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Testing the systemic induced resistance hypothesis with Austrian pine and *Diplodia sapinea*

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ABSTRACT

Systemic induction of defenses (e.g. phenolic metabolites) is considered vital in conifer resistance to pathogens and insects, and forms the mechanistic basis of the systemic induced resistance hypothesis (SIRH). In this study, the SIRH was tested on juvenile Austrian pine. Main stems expressed SIR in a manner that was consistent with the SIRH, while shoots became uniformly more susceptible to subsequent inoculations, demonstrating clear organ specificity in the tree's response. The majority of phenolic metabolites were poorly correlated with phenotype. Thus, the defensive system of Austrian pine is highly plastic and organ specific, and cannot be predicted by phenolic profiles alone.

1. Introduction

Plant constitutive defenses are sufficient against most attacking organisms, but are energetically expensive to build and maintain in the absence of an attack. Induced defenses are thought to minimize these fitness costs by engaging only during times of attack [1]. These induced defenses can be biochemical, anatomical, indirect (e.g. tritrophic interactions), or manifested as a tolerance response, and can be induced at the site of infection or systemically [2]. Induced responses can have important ecological consequences [3]. For instance, when induced defenses are expressed in distal tissues, the phenomenon of systemic induced resistance (SIR) can occur, whereby the whole plant has heightened resistance to subsequent biotic challenges. This phenomenon has been well studied in model plants but comparatively less so in trees [2]. However, SIR against some fungi and insects has been observed in certain conifer species [4–10].

Trees possess a multi-layered array of mechanisms that help protect them against herbivores and pathogenic microbes [11]. Induced defenses are considered vital components of tree defense [2], and may explain why, for most forest ecosystems, the majority of trees at any given time are healthy, despite being continually exposed to pathogens and pests. However, despite the induction of systemic defenses, trees symptomatic of infection are frequently weaker against further attacks, a phenomenon known as systemic induced susceptibility (SIS). Thus, a fundamental question arises: if trees express induced defenses after an initial attack, why are symptomatic trees more susceptible to subsequent attack? The systemic induced resistance hypothesis (SIRH) explores this conundrum [12]. The SIRH predicts that an induction event will trigger systemic defenses that increase resistance to subsequent attacks, and this increase in resistance will manifest over time and persist if the induction event is minor enough to not directly compromise the plant's overall ability to respond to further attack. However, if the induction event is severe, a tree's resistance will drop below constitutive levels, as energy reserves are depleted. At this point, trees will become symptomatic and express SIS if challenged by another attack. Thus, the SIRH predicts a quadratic response over time for the strength of induced resistance when induction events are severe enough to compromise overall tree vigor [see Fig. 1 in Bonello et al. (2006)]. To capture this temporal aspect, SIR must be assessed at multiple time points following an induction event. Bonello et al. (2006) also propose that specialized metabolites, like phenolics, are important for determining whether trees express SIR or SIS, and that the levels of these metabolites can be correlated to resistance in the plant. While a useful framework for predicting plant-pathogen interaction outcomes, the SIRH

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remains to be experimentally validated in any plant system.

The Austrian pine – *Diplodia sapinea* pathosystem has been used to examine SIR in trees, and to characterize the signaling and biochemical aspects of SIR [8,13–15], making it an ideal model system for investigating the SIRH. For this pathosystem, trees are first induced with inoculations of *D. sapinea* on the main stem. In all work conducted to date, a second, challenge inoculation of *D. sapinea* is then applied to a distal tissue at one single time (usually three weeks post induction), while the tree still shows no crown symptoms from the induction inoculation. This work has shown that when trees are challenged in a distal portion of the main stem, SIR is expressed, as denoted by shorter challenge lesion lengths compared to non-induced controls [6,8]. Interestingly, while the stem expresses SIR following induction, the shoots of similarly induced trees express SIS [6]. A similar increase in shoot susceptibility to *D. sapinea* was observed in Stone pine (*Pinus pinea* L.) following induction of the main stem with *Heterobasidion* species [16]. The underlying mechanisms conferring SIR or SIS are not known, but it is hypothesized that the synthesis and translocation of defensive metabolites in and out of the distal tissues are critical in this response [6,14,16].

Here the Austrian pine – *Diplodia sapinea* pathosystem was used to test whether (1) SIR or SIS are characterized by a temporal component following induction, as predicted by the SIRH; (2) conformance to the SIRH is organ specific; and (3) phenolic metabolism is associated with the SIR or SIS phenotypes.

2. Materials and methods

2.1. Plant and fungal material

Four year-old, open pollinated Austrian pine trees growing in 1-gallon plastic pots (plants donated from Willoway Nursery Madison, OH) were arranged outdoors on an ornamental nursery gravel lot. Trees received watering via drip irrigation twice daily throughout the duration of the experiment. A strain of *D. sapinea* used in previous, published work was isolated from symptomatic pine cones of an Austrian pine tree growing on The Ohio State University campus in Columbus, Ohio [17].

2.2. Experimental design and layout

To test the SIRH, trees were first induced by inoculating the lower stem, and then challenged with distal inoculations on either the main stem or the shoots. The resulting challenge lesions were measured to assess the strength of SIR or SIS. To investigate the temporal component of the SIRH, the application times of the induction inoculations were staggered to create different intervals of time between induction and challenge. This staggering of induction time points allowed for all trees to be challenged on the same day, thereby controlling for the effect of potentially different environmental conditions at the time of challenge. Individual trees were assigned to a single induction time point, and no tree was induced more than once. Induction time points were 4, 8, and 12 days before challenge (dbc). A group of non-induced control (NIC) trees was also included.

The SIRH predicts that trees which become symptomatic in response to the initial induction will express SIS instead of SIR [12]. To test this aspect of the SIRH, a fifth induction treatment was included, where trees were induced 12 dbc like above, but received double the amount of induction inoculations. This greater inoculation amount was determined in preliminary trials to cause symptoms (shepherd's crooking) within 10–16 days, depending on the individual tree. Therefore, at the 12 dbc time point there were two groups of trees: (1) those that received an induction dose equivalent to trees in the 4 dbc and 8 dbc time points (hereafter referred to as 12 dbc asymptomatic), and (2) those that received the heavier induction treatment known to cause symptoms (hereafter referred to as 12 dbc symptomatic). Induction inoculations for 12 dbc asymptomatic and symptomatic trees were applied on July 15, 2013; 8 dbc induction inoculations were applied on July 19, 2013; 4 dbc induction inoculations were applied on July 23, 2013. All trees received challenge inoculations on July 27, 2013. A few exceptions to challenge inoculation timing were made for the 12 dbc symptomatic trees, and are explained below in the section on *Inoculations, Tissue Collection and Resistance Assessment*.

Trees were arranged in a randomized complete block design with four blocks, with each block containing 20 trees for a total of 80 trees. Trees were generally similar in size and appearance, but were blocked by total height based on visual inspection. Each block had four replicate trees for each of the five unique induction treatments. Two of the four replicates were then assigned a challenge inoculation tissue (stem or shoot) to give two replicates of each induction treatment by challenge tissue per block.

2.3. Inoculations, tissue collection and resistance assessment

Induction treatments consisted of two inoculations of *D. sapinea* on opposite sides of the main stem 15 cm above the soil line, except for the 12 dbc symptomatic trees, which received four inoculations spaced evenly around the stem 15 cm above the soil line. Inoculations were applied using a 5 mm cork borer to remove the outer bark and phloem, exposing the xylem. Plant tissue was replaced with 5 mm diameter agar plugs taken from the margins of an active *D. sapinea* colony growing on potato dextrose agar (PDA). These plugs were placed mycelium-side down on the wound, and sealed with duct-tape to minimize contamination and desiccation. Mock inoculations have never previously resulted in SIS/SIR or the systemic accumulation of phenolic compounds in this system [6,14], and so they were omitted in this study.

All NIC, 4 dbc, 8 dbc, 12 dbc asymptomatic trees, and thirteen of the 12 dbc symptomatic trees actually expressing symptoms received challenge inoculations on July 27, 2013. The remaining three 12 dbc symptomatic trees received challenge inoculations when they became symptomatic, which occurred on July 29, July 31, and August 2, 2013, depending on the individual tree; thus, the exact time between induction inoculation and challenge inoculation for the symptomatic trees varied between twelve and eighteen days. However, this treatment will continue to be referred to as 12 dbc symptomatic for conceptual consistency and ease of interpretation. The trees that became symptomatic on July 29 and July 31 were both shoot-challenged (blocks 1 and 4 respectively), while the tree that became symptomatic on August 2 was stem-challenged (block 3).

On the main stem, a single challenge inoculation was applied 30 cm above the soil line (i.e. 15 cm above the induction site) in a manner consistent with the induction inoculation. A 5 mm diameter phloem plug was also removed from the stem on the opposite side of the challenge inoculation to provide enough tissue for the phytochemical analyses, but this site was not inoculated. The challenge sites were sealed with duct tape. Shoots were challenge-inoculated following the methods of Blodgett and Bonello (2003) [18], and consisted of creating a small, circular wound of approximately 3 mm in diameter made on the current-year shoots using a scalpel at approximately 10 cm from the shoot tip. Needles immediately surrounding the inoculation site were removed, and a single 3 mm diameter agar plug, taken from the margins of an active *D. sapinea* colony growing on PDA, was placed mycelium-side down on the wound. Shoot inoculations were then wrapped with parafilm. For each tree, two shoots were inoculated. At the time of