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The Effect of Applied Salinity and Water Stress on Chemical Suppression of *Phytophthora ramorum* from Soilborne Inoculum in *Rhododendron*

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Abstract

A serious concern for nurseries is the potential for *Phytophthora ramorum* and other *Phytophthora* species to colonize roots without inducing aboveground symptoms in plants that then serve as cryptic reservoirs of inoculum. Episodic abiotic stresses that reduce plant water potential can compromise the host resistance to trigger disease development from root and crown infections during many *Phytophthora*–plant interactions. We conducted a series of experiments with root-inoculated *Rhododendron* plants in a potting soil mix to assess the influence of excess salt or water deficit on ramorum blight development and the potential for these abiotic stresses to affect the efficacy of oomycete-suppressive chemical soil treatments. During growth chamber trials, *P. ramorum* colonized roots of both nonsalt-treated and salt-treated plants. However, salt treatment offset the benefit realized from soil treatment with mefanoxam (Subdue Maxx) and mandipropamid (Micora), as evidenced by the enhanced pathogen colonization of roots. A 3-week episode of water stress imposed after chemical treatment but before inoculation eliminated protection against *P. ramorum* root colonization conferred by fosetyl-Al (Aliette). At an outdoor experimental nursery, foliar symptoms were apparent in 23% of root-inoculated plants during two trials and absent during one trial. However, the majority of inoculated plants in all trials had colonized roots with little or no aboveground symptoms. A single application of Subdue Maxx or Aliette reduced root colonization by *P. ramorum* in *Rhododendron* plants. Although salt stress did not enhance ramorum blight symptom expression at the nursery, salt partially offset protection from *P. ramorum* root colonization obtained by Subdue Maxx.

Keywords: Aliette, fosetyl-Al, cryptic infections, mandipropamid, mefenoxam, Micora, predisposition, Subdue Maxx

The contamination of plants by *Phytophthora* species is a widespread problem for nurseries throughout North America and Europe (Bienapfl and Balci 2014; Jung et al. 2016; Yakabe et al. 2009). Of particular concern in the western United States, Canada, and northern Europe is *P. ramorum* Werres, de Cock & Man in't Veld, which attacks or is associated with more than 130 plant species and causes foliar blight and dieback of many nursery species and sudden oak death and sudden larch death in forests and other landscapes (Blomquist et al. 2016; Brasier and Webber 2010; USDA-APHIS 2020a; Werres et al. 2001). A challenge for disease management as well as for nursery inspection and certification programs is that soil and roots contaminated by *P. ramorum* may serve as potential reservoirs of inoculum while aboveground portions of plants remain asymptomatic. *P. ramorum* can overwinter in forest soils as well as in commercial potting medium (Vercauteren et al. 2013). During a study conducted in Germany, two commercial woody ornamental nurseries were sampled for the presence of *Phytophthora* species over a period of 3 years (Junker et al. 2016). *P. ramorum* could be detected during all seasons of the year. Puddles along the pathways in the nurseries had the highest percentage of positive detections. Plant residues, wind-carried leaves, water, and sediment from water runoff were also conducive for survival of the pathogen.

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A systems approach, called Hazard Analysis of Critical Control Points and modeled based on U.S. Food and Drug Administration regulatory standards, was implemented for the management of pests and pathogens of nursery crops (Parke and Grünwald 2012). This approach identifies critical control points in the nursery, that is, the times and places for mitigating the risk of contamination from potential inoculum sources (e.g., ground, irrigation water, containers, and planting media). Significant efforts have been made to establish best management practices to detect and mitigate inoculum sources (Frankel et al. 2018; Swiecki et al. 2018a, b). Although different contamination hazards have been identified, additional factors may influence inoculum efficiency and host susceptibility. For example, abiotic stresses such as overfertilization and salinity or water deficit arising from inconsistent irrigation or improper transplanting may allow for infection by opportunistic pathogens or predispose plants to levels of pathogen inoculum that they would normally resist (Bostock et al. 2014; Hummel et al. 2013).

Because salt stress is relatively easy to impose and remove experimentally, we have used it in several pathosystems as a proxy for abiotic stresses known to reduce water potential (i.e., soil salinity, water deficit, root hypoxia from waterlogging). Reduced water potential can predispose plants to disease, particularly those caused by root and crown oomycete pathogens (Blaker and MacDonald 1981; Bostock et al. 2014; Dileo et al. 2010; Duniway 1977, 1983; MacDonald 1984; Pye et al. 2018). During an earlier study involving *Rhododendron* plants maintained in growth chambers under a modified hydroponic format, we found that a brief episode of acute salt stress compromises the host to facilitate ramorum blight development from root infections (Roubtsova and Bostock 2009). However, it is unknown how salinity influences susceptibility of rhododendrons in soil and under the environmental conditions of a nursery. It is also unknown how salt stress influences the efficacy of chemicals used by nurseries to attempt to manage ramorum blight and other oomycete diseases.

The objective of this study was to assess the impact of salt stress and water deficit on ramorum blight development in root-inoculated rhododendrons maintained in a standard potting soil. Experiments were conducted under the highly controlled conditions of a growth chamber (salt and water stress trials) and under containment in an outdoor experimental nursery (salt stress only). We also assessed the impact of these

stresses on the efficacy of fosetyl-Al (Aliette), mandipropamid (Micora), and mefanoxam (Subdue Maxx), which are chemicals used for oomycete disease management (i.e., systemic oomycete-suppressive chemicals) (Swiecki et al. 2018a); hereafter, we simply refer to these chemicals, albeit imprecisely but more generically, as fungicides. Portions of this research have been presented in abstract form (Roubtsova and Bostock 2013).

Materials and Methods

Plants. All plants were purchased as liners or as 1-liter (SP5) or 2.8-liter (#1) potted plants from Briggs Nursery LLC (Elma, WA). Liners of *Rhododendron* hybrid cultivar Cunningham's White, cultivar Roseum Elegans, or cultivar Cunningham's Blush were transferred to 10-cm diameter (0.5-liter) pots containing Sunshine Pro Premium potting soil (Sun Gro Horticulture Canada CM Ltd) and maintained in a greenhouse until they were used during experiments. Although cultivar Cunningham's White was used in most trials, cultivar Roseum Elegans was used in one of four growth chamber trials involving salt stress and one of three nursery trials, and cultivar Cunningham's Blush was used in two growth chamber trials involving water stress. This was because of the limited availability of cultivar Cunningham's White at the time of purchase when plants were needed for experiments. Cunningham's White and Roseum Elegans have similar susceptibility to *P. ramorum* (De Dobbelaere et al. 2010). Cunningham's Blush is a hybrid of uncertain heritage and susceptibility relative to the other cultivars.

Plants maintained in the greenhouse and growth chamber at University of California, Davis were watered with half-strength (0.5×) Hoagland's solution (Hoagland and Arnon 1950). Plants in 1-liter and 2.8-liter pots received 325 and 650 ml of 0.5× Hoagland's solution per pot, respectively, twice per week. Plants in 0.5-liter pots received 85 ml of the nutrient solution once per week. Plants grown at the experimental nursery received municipal water at a frequency to maintain field capacity or nearly field capacity without additional nutrients.

Pathogen. We used two isolates of *P. ramorum* originally found in diseased plants in Marin County, CA, and archived in the California Department of Food and Agriculture collection. NA1 isolates #1418983-2 (from *Rhododendron*) and #1418886 (from *Camellia japonica*) were used during growth chamber and nursery trials, as indicated in the detailed descriptions of each trial.

Experimental formats and stress regimens. *Growth chamber trials: Effects of salt and fungicide on leaf water potential and root colonization by P. ramorum.* Plants of *Rhododendron* cultivar Cunningham's White or cultivar Roseum Elegans were maintained in 10-cm-diameter pots (0.5 liter) in a greenhouse. For experimental treatments and incubation, plants were transferred to a growth chamber set at 20°C with a 12-h day length and 70% relative humidity under fluorescent and incandescent lights emitting a photon flux of 150 $\mu mol\ m^{-2}s^{-1}$. To assess the impact of soil applications of salt, fungicide, or the combination of salt and fungicide on leaf water potentials (reported in MPa), plants were treated by soil drench (0.1 liter per pot) with mefenoxam (Subdue Maxx; Syngenta; 22% a.i. SL; 0.16 ml per liter) or fosetyl-Al (Aliette; Bayer Crop Science; 80% WDG a.i.; 3.8 g per liter) 3 days before salt stress. Salt stress was imposed by soil drench with 0.2 M NaCl/0.02 M $CaCl_2$ (0.1 liter per pot; electrical conductivity ≈23 mS/cm) for 24 h. At the end of the salt stress episode, leaf water potentials were measured by excising one leaf from the midsection of each plant at the junction of the petiole with the stem using a razor blade. Midmorning potentials were determined with a pressure bomb (model 3005F01; Soil Moisture Equipment Corp., Santa Barbara, CA). Control leaves were from plants that had received a normal watering regimen without exposure to salt or fungicide. This experiment was performed once with measurements obtained from each of five plants for each treatment combination.

Four trials (GS1 to GS4) were conducted to assess the impact of soil applications of salt, fungicide, or the combination of salt and fungicide on root colonization by *P. ramorum*. Plants of *Rhododendron* cultivar Cunningham's White (three trials) or cultivar Roseum Elegans (one trial) in 10-cm-diameter pots (0.5 liter) were placed in a growth chamber at the aforementioned settings. Depending on the trial, four to six plants were used for each treatment group. Trials GS1 and GS2 used *P. ramorum* isolate #1418983-2, and trials GS3 and GS4 used isolate #1418886. Plants were inoculated by removing them from their pots, thoroughly mixing 100 cm^3 of rinsed *P. ramorum*-infested V-8 broth/vermiculite with potting soil in a ratio of approximately one part inoculum mix to two parts soil (1:2, vol/vol), and then repotting the plants surrounded with the V-8 broth/vermiculite–soil mix and adding soil as needed (Parke and Lewis 2007). Noninoculated controls were repotted with a noninfested V-8 broth/vermiculite–soil mix. A layer of clean potting soil was added to a depth of approximately 1.3 cm on the top surface of each pot to reduce the possibility of splash dispersal of inoculum onto the stem, leaves, or adjacent plants. Fungicides (Subdue Maxx and Aliette at the aforementioned concentrations) and mandipropamid (Micora; Syngenta; 250 SC; 250 g/liter a.i.; 0.63 ml per liter) were applied 10 days after inoculation (dai) in a volume of 0.1 liter per pot. Three days later, salt stress was imposed by applying 0.1 liter of 0.2 M NaCl/0.02 M $CaCl_2$ to the soil. After 24 h, plants were thoroughly watered to remove salt; then, they were watered once per week for 3 months. They were evaluated for root colonization and any aerial symptoms. Trial GS1 (January to April 2012) used cultivar Roseum Elegans, and trials GS2 (August to December 2012), GS3 (January to April 2013), and GS4 (July to November 2013) used cultivar Cunningham's White. Because of limited availability of suitable plants at the time of some trials, Micora and Aliette were each evaluated during three of the four trials (Micora, trials GS1, GS3, and GS4; Aliette, trials GS2 to GS4). Subdue Maxx was evaluated during all four trials.

Growth chamber trials: Effects of water deficit and fungicide on leaf water potentials and disease. We conducted experiments to determine whether an episode of water deficit influences genus *Rhododendron* susceptibility to *P. ramorum.* We also examined the potential interaction of water stress with fungicide treatments. Five trials were conducted in a growth chamber at the aforementioned settings using cultivar Cunningham's White (trials GW1 to GW3) or cultivar Cunningham's Blush (trials GW4 and GW5). Within each trial, each treatment set had five or six plants. All trials imposed the same treatments and a 3-week period with no water; however, trials used three different treatment sequences.

During trial GW1, Cunningham's White plants were inoculated with V-8 broth/vermiculite mixture colonized by *P. ramorum* inoculum as described. One week later, water was withheld from half of the plants (water stress), and the other half was watered once weekly with 85 ml 0.5× Hoagland's solution (control). This regimen continued for 3 weeks; at the end of the stress period, fungicides were applied as a soil drench at the rates previously described, with deionized water used as a soil drench for the control. All plants were watered weekly (0.5× Hoagland's solution) for 4 weeks or more; then, they were evaluated for aerial symptoms and root colonization by *P. ramorum*.

During trial GW2, plants were inoculated as described; 10 days later, they were treated with fungicides. One week later, water stress was imposed and continued for 3 weeks, as in trial 1. Plants were returned to a normal weekly watering regimen for 7 weeks before evaluation.

During trials GW3 to GW5, fungicides were applied on day 1 of the experiment; then, water was withheld for approximately 3 weeks. At the end of the water stress regimen, leaf water potentials were determined and all plants were inoculated by layering vermiculite–V8 broth mix infected with *P. ramorum* on the soil surface without incorporation into the soil. Plants were returned to a normal watering schedule with 85 ml per pot 0.5× Hoagland's solution each week and maintained for 8 weeks until evaluation. During these latter trials, the plants were bottom-watered to avoid disturbing the top-layered inoculum mix.

Nursery trials. Three trials (NS1 to NS3) to assess the potential interaction of a salt stress episode and a soil application of fungicide on *P. ramorum* development were conducted at the National Ornamental Research Site at Dominican University of California (NORS-DUC; San Rafael, CA; 37°58′51.12″ N, 122°31′04.20″ W; elevation 22.6 m). This outdoor nursery, established in 2009 by the U.S. Department of Agriculture Animal and Plant Health Inspection Service, California Department of Food and Agriculture, and Dominican University of California, is a contained experimental facility for research involving *P. ramorum* and other high-consequence, regulated pathogens of nursery plants. Fungicides were applied after inoculation, with the caveat

that our intent during these trials was not to ascribe preventive or curative actions of the chemicals. The first trial (NS1) was conducted from 10 June to 2 December 2011 (176 days). On day 1 of the trial, 96 plants of *Rhododendron* cultivar Cunningham's White were transferred from 2.8-liter (#1 container) to 6.1-liter (#2 container) pots amended with *P. ramorum*-infested vermiculite/potting soil mix (1:2, vol/vol), prepared as described. A layer of clean potting soil was added to a depth of approximately 1.3 cm on the top surface of each pot. Plants were placed on wooden pallets, six plants per pallet, in each of two blocks, with 48 plants per block watered with municipal water by drip irrigation with a single emitter in each pot. Each block had dimensions of approximately 3.6 m × 4.6 m. A completely random design was used for treatment assignments within each block. Ten days after transplanting and inoculation, mefenoxam (Subdue Maxx; 0.16 ml per liter), fosetyl-Al (Aliette, 3.8 g per liter), or water as a control were applied to the soil as a drench (0.5 liters per pot). Two days later, half of the plants receiving a chemical or water treatment received a soil drench of 0.2 M NaCl/0.02 $CaCl_2$ (0.5 liters per pot). After 24 h, all plants were irrigated extensively to reduce salt levels. Four weeks later, the conductivity of water runoff collected from salted and nonsalted noninoculated plants was measured with a conductivity meter (YSI Inc., Yellow Springs, OH).

The second trial (NS2) was conducted from 30 January to 23 June 2012 (146 days); its design was similar to that of the first trial, except cultivar Roseum Elegans plants were used and this trial included a third block of 48 plants (Supplementary Fig. 1A). Plants were transferred from 1-liter to 2.8-liter pots amended with the *P. ramorum*-infested vermiculite–soil mix on day 1. The third trial (NS3), conducted from 16 October 2012 to 27 March 2013 (163 days), had a format similar to that of trial NS2 but used cultivar Cunningham's White plants and had two blocks of 48 plants each, as in trial NS1 (Supplementary Fig. 1B).

P. ramorum isolate 1418983-2 (from *Rhododendron*) was used during trial NS1, and isolate 1418886 (from *C. japonica*) was used during trials NS2 and NS3. Archived local daily temperature and precipitation data were retrieved from the nearest University of California IPM weather station CIMIS #157 at Point San Pedro, San Rafael, CA (37°59′ N, 122°28′ W; elevation 1.5 m; http://ipm.ucanr.edu), which is located approximately 4 km southeast of the NORS-DUC site.

Disease evaluation. At the end of a nursery trial, plants were rated by visual evaluation of ramorum blight symptoms on foliage and/or stems using the following scheme: 0, no aerial symptoms, healthy plants; 1, mild leaf chlorosis; 2, scattered leaf lesions and blighting, widespread chlorosis; and 3, wilting, extensive leaf and stem symptoms, plant collapse, and death (Supplementary Fig. 2).

The presence of *P. ramorum* was confirmed by its isolation from roots, leaves, and/or stems of inoculated plants on pimaricin-ampicillin-rifampicin-pentachloronitrobenzene-hymexazol (PARP-H) agar and by culture morphology (Jeffers and Martin 1986). Aerial parts of plants were sampled from only a few plants with pronounced ramorum blight symptoms during several of the trials. Four to six 0.5-cm^2 pieces of symptomatic leaf or stem tissue were plated on PARP-H medium to confirm that lesions were caused by the pathogen. Root samples were taken from all plants and processed in a manner similar to that of Bhat et al. (2006), with some modifications for growth chamber and nursery trials.

For root analyses of growth chamber plants, at the end of a trial, plants were removed from their pots and soil was removed by gently shaking. Roots were excised from the shoot and rinsed with tap water to remove soil particles. Several intact roots were selected from the root mass of each plant, immersed in 0.6% NaOCl for 30 seconds, rinsed in deionized water, and sectioned with a razor blade. Ten root pieces, each approximately 0.5 cm long, from the sectioned roots were transferred to a plate of PARP-H selective medium. Plates were incubated for 7 to 10 days at room temperature (22 to 24°C) and observed for colony morphology and structures consistent with *P. ramorum* to estimate pathogen colonization (i.e., percent of root pieces infected).

For root analyses of nursery plants in 2.8-liter or 6.1-liter pots, soil cores were taken from each pot with a 2.54-cm-diameter stainless steel soil sampler that was surface-sterilized with a propane torch and cooled between each pot. For each soil core, the sampler was inserted approximately half the distance between the plant stem and pot edge and to the bottom of a pot. Two soil cores were taken from opposite sides from each 2.8-liter pot, and three soil cores evenly spaced around the plant were taken from the 6.1-liter pots. The soil cores containing roots from a pot were combined and mixed in a plastic bag to constitute a sample. The bags were placed in an ice chest for transport to the laboratory, where they were shaken and rinsed with tap water to remove soil particles. Several intact roots were selected from each sample and processed and evaluated for the presence of *P. ramorum* as described (ten 0.5-cm-long root pieces selected from each sample placed on a PARP-H plate).

Experimental design and statistical analyses. The experimental treatment design was complete randomized (growth chamber trials) or complete randomized within blocks (nursery trials). For the nursery trials, there were six individual plant replicates for each treatment in each of two blocks (trials NS1 and NS3) or three blocks (trial NS2). Sentinel *Rhododendron* plants were placed around the periphery and outside of the experimental area to monitor for *P. ramorum* escape from or introduction to the nursery section where trials were conducted. To check for the possibility of pathogen contamination, two growth chamber trials included treatment sets of noninoculated stressed or nonstressed plants with no fungicide, and all nursery trials included treatment sets of noninoculated nonsalted and salted plants with no fungicide. No roots from these treatments showed contamination by *P. ramorum*. During the final analyses, the negative root colonization data from the noninoculated sets were excluded from the model and means comparisons, except during the correlation analysis of root colonization and aerial symptom ratings. Percent root colonization data were converted using the arcsine square root transformation before analysis. Statistical analyses were performed using JMP PRO software version 15.0 (SAS Institute, Cary, NC).

Results

Growth chamber trials: Effects of the interactions of abiotic stresses and fungicides on ramorum blight. Biological materials and treatment sequences used during growth chamber trials to assess salt or water stress effects on ramorum blight development in *Rhododendron* plants and on the efficacy of fungicides to protect roots against *P. ramorum* colonization are reported in Table 1. During these trials, the effect of the cultivar was not significant.

Leaf water potential measurements: Effects of salt stress and fungicides on noninoculated plants. A mixed model analysis of leaf water potential with fungicide, salt, and fungicide × salt as fixed effects found that fungicide and salt were significant ($P < 0.0001$), but the interaction of fungicide × salt was not significant ($P = 0.424$). A means comparison using the Tukey's honestly significant difference (HSD) test with all pairwise comparisons indicated that imposition of a 24-h episode of salt exposure in *Rhododendron* roots significantly reduced leaf water potential with all treatments ($P < 0.0001$) (Fig. 1A). There was a small but statistically significant protection by Aliette against the salt-induced reduction in leaf water potential relative to the water/no-fungicide, salt-treated control ($P < 0.0001$). Plants treated with Aliette or Subdue Maxx without a salt stress episode showed slightly, but significantly, higher leaf water potential than the water/no-fungicide, no-salt control (Aliette, $P = 0.002$; Subdue Maxx, $P = 0.035$) (Fig. 1A).

Effects of the interaction of salt stress and fungicide on P. ramorum root colonization. We evaluated the effects of salt on the fungicide suppression of root colonization by *P. ramorum* (trials GS1 to GS4). A mixed model analysis with fungicide, salt, pathogen isolate, and fungicide × salt as fixed effects and trial as a random effect found that trial was not significant (Wald $P = 0.2474$). There was no significant effect of the *P. ramorum* isolate used during these trials ($P = 0.457$), with both isolates performing similarly. However, fungicide ($P < 0.0001$), salt ($P < 0.0001$), and fungicide × salt ($P = 0.0108$) were significant. Aliette and Subdue Maxx suppressed *P. ramorum* root colonization in both no-salt ($P \leq 0.0001$) and salt-treated plants ($P \leq 0.0002$) relative to the respective water-treated/no-fungicide controls (Dunnett's method) (Fig. 1B). Micora did not significantly reduce *P. ramorum* root colonization in either no-salt ($P = 0.138$) or salt-treated ($P = 0.977$) plants relative to the water-treated no-salt and

salt-treated controls, respectively (Dunnett's method). However, salt treatment negated the benefit observed with Subdue Maxx by enhancing *P. ramorum* colonization ($P = 0.03$, pooled t test), and it significantly enhanced root colonization in Micora-treated plants compared with no-salt/Micora-treated plants ($P = 0.0007$) (Fig. 1B). Salt treatment did not significantly enhance *P. ramorum* root colonization in water/no-fungicide (control) or in Aliette-treated plants compared with the corresponding plants treated with water/no salt or Aliette/no salt (Fig. 1B).

Leaf water potential measurements: Water stress and fungicides. We examined the interaction of fungicides and water stress imposed before inoculation with *P. ramorum* on leaf water potentials (trials GW3 to GW5). During these trials, fungicide or water (i.e., no fungicide/control) was applied immediately before the water deprivation period, and leaf water potentials were measured at the end of this period and before inoculation. A mixed model analysis with fungicide, water stress, and fungicide × water stress as fixed effects and trial as a random effect found that trial was not significant (Wald $P = 0.329$). However, fungicide ($P = 0.0147$), water stress ($P < 0.0001$), and fungicide × water stress ($P = 0.0004$) were significant. In the absence of water stress, fungicides did not significantly affect leaf water potentials relative to the water/no-fungicide-treated control (Dunnett's method, $P > 0.05$) (Fig. 2A). However, Aliette partially offset the water potential reduction resulting from the period of water deficit relative to the control (Dunnett's method, $P = 0.0054$) (Fig. 2A).

Effects of the interaction of water stress and fungicides on P. ramorum root colonization. Trials GW1 to GW5 tested Aliette and Subdue Maxx and different treatment sequences, with plants inoculated either before (GW1 and GW2) or after (GW3 to GW5) stress and fungicide treatments. A mixed model analysis of trials GW1 and GW2 with fungicide, water stress, and fungicide × water stress as fixed effects and trial as a random effect found that trial was not significant for root colonization. The effect of fungicide was significant ($P = 0.032$), with Subdue Maxx significantly suppressing root colonization relative to the untreated control according to Dunnett's method ($P = 0.043$). Water stress did not have a significant effect on root colonization by *P. ramorum* in all treatment sets (controls and fungicide-treated), and plants were largely asymptomatic, with average foliar ratings <1.

Table 1. Summary of materials and treatment sequences for growth chamber trials

Stress	Trial	Treatment sequence[a]	Trial duration, days[b]	Cultivar/*Phytophthora ramorum* isolate[c]
Salt	GS1	Inoculate → fungicide → salt → evaluate	92	Roseum Elegans/1418983-2
	GS2	Inoculate → fungicide → salt → evaluate	123	Cunningham's White/1418983-2
	GS3 and GS4	Inoculate → fungicide → salt → evaluate	91,124	Cunningham's White/1418886
Water deficit[d]	GW1	Inoculate → withhold water → fungicide → resume water → evaluate	54	Cunningham's White/1418886
	GW2	Inoculate → fungicide → withhold water → resume water → evaluate	93	Cunningham's White/1418886
	GW3	Fungicide → withhold water → leaf water ψ/inoculate/resume water → evaluate	82	Cunningham's White/1418886
	GW4	Fungicide → withhold water → leaf water ψ/inoculate/resume water → evaluate	84	Cunningham's Blush/1418886
	GW5[e]	Fungicide → withhold water → leaf water ψ/inoculate/resume water (IC)	84	Cunningham's Blush/1418886

[a] Additional details of the treatments, including controls and time between treatments in the sequence, are provided in Materials and Methods.
[b] Duration is the time between the first treatment and root collection for the evaluation of *P. ramorum* colonization.
[c] *Rhododendron* cultivar and *P. ramorum* isolate used for each trial.
[d] Water deficit was imposed by withholding water for 3 weeks. Leaf water potentials (ψ) were measured at the end of the water stress episode and before resuming water.
[e] Trial 5 included measurements of leaf water potentials, but root colonization was not evaluated at the end of the trial. IC = incomplete.

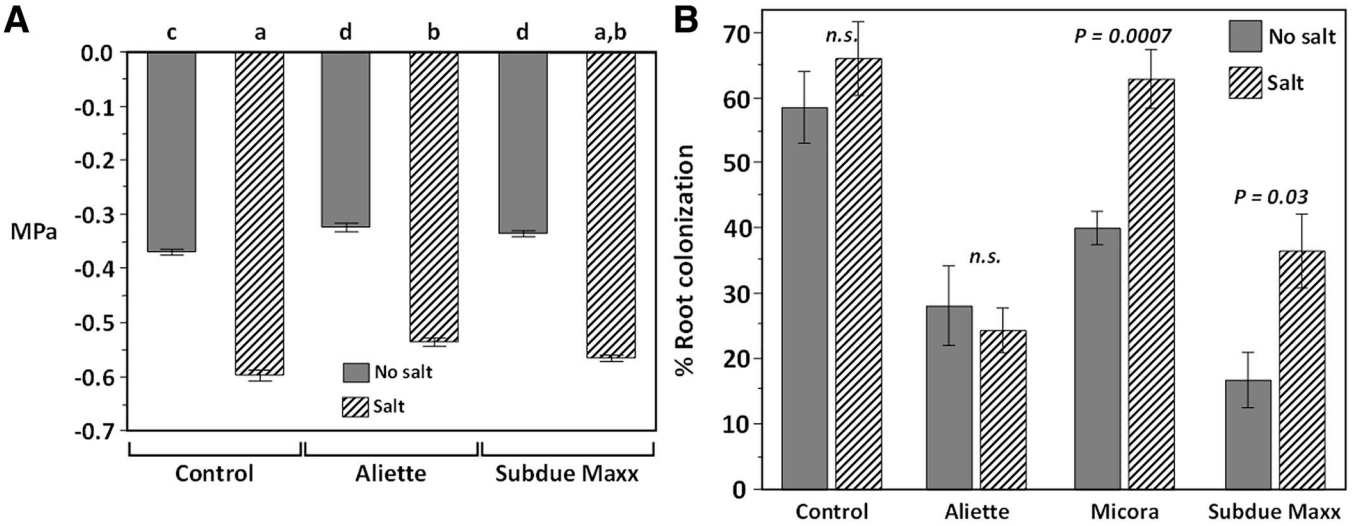

Fig. 1. A, The effects of salt and chemical treatments on midmorning leaf water potentials (MPa) in *Rhododendron* plants maintained in a growth chamber. Means and standard errors are indicated from one trial ($N = 5$ for each treatment mean). Letters on top of each bar indicate significant differences based on Tukey's honesty significant different (HSD) ($\alpha = 0.05$). **B,** The effects of salt and chemical treatments on *Phytophthora ramorum* colonization of roots of *Rhododendron* plants (growth chamber). Means and standard errors are from four trials (GS1 to GS4; $N = 20$ for each treatment mean). Significant differences between no-salt and salt treatments within each chemical treatment are indicated (t test; *n.s.* = not significant).

Because of the likely inhibitory effect of water stress on infection when imposed after inoculation, we reversed the treatment sequence during trials GW3 to GW5, with fungicide and water stress before inoculation and resumption of normal watering, a sequence that would seem to be more favorable for subsequent root infection and disease development than that during the earlier trials. Trials GW3 to GW5 also included leaf water potential measurements at the conclusion of the water stress period. Root colonization data were collected for trials GW3 and GW4. A mixed model analysis with the same fixed and random effects variables as described found that trial was not significant for root colonization (Wald $P = 0.4941$). The effect of fungicide and the interaction of fungicide × water stress were significant ($P < 0.0001$ and $P = 0.0214$, respectively), but water stress was not significant ($P = 0.2991$).

The arcsine square root-transformed root colonization data for trials GW3 and GW4 best corresponded to a fitted, normal, three-mixture distribution informed by using Akaike information criterion (AIC) values as performed with JMP. In the absence of water stress, Aliette and Subdue Maxx significantly reduced root colonization by *P. ramorum* relative to the water-treated (no fungicide) control by Dunnett's method (Aliette: $P = 0.0026$; Subdue Maxx: $P = 0.0002$) (Fig. 2B). After a water stress episode, root colonization in fungicide treatments was not different from that of the water-treated control. However, water stress significantly increased root colonization relative to the nonstressed control in Aliette-treated plants (pooled *t* test, $P = 0.046$) (Fig. 2B) and increased colonization in Subdue Maxx-treated plants, although this difference was not statistically significant.

Nursery trials: Interaction of salt stress and fungicides on ramorum blight. Three trials (NS1 to NS3) conducted at the NORS-DUC facility evaluated the effect of salinity on fungicide efficacy (Table 2). To determine if salt levels remained increased in soil after salt treatment and thorough watering, the conductivity of runoff was measured 4 weeks after salt application during trial NS1. Although the conductivity of water runoff from salt-treated plants was higher (486 ± 54 µS/cm; $N = 6$) than that of the no-salt controls (364 ± 23 µS/cm; $N = 6$) and similar to the irrigation water used at the facility (≈430 µS/cm), this difference was not statistically significant and well below the approximately 23 mS/cm of the applied salt solution.

A mixed model analysis of percent root colonization from the three trials was conducted with fungicide, salt, fungicide × salt, and pathogen isolate as fixed effects and trial and block as random effects. Trial was significant ($P < 0.0001$); trial NS1 was different from trials NS2 and NS3, with overall percent root colonization during trial NS1 significantly higher than that for trials NS2 and NS3. The arcsine square root-transformed root colonization data for the three nursery trials best corresponded to a fitted, normal, two-mixture distribution using AIC values as performed with JMP. Trial NS1 was further analyzed separately from trials NS2 and NS3.

A mixed model analysis of trial NS1 root colonization indicated that block (Wald $P = 0.50$), salt ($P = 0.85$), and fungicide × salt ($P = 0.23$)

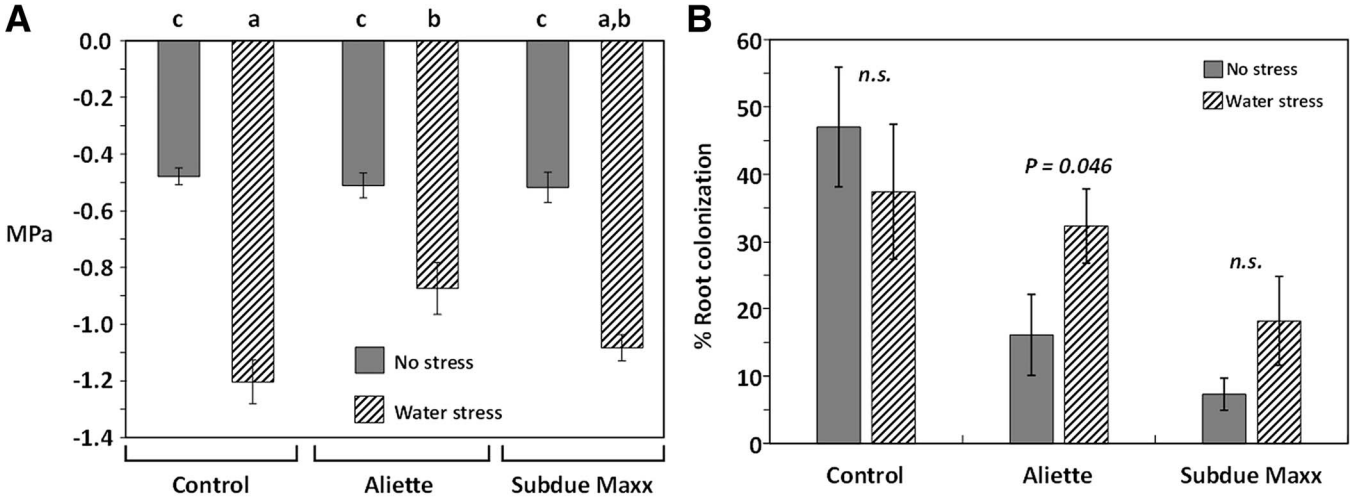

Fig. 2. A, The effects of water stress and chemical treatments on midmorning leaf water potentials (MPa) in *Rhododendron* plants maintained in a growth chamber. Means and standard errors are from three trials (GW3 to GW5; $N = 17$ for each treatment mean). Letters on top of each bar indicate significant differences based on Tukey's honestly significant difference (HSD) ($\alpha = 0.05$). **B,** The effects of water stress and chemical treatments on *Phytophthora ramorum* colonization of roots of *Rhododendron* plants (growth chamber). Means and standard errors are from two trials (GW3 and GW4; $N = 12$ for each treatment mean). Significant differences between no-stress and water stress treatments within each chemical treatment are indicated (pooled *t* test; *n.s.* = not significant).

Table 2. Summary of National Ornamental Research Site at Dominican University of California (NORS-DUC) nursery trials to assess the effects of salt stress and fungicides on *Phytophthora ramorum* development in *Rhododendron*

Trial[a]	Dates	Trial duration (days)[b]	Average high/low temp (°C) (range high/range low)[c]	Cumulative precipitation, mm[d]	Cultivar/ *Phytophthora ramorum* isolate[e]
NS1[f]	10 June–2 Dec. 2011	176	21.5/9.3 (15.0–35.0/1.1–12.2)	101	Cunningham's White/1418983-2
NS2	30 Jan.–23 June 2012	146	19.0/6.6 (13.3–31.7/–1.1–11.7)	267	Roseum Elegans/1418886
NS3	16 Oct. 2012–27 Mar. 2013	163	16.5/4.3 (14.4–28.9/–8.3–7.8)	309	Cunningham's White/1418886

[a] The treatment sequence for these trials was inoculate → fungicide → salt → evaluate. Additional details of the treatments, including controls and time intervals between treatments in the sequence, are provided in Materials and Methods.
[b] Duration is the time between inoculation and root collection for the evaluation of *P. ramorum* colonization.
[c] Average high and low temperatures and the ranges of daily high and daily low temperatures over the course of the trial.
[d] Cumulative precipitation (mm) over the course of the trial.
[e] *Rhododendron* cultivar and *P. ramorum* isolate used for each trial.
[f] Trial NS1 used 6.1-liter pots and trials NS2 and NS3 used 2.8-liter pots.

were not significant. However, fungicide was significant ($P = 0.015$). Means comparisons revealed that root colonization was not significantly reduced by Aliette or Subdue Maxx compared with the water-treated control in the absence of salt (Dunnett's method, $P > 0.35$) (Fig. 3A). However, with salt treatment, only Aliette significantly reduced root colonization compared with the salted control (no fungicide) and Subdue Maxx-treated plants (Dunnett's method, $P = 0.028$); root colonizations with the salt and no-salt Subdue Maxx treatments were not significantly different from each other (Fig. 3A). During trial NS1, there were no significant foliar or aboveground symptoms 6 months after inoculation (aerial ratings of 0 or 1 in a few plants) despite the significantly higher level of root colonization than that during subsequent trials (Supplementary Fig. 3).

Treatment effects in trials NS2 and NS3 were more distinct than those in trial NS1. A least squares analysis of root colonization during trials NS2 and NS3 indicated that salt treatment did not enhance root colonization in water/no-fungicide control or in fungicide-treated plants relative to their no-salt counterparts (Student's *t* test, threshold $\alpha = 0.05$) (Fig. 3B). However, at a less stringent significance threshold ($P = 0.07$), salt partially offset the reduction in root colonization observed in Subdue Maxx-treated plants. A single application of Aliette or Subdue Maxx significantly inhibited root colonization by *P. ramorum* relative to the water/no-fungicide control in both no-salt and salt-treated plants (Tukey's HSD, $\alpha \leq 0.05$) (Fig. 3B).

In contrast to trial NS1, which used 6.1-liter container plants, aerial symptoms were apparent 5 months after inoculation in 2.8-liter container plants in trials NS2 and NS3. Nonetheless, average symptom ratings ranged between healthy (0) and mild leaf chlorosis (1), with most plants appearing healthy (Table 3; Supplementary Fig. 4). Of 240 plants evaluated during trials NS2 and NS3, which also included noninoculated no-salt and salt-treated controls in the analysis, 33 symptomatic plants had a rating of 2 and 13 had a rating of 3. All other plants (194) had ratings of 0 or 1. Inoculation significantly increased aerial symptoms compared with the noninoculated, water-treated controls in both no-salt and salt-treated plants (Table 3). However, similar to the outcomes of root colonization during these trials, salt treatment did not significantly increase ramorum blight symptoms with any treatment combination. A correlation analysis of aerial symptom ratings and root colonization (based on arcsine square root-transformed data) yielded a Spearman's Rho correlation coefficient of 0.2743 ($P < 0.0001$). Of 60 noninoculated plants in the two trials, six plants displayed ramorum blight symptoms, with *P. ramorum* isolated from symptomatic foliage, but not from the stems or roots of those plants. Furthermore, roots of all asymptomatic, noninoculated plants were sampled and none was infected. The foliar infections were likely caused by cross-contamination from splash-dispersed inoculum from the soil surface of adjacent, asymptomatic, inoculated plants within the experimental blocks because noninoculated and inoculated plants were randomized within blocks and sentinel *Rhododendron* plants surrounding the experimental area remained healthy and free of *P. ramorum*.

Discussion

During previous work, we found that root stress imposed at the same salt concentrations used in the present study but applied in a hydroponic format predisposed small, root-inoculated *Rhododendron* and *Viburnum* plants to ramorum blight development (Roubtsova and Bostock 2009). This was similar to the predisposing effect of salinity observed in other *Phytophthora*–plant interactions (Blaker and MacDonald 1986; MacDonald 1982, 1984; Pye et al. 2018; Swiecki and MacDonald 1991). Based on the results of our initial study, we posited

Table 3. Average aerial symptom ratings of *Rhododendron* plants for trials NS2 and NS3 conducted at National Ornamental Research Site at Dominican University of California (NORS-DUC) ($N = 30$ plants for each mean and standard error). Foliar symptoms were rated approximately 146 days (NS2) or 163 days (NS3) after inoculation with *Phytophthora ramorum* using a scale of 0 to 3 as described in Materials and Methods. Inoculated and noninoculated treatment comparisons and no-salt and salt comparisons were significantly different from each other according to the Wilcoxon rank sum test, except when indicated.

Treatment	No salt	Salt
Water/noninoculated	0.6 ± 0.2[a]	0.2 ± 0.1[b]
Water/inoculated	1.2 ± 0.2[a]	1.0 ± 0.2[b]
Aliette/inoculated	0.9 ± 0.2	0.8 ± 0.2
Subdue Maxx/inoculated	0.7 ± 0.1	0.7 ± 0.1

[a] Significantly different at $P = 0.02$ according to the Wilcoxon rank sum test ($\chi^2 = 5.43$).
[b] Significantly different at $P = 0.0005$ according to the Wilcoxon rank sum test ($\chi^2 = 11.97$).

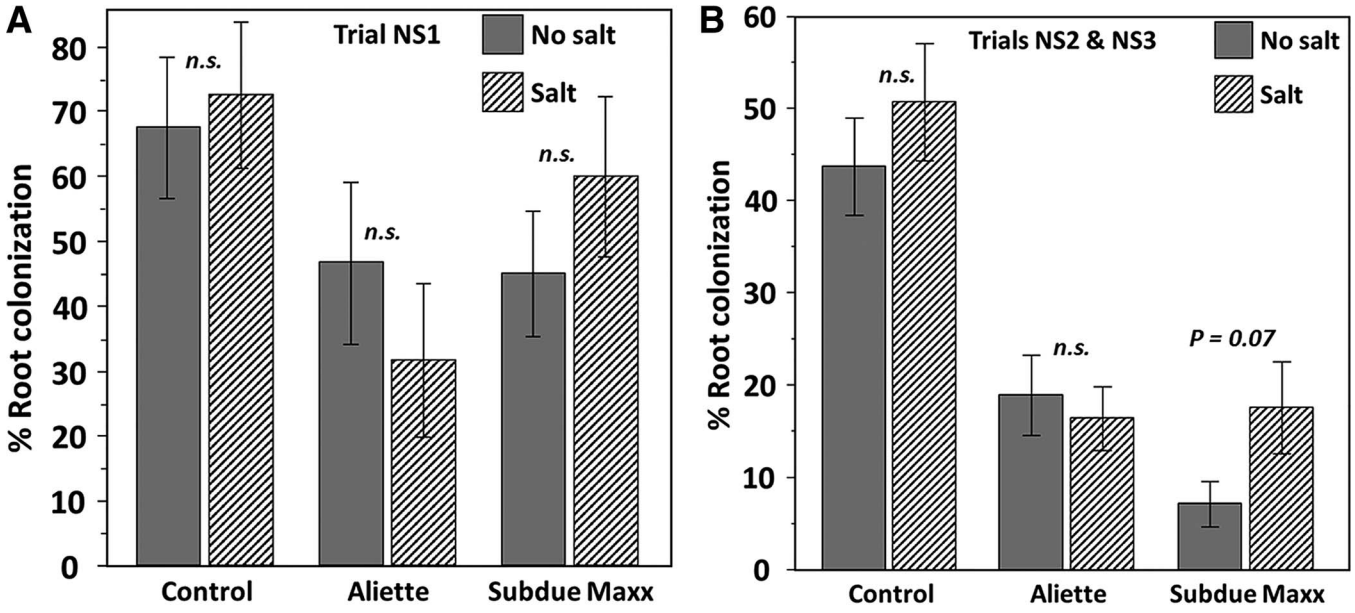

Fig. 3. The effects of salt and chemical treatments on *Phytophthora ramorum* colonization of roots of *Rhododendron* plants during three trials conducted in the outdoor experimental nursery at National Ornamental Research Site at Dominican University of California (NORS-DUC). Means and standard errors are indicated. Significant differences between no-salt and salt treatments within each chemical treatment are indicated (*t* test; *n.s.* = not significant). **A,** Results from trial NS1 ($N = 12$ for each treatment mean). **B,** Combined results from trials NS2 and NS3 ($N = 30$ for each treatment mean).

that episodic stress in other settings might explain the rapid onset of ramorum blight from asymptomatic infections in nursery plants, including fungicide-suppressed infections. During this study, which used larger plants maintained in a standard potting soil mix, we found that our experimental stress regimens (salt or water deficit) were insufficient to clearly predispose genus *Rhododendron* to ramorum blight. Root colonization by *P. ramorum* in the absence of fungicides was similar in both stressed and nonstressed plants, with extensive colonization in both cases (i.e., the salt and water deficit regimens did not significantly enhance root colonization by *P. ramorum* to a level greater than that in the nonstressed, inoculated controls). A single fungicide treatment significantly reduced root colonization compared with the nontreated controls during all growth chamber trials and two of three nursery trials. However, after an abiotic stress episode, fungicide treatment outcomes of inoculated plants were more complex.

During growth chamber trials, salt stress offset the reduction in root colonization observed in plants treated with Micora or Subdue Maxx (Fig. 1B), but it did not diminish Aliette performance. Aliette reduced root colonization in both no-salt and salt-treated plants by more than 50% compared with the corresponding controls. In contrast, water stress enhanced root colonization in Aliette-treated plants compared with Aliette-treated, nonstressed plants to a level similar to that of the no-fungicide controls (Fig. 2B). Again, Subdue Maxx strongly reduced *P. ramorum* root colonization, but water stress only partially offset Subdue Maxx performance; this effect was not statistically significant.

During the nursery trials, we also could not provoke a clear predisposition phenotype resulting in ramorum blight of the shoots with our salt stress regimen. During two of three nursery trials, Aliette and Subdue Maxx protected roots from *P. ramorum* colonization in both no-salt and salt-treated plants, although salt offset Subdue Maxx protection, albeit to a root colonization level that was still significantly less than the no-fungicide, salt-treated control (Fig. 3B). Although most differences among treatments did not reach statistical significance in trial NS1, treatment effects on root colonization for the three nursery trials trended similarly.

Our stress regimens clearly had a physiological impact on plants maintained in the growth chamber, as reflected in the significant decline in leaf water potentials compared with the nonstressed controls. In the case of salt treatment, the decline to approximately −0.6 MPa (Fig. 1A) likely only reached a predisposition threshold for genus *Rhododendron*, because water potentials in the range of −0.5 to −1 MPa have been shown to be thresholds for predisposition in other mesophytic species (Bostock et al. 2014). The significant salt-reversal of Micora and Subdue Maxx protection under the tightly controlled growth chamber environment suggests that a predisposition threshold was attained. The water stress regimen imposed in the growth chamber reduced leaf water potentials to approximately −1 MPa (Fig. 2A), and it also appeared to have attained the threshold, at least with Aliette, where water stress counteracted its protection (Fig. 2B).

The absent or, at best, weak salt effects during the NORS-DUC trials may reflect variable environmental conditions of an outdoor nursery and associated logistical challenges to experimentally achieving and sustaining a uniform predisposition threshold among the plants. Consideration of the salt solution volumes compared with pot volumes may also partially explain the differences between trials. Although plants in all three nursery trials each received 0.5 liters of salt solution, this was only 8% of the pot volumes in NS1 (6.1-liter pots) compared with 18% of the pot volumes in NS2 and NS3 (2.8-liter pots). The stress intensity experienced by plants in NS1 was likely less than that experienced by plants in NS2 and NS3, and it may partly explain the absence of any treatment effects and symptoms in NS1 and more distinctive symptoms in the latter trials.

The inability of salt or water stress regimens to enhance *P. ramorum* colonization in the no-fungicide controls also could be attributable to inoculum density, which, if too high (i.e., root colonization already at saturation in the no-stress controls), would obscure a stress-induced predisposition phenotype. Before use, we tested each inoculum batch by plating on PARP-H to ensure viability of *P. ramorum* in the mix. However, we did not determine the dynamics of zoospore production from the *P. ramorum*-infested soil mix in the pots throughout the course of the 3- to 6-month trials. Suitable qualitative and quantitative methods for baiting and zoospore capture from irrigation runoff from genus *Rhododendron* and other nursery plants have been reported (Rollins et al. 2016; Shishkoff 2009, 2011; Swiecki et al. 2018b). Such determinations might have provided additional insight regarding treatment outcomes but likely would be intrusive by introducing additional variation and exceeding the resources to conduct our study. In retrospect, salt stress treatments of greater intensity and more extensive root sampling with a robust assessment of root-to-root variability within a sample may have enabled a clearer assessment of treatment differences.

The ability of *Rhododendron* plants to sustain extensive root colonization by *P. ramorum* with little or no aboveground symptoms in the outdoor nursery environment is particularly striking. This was especially true during the first nursery trial with the larger plants (6.1-liter pots) with root systems that may compensate and tolerate greater colonization than smaller plants. Additionally, the weather conditions during that trial, initiated in late spring and terminated in late fall, were warmer and drier than the conditions during trials NS2 and NS3. This time period is also when *Rhododendron* leaves are least susceptible to *P. ramorum* (Tjosvold et al. 2008). Furthermore, the seasonal cooler, wetter conditions during the latter trials would likely favor *P. ramorum* root infections and, perhaps, greater invasive growth (DiLeo et al. 2014; Grünwald et al. 2019; Parke and Lewis 2007; Rizzo et al. 2005), thus resulting in the more visible foliar symptoms in those trials. We can only speculate about the effects on pathogen root-to-shoot movement because, except for a few plants, we did not systematically sample the stems and foliage of root-inoculated symptomatic or asymptomatic plants for the presence of *P. ramorum*. Nonetheless, there was a relatively weak positive correlation between root colonization and aerial symptoms.

Of the three tested fungicides, Subdue Maxx generally was most suppressive of root colonization during our experiments, consistent with its strong performance during studies of foliar protectants against *P. ramorum* in nursery ornamentals (Becker et al. 2010; Chastagner et al. 2008; Linderman and Davis 2008; Tjosvold et al. 2008). The chemicals we tested are more effective against *P. ramorum* as preventive treatments, and they are generally applied multiple times at nurseries that use fungicides to manage pathogenic *Phytophthora* spp. However, we found that even one treatment of a chemical during a trial, applied either before or after inoculation, significantly reduced root colonization by *P. ramorum*. Chemical management of *Phytophthora* spp. in the nursery environment is controversial because of concerns that fungicides suppress, but do not eliminate, the pathogen from nursery materials, thus suggesting they are not part of best management practices or confirmed nursery protocols (Swiecki et al. 2018a; USDA-APHIS 2020b)

The effect of abiotic stress on the performance of Aliette during our study depended somewhat on the type of stress. The protection from Aliette against root colonization by *P. ramorum* was not reversed by a salt stress episode, which was in contrast to water stress, which significantly offset its protection. Interestingly, root treatment with Aliette moderated both the salt stress-induced and water stress-induced impacts on leaf water potentials (Figs. 1A and 2A). The basis for this is unclear, but it is suggested that Aliette has physiological effects on rhododendrons beyond direct effects on the pathogen. The relative contributions of direct and indirect modes of action of Aliette and related phosphonic/phosphorous acid fungicides have been the subject of debate (Fenn and Coffey 1985), and potassium salts of phosphorous acid (i.e., Agrifos) have been shown to inhibit and limit *P. ramorum* in culture and in perennial hosts (Garbelotto et al. 2009). Nonetheless, phosphonate chemicals can engage or prime plant defense responses (Guest and Bompeix 1990; Tripathi et al. 2019). We did not explore this aspect of the action of Aliette in genus *Rhododendron* because it was beyond the scope of our study, but plant activators (i.e., elicitors of induced resistance) can protect plants against the exacerbating effect on disease of predisposing stress (Pye et al. 2013).

Conclusion. The full significance of soilborne inoculum and root infections by *P. ramorum* to the etiology and epidemiology of ramorum blight and sudden oak death in nurseries and natural landscapes is unresolved. However, there is strong evidence for the root-to-root

spread of the pathogen in the nursery (Chastagner et al. 2013), persistence of the pathogen in soil mix and roots of nursery ornamentals (Shishkoff 2007), and survival of the pathogen in nursery substrates, residues, water, and sediment (Junker et al. 2016). Our results affirm that soilborne *P. ramorum* can attack and extensively colonize roots of *Rhododendron* plants, with infected plants displaying few or no ramorum blight symptoms. Our results also show that an episode of salt stress or water stress can offset root protection afforded by some oomycete-suppressive chemicals without enhancing ramorum blight symptoms. Therefore, abiotic stresses encountered in the nursery as well as during shipment and transplanting can exacerbate root infections and reduce the efficacy of chemical and other control measures. These features of the disease and the proclivity of the pathogen for asymptomatic infections in some hosts further complicate sample selection for confirmatory diagnoses during nursery inspections and certification efforts and have implications for containment and mitigation of *P. ramorum* and other soilborne *Phytophthora* species of concern.

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