.004ab	0.035±0.005a*
	%	0.72±0.05	0.88±0.08	0.74±0.07	0.78±0.05	0.9±0.11	0.82±0.06
Germacren D-4-ol	[ ]	0.10±0.02	0.12±0.03	0.12±0.03	0.07±0.02b	0.09±0.02*b*	0.17±0.03a
	%	4.4±0.8	3.4±0.5	4.6±0.9	3.7±0.9	3.3±0.6	4.6±0.8
t-cadinol	[ ]	0.012±0.001b	0.024±0.003a	0.013±0.001b	0.018±0.005	0.025±0.005	0.028±0.005^
	%	0.47±0.05b	0.72±0.08a	0.42±0.03b	0.77±0.1	0.79±0.13	0.76±0.11
α-cadinol	[ ]	0.010±0.001b	0.018±0.001a	0.011±0.001b	0.017±0.004	0.020±0.004	0.027±0.005*
	%	0.37±0.04b	0.66±0.13a	0.36±0.03b	0.81±0.11^	0.76±0.15	0.73±0.13*
ß-springene	[ ]	0.021±0.002	0.024±0.002	0.021±0.002	0.019±0.001b	0.028±0.001a	0.033±0.003a*
Total MT	[ ]	12±1	10±1	11±1	10±1b	18±2a*	18±1a*
	%	80±1a	76±1b	81±1a	83±2	84±2*	79±2
Total ST	[ ]	2.8±0.2	3.2±0.4	2.7±0.3	2.3±0.2b	3.1±0.3ab	3.6±0.4a
	%	20±1b	24±1a*	19±2b	17±2	16±2	21±2
Total terpenes	[ ]	15±1	13±1	14±1	12±1b	21±2a*	22±1a*

Table 1. Mean concentrations (mg g^{-1} dry weight) and proportions (± SE) of the terpenes in the needles of the three attack states of *Pinus sylvestris* subsp. *nevadensis* and *P. sylvestris* subsp. *iberica.* Shaded cells indicate significant (one-way ANOVA, Tukey's post-hoc test, $P < 0.05$) or marginally significant ($P < 0.10$) differences. Asterisks (*) indicate significant ($P < 0.05$), and circumflexes (^) indicate marginally significant ($P < 0.10$), differences between the same attack states of the two *P. sylvestris* subspecies. MT, monoterpenes; ST, sesquiterpenes.

Statistical analyses

We first filtered the data to eliminate the outliers following the *very conservative* approach defined by Leys et al. (2013). We then performed Shapiro-Wilk and Levene's tests on all variables to assess the normality and homogeneity of the variances, respectively. Data that did not fulfil the normality requirements were log-transformed or were analysed with non-parametric methods (Kruskal-Wallis analysis of variance (ANOVA)). The statistical significance of differences amongst attack states and subspecies were assessed by one-way ANOVAs and Tukey's HSD post-hoc tests ($P < 0.05$). Simple regressions assessed the correlations between nutrient and needle terpene concentrations. The statistical analyses were performed with Statistica version 8.0. (StatSoft Inc. Tulsa, USA) and R version 2.15.2 (R Core Team 2013). Graphs were constructed with SigmaPlot v. 11.0 (Systat software, Chicago, USA).

Results

Needle terpene concentrations

The needles of both subspecies of Scots pine had large amounts (5-29 mg g^{-1} dw) and varieties of terpenes (Fig. 1, Table 1). We only statistically analysed the terpenes with values higher than 0.1% of the total peak area (21 compounds). The most abundant monoterpene was α-pinene and the most abundant sesquiterpene was germacrene D.

Differences in needle terpene concentrations amongst attack states

The concentrations amongst the attack states were similar for *nevadensis*, with a few differences (Table 1). The concentrations of the monoterpenes tricyclene, camphene, and β-pinene in *nevadensis* were higher in the Control than the Systemic needles, and the concentration of γ-terpinene was higher in the Control than both Systemic and Local needles (one-way ANOVA, Tukey's HSD post-hoc test, $P < 0.05$). The concentrations of sesquiterpenes τ- and α-cadinol were twice as high in the Systemic than the Control and Local needles (one-way ANOVA, Tukey's HSD post-hoc test, $P < 0.01$). In contrast to *nevadensis*, the concentrations of most terpenes in *iberica* varied significantly amongst the attack states. Local and Systemic needles had the highest

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concentrations, approximately double those of the Control needles for most of the terpenes studied, especially monoterpenes (one-way ANOVA, Tukey's HSD post-hoc test, $P < 0.05$ and $P < 0.01$) (Fig. 1, Table 1). The concentrations of sabinene, bicyclogermacrene, and germacren-4-ol were higher in the Local than the Systemic needles, but only marginally significantly (one-way ANOVA, Tukey's HSD post-hoc test, $P < 0.10$). The concentrations of most monoterpenes, but not sesquiterpenes except caryophyllene and α-caryophyllene, were significantly higher in the Systemic than the Control needles.

The Control concentrations of sabinene (one-way ANOVA, Tukey's HSD post-hoc test, $P < 0.05$), camphene, and β-myrcene ($P < 0.10$) were highest in *nevadensis* needles. The Systemic concentrations of most monoterpenes and total terpenes but not sesquiterpenes were significantly ($P < 0.01$) higher in *iberica* than *nevadensis*. The Local concentrations of six mono- and five sesquiterpenes were significantly higher in *iberica* than *nevadensis* ($P < 0.01$) (Table 1).

Proportional differences in needle terpenes amongst attack states

We analysed the changes in the proportions of needle terpenes (monoterpene compounds relative to total monoterpenes, and sesquiterpene compounds relative to total sesquiterpenes). The proportions were similar amongst the attack states within each subspecies. The proportions in *nevadensis* of camphene, γ-terpinene, and germacrene D were higher, but only with marginal significance, in the Control needles, and the proportions of both cadinols were significantly ($P < 0.01$) higher in the Systemic needles (Table 1). The proportion in *iberica* of γ-terpinene was marginally higher in the Control needles, and the proportion of bicyclogermacrene was marginally higher in the Local than the Systemic needles. Proportions differed significantly between the attack states of *nevadensis* and *iberica* only in the Control needles. The proportions of tricyclene, camphene, and β-myrcene were higher in the *nevadensis* Control than the *iberica* Control needles; the proportion of α-cadinol, though, was higher in the *iberica* Control needles. The proportions of sesquiterpenes were highest in the *nevadensis* Systemic needles (and consequently, the proportions of monoterpenes were lowest), and *iberica* had an opposite but non-significant trend (Table 1). The *nevadensis* Systemic needles thus had higher sesquiterpene proportions than the *iberica* Systemic needles (Table 1).
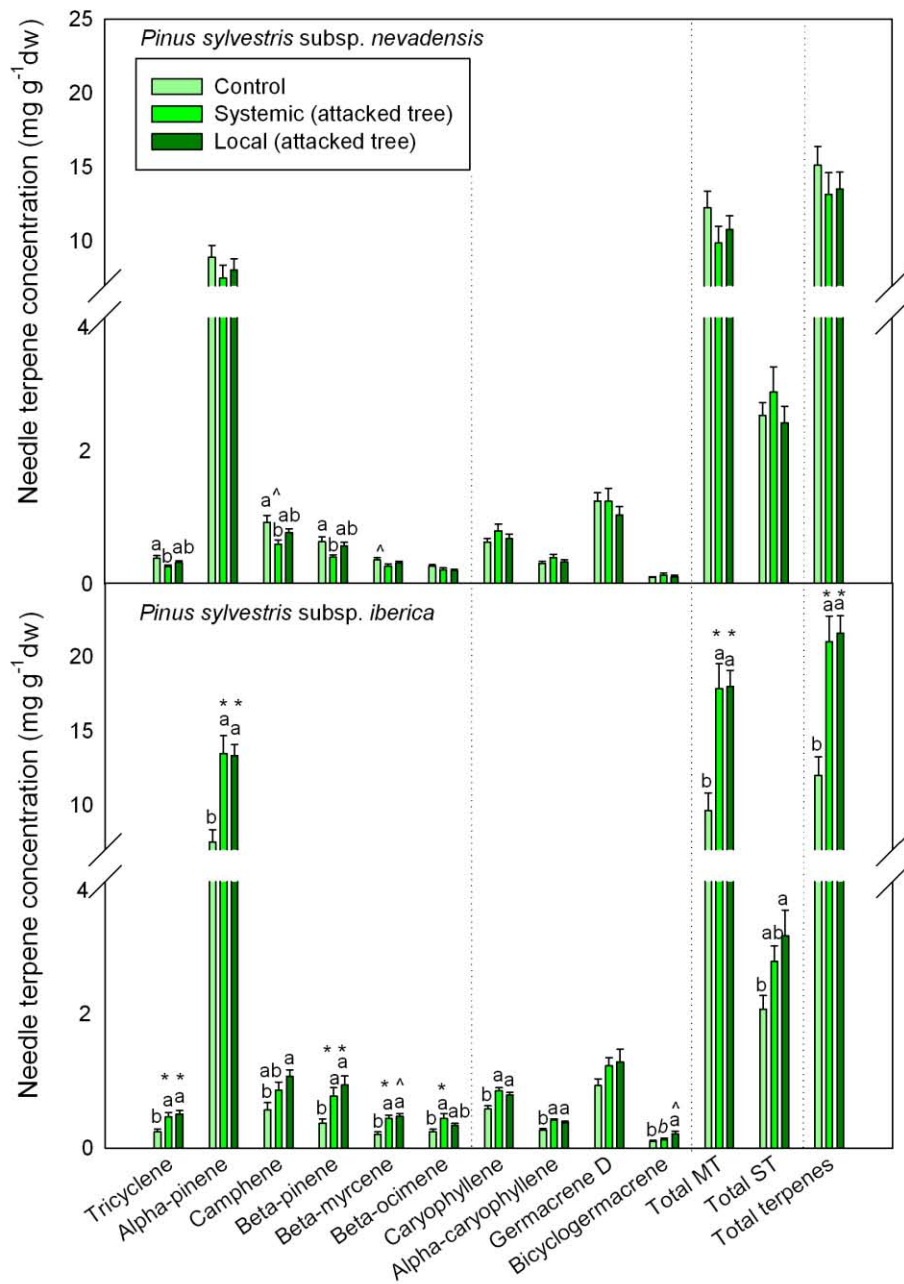
Figure 1. Mean concentrations (± SE) of the most abundant terpenes in the needles of the three attack states of *Pinus sylvestris* subsp. *nevadensis* and *P. sylvestris* subsp. *iberica.* Different letters indicate significant differences (one-way ANOVA, Tukey's post-hoc test, $P < 0.05$), and italicised letters indicate marginally significant differences ($P < 0.10$). Asterisks (*) indicate significant differences ($P < 0.05$), and circumflexes (^) indicate marginally significant differences ($P < 0.10$), between the same attack states of the two *P. sylvestris* subspecies. The concentrations and proportions of the least abundant terpenes are listed in Table 1. dw, dry weight; MT, monoterpenes; ST, sesquiterpenes.
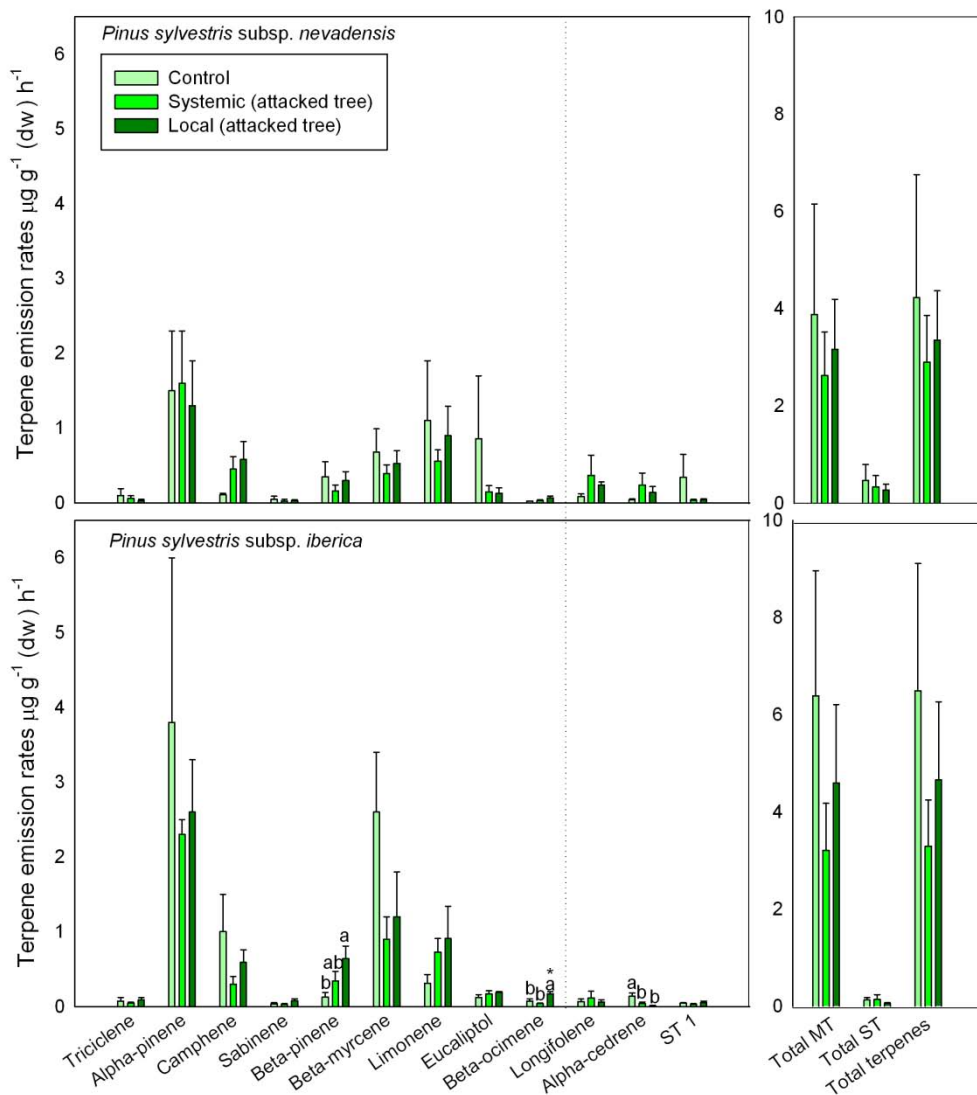
Figure 2. Mean emission rates (± SE) of the most abundant terpenes emitted by the three attack states of the two *Pinus sylvestris* subspecies. Different letters indicate significant differences (one-way ANOVA, Tukey's *post-hoc*, $P < 0.05$). Asterisks (*) indicate significant differences between the same attack states of the two *P. sylvestris* subspecies. The emission rates and proportions of the least abundant terpenes are listed in Table 2. dw, dry weight; MT, monoterpenes; ST, sesquiterpenes.

Rates of needle terpene emission

Absolute emissions

Twelve monoterpenes and three sesquiterpenes were clearly detected and were used for statistical analyses, with total terpene emission rates ranging from 0.21 to 16.9 mg g^{-1} dw h^{-1}. Monoterpenes represented ca. 95% of the total emissions, with α-pinene the most abundant monoterpene (Table 2). δ-3-carene was detected in most samples, but

the blanks often had higher values than the samples, so this terpene was discarded from the analyses. None of the terpenes emitted by *nevadensis* differed significantly amongst the attack states, but terpene emissions tended (not significantly) to be lower for the Systemic and Local needles compared to the Control needles. The general trends were similar in *iberica,* but some terpenes differed significantly amongst the attack states. The Local needles had higher emissions of β-pinene and ocimene than the Control and Systemic needles (Fig. 2), whereas α-cedrene emissions were higher for the Control than the Systemic and Local needles. The Local emission rates of β-ocimene were significantly higher in *iberica* than *nevadensis* (Fig. 2).

Proportions of terpene emissions

The proportions of terpene emissions did not differ significantly amongst attack states in *nevadensis*, whereas the proportion of β-myrcene emission in *iberica* was highest for the Control needles, but only with marginal significance (one-way ANOVA, Tukey's HSD post-hoc test, $P < 0.10$), and the proportion of limonene emission was significantly higher for Systemic than Control needles (Table 2).

Measurements of gas exchange

Photosynthesis and stomatal conductance did not differ significantly amongst the attack states or subspecies, despite the tendencies of higher levels in the Systemic and Local than the Control needles in both subspecies and in *nevadensis* than *iberica* (Fig. S1, Supplementary Data).

Nutrients

Needle C and N concentrations in *nevadensis* did not differ significantly amongst attack states, but P concentration was highest in the Systemic needles (one-way ANOVA, Tukey's post-hoc test, $P < 0.05$). The C:N ratio in *nevadensis* did not differ significantly amongst attack states, but the N:P and C:P ratios were highest in the Control needles (Figs. 3 and S2). C concentration in *iberica* was marginally highest (one-way ANOVA, Tukey's post-hoc test, $P < 0.10$) in the Local needles, which had the lowest N and P concentrations (one-way ANOVA, Tukey's post-hoc test, $P < 0.01$) (Fig. 3). The C:N and C:P ratios were highest in the Local and Systemic needles of *iberica*, but the N:P ratio did not vary significantly amongst attack states.
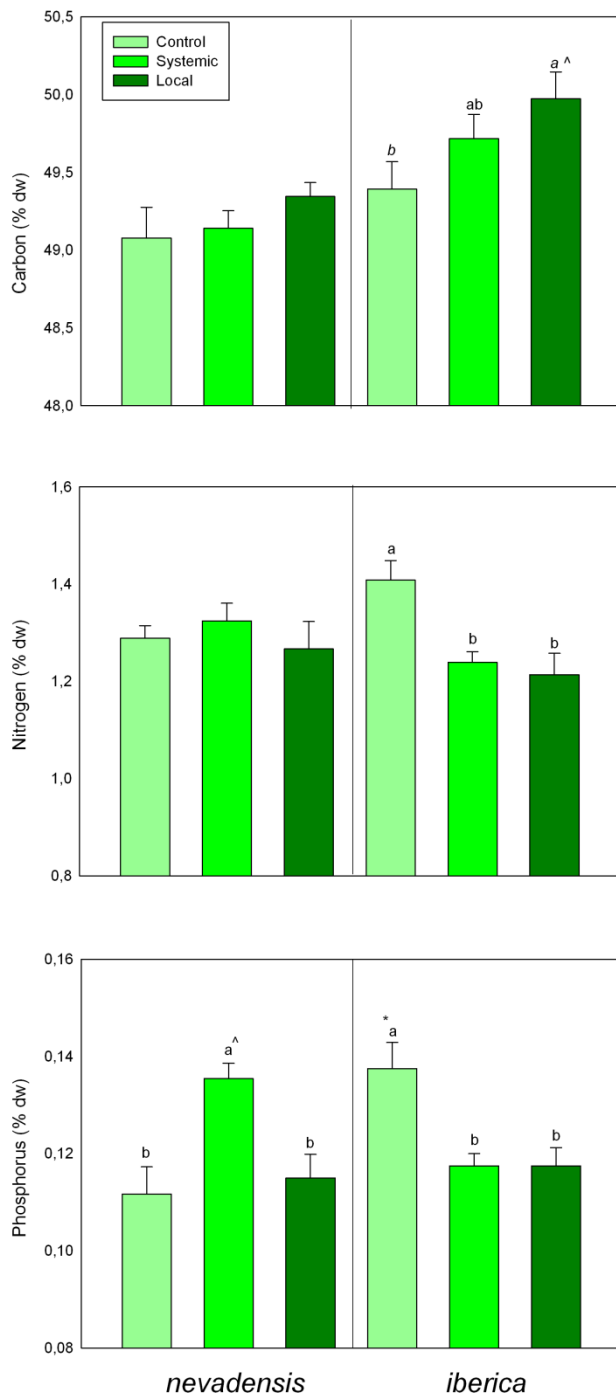
Figure 3. Needle concentrations (± SE) of carbon, nitrogen, and phosphorus of the tree attack states of *Pinus sylvestris* subsp. *nevadensis* and *P. sylvestris* subsp. *iberica*. Different letters indicate significant differences amongst the attack states within a subspecies (one-way ANOVA, Tukey's *post-hoc*, $P < 0.05$), and asterisks (*) and circumflexes (^) indicate significant ($P < 0.05$) and marginally significant ($P < 0.10$) differences amongst the same attack states of the two subspecies. dw, dry weight.

The concentrations of all the terpenes studied were tested for correlations with C, N, and P concentrations and their ratios. Only a few of the monoterpenes correlated significantly with these nutrients in *nevadensis*, but more terpenes in *iberica* were more strongly correlated with the nutrients, especially the monoterpenes (Figs. 4 and S3, Table S1). Total monoterpenes were significantly correlated with the C:N and C:P ratios in both subspecies (Fig. S3). We determined the differences between subspecies in the slopes of the regressions of total monoterpenes against nutrients using the "homogeneity of slopes" method (Figs. 4 and S3). The slopes were usually steeper in *iberica*, but these were only significant for C ($P < 0.05$) and P ($P < 0.01$) concentrations and the C:P ratio ($P < 0.001$).

Larval survival bioassay

First instar larval survival ranged between 35 and 65%, but no statistically significant differences were found amongst the attack states in none of both subspecies (Fig. 5). In *nevadensis*, survival tended to decrease in the Local needles (one-way ANOVA, Tukey's post-hoc test $P = 0.57$), while in *iberica* survival tended to be slightly higher in the Systemic and Local needles ($P = 0.64$). No differences were observed in the treatments comparison amongst subspecies, despite *iberica* showed slightly higher survival values than *nevadensis*. Correlations of larval survival and needle terpene concentrations or nutrients were not statistically significant, and did not suggest any clear trend (data not shown).

Discussion

Our results suggest that *iberica* reacts to previous defoliation by inducing the production of needle terpenes and reducing the concentrations of N and P, whereas no clear trends were found in *nevadensis* (Figs. 1-3). Despite the contrasting responses of both subspecies, no differences could be found in the PPM larval survival amongst attack states. The production of terpenes was strongly induced in *iberica*, with a substantial increase in needle terpene concentrations and changes in the emissions of

several terpenes reported to have electrophysiological (Zhang et al. 2003) and behavioural (Mateus 2009; Niccoli et al. 2008; Paiva et al. 2010; Tiberi et al. 1999) effects on adult PPM females. The concentration of C increased in *iberica*, and those of N and P decreased, in Local and Systemic needles, suggesting lowered nutrient quantity and higher C:N and C:P ratios as another possible defensive mechanism. Nevertheless, the lack of differences in larval survival suggest that the needle changes reported are weakly related to the survival of PPM, in agreement to some other PPM studies (Hódar et al. 2004; Hódar et al. 2015). The results are discussed in relation to previous studies of PPM and herbivory, including the possible roles of coevolutionary history and abiotic stressors in the contrasting reactions to previous defoliation observed between the subspecies.

Needle terpene concentrations

The terpene needle concentrations were within the range previously reported for *P. sylvestris* (Beyaert et al. 2012; Hodar et al. 2004; Moreira et al. 2013) but were lower than those in older needles from the same study site in winter (Achotegui-Castells et al. 2013), probably due to the lower terpene concentrations in young tissues (Hanover 1992). Terpene production was clearly induced in *iberica* Systemic and Local needles in response to previous defoliation but had no clear reaction pattern in *nevadensis*, with concentration decreases for some monoterpenes but two notable inductions of the minor sesquiterpenes τ-cadinol and α-cadinol in the Systemic needles. Control needles had similar concentrations for both subspecies, but *nevadensis* had higher concentrations of several terpenes. Comparing Local and Systemic needles of both subspecies, *iberica* had substantially higher concentrations than the corresponding attack states of *nevadensis* (Fig. 1, Table 1), suggesting that *iberica* was capable of inducing notably higher terpene production than *nevadensis* after the PPM attacks. Conversely, the lack of clear terpene shifts in *nevadensis* after PPM defoliation (in accordance with Hódar (2015; 2004) and the higher basal terpene concentrations in the Control needles, suggest that terpenes in this subspecies are a constitutive rather than an induced defence. Our results for *iberica*, however, are supported by those of Battisti (1988), who found an increase in needle chemical defences after previous defoliation by the PPM, despite the inability of the indirect measures (increase in energetic values) to identify these compounds.

Needle terpene emissions

Our measures of terpene needle emissions were quantitatively similar to those of the Control needles in our previous study (Achotegui-Castells et al. 2013) and other studies (Llusia and Penuelas 2000; Sabillón and Cremades 2001). Statistically significant differences amongst attack states were only found in *iberica*, although the low number of viable samples in *nevadensis* Control needles (n=4) may have impeded the observation of differences in emission rates amongst its attack states. Interestingly, most of the changes in terpene emission detected amongst the *iberica* attack states have been reported to play electrophysiological and behavioural roles in PPM host selection. These terpenes included (-)-β-pinene (Mateus 2009), trans-ocimene and cis-ocimene (Paiva et al. 2010; Zhang et al. 2003) (+)- and (-)-limonene (Mateus 2009; Niccoli et al. 2008; Tiberi et al. 1999; Zhang et al. 2003), and myrcene (Mateus 2009; Zhang et al. 2003) (see Table 3).

Our results thus show for the first time that previous PPM defoliation produces changes in terpene emissions of new pine needles that could be detected by the adult female, further supporting the hypothesis that VOCs may play a role in PPM host selection (Paiva et al. 2010, Jactel et al. 2011, Zhang et al. 2003). These changes in volatile emissions might also be perceived by antagonists of PPM, such as the parasitoids *Bariscapus servadeii* or *Ooencyrtus pityocampae* or other insect predators (Hilker et al. 2002; Mattiacci et al. 1995; Schmidt et al. 1999). Our analyses unfortunately could not determine the cis/trans isomerism nor the chirality of these terpenes, and PPM ovipositional preferences were not studied. We could thus not determine if the shifts of terpene emissions in previously defoliated trees would have attractive or repellent roles, if any, for the adult PPM females. A preference for hosts to select undefoliated trees has been observed for the PPM (Sbabdji and Kadik 2011) and other herbivorous insects (Johns and Leggo 2014; Leather et al. 1987). But a lack of host selection (Battisti 1988), or discrepancies between host selection and larval performance, however, have also been reported in the PPM (Stastny et al. 2006) and other tree-herbivore systems (Trewhella et al. 1997). These discrepancies have led some authors to consider the PPM (Hódar et al. 2002) and other epidemic insect pests (Tammaru et al. 1995) to be unselective. The contrasting observations reported in our study and in previous studies suggest that PPM may be more or less selective depending on several factors affecting the moth (e.g. flight capacity, native range,
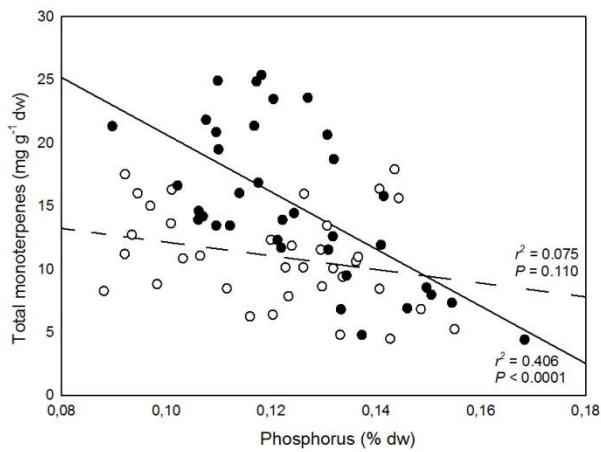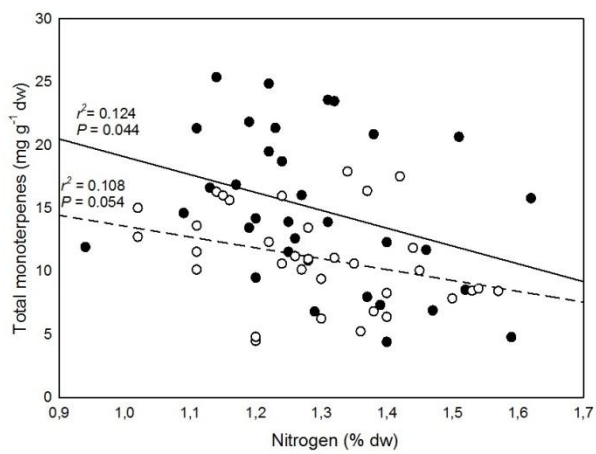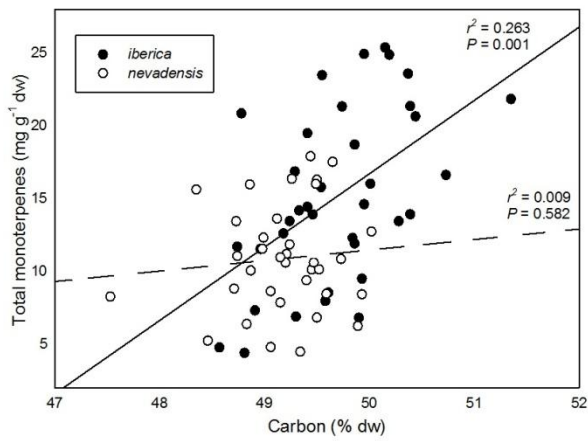
Figure 4. Correlations between nutrient and total monoterpene concentrations. Solid lines indicate significant correlations ($P < 0.05$), short-dashed lines indicate marginally significant correlations ($0.05 < P < 0.10$), and long-dashed lines indicate non-significant correlations ($P > 0.10$). dw, dry weight.

		P. sylvestris subsp. *nevadensis*			P. sylvestris subsp. *iberica*		
		Control	Systemic	Local	Control	Systemic	Local
Tricyclene	[ ]	0.10±0.09	0.061±0.036	0.028±0.018	0.075±0.047	0.045±0.013	0.089±0.03
	%	0.90±0.28	0.78±0.30	0.43±0.22	0.30±0.16	1.0±0.3	1.3±0.5
α-pinene	[ ]	1.5±0.8	1.6±0.7	1.3±0.6	3.8±2.2	2.3±0.2	2.6±0.7
	%	32±11	44±11	32±10	41±9	41±5	32±8
Camphene	[ ]	0.11±0.02	0.45±0.17	0.58±0.24	1.0±0.5	0.30±0.10	0.59±0.17
	%	5.5±0.7	9.7±3.0	6.9±1.4	6.8±1.5	5.8±1.9	8.1±2.6
Sabinene	[ ]	0.048±0.041	0.027±0.019	0.029±0.013	0.038±0.015	0.030±0.009	0.077±0.028
	%	0.41±0.16	0.54±0.26	2.5±1.5	0.50±0.15	2.4±1.0	1.0±0.5
β-pinene	[ ]	0.35±0.20	0.16±0.08	0.30±0.12	**0.13±0.06b**	**0.34±0.13ab**	**0.64±0.17a**
	%	4.8±1.3	3.6±1.0	8.6±2.1	3.4±0.9	4.9±0.7	7.4±1.9
β-myrcene	[ ]	0.68±0.31	0.39±0.12	0.53±0.17	2.6±0.8	0.90±0.30	1.2±0.6
	%	20±9	12±6	16±7	**44±12*a***	**18±5ab**	**13±4*b***
α-phellandrene	[ ]	0.081±0.057	0.015±0.005	0.026±0.008	0.040±0.023	0.029±0.008	0.063±0.018
	%	1.5±0.8	1.1±0.5	0.81±0.22	0.42±0.11	0.78±0.13	0.82±0.12
Limonene	[ ]	1.1±0.8	0.56±0.15	0.90±0.39	0.31±0.12	0.73±0.18	0.91±0.43
	%	15±2	17±5	16±4	**7.0±1.2b**	**19±5a**	**9.1±2.8ab**
Eucaliptol	[ ]	0.86±0.84	0.15±0.08	0.13±0.07	0.12±0.04	0.17±0.04	0.19±0.01
	%	5.8±5.0	3.9±0.6	4.4±1.5	2.8±0.9	5.4±2.0	2.0±0.3
Ocimene	[ ]	0.026±0.001	0.032±0.010	0.067±0.028	**0.075±0.029b**	**0.039±0.007b**	**0.17±0.03a***
	%	1.9±0.5	0.85±0.42	2.4±0.7	1.7±0.1	4.0±2.6	2.2±0.3
MT 1	[ ]	0.11±0.08	0.037±0.013	0.051±0.017	0.073±0.027	0.050±0.009	0.08±0.02
	%	1.8±0.8	1.1±0.2	1.6±0.1	1.6±0.5	1.3±0.3	1.0±0.2
Longifolene	[ ]	0.086±0.035	0.37±0.27	0.24±0.04	0.067±0.034	0.12±0.09	0.061±0.029
α-cedrene	[ ]	0.042±0.013	0.24±0.16	0.14±0.08	**0.14±0.04a**	**0.040±0.021b**	**0.011±0.003b**
ST 1	[ ]	0.34±0.31	0.040±0.010	0.041±0.015	0.045±0.007	0.029±0.011	0.056±0.019
Total MT	[ ]	3.9±2.3	2.6±0.9	3.2±1	6.4±2.6	3.2±1	4.6±1.6
	%	94±3	97±1	98±1	98±1	96±4	95±3
Total ST	[ ]	0.47±0.33	0.33±0.24	0.27±0.13	0.13±0.05	0.15±0.1	0.06±0.02
	%	8±3	9±6	3±2	2±1	7±6	5±3
Total terpenes	[ ]	4.2±2.5	2.9±1	3.4±1	6.5±2.6	3.3±1	4.7±1.6

Table 2. Mean emission rates ($\mu g\ g^{-1}$ dry weight h^{-1}) and proportions (± SE) of the terpenes emitted by the three attack states of the two *Pinus sylvestris* subspecies. Different letters indicate significant differences (one-way ANOVA, Tukey's *post-hoc*, $P < 0.05$), and italicised letters indicate marginally significant differences. Asterisks (*) indicate significant differences between the same attack states of the two *P. sylvestris* subspecies. MT, monoterpenes; ST, sesquiterpenes.

genetic preferences, or diapause) or the host (e.g. species and varieties, induced resistance, nutrient availability, drought, or health).

Differences in terpene emissions (Table 2)	Results	Electrophysiological response (Zhang et al. 2003)	PPM attraction	PPM repulsion	No effect
β-pinene	Increase in emission rates	No	Mateus (2009)	Niccoli et al. (2008)	—
β-myrcene	Decrease in proportion of emission rates	Yes (strong)	—	—	Mateus (2009)
Limonene	Increase in proportion of emission rates	Yes (weak)	—	Tiberi et al. (1999) Mateus (2009)	Niccoli et al. (2008)
Ocimene	Increase in emission rates	Yes. both *cis* - (weak) and *trans* - (strong)	Paiva et al. (2010)	—	—
Cedrene	Decrease in emission rates	No (?)	—	—	—

Table 3. Comparison of our results with published effects of terpene volatiles on electrophysiological and behavioural responses of *Thaumetopoea pityocampa* (PPM). The first column indicates the compounds for which we found emission changes, and those differences are specified in the second column. The Third column shows the electrophysiological responses of PPM antennae to these compounds, as described by Zhang et al. (2003). The last column organises the available literature based on the response reported (attraction, repulsion, or no effect).

Nutrients

The concentrations of C tended to increase in Local needles in both pine subspecies, but differences were only marginally significant in *iberica* (Fig. 3). The significant correlation between C and terpene concentrations in *iberica* (Fig. 4) could be partially due to the strong induction of these C-rich compounds ($C_{10}H_{16}$ (monoterpenes) and $C_{15}H_{24}$ (sesquiterpenes)) (Thomas and Malczewski 2007). The slight non-significant increases in N concentrations due to previous defoliation in *nevadensis* (Fig. 3) were similar to those reported by Hódar et al. (2004; 2015), and match the response to previous defoliation usually reported for evergreen trees (Hodar et al. 2004; Nykanen and Koricheva 2004; Šmits and Larsson 1999). In contrast, N concentrations in *iberica* decreased in the Local and Systemic needles, similar to the results reported by Battisti (1988) and Chen et al. (2002), who also observed negative correlations between terpene concentrations and N. Low N concentrations in needles have been proposed to account for reduced survival in the PPM (Battisti 1988; Hodar et al. 2002) and other herbivores that feed on conifer needles (Raffa et al. 1998; Roitto et al. 2009). The increase in the C:N ratios of both subspecies with herbivory (only significant in *iberica*), along with the significant correlations with terpene concentrations (Fig. S3), suggest a decrease in what some authors call food quality (Awmack and Leather 2002; Battisti 1988). The meta-analysis for evergreen species by Nykanen and Koricheva (2004) did not find clear changes in P concentrations after herbivory, which varied widely, due to contrasting reports of the effect of herbivory on P concentrations, which ranged from increases (Raffa et al. 1998) to decreases (Watt et al. 1991). Watt et al (1991) found a higher N:P ratio after previous defoliation, in contrast to our lower N:P ratio in Local

needles in *nevadensis* but more similar to the slight and non-significant increase in *iberica*.

Previous defoliation elicited systemic reactions in the Scots pines. A few concentrations differed amongst attack states in *nevadensis*, mostly in the Systemic needles, especially the concentrations of cadinols and the proportions of sesquiterpenes (Table 1). Similar systemic reactions were also observed at the same study site in winter (Achotegui-Castells et al. 2013). The only significant change in nutrients found in *nevadensis*, was also observed in the P concentrations of Systemic needles. Systemic reactions were more pronounced in *iberica*. Direct defences (needle terpene concentrations) were equivalent in the Systemic and Local needles (Fig. 1, Table 1), and systemic inductions were also observed in indirect defences (needle terpene emissions) (Fig. 2, Table 2). Systemic changes were also observed in N and P. These results, together with those of other studies (Hilker et al. 2002; Nykanen and Koricheva 2004), suggest that *P. sylvestris* is capable of eliciting systemic terpene reactions prior to herbivorous attack. Investment in systemic responses would theoretically protect the entire tree before future aggressions, thereby impeding larval survival or even emitting VOCs that could deter oviposition by the PPM (Hilker et al. 2002; Sticher et al. 1997).

Larval survival bioassay

None of the differences observed in needle terpene concentrations or nutrients had a determinant influence in early stages of PPM larval survival (Fig. 5). These results would contradict studies linking decrease of larval survival with increased chemical defences and decreased nutrient concentrations (Battisti 1988; Traw and Dawson 2002; Hódar et al. 2002; Riotto et al. 2009). However, there is an important number of studies that have failed to link herbivore survival and chemical and nutritional needle traits (Schuldt et al. 2012, Beyaert et al. 2012, Nykanen and Koricheva 2004), also in the PPM (Hódar et al. 2004; Hódar et al. 2015). Regarding the effect of needle terpene concentrations on PPM larvae, (2005) found that limonene or β-caryophyllene were related to inhibition of feeding in 4th instar PPM larvae, despite other main compounds seemed to have no remarkable effect on PPM survival. Two studies found plant essential oils mainly formed by terpenes show substantial larvicidal effects when are in contact with PPM larvae (Kanat and Alma 2004; Kesdek et al. 2014). In addition, survival is thought to be less dependent on toxicity than on growth, and maybe this

would explain why we failed to detect the effect of terpenes (Cornell and Hawkins 2003). Concerning nutrients, we expected that the decrease in *iberica* needle concentrations would have lowered larval survival, but maybe the nutrient decrease was not large enough to produce a detectable effect. PPM is a specialist herbivore, and may be adapted to deal with plant responses to herbivory (Cornell and Hawkins 2003; Mumm and Hilker 2006). Further studies should test if PPM is tolerant to terpenes, as it has been reported for other lepidoptera (Mao et al. 2007; Raffa and Powell 2004) or is capable of avoiding them (1st instar PPM larvae mostly eats the epidermis of needles, and may avoid the resin ducts, where most needle terpenes are stored). *In planta* larval survival (Hódar et al. 2004, Hódar et al. 2015) and performance tests at different larval stages would thus be necessary to verify if the observed changes in needle chemistry and nutrition have no effect on PPM larvae.
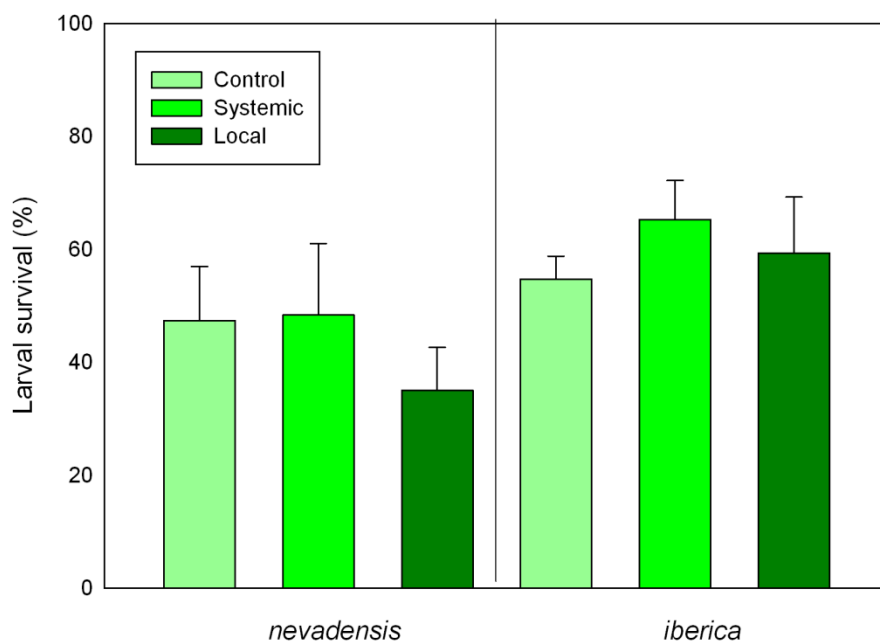
Figure 5. Mean percentages of survival (± SE) of 1st instar larvae of PPM of the three attack states of *Pinus sylvestris* subsp. *nevadensis* and *P. sylvestris* subsp. *iberica*.

Even though *nevadensis* and *iberica* belong to the same pine species and would be expected to respond similarly under the same environmental conditions or external pressures (Blomberg et al. 2003), they responded differently to previous attacks. Two factors may have contributed to these differences: the different historical relationships with the PPM and the degree of adaptation to local conditions. The two subspecies have had different historical relationships with the PPM, so their coevolution with the PPM has differed (Karban 2011; Rausher 2001). Two distinct haplotypes of the PPM coexist in the Iberian Peninsula and are associated with their glacial refugia and the host trees available during these periods of retraction (Kerdelhué et al.; Rousselet et al. 2010), with *iberica* within the area of one haplotype and *nevadensis* in the other (B and A2, respectively, in Rousselet et al. 2010). Which subspecies had a more intense coevolutionary history with the defoliator is not clear, but the geographic conditions suggest that the A2 haplotype occurred in thermic lowlands very close to high mountains, and the B haplotype occurred in the flat areas in the western Iberian Peninsula (see Rousselet et al. (2010) for the role of topography in shaping the distribution of PPM maternal lineages throughout the demographic history of its main host plants). The high constitutive levels of defensive compounds in the *nevadensis* Control needles and the lack of induced defence also suggest a selective environment of intense and frequent herbivory (Ito and Sakai 2009), which would favour constitutive over induced defences. Conversely, *nevadensis,* isolated on the top of the Sierra Nevada mountains, has been protected from attacks by the PPM until its recent uphill spread due to climate change (Battisti et al. 2005; Hódar et al. 2003), which could in turn relax the intensity of the constitutive defence during recent times. A much deeper knowledge of the Quaternary history of the PPM and its main host plants in the Iberian Peninsula, however, is needed to clarify the role of history in the defensive strategies of these plants. Regarding adaptation, *nevadensis* is endemic to the study site and is thus expected to be better adapted to the environmental conditions of Collado de Matasverdes (hot and dry summers and cold winters) than *iberica*, which has been planted for reforestation. A companion study by Rivas et al. (in preparation) found that *iberica* had clearly stronger metabolic responses and higher concentrations of compounds correlated with hydric stress than *nevadensis*. Several studies have found increased terpene defences when trees were under nutrient limitation (Moreira et al. 2015) or drought (Blanch et al. 2009; Kainulainen et al. 1992) but the defensive reactions of trees under drought remain uncertain (Jactel et al. 2012).

In summary, the increases in terpene concentrations, changes in terpene emissions, and decreases in nutrient concentrations in *iberica* suggest strong and multiple terpene reactions to previous defoliation in this Scots pine subspecies. Increases in terpene concentrations, together with decreased concentrations of N and P (and consequent increases in the C:N and C:P ratios), despite theoretically lowering food quality, did not seem to have a negative impact on PPM larval survival. However, changes in terpene emissions could be important to PPM and other insects. In contrast, no clear responses and no changes in larval survival were observed in *nevadensis*, as previously reported by Hódar (2015; 2004). The little information available for terpene reactions to previous defoliation by the PPM suggests that some pines can induce terpenes in response to herbivory, but not under all circumstances. The situation described here matches the contrasting reports of plant reactions and insect survival to previous defoliation and supports the premise that the response of plants to herbivory is a very complex matter that depends on tree genetics, plant-pest coevolution, and many biotic and abiotic stress factors. Further investigation should test larval survival at different larval stages by *in planta* tests to confirm that pine responses have no effect in larval survival. Olfactometric or experimental field assays should be performed to ascertain if PPM or their antagonists show preferences between blends of undefoliated and defoliated trees. Finally, it would be very interesting to determine if the contrasting reactions to herbivory of these subspecies are correlated with drought stress or nutrient limitations.

Acknowledgements

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Chapter 4. Strong induction of minor terpenes in Italian Cypress, *Cupressus sempervirens*, in response to infection by the fungus *Seiridium cardinale*

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Abstract

Seiridium cardinale, the main fungal pathogen responsible for cypress bark canker, is the largest threat to cypresses worldwide. The terpene response of canker-resistant clones of Italian cypress, *Cupressus sempervirens*, to two differently aggressive isolates of *S. cardinale* was studied. Phloem terpene concentrations, foliar terpene concentrations, as well as foliar terpene emission rates were analyzed 1, 10, 30, and 90 days after artificial inoculation with fungal isolates. The phloem surrounding the inoculation point exhibited *de novo* production of four oxygenated monoterpenes and two unidentified terpenes. The concentrations of several constitutive mono- and diterpenes increased strongly (especially α-thujene, sabinene, terpinolene, terpinen-4-ol, oxygenated monoterpenes, manool, and two unidentified diterpenes) as the infection progressed. The proportion of minor terpenes in the infected cypresses increased markedly from the first day after inoculation (from 10% in the control to 30-50% in the infected treatments). Foliar concentrations showed no clear trend, but emission rates peaked at day 10 in infected trees, with higher δ-3-carene (15-fold) and total monoterpene (10-fold) emissions than the control. No substantial differences were found among cypresses infected by the two fungal isolates. These results suggest that cypresses activate several direct and indirect chemical defense mechanisms after infection by *S. cardinale*.

Key Words: VOCs, cypress bark canker, sabinene, manool, oxygenated monoterpenes, *de novo*.

Introduction

Fungal pathogens infect trees by using enzymes, toxins, growth regulators, and by obtaining nourishment from the substances produced by the host. Conifers make use of chemical defenses, mainly terpenes and phenols (Franceschi et al. 2005; Phillips and Croteau 1999), to face pathogenic fungi and other threats. Terpenes are used in conifers as constitutive defenses (a first line of defense against any enemy) but also as induced defenses against pathogens; increases in absolute amounts, proportional changes, phytoalexin production and general or specific responses to an antagonist can appear at different time points following infection (Michelozzi 1999). Oleoresin is secreted from injured or infected tissues, thus deterring fungal pathogens or insects and sealing the wound at the same time (Trapp and Croteau 2001). Hundreds of studies have demonstrated that terpenes can strongly inhibit fungal spore germination and mycelial growth (see reviews by Bakkali et al. 2008, Boulogne et al. 2012 and references therein) by disrupting internal structures and permeabilizing fungal cells (Bakkali et al. 2008).

Plants can respond generally to pathogenic infections but may also react specifically to specific pathogens. Conifers can have distinct terpene reactions to different fungal pathogens (Raffa and Smalley 1995; Schiller and Madar 1991; Zamponi et al. 2007), but usually exhibit similar reactions to different fungal isolates or strains of the same fungus (Bonello et al. 2008; Faldt et al. 2006; Schiller and Madar 1991). In addition to the local terpene reactions to fungal infection, systemic responses have been found in non-infected tissues. Systemic changes in phloem terpene concentrations (Viiri et al. 2001), foliar terpene concentrations (Schiller and Madar 1991), and foliar terpene emission rates (Lusebrink et al. 2011) have been observed in conifers infected by fungi. These phenomena could enhance the defense of undamaged plant tissues, prepare the plant for new attacks related to the infection, or activate indirect defense strategies (Bonello et al. 2008).

Cypress bark canker caused by the mitosporic fungus *Seiridium cardinale* (Wagener) Sutton & Gibson is the most severe and widespread disease affecting Italian cypress (*Cupressus sempervirens* L.) worldwide (Battisti et al. 1999; Della

Rocca et al. 2011; Graniti 1998). This disease affects the cortical tissues (phloem and cambium but not xylem) of several members of the Cupressaceae family, causing severe diebacks and often death of the cankered trees over a time span of months to years (Graniti 1998). After the first outbreak reported in California in 1929 (Wagener 1939), cypress bark canker has spread rapidly to other regions of the world, having a relevant impact in the Mediterranean Basin (Graniti 1998; Panconesi 1991; Xenopoulos 1990). The disease spreads by dissemination, mainly by rainwater, of asexual spores of the fungus (conidia) produced in fruiting bodies on the surface of affected trees or by windborne raindrops and vectors (Battisti et al. 1999; Covassi et al. 1975; Zocca et al. 2008). Results from a 40-yr genetic improvement program have revealed a moderate variability in the response of some Mediterranean native and naturalized *C. sempervirens* populations to *S. cardinale* infections, with 1-2% of trees being resistant. Several resistant genotypes have been selected, and some varieties have been patented and successfully commercialized (Danti et al. 2006, 2013; Panconesi and Raddi 1991).

Italian cypress has an oleoresin rich in terpenoids and reacts to wounds or fungal infection by producing traumatic resin ducts in the phloem (Hudgins et al. 2004; Krokene et al. 2008). The composition of basic terpenes in several tissues and the reaction to some environmental changes have been studied for this tree (Gallis et al. 2007; Mazari et al. 2010; Piovetti et al. 1981; Piovetti et al. 1980; Yani et al. 1993; Yatagai et al. 1995). Two terpene phytoalexins, cupressotropolone A and B, were detected in Italian cypresses inoculated with *Diplodia pinea* f. sp. *cupressi*, another canker-causing fungal pathogen (Madar et al. 1995a; Madar et al. 1995b). These phytoalexins showed substantial activity against several fungal pathogens of cypress, including *S. cardinale* (Madar et al. 1995a). Moderate antifungal activity of the essential oil of *C. sempervirens* leaves was observed against fungal pathogens of other hosts (Mazari et al. 2010). The proportions of terpene contents of leaves of healthy and naturally infected *C. sempervirens* trees (by *D. pinea* f. sp. *cupressi* and *S. cardinale*) were studied by Schiller and Madar (1991), and although proportions differed among treatments, no specific compound was associated with fungal infection or resistance, and no clear differences in tree response among the two fungal pathogens were found.

In summary, little is known about conifer phytoalexin production, systemic reactions, or foliar emissions under fungal infection, especially for families other than

Pinaceae. As for the *C. sempervirens – S. cardinale* pathosystem, little is known about changes in the terpene composition of Italian cypress as a response to infection by the main cypress bark canker agent.

The goals of this study were thus: (*i*) to monitor the locally induced terpene response of the phloem of canker-resistant cypress clones to wounds and infection by two *S. cardinale* isolates during the first 90 days after artificial inoculation; *(ii)* to investigate the systemic response of cypress leaves to fungal infection, analyzing foliar concentration and emission rates and; *(iii)* to study the differential responses in cypress tissues induced by the two isolates of *S. cardinale* characterized by different pathogenicity.

Materials and Methods

Study Site

The study was performed in an experimental field of the Institute of Sustainable Protection of Plants – National Research Council (IPSP-CNR, in italian) in Cannara, Perugia, central Italy (42°58'29" N, 12°36'38" E). The field was at an elevation of 192 m a.s.l. and provided equal light, nutrient, and water availability for all trees. We used 64 four-yr-old grafted plants of *C. sempervirens*, planted with a 3 × 3 m spacing and belonging to four genotypes patented by IPSP-CNR for their resistance to cypress bark canker: Italico, Bolgheri, Agrimed and Mediterraneo (16 trees of each genotype) (Danti et al. 2006; Panconesi and Raddi 1991). Cypresses were watered twice a week during the first month after planting. Soil was a clayey reclaimed alluvial. The climate is moderately continental, with hot summers and cold winters with sporadic snowfall. The average rainfall is 815 mm yr^{-1} distributed on 80 rainy days with a peak in autumn. The yearly average annual temperature is 13.8 °C. The coldest month is January with an average minimum of 0 °C, and the warmest month is July with an average maximum temperature of 30 °C.

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Experimental Design

To monitor tree reactions against fungal infection, we applied four treatments to the cypresses: 1) control (no damage); 2) mildly virulent (Mv, wound + inoculation with a moderately aggressive *S. cardinale* isolate (ref. submitted)); 3) highly virulent (Hv, wound + infection with a more aggressive *S. cardinale* isolate); and 4) Wounded (wound only, without inoculation). Trees were inoculated following a standard procedure (Danti et al. 2006, Danti et al. 2013), which consists of removing a disc of bark from the stem with a sterile cork borer of 4 mm diam and filling the wound with a plug of the same size of malt extract agar (MEA). This plug was taken from the margin of a colony of the fungus grown on MEA 2% in the dark for 15 days at 25 °C. The inoculation site was covered with wet cotton wool and wrapped with Parafilm®.

Tissue samples were collected from 26 April to 25 July 2012, 1, 10, 30, and 90 d after applying the above treatments. The sampling method was destructive, so trees were used only once to avoid any effects from the wounds. Each treatment, for each sampling date, had four replicates (four treatments × four time points × four replicates = 64). Within the treatments, each of the four replicates contained each of the four tree genotypes.

Field sampling

Tissue Sampling

Three types of samples were collected from each tree: *i*) phloem removed from a segment of the inoculated stem containing the infected tissues (samples were taken from a height of ca. 80 cm); *ii*) foliar tissue from the closest branch to the inoculation point and; *iii*) foliar volatile organic compound (VOC) emission, from the same branch where foliar tissue was taken. Emissions were sampled first to avoid tree reactions to wounding. All sampled tissues were stored in liquid nitrogen in the field and then at -20 ºC in the laboratory.

VOC Sampling

Twigs immediately above the inoculation point (3.5-21 cm) were sampled to analyze VOC emissions. The selected twigs were wrapped first with Teflon ribbon a few days before the sampling to minimize effects of mechanical manipulation and alteration of the emissions.

The VOC emissions were sampled from 09:00 to 15:00 h (solar time) using the conifer chamber (a 230 cm^3 cuvette) of the LiCor 6400 Portable Photosynthesis System (Li-Cor Inc, Lincoln, NE, USA). The twig was carefully inserted into the chamber, placing its closure on the Teflon ribbon. Air flow rate inside the conifer chamber was set to 600 µmol s^{-1}. The chamber was allowed to stabilize for 15 min, as monitored by environmental and physiological parameters such as temperature, photosynthetic active radiance (PAR), photosynthesis, and stomatal conductance. When the twig had physiologically stabilized, we placed one end of a metallic VOC trap (Markes International Inc. Wilmington, DE, USA), filled with 115 mg of Tenax and 230 mg of Unicarb, in the chamber to collect the VOCs exhausted from the twig chamber. A QMAX pump (Supelco, Bellefonte, PA, USA) attached to the other end of the metallic trap pulled the air from the conifer chamber. A Defender 510 fluxometer (Bios International Corporation, Butler, NJ, USA) was placed between the QMAX and the VOC trap to control the air flux. Sampling time was 5 min, with an absorption flux of ca. 7 ml s^{-1}. The sampled VOC traps were stored in the field in a 4 ºC portable refrigerator until transferred to a -20 ºC freezer in the laboratory. Blank samples were collected after every two twig samples, as described above, but without a twig inside the conifer chamber. The VOC-sampled leaves also were stored, and once in the laboratory dried until constant weight, in order to refer the emission rates to g of dry weight (µg g^{-1} of foliar dry weight h^{-1}).

Sample analyses and terpene identification

Phloem and leaves were ground separately inside 50-ml Teflon tubes filled with liquid nitrogen to avoid the evaporation of VOCs and to facilitate their crushing. After samples had been pulverized, 1 ml of pentane containing 0.5 µl of dodecane (used as an internal standard) was added, and the Teflon tubes were stored for at least 12 h at -20 ºC. After extract stabilization to laboratory temperature, 300 µl of the supernatant were

stored in vials, for subsequent analysis in a gas chromatograph/mass spectrometer (GC/MS). The tubes, now containing only the unused extract, were dried to a constant weight and then weighed in a precision balance. Tubes were later exhaustively cleaned, dried and reweighed to tare them. One blank was analyzed after every five samples.

Two µl of the biomass extract were injected into a capillary column (HP 5MS, 30 m × 0.25 µm × 0.25 mm) in a GC (7890A, Agilent Technologies, Santa Clara, CA, USA) with a MS detector (5975C inert MSD with Triple-Axis Detector, Agilent Technologies). The temperature was maintained first at 35 ºC for 2 min, increased at 15 ºC min^{-1} to 150 ºC and maintained for 5 min, increased at 30 ºC min^{-1} to 250 ºC and maintained for 5 min, and finally increased at 30 ºC min^{-1} to 280 ºC and maintained for 5 min. Total run time was 29 min, and the helium flow was set to 1 ml min^{-1}.

Terpenes were identified by comparing the mass spectra with published spectra (libraries NIST 05 and Wiley 7n) and the spectra of known standards. Calibration curves for the quantification of each terpene were prepared with commercial standards of the most abundant compounds found in the samples. Four monoterpenes (α-pinene, sabinene, limonene, and γ-terpinene), three sesquiterpenes (caryophyllene, caryophyllene oxide, and cedrol), two diterpenes (phytol and totarol), and one non-terpene internal standard (dodecane) were used (Fluka Chemie AG, Buchs, Switzerland). All terpene calibration curves were highly significant ($r^2 \geq 0.99$) for the relationship between signal strength and terpene concentration. The most abundant terpenes exhibited similar sensitivities (differences <5%). Terpenes identified only by published spectra that were considered important for the experiment were later verified with standards: α-thujene (Chemos GmbH, Regenstauf, Germany) terpinolene, terpinen-4-ol, sabinene hydrate, camphor, α-terpineol (Fluka Chemie AG, Buchs, Switzerland), and manool (Sequoia Research Products Limited, Pangbourne, United Kingdom).

Terpene emission rates

The terpene emissions collected by the VOC traps were released with an automatic sample processor (TD Autosampler, Series 2 Ultra, Markes International Inc. Wilmington, DE, USA) and desorbed using an injector (Unity, Series 2, Markes

International Inc. Wilmington, DE, USA) in the GC/MS described above. A full-scan method was used for the chromatographic analyses. The desorbed sample was retained in a cryotrap at -20 °C. The split was 1:10. The sample was redesorbed at 250 °C for 10 min, injected into the column with a transfer line at 250 °C, and submitted to the same chromatographic process described above for the analysis of terpene concentrations.

No diterpenes were used as standards for the analyses of emission rates because they are not volatile at ambient temperature. The terpene emission rates were expressed in $\mu g\ g^{-1}$ (dry weight (dw)) h^{-1}. Even though the days of sampling were similar (sunny and warm), the terpene emission rates were standardized at 30 °C using an algorithm for terpene-storing species (Guenther et al. 1993):

$E = E_s \{\exp[\beta(T-T_s)]\}$

where E represents the emission rates in $\mu g\ g^{-1}$ (dw) h^{-1} of monoterpenes at temperature T (in degrees Kelvin, K), E_s is the emission factor in $\mu g\ g^{-1}$ (dw) h^{-1} at standard temperature T_s (303 K), and β represents an empirically determined coefficient, 0.09 K.

Statistical analyses

Data were analyzed using restricted maximum likelihood (REML), with the treatment (control, Wounded, Mv and Hv) as the fixed factor and the genotype (Agrimed, Bolgheri, Italico and Mediterraneo) as the random factor. Pairwise comparisons between treatments were performed using a Tukey's *post-hoc* test. Data that did not fit normality requirements were log transformed. Statistical analyses were conducted using R software version 2.15.2 (R Foundation for Statistical Computing, 2012) and Statistica version 8.0 (Statsoft Inc. Tulsa, OK, USA) and the graphics were generated using SigmaPlot version 11.0 (Systat Software, Chicago, IL, USA).

Results

Local Phloem

Phloem samples of cypresses had similar concentrations of monoterpenes and diterpenes, and sesquiterpenes represented only ca. 10% of the total terpene concentration. Sixty-eight terpenes represented more than 0.1% of the total peak area of the chromatograms, and those detected in more than 40% of all samples (27 terpenes) were selected for statistical analyses. The most abundant monoterpenes were α-pinene and δ-3-carene (ca. 90% of total monoterpenes in the control). α-Cubebene and longifolene were the principal sesquiterpenes, and totarol was the most abundant diterpene (ca. 60% of total diterpenes in the control).

Qualitative Differences among Treatments

Six terpenes appeared exclusively in the infected treatments (Mv and Hv) 30 and 90 days after inoculation. These six *de novo* terpenes were found in all four cypress genotypes. Four of these were oxygenated monoterpenes: oxygenated monoterpene *de novo 1* (detected in 15 of 16 samples of Mv and Hv at days 30 and 90, 0.093±0.02 mg g^{-1}, mean±SE), sabinene hydrate (16/16; 0.17±0.03 mg g^{-1}), camphor (10/16; 0.16±0.04 mg g^{-1}), and α-terpineol (13/16; 0.36±0.1 mg g^{-1}). The monoterpene *de novo 2* (14/16; 0.11±0.04 mg g^{-1}) and the diterpene *de novo* 3 (6/16; 5.4±1.7 mg g^{-1}) could not be identified. No differences in concentration were detected between treatment or time for the *de novo* compounds (REML, fixed=treatment, random=genotype, paired Tukey's *post-hoc* test, $P < 0.05$). Thymyl methyl ether (another oxygenated monoterpene) did not appear in the control but was detected in some of the Wounded samples and in all infected treatments from day 10 to day 90, reaching a mean concentration of 2.9±1.2 mg g^{-1} in Hv at day 30 (Table 1).

Quantitative Differences among Treatments

Total concentrations were lower in the infected treatments than in the control at days 1 and 10 but increased substantially after day 30 (Table 1). Total terpenes were nearly 4-fold higher in the infected treatments compared to control at day 30, and reached a maximum of 140 mg g^{-1} at day 90 (Table 1). This increase in total terpenes was due partly to increased concentrations of some of the most abundant compounds (α-

pinene, diterpene 1) but also to the strong increases in concentrations of several minor compounds. These changes led to a decrease in the proportions of the main compounds. α-Thujene was among the most induced compounds in the infected treatments (up to a 57-fold increase relative to the control), and presented differences from day 10, with concentrations and proportions rising steadily until day 90. Next in order of retention time was sabinene, whose concentrations (60-fold increase) had begun to differentiate by day 10 and whose proportions peaked between days 10-30, and then dropped slightly by day 90 (Fig. 1).

Terpinolene concentrations (18-fold increase) had higher proportions in the infected treatments throughout the experiment, reaching maximum proportion at day 1. Terpinen-4-ol (622-fold increase) retained a high concentration and proportional difference between treatments from days 10 to 90. Diterpene 2 was the most induced diterpene (164-fold increase) and increased its concentration steadily from day 1 to day 90 (Fig. 2). Diterpene 5 (43-fold), diterpene 6 (42-fold), and manool (11-fold) increased in concentration and proportions from day 10 to 90. Limonene (12-fold) and α-terpinene (15-fold) also notably increased, but the concentrations were significantly higher than the control only at day 90. Oxygenated monoterpenes (the sum of terpinen-4-ol, thymyl methyl ether, and bornyl acetate) were the most induced terpene class, with up to 1063-fold higher concentrations in the infected treatments than in the control (Fig. 1).

At day 1 post inoculation, total terpenes tended to decrease relative to control, as did all terpene classes (mono-, sesqui-, and diterpenes), despite the lack of statistical differences among treatments. Only cedrol exhibited differences, with Mv higher than Wounded and Hv (REML, fixed=treatment, random=genotype, paired Tukey's *post-hoc* test, $P < 0.05$) (Table 1). δ-3-Carene had a higher proportion in Wounded than in all other treatments, and terpinolene, the minor monoterpenes (sum of all monoterpenes except α-pinene and δ-3-carene), and diterpene 2 had higher proportions in the infected treatments than in the control or Wounded (Table 1, Figs. 2-3). Terpene concentrations decreased significantly at day 10 in both infected treatments relative to control for total terpenes and all terpene classes, except the oxygenated monoterpenes, that increased 75-fold. α-Pinene, α-fenchene, β-pinene, β-myrcene, δ-3-carene, total monoterpenes, all sesquiterpenes (including total sesquiterpenes), the majority of diterpenes (including total diterpenes), and total terpenes had the highest concentrations in the control. Terpinolene, terpinen-4-ol,

minor monoterpenes, and oxygenated monoterpenes, however, increased significantly in infected treatments compared to the control and Wounded (Table 1). α-Fenchene, δ-3-carene, total sesquiterpenes, and diterpenes 3, 4, and 7 also decreased in proportion in the infected treatments relative to the control. In contrast, α-thujene, sabinene, terpinolene, terpinen-4-ol, oxygenated monoterpenes, minor monoterpenes, α-cubebene, manool, diterpenes 2 and 5, and totarolone had higher proportions in infected treatments than in the control or Wounded (Table 1).

By day 30, concentrations tended to change relative to those at day 10, with total terpene, total mono-, total sesqui-, and total diterpene concentrations increasing non-significantly in the infected treatments. Concentrations of α-thujene, sabinene, terpinolene, terpinen-4-ol, minor and oxygenated monoterpenes, β-cedrene, manool, diterpenes 2 and 5, and totarolone were higher in infected treatments than control or Wounded (Table 1). Proportions showed similar trends, with the monoterpenes listed above increasing in proportion in the infected treatments. α-Cubebene, manool, and diterpenes 2, 5, and 6 also increased in proportion. In contrast, α-pinene, β-pinene, longifolene, totarol, diterpenes 3 and 7, and total diterpenes decreased in proportion (Table 1). Finally, the largest contrasts appeared by day 90, with concentrations in the infected treatments being the highest reported in the study. Concentrations of α-thujene, α-pinene, sabinene, β-pinene, β-myrcene, limonene, terpinolene, terpinen-4-ol, α-terpinene, oxygenated, minor and total monoterpenes, β-cedrene, cedrol, manool, diterpenes 1, 2, 5, and 6, totarolone, hinokione, total diterpenes, and total terpenes were all higher in infected treatments than in Wounded and/or the control. The proportions also were higher in the infected trees for α-thujene, sabinene, β-myrcene, limonene, terpinolene, terpinen-4-ol, oxygenated, minor and total monoterpenes, β-cedrene, manool, and diterpenes 2 and 6. In contrast, longifolene, total sesquiterpenes, totarol, diterpenes 3 and 7, totarolone, hinokione, and total diterpenes showed the opposite trend, having higher proportions in the control or Wounded than in the infected treatments (Table 1). No differences were found among the control trees from days 1 to 90, except for total diterpene concentrations at day 90, which were higher than on other sampling days.

Name	RT (min)		DAY 1				DAY 10			
			control	Wounded	Mildly virulent	Highly virulent	control	Wounded	Mildly virulent	Highly virulent
α-thujene	7.73	[ ]	0.027±0.006	0.025±0.013	0.038±0.016	0.011	0.035±0.012	0.085±0.053	0.035±0.013	0.072±0.035
		%	0.23±0.01	0.32±0.11	0.55±0.24	0.18	**0.24±0.05b**	**0.75±0.33ab**	**0.82±0.19ab**	**1.1±0.3a**
α-pinene	7.83	[ ]	6.3±0.1	2.9±1.2	2.7±2.1	1.1±1.0	**8.8±2.3a**	**6±2.4b**	**2.5±1.1b**	**3.6±1.9b**
		%	55±10	53±7	42±10	37±6	59±9	71±5	47±14	46±11
α-fenchene	8.04	[ ]	0.33±0.13	0.15±0.07	0.27±0.21	0.18	**0.38±0.15a**	**0.11±0.07b**	**0.048±0.024b**	**0.063±0.038b**
		%	2.7±0.7	2.3±0.5	2.4±0.3	2.8	**2.2±0.6a**	**0.98±0.38ab**	**0.76±0.24b**	**0.77±0.23b**
sabinene	8.33	[ ]	0.074±0.002	0.039±0.014	0.064±0.049	0.015±0.009	0.11±0.04	0.41±0.25	0.21±0.09	0.43±0.2
		%	0.65±0.09	0.9±0.17	2.1±0.8	2.7±1.4	**0.68±0.08b**	**3.4±1.6a**	**3.9±1.0a**	**5.3±0.5a**
β-pinene	8.39	[ ]	0.11±0.07	0.06±0.019	0.079±0.079	0.049±0.042	**0.16±0.03a**	**0.15±0.07ab**	**0.053±0.016b**	**0.088±0.049ab**
		%	0.88±0.43	1.7±0.6	2.5±1.2	3.8±1.8	1.4±0.5	1.7±0.2	1.1±0.2	1.0±0.3
β-myrcene	8.49	[ ]	0.14±0.07	0.059±0.021	0.097±0.084	0.042±0.035	**0.22±0.06a**	**0.14±0.08ab**	**0.069±0.031ab**	**0.14±0.08ab**
		%	1.1±0.4	1.2±0.2	2.7±1	3.8±2.2	1.4±0.1	1.3±0.5	1.3±0.2	1.3±0.5
δ-3-carene	8.78	[ ]	4.3±1.5	1.7±0.8	2.3±2	1.2±1.2	**5.4±2.5a**	**1.1±1.1b**	**0.29±0.26b**	**0.76±0.63b**
		%	**35±7ab**	**29±6a**	**19±10bc**	**19±18b**	**29±8a**	**7.9±7.9b**	**4.0±3.1b**	**8.7±4.1b**
limonene	9.01	[ ]	0.11±0.04	0.056±0.015	0.072±0.05	0.029±0.021	0.13±0.03	0.08±0.03	0.27±0.21	0.11±0.04
		%	0.9±0.2	1.5±0.4	3.7±1.5	4.2±2.8	0.90±0.11	0.90±0.07	6.4±5.6	1.0±0.2
terpinolene	9.67	[ ]	0.34±0.2	0.3±0.09	0.32±0.18	0.19±0.09	0.68±0.21	0.76±0.27ab	0.71±0.19	2.01±0.98a
		%	**2.7±1.3ab**	**10.7±5.8b**	**31±15a**	**34±16ab**	**5±1b**	**10±2ab**	**21±8a**	**19±7ab**
terpinen-4-ol	10.74	[ ]	NA	0.011	0.006	NA	**0.016±0.005b**	**0.051±0.036a**	**0.055±0.017a**	**0.13±0a**
		%	NA	0.13	2.0	NA	**0.08±0.01b**	**0.55±0.25ab**	**2.1±1.1a**	**1.8±0.8ab**
carvacrol methyl ether	11.50	[ ]	NA	NA	NA	NA	NA	1.234	0.97±0.78	1.08±0.4
		%	NA	NA	NA	NA	NA	10	26±13	19±13
α-terpinene	13.35	[ ]	0.044±0.023	0.018	0.062	NA	0.087±0.045	0.044±0.004	0.015±0.005	0.037±0.028
		%	0.42±0.26	0.22	0.35	NA	0.40±0.14	0.35±0.05	0.21±0.04	0.24±0.17
minor monoterpenes		[ ]	1.2±0.5	0.7±0.2	0.8±0.6	0.4±0.2	**1.8±0.5b**	**2.2±1ab**	**1.9±0.8ab**	**3.8±1.6a**
		%	**9.6±2.6ab**	**19±6b**	**44±17a**	**50±18ab**	**12±2b**	**24±2ab**	**49±15a**	**45±15a**
oxygenated monoterpenes		[ ]	NA	0.01	0.006	NA	**0.016±0.005b**	**0.46±0.45ab**	**1±0.8a**	**1.2±0.4a**
		%	NA	0.13	2	NA	**0.08±0.01b**	**4.0±3.7ab**	**28±13a**	**21±14a**
total monoterpenes		[ ]	12±2	5±2	5±4	2.3±2.1	**16±5a**	**9±3.7b**	**4.6±1.5b**	**8.1±3.7b**
		%	55±2	60±4	62±13	56±14	44±7	48±2	57±7	48±13
α-cubebene	13.47	[ ]	0.51	0.35±0.18	0.39±0.29	0.17±0.10	**1.47±0.31a**	**0.59±0.25b**	**0.29±0.11b**	**0.33±0.19b**
		%	25	43±5	51±25	68±24	**45±6b**	**44±8ab**	**68±11ab**	**66±10a**
longifolene	14.99	[ ]	0.84±0.45	0.45±0.23	1.1±0.9	1.4	**1.4±0.4a**	**0.5±0.4b**	**0.18±0.1b**	**0.14±0.12b**
		%	64±2	44±6	41±18	63	39±9	27±17	20±6	20±7
β-cedrene	15.10	[ ]	0.17±0.05	0.15±0.03	0.18±0.13	0.23	**0.45±0.08a**	**0.16±0.05b**	**0.064±0.025b**	**0.10±0.07b**
		%	15±5	10±2	8.5±0.8	10	11±3	16±6	11±1	12±2
cedrol	17.49	[ ]	**0.08±0.01ab**	**0.051±0.015b**	**0.21±0.09a**	**0.056±0.053b**	**0.35±0.11a**	**0.14±0.08b**	**0.076±0.02b**	**0.057±0.042b**
		%	9±5	19±10	24±21	8±3	9.2±3.7	13±5	11±6	11±2
total sesquiterpenes		[ ]	1.3±0.7	0.81±0.41	1.4±1	0.77±0.72	**3.4±0.7a**	**1.4±0.5b**	**0.48±0.22b**	**0.57±0.39b**
		%	6±2.8	8±2.3	11±1	16±5	**10±1a**	**7.9±0.6b**	**5.7±1.4b**	**3.6±1.2b**
manool	20.82	[ ]	0.17	0.11±0.06	0.2±0.06	0.14±0.13	0.33±0.07	0.47±0.32	0.37±0.22	0.37±0.13
		%	2.1±0.1	3.9±1.2	4±2.2	4.3±2.3	**2.3±0.3b**	**6.1±2.7ab**	**17±5a**	**10±4ab**
diterpene 1	22.26	[ ]	1.4±0.8	0.7±0.4	1.8±1.5	0.6±0.6	**2.2±0.7a**	**0.7±0.5b**	**0.22±0.17b**	**0.44±0.36b**
		%	18±11ab	18±5a	18±6ab	10±0b	15±5	8.0±3.8	5.0±2.5	8.7±4.8
diterpene 2	22.84	[ ]	0.04±0.04	0.042±0.005	0.11±0.07	0.038	0.09±0.03	1.2±0.9	0.77±0.5	0.87±0.28
		%	**0.53±0.45b**	**0.78±0.06ab**	**1.4±0.1a**	**0.30**	**0.64±0.23b**	**15±8a**	**27±9a**	**24±9a**
diterpene 3	22.96	[ ]	0.11±0.02	0.072±0.034	0.13±0.09	0.37	**0.37±0.07a**	**0.15±0.05b**	**0.12±0.04b**	**0.06±0.02b**
		%	1.4±0.2	1.7±0.2	1.6±0.1	2.8	**2.7±0.3a**	**2.3±0.3ab**	**2.2±0.7ab**	**1.8±0.4b**
totarol	23.31	[ ]	4.8±10	1.6±0.7	4.3±2.8	4.1±4	**7.7±1.3a**	**2.7±1.5b**	**0.92±0.64b**	**0.99±0.49b**
		%	59±11	54±4	58±7	49±12	54±2	38±10	28±9	29±14
diterpene 4	23.45	[ ]	0.25±0.09	0.074±0.032	0.19±0.13	0.46	**0.51±0.11a**	**0.31±0.14ab**	**0.1±0.04b**	**0.1±0.03b**
		%	3.1±1.1	2.8±0.8	2.4±0.1	3.5	**3.7±0.8a**	**4.8±1.2a**	**3.5±0.4b**	**2.9±0.5b**
diterpene 5	23.77	[ ]	0.018±0.005	0.028	0.061±0.011	0.029	0.063±0.01	0.18±0.15	0.16±0.07	0.41±0.16
		%	0.22±0.07	6.4	1.4±0.9	0.22	**0.43±0.11bc**	**2.1±1.4b**	**5.8±2.8ab**	**11±6a**
diterpene 6	24.64	[ ]	0.044±0.018	0.041	0.15	0.040	**0.091±0.023ab**	**0.100±0.003a**	**0.03±0.003b**	**0.04±0.025bc**
		%	0.55±0.24	0.93	1.1	0.27	0.65±0.27	1.0±0.1	0.54±0.03	0.72±0.27
diterpene 7	24.93	[ ]	0.73±0.06	0.34±0.16	0.75±0.62	0.82±0.81	**1.9±0.4a**	**0.5±0.3b**	**0.27±0.2b**	**0.22±0.17b**
		%	9.1±0.9	11±1	8±2	12±0	**14±2a**	**5.1±2.5b**	**8.6±3.3ab**	**4.7±2.1b**
totarolone	25.45	[ ]	0.04±0.01	0.03	0.043±0.025	0.10	0.09±0.013	0.17±0.08	0.06±0.03	0.079±0.03
		%	0.49±0.07	0.75	0.65±0.16	0.78	**0.64±0.11b**	**3.9±2.1a**	**1.9±0.6ab**	**2.4±1.0ab**
hinokione	25.55	[ ]	0.45±0	0.2±0.09	0.46±0.39	0.46±0.42	**0.95±0.21a**	**0.83±0.28ab**	**0.32±0.11b**	**0.34±0.112b**
		%	5.6±0.1	6.8±0.4	4.7±1.5	21±14	6.5±0.4	14±4	8.4±0.4	9.4±3.0
total diterpenes		[ ]	8.0±0.1	3.1±1.4	8.1±5.8	6.6±6.5	**14±2a**	**7.2±2.8b**	**3.1±1.4b**	**3.8±1.2b**
		%	39±5	32±2	40±0.1	42±19	46±7	42±2	28±6	41±14
total terpenes		[ ]	21±3	9.3±3.9	10±8	7.5±7.1	**34±6a**	**18±7b**	**9.0±3.3b**	**13±5b**

Table 1a Mean concentrations (±SE) in mg g^{-1} dry weight and mean proportions (±SE) in %, relative to the terpene category, of the terpenes in the local phloem of cypresses infected with *S. cardinale*. RT=retention time. [ ]=concentration, %=proportion, NA=not available. Numbers and letters in bold type indicate statistically significant differences (REML, fixed=treatment, random=genotype, paired Tukey's post-hoc test, *P* < 0.05) and marginally significant differences (*P* < 0.10, in *italics*)

Name	RT (min)		DAY 30 control	DAY 30 Wounded	DAY 30 Mildly virulent	DAY 30 Highly virulent	DAY 90 control	DAY 90 Wounded	DAY 90 Mildly virulent	DAY 90 Highly virulent
α-thujene	7.73	[]	0.018±0.008b	0.11±0.02a	0.49±0.07a	0.68±0.33a	0.032±0.011b	0.11±0.05b	1.5±0.5a	1.8±0.5a
		%	0.16±0.04b	0.52±0.11b	1.4±0.3a	1.3±0.3a	0.30±0.06b	0.70±0.06b	2.4±0.4a	2.2±0.6a
α-pinene	7.83	[]	7.8±2.4	18±4.3	26.2±8.7	32±15	8.7±3.1b	8.8±3.8bc	45±19ab	54±12a
		%	78±6ab	82±9a	61±6b	67±6ab	77±10	67±13	68±4	68±5
α-fenchene	8.04	[]	0.18±0.09	0.21±0.06	0.81±0.4	1.2±0.9	0.15±0.07	0.21±0.15	0.8±0.3	0.8±0.2
		%	1.4±0.5	1.1±0.4	1.6±0.6	1.7±0.6	1.4±0.5	1.1±0.2	1.1±0.2	1.1±0.1
sabinene	8.33	[]	0.05±0.02b	0.48±0.06a	2.02±0.29a	2.9±1.5a	0.066±0.027b	0.42±0.21b	3±0.5a	2.6±0.5a
		%	0.51±0.11b	2.3±0.4b	6.1±1.6a	5.3±1.6a	0.58±0.12c	2.3±0.7b	6.7±2.0a	3.5±0.5ab
β-pinene	8.39	[]	0.13±0.07	0.38±0.04	0.35±0.08	0.48±0.22	0.22±0.1bc	0.3±0.14b	0.97±0.31ab	1.1±0.3a
		%	1.3±0.5ab	1.8±0.4a	1.0±0.2b	0.9±0.1b	1.7±0.7	2.0±0.3	1.7±0.2	1.4±0.3
β-myrcene	8.49	[]	0.12±0.07	0.36±0.02	0.67±0.16	0.98±0.52	0.26±0.09b	0.36±0.18b	2.1±0.7a	2.4±0.7a
		%	1.1±0.3	1.7±0.3	1.7±0.1	1.5±0.4	2.2±0.4ab	2.0±0.6b	3.5±0.3a	3.0±0.7a
δ-3-carene	8.78	[]	1.9±1	1.3±1.3	2.6±1.8	1.1±0.9	1.3±1.1	3.5±3.3	1.1±0.8	1.7±0.9
		%	14±7	7.7±7.3	5.1±3.2	2.0±1.4	12±8	19±11	4.6±4.2	3.4±2.3
limonene	9.01	[]	0.066±0.032	0.19±0.02	0.4±0.11	0.67±0.43	0.12±0.04b	0.2±0.1b	1±0.4a	1.3±0.3a
		%	0.68±0.18	0.93±0.18	0.97±0.08	0.97±0.27	0.98±0.25b	1.2±0.2b	1.7±0.1a	1.6±0.3a
terpinolene	9.67	[]	0.22±0.04b	0.41±0.1a	3.29±1.04a	3.9±2.2a	0.3±0.06b	0.56±0.33b	2.5±1ab	4±0.8a
		%	2.6±0.8b	2±0.6b	7.6±1.4a	6.5±1.0a	3.1±0.8b	3.4±0.6ab	4.5±0.8ab	6.3±2.3a
terpinen-4-ol	10.74	[]	0.006±0.001b	0.018±0.006b	1.4±0.4a	3.5±2.7a	0.019±0.005b	0.058±0.014b	2.5±1.2a	3.5±1a
		%	0.09±0.04b	0.08±0.02b	3.3±0.4a	4.0±2.2a	0.20±0.07b	0.27±0.01b	3.2±1.0a	4.2±1.2a
carvacrol methyl ether	11.50	[]	NA	NA	2.9±1.2	2.5±0.9	NA	0.032	1.5±1.3	2.8±0.9
		%	NA	NA	9.8±4.2	8.8±4.6	NA	0.19	1.8±1.5	4.1±1.3
α-terpinene	13.35	[]	0.025±0.01	0.03±0.002	0.25±0.07	0.27±0.14	0.052±0.012b	0.13±0.1b	0.73±0.28a	0.8±0.19a
		%	0.17±0.06	0.14±0.04	0.58±0.09	0.43±0.09	0.52±0.12	0.71±0.29	1.1±0.1	1.0±0.1
minor monoterpenes		[]	0.8±0.3bc	2.2±0.1b	13±3a	17±8ab	1.2±0.4b	2.3±1.2b	17±6a	21±4a
		%	8±1b	11±2b	34±6a	31±5a	11±2b	14±2b	28±3a	28±5a
oxygenated monoterpenes		[]	0.006±0.001b	0.018±0.006b	4.3±1.2a	6±2.7a	0.019±0.005b	0.074±0.002b	4.12±2.4a	6.3±1.6a
		%	0.088±0.043b	0.080±0.02b	13±4a	13±4a	0.20±0.07b	0.37±0.08b	5.0±2.4a	8.3±2.0a
total monoterpenes		[]	11±4	22±3	41±11	50±24	11±3b	15±7b	63±23a	77±13a
		%	41±3	45±4	49±5	49±4	22±1c	42±5b	54±2a	55±3a
α-cubebene	13.47	[]	0.56±0.23	0.76±0.08	2.3±0.9	1.7±0.9	1.8±0.5	1.3±0.5	3.8±1.5	3.3±0.5
		%	31±5ab	22±3b	38±5a	29±5ab	40±7	40±7	45±7	40±7
longifolene	14.99	[]	0.68±0.29	2.03±0.73	1.8±1	1.7±1.3	1.6±0.8	2.2±1.1	2.8±1.7	2.1±0.6
		%	37±8ab	56±17a	28±8b	27±11b	35±12ab	44±16a	36±10ab	25±6b
β-cedrene	15.10	[]	0.26±0.05b	0.74±0.3ab	1.11±0.22a	1.3±0.6a	0.62±0.2b	0.23±0.19b	0.8±0.4b	1.9±0.4a
		%	20±6	21±12	22±3	28±6	14±3ab	11±3b	15±1ab	21±3a
cedrol	17.49	[]	0.14±0.01	0.25±0.16	0.61±0.09	0.77±0.38	0.53±0.18ab	0.13±0.09b	0.44±0.23b	1.2±0.2a
		%	12±6	7.1±5.1	13±2	16±3	12±2	9±6	8.1±2.8	14±2
total sesquiterpenes		[]	1.6±0.6	3.5±0.6	5.9±2.1	5.6±2.3	4.5±1.1	3.7±1.7	7.7±2.9	8.5±0.8
		%	6.5±0.6	7.6±1.3	6.8±1.1	5.9±0.8	9.3±1.5a	9.4±0.8a	7.0±1.0bc	6.4±0.8b
manool	20.82	[]	0.23±0.09b	0.61±0.16ab	2.2±0.69ab	2.5±0.9a	1.7±0.9ab	0.7±0.3b	3.4±1.7a	3.1±0.8a
		%	1.7±0.1cd	2.6±0.3bc	7.9±2.7ab	8.0±0.7a	4.3±1.0ab	3.7±0.2b	6.5±1.4a	6.0±0.8a
diterpene 1	22.26	[]	1±0.4	2.2±0.6	3.9±1.7	3.4±1.8	2±0.6ab	1.7±0.8b	3.1±1.9ab	7±1.7a
		%	8.0±3.0	9.2±1.5	11±3	9±2	5.8±1.5	9.7±2.3	9±3	14±2
diterpene 2	22.84	[]	0.05±0.034b	1.6±0.5a	6.4±1.8a	8.2±2.4a	0.27±0.2b	1.4±0.7b	8.5±3.7a	9.6±2.6a
		%	0.8±0.7b	6.6±1.2a	23±8a	29±2a	0.57±0.33b	6.1±3.1b	17±3a	19±3a
diterpene 3	22.96	[]	0.34±0.11	0.62±0.12	0.54±0.09	0.51±0.16	1.2±0.4a	0.49±0.27b	0.95±0.38a	1±0.2ab
		%	2.8±0.2a	2.7±0.1a	1.9±0.3b	1.7±0.1b	3.4±0.2a	2.5±0.3ab	2.2±0.2b	2.2±0.3b
totarol	23.3	[]	7.8±2.6	12±2	10±3	8.5±3	19±5	9±4	16±6	18±3
		%	62±2a	52±1a	33±6b	27±2b	56±2a	51±4a	40±4b	38±4b
diterpene 4	23.45	[]	0.36±0.07b	0.94±0.13a	0.65±0.1ab	0.79±0.2ab	1.5±0.6	0.95±0.67	1.5±0.6	1.3±0.4
		%	3.2±0.5	4.6±1.4	2.3±0.3	3.2±1.0	4.2±0.4	4.3±1.5	3.5±0.4	2.7±0.4
diterpene 5	23.77	[]	0.027±0.005b	0.42±0.2a	0.91±0.32a	1.1±0.4a	0.31±0.21b	0.48±0.05b	0.6±0.3ab	1.2±0.4a
		%	0.27±0.10bc	1.6±0.6b	3.0±0.7ab	4.0±0.7a	0.70±0.32	1.9±0.5	1.7±0.5	2.5±0.6
diterpene 6	24.64	[]	0.032±0.01	0.22±0.07	1.1±0.5	1.3±0.9	0.053±0.016b	0.27±0.1ab	1±0.4ab	1.4±0.7a
		%	0.4±0.23b	0.92±0.16ab	3.5±1.4a	3.1±1.4a	0.12±0.01b	1.1±0.6ab	2.0±0.3ab	2.7±1.3a
diterpene 7	24.93	[]	1.4±0.7	1.7±0.2	1.9±0.7	1.6±0.7	4.2±1.3a	1.8±0.8b	3.2±1.1ab	2.7±0.3ab
		%	10±2a	7.4±0.3ab	5.8±1.6ab	4.4±1.2b	12±0a	11±1ab	8.2±0.7b	5.9±0.8c
totarolone	25.45	[]	0.13±0.04b	0.41±0.09a	0.36±0.1ab	0.42±0.04a	0.74±0.21b	0.39±0.11a	0.51±0.19ab	0.55±0.15ab
		%	1.5±0.7	1.8±0.1	1.5±0.6	2.0±0.7	2.1±0.1a	1.4±0.1ab	1.9±0.6ab	1.1±0.2a
hinokione	25.55	[]	1.1±0.2	2.3±0.4	2±0.2	2.4±0.6	3.8±1.3b	1.7±0.8a	3.4±1.1ab	3.1±0.6ab
		%	9.3±1.3	10±1	7.2±0.9	9.2±1.6	11±1a	9.0±0.5a	8.6±1.1ab	6.3±0.4b
total diterpenes		[]	13±4	23±4	30±7	31±11	35±10ab	18±9b	42±16a	49±9a
		%	52±4a	47±4ab	38±4b	38±4b	69±3a	49±5b	36±1c	34±2c
total terpenes		[]	25±8	48±7	82±18	93±39	51±14b	37±16c	117±43ab	140±22a

Table 1b Mean concentrations (±SE) in mg g^{-1} dry weight and mean proportions (±SE) in %, relative to the terpene category, of the terpenes in the local phloem of cypresses infected with *S. cardinale*. RT=retention time. [ ]=concentration, %=proportion, NA=not available. Numbers and letters in bold type indicate statistically significant differences (REML, fixed=treatment, random=genotype, paired Tukey's post-hoc test, $P < 0.05$) and marginally significant differences ($P < 0.10$, in *italics*)
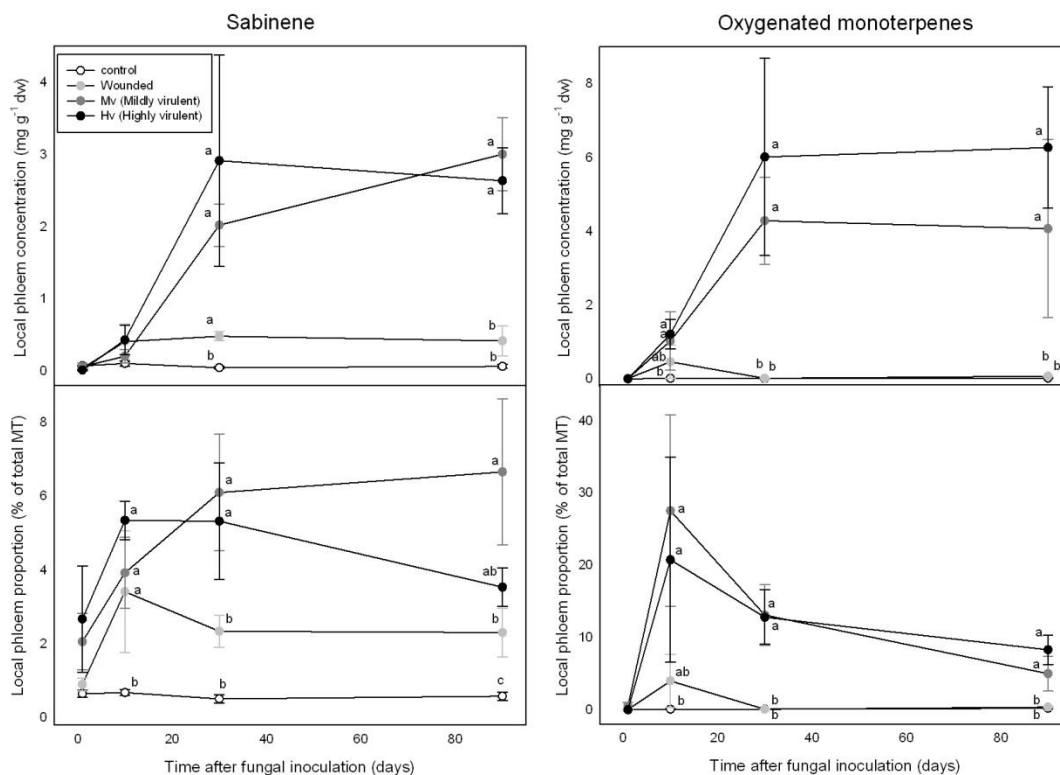
Figure 1. Mean phloem concentrations (±SE) and mean proportions (±SE) relative to total monoterpenes (MT) of sabinene and oxygenated monoterpenes (sum of terpinen-4-ol, thymyl methyl ether, and bornyl acetate), some of the most induced compounds in the infected treatments (Mv and Hv) relative to the control and Wounded. Different letters indicate statistically significant differences (REML, fixed=treatment, random=genotype, paired Tukey's post-hoc test, P < 0.05)

Two PCAs (Fig. 4) were conducted with phloem monoterpene concentrations and monoterpene proportions on days 30 and 90 as variables, to provide a general overview of the differences among treatments and infection times. In the concentration PCA, the first two PCs accounted for 69.1% and 11.0% of the total variance, respectively. PC1 distributed the cases by terpene concentration, separating Hv and Mv from Wounded and control treatments (two-way ANOVA of the PC scores, *P* < 0.05) and PC2 significantly separated the cases of day 30 from those of day 90 (*P* < 0.05). In the proportion PCA, the first two PCs accounted for the 36.3% and 20.4% of the total variance, respectively. PC1 significantly (*P* < 0.05) separated the cases with decreased proportion of main terpenes and increased proportion of minor terpenes, and PC2 also separated the cases of day 30 and day 90 (*P* < 0.05).
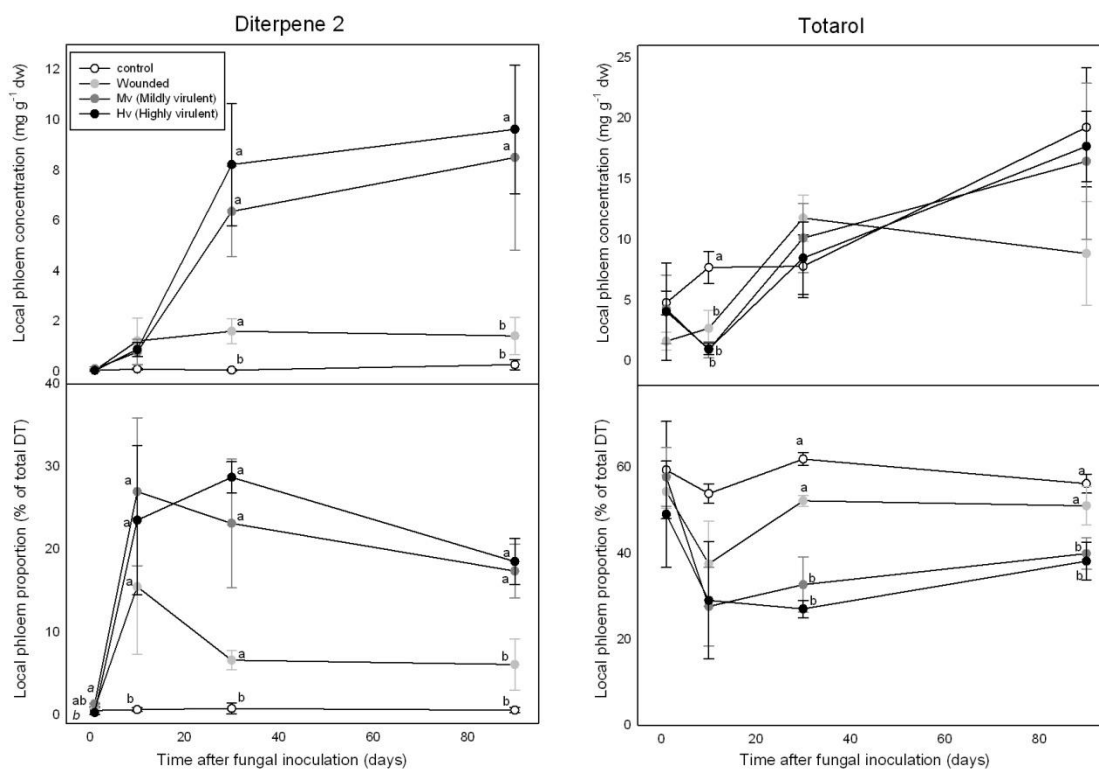
Figure 2 Mean phloem concentrations (±SE) and mean proportions (±SE) relative to total diterpenes (DT) of diterpene 2, and totarol. Different letters indicate statistically significant differences (REML, fixed=treatment, random=genotype, paired Tukey's *post-hoc* test, *P* < 0.05) and marginally significant differences (*P* < 0.10, in *italics*)

Fungal isolates

Mv and Hv did not elicit clearly different reactions. Statistically significant differences between terpene concentrations in the infected treatments were observed only for two sesquiterpenes. Cedrol was significantly higher in Mv than in Hv at day 1, and cedrol and β-cedrene were higher in Hv than in Mv at day 90 (Table 1).

Foliar terpene concentration

Leaves also presented abundant terpenes, with high concentrations of monoterpenes, moderate abundances of sesquiterpenes, and traces of diterpenes. No qualitative differences were found among treatments, and few quantitative differences in concentrations were observed (Table 2). No differences in concentration were detected

at day 1 (Table 2). At day 10, the control had higher concentrations of the sesquiterpenes α-cubebene, caryophyllene, germacrene D, α-muurolene, and total sesquiterpenes than did Hv. At day 30, no differences among treatments were found (Table 2). At day 90, the control had higher concentrations of β-myrcene, limonene, terpinolene, bornylene, and α-cubebene than did Wounded. No correlation was found between the concentrations (Table 2) and proportions (data not shown) of the terpene species analyzed. No direct differences were found between the fungal isolates. Hv had lower concentrations than the control in several occasions on day 10 (Table 2), while Mv concentrations were not different from the control or Wounded.

Foliar emission rates

The foliar emissions contained eight monoterpenes and two sesquiterpenes (Table 3, Fig. 5). No qualitative differences were found, but some quantitative differences appeared. The largest differences were in total monoterpene emissions and δ-3-carene (REML, fixed=treatment, random=genotype, paired Tukey's *post-hoc* test, $P < 0.05$), which were higher for the infected trees at day 10 than the control and Wounded. The proportions did not show any clear trend (data not shown).

At day 1, the emission rates of β-myrcene and limonene were higher in Wounded than in the control (Table 3). At day 10, δ-3-carene had a higher emission rate in Hv than the control and a marginally higher emission rate than in Wounded. α-Cedrene also had a marginally higher emission rate in Hv than in the control. Total monoterpenes showed higher emission rates in infected treatments than in the control. In contrast, the emission rate of β-pinene was marginally higher in the control than in Wounded. All compounds, except β-myrcene and δ-3-carene, had the highest emission rates in the Hv treatment at day 10. At day 30, differences were observed only in emission rates of sesquiterpenes; Hv had a higher foliar emission rate of longifolene than did Mv, and Wounded had a marginally significant higher emission rate of α-cedrene than did Mv. Finally, at day 90, α-cedrene had a higher emission rate in the control than in Wounded, and Mv, and β-pinene had a higher emission rate in Mv than in Hv (Table 3). Hv tended to elicit higher emissions and larger differences (sometimes statistically significant) relative to the control and Wounded than did Mv (Table 3, Fig. 5). Foliar concentrations and emissions appeared to be negatively correlated, but the correlations were not statistically significant. Only the correlation between total

monoterpene concentration and total monoterpene emission was significant for day 10 (simple regression; R^2 = 0.435, P < 0.05).
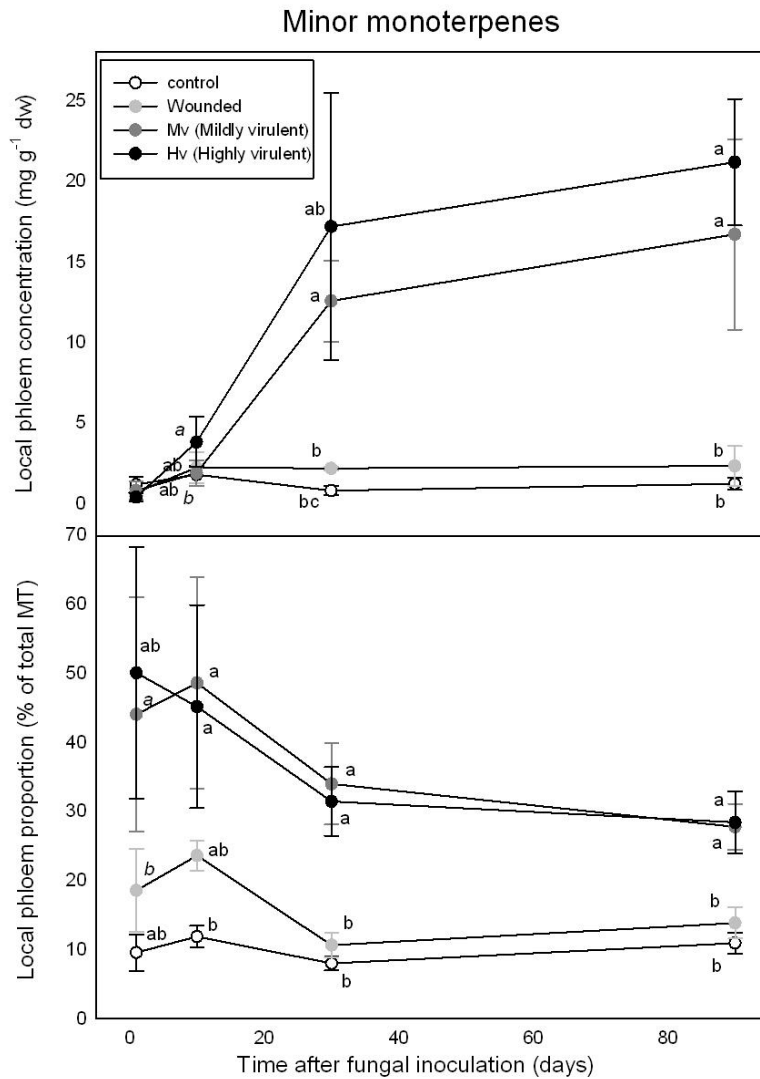
Figure 3 Mean phloem concentrations (±SE) and mean proportions (±SE) of minor monoterpenes (those <5% of total monoterpenes (MT): all except α-pinene at ca. 70% and δ-3-carene at ca. 20%). Different letters indicate statistically significant differences (REML, fixed=treatment, random=genotype, paired Tukey's *post-hoc* test, P < 0.05) and marginally significant differences (P < 0.10, in *italics*)
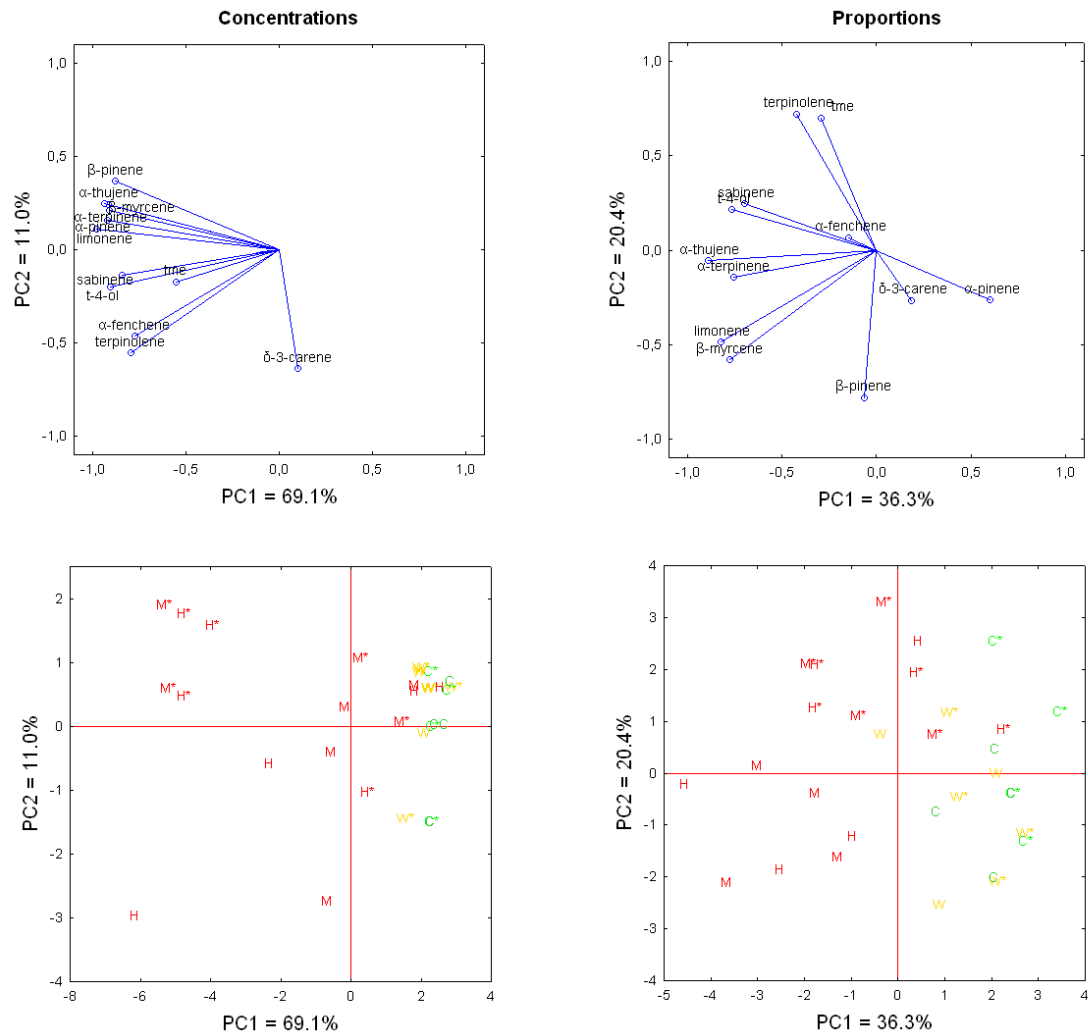
Figure 4 Principal Component Analysis (PCA) for the concentrations (mg g^{-1} of dry weight) (left panels) and proportions (% of total monoterpenes; right panels) of the 12 monoterpenes studied at days 30 and 90 after infection. The biplots depict loadings of PCA variables (above) and scores of PCA cases (below). T-4-ol = terpinen-4-ol, tme = thymyl methyl ether. Letters indicate the different treatments applied: C = Control (green), W = Wounded (yellow), M = Mildly virulent (red), H = Highly virulent (red). Samples of day 90 are marked with an asterisk (*), and samples of day 30 have no asterisk ( )

Discussion

Qualitative and quantitative changes in local phloem

Despite genotypic differences among trees and the different levels of pathogenicity of the fungal isolates, the same six terpenes appeared *de novo* only in the inoculated treatments at days 30 and 90, for all genotypes studied. Notably, four of these six compounds were oxygenated monoterpenes (oxygenated monoterpene 1, sabinene

Table first part (DAY 1 / DAY 10)

Name	RT (min)	DAY 1				DAY 10			
		control	Wounded	Mildly virulent	Highly virulent	control	Wounded	Mildly virulent	Highly virulent
tricyclene	7.68	0.053±0.022	0.041±0.017	0.059±0.005	0.054±0.014	0.074±0.041	0.032±0.014	0.058±0.025	0.028±0.020
α-thujene	7.72	0.20±0.15	0.24±0.21	0.05±0.02	0.04±0.01	0.84±0.79	0.40±0.37	0.34±0.31	0.16±0.15
α-pinene	7.82	12±5	8.7±3.9	11±4	12±5	15±7	7.8±3.0	13±5	6.2±4.1
α-fenchene	8.01	0.18±0.08	0.12±0.04	0.15±0.06	0.14±0.08	0.21±0.07	0.12±0.05	0.17±0.07	0.087±0.017
sabinene	8.32	0.63±0.48	0.67±0.59	0.23±0.05	0.17±0.04	1.9±1.7	1.0±0.9	1.1±0.9	0.46±0.39
β-pinene	8.39	0.17±0.06	0.11±0.04	0.17±0.05	0.15±0.06	0.23±0.08	0.13±0.04	0.18±0.06	0.081±0.044
β-myrcene	8.47	0.28±0.11	0.16±0.07	0.27±0.07	0.22±0.08	0.42±0.24	0.18±0.09	0.29±0.13	0.088±0.037
δ-3-carene	8.77	6.6±3.0	3.7±1.7	5.3±2.0	4.8±2.7	6.0±2.9	3.5±1.6	5.6±2.5	1.8±0.7
limonene	8.98	0.34±0.13	0.27±0.13	0.27±0.10	0.26±0.11	0.34±0.19	0.20±0.10	0.33±0.15	0.12±0.05
γ-terpinene	9.31	0.041±0.014	0.025±0.014	0.020±0.003	0.021±0.006	0.083±0.064	0.05±0.04	0.050±0.036	0.034±0.022
terpinolene	9.66	0.33±0.16	0.17±0.10	0.29±0.10	0.31±0.15	0.37±0.18	0.18±0.09	0.30±0.13	0.085±0.036
monoterpene 1	11.68	0.19±0.11	0.081±0.064	0.016±0.005	0.009±0.004	0.041±0.026	0.028±0.014	0.067±0.031	0.054±0.034
bornylene	13.13	0.062±0.038	0.037±0.03	0.045±0.018	0.046±0.026	0.061±0.034	0.037±0.021	0.046±0.04	0.011±0.005
α-terpinene	13.36	0.67±0.42	0.33±0.25	0.94±0.47	0.59±0.28	0.84±0.31	0.45±0.23	0.72±0.35	0.14±0.05
Total monoterpenes		21±9	15±6	18±7	19±8	26±9	14±5	22±7	9±4
α-cubebene	13.43	0.15±0.05	0.094±0.047	0.12±0.04	0.14±0.06	**0.32±0.17a**	**0.17±0.09ab**	**0.25±0.06ab**	**0.043±0.018b**
β-cedrene	15.1	0.12±0.03	0.070±0.032	0.17±0.09	0.22±0.06	0.18	0.14±0.12	0.14±0.06	0.105
caryophyllene	15.18	0.56±0.19	0.38±0.23	0.34±0.15	0.40±0.15	**0.85±0.46a**	**0.59±0.31ab**	**0.66±0.28ab**	**0.19±0.10b**
α-caryophyllene	15.74	1.3±0.6	0.89±0.58	0.81±0.4	1.2±0.5	2.2±1.2	1.8±1.0	2.2±1.2	0.56±0.29
germacrene D	16.13	2.6±0.9	1.6±0.9	2.0±0.8	3.3±1.1	**5.6±2.3a**	**3.4±1.4ab**	**4.0±1.3ab**	**1.5±0.8b**
α-muurolene	16.31	0.12±0.03	0.089±0.035	0.075±0.032	0.089±0.030	**0.25±0.10a**	**0.17±0.07ab**	**0.18±0.04ab**	**0.072±0.001b**
cedrol	17.48	0.32±0.06	0.16±0.07	0.40±0.16	0.44±0.22	0.41±0.25	0.36±0.20	0.32±0.18	0.15±0.12
Total sesquiterpenes		4.9±1.8	3.1±1.9	3.8±1.2	5.5±1.8	**9.5±4a**	**6.3±3ab**	**7.1±2.6ab**	**2.5±1.2b**
Total terpenes		26±10	18±7	22±8	24±9	36±12	20±7	29±9	11±5

Table second part (DAY 30 / DAY 90)

Name	RT (min)	DAY 30				DAY 90			
		control	Wounded	Mildly virulent	Highly virulent	control	Wounded	Mildly virulent	Highly virulent
tricyclene	7.68	0.071±0.035	0.081±0.015	0.13±0.026	0.11±0.04	0.091±0.008	0.083±0.021	0.092±0.01	0.06±0.027
α-thujene	7.72	0.29±0.23	0.31±0.27	0.07±0.02	0.06±0.02	0.21±0.16	0.063±0.021	0.040±0.003	0.04±0.02
α-pinene	7.82	16±8	16±5	23±8	19±8	19±5	17±6	24±2	17±8
α-fenchene	8.01	0.23±0.09	0.19±0.012	0.31±0.12	0.25±0.11	0.24±0.03	0.15±0.05	0.21±0.05	0.18±0.08
sabinene	8.32	1.1±0.9	1.1±0.8	0.40±0.03	0.34±0.13	0.83±0.61	0.26±0.06	0.19±0.03	0.15±0.06
β-pinene	8.39	0.24±0.10	0.23±0.04	0.36±0.12	0.35±0.16	0.28±0.06	0.15±0.07	0.30±0.03	0.23±0.11
β-myrcene	8.47	0.36±0.12	0.29±0.05	0.40±0.17	0.32±0.16	**0.41±0.04a**	**0.25±0.07b**	**0.37±0.04ab**	**0.28±0.12ab**
δ-3-carene	8.77	8.8±3.7	5.7±0.8	9.5±4.2	6.5±2.9	7.2±1.1	4.1±1.5	6.1±1.7	5.0±2.4
limonene	8.98	0.46±0.17	0.31±0.05	0.53±0.26	0.45±0.20	**0.51±0.10a**	**0.29±0.08b**	**0.41±0.12ab**	**0.36±0.18ab**
γ-terpinene	9.31	0.039±0.025	0.051±0.031	0.032±0.011	0.024±0.007	0.05±0.02	0.021±0.004	0.027±0.004	0.018±0.008
terpinolene	9.66	0.38±0.14	0.28±0.04	0.50±0.22	0.33±0.16	**0.48±0.05a**	**0.26±0.07b**	**0.39±0.07ab**	**0.31±0.15ab**
monoterpene 1	11.68	0.019±0.004	0.011±0.007	0.013±0.001	0.018±0.011	0.013±0.004	0.015±0.003	0.034±0.024	0.014±0.013
bornylene	13.13	0.074±0.032	0.049±0.014	0.094±0.046	0.053±0.025	**0.080±0.017a**	**0.029±0.014b**	**0.063±0.024ab**	**0.048±0.026ab**
α-terpinene	13.36	0.93±0.39	0.68±0.10	1.6±0.8	1.2±0.7	1.0±0.2	0.44±0.15	0.89±0.24	0.57±0.27
Total monoterpenes		29±11	25±3	37±14	29±12	30±5	23±7	33±3	18±10
α-cubebene	13.43	0.25±0.03	0.28±0.093	0.27±0.12	0.29±0.28	**0.16±0.03a**	**0.087±0.068b**	**0.15±0.03ab**	**0.13±0.06ab**
β-cedrene	15.1	0.19±0.10	0.14±0.02	0.42±0.29	0.38±0.29	0.19±0.04	0.11±0.05	0.25±0.09	0.15±0.09
caryophyllene	15.18	0.43±0.21	0.43±0.12	0.63±0.40	0.39±0.25	0.63±0.25	0.40±0.14	0.49±0.16	0.27±0.19
α-caryophyllene	15.74	1.3±0.7	1.3±0.4	1.6±0.9	1.0±0.6	1.9±0.8	1.2±0.4	1.2±0.5	0.83±0.62
germacrene D	16.13	2.2±0.9	3.0±0.3	3.7±1.3	2.4±0.8	3.7±1.0	3.3±1.0	3.2±0.8	1.7±0.8
α-muurolene	16.31	0.078±0.032	0.11±0.02	0.14±0.05	0.095±0.035	0.12±0.04	0.097±0.028	0.10±0.02	0.066±0.037
cedrol	17.48	0.69±0.32	0.45±0.05	1.3±0.7	1.2±0.89	0.56±0.14	0.24±0.13	0.55±0.26	0.4±0.3
Total sesquiterpenes		5.1±1.9	5.6±0.7	7.9±2.9	5.5±2	7.3±1.9	5.3±1.5	5.7±1.1	2.7±1.5
Total terpenes		34±12	31±3	45±17	34±14	38±5	29±9	38±4	28±15

Table 2 Mean concentrations (±SE) in mg g^{-1} dry weight of the terpenes in the leaves of cypresses infected with *S. cardinale.* RT=retention time. Numbers and letters in bold type indicate statistically significant differences (REML, fixed=treatment, random=genotype, paired Tukey's *post-hoc* test, $P < 0.05$)

hydrate, camphor, and α-terpineol), a class of terpenoids noted for strong antifungal activity, usually more fungistatic than non-oxygenated monoterpenes. (Bakkali et al. 2008b; Hussain et al. 2011; Jiao et al. 2012; Zouari et al. 2011). Most of the *de novo* compounds were detected in relatively low concentrations (0.09-0.36 mg g^{-1} dw) except for *de novo 3*, a diterpene that had a mean concentration of 5.4 mg g^{-1} but was rarely detected. We were not able to detect cupressotropolone A and B, two sesquiterpene phytoalexins of fungal-infected cypresses discovered by Madar et al. (1995a) using thin layer chromatography (TLC).

The scarce information that is available for the role of sabinene hydrate in tree defense and fungal inhibition (Ramos et al. 2011; Tomlin et al. 2000) suggests that this compound might have moderate defensive and antifungal activity. The role of camphor (Kotan et al. 2007; Marei et al. 2012; Pragadheesh et al. 2013; Ramsewak et al. 2003b) is ambiguous, being inhibitory for some fungi but not for others, suggesting slight fungal toxicity. α-Terpineol, however, is a powerful fungal inhibitor (Cakir et al. 2004; Hammer et al. 2003; Kossuth and Barnard 1983; Kotan et al. 2007; Kusumoto et al. 2014; Zhou et al. 2014) Thymyl methyl ether is among the least inhibitive chemical structures of thymol to several fungi (Kumbhar and Dewang 2001).

The only *de novo* terpenes known to be produced by Italian cypress in response to a fungal pathogen are the oxygenated sesquiterpenes cupressutropolone A and B, produced under infection by *Diplodia pinea,* another canker-causing fungus (Madar et al. 1995a)*.* These two sesquiterpenes are considered *C. sempervirens* phytoalexins, because they cause strong or total inhibition of mycelial growth and spore germination for *S. cardinale* and other cypress pathogens (Madar et al. 1995a). The *de novo* compounds we found could, thus, likely be antifungal phytoalexins because *i*) sabinene hydrate, camphor, and α-terpineol appeared exclusively in the infected treatments, *ii*) they are oxygenated monoterpenes, *iii*) their antifungal activity has been reported in literature (especially α-terpineol), and *iv*) the report by Madar et al. (1995a)*.* The possibility that these *de novo* compounds (especially α-terpineol and camphor) are a product or a biotransformation of the infecting fungal pathogen, however, cannot be discarded (Kusumoto et al. 2014; Leufvén et al. 1988; Siddhardha et al. 2011; Tan and Day 1998). Furthermore, any terpene concentration found in the infected treatments could have been altered by fungal biotransformation or production.

The increased terpene concentrations in the local phloem tissues of the infected treatments were expected because resinosis from the cracks of infected tissues is a common symptom of cankered cypresses (Graniti 1998). This phenomenon has been observed in numerous studies that address the reaction of conifer phloem and xylem to infection by fungal pathogens (Blodgett and Stanosz 1998; Bonello et al. 2008; Faldt et al. 2006; Raffa and Smalley 1995; Viiri et al. 2001). In our study, the monoterpenes, well-known inhibitors of fungi mycelial growth and spore germination (Bakkali et al. 2008b; Kalemba and Kunicka 2003), and diterpenes, which also have strong antifungal activity (Eberhardt et al. 1994; Kopper et al. 2005; Kusumoto et al. 2014a), were the most reactive terpenoid groups in the phloem. The oxygenated monoterpenes were the most induced terpenoid category (Table 1, Fig. 1), increasing their concentrations up to 1000-fold in infected trees relative to control and up to 333-fold relative to Wounded. The concentration decreases observed at day 10 for some of the major monoterpenes, all sesquiterpenes, and several abundant diterpenes (Table 1, Fig. 1) were unexpected. Concentration decreases for several compounds also have been observed, however, in other pathosystems (Boone et al. 2011; Davis and Hofstetter 2011), and at least one general decrease in terpene concentration also has been reported (Bonello et al. 2008). At day 10, the few compounds that increased in concentration showed an abrupt increase in proportion, and they were the same compounds that were most induced throughout this study, such as α-thujene, sabinene, terpinolene, manool, diterpene 2, and diterpene 5. By decreasing concentrations of the main compounds and by slightly increasing the concentrations of some induced terpenes, proportions of the induced compounds can increase drastically (see terpinolene and diterpene 2 in Table 1). This strategy might be a fast and cheap way of producing the desired terpene proportions rapidly, rather than by strongly increasing the concentrations of these induced compounds.

α-Thujene, sabinene, terpinolene, terpinen-4-ol, manool, and diterpenes 2 and 5 responded most to *S. cardinale* infection. The information available for α-thujene (Raffa and Berryman 1982b; Zhao et al. 2010) suggests that conifers do not use it as a defensive compound, but it may have some antifungal activity (Bajpai et al. 2007). Sabinene (De Alwis et al. 2009; Espinosa-garcia and Langenheim 1991; Kohzaki et al. 2009) and terpinolene (Davis et al. 2011; Viiri et al. 2001) are among the most induced compounds in some conifers under fungal attack, and possess antifungal properties against several phytopathogens and fungal endophytes (Bridges 1987; De Alwis et al.

2009; Espinosa-garcia and Langenheim 1991; Kohzaki et al. 2009; Paine and Hanlon 1994). Herbicide application also can increase the concentration of terpinen-4-ol in *P. ponderosa* (Kidd and Reid 1979), a compound with remarkable biological activity on fungi (Kusumoto et al. 2014; Morcia et al. 2013; Nenoff et al. 1996) and bacteria (Kotan et al. 2007). Manool concentrations can increase in conifers under biotic attack (Hanari et al. 2002; Tomlin et al. 2000), and can inhibit growth of several canker agents (Yamamoto et al. 1997) and pathogenic bacteria (Ulubelen et al. 1994). In our study, the concentrations and proportions of two unidentified compounds, diterpenes 2 and 5, increased substantially in infected trees (Table 1, Fig. 2) and may play a role in cypress defense, thus warranting further efforts to identify them.

The concentrations and proportions of the minor monoterpenes increased in the infected treatments at the expense of the two main monoterpenes, α-pinene and δ-3-carene (their sum represented more than 90% of the monoterpene fraction in the control), which significantly decreased in proportion to 50-70% (Table 1, Fig. 3). The proportions PCA (Fig. 4) corroborates these observations, showing the main monoterpenes going in opposite direction to minor terpenes. Proportional changes also were observed in the diterpenes, where that of totarol, the main compound of the diterpene fraction, decreased from 50-60% in the control to 30% in infected treatments (Table 1, Fig. 2) primarily in favor of diterpene 2 and manool. These results, thus, suggest that infected cypresses invest more in minor compounds than in major ones. This strategy had been observed in *Picea abies*, *Abies grandis*, and *Pinus resinosa*, where their main monoterpenes (pinenes), lowered proportions in infected trees in favor of minor monoterpenes such as sabinene and terpinolene (Klepzig et al. 1995; Raffa and Berryman 1982b; Zhao et al. 2010). Some tree terpenes (usually the main compounds) have low inhibiting effects (Kusumoto et al. 2014) or can even enhance the growth of some fungal pathogens (Bridges 1987; Cakir et al. 2004; Davis and Hofstetter 2011), because some pathogenic fungi have developed the ability to survive in the presence of the major compounds of their common hosts, detoxifying them or even exploiting them as carbon sources (Kusumoto et al. 2014; Wang et al. 2013). One plausible hypothesis accounting for our results is that a strong concentration and proportion increase of minor terpenes in infected cypresses would help to lower the success of *S. cardinale* infection or slow its growth considerably, thereby allowing the tree to react effectively, at least in resistant varieties.

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The absence of differences between Mv and Hv suggests that *C. sempervirens* cannot distinguish between these two *S. cardinale* isolates. The short time period that this conifer and fungus have coexisted suggests that co-evolution or a capacity to elicit specific responses in their interactions is unlikely. Hv tended to elicit slightly (non-significantly) higher reactions compared to Mv, but probably due to the aggressiveness of the isolate and not to a specific reaction of the tree against it. Further study should compare the terpene reaction of *C. sempervirens* to different canker species or similar fungal pathogens to determine if the tree reaction elicited by *S. cardinale* is species-specific or just a general pathogen defense.

The main mechanism of reaction to *S. cardinale* infections in cypresses is based on formation of a necrophylactic periderm, a quantitative (polygenic) trait that in resistant trees is able to compartmentalize and prevent fungal growth in bark tissues. Resistant and susceptible trees differ in the speed of reaction (how quickly they can build the barrier) and in the thickness (number of cell rows) of the barrier and its rate of suberization (Ponchet and Andreoli 1990). This mechanism is not specific against a particular fungus but is the same that is activated by cypresses as a consequence of a simple wound (without infection). This mechanism is disturbed by an invading fungus in infected trees. The production of inhibiting terpenes induced by infection in more resistant trees might affect the 'struggle' between host and pathogen, shifting this equilibrium by slowing fungal development and favoring the host to build an effective pathogen barrier. The terpene compounds found in the phloem of *C. sempervirens* were consistent with those found in previous studies (Gallis et al. 2007; Piovetti et al. 1981; Piovetti et al. 1980). Concentrations also were within the ranges of those in similar studies of other conifers infected by fungal pathogens (Blodgett and Stanosz 1998; Raffa and Berryman 1982b; Viiri et al. 2001).
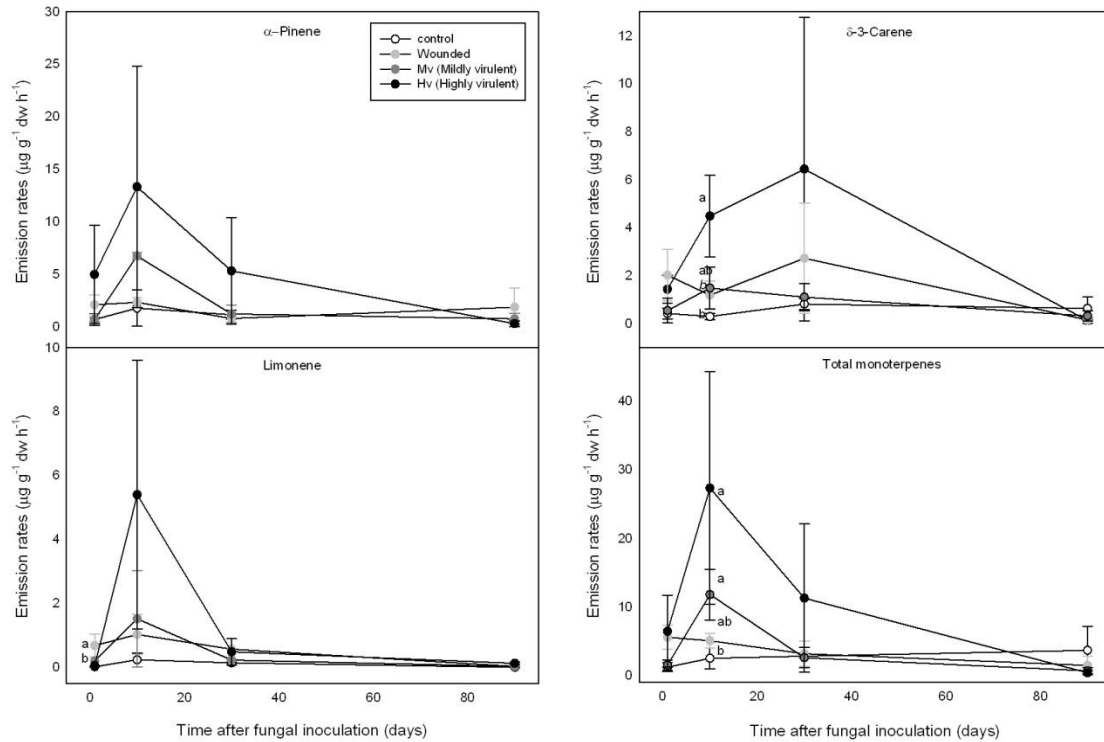
Figure 5. Mean rates of emission (±SE) of main monoterpenes emitted by leaves. Different letters indicate statistically significant differences (REML, fixed=treatment, random=genotype, paired Tukey's *post-hoc* test, $P < 0.05$)

Foliar terpene concentration

Terpene species and the foliar proportions in our study coincided with those in Schiller and Madar (1991), who reported that α-pinene and δ-3-carene were the most abundant terpenes. Mazari et al. (2010) also observed α-pinene as the main compound, but limonene was the second most abundant, and δ-3-carene was among the minor monoterpenes.

None of the compounds or tendencies for the infected treatments in our study, however, behaved similarly to those reported in Schiller and Madar (1991). The only trend in our study was a lower foliar concentration in Hv and Wounded than in the control cypresses (Table 2). No compound showed a consistent trend throughout the 90-day experiment. The inconsistencies between our study and that by Schiller and Madar (1991) suggest that leaves may not show a clear pattern of changes in terpene

Name	RT (min)	Day 1				Day 10			
		control	Wounded	Mildly virulent	Highly virulent	control	Wounded	Mildly virulent	Highly virulent
α-thujene	6.53	0.015±0.005	0.18±0.15	0.098±0.082	0.072±0.036	0.16	0.055±0.012	0.086±0.046	1.23±0.92
α-pinene	6.70	0.69±0.54	2.1±0.9	0.70±0.23	5.0±4.7	1.8±1.7	2.3±0.5	6.8±0.3	13±12
camphene	6.82	0.022±0.020	0.10±0.05	0.050±0.003	0.045±0.031	0.13±0.11	0.078±0.022	0.21±0.11	1.2±1.1
sabinene	7.15	0.031±0.017	0.32±0.28	0.29±0.28	0.28±0.22	0.12	0.15±0.11	0.084±0.032	1.1±1.0
β-pinene	7.17	0.077	0.089±0.011	0.059±0.023	0.18	**0.96±0.65a**	**0.22±0.15b**	**0.56±0.44ab**	**1.4±0.7ab**
β-myrcene	7.22	**0.012±0.004b**	**0.26±0.08a**	0.15	0.20±0.13	0.024	0.089±0.002	0.41±0.31	0.31
δ-3-carene	7.64	0.43±0.23	2.0±1.1	0.55±0.52	1.5±0.6	**0.30±0.13b**	**1.2±0.6b**	**1.5±0.9ab**	**4.5±1.7a**
limonene	7.70	**0.029±0.019b**	**0.69±0.36a**	0.21	0.069	0.24±0.22	1.0±0.6	1.5±1.5	5.4±4.2
longifolene	13.31	0.056±0.023	0.14±0.12	0.030	NA	NA	0.30±0.23	0.94	0.92±0.71
α-cedrene	13.42	0.37±0.34	0.51±0.38	0.11	0.139	**0.19±0.16b**	1.0	1.8	**1.7±1.2a**
Total monoterpenes		1.2±0.7	5.6±1.7	2.1±0.8	6.5±5.3	**2.5±1.5b**	**5.1±1.1ab**	**12±4a**	**27±17a**
Total terpenes		1.4±0.6	6.1±1.7	2.2±0.9	6.5±5.3	2.6±1.5	5.6±1.5	13±5	30±19

Name	RT (min)	Day 30				Day 90			
		control	Wounded	Mildly virulent	Highly virulent	control	Wounded	Mildly virulent	Highly virulent
α-thujene	6.53	0.13±0.06	0.046±0.031	0.14±0.13	0.10±0.087	0.001	0.022	0.020	NA
α-pinene	6.70	1.7±0.8	0.75±0.29	1.3±0.82	5.3±5.1	NA	1.9±1.8	0.76±0.55	0.30±0.29
camphene	6.82	0.27±0.24	0.027±0.015	0.031±0.026	0.14±0.12	0.053±0.05	0.016±0.014	0.027	0.013±0.012
sabinene	7.15	0.49±0.46	0.084±0.043	0.27±0.23	0.26±0.23	0.015±0.011	0.049±0.035	0.029±0.019	0.003±0.002
β-pinene	7.17	0.041±0.008	0.15	0.083±0.042	0.16±0.14	**0.029±0.027ab**	**0.025**	**0.027±0.025b**	**0.011±0.008a**
β-myrcene	7.22	0.22±0.11	0.25±0.10	0.15±0.021	0.47±0.45	0.010	NA	0.04±0.038	0.005
δ-3-carene	7.64	1.0±0.2	2.6±2.3	1.3±0.6	6.5±6.3	0.64±0.48	0.16±0.06	0.33±0.21	0.14±0.12
limonene	7.70	0.16±0.03	0.46	0.27±0.01	0.49±0.41	0.011±0.009	0.037	0.012±0.009	0.14±0.04
longifolene	13.31	**0.12±0.02ab**	0.052	**0.018±0.007b**	**0.25±0.22a**	0.024	0.006±0.001	0.16±0.16	0.008±0.007
α-cedrene	13.42	**0.19±0.11ab**	**0.27±0.13a**	**0.069±0.052b**	**0.57±0.49ab**	**0.064±0.004a**	**0.016±0.001b**	**0.012±0.002b**	0.026
Total monoterpenes		3.8±0.8	3.0±1.8	2.9±1.5	11.3±10.8	3.7±3.5	1.5±1.3	0.69±0.42	0.40±0.30
Total terpenes		3.9±0.7	4.6±1.9	3.0±1.5	12±11	3.7±3.5	1.5±1.3	0.82±0.46	0.42±0.30

Table 3 Mean terpene emission rates (±SE) in µg g^{-1} dry weight h^{-1} of terpenes emitted by leaves of cypresses infected with *S. cardinale*. RT=retention time. NA=not available. Numbers and letters in bold type indicate statistically significant differences (REML, fixed=treatment, random=genotype, paired Tukey's *post-hoc* test, $P < 0.05$) and marginally significant differences ($P < 0.10$, in *italics*)

concentrations when infected by *S. cardinale.* The lack of differences among our treatments may have several explanations. The constitutive foliar chemotype of Agrimed is very different from those of the other resistant genotypes, and reaction patterns seemed to differ among the genotypes. The distance of the twig from the fungal infection, which varied from 3 to 21 cm, also was not correlated with foliar terpene concentration. The lower terpene concentrations in leaves may have been due to increased foliar emission. However, only a statistically significant relationship, between total monoterpene emission and total monoterpene concentration of day 10, was found, so our results do not provide enough support for this hypothesis. In addition, the inhibition of photosynthesis caused by *S. cardinale* may have affected terpene concentrations (Muthuchelian et al. 2005; Penuelas and Llusia 1999).

Foliar emission rates

Foliar terpene emission rates of the control ranged between 2 and 4 µg g^{-1} dw h^{-1}, similar to rates reported by Yatagai et al. (1995) and Yani et al. (1993) for the same species. The compounds detected also were similar to those in the previous two

studies, but the monoterpene proportions were similar only to those in Yani et al. (1995). Yatagai et al. (1993) reported that limonene was responsible for 83% of the emission blend, however, limonene represented only ca. 4% of the emissions in the control in this current study (Table 3, Fig. 4).

The sampled leaves could represent only systemic responses to infection (twigs were up to 21 cm from the inoculated zone), but the infected plants usually displayed higher emissions than the control and sometimes the Wounded plants. These higher emissions were statistically significant, however, only at day 10 after inoculation (for δ-3-carene and total monoterpenes). Many other compounds showed a non-significant highest emission at day 10, possibly indicating that their maximum emission in response to *S. cardinale* infection occurs around this time. This change in volatile bouquet could be used by the vectors of cypress bark canker, such as *Phloeosinus aubei* (Covassi et al. 1975), *Megastigmus Watchli*, or *Orsillus maculatus* (Battisti et al. 1999; Zocca et al. 2008), or even parasitoids of these vectors (Adams and Six 2008; Boone et al. 2008; Sullivan and Berisford 2004).

In summary, all resistant genotypes of Italian cypress reacted strongly and similarly to *S. cardinale* infection by drastically increasing the phloem concentrations of several minor terpenes and moderately increasing the concentrations of major terpenes. This translated into moderate increases in total concentrations. Monoterpenes (especially the oxygenated monoterpenes, which increased quantitatively but also may be generated *de novo* in response to infection) and diterpenes were the most induced terpene classes in the infected trees, thus leading to a considerable proportional increase in minor monoterpenes and a consequent proportional decrease in the main monoterpenes. Such a strategy could help cypress defense, because some pathogens are adapted to the principal constituents of trees. Foliar concentrations did not show any clear trend apart from a concentration decrease in the infected treatments, which may have been due to a canker-induced inhibition of photosynthesis or a decrease due to increased emissions. Emission rates of foliar terpenes suggest that emission bouquets change under infection, opening the possibility of attracting *S. cardinale* vectors. The emission rates of foliar terpenes and several phloem proportions of oxygenated monoterpenes, terpinolene, and manool among others, reacted quite quickly, reaching their maximum proportions between days 1 and 10, while proportions of most phloem terpenes (α-thujene, α- pinene,

sabinene, or totarol) continued to increase during infection, peaking around day 30 or 90. No clear differences were found between the fungal isolates for any tissue examined, despite trends suggesting that a slightly stronger reaction was elicited by the more virulent fungal isolate (Hv).

This study is the first to describe the complex dynamics of the terpene reaction of *C. sempervirens* to *S. cardinale* in the early stages of infection. The results raise questions that warrant further research. Such studies should compare terpene and physiological reactions of *C. sempervirens* clones that are susceptible and resistant to bark canker, identify unknown induced compounds (e.g., diterpenes 2 and 5), and test Italian cypress terpenes against *S. cardinale* in experiments of growth inhibition and fungal biotransformation. In relation to indirect defenses, further research should study the emissions of cankered cypresses ca. 10 days after inoculation and test the attraction of several potential pathogen vectors to foliar terpene emissions.

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Chapter 5. Terpene arms race in the *Seiridium cardinale – Cupressus sempervirens* pathosystem

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Abstract

The canker-causing fungus *Seiridium cardinale* is the major threat to *Cupressus sempervirens* worldwide. We investigated the production of terpenes by canker-resistant and susceptible cypresses inoculated with *S. cardinale*, the effect of these terpenes on fungal growth, and the defensive biotransformation of the terpenes conducted by the fungus. All infected trees produced *de novo* terpenes and strongly induced terpenic responses, but the responses were stronger in the canker-resistant than the susceptible trees. *In vitro* tests for the inhibition of fungal growth indicated that the terpene concentrations of resistant trees were more inhibitory than those of susceptible trees. The highly induced and *de novo* terpenes exhibited substantial inhibition (more than a fungicide reference) and had a high concentration-dependent inhibition, whereas the most abundant terpenes had a low concentration-dependent inhibition. *S. cardinale* biotransformed three terpenes and was capable of detoxifying them even outside the mycelium, in its immediate surrounding environment. Our results thus indicated that terpenes were key defences efficiently used by *C. sempervirens* but also that *S. cardinale* is ready for the battle.

Keywords: Terpene induction – Antifungal activity- Growth inhibition-Biotransformation – Detoxification

Introduction

Terpenes, among the main defences of conifers, act as a first line of defence against biological agents and are usually strongly induced when trees are infected by bark-beetle/fungal pathogen complexes (Phillips and Croteau 1999; Raffa and Smalley 1995). Terpene profiles are strongly genetically controlled, and conifers can differ greatly in their constitutive terpenes and defensive responses, depending on tree provenance, population, or variety (Schiller 1990; Woodward et al. 2007). Some studies have attempted to correlate terpenes with resistance in Pinaceae tree varieties against fungal pathogens, and even though links between pathogen resistance and increased terpene concentrations have been reported (Raffa and Berryman 1982; Zhao et al. 2010), a consensus has not been reached due to other conflicting reports (Woodward et al. 2007; Rockwood 1973). The ability of terpenes to inhibit spore germination and the growth of fungal pathogens is well known (Bakkali et al. 2008; Dixon 2001). The inhibition caused by arbitrary concentrations of terpenes (especially monoterpenes (MTs)) has been tested on conifer pathogens, but experiments studying the effects of *in planta* concentrations are rare (Andrews et al. 1980; Sherwood and Bonello 2013). In the context of an arms race with trees, though, several specialised pathogenic fungi possess mechanisms of terpene biotransformation and detoxification (Kusumoto et al. 2014; Wang et al. 2014) and in some cases can even exploit these terpenes as carbon sources for their growth (Martínez-Iñigo et al. 2000; Wang et al. 2013). We still know little about terpenoid synthesis and biotransformation in fungi, with only three biotransformative pathways fully described genetically and enzimatically (Marmulla and Harder 2014). The biotransformation of terpenoids has been studied in only a few fungal pathogens of Pinaceae (Ekman and Sjoholm 1979; Kusumoto et al. 2014), *Grosmannia clavigera* in particular (Lah et al. 2013; Wang et al. 2013), so our understanding of fungal resistance to terpenes remains very poor, despite it is crucial to understand any conifer pathosystem.

Seiridium cardinale is the main agent of cypress canker, a severe pandemic disease responsible for significant mortality in *Cupressus sempervirens* and most species of Cupressaceae worldwide (Danti et al. 2013). The fungus is disseminated over short distances by airborne rainwater, and insect vectors may be responsible for its spread over longer distances (Battisti et al. 1999; Covassi et al. 1975) (Fig. 1). The hyphae of *S. cardinale* infect the phloem, parenchyma, and cambium, occupying

intercellular spaces and attacking cells with enzymes that degrade cell walls (Gonthier and Nicolotti 2013). *S. cardinale* secretes several phytotoxins (Magro et al. 1982), such as sesquiterpenes (STs) that cause systemic chlorosis and browning of leaves and uninfected plant tissues (Ballio et al. 1991; Evidente et al. 1993). The phloem of infected canker-resistant trees produce *de novo* MTs and strongly induce several minor MTs and diterpenes (DTs) (Achotegui-Castells et al. 2015), but information about non-resistant cypresses remains unavailable. Regarding fungal growth inhibition, only one study had described how two ST phytoalexins produced by cypresses infected by *Diplodia pinea* f.sp. *cupressi*, another canker-causing fungal pathogen, can strongly inhibit *S. cardinale* germination (Madar et al. 1995). To our knowledge, no other terpenes of *C. sempervirens* have been tested, and the terpene biotransformation capacity of this fungus has never been investigated. To fill these gaps in our understanding of the arms race between the tree and the fungus, we studied the terpenic composition and response of *C. sempervirens* trees selected for resistance against canker (Agrimed) and trees not selected for resistance (NR) to *S. cardinale* infection using gas chromatographic-mass spectrometric (GC-MS) analyses of control, wounded, and infected phloem tissues. We then used *in vitro* growth inhibition tests using both *in planta* and arbitrary concentrations to determine the antifungal activity of 15 terpenes in healthy and cankered *C. sempervirens*. We also studied the biotransformative and detoxificant capabilities of *S. cardinale* inside (hyphae, H) and outside (hyphae-free, HF) the mycelium with GC-MS analyses of *in vitro* inhibition test plugs*.*

Materials and methods

Terpene concentrations

Plant and fungal material

Thirty-six five-year-old grafted *Cupressus sempervirens* L. trees grown in pots were divided into two groups: 18 were not selected for resistance to cypress bark canker (NR) and 18 were the Agrimed nº1 (Panconesi and Raddi 1991) cultivar (hereafter Agrimed) patented for canker resistance. The plants were maintained under a shedding tunnel at ISZA-CRA in Firenze (Italy) and were watered daily. The *S. cardinale*

(Wagener) Sutton & Gibson standard isolate ATCC 38654 was used for the artificial inoculations and the inhibition and biotransformation tests. The fungus was grown on malt agar extract (MEA) at 25 ºC in the dark for 15 days.

Inoculation and sampling

Three treatments were applied to both tree groups in August 2013: control (no inoculation wound, no fungus), wounded (inoculation wound, no fungus), and infected (inoculation wound + fungus). The phloems of three randomly chosen main branches of the trees were inoculated following standard procedures (Achotegui-Castells et al. 2015). Each treatment had six replicates, and each replicate consisted of three sub-replicates (three branches). Phloem tissues were sampled 30 days after inoculation, kept in liquid nitrogen and stored in a -20 ºC freezer.

Sample analyses and terpene identification

The phloem sub-samples of each replicate were bulked and ground with a pestle in 50-ml Teflon tubes containing liquid nitrogen to avoid evaporation and facilitate the grinding. One ml of pentane containing dodecane (internal standard) was added to the ground tissues, and the solution was stored overnight at -20 ºC. Three-hundred µl of the supernatant were analysed by GC-MS. The Teflon tubes were dried to constant weights, weighed in a precision balance, cleaned thoroughly, dried, and reweighed to tare the tubes. One blank was analysed for every six samples.

Two microlitres of the phloem extract were injected into a capillary column (HP 5MS, 30 m × 0.25 µm × 0.25 mm) of a GC (7890A, Agilent Technologies, Santa Clara, USA) with an MS detector (5975C inert MSD with Triple-Axis Detector, Agilent Technologies). Initial temperature was maintained at 35 ºC for two minutes, increased at 15 ºC min^{-1} to 150 ºC and maintained for 5 min, thereafter at 30 ºC min^{-1} to 250 ºC and maintained for 5 min, and finally at 30 ºC min^{-1} to 280 ºC and maintained for 5 min. Total run time was 29 min, the helium flow was set to 1 ml min^{-1}, and the split was 1:10. The terpenes were identified by comparing the mass spectra with known standards and published spectra (NIST 05, NIST 08, and Wiley 7n libraries). Calibration curves for terpene quantification were prepared with dodecane and commercial standards of four MTs (α-pinene, sabinene, δ-3-carene and γ-terpinene), four STs (caryophyllene, caryophylene oxide, cedrol and farnesol) and two DTs (totarol and phytol). All terpenes

were purchased from Fluka Chemie AG, Buchs, Switzerland. Four different concentrations were used to perform terpene calibration curves, with correlations between signal and concentration always highly significant ($r^2 \geq 0.99$). The main terpenes had similar sensitivities (differences <5%).

Antifungal assays

The terpenes tested for antifungal activity were selected by their performance in our previous study (Achotegui-Castells et al. 2015), the current study, and compound chirality observations (Michelozzi, unpublished results). The tested terpenes were: (+)-α-thujene, (+)-α-pinene, (+)-sabinene, (+)-δ-3-carene, limonene (unknown chirality), terpinolene (unknown chirality), (+)-sabinene hydrate, (+)-camphor, (-)-terpinen-4-ol, (+)-α-terpineol, (-)-bornyl acetate, α-humulene (unknown chirality), (+)-cedrol, (+)-manool, and (+)-totarol. All compounds were obtained from Fluka Chemie AG, (Buchs, Switzerland), except (+)-α-thujene (Chemos GmbH, Regenstauf, Germany) and (+)-manool (Sequoia Research Products Limited, Pangbourne, UK). We used the broad-spectrum fungicide azoxystrobin (Quadris®, Syngenta), commonly used against cypress bark canker (Della Rocca et al. 2011), as a positive control in the antifungal tests.

The *in vitro* tests were performed in 6-cm Petri dishes containing 5 g of 2% MEA. The test solutions were prepared by mixing the terpenes with 60 μl of acetone, whereas the acetone controls contained only acetone. The solutions were gently shaken, pipetted, and then spread over the MEA surface with a spatula. A 5-mm disk of a *S. cardinale* colony was then placed in the centre of the Petri dishes, which were immediately tightly sealed with Parafilm® and incubated at 25 ºC for 6 d in the dark. All tests were replicated four to five times. Three *in planta* concentrations corresponding to those found in the *C. sempervirens* GC-MS (in mg g^{-1} phloem tissue) analyses were tested for each terpene *in vitro* (in mg g^{-1} MEA). The control (mean concentration of both groups of trees), infected NR, and infected Agrimed (Table 2) concentrations were tested. Three arbitrarily fixed concentrations were tested to compare the inhibitory powers of the terpenes: 0.25, 0.50, and 1.0 mg g^{-1} MEA. Two perpendicular diameters of the fungal colonies were measured after 3 d and 6 d. Growth-inhibition rates (%) were calculated by:

Growth-inhibition rate (%) = 100 x (1-Da/Db)

where *Da* is the average mycelial diameter of each sample test and *Db* is the average mycelial diameter of the acetone control.

Biotransformation

MEA plugs (5 mm diameter) from the 1.0 mg g^{-1} MEA inhibition tests (transformation, MEA + acetone + terpene + fungus) were sampled immediately after the measurement of fungal growth at day 6 to study fungal biotransformations. Three supplementary tests, agar (MEA + acetone, to detect MEA metabolites), fungus (MEA + acetone + fungus, to detect fungal metabolites), and terpene (MEA + acetone + terpene, to detect terpene oxidations or degradations), were also sampled 6 d after test application. No terpenes were detected in the agar and fungus tests. Three plugs were taken from the transformation test, one from the margin of the colony (hyphae, H), one of MEA 5 mm from the mycelial border (hyphae-free, HF), and one identical to HF to incubate for two weeks for verifying the absence of hyphae (no *S. cardinale* grew in the HF plugs for verifying the absence of hyphae). Plugs were extracted with a 5-mm cork borer and were placed in refrigerated Eppendorf tubes and immediately stored at -80 ºC. Sample analyses and identification were performed as described above but with individual calibration curves for each terpene and a split of 1:3 to maximise compound detection. The GC-MS analyses of the MEA found no MTs (Kusumoto et al. 2014), but most of the oxygenated MTs, STs, and DTs had consistent concentrations.

Detoxification

The inhibitory activity of the terpenes biotransformed by *S. cardinale* on the fungus mycelial growth was assessed *in vitro*. Two groups (detoxification and control) of 12 Petri dishes containing MEA and 1mg g^{-1} of (+)-camphor, or (-)-bornyl acetate or (+)-cedrol (commercial standards) solved in pentane (4 replicates for each terpene) were prepared following the procedure described before. In each Petri dish of the first group (detoxification treatment), a 5-mm plug of *S. cardinale* mycelium was added as described above. No *S. cardinale* plug was added to the second group of Petri dishes (control treatment). The Petri dishes were stored at the previously described conditions for 6 days. Then, a 10 mm width ring of MEA+terpene (hyphae free) surrounding the *S. cardinale* colony was trimmed from each Petri of the detoxification treatment. In the

control Petri dishes, we trimmed the equivalent ring of agar from an equivalent position. The trimmed rings of MEA from each Petri dish were separately soaked with 3 ml of pentane in hermetic vials and kept overnight at -20°C in constant shaking (150 rpm), in order to extract all the terpenes and the products biotransformed by the fungus. Next morning, we concentrated the resulting extract with a flux of gaseous nitrogen until we reached a final volume of 100 µl. This final solution was pipetted and spread in the surface of two new groups of MEA Petri dishes (4 replicates each): Biotransformed substrate (extract of MEA with biotransformation products of *S. cardinale*) and Non-biotransformed substrate (extract of MEA with non biotrasformed terpenes). Petri dishes were incubated under the previously described conditions and fungal growth was measured after 3 and 6 days.
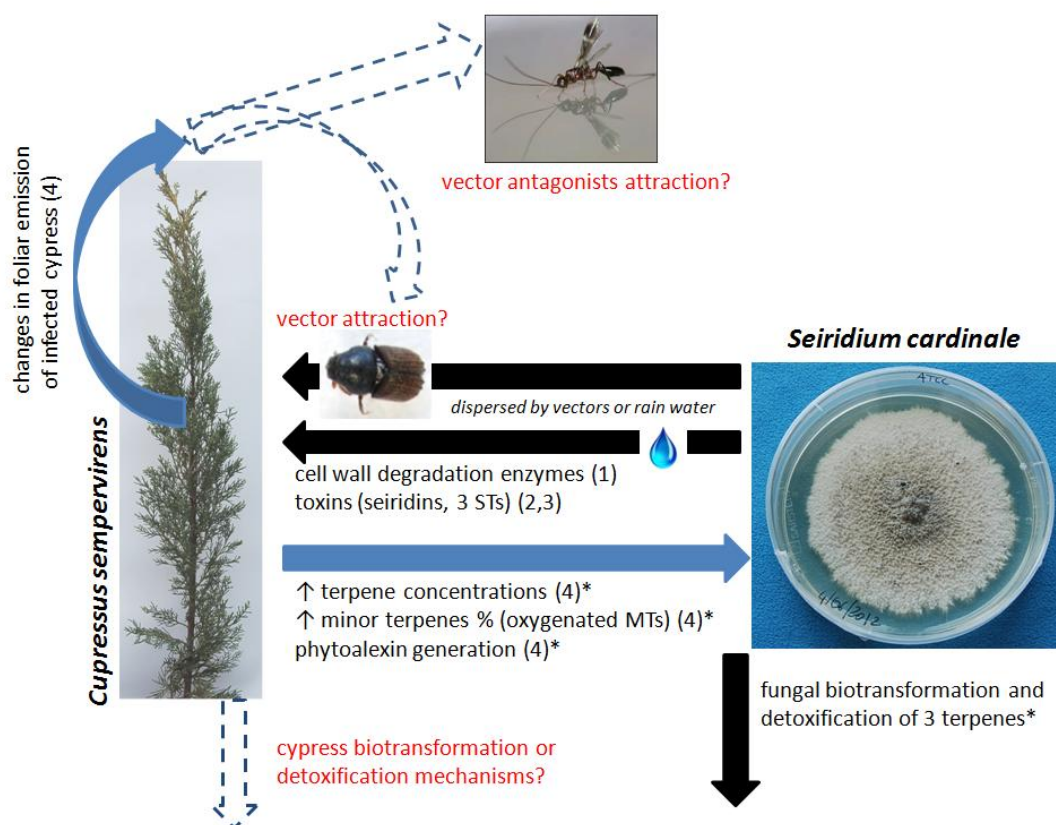
Figure 1 Scheme depicting the interactions between host and pathogen in which terpenes play or may play a role. Black arrows indicate fungal activity, blue arrows indicate tree activity, and dashed arrows with red labels indicate possible but yet unknown interactions. Asterisks (*) indicate the findings of the current study. References: 1 (Magro et al. 1982), 2 (Ballio et al. 1991), 3 (Evidente et al. 1993), 4 (Achotegui-Castells et al. 2015). Photograph credit: All photographies taken by Gianni Della Rocca, except "antagonist" (USDA). URL: https://en.wikipedia.org/wiki/Spathius_agrili#/media/File:Spathius_agrili.png

Statistical analyses

The data were analysed by one-way ANOVAs, and treatments and tests were compared with Tukey's *post hoc* tests ($P < 0.05$). Outliers were removed using absolute deviation around the median (Leys et al. 2013). Data not fitting the requirements of normality were transformed or analysed with non-parametric methods (Kruskal-Wallis one-way ANOVA). The statistical analyses used Statistica version 8.0 (StatSoft Inc. Tulsa, USA), SigmaPlot version 11.0 (Systat Software, Chicago, USA), and R software version 2.15.2 (R foundation for Statistical Computing, 2012).

Results

Terpenic composition differed substantially between tree groups and treatments 30 days after artificial inoculation. Sabinene hydrate, camphor, and oxygenated MT1 and 2, were *de novo* terpenes exclusively found in the infected states of both groups (oxygenated MT2 only in infected Agrimed). Other compounds, such as ocimene, thymyl methyl eter, and MT4 were only found in the wounded and infected states. The concentrations of these terpenes were usually low (Table 1). DTs were the main fraction (70-80% of total terpenes, led by totarol) in the phloem of both cypress groups, followed by MTs (20-30%, led by α-pinene and δ-3-carene) and STs (ca. 1%, led by cedrol) (Table 1). The concentrations of terpenes in both cypress groups, especially MTs and DTs, tended to be higher in the infected than the wounded and control treatments (one-way ANOVA, Tukey's *post hoc* test $P < 0.05$ or $P < 0.10$) (Fig. 2, Table 1). Infected Agrimed had higher concentrations than the wounded or control treatments (of 17 terpenes) more often than infected NR (of eight terpenes) (Table 1). Agrimed had higher concentrations than NR of longifolene, totarol, and total DTs in the control treatments and of ocimene in the infected treatments (Table 1, Fig. 2). The proportions of terpenes (relative to their class) followed similar trends but also decreased for some compounds, especially the most abundant terpenes (Table 1, Fig. 2). Agrimed was again more responsive to infection, with 25 terpenes significantly changing proportions (19 increases and six decreases) than NR, with 11 changes (10 increases and one decrease). Infected and wounded Agrimed had higher proportions than NR of 20 terpenes, mostly MTs.

The antifungal activity against *S. cardinale* of the *C. sempervirens* terpenes varied substantially when tested *in vitro*, ranging from complete growth inhibition (e.g. (+)-α-terpineol and (-)-terpinen-4-ol) to growth stimulation (e.g. (+)-α-pinene and limonene) (Fig. 3, Table 2). Inhibition appeared to be concentration-dependent for most terpenes, with several concentration-inhibition patterns (Fig. 3). Several of the simulated concentrations in the *in planta* tests showed different inhibition power among the control, infected Agrimed, and infected NR (one-way ANOVA, Tukey's *post hoc* test, $P < 0.05$) (Table 2, Fig. 3). The *in planta* concentrations of infected Agrimed were more inhibitory than the control for all compounds except (+)-α-pinene, (-)-bornyl acetate and limonene. Infected NR concentrations were more inhibitory than the control concentrations for (+)-sabinene, terpinolene, (+)-cedrol and (+)-manool, and infected Agrimed concentrations were more inhibitory than infected NR concentrations for (+)-sabinene, (+)-δ-3-carene, and (-)-terpinen-4-ol. The mean of all inhibitions of infected Agrimed (24.1%) was significantly higher than that of infected NR (18.4%) and the control (15.0%) (one-way ANOVA, Tukey's *post hoc* test, $P < 0.01$). Only some oxygenated MTs, (+)-cedrol, and the DTs had substantial effects on fungal growth in the fixed concentration tests (Table 2) at 0.25 mg g^{-1} malt agar extract (MEA). (+)-Totarol was more inhibitory than azoxystrobin, a reference fungicide. At 0.50 mg g^{-1} MEA, some MT hydrocarbons began to show moderate rates of inhibition (ca. 25%), the oxygenated MTs substantially increased their inhibition, whereas STs and DTs maintained similar inhibitions to growth. The most concentrated test, 1.0 mg g^{-1} MEA, exhibited the strongest inhibitions, led by oxygenated MTs, half of which inhibited growth completely and overcame the inhibition caused by the fungicide, followed by DTs, STs, and MTs. We calculated the concentration-dependence of inhibition for each terpene within that concentration range (Fig. 3d) by subtracting the inhibition in the 0.25 mg g^{-1} MEA test from the inhibition in the 1.0 mg g^{-1} MEA test. Oxygenated MTs were the most concentration-dependent class of terpenes compared to MT hydrocarbons, STs and DTs (one-way ANOVA, Tukey's *post hoc* test, $P < 0.05$). The most concentration-dependent compounds were the *de novo* terpenes (68.2%), followed by induced terpenes (22.1%), and the major terpenes (9.0%) (one-way ANOVA, Tukey's *post hoc* test, $P < 0.01$) (Fig. 3d).

Several biotransformations in both H (hyphae) and HF (hyphae free, 0.5 cm away from the mycelial border) plugs were detected in the biotransformation tests where *S. cardinale* grew on MEA plates containing (+)-camphor, (-)-bornyl acetate, or
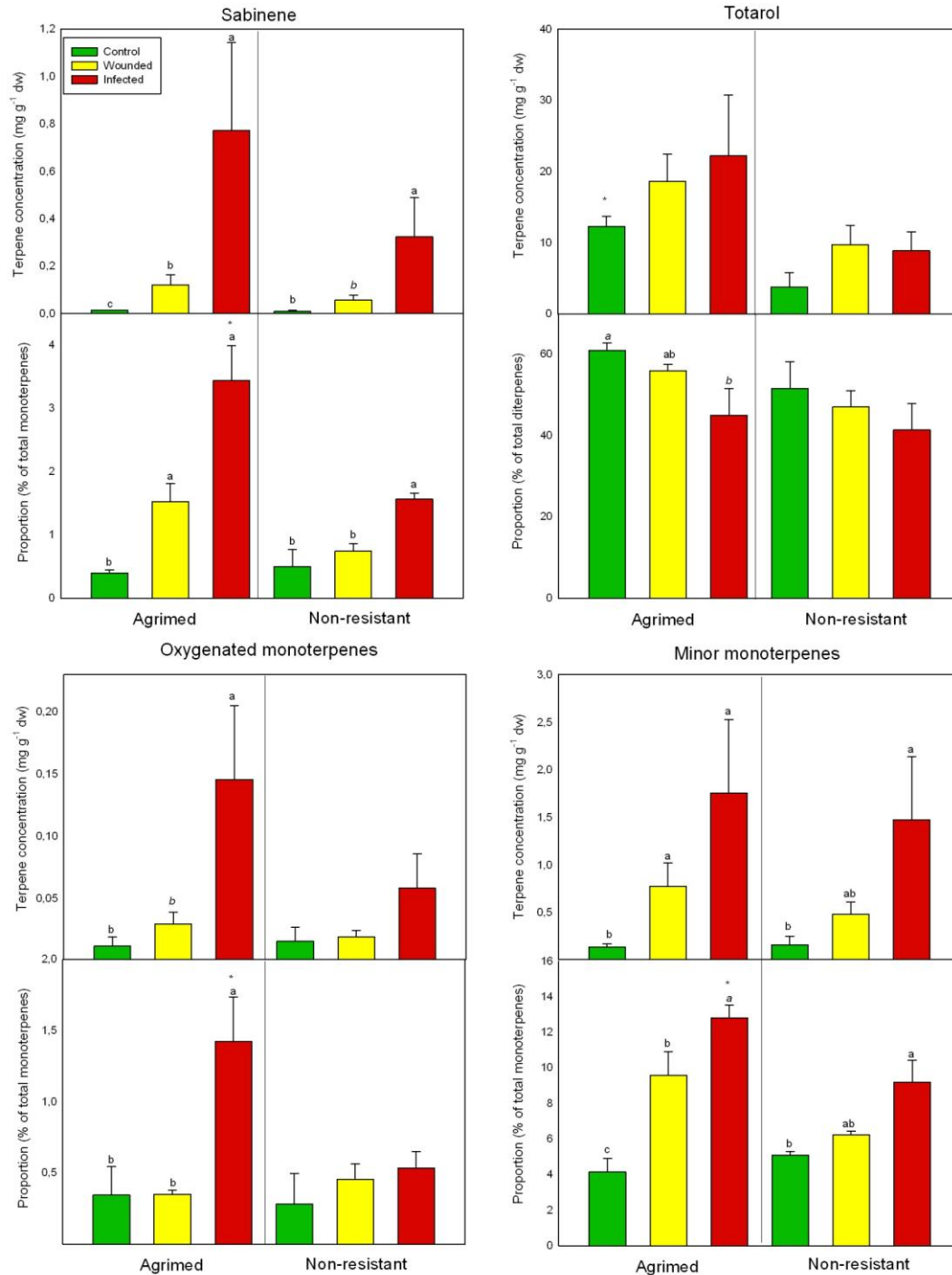
Figure 2 Mean phloem concentrations (mg g^{-1} dry weight ±SE) and proportions (% relative to the terpene class ±SE) of sabinene, totarol, oxygenated monoterpenes, and minor monoterpenes for the two *Cupressus sempervirens* groups, Agrimed (canker resistant) and non-resistant (NR). Different letters indicate significant differences among treatments of the same group (one-way ANOVA, Tukey's *post hoc* test, *P* < 0.05). Asterisks (*) indicate significant differences (one-way ANOVA, Tukey's *post hoc* test, *P* < 0.05) between the same treatments of both groups. dw, dry weight.

Terpenes	Agrimed			Non-Resistant		
	Control	Wounded	Infected	Control	Wounded	Infected
MT1	0.00065	0.0011±0.0001	0.0035±0.0005	0.0014±0.0001	0.002	0.0045±0.0025
	0.020	0.012±0.001	0.020±0.004	0.022±0.002	0.022	0.024±0.01
MT2	0.0024	0.0030±0.0007	0.0073±0.0024	0.0024±0.0002	0.0029±0.0022	0.0043±0.0016
	0.070	0.035±0.008	0.045±0.013	0.04±0.01	0.042±0.02	0.029±0.004
tricyclene	0.0088±0.0004	0.020±0.004	0.028±0.0073	0.010±0.005	0.028±0.0062	0.051±0.020
	0.27±0.03	0.28±0.03	0.28±0.01	0.29±0.05	0.30±0.02	0.35±0.02
α-thujene	**0.0081±0.0006b**	**0.043±0.012ab**	**0.20±0.09a**	0.011±0.005	0.027±0.002	0.039±0.018
	0.25±0.04b	**0.52±0.07b***	**0.92±0.09a***	**0.28±0.03b**	**0.24±0.04ab**	**0.43±0.08a**
α-pinene	2.9±0.3	6.6±1.6	7.6±1.8	2.9±1.7	7.2±2	12.1±3.9
	89±4a	**85±1ab**	**77±3b**	88±3	93±0	**90±1***
α-fenchene	0.020±0.004	0.044±0.011	0.13±0.05	0.048±0.028	0.071±0.026	0.14±0.05
	0.59±0.07	0.57±0.08	0.57±0.12	**1.7±0.4ab***	0.9±0.2b	1.2±0a
camphene	0.014±0.001	0.028±0.006	0.063±0.024	0.017±0.009	0.035±0.006	0.062±0.022
	0.43±0.09	0.37±0.04	0.42±0.03	0.55±0.17	0.39±0.02	0.42±0
sabinene	**0.013±0.002c**	**0.12±0.04b**	**0.77±0.37a**	**0.010±0.005b**	**0.058±0.02b**	**0.32±0.17a**
	0.40±0.05b	**1.5±0.3b**	**3.4±0.6a***	**0.5±0.27b**	**0.74±0.12b**	**1.56±0.1a**
β-pinene	**0.010±0.002b**	**0.049±0.012ab**	**0.10±0.03a**	0.053±0.007	0.079±0.021	0.14±0.05
	0.31±0.03b	**0.65±0.08a**	**0.81±0.10a**	**0.84±0.00***	**1.0±0.1***	1.0±0.1
β-myrcene	0.015±0.004	0.055±0.019	0.19±0.08	0.032±0.004	0.047±0.013	0.13±0.06
	0.45±0.09b	**0.65±0.07ab**	**1.2±0.2a**	0.52±0.01	0.6±0.03	0.6±0.09
δ-3-carene	0.26±0.12	0.45±0.16	1.1±0.48	0.17±0.131	0.22±0.009	0.17±0.09
	7.3±3.2	5.4±1.7	4.1±1.9	**6.4±2.6a**	**1.2±0.5b**	**1.2±0.4b**
limonene	0.012±0.002	0.033±0.009	0.11±0.04	0.021±0.001	0.039±0.009	0.090±0.041
	0.34±0.02b	**0.4±0.03b**	**0.59±0.05a**	0.34±0.04	0.40±0.03	0.55±0.08
ocimene	NA	0.010±0.005	**0.015±0.003***	NA	0.004±0.001	**0.007±0.002**
	NA	0.09±0.03	0.11±0.02	NA	0.03±0.01	0.04±0.01
γ-terpinene	0.0024±0.0002	0.0070±0.0015	0.0079±0.002	0.0022±0.0006	0.0042±0.0008	0.012±0.005
	0.074±0.01	**0.096±0.01***	0.10±0.01	**0.036±0.02b**	**0.048±0.01a**	**0.072±0a**
sabinene hydrate	NA	NA	0.013±0.007	NA	NA	0.0051±0.0032
	NA	NA	0.072±0.04	NA	NA	0.033±0.008
terpinolene	0.020±0.011	0.31±0.12	0.48±0.16	0.028±0.016	0.075±0.022	0.25±0.12
	0.56±0.3b	**3.8±1.0a***	**3.3±0.5a**	**0.49±0.32b**	**0.91±0.14ab**	**1.52±0.28a**
camphor	NA	NA	0.011±0.005	NA	NA	0.017±0.007
	NA	NA	0.060±0.02	NA	NA	0.10±0.011
terpinen-4-ol	**0.0048±0.02b**	**0.0081±0.006b**	**0.058±0.028a**	0.0086±0.007	0.0094±0.002	0.028±0.013
	0.16±0.02ab	**0.1±0.01b**	**0.78±0.28a**	0.16±0.13	0.18±0.04	0.28±0.09
thymyl methyl eter	NA	0.0074±0.0009	0.017±0.004	NA	NA	0.0064±0.0008
	NA	0.049±0.025	0.30±0.15	NA	NA	0.063±0.028
MT3	0.0097	**0.011±0.003b**	**0.065±0.038a**	**NA**	**0.0049±0.0004b**	**0.028±0.014a**
	0.081	0.15±0.02	0.46±0.18	NA	0.10±0.03	0.13±0.01
bornyl acetate	0.0014±0.001	0.0029±0.0010	0.0050±0.001	0.00046±0.0001	0.0017±0.0007	0.008±0.004
	0.039±0.01	0.04±0.01	0.055±0.006	0.0078±0.0035	0.03±0.0091	0.0474±0.0107
MT4	NA	0.0067	0.013±0.002	0.00082	0.0024	0.0071±0.0035
	NA	0.047	0.082±0.023	0.012	0.018	0.03±0.012
MT5	0.0037	0.0075±0.003	0.0084±0.004	**0.0025±0.0008b**	**0.0040±0.0008b**	**0.027±0.011a**
	0.1	0.086±0.01	0.095±0.022	**0.039±0.008b**	**0.047±0.006b**	**0.13±0.024a**
MT6	**0.0010±0.0001b**	**0.0037±0.001b**	**0.016±0.005a**	**0.0014±0.0006b**	**0.0021±0.0006b**	**0.0084±0.0005a**
	0.077±0.04	0.048±0.01	0.095±0.03	0.023±0.013	0.021±0.007	0.035±0.006
terpinyl acetate	**0.0069±0.0003b**	**0.018±0.005b**	**0.081±0.026a**	0.0067±0.0038	0.010±0.003	0.052±0.030
	0.20±0.17ab	**0.22±0.03b**	**0.52±0.1a**	0.12±0.1	0.13±0.02	0.27±0.1
minor MT	**0.14±0.03b**	**0.78±0.25a**	**1.8±0.8a**	**0.16±0.09b**	**0.48±0.13ab**	**1.48±0.66a**
	4.1±0.8c	**9.6±1.3b***	**13±1a***	**5.1±0.2b**	**6.2±2ab**	**9.2±1.3a**
oxygenated MT	**0.012±0.007b**	**0.030±0.009b**	**0.15±0.06a**	0.016±0.011	0.019±0.005	0.058±0.028
	0.34±0.20b	**0.35±0.03b**	**1.4±0.3a***	0.28±0.22	0.46±0.11	**0.53±0.11**
total MT	**3.4±0.4b**	**7.9±1.9ab**	**14.3±5a**	3.2±1.8	7.9±2.1	13.7±4.6
% of MT	19±5	19±2	24±1	20±7	21±2	30±2
α-cubebene	0.083±0.021	0.17±0.04	0.32±0.10	**0.035±0.013b**	**0.080±0.022ab**	**0.45±0.24a**
	22±6b	**34±3a**	**30±1ab**	27±5	21±2	35±8
copaene	0.0098±0.0025	0.020±0.004	0.030±0.011	0.0038±0.0017	0.0097±0.0022	0.013±0.004
	1.5±0.1b	**3±0.1a**	**2.9±0.1a**	1.9±0.1	1.5±0.1	1.6±0.4
ST1	0.0016±0.0009	0.0042±0.0006	0.0075±0.0025	0.0023±0.0013	0.0029±0.0004	0.015±0.008
	0.43±0.25	0.73±0.1	0.96±0.28	0.33±0.01	0.48±0.05	0.72±0.35
longifolene	**0.073±0.012***	0.060±0.014	0.13±0.06	**0.024±0.014**	0.061±0.02	0.068±0.023
	15±4	17±2	16±2	11±2	16±1	10±2
ST2	0.033±0.0033	0.033±0.01	0.068±0.024	0.015±0.008	0.041±0.013	0.056±0.021
	6.8±1.3	5.9±0.3	6.0±0.1	**9.1±0.7ab**	**9.5±0.5a**	**7.4±0.8b**
caryophyllene	0.0088±0.0008	0.010±0.003	0.0070±0.001	0.0074±0.0025	0.012±0.003	0.017±0.011
	1.8±0.4b	**1.8±0.3b**	**0.8±0.1a**	1.6±0.6	1.0±0.1	1.9±0.1
ST3	0.0099±0.0011	0.0095±0.0026	0.018±0.006	0.018±0.013	0.011±0.003	0.013±0.007
	2±0.4	1.7±0.1	1.7±0.1	2.5±0.3	2.5±0.2	2.2±0.2
α-caryophyllene	**0.0010±0.0001b**	**0.0026±0.0003a**	**0.0017±0.0005ab**	0.0025±0.001	NA	NA
	0.28±0.03ab	**0.4±0.06b**	**0.18±0.02a**	0.32±0.08	NA	NA
ST4	0.0025±0.0012	0.0098±0.0037	0.018±0.006	0.0055±0.0043	0.007	0.0025
	0.34±0.04b	**1.7±0.1a**	**1.4±0.1a**	0.62±0.06	0.74	1.07
cedrol	0.19±0.02	0.19±0.057	0.37±0.13	0.079±0.039	0.21±0.07	0.30±0.11
	44±3a	**33±2b**	**33±1b**	47±2	49±2	41±5
Total ST	0.38±0.02	0.55±0.159	1.1±0.4	0.16±0.08	0.42±0.13	0.93±0.43
% of ST	**2.1±0.4a**	**1.3±0.1b**	**1.7±0.2ab**	1.4±0.2	1.2±0.1	1.6±0.3

Table 1 (continues in next page)

Terpenes	Agrimed Control	Agrimed Wounded	Agrimed Infected	Non-Resistant Control	Non-Resistant Wounded	Non-Resistant Infected
manoyl oxide or epimanoyl oxide	0.039±0.009	0.082±0.015	0.13±0.03	0.021±0.011	0.078±0.027	0.074±0.037
	0.26±0.05	0.26±0.01	0.30±0.02	0.24±0.03	0.26±0.03	0.32±0.02
DT1	**0.056±0.003b**	**0.093±0.013ab**	**0.16±0.04a**	0.085±0.037	0.10±0.03	0.083±0.038
	0.33±0.01ab	**0.31±0.02b**	**0.37±0.02a**	0.28±0.03	0.36±0.04	0.37±0.04
manool	**0.15±0.06b**	**0.89±0.08a**	**1.5±0.4a**	0.14±0.05	1.0±0.3	1.2±0.5
	1.2±0.1b	**2.6±0.3ab**	**3.6±0.7a**	**2.3±0.6b**	**5.7±0.9a***	**5.8±1.0a**
DT2	0.014±0.002	0.027±0.006	0.026±0.008	0.018±0.009	0.013±0.001	0.023±0.006
	0.089±0.01	0.076±0.006	0.078±0.010	0.059±0.002	0.055±0.007	0.072±0.012
DT3	0.054±0.016	0.073±0.014	0.12±0.04	0.033±0.013	0.061±0.009	0.12±0.08
	0.41±0.02	0.39±0.07	0.35±0.07	0.37±0.02	0.24±0.03	0.36±0.09
DT4	**0.75±0.26b**	**1.8±0.3ab**	**2.5±0.5a**	1.4±0.7	1.8±0.5	1.6±0.7
	5.5±1.0b	**7.2±1.2ab**	**8.3±0.7a**	6.6±2.0	6.6±0.6	6.8±0.5
DT5	**0.014±0.004b**	**0.080±0.020a**	**0.068±0.013ab**	0.12±0.05	0.074±0.021	0.17±0.10
	0.078±0.017	0.20±0.04	0.32±0.06	0.43±0.20	0.47±0.11	0.72±0.18
DT6	**0.028±0.006b**	**1.7±0.3ab**	**2.7±1.0a**	**0.081±0.033b**	**1.8±0.5ab**	**3.6±1.5a**
	1.2±0.6b	**4.7±0.9a**	**12.7±5.2a**	**3.6±1.4b**	**11±3ab**	**17±4a**
DT7	0.74±0.09	0.94±0.16	0.97±0.32	0.26±0.14	0.61±0.17	0.63±0.25
	3.8±0.2	3.1±0.1	3.0±0.4	3.1±0.5	3.0±0.1	2.7±0.3
totarol	**12±1***	19±4	22±9	**3.8±2.0**	9.7±2.7	8.9±2.7
	61±2a	**56±2ab**	**45±7b**	52±7	47±4	41±6
ferruginol	0.43±0.05	0.7±0.06	0.97±0.33	**0.15±0.08b**	**0.86±0.26a**	**0.70±0.22ab**
	2.1±0.1	2.5±0.2	2.1±0.1	2.4±0.5	3.2±0.3	2.1±0.1
2,2'-Methylenebis(6-tert-butyl-4-methylphenol)	0.047±0.01	0.061±0.01	0.081±0.014	0.12±0.072	0.064±0.01	0.080±0.007
	1.7±1.0a	**0.20±0.04b**	**0.19±0.04b**	11±7	0.22±0.04	0.60±0.11
DT8	**0.026±0.006b**	**0.22±0.03ab**	**0.28±0.08a**	0.018±0.004	0.23±0.1	1.4±0.6
	0.46±0.28	0.61±0.10	1.4±0.5	**1.1±0.4b**	**1.4±0.4b**	**3.5±0.9a***
5E	1.7±0.5	3.2±0.8	4.0±1.5	1.5±0.9	1.2±0.3	1.9±0.7
	8.9±0.5	**9.8±0.5***	8.9±1	8.9±0.6	**5.9±0.9**	5.9±1.2
totarolone	0.28±0.08	0.49±0.05	0.42±0.09	0.29±0.19	0.58±0.15	0.26±0.11
	1.6±0.3	1.3±0.2	1.2±0.2	**0.82±0.23b**	**2.5±0.5a**	**1.2±0.5ab**
DT9	**1.5±0.4b**	**2.5±0.1ab**	**2.9±0.6a**	0.45±0.25	2.5±0.7	3.0±1.2
	9.6±0.2*	9.3±0.3	10±1	5.6±1.2	8.2±0.7	8.4±0.4
DT10	**0.02±0.01b**	**0.24±0.06a**	**0.32±0.07a**	0.10±0.07	0.33±0.09	0.2±0.07
	0.19±0.04b	**0.81±0.22ab**	**0.71±0.06a**	0.25±0.01	1.1±0.2	1.2±0.45
hinokiol	0.03	0.11±0.03	0.14±0.03	0.028±0.025	0.19±0.06	0.13±0.04
	0.10	0.25±0.07	0.38±0.09	0.056±0.032	0.86±0.25	0.46±0.19
DT11	0.052±0.017	0.058±0.009	0.048±0.006	0.071±0.023	0.066±0.012	0.056
	0.30±0.13a	**0.16±0.02ab**	**0.085±0.017b**	2.9±2.7	0.20±0.08	0.34
total DT	**20±3***	30±5	45±14	**6.4±3.4**	29±9	25±11
% of DT	79±5	80±2	75±1	78±6	78±2	69±2
Total terpenes	**16±4b**	**40±9ab**	**60±19a**	9.1±4.9	37±11	48±17

Table 1 Mean concentrations (mg g^{-1} dry weight ±SE) and proportions (% ±SE) relative to the class of the terpenes in the local phloem of canker-resistant (Agrimed) and non-resistant (NR) cypresses. Numbers and letters in bold type indicate significant (one-way ANOVA, Tukey's *post hoc* test, $P < 0.05$) and marginally significant ($P < 0.10$, in *italics*) differences within the treatments of a tree group. Asterisks (*) indicate statistically significant differences between the same treatment of different groups (one-way ANOVA, Tukey's *post hoc* test, $P < 0.05$). [ ], concentration; MT, monoterpene; ST, sesquiterpene; DT, diterpene; NA, not available.

(+)-cedrol (the transformation test), but only the terpene substrate was found on MEA plates containing these three terpenes but without the fungus (the terpene test) (Fig. 4). The Petri dishes with fungus grown on a substrate of (+)-camphor had six new compounds, three of which were identified as bornane-2,5-dione, bornane-2,3-dione (tentative identification), and bornane-2,6-dione. Fungus grown on MEA containing (-)-bornyl acetate generated three biotransformation products, two of which were identified as camphor and borneol. *S. cardinale* grown on (+)-cedrol produced six new compounds that could not be identified. The tests also produced quantitative differences among these three terpenes (Fig. 4), and in all cases, the terpene substrate concentrations were higher in the MEA from the plates of the terpene test than H and HF of the transformation test (one-way ANOVA, Tukey's *post hoc* test, $P < 0.01$). The

H and HF samples of the transformation test also presented several differences, with H usually having higher concentrations of biotransformation products than HF (Fig. 4). A test to assess detoxification (Fig. 5) showed how the HF substrate of the three biotransformed terpenes ((+)-camphor, (-)-bornyl acetate and (+)-cedrol) was significantly less inhibitive to fungal growth than the HF substrate of non-biotransformed terpenes (T-tests $P < 0.05$).

Discussion

Agrimed responded more strongly to infection than NR, producing an extra *de novo* oxygenated MT and more inductions in concentrations (including total terpenes) and proportions. Agrimed also had several higher concentrations and proportions of various terpenes than NR in the infected treatments (Table 1). Our results thus agreed with those from studies that correlated increased terpene concentration with infection resistance in conifers (Raffa and Berryman 1982; Zeneli et al. 2006; Zhao et al. 2010). The current results (branch inoculations) agreed with those of our previous study (Achotegui-Castells et al. 2015) (stem inoculations), despite some differences likely associated with the different phloem samples analysed (Bonello et al. 2008; Hanover 1992). A comparison of both studies suggests that branches, despite exhibiting a similar response, are less protected than the trunk, supporting field observations that found most of the cankers initiate in the axils of young branches (Danti et al. 2013). The terpenes found in Italian cypress tissues in response to *S. cardinale* infection may not only be produced by the tree, as other endophytic microorganisms could be contributing to tree defence (Soltani et al. 2015). However it is technically very difficult to separate the real effect of those microorganisms from the 'pure' response of the plant. Further research should try to ascertain the contribution of endophytes to *C. sempervirens* defence against *S. cardinale*.

The majority of terpenes showed a concentration-dependent inhibition of fungal growth (Espinosa-García et al. 1993; Kusumoto et al. 2014) (Fig. 3). Concentration thus determined the ultimate capacity of inhibition (*in planta* tests, Table 2, Fig. 3),

despite different inhibitions for some terpenes at equal concentrations (fixed tests). Agrimed responded to infection stronger than NR, and its concentrations also appeared to be more inhibitory to fungal growth in the *in planta* tests. Differences in inhibition between the concentrations of both infected groups occurred only for the MTs ((+)-sabinene, (+)-δ-3-carene, and (-)-terpinen-4-ol), so these results suggest that, by day 30, MTs could be the class of terpenes responsible for conferring the higher canker resistance to Agrimed (Table 2).

			In planta concentrations				Fixed concentrations		
			Control	Infected NR	Infected Agrimed		0.25 mg g^{-1}	0.50 mg g^{-1}	1.0 mg g^{-1}
Monoterpene hydrocarbons	(+)–α–thujene	[ ]	0.009	0.04	0.20	Inhibition	−10.7±1.7ef	−4±2.3g	−2.0±1.4g
		Inhibition	−9.1±2.5b	−8.0±0.7b	2.8±1.3a				
	(+)–α–pinene	[ ]	2.9	7.6	12.1	Inhibition	−10.1±1.3ef	−1.4±1.3g	0.3±0.6g
		Inhibition	100±0	100±0	100±0				
	(+)–sabinene	[ ]	0.012	0.32	0.77	Inhibition	12.9±1.0d	23.7±0.5de	24.6±0.5f
		Inhibition	1.4±1.4c	21.1±1.3b	29.3±2.0a				
	(+)–δ–3–carene	[ ]	0.21	0.17	1.1	Inhibition	10.4±1.6d	16.9±0.3f	19.9±1.3f
		Inhibition	9.1±1.4b	1.9±0.3b	24.8±2.8a				
	limonene	[ ]	0.016	0.09	0.11	Inhibition	−11.7±1.3f	−8.1±4.2g	−2.5±0.7g
		Inhibition	−2.0±0.6a	−4.8±2.6ab	−8.3±0.9b				
	terpinolene	[ ]	0.024	0.25	0.48	Inhibition	−0.6±0.9def	7.8±4.5f	16.0±2.4f
		Inhibition	−9.7±2.8b	−0.6±0.9a	6.6±1.5a				
Oxygenated monoterpenes	(+)-sabinene hydrate	[ ]	x	0.005	0.013	Inhibition	1.6±2.0def	35±2.7d	92.4±5.0ab
		Inhibition	NA	−2.2±1.4	4.3±0.5				
	(+)–camphor	[ ]	x	0.017	0.011	Inhibition	−0.9±1.7def	14.4±0.8de	59.7±3.4de
		Inhibition	NA	1.1±2.0	−1.1±0.5				
	(−)–terpinen–4–ol	[ ]	0.007	0.028	0.095	Inhibition	36.7±5.1c	81.5±2.3a	100±0a
		Inhibition	−1.8±0.3b	−2.6±1b	13.4±3.9a				
	(+)–α–terpineol	[ ]	not detected		0.36*	Inhibition	60.7±6.8b	98.9±0.7a	100±0a
		Inhibition			78.9±2.3*				
	(-)-bornyl acetate	[ ]	0.001	0.008	0.005	Inhibition	2.5±0.6de	33.3±0.0d	50.3±3.0e
		Inhibition	−5.1±2.6	−8.0±1.4	−10.3±0.5				
Sesquiterpenes	α–humulene	[ ]	0.0010	0.0009	0.0017	Inhibition	10.9±2.0d	15.0±1.3f	35.4±2.0f
		Inhibition	−15.4±1.2	−12.8±2.7	−14.2±1				
	(+)–cedrol	[ ]	0.12	0.30	0.37	Inhibition	50.3±1.9bc	60.6±1.2bc	62.7±3.1cd
		Inhibition	46.2±2.2b	56.1±2.4a	61.5±1.0a				
Diterpenes	(+)–manool	[ ]	0.15	1.22	1.45	Inhibition	47.9±1.6bc	49.7±1.2c	68.6±0.8cd
		Inhibition	46.6±1.2b	65.2±0.5a	64.4±1.8a				
	(+)–totarol	[ ]	not tested			Inhibition	83.6±0.8a	78.9±1.6ab	87.1±2.0bc
		Inhibition							
Fungicide	azoxystrobin	[ ]	not tested			Inhibition	59.0±1.7b	68.5±1.5b	76.8±1.4c
		Inhibition							

Table 2 Mean rates of growth inhibition (% ±SE) of *Seiridium cardinale* by 15 terpenes (11 monoterpenes, two sesquiterpenes, and two diterpenes) and one fungicide relative to the inhibition by acetone (control). *The in planta* concentrations tested the same terpene concentrations as those in the phloem (Table 1) in the different treatments (control, infected NR, and infected Agrimed) applied per gram of malt extract agar (MEA). The fixed concentrations tested three arbitrary concentrations (0.25, 0.50, and 1.0 mg g^{-1} MEA) for comparing the inhibitory power among several terpenes. Different letters indicate significant (one-way ANOVA, Tukey's *post hoc* test, $P < 0.05$) and marginally significant ($P < 0.10$, in *italics*) differences within the treatments of a tree group. Comparisons for the *in planta* concentration tests were performed between treatments (horizontal), and comparisons for the fixed concentrations were performed within the treatments (vertical). The asterisks (*) for (+)-α-terpineol indicate that this test was performed with the concentration found in a previous study (Achotegui-Castells et al. 2015), because we did not detect this compound in the current study.

The low concentrations of the oxygenated MTs (except terpinen-4-ol) and the low dependence of inhibition on the concentration of STs and DTs prevented these terpenes from causing significantly different inhibitions between groups by day 30, despite reports of being strong inhibitors of fungal growth (Becerra et al. 2002; Cheng et al. 2011).Despite being a useful tool for studying inhibition more realistically, *in planta* inhibition tests have two important limitations: *i*) mean concentrations in phloems are applied, which does not represent the real variability of concentrations, and *ii*) the application of the same concentrations in the MEA as those found in phloems may not be quantitatively appropriate. Our results suggested that X mg g^{-1} MEA were more inhibitory than X mg g^{-1} phloem. The fixed concentration tests allowed a comparison of the inhibitory powers of the terpenes and can help to predict inhibition in canker-infected cypress trunks or more advanced states of infection (e.g. day 90), which should exhibit higher concentrations (Achotegui-Castells et al. 2015) than those in the current study.

Our results suggest that the low inhibitory power of MT hydrocarbons is likely due to their high volatility and widespread occurrence in nature. In addition, several studies have reported that some terpenes, usually the most abundant compounds of a host, can enhance the growth of pathogens of conifers (Bridges 1987; Hofstetter et al. 2005). In our study, the oxygenated MTs, well-known inhibitors of fungal growth (Bakkali et al. 2008; Kusumoto et al. 2014), were the most inhibitory compounds at high concentrations. The *de novo terpenes* (+)-sabinene hydrate (Ramos et al. 2011), (+)-camphor (Pragadheesh et al. 2013; Ramsewak et al. 2003), and (+)-α-terpineol (Cakir et al. 2004; Kusumoto et al. 2014), known to exhibit antifungal activity, were among the most inhibitory compounds in the fixed concentration tests and thus should be considered as phytoalexins against *S. cardinale*. The *de novo* compounds produced by an infected conifer can have very strong inhibitory effects on the infecting pathogen (Bridges 1987; Madar et al. 1995). The oxygenated MTs had low inhibitory rates at the *in planta* concentrations but would likely have been strong inhibitors at the ca. ten-fold higher concentrations reported in our previous study (Achotegui-Castells et al. 2015), as suggested by their concentration-inhibition curves (Fig. 3) and the (+)-α-terpineol test (Table 2). STs represented only ca. 1% of the total terpene concentration in our study, but (+)-cedrol, the main ST, can be very inhibitory to fungi (Cheng et al. 2011) and maintained high rates of inhibition (ca. 60%) even at low concentrations. DTs also
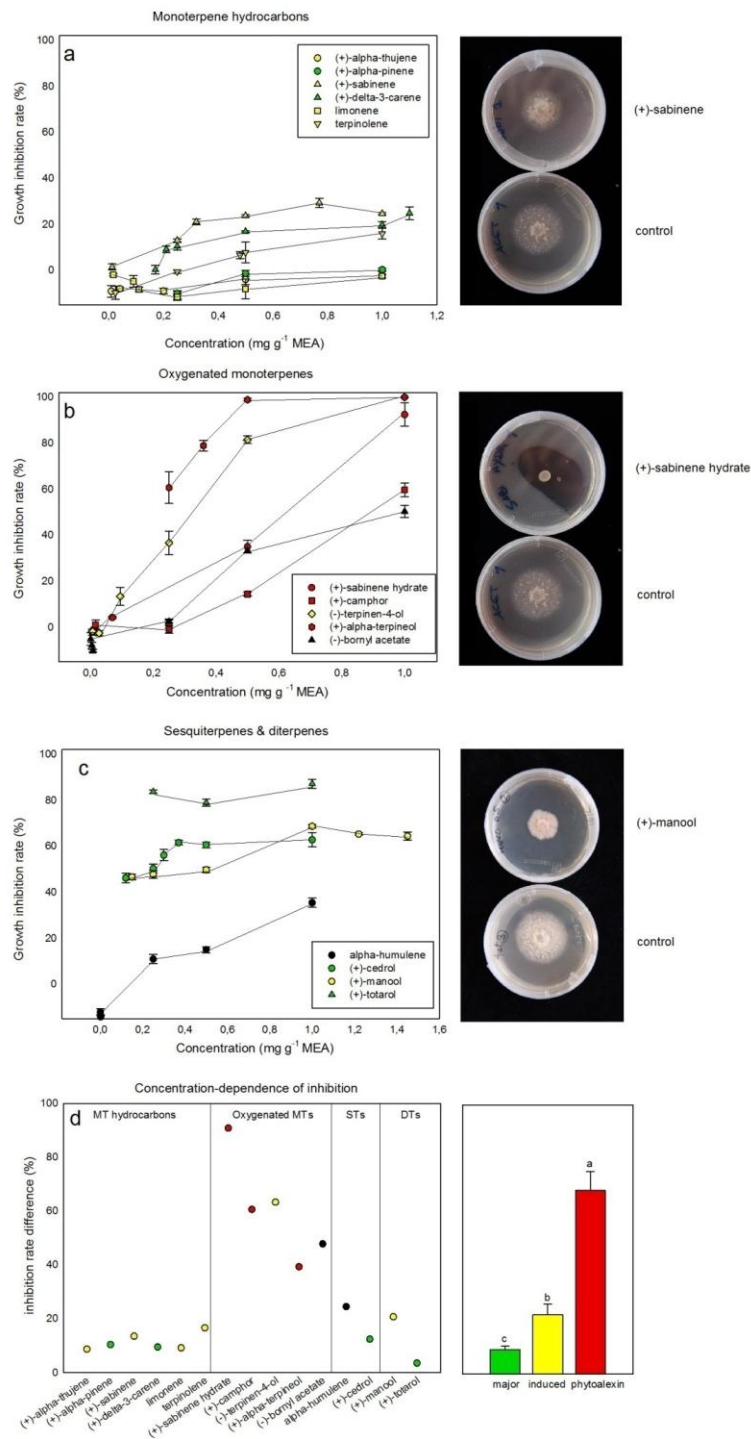
Figure 3 Inhibition-rate curves of fungal growth (mean ±SE) and photographs of growth inhibition for a) monoterpene hydrocarbons, b) oxygenated monoterpenes, and c) sesquiterpenes and diterpenes, and d) the results of a concentration-dependence test (difference between the inhibitions of the 1.0 and 0.25 mg g^{-1} MEA tests). Different letters in the histogram in d) indicate significant differences (one-way ANOVA, Tukey's *post hoc* test, $P < 0.05$). Green, main terpenes of each terpene class; yellow, canker-induced terpenes; red, phytoalexins; black, uncategorised. Photograph credit: Gianni Della Rocca.
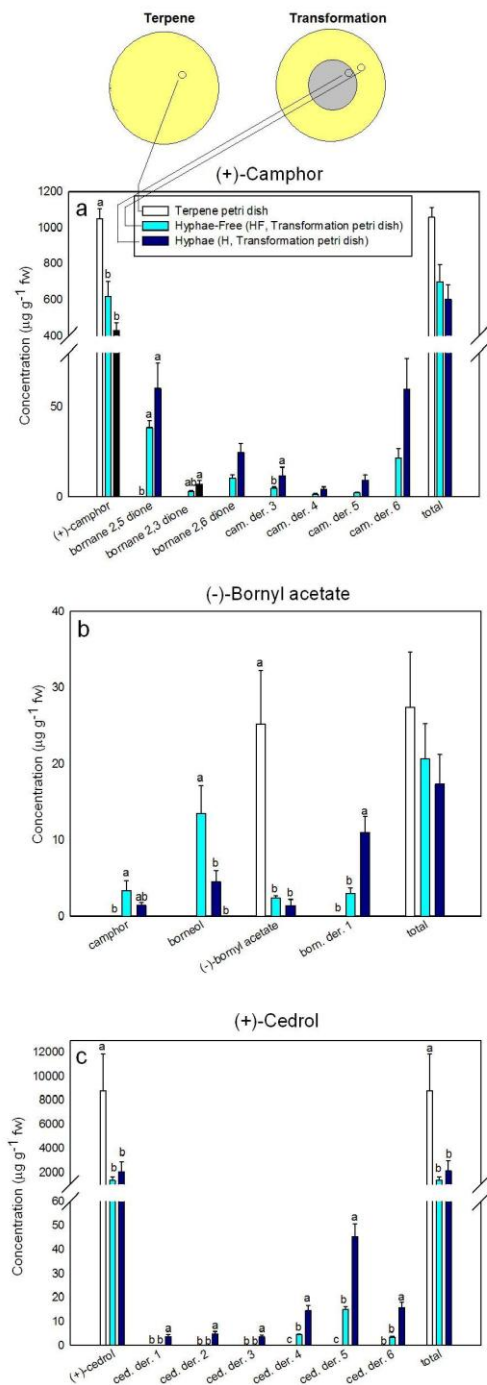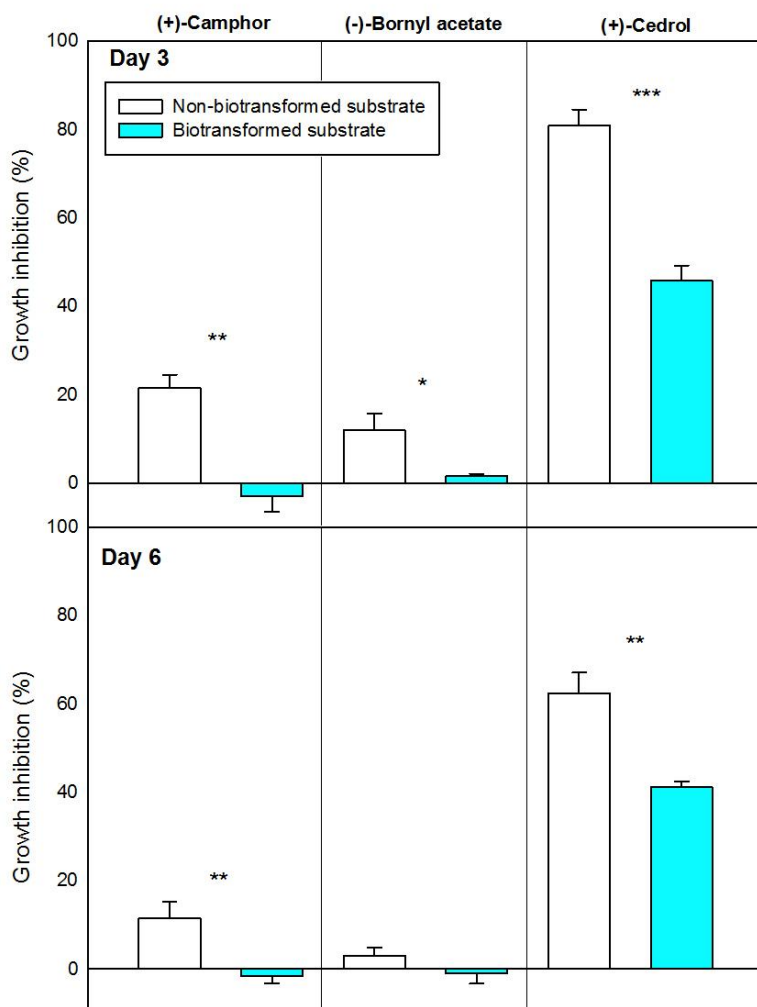
163

Figure 4 Mean concentrations (mg g^{-1} fresh weight ±SE) of compounds in the plugs of malt agar extract (MEA) extracted from tests performed with (+)-camphor, (-)-bornyl acetate and (+)-cedrol in the terpene (MEA + acetone + terpene) and transformation (MEA + acetone + terpene + fungus) tests. Different letters indicate significant differences (one-way ANOVA, Tukey's *post hoc* test, $P < 0.05$). fw, fresh weight; cam. der., (+)-camphor derivative; born. der., (-)-bornyl acetate derivative; ced. der., (+)-cedrol derivative. ?, tentative identification.

had strong inhibitory power, even at low concentrations. Constitutive totarol (higher in Agrimed (Fig. 2)) could be an effective first line of defence against fungal infection (Fig. 3). (+)-Totarol can inhibit efflux-pump activity in bacteria (Smith et al. 2007), which could be related to its low concentration-dependent inhibition of *S.cardinale* (Fig. 3).

The level of inhibition by the major terpenes of *C. sempervirens*, ((+)-α-pinene, (+)-δ-3-carene, (+)-cedrol, and (+)-totarol), differed little between 0.25 and 1.0 mg g^{-1} MEA (Fig. 3d), suggesting that their inhibitory capacities have a low dependence on concentration within this concentration range. In contrast, inhibition by oxygenated MTs (containing all three phytoalexins and the strongly induced terpene terpinen-4-ol) was very concentration-dependent (Fig. 3), perhaps accounting for the higher concentrations of several minor compounds such as the oxygenated MTs (Fig. 2), terpinolene, or manool rather than of major compounds. Differences in the concentrations and proportions of several specific terpenes between groups may partly account for the ability of Agrimed to resist cypress bark canker, which develops further in NR (eventually resulting in death). Our results thus support the hypotheses proposed in our previous study (Achotegui-Castells et al. 2015), which suggested that terpenes may function to slow fungal advance, enhance compartmentalisation by necrophylactic periderm, and ultimately stop the fungal infection.

The biotransformation of (+)-camphor to bornane-2,5-dione was observed for the first time in *Pseudomonas putida* (Bradshaw et al. 1959), and this biotransformative pathway has since been extensively studied, mainly in bacteria (Jones et al. 1993; Taylor and Trudgill 1986). The fungal biotransformation of bornyl acetate to camphor and borneol has also been described (Kusumoto et al. 2014; Miyazawa and Miyasato 2001). Detoxification was observed (Fig. 5) in HF substrate for (+)-camphor, (-)-bornyl acetate and (+)-cedrol, suggesting that *S. cardinale* is capable of altering its immediate environment on its behalf (Farooq et al. 2002).The biotransformations and detoxifications observed in the MEA free of fungal hyphae (HF) could be explained by two processes. *i) S. cardinale* excretes terpene substrates along with some biotransformed products away from fungal cells, which would act as a detoxification mechanism to lower the cellular terpene levels (Wang et al. 2013). This explanation, though, is inconsistent with the significantly lower concentrations of terpene substrates

in the HF plugs of the transformation tests relative to those of the terpene tests (Fig. 4). *ii) S. cardinale,* suggested to release exoenzymes that play a role in systemic pathogenesis (Graniti 1998), may also have secreted exoenzymes capable of degrading defensive terpenes before hyphal contact. Such a mechanism would be advantageous to *S. cardinale*, because the fungus would encounter partially detoxified defences, and thus a less aggressive environment to colonise.

Figure 5. Fungal growth inhibition (mean ±SE) measured at day 3 (top) and day 6 (bottom) provoked by the the application of pentane extracts of Non-biotransformed substrate and Biotransformed substrate, Asterisks (*) indicate statistical significance between the two treatments (T-tests, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Histogram colors are in accordance with those of figure 4.

Conclusions

The differences in constitutive and induced terpene responses to infection between NR and Agrimed, along with the inhibitory power of these compounds, suggest that part of the Agrimed resistance to cypress bark canker may be due to its stronger and more inducible terpenic profile. Inhibition tests suggest that *C. sempervirens* reacts to the early stages of infection (day 30) by increasing the concentration of MTs but may be preparing itself for more advanced stages by beginning to generate several phytoalexins and increasing the concentrations of the most inhibitory compounds currently known for this pathogen. Cypress devoted more resources to increasing the concentrations of minor than of major terpenes, corroborating the observations of our previous study (Achotegui-Castells et al. 2015), and we suggest that this strategy may be due to the high concentration-dependent inhibition of the most highly induced minor terpenes and the low concentration-dependent inhibition of the major terpenes. Nevertheless, *S. cardinale* may be able to tolerate some of the most inhibitory terpenes of *C. sempervirens*, detoxifying them by biotransformation and changing its immediate environment for its behalf. Further studies should determine the identity of the biotransformed compounds, and investigate the biotransformation and detoxification mechanisms of the fungus. It would also be very interesting to see if other pathosystems react similarly and corroborate the tree defence mechanisms suggested here. In more practical terms, the strong actions of the most inhibitory terpenes warrant further efforts to test their viability as natural fungicides against *S. cardinale*.

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General conclusions

General conclusions

- The conifer terpenes of the interactions studied clearly reacted to aggressions of biotic agents, confirming that the concentrations and emissions of these secondary compounds are sensible to herbivory and pathogenicity, in differnent plant tissues (Ch 1. – Ch. 5). However, the reactions were notably different in the two interactions studied: in Italian cypress, phloem concentrations and foliar emissions clearly increased in response to infection (Ch. 4, Ch. 5), whereas in herbivore-attacked Scots pines, needle terpene concentrations decreased (Ch. 1, Ch. 2) coupled to an increase of terpene emissions (Ch. 1). However, when growing new needles after previous defoliation (Ch. 3), one Scots pine subspecies was able to increase needle terpene concentrations and change foliar emissions, whereas the other subspecies showed no changes in these parameters.

- Our results in the *Thaumetopoea pityocampa - Pinus sylvestris* system indicate, coinciding with recent reports, that the needle terpene concentrations of Scots pines decrease in front of PPM attacks (Ch. 1, Ch. 2), and suggest this outcome could be due to a combination of terpene emission losses (Ch. 1) and weak terpene inductions (Ch. 2). However, slight terpene concentration increases were reported in systemic needles (Ch. 1, Ch. 2). New needles grown after an attack episode (Ch. 3) showed contrasting responses in the two Scots pine subspecies studied. The increased terpene concentrations, changes in terpene emissions and decrease of nutrient concentrations, in both attacked (local) and non-attacked (systemic) needles, were observed just in one subspecies. These results indicate that pines are capable of articulating a complex response to previous defoliation, but that this outcome could be dependent on other factors such as philogeny or abiotic stress. Nevertheless, our laboratory tests suggest these changes in needle properties were not capable of diminishing larval survival of *Thaumetopoea pityocampa*.

- The strong terpene inductions and substantial changes in terpene proportions observed in the *Seiridium cardinale – Cupressus sempervirens* pathosystem (Ch. 4, Ch. 5) indicate that terpenes play an important role in the defence strategy of Italian cypress against this pathogen. Changes in terpene emissions were also reported in systemic tissues (leaves) (Ch. 4). Accordingly, results of fungal inhibition tests suggested that terpenes have an important role in slowing down fungal growth (Ch. 5), indicating that these compounds can enhance the healing process leaded by the necrophylactic periderm. However, we observed that some terpenes stimulate fungal growth and that *Seiridium cardinale* is capable of biotransforming and detoxifying several of these compounds, evidencing that this fungus is relatively resistant to the terpene defences of Italian cypress (Ch. 5).

- We described for the first time the foliar terpene emissions resulting of these two interactions (Ch.1 , Ch. 3, Ch. 4), in local and systemic leaves. Significant changes in terpene emission rates and proportions were observed in both systems (Ch. 1, Ch. 3, Ch. 4), suggesting foliar terpene emissions are sensible to biotic attacks. The changes reported could be important cues for organisms related to the biotic attacks described.

- Clear and significant terpene systemic reactions were observed in both systems (Ch.1, Ch. 2, Ch.3, Ch. 4). Foliar concentrations and emissions of terpenes changed in response to herbivory (Ch.1, Ch.2, Ch. 3) and fungal infection (Ch. 4) in systemic tissues, highlighting the importance of defence regulation of close (Ch. 2, Ch. 4) and distant (Ch. 1, Ch. 2, Ch. 3) undamaged plant parts that are prone to be attacked soon.

- Comparisons between tree varieties were conducted for both systems (Ch. 1, Ch. 3, Ch.5), and demonstrated that very remarkable differences in terpene response to biotic attacks can occur within trees of the same species. In Scots pines (Ch. 1, Ch. 3) *Pinus sylvestris* subsp. *iberica* was clearly more prone to change needle terpene concentrations, especially after previous defoliation (Ch. 3), than *Pinus sylvestris* subsp. *nevadensis*. In Italian cypress, the comparison of trees susceptible and resistant to *Seiridium cardinale* showed significant differences in terpene responses to infection. Results suggested resistant trees induce stronger

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terpene defences in front of fungal infection (Ch. 5), and that part of their resistance to *S. cardinale* could be attributed to these compounds.

- Laboratory tests were performed to assess the effects of terpenes on the biotic agents studied (Ch.3, Ch. 5). Tests provided valuable information about larval survival (Ch. 3) and fungal growth inhibition, biotransformation and detoxification (Ch. 5), that were key to evaluate the defensive role of terpenes. Nevertheless, innovative and more realistic tests are needed to further understand the interactions between plants and biotic agents.

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Appendix

Appendix 1: Supplementary materials of Chapter 3
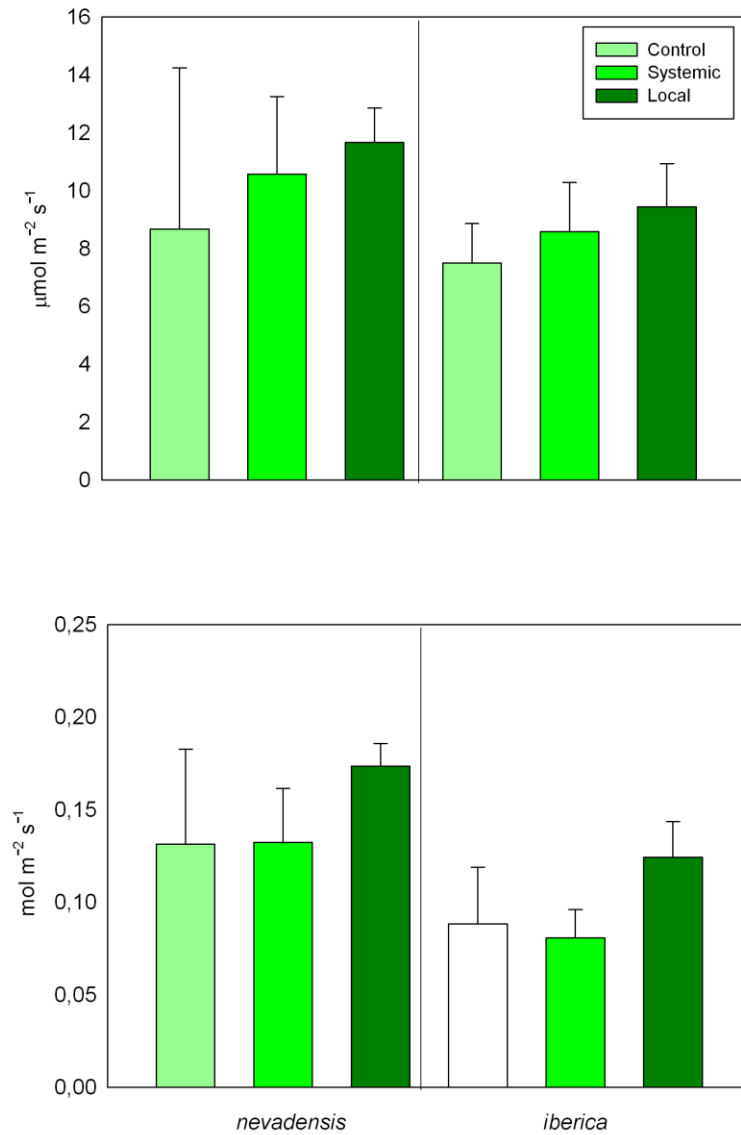
Figure S1. Mean (± SE) net photosynthetic rates (µmol m^{-2} s^{-1}) and stomatal conductances (mol m^{-2} s^{-1}) of the three attack states of the two *Pinus sylvestris* subspecies.
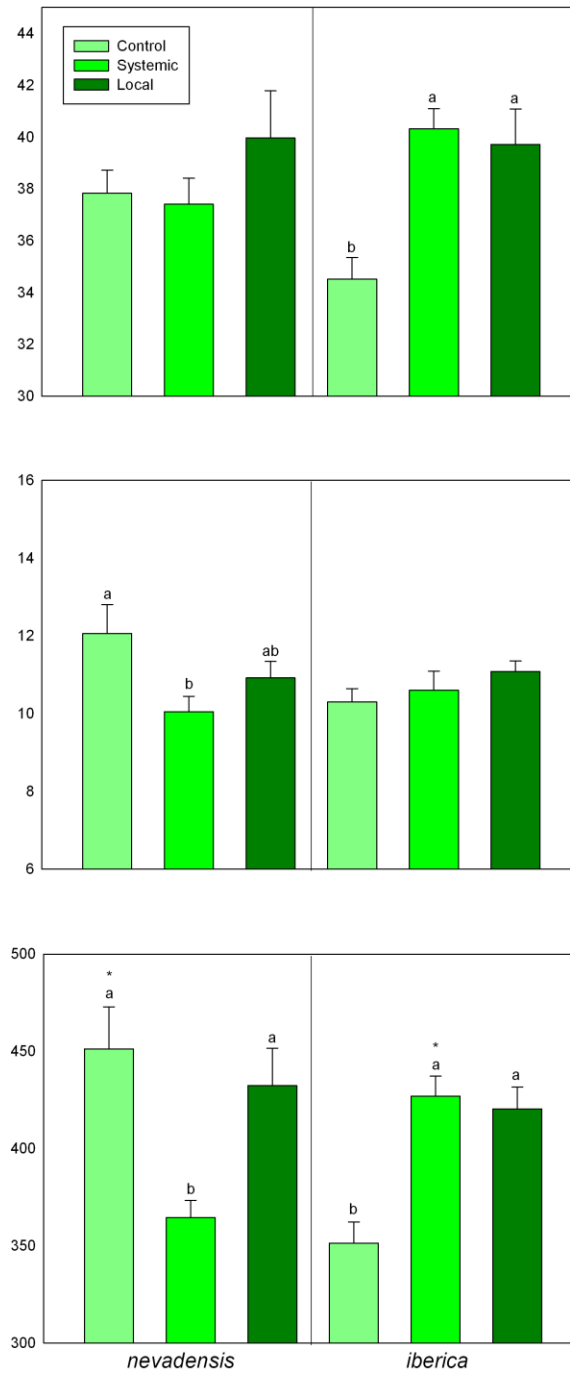
Figure S2. Mean (± SE) nutrient ratios. Different letters indicate significant differences (one-way ANOVA, Tukey's post-hoc test, $P < 0.05$), and italicised letters indicate marginally significant differences ($P < 0.10$). Asterisks (*) indicate significant differences between the same attack state of the two *P. sylvestris* subspecies.

Figure S3. Correlations between nutrient ratios (%dry weight) and total monoterpene concentrations. Solid lines indicate significant correlations ($P < 0.05$), short-dashed lines indicate marginally significant correlations ($0.05 < P < 0.10$), and long-dashed lines indicate non-significant correlations ($P > 0.10$).

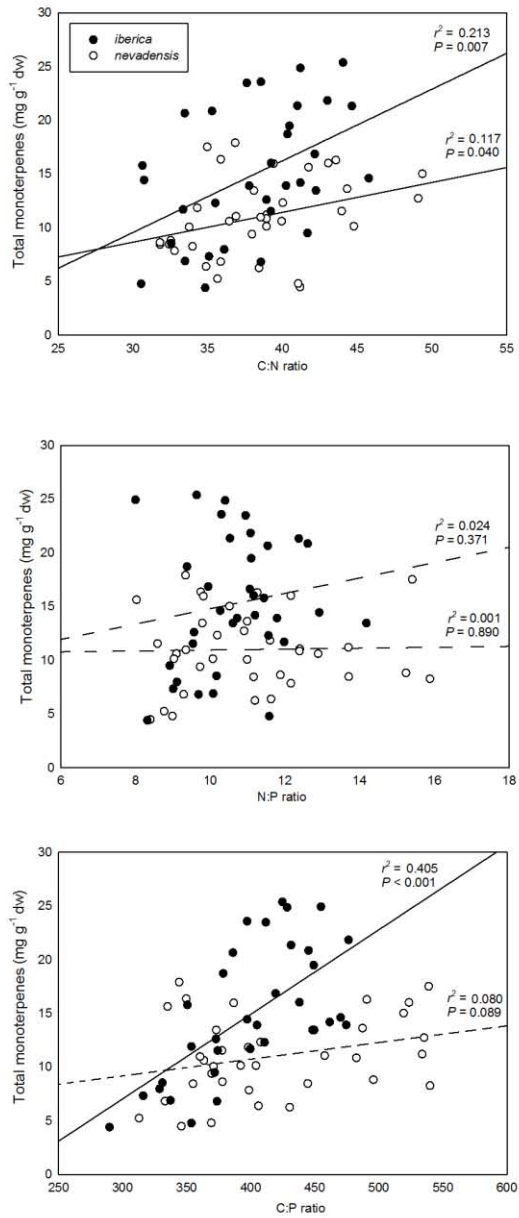| | | Carbon | | | | | | Nitrogen | | | | | | Phosphorus | | | | | |
| | | nevadensis | | | iberica | | | nevadensis | | | iberica | | | nevadensis | | | iberica | | |
		β	r²	P	β	r²	P	β	r²	P	β	r²	P	β	r²	P	β	r²	P
Monoterpenes	Triciclene	0.142	0.02	0.424	0.52	0.271	0.001	-0.164	0.027	0.354	-0.233	0.054	0.192	-0.239	0.057	0.173	-0.498	0.248	0.002
	α-pinene	0.069	0.005	0.692	0.507	0.258	0.002	-0.391	0.153	0.02	-0.384	0.148	0.027	-0.241	0.058	0.163	-0.588	0.345	>0.001
	Camphene	0.106	0.011	0.55	0.432	0.187	0.012	-0.071	0.005	0.69	-0.14	0.02	0.459	-0.243	0.059	0.167	-0.637	0.406	>0.001
	Sabinene	0.061	0.004	0.728	0.452	0.204	0.011	0.098	0.01	0.575	-0.112	0.012	0.565	-0.297	0.088	0.083	-0.461	0.212	0.009
	ß-pinene	0.147	0.021	0.423	0.675	0.455	>0.001	-0.297	0.088	0.099	-0.241	0.058	0.227	-0.428	0.183	0.015	-0.624	0.39	>0.001
	ß-myrcene	0.181	0.033	0.304	0.46	0.212	0.005	-0.111	0.012	0.534	-0.247	0.061	0.165	-0.369	0.136	0.032	-0.489	0.239	0.002
	Limonene	0.293	0.086	0.098	0.543	0.295	0.001	-0.108	0.012	0.551	-0.348	0.121	0.055	-0.45	0.202	0.009	-0.632	0.399	>0.001
	ß-ocimene	0.124	0.015	0.484	0.224	0.05	0.195	-0.158	0.025	0.371	-0.173	0.03	0.345	-0.063	0.004	0.725	-0.296	0.087	0.085
	γ-terpinene	0.032	0.001	0.853	0.234	0.055	0.175	-0.257	0.066	0.136	-0.048	0.002	0.796	-0.14	0.019	0.424	-0.35	0.122	0.039
Sesquiterpenes	ß-elemene	0.143	0.021	0.411	0.159	0.025	0.353	-0.194	0.038	0.264	-0.104	0.011	0.563	-0.097	0.009	0.58	-0.051	0.003	0.77
	Caryophyllene	0.178	0.032	0.306	0.213	0.045	0.242	-0.199	0.039	0.252	-0.139	0.019	0.471	0.212	0.045	0.221	-0.332	0.11	0.063
	α-caryophyllene	0.166	0.027	0.341	0.307	0.094	0.082	-0.186	0.035	0.284	-0.266	0.071	0.156	0.218	0.048	0.208	-0.376	0.141	0.031
	ST 1	0.224	0.05	0.196	0.193	0.037	0.259	0.013	0.000	0.94	-0.28	0.078	0.114	0.221	0.049	0.202	-0.067	0.004	0.698
	Germacrene D	0.239	0.057	0.167	0.238	0.057	0.168	-0.202	0.041	0.244	-0.245	0.06	0.177	0.064	0.004	0.714	0.098	0.01	0.576
	Bicyclogermacrene	0.149	0.022	0.394	0.211	0.044	0.217	-0.223	0.05	0.198	-0.039	0.001	0.831	-0.108	0.012	0.538	-0.047	0.002	0.786
	γ-cadinene	0.154	0.024	0.383	0.211	0.044	0.217	-0.146	0.021	0.41	-0.312	0.097	0.088	0.046	0.002	0.795	-0.1	0.01	0.575
	δ-cadinene	0.259	0.067	0.146	0.075	0.006	0.673	-0.093	0.009	0.608	-0.353	0.124	0.052	0.037	0.001	0.838	-0.116	0.014	0.512
	Germacren D-4-ol	0.241	0.058	0.185	0.086	0.007	0.629	-0.075	0.006	0.682	-0.204	0.041	0.272	-0.01	0.000	0.957	-0.098	0.01	0.581
	t-cadinol	0.081	0.007	0.676	0.053	0.003	0.77	-0.119	0.014	0.539	-0.229	0.052	0.224	0.196	0.038	0.308	0.135	0.018	0.452
	α-cadinol	0.131	0.017	0.515	0.044	0.002	0.809	-0.112	0.013	0.577	-0.198	0.039	0.293	0.04	0.002	0.842	0.236	0.056	0.186
Diterpene	ß-springene	0.188	0.035	0.28	0.414	0.171	0.013	-0.096	0.009	0.582	-0.333	0.111	0.063	0.098	0.01	0.575	-0.262	0.069	0.128
Totals	Total monoterpenes	0.096	0.009	0.582	0.513	0.263	0.001	-0.329	0.108	0.054	-0.352	0.124	0.044	-0.283	0.08	0.099	-0.614	0.377	>0.001
	Total sesquiterpenes	0.231	0.053	0.181	0.25	0.063	0.141	-0.232	0.054	0.179	-0.269	0.073	0.13	0.103	0.011	0.554	-0.143	0.02	0.407
	Total terpenes	0.138	0.019	0.428	0.522	0.272	0.001	-0.332	0.11	0.052	-0.375	0.14	0.032	-0.21	0.044	0.226	-0.595	0.353	>0.001
	%MT	-0.294	0.087	0.097	0.236	0.056	0.167	-0.143	0.021	0.426	-0.104	0.011	0.566	-0.37	0.137	0.034	-0.569	0.323	>0.001

Table S1. Correlations between all terpene and nutrient concentrations studied. Shaded cells indicate significant correlations, with the degree of shading corresponding to the strength of the correlation.