Heat-induced predisposition to *Phytophthora* root rot disease in *Rhododendron*

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Abstract

Resistance to pathogens may be an important attribute of plants that are adapted to warm climates, where disease pressure is often greater. However, resistance genes alone may not be sufficient to prevent disease because abiotic stress, including high temperatures, can reduce host resistance, a phenomenon known as 'predisposition'. This study compares the disease responses of diverse *Rhododendron* taxa to heat stress following inoculation with *Phytophthora cinnamomi* Rands, the primary pathogen causing root rot disease, in order to determine the variation in predisposition. The key experimental comparison was between non-stressed plants (26°C) and plants whose roots had received a high temperature treatment (40°C) prior to inoculation. Roots were sampled at time intervals (DAI, days after inoculation) and visually rated using a disease scale ranging from 1 (healthy) to 5 (dead). In highly susceptible taxa such as the cultivar *R.* 'Haaga' and the *Rhododendron* species *R. ponticum*, there was no significant effect of heat stress on disease development compared to non-stressed plants – severe root rot symptoms appeared early in both cases. Heat treatment significantly increased disease severity in the cultivars *R.* 'Ingrid Mehlquist' and *R.* 'Holden' based on comparisons between 14 and 48 DAI, a clear indication of heat induced predisposition. For two resistant Asian species, high temperature stress consistently increased root disease scores in *R. keiskei* while the opposite response pattern was observed in *R. hyperythrum*, where symptoms following heat stress were equal to or lower than non-stressed plants at most sampling times. These data suggest that the unusual ability of *R. hyperythrum* and its hybrids to adapt to warm climates (USDA hardiness zone 9) is due in part to a mechanism which prevents predisposition to disease at high soil temperatures.

Keywords: *Phytophthora cinnamomi*, high temperature stress, *Rhododendron hyperythrum*

INTRODUCTION

Root rot and dieback diseases caused by the invasive soil pathogen *Phytophthora cinnamomi* affect over 3000 species worldwide (Hardham, 2005), including many fine-rooted woody plants in agricultural and natural settings. Many important ornamental plants are susceptible to the pathogen, which limits disease management to cultural and chemical practices (Benson and Broembsen, 2001). Although it currently is most problematic in southern regions with warm, wet climates (Marcais et al., 1996), epidemiologists predict that global climate change will increase the activity of *P. cinnamomi* and its distribution towards the poles (Anderson et al., 2004; Bergot et al., 2004).

Non-scaly leafed or elepidote rhododendrons (subgenus *Hymenanthes*) are popular ornamental shrubs that are highly susceptible to root rot disease. Combined surveys of over 300 genotypes including cultivars and species have identified fewer than 3% with moderate to high levels of resistance to *P. cinnamomi* (Hoitink and Schmitthenner, 1974; Krebs and Wilson, 2002). Defense against the pathogen in *Rhododendron* and other woody plants is considered to be a partial resistance mechanism controlled by multiple genes (Butcher et al., 1984: Stuckley and Crane, 1994; Krebs, 2009). The resistant condition is modulated by the

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environment, and rhododendrons are predisposed to root rot disease by abiotic stresses such as drought and flooding (Blaker and MacDonald, 1981; Krebs, 2013).

This paper investigates the effect of heat stress on root rot disease in rhododendrons. Interest in this question stems from the general observation that disease resistance is common among rhododendrons and azaleas that are grown in the warmer climates of North America. Among cultivated taxa, the Asian evergreen azaleas (subgenus *Tsustusi*) are the most resistant to *P. cinnamomi* (Benson, 1980) and they are widely grown throughout the Gulf South (USDA hardiness zones 8-9). In contrast, the largely susceptible elepidote rhododendrons are generally restricted to cooler, northern latitudes. The few elepidotes known to perform well in the southern U.S. are all introductions from Asia (Thornton, 1990) and some of those have demonstrated *P. cinnamomi* resistance (Hoitink and Schmitthenner, 1974).

The association of heat tolerance and disease resistance is perhaps best documented in *R. hyperythrum*, an elepidote species from Taiwan that combines both traits and is being used to breed hybrid rhododendrons suitable for both northern and southern regions in the U.S. (Thornton, 1990; Krebs, 2009, 2014). In southern field trials of segregating F_1 populations, plant survivorship and vigor appear to be determined primarily by resistance to *P. cinnamomi*, although this has not yet been fully confirmed (Krebs, 2014). Resistance genes may be essential for survivorship under warm, wet conditions that favor pathogen activity and increase disease pressure. Heat tolerant rhododendrons may also have an additional layer of defense that enables them to be less predisposed by high temperatures to root rot disease. To test this, a genetically diverse group of rhododendrons, including *R. hyperythrum*, were subjected to root heat stress and then inoculated with *P. cinnamomi* in order to compare the disease response to inoculated plants that were not pre-stressed.

MATERIALS AND METHODS

Plants

Rhododendron species accessions at The Holden Arboretum were used to make intraspecific pollinations in spring 2013 and generate seed for analysis. The species used included *R. hyperythrum* (resistant), *R. keiskei* (resistant), and *R. ponticum* (susceptible), based on prior surveys of *P. cinnamomi* root rot disease in the genus (Hoitink and Schmitthenner, 1974). Seeds were sown in August 2014 using standard protocols (Rowe et al. 1994), germinated, and eventually planted five per pot in 11.4 cm^2 pots filled with a mixture of pasteurized peat moss and perlite (1:1 by volume), Aquagro wetting agent (Aquatrols, Paulsboro, NJ. 1.56 L m^{-3}), and pulverized dolomitic limestone (0.93 L m^{-3}). The pots were placed in greenhouse with supplemental lighting to provide a 16 h daylength and temperatures set to 21°C min/27°C maximum. Plants were watered as needed, including a weekly application of Peter's 21-7-7 Acid fertilizer at a rate of 200 ppm N. The seedlings were approximately 6 months old at the time of treatment (February 2015).

Clonal replicates of 3 rhododendron cultivars, *R.* 'Ingrid Mehlquist' (resistant), *R.* 'Haaga' (susceptible), and *R.* 'Holden' (unknown) were also included in the study based on prior root rot disease surveys (Krebs and Wilson, 2002). These were supplied as micropropagated liners (6.2 cm^2 plugs) by Briggs Nursery (Porter, WA) in spring 2014, transplanted singly into the same pots and media described above, then grown on using the same greenhouse and cultural conditions as the seedlings. These plants had been growing for 10 months at the time of the experiment, and had root systems that filled most of the pot volume.

Wild collected seeds from two populations of the North American species *R. minus* were germinated and grown for six months using the above protocol in order to provide material for root viability assays following heat treatment. The populations were from Hawksbill Mt., NC (northern high elevation) and Providence Canyon, GA (southern low elevation).

Heat stress treatment

Using a method adapted from MacDonald (1991), pots containing species seedlings (five per pot) or cultivars (one per pot) were placed in a large capacity water bath (Humboldt, Elgin, IL) to apply heat treatments to root systems. A grid constructed of steel reinforcement bars was used to submerge the buoyant pots, up to 16 at a time. Pots were first placed in polyethylene bags (open at the top) to prevent water from entering the root zone, then wedged into the steel grid and places in bath water to within 1 cm of the tops of the pots. Thermal data loggers were used to monitor pot media temperature for the duration of the treatment (Figure 1).

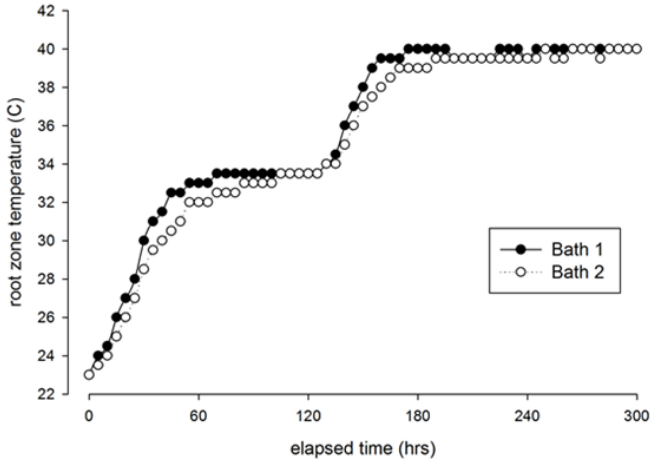

Figure 1. Temperature profiles used to heat rhododendron roots in two water baths operated concurrently. One data logger probe per bath was inserted into the media of a randomly selected pot halfway between the center and edge of the pot. Replicate pots were randomized between water baths.

The bath temperature settings required to generate the desired treatment profile were predetermined in trial runs. As the bath lacked programmable temperatures, set points were entered manually over the course of the treatment. Nonetheless a fairly consistent profile was established where pots starting at room temperature (24-26°C) were raised to 34°C within one hour and held at that temperature for one hour, then raised to 40°C with one hour and held at that temperature for two hours (Figure 1), a heat stress treatment totaling five hours. Control (non-stressed) pots were held at 26°C for an equal period of time. Two water baths were operated concurrently, and plant genotypes were randomized between them to allow for deviations in temperature exposure.

Inoculum

Three Ohio-derived isolates of *P. cinnamomi* were bulked by culturing them together on sterilized rice grains according to the protocol of Benson (2000). Two isolates – PC544 and PC986 – cultured from symptomatic rhododendrons were supplied by The Ohio State University (Dr. Anne Dorrance), and a third – MADC – was obtained from diseased rhododendrons at The Holden Arboretum by the author. All had been verified by growth on semi-selective PARPH-V8 media (Ferguson and Jeffers, 1999) and a subsequent match to the LPV gene sequence diagnostic for *P. cinnamomi* (Kong et al., 2003). Control inoculum consisted of autoclaved rice grains without the addition of the pathogen.

Inoculation

After removal from the heated water bath, pots were cooled at room temperature for two hours. For plants grown singly in pots (*Rhododendron* cultivars), a glass stir rod was used to create holes 4 cm deep, one along each side of the square pot approximately 1.2 cm from the edge of the pot. A single rice grain was placed in each hole and tamped down with a

stir rod to either inoculate (rice cultured with *P. cinnamomi*) or mock inoculate (control rice cultures) the plant. For the pots containing five seedlings (*Rhododendron* species), the same protocol was followed to place one rice grain adjacent to each seedling. Once inoculated the pots were watered to drip through and placed in trays with a water level half the height of the pots for 18 h to maintain the potting media at field capacity. Subsequently the pots were placed on greenhouse benches and watered daily for 1 week to keep conditions wet, and then three times weekly (including a fertilization at 200 ppm N) for the duration of the experiment.

Experimental design and data collection

For the cultivar comparisons (single plants per pot), the pots were arranged in a randomized complete block design with four blocks. Five pots of each treatment (cultivar × temperature × inoculum) were included in each block in order to allow sampling up to five time intervals (DAI, days after inoculation). For the species comparison, five pots of each treatment (species × temperature × inoculum) were arranged in a completely randomized design and sampled over time.

Samples were collected post-inoculation up to 36 DAI (species) or 48 DAI (cultivars). In the cultivar experiment, four pots of each treatment were randomly selected across blocks at each sampling date (n=4). Plants were pulled from the pots and moved to a sink so that most of the potting mix could be washed off the roots. The cleaned roots were then floated in water to allow observation of the full root structure, and assigned a disease score based on visual assessment of the extent of tissue necrosis; 1 (no root damage), 2 (fine root necrosis), 3 (coarse root necrosis), 4 (crown damage), or 5 (dead plant) (Hoitink and Schmitthenner, 1974; Benson, 1980; Krebs and Wilson, 2002).

The same method of disease scoring was used for the species experiment, with the difference that only one pot per treatment was randomly sampled at each date and each seedling per pot was counted as a replicate (n=5). Because the disease scores represent ordinal data, mean comparisons were made using the non-parametric Mann-Whitney U test (pairwise comparisons) or the Krusal-Wallis test (multiple comparisons).

Root viability assay

The effects of heat stress on root viability were determined by staining the tissue with triphenyltetrazolium chloride (TTC) using a modification of earlier protocols (Joslin and Henderson, 1984; Jiang and Huang, 2001). Seedlings from the two *R. minus* populations were given either a control (26°C) water bath treatment, or a heated bath treatment with the final 2-h ramp set at 40 or 50°C. After treatment the pots were cooled to room temperature, seedlings promptly removed, and roots were washed free of peat and perlite, with a final rinse in distilled water. Roots were cut at the crown, damp dried with paper towels, placed in 15 mL Corning tubes (Corning, NY), then filled to cover the tissue with a TTC solution (0.6% w:v in 0.05 M KH_2PO_4 buffer pH 7.4 with 2 drops of Tween – 20 per 500 mL buffer). The stain was infiltrated by placing the tubes in a vacuum chamber for 15 min at 50 kPa. Tubes were then stored in the dark for 20 h to allow the reduction of TTC to formazan. Boiled roots were included as a zero viability reference (non-staining).

After staining, complete roots (excluding the crown region) from individual seedlings were blotted dry and cut into short segments that were mixed to create a composite sample. Five samples from each root composite were taken with fresh weights ranging from 10-80 mg, a previously determined linear absorbance range for tissue from non-heat-stressed plants. The weighed samples were transferred to 13×100 mm test tubes containing 5 mL of 95% ethanol and immersed in a 75°C water bath for 15 min to extract the formazan. After cooling and vortexing the sample tubes (allowing the root tissue to settle), absorbance was measured spectrophotometrically at 480 nm against an ethanol reference sample. Root tissue was recovered from the tubes and dried (24 h at 50°C) to obtain dry weights.

Average absorbance per mg dry weight values were determined for each seedling based on the five composite samples. Mean comparisons (t-tests) were based on 5 seedlings (reps) for each population × temperature treatment.

RESULTS AND DISCUSSION

Effects of heat stress on root viability

Estimates of root viability using the vital stain TTC are shown in Figure 2. As expected, boiled roots (killed) had absorbance values near zero.

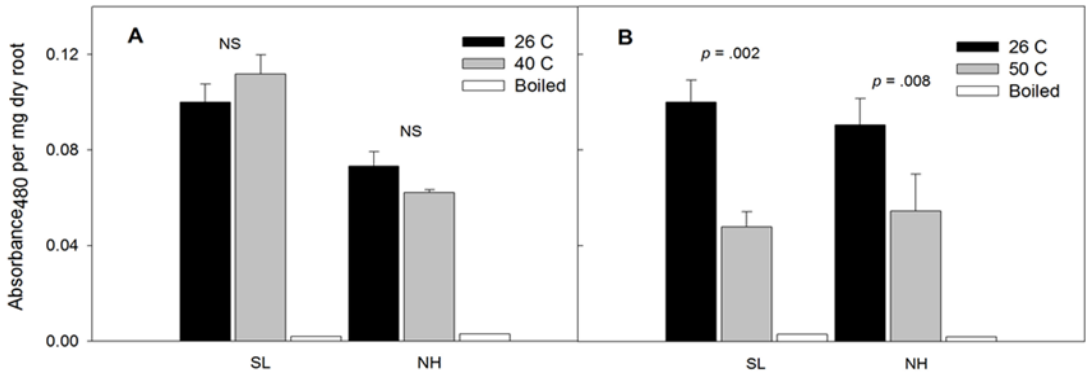

Figure 2. Effect of root heat treatment on viability. A) 26 versus 40°C treatments, B) 26 versus 50°C treatments. SL = southern low elevation *R. minus* (Providence Canyon, GA), NH = northern high elevation *R. minus* (Hawksbill Mt., NC). Mean comparisons (*t*-test) given for the 26/40°C and 26/50°C root samples.

At 40°C, there was no significant difference (*t*-test) in root viability in either population relative to controls, roots treated at a bath temperature of 26°C. Roots treated at 40°C had slightly higher (12%) viability in the southern population and somewhat lower (15%) viability in the northern population compared to tissue sampled at 26°C (Figure 1A). In the 50°C treatment, there was a significant loss of root viability relative to tissue at 26°C, with 51 and 39% reductions in the southern and northern *R. minus* populations, respectively (Figure 2B). Although the *R. minus* populations were from contrasting warm and cold climates, heat stress treatments at 40 and 50°C did not reveal potential adaptive differences in root viability under these test conditions.

The effect of high temperatures on root health also appeared to be minimal in the experiments where damage (necrosis) was visually estimated. In heat-stressed, noninoculated cultivars some fine root necrosis was observed (presented as disease scores of ~1.2 in Figure 3A). However, the species study showed that roots receiving the heat stress treatment were completely healthy (disease scores = 1) for the duration of the experiment (Figure 3). These visual root scores are in general agreement with the results of the TTC cell viability test (Figure 1), where high temperature exposure up to 40°C in the water bath did not significantly reduce root cell viability.

Other research on heat-induced disease predisposition has demonstrated the need for treatment parameters that enable temperature effects on root health to be separated from pathogen effects. The treatment protocol used in the present study was adopted from MacDonald (1991) who demonstrated that a 40°C root treatment for 30 min represented a 'critical stress threshold' in chrysanthemums inoculated with the pathogen *P. cryptogea*. It was a treatment which limited heat damage to root tissue compared to higher temperatures and strongly predisposed the plants to root rot disease compared to lower temperatures. A study of *Hibiscus* (Lyles et al., 1992) found that brief exposure of hydroponically-grown roots to 30 or 40°C temperatures prior to inoculation with the root rot pathogen *P. parasitica* clearly delineated the effects of high temperature on root necrosis from those caused by heat-induced predisposition, whereas those effects were not distinguishable at 50°C.

In experiments using a heat treatment prior to inoculation, root cell mortality caused by the stress could potentially alter pathogen behavior. Cell death in root tips, for example, could impede pathogenesis by preventing the attachment and encystment of zoospores in

the zone of elongation above the root cap. Alternatively, leaked sugars and amino acids from freshly killed root cells could accelerate infection by attracting zoospores with a stronger chemotactic signal than nonstressed roots (Osswald et al., 2014). However, these exudates could also be released under nonlethal levels of heat stress due to changes in membrane function, resulting in a stronger rhizosphere attraction to zoospores (MacDonald, 1991; Ingram et al., 2015) and acting as a predisposing mechanism for disease development.

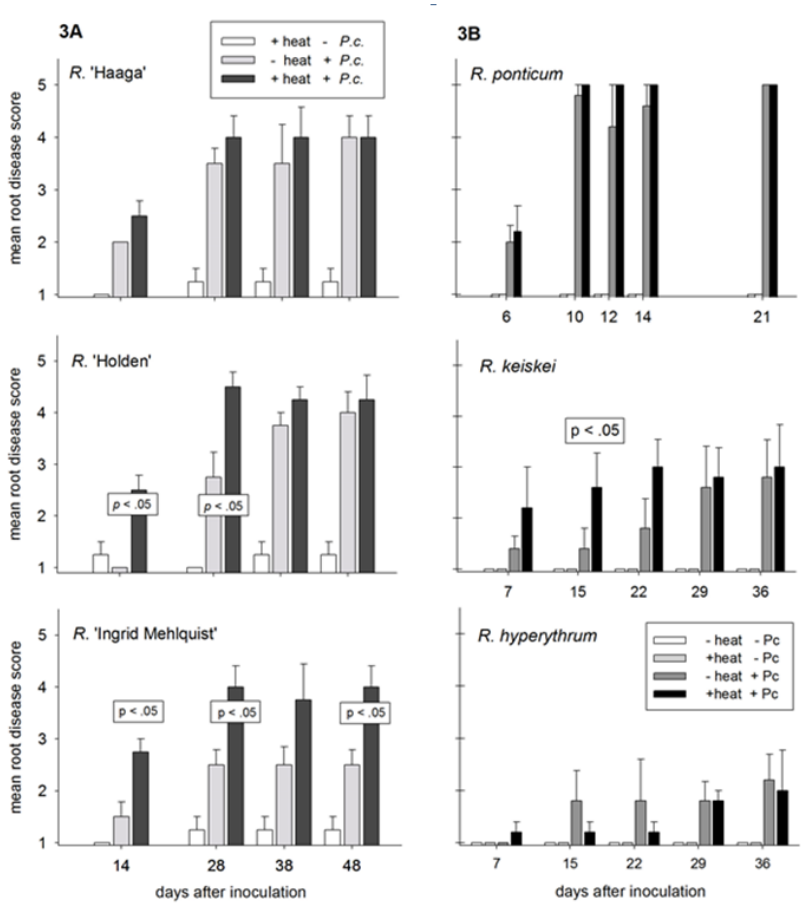

Figure 3. Mean root rot disease scores over time for three rhododendron cultivars (A) and species (B) that received 26 (-heat) or 40°C (+heat) root treatments followed by mock inoculation (-*P.c.*) or *P. cinnamon* inoculated rice grains (+*P.c.*). Unless otherwise indicated, mean comparisons of predisposing effects (-heat+*P.c.* versus +heat+*P.c.* treatments) are not significant (Mann Whitney U test).

Predisposing effects of heat stress on disease

Among cultivars, disease severity at all sampling times was highest in plants that were heat stressed prior to inoculation, but the magnitude of this effect varied among genotypes and over time (Figure 3A). Disease scores for stressed and non-stressed roots from the susceptible cultivar *R.* 'Haaga' were statistically equivalent over the time course. In contrast, significant predisposing effects of heat stress were observed in the susceptible cultivar *R.* 'Holden' at 14 and 28 DAI. Relative to inoculated plants that were not heat stressed, the increase in disease severity was 150% (14 DAI) and 61% (28 DAI), at which point maximum levels of disease were attained. The resistant cultivar *R.* 'Ingrid Mehlquist' was also clearly predisposed to disease by heat stress, where disease scores were significantly higher following heat stress in three of the four sampling times (p<.05). By 48 DAI, symptoms in non-stressed roots were limited to fine or coarse roots (disease score = 2.5) while those in heat-stressed roots had progressed into crown tissue (disease score = 4.0), an average 60%

increase in disease severity over the time course. The disease response over time in *R.* 'Ingrid Mehlquist' was similar to *R.* 'Holden', where maximum disease scores in heat stressed plants occurred by 28 DAI.

The predisposing effects of high temperature on disease were also examined in several *Rhododendron* species from Asia (Figure 3B). There were detectable but non-significant increases in disease scores throughout the time course following heat treatments in *R. ponticum,* a highly susceptible species where most inoculated seedlings died by 10 DAI. The resistant species *R. keiskei* was predisposed to root rot disease by heat stress, with the largest increases in disease severity occurring at the early sampling times 57% (7 DAI), 86% (15 DAI), and 67% (22 DAI). However, mean differences in stressed and non-stressed disease scores were only significant for the roots sampled 15 DAI (p<.05). In contrast to the other species and cultivars included in this study, the resistant species *R. hyperythrum* did not appear to be predisposed to disease by high temperature stress. With the exception of samples taken 7 DAI, heat stressed roots from *R. hyperythrum* had disease scores that were 0 to 33% lower than those from non-stressed roots in samples assessed between 15 and 36 DAI, and mean scores between the two treatments did not differ significantly.

The lack of statistical significance in the species group results from a number of sources. Unlike the cultivar group, replicate species plants were not clonal but grown from seed and potentially variable in their disease responses. This could contribute to the higher standard errors seen for the species than for the cultivars (Figure 3). In addition, the sample size (n=5) was too small in most cases to significantly separate means using nonparametric analysis of root disease ratings. Therefore, while the opposing disease responses of *R. keiskei* and *R. hyperythrum* to heat stress are noteworthy, the cultivar data provide a more robust example of temperature-induced predisposition in *Rhododendron*.

Similar instances of heat-induced susceptibility are reported. MacDonald (1991) used a water bath method to stress potted chrysanthemums prior to inoculation with *P. cryptogea* and observed a 4-fold increase in the proportion of necrotic roots following treatment temperatures of 40°C. Milder increases in temperature can also reduce host defenses. In *Arabadopsis*, high temperature inhibition of R and R-like genes occurs at 28°C, while plants grown at 22°C remain resistant to *Pseudomanas syringae* (Zhu et al., 2010). High temperature can also induce resistance, as in the case of heat shock factors that are associated with elevated levels of immunity in cucumber (Stermer and Hammerschmidt, 1987). The temperature level used in this study (40°C) could potentially generate heat shock proteins, but no enhanced resistance was observed, and the use of a gradual temperature ramp with prolonged set point exposures may have minimized their accumulation.

Beyond the laboratory, high temperature effects on disease are an important consideration for plants in nurseries and in the landscape (Ingram et al., 2015). Direct sunlight on black containers can create maximum temperatures in excess of 50°C in the root zone, and experiments with potted chrysanthemums (MacDonald, 1991) and hibiscus (Lyles et al., 1992) have demonstrated that shade-grown containers are much less susceptible to *Phytophthora* root rot than exposed ones. Benson (1986) found similar results in a potted azalea study, but also noted that plants in landscape beds, either exposed or shaded, had higher disease ratings than the container plants. This was attributed cooler temperatures and higher matric potentials in the beds, conditions favoring *P. cinnamomi* disease development. Although the temperatures used to heat stress rhododendrons in this study are well above those considered to be optimal for *Phytophthora* (~26°C), predisposing effects could have a duration extending into cooler diurnal periods when the pathogen is more active. For example, production of absisic acid (ABA) in response to abiotic stress is linked with weakened pathogen defense signaling pathways due to inhibition of salicylic acid or jasmonic acid signaling hormones. ABA levels in salt-stressed tomatoes were maintained for 24 h after removal from the stress, resulting in a prolonged predisposed condition (DiLeo et al., 2010).

The data provided by this study adds high temperature to a number of other abiotic stresses known to predispose rhododendrons to *Phytophthora* disease. Both flooding and drought stress resulted in the loss of *P. cinnamomi* resistance in the cultivar *R.* 'Caroline'

(Blaker and MacDonald, 1981). Salt (osmotic) stress of *R*. 'Cunningham's White' prior to inoculation with *P. ramorum* resulted in faster root symptom development and plant mortality (Roubtsova and Bostock, 2009). These studies used one or two host genotypes to isolate the effects of other variables such as stress treatment duration or inoculum density. The present analysis demonstrates that a genetically diverse group of *Rhododendron* taxa, including susceptible and resistant genotypes, are predisposed by heat stress to *Phytophthora* root rot disease.

The example of *R. hyperythrum* represents an important exception because it maintains a high level of resistance following heat stress, similar to the response of ABA-deficient tomato mutants (DiLeo et al., 2010). In addition, *R. hyperthrum* and hybrids derived from it are less predisposed to root rot under field flooding conditions than resistant cultivars with different genetic backgrounds such *R*. 'Caroline' (Krebs, 2013). Rhododendron breeders have successfully used *R. hyperythrum* to develop hybrids adapted to the Gulf South (USDA hardiness zone 9), a hot climate not suited for most rhododendrons (Thornton, 1990; Krebs, 2009, 2014). This adaptation may be due in part to above ground attributes such as a higher photosynthetic temperature optima in *R. hyperythrum* compared other species of *Rhododendron* with less heat tolerance (Ranney et al., 1995). However, below ground adaptations could be equally important, particularly in hot, wet conditions where there is high disease pressure from soil pathogen such as *P. cinnamomi*. In that environment, the adaptive success of *R. hyperythrum* may be due in large part to resistance genes combined with a mechanism that prevents predisposition to disease at high temperatures.

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