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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.

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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

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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.

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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-

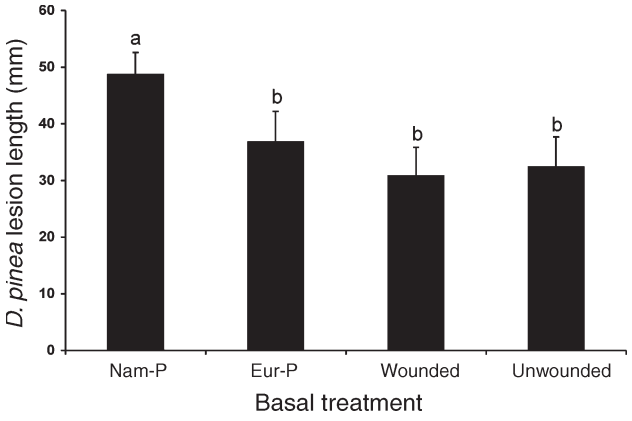

Figure 1. Lengths of lesions (means + SE, n = 14–16) caused by *Diplodia pinea* in *Pinus pinea* shoots. Different letters indicate significant differences ($P < 0.05$) according to the LSD analysis. Data were pooled across the two trials.

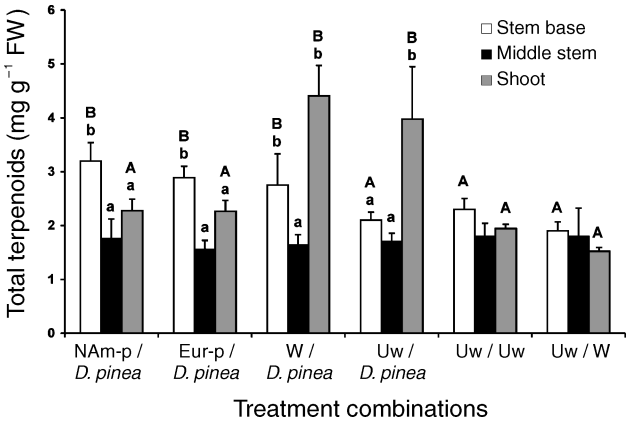

Figure 2. Total absolute amounts of terpenoids in seedlings of *Pinus pinea* (mean + SE). Terpenoids at the stem base and in the shoots were extracted from the reaction zones of *Heterobasidion annosum* (or the control wounds) and *Diplodia pinea* infection sites, respectively. Middle stems were not 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.

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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

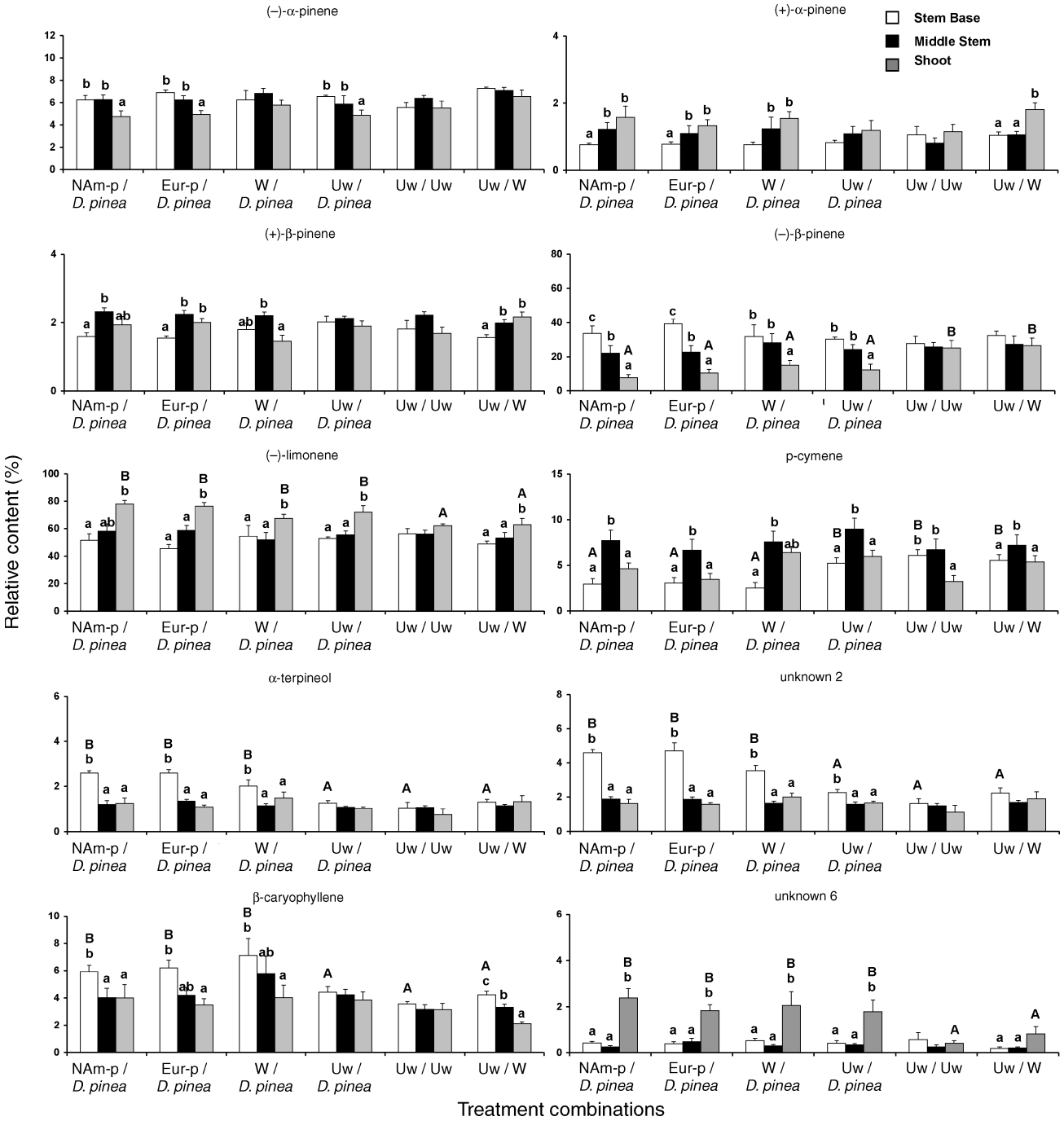

Figure 3. Enantiomeric profiles (mean percentage of total + SE) of terpenoids in *Pinus pinea* seedlings. Terpenoids at the stem base and in the shoots were extracted from the reaction zones of *Heterobasidion annosum* (or the control wounds) and *Diplodia pinea* infection sites, respectively. Middle 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.

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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;

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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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