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Physiological and Molecular Plant Pathology 63 (2003) 249–261

PMPP

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Pinus nigra–Sphaeropsis sapinea as a model pathosystem to investigate local and systemic effects of fungal infection of pines

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Accepted 9 February 2004

Abstract

The Austrian pine/*Sphaeropsis sapinea* pathosystem was tested as a model to study the secondary metabolism of both localized and systemically induced defense responses in pine. Compared to wounding alone, fungal inoculations in lower stems of five-year-old potted Austrian pines induced significant ($P < 0.01$) depletion or accumulation (0.4–101-fold) in reaction zone phloem of several soluble and cell-wall-bound phenolics, and a 1.8-fold increase in lignin deposition. Inoculations also resulted in significant ($P < 0.05$) accumulation (up to 3-fold) of several compounds in the phloem 25 cm above the inoculation site, indicating systemic induction of metabolic pathways. Taxifolin was among the induced metabolites, but was only observed in lower stems, irrespective of treatment. Another putative flavonoid was observed only when trees were inoculated with live mycelia, suggesting a phytoalexin-like role for this compound. In spite of these changes, lignin content and concentrations of several metabolites considered important in resistance (such as stilbenes) were either not correlated, or were positively correlated, with lesion size. Only three metabolites had negative correlations with lesion size, suggesting a potential role in resistance. Protein and lipid fractions of mycelial extracts, as well as killed mycelium of the pathogen, were also examined as potential elicitors. Among the treatments other than live inoculation, killed mycelium was the strongest inducer of host responses, followed by the protein extract. Lipids did not elicit host responses different from wounding alone.

Keywords: *Pinus nigra*; *Sphaeropsis sapinea*; Canker; Secondary metabolism; Chemistry; Phenolics; Proteins; Lipids; High pressure liquid chromatography; Induced defense; Lignification; Resistance

1. Introduction

Extensive research has highlighted the role of several defense molecules, including pathogenesis related (PR) proteins and a vast assortment of antimicrobial secondary metabolites, in localized as well as systemically induced disease resistance of herbaceous plant species [31,50,54]. Existing knowledge of localized defense responses of conifers to pathogenic attack suggests that some biochemical mechanisms may be shared by conifers and herbaceous angiosperms [18,25,26,35,40,45,53,55].

In many plant–pathogen systems, prior pathogenic infections have been shown to induce resistance in previously non-infected parts of the plant, a phenomenon generally termed systemic acquired resistance (SAR) [50]. Recently, SAR has become synonymous with salicylic acid (SA) mediated induction in herbaceous model plant systems [39]. A similar phenomenon, induced by plant growth promoting rhizobacteria, is called induced systemic resistance (ISR). ISR also results in an enhanced resistance phenotype, but is apparently dependent on signaling pathways mediated by jasmonic acid (JA) rather than SA [44].

In conifers, evidence exists that SA and JA can induce defense responses, both locally and distally/systemically [19,22,25,33,45], but it is presently unclear whether these signaling molecules are actually expressed in planta at functional levels. Because of uncertainty about the signaling role of SA and JA in conifers, we prefer to use the more general terminology of systemic induced resistance (SIR) to describe this pathogen-induced resistance phenotype [12]. SIR in its various forms has been known in herbaceous angiosperms for a few decades, but has only recently been reported for a conifer, in Monterey pine [12]. SIR in Monterey pine appeared to be sustainable for a relatively

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doi:10.1016/j.pmpp.2004.02.002

long time with multiple sequential inoculations, almost two years in that study. Consequently, SIR has been suggested as one of the possible mechanisms of remission in the pitch canker epidemic on Monterey pine in California [28].

A recent field study has shown that mature ponderosa pine responds systemically to artificial inoculation with a root and butt rot pathogen by accumulating ferulic acid glucoside in the phloem [15]. Similar systemic effects of root disease on pine secondary metabolism were shown in an investigation of natural root disease centers, but no attempts were made to identify induced molecules [34]. These responses might be involved in SIR in pines, but no detailed studies are known.

While evidence for pathogenic and chemical induction of selected defense pathways now exists, the nature of actual disease resistance in pines remains largely unexplained. This is due to lack of host material with defined genetic background: clonal systems are available for host–pathogen interaction studies in spruce [24], but not in pine. Furthermore, the long life cycles and large size of trees make working with these plants inherently difficult, especially under forest conditions. In an effort to develop a working model system for pine–pathogen interactions we have used greenhouse grown, potted Austrian pine (*Pinus nigra* Arnold) saplings infected with *Sphaeropsis sapinea* (Fr.:Fr.) Dyko & Sutton in Sutton. Expectations for a tree model system cannot be the same as for classic herbaceous model systems such as tobacco and *Arabidopsis*, for which genetic homogeneity can be easily achieved and for which defined host–pathogen interactions are available. However, this pathosystem is amenable to easy manipulation and exhibits responsiveness that is both detectable and reproducible. It is also biologically relevant, i.e. it reasonably represents other necrotrophic host–pathogen interactions at various host growth stages. Austrian pine is native to the Mediterranean basin [1], where it is a dominant forest species. Since its introduction into the USA, Austrian pine has become one of the most widely planted ornamental conifers [52]. However, shoot blight and canker caused by *S. sapinea* have caused extensive damage to Austrian pine and other conifers, both in the US and throughout the world. Seedlings, saplings (such as those used in this study), and mature trees are impacted to various degrees and can be killed at all ages in natural stands, nurseries, landscapes, plantations, and Christmas tree farms [17,27,49].

The aim of this study was to test the viability of the Austrian pine/*S. sapinea* pathosystem as a model to investigate localized and systemic host defense responses that might explain pine resistance to canker pathogens. The specific objectives of this study were to: (1) survey the induced, localized secondary metabolic responses that correlate with disease resistance; (2) establish whether fungal infection induces systemic changes in secondary metabolism of Austrian pine; and (3) identify likely classes of pathogen-derived elicitors that may be used in the future to further dissect the chemistry and molecular biology of the interaction.

2. Materials and methods

2.1. Plant material

Dormant, five-year-old Austrian pines were obtained from Ridge Manor Nursery (Madison, OH) in spring 2001. Trees were grown in an organic mix (5% hardwood bark, 60% pine bark, and 35% peatmoss) in 11 l plastic pots. The pines had a mean stem height of 83 ± 0.23 cm (standard error) and a mean stem diameter of 2.8 ± 0.03 cm. They were placed in a greenhouse with a mean temperature of 22 ± 0.05 °C, and a mean relative humidity of 54 ± 0.15%. The maximum recorded ambient greenhouse photon flux density was 1350 $\mu E\ s^{-1}\ m^{-2}$. We selected trees of uniform size and form to reduce some experimental variability. Trees were maintained in the greenhouse for the duration of the experiment. Since even a moderate level of water stress is known to affect canker development [5,6], trees were watered twice daily to field capacity to exclude water stress as a factor. Pre-dawn needle water potential measurements confirmed that the trees were never water stressed during the experiments, even as cankers developed.

2.2. Putative protein and lipid elicitors

A total protein extract and a total lipid extract of fungal mycelium were prepared to investigate the possible role of specific chemical categories of elicitors. A third elicitor category, comprising the whole fungal thallus, is described below under ‘killed mycelium’.

Mycelium of *S. sapinea* was grown on potato dextrose agar (PDA). There are two distinct isolate groups (morphotypes) of *S. sapinea* in the Midwest (A and B) that have been shown to differ in aggressiveness on other conifer hosts [8,10]. Recently, the two morphotypes have been elevated to species status: the A isolate group is now the type for *S. sapinea*, while the B isolate group is now called *Diplodia scrobiculata* [20]. Preliminary surveys in Ohio have resulted in the recovery of only *S. sapinea* and pathogenic tests revealed that the isolates are aggressive on Austrian pine [3] (Blodgett and Bonello, unpublished). Only *S. sapinea* was used in this study.

Five-millimeters diameter plugs from margins of actively growing colonies were used to inoculate flasks containing 100 ml of Czapek Dox medium. The liquid cultures were incubated for 12 days in a Innova 4330 incubator shaker (New Brunswick Scientific, Edison, NJ) at 150 rpm, in the dark at 24 °C. Flasks were then stored at −80 °C until use. Thawed cultures were filtered at 4 °C through a 1.5 mm mesh screen to separate the mycelium from the culture broth. The solid portion was rinsed with

cold (4 °C) demineralized, double distilled water. Each flask yielded 12–30 g mycelium wet mass.

Twenty grams (wet weight; water content 95%) of mycelium were mixed with 40 ml of protein extraction buffer at 4 °C. The protein extraction buffer consisted of 0.1 M Tris–HCl (pH 7.3), 0.15 M NaCl, 1 M sucrose and 3 mM dithiothreitol (DTT). The mycelium, with buffer, was homogenized for 3 min in a Sorvall Omni-mixer homogenizer (Norwalk, CT) at full speed. The homogenate reached a final temperature of 6–8 °C. This homogenate was centrifuged at approx. 20,600*g* for 15 min at 0 °C to obtain a supernatant of intracellular proteins. Sixty milliliters of the original culture broths were centrifuged at approx. 20,600*g* for 15 min at 0 °C to obtain a supernatant of extracellular proteins. The supernatant was filtered through four layers of cheese-cloth to remove the oily top layer.

To maximize the probability of detecting proteinaceous elicitors, the intracellular and extracellular extracts were combined to obtain 100 ml of solution in each of two trials (see experimental design); in the first trial the preparation was concentrated to approx. 9.2 ml, in the second trial to 11.0 ml. Proteins were concentrated using 3500 MW cutoff, regenerated cellulose dialysis tubing (Fisher Scientific, Pittsburgh, PA) at 3 °C. The dialysis tubing containing the extracts was placed in a tray and covered with carboxymethyl cellulose (medium viscosity; Sigma Chemical Company, St Louis, MO) for 16 h. The carboxymethyl cellulose was changed at 3, 6, and 14 h. The first trial yielded 43.5 mg total protein g^{-1} wet mycelium mass (94.6 mg total protein ml^{-1} solution), while the second yielded 32.7 mg total protein g^{-1} wet mycelium mass (59.4 mg total protein ml^{-1} solution). Total protein concentrations were quantified against a human albumin standard curve obtained with the Sigma microprotein Kit (Sigma, St Louis, MO). Protein preparations were stored at 3 °C for less than 22 h before being used as host treatments. Smearing of small aliquots of protein preparations on PDA plates using sterile bacterial loops showed that these preparations were free of live pathogen.

An intracellular lipid fraction was obtained by homogenizing 20 g (wet weight; water content 95%) of mycelium in 340 ml of 2:1 chloroform–methanol at 23 °C for 3 min at full speed in a Sorvall Omni-mixer homogenizer. The homogenate reached a final temperature of 42 °C. The homogenate was then sonicated for 3 min using the Biosonik III sonicator (Bronwill Scientific, Rochester, NY) at an intensity of 55. The homogenate reached a final temperature of 31 °C. The homogenate was then filtered through Whatman No. 1 filter paper (W&R Balston Limited, England) into a separating funnel, 68 ml (16.7% of total volume) of demineralized, double distilled water containing 0.73% NaCl were added, and the solution was thoroughly mixed. After separation, the lower phase was collected. This phase was concentrated to 6 ml using a rotary evaporator under reduced pressure at 35 °C. Two separate batches were prepared for use in the two separate trials described below. Lipid yield was determined gravimetrically and was 1.8 mg g^{-1} wet mycelium mass for trial one and 1.3 mg g^{-1} wet mycelium mass for trial two. Though unlikely, some of these intracellular lipid preparations may have lacked some of their original constituents, as the freeze–thaw cycle to which the whole mycelium was subjected could have resulted in partial leaking of cytoplasmic components.

The lipid preparation likely contained residual organic solvents. Thus, it was not applied directly to the trees. Instead, 65 μl of the lipid solution (corresponding to 0.39 and 0.28 mg of lipids in trials one and two, respectively) were placed in each of several 13 mm diameter wells created on PDA plates and the solvent was allowed to evaporate from the open Petri dishes in a laminar flow hood for approx. 20 min. The wells (approx. 0.5 mm deep) were produced by floating 13 mm diameter sterile filter paper disks on the molten medium surface and by removing the filters with forceps once the agar had solidified. Plates were stored at 4 °C for less than 40 h before being used as host treatments. Ten millimeters diameter PDA plugs were cut from the 13 mm diameter PDA-lipid wells, and were applied to each wound, lipid side toward the host tissue, as described below. Since each 10 mm diameter plug represented 59.2% of the total area of each 13 mm diameter well, each PDA plug carried 0.23 and 0.17 mg of fungal lipids in trials one and two, respectively.

2.3. Killed mycelium

In order to mimic live inoculations as close as possible we challenged the host with killed mycelium as an elicitor. While this approach may not exactly duplicate the effects of inoculation with a live pathogen, the procedure may mimic potential release of elicitors from hyphae of living fungi. To avoid physical changes to the fungal cell walls, instead of autoclaving the fungus we killed the mycelium chemically by exposing it to propylene oxide. Open culture plates of *S. sapinea* were placed in sealed plastic boxes in the presence of an open container of propylene oxide (Fisher Scientific) for 24 h. Plates were then left covered in a fume hood in opened plastic boxes for 2 days to allow for dissipation of propylene oxide. The killed cultures plates were then wrapped with Parafilm and used as treatments after seven additional days. Death of the pathogen was confirmed by subculturing.

2.4. Experimental design and protocols

A fully factorial design was used in which six treatments were tested at three different independent times and over two independent trials. Each unique treatment-time-trial combination consisted of five independent statistical units (trees), for a total of 180 trees. The six treatments were: wounded (i.e. mock inoculation), live inoculation, killed fungus (i.e. inoculation with killed fungus), lipids

(i.e. application of the lipid preparation), proteins (i.e. application of the protein preparation), and nonwounded (untreated) controls. In each treatment, other than the nonwounded controls, each tree was wounded at three equally spaced locations around the stem, 10 cm above the soil, by removing outer bark and phloem using a 12 mm diameter cork borer. In the protein treatment, 65 μl of fungal protein solution (corresponding to 6.1 and 3.9 mg of protein in trials one and two, respectively) were applied to each wound and the wound was covered with a sterile 10 mm diameter plug of PDA. In the lipid treatment, 10 mm diameter plugs taken from the PDA wells were inserted in the wounds. In the wounded treatment, sterile PDA plugs were inserted in the wounds. In the live inoculation treatment, 10 mm diameter plugs were taken from the margins of actively growing cultures, while in the killed fungus treatment the plugs were taken from the margins of killed cultures. In all cases, other than the nonwounded controls, the treatment locations were wrapped in Parafilm for the duration of the experiment.

Trees were sampled at 7, 14, and 28 days post-treatment. One, 7-mm-diameter bark/phloem tissue-plug sample was collected immediately above each of the three wounds. The tissues were surface-disinfested and processed for *S. sapinea* detection as described previously [4]. The pathogen was always reisolated from the lesions caused by the live inoculations, once from the 30 nonwounded trees, and from six of the 30 trees in the protein treatments. The pathogen was never detected in any of the other treatments. Detection in one nonwounded tree and in trees treated with protein extract may be attributable to presence of the pathogen as an endophyte in some trees [23].

Two 10 mm diameter phloem/bark plugs were collected 1 cm (plug center) above the upper wound margin and midway between wounds, where the reaction zone was most likely located (henceforth referred to as the 'lower site'), and two 10 mm diameter phloem/bark plugs were collected 25 cm above the wound on opposite sides of stems (henceforth referred to as the 'upper site'). Plugs were immediately frozen in liquid nitrogen and stored at −80 °C until processing for chemical analyses. One 10 mm diameter bark/phloem sample was also collected 12.5 cm above the wound for determination of bark/phloem dry weight and water content. The plugs were dried for 5 days at 50 °C.

After all tissue samples were collected, the outer bark was removed with a knife at each of the three inoculation sites of each tree. Lesion (canker) lengths were then measured, if present, above wounds and the three lesions on each tree were averaged to represent the response of that tree. Lesions consisted of necrotized phloem tissue as is common with canker diseases of trees. Other characters recorded included the amount of exuded resin (recorded as: 1: low, ≤5 mm from wound; 2: moderate, ≤ 4 cm; 3: high, >4 cm), sapwood discoloration (recorded as: 1: tan, 2: brown, or 3: black), and length of sapwood discoloration above the wound.

2.5. Chemical analyses

The two tissue samples taken from the same height on a tree were combined into a composite sample, ground to powder in liquid nitrogen, and processed for sequential extraction of soluble and cell wall-bound phenolics, and lignin, as described previously [13,15], with modifications. Centrifugation in all of the following steps was carried out in an Eppendorf 5415D centrifuge at 16,100*g* for 5–10 min. For methanol-soluble phenolics, the ground plant tissues (100 mg FW) were extracted twice in 0.5 ml HPLC grade methanol for 24 h at 3 °C, the supernatants were combined, and then stored at −20 °C until analyzed. For cell wall-bound phenolics, pellets from the soluble phenolics procedure were washed once each with 1 ml of HPLC grade water (Fisher) and HPLC grade methanol (Fisher), washed with 900 μl of *tert*-butyl methyl ether (Sigma), and dried overnight. Pellets were then hydrolyzed at 40 °C for 21 h on a shaker in 200 μl of 1 N NaOH containing 100 mM ascorbic acid (Sigma) and 0.2% (wt/vol.) sodium borohydride ($NaBH_4$) (Sigma). The reaction mixture was acidified with 200 μl of 1.5 M formic acid (Fluka Chemie, Buchs, Switzerland), followed by 400 μl of HPLC grade methanol. Supernatants were stored at −20 °C until analyzed. For lignin, the pellets from the cell wall-bound phenolics procedure were washed once each with 1 ml of HPLC grade water. Pellets were resuspended in 1 ml of 2 N HCl and 0.25 ml of thioglycolic acid (Sigma). Eppendorf tubes containing the solutions were placed in an oven at 86 °C for 4 h and the supernatants were discarded. Pellets were rinsed two times with 1.5 ml of water, resuspended in 1 ml of 0.5 M NaOH, and the tubes were placed on a shaker for 18 h at 200 rpm. The supernatants were saved. The pellets were again resuspended in 0.5 ml of 0.5 M NaOH for 18 h and the supernatants were pooled. The solutions were acidified with 0.3 ml of conc. HCl at 4 °C for 4 h and supernatants were discarded. The lignin–thioglycolic acid pellets were dried in a desiccator and then dissolved in 1 ml of 0.5 M NaOH. In all steps, Eppendorf tubes were vortexed after adding solutions, and supernatants and pellets were separated by centrifugation. Unless expressed, procedures were conducted at room temperature (approx. 22 °C). Lignin concentration was determined spectrophotometrically against a standard of spruce lignin (Sigma-Aldrich, St Louis, MO) [15].

Soluble and cell wall-bound phenolics were analyzed by HPLC, using a Waters (Milford, MA) 2690 separations module, 474 scanning fluorescence detector, and 996 photo diode array detector. The system was managed by a workstation running Waters Millennium HPLC software. A Waters Xterra™ RP18, 5 μm, 4.6 × 150 mm^2 column was used for sample separation. The autosampler temperature was 4 °C and column temperature was 30 °C for all

analyses. Injections consisted of 5 μl of either soluble or cell wall-bound phenolic extracts. The fluorescence detector was set at 300 nm excitation and 400 nm emission wavelengths. The photo diode array detector was set to scan between 237 and 376 nm, and two channels were selected for data processing, 280 and 308 nm. The former wavelength is commonly used for phenolic detection, but often generates poor peak separation with conifer samples. This is presumably due to the presence of soluble tannins that create drifting baselines with large humps. The 308 nm wavelength was selected based on preliminary 3D scans of actual Austrian pine phloem samples and various standards (described below under 'Peak identification and quantification'), because it offered clean peak separation/detection, and optimization of stable and flat baselines.

The acidified water–methanol solvent system developed by Rosemann et al. [46] was used. Composition of solvent A (per liter) was 980 ml dd H_2O, and 20 ml of 5% ammonium formate (Sigma) in formic acid (Fluka). Composition of solvent B (per liter) was 882 ml HPLC grade methanol, 96 ml dd H_2O, and 20 ml of 5% ammonium formate (Sigma) in formic acid (Fluka). The following linear gradient (cumulative run time (min), flow rate (ml/min), % solvent A) was used: 2.0, 1.0, 100.0; 4.0, 1.0, 90.0; 20.0, 1.0, 52.0; 38.0, 1.75, 0.0; 39.0, 1.75, 0.0; 41.0, 1.75, 100.0; 43.8, 1.75, 100.0; and 44.0, 0.5, 100.0 (total run time 44 min).

2.6. Peak identification and quantification

Peaks that showed a treatment-dependent behavior were detected using a preliminary screening of samples pooled within each treatment and time, and separately for each trial and sampling site. This was done with both soluble and cell-wall bound metabolites. In this screening phase, 20 μl samples from each of the five statistical units (trees) in each treatment were pooled to obtain an average chromatogram for each treatment, and the six chromatograms thus obtained (one for each treatment) were overlayed. In this way we could select peaks that behaved in a treatment-dependent fashion. Those peaks were then processed and quantified for all individual samples.

The spectral characteristics and retention times of the individual peaks were compared to those of the following standards: benzoic acid (Sigma), caffeic acid (Sigma), catechin (racemic) (Sigma), coniferyl alcohol (Sigma), ferulic acid, ferulic acid glucoside (synthesized-[15]), kaempferol (Roth, Karlsruhe, Germany), naringenin (racemic) (Sigma), pinosylvin (Apin Chemicals Ltd, Abingdon, UK), pinosylvin monomethyl ether (Apin Chemicals Ltd), quercetin dihydrate (Roth), SA (Sigma), taxifolin (racemic) (Sigma), *trans*-4-cinnamic acid (Apin Chemicals Ltd), *trans*-4-coumaric acid (Apin Chemicals Ltd), vanillic acid (Sigma), vanillin (Sigma), vanillin glucoside (Sequoia Research Products Ltd, Oxford, UK). Plant samples were also spiked with the standards to assure that peaks matched to standards by the HPLC software did indeed coelute with the standards. Identifiable peaks were quantified against standard calibration curves (all regressions linear with $P < 0.001$). Additionally, we considered the total soluble secondary metabolite (SumSol) and total cell wall-bound secondary metabolite (SumCW) fractions measured as sums of all peak areas in an HPLC chromatogram at 280 nm.

The minimum detectable peak area was set at 9000 peak area units in our instrument. To simplify statistical treatment of our data (for example, to avoid calculation of ratios with null denominators), we elected to treat all instances of undetectability as an arbitrary value of 7000 peak area units rather than zero.

2.7. Statistical analyses

The responses measured at the upper site depended on the treatments at the lower site. Therefore, the two sampling sites were analyzed independently due to the nature of the different questions being addressed. For example, only the lower site was inoculated and produced cankers and resin.

Data were analyzed using SPSS 11.0 for Windows and Prism 3.02 (GraphPad Software, Inc., San Diego, CA). When the assumptions of normality and variance homogeneity could be satisfied by either the raw, log-transformed or square root-transformed data, differences in lesion lengths and compound concentrations among treatments were tested using univariate ANOVA, followed by Fisher's LSD multiple range test for mean separation. When the ANOVA assumptions could not be satisfied, univariate Kruskal–Wallis test followed by Dunn's multiple range test was used. Trial (two levels), time (three levels), and treatment (six levels) were the factors. When the analysis showed no significant differences ($P > 0.05$) between different levels of the same factor, data sets were combined. In the results from both analyses, degrees of freedom (DF) are indicated as subscripts to the F_{DF} and χ_{DF} statistics.

Relationships among the various metabolites, and among metabolites and phenotypic characters, such as canker size and resinosis, were tested using Pearson product-moment correlation analysis. Where appropriate, homogeneity of independent correlations was tested using the method described by Sheskin [48].

To identify the elicitor preparations and treatments that most closely mimicked the effects of the live infection treatment we performed hierarchical cluster analyses of compound concentrations, under the assumption that similar treatments would induce similar secondary metabolite phenotypes. Data were pooled across trials and dates, since these were not significant factors for the majority of the variables. Mean lignin concentrations and peak areas for each compound in each treatment were standardized using a 0–1 scale. Cluster analysis was carried out for the lower site on the stem since it was directly affected by the treatments. Both squared Euclidean distances and Pearson product-moment correlations algorithms were used.

Table 1
Main effects of time and treatment on resinosis, lesion color, and length of discoloration (mean ranks and Kruskal–Wallis analysis)

Time	Resinosis	Lesion color	Length of discoloration
7 days	72	62	85
14 days	67	65	90
28 days	87	99	96
P	<0.05	<0.001	NS
N	150	150	180
Treatment			
Untreated	N/A	N/A	N/A
Wounding	57	55	68
Inoculation	91	104	156
Killed fungus	73	73	91
Proteins	104	91	122
Lipids	53	55	70
P	<0.001	<0.001	<0.001
N	150	150	180

3. Results

3.1. Canker lesions and other phenotypic characters

Canker lesions were never detected in the wounded, protein, lipid, and nonwounded treatments. Lesions were detected on two trees treated with the killed mycelium (0.5 and 0.9 cm). Lesions were always present at the sites of live inoculations, with mean lengths of 2.6 ± 0.3 cm (range: 1.1–4.1 cm), 4.1 ± 0.5 cm (range: 2–6 cm), and 5.0 ± 0.7 cm (range: 2.3–8.5 cm) at 7, 14, and 28 days, respectively. (Error statistic is standard error of the mean.)

Resinosis, lesion color, and length of discoloration were not significantly affected by trial. Resinosis and lesion color were affected by time, reflecting the progression of symptom development (Table 1). All three phenotypic traits were affected by treatment (Table 1), with live inoculation, killed fungus, and the protein treatments ranking at the top for all three response variables.

Water content of the phloem was 64 ± 0.2% and did not vary by trial, time, or treatment.

3.2. Secondary metabolism

Based on our screening process, we chose to evaluate 15 peaks (known and unknown compounds) that appeared to behave in a treatment-dependent fashion in our HPLC chromatograms of both soluble and cell wall-bound phenolics. All compounds evaluated in this study, listed by their names used in this paper, and their HPLC-related properties are listed in Table 2.

The compounds that were analyzed by Kruskal–Wallis test were: lower site, SumCW, pinosylvin, naringenin-like compound, pinosylvin monomethyl ether, and Pk34.3; upper site, SumCW, Pk8.9, taxifolin-like compound, pinosylvin, pinosylvin monomethyl ether, and Pk34.3. For all of these compounds, trial was a significant factor only for Pk8.9 ($\chi^2_1 = 7.2, N = 180, P < 0.01$) at the lower site. Time was a significant factor only for Pk34.3 ($\chi^2_2 = 7.7, N = 180, P < 0.05$) at the upper site.

ANOVA for data from the lower site indicated that trial was a significant factor only for Pk8.9 ($F_{1,179} = 7.1$; $P < 0.01$), catechin-like compound ($F_{1,179} = 19.6$; $P < 0.001$), and Pk3.9 ($F_{1,179} = 25.8$; $P < 0.001$). Time was a significant factor only for ferulic acid ($F_{2,179} = 3.3$; $P < 0.05$). For interactions, time × trial × treatment was significant only for Pk8.9 ($F_{10,179} = 2.1$; $P < 0.05$).

Table 2
Metabolites selected for analysis

Compound	Approx. retention time (min)	Cellular localization	Spectral match[a]	Detector channel for quantification	Response factor (RF)[b]
Pk8.9	8.9	Soluble	None	Fluorescence	NA
Pk11.3	11.3	Soluble	None	308 nm	NA
Pk12.2	12.2	Soluble	None	308 nm	NA
Ferulic acid glucoside	12.8	Soluble	Ferulic acid glucoside	Fluorescence	7573
Catechin-like compound	14.7	Soluble	Catechin	280 nm	NA
Taxifolin-like compound	18.9	Soluble	Taxifolin	Fluorescence	NA
Taxifolin	20.4	Soluble	Taxifolin	308 nm	9897
Pinosylvin[c]	28.3	Soluble	Pinosylvin	Fluorescence	50,948
Naringenin -like compound	30.3	Soluble	Naringenin	308 nm	NA
Pinosylvin monomethyl ether[c]	31.6	Soluble	Pinosylvin monomethyl ether	Fluorescence	65,512
Pk34.3	34.3	Soluble	None	Fluorescence	NA
Pk37.1	37.1	Soluble	None	280 nm	NA
Pk2.3	2.3	Cell wall-bound	None	280 nm	NA
Pk3.9	3.9	Cell wall-bound	None	280 nm	NA
Ferulic acid	18.3	Cell wall-bound	Ferulic acid	308 nm	19,689

[a] Match to spectra obtained for a suite of pure standards under the same separation conditions.
[b] Slope of linear calibration curve through zero: $y = \mathrm{RF} \times x$, where y is peak absorbance area and x is compound concentration in μg ml^{-1}. Injection volume was 5 μl.
[c] These compounds are collectively referred to as 'stilbenes' throughout the text.

The ANOVA for the upper site showed that trial was a significant factor only for Pk2.3 ($F_{1,179} = 9.0$; $P < 0.01$), and Pk3.9 ($F_{1,179} = 113.1$; $P < 0.001$). For interactions, time × trial was significant only for Pk2.3 ($F_{2,179} = 4.2$; $P < 0.05$), and Pk3.9 ($F_{2,179} = 5.0$; $P < 0.01$). Since there were few factor effects, other than treatment, only main treatment effects on molecular components are shown in Tables 3 and 4.

At the lower site the soluble compounds taxifolin-like compound, taxifolin, naringenin-like compound, pinosylvin, pinosylvin monomethyl ether, Pk34.3, and Pk37.1 were found at significantly higher concentrations at the live inoculation site than at all other treatment sites. Based upon either mean or median concentrations (depending on the distribution of the data, Table 3), most of these compounds accumulated several fold over the wounded control, and up to over 100-fold in the case of pinosylvin monomethyl ether and Pk34.3.

On the other hand, the soluble compounds Pk8.9, Pk11.3, ferulic acid glucoside, catechin-like compound, and the cell wall-bound compounds Pk3.9 and ferulic acid generally decreased in the live inoculation treatment compared to the other treatments. SumSol and SumCW were invariable across treatments. Generally, the killed fungus, lipid, and protein treatments caused variations in compound concentrations that were intermediate between the live inoculation and the wounded treatments, and with the exception of ferulic acid, peak behaviors in the wounded and nonwounded controls were statistically indistinguishable.

At the upper site, the compounds showing treatment dependence were soluble taxifolin-like compound, pinosylvin monomethyl ether, Pk34.3, and cell wall-bound Pk2.3. Based upon either mean or median concentrations (depending on the distribution of the data, Table 4), most of these compounds accumulated several fold higher in the live inoculation treatment over the wounded treatment (up to 2.9-fold in the case of taxifolin-like compound and at least 3-fold in the case of pinosylvin monomethyl ether). Cell wall-bound ferulic acid decreased in the live inoculation treatment relative to the nonwounded control, but not the wounded treatment. Again, the killed fungus, lipid, and protein treatments caused variations in compound concentrations that were generally intermediate between the live inoculation and wounded treatments, and behaviors in the wounded and nonwounded controls were statistically indistinguishable.

Lignin concentration was significantly higher in the live inoculation treatment than in all other treatments at the lower site. However, no significant treatment effects were detected at the upper site.

Cluster analysis of secondary metabolite data for the lower site using two different algorithms yielded dendrograms with identical topologies (Fig. 1). The killed fungus treatment was most closely related to the live inoculation, followed by the protein treatment, wounded, nonwounded, and lipid treatments. However, the overall similarity between live inoculation and killed fungus and protein treatments was rather low.

3.3. Canker correlations

The relationships between canker length and lignin accumulation in the live inoculation treatment at the different dates are shown in Fig. 2. Canker length across all dates was positively, but non-significantly correlated with lignin concentration. At seven days there was a significant positive correlation between canker and lignin ($r = 0.729$, $N = 8$, $P < 0.05$), while at 14 and 28 days the correlations were negative and non-significant (14 days: $r = -0.417$, $N = 9$, $P = 0.26$; 28 days: $r = -0.071$, $N = 9$, $P = 0.85$). The correlations among all three dates were not significantly different ($\chi^2_2 = 5.9$, $P < 0.1$), but between 7 and 14 days they were significantly different ($\chi^2_1 = 12.3$, $P < 0.001$). This suggests that a change had occurred in lignification response to the pathogen during the second week.

Over all dates, lesion length was negatively correlated with Pk8.9 ($r = -0.419$, $N = 30$, $P < 0.05$), Pk11.3 ($r = -0.382$, $N = 30$, $P < 0.05$), and ferulic acid glucoside ($r = -0.374$, $N = 30$, $P < 0.05$), but positively correlated with taxifolin ($r = 0.438$, $N = 30$, $P < 0.05$), pinosylvin monomethyl ether ($r = 0.367$, $N = 30$, $P < 0.05$), and SumCW ($r = 0.447$, $N = 30$, $P < 0.05$). However, no significant correlations were found between canker length and any of the compounds at each individual date. All other correlations were not significant.

4. Discussion

In this study we demonstrate that Austrian pine responds to infection of the lower stem by *S. sapinea* with significant, localized and systemic alteration in the concentrations of secondary metabolites putatively involved in defense. In particular, we show significant systemic accumulation of antimicrobial stilbenes and several unknown soluble phenolics, as well as a significant depletion in the amount of ferulic acid esterified to the cell walls of phloem tissues. Furthermore, killed mycelium and a fungal protein extract elicited visual responses, such as amount of resinosis and lesion color, that partly mimicked the live inoculation treatment. Killed mycelium and the fungal protein extract also elicited changes in host local secondary metabolism that were generally in the same direction, if not always of the same magnitude, as those elicited by a live inoculation. The non-randomness of these effects was reflected in the cluster analysis depicted in Fig. 1 and suggests that these elicitor preparations may be exploited in the future to dissect localized host responses in the absence of a live infection.

It could be argued that the systemic physiological changes induced by fungal infection affect the environment in which a challenge by *S. sapinea*, or other pathogens,

Table 3
Mean peak area or compound concentration by treatment at lower stem site

Compound	Treatments						
	Nonwounded	Wounded	Live inoculation	Killed fungus	Proteins	Lipids	Live inoculation/ wounded[a] (significance)
Lignin[b] (mg g FW^{-1})	9.6 bc (1.1)	8.4 bc (1.0)	15.3 a (1.6)	7.9 bc (0.9)	10.7 ac (1.4)	8.7 bc (1.5)	1.8 ($P < 0.001$)
SumSol[b]	2.3×10^7 a (1.5×10^6)	2.4×10^7 a (1.3×10^6)	2.3×10^7 a (1.2×10^6)	2.4×10^7 a (1.4×10^6)	2.6×10^7 a (1.1×10^6)	2.3×10^7 a (1.5×10^6)	1.0 (NS)
SumCW[c]	1.2×10^7 a (2.7×10^6)	1.2×10^7 a (2.9×10^6)	1.2×10^7 a (5.1×10^6)	1.1×10^7 a (2.3×10^6)	1.1×10^7 a (2.9×10^6)	1.2×10^7 a (3.4×10^6)	1.0 (NS)
Pk8.9[b]	3.0×10^5 b (1.2×10^4)	3.1×10^5 b (1.6×10^4)	2.0×10^5 a (1.9×10^4)	2.8×10^5 b (1.4×10^4)	2.6×10^5 b (1.0×10^4)	3.0×10^5 b (1.9×10^4)	0.6 ($P < 0.001$)
Pk11.3[b]	4.7×10^5 b (5.2×10^4)	4.7×10^5 b (4.6×10^4)	2.8×10^5 a (4.6×10^4)	3.7×10^5 ab (4.6×10^4)	4.4×10^5 b (6.0×10^4)	5.6×10^5 b (9.5×10^4)	0.6 ($P < 0.001$)
Pk12.2[b]	1.3×10^5 a (1.7×10^4)	1.7×10^5 a (2.7×10^4)	1.3×10^5 a (1.3×10^4)	1.6×10^5 a (2.2×10^4)	1.3×10^5 a (1.6×10^4)	1.6×10^5 a (2.1×10^4)	0.8 (NS)
Ferulic acid glucoside[b] (μmol g FW^{-1})	3.5 b (0.3)	3.9 b (0.4)	2.1 a (0.3)	3.1 b (0.2)	3.0 ab (0.3)	3.9 b (0.4)	0.5 ($P < 0.001$)
Catechin-like compound[b]	1.0×10^6 ab (8.9×10^4)	1.1×10^6 b (7.4×10^4)	7.8×10^5 a (7.9×10^4)	1.1×10^6 ab (8.7×10^4)	1.0×10^6 ab (8.0×10^4)	1.1×10^6 ab (1.1×10^5)	0.7 ($P < 0.01$)
Taxifolin-like compound[b]	6.9×10^4 b (7.7×10^3)	7.9×10^4 b (8.9×10^3)	2.0×10^5 a (3.3×10^4)	8.7×10^4 b (7.9×10^3)	9.1×10^4 b (1.2×10^4)	8.2×10^4 b (8.3×10^3)	2.9 ($P < 0.001$)
Taxifolin[b] (nmol g FW^{-1})	76.8 b (14.9)	67.8 b (10.2)	325.8 a (57.7)	189.6 b (46.9)	78.6 b (10.7)	69.9 b (11.3)	4.8 ($P < 0.001$)
Pinosylvin[c] (nmol g FW^{-1})	6.2 b (0.0)	6.2 b (0.0)	81.1 a (157.2)	6.2 b (12.2)	6.2 b (0.0)	6.2 b (0.0)	13.1 ($P < 0.001$)
Naringenin-like compound[c]	7.0×10^3 b (0)	7.0×10^3 b (0)	4.0×10^4 a (6.5×10^4)	7.0×10^3 b (0)	7.0×10^3 b (0)	7.0×10^3 b (0)	5.7 ($P < 0.001$)
Pinosylvin monomethyl ether[c] (nmol g FW^{-1})	4.5 b (0)	4.5 b (0)	456.3 a (657.7)	27.5 b (58.2)	4.5 b (7.9)	4.5 b (0.6)	101.4 ($P < 0.001$)
Pk34.3[c]	7.0×10^3 d (1.2×10^4)	1.3×10^4 b,d (2.8×10^4)	1.3×10^6 a (1.4×10^6)	7.9×10^4 c (1.3×10^5)	4.1×10^4 b,c (4.1×10^4)	1.2×10^4 b,d (2.1×10^4)	100.4 ($P < 0.001$)
Pk37.1[b]	4.5×10^5 cd (1.3×10^5)	9.3×10^5 cd (5.3×10^5)	6.3×10^6 a (8.0×10^5)	3.0×10^6 b (9.0×10^5)	1.2×10^6 bc (2.5×10^5)	5.8×10^5 d (1.1×10^5)	6.7 ($P < 0.001$)
Pk2.3[b]	9.0×10^5 a (3.0×10^4)	9.0×10^5 a (2.6×10^4)	9.4×10^5 a (3.3×10^4)	9.9×10^5 a (2.4×10^4)	9.3×10^5 a (2.6×10^4)	9.8×10^5 a (2.3×10^4)	1.0 (NS)
Pk3.9[b]	3.7×10^6 ab (1.2×10^5)	3.8×10^6 b (7.8×10^4)	3.3×10^6 a (1.3×10^5)	3.8×10^6 b (7.9×10^4)	3.8×10^6 b (9.5×10^4)	4.0×10^6 b (1.3×10^5)	0.9 ($P < 0.01$)
Ferulic acid[b] (μmol g FW^{-1})	3.4 d (0.1)	2.5 c (0.1)	1.1 a (0.1)	2.0 b (0.1)	2.1 b (0.1)	2.7 c (0.1)	0.4 ($P < 0.001$)

Except where indicated, values are peak area absorbance or fluorescence units per 5 μl injection (Table 1). 7.0×10^3 is used as an arbitrary peak area for undetected compounds (see text).

[a] Ratio is based on either mean or median, depending on values in the table (see footnotes b and c).

[b] Means (SE). Means were transformed for analysis where appropriate to satisfy ANOVA's assumptions. Values in each row followed by different letters are different at $P < 0.05$. Means with an SE of zero correspond to the arbitrary values used for undetected compounds.

[c] Medians (interquartile range). Medians were used in Kruskal–Wallis analysis. Values in each row followed by different letters are different at $P < 0.05$. Medians with an interquartile range of zero correspond to the arbitrary values used for undetected compounds.

Table 4
Mean peak area or compound concentration by treatment at upper stem site

Compound	Treatments						
	Nonwounded	Wounded	Live inoculation	Killed fungus	Proteins	Lipids	Live inoculation/ wounded[a] (significance)
Lignin[b] (mg g FW^{-1})	7.3 a (0.5)	7.6 a (0.5)	7.5 a (0.5)	7.1 a (0.4)	6.7 a (0.5)	6.9 a (0.5)	1.0 (NS)
SumSol[b]	1.5×10^7 a (9.5×10^5)	1.3×10^7 a (8.9×10^5)	1.5×10^7 a (9.9×10^5)	1.3×10^7 a (6.9×10^5)	1.3×10^7 a (1.1×10^6)	1.3×10^7 a (9.1×10^5)	1.1 (NS)
SumCW[c]	9.1×10^6 a (1.5×10^6)	9.0×10^6 a (2.3×10^6)	9.1×10^6 a (1.5×10^6)	9.2×10^6 a (1.7×10^6)	9.5×10^6 a (2.1×10^6)	9.1×10^6 a (2.0×10^6)	1.0 (NS)
Pk8.9[c]	3.0×10^5 a (8.7×10^4)	3.2×10^5 a (8.8×10^4)	3.2×10^5 a (9.4×10^4)	3.3×10^5 a (8.0×10^4)	3.0×10^5 a (7.7×10^4)	3.0×10^5 a (9.0×10^4)	1.0 (NS)
Pk11.3[b]	4.1×10^5 a (5.9×10^4)	4.7×10^5 a (5.6×10^4)	4.9×10^5 a (6.1×10^4)	4.1×10^5 a (3.9×10^4)	5.0×10^5 a (7.4×10^4)	5.3×10^5 a (8.8×10^4)	1.0 (NS)
Pk12.2[b]	1.5×10^5 a (2.0×10^4)	1.4×10^5 a (2.0×10^4)	1.6×10^5 a (2.2×10^4)	1.8×10^5 a (2.2×10^4)	1.4×10^5 a (1.7×10^4)	1.5×10^5 a (1.6×10^4)	1.1 (NS)
Ferulic acid glucoside[b] (μmol g FW^{-1})	3.1 a (0.3)	3.7 a (0.4)	3.4 a (0.3)	3.4 a (0.2)	3.2 a (0.3)	3.5 a (0.4)	0.9 (NS)
Catechin-like compound[b]	8.9×10^5 a (5.4×10^4)	7.4×10^5 a (4.9×10^4)	8.6×10^5 a (5.1×10^4)	7.9×10^5 a (3.9×10^4)	7.7×10^5 a (5.8×10^4)	7.4×10^5 a (5.0×10^4)	1.2 (NS)
Taxifolin-like compound[c]	3.3×10^4 b (1.4×10^4)	3.7×10^4 b (2.3×10^4)	1.1×10^5 a (3.2×10^4)	3.2×10^4 b (1.3×10^4)	3.2×10^4 b (2.2×10^4)	2.8×10^4 b (1.9×10^4)	2.9 ($P < 0.001$)
Pinosylvin[c] (nmol g FW^{-1})	6.2 a (0)	6.2 a (0)	6.2 a (0)	6.2 a (0)	6.2 a (0)	6.2 a (0)	1.0 (NS)
Pinosylvin monomethyl ether[c] (nmol g FW^{-1})	4.5 b (0)	4.5 b (0)	13.7 a (22.1)	4.5 b (0)	4.5 b (0)	4.5 b (0)	3.0 ($P < 0.05$)
Pk34.3[c]	7.0×10^3 b (0)	7.0×10^3 b (0)	1.5×10^4 a (3.0×10^4)	7.0×10^3 b (0.7×10^3)	7.0×10^3 b (0)	7.0×10^3 b (0)	2.2 ($P < 0.001$)
Pk37.1[b]	2.4×10^5 a (2.3×10^4)	2.7×10^5 a (4.9×10^4)	2.9×10^5 a (9.4×10^4)	2.7×10^5 a (4.0×10^4)	2.7×10^5 a (3.3×10^4)	2.3×10^5 a (2.3×10^4)	1.1 (NS)
Pk2.3[b]	9.0×10^5 b (2.5×10^4)	9.0×10^5 b (2.5×10^4)	1.0×10^6 a (1.9×10^4)	9.3×10^5 ab (3.4×10^4)	9.0×10^5 ab (2.2×10^4)	9.4×10^5 ab (2.6×10^4)	1.1 ($P < 0.01$)
Pk3.9[b]	3.1×10^6 a (1.1×10^5)	3.1×10^6 a (1.1×10^5)	3.2×10^6 a (9.9×10^4)	3.0×10^6 a (1.5×10^5)	3.2×10^6 a (9.8×10^4)	3.1×10^6 a (1.4×10^5)	1.0 (NS)
Ferulic acid[b] (μmol g FW^{-1})	3.7 b (0.1)	3.6 ab (0.1)	3.1 a (0.1)	3.5 ab (0.1)	3.6 ab (0.1)	3.7 b (0.1)	0.9 (NS)

Except where indicated, values are peak area absorbance or fluorescence units per 5 μl injection (Table 1). 7.0×10^3 is used as an arbitrary peak area for undetected compounds (see text).

[a] Ratio is based on either mean or median, depending on values in the table (see footnotes b and c).

[b] Means (SE). Means were transformed for analysis where appropriate to satisfy ANOVA's assumptions. Values in each row followed by different letters are different at $P < 0.05$. Means with an SE of zero correspond to the arbitrary values used for undetected compounds.

[c] Medians (interquartile range). Medians were used in Kruskal–Wallis analysis. Values in each row followed by different letters are different at $P < 0.05$. Medians with an interquartile range of zero correspond to the arbitrary values used for undetected compounds.

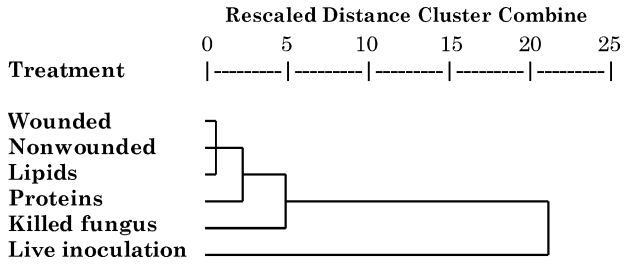

Fig. 1. Dendrogram resulting from cluster analysis of data from the lower stem site, grouped by treatment. Squared Euclidean distances applied to mean values in Table 3 were used to evaluate associations among treatments.

will operate. If some of the accumulated metabolites have fungitoxic or fungistatic properties against *S. sapinea*, they may account, at least partially, for localized resistance and for the SIR phenotype we have observed in this system in a preliminary study [2]. Perhaps, a way to evaluate the potential function of these metabolites in a systemic defense response against *S. sapinea* is by examining the role of the same compounds at the induction site, i.e. in the localized defense response. Our basic hypothesis is, if a compound or process is involved in resistance, the concentration of that compound or the intensity of that process at the margin of infected tissues (the reaction zone) would be negatively correlated with lesion length.

Induced lignification of cell walls is considered a main process by which plants resist pathogenic invasion. In our system, the correlation changed from positive to negative over the course of two weeks post-inoculation, suggesting that the host was attempting to wall off the pathogen with some success after an initial lag period of one week. By the fourth week, there was no correlation between canker size and lignin accumulation, with lignin content remaining at the levels found at two weeks (Fig. 2). This suggests that

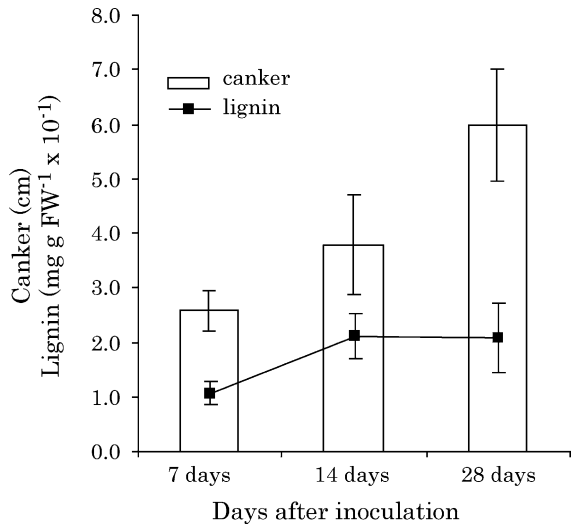

Fig. 2. Relationship between canker size and induced lignin accumulation in the live inoculation treatment. The correlation between canker size and lignin changed from positive to negative between the first and second weeks, but was not significant thereafter.

lignification might have reached a maximum at two weeks, after which plants were unable to contain the pathogen through an induced lignification process. Thus, while lignification might play a role in pathogen limitation in our system, it appears to only have a moderate overall effect in defense at the induction site.

In conifers, stilbenes are generally described as phytoalexin-like compounds or phytoanticipins, rather than true phytoalexins, because they are strongly antimicrobial in vitro but are often present constitutively in certain tissues rather than appearing de novo following infection [38]. Because of their behavioral properties, they have been implicated in defense of conifers against pathogens and wood decay fungi [11,16,32,37,47,56]. However, in our study, the correlations between stilbene concentrations and canker size were always non-significant and mostly positive, at all dates as well as at individual dates (data not shown). This occurred at a time when stilbene concentration increased 13-fold and 100-fold in live inoculations for pinosylvin and pinosylvin monomethyl ether, respectively. Thus, despite the fact that these compounds are strongly antifungal in vitro [7], their role as defensive molecules involved in actual resistance in planta in our system is questionable. This confirms previous results in which these compounds behaved more like markers of disease than like defensive chemicals involved in resistance [9], and were demonstrated to be autotoxic in an in vitro study of the Scots pine/*Heterobasidion annosum* pathosystem [13].

Most other soluble and cell wall-bound metabolites (including the total soluble and wall-bound fractions) also were either not correlated or positively correlated to canker size. This would exclude them as having a role in resistance under our hypothesis, despite increasing up to over 100-fold in the case of Pk34.3 at the induction site. Only three metabolites, Pk8.9, Pk11.3, and ferulic acid glucoside were negatively correlated with canker size, and significantly across all dates. Thus, these compounds may exert a negative effect on the pathogen in planta. However, until these compounds are tested in vitro against the pathogen and are demonstrated to have fungitoxic or fungistatic properties, it is not possible to know whether they might have a role in resistance. The negative correlations we found may simply be the result of the pathogen catabolizing these compounds, with depletion occurring where the pathogen is more abundantly present in host tissues.

Other than the stilbenes pinosylvin and pinosylvin monomethyl ether, only one compound appears to be absent in unwounded trees and present after infection. At the lower site, naringenin-like compound was consistently detected in the live inoculation treatment, but only in one or two trees in the wounded, killed fungus, lipid, and protein treatments, and never in the untreated group. If this compound displayed antifungal properties in vitro it could be classified as a phytoalexin. However, it is doubtful it has a role in

resistance as its concentrations were not negatively correlated with canker size.

Interestingly, the flavonoid taxifolin was present constitutively at the lower site and its accumulation was significantly induced there by fungal infection, similarly to other pathosystems [21]. However, it was never detected at the upper site. The significance of this longitudinal expression differential for this compound is unknown at present.

A lack of clear pathogen-limiting local effects by secondary metabolites in our system might appear surprising, as secondary metabolism has often been suggested to be implicated in resistance, particularly in the conifer literature. Three non-mutually exclusive hypotheses might explain this: (1) canker lesion is not related to actual disease resistance and therefore our assumption is flawed; (2) secondary metabolites and secondary metabolic processes are not involved in defense in our pathosystem, and other concurrent host defense responses are more important in resistance in Austrian pine; and (3) secondary metabolites do contribute to defense in this pathosystem by attaining anti-microbial concentrations at the infection front more quickly in more resistant plants even though, overall, concentrations are higher in more susceptible plants; however, the measurements made in this study at fixed distances from the inoculation site did not reflect the local concentrations at the infection front.

With respect to hypothesis 1, while canker length is commonly used to assess disease levels, it could be argued that lesion width, which is directly related to girdling potential, may be a better measure of disease susceptibility. We did not measure lesion width in our system. However, studies conducted on an etiologically similar pathosystem, pitch canker of Monterey pine (*Pinus radiata* D. Don) caused by *Fusarium circinatum* Nirenberg and O'Donnell, showed that lesion length is an excellent predictor of actual disease resistance, at time scales ranging from a few weeks to a few months [29]. In our experiments, lesion length ranged from 1.1–4.1 cm at one week, to 2.3–8.5 cm at four weeks. While at present we are not certain that this range of lesion lengths encompasses both highly susceptible and highly resistant phenotypes, we know that the seed sources used in this experiments are open pollinated families of wide geographic provenance in Turkey (Ridge Manor Nursery, personal communication). Pines are known to have highly heterogeneous genetic background even at local geographic scales in native stands [36] and this can be reflected in a wide range of resistance levels to canker pathogens [51]. Therefore, we believe that the lesion development responses in our pathosystem reflect a working range of quantitative resistance that is adequate for studies of defense mechanisms in Austrian pine. However, a definitive test of this hypothesis with a longer term evaluation of the relationship between lesion length and resistance is needed.

If our assumption of a direct relationship between canker length and disease susceptibility is valid, hypothesis 2 might be relevant to our results, because most studies have evaluated phenolic defense responses in conifers and other systems in isolation from other defense responses. Our study is no exception. Therefore, it is possible that the role in resistance generally attributed to phenolic metabolism in conifers was often masking other unexplored, but more significant defense mechanisms at the local level. These might include production of cell wall appositions [14], suberization of host cell walls [42], accumulation of PR-proteins [19,57], terpenoids [9,22,25,40], and deposition of phenolic polymers. Deposition of phenolic polymers might be the result of condensation of some of the soluble compounds we have investigated, such as the stilbenes [43]. Furthermore, while accumulation of secondary metabolites has been observed in innumerable host–pathogen interactions, in most cases this phenomenon has not been unequivocally correlated with resistance [54]. This is particularly true for conifers, where most studies on many other host–pathogen systems have been qualitative in nature, due to the difficulty of quantifying disease levels. Our model system allows for quantification of disease expression and therefore we are in a position to begin making predictions regarding the role of secondary metabolism in pine resistance to a necrotrophic canker pathogen.

If tissues were sampled at locations other than the reaction zones, chemical analyses might not reveal functional concentrations of antimicrobial compounds at the infection front (hypothesis 3) [41]. This might be a concern because sampling exactly at the infection front in our pathosystem is impossible in the absence of wounding to expose the lesion. We opted to avoid the wounding effect during sampling and instead maximized the probability of harvesting the reaction zone by taking bark samples midway between the treatment wounds, since the bark plugs were 10 mm in diameter and the treatment wounds were approx. 2–3 cm from each other around the stem. Using this sampling strategy we covered 33–50% of the space between the treatment wounds, making it plausible that we captured the reaction zone in most, if not all, cases. As for the possible faster accumulation of antimicrobial compounds in more resistant plants, this could have occurred before our first sampling date of one week after treatment. Consequently, we plan to conduct experiments to study the effect of infection on secondary metabolism during the first few hours/days after treatment.

Whatever the actual resistance mechanisms may be at the induction site, extrapolation of the localized responses to a systemic host response may be misleading. It is possible that after an initial infection, which may involve a hypersensitive reaction-type host response, the whole tree becomes pre-conditioned to respond more successfully to a subsequent infection [39]. In other words, the plant tissues may become competent and potentiated [30] at a systemic level.

The success of a resistance response involving secondary metabolism is often predicated on the speed of deployment, rather than its intensity [54]. Therefore, systemically elevated stilbene concentrations may play a role at the onset of a second pathogenic challenge, even though these compounds appear neutral in the localized response to the inducing infection. Additionally, the host might be able to respond to a challenge infection with a faster lignification response, even though baseline lignin content was not affected systemically in our study. Thus, systemic potentiation of defense responses based on secondary metabolism remains a possibility to explain SIR, which is expressed in our system [2].

It is not known at present whether the systemic effects observed in this study are due to mass upward metabolite translocation in the phloem or remote signaling and systemic up-regulation of gene activity. Other studies have presented convincing evidence that salicylates and jasmonates can have strong regulatory effects of defense responses when applied to conifers exogenously [19,22,25,45]. While signaling by these molecules cannot be excluded, SA was never detected in our study or in other pathosystems, including the Monterey pine pitch canker, and the ponderosa pine and Scots pine/*H. annosum* pathosystems (Bonello et al., unpublished results).

We believe the Austrian pine/*S. sapinea* model system will be valuable in the investigation of factors involved in local and systemic pine resistance to canker pathogens, particularly if host clonal populations of resistant and susceptible genotypes can be established. The relative ease with which this model system can be manipulated will make it an important tool for studying these interactions at the genomic and proteomic levels.

Acknowledgements

The authors wish to thank Ridge Manor nursery for providing the plant material used in this study, Laura Wutz for technical assistance, and Harold Brown for greenhouse assistance. Werner Heller is thanked for synthesizing ferulic acid glucoside for us. We are thankful to Brian B. McSpadden Gardener for assistance with parts of the statistical analysis, and Terry L. Graham, Brian B. McSpadden Gardener, and Steve Woodward for helpful comments on the manuscript.

Salaries and research support provided in part by State and Federal funds appropriated to the Ohio Agricultural Research and Development Center, the Ohio State University.

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