Enzyme systems of red alder and Douglas-fir in relation to infection by *Poria weirii*[1]

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Abstract

Poria weirii *hyphae secreted compounds, presumably phenoloxidases, that resulted in oxidation of catechol, DL-dopa, and hydroquinone but not P-cresol. Phenoloxidases with similar activity were found in red alder leaves but were lacking in Douglas-fir leaves. Leaves of both alder and Douglas-fir showed peroxidase activity, but at much higher levels in alder than in fir. Alder leaves were able to reduce nitrate nitrogen, presumably through presence of nitrate reductase, whereas leaves of Douglas-fir lacked apparent nitrate-reducing ability.*

These preliminary studies will be followed by studies on wood and roots of alder and Douglas-fir. If, as is likely, the phenols and phenoloxidase system in red alder leaves are also present in roots, a reason for alder's resistance to Poria weirii *can be hypothesized. On penetration by the fungus, the o-dihydric phenols in alder tissue would be oxidized into fungitoxic compounds through the catalytic action of the phenoloxidases. These compounds, deposited about the periphery of the initial infection, would inhibit further spread of the fungus. Peroxidase activity, too, might contribute to resistance. Nitrate reductase activity assumes possible importance in that* Poria weirii *cannot reduce nitrate, whereas many of its antagonists can. Thus, the high nitrate levels in stands containing alder are not directly usable by* P. weirii *but permit buildup of high populations of antagonists.*

Introduction

Red alder *(Alnus rubra* Bong.) is resistant to infection by *Poria weirii* Murr., one of the major root pathogens of conifers in western North America (Wallis and Reynolds, 1962 and 1965). In this paper, we report studies on (1) phenoloxidases, peroxidase, and nitrate reductase activities in red alder as compared to Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco), and (2) some phenols that are oxidized by phenoloxidases produced by *P. weirii*. The possible role of these enzyme systems in resistance of alder to *P. weirii* infection is hypothesized.

Certain phenoloxidases in wood-rotting fungi are related to lignin-decomposing ability (Etheridge, 1957; Käärik, 1965; Lindeberg, 1948; Schänĕl, 1967). *P. weirii* is a lignin decomposer, producing a yellow ring rot in conifer wood; when grown on gallic or tannic acid media, it produces a brownish diffusion zone (Nobles, 1948), indicating presence of phenoloxidases. On the other hand, activity of other phenoloxidases in higher plants has been related to browning of injured tissues (Burges, 1963; Sisler and Evans, 1958) and to defense against parasitic infection (Hare, 1966; Rubin and Artsikhovskaya, 1963).

The literature on phenolic content of Douglas-fir and red alder tissue is scant, but some leads on compounds of possible importance in resistance to *Poria weirii* appear in the compilations of Hegnauer (1962, 1964). Leaves of both species contain quercetin kaempferol, and myricetin. If present also in roots of both, these compounds would not account for differences in resistance between the two. The compounds taxifolin (which oxidizes to quercetin), catechin, and epicatechin, occurring in bark of Douglas- fir and other *Poria*-susceptible trees such as *Tsuga* spp., similarly do not appear to inhibit penetration by *P. weirii.* On the other hand, leaves of red alder contain compounds which have not been reported from Douglas-fir: chlorogenic, caffeic, gallic, and protocatechnic acids. Moreover, red alder wood and bark contains a phenolic xyloside that is responsible for the reddening of freshly exposed wood, and its bark contains taraxerol and teraxeron. Chlorogenic, caffeic, gallic, and protocatechnic acids or products of their oxidation as catalyzed by phenoloxidases are fungitoxic and are implicated in disease resistance of a number of plants, as are catechol and several other phenols and quinones (cf. Hare, 1966).

High peroxidase activity is related to field resistance of potatoes to blight caused by *Phytophthora infestans* (Mont.) deBary (Fehrmann and Dimond, 1967; Umaerus, 1963). Its role in resistance of tree roots to pathogenic infection is unexplored.

Activity of nitrate reductase, the enzyme necessary for assimilation of nitrate ions, has not been heretofore determined for either red alder or Douglas-fir. Studies of *P. weirii,* however, have shown that it cannot use nitrate and lacks nitrate reductase (Li et al., 1967).

The studies reported in this paper are preliminary in respect to the role of enzymes in resistance of trees to infection by *Poria weirii,* because enzyme activities were determined for leaf rather than root tissue of alder and Douglas-fir (leaves were easier to work with in developing techniques and in gaining initial data). Further studies will follow on root tissue, which likely, but not necessarily, contains the same or similar enzyme systems.

Materials and Methods

DETECTION OF SPECIFIC EXTRACELLULAR PHENOLOXIDASES PRODUCED BY *PORIA WEIRII*

A culture of *P. weirii* was grown in 250 ml of liquid synthetic medium (Table 1 of Trione, 1964, but substituting 10 gm glucose for sucrose). After

3 weeks of growth, 10 ml aliquots of the medium were pipetted into each of 18 Erlenmeyer flasks. The medium in 8 of the flasks was boiled to inactivate enzymes for controls. Meanwhile, solutions of the phenols P-cresol, DL-dopa, catechol, and hydroquinone were prepared at concentrations of 2.75 mg/ml double-distilled water and sterilized by glass filtration. Two ml of each phenol solution were then added to each of two flasks containing boiled and unboiled aliquots of medium on which *P. weirii* had been grown, while similar amounts of distilled water alone were added to two flasks of unboiled medium as additional controls. Brown to black discoloration of the mixtures indicated activity of enzymes on the specific phenols.

EXTRACTION OF PHENOLOXIDASES FROM RED ALDER AND DOUGLAS-FIR

The extraction procedure was essentially the same as that of Badran and Jones (1965). Four gm of leaves were collected from 2-year-old, greenhouse-grown trees and washed in cold, redistilled water. The leaves were cut into small fragments while immersed in 100 ml 0.1M-potassium phosphate buffer at pH 6.5, containing 1 percent polyethylene glycol (PEG), molecular weight 4,000 (Carbowax 4000, Union Carbide Chemicals Company). The fragments were vacuum infiltrated for 30 minutes, then removed and added to another 40 ml PEG-buffer solution. This mixture was homogenized in an Omni-Mixer at maximum speed for 3 minutes, after which the homogenate was poured into 80 ml cold acetone (-20°C), and centrifuged at 1,200 g for 10 minutes. The precipitate was immersed for 30 minutes in 20 ml of 0.1M-potassium phosphate buffer, pH 6.5, to solubilize the enzymes, and centrifuged at 3,000 g for 10 minutes. The supernatant was decanted and recentrifuged at 18,000 g for 10 minutes. The final supernatant was then tested for poly-phenoloxidase activity.

The assay mixture contained 0.1 ml enzyme extract and 2.9 ml of 10^{-3} M substrate — catechol, chlorogenic acid or DL-dopa. The reaction was followed by measuring light absorption at 15-second intervals in a Beckman DB spectrophotometer at 400 mμ for chlorogenic acid and catechol and at 495 mμ for DL-dopa. This 0.1 ml enzyme extract represented the enzyme activity per 18 mg fresh leaf tissue.

EXTRACTION OF PEROXIDASE FROM RED ALDER AND DOUGLAS-FIR

Six g of leaves of each species were collected from 2-year-old greenhouse-grown trees. The leaves were washed in cold, redistilled water and stored in a cold room at 0°C for 18 hours. They were then homogenized with 50 ml buffer at pH 7.0 (McIlvaine, 1921) containing 10^{-2} M cysteine and 6 g of dry polyclar AT (Loomis and Battaile, 1966) in an Omni-Mixer at maximum speed for 3 minutes. The homogenate was filtered through fine-mesh nylon cloth and the filtrate centrifuged at 3,000 g for 5 minutes. The clear supernatant was then tested for peroxidase activity. The assay mixture consisted of 0.5 ml enzyme extract, 0.1 ml of 0.2M pyrogallol, 1.0 ml McIlvaine's buffer, pH 7.0, 0.5 ml of 0.01M H_2O_2 and 0.9 ml redistilled water. Blanks

used in the spectrophotometric analysis were composed of the same assay mixture, but one lacked enzyme extract and the other, H_2O_2. Absorption at 430 mμ was measured at intervals of 15 seconds in a Beckman DB spectrophotometer. This 0.5 ml enzyme extract represented the enzyme activity per 60 mg fresh leaf tissue.

REDUCTION OF NITRATE TO NITRITE BY RED ALDER AND DOUGLAS-FIR

Ten grams of leaves were collected from Cascade Head Experimental Forest (maintained by the U. S. Forest Service, Pacific Northwest Forest and Range Experiment Station), Oregon. The leaves were surface-sterilized by dipping into 2.65 percent sodium hypochlorite solution for 5 minutes and washed twice with sterile distilled water. They were then immersed for 4 days at room temperature in 600 ml Hoagland solution I (Hoagland and Arnon, 1938), which had been diluted to ¼ strength with sterile distilled water. This step was devised on the basis that reduction of nitrate requires the enzyme nitrate reductase (Townsend and Blatt, 1966), whose production is induced in the presence of nitrate ions (Beevers and Hageman, 1963; Raghavan and Torrey, 1964; Rijien, 1958 and 1960). Two ml of streptomycin sulfate solution (10 mg/ml) were also added to the solution to minimize bacterial growth. Nitrite released into solution was tested by adding 1.0 ml of 1 percent sulfanilamide and 1.0 ml 0.02 percent N-(1-Naphthyl) ethylenediamine hydrochloride to 2 ml test solution. After 20 minutes, the color density was read at 540 mμ on the Beckman DB spectrophotometer. Hoagland solution without leaf inoculation, and sterile distilled water with leaf inoculation were used as blanks.

Results

Extracellular compounds produced in synthetic medium by *P. weirii* resulted in the oxidation of catechol, DL-dopa, and hydroquinone. No oxidation of *P*-cresol was detected. The secreted compounds presumably included the enzymes *o*-diphenoloxidase, which catalyzes oxidation of catechol and DL-dopa, and *p*-diphenoloxidase, which catalyzes oxidation of hydroquinone.

Red alder leaves possessed phenoloxidases which catalyzed the oxidation of *o*-dihydric phenols, such as chlorogenic acid, catechol, and DL-dopa, chlorogenic acid being the most reactive substrate. The extract from Douglas-fir leaves showed no phenoloxidase activity for these three substrates (Figs. 1 and 2). Leaves of both red alder and Douglas-fir possessed peroxidase, but the former showed much higher activity than the latter (Fig. 3). Red alder leaves possessed nitrate reducing capacity and, therefore, nitrate reductase: in 4 days 1 gm of leaf tissue was able to form 0.74 μ moles of nitrite (Table 1). However, no activity was detected in Douglas-fir leaves.

Discussion and Conclusions

Poria weirii can penetrate the wood of both Douglas-fir and red alder, but in the case of alder the bark must generally be injured first. Even then,
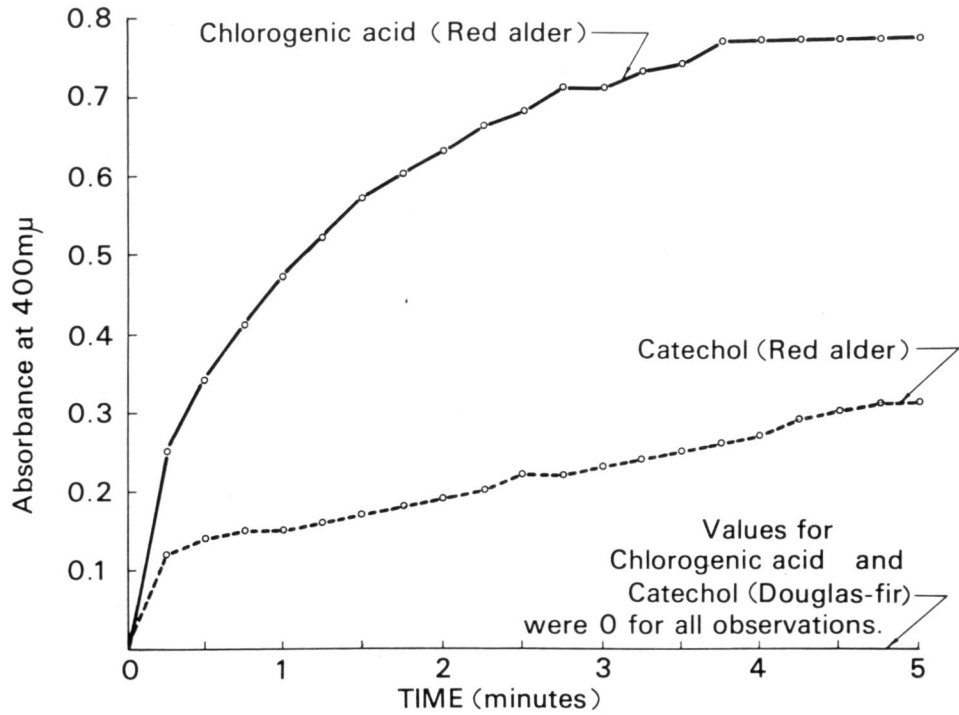
Figure 1. Phenoloxidase activity of leaf extracts obtained from red alder and Douglas-fir. Assay: substrates, chlorogenic acid and catechol 1 x 10^{-3}M, 2.9 cc, enzyme extract 0.1 cc.

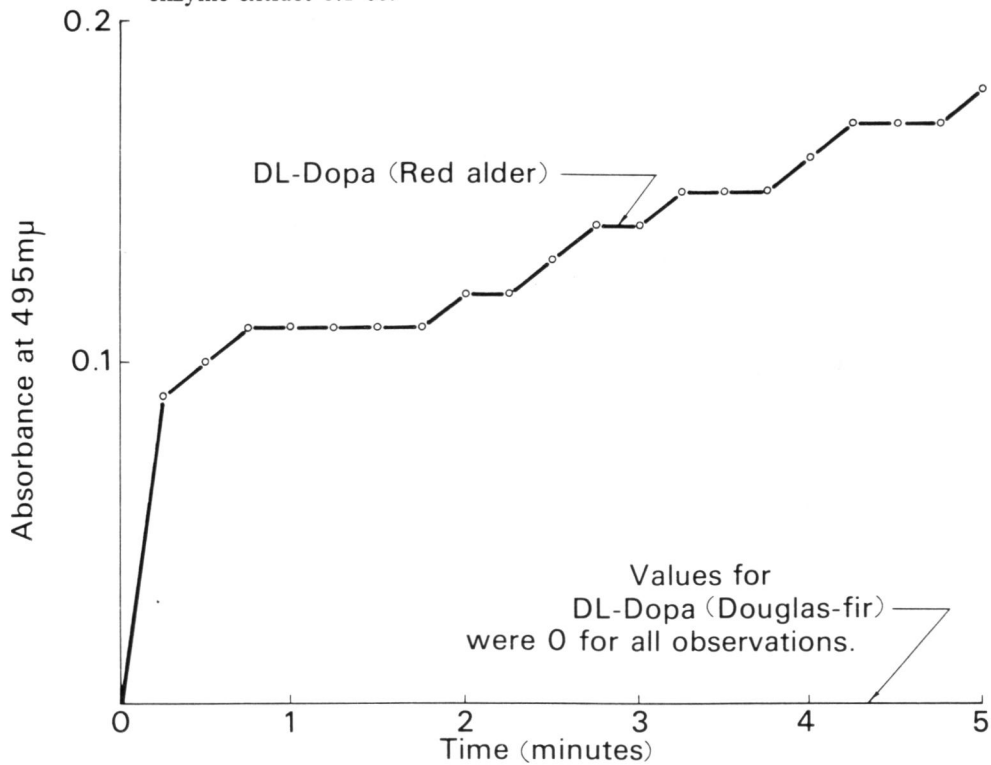**Figure 2.** Phenoloxidase activity of leaf extracts from red alder and Douglas-fir. Assay: substrate, DL-dopa, 1 x 10^{-3}M, 2.9 cc. enzyme extract 0.1 cc.
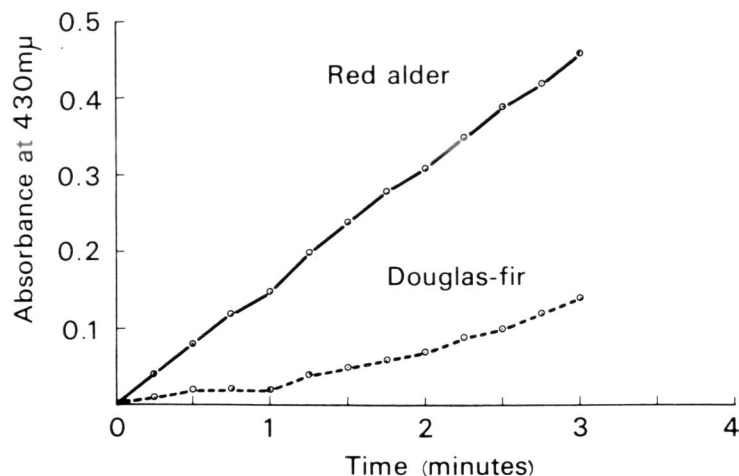
Figure 3. Peroxidase activity of leaf extracts obtained from red alder and Douglas-fir. Assay: pyrogallol, 0.2 M, 0.1 ml, McIlvaine's buffer, pH 7.0, 1.0 ml H_2O_2, 0.01 M, 0.5 ml, enzyme extract 0.5 ml and redistilled water 0.9 ml.

growth of the fungus is restricted to a small zone around the point of entry (Wallis and Reynolds, 1964). Heat labile compounds evidently account for this resistance of alder wood to growth of the fungus: *P. weirii* will grow well in the autoclaved wood (Wallis and Reynolds, 1962), but not in previously unheated wood (G. W. Wallis, personal communication).

The heat labile compounds prominently involved in alder's resistance to *P. weirii* probably include phenoloxidases that catalyze oxidation of chlorogenic acid and other phenols in the tissue into fungitoxic end-products (Hegnauer, 1964; Rubin and Artsikhovskaya, 1964; Hare, 1966). Initial penetration by the fungus into alder wood could activate the phenoloxidases in the tissue (Byrde et al., 1960; Rubin and Artsikhovskaya, 1964). The products of phenol oxidation would then accumulate in infected cells and polymerize, forming dark, resin-like substances which impregnate the tissue and form a barrier to further spread of the mycelium (Rubin and Artsikhovskaya, 1963 and 1964). It is also possible that these or other oxidized phenols in the infected tissue inactivate or precipitate the extracellular phenoloxidases produced by *P. weirii*, thereby preventing lignin degradation by the fungus (Byrde et al., 1960; Loomis and Battaile, 1966).

TABLE 1. Reduction of nitrate to nitrite in 4 days by leaves of red alder and Douglas-fir

Species	Absorbance of test solution at 540 mμ	Nitrite formed (μmole/g of tissue)	Mean
Douglas-fir	0	0	
	0	0	0
	0	0	
Red alder	0.385	0.770	
	.397	.794	0.784
	.390	.780	

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Red alder has high peroxidase activity, which might increase considerably in hypersensitive red alder reaction-tissue during invasion by *P. weirii* hyphae (Rubin and Artsikhovskaya, 1963 and 1964). Fehrmann and Dimond (1967) have postulated that peroxidase activity is one attribute of host tissue contributing to environmental inhospitality for a fungus.

One of the direct results of nitrogen fixation by alder nodules is a buildup of total soil nitrogen levels and, particularly, of nitrite nitrogen (Bollen and Lu, 1968). *Poria weirii* has previously been shown unable to assimilate nitrate, presumably for lack of nitrate reductase (Li et al., 1967). If our results with leaves are representative of the tree as a whole, Douglas-fir may also lack this enzyme. Red alder, in contrast, has the enzymatic ability to reduce nitrate to usable form as do many soil organisms likely to be strong antagonists of *P. weirii* (cf. Li et al. 1967). Consequently, *P. weirii* does not compete with its antagonists, such as *Streptomyces* species, for nitrate – the presence of which contributes to the buildup of antagonistic populations in presence of alder (Lu and Bollen, 1968). *P. weirii* survives relatively poorly where antagonistic and competing organisms thrive (Nelson, 1967); the circumstances of nitrogen fixation and presence or lack of nitrate reductase activity in the respective organisms can logically be expected to mitigate against *P. weirii* in stands containing alder as a major component.

To recap these interpretations, the phenols and phenoloxidases present in red alder but not detected in Douglas-fir can be hypothesized as a primary biochemical source of alder's resistance to *Poria weirii* by (1) resulting in oxidation of *o*-dihydric phenols into fungitoxins at the infection site, and (2) destroying the lignin decomposing ability of *P. weirii* by inactivating its extracellular enzymes. The relatively high peroxidase activity may further contribute to resistance against the fungus. Because *P. weirii* spreads by growth in infected root systems rather than by growth through the soil, spread from tree to tree is primarily by growth of healthy roots to an infection source (Wallis and Reynolds, 1965). The mere presence of resistant alder trees in a stand, therefore, reduces the chances for root contacts between susceptible trees. The high soil nitrate levels in stands containing alder, moreover, cannot be used by *P. weirii* but do encourage growth of organisms that antagonize or compete with it.

Additional research is planned to test these hypotheses as well as to determine whether the phenols or their fungitoxic oxidation products are present in soils of stands containing alder.

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