IMMUNOASSAYS TO DETECT AND QUANTIFY STAINING FUNGI IN MEDIA OR IN WOOD

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Abstract

The application of various immunological techniques to detect or quantify staining fungi in wood is reviewed. Brief explanations are given on the production of polyclonal and monoclonal antibodies; their use in enzyme-linked immunosorbent assays (ELISAs) and in immunocytochemistry for light or electron microscopy. A variety of rapid and simple detection systems (modified ELISA, dot immunoassay, immunogold silver staining for light microscopy) have been developed for the staining fungus *Ophiostoma piceae* grown in both artificial media or wood.

Introduction

Detecting fungi or their metabolites in wood before any damage has occurred is an important task in managing diseases and biodeterioration. Fungi in wood are traditionally detected by microscopic examination of the sections after histological staining or by isolation into pure culture in artificial media. Both procedures are tedious, time consuming, non-specific and may give results which do not reflect true ecological situations. More recently, DNA molecular probes, enzyme electrophoretic patterns and immunodetection have attracted special focus. Immunological methods are attractive because they are sensitive and specific. Widely used in medicine for several decades, these techniques are now being applied in forest and wood products pathology (2, 16, 9, 3, 7, 10). The specificity of antibodies for their antigens make them excellent probes for detecting microorganisms or their metabolites, which may be present in trace amounts in their natural environment. To date their applications have been largely for research purposes, but it is not unreasonable to forsee them being used as diagnostic tools. In this report we will review the different immunological assays that we have tested for detecting or quantifying staining fungi in artificial media or in wood.

Technology

1) Antibody production

Foreign substances, when introduced into a vertebrate animal in an appropriate manner stimulate the animal's immune response. One aspect of this stimulation is activation of specific B-lymphocytes to produce clones of plasma cells which secrete immunoglobulins (Igs) that bind to an antigen. Each clone secretes only one molecular species of immunoglobulin. The overall immune response to a given antigen results in the production of a variety of antibodies (Abs). Because of this variety, the mixture of Abs obtained from the serum fraction of the total blood

of the inoculated animal is referred to as polyclonal antiserum. It can contain many activities to various parts of the antigen.

For monoclonal antibodies (Mabs), individual B-lymphocytes are harvested from the spleen of the injected animal (usually a mouse). The cells are fused with a myeloma cell line to produce somatic cell hybrids (hybridomas) which produce immunoglobulins. Each individual hybridoma can be propagated in tissue culture and will secrete only one molecular species of immunoglobulin (1). Hybridomas can be frozen and retrieved at a later date. Each provides a highly reproducible source of a specific antibody. Once an antibody has been obtained, it may be used in a variety of immunological assays to detect (qualitatively or quantitatively), identify, localize or purify one antigen from a complex mixture.

2) Antigens

Almost any substance can be immunogenic. Many polysaccharides; virtually all proteins, nucleoproteins, lipoproteins, synthetic polypeptides; and even small molecules (i.e. chemicals) bound to proteins can behave as antigens. Most particulate or soluble antigens are multivalent; i.e. they contain several regions called antigenic sites or epitopes, and each epitope is able to induce the production of a specific antibody. An antigen has two properties: immunogenicity and the ability to react specifically with an antibody.

There are as many ways of producing fungal antigens as papers on the subject. Mycelia have been the most commonly used antigen (6, 9). Mycelia are produced in a liquid medium, washed, homogenised and centrifuged; the pellet is termed "cell wall" antigen and the supernatant "soluble" antigen (6). Spores, cell wall and more specific antigens such as enzymes and excreted metabolites also have been used as antigens (9, 2, 16, 12).

The staining fungus used in this study, *Ophiostoma piceae*, was originally isolated from *Pinus banksiana* and has been maintained in the Forintek culture collection of wood-inhabiting fungi. The organism was chosen because of its reliability in producing a dark stain in wood. Cell fragments from washed, broken and homogenized 4 day old mycelia, grown in a malt-yeast extract glucose broth, were used to produce the polyclonal antisera. Cell wall protein extracts were injected into mice to generate monoclonal antibodies.

3) Assays

Immunoassays exploit the reversible interaction of an antibody (Ab) with its antigen. The assays are based on two important biological principles: the specificity of an antibody (Ab) for a particular antigen (Ag) molecule, and the ability to detect the presence of this Ab:Ag complex. In most detection systems the antigen is first bound to a solid support (plate, membrane) or used directly if already present on a solid support (i.e. slice of wood). Secondly, the experimental antibody preparation produced from rabbit serum (polyclonal) or fused mouse spleenocytes (monoclonal), is applied to localized antigen. If antibodies specific for the antigen are present,

they will bind to form a complex. Thirdly, a commercially available, secondary antibody directed against the species Igs of the experimental antibody are used to detect the formation of the complex. Various labels may be attached to these secondary antibodies to assist observation of binding to the Ag:Ab complex (for example, enzymes, fluorochrome dyes such as fluorescein or Texas Red, heavy metals such as gold or gold in combination with silver, or radioactive compounds (Figure 1).

With the enzyme-labelled secondary antibodies, the choice of substrate solution is selected according to the application. Aqueous coloured products are required for assays which use spectrophotometer determination (ELISA). Insoluble reactions giving chromogenic products are used for immunological cytochemistry and for protein blotting, and radioautographic film for chemiluminescent products.

Detection of the Staining Fungi

To detect, identify or quantify fungi or their metabolites in wood, the antigens (fungi or metabolites) could be directly labelled on wood or extracted from wood.

1) Enzyme immunoassays

We have shown that fungal antigens could be removed effectively from solid wood by different physical treatments such as grinding thin sections of wood or sawdust in buffer. The release of fungal antigen from wood could be increased using non-ionic detergents (i.e. Tween or Triton X-100). However, the detergent had to be removed or diluted out, since it interferred and prevented the antigen binding on a solid support (4). These treatments are time consuming and do not increase significantly the sensitivity of the assay. Table 1 compares the results obtained by ELISA with stained microtome sections examined by light microscopy. The longitudinal growth rate of the fungus in the tracheids of the wood was 4.2 mm/day. Visible staining of the wood was not seen until day 6, at some distance behind the mycelial front. Fungal biomass increased with time, reaching a maximum value of 7.8 µg mycelium/mg of dry wood at day 12.

To improve the efficiency of the immunoassay and decrease the steps involved in the extraction of the wood, we evaluated the possibility of carrying out the assay directly on thin sections of wood. The results obtained in sequential sections (20 µm in thickness) of an infected wood block showed up to 13% variability (Table 2). When compared to the traditional ELISA (wood extracts), the values were slightly lower only on the wood section showing no stain. However, a much larger difference was observed in the staining section of the wood (Table 2). This difference in sensitivity is attributed to the fact that the antibodies could not reach the cytoplasm of the fungus and the fungal cell surface when embedded in the host cells. This method could be of great benefit if speed is more important than accuracy.

Although the ELISA is quantitative, highly specific, and sensitive relative to the conventional tests, it has been shown that an alternative, the dot immunoassay, is up to 25 times more sensitive and relatively easy to perform (14). Consequently, we chose to use this assay, after first establishing the optimal conditions for detecting *Ophiostoma piceae* antigens by examining the membrane type, blocking conditions, and secondary antibody detection systems. Best results (low background staining and high sensitivity) were obtained with nitrocellulose membrane (NPC), with 10% milk in PBS (pH 7.4) applied for 2 to 3 hours as a blocking agent. The dot immunoassay was more sensitive than the previously described ELISA. Only 2.5 µg of fungal dry weight could be detected by the ELISA. When the same *Ophiostoma piceae* crude mycelia were applied as 2 µL dots onto the NCP, as little as 40 ng of antigen could be detected with chemical or luminescent substrates respectively (Figure 2). In a comparative study with the fungal mycelium, when the chromogenic alkaline phosphatase substrate (5-bromo-4-chloro-3-indoyl phosphate with nitroblue tetrazolium) was utilized instead of the luminescent substrate (adamantyl-1,2-dioxetane phosphate; AMPPD) an immunoreactivity pattern similar to that of a 5 minute exposure with AMPPD was obtained. Preliminary investigations suggested that the dot immunoassay is a very sensitive method that can be performed rapidly with minimal laboratory equipment. We are presently modifying this methodology to detect fungi present on wood.

2) Cytoimmunochemistry

Antibodies were also used to localize antigens in fungal or wood tissues (*in situ*). The primary goal of any immunocytochemical method is to prepare tissue in a manner such that (a) antibodies have access to the antigens, (b) the tissue should be preserved, (c) the antigenicity of the component to be analyzed survives the fixation protocol. Immunofluorescence is ideal for locating antigens at the level of light microscopy. Unfortunately, wood shows some endogenous fluorescence resulting from lignin and phenolic compounds. Immunogold and immunogold silver staining techniques offer alternative ways to localize antigens for light and electron microscopy. Figure 3 illustrates the immunogold labeling of sections from *O. piceae* cells grown in liquid culture using the polyclonal serum produced against the staining fungus. The cell wall was strongly gold labeled, whereas the cytoplasm was only moderately gold labeled. When the sections were incubated with a monoclonal antibody specific to the staining fungus, most of the gold particles were localized on the outer surface of the cell wall (Figure 4). Sections from *O. piceae* infected wood blocks that were incubated with the polyclonal antiserum had uniform and intense label over the fungal cell wall (Figure 5). The protoplasm was labeled to a lesser extent, whereas the wood cell wall showed some gold particules. An additional extracellular layer was often seen on the outer surface of the hyphae, adjacent to the wood cell lumen (Figure 5); this structure was not observed in cells grown in liquid culture.

Colloidal gold was first introduced as a marker for electron microscopy immunochemistry (8). While such gold paricles are too small for demonstration at the light level, highly concentrated gold may stain red. Recently, a chemical enhancement method was developed that allows the visualization of colloidal gold particles by light microscopy (13). In this immunogold silver staining technique (IGSS), the small gold particles are made visible by coating them with

metallic silver. We applied this technique to unembedded thin wood sections infected with a biological control agent against staining fungi (3). We also used this technique to assess the specificity of different monoclonal antibodies to a variety of fungi grown on wood blocks. Sections were incubated with polyclonal or monoclonal antibodies produced against the staining fungus *O. piceae*. The primary antibody was detected by a secondary antibody coated with 1 nm gold particles and amplified by silver precipitation. With the polyclonal antiserum the intensity of the signal was very strong and the whole hyphae appeared black against the unstained wood cell lumen. On thin sections the intensity of the signal was more evenly distributed (Figure 7). We found that unfixed and untreated wood sections gave satisfactory reactivity with both polyclonal or monoclonal antibodies. This IGSS technique is easy to perform, since the wood sections do not require any fixation or embedding prior to immunodetection.

Conclusions

A number of important points emerge from this succinct review. Immunological techniques offer great potential to wood and tree pathology. Specific antibodies can be produced to detect specific fungal species. The ELISA system is an effective method for quantifying fungal colonization of wood, as well as detecting infection prior to appearance of typical sapstain symptoms. The use of IGSS with light microscopy provides a rapid and accurate screening method for handling large numbers of infected wood specimens. From those results it is apparent that the procedure which is chosen will always depend not only on the objectives, but also on the relative importance of accuracy and convenience.

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Table 1. Longitudinal growth and biomass estimation by ELISA of a staining fungus (*Ophiostoma*) in *Pinus banksiana* sapwood blocks.

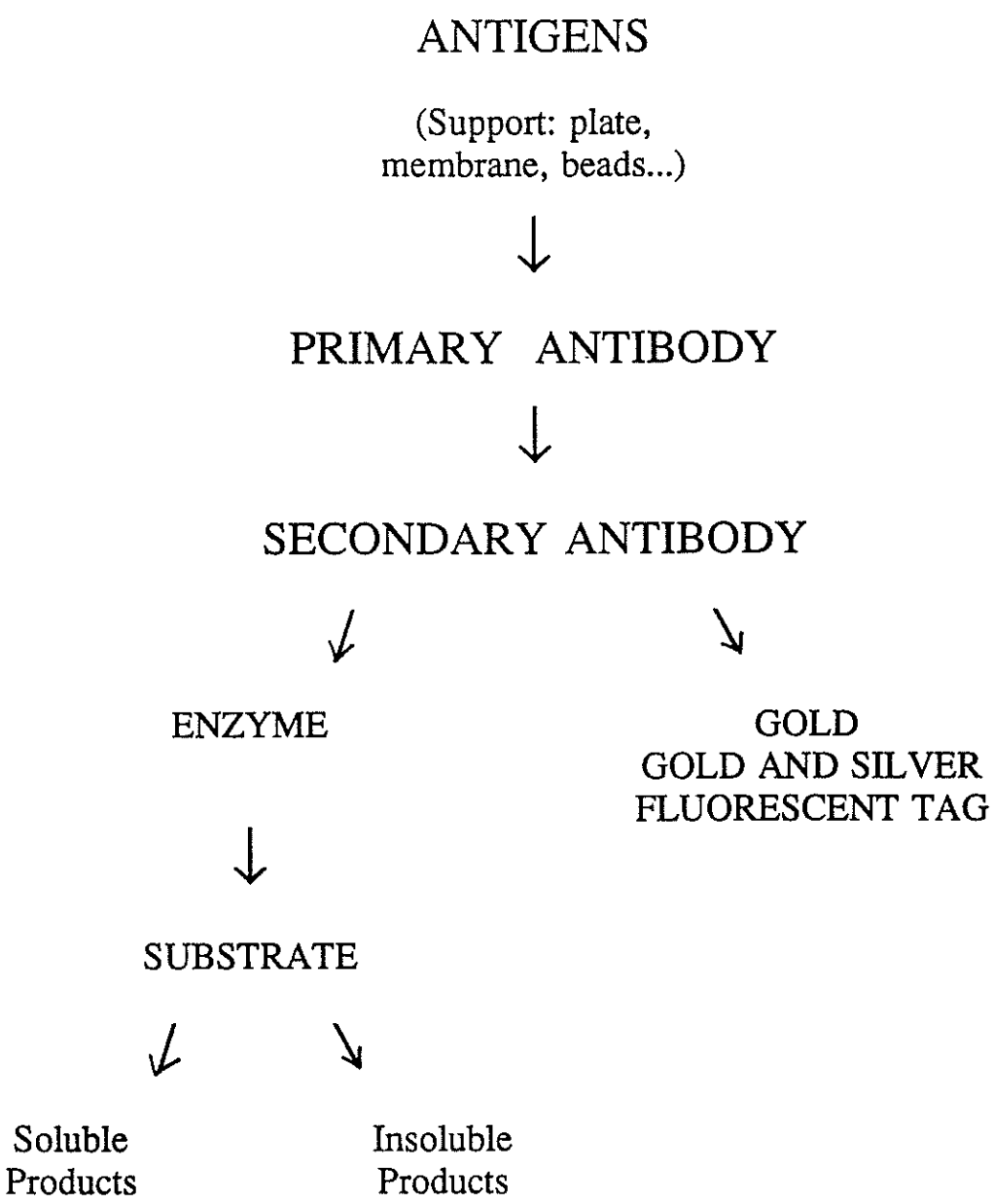
Time after inoculation (days)	Longitudinal growth (cm)	Fungal biomass measured by ELISA (µg/mg dry wood)
4	1.2	0.18
6	2.5	1.66
8	3.4	2.81
10	4.2	5.99
12	5.0	7.76

Table 2. Comparison of the traditional and direct ELISA methods for detecting *Ophiostoma piceae* in wood.

Localization of wood sections	µg mycelium/mg wood	
	Traditional ELISA	Modified ELISA
Upper	3.37	1.24 (13%)
Middle	1.25	0.86 (7.8%)
Lower	1.1	0.74 (11%)

Value in parenthesis = coefficient of variation for 4 sequential sections of wood.

Figure 1: Principle of basic immunoassays.



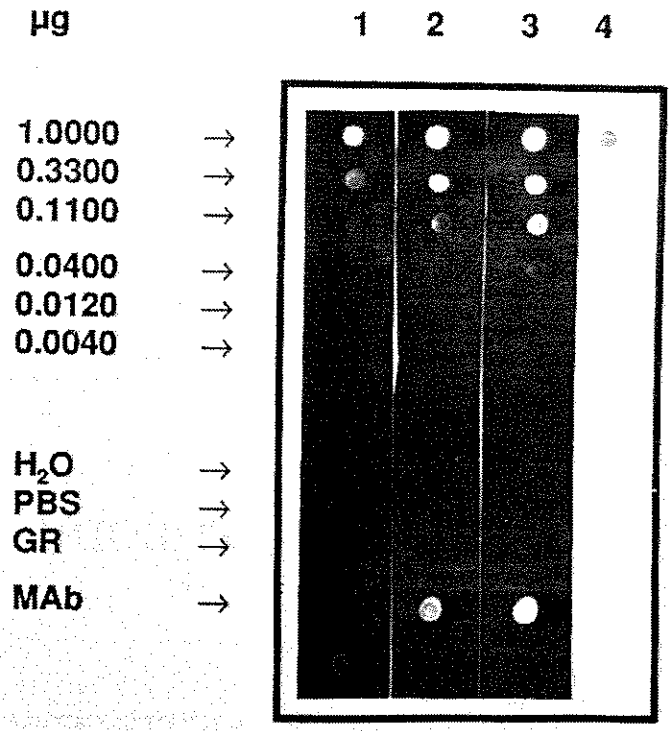
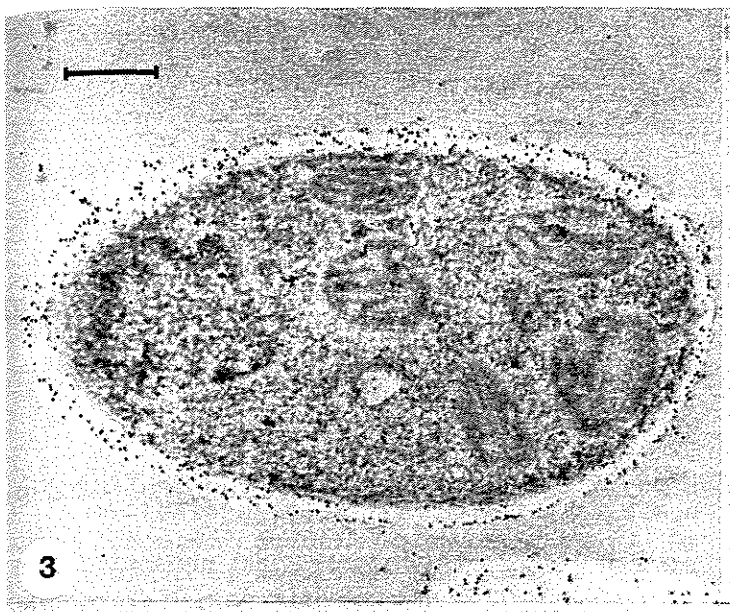


Figure 2. Dot immunoassay: *O. piceae* mycelia antigens. Fungal antigen were spotted onto nitrocellulose and incubated with Mab against the cell wall protein extract of the staining fungus. The anti-mouse alkaline phosphatase-labelled second antibody and the substrate are described in the text.



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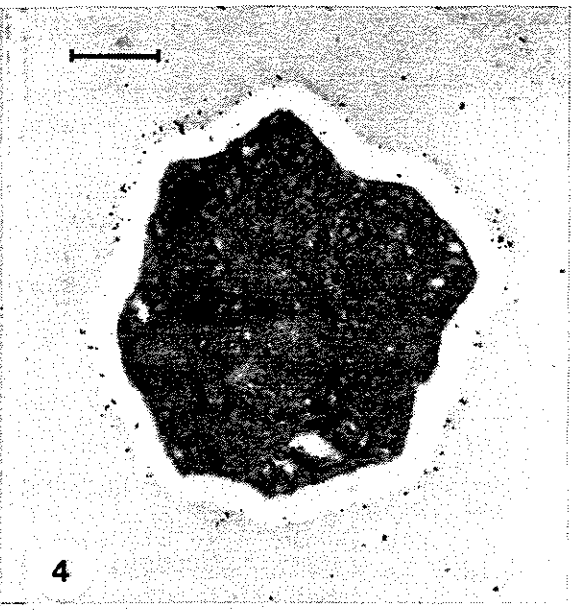

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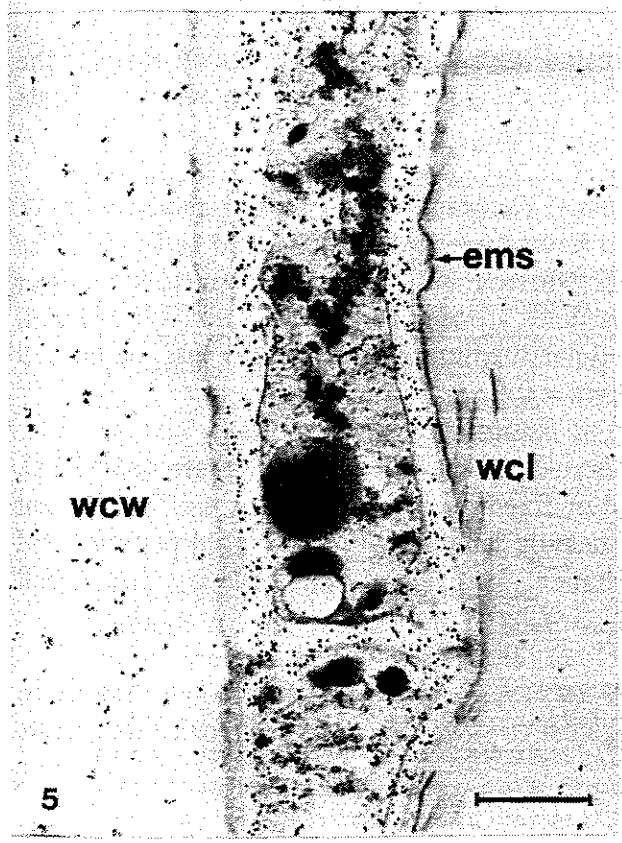

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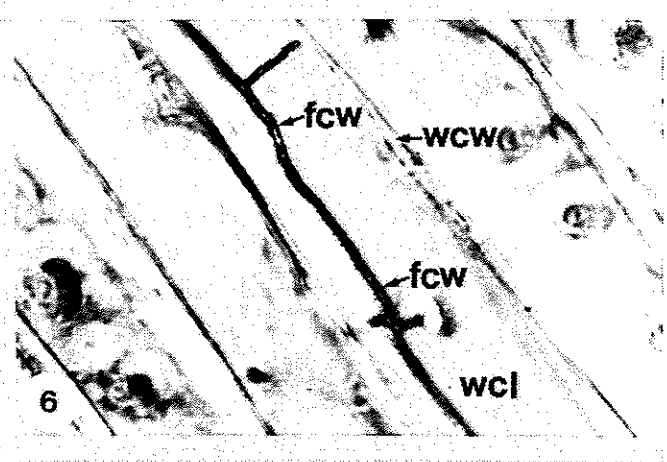

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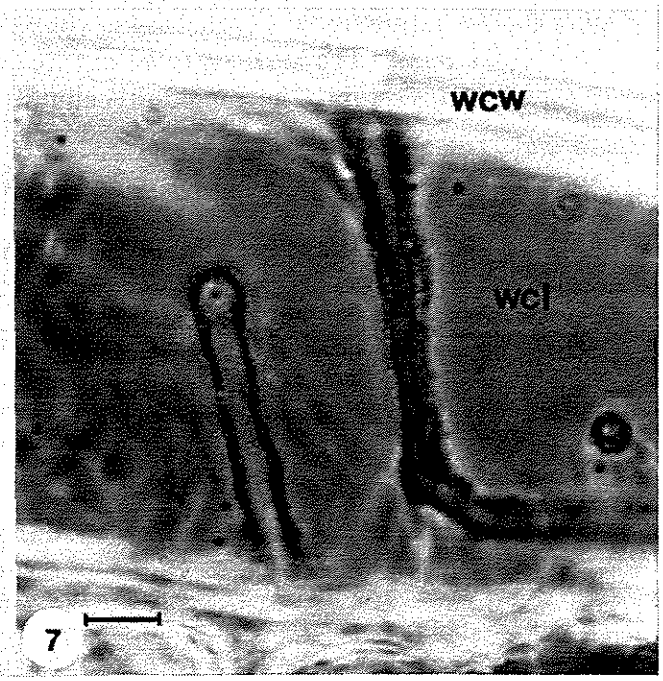

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Figures 3-4. Immunogold labeling of *O. piceae* cells fixed with glutaraldehyde and embedded in LR White medium.
Figure 3. Sections of *O. piceae* cell incubated with polyclonal antiserum. The labeling is localized mainly over the cell wall, with some gold particles randomly distributed over the protoplasm.
Figure 4. Sections of *O. piceae* treated with monoclonal antibody against the staining fungus. The label is localized on the outer surface of the cell wall. Scale bars = 0.5 •m.

Figure 5. Immunogold labeling of sections from *P. banksiana* wood blocks infected with *O. piceae*, fixed with glutaraldehyde-osmium tetroxide and embedded in Epon. The fungal cell wall is heavily labeled, whereas the protoplasm contains a lesser concentration of label. Some gold particles are also located on the wood cell wall and wood cell lumen. *ems*, extracellular membranous structure; *wlc*, wood cell lumen; *wcw*, wood cell wall. Scale bars = 0.5 •m

Figure 6. Immunogold silver staining (IGSS) from *P. banksiana* wood blocks infected with *O. piceae*. The sections were not treated or fixed prior to incubation with polyclonal antiserum. The fungal mycelium is heavily labeled with silver-gold particles and easily identified in the wood cell lumen. *fcw*, fungal cell wall; *wcl*, wood cell lumen; *wcw*, wood cell wall; X 615.

Figure 7. IGSS of semi-thin sections from *P. bianksiana* wood blocks infected with *O. piceae*. The primary antibody was a Mab against *O. piceae* cell wall proteinextracts. Scale bars = 5 •m.