USE OF SYRINGALDAZINE FOR DETECTION
OF LACCASE IN SPOROPHORES
OF WOOD ROTTING FUNGI

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SUMMARY

Fungal laccase can be detected with the nonautooxidizable laccase-specific compound, syringaldazine, in the presence of tyrosinase and peroxidase. Data are presented from tests with the azine derivative of numerous fungous sporophores and associated decayed wood tissues from various localities in Wisconsin and Oregon. Syringaldazine is shown to be a reliable replacement and more specific reagent for the detection of extracellular fungal laccase, and in the absence of laccase and in the presence of hydrogen peroxide, for peroxidase. Laccase is further implicated in fruit-body formation.

Since the publication of a technique by Nobles (1958), tincture of guaiac has been used for topical application to fungal cultures as a spot test for extracellular laccase (p-diphenol:oxygen oxidoreductase. EC 1.10.3.2). Development of the blue color ("guaiacum blue") in air at the site of application was taken as a positive indication of the presence of laccase. As indicated by Nobles (1958), tyrosinase (α-diphenol:oxygen oxidoreductase, EC 1.10.3.1) is as effective as laccase in oxidizing furoguaiacins, the active principle in tincture of guaiac, to guaiacum blue (Kratochvil et al., 1971). Thus, tyrosinase interferes with the use of the guaiac test as a means of detecting laccase.

Moreover, on standing in stock solutions some substances that accompany furoguaiacin in gum guaiac form organic hydroperoxides that can serve as hydrogen acceptors for oxidation of furoguaiacin to guaiacum blue by peroxidase (donor:hydrogen peroxide oxidoreductase. EC 1.11.1.7) (cf. Mauzy, 1954, particularly pp. 383-384). Thus, peroxidase can also interfere in tests for laccase with older solutions of tincture of guaiac.

Syringaldazine was recently introduced as an ideal substrate for

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rapid and unequivocal detection of laccase, for example in cultures of wood-rotting fungi (Harkin and Obst, 1973). Addition of a pale yellow 0.1% solution of syringaldazine in 95% ethanol to a purified fungal laccase (Fähreus and Reinhammer, 1967) in the presence of air produces an intense purple color or pigment. No color is formed with tyrosinase. Syringaldazine does not autoxidize and form organic hydroperoxides, so interference from peroxidase plus endogenous hydroperoxide is excluded.

We now report results of tests for laccase with the azine carried out on fungus sporophores from various sites in Wisconsin and Oregon. Tests on the wood beneath the sporophores were also conducted. Since the azine is not oxidized by tyrosinase, in the absence of laccase, tincture of guaiac can be used to detect tyrosinase (Harkin and Obst, 1973). The guaiac test was therefore also applied to the sporophores and underlying wood. Also, since in the absence of laccase, the combination of syringaldazine plus hydrogen peroxide can be used to detect peroxidase (Harkin and Obst, 1973), specimens that gave a negative or weak reaction with azine alone in air were later tested with azine plus peroxide or guaiac plus peroxide to test for peroxidase.

MATERIALS AND METHODS

Syringaldazine was prepared by the method of Bauer and Rupe (1971). The reagent is now available from Aldrich Chemical Co., Milwaukee, Wis. 53233. A saturated (0.05-0.1%) stock solution of azine was prepared by heating the crystalline substance in ethanol, cooling, and decanting the yellow supernatant from material that recrystallized. A drop of supernatant solution was applied to a freshly exposed surface of the fungus fruit body or the underlying wood. Dry specimens were wetted with 50% aqueous ethanol before reagent application.

Tincture of guaiac was prepared by refluxing 1 g of gum guaiac powder (Sargent-Welch Scientific Co., Skokie, Ill. 60076) in 50 ml of ethanol and filtering the solution after cooling. Stock solutions made in this way were always tested by addition of horseradish peroxidase (Type I, Sigma Chemical Corp., St. Louis, Mo. 63118) without hydrogen peroxide before use. In older solutions we obtained a blue color due to endogenous peroxide in the extract (cf. Maehly, 1954). Such solutions were discarded. The stock solutions were also tested with laccase (Fähreus and Reinhammer, 1967) or tyrosinase (Worthington Biochemical Corp., Freehold, N. J. 07728) to ensure that they still contained enough furoguaiacin (Kratochvil et al., 1971) to give a strong blue color. For more accurate assays of furoguaiacin content, thin-layer
chromatography (Auterhoff and Kuhl, 1966) was used, the chromatograms being sprayed with tyrosinase to reveal the furoguaiacin as guaiacum blue (Kratochvil et al., 1971). The tincture of guaiac was applied topically in the same way as the azine solution.

Hydrogen peroxide was used as a freshly prepared 0.03% solution, and added dropwise after azine application to areas where no laccase reaction was obtained.

RESULTS

Wherever laccase was present, a pink through red to purple color appeared. The rate of color formation and the color intensity suggested the amount of active enzyme present. With very high laccase activities, almost immediate purple coloration occurred, but the color faded again gradually. Generally the color was stable. If no color developed, the absence of laccase was indicated.

Wherever a pink-red or purple color appeared with azine due to laccase activity, a green-blue to intense blue color was observed with tincture of guaiac. The possible presence of tyrosinase in these areas was masked by the laccase reaction. This was the situation with most of the specimens tested. However, in a few tissues where the laccase reaction was negative, a blue color with guaiac was still obtained, giving positive evidence of tyrosinase. This may have been intracellular enzyme released by damaging the fungus tissue. On a few samples, peroxidase activity was apparently present as indicated by a reaction with azine only in the presence of hydrogen peroxide, or by a faster or more intense color development when peroxide was added.

As expected, sporophores of several fungi especially of species classically referred to as “brown rotters” did not react positively to any of our tests for laccase, peroxidase or tyrosinase. These include the following (asterisk indicates white rotters):

- Coniophora puteana (Schum. ex Fr.) Karst.
- *Cystostereum pini-canadensis* (Schw.) Gilbertson
- Dacryobolus karstenii (Bres.) Parm.
- *Hyphodontia pallidula* (Bres.) J. Erikss.
- Hypoxylon deustum (Hoffm. ex Fr.) Grev.
- Lactiporus sulphurosus (Bull. ex Fr.) Bond.
- *Peniophora sanguinea* (Fr.) Hoehn. & Litsch.
- Polyporus floriformis Qüél.
- Polyporus sericcomollis Rom.
- *Poria albolutescens* (Rom.) Egel.
- *Poria carbonica* Overh.
- *Poria myceliosa*Pk.
- *Poria xantha* (Fr.) Cke. sensu Lind
- Tyromyces caesius (Schrad. ex Fr.) Murr.
Some results of positive tests for the enzymes laccase, tryosinase, and peroxidase on fungus sporophores and associated decayed wood using syringaldazine, tincture of guaiac, and hydrogen peroxide are as follows:

<table>
<thead>
<tr>
<th>FUNGUS</th>
<th>TEST RESULTS</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Asterostroma andinum</em> Pat.</td>
<td>Staining confined to margin and hymenophore with s. Substratum stained with s and g.</td>
</tr>
<tr>
<td><em>Bjerkandera adusta</em> (Willd. ex Fr.) Karst.</td>
<td>Pileus and context next to substratum stained with s and g. Substratum stained transiently with s and g.</td>
</tr>
<tr>
<td><em>Daedalea unicolor</em> Bull. ex Fr.</td>
<td>Generalized weak staining of sporophore and substratum with s and g (specimens dry).</td>
</tr>
<tr>
<td><em>Favolus alveolaris</em> (DC. ex Fr.) Quél.</td>
<td>Pileus stained strongly with s, weakly with g. Substratum stained with s and g.</td>
</tr>
<tr>
<td><em>Fomes connatus</em> (Weinm.) Gill.</td>
<td>Pileus stained with s and g. Substratum and context above tubes stained strongly with s and g.</td>
</tr>
<tr>
<td><em>Fomes fomentarius</em> (L. ex Fr.) Kicks</td>
<td>Staining detectable on edge of pileus with s and g. Substratum stained with s and g.</td>
</tr>
<tr>
<td><em>Fomes ohiensis</em> (Berk.) Murr.</td>
<td>Generalized staining of sporophore and substratum with s and g. Faint staining of tubes with s + H₂O₂.</td>
</tr>
<tr>
<td><em>Hericium abietis</em> (Weir ex Hubert) K. Harrison</td>
<td>Staining with s at base of sporophore, on short stems, and at ends of dichotomies Substratum stained with s. g not tested</td>
</tr>
<tr>
<td><em>Hyphodentia arguta</em> (Fr.) J. Erikss.</td>
<td>Generalized staining in fruiting body only with s or g + H₂O₂. No visible staining in substratum.</td>
</tr>
<tr>
<td><em>Hypochnicium bombycinum</em> (Fr.) J Erolss.</td>
<td>Sporophore stained strongly with s, faintly with g. Substratum stained with s and g.</td>
</tr>
<tr>
<td><em>Inonotus radiatus</em> (Sow. ex Fr.) Karst.</td>
<td>Rapid staining with s on pilens, but not with g. Color faded rapidly indicating very active laccase. Rapid substratum staining with s and g.</td>
</tr>
<tr>
<td><em>Kavinia himantia</em> (Schw.) J. Erikss.</td>
<td>Generalized strong staining of sporophores and rhizomorphs with s and g. No discernible staining of substratum.</td>
</tr>
</tbody>
</table>
Pileus surface stained slowly with s and g along with edges of disseminations. Staining intense above tubes. Substratum stained with s and g.

*Lycoperdon perlatum* Pers.

Staining with s and g + H₂O₂ apparent in subtissues comprised of immature gleba. Staining strong with g + H₂O₂.

*Ryswolzercius coronus* (Fr.) Parm.

Pileus surface stained with s and g. Localized staining directly behind hymenophore with s and g. Marginal tissue stained strongly with s and g.

*Odonia penni* Lépich.

Parts of sporophore stained with s and g, not localized. Faint stain only with s in substratum (color development slow; specimen very dry).

*Odonia spatulata* (Fr.) Lépich.

Nonlocalized staining of sporophore with s and g. Substratum staining not observed (sample very dry).

*Peniophora laca* Burt

Margin staining only with s and g.

*Phlebia livida* (Fr.) Bres.

Staining faint with s and g on sporophore and substratum. Not localized.

*Phlebia radiata* Fr.

Generalized strong staining of substratum and subiculum of sporophore with s and g. Not discernible in pigmented parts.

*Pluteus cedreus* (Jacq. ex Fr.) Kummer

Localized strong staining with s and g on pileus and edges of gills. Substratum stained with s and g.

*Polyporus parmeus* Fr.

Disseminations stained rapidly and strongly with s and g; margin stained weakly with both. Substratum stained with s and g.

*Polyporus pubescens* Schum. ex Fr.

Context stained with s and g. Substratum stained slowly with s and g.

*Polyporus subpunctatus* (Schw.) Overbe

Staining with s and g in sporophore not localized. Substratum staining with s and g. Intenser reaction on H₂O₂ addition.

*Polyporus versicolor* L. ex Fr.

Hymenophore and concentric zone 2-3 mm behind margin stained with s. Substratum stained with s and g. All parts of sporophore stained with g.

*Poria brunia* (Kars.) Sacc.

Hymenophore stained with s and g in spots; sometimes yellowish. Margin and substratum stained with s and g.
Sporophore stained slowly but intensely with g. Staining with s strongest at margin and dissepiments. Substratum stained slowly but intensely with s and g (more rapidly with g).

*Russula caesica* (Fr.) Pers. Localized staining with s and g evident above gills, below pileus, and internal peripheral tissue of stipe.

*Schizophyllum commune* Fr. Staining in sporophore with s and g mostly at base; same on dissepiments. Substratum stained faintly with s and g.

*Selkodermum* sp. Staining with s and g at base of sporophore. Peripheral tissues stained with g.

*Scytinostroma galacticum* (Fr.) Donk All parts of sporophore stained with s and g, but apparently more intensely in hymenophore region. Substratum stained with s and g. Color fading and ease of regeneration indicate high laccase activity.

*Stereum frustulatum* (Pers. ex Fr.) Fckl. Staining with s and g in substratum; none observed on sporophores.

*Stereum gummatus* (Fr.) Fr. Rapid staining with s and g, localized in context and pileus surface. Intense staining with s directly above hymenophore. Substratum stained with s and g.

*Stereum ostrea* (Blume & Nees ex Fr.) Fr. Staining with s localized in context above hymenophore. Staining with g weak (fruit body dry).

*Trechispora verna* Libertia No localized staining with s and g. Color with s very strong, but faded rapidly, suggesting strong laccase. No visible staining in substratum.

*Trametes albolux* (Pil.) Bond. & Sing. Staining with s and g in context above hymenophore. Strong substratum stain with s and g.

*Variosia incrustans* (Schw.) Karst. Unlocalized strong and rapid staining of sporophore and substratum with s and g.

**DISCUSSION AND CONCLUSIONS**

The data indicate that syringaldazine is a reliable replacement for tincture of guaiac for routine field tests for extracellular laccase production by wood-destroying fungi. The majority of the fungi of the "white rot" type that we examined gave a positive test, although the
intensity of the response varied widely. A positive reaction was generally obtained on the substrata of the sporophores that gave a positive

test. These were a variety of wood species which are typical hosts of the fungi concerned. However, our test, like any field test designed to indi­
cate phenol oxidases, is subject to the potential drawback of poor spec­
imen condition, i.e., moisture content and stage of development. Dry or deteriorated sporophores and wood gave erratic results. Sporophores

that appeared to be at the peak of development, with high moisture con­
tents, usually gave the most pronounced and lucid results.

Koenigs (1972) reported the production of extracellular peroxidase
by fungi in axenic culture for a number of species that he examined, but he made no attempts to relate this phenomenon to more natural cir­
cumstances. Though we could not test unequivocally for peroxidases in all instances because of the presence of laccase, the enzyme was de­
tected in the sporophores of four species and in the substrata of two,
confirming that peroxidase is produced in the environment by wood-
destroying fungi.

A remarkable phenomenon is the apparent localization of laccase, as visualized with syringaldazine, in various parts of sporophores of most
species, particularly those with pale tissues. Localization occurred mostly where tissues were apparently undergoing morphogenesis or active growth (margins, dissepiments, hymenophore layers, rhizomorphs, apices of teeth, etc.). Of interest in this respect is the recent work of
Leonard (1971), on Schizophyllum commune, who states that “whether or not these enzymes [phenol oxidases] are involved directly, indirectly, or not at all in fruiting morphogenesis is not possible to ascertain at the present time,” but they “may do more than simply accompany the process of fruit body formation.” From our observations, the coincident localized occurrence of high concentrations of phenol oxidases with tissues that are obviously sites of high metabolic activity suggests that phenol ox­
idasess, particularly laccase, may be involved in the differentiation of fruit­
ing bodies following their initiation. Their specific role and mode of operation, if any, remain to be elucidated.

LITERATURE CITED


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