Systemic induction of phloem secondary metabolism and its relationship to resistance to a canker pathogen in Austrian pine

C. Wallis[1], A. Eyles[1], R. Chorbadjian[2], B. McSpadden Gardener[3], R. Hansen[4], D. Cipollini[5], D. A. Herms[2] and P. Bonello[1]

[1]Department of Plant Pathology, The Ohio State University, 201 Kottman Hall, 2021 Coffey Road, Columbus, OH 43210, USA; Departments of [2]Entomology, [3]Plant Pathology and [4]Agricultural Engineering, The Ohio State University/Ohio Agricultural Research and Development Center, 1680 Madison Ave, Wooster, OH 44691, USA; [5]Department of Biological Sciences, Wright State University, 208 Biological Sciences Building, 3640 Colonel Glenn Hwy, Dayton, OH 45435, USA

Author for correspondence:
C. Wallis
Tel: +1 614 688 5409
Fax: +1 614 292 4455
Email: wallis.23@osu.edu

Received: *5 October 2007*
Accepted: *6 October 2007*

Summary

- The mechanisms and conditions affecting expression of systemic induced resistance (SIR) in pine are not clearly understood. Two hypotheses were tested here: that SIR against a pathogen induced by either a pathogen or an insect involves coordinated shifts in phloem secondary metabolism; and that fertility affects the production of these compounds.
- To test these hypotheses, a tripartite system was used comprising Austrian pine (*Pinus nigra*) grown under three different fertility regimes, the fungal pathogen *Diplodia pinea*, and the defoliator *Neodiprion sertifer*.
- Fungal induction led to systemic accumulation of lignin, phenolic glycosides and stilbenes, whereas insect defoliation led to an increase in germacrene D concentration in branch phloem. Fertility affected the concentrations of only the phenolic glycosides. Multivariate analyses showed coregulation of compounds within at least three consistent groupings: phenolic glycosides, stilbenes and monoterpenes. As groups and as individual compounds, accumulation of phenolic glycosides and stilbenes was negatively correlated with disease susceptibility.
- The experimental manipulation of the phenolics and terpenoids metabolic networks achieved in this study by biotic induction and changes in nutrient availability suggests that lignin, phenolic glycosides and stilbenes are important biochemical factors in the expression of SIR against the pathogen in this system.

Key words: chemistry, defense, *Diplodia pinea*, *Neodiprion sertifer*, phloem, *Pinus nigra*, systemic induced resistance.

New Phytologist (2008) **177**: 767–778

No claim to original US government works.
Journal compilation © *New Phytologist* (2007)

doi: 10.1111/j.1469-8137.2007.02307.x

Introduction

Conifers have developed both constitutive and inducible defenses to ward off attack from pathogens (Franceschi *et al.*, 2005; Schmidt *et al.*, 2005). Constitutive defenses act to repel or inhibit pathogen access, whereas inducible defenses act to kill or compartmentalize invaders once an attack has begun (Franceschi *et al.*, 2005; Bonello *et al.*, 2006; Keeling & Bohlmann, 2006). Induced defenses comprise both structural and biochemical elements to slow pathogen progress. For example, cell wall appositions and accumulation of secondary metabolites (e.g. phenolics and terpenoids, lignin) and pathogenesis-related proteins are thought to contribute to pathogen containment (Bonello *et al.*, 2006; Keeling &

Bohlmann, 2006). There is evidence that this is sometimes followed by systemic induction of defenses to respond more quickly to further attacks elsewhere on the plant (Bonello *et al.*, 2003; Blodgett *et al.*, 2007). All these defenses, acting in concert, can directly antagonize the pathogen by sealing off its access to vulnerable tissue, limiting spread, or killing the pathogen through toxic activity (Bonello *et al.*, 2006).

Induced plant defense responses consist of two major types: those associated with local induced resistance (in tissues surrounding an infection site); and those associated with systemic induced resistance (SIR), which is a phenotype characterized by increased resistance to subsequent attacks on distal parts of a plant (Bonello *et al.*, 2006). While SIR phenotypes in conifers are known to occur (Christiansen *et al.*, 1999; Enebak & Carey 2000; Bonello *et al.*, 2001; Franceschi *et al.*, 2005; Zeneli *et al.*, 2006; Blodgett *et al.*, 2007; Swedjemark *et al.*, 2007), the chemistry that mediates this systemic response is poorly understood (Bonello *et al.*, 2006). As part of the SIR phenomenon, it is assumed that coregulation of plant defenses occurs, and this coregulation is important in mounting defenses against multiple threats (Stout *et al.*, 2006).

Plants are often attacked simultaneously by a variety of pests throughout the entire organism in any growing season, so there is increasing interest in studying host-mediated cross-effects between insects and pathogens (Franceschi *et al.*, 2005). Reviews that focus mostly on interactions between pathogens and insects mediated by herbaceous and woody angiosperms found that pathogen infection had varied effects on the behavior or performance of foliage-feeding insects, and insect attack had varied effects on the growth of fungal pathogens (Rostas *et al.*, 2003; Stout *et al.*, 2006). Insect herbivory on the same part of the plant (leaves) that was later infected by a fungal pathogen had negative effects (Hatcher *et al.*, 1994; Russo *et al.*, 1997; Moran & Schultz, 1998; Stout *et al.*, 1999; Hatcher & Paul, 2000); no effects (Russo *et al.*, 1997; Moran & Schultz, 1998); or positive effects (Padgett *et al.*, 1994; Simon & Hilker, 2003) on fungal growth or disease development.

An additional and often overlooked factor in the development and deployment of host defenses is nutrient availability to the host. Many studies have examined the effects of fertility on constitutive defenses, especially against insects, and their results have varied (Kytö *et al.*, 1999; Herms, 2002; vanAkker *et al.*, 2004; Koricheva *et al.*, 2004), in part because nutrient availability may have nonlinear effects on these responses (Herms & Mattson, 1992; Herms, 2002). The nutrient status of a plant determines the amount of resources that the plant can allocate to constitutive defenses (Franceschi *et al.*, 2005). The effects of nutrient availability on constitutive defense chemistry in conifers are little understood, but even less studied are the effects of nutrient availability on induced defense responses, both locally and systemically (Bonello *et al.*, 2006). The few studies that have addressed this question in conifers have found that increased fertilization often can lead to increased severity of fungal diseases (Entry *et al.*, 1991; Blodgett *et al.*, 2005).

In a companion paper, Eyles *et al.* (2007) showed that a fungal pathogen can affect insect performance and survival indirectly through their shared pine host and *vice versa*. To test that hypothesis, we used the Austrian pine (*Pinus nigra* Arnold)/*Diplodia pinea* (Desm.) Kickx. model system for SIR in conifers, as described by Bonello & Blodgett (2003); Blodgett *et al.* (2007). To this system we added the European pine sawfly (*Neodiprion sertifer* (Geoff.)), which is an insect defoliator of *P. nigra*. This tripartite system was subjected to variable nutrient availability. Included in the findings by Eyles *et al.* (2007), two SIR-related phenomena were apparent: fungal induction led to a reduction in subsequent fungal lesion development; and fertilization did not affect the expression of induced resistance to the pathogen (Eyles *et al.*, 2007).

The objective of this study was to profile systemic chemical changes in phloem tissues of induced plants and examine the extent to which they relate to fungal performance. We hypothesized that conifers rely predominantly on systemically inducible, coregulated chemical defenses (both phenolic and terpenoid compounds) to limit the growth of fungal pathogens; and that development of the chemical components of inducible resistance is influenced by the nutrient status of the trees. We evaluated the effects of previous fungal infection and insect defoliation on levels of phloem phenolic and terpenoid metabolism in relation to the development of a subsequent *D. pinea* infection across three fertilization regimes. We also evaluated potential trade-offs between different metabolic pools, such as those associated with the terpenoid and phenolic pathways.

Materials and Methods

Location of the study, plant material, experimental design, fertilization schemes, pathogen and insect induction treatments, and pathogen challenge bioassays are described in detail by Eyles *et al.* (2007). In brief, each of 240 4-yr-old trees was assigned to one of three different fertility treatments applied with irrigation (fertigation): 30, 75 or 150 ppm N fertilization level, using the following ingredients: $Ca(NO_3)_2$, NH_4, H_2PO_4, KNO_3. These were applied in a 3 N : 1 P : 2 K ratio, with all sources of nitrogen adding up to the required ppm. The trees were pretreated under these conditions for the 2004 growing season, and this continued through the 2005 season.

In spring 2005, the following induction treatments were applied in equal replication ($n = 60$ per induction treatment, assigned to five blocks) across the fertilizer-treated trees ($n = 20$ per induction and fertilizer combination): (1) fungal induction, conducted by making a wound with a 10-mm-diameter cork borer in the main stem 5 cm above the soil line and placing an 8-mm-diameter *D. pinea*-colonized agar plug into the hole and securing with duct tape; (2) mock-inoculation, where the fungal induction was mimicked using a noncolonized agar

 No claim to original US government works.
Journal compilation © *New Phytologist* (2007)

plug; (3) insect defoliation, conducted by introducing *c.* 150 *N. sertifer* larvae to each tree, which resulted in 75% defoliation (with 25% of the foliage protected by bagging branches); and (4) nonwounded, untreated control trees. After a 16-d incubation period, the fungal challenge (whereby a branch *c.* 15 cm from the soil line was inoculated using an infected agar plug similar to the fungal induction described above) was applied to half the trees in each induction treatment. At the same time, another branch was removed from every tree for phytochemical analysis. An exception was the insect-defoliated trees, for which only one third of the trees were assayed for phytochemical analysis, and another third underwent the fungal challenge. This was a result of the trees having only four total branches, with three defoliated to obtain the target of 75% defoliation. The remaining, protected branch (25% of foliage) was then utilized for the phytochemical analysis or the fungal challenge. Following a period of 10 d, the fungus-challenged branch was removed and the lesion length just under the outer bark (including the original 10-mm wound) was recorded as a measure of resistance according to the procedure described by Blodgett *et al.* (2007). The rare cases in which no lesion developed beyond the wound were excluded from the statistical analysis. No fungal reisolations from the lesions were attempted in this study, as all previous, similar experiments (e.g. Bonello & Blodgett, 2003) have always yielded the pathogen from lesions resulting from artificial inoculations.

Phytochemical analyses

Phloem samples were obtained from an excised, untreated branch at the same time as the pathogen challenge was initiated, and flash-frozen in liquid N. This tissue was ground to a powder in liquid N, and 100 mg (FW) ground tissue was used for extraction of phenolics and lignin, following the procedures described by Bonello & Blodgett (2003); Blodgett *et al.* (2007), with slight modifications. In brief, methanol-soluble phenolics were obtained by extracting the ground phloem tissue twice in 500 µl HPLC-grade methanol (Fisher, Pittsburgh, PA, USA) for 24 h at 4°C. The supernatants were combined and stored at −20°C until they were analysed by HPLC as described below. Total phenolic content was estimated using a modification of the Folin method of Bonello & Pearce (1993). 1.875 µl methanol extract were added to 73.1 µl methanol and 675 µl water, and mixed. To this, 37.5 µl Folin's phenol reagent (Sigma, St Louis, MO, USA) was added, followed 3 min later by 37.5 µl 1 M $NaHCO_3$ and thorough mixing. After 1 h incubation at room temperature, the samples were analysed spectrophotometrically against a standard curve of gallic acid at 725 nm.

HPLC analysis was conducted using a Waters (Milford, MA, USA) 2690 separations module equipped with a Waters Xterra RP18, 5 µm, 4.6 × 150-mm column and a 996 photodiode array detector. The Waters MILLENNIUM HPLC software was utilized for data acquisition and managing the separation gradient. A binary solvent system was used, with solvent A consisting of 2% glacial acetic acid (Fisher) in HPLC grade water, and solvent B consisting of 2% glacial acetic acid in HPLC-grade methanol. The linear gradient was as used by Bonello & Blodgett (2003). The photodiode array detector was set to scan wavelengths between 237 and 400 nm, with two channels selected for data processing at 280 and 308 nm.

To measure lignin concentration, pellets from the crude phenolic extraction were washed with 1 ml water and 0.9 ml *tert*-butyl methyl ether (Sigma). The pellets were left to dry overnight, then processed according to the methods of Bonello & Pearce (1993).

Terpenoids were quantified by extracting ground phloem tissue (100 mg) twice in 500 µl methyl chloride (Fisher) containing 0.1% (v/v) *p*-cymene (Sigma) (internal standard) for 24 h at 4°C. The supernatants were combined and analysed using gas chromatography (GC). GC analysis was conducted on a Hewlett Packard (Palo Alto, CA, USA) 6890 GC, equipped with an Agilent (Palo Alto, CA, USA) 19091B-102 Ultra 2 5% phenylmethylsiloxane column and a flame ionization detector. The gradient and data acquisition were managed using HP CHEMSTATION software. The oven gradient was based on the method of Martin *et al.* (2002) and was as follows: initial temperature of 40°C for 2 min; ramping at 10°C min^{-1} to 240°C; ramping at 50°C min^{-1} to 300°C, followed by a 5-min hold (total run time 28.20 min). A split-injection mode with a split ratio of 12 : 1 and an initial temperature of 220°C were used. Hydrogen was used as the carrier at a constant flow of 2.1 ml min^{-1}. The front detector was set at 220°C.

Compound identification

Phenolic peaks were identified by a combination of matching UV spectra and retention times with standards or through the use of liquid chromatography–mass spectrometry. Only three compounds that were quantified by HPLC remained unidentified, and these were labeled as unknowns 1–3 (uk1–uk3). An additional peak, labeled uk4, was subsequently discovered to be a coelution of levopimaric and abietic acids, which separated into distinct peaks only on occasion.

Monoterpenes were identified by retention time against the following standards: β-pinene, α-pinene, camphene, myrcene, limonene and bornyl acetate (all Sigma). Other terpenoids were identified using GC–MS and matching to the Wiley Registry of Mass Spectral Data and the NIST Library, using RSI values of at least 850 as a threshold for identification. Confirmation of the sesquiterpenes *trans*-caryophyllene and α-humulene were made by comparison with retention times of commercial standards (Sigma).

Statistical analyses

Statistical analyses were conducted using SPSS 14.0 for WINDOWS (SPSS Inc., Chicago, IL, USA). Treatment effects on lesion

No claim to original US government works.
Journal compilation © *New Phytologist* (2007)

length (which has been used as a measure of resistance: Blodgett *et al.*, 2007; Eyles *et al.*, 2007) and the relative abundance of each individual phytochemical were analysed by three-way univariate ANOVA. The model included three fertility levels, four induction levels, five blocks and all interactions. Fisher's least significant difference (LSD) multiple range tests were then used to separate treatment means ($\alpha < 0.05$).

Natural groupings of the compounds analysed were identified through a combination of cluster analysis and principal components analysis (PCA), as suggested by McSpadden Gardener (2006). Such groupings may be used to infer coordinated regulation when their levels change in concert (McSpadden Gardener, 2006). This methodology of grouping variables in response to multiple treatments has been utilized before (Benitez *et al.*, 2007). For these analyses, the HPLC-derived peak areas were scaled into quintiles. A hierarchical cluster analysis was performed using furthest-neighbor joining based on squared Euclidean distance for each of the separate induction treatments (four dendrograms). Compounds that grouped together in at least three of the four dendrograms were considered a natural group and combined into a cluster. Additionally, PCA was conducted on the data from all treatments by defining components based on eigenvalues > 1. Natural groupings were assessed by evaluating relative placement on ordination plots composed of the first three principal components. The compounds were grouped together by calculating a quintile mean of the clustered compounds for each tree. These groups were inferred to be coregulated, when their rescaled cluster distance from each other was ≤ 17, a distance chosen based on its ability to resolve compounds in part to what was known *a priori* about their chemical classification (phenolics or terpenoids), as well as correspondence with the PCA analysis.

Univariate ANOVAs were carried out on the defined clusters to measure the effects of induction treatment on each group of apparently coregulated compounds, and Fisher's LSD mean separation tests were used to separate effects of induction treatment on each of the cluster groups ($\alpha < 0.05$).

Spearman's ρ correlations were conducted between lesion size (resistance phenotype) and the scaled abundance of each of the individual compounds, and between lesion size and the scaled abundance of apparently coregulated groups defined by the multivariate analyses described above. Likewise, the relative abundances of the various natural groups were correlated with one another. Significant correlations were defined as having $P < 0.05$.

Results

Analysis of individual compounds

Total phenolics, lignin, coumaric acid hexoside, dihydroconiferin II, ferulic acid glucoside, taxifolin hexoside, uk1, taxifolin, pinosylvin and pinosylvin monomethyl ether were all characterized by significantly higher amounts in the fungal induction treatment compared with nonwounded and/or wounded (mock-inoculated) control trees (Table 1). The monoterpenes α-pinene, β-pinene and limonene were present in significantly lower amounts in the insect-defoliation treatment compared with the fungal inoculation treatment, with the controls having intermediate concentrations. (It should be noted that we did not conduct stereoisomer analysis and therefore these compounds were combinations of their enantiomers.) Finally, germacrene D was present in insect-defoliated trees in significantly higher concentrations than in all other treatments.

Fertilization had no significant effects on the concentrations of any of the phloem terpenoids (Table 2). Total phenolics, coumaric acid hexoside, hydroxypropiovanillone hexoside, ferulic acid glucoside and taxifolin hexoside were characterized by significantly lower concentrations in the 150-ppm N treatment compared with the other treatments (Table 2). Uk3 was significantly more concentrated at 150 ppm N compared with the other treatments.

A significant induction × fertilizer interaction was found only with the total phenolics, with greater total phenolic concentration in both fungal and insect defoliator-induced trees than controls in the 30- and 75-ppm treatments (although for 30 ppm the concentration increase was not statistically significant), but lowered phenolic concentration from insect defoliation in the 150 ppm treatment (with no increase/decrease for fungal induction in that treatment). Other significant interactions were found between fertilizer × block for total phenolics, dihydroconiferin I and neoabietic acid; and induction × fertilizer × block for neoabietic acid. The interactions with blocks did not change the patterns of the main effects.

Correlations between individual compounds and lesion size

Hydroxypropiovanillone hexoside, ferulic acid glucoside and uk1 were negatively correlated with lesion length, whereas uk3 and the labdadiene isomer were positively correlated with lesion length (Table 3). Lignin and β-pinene were additional compounds that were marginally ($P < 0.10$) negatively correlated with lesion length.

Coaccumulation of secondary metabolites

Cluster analyses revealed seven consistent groupings whereby compounds occurred together in at least three of the four induction treatments (Fig. 1). Cluster 1 consisted mainly of phenolic hexosides, taxifolin and lignin. Cluster 2 consisted of the monoterpenes except β-pinene. Clusters 3, 4 and 6 consisted of the remaining terpenoids. Cluster 5 consisted of the uk1, pinosylvin and pinosylvin monomethyl ether, and cluster 7 consisted of uk2, uk3 and uk4.

No claim to original US government works.
Journal compilation © *New Phytologist* (2007)

Table 1 Means (SE) for lesion length and concentration of each compound in branch phloem of Austrian pine (*Pinus nigra*); different letters indicate separation of means by LSD within row ($P < 0.05$)

	Phloem											
	Nonwounded			Mock-inoculated			Fungal inoculated			Insect-defoliated		
Lesion length (mm)	21.62	(1.37)	a	19.86	(1.32)	a	13.39	(1.20)	b	20.57	(1.12)	a
Lignin (mg g^{-1} FW)	11.50	(0.492)	b	10.59	(0.530)	b	14.75	(0.505)	a	12.63	(0.852)	b
Total phenolics† (mg g^{-1} FW)	2.57	(0.226)	ab	2.33	(0.225)	b	3.16	(0.217)	a	3.03	(0.370)	ab
Phenolics (AU, unless indicated)												
Dihydroconiferin I	1.52×10^5	(1.14×10^4)		1.25×10^5	(1.12×10^4)		1.41×10^5	(1.12×10^4)		1.28×10^5	(1.96×10^4)	
Coumaric acid hexoside	3.14×10^5	(3.96×10^4)	b	3.42×10^5	(3.79×10^4)	b	5.03×10^5	(5.87×10^4)	a	3.96×10^5	(8.51×10^4)	ab
Hydroxypropiovanillone hexoside	5.20×10^5	(5.04×10^4)		5.13×10^5	(4.48×10^4)		6.05×10^5	(5.28×10^4)		5.21×10^5	(6.92×10^4)	
Dihydroconiferin II	1.34×10^5	(1.44×10^4)	b	1.57×10^5	(1.44×10^4)	ab	1.93×10^5	(1.47×10^4)	a	1.58×10^5	(2.55×10^4)	ab
Ferulic acid glucoside (µg g^{-1} FW)	251	(27.7)	b	319	(28.3)	ab	393	(33.2)	a	377	(48.7)	a
Taxifolin hexoside	4.13×10^5	(3.85×10^4)	b	3.99×10^5	(3.18×10^4)	b	5.50×10^5	(4.65×10^4)	a	4.87×10^5	(6.03×10^4)	ab
Unknown 1	3.88×10^4	(2.08×10^4)	b	3.10×10^4	(1.72×10^4)	b	1.32×10^5	(1.65×10^4)	a	4.68×10^4	(2.95×10^4)	b
Taxifolin (µg g^{-1} FW)	42.0	(3.04)	b	40.1	(2.84)	b	63.3	(5.97)	a	43.8	(4.64)	b
Pinosylvin (µg g^{-1} FW)	24.2	(2.92)	b	22.1	(2.07)	b	57.6	(14.6)	a	19.6	(1.42)	b
Pinosylvin monomethyl ether (µg g^{-1} FW)	4.49	(0.696)	b	3.74	(0.198)	b	19.6	(5.83)	a	6.21	(2.37)	b
Unknown 2	2.78×10^4	(1.98×10^3)		2.65×10^4	(2.21×10^3)		3.29×10^4	(3.24×10^3)		2.16×10^4	(1.53×10^3)	
Unknown 3	1.58×10^4	(1.49×10^3)		1.39×10^4	(1.60×10^3)		1.38×10^4	(1.85×10^3)		1.86×10^4	(4.59×10^3)	
Resin acids												
Unknown 4 (AU)	2.67×10^4	(2.71×10^3)		2.59×10^4	(1.92×10^3)		2.99×10^4	(3.11×10^3)		2.42×10^4	(4.42×10^3)	
Neoabietic acid (AU)	1.41×10^5	(1.92×10^4)		1.40×10^5	(1.80×10^4)		1.51×10^5	(1.99×10^4)		2.27×10^5	(2.35×10^4)	
Labdene isomer (µg *p*-cymene equiv. g^{-1} FW)	319.1	(83.69)		344.6	(80.80)		35.24	(77.73)		212.7	(119.2)	
Labdadiene isomer (µg *p*-cymene equiv. g^{-1} FW)	4100	(742.2)		3871	(708.5)		3592	(703.6)		2942	(1073.6)	
Monoterpenes (µg *p*-cymene equiv. g^{-1} FW)												
α-pinene‡	11670	(891.7)	b	12770	(936.7)	ab	14150	(907.0)	a	9530	(1510)	b
Camphene	211.6	(16.72)		216.4	(16.84)		253.2	(16.97)		175.1	(27.90)	
β-pinene‡	670.8	(103.52)	bc	921.3	(95.93)	b	1287	(89.65)	a	523.9	(152.52)	c
Myrcene	309.1	(25.91)		302.7	(27.99)		337.9	(27.19)		211.8	(44.03)	
Limonene‡	1929	(241.4)	ab	2209	(251.3)	a	2627	(249.4)	a	997	(431.6)	b
Bornyl acetate	416.7	(38.39)		360.5	(40.97)		382.9	(40.64)		268.6	(66.36)	
Sesquiterpenes (µg *p*-cymene equiv. g^{-1} FW)												
Trans-caryophyllene	795.1	(116.4)		766.1	(118.2)		1005	(118.2)		1159.8	(180.3)	
α-humulene	239.4	(35.51)		222.4	(34.99)		193.6	(34.04)		223.0	(49.49)	
Germacrene D	4708	(648.6)	b	3706	(664.1)	b	4017	(638.1)	b	7917	(1028.8)	a
α-muurolene	207.6	(43.10)		251.4	(41.84)		211.0	(42.73)		260.7	(54.60)	

†Measured by Folin's method.
‡Exist as a combination of two stereoisomers analysed together.

No claim to original US government works.
Journal compilation © *New Phytologist* (2007)

Table 2 Means (SE) for lesion length and concentrations of phenolic compounds in branch phloem of Austrian pine (*Pinus nigra*); different letters indicate mean separation by LSD within row ($P < 0.05$)

	Nitrogen (ppm)								
	30			75			150		
Lesion length (mm)	18.25	(1.71)		20.36	(1.20)		18.19	(1.47)	
Lignin (mg g^{-1} FW)	12.55	(0.55)		12.51	(0.57)		11.94	(0.52)	
Total phenolics† (mg g^{-1} FW)	3.20	(0.23)	a	3.15	(0.23)	a	1.97	(0.24)	b
Phenolics‡ (AU, unless indicated)									
Dihydroconiferin I	1.34×10^6	(1.17×10^5)		1.33×10^6	(1.18×10^5)		1.44×10^6	(1.19×10^5)	
Coumaric acid hexoside	5.08×10^5	(5.85×10^4)	a	3.95×10^5	(4.03×10^4)	b	2.61×10^5	(2.54×10^4)	c
Hydroxypropiovanillone hexoside	5.92×10^5	(5.11×10^4)	a	5.91×10^5	(4.49×10^4)	a	4.46×10^5	(3.91×10^4)	b
Dihydroconiferin II	1.69×10^6	(1.47×10^5)		1.61×10^6	(1.50×10^5)		1.51×10^6	(1.52×10^5)	
Ferulic acid glucoside (μg g^{-1} FW)	380.2	(32.0)	a	359.0	(30.2)	a	242.3	(20.2)	b
Taxifolin hexoside	4.79×10^5	(3.60×10^4)	a	5.26×10^5	(4.24×10^4)	a	3.67×10^5	(3.09×10^4)	b
Unknown 1	4.99×10^4	(1.76×10^4)		8.64×10^4	(1.81×10^4)		5.38×10^4	(1.75×10^4)	
Taxifolin (μg g^{-1} FW)	42.23	(3.52)		55.11	(4.31)		45.29	(3.83)	
Pinosylvin (μg g^{-1} FW)	36.21	(11.00)		29.94	(3.82)		33.66	(8.17)	
Pinosylvin monomethyl ether (μg g^{-1} FW)	11.41	(4.88)		9.30	(2.03)		6.33	(1.92)	
Unknown 2	2.57×10^4	(2.47×10^3)		3.00×10^4	(2.44×10^3)		2.94×10^4	(2.19×10^3)	
Unknown 3	1.19×10^4	(9.53×10^3)	b	1.37×10^4	(1.21×10^3)	b	1.94×10^4	(2.46×10^3)	a

†Measured by Folin's method.
‡Mass provided for compounds quantified with standards.

The PCA on the complete data set yielded similar groupings, with four components altogether explaining 49% of the total variance. Compounds with component matrix scores > 0.50 for each component were grouped together. PC1 contained the monoterpenes α-pinene, camphene, myrcene and bornyl acetate with positive component matrix scores, and contained dihydroconiferin II, coumaric acid hexoside, hydroxypropiovanillone hexoside, ferulic acid glucoside, taxifolin hexoside and taxifolin with negative component matrix scores. Thus PC1 delineated groups of compounds very similar to clusters 1 and 2, and indicated that those clusters were negatively correlated with one another. Uk1, uk2 and uk4 were associated with PC2 with positive component matrix scores. Germacrene D, α-muurolene and neoabietic acid (which comprise cluster group 4) were associated with PC3 with negative component matrix scores, while α-pinene was associated with PC3 with positive component matrix scores. PC4 had only *trans*-caryophyllene and α-humulene (which made up cluster 3) associated positively with it. Component score plots of PC1 vs PC2 and PC1 vs PC3 (Fig. 2) clearly revealed groupings of compounds classified together in clusters 1, 2, 4 and, arguably, 5. Because such groupings appeared independently of the multivariate classification applied, they were considered natural groupings of compounds, a result that suggests coregulation in the phloem of Austrian pine.

Table 3 Spearman correlations between concentrations of individual compounds and lesion length in Austrian pine (*Pinus nigra*)

	Lesion length		
	ρ	*P*	*n*
Lignin	–0.209	0.095	65
Hydroxypropiovanillone hexoside	–0.242	0.042	71
Ferulic acid glucoside	–0.304	0.010	71
Unknown 1	–0.297	0.011	72
Unknown 3	0.253	0.032	72
β-pinene	–0.231	0.056	69
Labdadiene isomer	0.258	0.032	69

Only compounds with significant ($P < 0.05$) or marginally significant ($P < 0.10$) correlations are shown.

ANOVA of metabolite groups

Cluster 1, which consists mostly of phenolics, increased significantly in the fungal treatment over both control treatments, while insect defoliation resulted in a significant increase only over the nonwounded control. Cluster 2, which consists mostly of the monoterpenes, decreased significantly in the insect-defoliation treatment compared with the other

No claim to original US government works. Journal compilation © *New Phytologist* (2007)

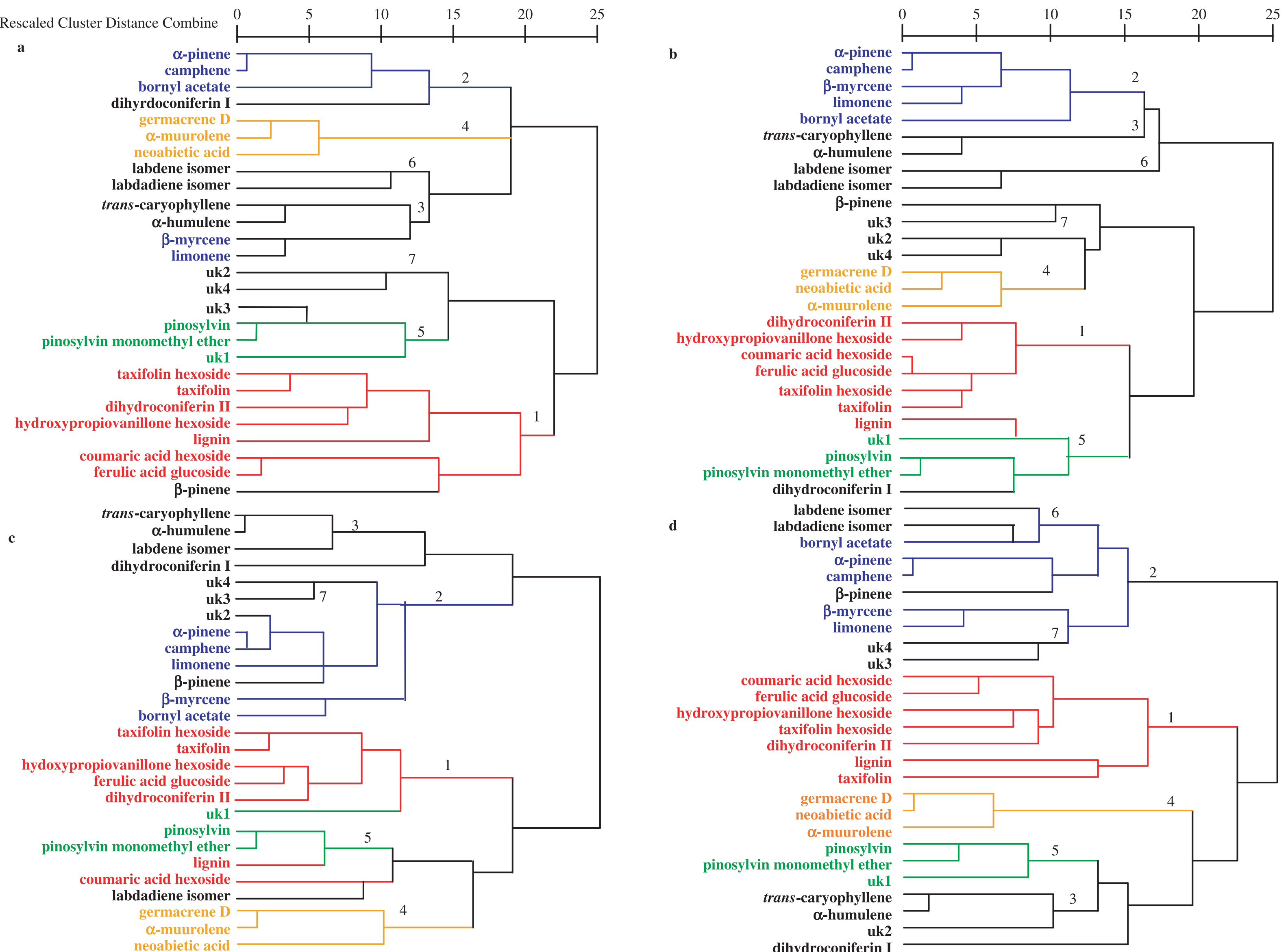

Fig. 1 Dendrograms based on furthest neighbor joining and squared Euclidean distance showing grouping of compounds based on patterns of expression in the (a) nonwounded treatment; (b) mock-inoculation treatment; (c) insect-defoliation induction treatment; (d) fungal induction treatment. Numbers above branches indicate cluster groupings as described in Table 4, determined by consistent grouping in three of the four dendrograms. Cluster 1 (phenolics) is highlighted in red; cluster 2 (monoterpenes) in blue; cluster 4 in orange; cluster 5 (stilbenes) in green. All other clusters are in black.

No claim to original US government works.
Journal compilation © *New Phytologist* (2007)

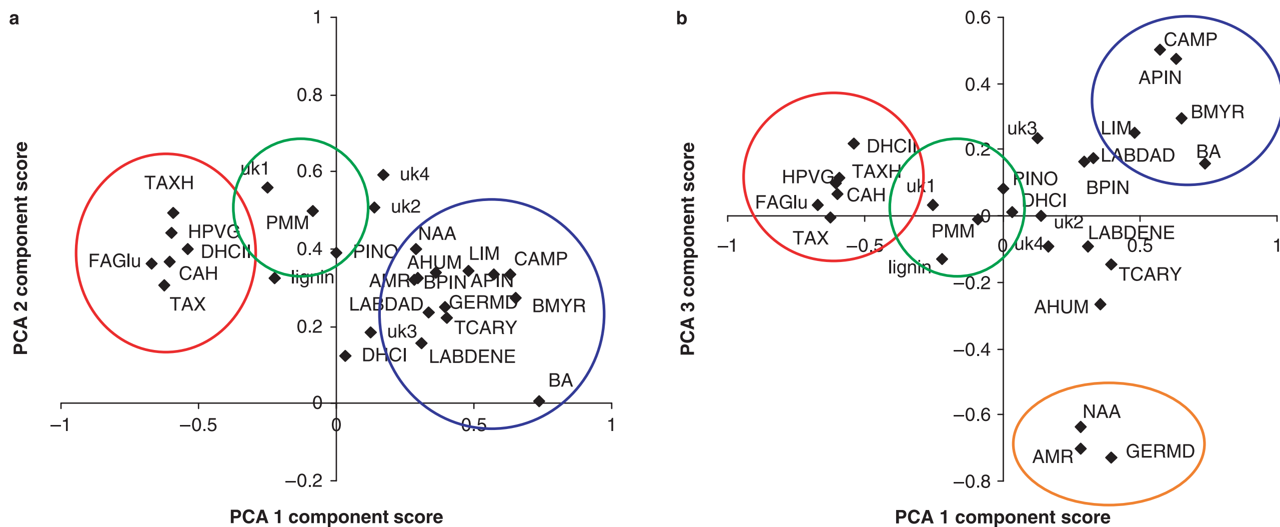

Fig. 2 Principal components analysis (PCA) component plots of (a) PCA 1 and PCA 2; (b) PCA 1 and PCA 3, based on an analysis that included all the treatments in this study. Circles represent defined, separated groups and correspond to clusters 1 (red); 2 (blue); 4 (orange); 5 (green) (Fig. 1). Abbreviations: AHUM, α-humulene; AMR, α-muurolene; APIN, α-pinene; BA, bornyl acetate; BMYR, β-myrcene; BPIN, β-pinene; CAH, coumaric acid hexoside; CAMP, camphene; DHCI, dihydroconiferin I; DHCII, dihydroconiferin II; FAGlu, ferulic acid glucoside; GERMD, germacrene D; HPVG, hydroxypropiovanillone hexoside; LABDAD, labdadiene isomer; LABDENE, labdene isomer; LIM, limonene; NAA, neoabietic acid; PINO, pinosylvin; PMM, pinosylvin monomethyl ether; TAX, taxifolin; TAXH, taxifolin hexoside; TCARY, *trans*-caryophyllene.

induction treatments. Cluster 4, which includes germacrene D, α-muurolene and neoabietic acid, increased significantly in the insect-defoliation treatment over controls. Cluster 5, which includes the stilbenes, increased significantly in the fungal induction treatment over the other treatments. Only cluster 1 was affected significantly by fertility level, with lower concentration at 150 ppm N than the other fertility groups ($F_{2,165} = 6.974$, $P = 0.001$). By contrast, the relative abundance of compounds defined in clusters 3, 6 and 7 did not change significantly in response to any of the treatments. For the most part, interactions among the main-effects variables (pathogen, insect and fertility) were not observed for any of these clusters of compounds, with the exception of significant ($P < 0.05$) interactions occurring for fertilizer × block for clusters 5 and 7.

Correlations of compound clusters with lesion size and with each other

The scaled concentrations of compounds present in clusters 1 and 5 were negatively correlated with lesion length, while cluster 6 was positively correlated with lesion length (Table 4). The combination of clusters 1 and 5 led to a stronger negative correlation with lesion length (Table 4). All other correlations with lesion size were not significant.

Correlations between compound clusters were computed to examine any potential resource trade-offs that might occur when Austrian pine mounts an induced defense response. Clusters 1 and 2 were significantly negatively correlated with each other, and cluster 1 was positively correlated with cluster 5 (Table 5). Cluster 2 was positively correlated with clusters 3, 6 and 7 (Table 5). Finally, clusters 3 and 4 were positively correlated, as well as clusters 3 and 6, 4 and 7, and 5 and 7 (Table 5).

Discussion

In this study we profiled systemic chemical changes in phloem tissues of Austrian pine in response to fungal infection and insect defoliation. We also examined the extent to which chemical changes related to fungal performance, and whether responses varied with fertility level. Fertility was previously found to have no effect on resistance to the fungal pathogen (Eyles *et al.*, 2007), but this study indicates that it influenced the accumulation of four phenolic glycosides (coumaric acid hexoside, hydroxypropiovanillone hexoside, ferulic acid glucoside, taxifolin hexoside), with the lowest concentration observed in the 150 ppm N treatment. There were no significant effects of fertility on the concentrations of any of the terpenes across all induction treatments.

As fertility did not alter the expression of resistance against the pathogen, but influenced the levels of some compounds, it might appear that phenolic glycosides or terpenes are not involved in resistance. However, we found that the systemic concentrations of eight phenolic compounds, two monoterpenes and one unknown compound increased significantly in trees that were induced by the fungal pathogen over nonwounded and/or mock-inoculated trees (Table 1). Furthermore, fertility and induction treatment interacted significantly to affect total phenolics, confounding the relationship between fertility treatment, phenolics and resistance.

No claim to original US government works.
Journal compilation © *New Phytologist* (2007)

Table 4 Spearman's correlations between mean quintile concentrations of each cluster group and lesion length in Austrian pine (*Pinus nigra*)

Cluster group	Description	Lesion length	
		ρ	*P*
1	Dihydroconiferin II, coumaric acid hexoside, hydroxypropionvanillone hexoside, ferulic acid glucoside, taxifolin hexoside, taxifolin, lignin†	–0.250	0.038
2	α-pinene, camphene, β-myrcene, limonene, bornyl acetate	0.033	0.790
3	*Trans*-caryophyllene, α-humulene	0.166	0.343
4	Germacrene D, α-muurolene, neoabietic acid	–0.043	0.728
5	Pinosylvin, pinosylvin monomethyl ether, unknown 1	–0.239	0.043
6	Labdene isomer, labdadiene isomer	0.275	0.022
7	Unknowns 2, 3, 4	–0.008	0.946
1 and 5	Dihydroconiferin II, coumaric acid hexoside, hydroxypropionvanillone hexoside, ferulic acid glucoside, taxifolin hexoside, taxifolin, lignin, pinosylvin, pinosylvin monomethyl ether, unknown 1	–0.283	0.016

For all correlations, $n = 69$.
†Lignin weakly grouped in cluster 1 according to the PCA, but met criteria of fitting in three of four of the dendrograms shown in Fig. 1. Without lignin, cluster 1 compounds are weakly correlated with lesion size (ρ = –0.171, $P = 0.150$).

Table 5 Pairwise Spearman's correlations between mean quintile concentrations of each cluster group

	Cluster 2		Cluster 3		Cluster 4		Cluster 5		Cluster 6		Cluster 7	
	ρ (*n*)	*P*	ρ (*n*)	*P*	ρ (*n*)	*P*	ρ (*n*)	*P*	ρ (*n*)	*P*	ρ (*n*)	*P*
Cluster 1 (phenolics)	–0.276 (166)	< 0.001	–0.124 (166)	0.113	–0.122 (165)	0.120	0.275 (166)	< 0.001	–0.092 (166)	0.238	0.038 (166)	0.630
Cluster 2 (monoterpenes)	–	–	0.262 (166)	0.001	0.114 (165)	0.145	–0.002 (167)	0.980	0.314 (166)	< 0.001	0.314 (167)	< 0.001
Cluster 3 (caryophyllene)			–	–	0.191 (165)	0.014	0.010 (166)	0.901	0.257 (165)	0.001	0.075 (166)	0.337
Cluster 4 (germacrene D)					–	–	0.048 (165)	0.539	0.078 (165)	0.317	0.248 (165)	0.001
Cluster 5 (stilbenes)							–	–	0.006 (166)	0.938	0.302 (181)	< 0.001
Cluster 6 (diterpenes)									–	–	0.120 (166)	0.123

Conversely, germacrene D was the only terpenoid that increased systemically following induction by *N. sertifer* defoliation, compared with the controls. This sesquiterpene was described previously as having no influence on the growth of *D. pinea* (Jurc *et al.*, 1999). However, it was described as increasing in concentration in the stem of methyl jasmonate-treated Douglas fir (*Pseudotsuga menziesii*) (Huber *et al.*, 2005). This is of biological interest because methyl jasmonate is known to mimic the induction of terpenoid metabolism that is often observed in several conifers following insect attack (Franceschi *et al.*, 2002; Martin *et al.*, 2002; Miller *et al.*, 2005; Erbilgin *et al.*, 2006).

While positive correlations between individual compounds and lesion length may be of some biological interest, negative correlations are of higher significance in SIR studies in which the basis of the observed resistance is investigated (Blodgett *et al.*, 2007). Among the individual compounds analysed in this study, only hydroxypropiovanillone hexoside, ferulic acid glucoside and uk1 were negatively correlated with lesion size (Table 3). Of these, only ferulic acid glucoside increased significantly in concentration as a response to pathogen induction, and this association suggests a role in resistance to *D. pinea*. It should also be noted that, while only marginally significant ($P < 0.10$), the correlations between lignin and lesion length, and β-pinene and lesion length, were also negative. Thus it is possible that they play a role in SIR observed here, confirming the implications of previous studies (Blodgett & Stanosz, 1997; Bonello *et al.*, 2003; Blodgett *et al.*, 2007).

Support for the interpretation that clusters 1 and 5 may be involved in resistance derives from the known *in vitro* antifungal activity of many of the compounds contained therein, some

No claim to original US government works.
Journal compilation © *New Phytologist* (2007)

of which were significantly affected by treatment. For example, some of the best examples of the involvement of phenolics in conifer defense involve the stilbenes pinosylvin and pinosylvin monomethyl ether, which have been found to increase in concentration, both locally and systemically, in response to pathogen attack in several pathosystems (Shain, 1967; Hart & Shrimpton, 1979; Lindberg *et al.*, 1992; Lieutier *et al.*, 1996; Bois *et al.*, 1999; Chiron *et al.*, 2000; Bonello & Blodgett, 2003). Pinosylvin and pinosylvin monomethyl ether have strong antifungal activity in *in vitro* assays against fungi, including *D. pinea* (Bonello *et al.*, 1993; Blodgett & Stanosz, 1997; Celimene *et al.*, 2001; Seppanen *et al.*, 2004). The role of stilbenes in disease resistance has been questioned because they were not individually correlated with resistance (Bonello & Blodgett, 2003; Blodgett *et al.*, 2007). However, additive but minor effects of individual stilbenes or their interactive effects can lead to the negative association of this group with fungal performance that was seen here. A similar argument could be made for the phenolic glycosides in cluster 1.

Thus phenolic glycosides may accumulate as part of the pathogen-induced, systemically resistant phenotype in anticipation of additional fungal attack. In some cases it is known that fungal pathogen attack results in cleavage of the sugar moiety of phenolic glycosides, and that the released aglycones have fungistatic/fungicidal activities (Woodward & Pearce, 1988). For example, coumaric acid may be released by pathogen hydrolases from its glycoside, and coumaric acid has been shown to be toxic *in vitro* against *Heterobasidium annosum* (Tomova *et al.*, 2005). Ferulic acid, which is known to have antifungal activity and may be implicated in the resistance of some plants to fungal infection (Sarma & Singh, 2003), may similarly be released from its glucoside.

While only a few individual phenolics were negatively correlated with lesion length, we found that several secondary metabolites were negatively correlated with lesion length as discrete groups. Clusters 1 (phenolic glycosides and lignin) and 5 (stilbenes) were negatively correlated with lesion length, suggesting that these groups of compounds act or are regulated in concert to contribute to disease resistance. In fact, the combination of clusters 1 and 5 resulted in a stronger correlation than either group by itself. Such additive effects are an often-overlooked aspect of studies of resistance mechanisms, although it is well known that regulation of the induced defense response occurs well upstream of individual components, so that whole groups of coregulated compounds may be involved (Metraux *et al.*, 2002; Koricheva *et al.*, 2004; Hale *et al.*, 2005; Schmidt *et al.*, 2005; Ralph *et al.*, 2006). The clusters of compounds correspond well with natural secondary metabolite groups: cluster 1 consisted mainly of phenolics, particularly phenolic glycosides; cluster 2 of monoterpenes; clusters 3 and 4 of the sesquiterpenes; cluster 5 of the stilbenes; and cluster 6 of the diterpenes. These groupings were consistent for at least three of the four induction treatment dendrograms, indicating that the relationships derived between the compounds were robust and that these compounds were indeed coregulated.

While not as clear cut, a role for terpenoids in resistance of Austrian pine to *D. pinea* is also supported by our analysis, because the fungal induction treatment resulted in significantly greater concentrations of α-pinene and β-pinene over the nonwounded control treatment, and there was a negative correlation between β-pinene and lesion length. Blodgett & Stanosz (1997) found that α-pinene and β-pinene had toxic effects *in vitro* on *D. pinea*, and these and other monoterpenes have previously been implicated in defense against complexes of bark beetles and associated fungi (Klepzig *et al.*, 1995; Raffa & Smalley, 1995; Klepzig *et al.*, 1996; Phillips & Croteau, 1999), as well as other fungi (Ennos & Swales, 1991; Himejima *et al.*, 1992) due to accumulation following a local attack. However, ours may be one of the first studies in which a negative correlation between experimentally manipulated *in planta* levels of a monoterpene and the extent of pathogen-induced tissue damage have been documented. It should be noted that three of the monoterpenes monitored in this study, α-pinene, β-pinene and limonene, are known to be combinations of stereoisomers. Thus detailed studies may be warranted to find out if the enantiomers respond differently to the treatments (Fäldt *et al.*, 2006), which might further support or refute their involvement in resistance.

Cluster 1 (phenolics) and cluster 2 (monoterpenes) were negatively correlated with each other (Table 5). This finding suggests a trade-off between the phenolic and terpenoid pathways, and has intriguing implications for how trees defend themselves against multiple threats from taxa as diverse as microbes and insects.

In summary, our work suggests that the systemic accumulation of 'anticipatory' compounds (phytoanticipins), especially phenolic glycosides, in response to the induction by a fungal infection, could be one of the bases of SIR in pine branches. We have also documented a trade-off between terpenoid and phenolic metabolism that may have important implications in our understanding of complex, multitrophic and multipartite systems. Furthermore, the only consistent fertility effect found in this study was lowered phenolic glycoside levels at the highest N addition, and this was not sufficient to affect lesion length in that treatment (the interaction between induction treatment and fertilizer was also not significant for cluster 1, which contained those phenolics). Trees may have been able to respond to the challenge attack by upregulating local defense responses in the branches, thus overcoming any shortfalls in phytoanticipins caused by the high-fertility treatment. The hypothesis that phenolic glycosides play an anticipatory, and perhaps essential, role in SIR could be tested through mathematical modeling in which these compounds would be used as predictors of relative resistance in naive trees, coupled with studies aimed at determining the levels of antifungal activity that these phenolic glycosides and their aglycones possess.

 No claim to original US government works.
Journal compilation © *New Phytologist* (2007)

Acknowledgements

We wish to thank Duan Wang, Anuprit Kaur, Justin Whitehill, Bryant Chambers, Diane Hartzler, Matt Solensky, Alejandro Chiriboga and Ilka Gomez for their technical assistance in this project. We also wish to thank Kurtz Bros Inc. and Smith Evergreen Nursery, Inc., Magnolia, OH, USA for their generous donations of planting media and the trees used in this study. Salaries and research support were provided in part by USDA National Research Initiative Competitive Grants Program No. 2004-35302-14667, an Interdisciplinary OARDC SEEDS Research Grant, and by State and Federal funds appropriated to the Ohio Agricultural Research and Development Center, the Ohio State University. All experiments comply with the laws of the USA.

References

vanAkker L, Alfaro RI, Brockley R. 2004. Effects of fertilization on resin canal defences and incidence of *Pissodes strobi* attack in interior spruce. *Canadian Journal of Forest Research* **34**: 855–862.

Benitez MS, Baysal Tustasa F, Rotenberg D, Kleinhenz MD, Cardina J, Stinner D, Miller SA, McSpadden Gardener BB. 2007. Multiple statistical approaches of community fingerprint data reveal bacterial populations associated with general disease suppression arising from the application of different organic field management strategies. *Soil Biology & Biochemistry* **39**: 2289–2301.

Blodgett JT, Eyles A, Bonello P. 2007. Organ-dependent induction of systemic resistance and systemic susceptibility in *Pinus nigra* inoculated with *Sphaeropsis sapinea* and *Diplodia scrobiculata*. *Tree Physiology* **27**: 373–382.

Blodgett JT, Herms DA, Bonello P. 2005. Effects of fertilization on red pine defense chemistry and resistance to *Sphaeropsis sapinea*. *Forest Ecology and Management* **208**: 373–382.

Blodgett JT, Stanosz GR. 1997. Differential inhibition of *Sphaeropsis sapinea* morphotypes by a phenolic compound and several monoterpenes of red pine. *Phytopathology* **87**: 606–609.

Bois E, Lieutier F, Yart A. 1999. Bioassays on *Leptographium wingfieldii*, a bark beetle associated fungus, with phenolic compounds of Scots pine phloem. *European Journal of Plant Pathology* **105**: 51–60.

Bonello P, Blodgett JT. 2003. *Pinus nigra–Sphaeropsis sapinea* as a model pathosystem to investigate local and systemic effects of fungal infection of pines. *Physiological and Molecular Plant Pathology* **63**: 249–261.

Bonello P, Gordon TR, Herms D, Wood DL, Erbilgin N. 2006. Nature and ecological implications of pathogen-induced systemic resistance in conifers: a novel hypothesis. *Physiological and Molecular Plant Pathology* **68**: 95–104.

Bonello P, Gordon TR, Storer AJ. 2001. Systemic induced resistance in Monterey pine. *Forest Pathology* **31**: 99–106.

Bonello P, Pearce RB. 1993. Biochemical defence responses in primary roots of Scots pine challenged *in vitro* with *Cylindrocarpon destructans*. *Plant Pathology* **42**: 203–211.

Bonello P, Storer AJ, Gordon TR, Wood DL. 2003. Systemic effects of *Heterobasidion annosum* on ferulic acid glucoside and lignin of presymptomatic ponderosa pine phloem, and potential effects on bark-beetle-associated fungi. *Journal of Chemical Ecology* **29**: 1167–1182.

Celimene CC, Smith DR, Young RA, Stanosz GR. 2001. *In vitro* inhibition of *Sphaeropsis sapinea* by natural stilbenes. *Phytochemistry* **56**: 161–165.

Chiron H, Drouet A, Lieutier F, Payer HD, Ernst D, Sandermann H. 2000. Gene induction of stilbene biosynthesis in Scots pine in response to ozone treatment, wounding, and fungal infection. *Plant Physiology* **124**: 865–872.

Christiansen E, Krokene P, Berryman AA, Franceschi VR, Krekling T, Lieutier F, Lonneborg A, Solheim H. 1999. Mechanical injury and fungal infection induce acquired resistance in Norway spruce. *Tree Physiology* **19**: 399–403.

Enebak S, Carey W. 2000. Evidence for induced systemic protection to fusiform rust in loblolly pine by plant growth-promoting rhizobacteria. *Plant Disease* **84**: 306–308.

Ennos RA, Swales KW. 1991. Genetic variation in a fungal pathogen response to host defensive chemicals. *Evolution* **45**: 190–204.

Entry JA, Cromack K, Hansen E, Waring R. 1991. Response of western coniferous seedlings to infection by *Armillaria ostoyae* under limited light and nitrogen. *Phytopathology* **81**: 89–94.

Erbilgin N, Krokene P, Christiansen E, Zeneli G, Gershenzon J. 2006. Exogenous application of methyl jasmonate elicits defenses in Norway spruce (*Picea abies*) and reduces host colonization by the bark beetle *Ips typographus*. *Oecologia* **148**: 426–436.

Eyles A, Chorbadjian R, Wallis C, Hansen R, Cipollini D, Herms D, Bonello P. 2007. Cross-effects of systemic induced resistance between an insect and a fungal pathogen in Austrian pine over a fertility gradient. *Oecologia* **153**: 365–374.

Fäldt J, Solheim H, Långström B, Borg-Karlson A-K. 2006. Influence of fungal infection and wounding on contents of enantiomeric composition of monoterpenes in phloem of *Pinus sylvestris*. *Journal of Chemical Ecology* **32**: 1779–1795.

Franceschi VR, Krekling T, Christiansen E. 2002. Application of methyl jasmonate on *Picea abies* (Pinaceae) stems induces defense-related responses in phloem and xylem. *American Journal of Botany* **89**: 578–586.

Franceschi VR, Krokene P, Christiansen E, Krekling T. 2005. Anatomical and chemical defenses of conifer bark against bark beetles and other pests. *New Phytologist* **167**: 353–375.

Hale BK, Herms DA, Hansen RC, Clausen TP, Arnold D. 2005. Effects of drought stress and nutrient availability on dry matter allocation, phenolic glycosides, and rapid induced resistance of poplar to two lymantriid defoliators. *Journal of Chemical Ecology* **31**: 2601–2620.

Hart JH, Shrimpton DM. 1979. Role of stilbenes in resistance of wood to decay. *Phytopathology* **69**: 1138–1143.

Hatcher PE, Paul ND. 2000. Beetle grazing reduces natural infection of *Rumex obtusifolius* by fungal pathogens. *New Phytologist* **146**: 325–333.

Hatcher PE, Paul ND, Ayres PG, Whittaker JB. 1994. The effect of an insect herbivore and a rust fungus individually, and combined in sequence, on the growth of two *Rumex* species. *New Phytologist* **128**: 71–78.

Herms DA. 2002. Effects of fertilization on insect resistance of woody ornamental plants: reassessing an entrenched paradigm. *Environmental Entomology* **31**: 923–933.

Herms DA, Mattson WJ. 1992. The dilemma of plants – to grow or defend. *Quarterly Review of Biology* **67**: 283–335.

Himejima M, Hobson KR, Otsuka T, Wood DL, Kubo I. 1992. Antimicrobial terpenes from oleoresin of ponderosa pine tree *Pinus ponderosa* – a defense mechanism against microbial invasion. *Journal of Chemical Ecology* **18**: 1809–1818.

Huber DPW, Philippe RN, Madilao LL, Sturrock RN, Bohlmann J. 2005. Changes in anatomy and terpene chemistry in roots of Douglas-fir seedlings following treatment with methyl jasmonate. *Tree Physiology* **25**: 1075–1083.

Jurc D, Bojovic S, Jurc M. 1999. Influence of endogenous terpenes on growth of three endophytic fungi from the needles of *Pinus nigra* Arnold. *Phyton – Annales Rei Botanicae* **39**: 225–229.

Keeling CI, Bohlmann J. 2006. Genes, enzymes and chemicals of terpenoid diversity in the constitutive and induced defence of conifers against insects and pathogens. *New Phytologist* **170**: 657–675.

Klepzig KD, Kruger EL, Smalley EB, Raffa KF. 1995. Effects of biotic and abiotic stress on induced accumulation of terpenes and phenolics in red pines inoculated with bark beetle-vectored fungus. *Journal of Chemical Ecology* **21**: 601–626.

No claim to original US government works.
Journal compilation © *New Phytologist* (2007)

Klepzig KD, Smalley EB, Raffa KF. 1996. Combined chemical defenses against an insect–fungal complex. *Journal of Chemical Ecology* **22**: 1367–1388.

Koricheva J, Nykanen H, Gianoli E. 2004. Meta-analysis of trade-offs among plant antiherbivore defenses: Are plants jacks-of-all-trades, masters of all? *American Naturalist* **163**: E64–E75.

Kytö M, Niemela P, Annila E, Varama M. 1999. Effects of forest fertilization on the radial growth and resin exudation of insect-defoliated Scots pines. *Journal of Applied Ecology* **36**: 763–769.

Lieutier F, Sauvard D, Brignolas F, Picron V, Yart A, Bastien C, JayAllemand C. 1996. Changes in phenolic metabolites of Scots pine phloem induced by *Ophiostoma brunneo-ciliatum*, a bark-beetle-associated fungus. *European Journal of Forest Pathology* **26**: 145–158.

Lindberg M, Lundgren L, Gref R, Johansson M. 1992. Stillbenes and resin acids in relation to the penetration of *Heterobasidion annosum* through the bark of *Picea abies*. *European Journal of Forest Research* **22**: 95–106.

Martin D, Tholl D, Gershenzon J, Bohlmann J. 2002. Induction of traumatic resin ducts, terpenoid resin biosynthesis and terpenoid accumulation in developing xylem of Norway spruce (*Picea abies*) stems. *Plant Physiology* **129**: 1003–1018.

McSpadden Gardener BB. 2006. Statistical analyses of microbiological and environmental data. In: van Elsas JD, Jannson J, eds. *Modern soil microbiology*, 2nd edn. Boca Raton, FL, USA: CRC Press, 555–585.

Metraux JP, Nawrath C, Genoud T. 2002. Systemic acquired resistance. *Euphytica* **124**: 237–243.

Miller B, Madilao LL, Ralph S, Bohlmann J. 2005. Insect-induced conifer defense. White pine weevil and methyl jasmonate induce traumatic resinosis, *de novo* formed volatile emissions, and accumulation of terpenoid synthase and putative octadecanoid pathway transcripts in Sitka spruce. *Plant Physiology* **137**: 369–382.

Moran PJ, Schultz JC. 1998. Ecological and chemical associations among late-season squash pests. *Environmental Entomology* **27**: 39–44.

Padgett GB, Russing JS, Snow JP, Boethel DJ, Berggren GT. 1994. Interactions among the soybean looper (Lepidoptera, Noctuidae), threecornered alfalfa hopper (Homoptera, Membracidae), stem canker, and red crown rot in soybean. *Journal of Entomological Science* **29**: 110–119.

Phillips MA, Croteau RB. 1999. Resin-based defenses in conifers. *Trends in Plant Science* **4**: 184–190.

Raffa KF, Smalley EB. 1995. Interaction of pre-attack and induced monoterpene concentrations in host conifer defense against bark beetle fungal complexes. *Oecologia* **102**: 285–295.

Ralph SG, Yueh H, Friedmann M *et al.* 2006. Conifer defence against insects: microarray gene expression profiling of Sitka spruce (*Picea sitchensis*) induced by mechanical wounding or feeding by spruce budworms (*Choristoneura occidentalis*) or white pine weevils (*Pissodes strobi*) reveals large-scale changes of the host transcriptome. *Plant, Cell & Environment* **29**: 1545–1570.

Rostas M, Simon M, Hilker M. 2003. Ecological cross-effects of induced plant responses towards herbivores and phytopathogenic fungi. *Basic and Applied Ecology* **4**: 43–62.

Russo VM, Russon BM, Peters M, Perkins-Veazie P, Cartwright B. 1997. Interaction of *Colletotrichum orbiculare* with thrips and aphid feeding on watermelon seedlings. *Crop Protection* **16**: 581–584.

Sarma BK, Singh UP. 2003. Ferulic acid may prevent infection of *Cicer arietinum* by *Sclerotium rolfsii*. *World Journal of Microbiology & Biotechnology* **19**: 123–127.

Schmidt A, Zeneli G, Hietala AM, Fossdal CG, Krokene P, Christiansen E, Gershenzon J. 2005. Induced chemical defenses in conifers: biochemical and molecular approaches to studying their function. In: Romeo J, ed. *Chemical ecology and phytochemistry of forest ecosystems*. Amsterdam: Elsevier, 1–28.

Seppanen SK, Syrjala L, von Weissenberg K, Teeri TH, Paajanen L, Pappinen A. 2004. Antifungal activity of stilbenes in *in vitro* bioassays and in transgenic *Populus* expressing a gene encoding pinosylvin synthase. *Plant Cell Reports* **22**: 584–593.

Shain L. 1967. Resistance of sapwood in stems of Loblolly pine to infection by *Fomes annosus*. *Phytopathology* **57**: 1034–1045.

Simon M, Hilker M. 2003. Herbivores and pathogens on willow: do they affect each other? *Agricultural and Forest Entomology* **5**: 275–284.

Stout MJ, Fidantsef AL, Duffey SS, Bostock RM. 1999. Signal interactions in pathogen and insect attack: systemic plant-mediated interactions between pathogens and herbivores of the tomato, *Lycopersicon esculentum*. *Physiological and Molecular Plant Pathology* **54**: 115–130.

Stout MJ, Thaler JS, Thomma B. 2006. Plant mediated interactions between pathogenic microorganisms and herbaceous arthropods. *Annual Review of Entomology* **51**: 663–89.

Swedjemark G, Karlsson B, Stenlid J. 2007. Exclusion of *Heterobasidion parviporum* from inoculated clones of *Picea abies* and evidence of systemic induced resistance. *Scandinavian Journal of Forest Research* **22**: 110–117.

Tomova L, Braun S, Fluckiger W. 2005. The effect of nitrogen fertilization on fungistatic phenolic compounds in roots of beech (*Fagus sylvatica*) and Norway spruce (*Picea abies*). *Forest Pathology* **35**: 262–276.

Woodward S, Pearce R. 1988. The role of stilbenes in resistance of Sitka spruce (*Picea sitchensis* (Bong.) Carr.) to entry of fungal pathogens. *Physiological and Molecular Plant Pathology* **33**: 127–149.

Zeneli G, Krokene P, Christiansen E, Krekling T, Gershenzon J. 2006. Methyl jasmonate treatment of mature Norway spruce (*Picea abies*) trees increases the accumulation of terpenoid resin components and protects against infection by *Ceratocystis polonica*, a bark beetle-associated fungus. *Tree Physiology* **26**: 977–988.

 No claim to original US government works.
Journal compilation © *New Phytologist* (2007)