Reducing infection and secondary inoculum of *Phytophthora ramorum* on *Viburnum tinus* roots grown in potting medium amended with *Trichoderma asperellum* isolate 04-22

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Abstract

Phytophthora ramorum is a concern of the ornamental nursery business because it is implicated in the movement on ornamental plants such as rhododendron and viburnum. Since there is a soil phase that infects the roots, above-ground symptoms are not often manifested and easily detected. Government agencies require that a *P. ramorum*-positive nursery be placed under some type of quarantine until it can demonstrate that it has mitigated *P. ramorum*. Typical methods of mitigation are often expensive, harmful to the environment, and impractical. Biological control using *Trichoderma asperellum* isolate 04-22 (Ta 04-22) has been

demonstrated to reduce *P. ramorum* soil populations to below detectable limits when incorporated into soil or potting mix. The purpose of this study was to determine whether Ta 04-22 would reduce *Viburnum tinus* root infection and reduce secondary sporulation from infected roots. When rooted *V. tinus* cuttings were planted into potting mix amended with three different final concentrations (1×10^5 , 1×10^6 , and 1×10^7 CFU/cm³ potting mix) of Ta 04-22 and drenched with either a *P. ramorum* sporangia or chlamydospore suspension, plants with any infected roots declined as the Ta 04-22 concentration increased. This was significant ($P = 0.039$) when *P. ramorum* chlamydospores were used as the inoculum propagule but not ($P = 0.143$) when sporangia were used. A second application of Ta 04-22 as a top-dressing after 4 weeks did not result in a decrease in plants with infected roots. When *P. ramorum*-infected *V. tinus* roots were treated with Ta 04-22 grown on wheat bran, Ta 04-22 formulated as a wettable powder, or RootShield® Plus WP, flow-through of *P. ramorum* secondary inoculum was significantly reduced compared to non-treated controls ($P < 0.001$). A second application did not reduce the secondary inoculum any further. These results demonstrate that various formulations of Ta 04-22 and, to a lesser degree, RootShield Plus WP can be used as a microbial biological control treatment to reduce infection of *V. tinus* roots by *P. ramorum* and reduce plant-to-plant spread by reducing secondary inoculum.

Key words: biological control, ornamentals, *Phytophthora ramorum*, *Trichoderma asperellum*

1. Introduction

47
48 *Phytophthora ramorum* is the causal agent of Sudden Oak Death and Ramorum Blight on
49 ornamentals. Ramorum Blight is considered to have only a moderate impact on ornamental
50 plants since it usually is not lethal to these hosts (Kliejunas, 2011) but can affect marketable
51 quality and salability of these crops as well as their aesthetic appearance in landscapes.
52 Symptoms on rhododendron, camellias, viburnum, and other hosts are usually non-distinctive
53 leaf necroses and blights that are not easily distinguishable from other diseases. However,
54 Ramorum Blight does have a serious impact on the nursery industry. Since shipment of infested
55 ornamental plants plays a role in the long distance spread of this pathogen (Goss et al. 2011;
56 Prospero et al. 2009). This is a cause for concern that the pathogen could spread from nurseries
57 into natural habitats (Davidson et al., 2005). A 2011 study (Kovacs et al., 2011) predicted a \$7.5
58 million economic cost and \$135 property value loss in California due to Sudden Oak Death
59 impact on oak trees alone. Therefore strict guidelines have been imposed on nurseries in areas
60 known to have *P. ramorum*. These restrictions have had substantial economic consequences on
61 the nursery industry (Kliejunas, 2011).

62 *Phytophthora ramorum* has been considered primarily a pathogen that infects above-
63 ground tissues (leaves, woody shoots, trunks) of the host (Werres et al., 2001) with a
64 demonstrated soil phase consisting of infected organic debris, infected roots, and persistent
65 chlamydospores (Fichtner et al, 2011; Parke and Lewis, 2007; Shishkoff, 2007). This ecology of
66 the habitat of *P. ramorum* is not unusual for *Phytophthora* spp. A good comparable example is
67 *P. megakarya*, causal agent of black pod disease of cacao. This species is similarly classified as
68 an above-ground pathogen (Coffey, 1991) but has been found on roots in low numbers (Opoku et
69 al., 2002) present in the soil as a reservoir of inoculum (Gregory et al., 1984). For many years,

researchers concentrated on aerial plant-to-plant spread, but it became clear that roots became infected and that rain splash of soil-borne inoculum could initiate above-ground infections (Asare-Nyako and Dakwa, 1974; Okaisabor, 1974). Other examples that have been cited are *P. palmivora* (Graham et al., 1998) and *P. cactorum* (Madden et al., 1992). Davidson et al. (2005) and Fichtner et al. (2011) both demonstrated splash dispersal as a transmission pathway of *P. ramorum* from infested soil to aerial plant parts. Demonstrating that *P. ramorum* has a soil phase is important epidemiologically because of the potential for movement after rain and in waterways. *Phytophthora ramorum* has been found in waterways, such as streams and rivers (Davidson et al., 2002; Sutton et al., 2009), and was recovered at a forested and riparian site 1 km downstream of an infested site that was connected via an ephemeral stream (Davidson et al., 2005). Therefore, it is worth considering that a viable pathway for spread in a nursery might occur when infested water comes in direct contact with roots. Studies have shown that *P. ramorum* can survive in potting medium that was exposed to containers of infected plants in a nursery (Jeffers, 2005; Tjosvold et al., 2009). Spread in a simulated nursery was greater via irrigation runoff than aerial spread for plants on a non-permeable ground cover (Heungens, et al., 2013).

Aerated steam and chemical fumigants are known methods to eliminate soilborne pathogens. Linderman and Davis (2008a) found that *P. ramorum* populations in potting media were killed by aerated steam heat treatments of 50 °C or higher or treatment with metam sodium concentrations of 0.25 ml/L of medium. However, using these techniques increases the chance of destroying beneficial microorganisms and of working in a hazardous environment. There have been numerous studies examining the use of fungicides to control *P. ramorum* on ornamentals (Heungens et al., 2006; Linderman and Davis, 2008b; Tjosvold et al., 2008; Pérez-Sierra et al.,

2011). Although fungicides are effective to some degree when applied as a preventative (Heungens et al., 2006; Tjosvold et al., 2008), they require repeated applications. Linderman and Davis (2008b) found that all chemicals tested in their study were fungistatic and not fungicidal. Besides environmental concerns, repeated applications potentially could lead to fungicide resistance by the pathogen as has been observed by other *Phytophthora* spp. (Grünwald et al., 2006; Pérez-Sierra et al., 2011). In addition, U.S. and European Union regulations prohibit the application of fungicides in the quarantine zones (USDA/APHIS, 2010b; Pérez-Sierra et al., 2011) due to potential masking of symptoms. These concerns have lead to the desire to investigate other control options, including biological control.

Some of the most studied and promising fungi used in a biological control system are *Trichoderma* spp. (Harman et al., 2004). Most *Trichoderma* spp. isolated from soil and composts are nutrient recyclers (Klein and Eveleigh, 1998). This may contribute to results that show some *Trichoderma* spp. increase plant growth when amended to soils (Doni et al., 2014; Hoyos-Carvajal et al., 2009; Ousley et al., 1994). In addition, specific populations of *Trichoderma* spp. when amended to composts and compost-amended media can suppress *Pythium* and *Phytophthora* root rots within days after their formulation (De Ceuster and Hoitink 1999). *Trichoderma* spp. are reported to suppress soilborne diseases caused by *Phytophthora* spp. in containerized systems (da S. Costa et al., 2000; Sharifi Tehrani and Nazari, 2004). Results from a study by Widmer (2014) demonstrated for the first time that a patented isolate (US Patent No. 9,320,283), 04-22, of *T. asperellum* has the potential to reduce soil populations of *P. ramorum* below detectable levels. However, that study was done without the presence of a host plant. The current study was done to determine whether *T. asperellum*-amended potting

mix could protect the model host *V. tinus* from root infection by *P. ramorum* propagules and reduce *P. ramorum* sporulation if it became infected.

2. Materials and Methods

2.1 Plant material

For experiments studying root infection in potting mix, *Viburnum tinus* ‘Compactum’ cuttings were rooted by first dipping the cut stem in Hormodin 2 (0.2% Indole-3-butyric Acid; OHP, Inc., Mainland, PA, USA) and then sticking the stem in Fafard 52 mix (Conrad Fafard Inc. Agawam, MA, USA), which consists of pine bark (60%), sphagnum peat, perlite, and vermiculite. For experiments studying production of root inoculum, cuttings of *V. tinus* ‘Compactum’ were produced by dipping the stem end in Hormodin 2 and then planting in 20 mm x 20 mm x 50 mm plastic pots containing Turface MVP (a calcined, montmorillonite clay substrate medium manufactured by Profile Products LLC, Buffalo Grove, IL). For both methods, cuttings were grown for 5-6 weeks under an intermittent mist tent in a greenhouse, until adventitious roots with lateral root development were present.

2.2 Culture maintenance

All work was conducted in a Biosafety level 3 containment facility under USDA-APHIS permit requirements for *P. ramorum*. *Phytophthora ramorum* isolates WSDA-1772, NS-5C, BS-2, BS-4, NA2-05166 and NA2-09053 were maintained on rye seeds in sterile water in a 20 °C incubator for long-term storage. Working cultures were prepared by removing a colonized rye seed and placing it on a *Phytophthora*-selective medium (PARPH+V8; Ferguson and Jeffers,

1999). Resulting mycelia growth was transferred to 20% V8 agar and stored in a 20 °C incubator. Isolates WSDA-1772 and NS-5C were originally recovered from *Viburnum* ‘Mariesii’ in Oregon and *Camellia sasanqua* ‘Bonanza’ in California, respectively. These isolates were found to be representative of *P. ramorum* infection on *Viburnum* spp. (Shishkoff, 2011; Widmer, 2010). Isolates BS-2 and BS-4 were collected from soil in Big Sur State Park. Isolates WSDA-1772, NS-5C, BS-2 and BS-4 contained the same single nucleotide polymorphism profile as known isolates of US clonal lineage NA1 typically found in California forests and nurseries (Grunwald, et al., 2009). Isolates NA2-1 and NA2-2 are isolates of US clonal lineage NA2 from Washington and California, respectively.

Trichoderma asperellum isolate 04-22 (Ta 04-22) was maintained on BBL™ cornmeal agar (CMA; Becton, Dickinson and Company, Sparks, MD, USA) in a 20 °C incubator for long-term storage. Working cultures were prepared by streaking half-strength Difco™ potato dextrose agar (1/2PDA; Becton, Dickinson and Company) and stored in a 20 °C incubator.

2.3 Formulation of *Trichoderma asperellum*

Ta 04-22 conidia were scraped from a culture grown on 1/2PDA for 1 to 2 weeks and transferred to autoclaved half-strength potato dextrose broth (1/2PDB; Becton, Dickinson and Company) in a 60-mm-diameter Petri plate. The culture was incubated in a 20 °C incubator for 3 days. The resulting culture in 1/2PDB was used to inoculate twice-autoclaved wheat bran (300 g plus 300 ml water) in spawn bags with a 0.2 µm filter patch (Fungi Perfecti LLC, Olympia, WA, USA) under sterile conditions. The bag was heat-sealed and placed under continuous lighting at 25 °C, occasionally mixing the contents by hand. After 10 days, the bag was opened up and the colonized wheat bran was allowed to air-dry for approximately 3 days. After drying, the

colonized wheat bran was ground to a coarse powder using a Waring model 7011HS blender (Waring Commercial, Torrington, CT, USA). The colonized wheat bran was stored in a closed bag at 4 °C until used. The colony forming units (CFU)/g of colonized wheat bran was measured by preparing several serial dilutions in 0.2% water agar and plating 1 ml of the final suspension onto each of 10 plates containing solidified Trichoderma Selective Medium (Askew and Laing, 1993).

Ta 04-22 -colonized wheat bran was formulated as a wettable powder (referred to as Ta 04-22WP) by a proprietary process from BioWorks, Inc. (Victor, NY). Final concentrations of Ta 04-22 conidia in 04-22WP ranged from 1.0 to 1.8×10^9 CFU/g of wettable powder.

2.4 Inhibition of *Phytophthora ramorum* root infection by *Trichoderma asperellum*

To investigate the effect of *T. asperellum* dosage on root infection, bags of standard potting mix (consisting of 41% peat, 11% bark, 23% perlite, 23% vermiculite, 2% sand, and a small amount of granular trace element blend) were either amended with wheat bran colonized by Ta 04-22 at final concentrations of 1×10^5 , 1×10^6 , or 1×10^7 CFU/cm³ potting mix. Non-amended potting mix was used as a control. The moisture was brought up to approximately 10% by adding the appropriate volume of water. The bags, closed but not sealed, were kept for 3 days at 20 °C.

Rooted cuttings were removed from the Fafard 52 mix and transplanted into 6.5 cm square pots containing the amended or non-amended potting mix (350 cm³ volume). There were 16 plants per treatment. The plants were placed on a greenhouse bench with drip irrigation. After 3 days, half of plants for each treatment were inoculated by drenching the pot with 25 ml

of either a *P. ramorum* sporangia suspension (56,000 sporangia per pot) or chlamydospore suspension (9500 chlamydospores per pot).

After 4 weeks, the plants were carefully removed from the pots. The potting mix in the pots was combined for each individual treatment. The population of *P. ramorum* was measured by plating a 1:5 dilution (v/v) of the potting mix in 0.2% water agar on PARPH+V8 and counting the colonies after 3 days. The roots were washed free of any potting mix. The detached roots were plated on PARPH+V8 after surface sterilizing in 70% ethanol for 20 s, rinsing three times in sterile water, and blotting dry on a sterile paper towel. The plates were sealed and placed in an incubator at 20 °C. After 5 days, the plates were examined and the individual root system recorded as positive for *P. ramorum* infection if a minimum of one colony grew from the roots. Verification for identification of *P. ramorum* was confirmed by visual observation of the characteristically large chlamydospores and semi-papillate sporangia on the agar (Werres et al., 2001). On the bottom of the Petri plate, infected root segments were outlined with a red marker and non-infected root segments with a blue marker. The plates were then scanned with an HP Scanjet 5500C model flatbed scanner (Hewlett-Packard; Palo Alto, CA). The length of infected and non-infected root segments were measured using ASSESS software (Version 2.0; American Phytopathological Society, St. Paul, MN) and the percentage of infected root length was calculated. The experiment was performed eight times.

To determine if a second application of *T. asperellum* will reduce root infection, the experiment was set-up in the exact same manner as described above incorporating an initial dosage of 1×10^7 CFU/cm³ potting mix. After 4 weeks, a second application of *T. asperellum*-colonized wheat bran (3.5×10^9 CFU/350 cm³ pot) was applied as a top-dressing. The plants were removed after an additional 4 weeks on the greenhouse bench and prepared and analyzed as

described above. The experiment was conducted a total of three times. The effect of adding a second application was analyzed as described above by comparing the percentage of infected plants at 4 and 8 weeks for each of the treatments.

2.5 Quantitation of *P. ramorum* inoculum from infected plant roots

2.5.1 Effect of wheat bran colonized by Ta 04-22 applied as a top-dressing

Phytophthora ramorum isolate NS-5C sporangia were prepared as described previously (Tooley et al., 2004), by placing mycelial plugs (10-mm-diameter) from 2-week-old cultures on V-8 agar into a sterile 1% soil extract solution for 48 h. Resulting sporangia were counted using a haemocytometer. Twelve *V. tinus* cuttings were inoculated by pouring 50 ml of a suspension of sporangia (500 sporangia/ml) over the surface of each 20 mm x 20 mm x 50 mm pot containing Turface MVP, a calcined, montmorillonite clay substrate medium (Profile Products LLC, Buffalo Grove, IL). Four additional plants were treated with water as negative controls. Inoculated cuttings were incubated overnight at 20 °C, then carefully uprooted, rinsed to remove all particles of infested Turface, and transplanted into 100 cm³ of clean Turface in square 20 mm x 20 mm x 50 mm pots with bottom plastic mesh inserts (mesh size No. 53) for easy drainage. After transplantation, six of the plants were treated with a top-dressing of 3-4 g of wheat bran colonized by Ta 04-22 so that the amount added was such that the concentration of *T. asperellum* was 1 x 10⁷ spores/cm³ of Turface. Six plants were treated with wheat bran alone. Two non-inoculated controls were treated with a top-dressing of *T. asperellum*-colonized wheat bran and two with wheat bran alone. After inoculation, plants were kept in a controlled-environment

chamber set at 20 °C with a 14-h photoperiod. Pots of each treatment were placed in their own plastic tray so that water from one treatment did not contact plants from another treatment. Trays from a given experiment were placed in the same growth chamber, but were moved randomly to different positions within the growth chamber at each sampling time. Flow-through was sampled weekly for 7 weeks after inoculation with *P. ramorum*. Sampling consisted of pouring enough distilled water through the pot to collect 25-30 ml of flow-through water in a plastic centrifuge tube. The collected flow-through was subsampled using a 3 ml plastic syringe to add 1 ml aliquots to each of three plates of PARPH+V8 medium. Plates were swirled to distribute a film of flow-through over the entire surface, and then incubated at 20 °C. Colonies on each plate were counted after 2 weeks using a dissecting microscope with dark-field illumination (under which colonies of *P. ramorum* are characteristically highly refractive) in order to quantify the CFU/ml of flow-through. At the end of the experiment, roots were surface-sterilized for 5 min in 0.025% sodium hypochlorite, rinsed and left to sit in distilled water for 30 min, and then 1-cm segments plated on PARPH+V8 medium. Ideally, 60-100 segments were plated; less if the root system was smaller. After a 1-week incubation at 20 °C, colonies from plated roots were counted to determine percent infection. This experiment was performed three times.

2.5.2 Effect of *T. asperellum* 04-22WP and Rootshield® Plus applied as a root drench

Phytophthora ramorum isolate NS-5C sporangia were prepared as described and counted using a haemocytometer. Formulated *T. asperellum* was compared with the commercial product Rootshield Plus™, both added at their label rates. Eighteen *V. tinus* cuttings were inoculated by

pouring 50 ml of a suspension of sporangia (500 sporangia/ml) over the surface of each pot. Four additional plants were treated with water as negative controls. Inoculated cuttings were incubated overnight at 20 °C, then transplanted into 100 cm³ of clean Turface in pots as described above. After transplantation, a time zero sample of flow-through water was taken from each pot. Afterwards, six plants were treated with Ta 04-22WP by pouring 50 ml of a suspension in water of the wettable powder over the surface of each pot for a final concentration of 1×10^7 CFU/cm³ of Turface potting media. Six plants were treated with Rootshield Plus™ in the same manner at the recommended rate for a final concentration of 9×10^3 CFU/cm³ of Turface potting media. The remaining six inoculated plants were non-treated to act as positive controls. Flow-through from the plants were sampled weekly for 8 weeks and then root segments were plated, as described above. This experiment was performed twice.

To determine if there was any added benefit to a second root drench with Ta 04-22WP or RootShield Plus, 30 *V. tinus* cuttings were inoculated as described above with *P. ramorum* sporangia, except using an equal mixture of five *P. ramorum* isolates (NS-5C, BS-2, BS-4, NA2-1 and NA2-2) at 500 sporangia/ml. Four plants treated with water alone were used as negative controls. After a time zero flow-through sample was taken, 12 inoculated plants were treated with Ta 04-22WP and 12 with Rootshield Plus as described above, with six inoculated plants non-treated to serve as positive controls. Samples of flow-through water were taken weekly through week four described above, at which time six of the Ta 04-22WP-treated cuttings were given an additional dose and six of the Rootshield Plus-treated cuttings were given an additional dose. Samples continued to be taken at weeks five, six, seven, and eight.

2.6 Statistical analysis

The effect of initial *T. asperellum* isolate 04-22 concentration on infection for each *P. ramorum* propagule type was compared by regression analysis using SigmaPlot Version 11.0 (SPSS Inc., Chicago, IL, USA). The data for the percentage of plants that had infected roots failed the normality test based on the Shapiro-Wilk test ($P \geq 0.05$) and were transformed with arcsine square root (Gomez and Gomez, 1984). The percentage of infected root segments were arcsine square root transformed prior to analysis. Data from non-infected plants were removed before analysis so as to not skew the data. The transformed values were subjected to analysis of variance (ANOVA) and any differences were determined by subjecting the means to least square means analysis with the pdiff option using Proc MIXED in SAS for Windows (version 9.2; SAS Institute, Cary, NC). Total root length was analyzed by subjecting the means to least square means analysis with the pdiff option using Proc GLM in SAS for Windows. Differences in the means were considered significant at $P \leq 0.05$.

Inoculum in flow-through over time was analyzed statistically after "colonies per pot" was transformed using a logarithmic (base e) transformation with "0" values replaced by "1" to retain low counts on the log scale. The transformed variable was analyzed by random coefficient analysis using the mixed model analysis of covariance program in SAS (PROC MIXED) to take into account that the same plants were sampled over time. The terms in the random statements (expt and day) were used as random in the random coefficient analysis while the test variable was considered fixed. Pairwise treatment differences were compared using the least significant difference (LSD) at each sampling time. The amount of inoculum produced by each plant could be totaled for the entire experimental time period to get "total colonies per pot" for each plant. This was analyzed by general linear models (GLM) in SAS and treatment means compared using

LSD to test the treatment differences. Percent root colonization at the end of the experiment was analyzed by GLM after performing an arcsin square root transformation to normalize data and treatments were compared by using LSD to test the treatment differences. Percent root colonization was analyzed by doing an arcsin square root transformation and comparing treatments using GLM and LSD.

3. Results

3.1 Inhibition of *Phytophthora ramorum* root infection by *Trichoderma asperellum*

Incorporating Ta 04-22 into the potting mix reduced the number of plants with *P. ramorum*-infected root systems. There was a decline in the percentage of plants that were infected with *P. ramorum* as the initial application rate of *T. asperellum* increased (Fig. 1). There was an effect on infected plants due to the *P. ramorum* propagule type at inoculation. Regression analyses showed a significant effect ($P=0.039$) when *P. ramorum* chlamydospores were used as the inoculum propagule, but not when sporangia were applied ($P = 0.143$). A second application of *T. asperellum* 4 weeks later did not significantly affect the percentage of plants that became infected when originally inoculated with sporangia (59% for one time application versus 50% for a second application; $P = 0.929$) or chlamydospores (29% for one time application versus 31% for a second application; $P = 0.998$).

Final populations of *P. ramorum* in the potting mix after 4 weeks declined as the initial rate of *T. asperellum* increased (Fig. 2), but the decline was not significant for chlamydospores ($P = 0.255$) or sporangia ($P = 0.206$) according to regression analyses.

When non-infected plants were removed from the analyses, there was no significant difference in the mean percentage of infected roots per plant between the controls and any of the treatments, regardless of the *P. ramorum* inoculum type (Fig. 3). There was no significant effect of *T. asperellum* on total root length of *V. tinus* roots, regardless of the inoculum type (Fig. 4).

3.2 Quantification of *P. ramorum* inoculum from infected plant roots

3.2.1 Effect of wheat bran colonized by Ta 04-22 applied as a top-dressing

In a regression analysis of flow-through over time for three trials, the variables day, day-treatment and day-day-treatment were significant at $P < 0.001$, indicating that the infected plants treated with Ta 04-22 differed from wheat bran-treated plants in their regression equations. At each sampling time, Ta 04-22-treated plants gave off significantly less inoculum than wheat bran-only plants (Fig. 5) according to tests of differences of Least Squares Means for plants at each sampling date ($P < 0.0001$). When total inoculum given off over the course of the experiment was analyzed by GLM, treatment was significant at $P < 0.0001$, with significantly less inoculum produced from *Trichoderma*-treated plants. A GLM of root colonization showed the variable treatment significant at $P < 0.001$, with *T. asperellum*-treated plants showing significantly less root colonization (average 32.7%) than plants treated with wheat bran alone (53.9%). In some samples very low levels of inoculum in flow-through were detected. No colony-forming units per pot were detected in flow-through from pots treated with *Trichoderma* at weeks 2 and 3 in the first trial, and 1.11 and 0.0 CFU/pot were detected in *Trichoderma*-treated pots in the 2nd trial at 4 and 5 weeks, respectively.

3.2.2 Effect of *T. asperellum* 04-22WP and Rootshield Plus applied as a root drench.

In a regression analysis of flow-through over time for two trials, the variables day, day-day, day-treatment and day-day-treatment were all significant at $P < 0.0004$, indicating that treatments differed in their regression equations both in the linear and quadratic components. Beginning at week one, Ta 04-22WP-treated plants gave off significantly less inoculum than Rootshield Plus-treated plants or non-treated plants (Fig. 6) according to tests of differences of Least Squares Means for plants at each sampling date ($P < 0.001$). From week three to week seven, both root drenches differed significantly from non-treated plants and each other ($P < 0.02$). When total inoculum given off over the course of the experiment was analyzed by GLM, treatment was significant at $P < 0.0001$, with most inoculum produced by nontreated diseased plants, significantly less by Rootshield Plus-treated plants and the least inoculum produced from Ta 04-22WP-treated plants. A GLM of root colonization showed the variable treatment significant at $P < 0.05$, with Ta 04-22WP-treated plants showing significantly less root colonization than non-treated plants (an average root colonization of 39.5% compared to 58.0%). Plants treated with Rootshield Plus were intermediate (50.2%). Inoculum levels in flow-through were high in this experiment: the lowest levels were seen at week two with 408 CFU/pot for Rootshield-treated pots and 206 CFU/pot for Ta 04-22WP-treated pots, compared to 1303 CFU/pot in untreated controls.

After one or two root drenches of infected plants with Ta 04-22WP or Rootshield Plus, regression analysis of flow-through over time for two trials, the variables day, day-day, day-treatment and day-day-treatment were all significant at $P < 0.0004$, indicating that treatments differed in their regression equations both in the linear and quadratic components. At time zero (just before drenches were applied) the amount of inoculum recovered from pot flow-through was low and did not differ significantly among the treatments. At week one and two, Ta 04-

22WP-treated plants gave off significantly less inoculum than Rootshield Plus-treated plants or non-treated plants (Fig. 7) according to tests of differences of Least Squares Means for plants at each sampling date ($P < 0.002$). From week three to week four, there was significant inoculum reduction for plants treated with Rootshield Plus ($P < 0.02-0.05$) and Ta 04-22WP ($P < 0.0001$). Just after the week four sample was taken, a second drench was applied to half the plants of each treatment group. This did not have an effect on inoculum production measured at weeks five through eight, when single- and double-dosed plants did not differ significantly. When total inoculum given off over the course of the experiment was analyzed by GLM, treatment was significant ($P < 0.0001$), with most inoculum produced by non-treated diseased plants, significantly less by Rootshield Plus-treated plants (with no significant difference between single- and double-dosed plants) and the least inoculum produced from Ta 04-22WP-treated plants (with no significant difference between single- and double-dosed plants). The lowest levels of inoculum in flow-through were seen in pots treated with Ta 04-22WP, with levels of 11.1-69.4 CFU for single-dosed pots, and 5.56-27.8 CFU/pot for double-dosed plants.

4. Discussion

In this study, we have shown that incorporating Ta 04-22 into the potting medium can protect *V. tinus* roots from becoming infected with *P. ramorum*. In addition, drenching the potting mix with Ta 04-22WP that contains *V. tinus*-infected roots reduces secondary production of *P. ramorum* sporangia. This is significant because it demonstrates for the first time that a biological control agent can be used to protect roots from *P. ramorum* and reduce spread of this pathogen.

In this study, we wanted to look at whether there was a difference in root infection and protection depending upon the *P. ramorum* propagule type. Chlamydospores would be the most persistent soilborne propagule produced in infected roots (Vercauteren et al., 2013). When these infected roots die or are left in the soil, these chlamydospores would be capable of infecting new roots. However, sporangia are also produced on the root surface and implicated in the spread from infected roots via runoff (Shishkoff, 2011). Results showed that propagule type was significant on root infection and the efficacy of Ta 04-22. This difference in the percentage of plants infected with *P. ramorum* between the inoculum propagule types is not surprising when considering the life cycle of *P. ramorum* and mechanism of biological control. Chlamydospores are resting structures that may not germinate immediately and asynchronously (Shaffer and Parke, 2013). Tooley et al. (2014) found after 8 days that only approximately 30% of the chlamydospores germinated at 25 °C, the approximate temperature maintained in the greenhouse for this study. This will give enough time for the *T. asperellum* to parasitize the propagule and biological control to start to be effective (Widmer, 2014). This would also lend to the preventative practice of applying the *T. asperellum* to the potting mix well before transplantation. Ta 04-22 populations are stable in potting mix and did not decline after 12 weeks at 20 °C (unpublished data). For *P. ramorum* sporangia, it has been observed that zoospores quickly release when suspended in water. After attraction to roots and encystment, germination will occur within hours (Ho and Zentmyer, 1977; Tippet et al., 1976; Widmer et al., 1998). This is too short of a time for the *T. asperellum* to parasitize the cysts before penetration of the roots. Better control might be achieved if the *T. asperellum* is mixed with some type of additive or other biological control agent that has an inhibitor mode of action. This has been observed in other systems (Hoitink and Boehm, 1999; Zhang et al., 2010).

Although final populations of *P. ramorum* in the potting mix after 4 weeks declined as the initial rate of *T. asperellum* increased (Fig. 2), the decline was not significant for chlamydospores ($P = 0.255$) or sporangia ($P = 0.206$) according to regression analyses. The lack of significance was due most likely to high variability among the trials. Although the direct soil plating technique used in this study is commonly used for evaluating populations of *Phytophthora* spp. in soil, it may not be the best technique for quantitative analysis (Eden et al., 2000). However, it does show a trend in population reduction. In addition, this data was recorded after 4 weeks, which was the length of the experiment. In a previous laboratory test, Widmer (2014) found that it took 7 to 8 weeks for *T. asperellum* isolate 04-22 to reduce *P. ramorum* levels to below detectable levels. The present study also involved a host plant in the potting medium, which could support higher levels of *P. ramorum* in the rhizosphere. *Phytophthora* spp. are considered to be poor competitive saprophytes and that temporarily removing vegetation is a potentially effective method of eradication (Dunstan et al., 2010). *Phytophthora cinnamomi* soil populations were much higher in soils with roots compared to adjacent bare soil (Marks et al., 1975). Graham (1995) showed that young citrus roots supported higher levels of *P. nicotianae* in the soil, but was affected by the tolerance of the rootstock. This highlights the importance of the results in this study that 04-22WP reduced secondary inoculum from roots, which reduces the threat of spreading this pathogen.

Despite that there was not a significant reduction in the percentage of plants with infected roots when inoculated with sporangia, the trend does show a reduction. This is most likely due to the effect that the secondary inoculum on infected roots is significantly reduced. Release of secondary inoculum of *P. ramorum*-infected roots was significantly reduced in potting medium amended with either one of the biological control agents compared to the control. The better

effect was observed when amended with 04-22WP. There was also a significant decline in the number of sporangia released when the potting medium was amended with the *T. asperellum*-colonized wheat bran demonstrating that the formulation was not a factor in suppression. A second application of the biological control agents after 4 weeks had no effect, although the assay used small rooted cuttings and more work would be required to find out the effect of single or multiple doses on larger plants. .

Results in this study did not show a growth stimulant to *V. tinus* roots when the potting mix was amended with Ta 04-22. Other *Trichoderma* spp. have been demonstrated to have growth stimulator effects (Doni et al., 2014; Harman et al., 2004; Hoyos-Carvajal et al., 2009; Ousley et al., 1994), especially under stress conditions (Azarmi et al., 2011; Bae et al., 2009). The plants in this study were not under abiotic stress and were only grown for 4 weeks. Hoyos-Carvajal et al. (2009) noted that growth stimulation in bean by *Trichoderma* spp. was strain specific and not characteristic for an individual species. Further investigations need to be done to study potentially longer term benefits and effects on other hosts. One of the proposed mechanisms for promoting plant growth by other *Trichoderma* spp. is the secretion of auxins and other secondary metabolites (Contreras-Cornejo et al., 2009; Vinale et al., 2012). Root growth promotion may be host specific and dependent upon interaction or production of plant-promoting auxins. Investigations have begun to study if Ta 04-22 secretes or has the genes to secrete any plant-promoting compounds.

Ta 04-22 applied as a wheat bran top-dressing reduced *P. ramorum* inoculum found in flow-through water from pots containing infected plants. Although plants were treated with *T. asperellum* 24 h after inoculation with the pathogen, there was a small but significant reduction in root infection by *P. ramorum* when measured at the end of the 8-week experiment. Possibly

this was due to the reduction in inoculum in potting media, which would have reduced the number of new infection sites on roots caused by sporangia diffusing through the potting media. Ta 04-22 formulated as a wettable powder reduced inoculum in flow-through water to a greater degree than Rootshield Plus, but a second drench of either product did not increase efficacy in this model system. This difference in efficacy between the two biological control agents is likely that the specific *T. asperellum* isolate in Ta 04-22 is a better biological control agent against *P. ramorum* than the active ingredient of RootShield Plus. However, both biological control agents were applied at the recommended rate and not varied so a dose effect cannot be ruled out. After 8 weeks, most of the rooted cuttings were badly rotted, and inoculum produced from positive controls was declining. If additional drenches were added to potted plants in a nursery setting, where the spread of disease and persistence of the biocontrol agent might follow different dynamics, it is possible a second treatment would be more effective. Probably more important than the effect of the drench on the treated pot itself is the effect on its neighbors if the reduced inoculum in flow-through water led to a reduction in plant-to-plant spread.

5. Conclusion

In the present study, we have demonstrated that *T. asperellum* isolate 04-22 does not only protect *V. tinus* roots from *P. ramorum* infection, but it can reduce secondary inoculum from already infected roots. RootShield Plus WP root drench was demonstrated also to reduce *P. ramorum* inoculum in flow-through water, though this effect was not as strong as with Ta 04-22WP root drench. This is the first time that a *Trichoderma* sp. has been demonstrated to protect roots from infection by *P. ramorum*. These observations are important as they give additional options for nursery growers to consider utilizing to reduce the spread of *P. ramorum* not only

within their own nursery settings but when transporting plant material to other geographical regions that may not have *P. ramorum* present.

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Figure 1. Regression analysis of the percentage of *Viburnum tinus* plants with *Phytophthora ramorum*-infected roots 4 weeks after inoculation with chlamydospores (●) or sporangia (▲) in *Trichoderma asperellum*-amended potting mix at different application rates. Error bars represent the standard error of the mean (95%).

Figure 2. Regression analysis of the population of *Phytophthora ramorum* in potting mix 4 weeks after inoculation with chlamydospores (●) or sporangia (▲) in *Trichoderma asperellum*-amended potting mix at different application rates. Error bars represent the standard error of the mean (95%).

Figure 3. Regression analysis of the mean percentage of *Viburnum tinus* root length per plant that is infected with *Phytophthora ramorum* 4 weeks after inoculation with chlamydospores (●) or sporangia (▲) in *Trichoderma asperellum*-amended potting mix at different application rates. Plants that had no infected roots were not included in the analysis. Error bars represent the standard error of the mean (95%).

Figure 4. Regression analysis of the mean total root length (cm) of *Viburnum tinus* plants 4 weeks after inoculation with *Phytophthora ramorum* chlamydospores (●) or sporangia (▲) in *Trichoderma asperellum*-amended potting mix at different application rates. Error bars represent the standard error of the mean (95%).

Figure 5. The amount of *Phytophthora ramorum* secondary inoculum found in flow-through water from pots containing root-inoculated plants of *Viburnum tinus* treated with a top-dressing

of wheat bran colonized by *Trichoderma asperellum* isolate 04-22 (Pram+Tasp) or as a top-dressing of wheat bran alone (Pram), shown as regression lines from logln-transformed data. Different letters at a sampling date indicate that inoculum differed significantly between treatments ($P < 0.05$) according to tests of differences of Least Squares Means for plants at each sampling date.

Figure 6. The amount of inoculum found in flow-through water from pots containing root-inoculated plants of *Viburnum tinus* treated with a drench of *Trichoderma asperellum* isolate 04-22 (“Pram +Tasp”) formulated as a wettable powder, drenched with Rootshield Plus (“Pram + Rsh”), or drenched with water (“Pram alone”), shown as regression lines from logln-transformed data. Different letters at a sampling date indicate that inoculum differed significantly between treatments according to tests of differences of Least Squares Means for plants at each sampling date.

Figure 7. The amount of inoculum found in flow-through water from pots containing root-inoculated plants of *Viburnum tinus* treated with a single or double drench of *Trichoderma asperellum* isolate 04-22 (“Pram +Tasp (single dose)” or “Pram +Tasp (double dose)”) formulated as a wettable powder, drenched with Rootshield Plus (“Pram + Rsh (single dose)” or “Pram + Rsh (double dose)”), or drenched with water (“Pram alone”), shown as regression lines from logln-transformed data. Arrow indicates time of second application of biological control treatment. Different letters at a sampling date indicate that inoculum differed significantly between treatments according to tests of differences of Least Squares Means for plants at each sampling date

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