Nutritional and pathogenic fungi associated with the pine engraver beetle trigger comparable defenses in Scots pine

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Conifer bark beetles are often associated with fungal complexes whose components have different ecological roles. Some associated species are nutritionally obligate fungi, serving as nourishment to the larvae, whereas others are pathogenic blue-stain fungi known to be involved in the interaction with host defenses. In this study we characterized the local and systemic defense responses of Scots pine (Pinus sylvestris L.) against Ophiostoma brunneo-ciliatum Math. (a blue-stain pathogen) and Hyalorhinocladiella macrospora (Franke-Grosm.) Harr. (a nutritional fungus). These fungi are the principal associates of the pine engraver beetle, Ips acuminatus (Gyll.). Host responses were studied following inoculation with the fungi, singly and as a fungal complex, and by identifying and quantifying terpenoids, phenolic compounds and lignin. Although the length of the necrotic lesions differed between control (wound) and fungal treatments, only two compounds (pinosylvin monomethyl ether and (+)-α-pinene) were significantly affected by the presence of the fungi, indicating that Scots pine has a generic, rather than specific, induced response. The fact that both nutritional and blue-stain fungi triggered comparable induced defense responses suggests that even a non-pathogenic fungus may participate in exhausting host plant defenses, indirectly assisting in the beetle establishment process. Our findings contribute to the further development of current theory on the role of associated fungal complexes in bark beetle ecology.

Keywords: blue-stain, induced response, Ips acuminatus, mutualism, phenolics, Pinus sylvestris, secondary metabolism, symbiosis, terpenoids.

Introduction

Most bark beetles (Coleoptera: Curculionidae, Scolytinae) that breed in conifers carry complexes of symbiotic fungi in mycangia, pits and setae occurring on the body surface (Francke-Grosman 1967, Kirisits 2004, Harrington 2005). Some of these associated fungi are known to have a direct mutualistic interaction with the beetles, whereby the insects disseminate the fungus and the fungus serves as nourishment to the larvae in the form of edible hyphae, spores and exudates (Harrington 2005). The nitrogen and phosphorus content of plant tissues is indeed very low, relative to insect metabolic requirements, but associated fungi are able to concentrate scarce nutrients and constitute an important source of sterols, which are essential elements of cellular structure, necessary precursors for hormone synthesis and critical factors in the production of viable eggs (Klepzig and Six 2004, Bentz and Six 2006). In this case the fungi, often referred to as nutritional or mycangial fungi, are typically non-pathogenic to the tree host and are usually transported in mycangia (Paine et al. 1997, Six 2003).

In the majority of cases, however, the effect of the fungi is thought to be indirect, whereby the fungi modify the quality of the host plant tissues in a way that facilitates brood establishment (Paine et al. 1997, Six 2003, Davis and Hofstetter 2011). In this case, bark beetle-associated fungi are often more or less aggressive tree pathogenic species (Kirisits 2004). Nearly
all of them are Ascomycetes belonging to the genera *Ophiostoma*, *Ceratocystis*, *Ceratocystipsis*, *Grosmannia* and their related anamorphs, which all form—with a few other genera—the morphologically homogenous group of the ophiostomatoid fungi (Wingfield et al. 1993, Kirisits 2004). These fungi are also known as ‘blue-stain’ fungi, on account of the bluish-grey color most of the species give to colonized sapwood (Harrington 2005).

The role of bark beetle-associated fungi and their importance in vector establishment is still widely debated. One hypothesis, recently called the Classic Paradigm (CP) (Six and Wingfield 2011), postulates that associated fungi have an indirect effect by aiding tree colonization by bark beetles. This hypothesis is divided into two components. The first suggests that tree invasion by aggressive, bark beetle-associated blue-stain fungi leads to tree death, primarily by blocking water conduction in the xylem and facilitating beetle colonization (Paine et al. 1997). The second, more recent concept, proposes that associated fungi play a role in beetle establishment on trees, but based on the stimulation and subsequent exhaustion of tree defenses by the fungi, resulting in assistance to their insect vectors in overcoming host plant defenses directed at them (Lieutier et al. 2009). By combining the views of Paine et al. (1997) and Lieutier et al. (2009), the CP states that beetle establishment proceeds in two successive steps: (i) defeat of tree defense in both the phloem and the external sapwood following pioneer beetle colonization and (ii) mass invasion of tree tissues by beetles (in the phloem) and fungi (in phloem and sapwood). Therefore, rather than fungal pathogenic potential, what is important for bark beetle populations is the ability of their associated fungi to stimulate the tree response and thus lower the critical threshold of attack density above which beetle attacks succeed (Lieutier et al. 2009).

Moreover, a highly pathogenic fungus would likely be detrimental, invading the tree too rapidly, outcompeting other mutualistic fungi, and making the tissues unsuitable for beetle brood development, as can occur in the case of *Dendroctonus frontalis* Zimm. (southern pine beetle) and *Ophiostoma minus* (H. et P. Syd. (Klepzig and Wilkens 1997, Hofstetter et al. 2006).

Recently, a new hypothesis contradicting the CP has been proposed, suggesting that, rather than playing a supporting role for the host beetle, phytopathogenicity performs an important role for the fungi, mediating competitive interactions in the fungal community and supporting survival in living and defended trees (Six and Wingfield 2011). Arguments for this hypothesis include the lack of consistent association of aggressive fungal associates with tree-killing bark beetles, the lack of correspondence between fungal growth in the host tree and the development of symptoms associated with a successful attack, and the ubiquity of similar associations of fungi with bark beetles that do not kill trees.

In this work we investigated the fungal complex associated with the pine engraver beetle * Ips acuminatus* (Gyll.), a small bark beetle infesting thin bark of Scots pine (*Pinus sylvestris* L.) that has been recently reported as a pest in many alpine forests (Colombari et al. 2012). *Ips acuminatus* is associated mainly with *Ophiostoma brunneo-ciliatum* Math. and *Hyalorhinocladiella macrosora* (Franke-Grosman) Harr. (syn. *Ambrosiella macrosora* Batra). The first species is a blue-stain pathogen thought to be involved in lowering the critical threshold of attack density for successful beetle colonization (Lieutier et al. 1991, Guérard et al. 2000, Kirisits 2004). *Hyalorhinocladiella macrosora*, whose ecological behavior and virulence have been poorly studied, is primarily known as a food source for the larvae. As with most nutritional fungi, this species is thought to be non-pathogenic (Francke-Grosman 1963, Batra 1967, Paine et al. 1997, Harrington et al. 2010).

Thus, the system *I. acuminatus*–*P. sylvestris* is potentially useful for testing hypotheses on inducible defenses against the fungal complex. The qualitative and quantitative secondary metabolic responses of Scots pine to blue-stain fungi have been studied extensively (e.g., Delorme and Lieutier 1990, Lieutier et al. 1996, Fäldt et al. 2006). However, to our knowledge, no data are available about tree responses to non-pathogenic and nutritional fungi in Scots pine, and only little information is available for other host trees (Raffa and Berryman 1982, Hofstetter et al. 2005, Davis and Hofstetter 2011). Consistent with the finding that pathogenicity is not related to a fungus’s ability to stimulate tree defenses (Krokene and Solheim 1997, Davis and Hofstetter 2011), our hypothesis is that *H. macrosora* triggers an inducible defense reaction comparable to the one induced by the blue-stain fungus *O. brunneo-ciliatum*. Moreover, we hypothesize that the fungal species involved in the complex have a synergistic effect on tree defense stimulation compared with the single species. To test our hypotheses, we characterized the defense responses of Scots pine, both locally and systemically, to inoculation with *O. brunneo-ciliatum* and *H. macrosora*, singly and as a fungal complex, by identifying and quantifying terpenoids, including resin acids, phenolic compounds and lignin.

**Materials and methods**

**Plant material, treatments and sampling**

In spring 2010, 50 Scots pines of similar size and age (~20 years old) were selected from a young alpine stand in San Vito di Cadore, Belluno Province, Italy (46°29′N, 12°10′29″E, 1105 m a.s.l.). The growing conditions and general health of each plant were assessed by measuring plant height and the length of the current year shoot of a randomly chosen branch located in the upper part of the tree crown (Lanner 1976). The experiment was carried out in two trials of 25 trees each, in order to minimize the effects of confounding factors that can
Nutritional and pathogenic fungi induced responses

Occur in a natural environment. Trees were assigned to five different treatments, with five replicates per treatment, in a completely randomized design in each trial. The first trial started on 27 May, the second on 30 May 2010, and they both lasted 3 weeks. Mean daily temperature during that period was 13.49 ± 4.8 °C (SD). The five treatments consisted of (i) inoculation with H. macrospora (certified isolate 367,53, CBS, Utrecht, The Netherlands) and (ii) O. brunneo-ciliatum (isolate CpR, Valcamonica, Italy), (iii) concurrent inoculation with both fungi, (iv) mechanical wounding, and (v) unwounded control. All treatments were performed at low density (50 points m\(^{-2}\) of bark), corresponding to four points in a 30 cm band around each tree trunk at 80 cm height (Fäldt et al. 2006). Inoculations were done by removing a plug of outer bark/phloem with a 5 mm cork borer and replacing it with a Scots pine outer bark/phloem plug of the same size previously sterilized and then colonized by the fungi. Full colonization of the bark/phloem plugs was achieved by placing them in a Petri dish containing 1.5% potato dextrose agar (Difco, BD, Franklin Lakes, NJ, USA), alongside plugs of 2% potato dextrose agar taken from fully colonized plates. After 7–10 days of incubation, all the bark/phloem plugs were completely covered with mycelium, homogeneously showing aerial vegetative hyphae, in the case of H. macrospora, and numerous synnemata in the case of O. brunneo-ciliatum. For treatment (iii), two half plugs, each colonized with the different fungi, were inserted in the same hole. In the wound treatment (iv) nothing was inserted after the plug removal. All of the inoculation sites and lesions were sealed with Scotch duct tape (3M, St Paul, MN, USA) to minimize contamination.

In order to measure constitutive lignin, terpenoids and phenolic compounds, the phloem and outer bark plugs resulting from the inoculation procedures (corresponding to time T\(_1\) of the experiment) were pooled for each plant, immediately frozen in liquid nitrogen and stored at ~80 °C until processing.

Three weeks after application of the treatment (corresponding to time T\(_2\) of the experiment), the vertical length of each lesion was measured on the external sapwood. In the three inoculation treatments, phloem tissue was taken from each lesion surrounding the inoculation site to re-isolate the fungi, as described in Bonello et al. (2008).

Samples for the measurement of locally induced lignin, terpenoids and phenolic compounds were taken at T\(_1\), with a 1 cm cork borer. Plugs contained both symptomatic and asymptomatic tissues from all of the four lesion points (position L, local), and were pooled for each plant for a cumulative amount of ~2 g FW. To measure systemic effects, the same number of plugs was taken at 20–30 cm above the inoculation sites, from a 10 cm band around each tree trunk (position S, systemic), and pooled for each plant. At this time, samples were also taken from the five untreated control plants, at the same trunk height of the other treatments. All sample plugs were immediately frozen in liquid nitrogen and stored at ~80 °C. All tools were rinsed with 90% ethanol after each use during manipulations in the field. In order to be processed for chemical analysis, all sample plugs were ground to a powder in liquid nitrogen.

Analysis of phenolics and resin acids

All solvents used for sample preparation and analysis were HPLC grade unless otherwise noted. 0.1 g FW of the ground tissue was freeze-dried and extracted twice in 0.5 ml of methanol (Fisher, Pittsburg, PA, USA) for 24 h at 4 °C (Wallis et al. 2008). Five-hundred microliters of combined supernatants were concentrated 2.5 times with a Vacufuge™ Concentrator 5301 (Eppendorf) and stored at ~20 °C until analyzed.

Liquid chromatography–mass spectrometry (LC–MS) with electrospray ionization (ESI-MS) (Varian 212-LC pumps and 500-MS, Palo Alto, CA, USA), in parallel with a photodiode array detector (PDA) (Varian ProStar 335), was employed in negative ion mode to identify phenolic compounds and resin acids.

Compounds from the plant extract were then quantified by high-performance liquid chromatography (HPLC-UV), which was performed using a Waters (Milford, MA, USA) Alliance 2690 separation module equipped with a 996 PDA and a 474 scanning fluorescence detector in serial mode. Detailed description of the operating conditions for both the LC-ESI-MS and the HPLC-UV can be found in the Supplementary material.

Authentic external standards of catechin, pinocembrin, pinosylvin and pinosylvin monomethyl ether (Apin Chemicals, Abingdon, UK), procyanidin B2 and taxifolin (Extrasynthese, Genay Cedex, France), vanillic acid, trans-coumaric acid, abietic acid, neoabietic acid and fericul acid (Sigma-Aldrich, St Louis, MO, USA) and pinoresinol (ArboNova, Turku, Finland) were used. Three-point standard calibration curves were used for pinoresinol, catechin, procyanidin B2, vanillic acid, pinosylvin and pinosylvin monomethyl ether; four-point standard curves were used for ferulic acid, pinocembrin, taxifolin and trans-coumaric acid; and five-point standard curves were used for neoabietic acid and abietic acid. In all standard curves, three technical replicates were averaged for each concentration. All linear regressions had R\(^2\) > 0.99 except for abietic acid which had R\(^2\) = 0.959. Additionally, relative standard error, with an upper limit of 5%, was used as a criterion to measure robustness of the curve (Massart et al. 1997). Data are reported as µg g\(^{-1}\) FW. Lack of suitable external standards made it impossible to quantify all compounds; hence, levels of identified compounds not having a direct match with a standard are expressed as standard equivalents, based on different compound classes. The apex-track algorithm (Empower2 software, Waters) was applied for all subsequent peak integrations. Minimum detectable peak area
for PDA was set to 80,000 peak area units, while for fluorescence it was set to 10,000 peak area units. Both values are well above the instrument detection limit. Calibration curves were extrapolated ±30% in both directions (linear range of the detector), and sample responses were quantified only if their peak area fell within the extrapolated range. The instrument calibration was verified periodically by analyzing a known concentration (0.5 mM) of catechin as check standard. To ensure consistency, standards, samples and check standards were run in the same session. A separate standard calibration curve (one replicate per point) was run at the end of the sample analysis. The observed $R^2$ values for this one replicate-based calibration curve were >0.99 for all standards except for abietic acid ($R^2 = 0.988$).

**Lignin analysis**

Pellets from the phenolic and resin acids extraction were processed for lignin extraction as described in Bonello and Blodgett (2003), with modifications. Centrifugation in all of the following steps was carried out in an Eppendorf 5415D centrifuge at 16,100 g for 5 min. Pellets were washed once with each 1 ml of water and methanol, washed with 0.9 ml of tert-butyl methyl ether (Sigma-Aldrich) and dried overnight. Pellets were then hydrolyzed at 40 °C for 21 h on a shaker in 400 μl of 1 N NaOH (Sigma-Aldrich). The reaction mixture was acidified with 200 μl of 1.5 M formic acid (Sigma-Aldrich), followed by 400 μl of methanol. Pellets were then washed once with 1 ml of water and resuspended in 1 ml of 2 N HCl and 0.3 ml of thioglycolic acid (Sigma-Aldrich). Eppendorf tubes containing the solutions were placed in an oven at 86 °C for 4 h and the supernatants were discarded. Pellets were rinsed twice with 1.5 ml of water, resuspended in 1 ml of 0.5 N NaOH, and the tubes were placed on a shaker for 19 h at 200 rpm. The supernatants were saved. The pellets were again suspended in 0.5 ml of 0.5 N NaOH for 19 h and the supernatants were pooled. The solutions were acidified with 0.3 ml of concentrated HCl at room temperature for 4 h and supernatants were discarded. The lignin–thioglycolic acid pellets were dried overnight and then dissolved in 1 ml of 0.5 N NaOH. In all steps, Eppendorf tubes were vortexed after adding solutions, and supernatants and pellets were separated by centrifugation. The solution was diluted 40 times with 0.5 N NaOH and lignin concentration was determined spectrophotometrically at 280 nm, against a five-point standard curve ($R^2 > 0.99$) of spruce lignin (Sigma-Aldrich) (Bonello et al. 1993).

**Analysis of terpenoids**

For terpenoids analysis, 0.1 g FW of the ground tissue was placed in a 20 ml glass vial and extracted for 24 h at 28 °C in 1.5 ml of n-pentane (Sigma-Aldrich) with tridecane 0.05 g l$^{-1}$ (Sigma-Aldrich) as internal standard (Raffa and Smalley 1995). Glass vials were sealed with a Teflon-coated crimped aluminum cap (Perkin-Elmer, Norwalk, CT, USA). One milliliter of extracted solution from each sample was then filtered and transferred to a sealed 2 ml glass vial (Perkin-Elmer). Vials were stored at −20 °C until analyzed. Analyses were performed by gas chromatography–flame ionization detection (GC-FID) with a Perkin-Elmer Autosystem XL GC equipped with an automatic sampler for liquid sample injections. The separation of the different enantiomeric monoterpenes was performed on a 30 m Cyclodex-B capillary, 0.25-mm-diameter column (J&W Scientific, Folsom, CA, USA). Cyclodextrins and their derivatives are useful in inducing asymmetric reactions and have been used extensively as stationary phase in chromatographic enantioselective separation of a wide variety of chiral compounds (Allahverdiev et al. 1999). Analysis conditions, modified from Bonello et al. (2008), were as follows: H₂ (carrier gas) at 2 ml min$^{-1}$; injector temperature 230 °C; flame ionization detector temperature 250 °C. The oven temperature programming started at 40 °C (isothermal, 3 min), and increased linearly to 200 °C at 1 °C min$^{-1}$; the final temperature of 200 °C was maintained for 10 min (total run time 173 min). For each sample run, 1 μl of extract was injected in the GC. Between each sample run, a blank run (only n-pentane) was performed. Data acquisition and subsequent processing were performed using TotalChrom™ 6.2.0.0.0:B27 chromatography software (Perkin-Elmer). Terpenoids (mono and sesquiterpenes) were identified by comparing their retention times with those of high-purity standards under the same conditions: ($\pm$)-α-pinene, ($\pm$)-α-pinene, ($\pm$)-β-pinene, ($\pm$)-β-pinene, ($\pm$)-limonene, ($\pm$)-limonene, ($\pm$)-sabinene, myrcene, p-cymene, γ-terpinene and terpinolene (Fluka, Sigma-Aldrich), Δ3-carene, (+)-camphene, β-caryophyllene and α-humulene (Sigma-Aldrich). The absolute amounts of individual terpenoids were determined by comparison with the internal standard, and expressed as μg g$^{-1}$ FW as tridecane equivalent. The minimum peak area detection limit for quantification was set to 1000 peak area units, which is 10 times above the instrument detection limit.

**Statistical analysis**

To test if the growing conditions were homogeneously distributed among the treatments we used one-way analysis of variance (ANOVA) on plant height and current year shoot length distribution.

To test the effects of treatment, plant height and shoot length on lesion length we used a general linear model. We initially tested interactions and main effects, simplifying the model if there were no significant interactions. If a significant treatment effect was observed, Tukey’s honestly significant difference (HSD) test was used to compare the different treatments.
For metabolites (i.e., lignin, individual phenolics and individual terpenoids), we first compared constitutive concentration among the treatments measured at $T_0$ and control measured at $T_1$, using one-way ANOVA. This preliminary analysis showed that constitutive metabolite concentration did not differ between treatments at $T_0$ (homogeneous starting conditions), and that all treatments at $T_0$ did not differ from the control at $T_1$, even though high variability was evident (data not shown). Consequently, we could assume that changes between $T_0$ and $T_1$ were due only to the treatment effect, and analyses of inducible variation were based on the difference ($\Delta$) between induced and constitutive metabolite concentration ($T_1 - T_0$), at both sampling positions (L and S). This experimental design, modified from Lombardero et al. (2000), was chosen in order to control for the high variability found in the constitutive metabolite profiles.

Main effects and interaction between treatment and sampling position on inducible variation were analyzed using linear mixed-effect models, incorporating sampling tree as a random factor, in order to account for the nested nature of the experimental design. If not significant, the interaction was removed.

To test if the induction (meant as a generic stress) had produced a significant variation of the constitutive amount of metabolites, irrespective of the treatments, $\Delta$ data for all treatments were pooled per sampling position and t-test was used to test if the $\Delta$ was different from 0, separately for L and S position.

Constitutive amounts and $\Delta$ of individual phenolics and individual terpenoids were $\ln(a+x)$ transformed to meet model assumptions, where $a$ was a natural number big enough to avoid negative values. Results were anti-$\ln(x)$ − $a$ back-transformed for graphical representation and discussion, and expressed as mean values and 95% confidence interval (CI), since standard error is not valid after anti-ln transformation (Bland and Altman 1996). Data were analyzed in R (R Development Core Team 2011).

For all the analyses described above, we preliminarily tested whether the results were consistent between the two trials. We therefore tested the interaction between trial (27 May vs. 30 May) and all the other factors in all of the analyses. As this interaction was never significant, the factor trial was excluded from the models, and all data were pooled, resulting in 10 replicates per treatment.

In all analyses we used a conservative significance threshold of $\alpha = 0.01$. No Bonferroni correction was used. The mathematical, logical and practical arguments against applying Bonferroni correction in ecological studies can be found in detail in Moran (2003) and references therein.

One tree was removed from all the analyses, after it was identified as an outlier with Grubb’s test ($P < 0.001$). This tree had extremely high amounts of constitutive metabolites (i.e., comparable to inducible levels in other plants) that were probably due to a pre-existing infection or stress condition of that plant.

**Results**

**Tree growth, fungal re-isolation and lesion length**

Plant height and shoot length (data not shown) did not vary in relation to the treatment ($F_{4,44} = 0.17$, $P = 0.953$ and $F_{4,44} = 0.94$, $P = 0.449$, respectively), confirming that the growing conditions were homogeneously distributed among the treatments.

The re-isolation rate of the inoculated fungi from the tissues surrounding the inoculum sites was 81.5% for *O. brunneo-ciliarium* and 17.4% for *H. macrospora*.

While relatively small, all lesions produced in response to the fungal inoculations were similar and significantly longer than those produced in response to wounding alone, independently of the inoculated species (Figure 1). There were no associations between plant height and lesion length, and between shoot length and lesion length ($F_{1,33} = 4.56$, $P = 0.040$ and $F_{1,33} = 0.07$, $P = 0.796$, respectively, data not shown). There were no interactions on lesion length among the factors ($P > 0.05$).

**Phenolics and resin acids**

We analyzed and quantified 20 different phenolic compounds (Table 1 and Supplementary Table S1), all of which have been previously detected in the phloem of Scots or Austrian pine (Lieutier et al. 1996, Sierota et al. 1998, Karonen et al. 2004a, 2004b, Wallis et al. 2008, 2011), except for unknown compound 1. Unknown compound 2, which had been previously detected also by Wallis et al. (2008), is likely another stilbene
Table 1. Constitutive amounts and inducible variation of identified phenolic compounds, resin acids and lignin in the outer bark and phloem of *P. sylvestris*.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Constitutive composition $T_0$ (μg g$^{-1}$ FW)</th>
<th>Inducible variation $T_1 - T_0$ (μg g$^{-1}$ FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean and CI95%</td>
<td>Local mean and CI95%</td>
</tr>
<tr>
<td>Vanillic acid hexoside</td>
<td>24.5 (18.8 to 30.3)</td>
<td>0.01 (−6.8 5 to 6.83)</td>
</tr>
<tr>
<td>Catechin hexoside</td>
<td>524.4 (447.2 to 620.2)</td>
<td>−173.1$^6$ (−247.1 to −98.5)</td>
</tr>
<tr>
<td>Procyanidin dimer (B type)</td>
<td>1071.8 (938.7 to 1206.5)</td>
<td>−56.8 (−163.1 to 50.6)</td>
</tr>
<tr>
<td>Epi/Catechin</td>
<td>3378.1 (2922.8 to 3849.6)</td>
<td>−173.1$^6$ (−247.1 to −98.5)</td>
</tr>
<tr>
<td>Procyanidin dimer (B2)</td>
<td>1170.4 (950.0 to 1395.4)</td>
<td>−56.8 (−163.1 to 50.6)</td>
</tr>
<tr>
<td>Procyanidin trimer (C type)</td>
<td>2099.5 (1858.8 to 2345.2)</td>
<td>−56.8 (−163.1 to 50.6)</td>
</tr>
<tr>
<td>Hydroxypropiovanillone hexoside</td>
<td>52.1 (39.6 to 64.5)</td>
<td>5.4 (−5.9 to 16.7)</td>
</tr>
<tr>
<td>Unknown 1</td>
<td>124,165$^1$ (103,966 to 146,186$^1$)</td>
<td>−14279$^1$ (−42,295 to 23,839$^1$)</td>
</tr>
<tr>
<td>Lignan hexoside</td>
<td>1349.5 (1078.4 to 1627.3)</td>
<td>−111.4 (−424.8 to 212.2)</td>
</tr>
<tr>
<td>Taxifolin hexoside</td>
<td>683.9 (601.6 to 766.9)</td>
<td>−153.8$^6$ (−226.8 to −80.3)</td>
</tr>
<tr>
<td>Taxifolin</td>
<td>982.0 (830.6 to 1135.4)</td>
<td>−56.1 (−108.4 to 223.3)</td>
</tr>
<tr>
<td>Procyanidin dimer (A type)</td>
<td>723.5 (585.2 to 863.7)</td>
<td>−56.1 (−108.4 to 223.3)</td>
</tr>
<tr>
<td>Pinosylv (B type)</td>
<td>0.3 (0.1 to 0.6)</td>
<td>58.8$^5$ (46.0 to 71.5)</td>
</tr>
<tr>
<td>Pinocebrin</td>
<td>3.4 (1.2 to 5.7)</td>
<td>17.0$^3$ (10.7 to 23.3)</td>
</tr>
<tr>
<td>Pinocebrin monomethyl ether</td>
<td>1.2 (0.3 to 2.2)</td>
<td>57.3$^4$ (44.7 to 70.0)</td>
</tr>
<tr>
<td>Unknown 2</td>
<td>35,030$^1$ (24,085 to 46,939$^1$)</td>
<td>68,162$^5$ (528,034 to 872,777$^1$)</td>
</tr>
<tr>
<td>Abietic acid</td>
<td>880.6 (346.6 to 1442.2)</td>
<td>23,797.8$^4$ (20,146.6 to 27,891.2)</td>
</tr>
<tr>
<td>Lignin</td>
<td>50,005.6 (45,500.3 to 54,510.9)</td>
<td>−13,347.8$^6$ (−17694.6 to −9001.0)</td>
</tr>
</tbody>
</table>

The constitutive amount corresponds to $T_0$. Inducible variation corresponds to the difference between induced and constitutive metabolite concentrations ($T_1 - T_0$). Since treatment did not have a significant effect on inducible variation for most compounds (see Table 2), data for all treatments were pooled for both sampling positions. Caffeic acid derivative, trans-coumaric acid and ferulic acid were below the PDA detection limit for quantification (80,000 peak area units). Neoabietic acid coeluted with an unidentified compound and was not quantified. Anti-ln back-transformed mean values (μg g$^{-1}$ FW) are shown.

$P$ values <0.01 are highlighted in bold.

$^1$Peak area.

$^2$Induced increase from 10 to 100% of constitutive value.

$^3$Induced increase from 100 to 1000% of constitutive value.

$^4$Induced increase from 1000 to 10,000% of constitutive value.

$^5$Induced increase >10,000% of constitutive value.

$^6$Induced decrease from 10 to 100% of constitutive value.
due to its strong autofluorescence and similarity of UV spectrum to those of pinosylvin and pinosylvin monomethyl ether (data not shown).

Treatment had no significant effect on inducible variation of most phenolics (Table 2). The only exception was pinosylvin monomethyl ether, for which the local increase in response to wounding alone was significantly lower than in response to inoculation with both fungi together (Figure 2). A similar but non-significant response was also observed for pinosylvin (Table 2). On the other hand, sampling position (local vs. systemic) had a strong effect on inducible variation of most phenolics, except for vanillic acid hexoside, catechin hexoside, ferulic acid hexoside, procyanidin trimer and hydroxypropiovanillone hexoside (Table 2). However, there was no significant interaction between treatment and sampling position.

After pooling all treatments, t-tests on the Δ data, separated by sampling position, showed that almost all phenolics responded significantly to at least one of the sampling positions, except for epi/catechin, hydroxypropiovanillone hexoside and lignan hexoside (Table 2). The stilbenoid pinosylvin, which was present only in trace amounts constitutively (0.3 μg g⁻¹ FW, CI₉⁵% = 0.1–0.6), had the strongest response at both sampling positions. The local increase was >10,000% of the constitutive amount, and the one in the systemic position was >600%. Pinosylvin monomethyl ether and unknown 2 showed strong increases as well (up to 5000%), but only locally. Pinocembrin also increased locally, up to 500% over the constitutive

![Figure 2. Effects of different treatments on the inducible variation of (a) pinosylvin monomethyl ether (F₃,₃₅ = 6, P = 0.002) and (b) (+)-α-pinene (F₃,₃₄ = 5.49, P = 0.003). Inducible variation corresponds to the difference between induced and constitutive concentrations (T₁ – T₀), in the outer bark and phloem of P. sylvestris. Anti-ln back-transformed mean values (μg g⁻¹ FW) are shown. Bars represent CI₉⁵%. Asterisks denote significant differences (α = 0.01) with the wound treatment (contrasts from the linear mixed-effect model).](https://academic.oup.com/treephys/article-abstract/32/7/867/1644630/Nutritional-and-pathogenic-fungi-associated-with)

### Table 2. Results from the linear mixed model testing the effects of treatment and sampling position on the inducible variation of phenolics, abietic acid and lignin (i.e., difference between induced and constitutive concentrations), in the outer bark and phloem of P. sylvestris. P values <0.01 are highlighted in bold.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Treatment df</th>
<th>F value</th>
<th>P value</th>
<th>Sampling position df</th>
<th>F value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vanillic acid hexoside</td>
<td>3, 35</td>
<td>0</td>
<td>0.899</td>
<td>1, 38</td>
<td>6</td>
<td>0.018</td>
</tr>
<tr>
<td>Catechin hexoside</td>
<td>3, 34</td>
<td>1.2</td>
<td>0.333</td>
<td>1, 37</td>
<td>0.3</td>
<td>0.561</td>
</tr>
<tr>
<td>Procyanidin dimer (B type)</td>
<td>3, 35</td>
<td>0.71</td>
<td>0.555</td>
<td>1, 38</td>
<td>14.43</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Epi/Catechin</td>
<td>3, 35</td>
<td>0.4</td>
<td>0.754</td>
<td>1, 38</td>
<td>10.82</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Procyanidin dimer (B2)</td>
<td>3, 35</td>
<td>0.79</td>
<td>0.510</td>
<td>1, 38</td>
<td>14.12</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ferulic acid hexoside</td>
<td>3, 35</td>
<td>1.1</td>
<td>0.347</td>
<td>1, 38</td>
<td>6.3</td>
<td>0.016</td>
</tr>
<tr>
<td>Procyanidin trimer (C type)</td>
<td>3, 35</td>
<td>0.33</td>
<td>0.807</td>
<td>1, 38</td>
<td>5.08</td>
<td>0.030</td>
</tr>
<tr>
<td>Hydroxypropiovanillone hexoside</td>
<td>3, 35</td>
<td>0</td>
<td>0.789</td>
<td>1, 38</td>
<td>0</td>
<td>0.667</td>
</tr>
<tr>
<td>Unknown 1</td>
<td>3, 35</td>
<td>0.85</td>
<td>0.478</td>
<td>1, 38</td>
<td>13.00</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Lignan hexoside</td>
<td>3, 35</td>
<td>0.22</td>
<td>0.879</td>
<td>1, 37</td>
<td>21.81</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Taxifolin hexoside</td>
<td>3, 35</td>
<td>2.8</td>
<td>0.055</td>
<td>1, 38</td>
<td>12.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Taxifolin</td>
<td>3, 35</td>
<td>1.2</td>
<td>0.324</td>
<td>1, 38</td>
<td>9.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Procyanidin dimer (A type)</td>
<td>3, 35</td>
<td>2.51</td>
<td>0.074</td>
<td>1, 37</td>
<td>27.02</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Pinosylvin</td>
<td>3, 35</td>
<td>3</td>
<td>0.037</td>
<td>1, 38</td>
<td>86</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Pinocembrin</td>
<td>3, 35</td>
<td>3</td>
<td>0.055</td>
<td>1, 37</td>
<td>20</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Pinosylvin monomethyl ether</td>
<td>3, 35</td>
<td>6</td>
<td>0.002</td>
<td>1, 38</td>
<td>66</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Unknown 2</td>
<td>3, 35</td>
<td>0.38</td>
<td>0.769</td>
<td>1, 38</td>
<td>239.09</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Abietic acid</td>
<td>3, 31</td>
<td>1.47</td>
<td>0.241</td>
<td>1, 25</td>
<td>184.15</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Lignin</td>
<td>3, 35</td>
<td>0.34</td>
<td>0.798</td>
<td>1, 38</td>
<td>7.64</td>
<td>0.009</td>
</tr>
</tbody>
</table>
amount. Catechin hexoside decreased slightly, but significantly, both locally and systemically, whereas taxifolin and taxifolin hexoside decreased slightly, but only locally. Vanillic acid hexoside, ferulic acid hexoside, unknown 1 and all procyanidin derivatives did not vary locally, but showed a slight increase, <70% of the constitutive amount, systemically (Table 1).

Among the resin acids, we identified abietic acid and neoabietic acid (Supplementary Table S1), but the latter coeluted with an unidentified compound, and therefore we were able to quantify only abietic acid (Table 1). Treatment had no significant effect on inducible variation of this resin acid, while sampling position did (Table 2). There was no significant interaction between treatment and sampling position ($F_{3,22} = 0.96$, $P = 0.430$). After pooling all treatments, t-tests on the $\Delta$ data, separated by sampling position, showed that after induction abietic acid increased very strongly, over 2000%, but only locally (Table 1).

**Lignin**

The constitutive amount of lignin were 50.0 mg g$^{-1}$ FW (Cl$_{95\%}$ = 45.5–54.5). Sampling position, but not treatment, had a significant effect on the inducibility of lignin concentration (Table 2), and there was no interaction between the two factors ($F_{3,35} = 1.2$, $P = 0.325$). After pooling all treatments, t-tests on the $\Delta$ data, separated by sampling position, showed that after the induction lignin decreased slightly, but significantly, both locally and systemically (Table 1).

**Terpenoids**

A total of 18 terpenoids were analyzed from the n-pentane extract (Table 3), all of which have been detected in previous studies in Scots, Austrian, jack and/or red pine (Delorme and Lieutier 1990, Raffa and Smalley 1995, Sadof and Grant 1997, Fäldt et al. 2006, Wallis et al. 2008), except for three unknown compounds (i.e., 3, 4 and 5).

Similar to lignin, abietic acid and phenolics, treatment had no significant effect on inducible variation of most terpenoids (Table 4), except for (+)-\(\alpha\)-pinene, for which the local increase in response to wounding alone was significantly lower than in response to the fungal complex (Figure 2). Similar responses were observed for (+)-\(\beta\)-pinene and \(p\)-cymene as well, but not significantly (Table 4). Sampling position had again a strong effect on inducible variation of almost all compounds, except for (−)-\(\beta\)-pinene (Table 4), but there was no interaction between sampling position and treatment.

After pooling all treatments, t-tests on the $\Delta$ data separated by sampling position showed that all terpenoids significantly increased at least in one of the sampling positions ($P < 0.01$) (Table 3). Locally, myrcene had the strongest response, with an increase higher than 100,000% of constitutive amount. (−)-\(\alpha\)-Pinene, (−)-\(\alpha\)-pinene, (−)-camphene, (−)-sabinene, \(\Delta\)-caryophyllene, (−)-limonene, (−)-limonene, \(p\)-cymene, terpinolene, \(\beta\)-caryophyllene, \(\alpha\)-humulene, unknown 4 and unknown 5 increased up to 3500% in the local position, whereas (+)-\(\beta\)-pinene and unknown 3 increased up to 1000%. (−)-\(\beta\)-Pinene was the only compound that did not increase locally ($P = 0.449$), but had a systemic increase higher than 150%. (−)-\(\alpha\)-Pinene, (+)-\(\alpha\)-pinene, myrcene, \(p\)-cymene, terpinolene and \(\beta\)-caryophyllene had a significant increase systemically as well, up to 200% of constitutive amount. \(\alpha\)-Humulene too had a similar systemical increase, although the significance was marginal ($P = 0.010$) (Table 3).

**Discussion**

In this paper we show that in the system Scots pine–\(I. acuminatus\)-associated fungi, the nutritional fungus \(H. macrospora\) triggers an inducible defense reaction comparable to the one induced by the blue-stain fungus \(O. brunneo-ciliatum\), since metabolic responses of the plant, even though strong, showed no differentiation according to the treatment. There was also no overall significant evidence that the fungal species had a synergistic effect on tree defense induction, even though some compounds suggest that behavioral pattern.

Most of the identified and quantified compounds responded locally and/or systemically to the induction. Among the phenolics, pinosylvin, pinosylvin monomethyl ether, unknown 2 and pinocembrin responded most strongly locally, showing an increase of up to 10,000% or more relative to constitutive concentrations. Stilbenes and flavonoids are well known to be widely involved in conifer defense mechanisms against pathogens (Bonello et al. 1993, Lietier et al. 1996, Bonello and Blodgett 2003, Franceschi et al. 2005), to be associated with disease states (Wallis et al. 2008) and to display strong antifungal activity in in vitro assays (Bonello et al. 1993, Seppanen et al. 2004). Moreover, pathogen-induced phenolics can have indirect effects on the performance of the beetle vectors, e.g., \(I. typographus\) (L) on spruce (Faccioli and Schlyter 2007) and \(I. cembrae\) (Heer) on larch (Rohde et al. 1996). Almost all other phenolic compounds found in this study also appeared involved in the induced response, although their inducible variation was lower than 100% of constitutive amount, perhaps suggesting a limited significance in defense. Lignin decreased after induction both locally and systemically, as found in a previous study (Bonello et al. 2003).

Among the terpenoids, myrcene had the strongest response, with the local increase higher than 100,000% of constitutive amounts. Still, all other terpenoids were also characterized by a strong increase after induction, generally only locally. Furthermore, the enantiomeric composition of \(\beta\)-pinene changed following induction. Locally, only (+)-\(\beta\)-pinene increased, becoming the most prevalent enantiomeric form, whereas (−)-\(\beta\)-pinene was the most prevalent form in the constitutive state. Conversely, only (−)-\(\beta\)-pinene increased...
Table 3. Chromatographic data, assigned identities, constitutive amounts and inducible variation of terpenoids isolated from outer bark and phloem of *P. sylvestris*.

<table>
<thead>
<tr>
<th>Peak number</th>
<th>RT (min)</th>
<th>Assigned identity</th>
<th>Constitutive composition $T_0$ (μg g$^{-1}$ FW)</th>
<th>Inducible variation $T_1 - T_0$ (μg g$^{-1}$ FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean and Cl$_{95%}$</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>10.57</td>
<td>(-)-α-Pinene</td>
<td>72.0 (48.9 to 95.5)</td>
<td>1381.5$^2$ (1071.6 to 1739.7)</td>
</tr>
<tr>
<td>2</td>
<td>10.80</td>
<td>(+)-α-Pinene</td>
<td>160.8 (109.6 to 214.5)</td>
<td>4028.6$^2$ (3057.2 to 5232.6)</td>
</tr>
<tr>
<td>3</td>
<td>12.12</td>
<td>(+)-Camphene</td>
<td>3.7 (1.9 to 5.5)</td>
<td>49.3$^2$ (37.7 to 61.1)</td>
</tr>
<tr>
<td>4</td>
<td>12.36</td>
<td>(-)-Sabinene</td>
<td>3.6 (1.9 to 5.4)</td>
<td>49.0$^2$ (34.2 to 63.9)</td>
</tr>
<tr>
<td>5</td>
<td>14.09</td>
<td>Myrcene</td>
<td>1.7 (0.7 to 2.7)</td>
<td>1915.5$^3$ (12470 to 2782.8)</td>
</tr>
<tr>
<td>6</td>
<td>14.35</td>
<td>(-)-β-Pinene</td>
<td>77.1 (48.3 to 106.6)</td>
<td>32.1 (-50.7 to 122.2)</td>
</tr>
<tr>
<td>7</td>
<td>15.09</td>
<td>(+)-β-Pinene</td>
<td>277.1 (19.4 to 35.9)</td>
<td>135.2$^1$ (105.3 to 165.8)</td>
</tr>
<tr>
<td>8</td>
<td>15.80</td>
<td>Δ-3-Carene</td>
<td>40.6 (14.3 to 67.7)</td>
<td>622.9$^2$ (332.5 to 976.5)</td>
</tr>
<tr>
<td>9</td>
<td>17.89</td>
<td>(-)-Limonene</td>
<td>3.9 (1.9 to 6.9)</td>
<td>73.6$^2$ (42.9 to 109.2)</td>
</tr>
<tr>
<td>10</td>
<td>18.01</td>
<td>(+)-Limonene</td>
<td>2.6 (1.4 to 3.7)</td>
<td>35.4$^2$ (22.3 to 48.8)</td>
</tr>
<tr>
<td>11</td>
<td>18.63</td>
<td>p-Cymene</td>
<td>8.0 (3.9 to 12.0)</td>
<td>149.1$^3$ (105.6 to 194.4)</td>
</tr>
<tr>
<td>12</td>
<td>20.40</td>
<td>γ-Terpinene</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>13</td>
<td>22.83</td>
<td>Terpinolene</td>
<td>3.9 (1.6 to 6.1)</td>
<td>119.1$^2$ (74.4 to 165.7)</td>
</tr>
<tr>
<td>14</td>
<td>42.75</td>
<td>Unknown 3</td>
<td>6.1 (2.6 to 9.6)</td>
<td>45.6$^1$ (27.1 to 64.5)</td>
</tr>
<tr>
<td>15</td>
<td>52.28</td>
<td>β-Caryophyllene</td>
<td>1.3 (0.5 to 2.2)</td>
<td>24.1$^2$ (13.2 to 35.1)</td>
</tr>
<tr>
<td>16</td>
<td>55.79</td>
<td>α-Humulene</td>
<td>1.0 (0.3 to 1.7)</td>
<td>34.4$^2$ (12.6 to 56.8)</td>
</tr>
<tr>
<td>17</td>
<td>58.22</td>
<td>Unknown 4</td>
<td>16.2 (9.7 to 22.9)</td>
<td>310.2$^2$ (215.1 to 412.7)</td>
</tr>
<tr>
<td>18</td>
<td>58.46</td>
<td>Unknown 5</td>
<td>17.2 (10.4 to 24.1)</td>
<td>177.6$^2$ (122.0 to 236.0)</td>
</tr>
</tbody>
</table>

Concentrations are expressed as tridecane equivalent. Compounds were identified using standards. The constitutive amount corresponds to $T_0$. Inducible variation corresponds to the difference between induced and constitutive metabolite concentrations ($T_1 - T_0$). Since treatment did not have a significant effect on inducible variation for most compounds (see Table 4), data for all treatments were pooled for both sampling positions. γ-Terpinene was below detection limit for quantification (1000 peak area units). Anti-ln back-transformed mean values (μg g$^{-1}$ FW) are shown.

P values ≤0.01 are highlighted in bold. RT, retention time.

1Induced increase from 100 to 1000% of constitutive value.
2Induced increase from 1000 to 10,000% of constitutive value.
3Induced increase >100,000% of constitutive value.
systemically, augmenting the difference between the two forms that was already present in the constitutive state. An induced systemic increase of (−)-β-pinene had already been reported in a study by Fäldt et al. (2006), which reports an analysis of the effects of fungal infection and wounding on contents and enantiomeric composition of monoterp enes in Scots pine. The role of terpenoids, including resin acids, in conifer defense against bark beetle-associated fungi is well known (Raffa and Smalley 1985, Franceschi et al. 2005, Zeneli et al. 2006), and several in vitro experiments have shown their toxic effects on fungi (Delorme and Lieutier 1990, Blodgett and Stanosz 1997, Kopper et al. 2005). γ-Terpinene, α-pinene and myrcene, for example, inhibit growth and germination of conifer pathogens (Raffa et al. 1985, Klepzig et al. 1996). Besides affecting associated fungi, many terpenoids are also involved in constitutive and induced defenses against the bark beetles themselves (Faccoli et al. 2005, Seybold et al. 2006). Lastly, the role of terpenoids in conifer defense does not only relate to their direct toxicity, but also, indirectly, to attraction/repellence of the beetles or their enemies (reviewed in Keeling and Bohlmann 2006).

In our experiment, we found a strong effect of sampling position. As expected, most compounds responded more strongly locally than systemically, but some phenolics behaved the opposite way (e.g., procyanidins), suggesting qualitative, besides quantitative, differences between localized and systemic-induced responses (Eyles et al. 2010). On the other hand, we found no defense differentiation according to the invading agent, either as a single species or as a fungal complex. Indeed, average lesion length was similar for all fungal species combinations; however, it was always longer than when resulting from wounding alone. This was true even for H. macrospora, in spite of very low re-isolation percentage from the tissues surrounding the lesion site. This suggests that low recovery was not a result of failed inoculation (escape) but was, instead, a reflection of the slow growth rate of H. macrospora (Batra 1967). However, induced chemical composition did not vary, not only among fungal species combination, but also between wounding and inoculations. The only exceptions were pinosylvin monomethyl ether and (+)-α-pinene, for which the local increase in response to the fungal complex was higher than in response to wounding alone. Under the conditions of our study, the lack of a specific response for most compounds can be interpreted as Scots pine having a generic, rather than specific, induced response to this bark beetle system. This is in accordance with other studies on Scots pine (Croisé et al. 1998, Kroken et al. 2000, Fäldt et al. 2006), and supports the hypothesis that although plants can specifically recognize the nature of pathogens, the optimal defense strategy is to activate all available defense mechanisms, so that at least some may be effective against a particular pathogen (Katagiri 2004). Furthermore, the inoculated species, even though performing different ecological roles, are taxonomically close (Rollins et al. 2001, Harrington et al. 2010) and could have triggered similar signaling molecules (Eyles et al. 2010). Some authors even maintain that chemical changes are involved in response to wounding rather than in response to the challengers themselves, and that the type of aggressor elicits only variations in rapidity and extension of the response (Mullick 1977, Lieutier 2004). This ‘shoot first—ask questions later’ strategy is, however, energetically
expensive, and may not be very advantageous for the plant in at least some antagonistic interactions (Keeling and Bohlmann 2006, Bolton 2009).

It must be noted that, working in a natural stand, we found very high levels of variability both in the constitutive and induced metabolite composition, similarly to Sierota et al. (1998) and Fäldt et al. (2006). Results, especially for monoterpenes, stilbenes and flavonoids, seem to follow a pattern in which the inducible response is least for wounding alone, increases with inoculations with single species and is greatest with the fungal complex, suggesting synergism of the two species. But due to high variability, this pattern is not statistically supported for most traits, except for lesion length, pinosylvin monomethyl ether and (+)-α-pinene. In some other cases, such as with pinosylvin, (+)-β-pinene and p-cymene, the non-significance is only marginal. The possibility of synergistic effects of the bark beetle fungal associates would give more importance to the complex itself, besides the roles that have been already confirmed for the single species (Lieutier et al. 2009).

In conclusion, our results reveal how even a non-pathogenic, nutritional fungus directly useful for larval development, may also indirectly assist in the beetle establishment process in Scots pine by participating in exhausting host plant defenses. Similar results have been found in other model systems, such as the southern pine beetle-associated complex (Hofstetter et al. 2005), Scolytus ventralis LeC. and Trichosporium symbioticum Wright (Raffa and Berryman 1982) and Dendroctonus brevicomis LeC. and its associated yeast Ogataea pini (Holst) Yam., Mat., Maed. et Mik. (Davis and Hofstetter 2011). All these systems show how fungi other than blue-stain fungi can strongly stimulate plant defense. In addition to these, our results therefore provide new implications in the debate on the importance of bark beetle-associated fungi (Lieutier et al. 2009, Six and Wingfield 2011). A new ecological role of non-pathogenic and nutritional fungi, that is the capacity to interact with the host plant in a way that facilitates brood establishment, is hence proposed. This model implies that, in stimulating plant responses to rapidly exhaust tree resistance, the lack of specificity in the host response to beetle-associated fungi can only be an advantage for the insects. Our study provides an initial assessment of the role potentially played by nutritional fungi such as H. macrospora. However, a better understanding of the virulence of the nutritional fungus would be helpful to better assess this system. Furthermore, confirmatory work, including more replicates and different associations of tree–bark beetle–fungi, is necessary in order to assess the generality of our findings.

Supplementary data
Supplementary data for this article are available at Tree Physiology Online.

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Conflict of interest

None declared.

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