Organic-induced induction of systemic resistance and systemic susceptibility in *Pinus nigra* inoculated with *Sphaeropsis sapinea* and *Diplodia scrobiculata*

JAMES T. BLODGETT, ALIETA EYLES and PIERLUIGI BONELLO

1 Department of Plant Pathology, The Ohio State University, Columbus, OH 43210, USA
2 USDA - Forest Service, Forest Health Management, Rapid City, SD 57702, USA
3 Corresponding author (jblodgett@fs.fed.us)

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**Summary** Systemic induced resistance (SIR) is a well-known host defense mechanism against pathogen attack in herbaceous plants, but SIR has only recently been documented in conifers. We tested if inoculation of Austrian pine (*Pinus nigra* Arnold) with *Sphaeropsis sapinea* (Fr.:Fr.) Dyko and Sutton or *Diplodia scrobiculata* de Wet, Slippers and Wingfield results in SIR or systemic induced susceptibility (SIS) to subsequent colonization by *S. sapinea*. Induction at the stem base resulted in significant (*P* < 0.01) SIR in the upper stem, and induction in the upper stem resulted in significant (*P* < 0.05) SIR at the stem base, indicating that SIR is bidirectional in Austrian pine. However, inoculation at the stem base resulted in significant (*P* < 0.01) SIS in shoot tips, demonstrating that, in the same host species, the expression of resistance can be organ-dependent, resulting in either SIR or SIS depending on the site of challenge infection. Systemic induced resistance in the stem was associated with induced lignification, supporting a potential role for this defense mechanism in disease resistance. Systemic induced susceptibility has been documented before, but this is the first demonstration of organ-dependent expression of both SIR and SIS in a tree or any other plant.

**Keywords:** Diplodia pinea, fungal pathogen, host defense, HPLC, lignification, predisposition, secondary metabolism, SIR, SIS, systemic induced resistance, systemic induced susceptibility.

**Introduction**

Systemic induced resistance (SIR) is the induction of resistance to pathogens in noninfected parts of a plant by prior infections or activity by various organisms elsewhere in the plant (*sensu* Bonello et al. 2001, Bonello and Blodgett 2003). Therefore, the outcome of SIR is functionally analogous to immunization in mammals. Extensive research, mostly in herbaceous species, particularly *Arabidopsis* (Durrant and Dong 2004), has shown that SIR originates as the result of a hypersensitive response that is mediated by the accumulation of one or more of the phytohormones salicylic acid, jasmonic acid and ethylene. Expression of SIR is correlated with the accumulation of pathogenesis-related (PR)-proteins and with the induction or enhancement of secondary metabolic responses, such as accumulation of soluble phenolics and cell wall lignification (Sticher et al. 1997, Heil 2001, Durrant and Dong 2004). Other hormones, such as abscisic acid, may also play a role in SIR (Dammann et al. 1997).

In addition to the large size and longevity of conifers, a differentiated resin system and the presence of secondary tissues set them apart from herbaceous plants. Thus, elucidation of SIR in coniferous trees would significantly contribute to our general understanding of how plants defend themselves against injurious agents and provide insights into the ecology of woody plants. Although the molecular basis of SIR is not well characterized in trees, it is known that pathogenic infection results in localized accumulation of PR-proteins (Graham and Bonello 2004), phenolics and terpenoids (the latter are often associated with formation of traumatic resin ducts) in several conifers (Franceschi et al. 2005). Many free or condensed phenolics have fungistatic or fungitoxic effects (Blanchette and Biggs 1992) and are precursors in lignin biosynthesis. Although induced cell-wall lignification is considered a major disease resistance mechanism (Vance et al. 1980, Hammerschmidt and Kuc 1982, Hammerschmidt 1999), its potential role in SIR has received little attention (Sticher et al. 1997).

Systemic induced resistance phenotypes occur in pine (Enebak and Carey 2000, Bonello et al. 2001, Kosaka et al. 2001, Schmale and Gordon 2003, Enebak and Carey 2004), and there is evidence for the involvement of selected signaling molecules in the localized and systemic induction of defense responses in pine as well as in other conifers. For example, exogenously applied salicylic or jasmonic acid induces accumulation of PR-proteins such as chitinase and a thaumatin-like protein in pine seedlings (Davis et al. 2002, Piggott et al. 2004) and increased biosynthesis of terpenoid compounds that

Bonello and Blodgett (2003) showed that, in the Austrian pine (Pinus nigra Arnold)—Sphaeropsis sapinea (Fr.:Fr.) Dyko and Sutton (syn. Diplodia pinea (Desm.) Kickx (Sutton 1980)) pathosystem, fungal inoculations induced significant local and systemic depletion or accumulation in the phloem of several soluble and cell-wall-bound phenolics, and a local increase in lignin deposition. Furthermore, Luchi et al. (2005) showed that wounding or inoculation of Austrian pine saplings with S. sapinea and Diplodia scrobiculata de Wet, Slippers and Wingfield (de Wet et al. 2003) induced an 8-fold increase in systemic resin flow and systemic induction of traumatic resin duct formation. However, these studies did not examine if systemic induction of Austrian pine by S. sapinea inoculation is correlated with SIR. Because no clear connection between alterations in secondary metabolism and the expression of SIR has been established in conifers, the primary objective of our study was to determine if and how these processes are related.

Sphaeropsis sapinea is an important agent of shoot blight, crown wilt, and canker diseases of conifer species throughout the world, in trees ranging in age from seedlings to fully mature (Gibson 1979, Palmer and Nicholls 1985, Farr et al. 1989, Nicholls and Ostry 1990, Swart and Wingfield 1991, Stanosz and Cummings Carlson 1996). Sphaeropsis sapinea and D. scrobiculata are phylogenetically similar and were considered two different morphotypes of S. sapinea until recently (de Wet et al. 2003). Sphaeropsis sapinea is an aggressive pathogen, whereas D. scrobiculata is less aggressive on most conifers (Blodgett and Stanosz 1997, Blodgett and Stanosz 1999), including Austrian pine (Blodgett and Bonello 2003). This difference in aggressiveness between two phylogenetically close fungal pathogens of Austrian pine provides an opportunity to examine the nature of disease resistance in the host species (Luchi et al. 2005). In the absence of genetically defined host populations differing in susceptibility, it is postulated that defense mechanisms expressed against the less aggressive pathogen but not against the aggressive sister species are at the basis of resistance.

We used the model pathosystem (Bonello and Blodgett 2003, Hammerschmidt 2003) comprising Austrian pine and the two canker pathogens S. sapinea and D. scrobiculata to: (1) test whether inoculation of Austrian pine with necrogenic pathogens results in SIR or systemic induced susceptibility (SIS), or both, following subsequent colonization by S. sapinea; (2) determine whether SIR in Austrian pine is unidirectional or bidirectional; and (3) correlate systemic resistance and susceptibility to changes in the host’s secondary metabolism to identify potential disease resistance mechanisms.

### Materials and methods

#### Plant material

Dormant, 5-year-old Austrian pines were obtained from Ridge Manor Nursery (Madison, OH) in spring 2001, and from Willoway Nursery (Avon, OH) in spring 2005. Trees were selected for uniformity in size and form to reduce experimental variability. Trees were maintained in a greenhouse (40°1’ N, 83°1’ W) for the duration of the experiment, as described in Bonello and Blodgett (2003). Because even moderate water stress is known to affect canker development (Blodgett et al. 1997), the trees were watered twice daily to field capacity to exclude water stress as a factor.

#### Study design

To assess the systemic effects of stem inoculation (stem induction) on resistance to S. sapinea, three experiments were conducted (Figure 1). Experiments 1 and 2 tested the SIR phenomenon in stems and whether induction is acropetal, basipetal or both. In Experiment 3, we examined if stem induction results in resistance to shoot tip fungal challenge. Chemical analyses were performed on tissue samples to identify potential mechanisms involved in systemic resistance or susceptibility.

#### Experiment 1 (lower stem induction, upper stem challenge)

Six treatment combinations were tested in two independent trials, beginning on June 7 and June 21, 2001. One of three induction treatments was applied to the stem of each tree. The induction treatments were applied by removing a disk of bark with a cork borer, followed by insertion of a sterile potato dextrose agar (PDA) plug (mock inoculation or wounding treatment), or a PDA plug colonized by S. sapinea isolate 3AP or D. scrobiculata isolate B1 (Blodgett and Bonello 2003). In each trial, there were 10 trees per induction treatment. Unwounded control trees were omitted from these trials because previous studies with Monterey and Austrian pines have shown that unwounded control trees do not differ from trees receiving a mock induction in terms of either SIR or secondary

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**Figure 1.** Schematic representation of the relative locations of the induction and challenge treatments on Austrian pine trees for Experiments 1–3.
metabolism (Bonello et al. 2001, Bonello and Blodgett 2003). Three weeks after induction, the trees were challenge-inoculated 25 cm above the induction site by wounding with a cork borer followed either by mock inoculation with sterile PDA plugs, or inoculation with mycelial plugs from PDA cultures of the S. sapinea isolate. One challenge inoculation treatment was used per stem, and half the trees in each induction treatment were either mock inoculated or inoculated with S. sapinea. The lengths of lesions above the challenge points were determined five weeks later and used as a measure of systemic host resistance. Phloem samples were collected from the margin of the challenge lesions (reaction zones), extracted and processed for chemical analyses.

Experiment 2 (upper stem induction, lower stem challenge) Experiment 2 was conducted in July 2005. There were two induction treatments: mock induction and inoculation with S. sapinea isolate 3AP, with five trees per treatment. In each induction treatment, trees were inoculated at 30 cm above the soil in the same manner as described in Experiment 1. Three weeks after induction, the trees were challenge-inoculated 25 cm below the induction site by wounding with a cork borer followed by mock inoculation or inoculation with the S. sapinea isolate. The lengths of challenge lesions below the challenge points were measured three weeks later. In this case, no chemical analyses were conducted.

Experiment 3 (lower stem induction, shoot tip challenge) Experiment 3 was conducted in two independent trials (beginning on June 7 and June 21, 2001). The induction treatments were as in Experiment 1, except that an unwounded control was also included. Challenge-inoculation treatments were performed about 2 cm below the shoot apex on expanding shoots as described previously (Blodgett and Bonello 2003). One of four randomly selected shoot tips on each tree was assigned to the following challenge-inoculation treatments: unwounded treatment, mock challenge, challenge with S. sapinea isolate 3AP and challenge with S. sapinea isolate 1SP (from Scots pine). The latter challenge was conducted to determine if the source of the isolate for the challenge affected the host response in relation to the induction treatments. Results of this challenge treatment did not differ from the challenge with S. sapinea isolate 3AP, and are not shown. Three weeks after the challenge-inoculation treatments, challenge lesions were measured as previously described (Blodgett and Bonello 2003), and shoot tip samples were collected, extracted, and processed for chemical analyses.

Chemical analyses Soluble and cell-wall-bound phenolics were analyzed by high-performance liquid chromatography (HPLC), and lignin was quantified spectrophotometrically as described by Bonello and Blodgett (2003). All but two of the phenolic compounds were positively identified with standards based on retention time and UV spectrum.

Statistical analyses 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 by univariate ANOVA. Means were separated by Fisher’s least significant difference (LSD) multiple range test at $P = 0.05$. When the assumptions of normality and variance homogeneity could not be satisfied, non-parametric Kruskal-Wallis tests were used. In Experiment 1, trial (two levels), induction treatment (three levels) and challenge treatment (two levels) were the factors. Comparisons were also made between fungal induction treatment (S. sapinea and D. scrobiculata) and mock induction giving two induction treatment levels. In Experiment 3, trial (two levels), induction treatment (four levels) and challenge treatment (four levels) were the factors. Comparisons were also made between fungal induction treatment (S. sapinea and D. scrobiculata) and noninoculation (mock induction and unwounded controls), giving two induction treatment levels. All factors were treated as fixed factors in the ANOVA. Degrees of freedom (DF) are indicated as subscripts to the $F_{DF}$ statistics in the results.

Relationships among the various metabolites and among metabolites and challenge lesion length were tested by non-parametric Spearman correlations on individual tree data and parametric Pearson’s product moment correlations on treatment means (Cipollini et al. 2004).

Results Induction treatment effects on systemic resistance and susceptibility to challenge inoculations Inoculation at the stem base with either S. sapinea or D. scrobiculata was equally effective in inducing an SIR response to a challenge-inoculation with S. sapinea at 25 cm above the induction site (Figure 1, Experiment 1) compared with the mock induction (treatment: $F_{2,28} = 7.241$, $P < 0.01$; trial: $F_{1,28} = 0.104$, $P = 0.750$; treatment × trial: $F_{2,28} = 2.566$, $P = 0.099$) (Figure 2A). On average, challenge lesions were 48% ($P < 0.01$) and 37% ($P < 0.05$) smaller in stems induced with S. sapinea and D. scrobiculata, respectively, compared with the corresponding lesions in stems of mock-induced trees. In Experiment 2, compared with mock-induced saplings, inoculation of potted Austrian pine saplings in the upper stem at 30 cm above soil with S. sapinea (Figure 1, Experiment 2) induced an SIR response to a challenge-inoculation with S. sapinea in the stem 25 cm below the induction site ($F_{1,9} = 8.253$, $P < 0.05$) (Figure 2B). On average, challenge lesions were 38% smaller in stems of trees induced with S. sapinea than the corresponding stem lesions of mock-induced trees. In Experiment 3, inoculation at the stem base with either S. sapinea or D. scrobiculata (Figure 1, Experiment 3) resulted in SIS in elongating shoot tips challenge-inoculated with S. sapinea about 2 cm below the shoot apex compared with shoot tips of mock-induced or untreated stems (treat-
ment: $F_{3.39} = 3.301, P < 0.05$; trial: $F_{1.39} = 4.417, P < 0.05$; treatment × trial: $F_{3.39} = 0.393, \text{NS}$) (Figure 2C). The challenge lesions were 45% (NS), 121% ($P < 0.05$; trial: $F_{1.39} = 4.371, P < 0.05$; treatment × trial: $F_{1.39} = 0.106, \text{NS}$).

**Lignin**

In Experiment 1, compared with the mock induction, induction with *S. sapinea* or *D. scrobiculata* resulted in a 25% increase in lignification of the reaction zone of lesions caused by fungal challenge in the upper stem; however, the increase in lignin concentration was not significant (treatment: $F_{1.29} = 3.493, P = 0.073$; trial: $F_{1.99} = 1.004, P = 0.326$; treatment × trial: $F_{1.99} = 0.668, P = 0.421$). Nevertheless, there was a strong negative linear relationship between challenge lesion length and lignin concentration in the reaction zone ($r^2 = 0.9956, P < 0.05, n = 3$) (Figure 3A). Furthermore, mean lignin accumulation in response to pathogenic challenge was positively correlated with lignin accumulation in response to a mock challenge, albeit not significantly ($r^2 = 0.8086, P = 0.288, n = 3$) (Figure 3B).

In Experiment 3, the induction treatments had no significant effects on lignin accumulation in response to challenge-inoculation with *S. sapinea* at the shoot tip. However, lignin concentrations were positively correlated with shoot tip lesion lengths (Table 1). In contrast to the stem challenge, there was no relationship between mean lignin accumulation and mean lesion length at the shoot tip (Figure 3C). In the induced trees, there was a positive linear relationship between lignin accumulation in response to a pathogenic challenge and lignin accumulation in response to a mock challenge ($r^2 = 0.9922, P < 0.066, n = 3$) (Figure 3D), as observed in response to a challenge in the upper stem (Figure 3B). In Experiment 3, among the induction treatments, induction with *D. scrobiculata* at the stem base resulted in the lowest lignin accumulation in the challenge lesion at the shoot tip. Lignin accumulation was 24–32% lower in lesions resulting from a mock challenge than in lesions caused by challenge-inoculation with *S. sapinea*, depending on induction treatment (Figure 3D). In trees that were not induced, the amount of lignin accumulated in response to a fungal challenge was roughly equivalent to the amount accumulated in response to a mock challenge (wound challenge), about 18 mg g$^{-1}$ fresh mass (data not shown).

**Phenolic compounds and other secondary metabolites**

The concentration of none of the soluble phenolic compounds analyzed was affected by the treatments. Furthermore, in Experiment 1, challenge lesion length was not correlated with the concentration of any of the secondary metabolites analyzed other than pinosylvin monomethyl ether (Table 1). In Experiment 3, the lengths of the lesions caused by a challenge inoculation with *S. sapinea* at the shoot tips were negatively correlated with concentrations of cell-wall-bound ferulic acid, free ferulic acid glucoside, a taxifolin-like compound, and two unknown compounds with retention times of 8.9 and 11.3 min (Table 1).
Discussion

We demonstrated that SIR occurs in stems of Austrian pines induced with two common fungal canker pathogens. We also showed that this phenomenon is bi-directional, i.e., induction of the lower stem induced resistance in the upper stem and vice versa. Furthermore, induction at the stem base resulted in SIS in pathogen-challenged shoot tips, and the less aggressive pathogen *D. scrobiculata* induced greater susceptibility than the more aggressive pathogen *S. sapinea*. Although SIR is known to occur in trees (Enebak and Carey 2000, Bonello et al. 2001, Enebak and Carey 2004), this is the first time that organ-dependent SIS by a pathogen has been documented in trees or any other plants, although general SIS has been observed in *Arabidopsis* (Cui et al. 2005).

The mechanisms underlying SIR and SIS are poorly defined (Cui et al. 2005) and are unknown in trees. Bonello and Blodgett (2003) reported that basal stem inoculations of Austrian pine saplings resulted in significant changes in secondary metabolite chemistry in the stem upstream of the inducing inoculation, and argued that such changes may provide a basis for potentiation of a host response to a subsequent pathogenic challenge. Distal and systemic potentiation of host defense responses by inducing pathogenic attacks is well known in herbaceous plants (Graham and Graham 1999), but has only recently been explored in conifers (Krokene et al. 1999, Franceschi et al. 2000, Nagy et al. 2000, Franceschi et al. 2002, Bonello and Blodgett 2003, Bonello et al. 2003, Hudgins et al. 2003, McNee et al. 2003, Luchi et al. 2005). All of these studies focused on characterization of host defense responses in stems, whereas we characterized systemic responses to pathogens in both stems and shoot tips.

One of these host defense responses, at least in the stem, may be induced lignification. We found that lignin concentration in the reaction zone of fungal-induced trees challenged with pathogens was about 25% higher (*P = 0.073*) than that of mock-induced (wounded) controls. Mean lignin concentration was negatively correlated with mean lesion length with a strong, significant linear relationship. The positive linear correlation between mean lignin concentration in reaction zones of fungal-challenged lesions and mock-challenged lesions (Cui et al. 2005) and are unknown in trees. Bonello and Blodgett (2003) reported that basal stem inoculations of Austrian pine saplings resulted in significant changes in secondary metabolite chemistry in the stem upstream of the inducing inoculation, and argued that such changes may provide a basis for potentiation of a host response to a subsequent pathogenic challenge. Distal and systemic potentiation of host defense responses by inducing pathogenic attacks is well known in herbaceous plants (Graham and Graham 1999), but has only recently been explored in conifers (Krokene et al. 1999, Franceschi et al. 2000, Nagy et al. 2000, Franceschi et al. 2002, Bonello and Blodgett 2003, Bonello et al. 2003, Hudgins et al. 2003, McNee et al. 2003, Luchi et al. 2005). All of these studies focused on characterization of host defense responses in stems, whereas we characterized systemic responses to pathogens in both stems and shoot tips.

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The positive correlation between length of challenge lesions...
in the stem and pinosylvin monomethyl ether, together with the lack of treatment effects on this compound and the related compound pinosylvins, provide indirect support for the suggestion that stilbenes play no major role in induced resistance (Bonello and Blodgett 2003). However, given the strong in vitro antifungal properties of stilbenes (e.g., Bonello et al. 1993) and the significantly higher concentrations of stilbenes in unchallenged phloem of induced trees (Bonello and Blodgett 2003), it is possible that these compounds contribute to reduced tissue invasion by the fungus at the time of challenge (Bonello and Blodgett 2003), with other defense responses becoming important later in the expression of SIR, such as induced lignification. Given the lack of treatment effects on these compounds in Experiment 3, the negative correlations between challenge lesion lengths and the concentrations of various phenolic compounds might reflect their catabolism by the fungus (Bonello and Blodgett 2003).

In conclusion, four key findings were obtained: (1) both SIR and SIS expression were organ-dependent; (2) the long-distance transport of signal molecules that elicit SIR was bidirectional, at least in the stem; (3) D. scrobiculata, the less aggressive of the two pathogens tested, induced a stronger SIS response; and (4) induced lignification might be one mechanism involved in the systemic defense responses of Austrian pine. Our data provide the basis for at least one testable hypothesis, namely that SIR induction in response to stem inoculation with a pathogen results in reallocation of transportable secondary metabolites (such as the glycosides of lignin precursors (Bonello et al. 2003)) from the needles and shoot tips to the stem phloem, where they participate in a defense response to the inducing infection. Our results also demonstrate the need for further studies of systemic plant responses to pathogens in gymnosperms, because herbaceous angiosperm model systems may not adequately represent this important taxonomic division of plants.

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References


Table 1. Correlations between lengths of Sphaeropsis sapinea challenge lesions and selected secondary metabolites in Pinus nigra. For Experiment 1, n = 29, and for Experiment 3, n = 40. Abbreviations: FA, ferulic acid; FAG, ferulic acid glucoside; TLC, taxifolin-like compound; PSM, pinosylvin monomethyl ether; and r; Pearson’s product moment correlation.

<table>
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<th>Statistic</th>
<th>Peak 8.9</th>
<th>Peak 11.3</th>
<th>FA</th>
<th>FAG</th>
<th>TLC</th>
<th>PSM</th>
<th>Lignin</th>
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<td>Experiment 3: Shoot tip lesions</td>
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1 Unidentified metabolites with retention times of 8.9 and 11.3 min.
2 For Experiment 1, only significant Spearman correlations between length of challenge lesions and concentrations of secondary metabolites analyzed are shown.


