In Vitro Response of *Heterobasidion annosum* to Manganese[1]

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ABSTRACT

Manganese (Mn) is postulated to play a role in wood decay caused by certain basidiomycetes. We determined the in vitro response of *Heterobasidion annosum* (Fr.) Bref. to Mn and compared differences between three isolates each of the S and the P intersterility groups from the Western United States. On manganese-amended malt agar plates, *H. annosum* produced a brownish-black pigment that increased with increasing concentration of Mn (1, 5, and 10 mM). The amount of pigment production varied among the isolates, but isolates of the S group clearly produced more pigment than those of the P group. In pigmented areas, hyphae appeared to be packed with black granules, and brownish-black granules/crystals were present in the medium. The black pigment implicates the presence of MnO_2, potentially resulting from the activity of Mn-dependent peroxidase (MnP). An assay for MnP did not detect the enzyme in crude culture filtrates of a chemically defined, nitrogen-limited, buffered medium of the six isolates. The differential response of S and P group isolates to Mn raises questions about a role for this transition metal in *H. annosum* physiology.

Keywords: Conifers, genetic diversity, manganese dependent peroxidases, root decay pathogens.

INTRODUCTION

Transition metals such as manganese (Mn) have been reported to be involved in fungal degradation of wood and forest diseases (Blanchette 1984; Illman and others 1988, 1989; Shortle and Shigo 1973). A Mn-dependent peroxidase (MnP) secreted into culture medium by white-rot fungi (Glenn and Gold 1985; Johansson and Nyman 1987; Paszcynski and others 1985) oxidizes Mn^{2+} to Mn^{3+}, which degrades lignin-model compounds in vitro. Autoxidation of Mn^{3+} results in an accumulation of Mn dioxide. Manganese accumulated in black regions of wood decayed by several white rot fungi, including *Heterobasidion annosum* (Fr.) Bref. (Blanchette 1984).

Heterobasidion annosum is an economically important pathogen of conifers throughout the Temperate Zone forests of the world. This fungus attacks root systems and causes death of living root tissues, woody tissue decay and mortality. Three intersterility groups (ISG's) or biological species *H. annosum* are known and are associated with varying degrees of host specificity. One group, designated P, attacks mainly pine species, incense cedar, and certain hardwoods. Another group, known as the S group, attacks mainly true firs and spruce species. The third group, the F group, is specific on *Abies alba* in the Italian Alps and in the Apennines (Capretti and others 1991).

Recent population genetic studies of this fungus revealed considerable genetic diversity within and genetic divergence between ISG's from North America and Europe (Chase and Ullrich 1990; Otrosina and others 1992, 1993). In light of known host specificity differences between ISG's and genetic divergence in the fungus, as well as the postulated role of Mn in wood decay by certain basidiomycetes, *H. annosum* was employed as a model system to determine the response of *H. annosum* S and P ISG's to Mn^{2+} in pure culture and to determine if a Mn^{2+}-dependent peroxidase is excreted by *H. annosum*.

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MATERIALS AND METHODS

Culture Conditions and Enzyme Assay

Six isolates of *H. annosum* were used. Isolates 201, 214, and 247 of the S ISG were obtained from *Abies concolor* (Gord. & Glend.) Lindl. ex Hildebr. in the central Sierra range of California. Isolates 114, 334, and 361 of the P ISG were obtained from *Pinus ponderosa* Douglas ex P. Laws. & C. Laws. hosts in Montana (114) and California (334 and 361). Isolates were grown in 125-ml Ehrlenmeyer flasks in a chemically defined, nitrogen-limited culture medium by a procedure developed for the white rot fungus *Phanaerochaete chrysosporium* Burds. (Kirk and others 1986). Each flask received a 10-mm mycelial plug from the margin of 7-day-old colonies growing on 1.25-percent malt extract agar. Flasks were kept stationary in incubation chambers for 10 days. Cultures of *P. chrysosporium* were also grown to provide a positive check on assay procedures. The culture filtrate from each isolate was assayed for the presence of Mn^{2+}-dependent peroxidase using vanillylacetone as substrate (Paszczynski and others 1985). One unit of enzymatic activity was defined as 1 µmol of substrate oxidized per minute.

Agar Culture Studies

Malt extract agar medium (1.25 percent Difco malt extract and 1.5 g agar per liter of distilled water) was amended for Mn^{2+} by adding $MnSO_4$ to yield final concentrations of 0.0, 1.0, 5.0, and 10.0 mM $MnS0_4$. $MnSO_4$ culture medium was sterilized by autoclaving for 15 minutes at 121 °C. The pH was determined potentiometrically for all media by mixing subsamples with an equal proportion of distilled 18.0 megohm water. Samples were measured after 20 minutes equilibration. The pH values for 0, 1.0, 5.0, and 10.0 mM $MnS0_4$ were 5.2., 5.1, 4.7, and 4.5, respectively.

Three replicate plates for each isolate and Mn^{2+} amendment combination were inoculated with a 4-mm diameter agar plug taken from the margins of 7-day-old malt extract agar stock culture plates. After inoculum was placed in the center of each plate, the plates were incubated on a laboratory bench at 24 °C for 8 days.

Cultures were visually and microscopically evaluated for production of a brown-black pigment in culture medium and hyphae, indicative of Mn^{4+} dioxide accumulation. A visual rating system was used to score cultures for presence, extent, and intensity of a brown-black pigment. The ratings ranged from (O) to (++++), where (O) indicates no dark pigment and (+) to (++++) indicates increasing amounts of pigment. The ratings were used to compare S and P ISG's (table 1). Samples of mycelia were transferred to glass slides, covered with a cover slip without staining, and observed at 400× for presence and location of a brown-black pigment.

Table 1.--*Relative intensity of dark precipitate formation in Mn^{2+}-amended malt extract agar for S and P intersterility groups of* Heterobasidion annosum

Mn^{2+} concentration	Relative intensity S group	P group
1.0 mM	++++	+
5.0 mM	++++	+
10.0 mM	+++	++
0.0 mM	0	0

* Plus (+) symbol represents relative precipitate extent and intensity at various Mn^{2+}concentrations. Four (+) symbols = highest extent and intensity.

RESULTS AND DISCUSSION

A brown-black pigment developed in $MnSO_4$-amended cultures of *H. annosum*. The in vitro response of *H. annosum* to Mn^{2+} is presumably due to the activity of Mn-dependent peroxidase and subsequent accumulation of Mn^{4+} dioxide.

Microscopic examination of hyphae from the dark areas of the cultures reveals granular crystals in the medium surrounding hyphae. The hyphae also appear dark and packed with this material (fig. 1). These observations are consistent

with the accumulation of manganese oxide-containing black flecks that have been reported for *H. annosum*-decayed wood in nature (Blanchette 1984).

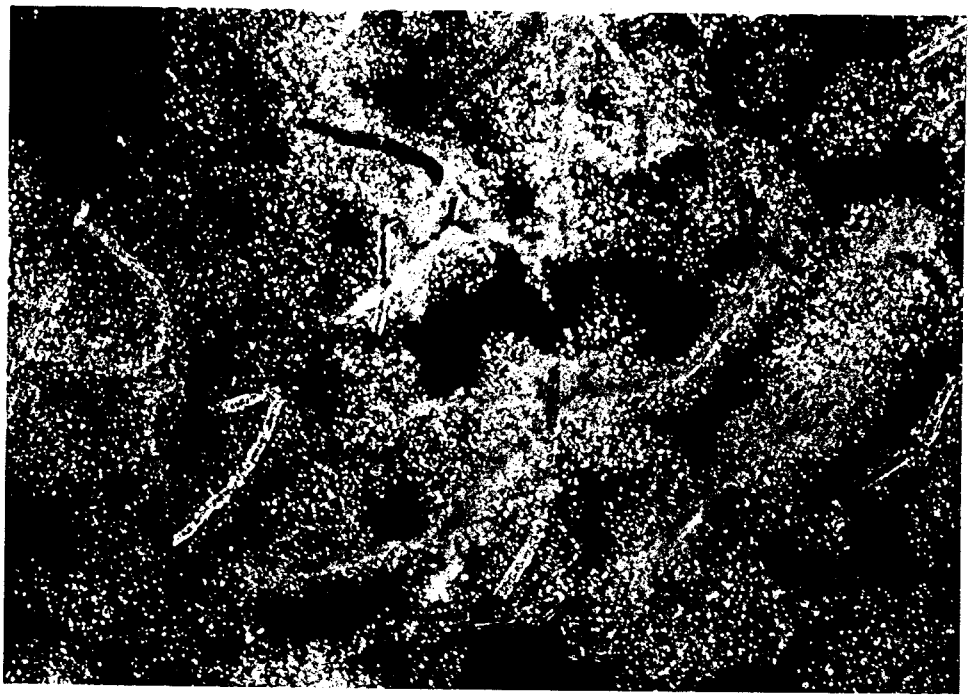

Figure 1.--*Putative oxidized manganese precipitate in* Heterobasidion annosum *hyphae. Note presence of refractile precipitate within and surrounding hyphae as it appeared under differential interference contrast. Hyphae were from 1.0 mM Mn^{2+}-amended 8-day-old malt extract agar culture. Magnification × 400.*

The S and P ISG's exhibited a differential response to Mn (table 1). The S group isolates had a qualitatively more intense development of dark pigment than did the P group isolates. This was consistent with all Mn concentrations with the exception of a P ISG that produced dark pigment in 10.0 mM $MnS0_4$. The S ISG produced more pigment in 1.0 and 5.0 mM $MnSO_4$ than in 10.0 mM, reflecting a concentration optimum for Mn. Growth of S and P ISG's appeared to be depressed in the 10.0 mM concentration relative to the 1.0 and 5.0 mM concentrations. Comparisons should be made between the growth rate and Mn oxide production for a larger number of isolates within intersterility groups of this fungus.

Extracellular manganese-dependent peroxidase activity was not detected in the crude culture filtrates of the *H. annosum* isolates. Peroxidase activity was detected in filtrates of *P. chrysosporium*. The culture conditions used for *P. chrysosporium* may not be appropriate for secretion of the peroxidase by *H. annosum* in liquid culture. However, observations of the dark pigment in agar plates may be due to secretion of the enzyme under those conditions.

CONCLUSIONS

There is considerable genetic divergence between the ISGs of *H. annosum* and this genetic divergence is associated with host specificity (Otrosina and others 1992, 1993). The apparent differential response to Mn^{2+} between the S and P ISGs suggests genetic divergence may also be manifested in some unknown differences in enzymes involved in oxidative lignin degradation. Lignins differ qualitatively between hardwood and softwood species (Highley and Kirk 1979, Sjostrom 1981). Although highly speculative, differences in Mn-dependent peroxidases in *H. annosum* may be a manifestation of the same

evolutionary forces that formed the basis for host specificity in this pathogen. Further studies are planned concerning response of various geographic sources and ISGs of *H. annosum* to Mn^{2+}, isozyme analysis of Mn-dependent peroxidase of the ISGs and isolation and characterization of Mn-dependent peroxidases from this forest pathogen. This fungus can serve as a model system to study wood decay mechanisms and evolutionary processes involved in host interactions.

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