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Detection of Brown-Rot antigens in Southern Pine

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DETECTION OF BROWN-ROT ANTIGENS IN SOUTHERN PINE

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SUMMARY

Brown-rot fungal antigens were detected by particle capture immunoassay (PCI) in southern pine 2 X 4's beyond visible or culturable hyphal growth. Further analysis of test samples revealed changes along the 2 X 4's that could be grouped into zones. Zone 1, the point of inoculation through 6 cm, showed low pH, measurable oxalic acid, high moisture, and high protein. Zone 2, through 16 cm, marked the end of visible hyphal growth, increased permeability, viable fungi isolated by culturing, and detection by microscopic examination. Zone 3, through 71 cm, revealed increased alkali solubility. Zone 4, through 84 cm, demonstrated positive PCI results (i.e. detection of fungal antigens). Further testing included mineral analysis which revealed iron accumulation. Movement of soluble fungal antigens beyond hyphal growth may account for the ability of the PCI to detect the presence of brown-rot fungi at zero percent wood weight loss.

KEY WORDS: Brown-rot fungi, particle capture immunoassay

INTRODUCTION

Brown-rot, the most destructive type of wood decay, is responsible for significant economic losses to in-service wood. Remedial treatments to arrest decay depend on the ability to diagnose decay in very early stages within the wood structure. Thus, more sensitive methods of detecting incipient decay are desired. Immunological methods for detection of brown-rot fungi have been developed by several groups (Goodell *et al.* , 1988; Jellsion and Goodell, 1989; Daniels and Nilsson, 1990; Breuil *et al.* , 1990; Clausen *et al.* , 1991; Clausen 1991), but most methods have either lacked specificity or convenience for adaptation as a field test. The particle capture immunoassay (PCI) (Clausen, 1994a, b), a two step field test is specific for brown-rot fungi. It has demonstrated sensitivity for fungal antigens at great distances from the fungal hyphae, both *in vitro* and *in vivo* . A field test using PCI, detected antigens to *Serpula lacrymans* nearly 1 meter from the antigen source. Likewise, PCI detected *postia placenta* antigen 84 cm from the antigen source in a scaled-up soil block test that accommodated dimension lumber.

The objective of this study was to look for chemical and morphological changes in the test units that would explain positive PCI results beyond the fungal hypha. Test units were sampled and examined for changes in wood structure, and accumulation of fungal by-products.

MATERIALS AND METHODS

Culture conditions : *Postia placenta* MAD 698 was cultured on southern pine 2 X 4s (86 cm long) by a modification of the ASTM soil block test (ASTM, 1991) referred to as the soil trough test (Clausen and Ferge, 1995). Briefly, 90 cm aluminum troughs containing 2 kg soil (50% moisture) held individual 2 X 4 southern pine test units that had a pre-test moisture content of 30 percent. The troughs were covered with foil and autoclaved for 2 hr. A liquid suspension of *P. placenta* was pipetted into predrilled holes in one end of each 2 X 4. Test units were incubated at 27° C, 80% RH, and sampled after 3 wk. Test units were sampled at 7.6 cm intervals with an alcohol-flamed 7 mm drill bit or core borer. Core borings were examined microscopically and wood shavings were extracted in 0.1% Triton X-100 (50 mg/ml) for further testing.

Test methods:

Moisture Moisture readings were taken immediately after incubation at sampling intervals with a Delmhorst RDX-1 moisture detector.

Hyphal growth Sample shavings were aseptically placed on 2% malt agar plates and incubated at 27° C, 80% RH for 3 wk.

pH Direct pH measurements were made from aqueous extracts of wood shavings.

Oxalic acid Oxalic acid was estimated using a micro-adaptation of a commercial assay (Sigma).

Protein Protein was determined by the BCA microassay (Pierce) using bovine serum albumin as a standard.

Microscopy Core samples taken at intervals were sectioned and stained with picro aniline blue-safranin (Wilcox, 1964) to differentiate wood structure from fungal hyphae. Selected wood samples were examined in SEM for visible damage to the bordered pit membranes.

Reducing sugar assay The enzymatic activities of xylanase and polygalacturonase were determined by a microadaptation of the Nelson-Somogyi reducing sugar assay (Green *et al.*, 1989; Clausen and Green, in press). A unit of enzyme activity was defined as the amount needed to liberate reducing sugars equivalent to 1 μ g of glucose per 24 h at 40° and 28°C, respectively.

Alkali solubility According to ASTM D1109-84 (1987), wood samples were NaOH extracted, and percent solubility was determined at each sample interval. Alkali solubility was expressed as the mean of 1% NaOH solubility per % dry weight of wood.

Particle capture immunoassay (PCI) PCI was conducted at sample intervals as previously described (Clausen 1994a, b; Clausen 1995). Fifty mg samples extracted in 0.1% Triton X-100, were tested with prepared PCI test strips.

Mineral analysis Inductive coupled plasma spectroscopy was used to analyze the mineral content in samples taken at intervals along the test units.

Permeability Permeability of 64mm diameter cores was measured by the method of Milota et al. , 1995.

RESULTS

The average moisture content at the site of inoculation was 55.6%, while moisture readings ranged from 27.7 to 33.7% along the remainder of the test units. Moisture in the control unit was uniformly 30.0%.

P. placenta was cultured from wood shavings at two sample intervals or 15.2 cm from the inoculation site. Hyphal growth was visible up to 12.7 cm in two test units and up to 17.5 cm from the inoculation site in the third test unit. Microscopic examination results paralleled culture results.

The pH of extracted wood shavings from the inoculation site was one unit lower (4.03) than other readings along the test units, which ranged from 4.67-5.00. pH of the control was 4.70.

Oxalic acid was measurable at the inoculation site (5.3 uM/L), but was not detectable in other samples. Likewise, reducing sugars were not detectable in extracted samples. Protein at the inoculation site was 729 mg/ml, while protein ranged from 186-437 mg/ml for all other sample intervals including the control (260 mg/ml).

Alkali volubility varied from 10-64% for the first 10 sample intervals. It was below 10% at the last sample site, the farthest point from inoculation. Longitudinal permeability was not altered in cores beyond the hyphal front. Likewise, electron microscopy revealed no damage to pit membranes in similar samples.

Based on test results, zones along three test units were identified that revealed certain characteristic changes in the wood (Figure 1). Mineral analysis showed accumulations of iron, ranging from undetectable to 230 ppm (Table 1). In general, Fe accumulations occurred in the first 23-38 cm and the last 8-15 cm with negligible accumulations in the center sections of the test units. These results did not correlate with positive PCI results.

Twenty-eight of the 33 samples were PCI positive over the entire length of the 3 test units. Two test units were PCI negative at the site of inoculation, but were positive at further sample sites. A negative PCI result has been seen in wood displaying late stages of decay in previous tests.

DISCUSSION

The particle capture, immunoassay (PCI) was shown to have an advantage over traditional methods of incipient decay detection (Clausen 1994a; Clausen and Ferge, 1995). PCI utilizes monoclonal antibody which is highly sensitive and

specific for an extracellular enzyme of brown-rot fungi. PCI can detect fungal antigens at zero percent wood weight loss beyond the fungal hypha. PCI has detected fungal antigens nearly 1 meter from the antigen source both in vitro and in vivo. Similar findings are seen for antigens to root rot fungi using ELISA. These antigens are detectable by polyclonal antibodies to unidentified fungal components at considerable distances from the infection in living trees (personal communication). Transport of fungal antigens in a living system is probable, but movement through kiln dried lumber is considered unlikely. Aspirated pits block the vertical passage through tracheids, although cracks and checks in kiln dried southern pine may facilitate fluid movement.

The ability to detect fungal antigens at considerable distances ahead of the hypha is repeatable in vitro and in vivo. It is theorized that this phenomena is due to antibody recognition of extracellular fungal enzymes. Other studies involving antibodies to cellular components of the hypha lack specificity and antigens cannot be detected prior to colonization by the fungus. A passive diffusion test using partially purified extracellular polysaccharidases showed that these enzyme were unable to simply diffuse through the wood structure (Clausen and Ferge, 1995). Additional tests were conducted to determine the mechanism of transport of the fungal antigens.

Many tests failed to indicate an explanation for transport of fungal antigens. One hypothesis was that pit membranes were opened by nonenzymatic decay agents, such as oxalic acid, or degradative enzymes, such as pectinases. Green et al. (1995a, b) demonstrated that polygalacturonase, in conjunction with oxalic acid production, facilitated hydrolysis of the bordered pit membranes in wood blocks infected with P. placenta prior to weight loss. Results in the current study indicated that beyond the hyphal front, this was not the case. Gas permeability was not altered as measured by the method of Milota et al.., 1995. No damage to the torus or pit membrane was revealed by SEM in samples beyond visible hyphal growth.

High moisture and protein values were noted only in the area of visible fungal growth, as were low pH values. Low reducing sugar activities were not surprising due to the sensitivity of the reducing sugar assay. Sensitivities of most test methods were at least 1000-fold higher than PCI, which is known to operate in the nanogram range of sensitivity.

Alkali volubility test results were indicative of cellulose alteration up to 71 cm from the site of inoculation. Mineral analysis showed iron accumulations 23 to 38 cm from the inoculation site and 8 to 15 cm from the opposite end of the test unit with negligible accumulations in the center of each test unit. P. placenta MAD-698 has been shown to accumulate iron in decayed wood (Green et al. 1996).

The mineral analysis and PCI support the theory that Postia placenta acts like a pump, converting woody substrates to CO and H₂O via an H₂O₂-Fe⁺⁺ system and pushes soluble proteins and minerals ahead of the hypha into the wood (Koenigs, 1974). A more sensitive detection system, such as HPLC may show that oxalic acid is also pushed ahead of the hypha until calcium accumulation causes it to precipitate as calcium oxalate (Green et al. 1995a; 1996).

Iron accumulation, alkali solubility, and PCI results indicate that some structural change has occurred in the test unit that allow small soluble molecules (<30 kDa of xylanase) to move longitudinally through wood structure. Further tests with soluble dyes or a more sensitive method of detecting reducing sugars may determine if soluble proteins would be capable of diffusion through wood structure.

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Table 1. Iron accumulation and particle capture immunoassay results at 11 sample sites along three southern pine 2 X 4s after infection with Postia placenta.

<u>Test unit</u>	<u>A</u>		<u>B</u>		<u>C</u>	
	<u>Fe (ppm)</u>	<u>PCI</u>	<u>Fe (ppm)</u>	<u>PCI</u>	<u>Fe (ppm)</u>	<u>PCI</u>
<u>Sample #</u> ^a						
01 ^b	23	+++	230	-	29	-
02	6	+	66	+	20	+
03	14	+++	43	+	7	+
04	2	-	76	+	13	+
05	<1	+++	<1	++	25	+
06	<1	+++	2	+	<1	+
07	4	+	<1	+	<1	+
08	<1	-	3	+++	<1	+
09	3	+	<1	++	<1	-
10	18	+	<1	++	<1	+
11	43	+	84	++	3	+
control	<1	-				

^a sample interval = 7.6 cm

^b site of inoculation

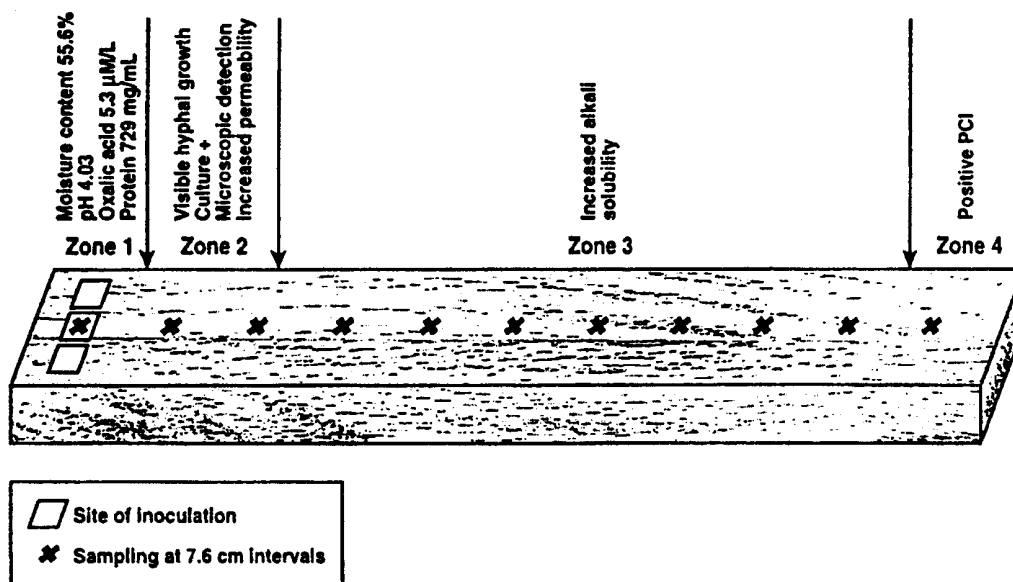


Figure 1. Schematic of 2 x 4 test unit, indicating zone of characteristic change in wood. Samples from zone 1 had low pH, measurable oxalic acid, high moisture content, and high protein. Zone 2 marks the end of visible hyphal growth, increased permeability, viable fungi, and detection by microscopic examination. Increased alkali solubility was noted through zone 3. PCI results were positive through zone 4.