AN ABSTRACT OF THE THESIS OF

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Title: ENZYME ACTIVITY AND PHYTOHORMONE PRODUCTION OF ECTOMYCORRHIZAL FUNGI

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Dr. James M. Trappe

The several thousand fungi known to form ectomycorrhizae have co-evolved with their host plants and have developed impressive physiological and ecological diversity. Exploration of some relationships of enzyme activities and phytohormone production of selected ectomycorrhizal fungi to distribution and tree hosts is reported in this thesis. The data have particular relevance to selection of isolates for inoculation of planting stock in forest nurseries, so variation between isolates within species was assessed as well as differences between species.

Enzymatic activity, selected isozyme patterns, and phytohormone production were assayed for the fungi cultured in liquid medium composed of minerals and dextrose. The mycelium was tested for acid and alkaline phosphatase and nitrate reductase activities and chromatographed for isozyme patterns. Culture filtrates were extracted and measured for extracellular cytokinins, IAA (indoleacetic acid) and, in two cases, gibberellins. Nine genera,

24 species and 46 isolates of mycorrhizal fungi were included in the experiments.

The level of enzymatic activity and the amount of extracellular phytohormones produced differed between species and within species of fungi. The data demonstrated that some of the variation among isolates was related to host tree species and environment.

Tricholoma ponderosum S-198 collected from Pinus contorta in coastal sand dunes showed high acid phosphatase and lower alkaline phosphatase than S-199 which was collected from Pseudotsuga menziesii at 1111 m elevation in the Oregon Coast Range. The two cultures also differed in pH optima. There were no significant differences between the two isolates in production of extracellular cytokinins or IAA (indoleacetic acid), and gibberellins were low for both.

Differences in enzyme activity and phytohormone production were prominent among the six isolates of Laccaria laccata. The patterns of acid phosphatase isozyme could be clearly divided into three host related groups. An isolate from a forest nursery differed strikingly in several characteristics from the other isolates, all of which were from a natural forest. This suggests that nursery soil management practices may select for particular edaphic ecotypes of mycorrhizal fungi.

Eight isolates of Pisolithus tinctorius showed generally low acid and alkaline phosphatase activities and also low nitrate reductase activity. These ectomycorrhizal fungi are characteristic of xeric sites with rocky soil that is low in organophosphorus.

Variation between isolates of *P. tinctorius* in phytohormone production was greater than for other fungi. This may account in part for the varied seedling response to ectomycorrhizal formation with this fungus.

Thirteen cultures spanning six species of the genus *Rhizopogon* showed patterns of phosphatase isozyme related to infrageneric groups of the species and also to host species. Host specific species resembled each other more than species associated with a broad range of hosts.

Of the other selected ectomycorrhizal fungi, an isolate of *Paxillus involutus* collected from association with *Corylus cornuta* showed higher nitrate reductase activity than any of the other fungi collected which were from coniferous forests.

These results support the concept that, for nursery inoculation mycorrhizal fungi must be carefully selected for specific sites and host species.

# Enzyme Activity and Phytohormone Production of Ectomycorrhizal Fungi

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# ENZYME ACTIVITY AND PHYTOHORMONE PRODUCTION OF ECTOMYCORRHIZAL FUNGI

## INTRODUCTION

Most vascular plants depend on mycorrhizal fungi for nutrient uptake and the effective use of nutrients (Harley and Smith 1983). Mycorrhizal fungi have coevolved with their host plants and have developed impressive physiological and ecological diversity (Trappe 1977). Phosphorus uptake by mycorrhizal fungi appears to be highly important for host nutrition (Harley and Smith 1983). The role of the fungi in nitrogen uptake appears somewhat less critical, and not all mycorrhizal fungi metabolize nitrate nitrogen (Lundberg 1970, Trappe 1976). Mycorrhizal fungi that have high nitrate reductase capability, however, could benefit host plant nutrition where nitrogen is limiting (Ho and Trappe 1980).

Ectomycorrhizal fungi profoundly affect rootlet morphogenesis and morphology by producing one or several growth regulating substances (Slankis 1973). Effects of the fungal extracellular phytohormones ethylene, IAA (indoleacetic acid), cytokinins and gibberellins on host plants has been observed in pot tests and in vitro. Marked interspecific variation in production of extracellular IAA, cytokinins and gibberellins and in promotion of mycorrhiza formation has been reported by Miller (1971), Gogala (1973), and Slankis (1973, 1974).

Mycorrhizal fungi differ in their effectiveness in improving host plant survival and growth under adverse conditions. In inoculation programs, fungi should be selected for traits that will improve the growth of host plants in nurseries and on reforestation sites. Little work on inter- and intraspecific differences in enzyme activity or phytohormone production by mycorrhizal fungi has been done either in the Pacific Northwest or elsewhere. To better define the physiological traits of candidate species for nursery inoculation the experiments reported here were conducted *in vitro* with 46 mycorrhizal isolates, including 24 species and 9 genera.

The research was designed to determine whether or not enzymatic activity and phytohormone production by ectomycorrhizal fungus isolates *in vitro* differ significantly either between species or within species. Where differences were evident, a second objective was to explore the possibility that those differences related either to the tree host with which the isolates were associated or to habitats in which they occurred. A survey across some key families of ectomycorrhizal fungi was first pursued as described in Chapter I. These studies established that different isolates within a species can vary as much in the traits examined as do different species. Moreover, the particular clarity of isozyme separation of phosphatases as compared to a number of other enzymes was revealed. Chapters II and III deal with intraspecific variation of two fungal species of particular interest for nursery inoculum. Chapter IV then examines relationships of host specificity to enzymes and phytohormones of several species within a single genus. Finally,

Chapter V describes studies of a mycorrhizal fungus that produces gibberellins and occurs in some distinctive habitats. These data provide the first basis for erecting and testing hypotheses on the mechanisms of differential host response to different mycorrhizal fungi.

# CHAPTER I

# Phosphatases, acid phosphatase isozymes, nitrate reductase, indoleacetic acid, and cytokinins produced by selected ectomycorrhizal fungi

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## SUMMARY

Seventeen isolates, encompassing five genera and eight species of ectomycorrhizal fungi, were compared for enzyme activity and phytohormone production. Isolates within species differed significantly in several of the factors measured, and differences between species were pronounced. The meaning of *in vitro* enzyme activity and phytophormone production of ectomycorrhizal fungi needs to be determined in relation to survival and growth of host seedlings in the field, so that the physiological traits of the fungi can be used in selection of inoculum for nurseries.

## INTRODUCTION

Nearly all tree species have co-evolved with mycorrhizal fungi in a strong symbiotic interdependency (Harley and Smith 1983). Trappe (1977) estimated that about 2,000 fungal species form ectomycorrhizae with Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) alone: these can vary markedly in their effects on hosts. Different species display physiological diversity that differentially influences the amount of mycorrhizal development and growth response of host plants (Harley and Smith 1983, Ho and Zak 1979, Hung and Trappe 1983, Laiho 1970, Lundeberg 1970, Trappe 1962). Mycorrhizal fungi are also credited with improving nutrient utilization by increasing root absorbing surfaces, producing extracellular enzymes, and exuding growth regulating substances

which not only affect mycorrhiza morphology but also induce morphogenesis of short roots (Slankis 1973, 1974). To determine if these factors relate to host specificity of the fungi, we selected 8 species with different host associations for determination of extracellular enzyme activities and production of cytokinin and IAA (indoleacetic acid). Five of the species were represented by two or more isolates to assess intraspecific variation.

## MATERIALS AND METHODS

The mycorrhizal fungi were isolated from fresh fruiting bodies and stored as stock cultures at the U.S. Forest Service Forestry Sciences Laboratory, Corvallis, Oregon (Table I.1).

Each isolate was transferred from stock culture to potato dextrose agar plates and incubated at 25°C for about one month. Inoculum plugs were then cut from the edges of the colonies with a copper loop 3 mm in diameter. For each isolate, one inoculum plug was added to each of the six replicate flasks of nutrient solution prepared for growing mycelium for each of the four enzyme and two growth regulator analyses to be performed.

For isozyme electrophoresis, acid and alkaline phosphatases, and nitrate reductase analyses, 250-ml Erlenmeyer flasks containing 100 ml of liquid medium were used for growing fungal colonies. For IAA and cytokinin analyses, 1-liter Erlenmeyer flasks containing 500 ml of the nutrient solution were used. The liquid medium was a modified Melin-Norkrans solution containing (per liter of distilled

water): 0.05 g $CaCl_2$, 0.025 g NaCl, 0.5 g $KHPO_4$, 0.25 g $(NH_4)_2$ $HPO_4$, 0.15 g $MgSO_4$ $7H_2O$, 0.02 g sequestrene (Geigy, 10 percent iron equivalent to 14 percent $Fe_2O_3$), 100 ug thiamine and 5 g dextrose per litre of distilled water. The medium was adjusted to pH 5.5 and autoclaved for 15 minutes at 115°C. All flasks of all isolates for a given analysis were inoculated and arranged randomly on a laboratory bench. After a month of growth at 20°C, contents of each flask were filtered through sterilized Buchner funnels with No. 1 filter paper, the retrieved colonies were washed aseptically with sterile demineralized water and then rinsed with sterile universal buffer (Skujin et al 1962) prepared as follows: a stock solution was made of 12.10 g tris (hydroxymethyl) aminomethane, 11.6 g maleic acid, 14.0 g citric acid, 6.28 g boric acid, 488 ml of 1-N NaOH, with distilled water added to make a volume of 1 litre. Twenty ml of the stock solution were adjusted to the desired pH after adding distilled water to bring the volume to 100 ml. The solution was then filter-sterilized by Buchner funnel with a UF fritted disc.

All results were subjected to analysis of variance. Significant differences between treatment means were tested by Tukey's test at P=0.05.

## Acid Phosphatase Activity

Each washed and buffer-rinsed colony was placed in a sterile 20 ml screw-cap tube with 4 ml of filter sterilized pH 5.5 universal buffer plus 1 ml of 0.1 M disodium p-nitrophenyl phosphate

tetrahydrate and incubated in a water bath at 30°C for two hours in the dark. After incubation, 5 ml of 0.5N NaOH was added to stop the reaction. Enzymatic activity was determined from the amount of p-nitrophenol released from the phosphatase substrate in the filtrate as measured with a Perkin-Elmer 552A UV/Vis spectrophotometer at 410 nm. The fungal colony was removed, rinsed and oven dried at 60°C for dry weight.

Alkaline Phosphatase

The same procedure was used as in acid phosphatase determination except with the universal buffer adjusted to pH 10.

Nitrate Reductase Activity

Each washed and buffer-rinsed colony was placed into a sterile 20 ml screw cap tube containing 3 ml phosphate buffer pH 7, 1 ml neutral succinic acid and 1 ml 0.1 M $KNO_3$. All reagents were filter sterilized through a Buchner funnel with a UF fritted disc. The tubes were incubated for 4 hours in a vacuum incubator at 30°C in the dark. They were then removed from the incubator and 2.5 ml 0.02 percent N-(1-naphthyl)-ethylenediamine dihydrochloride and 2.5 ml 1 percent sulfanilamide in 3N HCl were added. The amount of nitrite present in each suspension was determined a the spectrophotometer at 540 nm. The fungal colony was rinsed and oven dried for dry weight determination.

Electrophoretic Analysis

Each washed colony was rinsed with distilled water and suspended in 0.1 M tris-acetate buffer at pH 6.8 (containing 5 percent PVP 40, 5 percent DOW X1, 1 percent triton X 100 and 1 percent mercapto-ethanol) (D. Copes, personal communication), and stored at 0°C for not more than one month. To prepare for gel electrophoresis, the sample was ground and the suspension absorbed into a 5 x 10 mm paper wick. The starch gel (Scandalios 1969, Conkle et al. 1982) was prepared from 128 g hydrolyzed potato starch with 1.068 ml gel buffer solution (961 ml 0.2 M tris-citrate buffer, pH 8.3; 107 ml 0.2 M lithium-borate buffer, pH 8.3) to make two gel sheets 20 cm x 16 cm x 1.5 cm. A power supply unit was used to supply constant current of 100 mA for about 4 hours.

To develop each set of isozymes, gels were stained as follows: acid phosphatase: 100 ml of 0.2 M acetate buffer, pH 4; 100 mg α-naphthyl acid phosphate; 100 mg fast garnet GBC; 10 drops 10 percent $MgCl_2$; alkaline phosphatase: 100 ml 0.1 M tris-citrate buffer, pH 8.5; 100 mg α-naphthyl acid phosphate; 100 mg fast blue RR salt; 10 drops 10 percent $MgCl_2$; 10 drops 10 percent $MnCl_2$; esterase: 50 ml 0.2 M phosphate buffer, pH 4.3; 10 ml 0.2 M phosphate buffer, pH 9.2; 40 ml distilled water; 2 ml 1 percent α-naphthyl acetate in 1:1 acetone-water; 40 mg fast blue RR salt; cytochrome oxidase: 50 ml 0.1 M phosphate buffer pH 7.2; 35 mg α-naphthyl; 35 mg N,N-dimethylphenylenediamine; 35 mg sodium oxide; malate dehydrogenase: 50 ml 0.2 M Tris-HCl buffer, pH 8; 50 ml distilled water; 1.38 g DL-malic acid; 0.4 ml 0.1 M KCN; 1 ml 0.01 M

NAD; 1.6 mg phenazine methosulfate; 50 mg nitro blue tetrazolium; alcohol dehydrogenase: 50 ml 0.2 M Tris-HCl buffer, pH 8; 130 ml distilled water; 2 ml 0.002 M KCN; 2 ml 0.01 M NAD; 2 ml of phenazine methosulfate; 1 ml absolute alcohol; 50 mg nitro blue tetrazolium; and leucine aminopeptidase: 50 ml 0.2 M tris-maleate buffer, pH 3.3; 20 ml 0.2 M sodium hydroxide buffer, pH 14; 30 ml distilled water; 10 mg L-leucyl-β-naphthyl amide HCl; 20 mg black-K salt.

IAA (Indoleacetic acid) Production

Each culture filtrate was acidified to pH 2.0 and partitioned against reagent grade methylene chloride three times. The methylene chloride portions were taken to dryness in vacuo. The residue was dissolved in 10 ml ethanol and measured with a spectrophotometer at 280 nm (Galston and Davies 1970). The same sample was reduced in volume again for thin layer chromatographic examination. Thin layer chromatography was carried out according to Obreiter and Stowe (1964). Glass plates (20 cm x 20 cm) were coated with 1-mm-thick silicic acid: carboxymethylcellulose (28.5:1.5 w/w). The solvent was n-butanol:chloroform (3:2 v/v) saturated with 0.5 M formic acid. Ehrlich's reagent, 2 percent (w/v) p-dimethylamino-benzaldehyde added to a mixture of absolute ethanol: 10 N HCl (1:1 v/v), was used as detecting agent.

Cytokinin Production

Each culture filtrate was adjusted to pH 2 and extracted with reagent grade methylene chloride three times. The water phase was then adjusted to pH 8.6 with 1 N NaOH and extracted with 2 volumes of ethyl acetate. The ethyl acetate portion was evaporated to dryness under vacuum at 37°C and the residue was dissolved in 80 percent ethanol. The ethanol extract was reduced to water phase again in vacuo at 37°C and the water extract partitioned with 2 volumes of ethyl acetate. The ethyl acetate portion was discarded and the water phase was adjusted to pH 5.5 and extracted with 3 volumes of water-saturated n-butanol. The final water fraction was discarded. This procedure purportedly removes other plant growth substances such as auxins, gibberellins and abscisic acid (Shindy and Smith 1975). The n-butanol fraction was evaporated to dryness and the residue was taken up in double distilled water for thin layer chromatography, which was carried out according to Carlson and Larson (1977).

Glass plates (20 cm x 20 cm) were precoated with 1 mm thick silica gel F-254. Each extract was applied as a streak. The solvent for the separation was n-butanol:1N $NH_4OH$:Water (7:1:2). The standard was applied at both edges of each plate. The plates were air dried at the end of the run. The areas with Rf similar to the cytokinin standard at 254 nm were scraped off and extracted in ethanol for quantitative determination by spectrophotometer.

# RESULTS AND DISCUSSION

## Phosphatases

Acid phosphatase systems were more active than alkaline phosphatase systems (Table I.2), this is as expected because soils of coniferous forests in the Pacific Northest are generally acidic. Species differed significantly in activity. Within the five species represented by two or more isolates, only Amanita muscaria and Astraeus pteridis showed significant differences between isolates. Both are host generalists that form mycorrhizae with many tree species (Molina and Trappe 1982). The ratio of acid/alkaline phosphatases generally was significantly higher within host association groups for isolates that originated at east of the crest of the Cascade Range as compared to more western isolates, a phenomenon that at present we cannot explain. The pine-associated isolates tended to have significantly less acid phosphatase activity than the host generalists or the Pseudotsuga associates. Again, the explanation is not yet evident.

In electrophoresis the most consistent and cleanest band resolutions were produced by the acid phosphatase isozymes (Fig. I.1) The pattern of isozymes of acid phosphatase tended to cluster by host association groups. The four species and nine isolates of Suillus in this study show that trend particularly well (Fig. I.2).

Results with the other six isozymes (esterase, cytochrome oxidase, malate dehydrogenase, alcohol dehydrogenase, leucine aminopeptidase and alkaline phosphatase) were too inconsistent to

interpret meaningfully. Brewbaker et al. (1968) stated that most enzymes are not sharply defined by available stain procedures in electrophoresis. However, the choice of diverse substrates or inhibitors can provide more definitive evidence of isozyme specificity. The methods of Scandalios (1969) and Conkle et al. (1982) were developed for vascular plants and seeds; modifications need to be developed for fungal systems.

Nitrate Reductase

Previous studies indicate that ectomycorrhizal fungi do not effectively utilize nitrate nitrogen (Carrodus 1966, Harley et al. 1954, Lundeburg 1970), and Ho and Trappe (1980) showed that nitrate reductase activity of ectomycorrhizal fungi is low compared to Douglas-fir roots. All these fungi were from acidic forest soil. Negatively charged nitrate ions are not held by negatively charged soil particles, so nitrate nitrogen is easily leached from the root zone. Woolhouse (1969) stated that the chemical environment in the soil may be a dominant component in the selective pressure on a plant; in most forest soils, then, strong nitrate reductase activity would not be of major significance in the ability of a fungus to compete. Low levels of nitrate reductase activity of ectomycorrhizal fungi may have resulted from ecotypic selection through edaphic adaptation. In contrast, the one isolate that we examined from a nonforest soil (a hazel orchard), Paxillus involutus S-403, had significantly higher nitrate reductase activity than all other isolates tested. The irrigated, fertilized orchard may have selected for genotypes adapted for nitrate nitrogen metabolism.

Phytohormones

Ectomycorrhizal fungi produce extracellular ethylene IAA, cytokinins and gibberellins (Crafts and Miller 1974, Graham and Linderman 1980, 1981; Kampert and Strzelczyk 1978; Miller 1967, 1971; Slankis 1973, 1974; Ulrich 1960). The results of our study showed the amounts of extracellular IAA and cytokinins produced in liquid medium vary both between species and between isolates within species (Table I.3).

The highest IAA and cytokinin production occurred in Suillus brevipes S-256, which produced significantly more IAA and cytokinins than most other species or isolates. Among the three isolates of Amanita muscaria S-262 had significantly higher amounts of IAA than the other two.

Cytokinin and IAA regulate many physiological and biochemical processes of plants (Lethan and Palni 1983, Palni et al. 1984). A delicate balance apparently exists between cytokinins and IAA (Slankis 1973, Helgeson 1983). In higher plants cytokinins regulate endogenous IAA formation, activity and transport. Cytokinins at low concentration activate IAA oxidase while higher concentrations deactivate their activity (Hemberg and Larsson 1972, Rudawska 1980). Cytokinins are also reported to delay leaf senescence and relieve water stress (Kende 1984, Nissen 1983, Schistad and Nissen 1984). A shift in cytokinin and IAA balance may also change plant response and increase disease resistance (Helgeson 1983). In tissue culture experiments, high ratios of cytokinin to IAA stimulate formation of shoots, whereas low ratios favor formation of roots (Helgeson 1983, Skoog and Miller 1957, Skoog and Schmitz 1972). In

tree seedlings, cytokinin:IAA ratios thus have the potential to influence not only top/root ratios water stress and resistence to disease. The significantly higher cytokinin:IAA ratio was in Astraeus pteridis S-264, the lowest in Suillus tomentosus S-313.

## CONCLUSION

No clear differences emerged in phosphatase activities or phytohormone production between the different host specificity groups. Isozyme patterns did tend to be more similar within than between host specificity groups, as exemplified within the genus Suillus. The two Douglas-fir isolates resembled each other and the two pine isolates also resembled each other, but, the two host-related groups differed strikingly (Fig. I.2).

An ectomycorrhizal fungus is the sum total of its physiological responses to edaphic adaptation, a total that may differ as much between isolates of a species as between species. Trappe (1977) discussed the ecotypic variations between isolates of a single ectomycorrhizal fungus and stressed the need to test many ecotypes of a particular fungus in selecting specific isolates for nursery inoculation. Enzyme and phytohormone analysis seem promising for evaluating isolates as candidates for nursery inoculation, but first the results of in vitro tests must be related to effectivness on as well as survival and growth of host trees in nurseries and plantations.

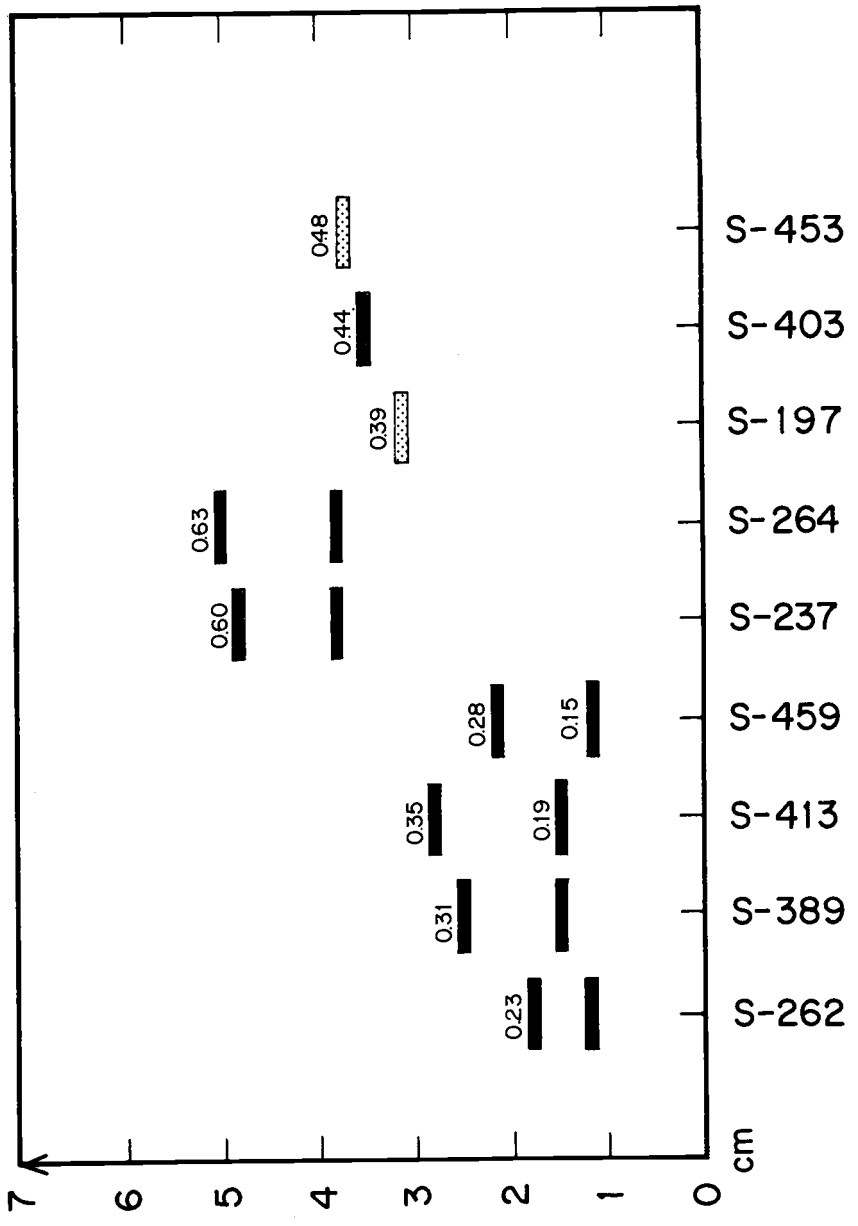


Figure I.1. Zymogram of acid phosphatase isozymes of selected ectomycorrhizal fungi. S-262, 389, 413, 459: Amanita muscaria; S-237, 264: Astraeus pteridis; S-197, 403: Paxillus involutus; S-453: Boletus edulis. The most intense bands are represented by solid lines, those of weak intensity by dotted lines.

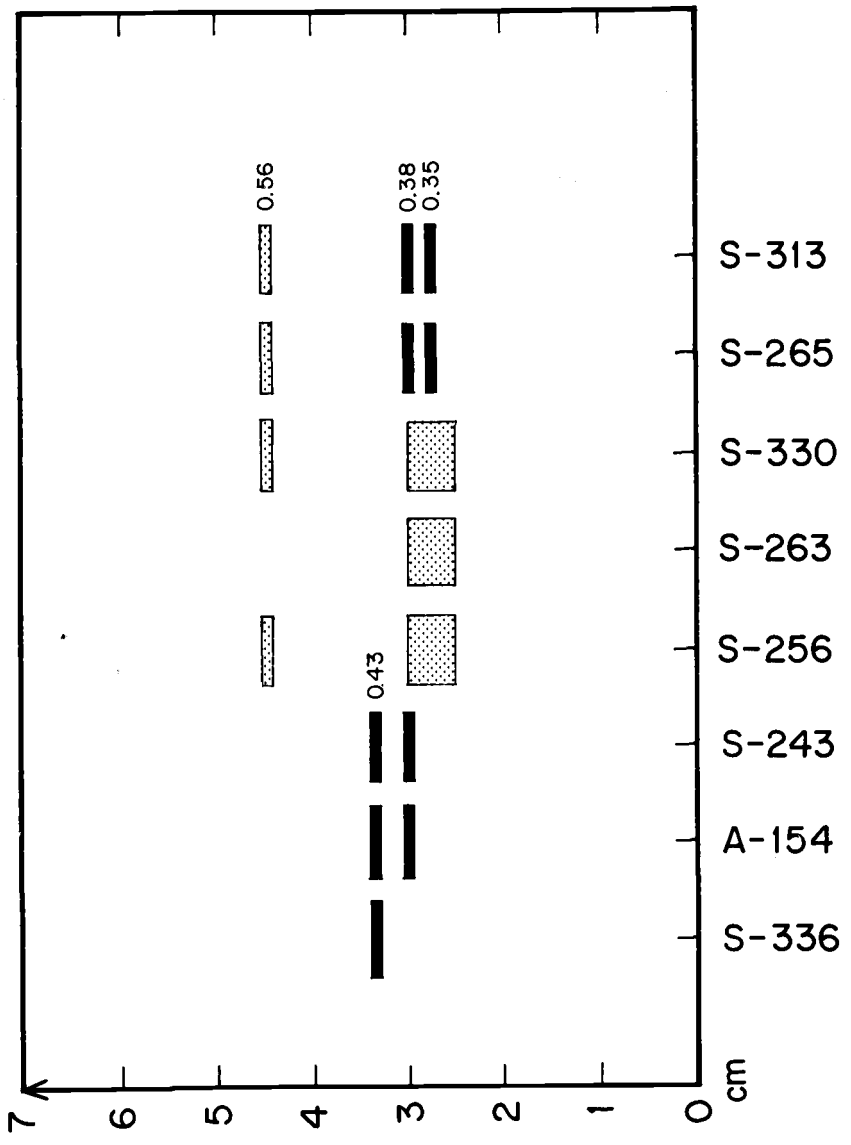


Figure I.2. Zymogram of acid phosphatase isozymes of selected ectomycorrhizal fungi. S-336: Suillus caerulescens; A-154, S-243: S. lakei; S-256, 263, 330: S. brevipes; S-265, 313: S. tomentosus. The most intense bands are represented by solid lines, those of weak intensity by dotted lines.

Table I.1. Species, associated hosts, geographic origin, and year of isolation of mycorrhizal isolates used in enzyme and growth regulator analyses.

| Species | Host | Location | Elevation (m) |
|---|---|---|---|
| *Amanita muscaria* (L. ex F.) Pers.:Hook. | | | |
| S-262 | Mixed conifer | Baker Co., OR | 2200 |
| S-389 | Mixed conifer | Skamania Co., WA | 1000 |
| S-413 | *Tsuga mertensiana* | Jackson Co., OR | 1100 |
| S-459 | *Picea sitchensis* | Glacier bay, AK | 20 |
| *Astraeus pteridis* (Shear) Zel. | | | |
| S-237 | *Pseudotsuga menziesii* | Jackson Co., OR | 1050 |
| S-264 | *Pseudotsuga menziesii* | Benton Co., OR | 920 |
| *Boletus edulis* Bull. ex Fr. | | | |
| S-453 | Mixed conifer | Juneau, AK | 50 |
| *Paxillus involutus* (Batsch ex Fr.)Fr. | | | |
| S-197 | *Salix* sp. | Linn Co., OR | 60 |
| S-403 | *Corylus* sp. | Linn Co., OR | 60 |
| *Suillus brevipes* (Peck) Kuntze | | | |
| S-256 | *Pinus ponderosa* | Union Co., OR | 1500 |
| S-263 | *Pinus contorta* | Lane Co., OR | 1500 |
| S-330 | *P. contorta* | Benton Co., OR | 60 |
| *S. caerulescens* Smith and Thiers | | | |
| S-336 | *Pseudotsuga menziesii* | Yamhill Co., OR | 300 |
| *S. lakeii* Smith and Thiers | | | |
| A-154 | Mixed conifer | Jefferson Co., OR | 920 |
| S-243 | *Pseudotsuga menziesii* | Benton Co., OR | 920 |
| *S. tomentosus* (Kauffm.) Sing., Snell and Dick | | | |
| S-265 | *Pinus contorta* | Lane Co., OR | 1500 |
| S-313 | Mixed conifer | Lane Co., OR | 10 |

Table I.2. Activity of acid and alkaline phosphatase expressed as millimoles of p-nitrophenol liberated per g fungal dry wt. and nitrate reductase as micromoles of nitrite reduced from nitrate per g fungal dry weight[1/].

| Species | Isolate number | Acid phosphatase mM/g | | Alkaline phosphatase mM/g | | Acid/alkaline phosphatase ratio | | Nitrate reductase µM/g | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Pseudotsuga associates | | | | | |
| Suillus caerulescens | S-336 | 355.7a | AB | 66.9a | ABC | 5.6a | ABCD | 1.6b | B |
| S. lakeii | A-154 | 141.1b | ABC | 90.2a | A | 2.8a | BCD | 0.8b | B |
| | S-243 | 77.8b | CD | 38.5a | ABC | 2.2a | CD | 3.6a | B |
| | | | | Pinus associates | | | | | |
| Suillus brevipes | S-256 | 39.0b | D | 3.4b | C | 17.1a | A | 2.1b | B |
| | S-263 | 17.2b | D | 3.3b | C | 6.8ab | ABCD | 3.9b | B |
| | S-330 | 21.6b | D | 21.6b | BC | 1.1b | D | 12.9a | B |
| S. tomentosus | S-265 | 139.5a | BCD | 56.1a | ABC | 2.9b | BCD | 6.0ab | B |
| | S-313 | 140.8a | BCD | 25.2b | ABC | 5.9b | ABCD | 7.7ab | B |
| | | | | Non-host-specific | | | | | |
| Amanita muscaria | S-262 | 277.7bc | ABC | 20.4d | BC | 13.7a | ABC | 4.4b | B |
| | S-389 | 460.0a | A | 32.5cd | ABC | 14.4a | AB | 2.2b | B |
| | S-413 | 40.0d | D | 22.3d | BC | 1.8c | CD | 5.8b | B |
| | S-459 | 86.5d | CD | 26.9cd | ABC | 3.4bc | BCD | 1.5b | B |
| Astraeus pteridis | S-237 | 275.5bc | ABC | 78.4a | AB | 3.6bc | BCD | 1.4b | B |
| | S-264 | 430.6ab | A | 55.4b | ABC | 7.8b | ABCD | 3.7b | B |
| Boletus edulis | S-453 | 284.5bc | ABC | 41.2bcd | ABC | 6.9bc | ABCD | 10.0b | B |
| Paxillus involutus | S-197 | 186.4cd | BCD | 38.7bcd | ABC | 5.2bc | ABCD | 1.9b | B |
| | S-403 | 176.3cd | BCD | 47.6bc | ABC | 3.8bc | BCD | 32.4a | A |

1/Within host-associate groupings, means within columns not sharing a lower case letter differ significantly by Tukey's test (P=0.05).
Among all isolates, disregarding host-associate groupings, means within columns not sharing a capital letter differ significantly by Tukey's test (P=0.05) with different hosts.

Table I.3. Extracellular IAA and cytokinin production of ectomycorrhizal fungi expressed as micromoles per g fungal dry weight[1/].

| Species | Isolate number | Cytokinins μ mole | | IAA μ mole | | Cytokinin/IAA ratio | |
|---|---|---|---|---|---|---|---|
| | | | | Pseudotsuga associates | | | |
| Suillus caerulescens | S-336 | 625a | BC | 1222a | BC | 0.3a | BC |
| S. Takei | A-154 | 521a | BC | 868ab | BCD | 0.5a | BC |
| | S-243 | 133b | B | 491b | BCD | 0.6a | BC |
| | | | | Pinus associates | | | |
| Suillus brevipes | S-256 | 1437ab | AB | 2814a | A | 0.5b | BC |
| | S-263 | 1922a | A | 1054b | BCD | 1.9a | BC |
| | S-330 | 746bc | ABC | 640b | BCD | 1.7a | BC |
| S. tomentosus | S-265 | 114c | C | 264b | CD | 0.4b | BC |
| | S-313 | 169c | C | 767b | BCD | 0.2b | C |
| | | | | Non-host-specific | | | |
| Amanita muscaria[2/] | S-262 | 547ab | BC | 1373a | B | 0.4d | BC |
| | S-389 | 318ab | BC | 422bc | BCD | 0.8bcd | BC |
| | S-413 | 300ab | BC | 399bcd | BCD | 0.8bcd | BC |
| Astraeus pteridis | S-237 | 220b | C | 143cd | D | 1.8bc | BC |
| | S-264 | 226b | C | 53d | D | 4.3a | A |
| Boletus edulis | S-453 | 221b | C | 423bc | BCD | 0.5cd | BC |
| Paxillus involutus | S-197 | 807a | ABC | 581b | BCD | 1.7bcd | BC |
| | S-403 | 719ab | BC | 337bcd | BCD | 2.0b | B |

[1/]Within host-associate groupings, means within columns not sharing a lower case letter differ significantly by Tukey's test (P=0.05). Among all isolates, disregarding host-associate groupings, means within columns not sharing a capital letter differ significantly by Tukey's test (P=0.05) with different hosts.
[2/]The stock culture of A. muscaria isolate S-459 died before this experiment began.

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# CHAPTER II

## Enzyme and phytohormone activity of six isolates of *Laccaria laccata*

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## SUMMARY

Six isolates of Laccaria laccata, S-167 from a forest nursery and S-238, S-283, S-326, S-444, and S-472 from natural forests were grown in liquid culture and analysed for acid and alkaline phosphatase and nitrate reductase activity, acid phosphatase isozyme patterns and IAA and cytokinin production. The isolates varied considerably in enzyme activity and growth regulator substances. Although no consistant relationships of the measured factors occurred with host or habitat sources of the forest isolates, the nursery isolates differed from the forest isolates in several significant ways. It is hypothesized that the nursery environment selects for edaphic ecotypes of L. laccata that are particularly adapted to cultivated, irrigated and fertilized soil.

## INTRODUCTION

Different strains of a mycorrhizal fungus species may vary strikingly in their influence on the growth of host plants (Mikola 1973). Suitability of a mycorrhizal fungus for its tree host for a particular site needs to be considered in nursery inoculation programs. Trappe (1977) emphasized that different strains or ecotypes of a mycorrhizal fungus should be tested in the process of selecting inoculum for the nursery.

The ectomycorrhizal fungus Laccaria laccata (Scop. ex Fr.) Berk. et Br. is common in coniferous forests of the Pacific

Northwest and is being developed for operational mycorrhizal inoculation in forest nurseries (Hung 1984, Molina and Chamard 1983). Its sporocarps may vary greatly in color and size of fruiting body. Isolates of L. laccata also vary in inoculum effectiveness and growth response of host seedlings (Molina 1982). The possibility that adaptation of a mycorrhizal fungus to a particular host-environment combination may evoke genetically controlled variations clearly requires more detailed study. Six isolates of L. laccata from different sites and associated with different conifer hosts were selected for comparison of enzymatic activity and growth regulator substance production. Four of the six isolates had been used previously to inoculate experiments with container seedlings and all formed mycorrhizae (Molina 1982).

## MATERIALS AND METHODS

Laccaria laccata was isolated from six habitats in Oregon (Table II.1) and kept as stock cultures. Procedures used for culture; acid and alkaline phosphatase, nitrate reductase, IAA, and cytokinin analyses; isozyme electrophoresis and statistical analysis were the same as described by Ho and Trappe (Chapter I).

# RESULTS

## Enzymes

Active acid phosphatase systems vary among species of ectomycorrhizal fungi (Bartlett and Lewis 1973, Williamson and Alexander 1975, Ho and Trappe in press; Ho and Zak 1979). Our data show that isolates from within a single species may vary as well (Table II.2). Isolate S-167 from a low elevation forest nursery had strikingly higher acid phosphatase activity than all other isolates. Significant differences in acid phosphatase activity occurred among the other isolates, but no relationships of activity level with host, elevation, or type of habitat emerged. Alkaline phosphatase activity was low for all isolates and the acid/alkaline phosphatase ratio was accordingly high in all cases. Significant differences occurred among isolates, but again these differences showed no trends in relationship to isolate host or habitat.

Nitrate reductase activity may differ significantly between species of ectomycorrhizal fungi (Ho and Trappe 1980). It differed as much or more between isolates of Laccaria laccata (Table II.2). The highest activity of the Laccaria isolates was still much lower than activity reported for nonmycorrhizal Douglas-fir root tips (Ho and Trappe 1980). As with phosphatases, no trends in relation to isolate host or forest habitat were evident.

## Acid Phosphatase Isozymes

The isozyme patterns of the six isolates of L. laccata can be separated into three distinct groups with some suggestion of relationship to host or habitat (Fig. II.1). Group 1 includes the two isolates associated with Douglas-fir in high quality, mesic sites, S-326 and S-444. Group 2 includes only S-238, collected from under Tsuga mertensiana in a cold, high elevation site with pumice soil. Group 3 includes isolates S-167, S-283 and S-472, originating from sources that differed greatly from each other in associated host, elevation, soil, and habitat.

## Phytohormones

Ectomycorrhizal fungi liberate extracellular ethylene, IAA, cytokinins and other metabolites (Gogala 1967, Graham and Linderman 1980, 1981; Miller 1967; Slankis 1973, 1974). The isolates of Laccaria laccata differed significantly in production of both IAA and cytokinins (Table II.3). S-238 and S-236 produced significantly more IAA than S-444, and the other isolates were intermediate between these extremes. S-167 produced significantly more cytokinins than S-283, S-444 and S-472 (Table II.4.)

In thin-layer chromatography IAA appeared at Rf 0.75; cytokinins appeared under UV light at 254 nm.

# DISCUSSION

*Laccaria laccata* isolate S-167, from a forest nursery in the Willamette Valley, stands apart from the other isolates (all from natural forests) in several respects. It produced the highest acid phosphatase activity, had the highest acid:alkaline phosphatase ratio, was the highest cytokinin producer, and had the highest cytokinin:IAA ratio. The other isolates differed significantly from each other in various ways, but at least in this limited sampling no trends towards relationships of isolate characteristics to host or forest habitat of origin were evident.

The particular differences between the nursery isolate and the forest isolates suggest that the cultivated, fertilized, and irrigated nursery environment may select for edaphic ecotypes of a mycorrhizal fungus, as has been proposed for vascular plants by Woolhouse (1969). *Laccaria laccata* should be an excellent species to experimentally test this hypothesis because of its abundance over a broad range of hosts and habitats and the relative ease with which it can be isolated, grown, and manipulated alone or with mycorrhizal hosts (Hung 1984, Molina 1982, Molina and Chamard 1983).

If the nursery environment selects for edaphic ecotypes of a mycorrhizal fungus, the implications for nursery practice need to be explored. For example, IAA and cytokinins have been shown to regulate division, expansion, and differentiation of cells, RNA and protein synthesis, and many other physiological and biochemical processes (Bates and Goldsmith 1983, Baulcombe and Kroner 1981,

Cleland 1982, Hagen and Kleinschmidt 1984, Letham et al. 1982, Palni et al. 1984, Zurfluh and Guilfoyle 1982). Ethylene also has root growth promoting properties similar to IAA (Graham and Lindermann 1980, 1981). These processes are apparently governed by a delicate balance of the quality and quantity of growth substances. A shift in this balance may strikingly alter plant response to plant morphology and to environmental stress (Slankis 1973, 1974; Helgeson 1983, Kende 1984). For instance, in experiments with tissue cultures, Skoog and Schmitz (1972) found that if the cytokinin:IAA ratio is maintained at high levels, cells that subsequently develop into buds, leaves and stems are produced in callus. But if the cytokinin:IAA ratio is lowered, root formation is favored. Helgeson (1983) found that in tissue culture the morphology of tissue was a major factor in resistance of plant callus to disease. Molina's (1982) inoculations of Douglas-fir seedlings with Laccaria laccata isolates are particularly noteworthy in this regard. He found that under standardized experimental conditions, different isolates of the fungus could produce significantly different top/root ratios.

The nursery isolate of our experiments, S-167, produced the highest cytokinin/IAA ratio and accordingly might be expected to produce a higher top/root ratio on host seedlings than the other isolates examined. This result would be the opposite from that generally desired by nurserymen, but it would be a reasonable distribution of growth from the standpoint of energetics. The seedlings, growing in an irrigated, fertile soil with a microbiont ecotype adapted to that soil could afford to put more of their total

photosynthate into photosynthesizing organs and less into absorbing organs. The nurseryman who wishes to reduce the top/root ratio of the seedlings might choose to replace the edaphic ecotype of the microbiont selected through soil management practices. To do so, cultured "wild" isolates carefully selected for more desirable physiological traits could be periodically inoculated in the nursery.

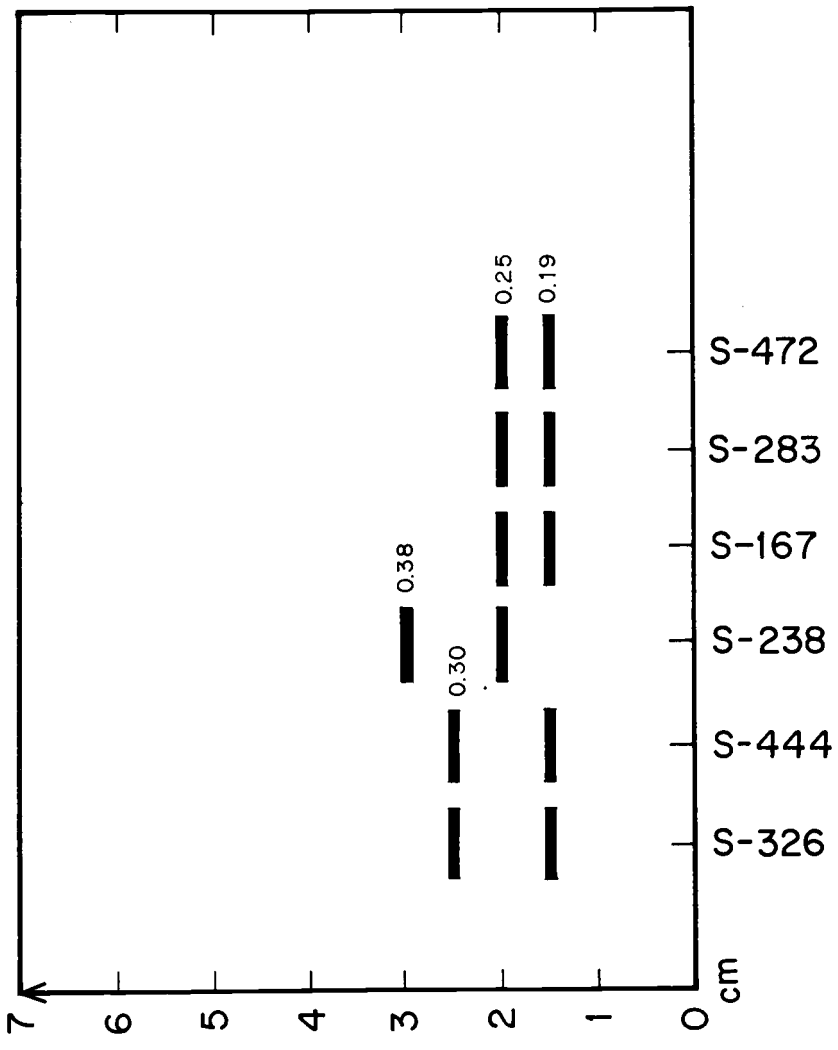


Figure II.1. Acid phosphatase zymogram for six isolates of Laccaria laccata. Darker bands indicate higher activity.

Table II.1. Source habitats of six isolates of *Laccaria laccata* from Oregon.

| Isolate No. | Habitat | Associated Host | Location | Elevation (m) | Year |
|---|---|---|---|---|---|
| S-167 | Tree nursery | *Pseudotsuga menziesii* | Clackamas Co., Willamette Valley | 100 | 1975 |
| S-238 | Pumice soil, cold mesic site | *Tsuga mertensiana* | Klamath Co., Cascade Range | 1525 | 1976 |
| S-283 | Xeric forest | Mixed conifer stand | Union Co., Blue Mountains | 1430 | 1976 |
| S-326 | High quality site | *Pseudotsuga menziesii* | Benton Co., Willamette Valley | 100 | 1976 |
| S-444 | High quality mesic site | *Pseudotsuga menziesii* | Benton Co., Coast Range | 300 | 1977 |
| S-472 | Sand dunes | *Pinus contorta* | Tillamook Co., Pacific Coast | 50 | 1978 |

Table II.2. Enzyme activity by isolates of L. laccata expressed as μM/g fungal dry weight*.

| Isolate No. | Acid phosphatase | Alkaline phosphatase | Acid/Alkaline ratio | Nitrate reductase |
|---|---|---|---|---|
| S-167 | 406 a | 5 b,c | 112 a | 3 b |
| S-238 | 67 c,d | 14 a | 5 b | 18 a |
| S-283 | 115 c | 7 b | 17 b | 8 b |
| S-326 | 112 c | 2 c | 60 a,b | 4 b |
| S-444 | 36 d | 3 c | 11 b | 4 b |
| S-472 | 171 b | 7 b | 25 b | 18 a |

*Means within column not sharing a common letter differ significantly by Tukey's test ($P \leq 0.05$).

Table II.3. Phytohormone production by isolates or *L. laccata* expressed as μM/g fungal dry weight*.

| Isolate No. | Cytokinins | IAA | C/I ratio |
|---|---|---|---|
| S-167 | 335 a | 518 a,b | 0.6 a |
| S-238 | 258 a,b | 665 a | 0.4 a,b |
| S-283 | 153 b | 631 a,b | 0.2 b |
| S-326 | 225 a,b | 662 a | 0.4 a,b |
| S-444 | 156 b | 297 b | 0.5 a,b |
| S-472 | 164 b | 584 a,b | 0.3 a,b |

* Means within column not sharing a common letter differ significantly by Tukey's test ($P \leq 0.05$).

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# CHAPTER III

## Comparison of eight *Pisolithus tinctorius* isolates for growth rate, enzyme activity and phytohormone production

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## SUMMARY

Eight isolates of Pisolithus tinctorius (Pers.) Coker and Couch (three each from Georgia and northern California and one each from Oregon and Washington) were compared in vitro for growth rate; alkaline and acid phosphatase and nitrate reductase activities; acid phosphatase isozyme patterns; and cytokinin, IAA, and gibberellin production. Significant differences appeared among isolates in each parameter examined. All isolates showed relatively low phosphatase and nitrate reductase activities. Isolate S-359 from northern California grew the slowest in culture and produced significantly more IAA than all other isolates and more cytokinin than six of the other seven isolates; this isolate also was the only one of the eight that did not share at least one acid phosphatase allele with the others in the isozyme analysis.

## INTRODUCTION

Pisolithus tinctorius is distributed around the world and has been recorded from at least 36 of the United States (Grand 1976). It can form ectomycorrhizae with a broad range of hosts (Marx 1977) in habitats such as mine spoils, sand dunes, sawdust piles, and eroded or rocky and shallow soils, characterized by environmental extremes such as high soil temperature in summer, extreme acidity, droughtiness, low fertility, or high concentrations of heavy metals, (Marx et al. 1982, Schramm 1966).

Isolates of P. tinctorius differ markedly in culture characteristics and in effectiveness as inocula in both bareroot and container nurseries (Marx 1981, Molina 1979). To better understand such differences, we compared eight isolates from the West Coast and from Georgia for growth rate, enzyme activity, acid phosphatase isozyme patterns, and phytohormone production.

## MATERIALS AND METHODS

The eight isolates used in this study were all isolated from sporocarps and maintained as stock cultures at the Forestry Sciences Laboratory, Corvallis (Table III.1). Three of the isolates (S-172, S-210, and S-360) had been previously used by Molina (1979) in mycorrhizal inoculation experiments. All cultures were grown at room temperature, completely randomized on a laboratory bench. Five replicate cultures were grown for each analytical procedure.

Analytical methods for monoester acid phosphatases, alkaline phosphatases, nitrate reductase, acid phosphatase isozymes, cytokinins, IAA, and gibberellins have been detailed elsewhere (Chapter 1 and II). The substrate for diesterphosphatase was bis-p-nitrophenyl phosphate (Eivazi and Tabatabai 1976); otherwise, its analysis was the same as for monoester phosphatase.

# RESULTS AND DISCUSSION

## Growth Rate

No geographic or host patterns were clear in terms of growth rate (Table III.2). Georgia and West Coast isolates both included representatives of the significantly fastest and slowest growing isolates. The same was true for isolates associated with Pinaceae vs. Fagaceae. The isolates used by Molina (1979) grew the same in relation to each other in both his and our studies. Time since original isolation is not a factor in growth or mycorrhiza formation by *P. tinctorius* (Marx 1981).

## Phosphatases

Isolates of *P. tinctorius* showed much lower acid and alkaline phosphatase activities than *Rhizopogon* species, *Laccaria laccata* (Ho and Trappe, unpublished data) and other ectomycorrhizal fungi (Ho and Zak 1979). Beckjord et al. (1984) found that both roots and leaves of nonmycorrhizal seedlings had significantly higher phosphorus content than those of *P. tinctorius*-inoculated seedlings. The weak phosphatase activity of this fungus (Table III.3) may account for ineffective phosphorus uptake in such circumstances.

*P. tinctorius* has proven especially effective as a symbiont of pines in harsh sites with low organic matter (Ruehle and Marx 1979). In forest soils of the Pacific Northwest, which typically abound in organic matter, *P. tinctorius* has generally not promoted

survival and growth of seedlings in the many plantations in which it has been tested (Castellano and Trappe, unpublished data). We hypothesize that its relatively poor capability to produce phosphatases limits its competitive ability where organophosphorus is a major phosphorus source. Release and utilization of organophosphorus by trees consumes both energy and nitrogen. Perhaps P. tinctorius particularly benefits its host metabolites more for mycelial growth than for enzyme production. Mycelial exploration of a large volume of soil for readily available, mineralized nutrients could be advantageous in low organic soils. In high organic soils, fungi with higher phosphatase and decompositional capability might have the competitive advantage in obtaining nutrients.

## Isozymes of Acid Phosphatase

The isozymes of acid phosphatase separated readily in starch-gel electrophoresis. All isolates except S-359 from California shared at least one allele (Fig. III.1). This indicates greater similarity in acid phosphatase loci than we have found in other ectomycorrhizal fungi (Chapter I and II).

## Nitrate Reductase

Nitrate reductase activity of the eight isolates of P. tinctorius (Table III.4) was generally lower than reported for other ectomycorrhizal fungus (Ho and Trappe, 1980; Chapter I and II). The fastest growing isolate showed the highest nitrate reductase

activity. The lower nitrate reduction capacity may reflect the adaptability and preference for soil chemical conditions in which this fungus thrives.

Phytohormones

The eight isolates of P. tinctorius produced various amounts of IAA, cytokinins, and gibberellins (Table III.5). The effects of these phytohormones on host plants have been discussed in our papers on Rhizopogon species, Tricholoma ponderosum and other ectomycorrhizal fungi (Chapter I and II). They strongly influence the morphology of mycorrhizae (Graham and Linderman 1980, 1981; Slankis 1973, 1974; Rupp and Mudge 1984). Variablility in ectomycorrhiza development among isolates of P. tinctorius has been reported by Molina (1979) and Marx (1981). These variations may be related to the hormones liberated by mycelium of P. tinctorius (Navratil and Rochon 1981). S-359 from northern California produced significantly higher amounts of IAA than other isolates; it also differed from the others in acid phosphatase isozyme patterns. S-431 from Washington showed high amounts of cytokinin and the significantly highest cytokinin:IAA ratio; it also produced the highest amount of gibberellins (Table III.4).

# CONCLUSIONS

Enzyme and phytohormone activity varied considerably between different isolates of *P. tinctorius*, confirming the need to evaluate many isolates of mycorrhizal fungi in selecting specific isolates for nursery inoculation. The significance of these *in vitro* variations to successful inoculation and desired host response now needs to be experimentally determined in fungus-host-soil systems.

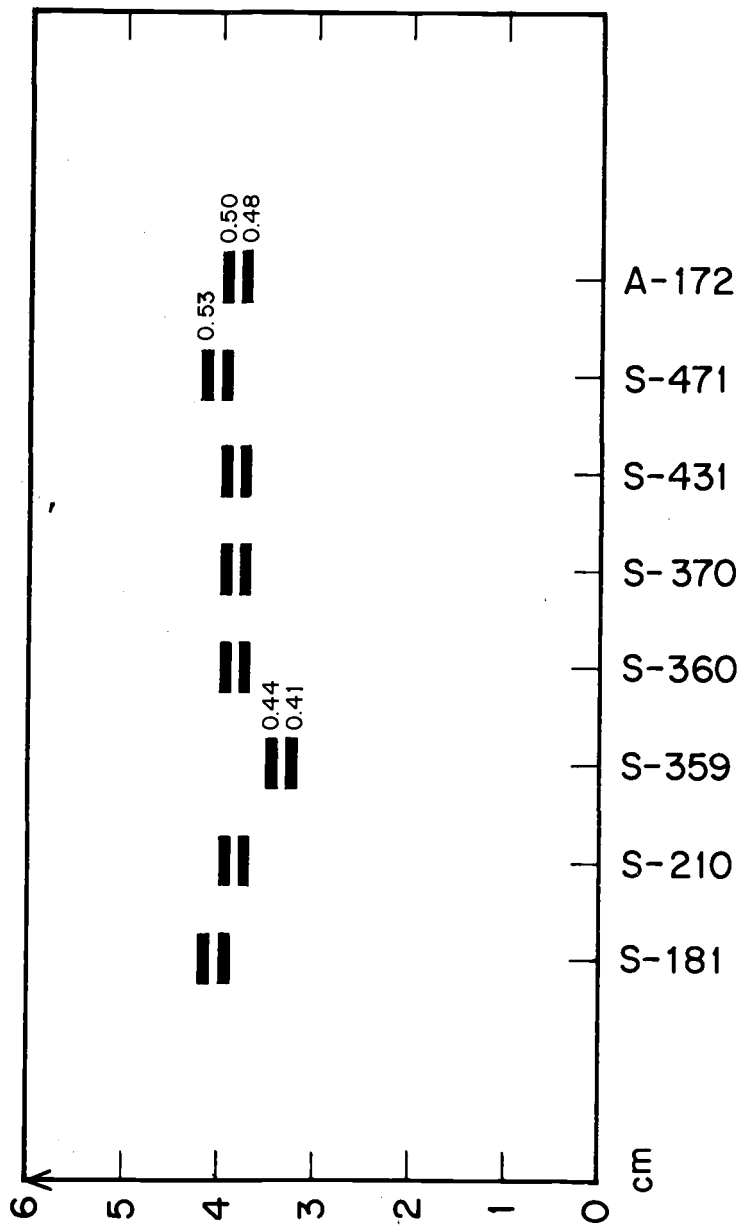


Figure III.1. Zymogram of acid phosphatase isozymes of eight isolates of Pisolithus tinctorius. Darker bands indicate higher activity.

Table III.1. Sources of isolates of Pisolithus tinctorius examined for enzyme activity and phytohormone production

| Location | Isolate no. | Associated host genera | Elev. (m) |
|---|---|---|---|
| Georgia | S-181 | Pinus taeda | μ |
| | S-210 | Pinus taeda | μ |
| | S-471 | Pinus taeda | μ |
| California | | | |
| Siskiyou Co. | S-359 | Pinus lambertiana | 330 |
| " " | S-360 | Lithocarpus densiflorus | |
| | | Arbutus menziesii | 600 |
| Trinity Co. | S-370 | Pseudotsuga menziesii | 400 |
| Oregon | | | |
| Benton Co. | A-172 | Quercus borealis | 200 |
| Washington | | | |
| Skamania Co. | S-431 | P. menziesii | 350 |

Table III.2. Growth of eight isolates of *Pisolithus tinctorius* for one month at room temperature.

| Isolate no. | Oven dry wt.* |
|---|---|
| Georgia: | |
| S-181 | 0.52[a] |
| S-210 | 0.40[a,b] |
| S-471 | 0.27[b,c,d] |
| California: | |
| S-359 | 0.05[d] |
| S-360 | 0.34[a,b,c] |
| S-370 | 0.17[c,d] |
| Oregon: | |
| A-172 | 0.22[b,c,d] |
| Washington: | |
| S-431 | 0.06[d] |

*Isolates not sharing a common letter differ significantly ($P<0.05$) by Tukey's test for differences among treatment means.

Table III.3. Acid and alkaline phosphatase activity of eight isolates of *Pisolithus tinctorius* expressed as millimole per g fungal dry weight*.

| Isolate source and No. | Acid monoester phosphatase | Alkaline monoester phosphatase | Ratio | Acid diester phosphatase | Alkaline diester phosphatase | Ratio |
|---|---|---|---|---|---|---|
| Georgia: | | | | | | |
| S-181 | 18.4[a] | 1.2[a] | 15.4[a] | 4.1[a] | 1.7[b] | 2.4[a] |
| S-210 | 3.9[b,c] | 2.3[a] | 1.7[b,c] | 0.5[c] | 1.3[b] | 0.4[c] |
| S-471 | 2.9[b,c] | 1.2[a] | 2.4[b,c] | 1.3[b,c] | 1.2[b] | 1.1[b,c] |
| California: | | | | | | |
| S-359 | 4.3[b,c] | 1.9[a] | 2.2[b,c] | 0.9[c] | 1.2[b] | 0.8[b,c] |
| S-360 | 7.3[b] | 3.2[a] | 2.3[b,c] | 0.8[c] | 0.9[b] | 0.9[b,c] |
| S-370 | 3.8[b,c] | 1.2[a] | 3.1[b] | 3.5[a,b] | 3.7[a] | 0.9[b,c] |
| Oregon: | | | | | | |
| A-172 | 3.6[b,c] | 3.8[a] | 1.0[c] | 1.7[b,c] | 1.0[b] | 1.7[b] |
| Washington: | | | | | | |
| S-431 | 2.0[c] | 1.4[a] | 1.4[b,c] | 0.8[c] | 0.9[b] | 0.9[b,c] |

*Isolates not sharing a common letter differ significantly ($P<0.05$) by Tukey's test for differences among treatment means.

Table III.4. Nitrate reductase activity expressed as um/g fungal dry weight*.

| Isolate source and No. | Nitrate reductase activity |
|---|---|
| Georgia | |
| S-181 | 28.1a |
| S-210 | 2.7b |
| S-471 | 2.1b |
| California | |
| S-359 | 1.3b |
| S-360 | 1.6b |
| S-370 | 0.7b |
| Oregon | |
| A-172 | 1.0b |
| Washington | |
| S-431 | 0.8b |

*Isolates not sharing a common letter differ significantly ($P<0.05$) by Tukey's test for differences among treatment means.

Table III.5. Phytohormones of eight isolates of P. tinctorius expressed as uM/g fungal dry weight*.

| Isolate source and no. | Cytokinin | IAA | Cy/IAA | Gibberellins |
|---|---|---|---|---|
| Georgia | | | | |
| S-181 | $22.1^{c}$ | $68.2^{b}$ | $0.3^{b}$ | $5.2^{c}$ |
| S-210 | $28.4^{c}$ | $56.0^{b}$ | $0.5^{b}$ | $8.5^{b,c}$ |
| S-471 | $60.9^{a,b,c}$ | $124.8^{b}$ | $0.5^{b}$ | $15.1^{b}$ |
| California | | | | |
| S-359 | $259.1^{a,b}$ | $1045.4^{a}$ | $0.2^{b}$ | $19.3^{b}$ |
| S-360 | $51.3^{b,c}$ | $64.3^{b}$ | $0.8^{b}$ | $9.7^{b,c}$ |
| S-370 | $66.3^{a,b,c}$ | $335.4^{b}$ | $0.2^{b}$ | $18.8^{b}$ |
| Oregon | | | | |
| A-192 | $63.7^{a,b,c}$ | $246.2^{b}$ | $0.3^{b}$ | $13.9^{b,c}$ |
| Washington | | | | |
| S-431 | $271.4^{a}$ | $139.9^{b}$ | $1.9^{a}$ | $44.3^{a}$ |

*Isolates not sharing a common letter differ significantly ($P<0.05$) by Tukey's test for differences among treatment means.

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# CHAPTER IV

## Enzymes and phytohormones of *Rhizopogon* species in relation to mycorrhizal hosts and infrageneric taxonomy

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## ABSTRACT

Acid and alkaline phosphatase and nitrate reductase activities, acid phosphatase isozyme patterns, and cytokinin and indoleacetic acid production were determined for 13 isolates encompassing 6 species and 3 sections of the genus Rhizopogon. Two of the species are mycorrhizal associates with Douglas-fir, one with pines, one a host generalist but usually associated with pines, and two broadly host-general. Isolates within species generally differed in as much from each other as did species or sections of the genus. Of all parameters examined, isozyme patterns were most consistent within species and differed most between sections.


## INTRODUCTION

Over 150 species of Rhizopogon have been described from North America (Harrison and Smith 1968, Hosford 1975, Smith 1964, 1968; Smith and Zeller 1966, Trappe 1975, Trappe and Guzman 1971). Members of this genus, largest of the hypogeous Basidiomycotina, appear to form mycorrhizae predominantly, if not exclusively, with members of the Pinaceae and Ericaceae (Kropp and Trappe 1982; Molina, 1980; Molina and Trappe, 1982a, 1982b; Trappe, 1962), Rhizopogon can be collected in abundance in the Pacific Northwest, and, for the most part, are easily isolated and grow well in pure culture. Accordingly, Rhizopogon is a good genus to study physiological traits in relation to mycorrhizal hosts and taxonomic

relationships. Our study was designed to examine differences between different isolates within a species and between species and sections of the genus in terms of acid phosphatase isozyme patterns, acid phosphatase, alkaline phosphatase and nitrate reductase activity, and phytohormone production.

## MATERIALS AND METHODS

Thirteen isolates encompassing six Rhizopogon spp. (Table IV.1) were used in the experiments. Most Rhizopogon spp. may be able to form mycorrhizae with nonspecific ericaceous hosts in the genera Arbutus and Arctostaphylos (Molina and Trappe 1982a). Other than that, Rhizopogon colossus and R. vinicolor fruit only in association with Pseudotsuga spp. and, in pure culture syntheses, form typical ectomycorrhizae primarily with that host genus. Rhizopogon occidentalis appears to be similarly related to pines. R. vulgaris fruits under pines or in mixed conifer forests and, in pure culture synthesis, forms mycorrhizae with Tsuga and Pinus spp. but not with Pseudotsuga. R. ellenae and R. subcaerulescens appear to be non-host-specific (Molina and Trappe, unpublished data). The isolates were grown in a liquid medium composed of 0.5 g $KH_2PO_4$, 0.25 g $(NH_4)HPO_4$, 0.15 g $MgSO_4 7H_2O$, 0.05 g $CaCl_2$, 0.025 g NaCl, 0.02 µg Geigy sequestrene, 100 µg thiamin, and 10 g glucose per liter of water. The medium was adjusted to pH 5.5 after it was autoclaved.

For enzyme analysis, isolates were grown in 100 ml of medium in 250 ml Erlenmeyer flasks; for growth regulator analysis, isolates were grown in 500 ml of medium in 1-liter narrow-necked Erlenmeyer flasks. Each isolate was represented by five replicate cultures for each experiment, the cultures being started with 2 mm plugs of mycelium from the edge of colonies grown on agar plates. Flasks were randomly arranged on a laboratory bench, incubated for 1 mo at room temperature (ca. 21°C) and the colonies then extracted for analysis.

For acid phosphatase, the mycelium from each flask was filtered from the medium and washed aseptically three times with sterile, deionized water, then rinsed with sterile universal buffer prepared as follows. A stock solution was first made of 3.025 g tris(hydroxymethyl)aminomethane, 2.9 g maleic acid, 3.5 g citric acid, 1.57 g boric acid, 122 ml 1 N NaOH, and distilled $H_2O$ to make a solution volume of 250 ml. Twenty ml of stock solution was titrated with 0.1 N HCl to lower the pH to 5.5, and distilled water was added to bring the volume to 100 ml. The solution was then sterilized by millipore filtration (0.22 μm). Each aseptically washed mycelium was then placed in a sterile 20 ml screw-capped tube with 4 ml of universal buffer and 1 ml of 0.1 M sodium p-nitrophenyl phosphate in universal buffer and incubated in a water bath at 30°C. After incubation, 4 ml of 0.5 M NaOH and 1 ml of 0.5 M $CaCl_2$ were added; the resulting suspension was filtered with Whatman No. 42 ashless filter paper. The fungal tissue was then oven dried, weighed, and the enzyme activity determined from the

amount of p-nitrophenol in the filtrate measured with a Perkin-Elmer 520 Vis/uv spectrophotometer at 410 nm. The results were calculated in micromoles of p-nitrophenol liberated/g dry wt.

Acid phosphatase isozymes were determined by starch gel electrophoresis (Conkle et al. 1982, Scandalios 1969, Smithies 1955). The gel was prepared from 84 g Sigma hydrolyzed starch and 700 ml gel buffer solution (630 ml 0.2 M Tris-citric buffer, pH 8.3, plus 70 ml of 0.2 M lithium borate buffer, pH 8.3). Each run was conducted at a constant current of 100 mA. The incubating solution for detecting acid phosphatase isozymes was composed of 100 ml 0.2 M, pH 4 acetate buffer, 100 mg α-naphthyl acid phosphate, 100 mg fast garnet GBC, and 10 drops of 10 percent $MgCl_2$ solution. Isozymes were recorded after 3 and 24 h incubation at room temperature.

For alkaline phosphatase, the filtered mycelium was washed aseptically with sterile deionized water and rinsed with universal buffer. Samples were placed into 20 ml screw-capped tubes together with 4 ml of universal buffer solution, adjusted to pH 10, plus 1 ml of 0.1 M sodium p-nitrophenyl phosphate in universal buffer at pH 10. The same procedures used in acid phosphatase analysis were then followed (Eivazi and Tabatabai 1976).

Nitrate reductase activity was assayed by standard methods with minor modifications (Ho and Trappe 1980). Mycelia were put into sterilized 20 ml screw-capped tubes containing 3 ml of millipore-filtered phosphate buffer (pH 7), 1 ml 0.1 M neutral succinic acid, and 1 ml 0.1 M $KNO_3$. The tubes were incubated in a

vacuum incubator at 30°C for 4 h, after which they were removed and the contents mixed with 2.5 ml 0.02 percent N-(1-naphthyl)-ethylenediamine dihydrochloride plus 2.5 ml 1 percent sulfanilamide. Amount of nitrite present in each mixture was determined spectrophotometrically at 540 nm on the spectrophotometer. Nitrate reductase activity was expressed as micromoles of nitrate reduced per gram dry fungal tissue wt.

For indoleacetic acid (IAA) determination, 1-mo-old cultures grown as described above were filtered and the ovendry wt of the mycelium was determined. Filtrates were acidified to pH 2 with phosphoric acid (Perley and Stowe 1966). Noninoculated medium served as the control treatment. Filtrates were extracted by partitioning against reagent-grade methylene chloride. The extracts were taken to dryness in a rotary evaporator; part of the residues were dissolved in a minimum volume of absolute methanol for thin-layer chromatrography, and the rest, in 10 ml absolute methanol for UV spectrophotometry. The thin-layer chromatography was carried out on 20 $cm^2$ glass plates coated with silicic acid:carboxymethyl cellulose (28.5:1.5, w/w). The solvent system was n-butanol:chloroform (3:2, v/v) saturated with 0.5 M HCOOH (Obreiter and Stowe 1964). For reference with each run, 10 mM IAA standards (Sigma) were spotted at both edges of the plate. Spectrophotometric determination were at 280 nm (Galston and Davies 1970).

For cytokinin determinations, cultures and filtrates were prepared and extracted as described for IAA. The methylene chloride

layers were discarded and the supernatant adjusted at pH 8, then extracted again by partitioning with n-butanol. The butanol extracts were evaporated to dryness (Kampert and Strzelczyk 1978). Extracts were then dissolved in 1 ml of methanol and applied as a streak on 20 $cm^2$ glass plates coated with 1.0 mm-thick layer of silica gel. The streak was separated with n-butanol:1 N $Nh_4OH:H_2O$, 7:2:1 volume basis (Carlson and Larson 1977). For reference with the run, 10 mM zeatin and zeatin riboside (Sigma), used as cytokinin standards, were spotted at both edges of the plate. For reference with the run, UV spectrophotometry was run at 254 nm with samples dissolved in 10 ml absolute methanol, with results calculated against a standard. Data were subjected to analysis of variance with significance of difference ($P \leq 0.05$) between treatment means evaluated by Tukey's test.

## RESULTS AND DISCUSSION

For the most part, the different isolates of individual Rhizopogon species did not differ significantly in enzyme activity or growth regulator production, although a few notable exceptions occurred (Table IV.2). Thus, R. vinicolor S-387 showed significantly greater alkaline phosphatase activity than S-214, and the two isolates of R. ellenae differed similarly. Acid phosphatase/alkaline phosphatase ratios were consistent within species. In nitrate reductase activity, the only significant differences between isolates within a species occurred with

R. vulgaris; in cytokinin production, only with R. occidentalis. No differences occurred within species for IAA. Striking within-species variation appeared in the acid phosphatase isozyme patterns of the two isolates of R. colossus (Fig. 1): only one band appeared for S-278, whereas 4 bands separated for S-347. Isozyme variation between isolates was less pronounced for R. vinicolor, R. ellenae, and R. subcaerulescens and was lacking for R. occidentalis and R. vulgaris. Isozyme patterns (Fig. 1) tended to differ more strongly between than within species. Even the closely related R. ellenae and R. subcaerulescens, which shared one band in common, differed in a second band that showed for both isolates of R. subcaerulescens but not for either isolate of R. ellenae.

In acid phosphatase activity, only two isolates of R. ellenae within a species differed significantly. Individual isolates of some species differed significantly from individual isolates of other species, but high variation masked significance of trends (Table IV.2). More pronounced differences between species appeared in alkaline phosphatase activity: both isolates of R. colossus and one of R. ellenae showed exceptionally high activity. The acid/alkaline phosphatase ratio was highest in the pine-specific R. occidentalis, with intermediate ratios showing for the non-host-specific R. vulgaris, which is commonly associated with pines. Except for the strikingly active R. vulgaris S-251, nitrate reductase activity did not differ between species. R. occidentalis was the strongest IAA producer, and its isolate S-252 produced the most cytokinins. The highest cytokinin/IAA ratio occurred with

R. vinicolor; there were no significant differences between the other species.

No clear differences between host specificity patterns or infrageneric groupings of Rhizopogon species emerge from these data, but some interesting possibilities deserve further exploration. The representative of section Villosuli (Smith 1964), R. colossus, shows a relatively high phosphatase activity evenly distributed between acid and alkaline phosphatases. This species is also host specific to Douglas-fir, as are several other members of section Villosuli (Molina and Trappe, unpublished data). R. vinicolor, also strongly associated with Pseudotsuga, has isozyme patterns of phosphatase quite different from those of R. colossus, but then it has been placed in section Fulviglebae by Smith. R. occidentalis, specific to pines, and R. vulgaris, commonly associated with pines, are both in the section Rhizopogon and have strikingly high acid/alkaline phosphatase ratios. R. ellenae and R. subcaerulescens, both host generalists in the section Amylopogon, show no distinctive activities or ratios and, indeed, tend to be on the low side in most of the parameters examined. The acid phosphatase isozyme patterns (Fig. IV.1) suggest closer relationship between the Pseudotsuga-associated R. colossus and R. vinicolor than either has for the other species. The pine-associated R. occidentalis and R. vulgaris form a second group with similar, one-band, low-Rf isozyme patterns. Finally, the host generalists R. ellenae and R. subcaerulescens resemble each other in isozyme patterns more than the other groups. Other representatives of these groupings need

similar study before broad conclusions can be drawn, but our data so far lend support to the infrageneric taxa proposed by Smith (1964).

The relationship of enzymatic activities and growth regulator production of mycorrhizal fungi to isolate distribution or ecotypic differentiation or the importance of these traits to survival and growth of host seedlings remains to be clarified. Woolhouse (1969) has proposed a relationship between phosphatases and edaphic ecotypes of vascular plants; the possibility of such a relationship in mycorrhizal fungi deserves consideration. Cytokinin and IAA regulate division and differentiation of cells, RNA and protein synthesis, and many other physiological and biochemical processes (Cleland 1981, Hagen et al. 1984, Letham et al. 1982, Schneider 1980, Thimann 1969, 1980). Ethylene also has whown root promoting properties similar to IAA (Graham and Linderman 1980, 1981). Shifts in the balance of growth regulators may affect a plant's physiological response (Helgesen 1983, Slankis 1973). For example, a high cytokinin/IAA ratio favors formation of shoots over roots in plant tissue cultures, whereas a lower ratio favors root growth (Helgesen 1983, Skoog and Miller 1957, Skoog and Schmitz 1972). Shoot/root ratios of ectomycorrhizal Pinaceae can differ with different, inoculated mycorrhizal fungi (Trappe 1975), a phenomenon that may relate to the cytokinin/IAA production of the fungi involved.

Fig. IV.1 Zymogram of acid phosphatse isozymes of Rhizopogon spp. The most intense bands are represented by solid lines, those of weak intensity by dotted lines.

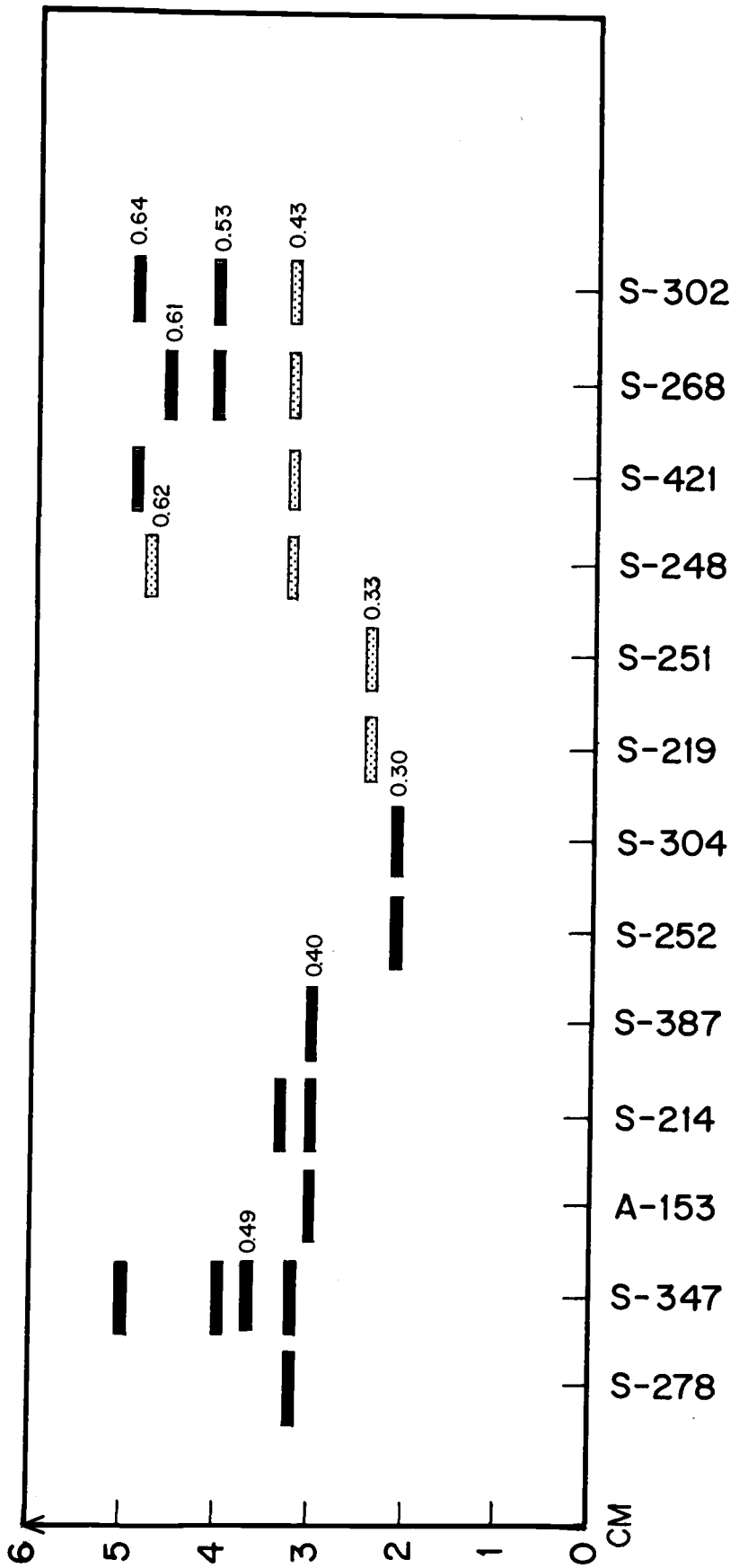
0.64
0.61
0.62
0.53
0.43
0.33
0.30
0.40
0.49
S-302
S-268
S-421
S-248
S-251
S-219
S-304
S-252
S-387
S-214
A-153
S-347
S-278
6
5
4
3
2
1
0
CM

fig. IV.1

Table IV.1 Isolates of Rhizopogon spp. used in enzyme and phytohormone analyses.

| Species and isolate number* | Host | Location (County and State) | Elevation (m) | Year of Collection |
|---|---|---|---|---|
| *Rhizopogon ellenae* | | | | |
| S-248 | Abies grandis | Union Co., OR | 1500 | 1976 |
| S-421 | Arbutus menziesii | | | |
| | Pinus ponderosa | Jackson Co., OR | 400 | 1977 |
| *R. subcaerulescens* | | | | |
| S-268 | Mixed conifer | Grant Co., OR | 1600 | 1976 |
| S-302 | P. contorta | Tillamook Co., OR | 30 | 1976 |
| *R. occidentalis* | | | | |
| S-252 | P. ponderosa | Union Co., OR | 1300 | 1976 |
| S-304 | P. contorta | Lane Co., OR | 45 | 1976 |
| *R. vulgaris* | | | | |
| S-219 | Tsuga mertensiana | Lane Co., OR | 1700 | 1976 |
| S-251 | P. ponderosa | Union Co., OR | 1300 | 1976 |
| *R. colossus* | | | | |
| S-278 | Mixed conifer | Yakima Co., WA | 900 | 1976 |
| S-347 | Pseudotsuga menziesii | Yamhill Co., OR | 300 | 1976 |
| *R. vinicolor* | | | | |
| A-153 | T. heterophylla | | | |
| | P. menziesii | Benton Co., OR | 450 | 1975 |
| S-214 | P. menziesii | Linn Co., OR | 1500 | 1976 |
| S-387 | T. heterophylla | Skamania Co., WA | 450 | 1976 |

*Voucher collections for each isolate are deposited in the Mycological Herbarium of Oregon State University.

Table IV.2. Activity of acid and alkaline phosphatase expressed as millimoles of p-nitrophenol liberated per g fungal dry wt., of nitrate reductase as micromoles of nitrite reduced from nitrate per g fungal dry wt., and production of extracellular IAA and cytokinin as micromoles per g fungal dry wt.[1/].

| Species and isolate | Phosphatases acid mm | | Phosphatases alkaline mm | | Nitrate reductase µM | | Cytokinin µM | | IAA µM | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Generalists** | | | | | | | | | | |
| *ellenae* | | | | | | | | | | |
| S-248 | 9.7b | C | 6.0c | D | 0.9a | B | 474.3a | BC | 2144.9a | ABC |
| S-421 | 147.1a | BC | 211.8a | AB | 1.0a | B | 276.9ab | BC | 1044.4a | BC |
| *subcaerulescens* | | | | | | | | | | |
| S-268 | 179.9a | BC | 58.6b | CD | 3.1a | B | 356.9ab | BC | 3371.5a | AB |
| S-302 | 105.4ab | C | 32.4bc | D | 4.2a | B | 148.0b | BC | 790.2a | BC |
| **Pine-preferring** | | | | | | | | | | |
| *occidentalis* | | | | | | | | | | |
| S-252 | 330.0a | AB | 15.9ab | D | 12.9b | B | 1176.8a | A | 3687.3a | A |
| S-304 | 511.6a | A | 20.7a | D | 6.1b | B | 132.8b | C | 1101.6b | ABC |
| *vulgaris* | | | | | | | | | | |
| S-219 | 85.5b | C | 6.4b | D | 3.9b | B | 479.3b | BC | 567.2b | C |
| S-251 | 62.7b | C | 4.5b | D | 109.9a | A | 584.7b | BC | 928.3b | BC |
| **Df-preferring** | | | | | | | | | | |
| *colossus* | | | | | | | | | | |
| S-278 | 258.0a | B | 249.2ab | AB | 1.7b | B | 588.4ab | BC | 1371.8bc | ABC |
| S-347 | 255.5a | B | 296.0a | A | 1.7b | B | 188.7bc | C | 1645.5ab | ABC |
| *vinicolor* | | | | | | | | | | |
| S-153 | 115.7b | C | 89.6c | CD | 0.8b | B | 612.1ab | BC | 585.4cd | C |
| S-214 | 106.2b | C | 22.4c | D | 4.9a | B | 162.5c | C | 244.8d | C |
| S-387 | 114.6b | C | 153.5bc | BC | 0.9b | B | 643.5a | B | 2286.5a | ABC |

[1/]Within host-associate groupings, means within columns not sharing a lower case letter differ significantly by Tukey's test ($P<0.05$). Among all isolates, disregarding host-associate groupings, means within columns not sharing a capital letter differ significantly by Tukey's test ($P<0.05$) with different hosts.

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CHAPTER V

# Enzymatic activity and phytohormone production of *Tricholoma ponderosum*, the "American Matsutake"

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## SUMMARY

*Tricholoma ponderosum* (Peck) Singer forms mycorrhizae with conifers in the Pacific Northwestern United States. Two Oregon isolates of *T. ponderosum*, S-198 from *Pinus contorta* on coastal sand dunes and S-199 from *Pseudotsuga menziesii* at 1000 m elev. in the Coast Range, were examined for pH optima, enzymatic activity and production of extracellular growth regulators. The pH optima did not differ significantly between the two isolates. S-198 was higher in acid phosphatase activity and lower in alkaline phosphatase activity than S-199. Both isolates were low in nitrate reductase activity and both showed similar patterns of acid phosphatase isozymes in electrophoretic analysis. Detectable extracellular growth regulators included gibberellins, cytokinins, and indoleacetic acid.

## INTRODUCTION

*Tricholoma ponderosum* (Peck) Singer forms mycorrhizae with Pinaceae in the northern U.S. and adjacent Canada (Kinugawa and Goto 1978). It is closely related to *T. matsutake* (Ito et Imai) Singer; the two differ only slightly in morphology (Zeller and Togashi 1934). In British Columbia, *T. ponderosum* is confined to certain bioclimatic zones: *Picea engelmannii* Parry, *Abies lasiocarpa* (Hook.) Nutt., interior *Tsuga heterophylla* (Raf.) Sarg., coastal *Pseudotsuga menziesii* (Mirb.) Franco and coastal *Tsuga heterophylla*.

All are characterized by dry summers followed by humid autumns and winters with mean annual precipitation exceeding 1000 mm, mostly falling from October to June. In Oregon T. ponderosum occurs on coastal sand dunes in association with Pinus contorta Dougl. and in the Coast and Cascade Ranges as high as 2500 m elev., usually under Pinus contorta, P. ponderosa Laws, Pseudotsuga menziesii, or Tsuga heterophylla. It also occurs in the Rocky Mountains, Great Lake States, northeastern United States and eastern Canada (Kinugawa and Goto 1978).

The vastly different terrain and hosts occupied by T. ponderosum suggest a possibility of edaphic ecotypes (Zeller and Togashi 1934, Woolhouse 1969). Accordingly, we compared two isolates for acid phosphatase, alkaline phosphatase, and nitrate reductase activity, acid phosphatase isozyme patterns, and production of free indoleacetic acid, cytokinins and gibberellins.

## MATERIALS AND METHODS

The isolates were obtained from sporocarp tissue and maintained in stock culture. Isolate S-198 was from a specimen collected from under Pinus contorta on coastal dunes at 25 m elev. at Sand Lake, Tillamook County, Oregon. S-199 was from Pseudotsuga menziesii in the Oregon Coast Ranges at 1,000 m elev. on Mary's Peak, Benton County.

## Fungus Culture and Experimental Design

Isolates were grown in 250 ml Erlenmeyer flasks with 100 ml of liquid medium for enzyme activity determination, and in 1 litre Erlenmeyer flasks with 500 ml of medium for analysis of free growth regulators. The liquid medium was modified Melin-Norkrans solution: 0.05 g $CaCl_2$, 0.025 g NaCl, 0.5 g $KH_2PO_4$, 0.25 g $(NH_4)_2HPO_4$, 0.15 g $MgSO_4\ 7H_2O$, 0.02 g sequestrene (Geigy), 100 µg thiamine HCl and 5 g dextrose per litre of distilled $H_2O$. It was adjusted to pH 5.5 and autoclaved at 115°C. For pH experiments, the pH was adjusted to 4,5,6,7, and 8, respectively after autoclaving. Ten replicate flasks for each isolate for each set of analyses were inoculated with plugs removed with a 3 mm-diameter brass loop from the edges of young colonies on agar plates. All treatments were arranged in a completely randomized design; the data were subjected to analysis of variance.

## Phosphatase Activity

After a month of growth at room temperature, the colony was filtered from each flask and washed aseptically with sterile deionized water followed by a rinse with sterile universal buffer (Skujins et al. 1962). A stock solution of universal buffer was made of 12.1 g tris (hydroxymethyl) aminomethane, 11.6 g maleic acid, 14.0 g citric acid, 6.28 g boric acid, and 488 ml 1N NaOH, with distilled water added to bring the total volume to 1 litre. Twenty ml of stock solution were adjusted to pH 5.5 for acid phosphatase and pH 10 for alkaline phosphatase determination (Eivazi

and Tabatabai 1976) and distilled water was added to bring the volume to 100 ml. The buffer solution was then sterilized by 0.22 μm Millipore filtration. After an aseptic rinse with sterile universal buffer, each colony was placed in a sterile 20 ml screw cap tube with 4 ml of buffer solution plus 1 ml filter-sterilized of 0.1 M disodium-p-nitrophenyl phosphate and incubated in a 30°C water bath for 2 hours in the dark. After incubation, 5 ml of 0.5 M NaOH were added to stop the reaction. The colony was removed, rinsed, and oven-dried at 60°C. Phosphatase activity of separate sets of acid phosphatase and alkaline phosphatase were determined from the amount of p-nitrophenol released in the filtrate from the phosphatase substrate as measured in a Perkin-Elmer 552A Vis/UV spectrophotometer at 410 nm.

Gel-electrophoretic Analysis

Each colony was washed, removed from liquid culture and suspended in 0.1 M tris-acetate buffer at pH 6.8 containing 5 percent of PVP 40, 5 percent of DOW X1, 1 percent Triton X100, and 1 percent 2-mercapto-ethanol and stored at 0°C.

Starch gel electrophoresis was performed according to methods of Scandalios (1969) and Conkle (1972). The colony was ground in buffer solution and the suspension was absorbed into a 5 x 10 mm paper wick. The gel was prepared from 128 g of hydrolyzed starch with 1067 ml of gel buffer solution (961 ml 0.2 M tris-citrate buffer at pH 8.3, 106 ml 0.2 M lithium-borate buffer at pH 8.3). The run was carried out at a constant current of 100 mA for 4

hours. The developing solution for detecting acid phosphatase isozymes consisted of 200 ml 0.2 M acetate buffer at pH 4, 100 mg α- naphthyl acid phosphate, 100 mg fast garnet GBC and 10 drops of 10 percent $MgCl_2$ solution. The isozymes were recorded after 3 and 24 hrs.

## Nitrate Reductase Activity

Each culture was filtered and washed aseptically with sterile deionized water followed by a rinse with sterile phosphate buffer at pH 7.0. After the rinse each colony was aseptically transferred to a sterile 20 ml screw cap tube containing 3 ml of filter-sterilized phosphate buffer at pH 7.0, 1 ml 0.1 M neutral succinic acid and 1 ml 0.1 M $KNO_3$. The tubes were incubated in a vacuum incubator at 30°C for four hours. After incubation, tubes were removed and the contents mixed with 2.5 ml 0.02 percent N-(1-naphthyl)-ethylenediamine dihydrochloride and 2.5 ml 1 percent sulfanilamide in 10 percent HCL. Amount of nitrate present in each mixture was determined on a Vis/UV-spectrophotometer at 540 nm.

## Indoleacetic Acid

The isolates were grown in 500 ml modified Melin-Norkran's liquid medium. Culture filtrates were acidified to pH 2.0 and partitioned against reagent grade methylene chloride in a separatory funnel. The methylene chloride portions were dried in a rotary evaporator. The residues were dissolved in 10 ml of ethanol for UV-spectrophotometric determination at 280 nm (Galston and Davies 1970).

Thin-layer chromatography was carried out by the methods of Obreiter and Stowe (1964) with 20 cm by 20 cm glass plates coated with silicic acid:carboxymethyl cellulose (28.5:1.5 w/w). The solvent system was n-butanol:chloroform (3:2 v/v) saturated with 0.5 formic acid. Ehrlich's detection reagent was prepared by adding 2 percent (w/v) p-dimethylaminobenzaldehyde to a mixture of absolute ethanol and 10N HCl (1:1 v/v).

Cytokinins

Each colony was filtered from 500 ml modified Melin-Norkran's liquid medium. Filtrates were adjusted to pH 2.0 and extracted with reagent grade methylene chloride three times. The water phase was then adjusted to pH 8.6 with 1N NaOH and extracted with two volumes of ethyl acetate three times. The ethyl acetate fraction was evaporated to dryness under vacuum at 37°C and the residue dissolved in 80 percent ethanol. The ethanol extract was reduced to water phase under vacuum at 37°C and then extracted with two volumes of ethyl acetate three times. The ethyl acetate phase was discarded and the water phase adjusted to pH 5.5 and extracted with three volumes of water-saturated n-butanol three times. The water fraction was discarded. This procedure purportedly removes all other plant growth substances, such as auxins, gibberellins and abscisic acid (Shindy and Smith 1975). The n-butanol fractions were vacuum dried and the residues taken up in 10 ml of deionized water for UV-spectrophotometric measurement at 254 nm. Thin-layer chromatography was carried out on 20 cm x 20 cm glass plates

precoated with silica gel to a thickness of 1 mm. Each extract was applied as a streak. The solvent system for separation was n-butanol: 1N $NH_4OH$: water (Carlson and Larson 1977). Zeatin and zeatin riboside (Sigma) used as cytokinin standards were spotted at both edges of the plates. The plates were air dried at the end of the run.

Gibberellins

The extraction of free gibberellins follows the procedure of Gaskin and MacMillan (1978). Culture filtrates were partitioned at pH 8 with ethyl acetate for neutral and basic ethyl acetate fractions (nonpolar gibberellic acids, e.g., $GA_9$, GA glucosyl esters), again partitioned at pH 2.5 with ethyl acetate for the acidic ethyl acetate fraction (free GA's) and finally partitioned at pH 2.5 with n-butanol for the acidic n-butanol fraction (polar GA's e.g. $GA_{32}$, GA glucosyl esters). The extracts were concentrated under reduced pressure at low temperature. The remaining traces of water were removed by azeotropic distillation in toluene. Finally the extracts were esterified (Reeve and Crozier 1978). For esterification, the extracts were dried over phosphorus pentoxide in vacuo for 6 hours. Dioxane and dimethylformamide dibenzylacetol were added through the septum at the rate of 100 $mm^3$ per milligram of samples and heated to 70°C water bath up to 4 hours for complete esterification. At the moment of completion of the reaction, GA benzyl esters separated from the excess of dimethylformamide dibenzylacetol. The extracts and standards were measured at 254 nm on a UV-spectrophotometer.

# RESULTS AND DISCUSSION

## pH

Neither isolate of Tricholoma ponderosum grew at pH 4. Both isolates grew within a pH range of 5 to 8: mean growth did not differ significantly either between isolates at a given pH or between pH's for a given isolate (Table V.1). Tricholoma matsutake isolates have been reported to grow at pH's of 4 or less, but best growth for some isolates occurs between pH 4 and 5.5, for others at pH 5 to 8 (Hamada 1950, Kuraishi 1953, Ogawa and Akama 1983). Variation between isolates of an ectomycorrhizal fungus species in response to pH in vitro is usual (Hung and Trappe 1983).

## Activity and Isozyme Patterns of Phosphatases

A major proportion of total phosphorus in forest soil is in organic forms. Organic matter in the floor of old-growth Douglas-fir forests can contain as much as 2,000 kg/ha of P; P content in young growth stands is considerably less (Youngberg 1979). The hydrolysis of complex organophosphorus compounds to inorganic phosphate readily available for uptake by plants is catalyzed mainly by phosphatases of soil organisms such as ectomycorrhizal fungi. The phosphatase activity of mycorrhizal fungi is a chief link in the phosphorus cycle of forest ecosystem: the soil of an old-growth Douglas-fir stand in Oregon has been estimated to contain 5,400 kg/ha of mycorrhizal root tips (Fogel, Ogawa and Trappe 1973). The soil of young, second growth

Douglas-fir stands has been estimated to contain fungal biomass ranging from 19,900 to 25,000 kg/ha (Fogel and Hunt 1979). Mycorrhizal fine roots account for 51-55 percent of organic matter uptake in temperat forest (Fogel and Hunt 1983). Phosphatase contribute to phosphorus turnover by catalyzing the hydroloysis of organic phosphorus compounds (Ho and Zak 1979).

The two isolates of T. ponderosum differed significantly in alkaline phosphatase activity but not in acid phosphatase activity (Table V.2); both have been in stock culture under the same cultural conditions for several years. The ratios of acid phosphatase to alkaline phosphatase also differed markedly.

Despite the differences in acid:alkaline phosphatase patterns, both isolates produced an identical band with an Rp value of 0.43 in starch-gel electrophoretic analysis of acid phosphatase isozymes. They appear to be homozygous for acid phosphatase.

## Nitrate Reductase Activity

Nitrification requires abundant exchangeable bases. After nitrification of ammonium negative nitrate ions are not held to any great extent by negatively charged soil particles. This accounts for the low rate of nitrification in acid forest soil. Thus, nitrate is readily leached from the root zone. In recycling nitrate ions where nitrogen is limiting, mycorrhizal fungi with high nitrate reductase activity would seem advantageous to host trees. Mycorrhizal fungi may also reduce the loss of nitrogen from leaching (Ho and Trappe 1980).

The nitrate reductase activity (Table V.3) of the T. ponderosum isolates was 47.3 uM nitrate reductase/g fungal dry wt. for S-198 and 45.2 for S-199, not a significant difference. This activity is quite higher than reported for most other species of ectomycorrhizal fungi (Ho and Trappe 1980, 1985).

Phytohormones

Structural modifications of ectomycorrhizae are induced by fungal growth hormones (Graham and Lindermann 1980, 1981; Slankis 1948, 1951, 1973, 1974; Rupp and Mudge 1984). Moser (1959), Ulrich (1960), Horak (1963), and Gogala (1967, 1970), Slankis (1973, 1974) showed that many ectomycorrhizal fungi liberate IAA and related indole compounds. Ectomycorrhizal fungi also produce extracellular ethylene, cytokinins, gibberellic acids and gibberellin-related compounds (Gogala 1967, 1970; Graham and Linderman 1980, 1981; Kampert and Strzelczyk 1978; Miller 1967a,b, 1971; Strzelczyk et al. 1975).

IAA and related indole compounds and cytokinins regulate division, expansion and differentiation of cells and synthesis of RNA and proteins (Bates et al. 1983, Cleland 1981, 1982; Hagen et al. 1984, Letham et al. 1982, 1983; Skoog and Schmitz 1972). Ethylene also has root growth promoting properties similar to IAA (Graham and Lindermann 1980, 1981). These physiological and biochemical processes are apparently regulated by a delicate balance of phytohormones, so that contribution of phytohormones by a mycorrhizal fungus can strikingly influence the mycorrhizal host (Slankis 1973, 1974). The two isolates of T. ponderosum produced

both IAA and cytokinins in different amounts under the same cultural conditions (Table V.3), and their ratios of cytokinins to IAA did differ significantly.

In tissue culture experiments, a high cytokinin-IAA ratio induced formation of buds, leaves and stems; when the ratio is lowered, root formation is favored (Helgeson 1983, Skoog and Miller 1957, Skoog and Schmitz 1972, Slankis 1973, 1974). In higher plants cytokinins act as regulators to endogenous IAA formation, activity and transport (Hemberg and Larsson 1972). Cytokinins activate IAA oxidase at low concentrations and inhibit its activity at higher concentrations (Rudawska 1980). We have found that the cytokinin-IAA ratio is relatively constant within species of many other ectomycorrhizal fungi (Ho and Trappe unpublished data). Probably this ratio governs the characteristic morphology of mycorrhizae.

Gibberellins have been known to stimulate cell division in shoot apices (Phinney and Spray 1982, Sponsel, 1983) to increase cell wall elasticity (Adams et al. 1975 Cleland 1981) and to induce the secretion of acid phosphatase in aleurone cells (Hooley 1984). Growth of stems, leaves, and roots is promoted by gibberellins (Sponsel, 1983). The action of exogenous gibberellins on polysome formations and translations of mRNA in germinating seeds also have been reported (Bernal Lugo et al. 1981, Martin et al. 1983), although direct application of gibberellic acid to roots usually is reported to have little effect (Salisbury and Ross 1978). Both isolates of T. ponderosum produced gibberellins at similar rates (Table V.3).

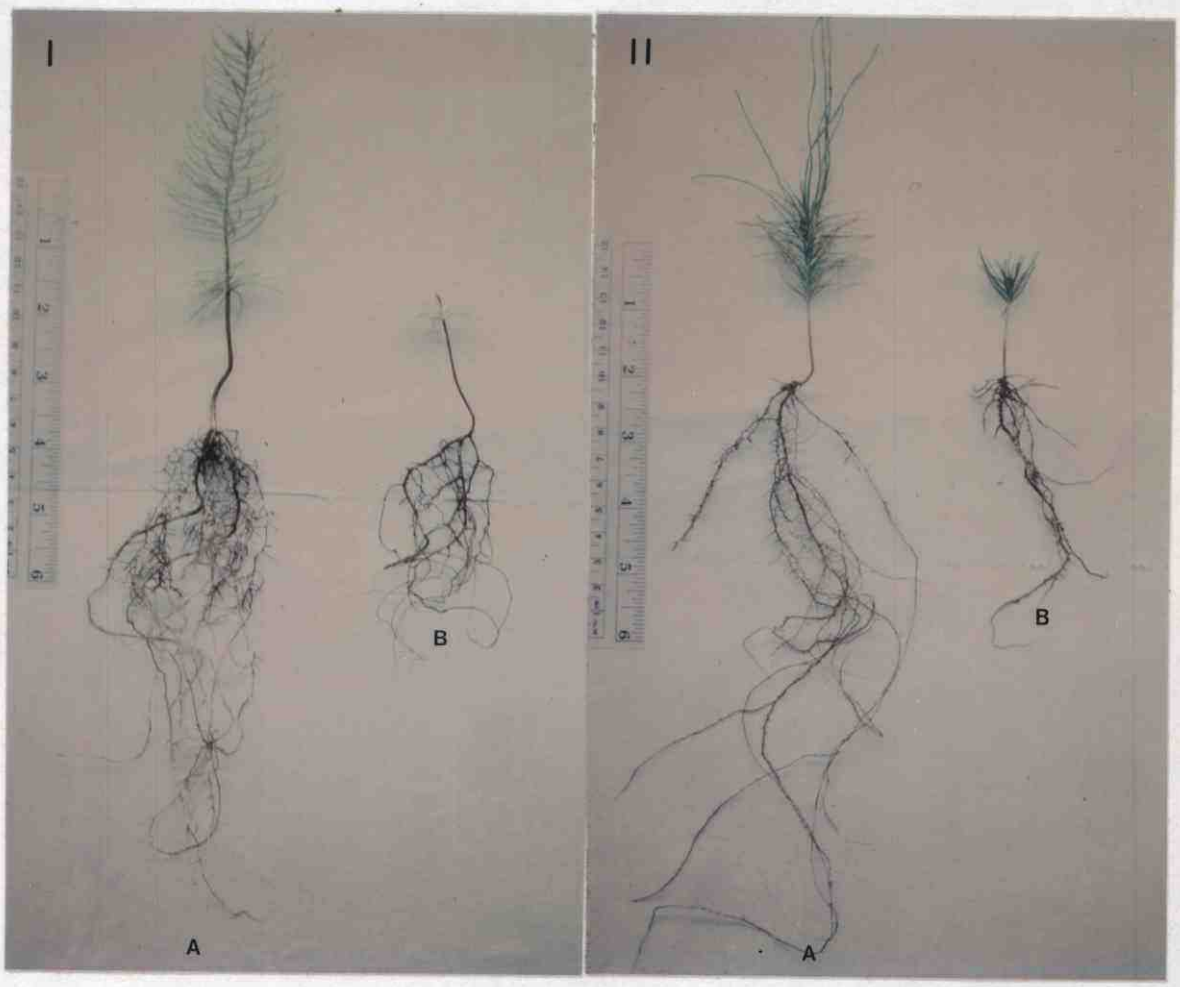


Figure. V.1 Mycorrhizae of T. ponderosum. I. Pseudotsuga menziesii. II. Pinus contorta. A. Control B. Mycorrhizae.

Table V.1. Mean dry weights of Tricholoma ponderosum isolates grown over a range of pH's[1/].

| | Isolate | |
|---|---|---|
| | S-198 | S-199 |
| pH | mg | mg |
| 4 | -0- | -0- |
| 5 | 488 | 352 |
| 6 | 421 | 534 |
| 7 | 467 | 459 |
| 8 | 360 | 390 |

[1/]Growth did not differ significantly between isolates at a given pH or between pH's for a given isolate.

Table V.2. Phosphatase activity of two isolates of Tricholoma ponderosum determined as millimoles of p-nitrophenol liberated per g dry weight of mycelium*.

| Isolate | Acid phosphatase m Mole/g | Alkaline phosphatase m Mole/g | Acid/Alkaline phosphatase ratio |
|---|---|---|---|
| S-198 | 1175[a] | 45[b] | 26[a] |
| S-199 | 881[a] | 119[a] | 7[b] |

*Means within columns not sharing a common postscript letter differ significantly by the Tukey test ($P \leq 0.05$).

Table V.3. Phytohormone production of two isolates of Tricholoma ponderosum determined as micromoles per g dry weight of mycelium*.

| Isolate | Cytokinins μM/g | IAA μM/g | Cytokinins/IAA | Gibberellins μM/g |
|---|---|---|---|---|
| S-198 | 732[a] | 119[b] | 6.15[a] | 98[a] |
| S-199 | 302[b] | 341[a] | 1.12[b] | 76[a] |

*Means within columns not sharing a common postscript letter differ significantly by Tukey's test ($P \leq 0.05$).

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# SUMMARY AND CONCLUSIONS

Mycorrhizal fungi have coevolved with host plants and have developed impressive physiological and ecological diversity (Trappe 1977). The fungi and their hosts may share similar genetic information in regard to their environment. Though ectomycorrhizal fungi in this experiment were cultured in artifical media without a host over many years, they nonetheless differed in enzyme activity and extracellular phytohormone production, even between isolates of a single species. The significance of the relationship between host specificity and fungal acid phosphatase isozyme patterns was convincingly demonstrated.

Woolhouse (1969) suggested that edaphic ecotypes of plants may result from active phosphatase enzymes in response to soil conditions. High phosphatase activity may be advantageous to some hosts in certain sites. In environmentally stressed sites, however, low phosphatase activities may be more suited for host survival, because a low metabolic rate may promote survival. Low nitrate reductase activity of the fungi studied perhaps reflects the acid soil environment usual in coniferous forests.

Extracellular phytohormones produced by the fungi represent a more complex situation. Not only the amounts of IAA and cytokinins produced but the ratio of one to the other varied between isolates. A slight shift in this balance may affect host growth patterns, in that the higher cytokinin/IAA ratios may produce high top/root ratios of seedlings, directly affecting the nutrient condition and

disease resistance, therefore survival and growth of hosts.

In enzyme activity and phytohormone production, the ectomycocrrhizal fungi studied varied considerably in relation to host tree species and habitat. These variations may reflect gene-controlled mechanisms for adaptation demonstrated in the isozyme patterns of acid phosphatase. The different adapted forms of isolates appear in some cases to be edaphic ecotypes located within ecologically defined areas e.g. nursery versus forest, Douglas-fir versus pine, or xeric versus mesic site. Patterns consitant for all fungi for any one or any combination of these adaptic situations did not appear clearly in these studies, although the nursery versus forsest comparison is similar for both Pisolithus and Laccaria isolates. Further studies should include many more isolates within a given species, with several replicate isolates from each host or habitat comparison. Other enyzmes may be critical and should be explored. Equally or perhaps more importantly, experiments are needed to define how isolate physiological differences affect host response. This can be done by pure culture inoculation of seedlings, at first for laboratory and greenhouse experiments and later for testing in nursery and plantation.

The importance of selecting mycorrhizal fungi for nursery inoculation has been emphasized in recent years by Trappe (1977). Forest nurseries grow seedlings for a particular area with seeds from that area. Selection of isolates from an area could become as routine as collecting the cones, if that kind of care significantly improves plantation performance of the seedlings. The selected

fungi could be physiologically tested and grown as inoculum. Eventually, we may have mycorrhiza-improvement programs just as comprehensive as the large-scale tree improvement programs that have been developed around the world (Trappe 1977).

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