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Applications of Defense Elicitors to Roots of Containerized Eastern White Pine (*Pinus strobus*) Stimulate Increased Defensive Enzyme Activities of Fine Roots

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Abstract. The expansion of the use of induced resistance (IR) has been, and remains, an attractive prospect for the management of woody plants, but little research has occurred assessing the ability of elicitors to induce the root defenses of woody plants. Eastern white pine (*Pinus strobus*) was used as a model plant to assess IR elicitation. Containerized plants were treated with phosphite (Phi), chitosan, curdlan (a β-1,3-glucan polymer), or silicon (Si) on 2022 June 7. The soluble phenolic levels, tissue levels of major resin acids (abietic and neoabietic), as well as the activities of peroxidase (POX), chitinase (CHI), and β-1,3-glucanase (βGLU) of fine roots were compared across elicitor treatments and nontreated controls on June 10, June 14, and June 27. There were no changes post-treatment to soluble phenolics or resin acids at any sampling point, but chitosan treatment resulted in an increase in POX and CHI activities, while curdlan increased CHI activity on June 10. On June 14, curdlan-treated plants had significantly higher POX and CHI activities, while Phi-treated plants had significantly higher POX activity. By June 27, curdlan- and Phi-treated trees had significantly higher CHI activities. Our data suggest that chitosan, curdlan, and phosphite stimulate biochemical responses and potentially prime root systems to respond to subsequent stresses, but there appears to be variation between these elicitors regarding rapid versus longer lasting IR effects.

Keywords. Defense Elicitors; Defense Induction; Induced Resistance; Pest Management; Root Defenses.

INTRODUCTION

Induced resistance (IR) is defined broadly as the activation of a plant's own genetically programed defenses, resulting in alterations that serve to reduce the impacts of ensuing pest or pathogen attack (Oostendorp et al. 2001; Percival 2001; Mauch-Mani et al. 2017). The utility and expanded use of IR in woody plant management has been of high interest for many years due to its potential cost-effectiveness and environmental friendliness (Percival 2001; Eyles et al. 2010; Llorens et al. 2017; Martín-García et al. 2019). Induced resistance-based strategies in woody plant industries take the form typically of the physical exposure of plants to a defense-eliciting substance prior to pest infestation or pathogen infection. For example, the management of woody plant diseases caused by various species of *Phytophthora* oomycetes commonly utilize phosphite elicitors (Pilbeam et al. 2011) such as against root rot of avocado (*Persea americana*)(e.g., Dobrowolski et al. 2008) or sudden oak death (caused by *Phytophthora ramorum*) of oaks (*Quercus* spp.) and tanoak (*Notholithocarpus densiflorus*)(e.g., Garbelotto et al. 2007).

Phosphites are conjugate bases of phosphorous acid, existing as salts (e.g., potassium phosphite), and are one of the more well-researched and developed IR elicitors in woody plant systems with several products commercially available, typically as fertilizers or fungicides. Phosphites have been shown to suppress pathogen infection of woody plants and are known to influence the physiology and biochemistry of trees in ways that would putatively increase

resistance (e.g., Cerqueira et al. 2017; Kasuga et al. 2021). However, the specific mechanisms involved in phosphite suppression of disease remain poorly understood and, beyond its ability to enhance resistance mechanisms, could involve several mechanisms including direct toxicity (e.g., Dalio et al. 2014) and competitive inhibition of phosphate metabolism of the attacking organism (e.g., Niere et al. 1994).

Other IR-eliciting substances such as chitosan and silicon (Si) have been studied in woody plants to varying degrees. Chitosan is a chemical derivative of chitin, a cell wall component of fungi, known to elicit IR to pathogens and relatively well-studied aboveground (e.g., Reglinski et al. 2004) but not belowground. The ability of chitosan to completely solubilize in slightly acidified water greatly enhances its utility in the context of its commercial use, where chitin is insoluble in most solvents (Roy et al. 2017), preventing its commercial use. Silicon is well studied in agricultural crop pathosystems (Wang et al. 2017) and has been shown to reduce foliar disease in rose (Gillman et al. 2003), yet there is limited research investigating the effects of Si on woody plant roots. The association of Si with increased levels of pest and disease resistance is partly physical, associated with the ability of certain plants to take up Si particles and incorporate them into tissues, which increases leaf toughness, but also partly due to the ability of Si to activate biochemical defenses (Wang et al. 2017). Chitosan- and Si-based products are available in the arboriculture industry, though the marketing and labelling of these products varies (e.g., fungicides, biostimulants, fertilizers).

Pathogen-derived substances are also well known to be defense elicitors. β-1,3-glucans are major constituents of *Phytophthora* cell walls, and though we are not aware of any research having taken place in woody plant systems, several researchers have reported defense responses and improved resistance in agricultural and model herbaceous plants upon exposure to these carbohydrates (Gozzo and Faoro 2013; Raaymakers and Van den Ackerveken 2016). Laminarin is a β-1,3-glucan that can exist in a water-soluble form and is derived from the brown algae *Laminaria digitata* that has been shown to elicit IR responses in several plants such as grapevine (*Vitis vinifera*)(Aziz et al. 2003). However, laminarin is prohibitively expensive to produce at commercial scale. For these reasons, pathogen-derived substances like cell wall components from *Phytophthora* or chemically relative substances like laminarin remain at the developmental stage regarding their use in woody plant industries. Insoluble β-1,3-glucans, such as curdlan, which is produced by the bacterium *Alcaligenes faecalis* (Harada et al. 1966), and *L. digitata* powders, can be found commercially and be less cost prohibitive, but how these substances would be applied and their effectiveness are unknown.

Eastern white pine (*Pinus strobus*) is particularly sensitive to *Phytophthora* root rot (Frampton et al. 2018). As a group, the biochemical defense mechanisms of pines are well characterized and rely on the accumulation of secondary metabolites such as phenolics and various types of terpenes (e.g., Trowbridge et al. 2016; Mason et al. 2017), as well as on protein-based defenses such as defensive enzymes (Barto et al. 2008). In this study, we used containerized white pine saplings as a model to evaluate the ability of phosphite (Phi), chitosan, Si, and insoluble curdlan to induce root phenolic-, terpene-, and enzyme-based defenses when these elicitors are applied to roots in a soil medium. Our aims were to detect a biochemical response to each of these elicitors, to elucidate potential differences in the type of biochemical response, and to assess if and/or how these biochemical responses were affected by time since application. We hypothesized that each of these substances would elicit a quantifiable defense response (e.g., phenolic accumulation), that plants would have unique biochemical responses to different eliciting substances (e.g., the increase in activity of different enzymes), and that elicitors likely have different temporal dynamics (e.g., one elicitor having a longer lasting quantifiable effect). Ultimately, information from this experiment can help guide future research and development of IR substances, products, and procedures within woody plant industries.

MATERIALS AND METHODS

Greenhouse Experiment

Eastern white pine saplings (approximately 10 to 12 mm stem diameter; seed sourced from Michigan; 120 plants) were purchased from Vans Pines Nursery (West Olive, MI, USA) and delivered in March of 2022 to The Morton Arboretum (Lisle, IL, USA) where all work took place. Greenhouse temperature was only controlled by fans and was approximately 12 to 30 °C from March to late June 2022. Plants were exposed to natural daylight conditions (approximately 12 hours daylight in mid-March to approximately 15

hours daylight in late June). Plants were potted in 1-gallon (3.8-L) pots with potting media (60:20:10:10 pine fines:peat moss:perlite:HydraFiber® EZ Blend [PROFILE Products LLC, Conover, NC, USA]; 5.5% dolomitic limestone; 1% gypsum; and a proprietary mix of macro and micronutrients). An additional 10-g dolomitic limestone were added to each pot to further resist pH change (Kamenidou et al. 2008, 2010). Plants were watered 2 to 3 times per week and allowed to acclimate to greenhouse conditions for 2 months (April through May 2022) until experiments began in June.

Plants were divided equally into the following 5 treatments (n = 24 plants/treatment): (1) potassium phosphite (Reliant®; Quest Products LLC, Linwood, KS, USA) at 940 µl l^{-1} (henceforth 'Phi'); (2) potassium silicate (K_2SiO_3)(AgSil® 16H; PQ Corporation, Valley Forge, PA, USA) at 500 mg l^{-1} (henceforth 'Si'); (3) chitosan (Sigma-Aldrich, Inc., St. Louis, MO, USA) at 250 mg l^{-1}; (4) curdlan (Sigma-Aldrich, Inc., St. Louis, MO, USA) at 200 mg l^{-1}; and (5) a control treatment consisting only of carrier solution. This number of replicates per treatment was used based on budgetary considerations. Carrier solution for all treatments was 0.1% acetic acid (Sigma-Aldrich, Inc., St. Louis, MO, USA) in de-ionized water. Solutions were prepared the day prior to application and allowed to incubate on a shaker overnight to ensure full dissolution. Application of elicitor solutions were applied at a volume of 100 mL per container. The application rate of Reliant® used reflects the low application rate on the label; the rate of AgSil® mirrors low dose rates of Si applied in similar experiments (Kamenidou et al. 2008, 2010). The dose rate of chitosan was experimental, though it is worth mentioning that experimental application rates and procedures of chitosan in the literature are incredibly variable (e.g., Benhamou and Thériault 1992; El Ghaouth et al. 1994; LaFontaine and Benhamou 1996; Zheng et al. 2021). The dose rate of curdlan was entirely experimental.

All products were applied on 2022 June 7 by digging 3 to 5 cm into pots right at the base of the plant to expose the roots and pouring elicitor solution directly onto the roots. This was done to ensure sufficient root contact with elicitors. For curdlan, the container containing solution was shaken vigorously for 3 to 5 seconds before uncapping and pouring directly onto the root systems in an attempt to reduce variability in dosage. This was done for each replicate. Plants were then allowed to respond to treatments, and subsets of 8 plants were harvested on June 10 (3 days post-treatment), June 14 (7 days post-treatment), and June 27 (20 days post-treatment). We chose to treat with each elicitor once rather than multiple treatments to reflect more of a practical tree management scenario where managers can often only perform a single treatment. To maintain a satisfactory level of replication (8 replicates per treatment per time point), we could only choose 3 time points for which to analyze induction responses. Therefore, we chose these 3 sampling points to attempt to capture both relatively rapid as well as longer-lasting induction responses.

Harvesting and Laboratory Preparation for Defense Induction Analyses

Plants were removed from pots, debris was washed from the root system using tap water, roots were dried with paper towels, and entire root systems were excised. Whole root systems were placed into plastic sandwich bags and stored at −20 °C until they could be processed. For processing, whole root systems were removed from −20 °C, and fine roots (i.e., thin, fibrous, nonwoody, approximately ≤ 2 mm thick) were removed from the whole root system by hand, placed directly into a mortar with liquid nitrogen, and ground into a powder in liquid nitrogen with a pestle. We elected to focus on fine roots because this is where new *Phytophthora* infections occur (Zwart and Kim 2012). Ground tissue was then partitioned into a 1.5-mL microtube (100 mg) to quantify total soluble phenolics and resin acids (i.e., abietic and neoabietic acids) as well as a 15-mL centrifuge tube (2 g) for enzyme activities. The resin acids, abietic acid and neoabietic acid, are 2 major constituents of conifer resins (Keeling and Bohlmann 2006; Kersten et al. 2006) which are known to have major roles in defense responses in pines. Tubes were stored at −20 °C until extractions and analyses could occur. Time points were all processed and analyzed separately. All solvents, buffers, and reagent material were prepared fresh for each set of plants. This experimental design and analysis procedure prohibited us from making direct treatment comparisons between time points. Therefore, treatments were compared only within a time point (statistical approach explained below).

Chemical and Enzymatic Defense Responses

Total soluble phenolics and abietane diterpenes (i.e., abietic and neoabietic acids) were extracted for 20

minutes in 700 µl of methanol containing 0.1-mg mL^{-1} internal standard (butylated hydroxyanisole; Sigma-Aldrich, Inc., St. Louis, MO, USA) in an ice bath using a sonicator, and the 21,000 g of supernatant (2 min) was used in analyses. Total soluble phenolic levels were quantified via the modified Folin–Ciocalteu procedure described by Cipollini et al. (2011) against a standard curve of gallic acid containing internal standard. Abietic and neoabietic acids were matched to external standards (Sigma-Aldrich, Inc., St. Louis, MO, USA) and quantified similarly as described by Kersten et al. (2006) via high performance liquid chromatography (HPLC) with slight modifications: an isocratic solvent system of 92.5% acidified methanol (0.1% acetic acid) and 7.5% acidified water (0.1% acetic acid) with a column temperature of 50 °C. The instrumentation used was a Shimadzu LC-2030C 3D Plus Prominence-*i* (Shimadzu Corporation, Kyoto, Japan) with a Nucleosil C_{18} column (250 mm × 4.6 mm; 5-µm particle size) equipped with a 20-mm precolumn of the same stationary phase.

For enzymatic defenses, powdered tissue was sonicated in 3 mL of 50 mM sodium phosphate buffer (pH 6.8) containing 3% (w:v) polyvinyl polypyrolidone (PVPP; The Vintner Vault, Paso Robles, CA, USA) for 10 min in an ice bath, and the 3,000-*g* (5 min) supernatant (1 mL) was used as the source of enzymes. The Bradford (1976) method was used to quantify the protein content of extracts using bovine serum albumin as standard. The activities of chitinase (CHI), peroxidase (POX), and β-1,3-glucanase (βGLU) were quantified according to Rigsby et al. (2016), Rigsby et al. (2018), and Barto et al. (2008), respectively. All materials for assays were sourced from Sigma-Aldrich, Inc. A Synergy HTX (BioTek Instruments, Winooski, VT, USA) plate reader and standard 96-well plates were used to quantify absorbance in all assays.

Statistical Analysis

Our experimental design (e.g., not a repeated measures design) and laboratory procedures (e.g., samples from a single time point were processed and analyzed separately) prevented us from directly comparing data between time points, necessitating that predictor impacts on responses be assessed within time points. Data were first inspected for normality via the Shapiro-Wilk test and transformed if necessary. The activities of βGLU on both June 14 and 27 required log transformations, and CHI activity on June 27 required a square root transformation. Abietic and neoabietic acid levels on June 27 also required a square root transformation. One-way ANOVA's were performed with treatment as the predictor variable. Models where elicitor treatment was a significant predictor were then subjected to a Tukey's post-hoc separation of means. The statistics software R was used in all analyses (R Core Team 2018).

RESULTS

There was no significant effect of elicitor treatment for total soluble phenolics, abietic acid, or neoabietic acid at any sampling time point ($P > 0.05$ for all). Also, there was no significant effect of elicitor treatment detected for βGLU activity at any sampling time point ($P > 0.05$ for all). The activity of POX was significantly affected by elicitor treatment on June 10 ($F_{4,35} = 4.38$, $P < 0.01$) and June 14 ($F_{4,35} = 4.23$, $P < 0.01$), but not on June 27 ($P > 0.05$). Specifically, on June 10, the Tukey separation of means showed that chitosan- and curdlan-treated fine roots had the highest POX activities, and the Phi-treated and control root systems had the lowest activities, while Si-treated root tissue were not differentiable from either group (Table 1). On June 14, both curdlan- and Phi-treated plants had higher levels of fine root POX activity, which was differentiable from control plants. Chitosan- and Si-treated plant root tissue POX activities were unable to be differentiated from other treatments (Table 1).

The activity of CHI was significantly affected by elicitor treatment on June 10 ($F_{4,35} = 17.43$, $P < 0.01$), June 14 ($F_{4,35} = 4.81$, $P < 0.01$), and June 27 ($F_{4,35} = 4.14$, $P < 0.01$). On June 10, chitosan-treated plants had the highest CHI activity, which was differentiable from all other treatments except for Si. Phi-treated plants had the lowest root tissue CHI activities, which was differentiable from all other treatments (Table 1). On June 14, curdlan-treated plants had the highest root tissue CHI activities, which was differentiable from control plants. All other treatments were not differentiable from controls (Table 1). On June 27, curdlan- and Phi-treated plants had the highest root tissue CHI activities, and control plants had the lowest, which was differentiable. Si- and chitosan-treated activities could not be differentiated (Table 1).

DISCUSSION

The elevated root POX and CHI activities of chitosan-, curdlan-, and Phi-treated plants likely denotes the

Table 1. Mean (± 1 SE) peroxidase and chitinase enzyme activities quantified 3 (June 10), 7 (June 14), and 20 (June 27) days post-treatment. Letters indicate a significant effect of elicitor, and different letters indicate statistical differences via post-hoc Tukey tests. SE (standard error); POX (peroxidase); CHI (chitinase); Phi (phosphite); Si (silicone).

Activity	Elicitor	June 10	June 14	June 27
POX (mmols min^{-1} mg^{-1})	Control	1.14 ± 0.17^{B}	0.72 ± 0.14^{B}	0.69 ± 0.08
	Chitosan	2.27 ± 0.30^{A}	1.08 ± 0.23^{AB}	0.66 ± 0.07
	Curdlan	2.57 ± 0.32^{A}	1.77 ± 0.27^{A}	0.64 ± 0.07
	Phi	1.11 ± 0.17^{B}	1.88 ± 0.23^{A}	0.65 ± 0.07
	Si	2.02 ± 0.15^{AB}	1.36 ± 0.30^{AB}	0.88 ± 0.08
CHI (ΔAbs hr^{-1} mg^{-1})	Control	0.036 ± 0.004^{B}	0.033 ± 0.006^{B}	0.032 ± 0.006^{B}
	Chitosan	0.070 ± 0.005^{A}	0.051 ± 0.006^{AB}	0.054 ± 0.007^{AB}
	Curdlan	0.042 ± 0.005^{B}	0.072 ± 0.007^{A}	0.082 ± 0.018^{A}
	Phi	0.016 ± 0.004^{C}	0.052 ± 0.006^{AB}	0.086 ± 0.012^{A}
	Si	0.051 ± 0.006^{AB}	0.052 ± 0.007^{AB}	0.065 ± 0.010^{AB}

induction of a defensive response, as POX and CHI enzymes and genes have been shown to respond to pathogen challenge in conifers (Hodge et al. 1995; Nagy et al. 2004; Jøhnk et al. 2005; Fossdal et al. 2006; Fossdal et al. 2007; Islam et al. 2010). Treatment with Si has no effect on enzyme activities, but it is possible that higher doses of Si may be required to capture differentiable responses. We anticipated the accumulation of secondary metabolites with increases in defensive enzyme activities as part of an induced response, given phenolic and terpene accumulation is so well documented in conifers (Franceschi et al. 2005; Moreira et al. 2016). It is not entirely clear why we did not observe this. It is possible that enzyme-based defenses of fine roots are more easily inducible and/or that chemical defenses are not part of fine root tissue resistance to biotic challenge, but this is speculative. Ultimately, it appears that these elicitors were all able to activate similar, seemingly broad-spectrum, enzyme-based defenses to variable extents.

Acute treatments that result in IR responses that endure for long periods of time (i.e., weeks or even months, if possible) are specifically of high interest to tree managers. We observed variation between elicitors with respect to how rapidly and prolonged they were able to elicit either POX or CHI activity. Chitosan rapidly (June 10 sampling date) increased both enzyme activities, while curdlan rapidly increased only POX activity. By June 14, plants treated with chitosan no longer had higher activities, but plants treated with curdlan had elevated activity for both enzymes, and plants treated with Phi only had elevated POX activities. On June 27, CHI activity for curdlan-treated plants remained elevated, and CHI activity of Phi-treated plants became statistically higher than controls. However, just because we may not have detected longer-lasting responses for certain elicitors doesn't mean that plants could not be primed for subsequent pathogen attack, which would not be known without an experiment involving treatment with elicitors and subsequent inoculation with pathogens. Priming is considered an intrinsic component of IR that enhances the sensitivity and responsiveness to pathogens, leading to increased resistance by way of enhanced pathogen perception, signal transduction, and defense responses without the associated fitness costs of full induction (Conrath 2009; Mauch-Mani et al. 2017). Importantly, a primed state is associated with a variety of physiological, transcriptional, and metabolic changes that may not manifest until subsequent challenge (e.g., Latunde-Dada and Lucas 2001). This very phenomenon is commonly reported in phosphite studies (e.g., Dalio et al. 2014; Felipini et al. 2016; Monteiro et al. 2016) and it is possible that chitosan and curdlan function similarly.

CONCLUSION

We showed that chitosan and curdlan were able to elicit elevated defense-associated enzyme activities relatively rapidly (e.g., 3 days post-treatment, samples on June 10), and that curdlan and Phi were able to elevate responses longer term (e.g., samples collected on June 14 and 27). The prospect that certain eliciting substances have the ability to stimulate long lasting induced responses is positive for tree managers, as it means that acute exposure could provide enhanced resistance for weeks or longer. Subsequent studies should include an inoculation component as well as a true repeated measures experimental design and the quantification of key genes and phytohormones in the induction response.

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