AN ABSTRACT OF THE THESIS OF

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To determine which isolates will most rapidly replace Phellinus weirii in stumps and roots, Trichoderma spp. were isolated from P. weirii-infested stumps and roots, identified, and examined for growth rates and lethal effects on P. weirii in vitro.

Phellinus weirii-infested Douglas-fir stumps and stump roots were sampled for microorganisms by aseptically transferring wood chips to malt agar plates. Trichoderma spp. were recovered infrequently (1.2 percent of all isolations), but their distribution suggested gradual invasion of the stumps and stump roots. Frequencies were greater in stumps of trees harvested eleven years previously than one year previously, in wood colonized than in wood not colonized by P. weirii, and in portions of roots further from the root collar. Trichoderma viride was the species most frequently isolated, followed by T. polysporum. No other Trichoderma species was isolated more than five times.

Isolates collected were tested for their linear growth rates on malt agar at 5, 10, 15, 20 and 25 C. Each species responded

differently to the temperatures tested and substantial variation also existed within species. Trichoderma viride and T. polysporum grew relatively rapidly at the lower temperatures, while T. harzianum and T. pseudokoningii grew rapidly at the higher temperatures.

Nine of the Trichoderma spp. isolates collected in the field were tested for their ability to kill two isolates of P. weirii growing on malt agar at 10 and 20 C. This test was made possible by the development of a medium incorporating three fungicides, which inhibited Trichoderma spp. but not P. weirii. Thus it was possible to differentiate rate of lethal effect on P. weirii from rate of overgrowth by the Trichoderma sp. At 10 C, the isolates of T. viride and T. polysporum killed P. weirii significantly faster than isolates of the other Trichoderma species. Significant differences also existed in the ability of the two P. weirii isolates to withstand the killing effect of Trichoderma spp.

It appears that T. viride and T. polysporum are the Trichoderma species which offer the greatest potential for reducing survival of P. weirii in the field. The ability of these species to colonize infested stumps and stump roots, to grow at temperatures normally found in the field, and to exert a lethal effect on P. weirii on artificial media has been demonstrated. Further tests are needed to thoroughly characterize antagonist and pathogen variability, and to compare results of antagonism tests on artificial media with those in the field.

Trichoderma spp.: Distribution in Stumps Infested With *Phellinus weirii* and Growth and Antagonism *In Vitro*

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INTRODUCTION

The fungus, *Phellinus weirii* (Murr.) Gilb., causes laminated root rot, a destructive disease of conifers in the Pacific Northwest of the United States and in British Columbia. Introducing antagonistic fungi, such as *Trichoderma* spp., into stumps and roots colonized by *P. weirii* has been proposed as a method of reducing survival of the pathogen, thus minimizing mortality in stands regenerating on infested sites. The research reported here attempts to increase our understanding of *Trichoderma* spp. as invaders of *P. weirii*-infested stumps and stump roots, and to identify some biological and environmental factors which may be important to consider when selecting antagonists.

Literature Review

Annual losses due to laminated root rot have been estimated at 4.4 million cubic meters of timber (Nelson et al., 1981). The disease is continued between rotations when roots of trees regenerating infested sites contact colonized roots of stumps from the previous stand (Wallis and Reynolds, 1965). *Phellinus weirii*

has been shown to survive in stumps and roots for up to 50 years (Hansen, 1979).

Control of laminated root rot can be achieved by reducing inoculum levels prior to planting susceptible species or by regenerating infested sites with immune or resistant species. No native conifers are immune although they vary in susceptibility (Hadfield, 1985). Of the tree species which are major components of western Oregon forests, Douglas-fir (Pseudotsuga menziesii [Mirb.] Franco) is highly susceptible, western hemlock (Tsuga heterophylla) is intermediate, and western red cedar (Thuja plicata) is relatively resistant. Choice of species for regeneration may be limited by silvicultural constraints. Hardwoods such as red alder (Alnus rubra) are immune and planting sites with this species to the exclusion of conifers in a crop rotation strategy prevents new infections and will eventually allow the inoculum to exhaust itself. It has been hypothesized that red alder actively reduces the longevity of buried P. weirii inoculum. Evidence is equivocal (Nelson et al., 1978), however, and in the most direct test to date, no reduction in survival was evident (Hansen, 1979). Economic analyses comparing Douglas-fir and red alder rotations (Tarrant et al., 1983) over the length of time necessary to exhaust inoculum indicate the greater economic value of Douglas-fir stands, but diseased sites must first be sanitized to minimize losses.

Minimizing infection in regenerated stands has most often been

attempted by reducing residual inoculum in stumps and roots of harvested, infected trees before replanting with susceptible trees. Several methods have been tested. Mechanical stump extraction removes much of the inoculum from the soil. Mortality has been reduced in the early years of stand development on some stumped sites (Thies, 1984). The procedure is expensive, however, and restricted to sites with moderate slopes. In addition, recent evidence indicates that small pieces of infested roots left in the soil may contain viable P. weirii inoculum for several years (Thies and Hansen, 1985). Fumigation of stumps has been studied (Thies and Nelson, 1982), and initial results indicate substantial reduction of P. weirii inoculum. Cost effectiveness and environmental side effects must be evaluated before this method can be employed on an operational basis. Reduction of inoculum might also be accomplished by introducing antagonistic fungi into infested stumps to eliminate the pathogen. Nelson and Thies (1985) introduced Trichoderma viride Pers. ex Gray. by inoculating P. weirii-infested stumps.

The antagonistic effects of Trichoderma spp. on plant pathogenic fungi are well documented (Cook and Baker, 1983). Weindling (1932) first observed hyphae of "Trichoderma lignorum" parasitizing hyphae of Rhizoctonia solani, Phytophthora parasitica, Sclerotium rolfsii, Pythium spp. and Rhizopus spp. Antagonism of soil-borne plant pathogens by Trichoderma spp. has been reviewed elsewhere (Papavizas and Lumsden, 1980; Cook and

Baker, 1983).

Antagonism of plant pathogens in woody substrates has also been studied. Gibbs and Smith (1978) distinguished two kinds of antagonists of plant pathogens in woody substrates: 1) Primary antagonists compete with the pathogen for uncolonized host tissue. Peniophora gigantea, which can be introduced into pine stumps to prevent invasion by Fomes annosus and subsequent spread to live trees, is a classic example (Rishbeth, 1963).
2) Secondary antagonists replace the pathogen in previously colonized host tissue. Gibbs and Smith (1978) considered Trichoderma spp. to be secondary antagonists. Trichoderma viride showed little ability to grow in fresh pine logs but could replace Fomes annosus and two other fungi occupying pine logs (Gibbs, 1967b).

The view that Trichoderma spp. are secondary antagonists is supported by reports of their detrimental effects on fungi inhabiting woody substrates. Armillaria root rot was controlled in citrus orchards by antagonism of Trichoderma spp. following fumigation with sub-lethal doses of carbon disulfide (Bliss, 1951). Investigations into the mechanism of this control showed that sub-lethal doses of fumigants, as well as heating or drying, stressed the pathogen, reducing its ability to withstand invasion. Following the stressing treatment, Trichoderma spp. were able to invade the Armillaria-colonized roots (Munnecke et al., 1981).

Other studies also demonstrate the ability of Trichoderma spp. to replace basidiomycetes in wood. Inoculation of T. viride into fruit trees infected with Stereum purpureum resulted in alleviation of symptoms associated with silver leaf disease (Dubos and Ricard, 1974; Corke, 1974). Lentinus edodes, the cultivated shiitake mushroom, is parasitized by Trichoderma spp. in oak bed logs (Komatsu and Hashioka, 1964; Komatsu, 1976). Trichoderma spp. invaded Phellinus weirii-colonized wood blocks buried in soil (Nelson, 1964 and 1976). Trichoderma viride preferentially colonized wood stained or decayed by P. weirii in infested stumps following inoculation (Nelson and Thies, 1985).

The latter study was conducted to determine the feasibility of establishing T. viride in P. weirii-infested stumps. One year after inoculation T. viride was successfully established in the upper portions of the stumps. Successful colonization was affected by the type of inoculum used, the season of inoculation, as well as the extent of stump decay by P. weirii. Inoculum formulated from fermented barley grains and T. viride spores was more effective than colonized wood dowels (Nelson and Thies, 1985). Inoculations conducted in June were less effective than those carried out in October or February (Nelson and Thies, 1986). No difference was observed in the extent of colonization by the three isolates of T. viride tested (Nelson and Thies, 1985).

The successful establishment of Trichoderma viride in P. weirii-infested stumps was encouraging, but the authors

concluded that to achieve a practical level of control, replacement had to be accelerated so that the lower stump and roots were colonized before the roots of the next generation of trees could make contact. One way to contribute to more rapid replacement would be to carefully select the Trichoderma spp. isolates used for inoculation. The three isolates of T. viride used in that study were all originally obtained from soil (Nelson and Thies, 1985). It is possible that other isolates of this species and even other species of Trichoderma might more rapidly replace P. weirii in stumps and roots.

Cook and Baker (1983) suggested that:

> "an antagonist sought for use in soil, on a plant surface, or within a plant tissue is most likely to be adapted to the niche where needed, if it is obtained from that niche originally."

The species and isolates of antagonists most likely to be adapted to conditions in P. weirii-infested stumps and stump roots will be those which are invading these tissues, even if the invasion is presently occurring at an unsatisfactory rate. The distribution of potential antagonists in P. weirii-infested stumps and stump roots should reveal if any are invading. An invading organism would increase in abundance over time. An invader particularly suited to replacement would preferentially colonize pathogen-colonized tissues. An invader might be distinguished from a prior occupant by a pattern of gradual colonization; that is tissues further from exposed surfaces would be colonized later

than those in close proximity to exposed surfaces.

Characteristics of isolates and species may affect the rate of replacement. Rapid growth rates of antagonists at appropriate temperatures may be important attributes. The lethal effect of isolates of Trichoderma spp. on Lentinus edodes was proportional to the amount of growth of the antagonist (Komatsu and Hashioka, 1964). Antagonism of Fomes annosus by T. harzianum Rifai and several other fungi was affected by growth rate, which varied with temperature (Lundborg and Unestam, 1980). Species of Trichoderma have been shown to differ in their temperature for optimum growth (Komatsu, 1976; Danielson and Davey, 1973b) and maximum temperature for growth (Danielson and Davey, op cit). Unfortunately, optimum and maximum growth temperatures are well above those found in forest soils in the western Cascades (Appendix I). Growth rates of isolates of Trichoderma spp. should be tested at appropriate temperatures to determine if there are differences between species and within species which will affect their competitive abilities in stumps and roots.

Another characteristic of isolates which could affect the rate of replacement is the degree of antagonism exhibited toward P. weirii. Antibiotic effects on pathogenic fungi differ considerably within species, as well as between species of Trichoderma (Dennis and Webster, 1971a and b). Variation in the antagonistic abilities of Trichoderma spp., and in the resistance of pathogens to them, necessitates screening isolates of this

species for specific situations rather than using one isolate for a wide range of applications (Bell et al., 1982).

Several parameters have been measured in studies assessing antagonism *in vitro*. The most common is the production of antibiotic compounds by the antagonist. Non-volatile antibiotics were measured by Dennis and Webster (1971a), Komatsu (1976), Sierota (1976), Magro et al. (1984), Tronsmo and Dennis (1978), Mitchell and Dix (1977), Klingstrom and Johansson (1973) and Gibbs (1967a and b). Volatile antibiotic production was measured by Mercer and Kirk (1984), Tronsmo and Dennis (1978), Bruce et al. (1984) and Dennis and Webster (1971b). Antibiotic production *in vitro*, however, was not always well correlated with effective antagonism *in vivo* (Lundborg and Unestam, 1980). Factors such as growth rate and ability to invade the substrate were also important. Hyphal interactions between target fungi and antagonists have also been studied (Dennis and Webster, 1971c; Tronsmo and Dennis, 1978; Elad et al., 1983). While qualitatively documenting hyperparasitism, these experiments do not easily lend themselves to quantification. Paired culture confrontations between target fungi and antagonists have also been used (Bell et al., 1982). The problems with assessing antagonism with this method are twofold. First, care must be taken to distinguish between superficial overgrowth and actual antagonistic effects, such as death of the target fungus. The most direct approach is to attempt to reisolate the target organism on a selective medium

following exposure to the antagonist. Bell et al. (1982) confirmed the viability or non-viability of Pythium spp., Rhizoctonia solani and Ceratobasidium cornigerum after exposure to Trichoderma spp. using media selective for the pathogens. The second problem is that antagonistic effects should be quantified. Simply scoring antagonistic isolates as "highly antagonistic, moderately antagonistic or poorly antagonistic" does not allow for direct numerical comparisons between isolates. The quantification should reflect, as closely as possible, the interactions which would take place under field conditions. In the present study we wish to determine which Trichoderma spp. isolates most rapidly kill P. weirii in a substrate previously colonized by the pathogen.

Dowding (1978) discussed two types of dual-culture methods, the chemostat method and the petri plate method, which can be used for measuring interactions between microorganisms in vitro. The chemostat method maintains nutrients and metabolic by-products at constant concentrations. The petri plate, or batch culture method, allows depletion of nutrients and accumulation of by-products. For investigations of interactions in decaying substrates, he concluded that the petri plate method more closely approximates field conditions.

Composition of the test medium can affect the degree of antagonism (Mitchell and Dix, 1977). Wood is the substrate of the eventual field application, but its heterogenous structure, uneven colonization by P. weirii and possible occupation by other

organisms could introduce a degree of variability in interactions which would make it unsuitable for rapid laboratory screening of a large number of isolates. Antagonism tests on malt agar closely approximated interactions between fungi in hardwood stumps (Rayner, 1978). Interactions on malt agar also were similar to those on karri wood blocks (Pearce and Malajcuk, 1983). These results combined with the common use of malt agar for culture of wood-decaying fungi, including P. weirii, make this medium a logical starting point for in vitro tests.

Temperature can affect the antagonism of fungal plant pathogens by Trichoderma spp. (Tronsmo and Dennis, 1978). Isolates should be tested for their antagonistic effects on P. weirii at expected field temperatures.

Investigations into controlling plant pathogens with antagonistic microorganisms

> "...should start in the field 'where the clues and the action are,' and after a phase of detailed laboratory study, should return to the field for testing and application" (Cook and Baker, 1983).

Reported here are the results of research which correspond to the initial field study, and first steps of laboratory study. A field survey was performed to identify potential antagonists and laboratory screening was begun to identify the isolates which have the most potential for effecting disease control.

Objective

The objective of this research was to determine which, if any, species of *Trichoderma* invade *Phellinus weirii*-infested stumps and stump roots, and to establish characteristics which will be useful in screening isolates of *Trichoderma* spp. for use as antagonists.

Approach

To achieve this objective a three-part study was conducted.

Field Survey

Phellinus weirii-infested stumps and stump roots of Douglas-fir trees were excavated and sectioned. Sample wood chips were transferred to culture media in petri plates to determine the presence of *P. weirii*, *Trichoderma* spp. and other microorganisms.

Growth Rate Study

Field-collected isolates of *Trichoderma* spp. were tested for their linear growth rates on artificial media at 5, 10, 15, 20, and 25 C.

In Vitro Antagonism Study

Selected field-collected isolates of *Trichoderma* spp. were tested for their lethal effect on *P. weirii* in paired culture on artificial media.

Hypotheses

The three-part study tested the following hypotheses.

Field survey.

The frequency of isolation of *Trichoderma* spp is:

a. Greater in stumps and stump roots of *P. weirii*-infested trees harvested 11 years ago than in those harvested 1 year ago.

b. Greater in wood colonized by *P. weirii* than in wood not colonized by *P. weirii*.

c. Greatest in stump wood which is closest to the stump surface.

d. Greatest in stump root wood which is farthest from the root collar.

Growth Rate Study

a. The species of *Trichoderma* collected from *Phellinus weirii*-infested stumps and roots have different mean linear growth rates at different temperatures.

b. Isolates within species of *Trichoderma* have different linear growth rates at different temperatures.

In Vitro Antagonism Study

a. Isolates of *Trichoderma* spp. differ in the rate at which they kill *P. weirii* on artificial media.

b. Isolates of *P. weirii* differ in their abilities to resist killing by *Trichoderma* spp. on artificial media.

c. The outcome of *in vitro* antagonism tests differs according to the temperatures at which the tests are conducted.

MATERIALS AND METHODS

Field Survey

Six sampling units were selected in four stands in the western Cascades, Marion County, Oregon (Table 1). The stands are part of the Santiam Resource Area, administered by the Salem District, Bureau of Land Management. All stands contained vegetation classified in the Tsuga heterophylla / Rhododendron macrophyllum / Berberis nervosa old growth association of the Tsuga heterophylla zone (Franklin and Dyrness, 1973). All stands had been salvage-thinned; symptomatic, diseased trees had been removed and apparently healthy trees remained. Three sampling units contained stumps of trees harvested one year previously and three contained stumps of trees harvested eleven years previously. In stands which contained stumps of different ages, different colored marking paint allowed for accurate dating of salvage-thinning.

Within each sampling unit, five Douglas-fir stumps were selected from among those which:

1. Contained Phellinus weirii stain or decay on the stump surface or mycelium on roots.
2. Were not hollow at the stump surface.
3. Were 60 cm or less in diameter at the stump surface.

On each stump the four largest lateral roots were excavated

Table 1. Location and physical characteristics of sampling units for field survey.

Sampling unit(s)	Stand	Location in Willamette meridian	Years since harvest 1	Years since harvest 11	Elevation (m)	Aspect (degrees)	Soil classification[a]
1	I	SE 1/4, Sec. 31, T 8S, R 4E	x		366	352	Typic cryorthod, coarse-loamy, mixed, orstein, Whetstone
2,3	II	NE 1/4, Sec. 1, T 9S, R 3E	x	x	366	330	Typic haplumbrepts fine-loamy, mixed, mesic, Horeb
4,5	III	SW 1/4, Sec. 1, T 9S, R 3E	x	x	366	337	Typic haplumbrepts, fine-loamy, mixed, mesic, Horeb
6	IV	NE 1/4, Sec. 5, T 9S, R 2E		x	567	210	Andic haplumbrepts, fine-loamy, mixed, mesic, Kinney

[a]Soil classifications from maps of the Reconnaissance Soil Survey of the Willamette Basin, Oregon, Segment III: Uplands Outside National Forests. Special Report 269. March 1969. Agricultural Experiment Station, Oregon State University, Corvallis, Oregon.

with hand tools. Using a chain saw, four 5 cm-thick transverse disks were cut from each root. The midpoints of the disks were located approximately 5, 25, 45, and 65 cm from the point where the root diverged from the bole (root collar). Four consecutive 5 cm-thick disks were also cut from the stem portion of the stump at midpoint distances of approximately 3, 9, 15, and 21 cm from the stump top.

In the field, immediately after sectioning, areas of each disk corresponding to the following "decay" classes were outlined in permanent ink with a felt-tipped marker:

a) sound wood--no stain or decay,

b) stained wood--stain and/or occasional pitting typical of *P. weirii*,

c) decayed wood--substantial pitting typical of *P. weirii* and

d) "other" wood--any wood not fitting into the other categories, including areas of resin-soaked wood, insect galleries and decay apparently caused by other fungi.

Disks were placed in clean plastic bags and transported to the laboratory where they were stored at 2 C for no more than 14 days before isolation. Field sampling was done between July 12, 1984 and September 14, 1984.

Ten sample points were chosen for each disk using a systematic grid and a random number table. At each sample point the "decay"

class was recorded (as defined above) and the disk was split longitudinally. Two adjacent wood chips were aseptically removed from the midpoint of the freshly exposed surface. One was transferred to malt agar (MA), prepared from 45 g Difco Malt Agar and 1 l distilled water, and the other to MA supplemented with 1 part per million (ppm) benomyl (active ingredient).

Organisms growing from wood chips were sorted on the basis of colony characteristics and microscopic examination into four groups: P. weirii, Trichoderma spp., miscellaneous fungi and bacteria. Representative cultures of P. weirii and miscellaneous fungi and all cultures of Trichoderma spp. were subcultured onto MA slants and stored at 2 C. All Trichoderma spp. isolates were also stored in liquid nitrogen and lyophilized and stored at 0 C. Trichoderma spp. isolates were identified to species aggregates according to Rifai (1969), Domsch et al. (1980) and Bissett (1984).

Frequencies were tabulated for the four groups of organisms in the following classifications: age, "decay" class, stump vs stump root, and location within the stump or root. A chi-square analysis test for goodness of fit was used to test a null hypothesis of random distribution, that is frequency of isolation was proportional to the number of isolations in each classification for each group of microorganisms. Continuity corrections were used for a conservative chi-square test with sample sizes of less than 200, as recommended by Sokal and Rohlf (1981). No tests were conducted for sample sizes of less than 25.

Growth Rate Study

All *Trichoderma* isolates collected in the field were tested for linear growth rate at 5, 10, 15, 20, and 25 C on MA. Twenty ml of culture medium was dispensed in each 100 X 15 mm plastic petri plate. Standardized inoculation was achieved by cutting a 3 mm plug with a flamed cork borer from the leading edge of a colony growing on MA at 20 C. Plugs were placed near the edge of fresh malt agar plates and incubated at laboratory temperature (approximately 23 C) for 4-9 hours to allow for colonization of the agar surface. The inoculated plates were placed in the 10, 15, 20, and 25 C incubators for 14-17 hrs and the 5 C incubator for three days, to allow for equilibration to the test temperature. At the end of these times, a line was marked on the bottom of the plate running through the inoculation plug and the center of the plate, roughly dividing the plate in half. The extent of mycelial growth along the line was marked as time zero. Growth along the line was measured in one direction at 24 h intervals for three days. Linear growth rate was calculated in mm/day. Some isolates, grown at 20 and 25 C, overgrew the plate in less than three days. In these cases growth rate was calculated using the 2-day, or if necessary, 1-day measurements. Each isolate was tested four times at each temperature, except for isolate 382-5 (*T. harzianum*) which because of contaminated plates

was tested three times at 15, 20, and 25 C.

The data for each temperature were subjected to nested analysis of variance to determine the percent of total variation attributable to differences between species, between isolates within a species, and between replicates (error). Analyses of variance and F-tests were used to test for significance in variation between species and between isolates within species. Tukey's studentized range test was used to test significant ($P \leq .05$) differences between species means at each temperature. A canonical plot was used to graphically depict the growth response of species and isolates to different temperatures. This analysis created two variables from linear combinations of growth rates at the five temperatures which maximized the separation between isolates (SAS Institute Inc., 1982). Each isolate is then plotted against these variables. A discriminant function analysis was performed to calculate the number (percent) of isolates placed in the correct morphological species solely on the basis of growth rates at the five temperatures (SAS Institute Inc., 1982).

In Vitro Antagonism Study

Nine of the field-collected *Trichoderma* spp. isolates were selected for *in vitro* antagonism tests. These included one isolate each of: *Trichoderma aureoviride* Rifai (301-0), *T. hamatum* (Bon.) Bain. (347-0), *T. harzianum* (382-5),

T. longibrachiatum Rifai (219-0), T. polysporum (Link ex Pers.) Rifai (320-6) and T. pseudokoningii Rifai (036-2); and three isolates of T. viride (036-3, 350-7 and 502-2).

Two field-collected isolates of Phellinus weirii, shown to be different clones by vegetative incompatibility tests (unpublished data), were also selected. Each P. weirii isolate was inoculated onto one edge of sixty petri plates, each containing 20 ml of MA and incubated at 20 C. After eleven days a line was drawn on the bottom of the plate, running through the inoculum plug and roughly dividing the plate in half. The extent of P. weirii mycelial growth along this line was then marked.

The plates were inoculated with the test Trichoderma sp. isolate (except 12 non-inoculated controls), using a 3 mm plug cut from the leading edge of a colony growing at 20 C on MA. The inoculum plug was placed on the center line, with its edge 10 mm from the marked extent of P. weirii growth. The inoculated plates were incubated at 10 or 20 C according to the following experimental design:

Experimental Design

9 isolates of Trichoderma spp. and 1 non-inoculated control

2 isolates of P. weirii

2 temperatures

3 replicates of each combination of Trichoderma sp. isolate, P. weirii isolate and temperature.

Plates incubated at 20 C were sampled after 4 days. Paired 3 mm plugs were cut with a flamed cork borer on either side of the center line, every 5 mm, up to 50 mm from the point of *Trichoderma* sp. inoculation. One plug of each pair was transferred to MA to test for the presence of the *Trichoderma* sp. The other plug was transferred to a medium containing MA supplemented with 1.5 ppm (active ingredient) each of benomyl, prochloraz and thiabendazole, to test for the presence of viable *P. weirii*. Preliminary tests had shown that this combination of three fungicides would differentially inhibit *Trichoderma* spp., achieving a consistent level of growth inhibition not possible with any one of the three. Plates incubated at 10 C were sampled in the same manner after 15 days. Reisolation plates were scored for the presence of *P. weirii* and *Trichoderma* sp. every other day for 10 days. The presence or absence of the fungi was used to calculate the rate of overgrowth by *Trichoderma* sp. and the rate of lethal effect on *P. weirii* in mm/day.

Tukey's studentized range test was used to test for significantly ($P \leq .05$) different mean rates between the *Trichoderma* spp. isolates and between the *P. weirii* isolates for each temperature. Analysis of variance was used to test for significant effects of the single factors and the two-way and three-way interactions.

Data from all studies were stored on the Forest Science Data Bank, Oregon State University.

RESULTS

Field Survey

Phellinus weirii was isolated from 3169 of 5970 (53.1 percent) sample points (Table 2) and from all 30 stumps. *Trichoderma* spp. were isolated 70 times (1.2 percent) (Table 2) and from 18 of 30 stumps. Seven species of *Trichoderma* were isolated (Table 3). The most prevalent of these were *T. viride* (36 isolates) and *T. polysporum* (19 isolates). No other *Trichoderma* species was isolated more than five times.

Stump Age

The distribution in the stumps and roots by age class differed for the groups of organisms. *Trichoderma* spp. were isolated significantly ($P \leq .005$) more frequently from 11-year-old stumps (1.7 percent) than from 1-year-old stumps (0.6 percent) (Table 2). *Phellinus weirii* was also isolated significantly ($P \leq .005$) more frequently from 11-year-old stumps. The differences in frequency of isolation for miscellaneous fungi and bacteria between older and younger stumps were not significant ($P \geq .05$).

Decay Class

The groups of organisms were differentially distributed in the "decay" classes. *Trichoderma* spp. were isolated most frequently

Table 2. Microorganisms isolated from stumps and roots of *Phellinus weirii*-infested Douglas-fir trees, one and eleven years following harvest ("decay" classes and locations within stumps combined).

Micro-organisms	Years since harvest	Number of isolations attempted	Isolation success		Expected frequency[a]	Chi-square (df)	Probability of a greater chi-square
			percent	frequency			
Trichoderma spp.	1	2990	0.6	19	35.1	13.9	.005
	11	2980	1.7	51	34.9	(1)	
	combined	5970	1.2	70			
P. weirii	1	2990	45.4	1356	1587.0	67.4	.005
	11	2980	60.8	1813	1582.0	(1)	
	combined	5970	53.1	3169			
Miscellaneous fungi	1	2990	12.2	365	368.1	0.1	.9
	11	2980	12.4	370	266.9	(1)	
	combined	5970	12.3	735			
Bacteria	1	2990	4.5	136	125.2	1.9	.25
	11	2980	3.8	114	124.8	(1)	
	combined	5970	4.2	250			

[a]Frequency expected if microorganisms were distributed randomly in stumps and roots of different age groups.

Table 3. Recovery of *Trichoderma* species from *Phellinus weirii*--infested stumps and roots.

Trichoderma species	No. of isolates	No. of stumps
T. viride	36	12
T. polysporum	19	7
T. aureoviride	5	3
T. harzianum	3	3
T. pseudokoningii	3	2
T. hamatum	3	1
T. longibrachiatum	1	1

from "other" wood (6.0 percent) and least frequently from sound wood (0.3 percent) (Table 4). Phellinus weirii was found most frequently in decayed and stained wood (93.9 and 81.3 percent, respectively). Miscellaneous fungi were isolated most frequently from "other" wood (61.6 percent), followed by sound wood (14.8 percent), stained wood (9.6 percent) and decayed wood (7.9 percent). Bacteria were most frequently isolated from "other" wood (9.3 percent).

Distribution trends are more apparent when the four "decay" classes are combined into two: stained wood and decayed wood constitute apparent Phellinus weirii-colonized wood (PW-wood), and sound wood and "other" wood constitute apparent non-Phellinus weirii-colonized wood (no-PW-wood). In this classification, Trichoderma spp. were significantly ($P \leq .005$) more abundant in PW-wood (1.6 percent) than in no-PW-wood (0.6 percent) (Table 5). Miscellaneous fungi were significantly ($P \leq .005$) more abundant in no-PW-wood (17.5 percent) than in PW-wood (8.3 percent). The difference in abundance of bacteria between PW-wood (3.8 percent) and no-PW-wood (4.7 percent) was not significant ($P \geq .05$).

Location Within Stump

The sampling scheme permits several comparisons to be made concerning the distributions of organisms within stumps and stump roots. Frequencies of isolation can be compared between stumps and stump roots, root disks proximal and distal to the root collar

Table 4. Recovery of microorganisms from wood of different "decay" classes in Phellinus weirii-infested stumps and roots (ages and locations within stumps and stump roots combined).

Micro-organisms	Wood "decay" class	Number of isolations attempted	Isolation success		Expected frequency[a]	Chi-square (df)	Probability of a greater chi-square
			percent	frequency			
Trichoderma spp.	sound	2437	0.3	7	28.6	51.0	.005
	stained	910	1.3	12	10.7	(3)	
	decayed	2472	1.7	42	29.0		
	"other"	151	6.0	9	1.8		
	combined	5970	1.2	70			
P. weirii	sound	2437	3.5	85	1293.6	2082.9	.005
	stained	910	81.3	740	483.0	(3)	
	decayed	2472	93.9	2321	1312.0		
	"other"	151	15.2	23	80.2		
	combined	5970	53.1	3169			
Miscellaneous fungi	sound	2437	14.8	360	300.0	354.5	.005
	stained	910	9.6	87	112.0	(3)	
	decayed	2472	7.9	195	304.3		
	"other"	151	61.6	93	18.6		
	combined	5970	12.3	735			
Bacteria	sound	2437	4.4	107	102.1	14.2	.005
	stained	910	2.8	25	38.1	(3)	
	decayed	2472	4.2	104	103.5		
	"other"	151	9.3	14	6.3		
	combined	5970	4.2	250			

[a]Frequency expected if microorganisms were distributed randomly in wood of different "decay" classes.

Table 5. Recovery of microorganisms from wood, colonized or not colonized by Phellinus weirii, from infested stumps and roots (ages and locations within stumps and stump roots combined).

Micro-organisms	Wood "decay" class	Number of isolations	Isolation success percent	Isolation success frequency	Expected frequency[a]	Chi-square (df)	Probability of a greater chi-square
Trichoderma spp.	PW-wood[b]	3382	1.6	54	39.7	11.08	.005
	no-PW-wood[c]	2588	0.6	16	30.3	(1)	
	combined	5970	1.2	70			
P. weirii	PW-wood	3382	90.5	3061	1795.2	2058.8	.005
	no-PW-wood	2588	4.2	108	1373.8	(1)	
	combined	5970	53.1	3169			
Miscellaneous fungi	PW-wood	3382	8.3	282	416.3	99.5	.005
	no-PW-wood	2588	17.5	453	318.6	(1)	
	combined	5970	12.3	735			
Bacteria	PW-wood	3382	3.8	129	141.6	2.6	.250
	no-PW-wood	2588	4.7	121	108.4	(1)	
	combined	5970	4.2	250			

[a]Frequency expected if microorganisms were distributed randomly in wood of different "decay" classes.

[b]PW-wood is wood colonized by P. weirii (stained and decayed wood).

[c]No-PW-wood is wood not colonized by P. weirii (sound and "other" wood).

and stump disks proximal and distal to the stump top. In addition, these classes can be sub-divided according to age of the stump to help elucidate invasion trends.

1)Stumps vs. Stump Roots.--*Trichoderma* spp., miscellaneous fungi and bacteria were significantly ($P \leq .005$) more abundant in stumps than stump roots (Table 6). The abundance of *P. weirii* did not differ significantly ($P \geq .05$) between stumps and stump roots.

2)Root Disks Proximal vs. Distal to the Root Collar.--*Trichoderma* spp., miscellaneous fungi and bacteria were significantly ($P \leq .005$) more abundant in root disks that were distal to the root collar than those proximal to it (Figs. 1, 2 and 3, respectively). *Phellinus weirii* was evenly distributed in root disks ($P \geq .05$) (Fig. 4).

3)Stump Disks Proximal vs. Distal to the Stump Top.--*Trichoderma* spp. were slightly more abundant in stump disks proximal to the stump top, although the difference was not significant ($P \geq .05$) (Fig. 1). Miscellaneous fungi were significantly ($P \leq .005$) more abundant in disks closer to the stump top (Fig. 2). Bacteria had a significantly ($P \leq .05$) uneven distribution within stumps (Fig. 3). They were most abundant in proximal stump disks and least abundant in distal disks, but their abundance at the intermediate distances was not positively

Table 6. Recovery of microorganisms from *Phellinus weirii*-infested stumps and stump roots (ages, "decay" classes and locations within stumps or stump roots combined).

Micro-organisms	Stump or root	Number of isolations attempted	Isolation success		Expected frequency[a]	Chi-square (df)	Probability of a greater chi-square
			percent	frequency			
Trichoderma spp.	stump	1180	2.1	25	13.8	10.3	.005
	root	4790	0.9	45	56.2	(1)	
	combined	5970	1.2	70			
P. weirii	stump	1180	50.9	601	626.5	1.3	.5
	root	4790	53.6	2568	2542.5	(1)	
	combined	5970	53.1	3169			
Miscellaneous fungi	stump	1180	29.5	348	145.3	352.5	.005
	root	4790	8.1	387	589.7	(1)	
	combined	5970	12.3	735			
Bacteria	stump	1180	5.8	69	49.4	9.7	.005
	root	4790	3.8	181	200.6	(1)	
	combined	5970	4.2	250			

[a]Frequency expected if microorganisms were distributed randomly between stumps and stump roots.

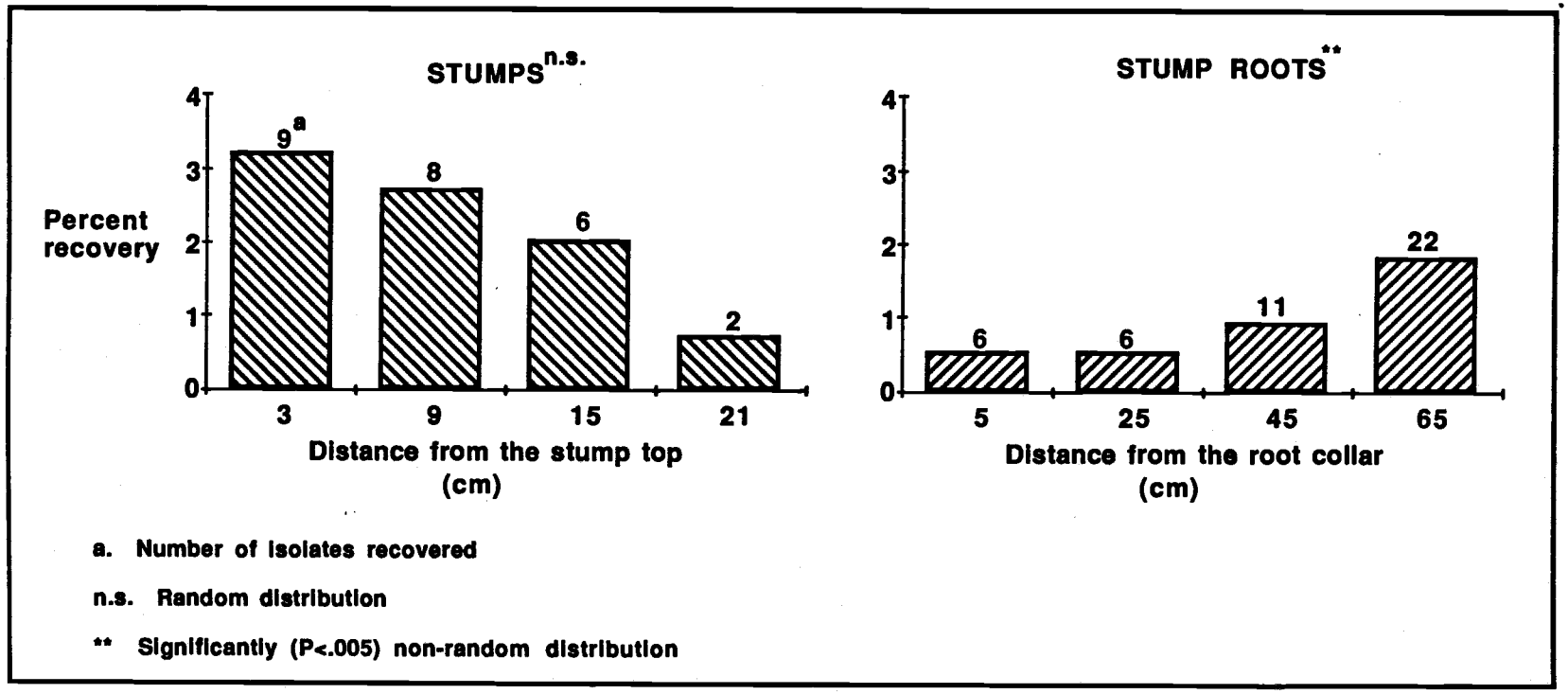


Figure 1. Recovery of Trichoderma spp. from within Phellinus weirii-infested Douglas-fir stumps and stump roots (ages and "decay" classes combined).

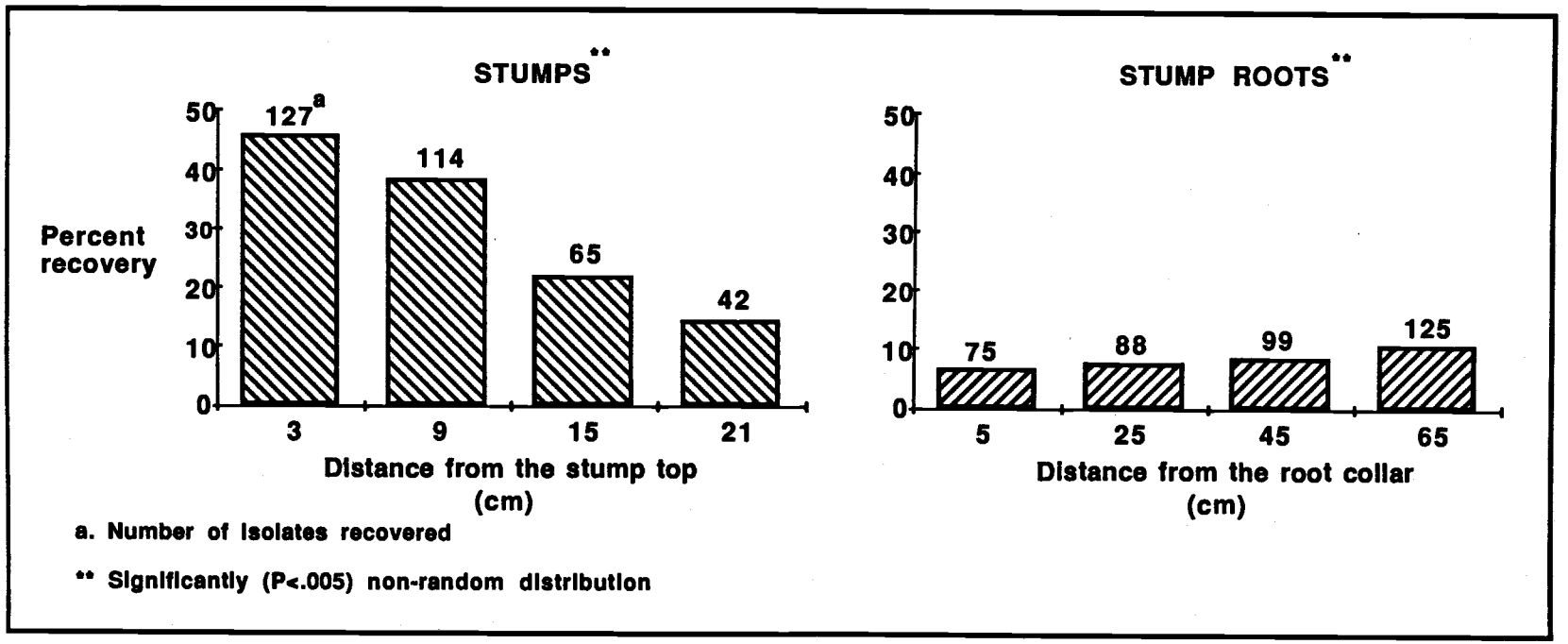


Figure 2. Recovery of miscellaneous fungi from within Phellinus weirii-infested Douglas-fir stumps and stump roots (ages and "decay" classes combined).

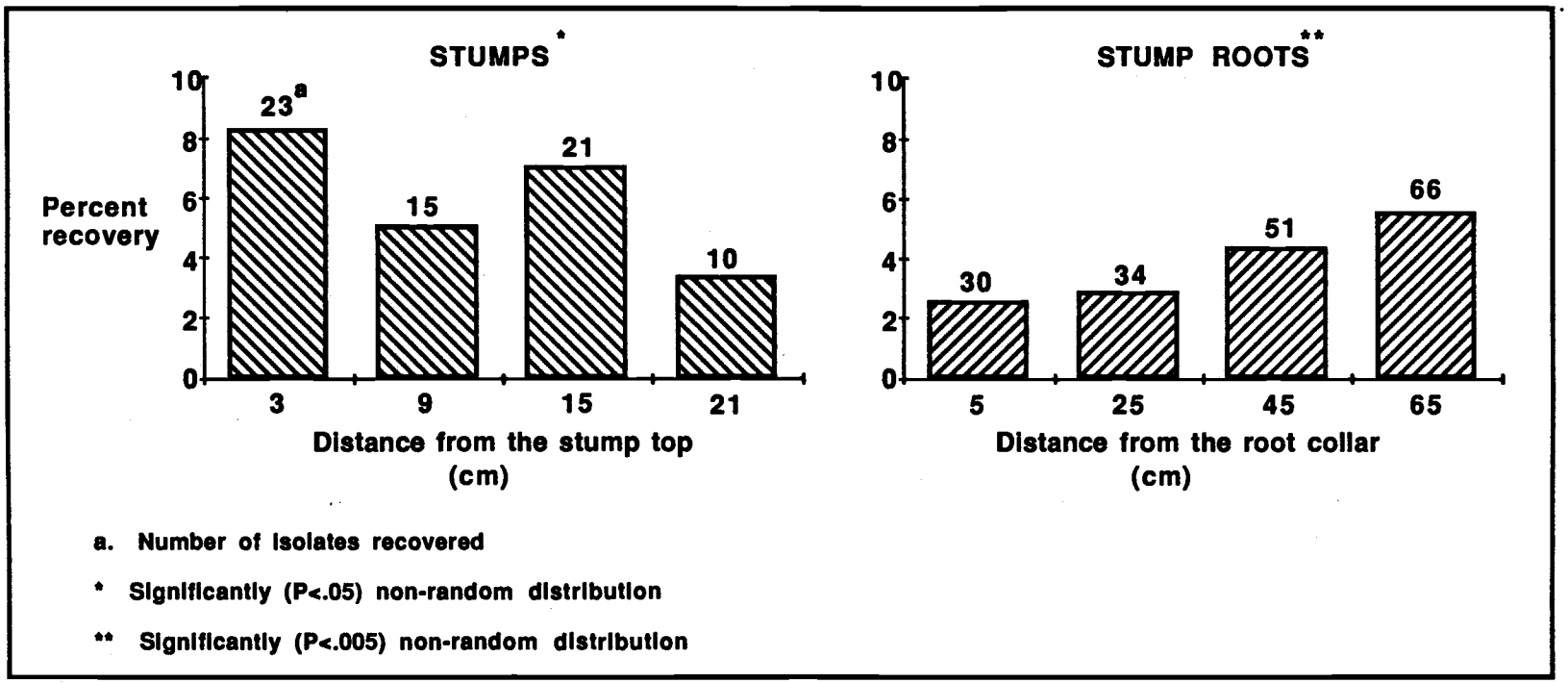


Figure 3. Recovery of bacteria from within Phellinus weirii-infested Douglas-fir stumps and stump roots (ages and "decay" classes combined).

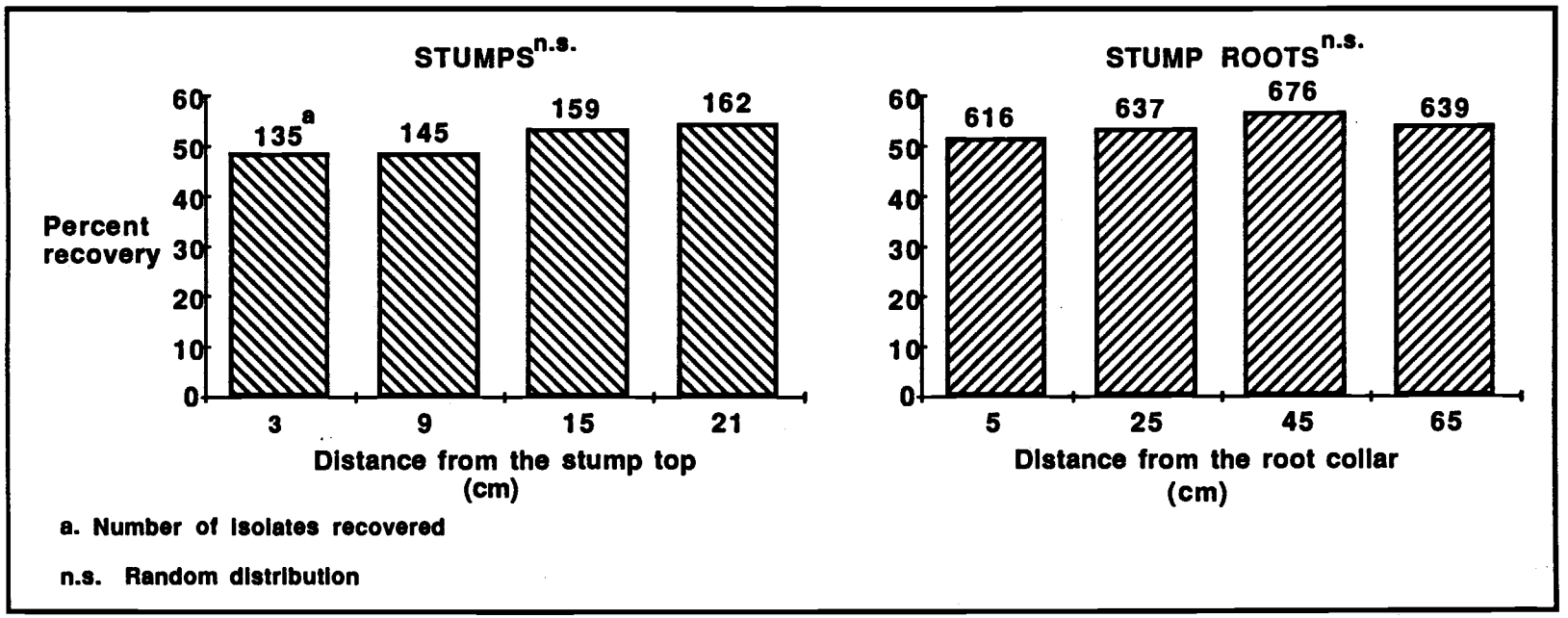


Figure 4. Recovery of Phellinus weirii from within P. weirii-infested Douglas-fir stumps and stump roots (ages and "decay" classes combined).

correlated with proximity to the stump top. Phellinus weirii was evenly distributed within stumps ($P \geq .05$) (Fig. 4).

4)Within Roots, by Age.--Phellinus weirii was significantly ($P \leq .005$) more abundant in 11-year-old than 1-year-old roots at each distance from the root collar (Fig. 5). Bacteria and miscellaneous fungi were not significantly ($P \geq .05$) more abundant in older root disks at any location (Figs. 6 and 7, respectively). Trichoderma spp. were slightly more abundant in older root disks that were close to the root collar, although the small sample size precluded statistical analysis (Fig. 8).

5)Within Stumps, by Age.--Phellinus weirii was significantly ($P \leq .05$) more abundant in older vs. younger stumps at each location except the uppermost disks. The greatest difference ($P \leq .005$) occurred in the stump disks furthest from the stump top (Fig. 5). Bacteria were more abundant in older stumps only in the top disk (Fig. 6). This difference was not tested for significance because of the small sample size. Miscellaneous fungi exhibited no significant ($P \geq .05$) differences in their abundance in 1 and 11-year-old stumps at any location (Fig. 7). Trichoderma spp. exhibited markedly different distribution patterns (Fig. 8), although small sample sizes precluded statistical analysis. In 1-year-old stumps they were infrequent in the top disks and absent in the other disks. In 11-year-old stumps they were present at each distance, but least abundant in the disks furthest from the stump top.

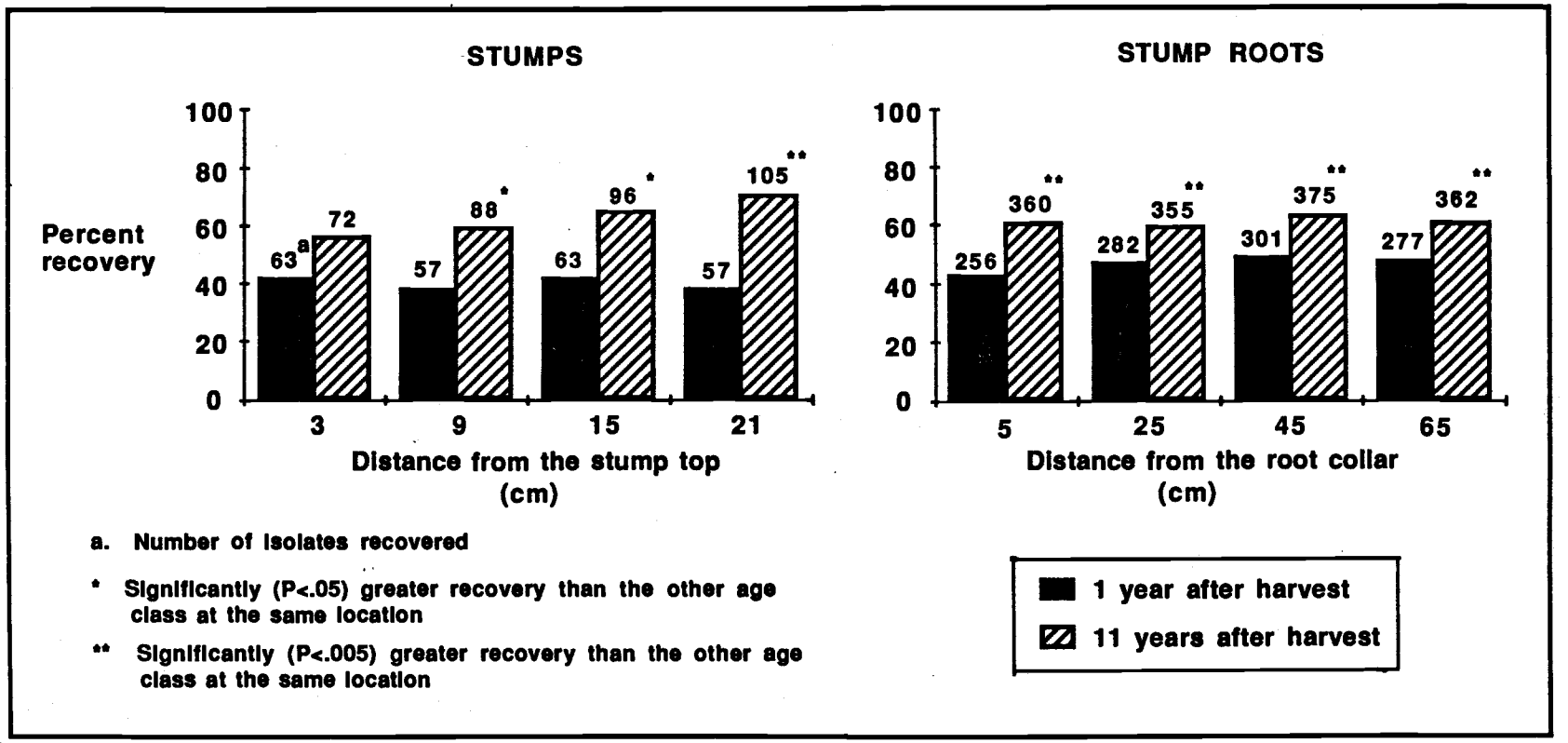


Figure 5. Recovery of Phellinus weirii from within stumps and stump roots of P. weirii-infested Douglas-fir trees harvested one and eleven years previously ("decay" classes combined).

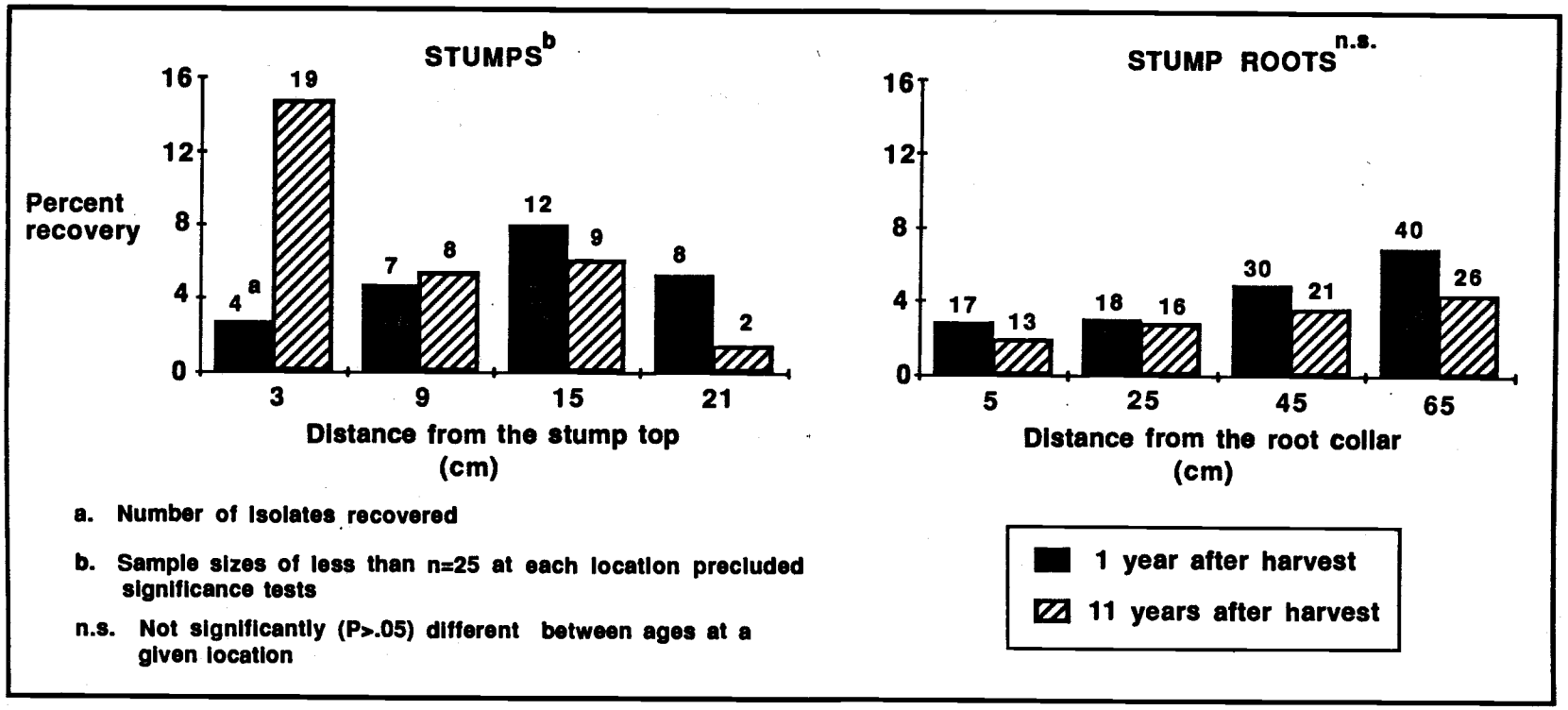


Figure 6. Recovery of bacteria from within stumps and stump roots of Phellinus weirii-infested Douglas-fir trees harvested one and eleven years previously ("decay" classes combined).

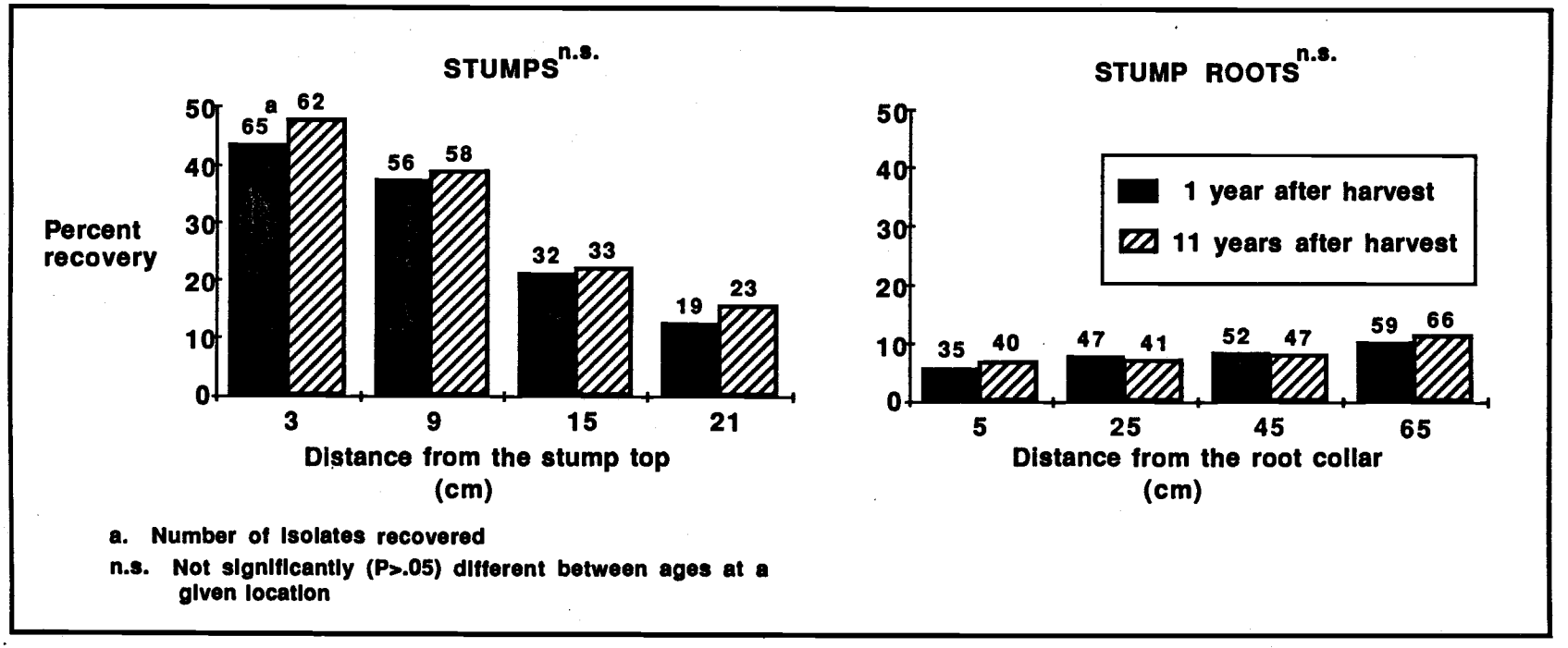


Figure 7. Recovery of miscellaneous fungi from within stumps and stump roots of Phellinus weirii-infested Douglas-fir trees harvested one and eleven years previously ("decay" classes combined).

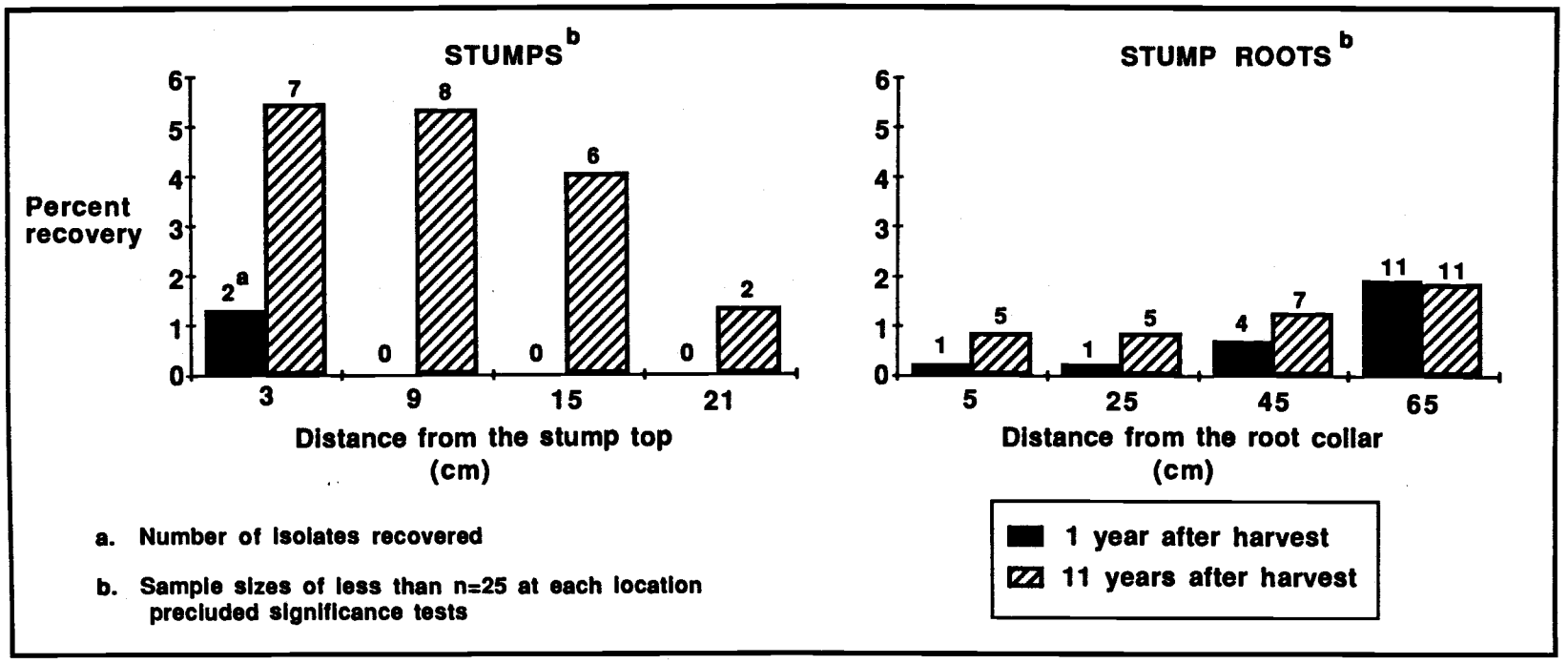


Figure 8. Recovery of Trichoderma spp. from within stumps and stump roots of Phellinus weirii-infested Douglas-fir trees harvested one and eleven years previously ("decay" classes combined).

Growth Rate Study

The *Trichoderma* species tested differed in their growth rates at different temperatures (Table 7). Three species, *T. pseudokoningii*, *T. harzianum* and *T. hamatum* grew fastest at 25 C, and four species, *T. polysporum*, *T. viride*, *T. aureoviride* and *T. longibrachiatum*, grew fastest at 20 C. Relative growth rates were calculated by comparing the mean growth rate of each species to the mean of all the species tested, at a given temperature (Fig. 9). *Trichoderma harzianum* and *T. pseudokoningii* grew slowest at 5 C and fastest at 25 C. In contrast, *T. polysporum* and *T. viride*, grew relatively well at 5 C, but slowly at 25 C.

The majority of variation in growth rate, at each temperature tested, occurred between species (Table 8). The percent of variation between replicates (error) was greatest at 10 C. For every temperature tested, analysis of variance showed that both between species and within species variation made significant ($P \leq .0001$) contributions to the total variation (Table 9).

Although some species could be distinguished on the basis of their growth rates at a single temperature (Table 7), analyses integrating growth rates at all five temperatures were necessary to separate all the species. The canonical plot shows that each species had a characteristic growth response to the five temperatures (Fig. 10). The discriminant function analysis placed 68 of the 69 isolates in the correct morphological species solely on the basis of the five growth rates (Table 10).

Table 7. Mean linear growth rate for species of Trichoderma on malt agar at 5, 10, 15, 20, and 25 C.

Trichoderma species	No. of isolates tested	Temperature (C) 5	10	15	20	25
		(mm/day)				
longibrachiatum	1	3.9[a]	7.3	13.3	18.3	16.2
polysporum	19	3.2±0.04 a[b]	5.2±0.06 b	9.6±0.09 e	12.7±0.09 d	8.9±0.31 e
hamatum	3	3.0±0.04 a,b	5.0±0.09 b,c	11.1±0.03 d	17.1±0.03 c	17.7±0.32 c
viride	36	2.8±0.08 b	6.3±0.11 a	12.9±0.16 b,c	17.7±0.21 c	12.1±0.28 d
aureoviride	5	2.4±0.18 b	5.8±0.27 a,b	14.1±0.23 a	21.3±0.11 a,b	19.8±0.08 c
harzianum	3	1.3±0.10 c	4.0±0.11 c	11.7±0.56 c,d	19.7±1.20 b	28.2±1.44 b
pseudokoningii	3	1.3±0.07 c	5.2±0.12 b,c	14.1±0.17 a,b	23.4±0.00 a	32.3±0.38 a

[a]Standard error of the mean and significant differences could not be calculated for T. longibrachiatum because only one isolate was tested.

[b]Species in the same column followed by the same letters do not have significantly different ($P \leq .05$) mean growth rates using Tukey's studentized range test.

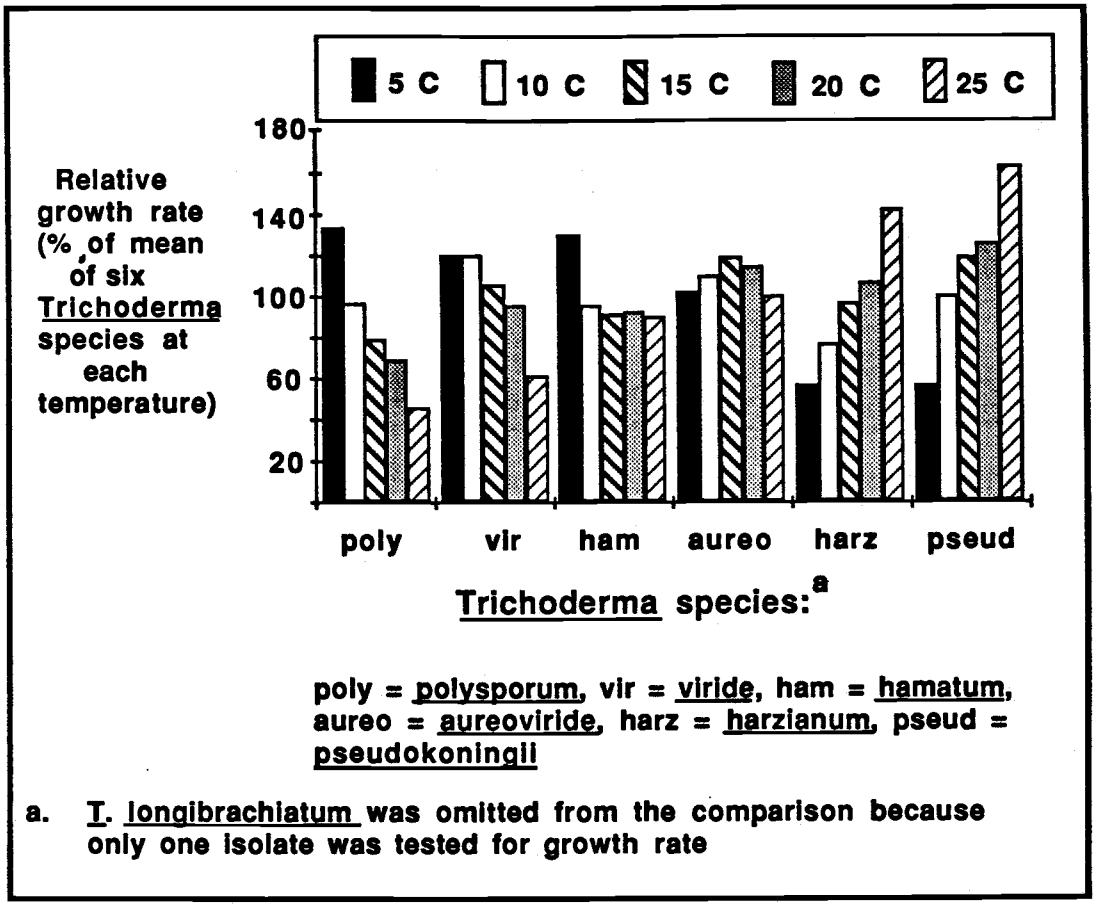


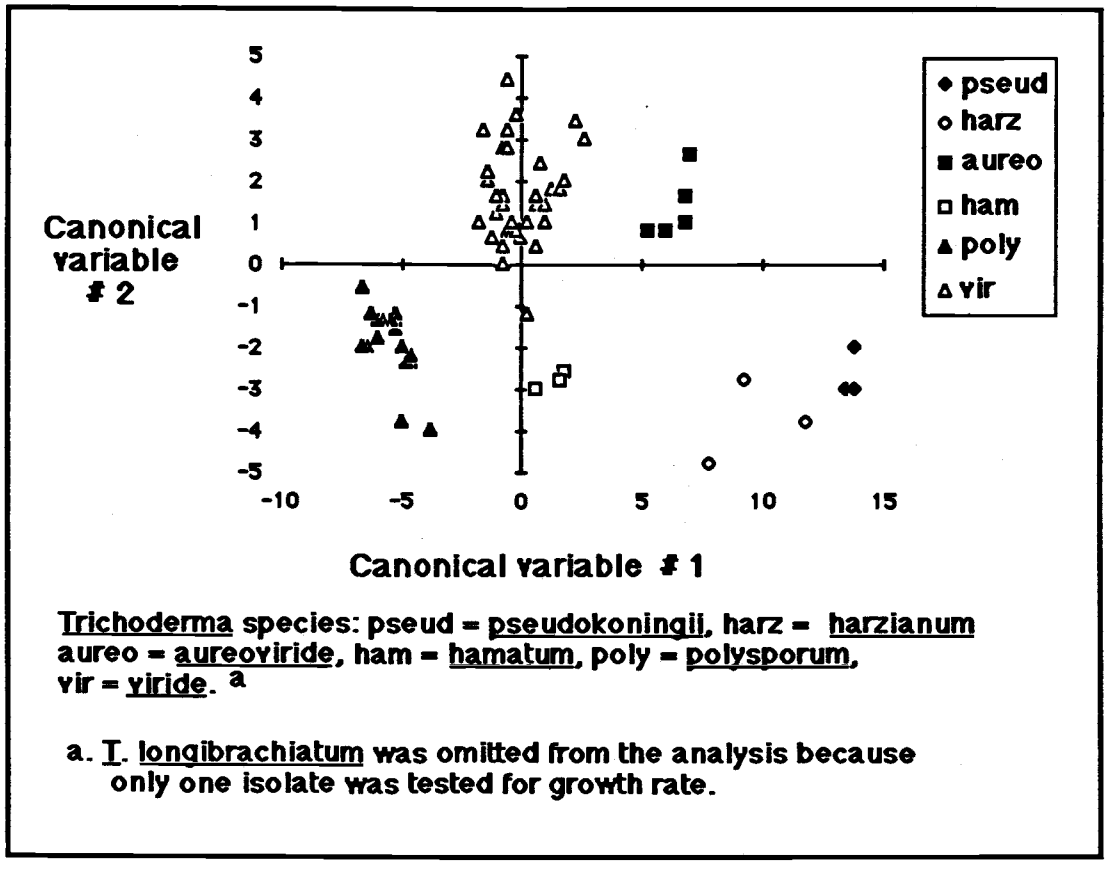
Figure 9. Relative linear growth rates on malt agar at five temperatures of Trichoderma species, from Phellinus weirii-infested Douglas-fir stumps and stump roots.

Table 8 Percent of total variation in linear growth rates found in species, isolates and replicates of Trichoderma spp. on malt agar at different temperatures.

Temperature (C)	Between spp.	Between isolates within spp.	Between replicates within isolates (error)
	percent of variation		
5	67.40	27.04	5.55
10	59.99	22.21	17.81
15	80.51	9.72	9.77
20	87.78	5.39	6.83
25	94.12	3.67	2.22

Table 9. Analysis of variance of linear growth rates of Trichoderma spp. on malt agar at different temperatures.

Temperature (C)	Source	DF	F	Probability of a greater F
5	Between species	6	389.11	.0001
	Between isolates	63	20.47	.0001
10	Between species	6	108.37	.0001
	Between isolates	63	5.99	.0001
15	Between species	6	253.66	.0001
	Between isolates	63	4.97	.0001
20	Between species	6	391.86	.0001
	Between isolates	63	4.15	.0001
25	Between species	6	1287.58	.0001
	Between isolates	63	7.59	.0001



Trichoderma species: pseud = pseudokoningii, harz = harzianum aureo = aureoviride, ham = hamatum, poly = polysporum, vir = viride. [a]

a. T. longibrachiatum was omitted from the analysis because only one isolate was tested for growth rate.

Figure 10. Trichoderma spp. isolates from Phellinus weirii-infested stumps and stump roots plotted on axes of two canonical variables created from linear combinations of growth rates at 5, 10, 15, 20, 25 C.

Table 10. Discriminant function analysis of growth rates of Trichoderma spp. isolates at five temperatures on malt agar.

Morphological species[a]	-----Number (percent) of isolates placed in each species by growth rate analysis------						
	viride	polysporum	aureoviride	hamatum	pseudokoningii	harzianum	total
viride	35 (97)	0 (0)	0 (0)	1 (3)	0 (0)	0 (0)	36 (100)
polysporum	0 (0)	19 (100)	0 (0)	0 (0)	0 (0)	0 (0)	19 (100)
aureoviride	0 (0)	0 (0)	5 (100)	0 (0)	0 (0)	0 (0)	5 (100)
hamatum	0 (0)	0 (0)	0 (0)	3 (100)	0 (0)	0 (0)	3 (100)
pseudokoningii	0 (0)	0 (0)	0 (0)	0 (0)	3 (100)	0 (0)	3 (100)
harzianum	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	3 (100)	3 (100)

[a] T. longibrachiatum was omitted from the analysis because only one isolate was tested for growth rate.

In Vitro Antagonism Study

Use of the dual isolation/dual culture media system proved to be a reliable method for assessing the presence and viability of *P. weirii* and *Trichoderma* spp. simultaneously. One hundred forty-four of 1080 sample points yielded both a *Trichoderma* sp. isolate on MA and *P. weirii* on the differential medium. *Phellinus weirii* was isolated from all sample points on both media from control plates which had not been inoculated with a *Trichoderma* sp.

The *Trichoderma* isolates tested differed in the rate at which they killed *P. weirii* on MA (Table 11). They can be grouped into three efficiency categories according to the ratio of the rate at which they killed *P. weirii* to the rate at which they overgrew the colony (kill:grow ratio). Group 1 isolates (*T. viride*--502-2, 036-3, and 350-7; *T. polysporum*--320-6; and *T. harzianum*--382-5) killed *P. weirii* at about the same rate as they overgrew the colony (kill:grow ratio range 0.9-1.2). Group 2 isolates (*T. pseudokoningii*--036-2, *T. aureoviride*--301-0, and *T. hamatum*--347-0) killed *P. weirii* somewhat more slowly than they grew over the colony (kill:grow ratio range 0.4-0.8). The group 3 isolate (*T. longibrachiatum*--219-0) killed *P. weirii* very slowly or not at all (kill:grow ratio range 0.0-0.1).

The temperature at which the test was conducted affected the relative performance of some of the isolates. *T. harzianum* killed *P. weirii* the fastest of any of the isolates at 20 C, but only the

Table 11. Antagonism of *Phellinus weirii* by *Trichoderma* spp. on malt agar at 10 and 20 C.

Trichoderma species (isolate number)	10 C: Mean[a] rate of lethal effect on *P. weirii* (mm/day)	10 C: Mean rate of overgrowth by *Trichoderma* (mm/day)	10 C: Mean kill:grow ratio[b]	20 C: Mean rate of lethal effect on *P. weirii* (mm/day)	20 C: Mean rate of overgrowth by *Trichoderma* (mm/day)	20 C: Mean kill:grow ratio
polysporum (320-6)	2.7 ± 0.1 a[c]	2.3 ± 0.0 ab	1.2 ± 0.0 ab	5.8 ± 0.3 cd	6.3 ± 0.0 de	0.9 ± 0.0 ab
viride (502-2)	2.6 ± 0.1 a	2.5 ± 0.1 a	1.1 ± 0.0 ab	7.1 ± 0.3 ab	7.7 ± 0.2 ab	0.9 ± 0.0 ab
viride (036-3)	2.2 ± 0.1 b	2.2 ± 0.1 abc	1.0 ± 0.1 ab	6.0 ± 0.4 bc	6.7 ± 0.4 cd	0.9 ± 0.0 ab
viride (350-7)	2.1 ± 0.1 b	2.0 ± 0.1 bc	1.1 ± 0.1 ab	6.7 ± 0.3 bc	7.3 ± 0.4 bc	0.9 ± 0.0 ab
harzianum (382-5)	1.8 ± 0.2 c	1.9 ± 0.2 cd	0.9 ± 0.0 b	8.1 ± 0.3 a	8.3 ± 0.3 a	1.0 ± 0.0 a
aureoviride (301-0)	0.9 ± 0.2 d	1.6 ± 0.2 d	0.5 ± 0.1 cd	4.8 ± 0.4 de	6.3 ± 0.6 de	0.8 ± 0.1 b
hamatum (347-0)	0.8 ± 0.1 d	2.0 ± 0.1 bc	0.4 ± 0.0 d	2.9 ± 0.4 f	5.8 ± 0.3 de	0.5 ± 0.1 c
pseudokoningii (036-2)	0.7 ± 0.0 d	1.0 ± 0.1 e	0.7 ± 0.1 c	4.4 ± 0.3 e	5.6 ± 0.3 ef	0.8 ± 0.0 b
longibrachiatum (219-0)	0.3 ± 0.1 e	2.3 ± 0.1 ab	0.1 ± 0.0 e	0.0 ± 0.0 g	4.8 ± 0.4 f	0.0 ± 0.0 d

[a]Means and standard errors of means of three replicates of each combination of *Trichoderma* sp. isolate and temperature (*P. weirii* isolates combined).
[b]Ratio of the rate of lethal effect on *P. weirii* to the rate of overgrowth by the *Trichoderma* sp. isolate.
[c]Means in the same column followed by the same letter are not significantly ($P \leq .05$) different using Tukey's studentized range test.

fifth fastest at 10 C. Alternatively, T. polysporum was the fifth fastest at 20 C and the fastest at 10 C. The kill:grow ratios of these isolates were relatively constant between temperatures, but their rates of overgrowth were markedly different (Table 11).

The three isolates of T. viride did not differ significantly at 20 C, but at 10 C one isolate (502-2) killed P. weirii significantly ($P \leq .05$) faster than the other two (Table 11).

The two isolates of P. weirii differed significantly ($P \leq .05$) in the rate at which they were killed by the Trichoderma spp. (Table 12) They also differed in the rate of overgrowth by the Trichoderma spp. Their kill:grow ratios did not differ significantly.

Analysis of variance of the rates of overgrowth by Trichoderma spp. indicated that temperature, P. weirii isolate, Trichoderma sp. isolate, and all the two-way and three-way interactions contributed significantly to the variation (Table 13). Analysis of variance of the rates of lethal effect on P. weirii indicated that all but the three-way interaction contributed significantly (Table 14).

Table 12. Antagonism of two *Phellinus weirii* isolates by *Trichoderma* spp. on malt agar at 10 and 20 C.

P. weirii isolate number	10 C			20 C		
	Mean[a] rate of lethal effect on *P. weirii* (mm/day)	Mean rate of overgrowth by *Trichoderma* (mm/day)	Mean kill:grow ratio[b]	Mean rate of lethal effect on *P. weirii* (mm/day)	Mean rate of overgrowth by *Trichoderma* (mm/day)	Mean kill:grow ratio
353-7	1.5 ± 0.2 b[c]	1.8 ± 0.1 b	0.8 ± 0.1 a	4.7 ± 0.4 b	6.0 ± 0.3 b	0.7 ± 0.1 a
591-5	1.7 ± 0.2 a	2.1 ± 0.1 a	0.8 ± 0.1 a	5.5 ± 0.5 a	7.0 ± 0.2 a	0.8 ± 0.1 a

[a]Means and standard errors of means of three replicates of each combination of *P. weirii* isolate and temperature (*Trichoderma* spp. isolates combined).

[b]Ratio of the rate of lethal effect on *P. weirii* to the rate of overgrowth by *Trichoderma* spp.

[c]Means in the same column followed by the same letter are not significantly ($P \leq .05$) different using Tukey's studentized range test.

Table 13. Analysis of variance of the rate of overgrowth of *Phellinus weirii* by *Trichoderma* spp. on malt agar at 10 and 20 C.

A N O V A

Source	df	F	Probability of a greater F
Temperature	1	4211.45	0.0001
P. weirii isolate	1	85.95	0.0001
Trichoderma sp. isolate	8	36.92	0.0001
Temp. X *P. weirii*	1	27.34	0.0001
Temp. X Trich.	8	27.19	0.0001
P. weirii X Trich.	8	5.05	0.0001
Temp. X *P. weirii* X Trich.	8	4.29	0.0003

Table 14. Analysis of variance of the rate of lethal effect on *Phellinus weirii* by *Trichoderma* spp. on malt agar at 10 and 20 C.

A N O V A

Source	df	F	Probability of a greater F
Temperature	1	1770.96	0.0001
P. weirii isolate	1	36.84	0.0001
Trichoderma sp. isolate	8	163.71	0.0001
Temp. X *P. weirii*	1	14.92	0.0002
Temp. X Trich.	8	53.14	0.0001
P. weirii X Trich.	8	2.65	0.0132
Temp. X *P. weirii* X Trich.	8	0.91	0.5093

DISCUSSION

Field Survey

The low frequency of Trichoderma spp. recovered from Phellinus weirii-infested stumps and stump roots (Table 2) is not suprising considering the longevity of P. weirii in these substrates (Hansen, 1979). If Trichoderma spp. were rapidly invading P. weirii-infested stumps, the continuation of disease between rotations would not be the serious problem that it is. The fact that Trichoderma spp. are present in measurable numbers is important, however, and shows that they are capable of surviving and growing in P. weirii-infested stumps and roots of Douglas-fir.

Species Composition

Two species, Trichoderma viride and T. polysporum, were the most prevalent (Table 3). Other workers have found these species to be common in cool temperate forest soils. Trichoderma viride and T. polysporum were the most abundant Trichoderma spp. in cool forest soils in Washington and North Carolina (Danielson and Davey, 1973a). They were common in spruce forest soils in southern Quebec (Widden, 1979), and southern Sweden (Soderstrom, 1975). These species were also common in a Douglas-fir forest soil in Oregon's western Cascades (Nelson, 1982). The relative abundance of T. viride and T. polysporum in this study shows the

ability of these species to colonize P. weirii-infested wood located in a temperate forest.

T. hamatum was isolated only 3 times in this study (Table 3). This low frequency differs from that found in soil isolations from a Douglas-fir forest, where it was the second most frequently recovered Trichoderma species (Nelson, 1982). The scarcity of T. hamatum recovered from stumps and roots may be explained in two ways. Either 1) it is not abundant in the soil of these stands and therefore little inoculum was present to invade infested wood, or 2) it is abundant in the soil but is not particularly successful at invading P. weirii-infested wood. The scarcity of T. hamatum cannot be definitively explained from the available data, but other studies suggest some possibilities. Nelson's (1982) study site is geographically and vegetationally similar to the sites in this study. It seems unlikely that T. hamatum abundance in soil would differ so sharply in similar sites. Additionally, in a survey of fumigated, P. weirii-infested Douglas-fir roots, T. hamatum was frequently isolated from disks which no longer contained viable P. weirii (presumably killed by the fumigant), but seldom from disks in which P. weirii was still viable (Nelson, personal communication). Results from in vitro antagonism tests reported here show that the isolate of T. hamatum tested was a less efficient antagonist than the isolates of T. viride and T. polysporum tested (Table 11). If subsequent tests show that most T. hamatum isolates show only moderate

antagonism toward P. weirii, the explanation that T. hamatum is present in soil but not successful at invading P. weirii-infested wood would be supported.

Distribution

The distributions of Trichoderma spp. and other microorganisms in P. weirii-infested stumps and stump roots according to age, "decay" class, and location within the stump provide information important for determining which of these organisms are invading. Because individual species of Trichoderma provide sample sizes which are too small to elucidate distribution patterns, all Trichoderma spp. are considered together.

1)Age.--The higher frequency of Trichoderma spp. in 11-year-old stumps and roots compared to 1-year-old stumps and roots (Table 2) indicates these fungi are invading, especially when compared with the distribution of the other groups of organisms in the two age classes. Miscellaneous fungi and bacteria were not more abundant in the older stumps and roots, indicating that these organisms were not very successful at occupying additional tissue after initial colonization. They may therefore be considered non-invaders in the present context. It should be recognized that there may be one or more species within these groups which were more abundant in older stumps and roots and might therefore be invading, but their presence would have

been masked when combined in the large groups, miscellaneous fungi and bacteria.

Phellinus weirii was more abundant in the older stumps and roots (Table 2). This observation indicates that the pathogen may continue to colonize new tissue after the death of the tree, but the present study was not designed to test this. The sample may have been biased because the original criteria for stump selection included the presence of P. weirii and the extent of decay. The hypothesis that P. weirii continues to colonize new wood after the death of infected trees is worthy of further testing.

2)Decay class.--The high percentage of isolations from stained and decayed wood which yielded P. weirii (81.3 and 93.9 percent, respectively) and the low percentage from apparently sound wood (3.5 percent) (Table 4) demonstrates that visual assessment of "decay" classes is a reliable indicator of the presence of P. weirii. The heterogenous "decay" class called "other wood" yielded 15.2 percent P. weirii. This class contained areas of resin-soaked wood, and these areas may have been the source for a portion of the positive P. weirii isolations. In addition, some areas were classified as "other wood" because of the presence of other decay organisms such as brown rot fungi, and these may have contained viable P. weirii as well.

The four groups of organisms exhibited different patterns of colonization in the different "decay" classes. These patterns are

best demonstrated by examining the combined "decay" classes (Table 6). Trichoderma spp. were more abundant in PW-wood, while miscellaneous fungi were more abundant in no-PW-wood. Bacteria were slightly more abundant in no-PW-wood, but not significantly so. Two explanations are possible for the greater frequency of miscellaneous fungi recovered from no-PW-wood. 1) Phellinus weirii excluded other fungi by competition for essential nutrients, or 2) P. weirii excluded other fungi from its colonized food base by some active means. While data from this study cannot support either of the two explanations, some evidence exists in the literature for active exclusion. Phellinus weirii produces pseudosclerotial envelopes (zone lines) (Nelson, 1964, 1967, 1968, 1973 and 1975). These structures surround the volume of wood occupied by the fungus. The presence of zone lines is positively correlated with P. weirii survival in infested wood blocks buried in soil, and the exclusion of invading microorganisms. (Nelson, 1964, 1967, 1970, 1973 and 1975). Although the presence of zone lines was not quantitatively recorded in this study, they were frequently observed in the uppermost several centimeters of the stump top. The presence of Trichoderma spp. in PW-wood is due to their ability to either penetrate zone lines or to invade prior to zone line formation (Nelson, 1973). Nelson (1976) found that when P. weirii could not be reisolated from previously colonized wood blocks buried in soil, zone lines had not formed, and Trichoderma spp. were the most frequently isolated organisms.

The preferential colonization of P. weirii-infested wood by Trichoderma spp. in this study (Table 5) supports Gibbs and Smith's (1978) view that they are secondary antagonists; that is, better able to replace an existing pathogen than to prevent an initial infection. The preference of Trichoderma spp. for wood colonized by other fungi has been reported by other researchers. Trichoderma spp. were restricted to decayed wood in Norway spruce (Picea abies) stems infected with Fomes annosus (Delatour, 1976). In P. weirii-infested Douglas-fir stumps inoculated with T. viride, reisolations yielded T. viride from 38 percent of isolations from advanced decayed wood, 29 percent of isolations from stained wood, and only 7 percent of isolations from sound wood (Nelson and Thies, 1985). The apparent preference of Trichoderma spp. for P. weirii-colonized wood, observed in this research as well as other studies, has three possible explanations. 1) Trichoderma spp. might be "obligate hyperparasites", that is they could require a living fungus within the wood. 2) Trichoderma spp. could be limited nutritionally in non-colonized wood, and prior colonization by P. weirii could make the limiting nutrients available. Nitrogen is present in low concentrations in conifer wood (Cowling and Merrill, 1966), and may be the limiting nutrient. Trichoderma viride failed to grow in surface sterilized wood strips, until treated with ammonium sulfamate (Rishbeth, 1976), and treatment of stumps with ammonium sulfamate increased natural colonization by T. viride (Rishbeth,

1979). Although this compound is applied to prevent stump regrowth, it is possible that it stimulates T. viride because of the nitrogen it supplies. 2,4,5-T, also applied to prevent regrowth, failed to encourage growth by saprophytic fungi such as T. viride (Rishbeth, 1976). 3) Trichoderma spp. could be inhibited by some secondary substance present in the wood which is degraded by P. weirii to allow subsequent invasion. The data from this study do not indicate which of the explanations is most likely.

3)Location Within Stumps and Stump Roots.--The most likely explanations for the higher frequency of Trichoderma spp., miscellaneous fungi and bacteria isolated from stumps as compared with stump roots (Table 6) are that the exposed wood on the stump surface served as the primary point of entry, or that conditions near the stump surface were more conducive to microbial colonization. It is also conceivable that these organisms were present in greater numbers in the stem prior to harvest.

Within roots, Trichoderma spp., miscellaneous fungi and bacteria were more abundant in disks distal than proximal to the root collar (Figs. 1, 2 and 3, respectively), but frequencies of isolation by age class reveal differing invasion activity. Miscellaneous fungi (Fig. 7) and bacteria (Fig. 6) were not more abundant in older vs. younger stump roots at any location, whereas Trichoderma spp. were slightly more abundant in older stump roots

proximal to the root collar (Fig. 8). The small sample size of Trichoderma spp. at each location within the roots precluded statistical analysis but the apparent differences suggest that Trichoderma spp. are capable of colonizing P. weirii-infested root tissue if they are suitably introduced.

The invasion of P. weirii-infested roots by all microorganisms occurred very slowly during the first eleven years following harvest. In fact, the frequency of isolation of P. weirii was greater at each location in older roots (Fig. 5), although this observation is possibly the result of sampling bias as discussed above. Stump and root colonization by Trichoderma spp. must be accelerated for the introduction of antagonistic fungi to successfully control the disease.

Within stumps, the organisms exhibited markedly different distribution patterns. Miscellaneous fungi were more abundant in disks closer to the stump top (Fig. 2), but at no location were they more abundant in older stumps than younger (Fig. 7). Bacteria were more abundant in older stumps only in the uppermost disks (Fig. 6). It appears that while bacteria and miscellaneous fungi were able to colonize upper portions of P. weirii-infested stumps, they were not able to penetrate interior portions to an appreciable extent. Conclusions from the analysis of the distribution of Trichoderma spp. within stumps are limited by small sample sizes, but the greater abundance of these fungi in older vs. younger stumps at each location, and gradually

decreasing abundance away from the stump top in older stumps (Fig. 8) suggests they are slowly invading P. weirii- infested stumps. Phellinus weirii was more abundant in older vs. younger stumps at each location except the uppermost disks (Fig. 5). The lack of greater abundance at this location may be the result of either increased occupation by invading organisms, or environmental constraints on P. weirii such as dessication or temperature extremes at the stump top.

Growth Rate Study

Analyses of the data from growth rate experiments demonstrate that species of Trichoderma responded differently to the temperatures tested. The analyses of variance showed that variation between species was highly significant at each of the five temperatures (Table 9). The canonical plot graphically shows the characteristic growth response of the different species (Fig. 10). Discriminant function analysis, using growth rates at five temperatures, correctly placed 68 of 69 isolates in the correct morphological species (Table 10). The incorrectly grouped isolate was morphologically identified as T. viride, but despite obvious morphological differences, was placed with T. hamatum by the analysis. Inspection of the canonical plot (Fig. 10) reveals that this isolate was only slightly closer to the T. hamatum center than to the T. viride center. The comparison of mean growth rates

of species also demonstrates differential growth response. At each temperature there were several species or groups of species which had significantly different mean growth rates (Table 7).

The differences in growth rates between species are important factors to consider when selecting fungi for inoculation of P. weirii-infested stumps. Other researchers have shown that antagonistic activity is related to growth rate (Komatsu and Hashioka, 1964; Lundborg and Unestam, 1980). Soil temperatures in an area comparable to that under study usually range from 3-16 C (Appendix I). These then are the critical temperatures for examining growth rates of candidate antagonists. Trichoderma polysporum and T. viride grew best relative to other species at the lower temperatures (Fig. 9). This is consistent with lower optimum and maximum growth temperatures reported for these species (Danielson and Davey, 1973b). The ability of these species to grow at low temperatures is probably an important factor in their widespread occurrence in cool temperate forest soils (Danielson and Davey, 1973a; Widden, 1979; Soderstrom, 1975; Gibbs, 1967a; Nelson, 1982) and in P. weirii-infested stumps and roots located in a cool temperate forest in the present study.

Trichoderma hamatum grew best relative to other species at 5 C. Above this temperature it consistently grew slightly slower than the average of all the species (Fig. 9). More isolates than the three tested here should be examined before concluding that this species generally grows well at 5 C. Intermediate optimum

and maximum growth temperatures and a high variability were reported for this species (Danielson and Davey, 1973b). Trichoderma harzianum and T. pseudokoningii grew rapidly at the higher temperatures. This finding agrees with high optimum and maximum growth temperatures reported for these species (Danielson and Davey, 1973b) and their widespread occurrence on warmer sites (Danielson and Davey, 1973a). Trichoderma longibrachiatum was omitted from the analysis of relative growth rates because only one field-collected isolate was available for testing, however, it appeared to grow well at 5 and 10 C (Table 7).

Analysis of variance reveals that substantial differences exist between isolates of a given species as well as between species (Table 9). At each temperature F-values were highly significant for within species variation. This indicates that candidate isolates should be tested for their growth rates at appropriate temperatures as part of a screening protocol when selecting isolates for inoculation into P. weirii-infested stumps.

The majority of variation between the hierarchical levels at each temperature was between species (Table 8). The percent of variation attributable to between replicates (error) was highest at 10 and 15 C. This may be the result of the length of time allowed for temperature equilibration before beginning the test. The error term increases as the temperature decreases from 25 to 10 C, but at 5 C it decreases. Three days were allowed for equilibration at 5 C instead of the 14-17 hours allowed for the

other temperatures. Increasing equilibration time, especially at 10 C, should reduce the amount of error in future tests, and still permit three days of growth rate measurements before the colony overgrows the margin of the petri plate.

While these tests provide a relative measure of growth at various temperatures, it should be recognized that malt agar is not the substrate found in nature. Results of these tests should be compared with tests conducted on a more wood-like medium to ascertain whether they can be unequivocally applied to field conditions.

In Vitro Antagonism Study

The many instances where *Phellinus weirii* was recovered from the same sample pair as a *Trichoderma* sp. using the differential medium, suggests that this medium is a useful tool for elucidating antagonism in these and future tests. Isolations from mixed cultures onto malt agar will almost always yield the faster growing *Trichoderma* sp., masking the presence of *P. weirii*. Use of malt agar and the differential medium, along with dual sampling, allowed for discrimination between rate of overgrowth and rate of actual lethal effect. For example, *T. longibrachiatum* overgrew the *P. weirii* colonies at a mean rate of 2.3 mm/day at 10 C, but only killed *P. weirii* at a rate of 0.3 mm/day.

The medium was developed because of the need for determining

viable P. weirii in the presence of Trichoderma spp. Preliminary tests had shown that Trichoderma spp. possessed an unacceptable level of resistance to all individual fungicides tested. In addition, different Trichoderma isolates were resistant to different fungicides. Therefore three different fungicides were incorporated into the medium. Phellinus weirii was not substantially inhibited by any of the fungicides or the combination of all three. Further preliminary tests and the subsequent in vitro antagonism tests reported here showed that the medium supplemented with three fungicides would not allow appreciable growth of any of the Trichoderma spp. isolates tested, and P. weirii could be consistently isolated even in the presence of Trichoderma spp. These fungicides can possibly be employed in future antagonism tests on other substrates.

These tests are the first attempt at quantifying the lethal effect of different Trichoderma species and isolates on P. weirii. Nine isolates representing six species were tested, but no attempt is made to draw definitive conclusions about the differences in antagonism by species. Dennis and Webster (1971a, b and c) and Bell et al. (1982) have emphasized the necessity of testing a large number of isolates of each species because of the high level of intraspecific variability. Nevertheless, significant differences were observed between isolates and further tests may substantiate species differences.

The trends that do emerge are consistent with the data from

the field survey and the growth rate tests and therefore merit further testing. The isolates of T. viride and T. polysporum killed P. weirii at the fastest rate at 10 C. These species were also the most abundant colonizers of P. weirii-infested stumps and roots, and had relatively rapid growth rates at cooler temperatures. From the available data, isolates of these two species seem to be the best candidates for further laboratory testing and eventual field trials. The isolate of Trichoderma harzianum was an efficient antagonist at both temperatures, but had a lower relative rate of overgrowth at 10 C. This observation is consistent with the growth rate tests in which T. harzianum grew best at warmer temperatures. If efficient antagonism toward P. weirii proves to be a species characteristic then isolates of T. harzianum should be considered for use on warmer sites.

The differences observed between the two temperatures in the level of antagonism of Trichoderma isolates, and the significant temperature effect and temperature X isolate interactions revealed by analysis of variance, emphasize the importance of screening potential antagonists at appropriate temperatures. These observations are consistent with previous reports on the importance of temperature in antagonism by Trichoderma spp. (Tronsmo and Dennis, 1978).

Differences in antagonism between isolates of the same species were observed. The three isolates of Trichoderma viride were efficient antagonists at both temperatures, but at 10 C, one

isolate killed P. weirii faster than the other two. This suggests that tests such as these could be used for screening isolates within species as well as between species.

The significant differences between the rates of lethal effect on the two Phellinus weirii isolates and the significant P. weirii isolate X Trichoderma sp. isolate interaction emphasize the importance of screening antagonists against pathogens on a site by site basis. These two P. weirii isolates were different clones (determined by vegetative incompatibility tests), but were obtained from a relatively small geographical area. Possibly more diverse P. weirii isolates such as those obtained from: 1) the coast ranges vs. the Cascades, 2) east of the Cascade crest vs. west of the crest, and 3) cedar hosts in the intermountain region vs. Douglas-fir hosts in the Pacific region would exhibit an equal or larger amount of variability in resistance to antagonism by Trichoderma spp. isolates.

These tests were conducted on an artificial medium (MA). Although previous work has shown that interactions between fungi on MA closely resemble those in wood (Rayner, 1978; Pearce and Malajcuk, 1983), this should not be universally assumed. Results from the antagonism tests reported here and those from future tests on artificial media should be compared to tests carried out in the field. The rapidity of in vitro tests on artificial media needs to be balanced against the fidelity of in vivo field tests. One strategy for achieving the proper balance would be to conduct

a series of tests which represent points on the continuum between artificial media and field tests. This series could include tests:

1) on artificial media such as MA,

2) on artificial media which contain nutrients which are major components of wood, such as cellulose, xylose and lignin,

3) on powdered wood or wood chips,

4) in wood blocks

5) in stumps in the field.

All tests could be conducted with the same groups of pathogens and potential antagonists. Comparison of the results of the laboratory tests with those of field tests could identify the level which optimizes both fidelity and rapidity. The differential medium reported above can be an important element in any or all of these tests, because it provides the capability of determining the viability of P. weirii despite the presence of Trichoderma spp.

General Discussion

The results from the three-part study show that:

1) Trichoderma spp. are capable of surviving in, and appear to be gradually invading, P. weirii-infested stumps and roots of Douglas-fir trees.

2) Two species of Trichoderma, T. viride and T. polysporum, were the most prevalent, and these species are well adapted for growth at temperatures common in the field.

3) Most Trichoderma spp. exert a lethal effect on P. weirii on artificial media and the rate can be measured.

4) Substantial variation exists between and within species in their rates of growth and antagonistic effects on P. weirii.

Further tests are needed to screen large numbers of isolates to identify the most efficient antagonists. The selected isolates should be used in field trials to determine the practicality of controlling laminated root rot by introducing antagonistic fungi into infested stumps and roots.

This research has focused on selecting antagonist isolates which will most rapidly replace P. weirii in infested stumps and stump roots. In order to achieve the maximum rate of replacement, several other aspects of the system should be studied.

1) The formulation of inoculum can affect the effective inoculum potential of introduced antagonists. Inoculum formulated with fermented barley grains was superior to colonized wood dowels (Nelson and Thies, 1985). Establishment of T. viride in beech tree trunks was improved by adding glycerol to the formulation and by covering the wound (Mercer and Kirk, 1984). The placement of inoculum is also important, considering the gradual colonization by Trichoderma spp. found in this study. It may be important to introduce antagonists deep into the interior of stumps and may be necessary to expose proximal sections of major roots for inoculation.

2) Nutritional amendments could affect interactions between P. weirii and introduced antagonists. Nitrogen in particular could

be important. The addition of ammonium sulfamate increased colonization of pine stumps by T. viride (Rishbeth, 1979). Although nitrate-nitrogen is not as stimulatory to growth as ammonium-nitrogen, Danielson and Davey, (1973c) found that all but one of the isolates they tested were able to sustain growth on a medium in which nitrate was the only form of nitrogen. Phellinus weirii was not able to utilize nitrate to an appreciable extent (Li and Bollen, 1975). This apparent differential ability to utilize nutrients should be studied further to determine if antagonists can be favored by nutrient amendments.

3) Stressing agents represent another possibility. Bliss (1951) and Munnecke et al. (1981) found that sub-lethal doses of fumigants inhibited Armillaria mellea more than Trichoderma spp. It is possible that fumigants or other substances more inhibitory to P. weirii than to Trichoderma spp. could be introduced prior to or with antagonists. Several species of Trichoderma were found in greater numbers in P. weirii-infested stumps treated with chloropicrin than in untreated stumps (Nelson, personal communication). Laboratory and field tests are currently underway to determine relative susceptibilities of P. weirii and Trichoderma spp. to chloropicrin (Nelson, personal communication).

Understanding the factors which affect the interactions between P. weirii and Trichoderma spp. in stumps and stump roots may contribute to the development of an important tool for controlling laminated root rot.

SUMMARY

Trichoderma spp. are colonists of *P. weirii*-infested stumps and roots of Douglas-fir, but their abundance is low (1.2 percent of all sample points) within the first 11 years following harvest. Two species, *T. viride* and *T. polysporum* were the most frequently isolated *Trichoderma* species. Invasion by *Trichoderma* spp. is demonstrated by their:

a) greater abundance in stumps and roots of *P. weirii*-infested trees harvested 11 years previously than in those harvested 1 year previously,

b) greater abundance in wood colonized by *P. weirii* than in wood not colonized by *P. weirii*,

c) gradual penetration into less exposed portions of stumps and roots.

The other groups of microorganisms did not exhibit these trends.

The species of *Trichoderma* collected from *P. weirii*-infested stumps and roots had characteristic growth responses to temperature. Relative to other *Trichoderma* species, *T. viride* and *T. polysporum* grew rapidly at cooler temperatures. This may partly explain their abundance in cool temperate forest soils and in *P. weirii*-infested stumps and roots in this study. Significant variation in growth rate exists within species and this may be an important characteristic to consider when screening for antagonists.

Use of the dual isolation/dual culture medium system for *in vitro* antagonism tests, enabled discrimination between rate of overgrowth by *Trichoderma* spp. and rate of lethal effect on *P. weirii*. Differences were observed in the rate at which the *Trichoderma* spp. isolates tested killed *P. weirii*. Further tests are needed to determine if these are consistent species differences. The two isolates of *P. weirii* tested differed in their ability to withstand killing by *Trichoderma* spp., emphasizing the importance of further characterization of pathogen variability and selection of antagonists for use in specific situations. Temperature affected the outcome of the tests, demonstrating the necessity of conducting screening tests at field temperatures.

Further screening is necessary to identify the isolates which are the most efficient antagonists under field conditions. Besides selecting efficient antagonists, other factors which merit study include formulation and placement of inoculum and possible use of nutritional amendments or differential inhibitors. A deeper understanding of all these factors is needed in order to achieve the maximum rate of replacement of *Phellinus weirii*.

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APPENDIX

Appendix I. Soil temperatures[a] on the H. J. Andrews Experimental Forest[b] in the western Cascades of Oregon.[c]

Site	Vegetation	Elevation (m)	Aspect (degrees)	Mean Yearly	Mean Jan.	Mean July	Mean Yearly Maximum	Mean Yearly Minimum
				Temp. (C)				
RS1[d]	forested	490	200	9.9	4.4	15.7	18.4	2.6
RS75[e]	forested	475	355	8.3	3.6	13.0	15.2	1.9
RS89[e]	clearcut	460	315	8.7	2.6	16.1	18.1	0.8

[a]Soil temperatures at a depth of 20 cm.

[b]Meteorological data is from the Primary Meteorological Station on the H. J. Andrews Experimental Forest, maintained by Oregon State University under sponsorship of NSF grant BSR 83-00370.

[c]The H. J. Andrews Experimental Forest is located approximately 34 km and 167° from the field study sites.

[d]Temperature data for 9 years.

[e]Temperature data for 6 years.