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

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ABSTRACT

Peroxidase (EC 1.11.1.7) activity is associated with suberization during endodermal development and metacutinization 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(13), and ultrastructure and permeability of the phi and dormancy layer (A. M. Johnson-Flanagan, J. N. Owens, unpublished data).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) seedlings 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, dried-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 H₂O₂ according to the methods of Hepler et al. (11). Modifications were as follows: Tris rather than Tris buffer was used; H₂O₂ 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 (final concentration, 2 mM) or disulfiram (final concentration, 100 μM). Controls 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 H₂O₂ 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. O₂ 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 H2O2 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 H2O, 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. Ridgway’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 H2O2 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 
H2O2 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 Grossman (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 steller 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 
dermis and metacutization layer (collectively termed dorn 
ancy layer) (A. M. Johnson-Flanagan, J. D. Owens, unpublished 
data) of brown roots. Peroxidase activity correlated to

**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.” (○—○), peroxidase activity; (●—●), residual 
respiration. Values are the mean of at least three measurements. 
**se of peroxidase activity is shown. Maximum se of residual respiration was 
±5%.

**Table 1. Comparison of Titration Results with SHAM or Disulfiram**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Ki (mM)</th>
<th>p</th>
<th>Valt</th>
<th>Valt × p</th>
<th>V cyt</th>
<th>V res</th>
<th>% V r</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM</td>
<td>1.0</td>
<td>0.65</td>
<td>49</td>
<td>32</td>
<td>53</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Disulfiram</td>
<td>10 µM</td>
<td>1.0</td>
<td>39</td>
<td>39</td>
<td>57</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 2.** Effect of SHAM on peroxidase activity in roots of white spruce 
seedlings. Peroxidase was extracted from a mixture of root classes and 
asayed as described in “Materials and Methods.” (○—○), K1, 20 µM 
H2O2; (●—●), 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%.

**Fig. 3.** Effect of disulfiram on peroxidase activity. Peroxidase activity 
inhibited similarly 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 1). 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 
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

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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 metacutulation 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 bispetally 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. O2 consumption resulted in H2O2 production required for peroxidase-initiated phenylpropanoid polymerization (7, 20). The alternative pathway was once thought to produce H2O2 (22), however, this has since been disproven (18). Therefore, our results may indicate that H2O2 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 H2O2 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 O2 consumption resulting in H2O2 production may be mediated by peroxidase (9, 20). Disulfiram and SHAM have been used to study these possibilities.

![Fig. 4. Isozyme pattern of peroxidase extracted from roots of white spruce seedlings. Extraction and electrophoresis were as described in "Materials and Methods."](image-url)
penoid polymerization does not produce isozymes in comparison to the inhibition of residual respiration (8). 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, Wal 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 O2 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 respiration 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 H2O2 production at the expense of NADH and initiation of phenylpropanoid polymerization at the expense of H2O2 (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 phenylpropanoids (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 H2O2 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 as 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 procendoderm 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.

LITERATURE CITED

2. Conkle MT 1972 Analyzing genetic diversity in conifers isozyme resolution by starch gel electrophoresis. U S Dep Agric For Serv For Prod Lab Rep PSW-264
5. Fielding JL, J Hall 1978 A biochemical and cytological study of perox-
PEROXIDASE ACTIVITY IN ROOTS OF WHITE SPRUCE SEEDLINGS

and suberin. Science 208: 990–1000