ABSTRACT

RICHTER, BRANTLEE SPAKES. Cultural and Biological Control Methods for Phytophthora Root Rot in Fraser Fir. (Under the direction of D. Michael Benson and Kelly L. Ivors).

Phytophthora root rot of Fraser fir caused by *Phytophthora cinnamomi* and several other *Phytophthora* spp. is a severe problem in Christmas tree production. Since fungicides are ineffective in disease control and host resistance is not yet available, cultural control methods are under investigation as a means of reducing disease pressure on infested production sites. Mulching systems with raised beds of pine bark, wood chips, or wood chips blended with compost were tested, along with compost or sulfur as soil amendments, at five sites spanning the western North Carolina growing region. Microbial populations and activity in soils and mulches were characterized over a two year period, using dilution plating with calculation of a log series diversity index for counts of bacteria, fungi, and cellulose-degrading micoorganisms, analysis of fluorescein diacetate hydrolysis for estimation of total microbial activity, and quantification of reducing sugars after incubation with carboxymethyl-cellulose for estimation of total cellulase enzyme activity. Bacterial and fungal counts, microbial activity, and cellulase activity were higher in mulch than in soil at all sites and times ($P<0.01$), and generally did not differ among mulch types nor among soils. Treatments significantly affected disease ratings and tree survival at three of five sites, with one or more mulch treatments yielding lower disease ratings and greater survival than controls. Tree mortality at each time point varied significantly with cellulase activity in the upper portion of the root zone ($P=0.005$). Other biological variables did not show significant relationship with disease ratings or mortality.

To further investigate the role of cellulase enzymes in suppression of *P. cinnamomi*, a commercial formulation of cellulase was used to generate a standard curve which could be used to correlate cellulase activity levels in field samples with the enzyme unit concentrations commonly used in laboratory studies. Two isolates of *P. cinnamomi* were exposed to a range of enzyme concentrations, and data were collected on biomass and sporangia production. Cellulase exposure reduced sporangia production but did not reduce biomass within the range analogous to that observed in field-applied mulch. In a bioassay

with lupine, cellulase applied to soil containing infested root fragments did not reduce disease progress.

Container trials were also used to assess the impacts of a wide range of organic and inorganic amendments on Phytophthora root rot in Fraser fir seedlings, and to examine the contributions of compost, microbial inoculants, and isolates of cellulytic fungi to disease suppression in wood chip mulches. In trials with wood chips blended into soil and trials with seedlings planted directly into wood chip mulch, seedling survival was greater when wood chips were amended with compost, a soil inoculant, or a biocontrol agent. If wood chips were amended with compost, the addition of a cellulytic fungus, including a known biocontrol agent, did not further enhance plant survival, and in most cases did not significantly increase cellulase activity over compost amended wood chips alone.

Cultural and Biological Control Methods for Phytophthora Root Rot in Fraser Fir

by
Brantlee Spakes Richter

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APPROVED BY:

_____	_____
Dr. D. Michael Benson Committee Co-Chair	Dr. Kelly L. Ivors Committee Co-Chair
_____	_____
Dr. H. David Shew	Dr. Wei Shi

DEDICATION

To my pack.

BIOGRAPHY

Brantlee Spakes Richter was born in Columbia, SC, and educated in the public school systems of Madison, WI, and Greensboro, NC. She attended The University of North Carolina at Greensboro, and then transferred to Arizona State University, where she received B.A. degrees in English and Women's Studies in 1993. After working and continuing coursework in Asheville, NC, and Lansing, MI, she returned to Arizona State in 1996 to pursue a Masters of Science in Botany/ Plant Biology, which she received in 1999. She worked for six years at a private environmental microbiology laboratory as a plant pathologist and laboratory manager prior to commencing her doctoral program at North Carolina State University.

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INTRODUCTION

Phytophthora Root Rot on Fraser fir

The fresh-cut Christmas tree market in the United States is a multi-million dollar industry, of which Fraser fir (*Abies fraseri* (Pursh.) Poir.) accounted for $67.9 million in annual sales by 1998 (56). Though annual sales have increased, more recent sales statistics are not available for individual species, as the current census figures combine sales of cut Christmas trees with other short-rotation woody crops. North Carolina ranks second in the nation in number of Christmas trees produced, but first in dollar value for this crop, largely due to the production of Fraser fir as the primary crop species (44, 57). Christmas tree production is an important part of North Carolina's economy, accounting for $125 million in cash receipts in 2007, ranking as the state's seventh largest crop (43). Production is centered in the mountain region, where the median household income in the top three Christmas tree producing counties averages 22% below the state-wide median (55). Christmas trees represent an economic boon in this area, where geography and climate limit crop choices. About 60% of North Carolina growers are small farmers, with fewer than 10 acres of trees, and there are over 1200 Christmas tree growers in the state, as compared with about 390 growers of sweet potatoes, the sixth largest crop (57). Income generated from the production of cut trees and peripherals (wreaths, ropes, and other greenery) is thus distributed across a comparatively large population segment, relative to that of other crops, creating an opportunity for profitable enterprise among a large number of family farms.

The native range of Fraser fir is limited to the southern Appalachian Mountains at elevations above 1372 m, spanning parts of Virginia, North Carolina, and Tennessee. Natural stands are found on shallow, rocky, acidic soils with a pH range of 3.5-4.2 (6). In North Carolina, commercial Fraser fir plantations are generally established at elevations below 1200 m, on soils with lower organic matter and higher pH. These sites are more accessible, have greater soil nutrient availability, and avoid problems associated with exposure, such as lodging, hail damage, and slow growth. Trees are usually 12-15 years old at harvest, with production occurring in three stages: 2 years in seedbeds, 1-3 years in line-

out beds, and 6-9 years in the field. Increasingly, greenhouse produced seedlings may be used rather seedbed grown seedlings at the lineout stage of production. The greatest threat to native populations of Fraser fir has been insect damage from the balsam woody adelgid, *Adelges piceae* (6). In production systems, insecticides are used successfully to keep insect infestations under control, and the greatest limitation to production is root rot caused by *Phytophthora* spp. Phytophthora root rot is a problem in every stage of production, and can kill trees of all ages. In field plantings, this disease is associated with poorly drained areas (24), and once the pathogen becomes established in a conducive site, the site is rendered unfit for future production. A 1997-1998 disease survey found an average of 9% disease incidence across 58 sites in western North Carolina, with a range of 0-75% (8). While disease incidence numbers within production sites have remained relatively stable over a 25-year span (8, 24), these numbers do not reflect the loss of productivity due to abandonment of sites infested with *Phytophthora*. In North Carolina Christmas tree pest management surveys, 72% of growers reported field mortality due to Phytophthora root rot in 2006, up from 60% in 2001 (49, 51). In an informal follow-up survey, fewer than half of growers indicated that they intended to continue trying to grow Fraser fir in areas where they had experienced losses (50).

Phytophthora cinnamomi was first identified as the causal agent of root rot in Fraser fir in 1963 (35), and remains the primary species associated with the disease, though *P. drechsleri*, *P. citricola*, and *P. cactorum* have also been confirmed as causal agents of root or crown rots on Fraser fir (1, 9, 48). *P. medicaginis*, *P. europea*, *P. gonapodyides*, and *P. nicotianae* have been found associated with diseased roots on *A. fraseri* in Michigan, but have not yet been examined for pathogenicity (22). In a 2000 study of North Carolina production sites, *P. cinnamomi* accounted for nearly all isolates of *Phytophthora* recovered from Fraser fir in the field, representing 91% of the isolates recovered across both field samples and nursery transplant beds, with *P. cactorum* and *P. drechsleri* also occasionally isolated (8). *Phytophthora cinnamomi* survives in nursery and field sites as chlamydospores and as vegetative hyphae in infected root fragments, and has been shown to survive in soil for at least 6 years (30, 58, 62). Some saprophytic potential has been observed with *P.*

cinnamomi, as well; chlamydospores, and to a lesser extent zoospores and encysted zoospores, have been found capable of colonizing dead host tissue buried in soil (30). Because only the A2 mating type has been found on Fraser fir in North Carolina, oospores are not a factor in survival or infection by *P. cinnamomi* in this system, although they may play a role in other species capable of causing Phytophthora root rot. Spread of the pathogen within a site is primarily through the production of motile zoospores, produced within sporangia either on the surface of infected roots or on germinating chlamydospores. Under favorable conditions, zoospores may form within 2 or 3 days after root infection, allowing for rapid build-up of propagules within an infested area (26). As *P. cinnamomi* is an introduced species, infestation of nursery beds during seedling production represents a major potential contributor to incidence in the field (8), and control strategies are needed to address both nursery and field incidence of the disease.

Current available controls include use of chemicals and avoidance of planting sites which are most conducive to disease development. While symptomatic transplant materials are avoided, this has not proven to be a highly effective preventative measure, as 1.2% of asymptomatic nursery plants have also been shown to harbor *P. cinnamomi* (8). Although this percentage appears small, it can translate to approximately 50 infected trees per hectare in a standard 5 ft by 5 ft spacing (1.52 m by 1.52 m). When infected trees are planted within an area that experiences surface flow during rain events, other trees down-slope of introduction points can readily become infected. Once the pathogen becomes established on a site, it can persist in the soil for long periods of time and the site can no longer be used for Fraser fir production. Therefore, each introduction of the pathogen has the potential to limit future production potential of a particular site. Metalaxyl is frequently used to control Phytophthora root rot in nursery production, with some use of fosetyl-aluminum as well. However, chemical controls are generally prohibitively expensive for field use and do not completely eliminate the pathogen. At present, their use in the field is relegated to containment of an infestation to allow for immanent harvest of surrounding trees. Disease resistance is rarely observed in Fraser fir and is not currently available as a control option, but resistance has been identified among other fir species, and grafting of Fraser fir onto root

stocks of more resistant species may be a viable component of future disease control strategies (21, 28, 29).

Biological and Cultural Controls

Use of organic soil amendments, such as compost, pine bark or wood chips, has proven beneficial in reducing *Phytophthora* disease incidence in greenhouse (3, 34, 52, 53) and field (15, 37, 47) production systems of other crops. However, attempts to use compost or other organic amendments for control of soilborne pathogens have yielded variable results, and modes of action have generally not been determined in cases for which disease suppression has been achieved. One of the best characterized amendment-based systems for control of *P. cinnamomi* is used in avocado production and is based on the "Ashburner system," named for Australian avocado grower, Guy Ashburner. The Ashburner system, as adapted for avocado production in the US, uses wood chip or yard-trimming mulch as an organic matter source and gypsum as a calcium source. The gypsum-amended mulch is mounded around the base of individual trees, providing a suppressive zone in which feeder roots proliferate. Suppression of *P. cinnamomi* in mulches and in naturally suppressive soils has consistently been attributed to microbial activity (13, 39, 45, 59). Initially, observations of increased numbers of bacteria and actinobacteria in suppressive soils, relative to disease-conducive soils, and the association of these organisms with lysed *Phytophthora* hyphae led to investigations of bacterial species as agents of disease suppression (12, 38). Numerous isolates of actinobacteria (19, 32, 42, 60), and fungi (14, 19, 23, 27, 38, 42, 46) have since been identified as having antagonistic ability against *P. cinnamomi*. Modes of action for suppression of *Phytophthora* by mulch-inhabiting organisms may include antibiotic production, enzyme production, competition, induced resistance, and hyperparasitism. While these mechanisms have been widely demonstrated on the level of individual strains under investigation for biological control, little work has been done to elucidate the relative roles of these various mechanisms in mulch systems. Most recent investigations into the mechanisms of suppression in the Ashburner system have focused on cellulase production by wood-decay

fungi associated with the mulch, and on effects of calcium on soil physical properties and pathogen biology as the primary sources of inhibition (17, 18, 20, 40).

Phytophthora cell walls are composed primarily (80-90%, dry weight) of β-linked glucose polymers, with an assortment of proteins, lipids, and other polysaccharides comprising the remainder of the wall mass (5). The β-glucan component is further divided into cellulosic (primarily β-1,4-linked) and non-cellulosic (primarily β-1,3- and β1,6-linked) glucans. Cellulose content is higher in sporangial and cyst walls than in hyphal walls (4, 5, 54). Cellulytic enzymes produced by cellulose-degrading fungi have been shown to degrade *Phytophthora* cell walls (18, 19, 31), and *Phytophthora* suppression in avocado mulch systems has been associated with cellulytic enzyme activity (17). Numerous fungi that are inhibitory to *Phytophthora*, including *Myrothecium roridum* (23), *Penicillium janthinellum* (46), *Chaetomium globosum*, *Gliocladium virens*, *Trichoderma viride* (27), and *Micromonospora carbonacea* (19), are also known cellulase producers (2, 19, 31, 33, 36). Cellulase produced by *Penicillium funiculosum* has been shown to reduce production of sporangia, zoospores, and chlamydospores by *P. cinnamomi* at concentrations of 10 units/ml or greater in soil extract (18). In a field study, cellulase enzymes were found to be most abundant in middle and upper mulch layers, with levels in underlying soil not varying significantly from those measured in unmulched soil (17). The same study showed no recovery of *Phytophthora* propagules and no root infection within mulch layers, and reduced levels at the soil-mulch interface, relative to those within soil at 7.5 and 15 cm depth. *Phytophthora* inoculum potential and root infection were similar between mulched and unmulched treatments at soil depths of 0-15 cm, indicating that the benefits of mulch do not extend into the underlying soil. When *Phytophthora* mycelia were incubated within mulch and soil layers, lysis of hyphae was greater in mulched than in unmulched soils, and was greatest at the mulch-soil interface (17). When grown in mulched systems, avocado produces abundant roots within the mulch layers, such that even though roots within the soil become infected with *Phytophthora*, the tree remains productive (16, 17). Because Fraser fir has a very shallow root system (6), it would also be likely to produce plentiful roots within a

surface mulch layer and may be a good candidate for translation of the Ashburner system for Christmas tree production systems.

In addition to the use of high C:N organic mulches, the other factor within the Ashburner system which has been utilized in avocado production is the addition of calcium, typically as gypsum. Calcium amendments may have two potential effects which would lead to reduction of Phytophthora root rot disease: improvement of water infiltration into soil, and interference with pathogen physiology. As a soil conditioner, gypsum ($CaSO_4 \cdot 2H_2O$) can prevent surface crust formation by releasing electrolytes which prevent clay particles from dispersing under rain impact (11). Addition of gypsum has been shown to increase infiltration rates by as much as 100% under simulated rainfall conditions (61). Improved drainage alone could decrease disease incidence at a site by reducing the duration of saturation within the root zone of infected plants, and thus reducing the potential for formation, release, and movement of zoosporangia. Recent research, however, suggests a greater role of calcium in its direct impact on *P. cinnamomi* physiology. In pot studies with avocado, soil amendment with 10% fine or coarse gypsum did not significantly increase drainage, but gypsum added at 1%, 5%, or 10% did decrease root infection by *P. cinnamomi*, suggesting that the primary mode of action in this system was not related to soil infiltration rates (41). In further pot studies with the same soil, it was shown that gypsum amendment decreased sporangial production, sporangial volume, zoospore production, and colony-forming units (40). The reduction in zoospore production was also observed when soil was amended with calcium carbonate ($CaCO_3$) or calcium nitrate ($CA(NO_3)_2$) (40). In *in vitro* studies, calcium has been shown to promote *P. cinnamomi* sporangia production at concentrations below 0.71m*M*, but to inhibit production at increasing concentrations above this optimum (25).

While the original Ashburner system included the use of lime to maintain soil pH above 6.0, some research suggests that lower pH levels may be more beneficial in inhibiting soilborne Phytophthora diseases. Optimum pH for sporangia formation and zoospore release by *P. cinnamomi* in potting medium is around 5.0-6.0 , with minimal sporangia production below pH 4.0 (7, 10, 53). Lowering growth medium pH has been shown to prevent

development of Phytophthora root rot in rhododendron, though *P. cinnamomi* propagules remained present and viable within the medium (10). In studies with pine bark-amended potting soil, artificially raising pH from 4.5 to 6.5 negated the disease-suppressive effects of the pine bark under saturated conditions but not at a lower moisture regime (53). As Fraser fir is well adapted to low pH soils, maintenance of low soil pH may be a practicable component of cultural practices to reduce Phytophthora root rot in this species.

Research Objectives

Phytophthora cinnamomi has been the focus of much research, and its basic biology and life cycle are well documented; yet, we still have great difficulty predicting its behavior within the environment, or curtailing its impacts on its various host species. Understanding of the factors involved in biological suppression of *Phytophthora* species remains rudimentary, based on fragments of knowledge crystallized around *in vitro* observations, and on broad associations with field-scale variables. Many more fragments and associations will need to be generated and linked before we are able to use biological suppression effectively and consistently in plant production systems.

The aims of this research were two-fold. The first was to apply the body of knowledge generated around the Ashburner system and its successors towards developing a viable production system for Fraser fir in sites where *Phytophthora* has been introduced, and where conditions are conducive for disease development. Field studies were conducted to assess the potential value of mulching systems and soil pH adjustment for suppression of Phytophthora root rot in Fraser fir grown on infested sites. These studies will provide the basis for development of effective cultural controls, and also contribute to the reservoir of field-scale observations on the biological and environmental variables associated with disease progression or suppression.

The second aim of this research was to contribute to our understanding of the mechanisms of suppression at work in mulch-based cultural systems. Laboratory and container trials were conducted to further our understanding of the role of cellulase enzyme activity as an agent of pathogen suppression, and to link the knowledge generated from *in*

vitro assays with the observations of cellulase activity and pathogen suppression in the field. It is anticipated that better understanding of the contributions of individual mechanisms to the overall phenomenon of pathogen suppression will enable us to more effectively utilize these ecological interactions in development of biocontrol techniques.

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CHAPTER 1

Cultural systems for suppression of Phytophthora root rot in Fraser fir: Microbial profiling and impacts on disease progression

ABSTRACT

Phytophthora root rot of Fraser fir, caused by several *Phytophthora* spp., is a severe problem in Christmas tree production. Since fungicides are not economically viable for disease control in field plantings and host resistance is not yet available, cultural control methods were investigated. Mulches, compost, and pH adjustment were tested on field planted trees at five sites. Treatments included wood chips, wood chips plus compost, or pine bark as raised beds, and compost or sulfur tilled into soil. Soil and mulch microbial populations were characterized by dilution plating and calculation of a log series diversity index, and by enzyme analyses at 5, 12, 17, and 24 months after planting. Bacterial and fungal counts, microbial activity, and cellulase activity were higher in mulch than in soil at all sites and times ($P<0.01$), and generally did not differ among mulch types nor among soils. Treatments significantly affected disease ratings and tree survival at three of five sites, with one or more mulch treatments yielding lower disease ratings and greater survival than controls. Tree mortality at each time point varied significantly with cellulase activity in the upper portion of the root zone ($P=0.005$). Other biological variables did not show a significant relationships with disease ratings or mortality.

INTRODUCTION

Phytophthora root rot, caused by *P. cinnamomi* and other species, is the primary limiting disease in production of Fraser fir (*Abies fraseri*) for the fresh cut Christmas tree market in North Carolina. Phytophthora root rot is a problem in every stage of production, and can kill trees of all ages. *P. cinnamomi* is frequently introduced into fields on infested transplants; one survey recovered the pathogen from 1.2% of asymptomatic transplants sampled from nursery transplant beds (6). In field plantings, disease development is associated with poorly drained areas (17), and once the pathogen becomes established in a

conducive site, the site is rendered unfit for future production. In a 2007 North Carolina Christmas Tree Pest Management Survey, 72% of growers reported field mortality due to Phytophthora root rot, up from 60% in a 2001 survey (32). While average disease incidence in individual production sites remained relatively stable at 9-10% from 1974 to 2000 (6, 17), these numbers do not reflect the loss of productivity due to abandonment of sites infested with *Phytophthora*.

Mefenoxam and fosetyl-AL are registered for use in Fraser fir plantations; however, these fungistatic formulations are useful only to contain outbreaks and slow the spread of the disease, allowing for immanent harvest of surrounding trees. Long-term use of fungicides is not economically viable for this perennial crop, which is typically planted in a seven to nine year cycle, and is not effective enough to allow continued productivity of infested land. While some disease resistance has been identified among other fir species, it is rarely observed in Fraser fir, and is not currently available as a control option (16, 19, 20). Although Phytophthora root rot has been sited as the number one pest problem by over 35% of growers surveyed, fewer than 2% reported using either of these fungicides (32). Affected areas are often left fallow, or planted in more resistant but less profitable species. Alternative methods are needed to allow continued production of Fraser fir, which has the highest customer demand among fresh-cut Christmas tree species (39).

Current cultural control recommendations center around avoidance strategies such as selection of transplant stock and nursery irrigation source, placement of seed and line-out beds to minimize periods of high soil moisture, and avoidance of high-risk field sites, characterized by their propensity to experience standing or running water during rainfall events. There are currently no cultural recommendations available for managing a site after *Phytophthora* has been introduced. This research was conducted to test mulch-based cultural systems for Fraser fir production under Phytophthora root rot disease pressure. The approach was based on the "Ashburner system" developed for avocado production in Australia and California. This system utilizes woody mulches in combination with animal manures and/or fertilizers to create a pathogen inhibitory zone in which feeder roots may develop. Disease reduction has been associated with the presence of inhibitory biota and enzyme activity

within the mulch (8, 13, 40). Although roots which extend into underlying soil are vulnerable to infection, roots produced within the mulch avoid infection and allow the trees to remain productive. Use of this system, in combination with development of resistant root stocks, has allowed continued production of avocados on *Phytophthora* infested land, and it shows potential to play a similar role in Fraser fir production systems.

In addition to mulch-based cultural options, soil pH management may be an option for reducing Phytophthora root rot disease pressure. Optimal pH for development of Phytophthora root rot on avocado was identified at approximately pH 6.5, with less disease at both low (3.5-5) and high (8+) pH (42). In a survey of Hawaiian soils, Ko and Shiroma (1989) found that suppression of chlamydospore germination in *P. cinnamomi* occurred more frequently in soils with pH near 4 or 8 than in soils with pH near 6, and inhibition of sporangium formation has also been observed in peat-based mix and pine bark at pH <4 (5, 7). Reduction of soil pH has shown mixed results as a control for Phytophthora root rot, with successful disease reduction in field-planted pineapple (28) and container-grown rhododendron (7), but unsuccessful results in nursery and field-grown avocado (42). While low pH may directly inhibit *P. cinnamomi* in soil, it may also disrupt potentially suppressive microbial communities. Fraser fir is native to high elevation mountain soils with pH 3.5-4.2, but production sites are typically limed to maintain pH at or above 5.5 (4). Because Fraser fir is naturally adapted to low pH, reduction of soil pH to a range inhibitory to *P. cinnamomi* may also be a viable cultural control strategy.

This study examined three mulch types and two soil treatments in field plantations of Fraser fir for their effects on Phytophthora root rot development, and for the development of an enhanced microbial community, which has in the past been associated with successful mulching systems. Objectives were to (i) evaluate the efficacy of mulches, compost, and pH adjustment for reducing Phytophthora root rot disease in the field, (ii) track changes in microbial numbers, activity, and cellulase production over time in mulches and treated soils, and (iii) examine the relationships between disease inhibition and biological factors across multiple field sites.

MATERIALS AND METHODS

Field Study. Field trials were initiated in the spring of 2006 at five sites located within established Fraser fir plantations in Mitchell, Avery, and Watauga counties in North Carolina, and Grayson County, Virginia (Figure 1). All sites had a history of Phytophthora root rot within the study areas. Soil samples were collected from each site prior to study establishment and analyzed for basic soil chemistry (Humic matter, CEC, base saturation, pH, P, K, Ca, Mg, Mn, Al, Zn, Cu, and S) by the NC Department of Agriculture Agronomic Services lab (Table 1). Rainfall data was collected for each site throughout the duration of the study, using a tipping bucket collector with a data logger.

Field treatments included wood chip mulch (WC), compost-amended wood chip mulch (WCC), or pine bark (PB) applied on top of existing soil in rows as "raised beds," and compost or soil sulfur incorporated into the soil in rows by roto-tiller. Each site contained four treatments plus one unamended control, arranged in a randomized complete block design. The compost, wood chip, and compost-amended wood chip treatments were applied at all sites; the remaining treatments were divided to include pine bark beds at three sites and sulfur incorporation at two sites. Each treatment consisted of five replicate plots of 16 trees (four by four) per plot, for a total of 80 trees per treatment and 400 trees per site, with the exception of one site, Avery-2, which contained only four replicates due to space constrictions. Trees were planted on a standard 5 ft by 5 ft (152 cm) grid with an unplanted buffer strip in between treatment blocks. All mulch materials were fresh-milled and delivered to each site within five weeks before planting. Wood chips for all sites were white pine, chipped with bark attached. Two suppliers were used to minimize transport distances. Chips used at the Mitchell and Avery county sites were mixed length (most chips less than 10 cm) and approximately 5-10 mm thick. Chips used at the Watauga and Grayson county sites were a mixture of mixed-length chips and fine shavings. Pine Bark (Avery-1, Mitchell, and Watauga sites) was a shredded product, rather than chipped, consisting primarily of longer bark strips (10 cm or greater) with minimal wood attached. Mulch beds were constructed by applying a layer of material approximately 30 cm deep to a 58 square meter plot (7.62m x

7.62m), and then excavating 30 cm wide aisles to create four bed rows aligned parallel to the predominant slope direction. For the WCC treatments, bagged composted dairy manure (Daddy Pete's, Statesville, NC) was blended in to wood chip beds on site with a roto-tiller at a rate of 15% compost, by volume. An equivalent volume of compost was tilled into soil for the compost-amended soil treatment. Sulfur was incorporated into soil at a rate of 1.09 kg per row of four trees; this rate was calculated to reduce soil pH within the root zone to a target of approximately pH 3.5-4. Plots were maintained by individual growers, per their standard growing practices, to include fertilization, weed control, and tree trimming. Glyphosate was used for weed control, with some additional mowing and hand-weeding performed during plot assessments. At twelve months after planting, one additional fertilizer application (18-46-0 diammonium phosphate, 1.7 kg per plot) was made at all sites using a broadcast spreader.

Disease assessments were conducted at the beginning and end of each growing season (May and September) through the first two years at all sites. Disease severity was rated on a four point scale, on which "1" was apparently healthy, "2" was showing significant chlorosis, "3" was showing wilting, flagging, severe chlorosis, and/or branch necrosis, and "4" was dead. Survival within each plot (16 plants) was calculated from ratings data by assigning a status of "1" to ratings 1-3, and a "0" to plants with a rating of 4, and then calculating mean survival status. Bed height measurements were also conducted on all sample dates to monitor settling rates and mulch loss.

Soil and mulch samples for biological analysis and pH monitoring were collected at three of the five sites: Avery-1, Watauga, and Grayson. Plots were sampled at three levels: upper mulch (top half of the mulch profile), lower mulch (bottom half of the mulch profile), and soil (0-15 cm depth). Samples were collected 30 cm from the base of each of the four interior trees in a plot, and the four samples at each level were combined for analysis. The sample point for each tree was rotated by compass direction for each sampling date. During the second season, plant tissue samples were also collected from each plot and analyzed in order to determine effects of treatments on plant nutrient status. At the end of the third growing season, a small subset of plants at the Avery-1 site was selected for sacrificial

sampling, to include two living and two dead plants from each treatment. All roots were removed and sorted by layer, then quantified by dry weight, and by image analysis using APS Assess software (Version 2.0, APS Press, St. Paul, MN). For each sample (plant x layer), 50 1-cm segments were plated on PARPH-V8 agar (15) for detection of *Phytophthora*, and the proportion of positive segments was calculated. If the sample consisted of less than 50 cm of total root length, the entire sample was divided into 1-cm segments and plated.

Biological Analyses. Soils and amendments collected at each sample date were processed for enumeration and diversity of culturable bacteria, fungi, and cellulose-degrading microorganisms, and for total microbial activity and cellulase enzyme activity. Analysis of culturable organisms was performed by dilution plating on R2A (Difco) for bacteria, modified rose bengal for fungi (33), and cellulose medium for cellulose degraders (3). Three subsamples were drawn from each bulked sample, and each was diluted in log-series and plated in a 3-log range for each media type. After counts were obtained, fifty colonies were selected from a countable plate for each sub-sample, and were used to obtain sample richness based on colony morphology. Diversity was then calculated using a log series index (18, 26). Where 50 distinct colonies were not available, smaller sets were used, but because the log series index is a poor predictor of diversity with very small sample size (18), samples with fewer than 30 colonies were omitted from analysis. Results from three subsamples were averaged for each plot at each layer.

Samples were analyzed for total microbial activity by fluorescein diacetate hydrolysis (1). Samples were incubated with 30.2 µg/ml fluorescein at 30ºC for 10 minutes, shaken briefly, and incubated an additional 10 minutes. After the 20 minute incubation, samples were shaken, and a 5-ml aliquot was transferred to 5 ml of 2:1 chloroform/methanol solution to stop the reaction. Samples were centrifuged, the aqueous fractions were filtered into spectrophotometer tubes, and absorbance was read at 490 nm. Four sub-samples and one substrate negative control were processed for each sample point at each layer.

Cellulase activity was assessed by a modified Schinner and von Mersi method (31, 36). Samples were incubated at 37ºC for 24 hours in 0.35% w/v carboxymethyl cellulose in

acetate buffer. After incubation, aliquots were transferred to microcentrifuge tubes and centrifuged for 3 minutes. Supernatants were diluted and processed for photometric analysis of reducing sugars. Three sub-samples and one substrate negative control were processed for each sample point at each layer.

Soil baiting and pathogen quantification by sieving and plating on selective medium were conducted during the first season (15, 21, 30), but detection levels were insufficient for resolution of pathogen populations, and these analyses were discontinued.

Statistical Analyses. SAS® software (Version 9.1, SAS Institute Inc., Cary, NC, USA) was used for all statistical analyses. To discern treatment and time effects for all biological data sets, repeated measures analysis of variance was conducted using a linear mixed model, with the SLICE option used to slice data sets by time and by treatment and Tukey's adjustment applied for least squares means separations. For determination of the covariance structure for each data set, analysis was repeated with each of eleven different models for covariance structure, and the structure which resulted in the lowest value for the Akaike Information Criterion (AIC) was selected. Mixed model repeated measures analysis was also used with biological variables as covariates to determine relationships between biological parameters and disease progress, expressed as change in survival for each block at each time period. For this analysis, data from all sites were used, with site as a random variable, and only the four treatments common to all sites were included. Correlation analysis was also used to detect correlations among biological data. Area under the disease progress curve (AUDPC) values were calculated for both ratings and survival data, and these were compared by analysis of variance using the general linear model (GLM) procedure in SAS. Ratings and survival at the conclusion of the study period (24 months) were also examined using Friedman's non-parametric analysis; means comparisons were performed using an F-approximation to Friedman's, by ranking data within blocks and using the GLM on ranked data with Tukey's HSD test. The GLM was used for analysis of variance on nutrient data, and the Kruskal-Wallis nonparametric analysis for root infection data. Data transformations were applied where deviations from normality could be reduced. An alpha level of α=0.05 was used for all tests.

RESULTS

Biological Data. All of the biological analyses conducted (plate counts, FDA, and cellulase activity) were positively and significantly correlated to one another, and to sample moisture content (Table 2). FDA and cellulase activity were more strongly correlated with one another ($r = 0.545$) than with colony forming units detected on any media type. Enumerations on cellulose agar showed the weakest correlation with all other variables with the exception of bacterial enumeration, with which it correlated well ($r = 0.469$).

Microbial Enumerations and Diversity. Bacterial and fungal counts were significantly higher in mulch than in soil at all sites and sample times ($P<0.01$, Figure 2). Cellulose degrader counts were more variable, with no significant difference between soil and mulch at the Grayson site, significantly higher counts in mulch at a single time point at the Watauga site, and at three of four sample dates at the Avery-1 site. Colony diversity was higher from soil than mulch for cultured fungi and bacteria at all sites ($P<0.01$ at each site), and for cellulose-degraders at two of three sites (Avery-1 and Grayson, $P<0.01$, $P=0.554$ at Watauga site). Among soils, bacterial and fungal counts varied by treatment and over time at all sites. Bacteria in soil tended to show seasonal fluctuations, with higher counts at the spring sample dates. Though not always statistically significant, sulfur-treated soil (Grayson site) consistently gave higher fungal counts and lower fungal and bacterial diversity than other soils. Cellulose-degrader counts in soils varied significantly by treatment only at one site, at two of four sample times (Grayson at 12 and 24 months, $P= 0.002$ at both times), but counts varied over time at all sites ($P<0.01$).

Among the three mulch types, bacterial counts differed significantly at all sites through the first year ($P<0.05$ at each of three sites and two sample dates), with highest counts from WCC mulch. Differences were less consistent during the second year, with significant differences at both time points at one site (Grayson), neither time point at one site (Avery-1), and only the spring time point at one site (Watauga). Bacterial counts within mulch differed significantly over time in all mulch types at all sites, with the single exception of WCC mulch at the Watauga site, but patterns over time were less consistent in mulch than

in soil. Fungal counts in mulches differed by mulch type at only one site (Avery-1), where lower counts were observed in PB than the other two mulches at all time points. Fungal counts changed significantly over time only in the WC mulch at two of three sites (Avery-1 and Grayson, P= 0.005, 0.001). Cellulose-degrader counts differed by mulch type at all sites; one of these sites (Grayson) showed differences at every time point, and the other two sites at only two of four sample dates, each. Among the mulch types, WCC generally yielded the highest cellulose-degrader counts across sites and over time. Counts changed over time only at the Avery-1 site in the PB and WC mulches. Differences in bacterial colony diversity were observed among mulch types at the Grayson site at three of four time points, but only at one (Avery-1) or two (Watauga) time points at the other sites. Where these differences occurred, WCC mulch had higher diversity than one or both of the other mulch types. Fungal colony diversity did not differ among mulch types, with the exception of two time points at a single site (Avery-1), where PB showed higher diversity than WC and WCC mulches. Cellulose-degrader diversity generally did not differ among mulch types.

Bacterial counts differed between upper and lower mulch layers at two of three sites, and fungal and cellulose-degrader counts at one site each. These differences did not appear to be related to season or rainfall, but complete analysis of weather factors was not conducted.

Total Microbial Activity. Microbial activity (fluorescein diacetate hydrolysis) was significantly higher in mulch than in soil at all three sites and all four sampling dates ($P<0.001$, Figure 3). Comparisons among soils differed across sites. At the Avery-1 site, soil activity did not vary significantly by treatment or over time. At the Watauga site, microbial activity varied by treatment at both of the spring samplings (12 and 24 months, P= 0.003, 0.043), but not at the fall sampling times (5 and 17 months), and activity in all treatments varied over time ($P<0.001$). At the Grayson site, soil activity varied by treatment through the first, but not the second, year (P= 0.045, 0.010 at 5 and 12 months), and activity varied over time in soil under mulch (P= 0.011, 0.002 under WC, WCC), but not in the control, sulfur or compost-treated soil.

Activity was generally similar among mulch types, with significant differences observed only during the second year at two of three sites (Avery-1 and Watauga, P= 0.002 and P= 0.011 at 17 months, P= 0.054 and P< 0.003 at 24 months), and with no mulch type consistently yielding higher activity across sites. Activity within mulches did vary significantly over time with the single exception of WCC mulch at the Avery-1 site (P<0.05 for all mulch types at all sites, except P= 0.226 for WCC at Avery-1), generally increasing over the two year study period. Activity was different between upper and lower mulch layers only at the 12-month time point, at two sites.

Cellulase Enzyme Activity. Cellulase activity was significantly higher in mulch than soil at all sites and times (P<0.001, Figure 4). Among soils, activity differed among treatments on only one or two sample dates at each site; where significant differences occurred, compost-amended soil consistently showed the highest activity. Activity did not change significantly over time in soils, with the exception of soil under WC mulch at two sites.

Cellulase activity did not differ among mulch types at any site or date. Differences in activity within mulch over time were discernable only in the WCC mulch at the Avery-1 site (P= 0.033). Cellulase activity also did not generally differ between upper and lower mulch layers; significant differences were observed between layers at only one or two sample dates at each site (17 months at Avery-1 and Grayson, P= 0.041, 0.043; 5 and 17 months at Watauga, P= 0.047, 0.052). While activity did not change significantly over time within lower mulch, it did vary significantly with time in upper mulch (P<0.01 at all sites).

Plant Disease Data. Comparison of AUDPC (Figure 5, values listed in Table 3) using the general linear model showed significant treatment effects at only one site each for mortality (Avery-2, P= 0.053) and disease ratings (Watauga, P= 0.036). Analysis of final disease ratings and mortality, using Friedman's nonparametric analysis, yielded clearer separation among treatments, with significant treatment effects (P<0.001) at three sites (Mitchell, Avery-2, and Watauga) for both mortality and disease ratings. At the Mitchell site, WC and WCC mulch treatments had significantly lower mortality and disease ratings than control and compost-treated soil. At the Watauga site, PB and WCC had lower mortality and disease

ratings than control and compost-treated soil, and PB also had lower ratings than WC mulch. At the Avery-2 site, trees in WC mulch had lower disease ratings than all non-mulch treatments, and sulfur-treated soil had higher ratings than compost-treated soil; WCC also separated from control, sulfur and WC treatments for mortality but not disease rating data.

Tree mortality at each time point, measured as change in survival since previous sample date, varied significantly with cellulase activity in the upper portion of the root zone (P=0.005). Covariance between tree mortality and FDA was not significant at the 0.05 level (P=0.121). Neither cellulase nor FDA levels among soils showed significant covariance with tree survival.

Within the small subset of trees sampled for root distribution at the Avery-1 site, treatment did not significantly affect percentage of roots from which *Phytophthora* was recovered (P=0.214). Within mulched treatments, infected roots were found in both soil and mulch layers. Though infection rates tended to be lower in mulch than soil, differences were not significant (P=0.114). Among mulch-planted trees, living trees showed higher mean proportions of root length within mulch than dead trees for all three mulch types, but the relationship between mortality status and percentage of root system in mulch was not significant, by length or dry mass (P=0.423). There was a significant difference detected among mulch types in proportion of root length within mulch (P=0.039); WC and WCC mulches had similar proportions of root length in mulch, which were approximately eight times those found in PB mulch (Table 4).

Abiotic Data. Plant tissue samples collected 17 months after planting indicated that treatments significantly impacted tissue N, P, K, Mg, Mn, and B (P = 0.007, 0.003, <0.001, 0.025, 0.024, 0.005), but did not impact Ca, S, Fe, Zn, or Cu (Table 5). Trees in sulfur-amended plots had the highest tissue N and Mn, and the lowest P, K, and B. WCC mulched trees had the highest tissue P and K. Trees in mulch treatments did not generally vary significantly from control trees in unamended soil, with the exceptions of K (WCC higher than controls), Mg (Controls higher than WC and PB) and B (WCC higher than controls).

Soil pH (1:2 slurry by volume in 0.01 M $CaCl_2$) differed among treatments and over time at all three monitored sites, with a pH range of 4.1-6.8 in all non-sulfured soils, 3.6-6.8

in lower mulches and 3.4-6.2 in upper mulches across the duration of the study. At the site which included a soil sulfur treatment, pH in sulfur treated soil ranged from 3.1 to 4.3, with a single outlier of 5.1 at the third sample date. With the exception of that date, sulfur treated soil had significantly lower pH than all other treatments. There was no significant covariation between either soil pH or pH in the upper portion of the root zone (soil or mulch) and tree survival within the four treatments common to all three sites. There was also no significant covariation between pH and tree survival within the one site that included the sulfur treatment.

Both summer (May-September) and winter (September-May) rainfall during the first year were positively correlated with disease ratings at one year after planting ($r = 0.655$, 0.736; $P = 0.008$, 0.002). After the first year, no further correlations with rainfall were observed. Soil moisture was significantly higher under mulch ($P<0.001$), but tree survival at each time point did not covary with soil moisture ($P=0.309$), nor was there a clear relationship between tree survival and moisture within the just upper portion of the root zone (soil or mulch).

DISCUSSION

Organic mulch and compost based growing systems have met with mixed success for suppression of soil borne Phytophthora diseases in the field, with results ranging from significant suppression to enhancement of disease development (9, 10, 12, 24, 25, 34, 35, 37, 38). Where systems have been successful, control has often been associated with the biological fraction of organic amendments (11, 13, 22, 40, 41), and disease suppression has been shown through reduced inoculum density (24), decreased disease incidence or severity (10, 35), and increased plant productivity (12, 37). Where organic amendments have been detrimental, these effects have been associated with increased moisture in the root zone, which favors zoospore production by *Phytophthora* species (35, 38). The relationship with moisture is complicated, however, because high moisture levels can also support larger populations of antagonistic microbes. In trials with Fraser fir, we found that mulches reduced disease incidence at three of five sites, while a single compost application tilled into

soil did not impact disease incidence at any site. Disease suppression was related to microbial enzyme activity within the upper portion of the root zone, with a trend for total activity (FDA, non-significant at α=0.05) and significant covariance with cellulase activity. Mulches did retain moisture and significantly increased the moisture content of underlying soils, relative to unmulched soils, but there was no clear association between this increased moisture and changes in plant survival at each time point, which supports the premise of a dual role for moisture in both suppression and exacerbation of disease.

Biological analyses (plate counts on three media, FDA, and cellulase activity) were positively correlated to one another, but only the comparison of FDA and cellulase activity had a correlation coefficient above 0.50. Enzyme analyses were generally more useful than plate counts for describing differences in biological activity among our field samples, yielding clearer patterns in data and more significant differences among treatments. Plate counts on cellulose agar proved the least valuable, with few significant differences among treatments and generally weak correlation to other analyses. This may be due in part to the presence in all samples of microorganisms which were capable of degrading cellulose in absence of other carbon sources, but which may not have been contributing significantly to overall cellulase activity within the original sample. Dilution plating is often cited as under-representing total populations and over-representing subsets which can flourish on laboratory media, where both nutritional and competitive conditions are very different from those in the original sample (2). However, even though limited to a culturable subset of the population, these assays can be useful for tracking broad categories of organisms and substrate utilization potential. The differences in our results between plating on cellulose agar and tracking cellulase activity suggest that all of our sample types served as reservoirs for organisms able to produce cellulase enzymes, but as one would expect, actual production of cellulase varied by sample type, reflecting differences in substrate availability. We were also able to see some differential effects of treatments on bacterial versus fungal components of the microbial populations, particularly in the sulfur-treated soil. Dilution plating also provides a relatively simple and inexpensive means of gauging diversity. Again, the diversity indices derived from plates are limited by culture bias, but richness from this subset is frequently as high or

higher than those derived from culture-independent methods, each of which has its own biases (14, 29). In this study, we observed higher diversity among colonies from soils than from mulches, which is expected due to the high availability of a single substrate type in mulch, versus the higher competition and niche diversification in soil. We did not find consistent differences in diversity among mulch types, nor were there clear differences in suppression among mulch types, so we were unable to draw any conclusions as to the role of microbial diversity in disease suppression. Only one of the biological variables, cellulase activity, covaried significantly with tree survival. It is possible that clearer relationships may have been observed had we collected biological data at all five sites, especially given that differences in survival were observed at three sites overall, but only at one of the three sites for which we have biological data.

The protection provided by mulches is dependent upon the suppressive microbial community resident in the much, and upon the balance among rates of root infection and deterioration of infected roots and production of new healthy roots. For the system to succeed, roots within the mulch must be protected from new infections, infections which occur within the soil must not progress to destroy roots within the mulch, and an adequate portion of the root system must lie within the mulch. The proportion of roots which must be maintained within the mulch will then depend upon the rates of infection and disease progression within the soil. In earlier studies conducted with avocado on a resistant rootstock, it was observed that roots in the soil became infected with *P. cinnamomi*, but roots within mulch and at the soil-mulch interface remained relatively disease-free, and both total microbial activity (FDA) and cellulase activity were negatively correlated with *Phytophthora* inoculum potential (13). In the current study, high levels of total microbial activity and cellulase activity were achieved with all mulches, averaging 3.2 and 7.8 times higher, respectively, than levels within underlying soil. These ratios are not as high as those observed by Downer and Menge (13), where mulches averaged 5.3 and 17.3 times higher FDA and cellulase activity than soil. The differences may be due to variations in methods used, but also may reflect differences in locally available mulch substrates. Though inoculum levels and infection rates were not tracked over time in this study, changes in tree

survival over time were significantly related to cellullase activity within the upper portion of the root zone (soil or mulch), suggesting that microbial cellulase production did provide some disease suppression. It is also likely that other organisms, aside from cellulase-producers, may contribute to disease suppression within the mulch, even though the relationship between total microbial activity and tree survival was not significant in this study. In an evaluation of *P. cinnamomi* suppressive soil, McDonald *et al* found no relationship between suppression and either total microbial activity or cellulase activity, and yet found that the suppression was microbially-mediated, with both general and specific types of suppression (27). While our work supports a role for cellulase activity in suppression of Phytophthora root rot, measurement of enzyme activity provides a relatively coarse-resolution description of potential mechanisms, and it is likely that multiple types of suppression are acting simultaneously within any successful mulch-based system.

While all three mulch types sustained high microbial populations, activity, and cellulase production across all sites, our mulch systems failed to meet the criterion that roots within the mulch remain disease free. Within the small sample of trees analyzed in this study, infected roots were frequently found within lower mulch layers. As Fraser fir is highly susceptible to *P. cinnamomi*, this is likely due to the rapid progression of infections which were initiated within soil and then expanded upward along individual roots. It is also possible that zoospores from soil may have been able to move into lower mulch during saturation events and initiate infections there; however, similar mulches have previously been found to inhibit zoospore release and reduce the ability of zoospores to reach and infect host tissue (11). While further study could clarify which of these scenarios is responsible for the occurrence of root infections within our mulches, either case suggests that development of resistant rootstocks will likely be required to limit the spread of *Phytophthora* within the root system. This work also suggests that some mulch substrates may encourage better root proliferation than others. As resistant root stocks and grafting techniques become available, further research is warranted to examine substrate selection and planting methods that will encourage and sustain root growth within the mulch layers, as well as to minimize settling and wash-out of mulch and determine requirements for substrate replenishment.

While mulches provided significant suppression of Phytophthora root rot in field planted Fraser fir at three of five sites, the level of protection was inconsistent across sites, and inadequate as a stand-alone solution for growers. In the avocado system, upon which this research was based, mulch is also not used alone, but rather in conjunction with a set of cultural practices, including site preparation, irrigation, and grafting with resistant root stocks. A combination of cultural controls and pathogen resistance will likely be required to attain adequate control of Phytophthora root rot in Fraser fir, as well, and the disease suppression observed in this study indicates that mulches may be a valuable contribution to any future integrated management systems.

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Table 1. Selected soil chemistry values for five Fraser fir study sites in North Carolina and Virginia.

Site	Humic Matter (%)	CEC	BS[a] (%)	pH	P (ppm)	K (ppm)	Ca (%)[b]	Mg (%)[b]	Mn (ppm)	Cu (ppm)	S (ppm)
Mitchell	0.36	6.7	89	5.6	108.0	314.8	68	15	102.5	0.70	8.22
Avery-1	0.71	12.6	86	5.2	198.6	469.2	64	13	35.7	1.2	2.12
Avery-2	1.28	12.8	86	5.4	6.0	285.4	54	26	14.5	1.0	1.56
Watauga	1.11	12.9	84	5.0	122.4	268.8	61	17	87.8	25.6	1.94
Grayson	0.91	12.5	81	4.8	281.4	382.2	49	24	32.5	0.97	1.96

[a] Base Saturation

[b] Percent of CEC occupied by nutrient listed

Values are averages of two samples from each site. Analyses performed by the NC Department of Agriculture Agronomic Services lab, Raleigh, NC.

Table 2. Correlation among biological variables.

	Moisture[a] (%)	CEL (CFU)	RB (CFU)	R2A (CFU)	CEA µg GE ·g^{-1}	FDA µg·g^{-1}h^{-1}
Moisture	1.000	-	-	-	-	-
CEL	**0.1466** 0.0003	1.000	-	-	-	-
RB	**0.3203** <.0001	**0.1902** <.0001	1.000	-	-	-
R2A	**0.3993** <.0001	**0.4369** <.0001	**0.4694** <.0001	1.000	-	-
CEA	**0.7772** <.0001	**0.1099** 0.0067	**0.2358** <.0001	**0.2637** <.0001	1.000	-
FDA	**0.6378** <.0001	**0.1727** <.0001	**0.4272** <.0001	**0.4671** <.0001	**0.5449** <.0001	1.000

[a] Headings indicate sample moisture, colony enumerations on three media types (CEL for cellulose-degreaders, RB for fungi, and R2A for bacteria), cellulase enzyme activity (CEA, glucose equivalents, GE, micrograms per gram dry sample), and fluorescein diacetate hydrolysis (FDA, micrograms per gram, per hour). The P-value is shown beneath the Pearson correlation coefficient (r) in each cell.

Table 3. Area under the disease progress curve for Fraser fir mortality at five sites.

Site	Treatment					
	Control	**Compost**	**Sulfur**	**WC**	**WCC**	**PB**
Mitchell	7.032	6.763	-	3.213	2.706	4.400
Avery-1	6.056	7.175	-	4.738	4.902	7.738
Avery-2	10.195	8.445	12.023	1.714	7.258	-
Watauga	8.338	8.931	-	5.663	3.369	1.188
Grayson	7.478	5.075	3.998	4.446	3.686	-

Values are calculated areas under disease progress curves, based on percent mortality in each treatment (mortality/month). Treatments included unamended control plots, soil amendment with compost, and wood chip (WC) or wood chip with compost (WCC) mulch as raised beds at all sites; two sites included soil amendment with sulfur, and three sites included pine bark mulch beds (PB).

Table 4. Root distribution of Fraser fir in soil and mulch layers at an Avery County production site.

Treatment	Root Length (cm)			Root Mass (g, dry)		
	Upper	Lower	Soil	Upper	Lower	Soil
Living trees						
PB Mulch	0	354.8	7664.5	0	1.360	47.326
WC Mulch	90.1	2894.9	5499.9	0.089	7.055	28.370
WCC Mulch	556.0	1560.8	4229.5	1.459	5.202	26.749
Dead trees						
PB Mulch	0	66.4	2835.3	0	0.827	25.344
WC Mulch	4.5	1050.1	2162.1	0.002	1.758	22.157
WCC Mulch	0	653.8	2042.9	0	4.191	14.200

PB= pine bark mulch, WC= wood chip mulch, WCC= wood chip mulch with 15% dairy compost, by volume. Roots of two living and two dead plants from each treatment were excavated sequentially from the upper half of the mulch, the lower half of the mulch, and the underlying soil. Length was assessed using APS Assess software (Version 2.0, APS Press), and mass is reported as weight after drying at 80ºC for 48 h. Numbers reported are averaged from two living or dead trees per treatment.

Table 5. Plant tissue nutrient status of Fraser fir needles 17 months after site establishment.

Treatment	N (%)	P (%)	K (%)	Ca (%)	Mg (%)	S (%)	Fe (ppm)	Mn (ppm)	Zn (ppm)	Cu (ppm)	B (ppm)
Control	2.18 b	0.19 ab	0.50 bc	0.40	0.11 a	0.12	65.2	177 b	27.8	7.8	25.9 bc
Compost	2.09 b	0.21 b	0.48 bc	0.44	0.13 ab	0.13	71.8	215 b	29.7	7.9	24.3 abc
Sulfur	2.40 a	0.15 b	0.42 c	0.37	0.10 ab	0.16	89.7	932 a	29.9	7.3	20.3 c
PB Mulch	2.20 ab	0.23 ab	0.56 ab	0.47	0.08 b	0.13	67.9	538 ab	32.1	8.2	35.5 ab
WC Mulch	2.19 b	0.20 b	0.51 bc	0.44	0.09 b	0.13	69.0	432 ab	30.8	8.2	29.4 abc
WCC Mulch	2.15 b	0.27 a	0.59 a	0.44	0.10 ab	0.14	72.2	285 ab	30.6	8.5	35.2 a

Values are means across five sites; means with the same letter within a column are not significantly different (α=0.05) using Tukey's HSD test. Columns with no letters showed no significant differences among treatments.

Figure 1. Site map, showing locations of five field trials conducted on commercial production sites for Fraser fir in Mitchell County, Avery County, Watagua County, and Ashe County, NC, and Grayson County, VA.

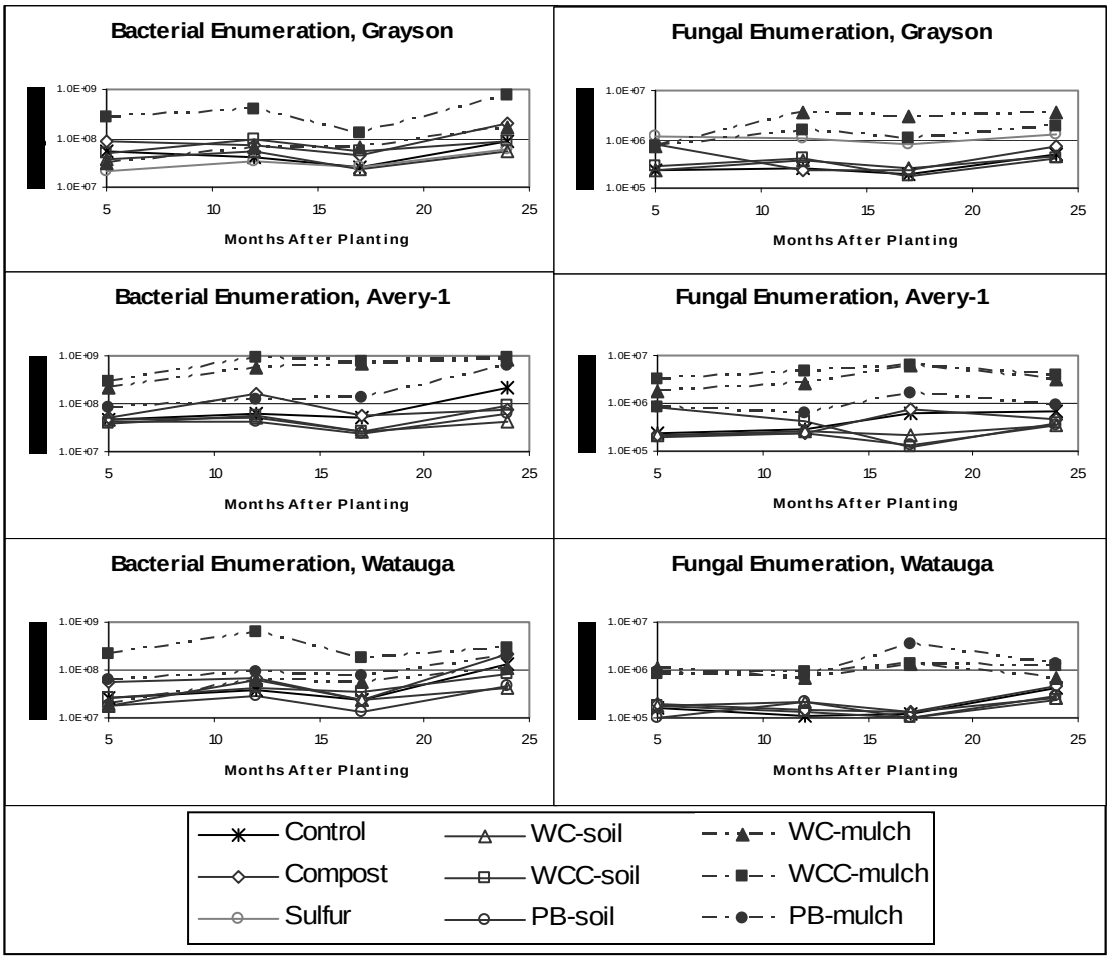


Figure 2. Bacterial and fungal enumerations in soil and mulch samples from three Fraser fir study sites. Counts are reported as colony forming units (CFU) per gram dry sample. Mulch beds were established at time of planting, and samples were taken at 5, 12, 17, and 24 months after planting.

Figure 3. Microbial activity by fluorescein diacetate hydrolysis (FDA) in soil and mulch samples from three Fraser fir study sites. Activity is reported as micrograms fluorescein released per gram dry sample, per hour. Treatments included unamended control plots, compost or sulfur soil amendment, and wood chip (WC), wood chip with compost (WCC), or pine bark (PB) mulch, as raised beds. Sample types shown are soil (S), lower mulch (L), and upper mulch (U). Mulch beds were established at time of planting, and samples were taken at 5, 12, 17, and 24 months after planting.

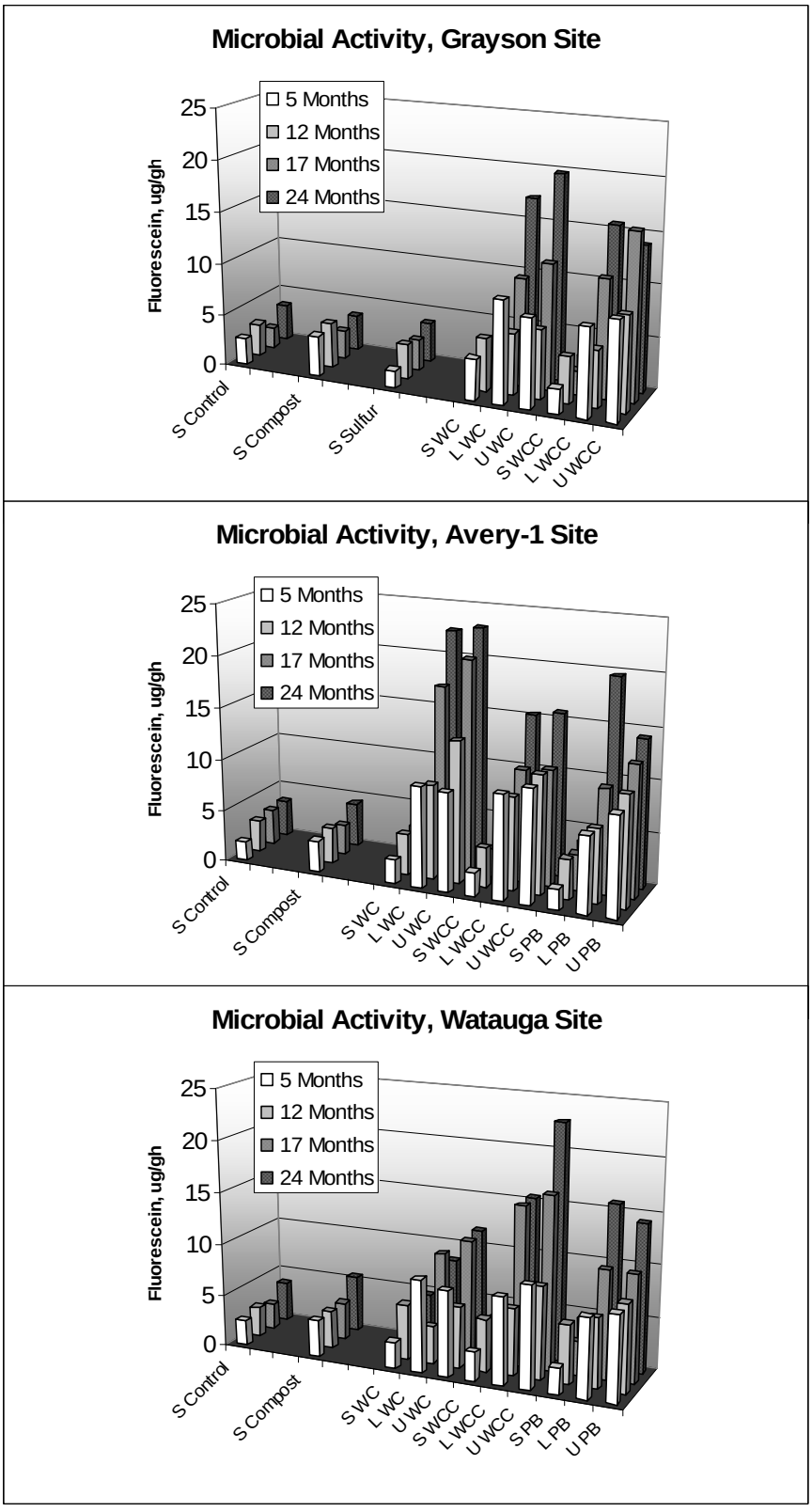

Microbial Activity, Grayson Site
Fluorescein, ug/gh
25
20
15
10
5
0
5 Months
12 Months
17 Months
24 Months
S Control
S Compost
S Sulfur
S WC
L WC
U WC
S WCC
L WCC
U WCC
Microbial Activity, Avery-1 Site
Fluorescein, ug/gh
25
20
15
10
5
0
5 Months
12 Months
17 Months
24 Months
S Control
S Compost
S WC
L WC
U WC
S WCC
L WCC
U WCC
S PB
L PB
U PB
Microbial Activity, Watauga Site
Fluorescein, ug/gh
25
20
15
10
5
0
5 Months
12 Months
17 Months
24 Months
S Control
S Compost
S WC
L WC
U WC
S WCC
L WCC
U WCC
S PB
L PB
U PB

Figure 4. Cellulase activity in soil and mulch samples from three Fraser fir study sites. Activity is reported as micromoles glucose equivalents (GE) released per gram dry sample after incubation with 0.35% CM-cellulose at 37ºC for 24 hours. Treatments included unamended control plots, compost or sulfur soil amendment, and wood chip (WC), wood chip with compost (WCC), or pine bark (PB) mulch, as raised beds. Sample types shown are soil (S), lower mulch (L), and upper mulch (U). Mulch beds were established at time of planting, and samples were taken at 5, 12, 17, and 24 months after planting.

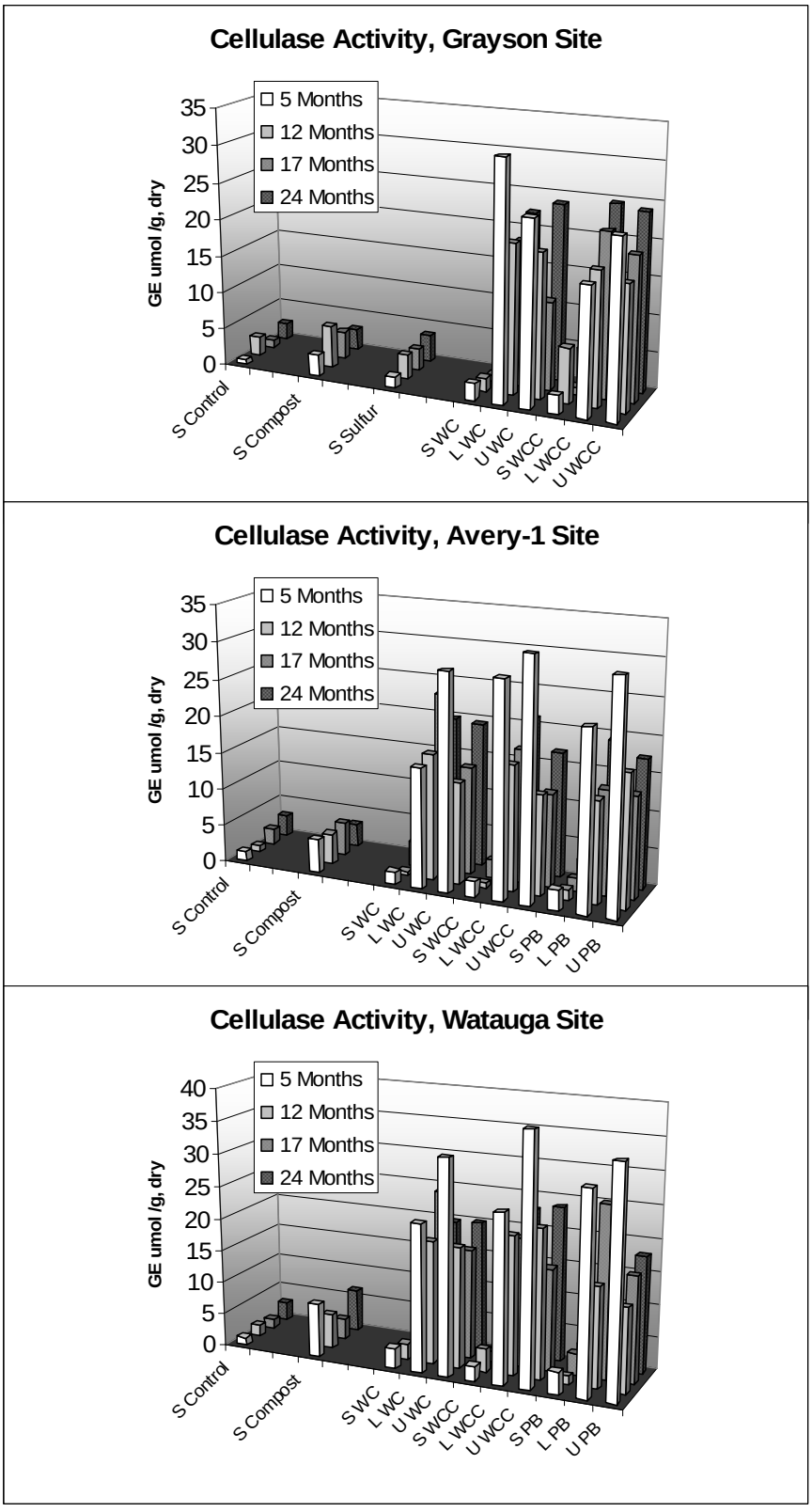
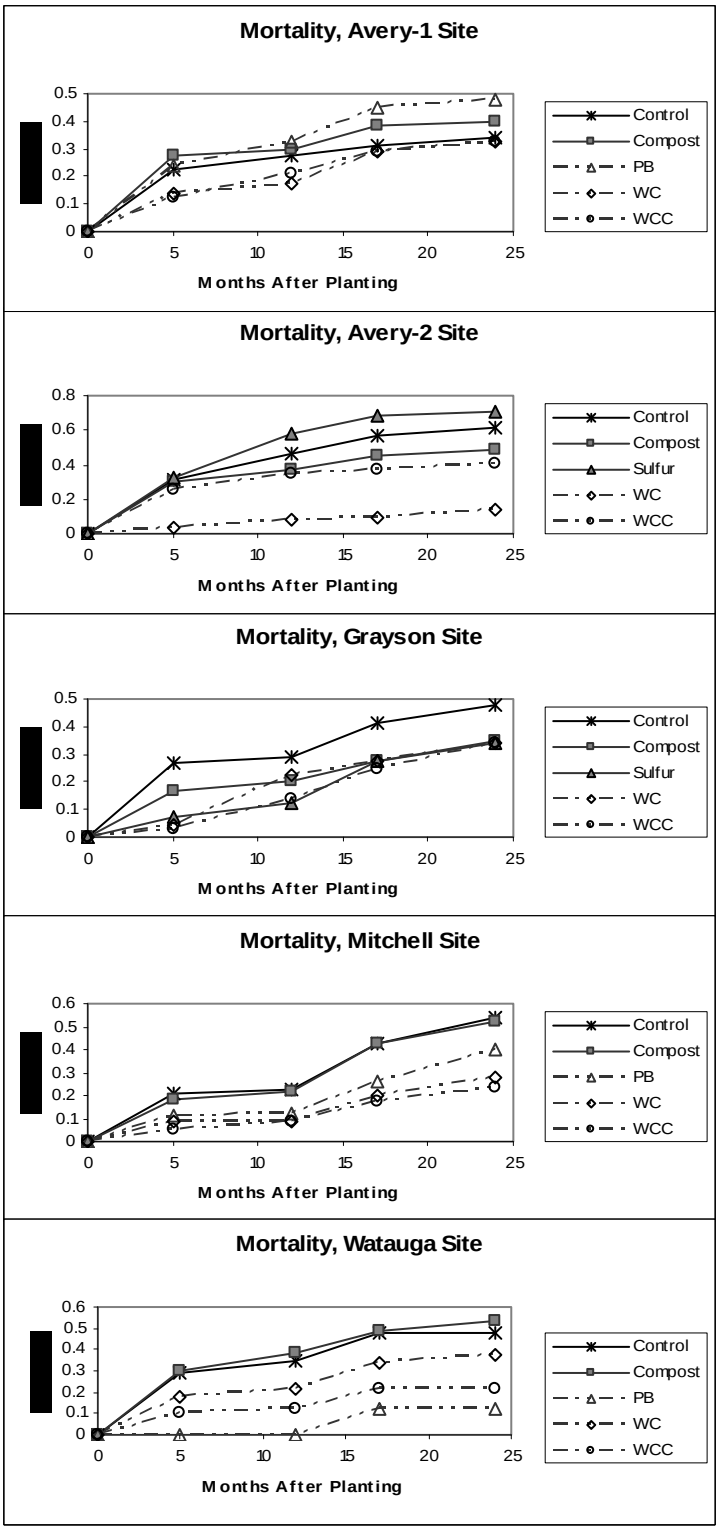

Cellulase Activity, Grayson Site
5 Months
12 Months
17 Months
24 Months
GE umol /g, dry
0
5
10
15
20
25
30
35
S Control
S Compost
S Sulfur
S WC
L WC
U WC
S WCC
L WCC
U WCC
Cellulase Activity, Avery-1 Site
5 Months
12 Months
17 Months
24 Months
GE umol /g, dry
0
5
10
15
20
25
30
35
S Control
S Compost
S WC
L WC
U WC
S WCC
L WCC
U WCC
S PB
L PB
U PB
Cellulase Activity, Watauga Site
5 Months
12 Months
17 Months
24 Months
GE umol /g, dry
0
5
10
15
20
25
30
35
40
S Control
S Compost
S WC
L WC
U WC
S WCC
L WCC
U WCC
S PB
L PB
U PB

Figure 5. Disease progress curves from five Fraser fir production sites, with mortality due to Phytophthora root rot expressed as proportion of trees deceased at each of four sample dates.


Mortality, Avery-1 Site
Months After Planting
Control
Compost
PB
WC
WCC
Mortality, Avery-2 Site
Months After Planting
Control
Compost
Sulfur
WC
WCC
Mortality, Grayson Site
Months After Planting
Control
Compost
Sulfur
WC
WCC
Mortality, Mitchell Site
Months After Planting
Control
Compost
PB
WC
WCC
Mortality, Watauga Site
Months After Planting
Control
Compost
PB
WC
WCC

CHAPTER 2

Cellulase activity as a mechanism for suppression of Phytophthora root rot, caused by *Phytophthora cinnamomi*, in mulch-based cultural systems

ABSTRACT

Phytophthora cinnamomi is a limiting factor in many perennial production systems. Wood-based mulches are currently used in avocado production and are being tested in Fraser fir production for reduction of Phytophthora root rot. Research with avocado has suggested a role of cellulase enzymes produced by mulch-inhabiting organisms in providing disease protection, through their effect on the cellulosic cell walls of *Phytophthora*. This work was conducted to determine whether cellulase production in mulch could account for disease suppression in these systems. A standard curve was developed to correlate cellulase activity in mulches with concentrations of a commercial cellulase formulation. *Phytophthora cinnamomi* was exposed to a range of enzyme concentrations, and data were collected on biomass and sporangia production. Sustained exposure to cellulase at concentrations of 10-50 U/ml significantly reduced sporangia production, but biomass was only reduced with concentrations over 100 U/ml. Based on the standard curve, exposure to 14 U/ml or greater was equivalent to cellulase activity in the aqueous fraction of field-applied mulches from two-year old Fraser fir study sites. In a bioassay with lupine, cellulase was applied at rates of 100 or 1000 U/ml, with three application timings, to soil containing Phytophthora-infected root fragments. Cellulase activity diminished by 47% between day one and day 15 after application. Cellulase applied to soil at 100 U/ml two weeks before planting resulted in cellulase activity of 20.08 µmol glucose equivalents per gram soil water (GE/g aq) at time of planting. This level was equivalent to two-year-old field applied mulches, which averaged 20.34 µmol GE /g aq. Cellulase activity at planting ranged from 3.35 to 48.67 µmol GE /g aq, but no treatment significantly affected disease progress in lupine plants.

INTRODUCTION

The use of mulches to reduce disease caused by soilborne *Phytophthora* species has been investigated in a number of cropping systems (6, 10, 18, 28). The efficacy of mulches, however, is variable, and the roles of various potential control mechanisms in these systems remain elusive. Where suppression has been achieved with mulches, it has been associated with the microbial component of the mulch (11, 30, 31). The resulting biological effects on the pathogen have included reduced differentiation and release of zoospores, reduced movement of zoospores, reduced inoculum potential, and increased lysis of hyphal cell walls (8, 11). Mulch-inhabiting microorganisms may directly inhibit *Phytophthora* spp. through production of antibiotics and enzymes, and may also induce host resistance. Multiple types of inhibition may operate simultaneously in a given system, and understanding which modes of action predominate in successful systems may help improve success rates and predictability of mulches for disease suppression.

Cellulose-rich mulches have been used successfully in Australia (5, 30, 31) and California (10, 13) to reduce Phytophthora root rot disease, caused primarily by *Phytophthora cinnamomi* Rands, in avocado orchards, and similar systems are under evaluation for disease suppression in Fraser fir farms in North Carolina and Virginia. Previous research has found that suppression of Phytophthora root rot with mulches is associated with microbial activity in the mulch (8, 11, 30), and it has been postulated that cellulases produced by mulch-inhabiting organisms may degrade the cellulosic component of *Phytophthora* cell walls (12). Previous research has shown that activities of cellulase and other enzymes are typically elevated in mulch, relative to un-mulched soils (9, 11). Downer *et al.* (2001) determined that enzyme activities in mulch and soil were positively correlated with microbial activity and fungal counts but negatively correlated with *Phytophthora* recovery (11). Cellulase is also known to directly impact *Phytophthora* cell walls; in fact, the cellulosic component of *Phytophthora* cell walls was originally identified through the use of cellulase enzymes (1). Laboratory research has also been conducted to quantify the impact of cellulase on cell walls of *P. cinnamomi*; Downer *et al* (2001) showed that addition of 10-25 U/ml cellulase to *Phytophthora* cultures in soil extract impaired zoospores and

chlamydospore development, and concentrations greater than 25 U/ml disrupted mycelium (12). Due to differences in units between the field and lab studies, it is not possible to determine whether the cellulase levels observed in field-applied mulches would be sufficient to achieve the effects observed in the *in vitro* assays. Soil assays for cellulase activity measure the mass of "glucose equivalents" (GE) released per unit mass of sample per unit time, while purified enzyme concentrations are expressed as enzyme units (U), where one unit is the amount of enzyme that catalyzes the conversion of one micromole of substrate per minute, under defined conditions. The mass of cellulose is unknown for any given soil or mulch sample, as are the relative quantities and molecular weights of each sugar released in the hydrolysis. Therefore, data from *in vivo* studies cannot be compared with *in vitro* assays.

While prior research has provided support for microbial cellulase as a primary mode of suppression in mulch, additional work is needed to interpret the existing research and determine the effects of cellulase on *Phytophthora* propagules at concentrations analogous to activity levels achieved from mulch applications. If disease suppression can be related to a threshold level of cellulase activity which is achievable in mulch, mulch formulations and cellulolytic microbes utilized in these systems could be optimized to achieve target cellulase activity levels. Further, tracking of cellulase levels in mulch over time could be used to determine optimal mulch age, and re-application times and rates. The objectives of this research were to: *(i)* establish levels of cellulase enzyme products (Units/ml) *in vitro* which yield enzyme activities (µmol Glucose Equivalents/g·h) similar to those achieved in organic field mulches, and determine the impact of these cellulase levels on *P. cinnamomi*; *(ii)* ascertain the effects of exogenously applied cellulases on *Phytophthora* inoculum potential in soil; and *(iii)* track the duration of cellulase activity associated with exogenous cellulase applications.

MATERIALS AND METHODS

Isolates and Enzymes. Two isolates of *Phytophthora cinnamomi*, designated 208 and 959, were used in all *in vitro* and container assays. Both strains were isolated from Fraser fir *(Abies fraseri)* production sites in Watauga County and Avery County, North Carolina,

respectively. In two *in vitro* trials, a reference culture from UC Riverside, designated M-295, was also included (12). The selection of cellulase source was based on commercial availability and compatibility with assay methods. Seven commercial enzyme products were initially tested in the preparation of enzyme activity curves. Bulking and stabilizing agents in four of these caused anomalous readings in the activity assays. Ecostone HPP5000, a powdered formulation of cellulases from *Trichoderma reesei* (AB Enzymes, Darmstadt, Germany), was selected for use in the remainder of the study.

Development of Standard Curve. Seven enzyme products were analyzed for cellulase activity using a modified Schinner & Von Mersi method commonly used for soil analysis (25, 29). To determine enzyme compatibility with the soil assay technique, each enzyme was diluted to create a stock solution, filter sterilized through a 0.2 µm syringe filter, and then further diluted in sterile deionized water to create solutions of 1, 10, 25, 50 and 100 U/ml. One milliliter of each diluted enzyme solution was incubated in 4.0 ml 0.35% carboxymethyl cellulose (CM-cellulose) in acetate buffer at 37ºC, with three replicate sample tubes and two buffer control tubes for each solution. Samples were drawn at five and 24 hours for analysis of reducing sugars by reduction of potassium hexacyanoferrate(III) and subsequent development of ferric hexacyanoferrate(II) (Prussian blue). The color intensity was measured by spectrophotometry (Spectronic 20D+, Thermo Scientific) at 690 nm. Each enzyme was assayed twice, and each set of assays included an internal control series of glucose samples at 0, 5, 10, and 15 µg/ml. Products were evaluated for consistent performance under assay conditions, and relatively low background color development. Based on the results of these analyses, two enzyme products were tested further with increased CM-cellulose concentrations, in order to ensure that substrate was not limiting in the assay. Ecostone HPP5000, incubated for 24 hours in 0.7% CM-cellulose, yielded the most consistent results and the steepest activity curve with this assay procedure, and was therefore selected to develop the standard activity curve.

For comparison with activity in field established mulch beds, six mulch samples were processed using the same concentration ratios (by weight) and incubation conditions described above. Mulch samples were from two year old beds at field locations in Avery

County and Watauga County, NC, and Grayson County, VA (Chapter 1), and were selected to provide a wide range of enzyme activity levels, based upon analyses from field studies. One of these was a pine bark mulch, and the remainder were pine wood chips blended with 15% dairy compost, by volume. Because *in vitro* assays are typically performed in liquid culture, and *in vivo* exposure to cellulase enzymes within mulches or soils would be most likely to occur within the aqueous fraction, rather than within solids, results were reported as glucose equivalents per gram aqueous fraction (µmol GE/g aq). This was calculated as: (GE µmol)(V_t)(*df*) / V_s , where GE was calculated from the absorbance value, based on a calibration curve, V_t was total volume of liquid in the incubation vessel (buffer, substrate, and V_s), df was the dilution facter, and V_s was the volume of liquid in the aqueous portion of sample (assuming 1 g water ~ 1 ml).

In Vitro Assays. Assays for cellulase effects on biomass and sporangia production were conducted with both single enzyme applications and sustained enzyme exposure. Cultures were grown in sterile buffered pea broth, pH 5.8 (BPB), produced by adding 8.5 ml K_2HPO_4 and 91.5 ml KH_2PO_4 to pea broth (120 g peas in 500 ml water, autoclaved 5 minutes and strained through cheese cloth), and filling to one liter with deionized water. Single applications were performed by replacing the BPB medium at the end of an initial growth phase with solutions containing the cellulase enzyme product at various concentrations. Sustained applications were also initiated after an initial growth phase, with enzyme solutions replaced twice daily with fresh solution in order to maintain enzyme activity levels through the duration of the trial. Four sample replicates were used for each isolate at each enzyme level, and each trial was replicated twice.

For biomass assays, 7-mm plugs were cut from the margins of actively growing cultures of *P. cinnamomi* isolates 208 and 959 on V8 juice agar and transferred to four replicate sterile 9-cm Petri dishes for each isolate by enzyme level combination. Dishes were filled to level with the top of the plug with BPB and incubated at 27ºC until colony diameters reached approximately 3 to 4 cm. Fresh enzyme working solutions were prepared by creating stock solutions that were filter sterilized through a 0.2 µm syringe filter and diluted in BPB to achieve final concentrations. Growth solution was then drained from the dishes

and replaced with enzyme solutions. For single-exposure trials, two enzyme concentration ranges were used, and each range was replicated twice. The first trials used a series of 0, 1, 5, 10, 25, 50, and 100 U/ml, and the second trials used a log series from 1 to 1000 U/ml, also with a 0 U/ml control. After enzymes were added, dishes were placed in the incubator in a completely randomized design and allowed to continue incubating at 27ºC until control colonies reached the dish edge. Sustained exposure trials used a series of 0, 10, 50, 100, and 500 U/ml. To facilitate solution replacement, dishes were not randomized. Dishes were grouped by isolate in open bins, and by concentration in stacks within bins. Location effects were minimized by rotating dish position within stacks, rotating stack position within the bins, and rotating bin position within the incubator after each enzyme replacement (twice daily). For all biomass calculations, the original plug was excised from each colony, and the colony was then washed onto a pre-weighed P5 grade filter paper (medium porosity, slow flow, 5-10µm particle retention) in a Buchner funnel over vacuum and rinsed twice with a stream of deionized water. Papers were removed, folded to contain the colony, and placed in a drying oven at 70ºC for a minimum of 24 hours. Three control papers were processed with no colony for each trial. Colony biomass was calculated as the difference between initial filter paper weight and dry weight with colony, after subtracting the average control paper loss from all initial weights.

For sporangia assays, *P. cinnamomi* isolates were tested in a range of sporulation solutions, including three soil extracts, a potting mix extract, and two modifications of the Chen-Zentmyer mineral salts solution (7, 24). The greatest number of sporangia were produced in the potting mix extract (FME), a 2% w/v suspension of Fafard Mix 2 (Conrad Fafard, Inc., Agawam, MA) filtered through diatomaceous earth and adjusted to pH 5.8. This solution was used as the diluent for all sporulation assays. To prepare for assays, 5-mm plugs were cut from the margins of actively growing cultures of *P. cinnamomi* on V8 juice agar and transferred to four replicate sterile 6-cm Petri dishes for each isolate by enzyme level combination. Dishes were filled to level with the top of the plug with BPB and incubated at 27ºC for three days. At the end of this growth stage, enzyme solutions were prepared by diluting the enzyme product in FME to achieve final dilution rates of 0, 10, 50,

100 and 500 U/ml. BPB solution was drained from the dishes, colonies were washed three times in deionized water, and 8 ml of the appropriate enzyme dilution was added to each dish. Dishes were incubated for two days at room temperature under fluorescent light. As with biomass trials, dishes for single application trials were arranged in a completely randomized design, while those for sustained enzyme trials utilized a rotation scheme to minimize positional effects. At the end of the sporulation period, the entire contents of each dish was washed into a blender canister with 20 ml physiological saline solution (0.9% NaCl) and blended for 25 seconds. Two 1.5-ml aliquots were immediately transferred into microcentrifuge tubes for short-term storage at 5ºC. Sporangia were counted by transferring 500µl from each of the two microcentrifuge tubes into a chamber of an inverted two-chamber McMaster counting slide (Chalex, Wallowa, OR). Slides were examined under a microscope at 40-100x magnification, and counts from the two sub-samples on each slide were averaged.

Soil Application Trials. Cellulase (Ecostone HPP5000) was applied to soil in 150-cc nursery cells (Ray Leach "Cone-tainer," Stuewe & Sons, Inc., Tangent, OR). Treatments included 100 U/ml and 1000 U/ml cellulase concentrations, applied once either one day or 15 days before planting, or applied three times at 7-day intervals from one to 15 days before planting, heat-treated enzyme (1000 U/ml, autoclaved 15 min at 121ºC) applied 15 days before planting, and a no-enzyme control. Field soil was collected from five sites in Avery County, NC. Each collection point was adjacent to a symptomatic Fraser fir tree within an area known to be infested with *P. cinnamomi*. Soil from all sites was blended together, sieved through a 5-mm mesh wire screen, and blended with 15% sand, by volume for use in container assays. Inoculum was artificially enhanced by blending infested root fragments into the soil mixture. These were produced by placing 20 to 30 rice grains colonized by one of three isolates of *P. cinnamomi* into the root zone of Fraser fir seedlings grown in one liter Anderson bands, and then exposing the plants to repeated flood cycles. Two weeks after inoculation, the shoots were clipped, and the root systems were washed and chopped into 1-cm segments. Chopped segments from trees with each of the three different isolates of *P. cinnamomi* (culture numbers 208, 959, and 2378) were blended, and added to the field soil mixture at a rate of two grams wet weight per liter of soil mix. Cells were filled with the

spiked soil mix and placed in racks in a greenhouse in a randomized complete block design, with eight cones per treatment, eight treatments per block (one rack), and five blocks. Two replicate trials were conducted. A set of three test cells was used to determine total soil water content at saturation; based upon the results of this test, 50 ml of enzyme or control solution was added to each cell, in order to completely saturate the soil. Cells were maintained on a misting bench throughout the 15-day treatment period to maintain soil moisture.

Three days before the final treatments were applied to soil cells, lupine seeds (*Lupinus angustifolius*, Adams-Briscoe Seed Company, Jackson, GA) were surface sterilized by rinsing in 95% ethanol, then soaking for three minutes in 5% commercial bleach, and were placed on sterile moist sand to germinate. One day after the final treatments, seedlings were transplanted into the soil cells. Plants were maintained under mist for the first three days, then hand-watered as needed for the duration of the study. Mortality data were collected daily, and at each date new mortalities were removed and the roots plated on PARPH medium for confirmation of *Phytophthora* infection (14). Because recovery rates on selective medium were commensurate with expected rates (16, 19, 21), all mortalities showing symptom progression indicative of Phytophthora root rot were included in data analysis.

In order to track cellulase activity in soil over time, 12 additional soil cells were set up as above for each of the single-application treatments (0, 100, and 1000 U/ml, and 1000 U/ml heat-treated), and six additional cells were set for the three-application treatments (100 and 1000 U/ml). At 24 h after the first treatment, six randomly selected cells were pulled from each of the single-application treatments and processed for enzyme activity. At 24 h after the final treatment, the remaining cells were processed. Enzyme activity was measured using the modified Schinner & Von Mersi method, with 6.0 g soil mix incubated in 24 ml 0.7% CM-cellulose solution at 37ºC for 24 hours. Remaining soil in each cell was used to calculate moisture content after drying at 110ºC for 24-48 h. For comparisons of cellulase concentrations in soil over time, activity results were adjusted to a dry matter basis. Results are also reported on a soil water basis for comparison with enzyme and mulch samples, based on the standard curve generated with the Ecostone HPP5000 cellulase product.

Statistical Analyses. Analysis of variance was conducted on data from *in vitro* assays using the general linear model function of SAS® software (SAS institute, Inc., Cary, NC), with Tukey's HSD test applied for means separations. Where data were non-normal and transformations did not normalize them, non-parametric analyses were used, as noted. GLM analysis on ranked data was used to conduct post hoc analyses on non-normal data sets. For each pair of replicated experiments, combined data were subjected to analysis of variance with repetition defined as an independent variable, and if repetition showed a significant treatment effect, data were treated separately in all further analyses. To determine whether there was a linear relationship between enzyme concentration and either biomass or sporangia formation, regression analysis was also conducted with each of these data sets, using the REG procedure in SAS. For cellulase activity data from soil application trials, mixed model repeated measures analysis was conducted using the PROC MIXED function in SAS, with the SLICE option used for comparisons across treatments and over time, and Tukey's adjustment applied for least squared means separations. For the repeated measures analysis, data from both repetitions were combined, with repetition defined as a random variable, and the covariance structure was determined by repeated analysis with each of eleven possible models and selection of the structure which yielded the lowest AIC value. Plant mortality data and area under the disease progress curve (AUDPC) across treatments were analyzed using Friedman's nonparametric analysis.

RESULTS

Standard Curve. An enzyme activity curve was generated to relate enzyme concentrations (units per milliliter) to activity levels (micromoles glucose equivalent, GE, per gram sample water) (Figure 1). The resultant curve has a function of $y = 3.2063\text{Ln}(x) + 3.8059$ ($R^2 = 0.9956$). Mulch samples from a two-year established field site ranged in activity from 12.3 to 24.1 µmol GE /g aq, which corresponds to an enzyme concentration range of approximately 14-553 U/ml (Table 1).

In Vitro Assays. Colony biomass did not decrease with increasing enzyme concentration in trials using a series of 0, 1, 5, 10, 25, 50, and 100 U/ml, but did vary using a series of 0, 1,

10, 100 and 1000 U/ml (Figure 2). In an analysis of variance using the GLM procedure on arcsin transformed data, no significant difference in response was detected between two repetitions of the experiment, so means of the two repetitions were used for analysis. With the lower concentration range (0-100 U/ml), analysis of variance did show significant differences in mean dry weights with isolate 208 (P=0.002), but there was no clear pattern discernable between enzyme concentration and dry weights. Mean separation (Tukey's HSD test) showed that no treatment differed significantly from the control (0 U/ml). Regression analysis for isolate 208 data also yielded significant results, but these showed a slight increase of dry weight with increasing enzyme concentration (model: Y=0.05042 + 0.00010282X , P=0.008, R^2 = 0.242). Data from isolate 959 did not show significant effects of enzyme concentration on dry weight with either the GLM (P=0.075) or regression analysis (P=0.087), but the regression model did show a negative relationship between enzyme concentration and dry weight (Y=0.09017 -0.00011055X , R^2 = 0.1085). With the higher concentration range (0-1000 U/ml), data were non-normal and non-parametric analyses were used. A Kruskal-Wallis test showed that there were significant treatment effects within each isolate in each repetition (P= 0.049, 0.027, 0.012, 0.008 for isolates 208 and 959 first trial, isolates 208 and 959 second trial, respectively). GLM analysis was performed on ranked data from both repetitions for means separation, and indicated that dry weights from the 1000 U/ml enzyme level were significantly different than all other treatments, and no other treatment levels differed from one another. *P. cinnamomi* colonies under sustained enzyme exposure at a range of 0-500 U/ml cellulase showed a pattern of decreasing dry weight with increasing enzyme concentration (Figure 3). Because experimental repetitions differed significantly from one another in a GLM analysis of combined data (P=0.002), data were analyzed separately for each repetition. Significant treatment effects on dry weight were observed in both trials (P<0.001), with the 500 U/ml treatment causing significant reduction in dry weight relative to all other treatments in both trials (P<0.05). Linear regression analysis conducted on data from each isolate yielded a significant negative relationship between enzyme concentration and dry weight for isolates 208 (P<0.001) and M295

(P=0.002) in the first repetition, and isolates 208 (P<0.001), 959 (P=0.050) and M295 (P<0.001) in the second repetition.

In sporulation trials with a single enzyme application, sporangia production increased with enzyme concentration up to 100 U/ml, then decreased at 500 U/ml (Figure 4). Because a GLM analysis of combined data showed no effect of repetition (P=0.272), data from both trials were combined for analysis. Enzyme concentration significantly affected sporangia production (P<0.001), and the 500 U/ml enzyme level significantly reduced sporangia production relative to 10, 50, or 100 U/ml (P<0.05). In trials with sustained enzyme application, sporangia production decreased sharply with increasing enzyme concentration (Figure 5). Due to the large number of zero values in the data set, data were non-normal and non-parametric analyses were used. Friedman's test was conducted on each repetition with isolate as a blocking variable, and treatment effects were found to be significant (P<0.001) in both trials. A GLM analysis on ranked data with Tukey's adjustment was used for means separation, and indicated that every treatment level was significantly different from each other level (P<0.01). Regression analysis was performed on data from each isolate at enzyme concentrations of 0-100 U/ml. All regressions showed a significant negative relationship between enzyme concentration and sporangia production (number of sporangia per colony), with the following models:

Isolate 208: $y = 8105.651 - 94.089x$ ($R^2 = 0.670$, $P<0.001$),

Isolate 959: $y = 11194.000 - 120.513x$ ($R^2 = 0.753$, $P<0.001$),

Isolate M295: $y = 8229.648 - 90.108x$ ($R^2 = 0.742$, $P<0.001$).

Soil Application Trials. Cellulase activity among enzyme-treated soils was compared on a dry mass basis (µmol GE/g) in order to compensate for slight moisture differences among samples. Cellulase activity varied across treatments and over time (Figure 6). Because analysis of variance with GLM on combined data showed no effect of repetition (P=0.665), data from both trials were combined for analysis, and repeated measures analysis was performed with trial as a blocking variable. Activity in cells treated with autoclaved enzyme solution was similar to that observed in control cells. In cells which received a single

enzyme application, activity diminished significantly ($P<0.001$), to an average of 53% of original values over the course of two weeks. Cells which received three enzyme applications had significantly lower activity one day after the final treatment than cells which received a single application had one day after the single treatment ($P=0.008$ for 100U/ml, $P<0.001$ for 1000U/ml). For comparison between treated soils and mulch activity levels, mulches were included in a repeated measures analysis of soil samples, and pair wise comparisons were made with least squares means applying Tukey's adjustment (Table 2). On a soil water basis (activity data as micromoles per gram soil or mulch water) mulch samples differed significantly ($P<0.05$) from all soil treatments except for soil treated with a single application of 100 U/ml enzyme and tested at 15 days after application ($P>0.999$). At time of planting, cellulase activity in the aqueous fraction of this treatment could be considered analogous to that in an average two year field-applied mulch.

In lupine bioassays with *P. cinnamomi*-infested root fragment inoculum, cellulase applications did not affect disease progress over a seven week study period (Figure 7). Using Friedman's nonparametric analysis, no significant differences were detected among treatments in either of two replicate trials for percent mortality at the end of seven weeks ($P=0.951$, 0.525) or for AUDPC ($P=0.903$, 0.482). *P. cinnamomi* was successfully recovered from 85% of symptomatic mortalities. This recovery rate was considered adequate indication that symptoms were caused by *Phytophthora* infection, and all symptomatic plants were used in the analysis.

DISCUSSION

Lysis of hyphal and sporangial walls has long been associated with suppression of *P. cinnamomi* in mulch-based cultural systems, and has led to repeated postulations that the degradation of cell wall components by mulch-inhabiting organisms is primarily responsible for pathogen suppression in these systems (4, 5, 11, 12, 20). Other control mechanisms have been identified which impact reproductive potential of *P. cinnamomi* in mulch (3, 22), and it remains unclear whether microbial lysis of cell walls is a primary mechanism of suppression, or is primarily *ex post facto* degradation of cells compromised by the actions of some other

mechanism. If cell wall degradation in mulches is the result of direct attack by microorganisms, or at least a side-effect of their enzyme production for other purposes, one would expect that observations of lysis would be associated with the presence of cell wall-degrading enzymes in concentrations sufficient to cause lysis in the absence of other factors. Although a positive association of lysis with cellulase enzyme activity has been demonstrated previously (11), differences in units of measurement between field and laboratory studies have precluded comparisons of enzyme levels observed in the field with those known to cause lysis of *Phytophthora* cell walls.

The standard curve generated in this study suggests that the cellulase activity in bulk samples of field-applied mulch two years after application is equivalent to an enzyme concentration range of 14-553 U/ml of a commercial cellulase enzyme product, under the test conditions described. In field trials, cellulase activity within mulches diminished over time, such that activity at two years after establishment was significantly lower than levels observed at five months (Chapter 1). Given the range of enzyme activity observed over time in field samples, cellulase activity in mulches generally may be expected to fall within or greatly exceed the range measured in the current study, depending on age of the mulch and environmental conditions. In the lower half of the range observed in two year old mulches, in vitro assays did not show an effect on biomass in *P. cinnamomi*, either with short-term or sustained enzyme exposure. Lysis of hyphae and associated biomass reduction were observed at sustained enzyme concentrations of 500 U/ml or more, which may be equivalent to activity levels in some mulches.

In previous studies on the effects of cellulase on *P. cinnamomi*, lysis was observed at much lower enzyme concentrations; Downer *et al* reported some lysis of hyphae with exposure rates above 25 U/ml, and complete disintegration at 100 U/ml (12). The enzyme product used in the earlier experiments was no longer available for inclusion in the current study, so it remains unclear whether these differences reflect differences in enzyme efficacy under the same conditions, or if they are the result of variations in product testing and labeling. Because enzyme units are defined by particular testing conditions, label descriptions of enzyme activity may vary from one product to another, depending on the test

method used. Nieves *et al* (1998) tested 13 commercial enzyme products using two common methods for determining enzyme activity, both recommended by the International Union of Pure and Applied Chemistry (IUPAC), and found that the product Units per milligram varied between methods, both in terms of total values reported for U/ml and in terms of product ranking by activity (23). One of these standard assays uses filter paper as the cellulose substrate, incubated in citrate buffer, pH 5, at 50ºC for one hour, and the other uses CM-cellulose as the substrate, incubated in citrate buffer, pH 4.8, at 50ºC for 30 minutes (15). The Ecostone HPP 5000 product used in the current study was labeled based on testing with CM-cellulose as a substrate, incubated at pH 4.0 and 60ºC. Kabel et al (2006) further explored the difference in cellulase enzyme performance on different substrates by testing 14 commercially available products with the IUPAC filter paper assay, and then testing them with natural cellulose fractionated from either wheat bran or grass (17). They found that the filter paper assay was a poor predictor of performance on natural substrates, and suggested that cellulase degradation is more dependent upon substrate characteristics than on enzyme activity, as determined in standard assays. To further complicate efforts to describe enzyme activity in biological assays, products which are standardized against one another at 50-60ºC may also perform very differently at temperatures below 35ºC, conditions under which they are likely to be operating in laboratory, greenhouse, or field conditions. This makes direct comparison across studies difficult; as with many types of bioassays, the only comparisons that can be made with certainty are those conducted within the same trial, under identical conditions. However, both the associative observations made in previous work (11) and the results of the current trials suggest that the bulk cellulase activity in a wood chip mulch can be sufficient to cause lysis of *P. cinnamomi* hyphae. Current results, however, suggest the caveat that not all mulches will maintain sufficient activity levels to cause lysis in the absence of other biocontrol mechanisms.

Within the concentration range analogous to mulch activity levels observed in this study, the primary impact of cellulase on *P. cinnamomi* was on the pathogen's ability to produce sporangia. We observed differential effects of single versus sustained enzyme applications on sporangia production in *P. cinnamomi*. At enzyme concentrations between

zero and 100 U/ml, a single enzyme application increased sporangia production, while sustained exposure decreased production. This differential response, if it occurs in field settings as well, provides mechanistic support for a phenomenon observed repeatedly in biocontrol trials: the biocontrol agent(s) must remain present and active in the infection zone over time in order to provide suppression. With a single application of cellulase, enzyme activity diminishes over time as the enzyme molecules are bound, degraded, or inactivated in the soil (26). In this study, short-term exposure to cellulase enzyme, which was allowed to subsequently degrade, may have caused some stress to the *Phytophthora* isolates, but was insufficient to suppress sporulation or growth at the same concentrations that showed suppression with longer exposure. The increase in sporulation observed with brief exposure may represent a physiological stress response which leads to a shift from a vegetative to a reproductive stage, consistent with Klebs' principle, as also observed by Malajczuk in studies with *P. cinnamomi* (20). Downer *et al* also noted that sporangia development was stimulated with very low levels of cellulase exposure, at 1 U/ml, in one of two trials, and that chlamydospore germination was stimulated with slightly higher exposure, at 10 U/ml (12). As with hyphal lysis results, the Downer *et al* study yielded reductions in sporangia development at lower stated enzyme concentrations than those observed in the current study, with sporangial counts approaching zero at 25 U/ml cellulase, rather than at 50-100 U/ml.

Cellulase-mediated reduction in sporangia production has the potential to slow the spread of *Phytophthora* within a site, and within a mulch may be a large factor in protecting roots from new infections. In soils, however, *P. cinnamomi* inoculum is frequently present in the form of infected root fragments. Even if formation of sporangia on these fragments can be reduced, lysis of chlamydospores and vegetative hyphae within root tissue is unlikely to occur, because they are protected within the tissue. Additionally, vegetative hyphae have a lower cellulose content than sporangia and chlamydospores (1, 2, 27), and are therefore less likely to be affected by enzyme exposure at low concentrations. The effects of cellulase on chlamydospores within roots has been investigated in artificially infected root tips (12), but not in mature roots, which would represent the majority of such inoculum in the field. In the present study, application of cellulase to soil with root fragment inoculum did not reduce

disease progress on lupine plants, even when applied at levels expected to inhibit sporangia production and lyse hyphae, indicating that survival of the pathogen within infested roots was likely not significantly reduced.

Microbial cellulase enzyme activity appears to play a significant role in suppression of *Phytophthora* with mulches and organic amendments, as evidenced by correlative field observations ((11), Chapter 1) and physiological studies *in vitro* (12). Though it appears that the enzyme activity levels observed in mulch samples are not always adequate for direct lysis of otherwise healthy *Phytophthora* hyphae, cellulase in the concentration range analogous to enzyme activity in mulch does have a clear impact on the pathogen's ability to produce the motile stage of its life cycle. It is also possible that by analyzing bulk samples, these studies underestimate the impact of cellulase enzymes at the pathogen cell surface. Direct attack and lysis of *Phytophthora* hyphae by microbial cellulases may be occurring at a much higher rate on a microcosmic scale, which we are unable to measure with currently available assays. Our ability to measure enzyme activity in environmental samples is currently limited to "bulk" activity levels within a sample; we can measure differences in activity among soil or mulch samples at the scale of one or several grams, but we lack the ability to track these activities on the scale of individual hyphae or chlamydospores, which may be subject to much larger concentrations of microbial enzymes than the averages that we see across the bulk soil or mulch. Bulk analysis of enzyme activity levels can be useful indicators of microbial populations and processes, but they are a relatively low-resolution approach to identifying groups of organisms that may play a role in plant disease suppression.

One of the challenges in studying biological and biochemical interactions within soil and other environmental samples is identifying comparable units of measurement that are both biologically meaningful and translatable across sample types and over time. Reporting of cell counts and enzyme concentrations has typically been done on a dry weight basis, to allow comparisons with a consistent frame of reference that changes little over time and is not affected by moisture content, which in most soil environments is a constantly fluctuating factor. However, the soil environment from a biological standpoint is an aqueous habitat in a constant state of flux. With changes in moisture, the entire habitat shrinks and swells, and

cells, chemicals, and enzymes contained in soil water become more or less concentrated. Thus measurement based on dry matter content is perhaps a better representation of total potential concentration than of the actual concentration experienced by the soil organisms and plant roots at a given moment. The difference between these frames of reference may be substantial, and should be considered when interpreting biological data. This study used both dry matter and sample water as platforms for comparison of enzyme concentration. When comparing among soils in a greenhouse study, results were similar using either measurement, but dry mass was considered more suitable, since the sum presence of enzyme over time was the point of interest. However, when comparing soil to mulch, dry matter is less equitable as a reference; the pore volume and surface area represented by a gram of soil are likely to be quite different than in a gram of mulch or compost. When considering the actual enzyme concentrations to which *Phytophthora* would be exposed in its environment, and in comparing those concentrations to *in vitro* assays, sample water mass (or volume, assuming the density of water to be a constant) was considered to be the more appropriate base.

This work has bridged earlier field and laboratory studies to provide evidence that cellulase may play a central role in suppression of soilborne Phytophthora diseases with mulch-based cultural systems. Variability in enzyme activity among mulches and over time may contribute significantly to the function of this mechanism in field applications. While cellulase activity may be sufficient in some mulches to cause direct lysis of pathogen mycelium and propagules, this mechanism may not function in all mulch systems, and is likely to be diminished as mulches age. At lower activity levels, cellulase may be playing smaller but more varied roles in contributing to disease suppression. In systems with lower cellulase activity, other biocontrol mechanisms may be more prominent, and are likely required to maintain pathogen suppression.

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Table 1. Cellulase activity in mulch samples from two year established field plots.

Mulch Type	Site	Moisture (%)	GE, (µmol/g aq)	GE, U/ml
Wood chip/ compost	Avery	71.87	12.28	14.07
Pine Bark	Watauga	61.16	24.06	553.07
Wood chip/ compost	Watauga	69.41	22.51	341.20
Wood chip/ compost	Watauga	73.50	19.88	150.55
Wood chip/ compost	Grayson	71.95	22.92	387.78
Wood chip/ compost	Grayson	69.19	20.38	175.53

Cellulase activity values are reported in micromoles glucose equivalents per gram sample water (µmol GE/g aq). Unit translations (GE, U/ml) were calculated using a standard curve generated with Ecostone HPP5000, a powdered formulation of cellulases from *Trichoderma reesei*. Mulch samples were either shredded pine bark or a blend of pine wood chips and dairy compost (15% by volume), and were collected from plots at three study sites in the North Carolina counties shown.

Table 2. Cellulase activity and moisture content of soils treated with Ecostone HPP5000, a powdered formulation of cellulases from *Trichoderma reesei,* as compared with field mulch samples.

Day	Treatment	Moisture (%)	GE, dry (µmol)	GE, aq (µmol)
1	0x1	28.12	1.20 a	3.35 a
1	1000htx1	27.76	2.13 a	5.89 a
1	100x1	27.79	13.83 c	38.08 c
1	1000x1	27.82	20.06 e	55.47 d
15	0x1	27.17	1.32 a	3.90 a
15	1000htx1	26.52	1.48 a	4.35 a
15	100x1	26.75	6.63 b	20.08 b
15	1000x1	26.58	11.53 c	34.43 c
15	100x3	26.79	11.73 c	34.79 c
15	1000x3	25.85	16.91 d	48.67 c
-	Mulch	69.52	42.44 f	20.34 b

Values are means from six samples per treatment, or six mulch samples collected from two year old established beds at field sites. Cellulase activity values are reported in micromoles glucose equivalents per gram dry sample (GE, dry) and per gram sample water (GE, aq). Treatments included water control (0x1), heat-treated enzyme at 1000 U/ml (1000htx1), 100 and 1000 U/ml enzyme (100x1, 1000x1) applied once at time 0 days, and 100 and 1000 U/ml enzyme applied three times (100x3, 1000x3) at times 0, 7, and 14 days. Analyses were conducted one day after the first applications (1 day) and 1 day after the third applications (15 days). Values sharing the same letter within a column are not significantly different from one another at α=0.05.

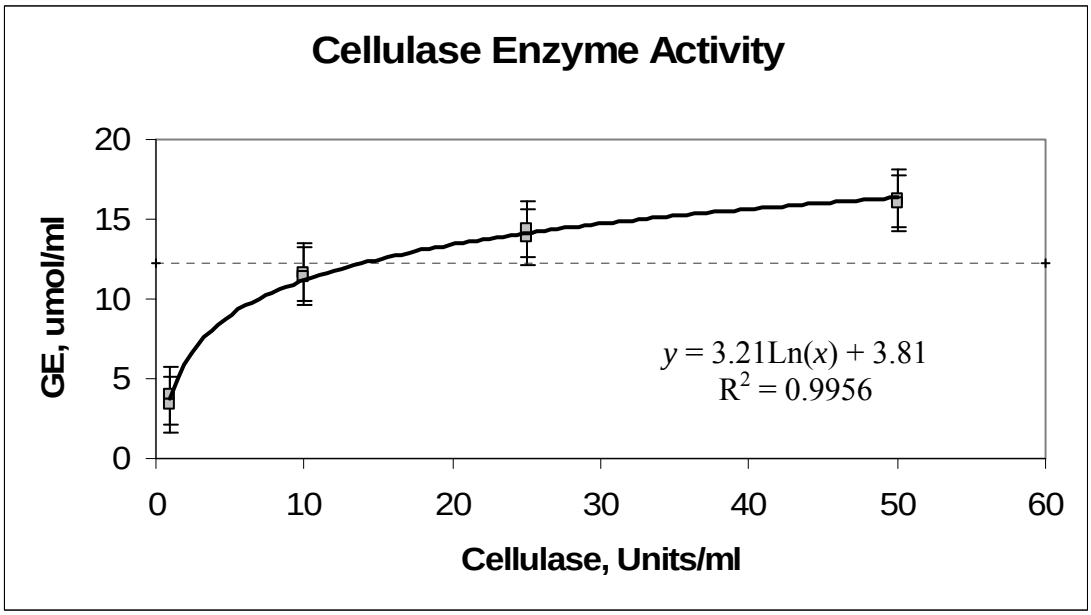

Figure 1. Cellulase enzyme activity curve for activity of Ecostone HPP5000, a powdered formulation of cellulases from *Trichoderma reesei*, tested at 1, 10, 25, and 50 enzyme units per milliliter (Units/ml). Reducing sugars were assayed after incubation at 37ºC for 24 hours in 0.7% CM-cellulose and are reported on the y-axis as micromoles of glucose equivalents (GE) per milliliter of enzyme solution. The dotted line represents the low end of the range of activity observed in six mulch samples from two-year established field sites, processed under the same conditions as the commercial enzyme product. Points shown are from two replications of the experiment, and bars represent standard error of the mean from the combined replications. The equation calculated for this curve was used to estimate the relationship between commercial enzyme units and field sample assays conducted under the same experimental conditions.

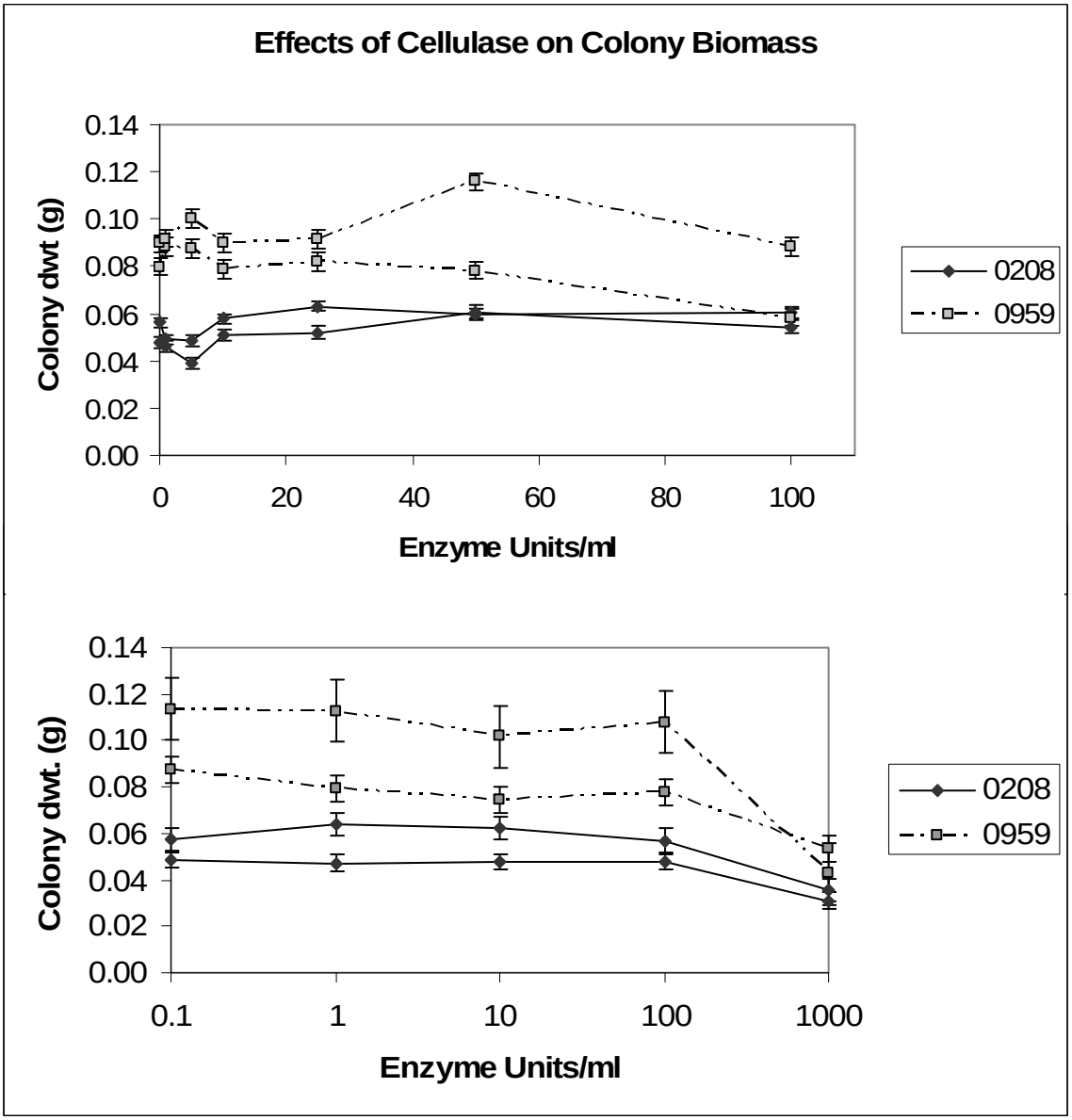

Figure 2. Effects of a single application of Ecostone HPP5000, a powdered formulation of cellulases from *Trichoderma reesei*, on colony biomass of two isolates of *Phytophthora cinnamomi* at two concentration ranges. Mean colony dry weights are shown for two replicate experiments conducted with each isolate. Bars represent standard error of the mean.

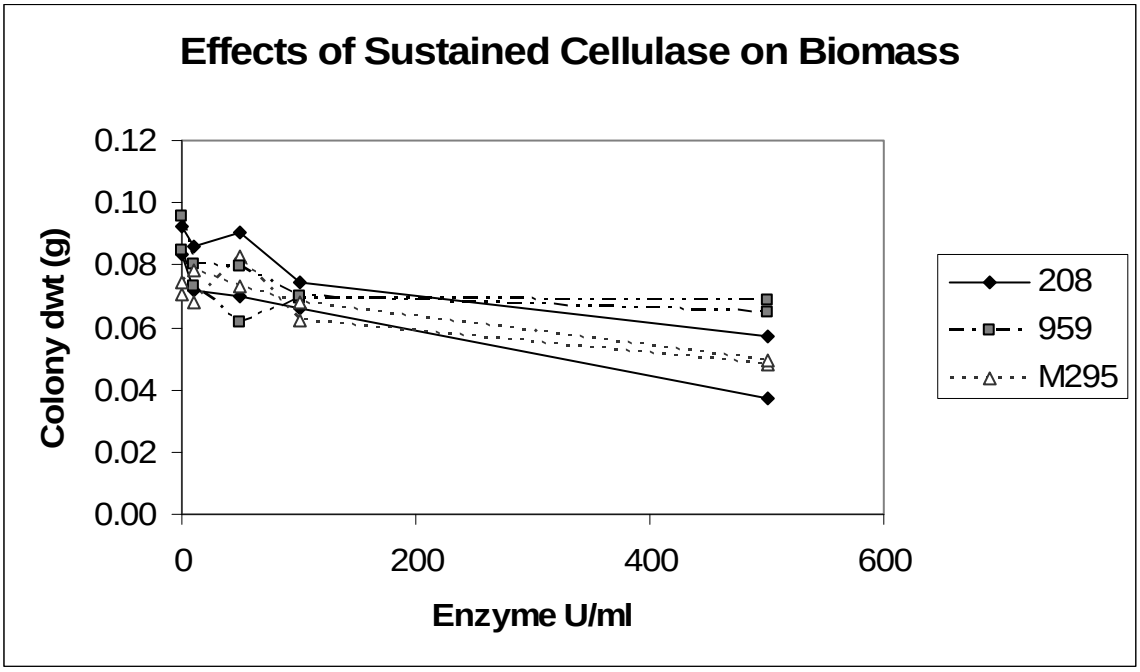

Figure 3. Effects of sustained exposure to Ecostone HPP5000, a powdered formulation of cellulases from *Trichoderma reesei*, on colony biomass of three isolates of *Phytophthora cinnamomi*. Mean colony dry weights are shown for two replicate experiments conducted with each isolate.

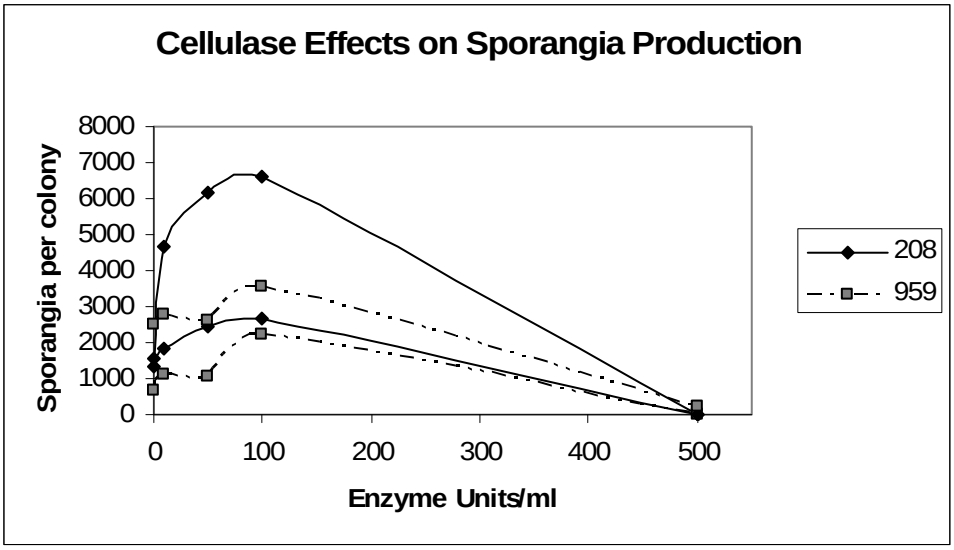

Figure 4. Effects of a single application of Ecostone HPP5000, a powdered formulation of cellulases from *Trichoderma reesei*, on sporangia production in two isolates of *Phytophthora cinnamomi*. Results shown are mean numbers of sporangia per colony across five enzyme concentrations, from two replicate experiments with each isolate.

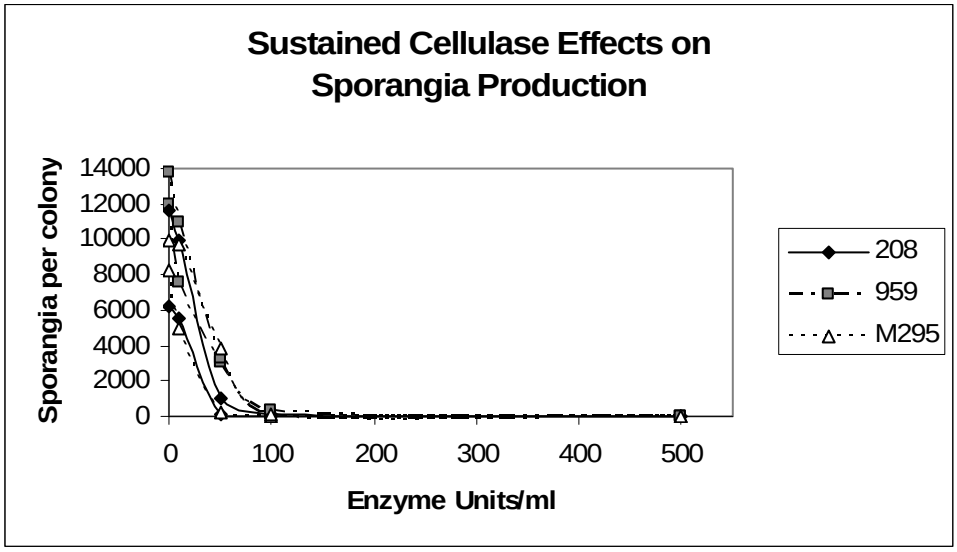

Figure 5. Effects of sustained application of Ecostone HPP5000, a powdered formulation of cellulases from *Trichoderma reesei*, on sporangia production in three isolates of *Phytophthora cinnamomi*. Sporangia production was recorded after three days under light at room temperature. Results shown are mean numbers of sporangia per colony across five enzyme concentrations, from two replicate experiments with each isolate.

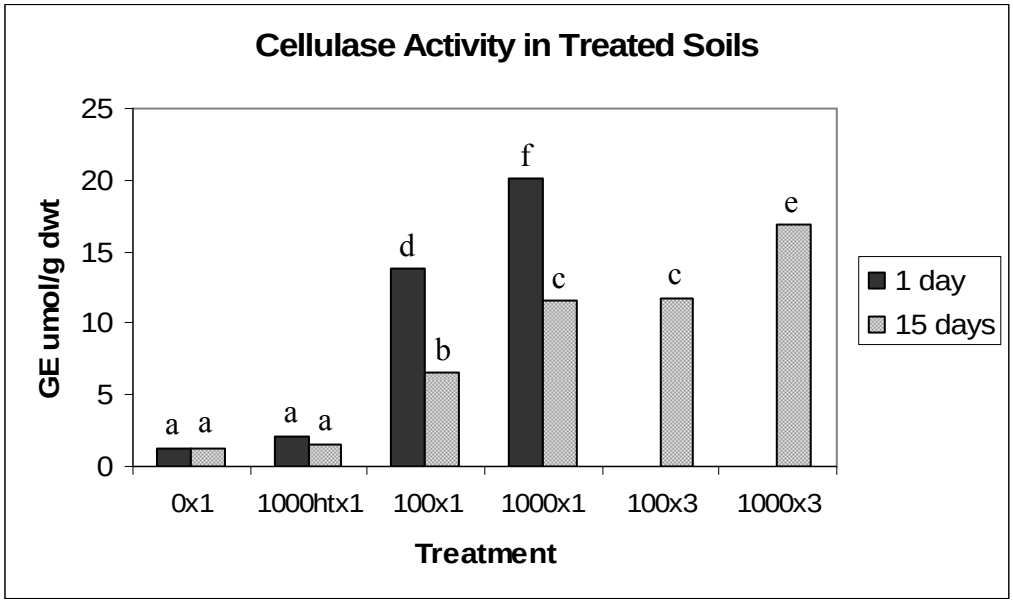

Figure 6. Cellulase activity (glucose equivalents, GE, per gram dry soil) in soils treated with Ecostone HPP5000, a powdered formulation of cellulases from *Trichoderma reesei*. Treatments included water control (0x1), heat-treated enzyme at 1000 U/ml (1000htx1), 100 and 1000 U/ml enzyme (100x1, 1000x1) applied once at time 0 days, and 100 and 1000 U/ml enzyme applied three times (100x3, 1000x3) at times 0, 7, and 14 days. Analyses were conducted one day after the first applications (1 day) and 1 day after the third applications (15 days). Bars with the same letter are not significantly different from one another at α=0.05.

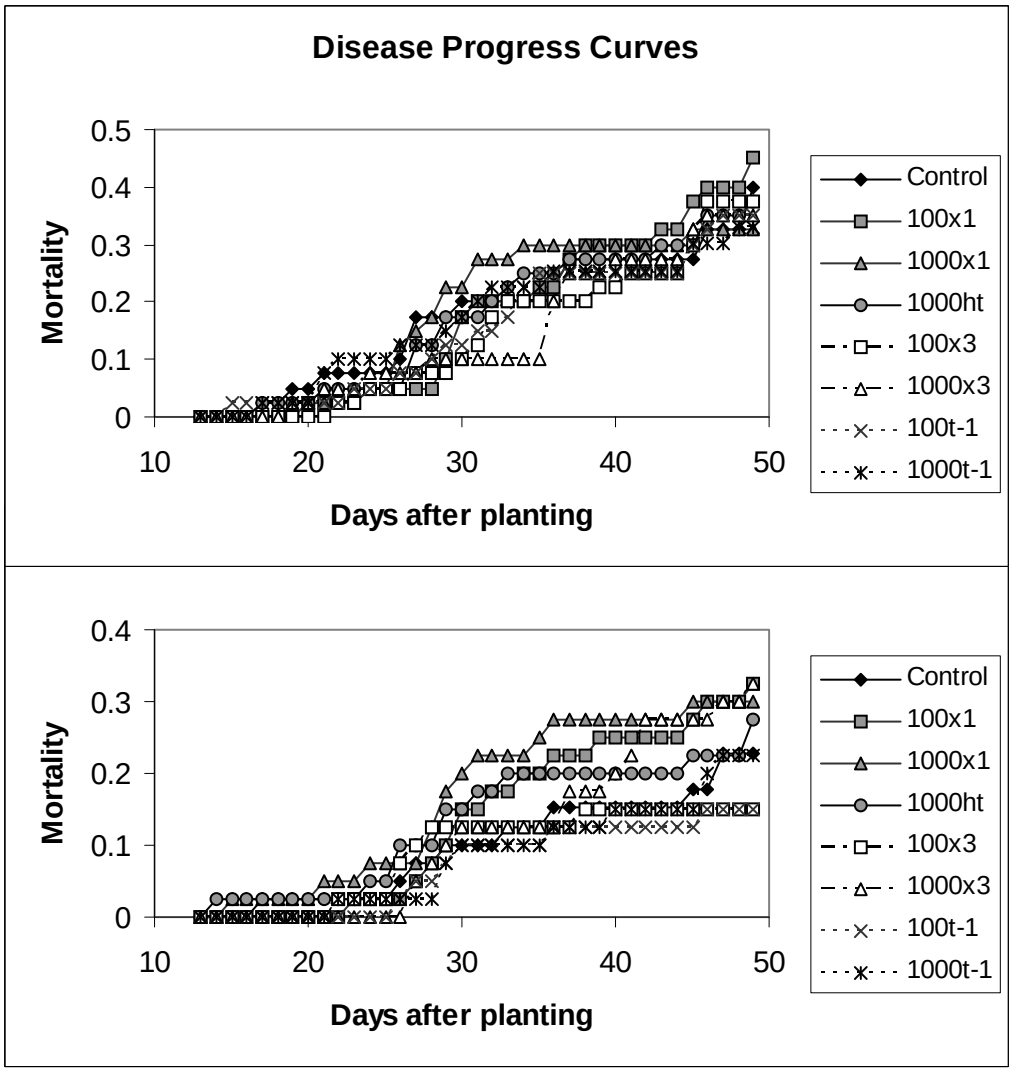

Figure 7. Disease progress curves from two replicate lupine bioassays in soils infested with *Phytophthora cinnamomi* and treated with Ecostone HPP5000, a powdered formulation of cellulases from *Trichoderma reesei*. Treatments included water control (Control), heat-treated enzyme at 1000 U/ml (1000ht), and 100 or 1000 U/ml enzyme applied once at 15 days before planting (100x1, 1000x1), applied three times at 1, 8, and 15 days before planting (100x3, 1000x3), or applied once one day before planting (100t-1, 1000t-1). Lupine seedlings were transplanted into treated soils and monitored for mortality every day for seven weeks.

CHAPTER 3

Soil and mulch amendments for suppression of *Phytophthora cinnamomi*: Effects of amendment type, microbial inoculation, and enhancement with cellulytic fungi

ABSTRACT

Numerous investigations have been made into the potential for biological control of Phytophthora root rot, caused by *Phytophthora cinnamomi* and other *Phytophthora* species, using mulches and organic soil amendments. Because field trials are limited in scope, container studies were established in order to examine a variety of amendment types and applications. A series of trials assessed organic amendments, with and without biological control agents, as well as gypsum and soil pH adjustment, for their impacts on Phytophthora root rot in Fraser fir. Isolates of cellulytic fungi were also investigated as potential biological control enhancers in a wood chip and dairy compost mulch. In trials with wood chips blended into soil and trials with seedlings planted directly into wood chip mulch, seedling survival was greater when wood chips were amended with compost, a soil inoculant, or a biocontrol agent. If wood chips were amended with compost, the addition of a cellulytic fungus, including a known biocontrol agent, did not further enhance plant survival, and in most cases did not significantly increase cellulase activity over compost amended wood chips alone.

INTRODUCTION

Organic amendments are often used in plant production systems to enhance growth, provide nutrients, and suppress disease. As a means of achieving suppression of soil borne Phytophthora diseases, a variety of soil amendments and mulches have been investigated, including yard waste (7, 11), sawdust (28), animal manures (1), sewage sludge (17), and various types of composts (1, 15, 18, 30). Some of these have also been investigated as carriers for specific biological control organisms (6, 9, 10, 16).

In addition to organic matter and the biological activity that it supports, soil chemical characteristics can contribute to suppression of soil borne Phytophthora diseases. In particular, calcium content and soil pH are factors with well-described impacts on *Phytophthora* species. In *in vitro* studies, calcium is known to promote sporangia production in *P. cinnamomi* at low concentrations, but to inhibit production at concentrations above 0.71m*M* (14), and in container studies, calcium amendment also reduced sporangial numbers and volume, zoospore production, and colony forming units (20). Calcium amendment can also impact soil drainage qualities, reducing surface crusting and increasing infiltration rates (5, 31), which may in turn reduce disease pressure by decreasing the occurrence of sustained matric potentials favorable to *Phytophthora* reproduction. A soil pH range between 5.0 and 6.0 is also favorable to *Phytophthora* reproduction, and pH levels below 4.0 are detrimental to production of sporangia (3, 4, 26). Low pH-dependent suppression has been demonstrated in container studies with rhododendron and lupine (4, 26).

Fraser fir (*Abies fraseri*) is one of over 1,000 host species susceptible to root rot caused by *Phytophthora cinnamomi*. Phytophthora root rot causes greater losses in Fraser fir production than all other diseases combined, and more North Carolina growers cite it as the most important pest management concern than any other production problem (25). Native to high mountain ridges in western North Carolina and Virginia, it is adapted to well drained, organic soils with low pH. Production areas, however, tend to be located in lower elevation sites, with more poorly drained mineral soils, and growers routinely lime soils to a target pH range of 5.5-5.8. Studies are currently underway to examine the potential utility of a mulching system for suppression of Phytophthora root rot in Fraser fir.

Field sites were established in the spring of 2006 to test the effects of raised mulch beds, as well as soil pH reduction in new plantings on previously infested sites. Due to constrictions on space and availability of various mulches in sufficient quantities to conduct field studies, a limited number of treatments were selected for use in these sites. These included pine wood chips, wood chips blended with compost (85% pine chips, 15% dairy compost, by volume), or shredded pine bark, applied on the soil surface, and dairy compost, tilled into the soil. Container studies were established in order to examine a wider variety of amendment types

and applications. These studies consisted of a series of three trials, with the objectives to *(i)* assess a variety of organic amendments, with and without biological control agents, gypsum, and pH adjustment for their impacts on Phytophthora root rot in Fraser fir; *(ii)* examine the contribution of dairy compost and a biological control agent to disease suppression in a wood chip mulch; and *(iii)* investigate the contribution of cellulytic fungi as potential biological control enhancers in wood chip mulch.

MATERIALS AND METHODS

Three studies were initiated during three consecutive years from 2006 to 2008. All trials were conducted at the Horticultural Field Laboratory at North Carolina State University (Raleigh, NC) on a gravel based container bed, under shade cloth, with overhead irrigation.

Study #1: Treatment of soil with various types of amendments.

Twelve treatments were established to test a range of organic amendments, biological agents, gypsum, and pH reduction. Treatments included: commercial dairy compost (Daddy Pete's Plant Pleaser, Stony Point, NC), hog manure compost (Super Soil Systems, Clinton, NC), hog lagoon compost (North Carolina State University, Raleigh, NC), vermicompost (Vermicycle Organics, Wilson, NC), greenwaste compost (City of Raleigh), pine wood chips (Keener Lumber Company), pine wood chips with *Trichoderma hamatum* T-382 (Sylvan Bioproducts, Cabot, PA), pine wood chips with SC27 soil inoculant (Natural Science Center, Steele, AL), coarse gypsum, low pH, low pH with gypsum, and an unamended control. A 1:1:1 blend of steamed soil, sand, and perlite was used as the base medium, to minimize background organic matter and allow good drainage in pots. All organic amendments were blended with the soil mix at a rate of 20%, by volume. The *Trichoderma hamatum* T-382 product was blended into soil-woodchip mix at a rate of 0.11g/L. SC27 (1:10 dilution) was applied as a drench at 200 ml per pot, one week prior to inoculation. Gypsum was blended at a rate of 5%, by volume. The "low pH" pots received four weekly drenches of pH 1.7 sulfuric acid; however, this treatment did not successfully decrease measured pH values below control soil values.

Fraser fir seedlings were planted into soil mixtures in 4-Liter pots, and were maintained under shade cloth with sprinkler irrigation for the duration of the study. Plants were arranged in a randomized complete block design with four uninoculated and four inoculated blocks; each inoculated block contained four plants per treatment, and each uninoculated block contained two plants per treatment. Blocks of uninoculated plants were placed upslope of inoculated plants to prevent spread of the pathogen during heavy rainfall events. Plants were inoculated three weeks after potting, using rice grain inoculum. *P. cinnamomi* (isolate 0146, from Fraser fir, Watauga County, NC) was grown on sterile white rice for one week, and two colonized rice grains were placed into the soil at each of three points around the base of each seedling, midway between the stem and the pot edge. Plants were monitored and rated weekly from 48 to 120 days after potting for disease symptoms and mortality. Disease was rated on a four point rating scale, where "1" was a healthy plant, "2" was exhibiting chlorosis, "3" showed chlorosis with wilting and/or branch death, and "4" was a dead plant.

Study #2: Amendment of wood chips with compost and a biocontrol agent.

Because wood chips with either microbial amendment or a biocontrol fungus showed greatest survival of Fraser fir in the first trial, and wood chips alone showed the lowest survival, the second trial was designed to examine the effects of microbial enhancement of wood chip mulch. In field trials, wood chips were applied as a raised bed, and compost was used to provide a larger and more diverse microbial population at the onset of the study than might be found in wood chips alone. In this container trial, Fraser fir seedlings were planted directly into wood chips, wood chips with dairy compost (15%, by volume), or wood chips with dairy compost and *Trichoderma hamatum* T-382 in 4-Liter pots. A standard potting mix (six parts pine bark, one part peat, four pounds lime per cubic yard) was used as a known disease conducive control. Twelve inoculated and six uninoculated plants per treatment were arranged in a randomized complete block design, with uninoculated blocks placed up-slope of the inoculated blocks. Plants were inoculated with colonized rice grains, as above, ten days after potting, and were rated biweekly for disease severity and mortality through 96

days. Pots were allowed to overwinter, and data were collected again the following spring, at days 300 and 340.

Because the *Trichoderma hamatum* T-382 product used in this trial was more than two years old, a sample of the inoculum was plated for determination of propagule density at the time of study establishment. The product sample was diluted in sterile water and plated on three replicate sets of Modified Rose Bengal agar (27) and Trichoderma Medium (8). Plating was repeated twice, and *Trichoderma* was not detected at the 10^3 CFU/g level in either test. The second test included a control sample from another product batch, which did provide positive results. At 20 days after establishment, samples were taken from one *Trichoderma*-amended and one non-amended pot, and two replicate samples from each treatment were plated on Trichoderma Medium. *Trichoderma* spp. were observed at approximately $2x10^4$ CFU/g dry sample in both amended and non-amended treatments.

Study #3: Amendment of wood chip mulch with cellulytic fungi.

In both previous trials, wood chips blended with compost provided significant suppression of Phytophthora root rot in Fraser fir. Because suppression in woody mulches has been associated with cellulase activity (12), it was reasoned that efficacy of the wood chip mulch might be enhanced by the addition of known cellulytic fungi. "Bio-enhanced" mulches have been investigated elsewhere for control of Phytophthora root rot (9, 10); these systems attempt to combine the specific suppression of a known biocontrol agent with the general suppression provided by the background microbial population, while also using the mulch base to provide a nutrient source for the biocontrol organism. This study was conducted to examine the effects of different cellulytic fungi on cellulase activity within the mulch and on suppression of Phytophthora root rot.

Eleven treatments were established in a base of wood chips with 15% compost, by volume, to include nine fungal isolates, gypsum, and an untreated control. Five of the fungal isolates were unidentified isolates recovered from field-applied wood chip mulch samples (Chapter 1), three were compost isolates acquired from the Pennsylvania State University disease collection (*Chaetomium* sp. DC16, *Chaetomium olivaceum* DC19, and *Scytalidium*

thermophilum DC295), and one was isolated from the *Trichoderma hamatum* T-382 product used in previous trials. Fungi were initially isolated from wood chip mulch either by dilution plating on cellulose agar (2), or by placing whole wood chips on sterile, moist filter paper in Petri dishes. Fungi which showed good clearing of cellulose on agar, or which colonized filter papers, were transferred to RB agar and malt extract agar with 250 mg/L streptomycin for isolation. Thirteen wood chip isolates, four PSU isolates, and the T-382 isolate were then transferred to potato dextrose agar, malt extract agar, and CMCA (22). Growth rates were monitored on each medium, and cellulose plates were stained with 1mg/ml-Congo Red (19) at two to nine days after transfer, depending upon colony growth rate. Cellulose clearing zones were measured by drawing two perpendicular lines through the center of each colony and measuring the radius from the plug edge to the colony edge (A) and the plug edge to the edge of the clearing zone (B) along each of the four transect lines. The average of the four (*A*-*B*) differences was recorded as the size of the clearing zone. Many of the isolates had distinct clearing under the colony and between hyphae, but the clearing zone did not extend beyond the colony edge; this appeared to be particularly true of fast-growing colonies with thin or feathery margins. To account for this clearing, isolates were also rated as (+) weak, (++) strong, or (+++) very strong for clearing underneath the colony. Isolates with a combination of prolific growth and good cellulytic activity were selected for use in the mulch trials. Each of the nine selections was transferred to a fungal spawn bag (Fungi Perfecti, Olympia, WA) containing a sterile blend of 500 cm^3 vermiculite, 40 cm^3 ground whole oats, and 250 ml V8 broth (23). Bags were placed in individual plastic bins and incubated in the dark at room temperature for three weeks prior to use.

Colonized vermiculite inoculants and gypsum were blended into wood chip compost mix at a rate of 5%, by volume. Fraser fir seedlings were potted into the treated mixtures in one-liter Anderson bands, and placed in a greenhouse in a complete randomized block design with seven inoculated and three uninoculated blocks. Inoculated pots received two *P. cinnamomi* infested rice grains (isolate 208, from Fraser fir, Watauga County, NC) at each of two opposite corners, placed approximately one centimeter from the pot corner and two centimeters under the mulch surface, at 14 days after planting. At eight weeks after planting,

the containers were moved from the greenhouse to the Horticultural Field Lab facility, where they were maintained with daily irrigation under shade cloth for the duration of the study. At 100 days after planting, mortality was low across all treatments, and inoculation with *P. cinnamomi* was repeated, as above. Plants were monitored for an additional 60 days.

At the end of the 160-day growth period, mulch was collected from all containers in odd-numbered blocks, to yield four samples per treatment. Cellulase activity was assessed in mulch samples by a modified Schinner and von Mersi method (24, 29). Samples were incubated at 37ºC for 24 hours in 0.35% w/v carboxymethyl cellulose in acetate buffer. After incubation, aliquots were transferred to microcentrifuge tubes and centrifuged for 3 minutes. Supernatants were diluted and processed for photometric analysis of reducing sugars. Three sub-samples and one substrate negative control were processed for each mulch sample.

Statistical Analyses. SAS® software (Version 9.1, SAS Institute Inc., Cary, NC, USA) was used for all statistical analyses. Area under the disease progress curve (AUDPC) was calculated for both ratings and survival data from each study, and these were compared using the general linear model analysis (GLM). Ratings and survival at the conclusion of each trial were also examined using Friedman's non-parametric analysis; means comparisons were performed using an F-approximation to Friedman's, by ranking data within blocks and using the GLM on ranked data with Tukey's HSD test. An alpha level of α=0.05 was used for all tests.

RESULTS

Study #1: soil mix treated with various types of amendments.

Within five weeks of potting, root rot symptoms were evident on both inoculated and uninoculated plants. Further testing of both study plants and extra seedlings not included in this study revealed that the seedling lot purchased for use in this study had contained plants which were infected with *Phytophthora* but were asymptomatic. Results of this study were somewhat compromised, in that some portion of the seedlings were already infected, and the treatments were all designed to have prophylactic, rather than curative, effects. In the

undefined portion of plants which were already infected, the treatments did not have opportunity to break the infection cycle. Despite the reduction in statistical strength of this test, some significant differences were observed (Figure 1). Area under the disease progress curve (AUDPC) was calculated for ratings within each inoculated block, yielding four ratings per treatment. A GLM analysis conducted on these AUDPCs was significant ($P<0.001$) for treatment effects, and means separation with Tukey's HSD test showed that wood chips with T-382 and wood chips with SC27 had significantly lower AUDPC for disease ratings than did wood chips alone or acid-treated soil with gypsum. AUDPCs from mortality data did not constitute a normal data set, so these were analyzed with Friedman's test, and means separations were calculated from a GLM analysis on ranked data. This analysis yielded the same results as for AUDPC based on disease ratings ($P<0.001$). Friedman's analysis of final disease ratings (day 120) also showed a significant treatment effect ($P=0.004$), and means separation from a GLM analysis of ranked data confirmed the same differences observed with AUDPCs.

Study #2: amendment of wood chips with compost and a biocontrol agent.

Areas under the disease progress curve were calculated for disease ratings and mortality with data through day 96, and again with data through day 340. All AUDPC data were analyzed with Friedman's test, followed by a GLM on ranked data with Tukey's adjustment used for means separations. For both disease rating and mortality data through the first season, there was disagreement between the two tests, wherein Friedman's test did not detect a significant treatment effect at $\alpha=0.05$ ($P=0.058$ for ratings, 0.0623 for mortality), but GLM analysis on ranked data did ($P=0.026$ for ratings, 0.031 for mortality). Means separation on ranked data indicated a significant difference in both disease ratings and mortality between trees grown in wood chips and wood chips blended with compost. For AUDPCs calculated on disease rating data through day 340, both Friedman's and GLM analysis of ranked data showed significant treatment effects ($P=0.034$, $P=0.007$). Plants in wood chips alone had greater AUDPC than those in wood chips with compost, with or without T-382, and plants in wood chips with compost had the lowest AUDPC, significantly

lower than both control and wood chip treatments (Figure 2). For AUDPCs calculated on mortality data through day 340, tests again disagreed on significance at α=0.05 (Friedman's P=0.072, GLM on ranked data P = 0.041). Means separations from GLM analysis on ranked data only detected a difference between plants grown in wood chips with or without compost.

Study #3: wood chip mulch amended with cellulytic fungi.

Areas under the disease progress curve were calculated for disease ratings with data through day 88, at which time plants were re-inoculated, and again with data through day 144. There was no significant difference among treatments for AUDPC based on ratings at day 88 (P=0.824) or day 144 (P=0.416). At day 88, only 12% of the seedlings had died across all treatments, with a maximum mortality of 33% observed in plants with T-382, and no mortality at all in five of the eleven treatments. After the second inoculation, plants across all treatments declined rapidly, with an overall mortality rate of 78% by day 144. The highest final survival rate was 50% in the plants which received wood chip isolate number 775.3, followed by 43% survival in plants which received gypsum (Figure 3). A GLM analysis of cellulase activity in mulches at the end of the study was significant for treatment effects (P<0.001), with higher cellulase activity in mulch amended with wood chip isolate number 767.3 than in all other treatments (Figure 4). The lowest cellulase activity was observed in mulch amended with gypsum, but this difference was not significant.

DISCUSSION

Despite numerous technical difficulties, which likely obscured treatment effects in two out of three container trials, several significant differences and trends emerged through this series of studies. The first of these was a consistent difference in disease progression between plants treated with wood chips alone, versus those treated with wood chips and a source of microorganisms, whether from dairy compost, an undefined general microbial inoculant, or a known single biocontrol agent. Because disease control with mulches and organic amendments is typically tied to the increased biological activity associated with the organic substrate, there may be value in providing not only organic matter, but also a source

of microorganisms to rapidly initiate biocontrol processes within the root zone. In field studies, the microbial component indigenous in the soil may be sufficient to quickly supply a diverse array of organisms, with a high probability that at least some of them will perform one or more biocontrol functions. In field studies with Fraser fir (Chapter 1), we did not see consistent differences among mulch types in microbial numbers, diversity, or activity by the first sample date, implying that within five months, similar population levels were established with or without the addition of a microbial source. In these container studies, however, the mulch materials were enclosed within plastic pots and were not in contact with underlying soil; there was little opportunity for the materials to become rapidly colonized, and this may have contributed to the differences in disease suppression observed between microbial-amended and non-amended wood chip treatments.

In studies with avocado mulches, gypsum has been shown to be an important component of the mulching system (20, 21). Results in these container studies did not provide any conclusive evidence for the role of gypsum in disease control. Poor performance of gypsum in the first container study may be attributed to the prophylactic nature of the mechanisms behind calcium-mediated disease suppression. Since many of the trees had preexisting *Phytophthora* infections, an amendment that works by impairing the pathogen's ability to reproduce and infect the host would not be likely to have a significant impact on disease progression. In the third container study, gypsum was combined with both organic matter and a microbial source, and this treatment yielded the second-highest survival among eleven treatments, but the differences were not statistically significant. Further work will be needed to determine whether gypsum would be a valuable component of mulching systems for Fraser fir.

Cellulase enzyme activity is a prominent microbial component which has been repeatedly associated with suppression of Phytophthora root rot (12, 13). If mulch systems could be managed to maintain a high level of cellulase activity, by selection of substrates and by amendment with known high-cellulase producers, there is potential that this mechanism could be enhanced to provide greater disease suppression. In the third container study, known cellulytic fungi were added to the best performing mulch from the earlier studies.

After the initial inoculation, all treatments were performing well, and there was low mortality across the entire study. After the second inoculation, plants in all treatments declined, still with no treatment effects. It appears that the wood chips with compost, which had provided suppression in the earlier container trials, continued to provide reasonably good suppression with or without the addition of specific cellulytic fungi, and that when the disease pressure was increased, the protection broke down regardless of the addition of either cellulytic fungi or gypsum. Cellulase activity levels were high across all treatments, but were significantly higher with one fungal isolate, wood chip isolate number 767.3, than in any other treatment. This increase in cellulase activity did not translate into an increase in disease suppression. The relative contribution of cellulase enzyme activity, among all possible mechanisms which may contribute to disease suppression in mulches, is not well understood. To whatever extent cellulase activity is responsible for suppression of Phytophthora root rot of Fraser fir grown in mulch, it appears that the level of activity provided in a pine wood chip and dairy compost mulch is sufficient for the full expression of this mechanism. While we were able to further enhance cellulase activity with the addition of one of the cellulytic fungal isolates, the additional cellulase did not result in additional disease suppression.

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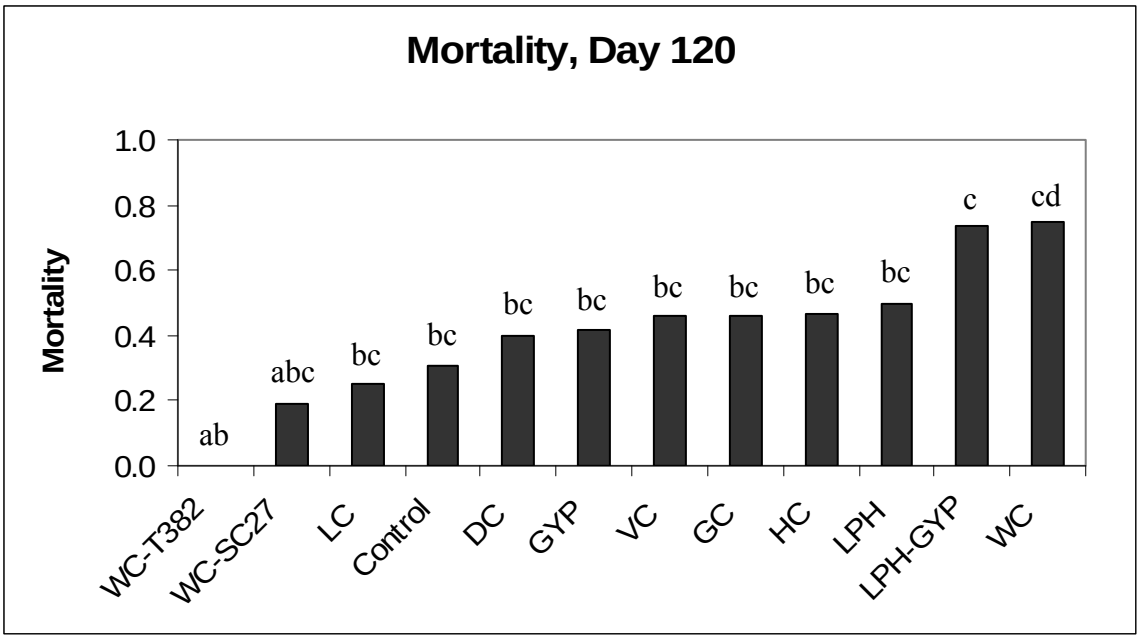

Figure 1. Final mortality ratings of Fraser fir seedlings grown in soil with various amendments and inoculated with *Phytophthora cinnamomi*. Mortality is shown as proportion of plants deceased at 120 days after planting. Treatments shown are: wood chips with *Trichoderma hamatum* T-382 (WC-T382), wood chips with soil inoculant SC27 (WC-SC27), hog lagoon compost (LC), untreated control soil (Control), dairy compost (DC), 5% gypsum amendment (GYP), vermicompost (VC), greenwaste compost (GC), hog manure compost (HC), acid-treated soil (LPH), acid-treated soil with 5% gypsum (LPH-GYP), and wood chips. All organic amendments were incorporated in soil at a rate of 20%, by volume.

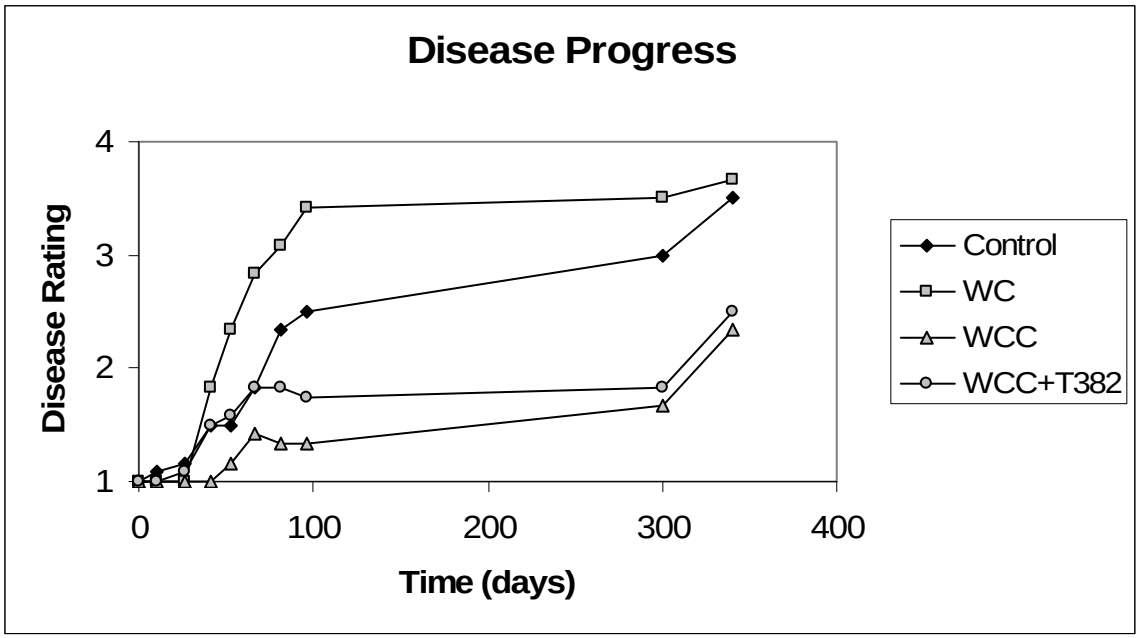

Figure 2. Disease progress curves based on average disease ratings for Fraser fir seedlings grown in a disease-conducive potting mix (Control), pine wood chips (WC), pine wood chips amended with dairy compost (WCC), and amended wood chips treated with a *Trichoderma hamatum* T-382 product (WCC+T382). Disease ratings were conducted on a four-point rating scale, where a rating of "1" was a healthy plant and "4" was a dead plant. Areas under the disease progress curve were: 1047.4 (WC), 846.2 (Control), 593.2 (WCC+T382), 497.3 (WCC).

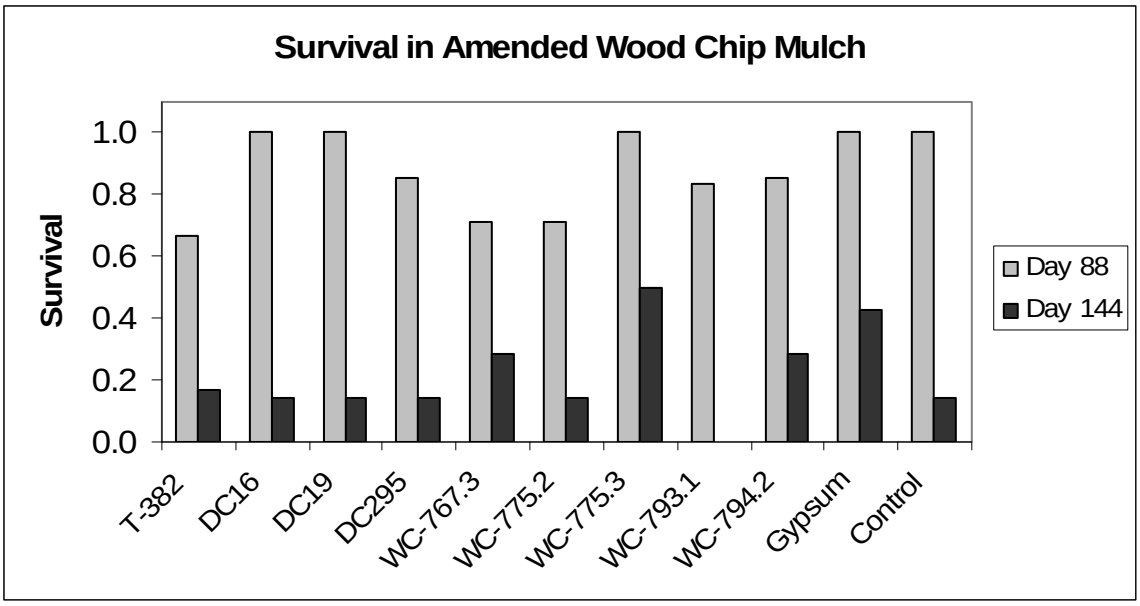

Figure 3. Survival of Fraser fir seedlings at 88 and 144 days after initial inoculation with *Phytophthora cinnamomi*. Survival is shown as proportion of living plants in each treatment. Treatments included nine fungal isolates or gypsum, blended into a wood chip and dairy compost mulch. Plants were inoculated with *Phytophthora cinnamomi* infested rice grains once at time zero and again at 88 days.

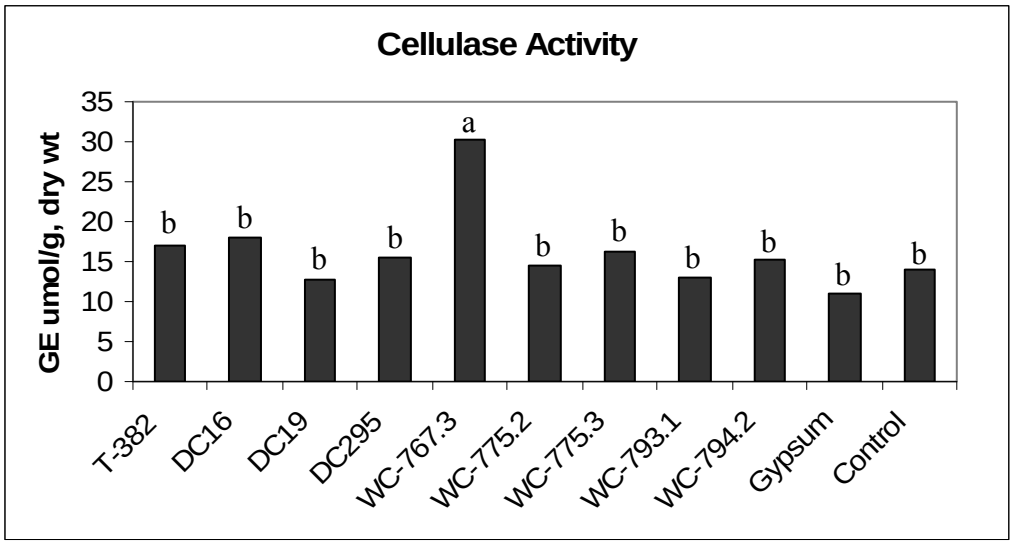

Figure 4. Cellulase activity in wood chip and dairy compost mulch, amended with each of nine fungal isolates or gypsum, or unamended. Cellulase activity is reported as concentration of reducing sugars (micromoles glucose equivalents, GE) per gram dry sample after incubation with 0.35%CM-cellulose at 37ºC for 24 hours.