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Peroxidase Activity in Relation to Suberization and Respiration in White Spruce (*Picea glauca* [Moench] Voss) Seedling Roots[1]

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ABSTRACT

Peroxidase (EC 1.11.1.7) activity is associated with suberization during endodermal development and metacutization in roots of white spruce (*Picea glauca* [Moench] Voss) seedlings. Histochemical analysis indicates a relationship between suberization and peroxidase activity, but peroxidase is ubiquitous. Increased peroxidase activity results from the induction of four anodic peroxidase isozymes in addition to quantitative increases in two anodic peroxidase isozymes. Four of these polymerized eugenol. Cold temperatures induce formation of two anodic isozymes and result in suberization. The increased peroxidase activity associated with suberization is correlated to residual respiration. In an attempt to elucidate this relationship, the effect of respiratory inhibitors on respiration and peroxidase activity are compared.

Freudenberg *et al.* (6) hypothesized that peroxidase catalyzed lignification and several studies have supported this role (5, 10). Peroxidase activity also has been implicated in the closely related process of suberization (1, 16). However, all of these studies pertain to the wounding response. Van Fleet (28) noted the presence of peroxidase in the endodermis, but there has been little other work on the relationship between peroxidase and suberization of discrete tissues.

This study forms part of a larger study of growth and development in roots of white spruce seedlings. Previous studies have reported on root development (14), root growth and root growth capacity (15), root respiration (13), and ultrastructure and permeability of the phi and dormancy layer (A. M. Johnson-Flanagan, J. N. Owens, unpublished data). This study examines peroxidase activity in white spruce (*Picea glauca* [Moench] Voss) seedling roots in relation to the development of the primary endodermis and dormancy layer (14). Peroxidase activity is located histochemically and quantified. Activity is correlated to changing enzyme patterns. The ability of the isozymes to initiate polymerization is assessed through the use of eugenol. A possible relationship between peroxidase activity and residual respiration is examined.

MATERIALS AND METHODS

Plant Material. All tissue was from 1.0 (1 year old) white spruce seedlings. Seeds were planted in styroblocks in the spring and the seedlings were maintained in a lathhouse. During the growing season, seedlings were fertilized bimonthly with 20-20-20 and kept moist. All roots studied were classified as long laterals and included white absorbing (bearing root hairs), white elongating (without root hairs), and brown roots (14). The three root segments from white roots were the tip (root apex with or without the root cap), the middle, (zones of elongation and differentiation), and the top (the suberizing segment) (13).

Induction of Suberization. Seedlings were transplanted into 95- × 230-mm pots containing sand:peat:vermiculite (2:1:1) and grown for 7 d at 4°C with a 16-h photoperiod of 400 μE m^{-2} s^{-1}.

Histochemistry. Fresh root tips were frozen at −12°C. Serial longitudinal sections were cut on a cryostat at 18 μm, dry-mounted on slides, and treated with phloroglucinol/HCl for lignin or saturated Sudan IV in 70% alcohol for suberin (12). Peroxidase activity was located with 0.05%, 3,3′-diaminobenzidine and H_2O_2 according to the methods of Hepler *et al.* (11). Modifications were as follows: Tes rather than Tris buffer was used; H_2O_2 was 0.03% rather than 0.01%; incubation was for 15 min rather than 15, 30, or 60 min; and aminotriazole was excluded from the controls.

Inhibition of Suberization. Root segments approximately 1 cm long were bathed in the respiratory reaction medium (13) containing SHAM[3] (final concentration, 2 mM) or disulfiram (final concentration, 100 μM). Collections were made for up to 4 h and were frozen, sectioned, and treated with Sudan IV as above. Controls either lacked inhibitors or contained 1 mM KCN.

Peroxidase Activity. Peroxidase was extracted and assayed at 460 nm in a Beckman Recording Spectrophotometer (1). Tissue was homogenized in 10 times its weight of ice-cold 20 mM borate buffer (pH 8.8) containing 5 mM bisulfite and centrifuged at full speed for 5 min on an International model HN centrifuge (International Equipment Co., Needham Heights, MA). The supernatant was diluted with an equal volume of ice-cold 0.1 M sodium acetate buffer (pH 5.4) and used immediately or frozen for later use. The assay mixture contained 1 ml of sodium acetate buffer (pH 5.4), 1 ml of 50 mM guaiacol (freshly prepared), and 1 ml of peroxidase extract adjusted to produce a change in absorbance of 0.5 to 0.8 in 5 min at 460 nm. The reaction was initiated by the addition of H_2O_2 to a final concentration of 200 μM. Results were expressed as the basis of protein content because a preliminary study showed that it was proportional to fresh weight.

Respiration. O_2 consumption in 100 mg of 1-cm root segments was measured polarographically as described previously (13). The methods for titrating with disulfiram concentrations between 0 and 100 μM was the same as that for titrating with SHAM (13).

Inhibition of Peroxidase Activity. Peroxidase was extracted from a mixture of root classes and assayed as above except that

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[3] Abbreviations: SHAM, salicylhydroxamic acid; disulfiram, tetraethylthiuram disulfide.

the H_2O_2 concentrations used to measure *K4* were 200 and 100 μM, and 20 and 30 μM, to measure *K1*. SHAM final concentrations were between 0 and 500 μM and disulfiram concentrations ranged from 0 to 100 μM.

Electrophoresis. Peroxidase was extracted as above and frozen for later use. The starch gel consisted of 315 ml H_2O, 35 ml gel buffer (3 mM Tris and 5 mM citric acid adjusted to pH 8.5), and 43.75 g starch. Samples were thawed and loaded onto the center of the gel with filter paper wicks. Ridgeway's electrode buffer was used (60 mM lithium hydroxide and 0.3 M borate, adjusted to pH 8.1) (2). Electrophoresis was carried out at 250 to 300 v and maximum of 100 mamp for 2 to 4 h at which time a food coloring marker had migrated approximately 8 cm. Gels were sliced into layers and stained with benzidine HCl (250 mg EDTA, 50 mg benzidine HCl, and 0.15 μM H_2O_2 in 50 ml of 0.2 M acetate buffer adjusted to pH 5.0) (2) or eugenol (75 mg eugenol, 100 ml of 0.05 M sodium phosphate buffer [pH 6.0], and 60 μM H_2O_2 and was freshly prepared) (19). Eugenol polymerization was verified by UV light excitation of the fluorescent product. The gels were scanned with a Spectrodensitometer, model SD 3000, at 450 and 325 nm, respectively. Horseradish peroxidase was used as the control.

Protein. Protein was determined according to the method of Sedmak and Grossberg (25).

RESULTS

Histochemistry. Peroxidase was located throughout the root apex, cortex, and stele of brown and white roots. The cell walls and protoplasts stained uniformly and positively in the root apex, whereas activity was restricted to the periphery of the cortical and stelar cells. Relative concentrations could not be determined because of interference by naturally brown cell walls in the root cap and cortex of the root. Peroxidase activity was associated with the primary endodermis in white roots and the secondary endodermis and metacutization layer (collectively termed dormancy layer) (A. M. Johnson-Flanagan, J. D. Owens, unpublished data) of brown roots. Peroxidase activity correlated to lignification of the metaxylem and suberization of the primary endodermis and dormancy layer. Exposure of seedlings at 4°C caused suberization of white roots leading to formation of the dormancy layer.

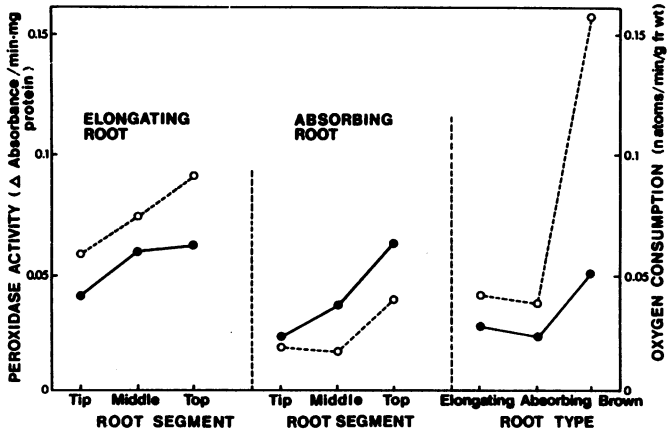

FIG. 1. Peroxidase activity and residual respiration in roots of white spruce seedlings. Tip, middle, and top refer to the root apex without the root cap, the zones of elongation and differentiation, and the segment of the root undergoing suberization, respectively. Activity in the three root classes was compared in the tip including the root cap. Peroxidase was extracted and assayed as described in "Materials and Methods." Residual respiration was the respiration remaining following SHAM and cyanide addition (final concentrations, 1.0 mM) and was assayed as described in "Materials and Methods". (O- - -O), peroxidase activity; (●——●), residual respiration. Values are the mean of at least three measurements. SE of peroxidase activity is shown. Maxium SE of residual respiration was ±5%.

Table I. *Comparison of Titration Results with SHAM or Disulfiram*

Root segments were titrated as described in the "Materials and Methods." O_2 uptake was 160 ± 24 natoms/min·g fresh weight. Values are the mean of at least three measurements. Data for SHAM titration are from Johnson-Flanagan (13).

Inhibitor	*Ki*	*p*	*Valt*	*Valt* × *p*	*Vcyt*	*Vres*
				% V_T		
SHAM	1.0 mM	0.65	49	32	53	15
Disulfiram	10 μM	1.0	39	39	57	2

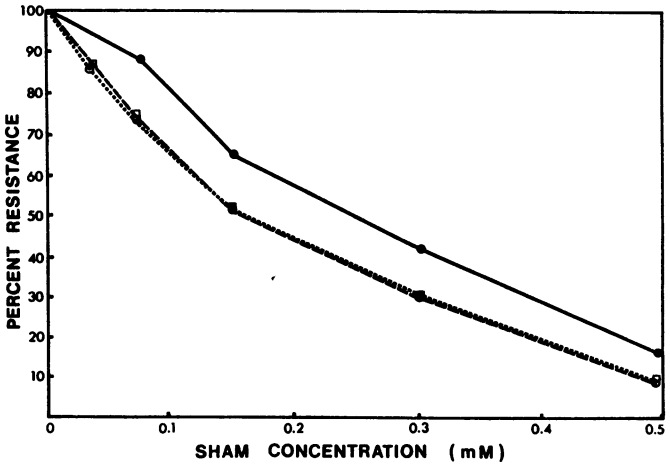

FIG. 2. Effect of SHAM on peroxidase activity in roots of white spruce seedlings. Peroxidase was extracted from a mixture of root classes and assayed as described in "Materials and Methods." (O- - -O), *K1*, 20 μM H_2O_2; (Δ- - -Δ) *K1*, 30 μM H_2O_2; (●——●), *K4*. Values are the mean of at least four separate preparations. 100% peroxidase activity was 0.03 to 0.06/min·mg protein. Maximum SE was ±3%.

Peroxidase Activity. Peroxidase activity increased basipetally in elongating and absorbing roots (Fig. 1). Peroxidase activity was significantly higher in elongating root segments than in the comparable absorbing root. However, if the root cap was included in the tip segment, peroxidase activity was not significantly different in elongating and absorbing roots. Peroxidase activity was very high in the tip segments of brown roots (Fig. 1). Residual respiration (in the presence of cyanide and SHAM) in the three root classes was related to peroxidase activity.

Inhibition of Suberization. Both SHAM and disulfiram suppressed suberization in root segments. Although this could not be quantified, suppression was less than in the roots treated with KCN.

Effect of Inhibitors on Respiration. Both SHAM and disulfiram were effective inhibitors of the alternative pathway (Table I). The *Ki* for disulfiram was 100-fold lower than the *Ki* for SHAM. Residual respiration was very low in the presence of disulfiram.

Effect of Inhibitors on Peroxidase Activity. The relationship between residual respiration and peroxidase activity was investigated further by measuring the inhibition of peroxidase by SHAM and disulfiram with respect to guaiacol. Increasing SHAM concentrations increased inhibition of the velocity constant for the formation of the enzyme-substrate complex (*K1*) and the velocity constant for the reaction of the secondary complex and the hydrogen donor (*K4*), although *K4* inhibition was approximately 10% lower (Fig. 2). Complete inhibition was

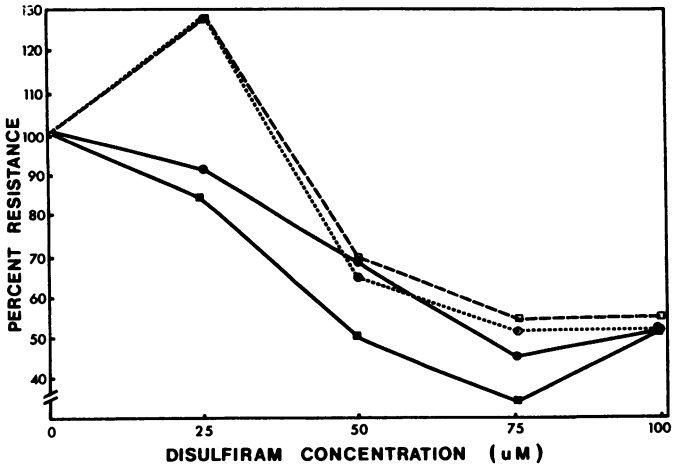

FIG. 3. Effect of disulfiram on peroxidase activity in roots of white spruce seedlings. Peroxidase was extracted from a mixture of root classes and assayed as described in the "Materials and Methods." (○– – –○), *K1*, 20 μM H_2O_2; (□– – –□), *K1*, 30 mM H_2O_2; (●——●), *K4*, 100 μM H_2O_2; (■——■), *K4*, 200 μM H_2O_2. Values are the mean of three separate preparations. 100% peroxidase activity was 0.03 to 0.06/min · mg protein. Maximum SE was ±3%.

caused by 825 μM SHAM. Disulfiram inhibited *K1* and *K4* (Fig. 3). *K1* inhibition increased as disulfiram concentrations increased to 75 μm disulfiram. *K4* was stimulated by 25 μM disulfiram and inhibited by concentrations up to 75 μM. Inhibition of *K1* and *K4* was not increased above 50% by 100 μM disulfiram.

Peroxidase Isozymes. Peroxidase isozyme patterns changed during suberization (Fig. 4). The fast migrating cathodic band was associated with white roots (either elongating or absorbing), whereas the slow migrating cathodic band was associated with suberization. The exception to this pattern occurred in white roots from natural conditions in the winter where there were numerous anodic bands.

White roots collected during periods of root growth lacked isozymes at RF 04, 0.45, 0.75, and 0.95. These bands were associated with roots undergoing suberization. Exposure to cold temperature induced bands 0.4, 0.75, and 0.95 in white and brown roots. Eugenol was polymerized into a fluorescent compound by the anodic bands at 0.075, 0.25, and 0.45. An additional band was resolved with eugenol at 0.59 in roots undergoing suberization. Densitometer scans of the gels indicated that the relative proportions of the isozymes changed as roots underwent the transformation from white to brown (Fig. 5). The relative concentration of the anodic band at RF 0.075 increased dramatically, while new bands were resolved and the band at 0.5 was lost. The bands at 0.25 and 0.4 to 0.45 increased during suberization but were low in brown roots.

DISCUSSION

Histochemistry. Peroxidase activity was associated with suberization and lignification in white spruce roots. Peroxidase was ubiquitous, however, activity was highest in the cell walls of the endodermis, the metacutization layer, and the metaxylem. The endodermis and metacutization layer are suberized and the metaxylem is lignified (14). Other studies have demonstrated the same relationships between peroxidase activity and suberization (1, 16, 28) and lignification (11). Peroxidase activity is positively correlated to endodermal development where it is thought to function in suberization (28). In *Coleus* wound vessel members, peroxidase occurs in the presence or absence of lignification (11) indicating that peroxidase has functions other than the initiation of lignin polymerization (5, 27).

Peroxidase Activity. Peroxidase functions in the initiation of suberin polymerization (17). Peroxidase activity increased basipetally in pea roots, where it correlated with lignification (5), and in white spruce roots, where it was associated with suberization. In white spruce roots, extensive suberization occurred in the middle and top segments of white roots. Lignification occurred only in the top segment and was restricted to the metaxylem (13). Peroxidase activity was also high in the tip of brown roots where the metacutization layer, which is suberized but not lignified, envelopes the root apex (13).

In white spruce roots, the parallel changes in residual respiration and peroxidase activity suggest a relationship between these two activities. O_2 consumption resulted in H_2O_2 production required for peroxidase-initiated phenylpropanoid polymerization (7, 20). The alternative pathway was once thought to produce H_2O_2 (22), however, this has since been disproven (18). Therefore, our results may indicate that H_2O_2 production is a component of residual respiration.

Inhibition of Respiration and Peroxidase. The suppression of suberization by SHAM and disulfiram could result from the direct inhibition of peroxidase, the inhibition of H_2O_2 production as a substrate for the peroxidase-initiated polymerization of suberin (20), or a combination of these two. This is further complicated by the possibility that the O_2 consumption resulting in H_2O_2 production may be mediated by peroxidase (9, 20). Disulfiram and SHAM have been used to study these possibili-

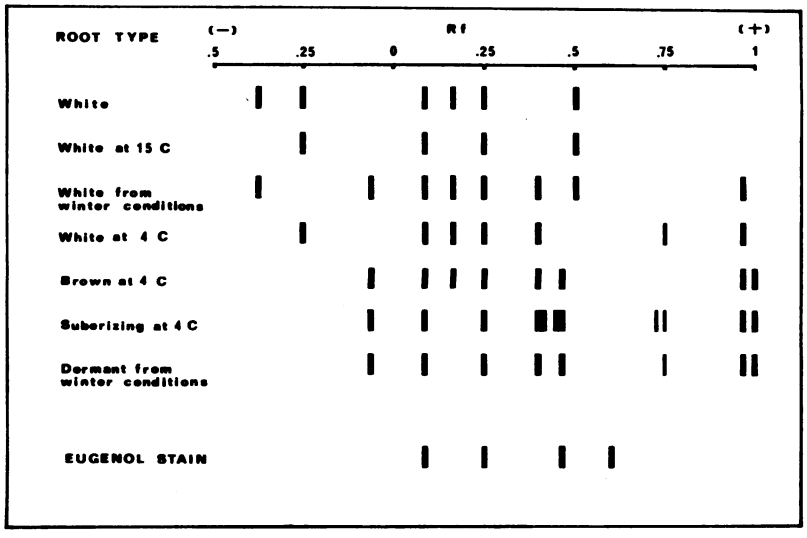

FIG. 4. Isozyme pattern of peroxidase extracted from roots of white spruce seedlings. Extraction and electrophoresis were as described in "Materials and Methods."

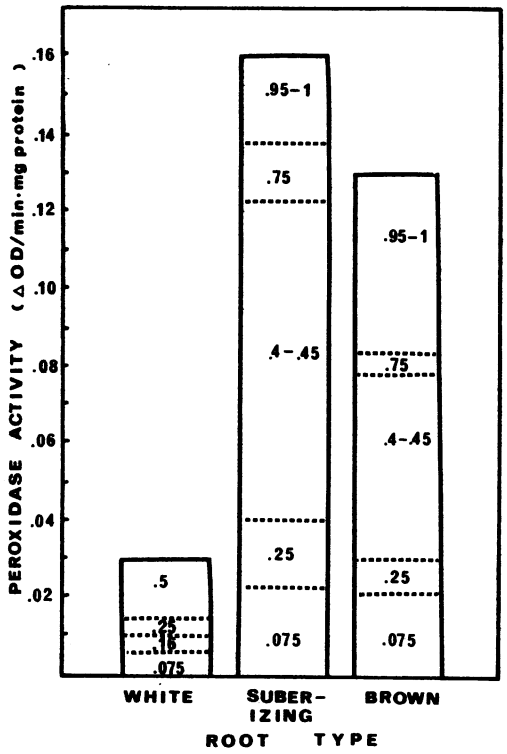

FIG. 5. Total peroxidase and relative activities of the anodic peroxidase isozymes during the transformation of a white root to a brown root. Total activity was measured spectrophotometrically at 460 nm in crude extracts and relative activities were obtained from densitometer scans as described in the "Materials and Methods." RF values are shown.

ties.

Disulfiram specifically inhibits the alternative pathway in isolated mitochondria (21). Although disulfiram is ineffective in red sweet potato tuber respiration (8), the *Ki* in white spruce roots was lower than the *Ki* reported for mitochondria isolated from red sweet potatoes (8), therefore, disulfiram is an effective inhibitor of white spruce root respiration.

Disulfiram may reduce residual respiration (8, 21). Therefore, decreased suberization in the presence of disulfiram could be attributed to inhibition of residual respiration. In white spruce roots, *Valt* and *Vres* were lower when titrated with disulfiram in comparison to SHAM. This resulted in an increase in *p*. Other workers have noted the increase in *p* and have attributed it to the inhibitory effect of disulfiram on state 3 respiration (21). This does not explain the inhibition of O_2 uptake during state 4 respiration noted in their study. Decreased *Vres* would account for these changes.

The relationship between respiration and peroxidase activity could not be studied because both SHAM and disulfiram effectively inhibited peroxidase in white spruce roots. *K1* and *K4* were completely inhibited by 825 μM SHAM, which is lower than the concentration used to inhibit white spruce root respiration (13). This is in agreement with other results (23, 24). Disulfiram concentrations of 25 μM stimulated *K1* and inhibited *K4* peroxidase activity, but the disulfiram concentration used in the respiratory study inhibited *K1* and *K4* peroxidase activity. It is apparent, therefore, that *Vres* is not a result of peroxidase activity.

Peroxidase Isozymes. Suberization in white spruce roots cannot be attributed to increased peroxidase activity until the isozymes are studied and quantified. This is because peroxidase functions in a number of catalytic capacities other than H_2O_2 production at the expense of NADH and initiation of phenylpropenoid polymerization at the expense of H_2O_2 (5, 27). These functional differences are believed to result from peroxidase isozymes (1, 4, 20). In white spruce roots, isozyme variation occurred during suberization and low temperature induction of suberization. Low temperature induction of peroxidase isozymes has been reported previously (3), but was not associated with suberization.

In white spruce, the fast-moving cathodic band was associated exclusively with white roots where cell division and elongation occur. A similar fast moving cathodic band was isolated from dividing cells of wounded potato tubers (1). Therefore, this band may be associated with growth promotion (29). A slow-moving cathodic band has been associated with lignification (27) and suberization (16). Eugenol polymerization has been used to indicate an ability to polymerize other phenylpropenoids (19), therefore, the lack of eugenol polymerization by this isozyme in the present study does not support a direct role in either process. However, the isozyme may correspond to GIII, the ionically bound isozyme that is a slow polymerizer but produces H_2O_2 rapidly (20).

Suberization was associated with induction and increasing proportions of numerous anodic isozymes and the loss of an anodic isozyme at 0.5. Lui (19) demonstrated the induction of anodic isozymes during the development of pea roots. Others have demonstrated a relative increase in the anodic bands during suberization (1, 16) and lignification (5). The anodic bands are efficient initiators of phenylpropenoid polymerization and of these, the fast migrating isozyme (GI) is most efficient (20). Although other results indicate that this fast-migrating band increases the most during suberization (1) and lignification (5), this was not the case in our study. Further, the fast-migrating band was ineffective in eugenol polymerization, whereas the intermediate bands were effective initiators of polymerization.

The intermediate bands increased the most during suberization, therefore, results from white spruce roots indicate that the differences between peroxidase activity in actively growing tissue and suberizing tissue may result from peroxidase isozymes that initiate polymerization of suberin. The lack of lignin in the root apex, the extensive suberization in the root apex, and the low proportion of lignin in comparison to suberin in the proximal root segments (14) indicate that it is suberization rather than lignification that is mediated by these isozymes in white spruce roots.

In a previous study (A. M. Johnson-Flanagan, J. N. Owens, unpublished data), a PAS-positive layer enveloping white spruce seedling root apices was thought to be a precursor to the metacutization layer. Smith and O'Brien (26) noted that peroxidase activity was localized in the epidermis of wheat roots and a slime layer was present. They suggested that the peroxidase functioned to cross-link the carbohydrates of the slime layer to phenolics. This resulted in autofluorescent cell walls such as occur in the metacutization layer. Van Fleet (28) also reported the presence of mucoprotein in the proendodermis and peroxidase activity in the endodermis. Therefore, this suggests that peroxidase functions in metacutization and the PAS positive layer is a precursor to the metacutization layer.

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