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THE ROLE OF TERPENES IN CHEMICAL DEFENCES OF FOREST PLANTS

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ABSTRACT

The role of terpenes in chemical defences of forest plants

Keywords: terpene, essential oil, constitutive and induced defences, systemic induced resistance, *in-vitro* test, *Alternaria alternata, Rosmarinus officinalis*, *Picea sitchensis*, *Heterobasidion annosum*.

Aims

The general goal of the thesis was to investigate the role of terpenes in the constitutive and induced chemical defenses of *Rosmarinus officinalis* L. and *Picea sitchensis* (Bong.) in response to *Alternaria alternata* (Fr.) Keissel and *Heterobasidion annosum* (Fr.) Bref. respectively.

Part A. *R. officinalis* is an important pioneer species of the Mediterranean maquis and is widely cultivated all over the world as ornamental and aromatic plant. The interest towards its cultivation is due to the many biological activities of the essential oil extracted from the flowering aerial tops: leaves, twigs and flowers. *A. alternata* is a pathogen of rosemary causing: 'alternaria leaf spot of rosemary'. This disease has been reported in various Italian regions; it causes the appearance of black spots on leaves and stems and even the defoliation of plants. Severe attacks can be particularly observed on plants growing in humid and poorly ventilated areas. The aims of this part of the thesis were (1) to investigate the geographical variation of constitutive terpenoid defenses in different provenances of *R. officinalis;* (2) to evaluate the antifungal activity *in-vitro* of some monoterpenes of the rosemary essential oil against *A. alternata.*

Part B. *P. sitchensis* is the most important commercial timber species in the United Kingdom, occupying 40% of the coniferous forest area. It is very susceptible to the *H. annosum* infection, which is a serious root and butt-rot fungal pathogen of conifers that causes significant economic losses when monoculture plantations are attacked. The objective of this study was: (3) to determine variations in constitutive and induced terpene contents in response to *H. annosum* attack in *P. sitchensis* clones.

Methods and Results

Part A. (1) **Methods:** Samples of rosemary were collected from four different natural populations and analyzed by gas chromatography–flame ionization detection (GC-FID). Enantiomeric monoterpenes were separated on a Cyclodex-B capillary column. **Results:** Three chemotypes were identified: α-pinene/verbenone (Sardinian plants), 1,8-cineole (Alberese plants) and α-pinene/1.8-cineole (Giglio and Elba islands plants). (2) **Methods:** *In-vitro* inhibiting activities of (+)-α-pinene, (-)-α-pinene, myrcene, (-)-β-pinene, (+)-β-pinene, (+)-limonene, (-)-limonene, 1,8-cineole and linalool were evaluated as inhibition of the mycelial growth of *A. alternata*. The terpenes previously identified in the essential oil of rosemary, were added in glass vials containing 100 ml of Potato Dextrose Broth and one *A. alternata* plug. The vials were sealed with teflon septums and crimped with aluminium caps; then they were put into a shaker for about ten days. **Results:** (2) The lowest values of the minimum inhibitory concentration (MIC) were observed for myrcene and (-)-α-pinene, whereas the least toxic compounds were (+)-β-pinene, 1.8-cineole and linalool. **Part B.** (3) **Methods:** Terpene composition was analyzed in cortical tissue samples of Sitka spruce. Each tree stem was inoculated at about 20-30 cm above ground. Four holes were drilled and the wood inocula together with the control treatment randomly inserted with a hammer. Reference samples were collected at day 0 (i.e. on the day of wounding and inoculation); bark samples were collected 3 and 43 days after wounding and inoculation. In order to investigate the systemic induced effect on terpene response to attack, bark samples were collected 43 days after the inoculation, at 15 and 100 cm from the site of infection. Samples of bark of Sitka spruce were analyzed by gas chromatography–flame ionization detection (GC-FID) and enantiomeric monoterpenes were separated on a Cyclodex-B capillary column. **Results:** Generally, the relative contents of constitutive terpenes did not vary among the four clones. The concentration of several terpenes increased following either wounding and wounding plus inoculation, reaching the highest values in infected tissues 43 days after the inoculation. There weren't significant differences between the terpene profiles of bark tissues collected at 15 and 100 cm from the wounded plus inoculated and the control tissues.

Conclusion

Part A. Based on the results of the studies on geographical variability of terpene content of *R. officinalis* and the antifungal activity of single monoterpenes, the plants from Alberese population, characterized by a high concentration of 1.8-cineole and a low concentration of myrcene and (-)-α-pinene, could be defined as "more susceptible" to the attack of *A. alternata*.

On the contrary, the Sardinian plants, with the content of 1.8-cineole lower than the content of (+)-α-pinene, could be "relatively resistant" to the fungus attack. Results also showed that this population was characterized by a high content of verbenone that, based on literature, has antimicrobial and antifungal activities. This supported the "relative resistance" of rosemary from Sardinia.

Part B. Data showed that terpene metabolism is involved in chemical defence of *P. sitchensis* in response to attack by *H. annosum*.

Total terpenoid concentrations showed the highest amounts in infected tissues collected 43 days after the inoculation. The concentration of several terpenes increased following wounding and wounding plus inoculation, reaching the highest values in infected tissues 43 days after the inoculation. No systemic induction of a monoterpene effect was observed.

Significance and Impact of the Study

Nowadays, the importance of terpenes in medicine, agriculture and industry has led to numerous studies on the synthesis, biosynthesis and biological activities of these substances. Yet we still know little about their actual roles in nature.

However, starting in the 1970s, a number of terpenes were demonstrated to be toxins, repellents or attractants to other organisms, which led to the belief that they have ecological roles in antagonistic or mutualistic interactions among organisms. Many terpene natural products have been reported to act as toxins, growth inhibitors, or deterrents to microorganisms and animals. Protection against enemies may indeed be their primary role in nature. They are also important in resistance to diseases caused by fungi and bacteria. Several terpenes are also known as antimicrobial agents and they are found in the areas of food preservation and in the manufacturing of medicinal antimicrobial agents and disinfectants. There also is an increasing

interest in research concerning the use of toxic natural products including plant essential oils, extracts and secondary metabolites for alternative pest and disease control in agroforestry.

This study could have significant implications for forest health and cultivated plants protection, including a rationale for reinforcing preventative measures to exclude biological invasions.

RIASSUNTO

Il ruolo dei terpeni nella difesa chimica di piante forestali

Parole chiave terpeni, olio essenziale, difese costitutive ed indotte, induzione sistemica, test *in-vitro*, *Alternaria alternata, Rosmarinus officinalis*, *Picea sitchensis*, *Heterobasidion annosum*.

Scopi

Obiettivo generale di questo lavoro è stato quello di studiare la relazione tra il ruolo dei terpeni nelle difesa chimica costitutiva ed indotta di *Rosmarinus officinalis* L. e *Picea sitchensis* (Bong.) in relazione, rispettivamente, all'attacco di *Alternaria alternata* (Fr.) Keissel e *Heterobasidion annosum* (Fr.) Bref.

Parte A. *R. officinalis*, specie pioniera della macchia Mediterranea, è oggi coltivato in tutto il mondo grazie alle sue qualità organolettiche e alla presenza di numerose sostanze terpeniche e fenoliche. Dalla distillazione della pianta si ricava un olio essenziale con proprietà biologiche. *A. alternata,* fungo patogeno del rosmarino, è agente di necrosi fogliare; attacchi più violenti, che possono portare al completo disseccamento della pianta,sono frequenti in piante cresciute in aree umide e poco ventilate. Gli scopi in questa parte della tesi sono stati: (1) studiare le variazioni geografiche nei profili terpenici costitutivi di diverse popolazioni di rosmarino; (2) Testare *in-vitro* l'attività antifungina di alcuni monoterpeni, costituenti l'olio essenziale di rosmarino, sulla crescita miceliare di *A. alternata.*

Parte B. *P. sitchensis* è la specie più importante per la produzione di legname in Gran Bretagna, occupando il 40% dell'area boschiva. Questa specie è molto suscettibile all'attacco di *H. annosum,* agente di marciume radicale, che causa importanti perdite economiche. Obiettivo in questo studio: (3) determinare le variazioni nel profilo terpenico costitutivo e indotto in 4 cloni di P. *sitchensis* inseguito all'attacco fungino.

Metodi e Risultati

Parte A. (1) **Metodi:** Campioni naturali di rosmarino sono stati raccolti da quattro provenienze diverse e analizzati secondo tecniche di gascromatografia (GC-FID); è stata utilizzata una colonna capillare Cyclodex-B per la separararazione dei composti enantiomerici. **Risultati:** le popolazioni sono state classificate in tre chemotipi diversi: α-pinene/verbenone (piante della Serdagna), 1,8-cineolo (piante del parco di Alberese) e α-pinene/1.8-cineolo (piante dell'isola del Giglio e dell'Elba). (2) **Metodi:** L'attività inibitoria sulla crescita del micelio di *A. alternata* è stata testata *in-vitro* per i seguenti monoterpeni: (+)-α-pinene, (-)-α-pinene, mircene, (-)-β-pinene, (+)-β-pinene, (+)-limonene, (-)-limonene, 1,8-cineolo e linalool. I monoterpeni, precedentemente identificati nell'olio essenziale del rosmarino, sono stati aggiunti in vials di vetro conteneti 100 ml of PDB e un tassello di *A. alternata*. Le vials, dopo essere sigillate, sono state poste in agitazione per circa dieci giorni. **Risultati:** Mircene e (-)-α-pinene sono risultati i composti più attivi, mentre (+)-β-pinene, 1.8-cineolo e linalolo, i meno tossici.

Parte B. (3) **Metodi:** Sono stati analizzati tessuti di corteccia feriti, feriti e infettati con *H. annosum,* e tessuti non trattati (controllo). Ogni fusto è stato inoculato a circa 20-30 cm dal suolo. I campioni sono stati raccolti: al momemto dell'inoculazione, per il controllo e 3 e 43 giorni dopo l'inoculazione, per i campioni feriti e per quelli feriti e infettati. Per verificare una possibile risposta sistemica indotta il campionamento è stato effettuato anche ad altezze diverse (15 e 100 cm dal sito di infezione) 43 giorni dopo l'inoculazione. Le analisi sono state effettuate mediante gascromatografo (GC-FID), dotato di colonna capillare Cyclodex-B, in grado di separare i composti enantiomerici. **Risultati:** In generale non sono state osservate differenze significative nei contenuti relativi dei terpeni costitutivi, fra i quattro cloni. La concentrazione dei terpeni incrementa inseguito alle ferite e alle inoculazioni, raggiungendo il massimo 43 giorni dopo l'infezione. Non sono state rilevate differenze significative fra i campioni raccolti ad altezze diverse e il controllo.

Conclusioni

Parte A. In base ai risultati sullo studio della variazione geografica nel profilo terpenico di rosmarino e l'attività antifungina di singoli monoterpeni, le piante

provenienti da Alberese, caratterizzate da un alto contenuto di 1.8-cineolo e un basso contenuto di mircene e (-)-α-pinene, potrebbero essere "più suscettibili" all'attacco di *A. alternata*. Al contrario, quelle provenienti dalla Sardegna, con un contenuto maggiore di (+)-α-pinene e verbenone potrebbero essere "più resistenti" all'attacco fungino. **Parte B.** I terpeni risultano essere coinvolti nella difesa chimica di *P. sitchensis* in seguito all'attacco di *H. annosum*, infatti la concentrazione totale dei terpeni ha rivelato il più alto incremento in tessuti infetti, raccolti 43 giorni dopo l'inoculazione. In seguito alle ferite e alle infezioni, si ha un incremento generale nel contenuto dei singoli terpeni, ma l'aumento più alto è risultato, anche in questo caso, nei tessuti infetti e raccolti al 43° giorno. Non è stata rilevata un risposta sistemica indotta.

Significato ed Impatto dello Studio

L'importanza dei terpeni in medicina, agricoltura e nell'industria alimentare ha portato allo sviluppo di numerosi studi sulla sintesi, biosintesi ed attività biologica di questi composti. Nonostante molte delle loro funzioni naturali siano ancora sconosciute, è stato dimostrato che, per alcuni organismi, certi terpeni possono essere tossici, repellenti od attrattivi, formulando l'ipotesi che i terpeni svolgano un ruolo ecologico in interazioni antagonistiche o mutualistiche tra organismi e siano coinvolti nella difesa da attacchi patogeni.

Ampiamente utilizzati per la loro attività antimicrobica, sono utilizzati come conservanti per il cibo, in cosmesi e in campo farmaceutico. Oli essenziali, estratti e metaboliti secondari, vengono impiegati come pesticidi alternativi in campo agroforestale.

Questo studio potrebbe avere importanti implicazioni per la gestione e valorizzazione di boschi e per la protezione di piante coltivate, includendo misure preventive come la selezione ed il miglioramento genetico. Tali misure possono essere di grande ausilio per far fronte alle varie esigenze di ordine sia produttivo, in senso lato, sia fitosanitario, in particolare. Pertanto, la possibilità di ricorrere a specie, cultivar o cloni resistenti rappresenta un'efficace arma di lotta contro numerose malattie biotiche anche nel settore forestale.

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BACKGROUND AND AIMS OF THE THESIS

Terpenoids perhaps are the most structurally varied class of plant natural products. The name terpenoid, or terpene, derives from the fact that the first members of the class were isolated from turpentine ("terpentin" in German) (Croteau *et al*. 2000).

All terpenoids, including both primary metabolites and more than 25,000 secondary compounds, are derived from the five-carbon precursor isopentenyl diphosphate (IPP). The 12,000 or so known alkaloids, which contain one or more nitrose atoms, are biosynthesized principally from amino acids. The 8000 or so phenolic compounds are formed by way of either the shikimic acid pathway or the malonate/ acetate pathway.

These compounds are derived by ripetitive fusion of branched five-carbon units based on isopentane skeleton. These monomers generally are referred to as isoprene units because thermal decomposition of many terpenoid substances yields the alkene gas isoprene as a product and because suitable chemical conditions can induce isoprene to polymerize in multiples of five carbons, generating numerous terpenoid skeletons. For these reasons, the terpenoids are often called isoprenoids, although researchers have known for well over 100 years that isoprene itself is not the biological precursor of this family of metabolites (Croteau *et al*. 2000).

The smallest terpenes contain a single isoprene unit; as a group, they are named **hemiterpenes** (half-terpenes). The best known hemiterpene is isoprene itself, a volatile product released from photosynthetically active tissues.

C_{10} terpenoids, although they consist of two isoprene units, are called **monoterpenes;** as the first terpenoids isolated from turpentine in the 1850s, they were considered to be the base unit from which the subsequent nomenclature is derived. The monoterpenes are best known as components of the volatile essences of flowers and of the essential oils of herbs and spices, in which they make up as much as 5% of plant dry weight. Monoterpenes are isolated by either distillation or extraction and find considerable industrial use in flavors and perfumes.

The terpenoids that derive from three isoprene units contain 15 carbon atoms and are known as **sesquiterpenes.** Like monoterpenes, many sesquiterpenes are found in essential oils. In addition, numerous sesquiterpenoids act as phytoalexins, antibiotic compounds produced by plants in response to microbial challenge, and as antifeedants that discourage opportunistic herbivory.

The **diterpenes,** which contain 20 carbons (four C_5 units), include phytol (the hydrophobic side chain of chlorophyll), the gibberellin hormones, the resin acids of conifer and legume species, phytoalexins, and a host of pharmacologically important metabolites, including taxol, an anticancer agent found at very low concentrations (0.01% dry weight) in yew bark, and forskolin, a compound used to treat glaucoma.

The **triterpenes,** which contain 30 carbon atoms, are generated by the head-tohead joining of two C_{15} chains, each of which constitutes three isoprene units joined head-to-tail.

The most prevalent **tetraterpenes** (40 carbons, eight isoprene units) are the carotenoid accessory pigments which perform essential functions in photosynthesis.

The **polyterpenes,** have more than eight isoprene units,

Despite great diversity in form and function, the terpenoids are unified in their common biosynthetic origin. The biosynthesis of all terpenoids from simple, primary metabolites can be divided into four overall steps: *(a)* synthesis of the fundamental precursor IPP; *(b)* repetitive additions of IPP to form a series of prenyl diphosphate homologs, which serve as the immediate precursors of the different classes of terpenoids; *(c)* elaboration of these allylic prenyl diphosphates by specific terpenoid synthases to yield terpenoid skeletons; and *(d)* secondary enzymatic modifications to the skeletons (largely redox reactions) to give rise to the functional properties and great chemical diversity of this family of natural products (Croteau *et al*. 2000).

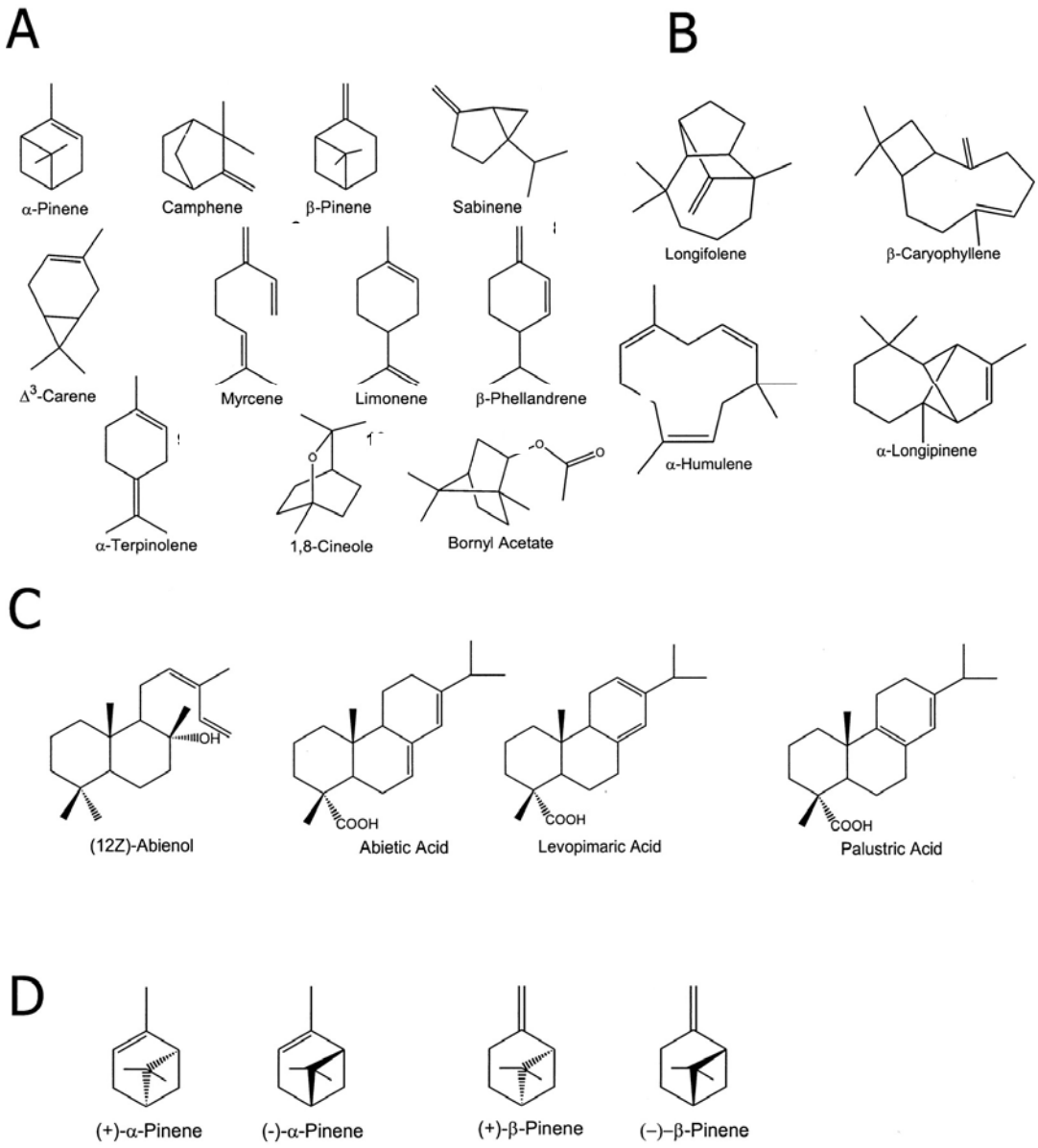

Figure 1: Representative structures of same terpenoids. A and D, Monoterpenes (10 carbon atoms). B, Sesquiterpenes (15 carbon atoms). C, Diterpene resin acids (20 carbon atoms) (Martin *et al.* 2002).

THE BIOSYNTHESIS OF TERPENES

The initial substrates for the biosynthesis of terpenes are the simple C_5-unit isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). The activity of three prenyltransferases produces the direct precursors of terpenes, the linear prenyl diphosphates geranyl diphosphate (GPP, C_{10}), farnesyl diphosphate (FPP, C_{15}) and geranylgeranyl diphosphate (GGPP, C_{20}). Terpene synthases (TPS) are the primary enzymes responsible for catalyzing the formation of hemiterpenes (C_5), monoterpenes (C_{10}), sesquiterpenes (C_{15}) or diterpenes (C_{20}) from the substrates DMAPP, GPP, FPP or GGPP, respectively (Dorothea Toll 2006). (Fig.2).

Important differences exist among the terpenoid biosynthesis. In particular, plants produce a much wider variety of terpenoids than do either animals or microbes, a difference reflected in the complex organization of plant terpenoid biosynthesis at the tissue, cellular, subcellular, and genetic levels.

The sesquiterpenes, triterpenes and polyterpenes appear to be produced in the cytosolic and endoplasmic reticulum (ER) compartments, whereas isoprene the monoterpenes, diterpenes, tetraterpenes, and certain prenylated quinones originate largely, if not exclusively, in the plastids. Terpene biosynthesis is probably the most expensive among the secondary metabolic processes and plants cannot maintain high concentrations of these defensive substances in all the tissues and organs at the same time (Gershenzon 1994; Lenghnheim 1994). The production of terpenoids is under genetic control (Gershenzon 1984; Gershenzon and Croteau 1990; Gershenzon and Croteau 1991; Gershenzon 1993; Croteau and Gershenzon 1994).

Terpenoids are synthesized in various cellular organelles, but are then stored in specialized secretory structures, thus protecting the plant's metabolic processes from their toxic effects. These structures are generally located in areas where they would most likely be effective in defense of various organs, for example trichomes on the surface of leaves, resin ducts and laticifers throughout tissues of trees, pockets near the epidermis of primary stems, in fruits (Gershenzon and Croteau 1991).

They are accumulated in target tissue which is generally more vulnerable than older leaves to herbivore attack and more important for the plant because photosynthetic rates are higher in young than in old leaves (Wahid *et al*. 1997).

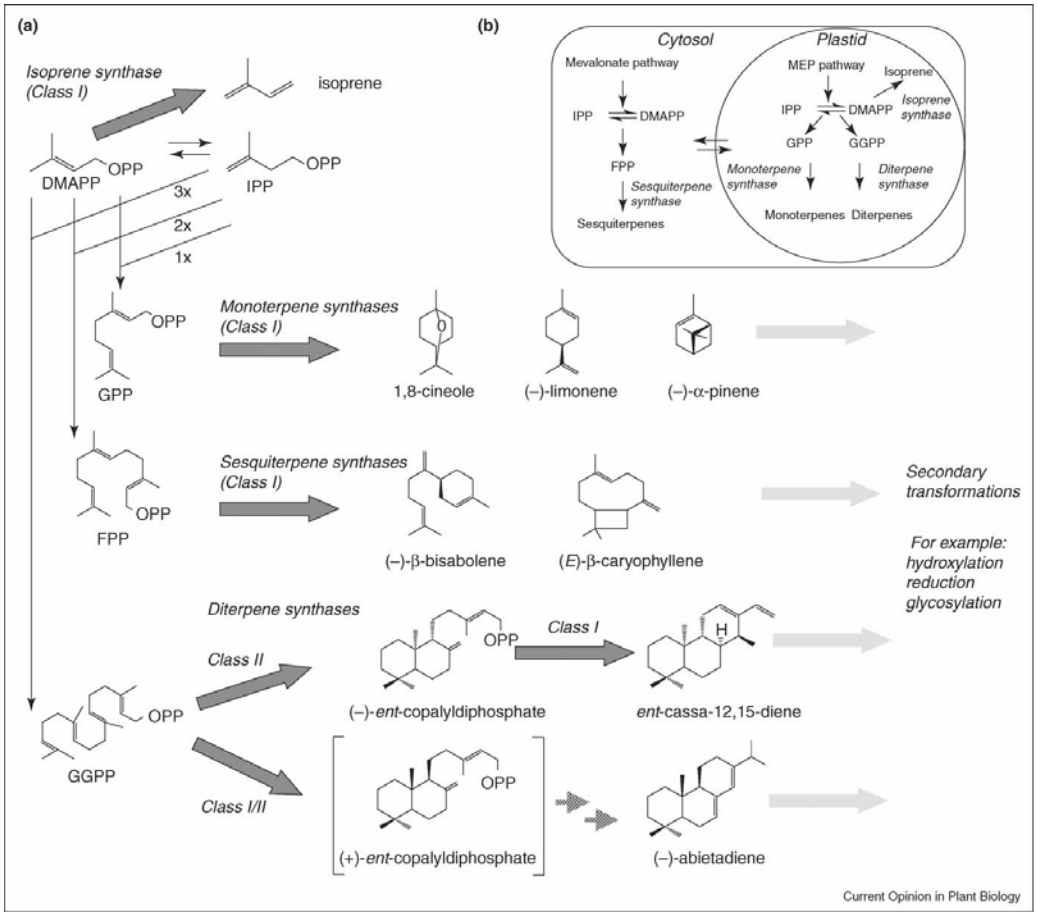

Figure 2: The biosynthesis of terpenes (Tholl 2006). (a). The initial substrates for the biosynthesis of terpenes are the simple C_5-unit isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). The action of various prenyltransferases then generates from the precursor the higher order terpenoid building blocks, geranyl pyrophosphate (GPP; C_{10}), farnesyl pyrophosphate (FPP; C_{15}), and geranylgeranyl pyrophosphate (GGPP; C_{20}). All terpene synthase products can be subject to further secondary transformations. (b) Compartmentation of terpene biosynthesis in the plant cell. Two independent pathways, the mevalonate and the methylerythritol phosphate (MEP) pathway, form the C_5-units IPP and DMAPP in the cytosolic and plastidic compartments, respectively. The biosynthesis of FPP and sesquiterpene metabolites occurs primarily in the cytosol, whereas the enzymes responsible for isoprene, monoterpene and diterpene formation are mostly located in plastids. OPP indicates the diphosphate moiety

VARIABILITY IN PLANT TERPENE MIXTURES

There are variations in terpene composition among tissues in a single species epigenetic variations), for example differences can be found between juvenile and mature tissues (Hanover 1992).

Bernard-Degan (1988) found that in *Pinus pinaster* the chlorophyllous tissues are varied in the relative amount of myrcene, limonene and 3-carene whereas cambium tissues are unable to elaborate these compounds ad produce only α- and β-pinene, that are accumulated in secondary resin ducts. So the relative compositions of volatile compounds differ among species, individuals and tissues (epigenetic variations) as well as between uninjured and injured tissues by fungi.

Differences between tissues were also detected in the enantiomeric composition for example, in Scots Pine, Sjödin *et al*. (2000) found that there was a higher ratio of (-)-α-pinene, (-)-β-pinene and (-)-limonene in the resin compared to the xylems.

Many studies showed that the differences in the terpene profile are within trees of the same species, so there are geographical and population variations. Hiltunene *et al*. (1975a) found large variations among *P. sylvestris* trees, and established two chemotypes designated as high and low 3-carene trees were growing in the north of Finland (high 3-carene) and in the south of Finland (low 3-carene). According to Agioni *et al*. (2004), inside *Rosmarinus officinalis* L. many chemotypes may be distinguished, the most important being traditionally cineoliferum (high content in 1,8-cineole), camphoriferum (camphor > 20%) and verbenoniferum (verbenone> 15%). According to the relative abundance of α-pinene, myrcene, or others relevant compounds many other chemotypes could also be recognized. Terpene profiles have been also widely used in chemosystematic studies to characterize coniferous species, provenances, clones and hybrids (Baradat and Yazdani 1988; Hanover 1992; Adams *et al*. 1993; Lang 1994; Baradat *et al.* 1996).

THE FUNCTION OF TERPENE NATURAL PRODUCTS

Plants produce a vast and diverse assortment of organic compounds. Many of these have no apparent function in the basic processes of growth and development, so they have been historically referred to as natural products or secondary metabolites.

The primary metabolites, such as phytosterols, acyl lipids, nucleotides, amino acids, and organic acids, in contrast are found in all plants and perform metabolic roles that are essential and usually evident (Croteau *et al*. 2000).

Although noted for the complexity of their chemical structures and biosynthetic pathways, from natural products have been widely perceived as biologically insignificant and have historically received little attention most plant biologists. Nowadays, the importance of natural products in medicine, agriculture and industry has led to numerous studies on the synthesis, biosynthesis and biological activities of these substances. Yet we still know little about their actual roles in nature.

Such knowledge is especially lacking for terpenes (also known as terpenoids or isoprenoids), the largest group of natural products. Of the approximately 25,000 terpene structures reported (Buckingham 1994), very few have been investigated from a functional perspective. In part this is a legacy of the once widely held belief that all natural products are metabolic wastes.

However, starting in the 1970s, a number of terpenes were demonstrated to be toxins, repellents or attractants to other organisms, which led to the belief that they have ecological roles in antagonistic or mutualistic interactions among organisms (Langenheim 1994).

Certain specialized groups of terpenes have been well-characterized for physiological functions, for example, sterols (membrane components, hormones) and carotenoids (photosynthetic pigments and antioxidants).

Many terpene natural products have been reported to act as toxins, growth inhibitors, or deterrents to microorganisms and animals so that their protection activity against enemies may be defined as their primary role in nature. For example, various monoterpenes (C_{10}) are toxic to insects (Lee *et al*. 2003), fungi (Hammer *et al*. 2003) and bacteria (Friedman *et al*. 2002) and serve as feeding deterrents to mollusks (Frank *et al*. 2002), insects (Szczepanik *et al*. 2002) and mammals

(Vourc'ch *et al*. 2002). EvidenceS for the defensive roles of terpenes are increasing with the development of the new discipline of chemical ecology.

Structurally similar terpenes often have very different ranges of biological activities, so it is no correct to make broad generalizations about their activity. This can be dangerous not only at the level of the whole class, but also when considering similar structures within a class. This is exemplified by the dimeric sesquiterpene gossypol, whose two atropoisomers (due to restricted rotation around the central binaphthyl bond) differ significantly in their effects on herbivores, pathogens and isolated cells. This is found in cotton and it is formed from two cadinane units (Gershezon and Dudereva 2007). Wild and domesticated cottons contain gossypol and related terpenoids in glands on their foliage, flower parts, bolls and roots (Hedin *et al*. 1992; Stipanovic *et al*. 1999). Gossypol occurs as a mixture of two enantiomers because of restricted rotation around the central binaphthyl bond. The ratio of (+)- to (–)-gossypol varies widely among cotton cultivars, and each enantiomer has different biological activities. For non-ruminant animals, such as rodents, chickens and humans, (–)-gossypol is significantly more toxic than the (+) enantiomer (Stipanovic *et al*. 2005). In fact, most biological activities of gossypol seem to be a consequence of this enantiomer. (–)-Gossypol inhibits the growth of cancer cells more than the (+) enantiomer (Liu *et al*. 2002), is a more effective antiamoebic agent (Gonzalez-Garza *et al*. 1992), and inhibits male fertility in humans (Matlin *et al*. 1995).

In contrast to (–)-gossypol, the (+) enantiomer shows little if any toxicity to non-ruminant animals. But cotton plants containing high levels of (+)-gossypol are also resistant to insect damage. Diet feeding studies on the generalist lepidopteran herbivore *Helicoverpa zea* showed that (+)-gossypol is as inhibitory as racemic or (–)-gossypol (Stipanovic *et al*. 2006). Similarly, the two enantiomers are equally effective in inhibiting the growth of the cotton fungal pathogen *Rhizoctonia solani* (Puckhaber *et al*. 2002). The toxicity of (–)-gossypol makes cottonseeds, which are excellent sources of oil and protein, unsafe for consumption by humans and monogastric animals. Plant breeders have long been attempting to remove gossypol from cottonseed without decreasing its levels in parts of the plant usually attacked by insects or pathogens. A recent demonstration of the tissue-specific RNA interference silencing of a gossypol biosynthetic gene encoding (+)-δ-cadinene synthase was quite successful in this regard (Sunikumar *et al*. 2006). Breeders may also wish to

maximize the level of (–)-gossypol in seeds for its use as a male contraceptive in humans.

Terpenes may also have critical roles in interactions among organisms by serving as a medium of communication among species. Most monoterpenes and sesquiterpenes are good conveyors of information over distances because they are low-molecular-weight, lipophilic molecules with high vapor pressures at ordinary temperatures. In addition, the existence of a vast structural variety of terpenes present allows messages to be very specific.

There are many examples of terpene-mediated communication in plant-insect interactions (Duderava *et al*. 2006), but terpenes also have important functions as pheromones. Among insect species, they serve as sex, aggregation, trail and alarm pheromones (Francke *et al*. 2005; Hick *et al*. 1999). For example, the sesquiterpene (E)-β-farnesene acts as an alarm pheromone in aphids. Released during predator attack, this acyclic hydrocarbon causes aphids to stop feeding, disperse, and give birth to winged (rather than wingless) forms, which leave their host plants (Hardie *et al*. 1999; Kunert *et al*. 2005). By releasing their own (E)-β-farnesene, plants could exploit these effects to repel aphids and attract aphid enemies, although this has not yet been clearly documented under natural conditions (Beale *et al*. 2006; Gibson *et al*. 1983).

Plants are immobile for their life cycle and often need other organisms to disperse pollen and seeds, so volatile compounds released from flowers and fruits seem to serve as attracting pollinators and dispersal agents. Terpenes are one of the major components of fruit and flower volatiles (Knudsen *et al*. 2006), but proof that a specific terpene attracts a specific animal under natural conditions has not often been obtained (Wright *et al*. 2005).

Gaschromatography in combination with electroantennogram detection has shown that terpenes are indeed perceived by pollinating insects (Raguso *et al*. 1983).

Researchers have discovered that herbivore feeding induces, on foliage, the emission of blends of volatiles in which terpenes are major components (Dicke *et al*. 1990; Turlings *et al*. 1990).

These blends serve as an odoriferous call for help, attracting predators and parasitoids that attack herbivores (Dicke *et al*. 1990; Kessler *et al*. 2001). Recent field and laboratory experiments have helped identify specific monoterpenes and

sesquiterpenes that are involved in mediating this attraction (Kessler *et al*. 2001; Schnee *et al*. 2006). It is not just feeding by herbivores that attracts their enemies to plants. Amazingly, the mere act of laying an egg can have the same effect. For example, when the pine sawfly (*Diprion pini*) lays its eggs on pine twigs, the volatiles released attract a wasp that parasitizes the sawfly eggs (Hilker *et al*., 2002). The sesquiterpene (E)-β-farnesene seems to be the principal attractive component of this blend (Mumm *et al*. 2005), but it is only active against a background of other pine terpenoids (Mumm *et al.* 2003).

Volatile terpenoid communication is not restricted to the aboveground parts of plants. Recently it was reported that insect attack on maize roots triggers the release of a sesquiterpene, (E)-β-caryophyllene, which attracts nematodes that prey on insect larvae (Rasmann *et al*. 2005).

Terpene messages from plants may also have other, unintended recipients. Enemies, such as herbivorous insects or parasitic plants, may use terpenes to locate their hosts. For example, larvae of the lepidopteran *Spodoptera frugiperda* use volatile terpenes released upon wounding to help find their food plants (Carroll *et al*. 2006). Seedlings of the parasitic plant dodder (*Cuscuta pentagona*) grow toward nearby tomato plants guided by a blend of monoterpenes (Runyon *et al*. 2006).

Finally, terpene volatiles can even alert other plants in the vicinity to the presence of herbivores. Plants were found to respond to aerial cues put out by herbivore-attacked neighbors by increasing their own defenses or priming the machinery involved in defense production (Baldwin *et al*. 2006; Dicke and Bruin 2001; Ton *et al*. 2007).

Much work on terpene defensive properties has centered on plant terpenes. They are also important in resistance to diseases caused by fungi and bacteria. Triterpenoid saponins are terpene glycosides with detergent properties that are toxic to fungi because of their ability to complex with sterols in fungal membranes, which leads to the loss of membrane integrity (Morrissey and Osbourn 1999). Mutants of an oat species (*Avena strigosa*) that are deficient in producing saponins are severely compromised in resistance to fungal pathogens compared with wild-type lines (Papadopoulou *et al*. 1999).

Organisms usually produce complex mixtures of terpene natural products instead of just one or two compounds. At the molecular level, the prevalence of terpene

mixtures may be a consequence of the properties of the biosynthetic pathway that produces them. However, at the organism level, the production of mixtures may be thought of as a direct way to enhance terpene function. If terpenes are used in communication, for example, the release of mixtures may result in messages with more specificity and a higher information content. For terpenes used in defense, for example, for organisms with a wide range of enemies, a diverse combination of terpene defenses may help achieve simultaneous protection against numerous predators, parasites and competitors. Mixtures have also been suggested to impede the ability of enemies to evolve resistance (Pimentel and Bellotti 1976). The presence of complex mixtures also increases the probability that individual organisms in a population will have a unique composition of defenses. Possession of a novel terpene composition may have defensive value against enemies already adapted to circumvent some of the terpene defenses prevalent in a given population (Feeny 1992).

Alternatively, mixtures of defenses may be deterrent to enemies for longer periods than single compounds as a result of effects at the sensory level (Akhatar and Isman 2003). Mixtures of terpenes containing compounds with different physical properties may allow more rapid deployment or longer persistence of defenses.

Until now it has been discussed on the function of terpenes as natural products in the natural world, but it also is very important to consider the role of terpenes in the industry. Thanks to their many properties, terpens are widely used as such or as raw materials for producing a variety of products (fragrances, flavors, perfumes, pharmaceuticals) and as it was said, before terpenes, mainly mono and sesquiterpenes, take part in the composition of essential oils in the aromatic plants.

They are also known as antimicrobial agents (Zygadlo and Juliani 2000), and they are found in the areas of food preservation and in the manufacturing of medicinal antimicrobial agents and disinfectants (Voda *et al*. 2003). There also is an increasing interest in research concerning with the development of new alternative pesticides, such as insect, growth regulators, fungal pathogens, toxic natural products including plant essential oils, extracts and secondary metabolites for pest control in agriculture (Hoffmann and Frodsham 1993; Gonzalez-Coloma *et al*. 1995, 2002, 2004; Hu *et al*. 1999; Isman 2000; Chiasson *et al*. 2001; Zolotar *et al*. 2002; Scott *et al*. 2003, 2004).

Zamponi *et al*. (2006) studied *in-vitro* the inhibitory effects of four monoterpenes (-)-α-pinene, (-)-β-pinene, δ-3-carene and myrcene, in both the contact and the volatile phase, on *Heterobasidion* spp., *Leptographium wingfieldii* and *Leptographium serpens*. They found that myrcene was the most strongly inhibiting compound for *H. annosum,* followed by δ-3carene; the *Leptographium* spp. were inhibited most strongly by δ-3-carene.

Many works have showed that a wide variety of essential oils and many of their compounds have antimicrobial activity (Morris *et al*. 1979; Andrews *et al*. 1980; Yousef and Tawil 1980; Deans and Ritchie 1987; Knobloch *et al*. 1989; Carson and Riley 1995; Sivropoulou *et al*. 1995; Bagamboula *et al*. 2003; Cosentino *et al*. 2003; Faleiro *et al*. 2003; Burt 2004). The type of antimicrobial activity shown by essential oils and their components varies from partial or complete inhibition of growth to bacterial activity (Andrews *et al*. 1980; Yousef and Tawil 1980).

DEFENSE MECHANISM IN CONIFERS

Biotic stress, in terms of herbivore attack or and fungi development, negatively affects the health of the tree. Clearly, trees have evolved effective defensive mechanisms against pathogen/insect colonization, a feature that has allowed these plants, as a group, to survive for millions of years (reviewed by Franceschi *et al*. 2005). One contributor to their persistence may be their ability to respond to fungal infections with localized defense responses that make induced tissues more resistant to a subsequent insect attack (Franceschi *et al*. 2005; Klepzig *et al*. 1995; Phillips and Croteau 1999). However, when the herbivores attack or fungi mycelia starts to grow in the plant's tissue, plants defend themselves against pathogens by a combination of: (1) structural characteristics that act as physical barriers and inhibit the pathogen from gaining entrance and spreading through the plant and (2) biochemical reactions that take place in the cells and tissues of the plant and produce substances that are toxic to the pathogen or create conditions that inhibit growth of the pathogen in the plant.

The combinations of these reactions employed in defense of plants are different in different host-pathogen systems. Besides, the combinations vary with the age of the

plants, the kind of plant organ and tissue attacked, the nutritional condition of the plant and the weather conditions.

Thus, plants have two basic types of defense strategies: **constitutive defenses** that are present in the tree without any challenge, and **inducible defenses** that are generated upon perception of a foreign challenge. A third strategy, **acquired** or **systemic defense**, can be considered to be a variation of inducible defenses but at some distance from the attack, temporally displaced with respect to the initial event, and with persistent properties. The 'choice' of strategy has been hypothesized to vary with the type of challenge (Matson and Hain 1985; Christiansen *et al*. 1987; Bonello *et al*. 2001).

There are four basic phases of defense systems in plants that are independent of the attacking organism. *(1)* The first is an effective constitutive defense that can repel or inhibit invasion of tissues. If this is not effective, *(2)* the next stage is to kill or compartmentalize the invading organism. *(3)* A third phase of defense is to seal and repair damage incurred so that the plant can continue to function normally, and so that opportunistic infections are prevented. *(4)* Finally, acquired or systemic resistance can be induced so that future attacks are more easily defended against. In addition, once an invading organism is identified, more specialized inducible defense responses can be elicited. The combination of constitutive and inducible systems provides a potent defense against attack.

Constitutive and inducible defenses are under different regulation, but must be coordinated to achieve optimal effect. While each of the constitutive defenses are at a set resistance that is determined by genetics and prior history, the inducible defenses are of variable resistance, the value being determined by the nature of the attack as well as the genetic ability of the individual.

CONSTITUTIVE DEFENSES

Constitutive defenses are of two basic types: **mechanical** and **chemical**.

Mechanical defenses are made of structural elements that provide a toughness or thickness to the tissues and inhibit chewing or pulling apart of the bark. They may also include 'spines' that can be effective against larger animals. Impregnation of tissues with polymers such as lignins and suberin can add to the mechanical properties and enhance resistance to penetration, degradation and ingestion/ chewing, particularly by smaller organisms.

Constitutive **chemical defenses** often occur as pools of stored chemicals (e.g. phenolics, terpenoids and alkaloids) that can be released upon attack. Chemical defenses include antifeedants, toxins, defensive proteins and enzymes, and reservoirs of chemicals such as resins that can flush away, repel or physically entrap small organisms. These defenses are dispersed throughout the various tissues of the bark, which include the periderm, cortex (if the stem is young) and secondary phloem. The secondary xylem can also contain some of these defenses as constitutive products (Pearce 1996; Fäldt *et al*. 2003).

The bark of conifers, which includes the periderm and secondary phloem, provides a sophisticated defensive barrier to invading organisms. The basic function of bark defenses is to protect the nutrient and energy-rich phloem, the vital meristematic region of the vascular cambium, and the transpiration stream in the sapwood.

This barrier includes static and constitutive defenses such as suberized and/or lignified periderm derivatives, sclerified cells, or cell layers (Wainhouse *et al.* 1990), cells with calcium oxalate crystals (Kartuch *et al*. 1991), parenchyma cells filled with secondary products such as phenolics (Cheniclet *et al*. 1988; Franceschi *et al*. 1998), and multicellular ducts or canals with constitutive resins and other products (Wu and Hu 1997). The defensive roles of these structures are deduced from their anatomy and chemical composition. The constitutive defense mechanisms provide an immediate resistance to invasion of the bark, but may be overcome by organisms that have become adapted to these structures.

Bark is composed of:

(1) The periderm, that provides a permeability barrier for control of gas exchange in the stem, and also is the first line of defense from adverse biotic and abiotic factors. It consists of the lateral meristem of the periderm (the phellogen or cork cambium) and its products (phellem or cork tissue outwards and phelloderm inwards). Periderms are quite variable among species but have some common components. They are generally characterized by the presence of multiple layers of structurally and chemically distinct cells. In addition, the cells may contain large amounts of phenolic materials, and it is also common to have one or more layers of cells containing calcium oxalate crystals. The combination of the mechanical properties of tough lignified walls and crystals, the suberized walls that provide a hydrophobic barrier and the chemical properties of phenolics, presents a multifunctional barrier to the external environment.

(2) The cortex that is produced during the primary development of the stem, and that may stay alive for a number of years of secondary growth. It commonly contains large amounts of phenolics within vacuoles of cortical parenchyma, and in many of the Pinaceae axial resin ducts are present. Sclerenchyma and calcium oxalate crystals may also be formed within the cortex. Although the cortex is an important defensive barrier during early stages of growth of the stem, the secondary phloem takes over this role once it develops more extensive layers over a number of years.

(3) The secondary phloem, that is a major site of constitutive defense mechanisms. Most conifers share three common constitutive defense components in their secondary phloem: sclerenchyma, calcium oxalate crystals and phenolic bodies, although the relative amount of each varies considerably among species (Hudgins and Franceschi 2004). It is common in certain taxa the presence of resin producing structures, as a major constitutive defense of particular importance. These include radial resin ducts derived from radial rays, axial resin ducts or canals, resin blisters and resin cells. The ducts and blisters have a lining of plastid-enriched epithelial cells that synthesize terpenoid resins and secrete them into an extracellular lumen where they accumulate pressure and expand into fairly large structures. Upon damage by wounding or by an invading organism, the pressurized resin is

released and can repel or flush the organism out of the bark, entrap the organism in sticky resin or otherwise kill the invader due to the toxic nature of the resin. Resin is synthesized in these specialized secretory structures. Resin cells are common in fir (*Abies* spp.), cedar (*Cedrus* spp.) and hemlock (*Tsuga* spp.); resin ducts are characteristic of spruce (*Picea* spp.), pine (*Pinus* spp.), larch (*Larix* spp.)and Douglas fir (*Pseudotsuga* spp., Hudgins *et al*. 2003). Resin ducts are further differentiated into two groups, "radial ducts" and "axial ducts", depending on their orientation in the plant tissues. Radial ducts are generally more common in the phloem, while axial ducts are usually scattered in the xylem (Wu and Hu 1997; Hudgins *et al*. 2003). Resin flow from radial phloem ducts can be enhanced by their connection to constitutive or induced axial resin ducts in the xylem (Christiansen *et al*. 1999a; Nagy *et al*. 2000). When the volatile components of the released resin evaporate, the nonvolatile components will crystallize to sterilize and seal the wounded region effectively. Whereas resin-producing structures are found in all Pinaceae, they do not occur constitutively in the secondary phloem of some other conifer taxa (Hudgins *et al*. 2003a, 2004). Although resin is considered to be a defense against bark-boring insects, and although a correlation between resin duct number and resistance to pine weevil has been shown (Alfaro *et al*. 1997), it is clear that other strategies have evolved that can work equally or more effectively (Hudgins *et al*. 2004). In conifers there are also axial phloem parenchyma cells that are specialized for synthesis and accumulation of phenolic compounds (Hudgins *et al*. 2003a; Hudgins *et al*. 2004). These are referred to as polyphenolic parenchyma cells (PP cells; Franceschi *et al*. 1998; Krekling *et al*. 2000). Within their vacuoles, PP cells contain variable amounts of phenolic bodies that are thought to serve as antifeedants and as antifungal agents (Beckman 2000). In addition to their phenolic contents, they also have thickened cell walls, albeit with abundant plasmodesmata that allow for axial and tangential exchange of information, and possibly for defense signaling (Krekling *et al*. 2000).

(4) The xylem involved in constitutive defenses too. Xylem parenchyma, both axial and radial systems, is present in the secondary xylem and can be involved in synthesis and storage of phenolics and resin, as well as other

secondary products. Xylem parenchyma is also involved in synthesis and secretion of 'extractives', such as lignans, into the heartwood of conifers (Kwon *et al*. 2001), which provide a defense against wood rotting fungi and other organisms. Constitutive axial resin ducts are also present in the xylem of some conifers (Wu and Hu 1997), and may contribute to resin flow when they are connected to radial resin ducts that traverse between xylem and phloem.

INDUCIBLE DEFENSE

Inducible defenses include a combination of responses to the specific organism, increased generalized responses, and processes aimed at limiting the extent of the damage inflicted by an organism. Acquired resistance is a long-term consequence of inducible defense. Inducible defenses are diverse and include **structural changes** and **synthesis of chemical** and **biochemical agents**.

Structural defenses occurring in the bark are important in containing and isolating an invasive organism, repairing damaged tissue, and limiting opportunistic or subsequent attack or invasions. The hypersensitive response can be thought of in part as a structural defense, although the endpoint is dead tissue. The hypersensitive response occurs locally at the site of infection or attack (Bleiker and Uzunovic 2004) and results in production of reactive oxygen species, and rapid cell death that is intended to kill and contain organisms such as fungal pathogens, bacteria and viruses. This 'scorched earth' defense (Berryman 1972) sacrifices a small volume of tissue in an attempt at rapid containment of invading organisms. Beyond this highly localized response, a more generalized response to wounding is the formation of callus tissue that can subsequently become lignified, suberized or impregnated with phenolics to provide a barrier, and may form part of the wound periderm. This reaction provides protection against further intrusion as well as walling off an organism such as a fungal pathogen. Wound callus also can repair damaged tissue so that continuity of function can be re-established – for instance, by reorganization of a region of damaged cambium.

Wound periderms are induced to form around an invaded or damaged region of bark, and serve to wall off the region as well as to re-establish a continuous surface barrier in the previously disrupted area. Wound periderms are produced by activation of PP cells in the secondary phloem, as well as callus tissues in severe wounds, which begin to divide to form a wound phellogen.

The wound periderm essentially isolates the damaged area and effects a permanent repair of tissues. Wound periderms form at the boundaries of lesions induced by bark beetle (Christiansen and Kucera 1999) or fungal attacks in the stems of conifers, but will form around any damaged tissue. Damaged or infected tissue is isolated from nutrient supplies by the wound periderm, and will eventually die if not already killed by an invasive organism.

Another induced structural defense is early lignifications of fibers (Hudgins *et al*. 2003a, 2004). Incipient fiber rows are produced yearly by the activity of the vascular cambium in many conifer species, but they do not become fully lignified until they are *c*. 3 yr old. Recent studies on induced defense responses demonstrated that these incipient fibers can be induced to become fully lignified after methyl jasmonate treatment. This compound generates a response similar to that of wounding, and is thought to be a signaling agent in normal wound responses of the bark of conifers (Franceschi *et al*. 2002), although the response may actually be mediated by ethylene induced by methyl jasmonate (Hudgins and Franceschi 2004).

Whereas constitutive chemical defenses are generally nonselective with respect to the pest species, **inducible chemical defenses** include both broad-spectrum and specific compounds. Chemical defenses are extremely diverse and can thus cover a range of pests. Non-protein chemicals such as products of the phenylpropanoid pathway (phenolics) and isoprenoid pathway (terpenoid resins) as well as alkaloids can have potent effects on invasive organisms. However, some of the biochemical pathways are created *de novo* in newly generated tissues or structures as well, a good example being resin produced in traumatic resin ducts (Thomson and Sifton 1926; Bannan 1936; Cheniclet 1987; Christiansen *et al*., 1999a; Nagy *et al*. 2000; Martin *et al*. 2002; Fäldt *et al*. 2003; Hudgins *et al*. 2003a, 2004). Another advantage of these chemical defenses is that they are often effective against a broad range of organisms, and thus can slow down an attack while recognition mechanisms come into play to identify the organism and activate specific defenses against it. Most work with

conifers has been done on phenolics, terpenoids and enzymes such as some of the PR proteins.

Phenolics are abundant in the bark of conifers (Pan and Lundgren 1995, 1996; Viiri *et al*. 2001), Phenolics and tannins can act as antifungal agents and antifeedants, and can bind hydrolytic enzymes secreted by pests, thus inhibiting their advancement into tissues (Hunter 1974; Nicholson and Hammerschmidt 1992; Appel 1993).

Various chemical studies have shown that production of phenolics, or upregulation of enzymes of the pathway (Richard *et al*. 2000), is rapidly induced in invaded bark and that the amount and type of phenolic compounds produced during invasion may be quite different from the constitutively produced phenolics (Brignolas *et al*. 1995; Lieutier *et al*. 1996; Bois and Lieutier 1997; Viiri *et al*. 2001; Bonello and Blodgett 2003; Bonello *et al*. 2003). The implication is that the induced phenolics are more toxic or more specific to an invasive organism than are the constitutive phenolics.

Terpenoid resin production can also be induced by attack. During and following an attack, resin flow from the wound can be quite extensive, especially in members of the Pinaceae. Part of this resin is from stored resin in existing resin-producing structures, and there is evidence that the constitutive ducts can be activated to produce more resin (Ruel *et al*. 1998; Lombardero *et al*. 2000; Hudgins and Franceschi 2004). Within 2–3 wk after attack, new resin ducts can also be induced to form, referred to as traumatic resin ducts (Alfaro 1995; Alfaro *et al*., 1996; Tomlin *et al*. 1998; Byun McKay *et al*. 2003), and the resin formed by traumatic ducts can be different than constitutive resin (Nault and Alfaro 2001; Martin *et al*. 2002; Miller *et al*. 2005). Many works on Norway spruce showed that hese ducts are formed above and below a damaged site or induced point on the stem (Franceschi *et al*. 2000; Nagy *et al*. 2000; Hudgins and Franceschi 2004; Hudgins *et al*. 2004; Krekling *et al*. 2004).

SYSTEMIC INDUCED RESISTANCE

Systemic induced resistance (SIR) is the induction of resistance to pathogens in noninfected parts of a plant by prior infections or activity by various organisms elsewhere in the plant (Bonello *et al*. 2001; Kuc 2001; Bonello and Blodgett 2003). And it is functionally analogous to immunization.

Extensive research in the last 20 years has studied the biochemical and molecular bases of SIR, mostly in herbaceous model species, particularly tobacco, tomato and *Arabidopsis*.

In model hosts development of SIR begins with a hypersensitive response to the initial infection and is mediated by the accumulation of the hydroxybenzoic acid derivative salicylic acid, the linolenic acid derivative jasmonic acid, and ethylene (Sticher *et al.* 1997; Stout *et al*. 1998, 1999; Heil 2001; Durrant and Dong 2004; Graham and Bonello, 2004). Other hormones, such as abscisic acid, can also play a role in SIR (Dammann *et al*. 1997; Bostock 1999).

Specifically, enhanced resistance to biotrophic pathogen infection appears to be mediated by salicylic acid, while enhanced resistance to insect feeding, via the wound response pathway, is mediated by jasmonic acid. Expression of SIR is correlated with the accumulation of pathogenesis-related (PR)-proteins and with the induction or enhancement of secondary metabolic responses, such as accumulation of soluble phenolics and terpenoids (with the formation of traumatic resin ducts), and cell-wall lignification (Sticher *et al*. 1997; Heil 2001; Durrant and Dong 2004; Graham and Bonello 2004; Franceschi *et al*. 2005).

However, evidence suggests that this distinction may not be so well defined and cross-talk may occur among signaling pathways (Bostock 1999; Paul *et al*. 2000; Durrant and Dong 2004; Hatcher *et al*. 2004; Beckers and Spoel 2006).

Although such extensive knowledge is not available for conifers, SIR phenotypes against stem and branch pathogens have been observed in pine in response to plant growth promoting rhizobacteria (Enebak and Carey 2000) and pathogen (Bonello *et al*. 1991; Schmale and Gordon 2003; Blodgett *et al*. 2007). However, limited evidence suggests that the signaling pathways in pine when attacked by necrotrophic pathogens might differ from those of most herbaceous plants/pathogen systems studied to date. For example, local and systemic changes in phenolic composition of

Scots pine (*Pinus sylvestris* L.) needles and ponderosa pine (*Pinus ponderosa* Dougl. ex Laws.) phloem in response to a root pathogens (Bonello *et al*. 1993; Bonello *et al*. 2003, respectively) and Austrian pine (*P. nigra* Arnold) needles and phloem in response to a canker pathogen, *Sphaeropsis sapinea* (Fr.:Fr.) Dyko and Sutton (Bonello and Blodgett 2003; Blodgett *et al*. 2007) were never associated with the accumulation of salicylic acid, a rather common theme in herbaceous hosts. However, exogenous treatment of conifer tissues with either salicylic acid and its derivatives, or methyl jasmonate, can result in induction of expression of some defense-related genes (Davis *et al*. 2002) and enhanced localized resistance to pathogens and bark beetles (Reglinski *et al*. 1998; Franceschi *et al*. 2002; Hudgins *et al*. 2003; Schmidt *et al*. 2005; Erbilgin *et al*. 2006; Zeneli *et al*. 2006).

An important ecological consequence of SIR may be cross induction of resistance between different host antagonists co-occurring on the same plants, for example, fungal pathogens and insects pests (Eyles *et al*. 2007). In such interactions, early colonization by a primary insect or pathogen is thought to induce changes in host biochemistry and physiology that make the plant less susceptible to further attacks (Stout *et al*. 2006). For example, changes in feeding behavior by an insect, resulting in reduced damage, may be induced in plant parts distant from the site of an earlier pathogenic attack (Rostas *et al*. 2003; Bonello *et al*. 2006).

Although SIR is of great interest for its potential application in disease and pest management, there may be situations in which the opposite phenomenon occurs

This demonstrates that spruce trees possess systemically inducible signaling pathways that result in major anatomical reorganization.

Krekling *et al*. 2004 showed that in Norway spruce, traumatic resin ducts (TD) induction was triggered by a signal, which propagates a developmental wave in the axial direction at about 2.5 cm per day. TDs are formed at least 30 cm above single inoculations within 16-36 days after inoculation.

Then they demonstrated that the amount of TDs and the degree of the TD development decreased with increasing distance from the inoculation site. Thus, the inductive stimulus for TD formation arrives sooner and/or is stronger closer to the inoculation point, resulting not only in more rapid development but also in the formation of the more TDs closer to the origin of the stimulus.

The signaling agent responsible for induction of TD formation has not been established, but various phytohormones have been shown to be capable of inducing TD formation and thus are possible candidates for control of this process (Fahn and Zamski 1970; Fahn *et al.* 1979; Pardos 1980). Jasmonates, which are endogenous plant phytohormones (Koda 1992; Creelman and Mullet 1997), are potent elicitors or signaling agents, and it is well known that they are involved in a host of defense responses (Seo *et al.* 1997; Thaler *et al*. 2001).

Little is known about jasmonate signaling in conifers. Recent work has shown that PP cell activation and TD development can be strongly elicited in the absence of wounding with exogenous treatments of methyl jasmonate (MJ) in the Pinaceae (Franceschi *et al*. 2002; Martin *et al*. 2002, 2003; Faldt *et al*. 2003; Hudgins *et al*. 2003a) and Taxodiaceae (Hudgins *et al*. 2004). While there is little information on natural jasmonates in conifers, jasmonic acid has been shown to be induced in conifer cell culture (*Taxus baccata*) following elicitation with fungal cell wall fragments (Mueller *et al*. 1993). By contrast, jasmonates are known to modulate many activities in angiosperms, including defense responses (Creelman and Mullet 1997; Cheong and Choi 2003; Farmer *et al*. 2003). A few investigations have also shown that MJ is capable of inducing terpenoid biosynthetic enzymes and their associated genes in Norway spruce (Martin *et al*. 2002, 2003; Faldt *et al*. 2003) and chalcone synthase, a key enzyme in the phenylpropanoid pathway, in white spruce (*Picea glauca*; Richard *et al*. 2000). Thus, MJ appears to be involved in signal transduction pathways for both the phenylpropanoid and terpenoid defense responses in conifer stems.

Application of MJ resulted in TD formation some distance from the site of application, and so probably jasmonate or some compound induced by jasmonates, are the signaling agents responsible for TD induction. It is possible that a methyl jasmonate derivative acts as a transportable signaling agent inducing TD formation in the cambial region. This is also consistent with the attenuation of TD development further away from the inoculation site as seen here, or away from the MJ application zone as seen by Franceschi *et al*. (2002).

Kozlowski *et al.* (1999) found that methyl jasmonate treatment enhanced resistance of Norway spruce seedlings to *Pythium ultimum* via activation of the salicylic acid pathway and subsequent induction of chitinase. Other studies have

shown induction of various potential defense-related proteins in white spruce by jasmonate treatment (Richard *et al*. 1999, 2000; Lapointe *et al.* 2001), but in neither of these studies was the cell biology of the process determined. Wheares Franceschi *et al*. (2002) indicated jasmonates may be involved in defense-related signaling in Norway spruce and specifically in the regulation of complicated anatomical changes that can occur in bark and cambial zone tissues in response to attack.

Traumatic resin ducts are formed through activation of the cambial zone, which results in an altered developmental program for cells that would normally become xylem. It is unlikely that methyl jasmonate is directly inducing this change, as auxins seem to be more directly involved in TD induction (Fahn and Zamski 1970; Fahn *et al.* 1979; Pardos 1980). Alternatively, ethylene, either induced by auxin (Yamamoto and Kozlowski 1987) or directly by elicitor (Popp *et al.* 1996) in conifer systems, may be the agent initiating TD formation. Thus, it is possible that methyl jasmonate induces changes in auxin or ethylene production that in turn result in activation of TD formation, but this remains to be proven.

Franceschi *et al*. (2002) showed the methyl jasmonate-induced response was not persistent, as only a single layer of TDs was formed the year of treatment and no new TDs were formed in the subsequent year. In contrast, bark-beetle attacks and fungal inoculation can lead to formation of multiple rows of TDs close to the wound in the year of damage and also in subsequent years (Franceschi *et al*. 2000). In the immediate vicinity of a wound, an active callus-like tissue is formed and the signaling agent for TD formation is likely continuously produced over a long period of time giving rise to the potential for multiple bands of TDs within a season and during subsequent seasons. Exogenously applied methyl jasmonate, however, appears to act as a one-time signal. Methyl jasmonate treatment elicited defense responses, but the compound, or its active products, were either sequestered or metabolized quickly and thus could not induce sequential activation of the cambial zone after it is reorganized following TD formation.

Polyphenolic parenchyma cell activation was also seen after external application of methyl jasmonate to bark. Jasmonates have been shown to induce chalcone synthase expression in white pine (Richard *et al.* 2000) and phenylalanine ammonia lyase in cultured tobacco cells (Sharan *et al.* 1998), and both of these enzymes are important in phenolic compound biosynthesis in Norway spruce (Brignolas *et al.*

1995; Nagy *et al.* 2000). Polyphenolic parenchyma cells are the primary site of phenolic biosynthesis in the secondary phloem, and so the activation of PP cells and increase in their phenolic contents is consistent with studies showing jasmonate induction of enzymes of the phenylpropanoid pathway (Sharan *et al.* 1998; Richard *et al.* 2000).

Franceschi *et al*. (1998). have suggested that the PP cells represent a primary site of both constitutive and induced defenses based upon their phenolic contents, high constitutive expression of phenylalanine ammonia lyase (PAL, a key enzyme in phenolic biosynthesis), and dynamic nature with respect to cellular changes after wounding.

A number of studies have revealed qualitative alterations in phenolic compounds following fungal inoculations, including increased activity of the flavonoid pathway (Brignolas *et al*. 1995; Lieutier *et al*. 1996; Bois and Lieutier 1997; Bonello *et al*. 2003), and accumulation of phenolics is considered a significant part of induced defense responses in the bark (Nicholson and Hammerschmidt 1992; Schultz *et al*. 1992; Viiri *et al*. 2001).

The phytohormone ethylene is involved in a diverse array of plant growth and developmental processes (Abeles *et al*. 1992; Kende 1993; Bleecker and Kende 2000) and is a possible candidate effector.

Recent evidence indicates that jasmonate-induced ethylene production is responsible for reprogramming of the cambial zone for traumatic duct formation, and ethylene may also induce activation of existing radial resin ducts to produce additional resin (Hudgins and Franceschi 2004; Hudgins *et al*. 2004). The end result of traumatic resin duct development is increased resin formation and accumulation (Martin *et al*. 2002; Miller *et al*. 2005) and enhanced resin flow. Increased resin flow can help to kill or flush out invaders as well as seal the wound, and resin-soaked regions of bark and sapwood may also be more resistant to introduced microbial activity. In addition, there is evidence that traumatic ducts may impart acquired resistance to subsequent attack (Christiansen *et al*. 1999b; Krokene *et al*. 2003) and that the resin in traumatic ducts may be more toxic through changes in terpenoid components or addition of phenolics (Nagy *et al*. 2000).

Another phytohormone that needs to be considered with respect to PP cell activation and TD formation in conifers is salicylic acid (SA), a known mediator of

the expression of various defense-related genes (Ryals *et al*. 1996; Sticher *et al*. 1997). In conifers, SA has been shown to accumulate in response to pathogen challenge (Franich *et al*. 1986; Kozlowski and Metraux 1998; Kozlowski *et al*. 1999) and Davis *et al*. (2002) showed that following either pathogen challenge or salicylic acid treatments, *Pinus* species were induced to express three chitinase homologs. Kozlowski *et al*. (1999) found that MJ induces the accumulation of salicylic acid in Norway spruce seedlings, establishing a link between the MJ and SA pathways in conifers. Although evidence indicates SA to be an important activator of genes encoding pathogenesis-related proteins (Durner *et al*. 1997), various proteins induced in the host plant in response to pathogens (van Loon *et al*. 1994), there is no information for its role in induction of other defense responses important to conifers, such as terpene and phenolic synthesis.

Protein-based chemical defenses in trees include enzymes such as chitinases and glucanases that can degrade components of invasive organisms, toxic proteins such as porins and lectins, and inhibitors of enzymes such as proteinase and amylase inhibitors. The enzyme inhibitors interfere with the ability of the organism to utilize resources of the invaded tissue. Other inducible enzymes such as peroxidases and laccases can make cell walls tougher through crosslinking reactions or promotion of lignification, or can be involved in directly affecting the invasive organism. Protein-based defenses can be highly specific to a particular organism. For example, in Norway spruce, chitinases exist as a large family of proteins, but only a small subset of them may be up-regulated during attack by a specific fungal pathogen (Hietala *et al*. 2004; Nagy *et al*. 2004) and it is presumed that these are effective against cell walls of that particular organism.

Overall, inducible chemical defenses follow a similar mechanistic concept as inducible structural defenses; that is, multiple overlapping strategies. Production of a toxic cocktail of diverse chemical components will maximize the potential to halt or destroy an aggressive or virulent invading organism, in contrast to a more conservative production of one or a few more directed defenses.

Bonello and Blodgett (2003), Luchi *et al*. (2005) and Blodgett *et al*. (2007) confirmed that SIR occurs in the Austrian pine/*S. sapinea* model pathosystem. In these studies, prior infection by the pathogen resulted in SIR to *S. sapinea*, as evidenced by significantly reduced challenge lesions in the main stem of inoculated

trees compared with trees receiving mock inoculations (Blodgett *et al*. 2007). SIR in Austrian pine is associated with clear systemic effects on phenolic and resin metabolism (Bonello and Blodgett 2003; Luchi *et al*. 2005; Blodgett *et al*. 2007), the production of differentially expressed proteins (Wang *et al*. 2006) and is dependent upon the relative locations of the inducing and subsequent challenge inoculations (Blodgett *et al*. 2007). Specifically, when the base of the main stem was induced with *S. sapinea*, SIR to the same pathogen occurred in the stem, while systemic induced susceptibility occurred in the shoot tips, suggesting that the end result of at least some host-mediated interactions may be both time and organ dependent (Blodgett *et al*. 2007).

A similar result, on SIS phenotype, was observed by Bonello *et al*. (2008). Three-year-old seedlings of *Pinus pinea* L. were inoculated near the stem base with one of two *Heterobasidion annosum* (Fr.) Bref. *sensu stricto* (*s.s.*) strains belonging to two populations: the North American P-group (NAm-P) and the European P-group (Eur-P). Three weeks after the stem inoculations with *H. annosum*, apical shoots were inoculated with *Diplodia pinea* (Desmaz.) J. Kick.

This study represents the first example of controlled cross-induction of SIS in trees between fungal pathogens belonging to different taxonomic groups (*D. pinea*, Ascomycota; *H. annosum*, Basidiomycota). This suggests that trees affected by root rots in the field may become predisposed to other diseases, such as shoot blights, even before their crowns become symptomatic for the root disease, which is the stage at which a connection between root rot and predisposition to other diseases is usually made (Bonello *et al*. 2008). This conforms with the hypothesis of Bonello *et al*. (2006) that the outcome of systemic interactions in conifers may have strong spatio-temporal components. Furthermore, Blodgett *et al*. (2007) and Wallis *et al*. (2008) showed that whether a fungal infection of Austrian pine stem induces SIR or SIS depends on the target organ of the subsequent challenge, with stems and branches becoming more resistant whereas shoots become more susceptible.

Bonello *et al*. (2001) demonstrated that resistance against the pitch canker pathogen, *Fusarium circinatum* Nirenberg and O'Donnell, can be induced systemically in Monterey pine (*Pinus radiata* D. Don) in the field using mechanical inoculations with the same pathogen. Induced resistance was sustained and intensified with boost inoculations over the course of at least one and a half years.

The natural occurrence of induced resistance to pitch canker has been documented in long-term monitoring plots. At these sites, a number of Monterey pines that were severely affected by pitch canker in 1996 was shown to be free of disease in 1999 (Gordon *et al.* 2001). Furthermore, existing infections had become contained and no new infections had been recorded, which suggested that trees in remission were more resistant to the pathogen. Resistance to pitch canker was confirmed in a subset of the trees in remission by direct challenge with the pathogen. In a separate study, Monterey pines in areas where pitch canker was well established were shown to be significantly more resistant than trees of this species in areas where the disease was a more recent occurrence (Gordon *et al.* 2006). This result suggests that exposure to the pathogen resulted in enhanced disease resistance over time.

One interesting implication of SIR in conifers is that it may provide an alternative explanation for what is known as ontogenetic disease resistance (ODR) (also known as age-related resistance, Panter and Jones 2002). ODR refers to resistance to a pathogen that changes with the developmental stage of the host, with resistance usually increasing with age. For example, ODR to white pine blister rust (WPBR), caused by the exotic and invasive pathogen *Cronartium ribicola* J.C. Fisch, has been reported in five-needled pines. ODR appears to be a significant factor in development of this disease, at least in sugar pine (*Pinus lambertiana* Dougl.). In particular, older trees may suffer little damage from WPBR, whereas young trees tend to be severely affected and are often killed (Schoettle 2003) To the best of our knowledge, no specific physiological, molecular or anatomical studies have been conducted to characterize this phenomenon. Nonetheless, this age-dependent expression of resistance has generically been attributed to developmental changes in the host. An alternative explanation is that some trees challenged with the pathogen manifest SIR and this, rather than ODR, is responsible for lesser impacts of the disease on older trees. In fact, ODR to WPBR observed in sugar pine may reflect the cumulative induction of resistance not only by the rust pathogen but also by other microbes, such as endophytes and mycorrhizal fungi. Ontogenetic resistance against insects has also been documented, for example in ponderosa pine against the tip moth *Rhyacionia neomexicana* (Dyar) (Lepidoptera: Tortricidae) (Wagner and Chen 2003), and might also be the result of cross-induction of resistance over the life of the tree/tissues by resident microorganisms. An SIR basis for ODR is an intriguing

concept, particularly in light of the fact that induced resistance (and more generally plant responses to stress) appears to have epigenetic components, making it durable and also heritable (Agrawal *et al.* 1999; Agrawal 2001; Molinier *et al*. 2006). Such transgenerational adaptive plasticity could be advantageous for the host tree species by generating seedlings that are primed to respond more forcefully to pathogens and insects. However, at present there is no experimental support for this concept in trees.

Very few tripartite studies investigating cross-induction of SIR have been documented for trees (Bonello *et al*. 2007). Simon and Hilker (2003) provided some evidence that feeding by the willow leaf beetle *Plagiodera versicolora* Laicharting (Col.: Chrysomelidae) increased its systemic susceptibility toward rust infection. McNee *et al*. (2003) demonstrated that *Heterobasidion annosum* (Fries) Brefeld induced decreased feeding by the bark beetle *Ips paraconfusus* Lanier on presymptomatic ponderosa pine (*Pinus ponderosa* Lawson) in areas away from the site of infection. This study demonstrated that systemic resistance against a bark beetle induced by a root pathogen in pre-symptomatic trees may be operating in this system. This phenomenon was associated with biochemical changes in the phloem of similarly inoculated trees (Bonello *et al.* 2003), but the exact biochemical and/or anatomical mechanisms underlying this cross-induction of resistance remain unknown.

Based on the evidence provided above, as well as studies in angiospermous tree/folivore systems (Lappalainem and Helander 1997; Raps and Vidal 1998), it is clear that trees possess defense traits that are locally and systemically inducible by pathogens and that can potentially affect insect behavior. However, such host-mediated interactions between pathogens and insects are not often studied in an integrated manner (Hatcher *et al*. 2004). In the context of host-mediated interactions between root pathogens and treekilling bark beetles, for example, it appears that such an integrated approach might provide significant insight into factors that can either enhance or diminish stability in conifer-dominated ecosystems. An improved mechanistic understanding of these associations could contribute to development of better management strategies, the practical value of which could be considerable, given that an estimated 6 millionm^3 of timber are lost every year to root disease-bark beetle complexes in the western US alone (Goheen and Hansen 1993).

AIM OF WORK

General aim of these studies was to investigate the role of terpenes in the constitutive and induced chemical defenses of forest plants against diseases. In particular the objective of these studies were:

(1) to investigate the geographical variation of constutive terpenoid defenses in different provenaces of R*osmarinus. officinalis* L.;

(2) to evaluate the antifungal activity *in-vitro* of some monoterpenes of the rosemary essential oil against *Alternaria alternata* (Fr.) Bref.;

(3) to determine variations in constitutive and induced terpene contents in response to *Heterbasidion annosum* (Fr.) Bref. attack in *Picea sitchensis* (Bong.) clones.

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PART A: RELATIONSHIP BETWEEN TERPENE COMPOSITION AND THE ATTACK OF *ALTERNARIA ALTERNATA* IN *ROSMARINUS OFFICINALIS*

GEOGRAPHICAL VARIATION IN THE CONSTITUTIVE TERPENE BASED DEFENSES OF FOUR DIFFERENT PROVENANCES OF *ROSMARINUS OFFICINALIS*

Introduction

Rosmarinus officinalis L. (rosemary) is a member of the most important family of the Labiatae which comprises up to 200 genera and about 3500 species, and it is naturally found in all of the coastal regions of the Mediterranean Sea (Do Amaral Franco and Rocha Afonsa 1972). In Italy it can be found mainly in coastal "macchia", garigues of the whole peninsula, with the exception of the North and Middle Adriatic (Pignatti 1982). It grows in every soil type, but it prefers a sandy, arid, calcareous, humus-poor soil. Rosemary is an evergreen shrub with aromatic and linear leaves, and its name derives from the Latin name *rosmarinus*, which literally means "dew of the sea".

Anthropologists and archaeologists have found evidence that rosemary herbs were used as medicinal, culinary and cosmetic virtues in the ancient Egypt, Mesopotamia, China and India (Stefanovits-Bányai et *al.* 2003).

Nowadays, this plant is widely cultivated all over the world, as ornamental and aromatic plant and the interest towards its cultivation is due to the many biological activity of the essential oil extracted from the flowering aerial tops: leaves, twigs and flowers, collected from spring to late autumn. Inside the species many chemotypes may be distinguished, the most important being traditionally: cineoliferum (high content in 1,8-cineole), camphoriferum (camphor > 20%) and verbenoniferum (verbenone> 15%). According to the relative abundance of α-pinene, myrcene, or

others relevant compounds many other chemotypes could also be recognized (Agioni *et al.* 2004).

Tuberso *et al.* (1998) reported that monoterpenes constitute 50% of the oil of rosemary, expecially α-pinene (>30%), camphene, and limonene; alcohols ~ 7%; and ketons ~10%. Considerable differences have been reported in the oil yield and the amount of its main constituents: α-pinene, verbenone, camphor and 1.8-cineole.

Many studies showed that terpenes are used in chemosystematic studies to characterize species, determine provenances, and to identify clones and hybrids because they are strongly inherited and little influenced by environmental factors (Baradat and Yazdani 1988; Hanover 1992; Adams *et al.* 1993; Lang 1994; Baradat *et al.* 1996; Tognetti *et al.* 1997). They can be used also as biochemical markers in varietal tests and are routinely used to identify origin of stands or seed lots of *P. pinaster* (Baradat and Marpeau-Bezard 1988; Baradat *et al.* 1991).

The aims of the present paper were to characterize the geographical distinction in terpene profile between provenances.

Materials and methods

Plant material

To characterize the geographical distinction in terpene profile between provenances, samples of rosemary were collected from five different natural stations, two from Sardegna and three from Tuscana. 15-20 cm-long branches were collected from 30 plants for each provenance and were carried to the laboratory for the analysis.

Gas chromatographic technique

Foliar samples (0.2 g) were ground in liquid N_2 and extracted in 1,5 ml of n-pentane with tridecane as an internal standard (Raffa and Smalley, 1995), placed in glass vials, sealed with teflon septums and crimped with aluminum caps.

The vials were stored at -20°C until analysed.

Terpenoids were analyzed by gas chromatography–flame ionization detection (GC-FID) with a Perkin-Elmer Autosystem XL GC, and enantiomeric monoterpenes were separated on a 30 m Cyclodex-B capillary column, 0.25 mm diameter, (J & W

Scientific, CA). Analysis was carried out under the following conditions: H_2 (carrier gas) at 69 kPa; injector temperature at 230 °C; detector temperature at 250 °C. The oven temperature programming started at 40 °C (isothermal 5 min), and increased to 200 °C, at 1.5 °C min^{-1}; the final temperature of 220 °C was maintained for 5 min.

Terpenoids were identified by comparison of retention times with those of standards under the same conditions.

Data analysis

The amount of each monoterpene (in sufficient quantities to be considered in ANOVA) was expressed as a percentage of total monoterpenes and in mg monoterpene / g of tissues. Percentages of various components were transformed to arcsin-square root functions on the plot mean basis to fulfil the normality assumption. The transformed plot means were used for ANOVA and discriminant function analysis.

Results

Changes in terpenoid proportions of the four provenances

Gaschromatographic analyses showed that the main constituents were 1.8-cineole (37,93%), (+)-α-pinene (28,46%), (-)-α-pinene (6%) and camphene (5,15%).

Sabinene, (-)-β-pinene, (-)-limonene, (+)-limonene, *p*-cymene, and linalool were present at less that 5%, while myrcene, (+)-β-pinene, α-phellandrene, γ-terpinene and unknown compounds were present in traces (Fig. 3).

Results showed a large variation in the constitutive terpene profiles of foliar tissues between different provenances (Fig.3).

The content of (-)-α-pinene was significantly higher in Alberese (6,87%), in Giglio (5,72%) and in the Elba (7,27%) islands provenances than in rosemary of Sardinia island (4,15%).

Plants from Sardegna and Giglio islands were characterized by the highest content of (+)-α-pinene (43,65% and 37,07%), while the content of this terpene was higher in rosemary from Elba island than in Alberese rosemary.

The population of Elba island was characterized by the highest content of camphene (7.42%). The content of camphene was significantly higher in Alberese

provenances (5.32%) than Giglio island provenance (3.16%); no significant differences were detected between plants from Sardinia (4.68%) and Giglio islands (3.16%).

The highest content of sabinene was detected in plants from Sardinia islalnd (4.21%) while the lowest content of this monoterpene was detected in Alberese plants.

The highest content of (+)-β-pinene was found in rosemary from Alberese (1.43%), the others provenances didn't show significantly differences.

The rosemary from Sardinia island was characterized by the highest content (-)-β-pinene, (6.71%).

The highest content α-phellandrene was found in Alberese provenance (0.57%).

Rosemary from Sardinia was characterized by the highest content of (-)-limonene (3.24%), while the lowest content was detected in plants collected Elba islands (0.07%).

The content of the (+)-limonene was significantly higher in Alberese and Sardinia ecotypes (4.26% and 3.39% respectively) than those of Elba and Giglio (2.70% and 1.91%).

The plants from Elba island were characterized by the highest content of *p*-cymene (4.26%), Alberese population has shown the lowest concentration of this terpene.

The provenances of Alberese (53.87%) was characterized by the highest content of 1.8-cineole, while the lowest content of this monoterpene was detected in plants from Sardinia island (5.05%).

Significant differences were detected in plants collected in Giglio and Elba islands.

The content of linalool was significantly higher in rosemary from Sardinia and Giglio island (2.03% and 1.61%) than the content in rosemary from Alberese and Elba island (0.74% and 0.67%).

The highest content of the camphor was detected in plant collected in Giglio island (3.16%).

Analysis detected that bornyl acetate was significantly higher in plants from Sardinia and Elba island (6.34% and 4.67%) than plants from Giglio island and Alberese (2.23% and 1.77%).

The highest content of verbenone was detected in plants from Sardinia (37.25%). The content of terpene was higher in rosemary from Giglio island (6.78%) than rosemary from Alberese (1.89%).

There were no significant differences between plants from Elba island (4.23%) and those from Giglio island and Alberese (6.78% and 1.89%).

The highest content of β-caryophyllene resulted in population of rosemary from Alberese (5.26%). No significant differences resulted in the others provenances.

Results of the analysis of variance indicate that all the terpenes were significantly different between the provenances. In particular, leaves of Sardinian plants showed the highest amount of (+)-α-pinene and verbenone, while Alberese provenance was characterised by the highest proportion of 1,8-cineole. (+)-α-pinene and 1.8-cineole were the major compounds in both Giglio and Elba populations.

Figure 4 shows the position of the four populations on the plane of the first two canonical axes. The analysis of enantiomeric terpenes correctly classified all plants belonging to each provenance and suggested four groupings.

Total terpene concentrations

The highest concentrations in total absolute amounts of terpenes were detected in the rosemary from Elba island (11.54 mg/g fresh weight).

The concentrations in total absolute amounts of terpenes were higher in plants from Sardinia island and Alberese park (7.27% and 7.79%) than in plants from Elba island (4.89%) (Fig. 5).

Quantitative changes in terpenoid profiles of the four provenances

Results showed a significant variation in the concentration the constitutive terpene profile of foliar tissues (expressed in mg monoterpene / g of tissues) between different provenances (Fig. 6).

The content of (-)-α-pinene was significantly higher in Alberese and in Giglio plants than in rosemary collected in Sardinia and in Elba island.

Plants from Giglio island were characterized by the highest content of (+)-α-pinene, followed by the plants from Sardinia island. There weren't significant differences in the concentration of this terpene between the populations from

Sardinia and Elba island. (+)-α-pinene had the lowest content in the rosemary from Alberese park.

The highest concentration of 1,8-cineole was detected in populations of rosemary from Alberese park and Giglio island. The plants from Elba island showed a concentration of this terpene higher than Sardinian plants.

Bornyl acetate was significantly higher in trees from Sardinia island than rosemary from Giglio, Elba islands and Alberese park.

Results showed that the highest concentrations of verbenone was detected in plants from Sardinia island. The content of this compound was higher in rosemary from Giglio island than rosemary from Elba island and Alberese park.

The population of Alberese was characterized by the highest content of β-caryophyllene. The concentration of this terpene was significantly higher in rosemary from Giglio island than rosemary from Sardinia and Elba islands.

No significant differences resulted in the concentration of the other terpenes between provenances.

Discussion

The chemical composition of rosemary oil has been the subject of considerable study, reviewed by Lawrence (Lawrence 1976-1977, 1979-1980, 1981-1987, 1988-1991, 1992-1994 and 1997). The reported components were mostly monoterpenes, the major ones being α-pinene, 1.8-cineole and camphor (Bauer *et al.* 1997) associated with variable amounts of camphene, limonene, borneol, verbenone, bornyl acetate.

According to Agioni *et al.* (2004) in *R. officinalis* many chemotypes may be distinguished, the most important being traditionally: cineoliferum (high content in 1,8-cineole), camphoriferum (camphor > 20%) and verbenoniferum (verbenone> 15%). According to the relative abundance of α-pinene, myrcene, or others relevant compounds many other chemotypes could also be recognized.

Terpene profiles have been also widely used in chemosystematic studies to characterize many species, provenances, clones and hybrids (Baradat and Yazdani 1988; Hanover 1992; Adams *et al.* 1993 Lang 1994; Baradat *et al.* 1996). Many studies showed that the differences in the terpene profile are within trees of the same species, so there are geographical and population variations.

Hiltunene *et al.* (1975a) found large variations among *Pinus sylvestris* trees, and established the two chemotypes designated as high- and low-3-carene trees growing in the north of Finland (high-3-carene) and in the south of Finland (low-3-carene).

Significant variations in the chemical composition of rosemary oil have been reported with relation to the geographic origin (Tuker and Maciarello 1986; Tewari and Virmani 1987; Mizrahi *et al.* 1991; Svoboda and Deans 1992; Chalchat *et al.* 1993; Lawrence 1995; Rao *et al.* 1997; Dellacassa *et al.* 1999). Two major types of rosemary oil can be distinguished with respect to these main constituents: oils with over 40% of 1.8-cineole (oils from Marocco, Tunisia, Turkey Greece, Yougoslavia, Italy and France) (Lawrence 1995; Mastelic *et al.* 1997, Boutekedjiret *et al.* 1998; Rezzoug *et al.* 1998; Boutekedjiret *et al.* 1999) and oils with approximately equal ratios (20-30%) of 1.8-cineole α-pinene and camphor (oils from France, Spain Italy, Greece and Bulgaria) (Lawrence 1997; Mastelic *et al.* 1997; Domokos *et al.* 1997; Tomei *et al.* 1995). One other chemical composition could be defined according to

the comparatively higher amount of myrcene in oils from Argentina, Portugual and Spain (Lawrence 1995; Dellacassa *et al.* 1999).

Data showed that the four populations of rosemary are different for the relative contents of three terpenes: 1.8-cineole, (+)-α-pinene and verbenone.

Generally they are in agreement with literature.

Plants from Alberese park could be considered a 1,8-cineole chemotype, because of their high content of this terpene (over 40 %), while, the plants from Giglio and Elba island, despite the absence of a high percentage of a single constituent, could be considered as 1,8 cineole/α-pinene chemotypes.

All the other volatiles were found in amounts inside the limits reported by literature (Carruba *et al.* 2006).

Results showed that the Sardinian rosemary could be defined as an α-pinene chemotype, these data being confirmed by others works (Granger *et al.* 1973; Falchi-Delitala and Soccolini 1980). According to Pintore *et al.* (2002) and Angioni *et al.* (2004) these plants could be also considered as an α-pinene\borneol\bornylacetate\verbenone chemotype because of their high content of these terpenes.

Besides, wild rosemary growing in Sardinia island (and in Corsica too), produces one oil which it seems to be suitable for aromatherapy (Penoel and Franchomme 1991): it exhibits a different composition, with high amounts of α-pinene, verbenone and bornyl acetate.

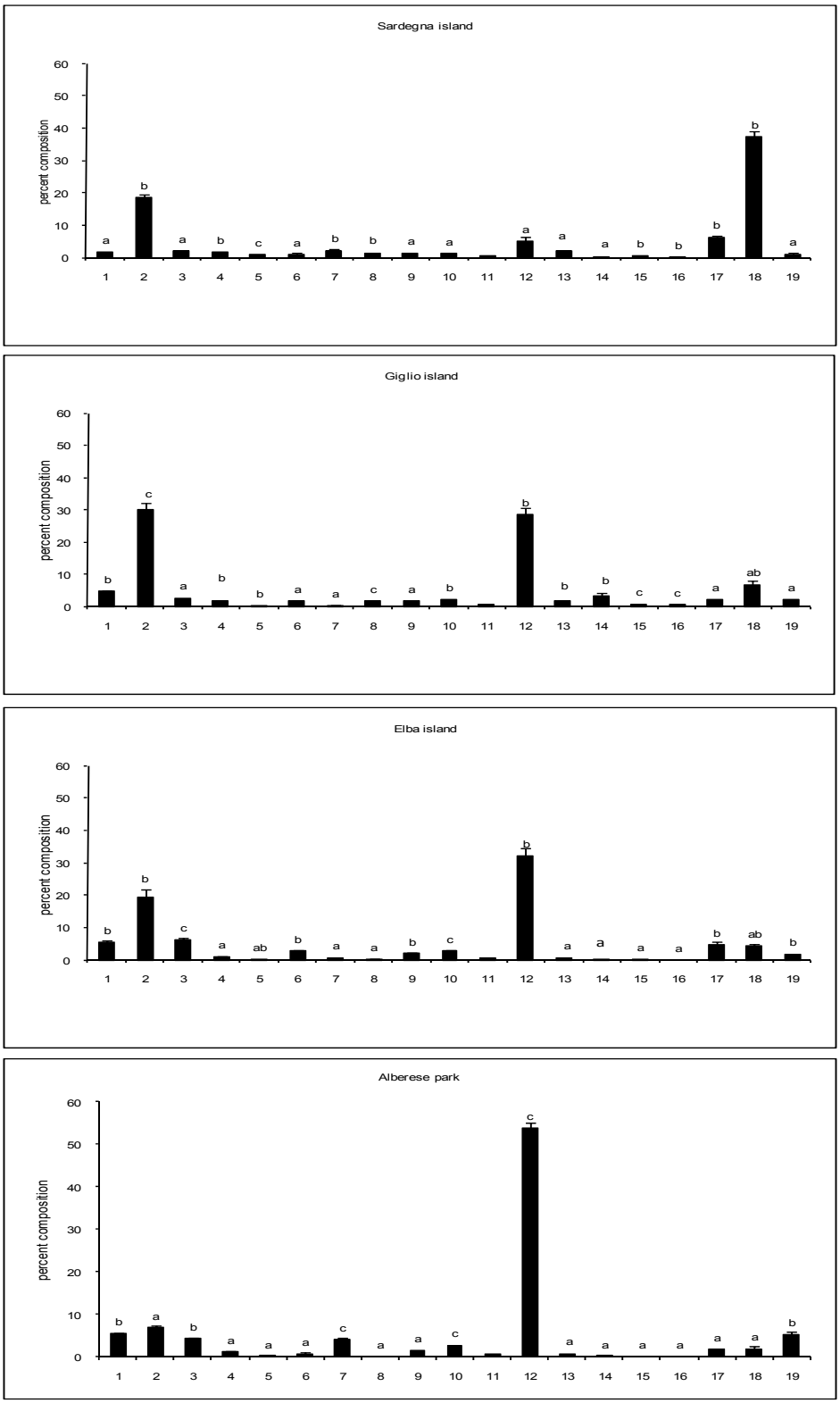

Figure 3: Relative content of terpenes in four provenances of *Rosmarinus officinalis* L.

Legend: (-)-α-pinene; 2: (+)-α-pinene; 3: camphene; 4: sabinene; 5: myrcene; 6: (+)-β-pinene; 7: (-)-β-pinene; 8: (-)-limonene; 9: (+)-limenone; 10: ρ-cymeme; 11: γ-terpinene; 12: 1,8-cineole; 13 : linalool; 14: camphor; 15: terpinen-4-ol; 16: borneol;17: bornyl acetate; 18: verbenone; 19: β-caryophyllene.

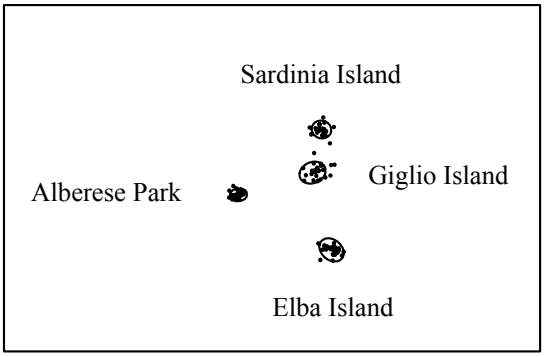

Figure 4: Discriminant analysis on aromatic profile and scatter diagram of four provenances of *Rosmarinus officinalis* L. in the plane of the first two canonical functions.

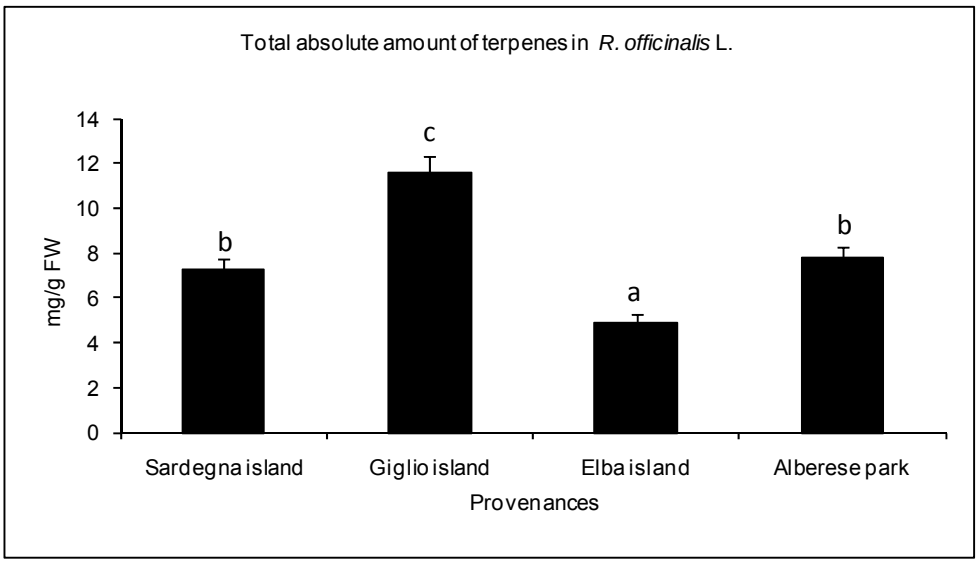

Figura 5: Total absolute amounts of terpenes in leaves of four provenances of rosemary

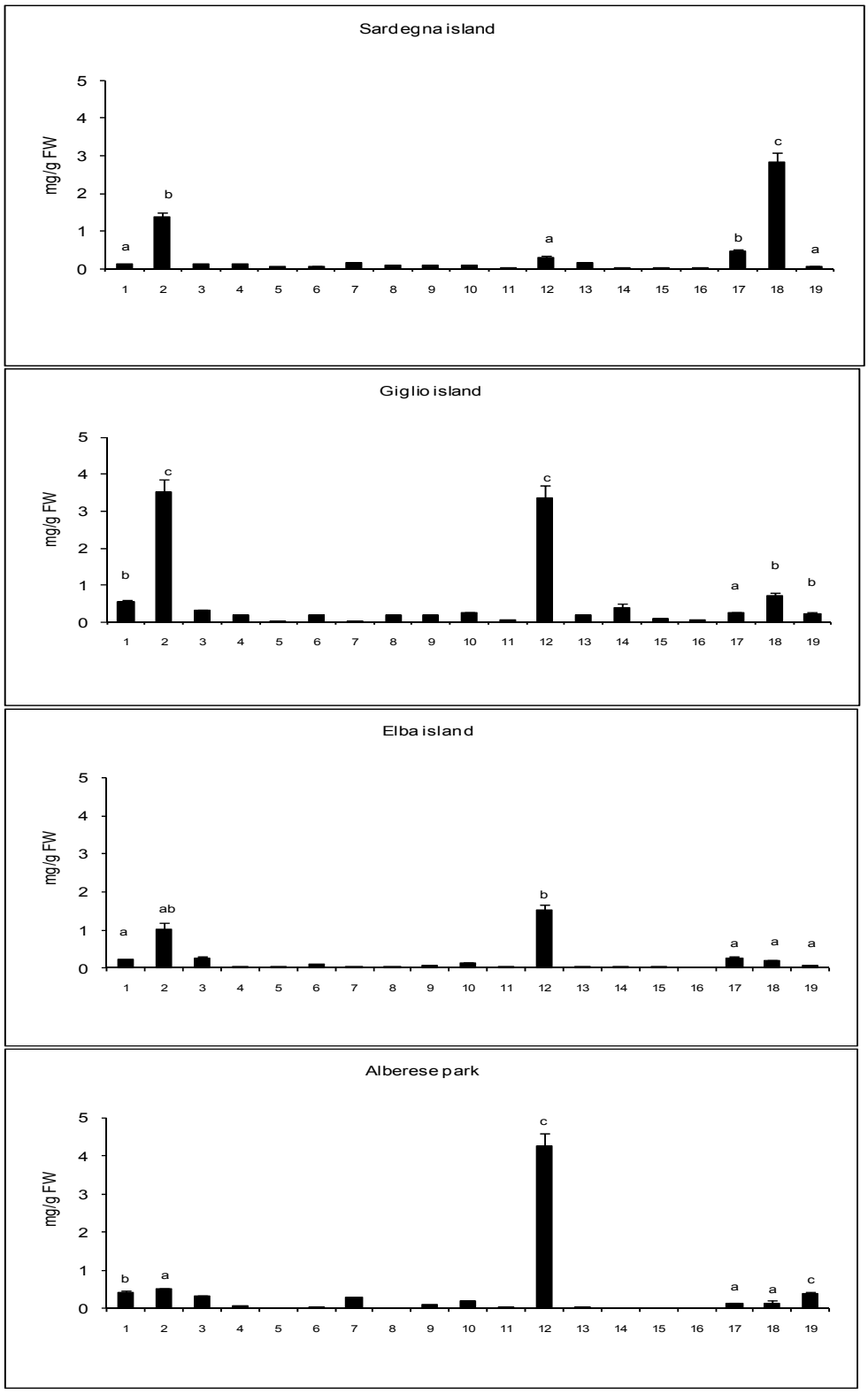

Figure 6: Concentrations of terpenes in four provenances of *Rosmarinus officinalis* L.

Legend: 1: (-)-α-pinene; 2: (+)-α-pinene; 3: camphene; 4: sabinene; 5: myrcene; 6: (+)-β-pinene; 7: (-)-β-pinene; 8: (-)-limonene; 9: (+)-limenone; 10: *p*-cymeme; 11: γ-terpinene; 12: 1,8-cineole; 13 : linalool; 14: camphor; 15: terpinen-4-ol; 16: borneol;17: bornyl acetate; 18: verbenone; 19: β-caryophyllene

ANTIFUNGAL ACTIVITY OF TERPENES IDENTIFIED IN LEAVES OF *ROSMARINUS OFFICINALIS* ON *ALTERNARIA ALTERNATA*

Introduction

Rosemary contains a great quantity of the essential oil and the yields range from 0.5% to up to 1%, (Tewari and Virmani 1987; Pintore *et al.* 2002).

The oil is widely used by the cosmetic industry as a fragrance component of soaps, creams, lotions and perfumes. Because of its antioxidative properties, due to the presence of phenolic diterpenes, rosemary is used in food industry as a food preservative. Moreover in medicine and in pharmacy it is used for its stimulatory activity on blood circulation for its hypoglycemic activity on the heart, on the nervous system, maybe for its camphor content; it also used as pulmonary antiseptic for treatment of respiratory diseases (Mentreddy *et al.* 2005; Murari Colalongo 1988; Oury, 1984; Weiss 1988). The antimicrobial activity of essential oil of *Rosmarinus officinalis* L. was investigated by many authors (Baratta *et al.* 1998; Milahu *et al.* 1997; Pintore *et al.* 2002; Angioni *et al.* 2004; Almela *et al.* 2006).

Rosemary has insecticidal (Katerinopoulus 2005), fungicidal (Pauli and Knobloch 1987) and antimicrobial (Pintore *et al.* 2002) activity; the main active components are 1,8-cineole, camphor and pinene (Hethelyi *et al.* 1989; Panizzi *et al.* 1993; Caccioni and Guizzardi 1994; Perrucci *et al*. 1994; Biavati *et al.* 1997).

Aim of this work was to evaluate the antifungal activity *in-vitro* of some compounds of the rosemary essential oil against *Alternaria alternata* (Fr.) Keissel.

Alternaria species are mainly saprophytic fungi. However, some species have acquired pathogenic capacities collectively causing disease with economic impact on a large variety of important agronomic host plants.

Some species are of clinical significance for the production of toxic secondary metabolites, some of which are powerful mycotoxins that have been implicated in the development of cancer mammals. *A. alternata,* in particular, is a human pathogen, especially in immunocompromised patients. In addition, alternaria spores are one of the most common airborne allergenes. (Thomma 2003).

Alternaria alternata (Fr.) Keissel. is also a pathogen of rosemary causing the 'alternaria leaf spot of rosemary', which has been reported in several Italian regions. This disease, causes the appearance of black spots on leaves and stems and the consequent defoliation of the plants. On leaves, lesions may easily expand, due to the production of a host-specific toxin by the pathogen. Severe attacks can be observed on rosemary plants, particularly on those growing in humid and poorly ventilated areas.

Materials and methods

Fungus material

The fungus was isolated from plants of rosemary from Antella (FI) and cultures were maintained in Petri dishes on Potato-Dextrose Agar (PDA).

In-vitro inhibiting activities of monoterpenes

In-vitro inhibiting activities of (+)-α-pinene, (-)-α-pinene, myrcene, (-)-β-pinene, (+)-β-pinene, (+)-limonene, (-)-limonene, 1,8-cineole and linalool were evaluated as inhibition of the mycelial growth of *A. alternta*, using vials containing Potato Dextrose Broth and increasing concentrations (0.025, 0.1, 0.4, 1.6 and 6.4, mM) of terpenes.

Terpenes, that were identified in essential oil of rosemary, were added in glass vials containing 100 ml of Potato Dextrose Broth and an *A. alternata* plug. The vials were sealed with teflon septums and crimped with aluminium caps, after they were put into a shaker for about ten days.

These methodology was used to assess the antimicrobial activity of terpene avoiding the limited water-solubility of these compounds (Janssen *et al.,* 1987; Rios *et al*., 1988). In literature, it is known that terpenoids have different antiseptic potency depending on their solubility in water.

Results

The minimum inhibitory concentration of (+)-α-pinene, (-)-α-pinene, myrcene, (-)-β-pinene, (+)-β-pinene, (+)-limonene, (-)-limonene, 1,8-cineole and linalool ranged from 0.025 to 0.4 mM.

Results showed that myrcene and (-)-α-pinene reduced significantly the mycelial growth of *A. alternata* at the lowest concentration, (0.025 mM). Increasing the concentration of terpenes (0.1 mM), (-)-α-pinene reduced the mycelial growth, whereas myrcene didn't show a significant reduction in the growth of fungus. At the following concentration, 0.4 mM, these compounds didn't show significant differences in the mycelial growth compared to the previous concentration.

For (-)-β-pinene, (+)-β-pinene, (+)-limonene, (-)-limonene, 1.8-cineole and linalool, it wasn't detected a significant reduction of the fungus at 0.025 mM concentration. At 0.1 mM concentration, 1.8-cineole, linalool and (+)-β-pinene didn't result to have an inhibitory effect against alternaria. At the same concentration, (-)-β-pinene, (+)-limonene and (-)-limonene showed a significant decrease of fungal growth.

At 0.4 mM concentration, (+)-β-pinene, (+)-limonene, 1.8-cineole and linalool showed a significant reduction of the fungal mycelia whereas for (-)-α-pinene, (+)-α-pinene, myrcene, (-)-β-pinene, (-)-limonene any significant difference in the mycelial growth was detected (Tab.1).

Higher concentrations ompletely inhibited the mycelial growth of *A. alternata*.

It was interesting to observe that the α-pinene enantiomers showed a different behavior towards alternaria: (-)-α-pinene was more active than (+)-α-pinene against the fungus at 0,025 mM concentration. This also happened for (+)-β-pinene and (-)-β-pinene, where the form plus was less active on the fungal growth.

About limonene enantiomers, we detected that (+)-limonene was still active at 0.4 mM concentration on the fungal growth: besides, (-)-limonene was significantly more active at the previous concentrations.

In table 2 we reported the highest concentration in which the terpene were soluble in water.

1.8-cineole and linalool resulted more soluble than the others compounds. The lowest solubility in water was detected in α-pinene enantiomers followed by β-pinene enantiomers.

Discussion

Many researchers studied the antifungal and the antimicrobial activity of the essential oil of rosemary. Angioni *et al*. (2004), tested the activity of essential oil and their main compounds (α-pinene, (-)-camphene, verbenone, camphor, bornyl-acetate and borneol) obtained from plants of Sardinian rosemary, finding low activity against *Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Pseudomonas aeruginosa and Candida albicans.* They didn't observe any inhibitory effect on the growth of bacteria and fungi; even if they found an induction effect of the essential oil, especially toward the growth of *Fusarium graminearum*. Also Pintore *et al*. (2004), showed that rosemary from Sardinia and their main compounds (α-pinene, (-)-camphene, verbenone, bornyl acetate, camphor and borneol) had a weak antimicrobic/fungitoxic activity. Before, also Masatoshi *et al.* (1997) had already found that the Sardinian and Corsican chemotypes (α-pinene/verbenone/bornylacetate) exhibited a non-significant activity, against both Gram (+) (*S. aureus* and *S. epidermidis*) and Gram (-) (*E. coli*, *P.aeruginosa*). On the contrary, they found that these oils and their major compounds had high repellency effect against the onion aphid *Neotoxoptera formosana.*

Baratta *et al.* (1998) tested a commercial sample (α-pinene, 1.8-cineole, camphor, α-terpineol chemotype) on twenty-five different genera of bacteria and one fungal species. They found a low activity except against *Staphylococcus aurens.*

Our data showed that the lowest values of MIC were observed for myrcene and (-)-α-pinene, whereas the least toxic compounds were (+)-β-pinene, 1.8-cineole and linalool.

The antiseptic potency of this compounds depends on their solubility in water. A high solubility makes an high antifungal activitiy. On this subject, however, Knobloch *et al.* (1985), reported that there are some anomalies with compounds such as thymol, carcvacrol and eugenol, as well as aromatic aldheydes, especially

cinnamaldehyde, which are of low water solubility but are highly antiseptic. We also found these anomalies for (-)-α-pinene and myrcene which have a low water solubility and resulted the most active against the growth of alternaria.

On the contrary, we observed that 1.8-cineole and linalool, with a high solubility in water, were less effective than other compound: (+)-β-pinene showed a low antifungal activity and a low solubility in water.

Besides, we found also a different behaviour between enantiomeric forms of α-pinene and β-pinene. Both the minus forms were more active than the positive forms.

In literature the activity of these compounds were investigated at different concentrations (Hammer *et al.* 2003; Cosentino *et al.* 1999). Angioni *et al.* (2004), added the essential oils, in high quantity (450 and 900 µl/ml) in Petri dishes containing PDA. In this study concentrations of terpenes tested were more similar to the real concentrations of these in the plants to have a more accurate test.

Besides, taking into account the low solubility in water of terpenes, we didn't add terpenes to agarized solutions, but to a PDB (Potato Dextrose Broth) liquid medium, keeping the vials in continuous agitation for the duration of the experiment.

However in this work single terpene were tested. It is known that the mixture add in a different way from the single, even if the individual terpenes have a broad-spectrum effects, the effectiveness of these compounds is not only dosage-dependent but is also enhanced by interaction with other terpenoids, wich may act additively or synergistically with them (Langenheim 1994).

For example rosemary oil repelled the aphids at a dose of 1 µl, while its single components did not repel the insects at 1 µl or 1 mg dose (Masatoshi 1998).

The type of antimicrobial activity shown by essential oils and their components varies from partial or complete inhibition of growth to bactericidal activity. (Andrews *et al*. 1980; Yousef and Tawil 1980). The spectrum of this antimicrobial activity and growth inhibition, in particular, seems to be quite broad, with some of the more commonly studied essential oils, such as tree oil. Several correlations have been made between the chemical composition of the essential oils and their antimicrobial activity (Chalchat *et al.* 1997; Chopineau 1997; Griffin *et al.* 1998).

At physiological level of the plant the combinations of the defense mechanisms used are different in different host-pathogen systems (Franceschi *et al.* 2005).

These data warrant further study on the defensive role of terpenes in response to the infection of *A. alternata.* It is possible that both constitutive and induced terpenes play direct roles in the defence of rosemary against the pathogen attack. Probably the results depend on the pathogen's activity and on the mixture of terpenes, rather than on the individual compounds; it has been assumed, therefore, that mixture of terpenes are important in the response of *R. officinalis* to infection with *A alternata* (Cates, 1996). In fact, the increase in monoterpenes in the infected tissues, coupled with the change from constitutive to stress monoterpene profiles could increase the toxicity of the resin to pathogens (Schmidt *et al*. 2005; Raffa and Smalley 1995).

TERPENE	CONTROL	0.025 mM	0.1 mM	0.4 mM
(-)-α-pinene	13.70 ± 1.28 [a]	9.53 ± 2.57 [b]	0.85 ± 0.58 [c]	0.11 ± 0.08 [c]
(+)-α-pinene	12.01 ± 1.23 [a]	11.36 ± 2.87 [a]	0.63 ± 0.77 [b]	0.09 ± 0.15 [b]
Myrcene	10.56 ± 1.64 [a]	2.30 ± 2.08 [b]	0.24 ± 0.12 [b]	0.04 ± 0.02 [b]
(-)-β-pinene	11.95 ± 1.44 [a]	11.84 ± 3.63 [a]	0.98 ± 0.95 [b]	0.02 ± 0.05 [b]
(+)-β-pinene	11.90 ± 1.25 [a]	11.80 ± 2.30 [a]	11.65 ± 3.99 [a]	0.05 ± 0.93 [b]
(-)-limonene	12.15 ± 1.06 [a]	11.82 ± 4.90 [a]	1.10 ± 1.19 [b]	0.11 ± 0.13 [b]
(+)-limonene	12.05 ± 1.46 [a]	12.47 ± 1.33 [a]	3.87 ± 0.25 [b]	0.07 ± 0.23 [c]
1.8-cineole	12.21 ± 1.33 [a]	9.53 ± 1.96 [a]	7.23 ± 4.12 [a]	0.11 ± 0.05 [b]
Linalool	11.56 ± 1.69 [a]	11.12 ± 0.97 [a]	8.80 ± 5.44 [a]	0.09 ± 0.04 [b]

Table 1: *A. alternata* mycelial mass dry weight (mg).

Terpenes	Molecular weight	Maximum concentration in which the terpene sare soluble in water. (Mm)
(+)-α pinene	136.24	0.037
(-)-α-pinene	136.24	0.037
(+)-limonene	136.24	0.15
(-)-limonene	136.24	0.15
Myrcene	136.24	0.22
1.8-cineole	154.25	22.6
(-)-β-pinene	136.24	0.081
(+)-β-pinene	136.24	0.081
Linalool	154.25	10.11

Table 2: water solubility (mM) of compounds

GENERAL CONCLUSIONS

The study on geographical variability of terpene content of *R. officinalis* showed three different chemotypes: α-pinene/verbenone (Sardinian plants), 1,8-cineole (Alberese provenance) and α-pinene/1.8-cineole (Giglio and Elba islands populations).

Results on antifungal activity of single monoterpenes showed that myrcene and (-)-α-pinene were the compounds with the highest inhibitory activity, whereas the least toxic compounds were (+)-β-pinene, 1.8-cineole and linalool. Based on these data, plants collected from Alberese park, that were characterized by a high concentration of 1.8-cineole and a lowconcentration of myrcene and (-)-α-pinene, could be "more susceptible" to the attack of *A. alternata.*

On the contrary, Sardinian plants, with the content of 1.8-cineole lower than the content of (+)-α-pinene, could be "relatively resistant" to the fungus attack. Results also showed that this population was characterized by a high content of verbenone, a bicyclic monoterpene with α, β-unsaturated carbonyl group, characterized by antimicrobial and antifugal activities (Griffin *et al.* 1999). This supported the "relatively resistance" of rosemary from Sardinia. Researchers found that verbenone showed an antifungal activity against *Botrytis cinerea* (*ca*. 80% growth inhibition), a lower activity against *Colletotrichum fragariae* (20% growth inhibition) (Meepagala *et al.* 2003) and against *Aspergillus niger* (Santoyo *et al.* 2005).

Antimicrobial activities were detected against gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*), gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*), a yeast (*Candida albicans*).

Moreover verbenone has been identified as the primary antiaggregation pheromone of mountain pine beetle (*Dendroctonus ponderosae* Hopkins), southern pine beetle (*Dendroctonus frontalis* Zimmermann (Renwick and Vite 1970; Brand *et al*. 1975) and Dendroctonus brevicomis LeConte.

Verbenone is now available commercially in a slow-release polyethylene pouch (Phero Tech Inc., Delta, BC) that has received U. S. Environmental Protection Agency (EPA) registration for use in forest stands containing southern pines (EPA Reg. No. 56261-CN-1 (1999) (Fetting 2005).

Following our result, rosemary from Elba and Giglio islands, results found that these plants can be classified as a chemotype α-pinene/1.8-cineole. and also show a low content of myrcene and verbenone. Generally these population could show an intermediate resistance to the *A. alternata* attack, with a greater "relatively resistance" in Giglio island plants: contents of (-)-α-pinene, (+)-α-pinene and verbenone were significantly higher in plants collected from Giglio than in plants from Elba islands.

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PART B: RELATIONSHIP BETWEEN TERPENE COMPOSITION AND THE ATTACK OF *HETEROBASIDION ANNOSUM* IN *PICEA SITCHENSIS*

THE ROLE OF VOLATILE COMPOUNDS IN THE CHEMICAL DEFENSE SYSTEM OF *PICEA SITCHENSIS* AGAINST *HETEROBASIDION ANNOSUM* ATTACK

Introduction

Picea sitchensis (Bong.) Carr. occurs naturally in a narrow and more or less continuous belt extending up the west coast of North America from the northern California to Alaska (Forrest 1982). Nowadays, sitka is the most important commercial timber species in United Kingdom, occupying 40% of the coniferous forest area.

It is very susceptible to the *Heterobasidion annosum* (Fr.) Bref. infection; this is a serious root and butt-rot fungal pathogen of conifers, but can also attack some angiospermous trees (Korhonen *et al.* 1998; Lygis *et al*. 2004). It usually enters the host through wounds or stumps, causing wood decay, with significant economic losses when monoculture plantations are attacked (Woodward *et al*. 1998). In some cases, host tree population may remain free of infection (Delatour *et al*. 1998); the basis for this apparent resistance to the pathogen is generally unknown (Karjalainen *et al.* 1998; Asiegbu *et al.* 2005). Plants produce a vast array of secondary metabolites such as terpenoids and phenolics, to defend themselves against their natural enemies (Croteau *et al.* 2000) but, in addition to a chemical response, there is also a physical reaction: in the inner bark tissue, the pathogen attack leads to the formation of a ligno-suberised boundary zone (LSZ, Woodward *et al.* 2007).

The aim of this study was to examine the variation in the chemical responses to *H. annosum* attack in *P. sitchensis*.

Material and Methods

Plant material

Terpene composition was analyzed in cortical tissue samples of 4 20 years old Sitka spruce clones, growing at the Scoot More site (Ref: NJ172392; Moray, Scotland, UK).

In this trial, the Sitka spruce clones included the two that in a previous screening of about 30 clones proved to develop the shortest stem bark lesions (Clones 20198 and 20206) and the two forming the longest stem bark lesions (Clones 20179 and 20204) following inoculation with *H. annosum*.

Fungal material

An isolate of *H. annosum* (O27_21) originally obtained from Sitka spruce trees growing in Bennachie Forest, Aberdeenshire (Ref: NJ690210; 57°16′43″ N, 2°30′57″ W; Bodles et al. 2005) was sub-cultured on 2% malt extract agar (MEA). Fresh cultures were stored in 90 mm diameter Petri dishes at 20°C and 65% RH in the dark.

Pine dowels (10 mm length, 6 mm diameter) cut from preformed dowels were submerged in distilled water for 24 h and subsequently autoclaved at 105 kPa for 30 min. After cooling, the water was discarded and the dowels submerged in fresh distilled water. The autoclaving procedure was repeated, the water discarded and the dowels cooled before being transferred onto Petri dishes containing *H. annosum* cultures. The dowels were incubated on the cultures at 22 °C for 4 weeks to allow colonization to occur. Control dowels were placed on sterile malt extract agar.

Experimental design

In August 2008, vigorous trees were randomly chosen from the dominant and co-dominant tree levels by examining the appearance and structure of the crown in the site. Similar growth conditions were ascertained with respect to the soil type, temperature, and rainfall values.

Each tree stem was inoculated at about 20-30 cm above ground. Four holes were drilled with a Maktek® cordless driver drill, and the wood inocula, together with the control treatment, randomly inserted with a hammer. To avoid any bruising damage

due to hammering, inoculum insertion was carried out with an extra piece of wood. Immediately after inoculation, each wound surface was sealed with vaseline. Four different Sitka spruce clone with 5 replicate trees per clone were inoculated.

Reference samples were collected at day 0 (i.e. on the day of wounding and inoculation); bark samples were collected 3 and 43 days after wounding and inoculation (in August and September 2008, respectively) with a 25 mm wood chisel by removing a piece of bark. A diagonal cut was performed on the upper side of the sample to discern it from the lower side. Sample length differed between 3 and 43 days sampling (approx 6 cm and 10 cm long in 3 and 43 days' samples, respectively) in order to include approx 2-3 cm of healthy tissue beyond the lesion boundary. Moreover bark samples were collected 43 days after the inoculation, at 15 and at 100cm from the site of infection.

All samples were stored at -80 °C until further use.

Analyses of terpenoids

Cortical samples were finely ground with a pestle and a mortar containing liquid nitrogen. For each sample, a 0.1 g subsample of the fine poweder was placed in a 2 ml glass vial, covered with a Teflon-coated screw cap (Perkin –Elmer, Norwalk, CT) and extracted in 2 ml of *n*-pentane with tridecane as an internal standard (Raffa and Smalley 1995). The extracts were placed in glass vials and stored at -20 °C until analysed.

Analysis have been performed using a gas chromatography-flame ionization detection (GC-FID) with a Perkin-Elmer Autosystem XL GC. Separation of enantiomeric monoterpenes was performed on a Cyclodex-B capillary column 30-m-long and 0.25-mm-diameter supplied by J & W Scientific (CA, U.S.A.), (Silvestrini *et al.* 2004). Terpene were identified by comparison of their retention times with those of standards under the same conditions. Absolute amounts of terpenoids were determined by comparison with the tridecane internal standard, and expressed as mg g^{-1} dry mass (DW). The relative amount (proportion of profile) of each monoterpene was expressed as a percentage of total monoterpenes.

Statistical analyses

Concentrations and proportions (%) of various terpenoid components were not normally distributed (Kolmogorov-Smirnov one sample test) and were analyzed by Friedman and the non-parametric Kruskal-Wallis ANOVA followed by the Mann-Whitney U Test for multiple comparisons.

Results

Constitutive profiles

Thirteen confirmed monoterpenes were detected in the cortical tissue of Sitka spruce.

Overall, the proportions of the main monoterpenes were: β-phellandrene (47,69%), (-)-β-pinene (14,21%), (–)-α-pinene (13,95%), sabinene (9,28%) and myrcene (4,57%), but (+)-α-pinene, (+)-β-pinene, (+)-limonene, γ-terpinene and terpinolene were also detected. Camphene, δ-3-carene and (–)-limonene, were in traces (Fig. 7).

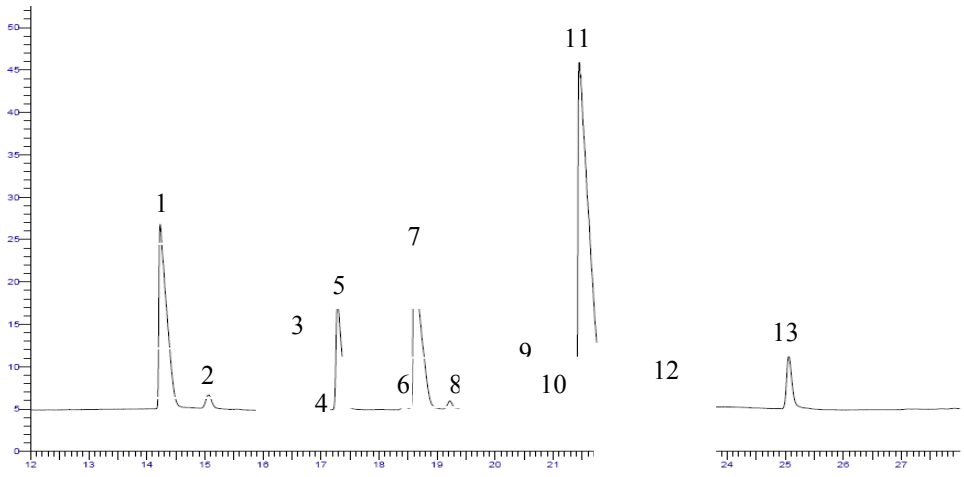


Figure 7: Typical terpene profile obtained from Sitka spruce.

Legend: 1: (–)-α-pinene; 2: (+)-α-pinene; 3: myrcene; 4: camphene; 5: sabinene 6: δ-3-carene; 7: (-)-β-pinene; 8: (+)-β-pinene; 9: (–)-limonene; 10: (+)-limonene; 11: β-phellandrene; 12: γ-terpinene; 13: terpinolene.

Relative percentage of (+)-α-pinene, myrcene, sabinene and terpinolene showed significant differences between the four clones.

(+)-α-pinene and myrcene were higher in clone 20179 than in the three other clones.

The relative percentage of sabinene and terpinolene was higher in clones 20204, 20198 and 20206 than clone 20179 (Fig. 8).

These results were confirmed by data expressed in mg monoterpenes / g tissues.

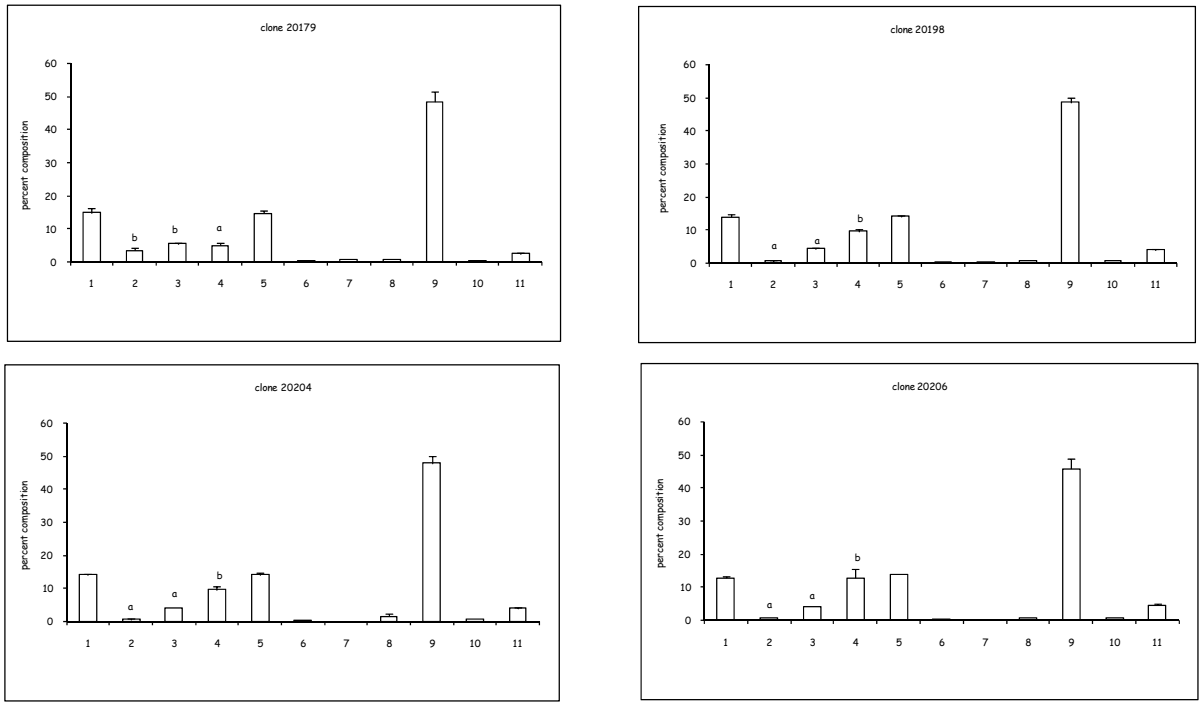


Figure 8: Relative percentage of constitutive terpene profile in four clones of Sitka spruce

Legend: 1: (–)-α-pinene; 2: (+)-α-pinene; 3: myrcene; 4: sabinene 5: (-)-β-pinene; 6: (+)-β-pinene; 7: (–)-limonene; 8: (+)-limonene; 9: β-phellandrene; 10: γ-terpinene; 11: terpinolene.

Changes in total terpene concentrations after inoculation

Total terpenoid concentrations showed significant differences between treatments in all the four clones.

Both wounded only and wounded plus inoculated tissues resulted in increased total quantities of terpenes produced in all plants. This effect was more evident in

infected tissues collected 43 days following inoculation in clones 20179, 20198 and 20206 (Fig. 9).

Total terpene concentration significantly increased in wounded only tissues collected on day 3 in all the clones and it remained stable on day 43 in clones 20179, 20204 and 20206. No significant differences were detected in clone 20198 between samples collected on day 43 and the control.

Total terpene concentration in wounded plus inoculation tissues significantly increased on day 3 in all clones and reached the highest values on day 43 in clones 20179, 20198 and 20206.

No significant differences were detected in clone 20204 between samples collected on day 3 and day 43.

No significant differences were observed between wounded only and wounded plus inoculation tissues collected on day 3 except to clone 20198 that showed higher terpene concentration in wounded only than wounded plus inoculation tissues.

On day 43 wounded plus inoculation tissues showed higher terpene concentrations than wounded only samples in clones 20179, 20198, and 20206. No differences in total amounts were detected between wounded only and wounded plus inoculation tissues from clone 20204.

No significant differences were detected between samples collected 15 and 100 cm above the lesion collected 43 days after the inoculation and the control tissues (day 0).

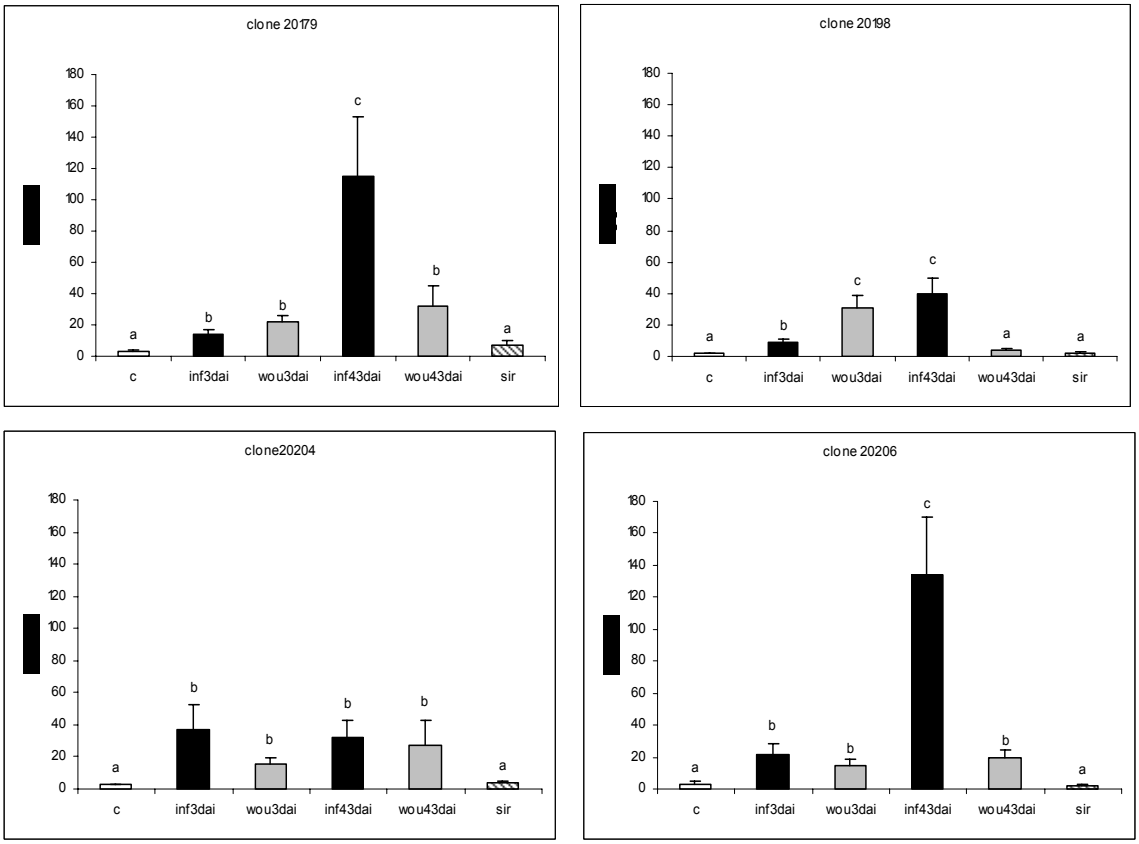


Figure 9: Total absolute amounts of terpenes in four clones of Sitka spruce (mean +SE).

This graph showed that 43 days following inoculation there was an increase in the total concentration of terpenes than the others treatments.

Different letters indicate significant differences ($p<0.05$).

□ control ■ infection ▒ wound ▨ sir15-100cm

Changes in concentrations of single terpene constituents after inoculation

Samples collected on day 3 showed a significant higher concentration of (-)-α-pinene, myrcene, β-phellandrene and γ-terpinene in both wounded and wounded plus inoculated tissues than the control tissues in clones 20179, 20204 and 20206. In clone 20198 the concentrations of (-)-α-pinene, myrcene and γ-terpinene were significantly higher in wounded than in wounded plus inoculated tissues and in the control, whereas the highest concentration of β-phellandrene was detected in wounded tissues.

The concentration of (+)-α-pinene significantly increased in tissues following the wounding and the wounding plus inoculation in clones 20198, 20204 and 20206,

whereas the highest value of this monoterpene content was riched in wounded tissue of clone 20179.

Sabinene concentration significantly increased after the treatments in all the four clones.

Concentration of (-)-β-pinene significantly increased in both wounded and wounded plus inoculated tissues in clones 20179 and 20206. Whereas the highest content of the terpene was detected in wounded only tissues in clone 20198 and in wounded plus inoculated tissues in clone 20204.

Terpinolene concentration showed a significant increase in both treated tissues in clones 20179 and 20206. In clone 20198, this terpene showed a significantly higher concentration in wounded tissues than in wounded plus inoculated and control tissues. Moreover, in clone 20204 terpinolene showed the highest concentration in infected tissues (Fig. 10).

Samples collected 43 days after the treatment, showed that concentrations of (–)-α-pinene, myrcene, sabinene, (-)-β-pinene, β-phellandrene, γ-terpinene and terpinolene significantly increased in the wounded only and in wounded plus inoculated tissues, reaching the maximum values in the infected tissues of clones 20179 and 20206. In clone 20198, the concentration of these compounds were significantly higher in wounded plus inoculated tissues than in wounded only and control tissues. Moreover in clone 20204 (–)-α-pinene, myrcene, sabinene, β-phellandrene and γ-terpinene showed a significant increase in both wounded and infected tissues. Regarding to (-)-β-pinene, this compound showed a significant increase in infected tissues. For terpinolene, it was detected a significant increase in both treated tissues, with the highest concentration in wounded plus inoculation tissues.

(+)-α-pinene showed a significantly higher concentration in wounded and wounded plus inoculated tissues than control, and the highest content in the infected tissues of clone 20179. In clone 20198, the concentration of (+)-α-pinene was significantly higher in infected tissues than wounded only and control tissues. Furthermore, the content of (+)-α-pinene significantly increased in wounded only and in wounded plus inoculated tissues in clones 20204 and 20206 (Fig. 10).

(-)-α-pinene, (+)-α-pinene, myrcene, sabinene, β-phellandrene, γ-terpinene and terpinolene increased significantly in wounded only tissues collected on day 3 and 43 from clones 20178, 20204 and 20206. In clone 20198, the highest content was detected in wounded tissues collected on day 3.

Regarding to (-)-β-pinene, there weren't significant differences between wounded tissues and control tissues, in clone 20204. In clones 20179 and 20206, there was a significant increase in wounded only tissues collected on day 3 and on day 43 compared to control tissues. Moreover, in clone 20198 the highest content of (-)-β-pinene resulted in samples collected on day 3 (Fig 10).

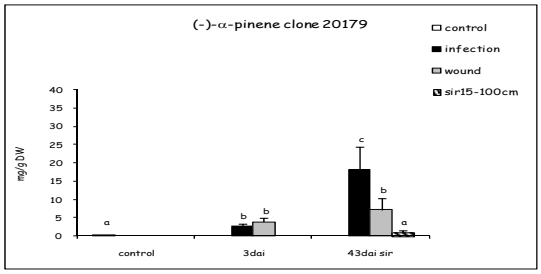
Wounded plus infected tissues showed significant differences in terpene concentration between samples collected on day 3 and on day 43.

(-)-α-pinene, myrcene, sabinene, (-)-β-pinene, β-phellandrene, γ-terpinene and terpinolene, showed a significantly higher concentration in infected tissues than the control, with the highest increase in samples collected on day 43 in clones 20179 and 20206. In clone 20204, the concentration of these compounds was significant higher in infected samples than in control tissues, whereas no significant differences were detected between infected tissues. In clone 20198, the highest amount of (-)-α-pinene, myrcene,(-)-β-pinene, γ-terpinene and terpinolene was detected in infected tissues collected 43 days after the inoculation. There were no significant differences between control and infected tissues collected on day 3.

Concentration of sabinene and β-phellandrene was higher in infected tissues than control tissues, reaching the maximum values in infected tissues collected on day 43 in clones 20179, 20189 and 20206. In clone 20204 the concentration of these compounds was higher in infected tissues than in control tissues.

The highest increase of (+)-α-pinene was detected in infected tissues collected 43 days after the inoculation in clone 20179. This compound showed also a concentration significantly higher in infected tissues collected on day 3 and on day 43 than in control tissues of clones 20198, 20204 and 20206 (Fig. 10).

No significant differences in terpene concentrations were observed between samples collected on day 43, at 15 and at 100 cm from the site of infection and the control tissues, (Fig. 10).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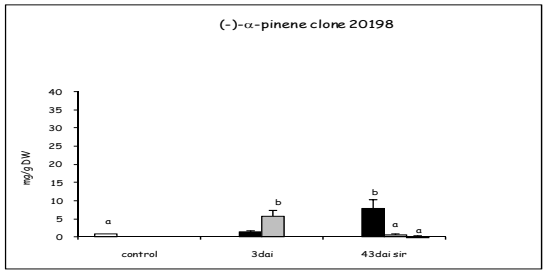
control
infection
wound
sir15-100cm
mg/g DW
control
3dai
43dai sir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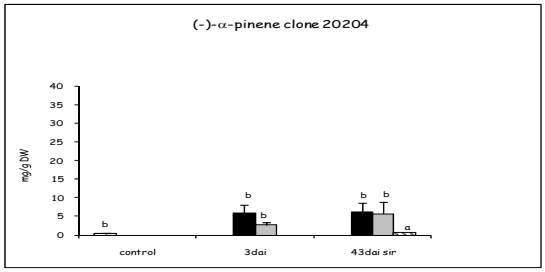
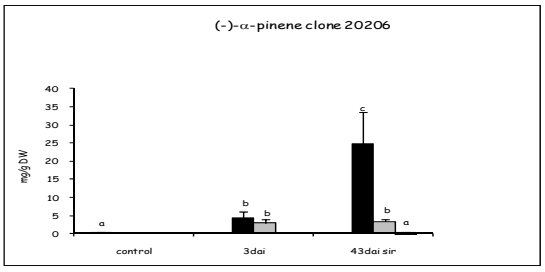
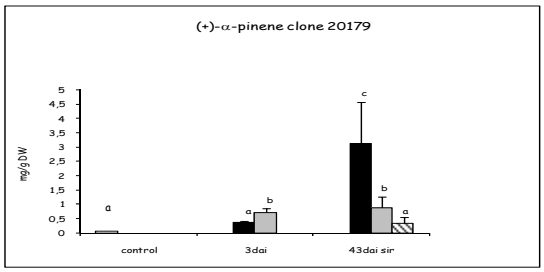
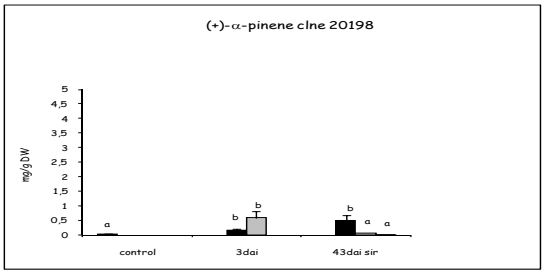
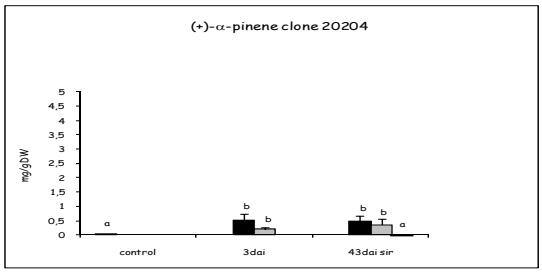
mg/g DW
control
3dai
43dai sir

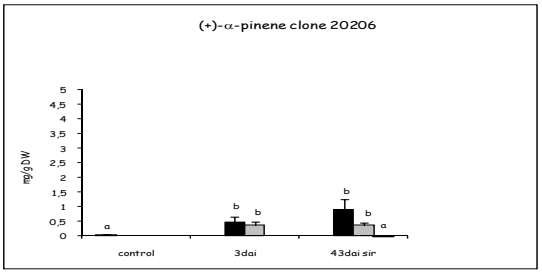

(-)-α-pinene clone 20204
mg/g DW
control
3dai
43dai sir

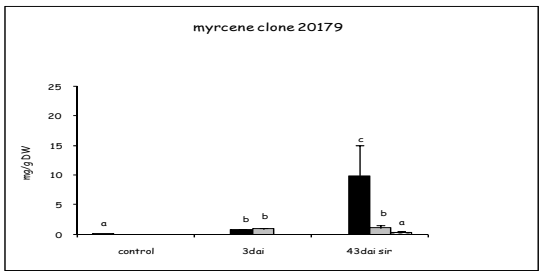

(-)-α-pinene clone 20206
mg/g DW
control
3dai
43dai sir

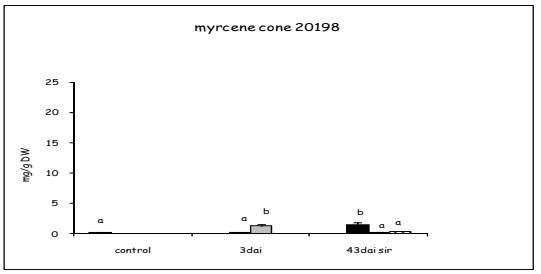

(+)-α-pinene clone 20179
mg/g DW
control
3dai
43dai sir

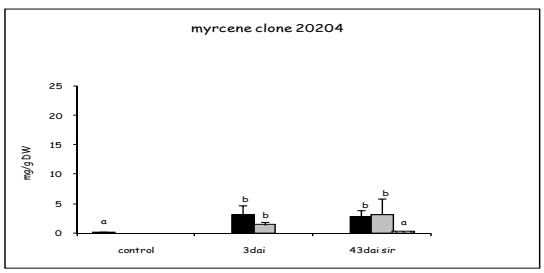

(+)-α-pinene clne 20198
mg/g DW
control
3dai
43dai sir

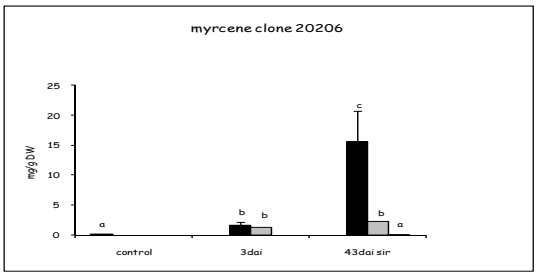

(+)-α-pinene clone 20204
mg/g DW
control
3dai
43dai sir

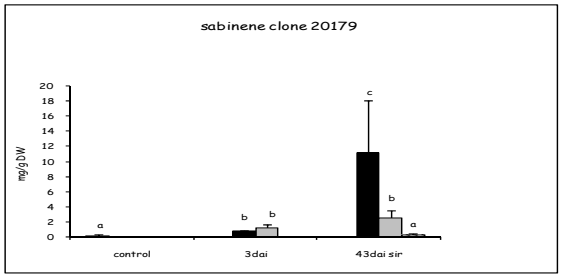

(+)-α-pinene clone 20206
mg/g DW
control
3dai
43dai sir

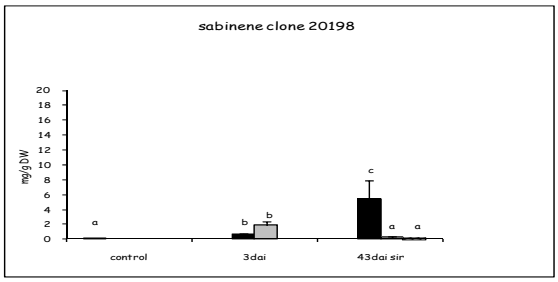

myrcene clone 20179
mg/g DW
control
3dai
43dai sir

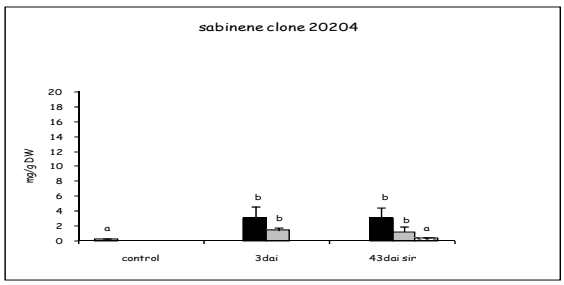

myrcene cone 20198
mg/g DW
control
3dai
43dai sir

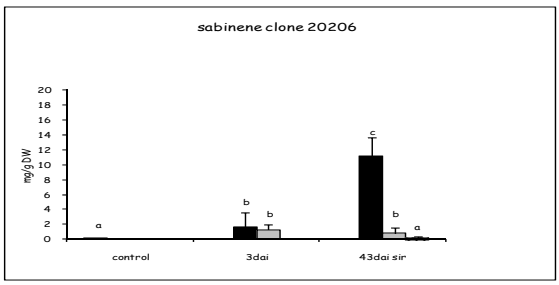

myrcene clone 20204
mg/g DW
control
3dai
43dai sir

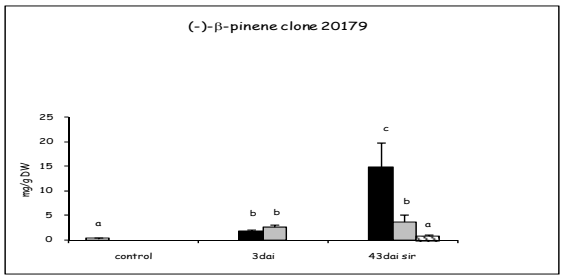

myrcene clone 20206
mg/g DW
control
3dai
43dai sir


sabinene clone 20179
mg/gDW
control
3dai
43dai sir


sabinene clone 20198
mg/gDW
control
3dai
43dai sir


sabinene clone 20204
mg/gDW
control
3dai
43dai sir


sabinene clone 20206
mg/gDW
control
3dai
43dai sir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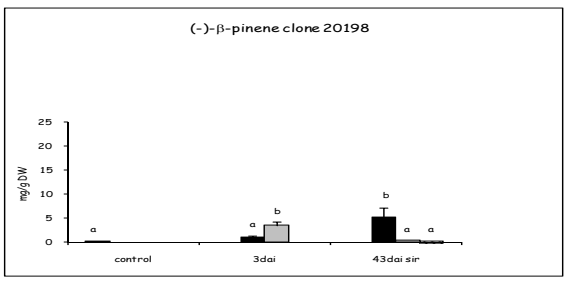
mg/gDW
control
3dai
43dai sir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mg/gDW
control
3dai
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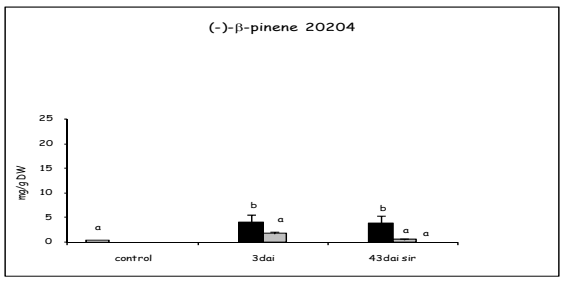
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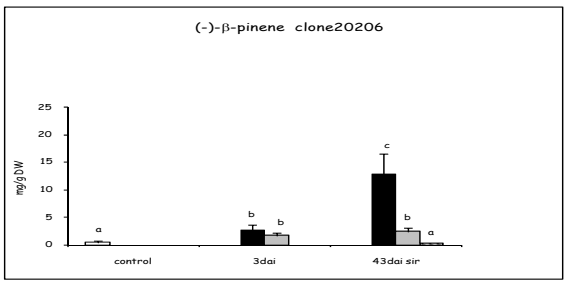
mg/gDW
control
3dai
43dai sir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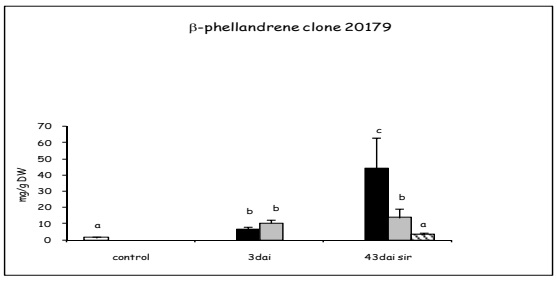
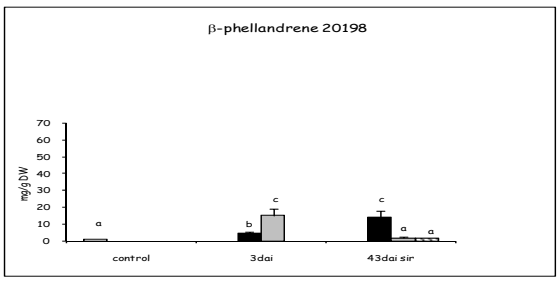
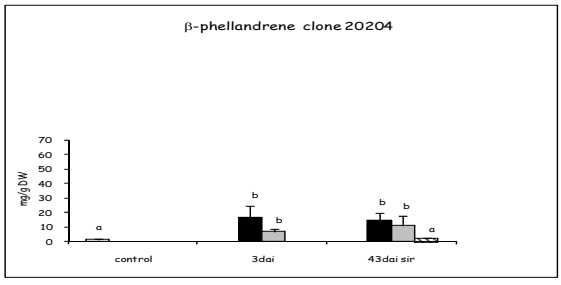
mg/gDW
control
3dai
43dai sir


β-phellandrene clone 20179
mg/gDW
control
3dai
43dai sir


β-phellandrene 20198
mg/gDW
control
3dai
43dai sir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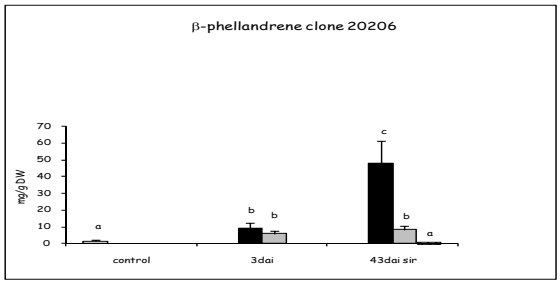
mg/gDW
control
3dai
43dai sir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mg/gDW
control
3dai
43dai sir

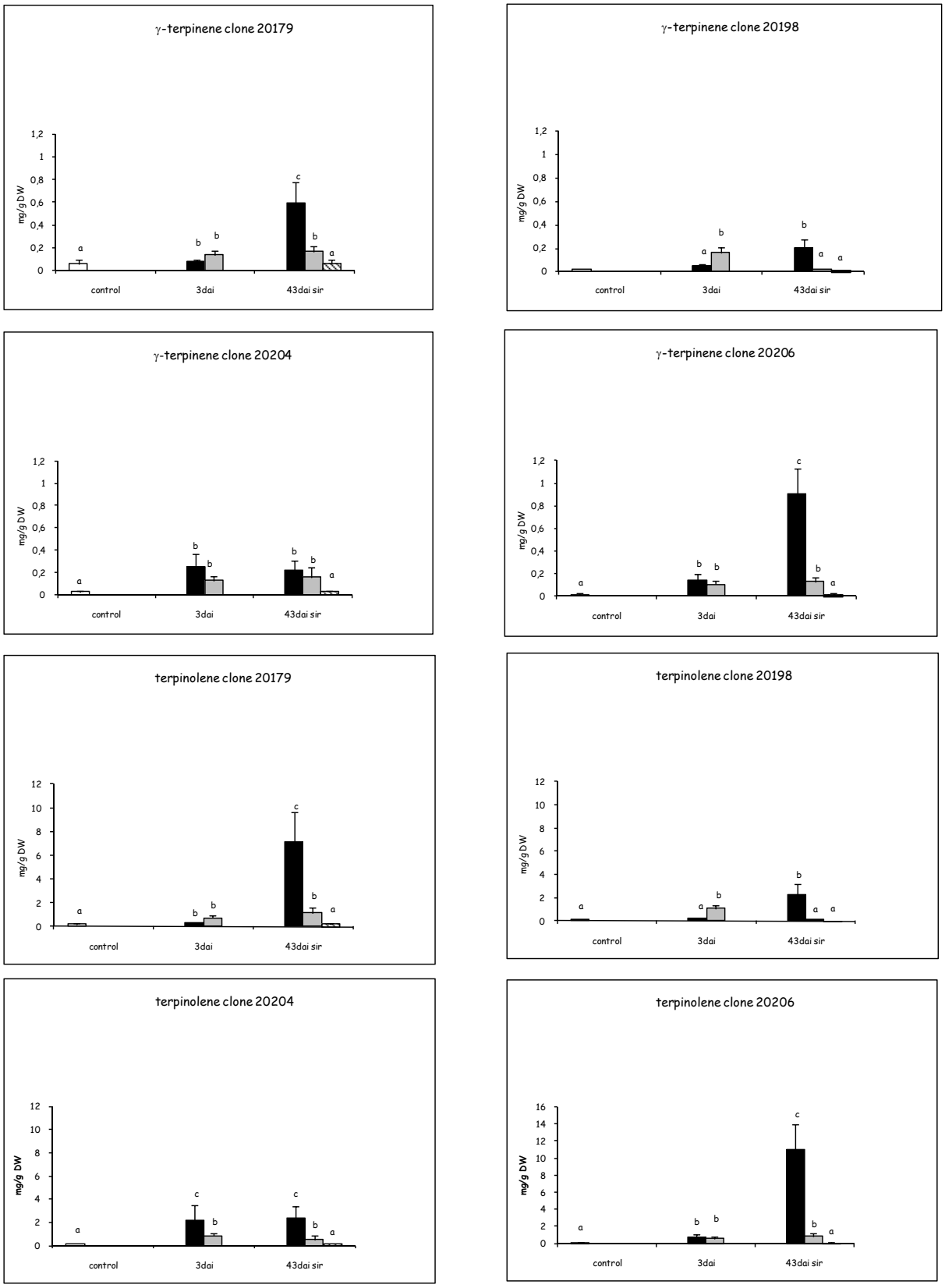

Figure 10: Enantiomeric profiles (mg moterpenes/ g tissue) of terpenes in Sitka spruce clones. Terpenes increased in inoculated tissues. Letters indicate significant differences ($p < 0.05$)

Discussion

In this work gaschromatographic analyses detected thirteen monoterpenes in the cortical tissue of Sitka spruce clones.

β-phellandrene was the compound present at the highest percentage; (–)-α-pinene and (-)-β-pinene, sabinene and myrcene were major constituents, while.(+)-α-pinene, (+)-β-pinene, (–)-limonene, (+)-limonene, γ-terpinene, terpinolene were minor components. Camphene and δ-3-carene were found in traces.

Proportions of these monoterpenes, in the constitutive profile, were similar to those reported in other studies (Forrest 1982; Forrest and Samuel 1986 and Woodward *et al.* 2007).

We didn't include sabinene in minor compounds, this was in disaccord with Woodward *et al*. (2007), besides, Forrest (1982) and Forrest and Samuel (1986), didn't detected sabinene.

Generally, the relative contents of constitutive terpenes did not vary between our test clones and, thus between more susceptible and relatively "resistant" trees (Woodward *et al.* 2007). Woodward *et al.* (2007), in fact found that the relative contents of several terpenes varied significantly between resistant and susceptible clones of Sitka spruce. They found that resistant clones had higher relative proportions of (+)-α-pinene, (-)-β-pinene and an unknown compound than the susceptible clones, whereas the latter showed higher relative proportions of (-)-limonene and three unknown compounds.

Total terpenoid concentrations showed significant differences between treatments in all the four clones. In general, we observed an increase in treated tissues, with the highest amount in infected tissues collected 43 days after the inoculation, particularly in clones 20179 and 20206.

This result is in accord with Woodward *et al.* (2007); they also found that total absolute quantities of monoterpenes differed between treatments and were significantly higher in the wounded plus inoculated and wounded only trees, than in samples collected without treatments. This effect was more evident in tissues following inoculation than in wounded only samples, and was more pronounced in the more susceptible clones.

According to our results, there was no relationship between changes in total amount of terpenes and length of the lesion. In fact, no similar trend in terpene response to infection was observed within each class of clones classified as relatively "resistant" and susceptible plants.

In general, higher concentrations of terpenes were detected in wounded only and in wounded plus inoculated tissues than in control tissues. The higest contents of terpenes were observed in tissue samples analyzed 43 days after the inoculation.

The highest increase of (-)-α-pinene was detected in infected tissues in clone 20179 and 20206; (+)-α-pinene resulted to have the highest increase in clone 20179.

Woodwarth *et al.* (2007) showed that the relative percentage of (-)-α-pinene and (+)-α-pinene didnt't vary in response to infection. These Authors reported data of terpenes expressed as relative content and they stated that similar trends were observed when data were expressed as proportion or absolute amounts.

Forrest (1982) found that Sitka spruce trees infected with *H. annosum* had a relative content of α-pinene in the constitutive profile lower than healthy trees. He suggested that plants characterized by a high content of α-pinene were less susceptible to the attack of *H. annosum* because of the fungitoxic properties of α-pinene. Indeed, the antifungal properties of (-)-α-pinene on the growth of *H. annosum* were observed in *in-vitro* test even if the inhibition of the mycelial growth with this terpene was less effective than other tested compounds (Zamponi *et al.* 2006).

Also (-)-β-pinene, showed the highest increase in infected tissues collected 43 days after the inoculation in clones 20179 and 20206. This was in accord with Woodward *et al.* (2007) who found an increase of the relative percentage of this compound after the treatments. They found that (-)-β-pinene was higher in wounded only samples than in wounded plus inoculation in the less susceptible clones, whereas it increased in both wounded plus inoculation tissues and wounded only in the more susceptible ones. In the work of Zamponi *et al.* (2006) *in-vitro* tests showed that (-)-β-pinene reduced significantly the mycelial growth of the fungus in contact phase and in vapour phase.

Myrcene increased in response to the inoculation, particularly in clones 20179 and 20206; these data are in agreement with the study performed by Zamponi *et al.*

(2006), that showed myrcene as the most active monoterpene on the mycelial growth of the *H. annosum*.

Also β-phellandrene increased in tissues infected with the fungus reaching the maximum values in samples collected from clones 20179 and 20206 on day 43. Woodward *et al*. (2007) didn't find a significant increase following the inoculation with H. annosum. Forrest (1982) reported that Sitka spruce trees with a high constitutive content of β-phellandrene were more susceptible to *H. annosum* attack.

Sabinene showed a significant increase in response to inoculation and pseudo-inoculation (wounded only) in clones 20179, 20198, and 20206; the highest amount was detected in tissues collected 43 days after the inoculation. Also Woodward *et al.* (2007) found that sabinene increased following the infection with *H. annosum*.

γ-Terpinene and terpinolene increased in response to the the inoculation, particularly in samples collected on day 43 in clones 20179 and 20206, results in agreement with Woodward *et al.* (2007) who found that γ-terpinene increased significantly following wounding plus inoculation with *H. annosum,* whereas they didn't find significant variations in the relative proportions of terpinolene.

No systemic induction was observed since terpene profiles did not change in cortical tissues at 15 and 100 cm from the inoculation site.

Systemic induced resistance is known to occur in many plants, including conifers (Bonello *et al.* 2006). It is a defense mechanism in which the activation of unknown signals produced at the site of initial infection (also defined as induction) primes the host against further pathogenic attacks (defined as challenges) in tissues located remotely from the site of initial infection. These signals induce the synthesis or accumulation, or both, of defence metabolites, such as terpenes and phenolics, including lignin, either before and after the challenge (Evensen *et al.* 2000; Bonello and Blodgett 2003; Hudgins *et al.* 2003; Theis and Lerdau 2003; Luchi *et al.* 2005; Phillips *et al.* 2006; Blodgett *et al.* 2007). Many of these responses are reflected in major anatomical reorganization, including the formation of polyphenolic parenchyma cells and traumatic resin ducts (Franceschi *et al.* 2005; Luchi *et al.* 2005), and cell wall lignifications (Hudgins and Franceschi 2004; Nagy *et al.* 2006; Blodgett *et al.* 2007). In this current work, systemic induction of a monoterpene effect was not observed, in agreement with data reported by Woodward *et al.* (2007) showing no systemic induction of monoterpenes of spruce trees.

Conclusions

In conclusion, these data show that terpene metabolism is involved in chemical defence of *P. sitchensis* in response to attack by *H. annosum*:

(1) Generally, the relative contents of constitutive terpenes did not vary between clones.

(2) Total terpenoid concentrations showed the highest amounts in infected tissues collected 43 days after the inoculation.

(3) The concentration of several terpenes increased following wounding and wounding plus inoculation, reaching the highest values in infected tissues 43 days after the inoculation.

(4) No similar trend in terpene response to infection was observed within each class of clones classified as relatively "resistant" and susceptible plants.

(5) No systemic induction of a monoterpene effect was observed. There weren't significant differences between terpene profiles of bark tissues collected 15 and 100 cm from the wounded plus inoculated and the control tissues.

This work warrant further studies in order to investigate the possibility to use terpenes as an aid to selecting of chemotypes less susceptible to infection by this pathogenic fungus.

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CONCLUSIONS

The study on geographical variability of terpene content of *R. officinalis* showed three different chemotypes: α-pinene/verbenone (Sardinian plants), 1,8-cineole (Alberese provenance) and α-pinene/1.8-cineole (Giglio and Elba islands populations). Results on antifungal activity of single monoterpenes showed that myrcene and (-)-α-pinene were the compounds with the highest inhibitory activity, whereas the least toxic compounds were (+)-β-pinene, 1.8-cineole and linalool.

Based on the results of these studies:

- Plants collected from Alberese park, that were characterized by a high concentration of 1.8-cineole and a lowconcentration of myrcene and (-)-α-pinene, could be "more susceptible" to the attack of *A. alternata.*
- Sardinian plants, with the content of 1.8-cineole lower than the content of (+)-α-pinene, could be "relatively resistant" to the fungus attack. Results also showed that this population was characterized by a high content of verbenone, characterized by antimicrobial and antifugal activities This supported the "relatively resistance" of rosemary from Sardinia.
- Rosemary from Elba and Giglio islands could show an intermediate resistance to the *A. alternata* attack, with a greater "relatively resistance" in Giglio island plants.

Study, on relationship between terpene composition and the attack of *H. annosum* in *P. sitchensis* clones, showed that terpene metabolism is involved in chemical defence of Sitka spruce in response to attack by fungus:

- Generally, the relative contents of constitutive terpenes did not vary among the four clones;
- Total terpenoid concentrations showed the highest amounts in infected tissues collected 43 days after the inoculation.

- The concentration of several terpenes increased following wounding and wounding plus inoculation, reaching the highest values in infected tissues 43 days after the inoculation.
- No similar trend in terpene response to infection was observed within each class of clones classified as relatively "resistant" and susceptible plants.
- There weren't significant differences between terpene profiles of bark tissues collected 15 and 100 cm from the wounded plus inoculated and the control tissues.

This work warrant further studies in order to investigate the possibility to use terpenes as an aid to selecting chemotypes less susceptible to infection by this pathogenic fungi. It could have significant implications for forest health and cultivated plants protection, including a rationale for reinforcing preventative measures to exclude biological invasions.

PAPERS

Papers related to thesis:

- Previati, A., V. Martini, T. Comunian, F. Mencarelli, N. Mulinacci and M. Michelozzi. 2009. Indagine preliminare sulla frazione volatile di 4 cloni italiani di aglio provenienti da coltura in campo e da micropropagazione. Italus Hortus. 16 (2): 213- 216.
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Tree Physiology 28, 1653–1660

Systemic effects of *Heterobasidion annosum s.s.* infection on severity of *Diplodia pinea* tip blight and terpenoid metabolism in Italian stone pine (*Pinus pinea*)

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Summary Three-year-old seedlings of *Pinus pinea* L. were inoculated near the stem base with one of two *Heterobasidion annosum* (Fr.) Bref. *sensu stricto* (*s.s.*) strains belonging to two populations: the North American P-group (NAm-P) and the European P-group (Eur-P). The NAm-P strain caused smaller *H. annosum* stem lesions than the Eur-P strain. Three weeks after the stem inoculations with *H. annosum*, apical shoots were inoculated with *Diplodia pinea* (Desmaz.) J. Kick. Basal stem infection with *H. annosum* resulted in *D. pinea* causing longer necrotic lesions in the shoots, indicating systemic induced susceptibility (SIS) to this shoot blight pathogen. Furthermore, stem induction with the NAm-P strain resulted in higher susceptibility to *D. pinea* than stem induction with the Eur-P strain. Total terpene accumulation was suppressed by about 50% in the shoots under attack by *D. pinea* when seedlings were induced with *H. annosum*. Total terpene concentration in shoots inoculated with *D. pinea* was negatively correlated with lesion size, both overall and by stem treatment. Stem base inoculation with *H. annosum* induced whole-plant changes in terpenoid profiles, but these were not associated with the SIS phenotype. We discuss our findings on modulation of systemic response of *P. pinea* to fungal attack in the context of tripartite ecological interactions.

Keywords: cross-induction, fungal pathogens, host-mediated interactions, systemic induced resistance, systemic induced susceptibility, terpenes.

Introduction

Plants have several resistance mechanisms protecting them against fungal infection and insect attack. Defenses are expressed both locally, at the site of primary infection, and systemically, a phenomenon known as systemic induced resistance (SIR). (Because nothing is known about the signaling system in pines, SIR is used in this paper as a general pathway-independent term that includes forms of pathway-specific systemic resistance such as systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Bonello et al. 2001)). Systemic induced resistance is known to occur in many plants, including conifers (Bonello et al. 2006). In SIR against pathogens, the activation of unknown signals produced at the site of initial infection (also defined as induction) primes the host against further pathogenic attacks (defined as challenges) in tissue located remotely from the site of initial infection. These signals induce the synthesis or accumulation, or both, of defence metabolites, such as terpenes and phenolics, including lignin, both before and after a challenge (Evensen et al. 2000, Bonello and Blodgett 2003, Hudgins et al. 2003, Theis and Lerdau 2003, Luchi et al. 2005, Phillips et al. 2006, Blodgett et al. 2007). Many of these responses are reflected in major anatomical reorganization, including the formation of polyphenolic parenchyma cells and traumatic resin ducts (Franceschi et al. 2005, Luchi et al. 2005), and cell wall lignification (Hudgins and Franceschi 2004, Nagy et al. 2006, Blodgett et al. 2007).

An important ecological consequence of SIR may be cross-induction of resistance between different host antagonists co-occurring on the same plant, for example, fungal pathogens and insect pests (Eyles et al. 2007). In such interactions, early colonization by a primary insect or pathogen is thought to induce changes in host biochemistry and physiology that make the plant less susceptible to further attacks (Stout et al. 2006). For example, changes in feeding behavior by an insect, resulting in reduced damage, may be induced in plant parts distant from the site of an earlier pathogenic attack (Rostas et al. 2003, Bonello et al. 2006).

Although SIR is of great interest for its potential application in disease and pest management, there may be situations in which the opposite phenomenon occurs. For example, in Austrian pine (*Pinus nigra* Arn.), inoculation of young saplings at the stem base with *Diplodia pinea* (Desmaz.) J. Kick (syn.

Sphaeropsis sapinea (Fr.:Fr.) Dyko and Sutton) or *Diplodia scrobiculata* de Wet, Slippers and Wingfield resulted in contrasting systemic phenotypes, with SIR of stem tissues but systemic induced susceptibility (SIS) of shoot tips (Blodgett et al. 2007), suggesting that the end result of at least some host-mediated interactions may be both time and organ dependent (Blodgett et al. 2007). To further explore these phenomena, we assessed whether stem inoculation with *Heterobasidion annosum* (Fr.) Bref. *sensu stricto* (*s.s.*), results in increased or decreased shoot susceptibility of Italian stone pine (*Pinus pinea* L.) seedlings to *D. pinea*.

Heterobasidion annosum s.s. is a serious root and butt-rot fungal pathogen of conifers, especially *Pinus* species, but can also attack some angiospermous trees (Korhonen et al. 1998, Lygis et al. 2004). This fungus usually enters the host through wounds or stumps, causing wood decay, with significant economic losses when monoculture plantations are attacked (Woodward et al. 1998). During the last few years a new *H. annosum s.s.* introduction, belonging to the North American P-group, has been recorded along the coastal Latium Region of Italy, where it coexists with the native European P-group in forests dominated by Italian stone pine (D'Amico et al. 2007, Gonthier et al. 2007). The introduction of this exotic strain may predispose trees to damage by other pathogens, such as *D. pinea*, a cosmopolitan fungus that causes shoot blight and stem canker disease in many conifers (Swart and Wingfield 1991, Stanosz et al. 1996, de Wet et al. 2003). In southern Europe and in the Mediterranean basin this pathogen is particularly injurious to Austrian pine, causing blight of extending shoots and tree death, but it occurs also on some other *Pinus* species, including *P. pinea* (Maresi et al. 2001). In the latter host, *D. pinea* may induce abortion of seed cones (Vagniluca et al. 1995). Damage appears to be exacerbated by unfavorable environmental conditions for the host, such as alternating dry and wet periods that occur during the spring, particularly along the Tyrrhenian coast of Tuscany. High stocking densities in old plantations also appear to facilitate epidemics (Vagniluca et al. 1995).

To investigate possible host-mediated effects of *H. annosum* stem infection on shoot susceptibility to *D. pinea*, we tested three hypotheses: (1) infection of the stem base of *P. pinea* with *H. annosum s.s.* induces greater susceptibility of shoots to *D. pinea* (Blodgett et al. 2007); (2) stem infections with *H. annosum* reduce total concentrations, and change the composition, of terpenoids accumulated in response to *D. pinea* in the shoots (systemic induced response); and (3) resistance to the pathogens, on the stem (*H. annosum*) and on the shoots (*D. pinea*), associated with changes in terpenoid concentration and composition.

Materials and methods

Plant material

In early spring 2006, 3-year-old Italian stone pines were purchased from Umbraflor s.r.l. (Regional Forest Nursery, Perugia, Italy). Plants were obtained from seeds (Seedlot no. 7/2001) of selected mother trees in Montebello Ionico (Reggio Calabria, Italy (37°59′ N, 15°46′ E). Each seedling was lifted with its root ball, planted in a 7.6-l plastic pot filled with a 1:1 (v/v) sand:peat mixture, and grown outdoors in a nursery near Florence, Italy (43°44′ N, 11°19′ E), with daily irrigation. Three weeks after transplanting, the most vigorous of the potted trees were randomly grouped into two trials (see below). The seedlings had a mean stem height of 74.4 ± 0.4 cm (SE) and a mean stem diameter, measured 3 cm above soil, of 0.6 ± 0.1 cm. Apical shoots had a mean length of 12.5 ± 3.6 cm, and diameter of 0.2 ± 0.06 cm. All size measurements were made on a subsample (n = 40) of seedlings selected at random.

Experimental design

Nine seedlings were used in each of six treatments. In the following treatment descriptions, the first treatment was applied as a basal stem treatment and the second treatment as a shoot treatment: (1) NAm-P *H. annosum s.s.* + *D. pinea*; (2) Eur-P *H. annosum s.s.* + *D. pinea*; (3) wounding (W) + *D. pinea*; (4) unwounded (Uw) + *D. pinea*; (5) unwounded + wounding; (6) unwounded + unwounded. The experiment was carried out in two trials, for a grand total of 108 seedlings: the first trial started on May 26, 2006, the second on June 1, 2006. Trees were assigned to different treatments in a completely randomized design in each trial.

Fungal inoculation

Two 8-day-old *H. annosum s.s.* strains growing on 2% malt extract (Liofilchem, Teramo, Italy) and 1.5% agar (Mallinckrodt Baker, Phillisburg, NJ) were used for the basal stem inoculations: NAm-P was represented by isolate CFUS16 (Castel Fusano, Italy) and Eur-P was represented by isolate 921013 1.1 (Tirrenia, Italy) (D'Amico et al. 2007). Basal stem inoculations with *H. annosum* were carried out 8 cm above the soil by using a cork borer previously dipped in 95% ethanol. A plug of outer bark and phloem was removed and a 5-mm diameter disk taken from the margins of actively growing cultures of *H. annosum s.s.* was inserted in the wound, mycelium side against the sapwood.

Three weeks after the stem treatments, the apical shoots were inoculated with a monoconidial *D. pinea* culture (isolate S79, Florence, Italy) grown on 2% water agar. At each inoculation site, located 3-cm above the basal portion of the apical shoot, the green periderm was wounded with a sterile scalpel to remove a needle fascicle. A 5-mm plug with inoculum side down was placed on the wound. Each treatment site was firmly wrapped with Parafilm to retain the inoculum plug and limit contamination and desiccation. All wounding controls consisted of application of non-colonized sterile plugs of malt extract agar in the stem, and 2% water agar in the shoots.

Lesion measurements and fungal re-isolations

For each trial, 10 days after inoculation with *D. pinea* (corresponding to 28 days after the *H. annosum* inoculations), shoot and stem lesions were measured upward and downward from each treatment site. Because mock inoculations of the stem did

not result in lesions beyond the wound itself, they were excluded from the statistical analysis of lesion lengths.

To confirm (or exclude in control samples) the presence of the pathogens at the treatment sites, small pieces of tissue were removed close to the necrotic areas, sterilized following Stanosz et al. (2001), and placed in 90-mm petri dishes containing 2% malt agar. Plates were incubated in the dark for 7 days at 20 °C.

Analysis of terpenoids

Terpenoids were analyzed in trees from Experiment 2. Ten days after inoculation with *D. pinea* (corresponding to 31 days after the *H. annosum* inoculations), tissue samples were collected from three positions on each seedling: (1) basal stem treatment site; (2) shoot treatment site; and (3) middle portion of the stem (between the two treatment sites), about 30 cm above the soil.

Stem phloem and shoot samples, about 3 cm in length, were collected around each treatment site, placed in 1.5-ml Eppendorf tubes and frozen in liquid nitrogen. Each sample was finely ground with a pestle and a mortar containing liquid nitrogen. For each sample, a 0.1 g subsample of the fine powder was placed in a 2-ml glass vial, covered with a Teflon-coated screw cap (Perkin-Elmer, Norwalk, CT), and extracted in 1 ml of *n*-pentane with tridecane as an internal standard (Raffa and Smalley 1995).

Terpenoids were analyzed by gas chromatography–flame ionization detection (GC-FID) with a Perkin-Elmer Autosystem XL GC, and enantiomeric monoterpenes were separated on a 30 m Cyclodex-B capillary column, 0.25-mm-diameter, (J & W Scientific, CA). Analysis was carried out under the following conditions: H_2 (carrier gas) at 69 kPa; injector temperature at 230 °C; detector temperature at 250 °C. The oven temperature programming started at 40 °C (isothermal, 5 min), and increased to 200 °C, at 1.5 °C min^{-1}; the final temperature of 220 °C was maintained for 5 min.

Terpenoids (mono- and sesquiterpenes) were identified by comparison of retention times with those of standards under the same conditions. Absolute amounts of terpenoids were determined by comparison with the tridecane internal standard, and expressed as mg g^{-1} fresh mass (FW). The relative amount (proportion of profile) of each monoterpene was expressed as a percentage of total monoterpenes, whereas each sesquiterpene was calculated as a percentage of total monoterpenes plus sesquiterpenes.

Statistical analysis

Mean lesion length served as a measure of resistance to the pathogens (Blodgett et al. 2007). Differences in mean lesion length among treatments were detected by analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) test. Data were log-transformed to achieve homogeneity of variance, which was confirmed by Levene's test.

Proportions (%) of various terpenoid components were transformed with arcsine-square root functions to correct for unequal variance and departures from normality. Terpene measurements were subjected to ANOVA and LSD post hoc tests. Data that were not normally distributed (Kolmogorov-Smirnov one sample test) were analyzed by the non-parametric Kruskal-Wallis ANOVA followed by the Mann-Whitney U Test for multiple comparisons.

To assess if terpenoids induced by *D. pinea* in the shoots (irrespective of the influence of *H. annosum* at the stem base) were related to shoot resistance to the pathogen, we conducted linear correlation and regression analyses between amounts of terpenoids and the lengths of lesions caused by *D. pinea*. If these compounds are related to resistance there should be a negative correlation between the variables, at least based on mean values (Blodgett et al. 2007). A positive correlation or the absence of a correlation would argue against a role in resistance for these compounds (Bonello and Blodgett 2003, Wallis et al. 2008). Relationships between lesion lengths and terpenoids were determined by Pearson's correlation in independent analyses. Pairwise correlations were calculated between lesion lengths and individual terpenoids, as well as between total terpenoid concentrations and lesion lengths, and mean total terpenoid concentrations and mean lesion lengths for the individual treatments. All analyses were conducted at $\alpha = 0.05$.

Results

Fungal inoculations and cross-induction of systemic susceptibility

No lesions were observed in the negative controls (mock inoculations and uninoculated trees). Seedlings inoculated with *H. annosum* exhibited necrosis and resin flow from the wound, whereas shoots inoculated with *D. pinea* showed tip-blight and necrosis. The presence of these pathogens in the symptomatic tissues was confirmed by re-isolation. Mock-inoculation and unwounded samples yielded neither pathogen.

Unless specifically indicated, trial was not a significant factor in the analyses, therefore data were pooled across trials. Mean lesion lengths varied significantly between *H. annosum* strains, with NAm-P causing significantly shorter stem lesions than Eur-P: 12.8 ± 1.0 versus 17.1 ± 2.1 mm for the two trials combined ($F_{1,25} = 7.52$, $P < 0.05$). However, this difference was driven entirely by the results of the first trial (trial: $F_{1,25} = 14.6$, $P < 0.01$; isolate × trial: $F_{1,25} = 13.9$, $P < 0.01$). Lesions caused by NAm-P and Eur-P in the first trial were 12.9 ± 1.2 and 23.0 ± 1.8 mm compared with 12.7 ± 2.0 and 11.2 ± 1.5 mm, respectively, in the second trial.

Basal stem treatments had significant effects on shoot resistance to *D. pinea* ($F_{3,58} = 3.42$, $P < 0.05$) (Figure 1). When seedlings induced with *H. annosum* (data from NAm-P and Eur-P strains combined) were compared with seedlings not induced with *H. annosum* (mock-inoculated and uninoculated stem treatments combined), the former had significantly longer necrotic shoots lesions in response to inoculation with *D. pinea* ($F_{1,58} = 6.88$, $P < 0.05$). Moreover, when only NAm-P and Eur-P inoculated trees were included in the analysis, *D. pinea* caused significantly longer lesions ($F_{1,28} = 5.03$, $P < 0.05$) in seedlings inoculated with NAm-P than with Eur-P.

Quantitative changes in total terpenoids

At the stem base, total terpenoid concentrations (mono- + sesquiterpenes) were significantly higher in *H. annosum* and mock-inoculated (wounded) trees than in unwounded controls ($F_{5,23} = 3.465$, $P < 0.05$) (Figure 2). In the intermediate stem portion, about 30 cm above the stem inoculation, there were no significant differences in total terpenoid concentrations between treatments (Figure 2).

Total terpenoid concentrations in shoots of trees inoculated with *H. annosum* were about 50% of those in shoots of corresponding mock-inoculated and unwounded stem controls ($F_{5,22} = 5.401$, $P < 0.01$) (Figure 2). Furthermore, total terpenoid concentrations did not differ between *D. pinea*-infected shoots of trees induced with *H. annosum* and shoots of non-induced and unchallenged (i.e., healthy) control plants (Figure 2). In shoots infected with *D. pinea*, there were no significant differences in total terpenoid concentrations between trees treated with NAm-P and Eur-P isolates of *H. annosum*, or between trees that were either mock-inoculated or unwounded (Figure 2).

Qualitative changes in terpenoid profiles

Eleven confirmed monoterpenes, one sesquiterpene (β-caryophyllene), and eight unknown compounds were detected in the phloem of *P. pinea*. Overall, the proportions of the main monoterpenes were: (–)-limonene (59.2%), (–)-β-pinene (24.1%), and unknown (uk)-8 (20.3%), but (–)-α-pinene (6.1%), *p*-cymene (5.5%), β-caryophyllene (4.2%), uk-2 (2.1%), (+)-β-pinene (1.9%), α-terpineol (1.4%), (+)-α-pinene (1.1%) were also detected. Unknown compounds uk-1 and uk-3–7 were present at less that 1%, and (+)-limonene was present in traces.

The profiles of these terpenoids differed among treatments. Kruskal-Wallis ANOVA between treatments at the same location on the tree showed significant changes in proportions of several terpenes, except in samples collected 30-cm above the basal inoculations. Relative amounts of (–)-β-pinene, (–)-limonene, and uk-6 varied significantly with basal treatment in shoots inoculated with *D. pinea*, whereas *p*-cymene, α-terpineol, uk-2, and β-caryophyllene showed significant differences only in the basal stem portion between treated (*H. annosum* and mock-inoculated) and untreated stems (Figure 3).

Significant differences in terpenoid profiles were also observed among different locations on the tree within each treatment combination. Lower relative amounts of (–)-α-pinene, (–)-β-pinene, α-terpineol, and β-caryophyllene were found in shoots inoculated with *D. pinea* compared with stems that were either wounded or inoculated with *H. annosum* (Figure 3). The opposite effect was found for (–)-limonene and uk-6 (Figure 3).

Relationship between D. pinea lesion lengths and terpenoids in the shoots

Linear regression of lesion length over total terpene concentration was negative and significant (lesion length = –4.49[total terpenes] + 48.1; $r^2 = 0.176$; ANOVA: $F_{1,21} = 4.471$, $P = 0.047$). Bivariate correlation was also negative and significant: $r = -0.419$, $n = 23$, $P = 0.023$. The correlation between mean *D. pinea* lesion lengths from the four basal treatments (Figure 1) and mean concentration of total terpenoids in the same shoot tissues (Figure 2) was also negative and significant ($r = -0.916$, $n = 4$, $P = 0.042$). No correlations were found between *D. pinea* lesion lengths and the concentrations of individual terpenoids in the shoots.

Discussion

Terpenoids and systemic induced susceptibility

We found that infection of the lower stems of *P. pinea* seedlings with *H. annosum* made the shoots more susceptible to in-

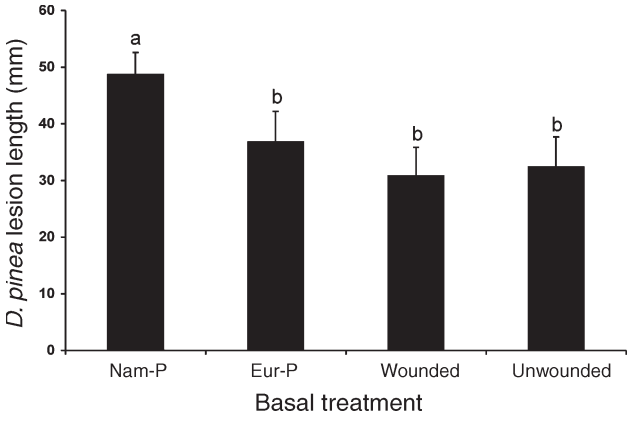

Figure 1. Lengths of lesions (means + SE, n = 14–16) caused by *Diplodia pinea* in *Pinus pinea* shoots. Different letters indicate significant differences ($P < 0.05$) according to the LSD analysis. Data were pooled across the two trials.

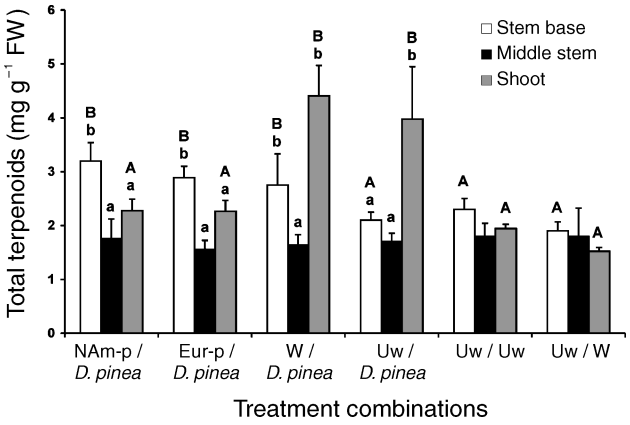

Figure 2. Total absolute amounts of terpenoids in seedlings of *Pinus pinea* (mean + SE). Terpenoids at the stem base and in the shoots were extracted from the reaction zones of *Heterobasidion annosum* (or the control wounds) and *Diplodia pinea* infection sites, respectively. Middle stems were not treated with a pathogen. Treatment combinations are defined as basal stem treatment/shoot treatment (W = wounded; Uw = unwounded controls). Different letters indicate significant differences ($P < 0.05$) by LSD analysis: lowercase letters refer to the analysis within a treatment combination; uppercase letters refer to the analysis within a sampling location on the tree.

fection by *D. pinea*, i.e., it elicited SIS. The overall increase in lesion length was about 1.2 cm, or 37%. Increases of this magnitude may be sufficient to tip the balance toward shoot mortality in shoots that might otherwise survive infection (Gordon et al. 1998). Furthermore, *H. annosum* infection in the lower stem reduced the concentration of total terpenoids in the shoots in response to *D. pinea* innoculation to the concentrations found in healthy shoots (Figure 2). This is the first report of systemic suppression of terpene accumulation in conifer shoots in response to a shoot pathogen by stem inoculation with a different pathogen. Our data suggest that terpenes, as a group, may be a source of resistance against this shoot blight

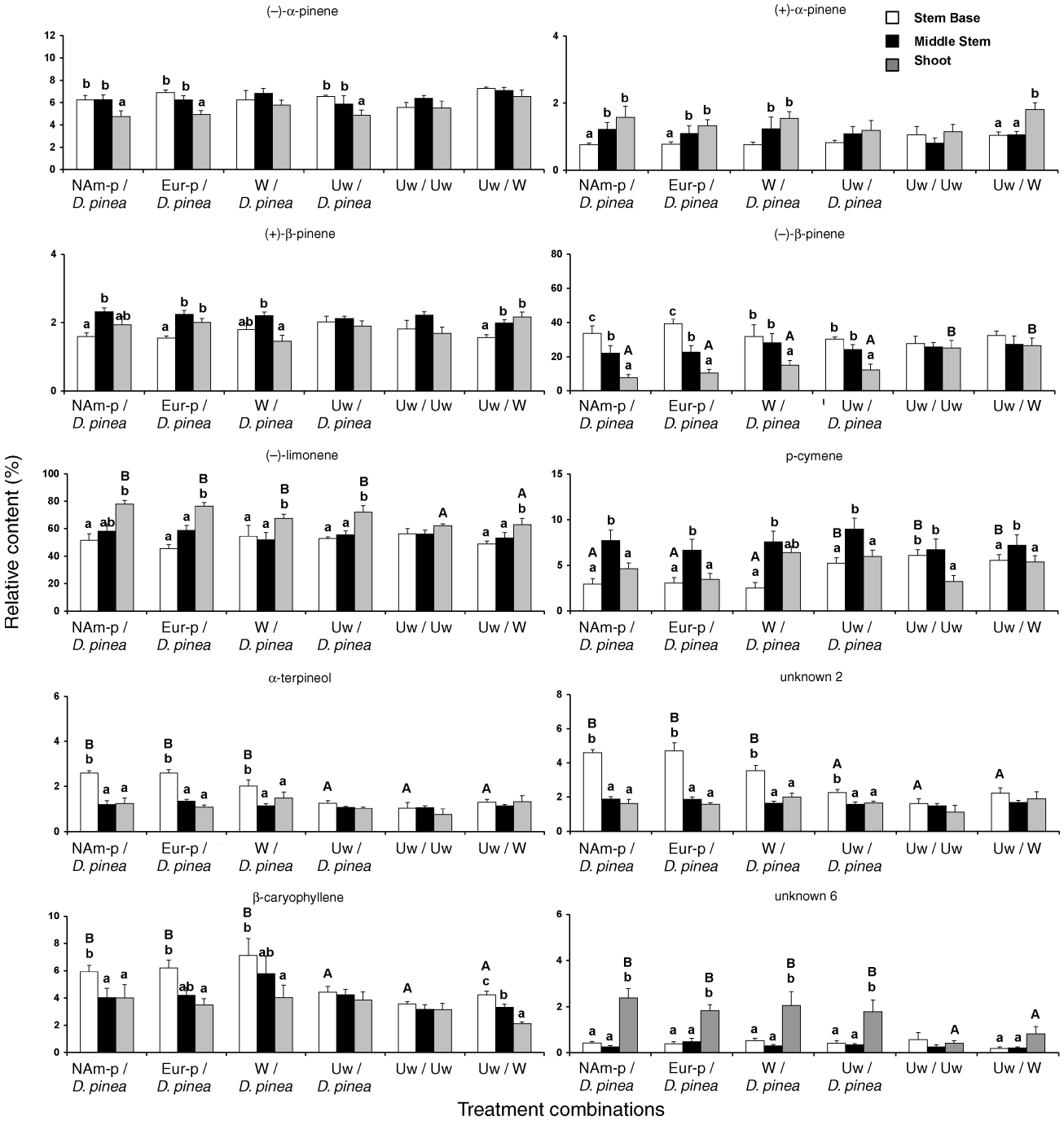

Figure 3. Enantiomeric profiles (mean percentage of total + SE) of terpenoids in *Pinus pinea* seedlings. Terpenoids at the stem base and in the shoots were extracted from the reaction zones of *Heterobasidion annosum* (or the control wounds) and *Diplodia pinea* infection sites, respectively. Middle stems were not treated with a pathogen. Treatment combinations are defined as basal stem treatment/shoot treatment (W = wounded; Uw = unwounded controls). Different letters indicate significant differences ($P < 0.05$) by LSD analysis: lowercase letters refer to the analysis within a treatment combination; uppercase letters refer to the analysis within a sampling location on the tree.

pathogen, because lower concentrations of total terpenes were present in shoots that became more susceptible to *D. pinea* and this was reflected in negative correlations between total terpene concentration and lesion size, both overall and by treatment. Our manipulation of terpene concentrations in shoots by stem induction with *H. annosum* supports the view that terpenoids are involved in localized resistance (Cheniclet 1987, Lieutier et al. 1993, Schmidt et al. 2005), and may represent a first line of defense against fungal and insect attack, besides being involved in wound healing (Phillips and Croteau 1999).

Terpene biosynthesis is probably the most expensive among the secondary metabolic processes and plants cannot maintain high concentrations of these defensive substances in all tissues and organs at the same time (Gershenzon 1994). Therefore, it is possible that, although the Italian stone pine seedlings we studied accumulated terpenoids in the stem in response to attack by *H. annosum*, smaller pools of carbon were available for local synthesis in the shoots, three weeks later, at the time of *D. pinea* infection.

The intermediate stem portions (about 30 cm above stem treatments) had the lowest absolute terpenoid concentrations and these did not vary with treatment (including controls). These results seem surprising and may be related to the timing of our sampling. For example, at 35 days after inoculation with *H. annosum*, terpene composition of *Picea sitchensis* (Bong.) Carr. changed in tissues surrounding the lesions, whereas it was not altered significantly in cortical tissues excised from points 25 cm from the wound, whether the tissues were wounded and inoculated or wounded only (Woodward et al. 2007). In a study on *Pinus sylvestris* L. (Faldt et al. 2006), pretreatment with *Leptographium wingfieldii* Morelet resulted in lower absolute monoterpene concentrations 20 cm above the fungal infection, with the highest concentrations at the infection site. A systemic induced response in terpene composition was observed at Day 124, whereas minor effects of pretreatment were detected at Day 28.

Often, an induced systemic response to fungal colonization or insect attack is marked by alteration in the relative amounts of terpenoids (Tomlin et al. 2000, Faldt et al. 2006). It is also known that qualitative differences in terpenoids can be significant factors in disease resistance. For example, studies on the relationships between monoterpenes and the susceptibility of slash pine (*Pinus elliottii* Engelm. var. *elliottii*) and loblolly pine (*Pinus taeda* L.) to *Cronartium quercuum* (Berk.) Miyabe ex Shirai f. sp. *fusiforme* (causal agent of fusiform rust) showed that certain constitutive combinations of monoterpenes were more indicative of resistance to the canker than β-phellandrene alone, the only major monoterpene showing significant variation between the "resistant" and the susceptible clones. In addition, some chemotypes were more effective than others against the canker caused by this pathogenic fungus (Michelozzi et al. 1990, 1995). In contrast, we found no terpene pattern specific for the cross-interaction between *H. annosum* (for either the NAm-P or Eur-P strain) and *D. pinea*: all pine shoots inoculated with *D. pinea* exhibited the same terpenoid profile irrespective of induction treatment at the stem base. This further supports the view that it is the whole complement of terpenoids that is best associated with (and might determine) resistance to *D. pinea*, rather than individual compounds, however they might behave under different treatment combinations.

Although no specific systemic effects of treatment on terpenoid profiles were found, we observed differences in the enantiomeric monoterpene profiles of *P. pinea* following mock-inoculation and inoculation with *H. annosum* and *D. pinea* (Figure 3). The proportions of (–)-α-pinene were unaffected by infection with the two *H. annosum* strains, whereas the relative content of (+)-α-pinene decreased in response to these pathogenic fungi. These results might be expected in the context of defense, given the lack of putative antimicrobial activity by (–)-α-pinene in in vitro tests of four monoterpenes against several *Heterobasidion* spp. (Zamponi et al. 2006).

The amount of (–)-α-pinene decreased in response to *D. pinea*, whereas the percentage of (+)-α-pinene increased. Blodgett and Stanosz (1997) found that α-pinene reduced the growth of *D. pinea*; however, they did not differentiate between the two enantiomers. Chou and Zabkiewicz (1976) demonstrated toxicity of (+)-α-pinene on *D. pinea* spores. Thus, a possible explanation for our results is that (+)-α-pinene is more toxic than (–)-α-pinene and that by increasing its relative concentration of secondary resin the host increasingly inhibits *D. pinea* in the challenged shoots.

The relative amounts of (+)-β-pinene decreased in response to attack by the *H. annosum s.s.* strains and in response to infection by *D. pinea* except in NamP/*D. pinea* and Uw/*D. pinea* seedlings, whereas the proportions of (–)-β-pinene increased in response to the two *H. annosum* strains and decreased in tissues infected with *D. pinea*. These data partially support the conclusions of previous studies. Zamponi et al. (2006) found that (–)-β-pinene significantly reduced mycelial growth of *Heterobasidion* spp., thus higher amounts of this compound would be expected in response to *H. annosum*. Although, Blodgett and Stanosz (1997) observed that β-pinene had an inhibitory effect on the growth of *D. pinea*, the amount of (–)-β-pinene was lower in infected shoots than in shoots in the other treatments (Figure 3).

The proportions and absolute amounts of (–)-limonene increased in shoots inoculated with *D. pinea* (Figure 3). However, previous studies have shown low toxicity of this monoterpene to *D. pinea* spores (Chou and Zabkiewicz 1976), whereas (+)-limonene was extremely toxic. Whatever its potential antifungal role, (+)-limonene occurred only in trace amounts in our study.

Potential ecological significance of SIS

Although an SIS phenotype similar to that observed in our study was previously described in Austrian pine challenged with *D. pinea*, the phenomenon was induced by stem infection with both *D. pinea* and a closely related fungal species, *D. scrobiculata* de Wet, Slippers and Wingfield (Blodgett et al. 2007). Our study represents the first example of controlled cross-induction of SIS in trees between fungal pathogens belonging to different taxonomic groups (*D. pinea*, Ascomycota;

H. annosum, Basidiomycota), with different life histories and ecological niches. This suggests that trees affected by root rots in the field may become predisposed to other diseases, such as shoot blights, even before their crowns become symptomatic for the root disease, which is the stage at which a connection between root rot and predisposition to other diseases is usually made. This conforms with the hypothesis of Bonello et al. (2006) that the outcome of systemic interactions in conifers may have strong spatiotemporal components (although their discussion related mainly to the systemic induced resistance (SIR) phenomenon). Furthermore, Blodgett et al. (2007) and Wallis et al. (2008) showed that whether a fungal infection of Austrian pine stem induces SIR or SIS depends on the target organ of the subsequent challenge, with stems and branches becoming more resistant whereas shoots become more susceptible. Our study on Italian stone pine provides further support for generalizing some of these novel concepts.

Trees infected with the exotic isolate of *H. annosum* became more susceptible to subsequent shoot infection by *D. pinea*. It is possible that the smaller stem lesions produced by NAm-P compared with Eur-P may be the result of a stronger stem defense response against the exotic strain that depletes resources for defense in the shoots, although that was not reflected in terpenoid concentrations in the stems or shoots of trees treated with both *H. annosum* strains. However, other defensive compartments not analyzed in this study, e.g., phenolics, may account for the observed differences (cf. Bonello and Blodgett 2003, Blodgett et al. 2005, Wallis et al. 2008). Our data are based on only one isolate of each of the two *H. annosum* populations. However, Garbelotto et al. (2007) have shown that several isolates from within the North American population of *H. annosum* found in central Italy (from which our NAm-P isolate originated) did not differ in aggressiveness when tested on Scots pine, suggesting a relatively recent introduction followed by a bottleneck that has rendered the population rather homogeneous in terms of aggressiveness. Thus, our isolate may be a good proxy of the current population. These results conform with the general expectation that exotics can be deleterious to the ecosystems they invade, in this case by making their host trees more susceptible to an indigenous pathogen (e.g., *D. pinea*). However, confirmation of this hypothesis would require extensive field tests with several different isolates of the two root rot pathogen strains.

In conclusion, although preliminary, our study yielded four results. First, our data corroborated previous work showing that the outcome of systemic cross-interactions mediated by a pine tree is contingent on which organs are induced and challenged (Blodgett et al. 2007). This has significant implications for the way we understand host-mediated interactions in trees (Bonello et al. 2006). Second, local and systemic induced pine defense against pathogens and insects is a highly coordinated process characterized by integration of several fundamental mechanisms (Bonello et al. 2006). Our study provides support for a significant role of terpenoids, as a group, in defense of Italian stone pine tissues against a fungal pathogen. Although this may appear an obvious conclusion, it is based on one of the first examples of manipulation of terpenoid concentrations in a conifer achieved by harnessing the endogenous systemic machinery of the host. Third, our results on the potential role of terpenoids as a group in resistance of Italian stone pine to shoot blight caused by *D. pinea* suggest that total terpenoids can be used as biomarkers for resistance in Italian stone pine and perhaps in other host species that are highly susceptible to *D. pinea*. Finally, an exotic strain of an indigenous pathogen may be rendering Italian stone pine, an important Mediterranean tree species, more susceptible to an indigenous pathogen. This could have significant implications for forest health protection policies, including a rationale for reinforcing preventative measures to exclude biological invasions. Such information is fundamental for the development and refinement of new models of how trees survive and mediate mutualistic or detrimental interactions with fungi and insects (Bonello et al. 2006).

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INDAGINE PRELIMINARE SULLA FRAZIONE VOLATILE DI 4 CLONI ITALIANI DI AGLIO PROVENIENTI DA COLTURA IN CAMPO E DA MICROPROPAGAZIONE

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Preliminary investigation on aromatic fraction of 4 Italian garlic clones from in vivo and in vitro culture.

Variation in aromatic fraction was investigated between 4 selected garlic clones from in vivo and in vitro cultures. The selected plant materials included one Polesano white-type garlic clone and 3 Sulmona red-type garlic clones.
The aromatic fraction was determined by means of headspace gas chromatography (HS-GC). Major compounds were diallyl disulphide and allyl methyl sulphide. The results of this preliminary work showed a large variation in several compounds between *in vivo* and *in vitro* samples: Analyses of the aromatic fraction revealed differentiation between the different clones and showed three groupings, corresponding to the pooled Sulmona red-type garlic clones (Sulmona 3 and Sulmona 6) and the other individually separated clones.

Key words: *Allium sativum* ; gas chromatography; diallyl disulphide; allyl methyl sulphide; micropropagation

Introduzione

L'impiego dell'aglio nella cucina e nella medicina tradizionale era già noto sin dall'antichità. Sembra che fosse dispensato in grandi quantità ai lavoratori che costruivano le piramidi in Egitto intorno al 4500 AC ed è citato nel Vecchio Testamento come uno di quei cibi di cui gli ebrei sentivano particolarmente la mancanza durante la traversata del deserto (Moyers 1996; Bibbia Numeri XI, 5). Anche Plinio vanta le importanti proprietà di questa pianta bulbosa nella sua Naturalis Historia. La pianta ha una lunga storia popolare di utilizzo nella dieta e per la cura di una vasta gamma di disturbi, in particolare micosi cutanee, candida e vaginite, dove le sue proprietà fungicide, antisettiche, toniche e parassiticide hanno mostrato di essere di beneficio (Ross *et al.*, 2001; Lawson, 1998). In effetti, fino alla scoperta della penicillina ed allo sviluppo dei farmaci antibiotici, l'aglio fu il trattamento di prima scelta per tutti i tipi di infezioni, dalla tubercolosi al tifo. E' coltivato

in tutto il mondo e le maggiori produzioni provengono da Cina e Stati Uniti, mentre in Europa i principali produttori sono Spagna, Italia e Francia. Esistono numerose varietà con proprietà chimiche ed organolettiche molto diverse; risulta quindi di estremo interesse la caratterizzazione dell'aglio italiano come prodotto tipico di qualità e, a tale scopo, è stata svolta un'indagine preliminare sulla variazione nella frazione aromatica di 4 linee italiane. Inoltre, sono state analizzate le differenze nella componente volatile della composizione chimica di aglio *in vivo* ed *in vitro*.

Materiali e Metodi

E' stata svolta un'indagine preliminare sulla frazione volatile di una linea di aglio bianco Polesano selezionato presso il Centro Sperimentale Ortofloricolo "Po di Tramontana" di Veneto Agricoltura e di 3 linee di aglio rosso di Sulmona (Sulmona 3, Sulmona 6, Sulmona 9).

I bulbilli di aglio rosso di Sulmona e di aglio bianco Polesano sono stati prelevati nel medesimo giorno. I bulbilli sono stati sterilizzati in una soluzione al 15% di ipoclorito di Na, (5% di cloro attivo) per 15 minuti, con 3 risciacqui in acqua sterile, il tutto sotto cappa a flusso laminare; dopodiché si è proceduto al prelievo degli apici meristematici. Gli espianti, di circa 0.5-0.6 mm, sono stati messi in provette sterili di policarbonato espanso contenenti circa 15 ml di substrato MS (Murashige e Skoog, 1962), con l'aggiunta di 0,2 mg l^{-1} di BAP e 0,01 mg l^{-1} di NAA, 30gr l^{-1} di saccarosio e 6 g l^{-1} di agar B&V, il tutto a pH 5,5 – 5,7. Le provette sono state poste in camera di crescita alla temperatura di 22 ± 1°C, con fotoperiodo di 16 ore di luce e 8 di buio ed intensità luminosa di 2500 / 3000 lux. Dopo 3 settimane circa i germogli sono stati rimessi in coltura con il medesimo substrato nutritivo, ma con 100 g l^{-1} di saccarosio, anziché 30, al fine di ottenere, dopo circa 2 mesi, un bulbillo di dimensioni idonee per le analisi.

Campioni di aglio delle stesse linee sono stati prelevati da piante coltivate *in vitro* epiante allevate in campo.

Circa 0.2 grammi di aglio macinato con azoto liquido sono stati posti in fiale, chiuse ermeticamente con setto in teflon e conservate a -20 °C, fino al momento dell'analisi.

Le analisi sono state eseguite con tecniche di campionamento di spazio di testa (HS-GC), con gascromatografo Perkin-Elmer AutoSystem, dotato di campionatore automatico Perkin-Elmer TurboMatrix 40. La colonna utilizzata, DB-WAX (J & W Scientific, CA, U.S.A.) ha una lunghezza di 30 m, con 0,25 mm di diametro.

Le analisi sono state eseguite nelle seguenti condizioni: flusso di H_2 (gas di trasporto) 0.68 atm; temperatura iniettore 160 °C; temperatura detector 250 °C. programma di temperatura del forno: 5 minuti a 40 °C; tempo; gradiente di: 1,5 °C/min fino a 160 °C; gradiente 20 °C/min fino a 200°C; tempo per 5 minuti.

I composti sono stati identificati mediante confronto con i tempi di ritenzione di standard puri, nelle stesse condizioni di analisi. Si è proceduto ad analisi della varianza per ciascuno dei composti e si sono eseguiti confronti a coppie, mediante il test di Tuckey. I dati sono stati elaborati secondo le procedure dell'analisi discriminante. Le analisi statistiche sono state eseguite utilizzando il programma di calcolo SYSTAT 11.0 (Systat Software Inc., Richmond, California, USA).

Risultati e Discussioni

In base alle nostre conoscenze, questo è il primo contributo alla determinazione della frazione aromatica di aglio coltivato *in vitro*. Le analisi hanno messo in evidenza differenze marcate nei profili aromatici tra aglio proveniente dal campo e quello da micropropagazione. I bulbilli allevati *in vitro* hanno mostrato un maggior contenuto di metil allil solfuro e dei composti non identificati 2 e 7, mentre l'aglio *in vivo* è risultato caratterizzato da un contenuto più elevato di allil disolfuro, metil allil trisolfuro e diallil trisolfuro (fig. 1). I dati riportati in bibliografia sul confronto tra materiale allevato *in vitro* e quello proveniente da colture in campo sono contrastanti; ad esempio Rapparini *et al.* (2004) non hanno osservato differenze nei profili terpenici volatili di *Myrtus communis* L. tra piante madri e piante *in vitro*, ad eccezione di 1,8 cineolo. Al contrario, Arikat *et al*. (2004) hanno messo in evidenza differenze quantitative nella produzione *in vivo* ed *in vitro* di olio essenziale di *Salvia fruticosa* Mill. Kintzios *et al.* (1998) hanno determinato un effetto del mezzo di coltura sulla produzione qualitativa di metaboliti in *Malva sylvestris* L. E' noto che il sistema *in vitro* provoca alterazioni nei processi fotosintetici e nel metabolismo in generale della pianta (Kozai e Nguyen, 2003). Variazioni epigenetiche dovute al ringiovanimento delle piantine micropropagate possono determinare variazioni qualitative e quantitative sulla produzione di metaboliti secondari (Hanover 1992).

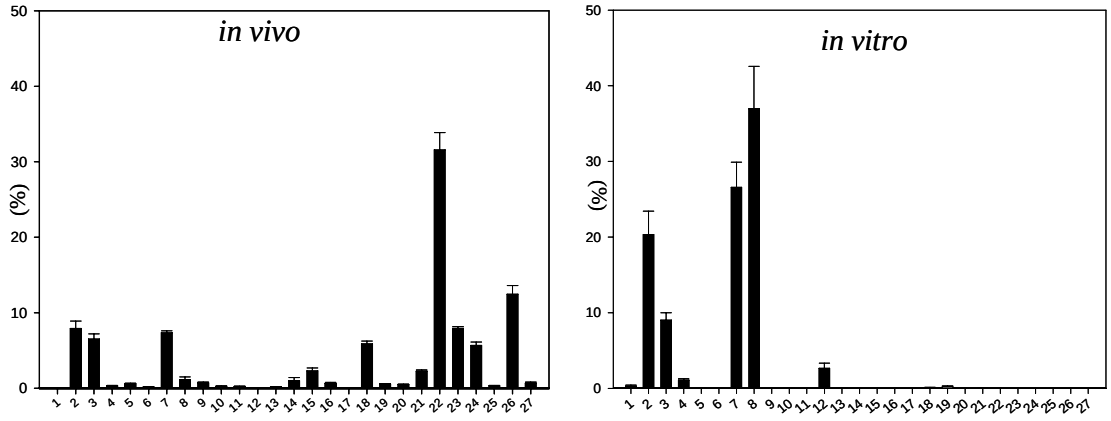

*Figura 1: P**rofilo aromatico di* **Allium sativum** ***L. coltivato in*** **vivo** ***e in*** **vitro.**

Legenda 1: 1: ***disolfuro di carbonio;*** *2:* ***composto non identificato 2;*** *3:* ***acetaldeide;*** *4:* ***dimetil solfuro;*** *5:* ***composto non identificato 9;*** *6:* ***allil mercaptano;*** *7:* ***composto non identificato 12;*** *8:* ***metil allil solfuro;*** *9:* ***composto non identificato 14;*** *10:* ***crotonaldeide;*** *11:* ***dimetil disolfuro;*** *12:* ***composto non identificato 23;*** *13:* ***aldeide tiglica;*** *14:* ***allil solfuro;*** *15:* ***alcol allilico;*** *16:* ***composto non identificato 29;*** *17:* ***piridina;*** *18:* ***composto non identificato 39;*** *19:* ***composto non identificato 41;*** *20:* ***dimetil trisolfuro;*** *21:* ***composto non identificato 47;*** *22:* ***allil disolfuro;*** *23:* ***composto non identificato 50;*** *24:* ***metil allil trisolfuro;*** *25:* ***composto non identificato 60;*** *26:* ***diallil trisolfuro;*** *27:* ***composto non identificato 65.***

Le diverse linee di aglio hanno mostrato profili aromatici diversi (fig. 2).

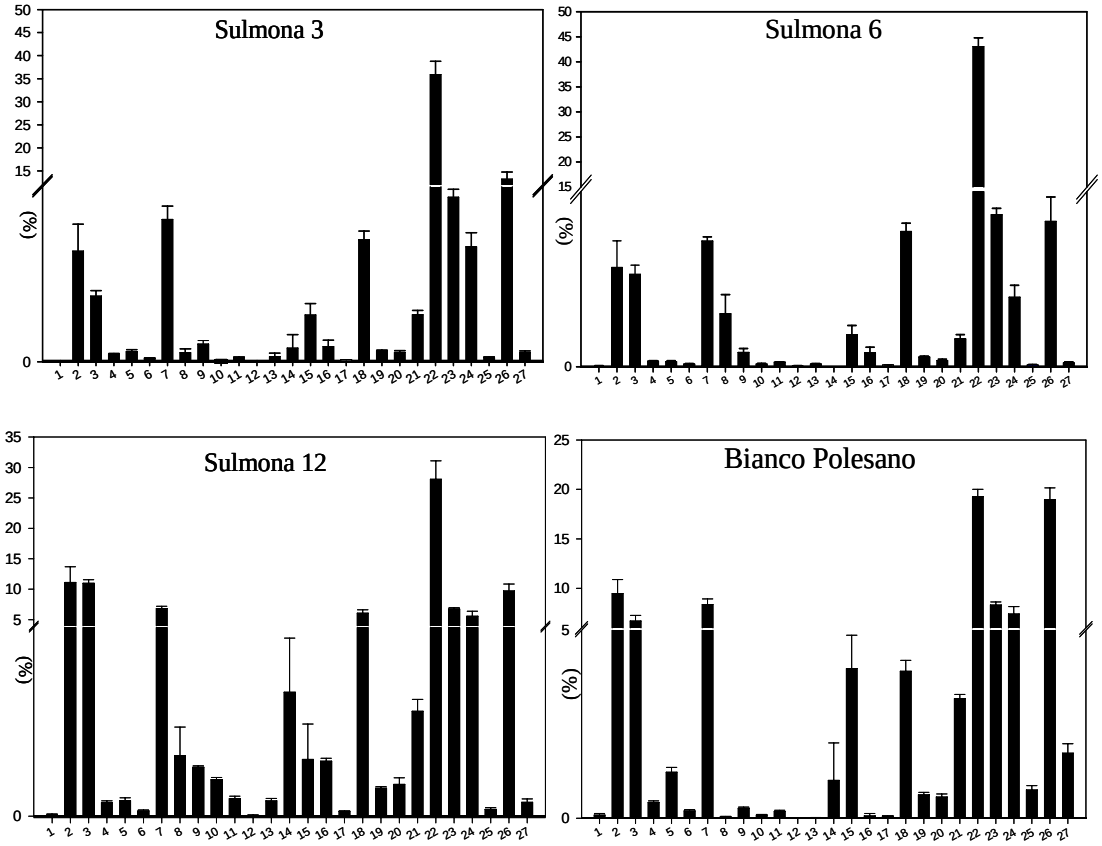

*Figura 2: **Profilo aromatico di linee diverse di* Allium sativum *L.***

Legenda 2: vedi Fig. 1

L'analisi discriminante ha consentito di esprimere graficamente la variabilità complessiva nel profilo aromatico fra le diverse linee di aglio. Dalla Figura -3 si può osservare nel piano delle prime due funzioni discriminanti una chiara separazione tra le linee Sulmona 12, Bianco Polesano e le due linee Sulmona 6 e Sulmona 3 che risultano molto simili.

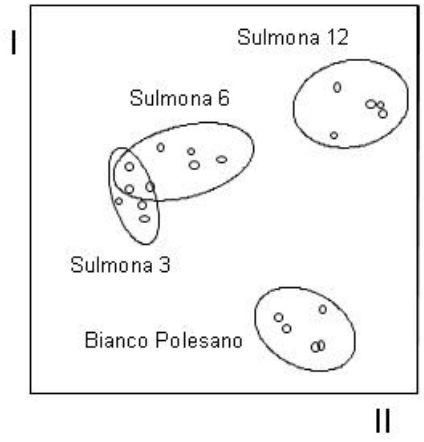

*Figura 3: **Rappresentazione grafica della dispersione di linee diverse di* Allium sativum *L. nel piano delle prime due funzioni discriminanti.***

Conclusioni

Questi risultati, ancora preliminari, potranno consentire la definizione di strategie per il miglioramento di aglio di provenienza italiana.

La caratterizzazione del profilo volatile potrà consentire la valorizzazione di linee di aglio di pregio da utilizzare nell'industria agro-alimentare e per usi medicinali.

Riassunto

E' stata svolta un'indagine preliminare sulla frazione volatile di un clone di aglio bianco Polesano e di 3 cloni di aglio rosso di Sulmona. Campioni di bulbi provenienti da coltura in campo e da micropropagazione sono stati analizzati mediante gas cromatografia con tecniche di campionamento di spazio di testa. Allil disolfuro e metil allil solfuro sono risultati i maggiori costituenti nei bulbilli provenienti rispettivamente da coltura in campo e micropropagazione**.** I risultati hanno mostrato differenze marcate nel contenuto relativo di numerosi composti tra campioni di aglio *in vivo* ed *in vitro*. L'analisi discriminante ha mostrato una chiara separazione tra le linee Sulmona 12, Bianco Palesano e le due linee Sulmona 6 e Sulmona 3 che risultano molto simili.

Parole chiave: *Allium sativum*; gascromatografia; allil disulfuro; metil allil solfuro; micropropagazione

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