Cellulase Activity as a Mechanism for Suppression of *Phytophthora* Root Rot in Mulches

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ABSTRACT

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Wood-based mulches are used in avocado production and are being tested on Fraser fir for reduction of *Phytophthora* root rot, caused by *Phytophthora cinnamomi*. Research with avocado has suggested a role of microbial cellulase enzymes in pathogen suppression through effects on the cellulosic cell walls of *Phytophthora*. This work was conducted to determine whether cellulase activity could account for disease suppression in mulch systems. A standard curve was developed to correlate cellulase activity in mulches with concentrations of a cellulase product. Based on this curve, cellulase activity in mulch samples was equivalent to

a cellulase enzyme concentration of 25 U ml⁻¹ or greater of product. Sustained exposure of *P. cinnamomi* to cellulase at 10 to 50 U ml⁻¹ significantly reduced sporangia production, but biomass was only reduced with concentrations over 100 U ml⁻¹. In a lupine bioassay, cellulase was applied to infested soil at 100 or 1,000 U ml⁻¹ with three timings. Cellulase activity diminished by 47% between 1 and 15 days after application. Cellulase applied at 100 U ml⁻¹ 2 weeks before planting yielded activity of 20.08 µmol glucose equivalents per gram of soil water (GE g⁻¹ aq) at planting, a level equivalent to mulch samples. Cellulase activity at planting ranged from 3.35 to 48.67 µmol GE g⁻¹ aq, but no treatment significantly affected disease progress. Based on in vitro assays, cellulase activity in mulch was sufficient to impair sporangia production of *P. cinnamomi*, but not always sufficient to impact vegetative biomass.

The use of mulches to reduce disease from soilborne *Phytophthora* species has been investigated in a number of cropping systems (7,8,12,25,27,39). Efficacy of mulches for disease suppression is variable, with decreases in inoculum levels or disease incidence in some cases (8,12,25,39), and either no effect or enhanced disease effects in others (7,27). The best documented successes in suppression of *Phytophthora* diseases with mulch have come from variations of the “Ashburner system,” wherein cellulose-rich mulches have been used in Australia (5,41,42) and California (12,15) to reduce *Phytophthora* root rot disease in avocado, caused primarily by *Phytophthora cinnamomi* Rands. Similar systems are also under evaluation in North Carolina Christmas tree plantations for suppression of *Phytophthora* root rot in Fraser fir, also caused primarily by *P. cinnamomi* (35). Understanding the modes of action that predominate in successful systems, such as the Ashburner system and its variants, may help improve success of mulches for suppression of soilborne *Phytophthora* diseases in other crops.

In examinations of avocado mulching systems, impacts of mulch on *P. cinnamomi* have included reduced differentiation and release of zoospores, reduced movement of zoospores, reduced inoculum potential, and increased lysis of hyphal cell walls (10,13). Disease suppression has been consistently associated with the microbial component of the mulch (10,13,41,42) and may be affected through any combination of biocontrol modes of action: antibiosis, competition, hyperparasitism, and plant defense induction.

Early 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 (4,28). Numerous isolates of actinobacteria (16,24,31,43) and fungi (6,16,18,20,28,31,33) have since been identified as antagonistic against *P. cinnamomi*, but no single organism or taxonomic group has been consistently linked with suppression, nor shown capable of causing suppression equivalent to that observed in mulches. More recently, it has been postulated that cellulase enzymes produced by mulch-inhabiting organisms may create a suppressive environment by degrading the cellulosic component of *Phytophthora* cell walls (14). Activities of cellulase and other enzymes are typically elevated in mulch, relative to unmulched soils (11,13,35), and Downer et al. (13) reported that enzyme activities in mulch and soil were positively correlated with microbial activity and fungal counts but negatively correlated with *Phytophthora* recovery. 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). Downer et al. (14) showed that addition of 10 to 25 U ml⁻¹ of a cellulase product to *Phytophthora* cultures in soil extract impaired zoospores and chlamydospore development, and concentrations greater than 25 U ml⁻¹ disrupted mycelium.

Although results from these earlier field and laboratory studies suggest a possible role of cellulase enzymes in pathogen suppression within mulch, differences in enzyme units between the field and lab studies preclude direct comparisons between them. At present 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 (40), while purified enzyme concentrations are expressed as enzyme units (U), where one unit is the amount of enzyme that catalyzes the conversion of one

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micromole of substrate per minute, under defined conditions (22). 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 translated for comparison with in vitro assays.

This work was conducted to determine whether cellulase activity in mulch can be sufficient to cause pathogen suppression in the absence of other mechanisms. The primary objectives of this research were to 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 to determine the impact of these cellulase levels on *P. cinnamomi*. Because earlier field work has shown little or no enhancement of cellulase activity in soil beneath mulch, and has correlated this stark difference in enzyme levels with incidence of root infections (13,35), this work also undertook a second objective to ascertain the effects of exogenously applied cellulases on *Phytophthora* inoculum potential in soil and to track the duration of cellulase activity associated with exogenous cellulase applications.

MATERIALS AND METHODS

Isolates and enzyme products. Two isolates of *P. 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 University of California-Riverside, designated M-295, was also included (14). The selection of cellulase source was based on commercial availability and compatibility with assay methods. Since the cellulase product used by Downer et al. (14) was no longer available, seven commercial enzyme products were initially tested in the preparation of enzyme activity curves (described below), and one product, 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 and Von Mersi method commonly used for soil analysis (36,40). To determine cellulase product compatibility with the soil assay technique, each product was diluted to create a stock solution, 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^{-1} . One milliliter of each diluted enzyme solution was incubated in 4.0 ml of 0.35% carboxymethyl cellulose (CM-cellulose) in 2 M acetate buffer, pH 5.5, at 37°C, with three replicate subsample tubes and two buffer control tubes. Samples were drawn at 5 and 24 h 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, Madison, WI) at 690 nm. Each commercial enzyme product was assayed twice, and each set of assays included an internal control series of glucose samples. Products were evaluated for consistent performance under assay conditions, and relatively low background color development due to the presence of bulking and stabilizing agents. Two enzyme products were further tested for optimal CM-cellulose concentration and incubation time. Ecostone HPP5000, incubated for 24 h in 0.7% CM-cellulose, yielded the most consistent results, and its activity showed the greatest sensitivity to changes in product concentration. Thus, it was used to develop the standard activity curve which allowed us to convert product concentration to mulch assay-associated activity, and vice-versa.

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 2-year-old beds in Christmas tree plantations at field locations in Avery County and Watauga County, NC, and Grayson County, VA (35), and were selected to provide a wide range of enzyme activity levels, based upon analyses from field studies. One of the six mulch samples was made of shredded pine bark, and the others 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, at particle surfaces and between solid particles rather than within the solids themselves, results were reported as glucose equivalents per gram aqueous fraction ($\mu\text{mol GE g}^{-1} \text{aq}$). This was calculated as $(\text{GE } \mu\text{mol})/(V_i)(df)/V_s$, where GE was calculated from the absorbance value, based on a calibration curve, V_i was total volume of liquid in the incubation vessel (buffer, substrate, and V_s), df was the dilution factor, and V_s was the volume of liquid in the aqueous portion of sample (assuming 1 g of water $\sim$ 1 ml).

In vitro assays. Cellulase effects on *Phytophthora* biomass and sporangia production were examined by single applications of or sustained exposure to the enzyme product Ecostone HPP5000. Cultures were grown in sterile buffered pea broth (BPB), pH 5.8, produced by adding 8.5 ml of 1 M K_2HPO_4 and 91.5 ml of 1 M KH_2PO_4 to pea broth (120 g of peas in 500 ml of water, autoclaved 5 min, and strained through cheesecloth), and filling to 1 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 analytical replicates were used for each isolate at each enzyme level, and each trial was performed in duplicate. All single-application trials used a completely randomized design. To facilitate solution replacement in sustained-application trials, dishes were not randomized; instead, location effects were minimized by rotating dish positions within the incubator after each enzyme replacement (twice daily).

For colony 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 (50 ml of clarified V8 juice and 15 g of Bacto agar per liter; clarified V8 juice produced by adding 1 g of CaCO_3 to 100 ml of V8 vegetable juice, hand shaking, and then centrifuging at 4,000 rpm for 20 min) 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 27°C until colony diameters reached approximately 3 to 4 cm. Growth solution was then drained from the dishes and replaced with enzyme product solutions that were prepared with BPB as described above. For single-application trials, two enzyme concentration ranges were tested, with one including 0, 1, 5, 10, 25, 50, and 100 U ml^{-1} , and the other including 0, 1, 10, 100, and 1,000 U ml^{-1} . Sustained exposure trials used a series of 0, 10, 50, 100, and 500 U ml^{-1} . After enzyme addition, dishes were incubated at 27°C until control colonies reached the dish edge. For all biomass calculations, the original plug was excised from each colony, and the colony was washed onto a preweighed P5 grade filter paper (medium porosity, slow flow, 5 to 10 μm particle retention) in a Buchner funnel over vacuum and rinsed twice with a stream of deionized water. Papers with colonies were dried at 70°C for a minimum of 24 h, and colony dry biomass was calculated after subtracting the average control paper (no colony) loss from all initial weights.

For sporangia assays, a preliminary trial was conducted to select the most suitable sporulation solution for the *P. cinnamomi* isolates. Solutions tested included three soil extracts, a potting

mix extract, and two modifications of the Chen-Zentmyer mineral salts solution (9,34). The greatest numbers of sporangia were produced in the potting mix extract (FME), a 2%, wt/vol, 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. Five-millimeter plugs of each isolate were cut from the margins of actively growing cultures of *P. cinnamomi* on V8 juice agar and transferred to sterile 6-cm petri dishes. Dishes were filled to level with the top of the plug with BPB and incubated at 27°C for 3 days. The BPB was then drained from the dishes, colonies were washed three times in deionized water, and 8 ml of the appropriate enzyme dilution (0, 10, 50, 100, or 500 U ml⁻¹ prepared in FME) was added to each dish. After incubation for 2 days at room temperature under fluorescent light, the entire contents of each dish was blended for 25 s in 20 ml of physiological saline solution (0.9% NaCl). Two 1.5-ml aliquots were immediately transferred into microcentrifuge tubes for short-term storage at 5°C. Sporangia were counted by transferring 500 µl from each of the two microcentrifuge tubes into a chamber of an inverted two-chamber McMaster counting slide (Chalex, Wallowa, OR). Slides were examined at 40× to 100× magnification, and counts from the two subsamples were averaged.

Soil application trials. In order to test the effects of exogenously applied cellulase on infectivity of *Phytophthora*-infested root fragments as propagules, cellulase (Ecoston HPP5000) was applied to soil in 150-cc nursery cells (Ray Leach “Cone-tainer,” Stuewe & Sons, Inc., Tangent, OR). Eight treatments were applied, including 100 and 1,000 U ml⁻¹ cellulase concentrations, applied once either 1 day or 15 days before planting, or applied three times at 7-day intervals from 1 to 15 days before planting, heat-treated enzyme (1,000 U ml⁻¹, autoclaved 15 min at 121°C) applied 15 days before planting, and a no-enzyme control. Field soil was collected from five sites in Avery County, NC, at points adjacent to symptomatic Fraser fir trees within areas known to be infested with *P. cinnamomi*. All soils were classified as loams, with one sample each from the Rosman loam, Saunook loam, and Dellwood cobbly sandy loam series, and two samples from within the Edneytown-Pigeonroost complex. 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* (culture numbers 208, 959, and 2378) into the root zone of Fraser fir seedlings grown in 1 liter Anderson bands (Stuewe and Sons, Inc., Portland, OR), and then exposing the plants to repeated flood cycles. Two weeks after inoculation, the root systems were washed and chopped into 1-cm segments. Chopped segments from all trees were blended and added to the field soil mixture at a rate of 2 g wet weight liter⁻¹ of soil mix. Cells were filled with the root-infested soil mix and placed in racks in a greenhouse in a randomized complete block design, with five blocks, eight treatments, and eight subsamples for each treatment, for a total of 320 cells. Fifty milliliters of enzyme or control solution was added to each cell in order to completely saturate the soil. Cells were maintained on a bench with intermittent mist 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* ‘Tifblue’) were surface sterilized by rinsing in 95% ethanol, followed by 3 min in 5% commercial bleach, and then placed on sterile moist sand to germinate. One day after the final treatments, lupine germlings were transplanted into the soil cells. Plants were maintained under intermittent mist for the first 3 days, and 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 (17). Because the recovery rate on selective medium, at 85% recovery from root samples of symptomatic plants, was commensurate with expected rates (21,26,29), all mortalities showing symptom progression indicative of *Phytophthora* root rot were included in data analysis. The entire experiment was conducted twice, with mortality tracked over a 7-week period for each replicate experiment.

In order to track cellulase activity in soil over time, additional soil cells were set up as above for each of the single-application treatments (0, 100, and 1,000 U ml⁻¹, and 1,000 U ml⁻¹ heat-treated) and three-application treatments (100 and 1,000 U ml⁻¹). 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 (six cells for each treatment) were processed. Enzyme activity was measured using the modified Schinner and Von Mersi method, with 6.00 g of soil mix incubated in 24 ml of 0.7% CM-cellulose solution at 37°C for 24 h (36,40). Remaining soil from each cell was used to calculate moisture content after drying at 110°C for 24 to 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 Ecoston HPP5000 cellulase product.

Statistical analyses. Analysis of variance was conducted on data from in vitro assays using the general linear model (GLM) function of SAS software (SAS Institute, Inc., Cary, NC), with Tukey’s HSD test applied for means separations. Where data were nonnormal and transformations did not normalize them, nonparametric analyses were used, as noted. GLM analysis on ranked data was used to conduct post hoc analyses on nonnormal 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 GE per gram of sample water) (Fig. 1). The resultant curve had a function of $y = 3.206\ln(x) + 3.806$ ($R^2 = 0.996$). Mulch samples from a 2-year established field site ranged in activity from 12.3 to 24.1 µmol GE g⁻¹ aq, which corresponded to an enzyme concentration range of approximately 14 to 553 U ml⁻¹ (Table 1).

In vitro assays. Colony biomass (as dry weight) did not decrease with increasing enzyme concentration in trials using a series of 0, 1, 5, 10, 25, 50, and 100 U ml⁻¹ (Fig. 2A), but did vary using a series of 0, 1, 10, 100, and 1,000 U ml⁻¹ (Fig. 2B). With

the lower concentration range (0 to 100 U ml⁻¹), analysis of variance detected 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 and no treatment differed significantly from the control (0 U ml⁻¹). Data from isolate 959 did not show significant effects of enzyme concentration on dry weight ($P = 0.075$). With the higher concentration range (0 to 1,000 U ml⁻¹), data were nonnormal, hence nonparametric analyses were used. A Kruskal-Wallis test showed significant treatment effects within each isolate in each repetition ($P = 0.049, 0.027, 0.012$, and 0.008 for isolates 208 and 959 first trial, isolates 208 and 959 second trial, respectively). GLM analysis of ranked data from both experiments indicated that dry weights from the 1,000 U ml⁻¹ enzyme level were significantly different from all other treatments, and no other treatment levels differed from one another. Colonies of *P. cinnamomi* under sustained enzyme exposure at a range of 0 to 500 U ml⁻¹ cellulase showed a pattern of decreasing dry weight with increasing enzyme concentration (Fig. 3). Significant treatment effects on colony dry weight were observed in both trials ($P < 0.001$), with the 500 U ml⁻¹ treatment causing significant reduction in dry weight relative to all other treatments ($P < 0.05$). Linear regres-

sion 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 (number of sporangia per colony) increased with enzyme concentration up to 100 U ml⁻¹, and then decreased at 500 U ml⁻¹ (Fig. 4). Enzyme concentration significantly affected sporangia production ($P < 0.001$), and the 500 U ml⁻¹ enzyme level significantly reduced sporangia production relative to 10, 50, or 100 U ml⁻¹ ($P < 0.05$). In trials with sustained enzyme application, sporangia production decreased sharply with increasing enzyme concentration (Fig. 5). 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 for means separation 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 to 100 U ml⁻¹. All regressions showed a significant negative relationship between enzyme concentration and sporangia production, 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 ($\mu\text{mol GE g}^{-1}$) in order to compensate for slight moisture differences among samples. Cellulase activity varied across treatments and over time

Fig. 1. Cellulase enzyme activity curve for Ecoston 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 37°C for 24 h in 0.7% carboxymethyl-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 2-year established field sites, processed under the same conditions as the commercial enzyme product. Points shown are from two experiments; 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.

TABLE 1. Cellulase activity in mulch samples from 2-year established field plots

Mulch type ^y	Site	Moisture (%)	GE, ($\mu\text{mol g}^{-1} \text{aq}$) ^z	GE, U ml ⁻¹
Wood chip/compost	Avery	72	12.3	14.1
Pine bark	Watauga	61	24.1	553.1
Wood chip/compost	Watauga	69	22.5	341.2
Wood chip/compost	Watauga	74	19.9	150.6
Wood chip/compost	Grayson	72	22.9	387.8
Wood chip/compost	Grayson	69	20.4	175.5

^y Mulch samples were either shredded pine bark or a blend of pine wood chips and dairy compost (15% by volume) collected from plots at three study sites.

^z Cellulase activity values are reported in micromoles glucose equivalents (GE) per gram of sample water ($\mu\text{mol GE g}^{-1} \text{aq}$). Unit translations (GE, U ml⁻¹) were calculated using a standard curve generated with Ecoston HPP5000, a powdered formulation of cellulases from *Trichoderma reesei*.

Fig. 2. Effects of a single application of Ecoston HPP5000, a powdered formulation of cellulases from *Trichoderma reesei*, on colony biomass of two isolates of *Phytophthora cinnamomi* (0208 and 0959) at two concentration ranges (A and B). Mean colony dry weights are shown for two replicate experiments conducted with each isolate. Bars represent standard error of the mean.

(Fig. 6). Activity in cells treated with autoclaved enzyme solution was similar to that observed in control cells. In cells which received a single enzyme application, cellulase activity diminished significantly ($P < 0.001$), to an average of 53% of original values over the course of 2 weeks. Cells which received three enzyme applications had significantly lower activity 1 day after the final treatment than cells which received a single application 1 day after treatment ($P = 0.008$ for 100 U ml⁻¹, $P < 0.001$ for 1,000 U ml⁻¹). 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 of 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⁻¹ enzyme and tested at 15 days after application ($P >$

Fig. 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* (0208, 0959, and M295). Mean colony dry weights are shown for two replicate experiments conducted with each isolate. Dry mass of colonies treated with 500 U ml⁻¹ cellulase was significantly reduced relative to all other treatments ($P < 0.05$).

Fig. 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* (0208 and 0959). Results shown are mean numbers of sporangia per colony across five enzyme concentrations, derived from two replicate experiments with each isolate. The 100 U ml⁻¹ enzyme level increased sporangia production relative to no enzyme (differences significant in one of two trials with each isolate), while the 500 U ml⁻¹ level significantly reduced sporangia production relative to 10, 50, or 100 U ml⁻¹ ($P < 0.05$) for both isolates.

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 7-week study period (Fig. 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 7 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.

Fig. 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* (0208, 0959, and M295). 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, derived from two replicate experiments with each isolate. In a general linear models analysis on ranked data with Tukey's adjustment for means separation, every treatment level was significantly different from each other level ($P < 0.01$) for all isolates tested.

Fig. 6. Cellulase activity (glucose equivalents [GE] per gram of dry soil) in soils treated with Ecostone HPP5000, a powdered formulation of cellulases from *Trichoderma reesei*. Treatments included water control, heat-treated enzyme at 1,000 U ml⁻¹ (1,000htx1), 100 and 1,000 U ml⁻¹ enzyme (100x1, 1,000x1) applied once at time 0 days, and 100 and 1,000 U ml⁻¹ enzyme applied three times (100x3, 1,000x3) 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 $\alpha = 0.05$.

DISCUSSION

Lysis of hyphal and sporangial walls has long been associated with suppression of *P. cinnamomi* in mulch-based cultural systems, leading to repeated postulations that the degradation of cell wall components by mulch-inhabiting organisms is primarily responsible for pathogen suppression in these systems (4,5,13,14,28). Other control mechanisms have been identified which impact reproductive potential of *P. cinnamomi* in mulch (3,30), and it has remained 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, observations of cell 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 (13), 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 2 years after application is equivalent to an enzyme concentration range of 14 to 553 U ml⁻¹ 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 2 years after establishment was significantly lower than levels observed at 5 months (35). 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 2-year-old mulches (equivalent to less than 200 U ml⁻¹ GE), *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⁻¹ or

more, which this work suggests may be equivalent to activity levels in some mulches, even at 2 years after application.

In previous studies on the effects of cellulase on *P. cinnamomi*, lysis was observed at much lower stated enzyme concentrations; Downer et al. (14) reported some lysis of hyphae with exposure rates above 25 U ml⁻¹, and complete disintegration at 100 U ml⁻¹. 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 variation in efficacy of the enzyme systems in the two products under the same conditions, or if they are the result of variations in product testing and labeling. Enzyme products may vary in relative abundance of exoglucanase, endoglucanase, and β -glucosidase, differences which may be obscured by testing and labeling of product enzyme units. Because enzyme units are defined by particular testing conditions, label descriptions of enzyme activity may also vary from one product to another depending on the test method used (32). Standard assays recommended by the International Union of Pure and Applied Chemistry (IUPAC) differ in substrate type, pH, and duration of incubation (19). Comparisons of commercial products using a standard substrate versus natural cellulose sources have also demonstrated that the standard assay was a poor predictor of performance on natural substrates, suggesting that cellulose degradation is more dependent upon substrate characteristics than on enzyme activity, as determined in standard assays (23). To further complicate efforts to describe enzyme activity in biological assays, products which are standardized

TABLE 2. Cellulase activity and moisture content of soils treated with Ecstone HPP5000, a powdered formulation of cellulases from *Trichoderma reesei*, compared with field mulch samples^y

Day	Treatment ^z	Moisture (%)	Cellulase activity	
			GE, dry (μ mol)	GE, aq (μ mol)
1	0x1	28	1.2 a	3.4 a
1	1,000htx1	28	2.1 a	5.9 a
1	100x1	28	13.8 c	38.1 c
1	1,000x1	28	20.1 e	55.5 d
15	0x1	27	1.3 a	3.9 a
15	1,000htx1	27	1.5 a	4.4 a
15	100x1	27	6.6 b	20.1 b
15	1000x1	27	11.5 c	34.4 c
15	100x3	27	11.7 c	34.8 c
15	1,000x3	26	16.9 d	48.7 c
—	Mulch	70	42.4 f	20.3 b

^y Values are means from six samples per treatment, or six mulch samples collected from 2-year-old established beds at field sites. Cellulase activity values are reported in micromoles glucose equivalents (GE) per gram of dry sample (GE, dry) and per gram of sample water (GE, aq). Values sharing the same letter within a column are not significantly different from one another at $\alpha = 0.05$ in a mixed model repeated measures analysis using least squared means separations with Tukey's adjustment.

^z Treatments included water control (0x1), heat-treated enzyme at 1,000 U ml⁻¹ (1,000htx1), 100 and 1,000 U ml⁻¹ enzyme (100x1, 1,000x1) applied once at time 0 days, and 100 and 1,000 U ml⁻¹ enzyme applied three times (100x3, 1,000x3) at times 0, 7, and 14 days. Analyses were conducted 1 day after the first applications (1 day) and 1 day after the third applications (15 days).

Fig. 7. Disease progress curves from two replicate lupine bioassays (A and B) in soils infested with *Phytophthora cinnamomi* and treated with Ecstone HPP5000, a powdered formulation of cellulases from *Trichoderma reesei*. Treatments included water control (control), heat-treated enzyme at 1,000 U ml⁻¹ (1000ht), and 100 or 1,000 U ml⁻¹ 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 1 day before planting (100t-1, 1000t-1). Lupine seedlings were transplanted into treated soils and monitored for mortality every day for 7 weeks.

against one another at 50 to 60°C, the typical range for standard assays, may also perform very differently at temperatures below 35°C, 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 may be made with certainty are those conducted within the same trial under identical conditions. However, both the associative observations made in previous work (13) 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. The range of activity measured in mulch samples within this study, however, suggests 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, cellulase primarily impacted 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 0 and 100 U ml⁻¹, 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 continuous suppression. With a single application of cellulase, enzyme activity diminishes over time as the enzyme molecules are bound, degraded, or inactivated in the soil (37). In this study, short-term exposure to cellulase enzyme, which was allowed to subsequently degrade, may have caused 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 (28) in studies with *P. cinnamomi*. Downer et al. (14) also noted that sporangia development was stimulated with very low levels of cellulase exposure, at 1 U ml⁻¹, in one of two trials, and that chlamydospore germination was stimulated with slightly higher exposure, at 10 U ml⁻¹. 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⁻¹ cellulase, rather than at 50 to 100 U ml⁻¹.

Cellulase-mediated reduction in sporangia production has the potential to slow the spread of *Phytophthora* within a site, and within a mulch may play a role 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 (1,2,38), 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 (14), 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 yielded only a short-term increase in cellulase activity and 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 before the enzymes were bound or inactivated within the soil.

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 (13,35) and physiological studies in vitro (14). Though it appears that the enzyme activity levels observed in mulch samples are not always adequate for direct lysis of *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. While not tested in the current study, cellulase activity at levels lower than those required to lyse hyphae may impair chlamydospore formation and zoospore encystment at the root surface, impacting both the survival and infection stages of the pathogen life cycle as well (14). 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 limited to "bulk" activity levels within a sample; differences in activity among soil or mulch samples can be measured 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 bulked soil or mulch. Enzyme activities determined in bulk soils can be useful indicators of microbial populations and processes, but bulk analysis is a relatively low-resolution approach to studying microbial interactions within soil and may provide only limited utility for elucidating the roles of soil organisms in plant disease suppression.

One challenge 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 is not affected by moisture content, which in most soil environments is a continually 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 target interest. However, when comparing soil to mulch, dry matter is less suitable as a reference; the pore volume and surface area represented by 1 g of soil are likely to be quite different than in 1 g 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 bridges earlier field and laboratory studies to provide preliminary evidence for the role of cellulase enzymes in suppression of soilborne *Phytophthora* diseases within 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, and further testing is needed to examine correlations between enzyme activity and disease suppression. While cellulase activity may be sufficient in some mulches to cause direct lysis of the pathogen

mycelium and propagules, this mechanism may not function in all mulch systems, and is likely to be diminished as mulches age. Our results suggest that in systems with lower cellulase activity, cellulases may play smaller but more varied roles in disease suppression, such that other biocontrol mechanisms are likely necessary to maintain pathogen suppression.

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