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Electron Microscopic Systems Imaging of C, P and Fe Localizations in the Brown-rot Fungus, *Postia placenta*

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Summary

Recently, oxalic acid, a possible product of oxaloacetase's action upon oxaloacetate and/or glyoxylic acid oxidase's attack upon glyoxylic acid, has been the focus of considerable research in brown-rot degradation of cellulose in wood. Oxalic acid, a low molecular weight organic acid, could serve as a proton donor for hydrolytic or an electron donor for oxidative (Fenton's mechanism involving Fe^{2+} and H_2O_2) cleavages of the structural, microfibrillar ß, 1--4 linked glucan. Here are reported the results of an electron microscopic systems imaging investigation concerned with detecting Fe in *Postia placenta* hyphae cultured on or in a defined medium either containing or lacking agar, respectively, or grown upon southern pine wood blocks. Ultrastructural analysis of hyphae grown upon blocks demonstrated hyphal sheaths closely appressed to extensively degraded wood cell walls. In addition, hyphae were sometimes observed within wood cell lumens confirming previous investigators' results. Comparative electron microscopic systems imaging at 25 (C), 59 (control-general survey), 110 (P), 222, and 710 (Fe) eVs of 20nm sections of glutaraldehyde-fixed and quetol-embedded hyphae cultured on either an agar-solidified medium or wood blocks revealed hyphal Fe (710 eV) and Fe within the hyphal sheath. While Fe was not visualized in wood cell walls of uninoculated wood blocks, it was in certain walls of inoculated blocks. The observed Fe localization, which differed from those of C and P, were qualitative. When available, evolving computer-assisted background image analysis employing element-specific ionization edges or coembedded Fe standards will permit rigorous quantitation, Nevertheless, these results derived from a novel electron microscopical technique constitute limited but additional support for an oxalic acid, Fe^{2+}/H_2O_2-Fenton's mechanism for brown-rot cellulose degradation in wood.

Introduction

White- and brown-rot fungi are the major wood destroying fungi. Wood destroying fungi can be classified as either white- or brown-rot fungi (Nilsson 1979; Eriksson *et al.* 1990). Whereas the former degrade both cellulose and lignin, the latter decompose only cellulose leaving the lignin intact (Highley and Kirk 1979) but demethylated (Kirk and Adler 1970; Kirk 1975; Highley *et al.* 1994). The decomposition of wood polymers (Eaton and Hale 1993) by brown-rot fungi has been reviewed many times and most recently by Illman and Highley (1989) as well as Highley and Flournoy (1994).

To develop new methods to protect wood from fungal attack, it is necessary to completely understand the chemical reactions underlying fungal decay of wood polymers. Therefore, considerable time and effort have been invested in defining the temporal syntheses and secretion of wood-destroying enzymes such as cellulases and hemicellulases as well as their isolations and characterizations (Highley *et al.* 1994).

The capacity of brown-rot fungi to initiate decay of the secondary cell wall and their widespread depolymerization of cellulose do not appear to be due to an enzymatic mechanism. To account for such a drastic decrease in the degree of cellulose polymerization, Koenigs (1972, 1974) hypothesized that wood cellulose could be oxidatively broken down by the [OH] radical produced by a Fenton type system (H_2O_2/Fe^{2+}) in brown-rot fungi (Poli *et al.* 1993; Evans *et al.* 1994). Oxalic acid is secreted by brown-rot fungi (Takao 1965) and has been implicated in their induced wood decay by either directly depolymerizing wood cellulose (Shimada *et al.* 1991, 1994; Green *et al.* 1992) and hemicellulose (Bech-Anderson 1987) or by reducing Fe^{3+} to Fe^{2+} (Schmidt *et al. 1981).*

This paper and the accompanying paper (Jordan *et al. 1996)* report the results of a combined biochemical and electron microscopic systems imaging investigation designed to test the hypothesis that brown rot-induced wood decay involves an oxaloacetase and/or glyoxylic acid oxidase, -oxalic acid H_2O_2/Fe^{++} Fenton-type mechanism. Here are reported the results

of the ESI investigation. Certain of the results have been presented in preliminary form (Jordan *et al.* 1994).

Experimental

Liquid culture of Postia placenta

Mycelia of a *P. placenta* isolate MAD 698 (Forest Products Laboratory, Madison, WI) maintained on agar slants were aseptically transferred to sterile Petri plates containing sterile-basal salts medium (Highley 1973) supplemented with 1% glucose (w/v) and solidified with 2% Bacto-Agar (Difco, Detroit, MI). The plates were incubated at 27 ± 2°C and 70% relative humidity for 5 days in the dark within a Labline gyrotory shaker. Thereafter, 20, 5mm mycelial discs were aseptically excised from the plate cultures and homogenized into 11 sterile medium containing 1% glucose. Then, 40ml of the homogenate were inoculated into 11 Erlenmeyer flasks containing 11 sterile Bailey's medium. These flasks were swirled and divided into 500ml volumes for each 11 flask. The inoculated flasks were incubated in the shaker for 25 days in the dark at 80-100rpms.

Separation of hyphae from culture medium

The 500ml contents of the 11 flasks were collected by gentle vacuum filtration (on ice) onto Whatman number one qualitative 9.0cm filter papers (Whatman Paper Ltd., Maidstone, England). The fungal hyphae and media were stored on ice for further analysis.

Growth of Postia placenta on agar and southern yellow pine

Two percent Bacto-Agar in basal salts medium containing 1% glucose was sterilized at 121°C for 20 min and then allowed to cool slightly. Next, sterile Petri plates were filled with the medium which was permitted to cool completely. Some plates were aseptically inoculated with *P. placenta* (MAD 698) from an agar slant. The inoculated plates were incubated in the dark 12–14 days at 27 ± 2°C and 70% relative humidity in a Labline gyrotory shaker.

Southern Yellow Pine blocks (6.35 × 6.35 × 3.18mm; the small dimension in the fiber direction) were decayed by *P. placenta* employing the standard American Society for Testing and Materials soil-block method (ASTM 1991) or an agar-block method. Wood decay chambers for the latter method were constructed by placing wood wafer feeders on agar in Petri dishes and subsequent inoculation with *P. placenta.* Chambers were incubated at 27 ± 2°C and 70% relative humidity in the dark for 3 weeks. After incubation, test blocks were again sterilized at 121°C, weighed and the percent weight loss determined.

Preparation of agar-cultured hyphae and uninoculated and inoculated wood blocks for electron microscopy

Plugs of agar-cultured *P. placenta* or inoculated wood blocks dissected into 0.5 to 1.0mm^3 pieces (controls = uninoculated blocks) were prepared for transmission electron microscopy (TEM) and electron microscopic systems imaging ESI (Ahn and Krivanek 1983).

Fixation

Hyphal plugs and wood pieces were pre-fixed in 2% glutaraldelyde in 0.1M potassium phosphate buffer, pH 6.8, for 30min (Sabatini *et al.* 1963) under vacuum at room temperature and then overnight at 4°C. Thereafter, hyphae were washed three times (15 min each time) in phosphate buffer and two times for 15 min each in dH_2O. For TEM, samples were subsequently post-fixed in phosphate-buffered 2% OsO_4 for 2h at room temperature. Post-fixed samples were washed three times (15 min each time) in phosphate buffer and two times for 15 min each in dH_2O.

Dehydration

Hyphal or wood samples were dehydrated through a graded alcohol series for 30 min (hyphae) or 1h (wood) each in 25, 50, 60,70, 80, 90, 100, 100, and 100% EM grade EtOH. Thereafter. the EtOH was exchanged with n-butyl glycidyl ether (n-BGE) dehydrant (intermediate solvent) as follows: 1:1 alcohol/n-BGE for 15 min and then n-BGE alone for 15 min.

Infiltration

Hyphae and inoculated or uninoculated wood samples were infiltrated under vacuum with a graded fresh quetol resin (Ted Pella Inc., Redding, CA) series consisting of quetol in n-BGE as follows: 25, 50, 60, 70, 80, 90, 100% quetol each for 30 min. Additionally, fungal and wood samples were placed into second and third 100% quetol changes under vacuum, respectively. Samples were then drained and resuspended in freshly-prepared 100% quetol for embedment in beem capsules. The samples were cored at 60°C for either 18h or overnight.

Preparation of Postia placenta hyphae for electon microscopy

Plugs of *P. placenta* on agar were excised from the Petri dishes for transmission electron microscopy and electron systems imaging (Ahn and Krivanek 1983)

Fixation

Hyphal plugs were pre-fixed in 2% glutaraldehyde in 0.1M potassium phosphate buffer, pH 6.8, for 30 min under vacuum at room temperature and then overnight at 4°C. Thereafter, hyphae were washed three times (15 min each time) in phosphate buffer and two times for 15 min each in distilled water. TEM samples were then post-fixed in 2% phosphate buffered osmium tetroxide phosphate buffer, and two times for 15 min each time in distilled water.

Dehydration

Fungal samples were dehydrated through a graded alcohol series for 30 min each time in 25%, 50%, 60%, 70%. 80%, 90%, 100%, 100%, 100% ethanol. The ethanol was then exchanged with n-butyl glycidyl ether (n-BGE) dehydrant (intermediate solvent) as follows: alcohol/n-BGE in a 1 : 1 ratio for 15 min and then n-BGE alone for 15 min.

Infiltration

Hyphae were infiltrated under vacuum with a graded fresh quetol resin (Ted Pella, Inc., Redding, CA) series consisting of quetol in n-BGE as follows: 25%, 50%, 60%, 70%, 80%. 90%, 100% quetol each for 30 min.

Additionally, fungal samples were placed into fresh 100% quetol resin overnight under vacuum. Next, samples were drained and resuspended in freshly-prepared 100% quetol for embedment in beem capsules. Samples were cured at 60°C for either 18h or overnight.

Preparation of Postia placenta-inoculated southern yellow pine blocks, for electron microscopy

Postia placenta -inoculated wood test blocks were cut into 0.5 to 1.0mm^3 pieces. Uninoculated sterile wood blocks served as controls and were also cut into the same size pieces which were prepared for TEM and ESI as follows:

Fixation

The wood pieces were pre-fixed in 2% glutaraldehyde in 0.1M potassium phosphate buffer, pH 6.8. Pre-fixation was carried out for 30min under vacuum at room temperature and then overnight at 4°C. Then samples were washed three times for 15min each in the buffer, and two times (15min each) in distilled water. Transmission electron microscopy samples were then post-fixed in

phosphate buffered 2% osmium tetroxide for 2h at room temperature. Post-fixed samples were again washed three times for 15 min each in phosphate buffer, and two times for 15 min each in distilled water,

Dehydration

As for fungal samples, wood samples were dehydrated in a graded alcohol series for 1h each in 25%, 50%, 60%, 70%, 80%, 90%, 100%, 100%, 100% ethanol. Subsequently, the ethanol was exchanged with n-BGE dehydrant as previously described.

Infiltration

Wood samples were infiltrated under vacuum as previously described for hyphae.

Sectioning and viewing for TEM

Ultrathin sections, 70nm (interference color, silver), were cut on a LKB 8800 Ultrotome III (LKB, Munich) using a diamond knife. The sections were collected from a water surface onto uncoated copper (200 mesh) and formvar coated grids (single slot) and examined at an accelerating voltage of 50-70kV using an Hitachi H-600 electron microscope (Hitachi, Tokyo) after staining with lead citrate and uranyl acetate (Reynolds 1963).

Electron microscopic systems imaging was performed with a Zeiss EM 902 transmission electron microscope (Carl Zeiss, Oberkochen). Very thin unstained sections, 30nm, of tissue blocks were collected upon uncoated 200 mesh copper grids and examined at an accelerating voltage of 80kV, The inelastically scattered electrons with element-specific energy losses (Ahn and Krivanek 1983) were employed to obtain high resolution imaging (Egerton 1986; Budd 1988) of the iron distribution in the sections. Control images were also obtained for carbon and phosphorus. Computer-assisted imaging was performed utilyzing a low scan charge-coupled device (Gatan, Warrendale, PA), Digital Micrograph 1.1.1 software (1024 × 1024 pixels) and a Quadra 950 Apple computer.

Statistical analyses

Where appropriate, significant differences between means were analyzed by an analysis of variance (Snedecor and Cochran 1979).

Results

Weight loss in wood blocks

Weight loss determinations were carried out over 3 weeks for southern yellow pine blocks. A mean percent weight loss of 55 ± 17% was obtained.

Transmission electron microscopy of Postia placenta hyphae

Transmission electron micrographs of hyphae cultured on agar revealed growing and non-growing regions typically exhibited by fungal hyphae. Hyphae showed polymorphism in that they were of various sizes and shapes (Fig. 1) and were also characterized by septa with clamps as revealed by high magnification of a septum (Fig. 2).

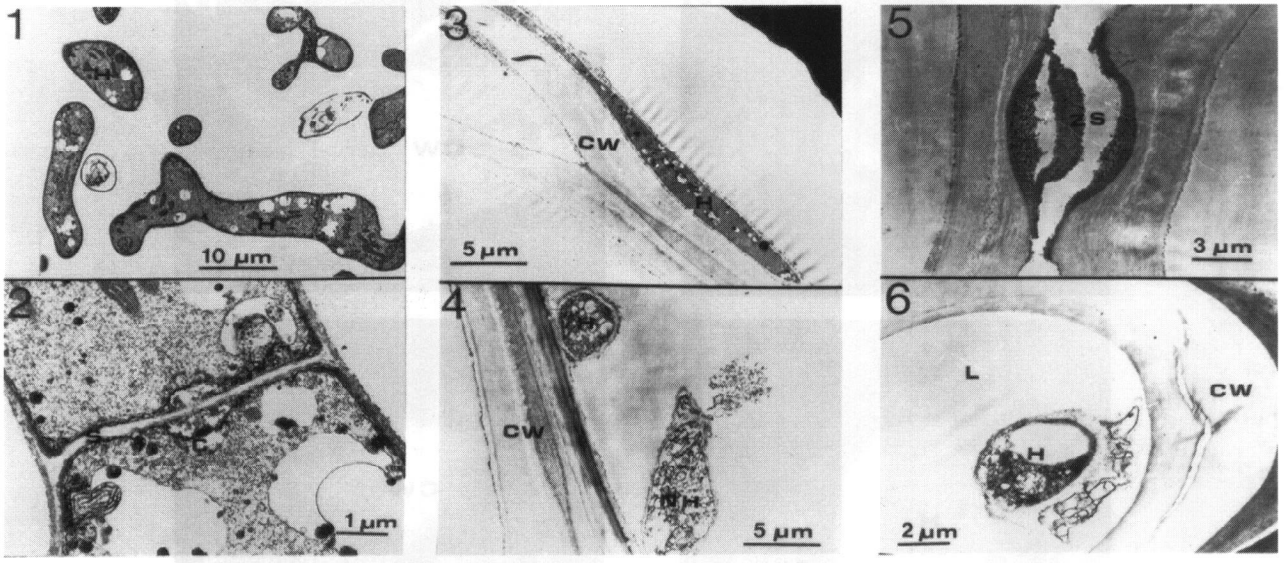
Transmission electron microscopy of Postia placenta-inoculated southern yellow pine

Micrographs of inoculated southern yellow pine depicted *P. placenta* growing adjacent to the wood cell wall (Fig. 3). Additionally, fungal remnants (Fig. 4) possessed putative hyphal sheath elements closely appressed to the wall. At 3 weeks post-inoculation, there was substantial degradation of the wood cell wall with numerous electron dense particles exterior to the wall and within a zone of separation (Fig. 5). Also, hyphae were apparent within the lumen of certain wood cells possibly surrounded by electron dense particles and a sheath (Fig. 6).

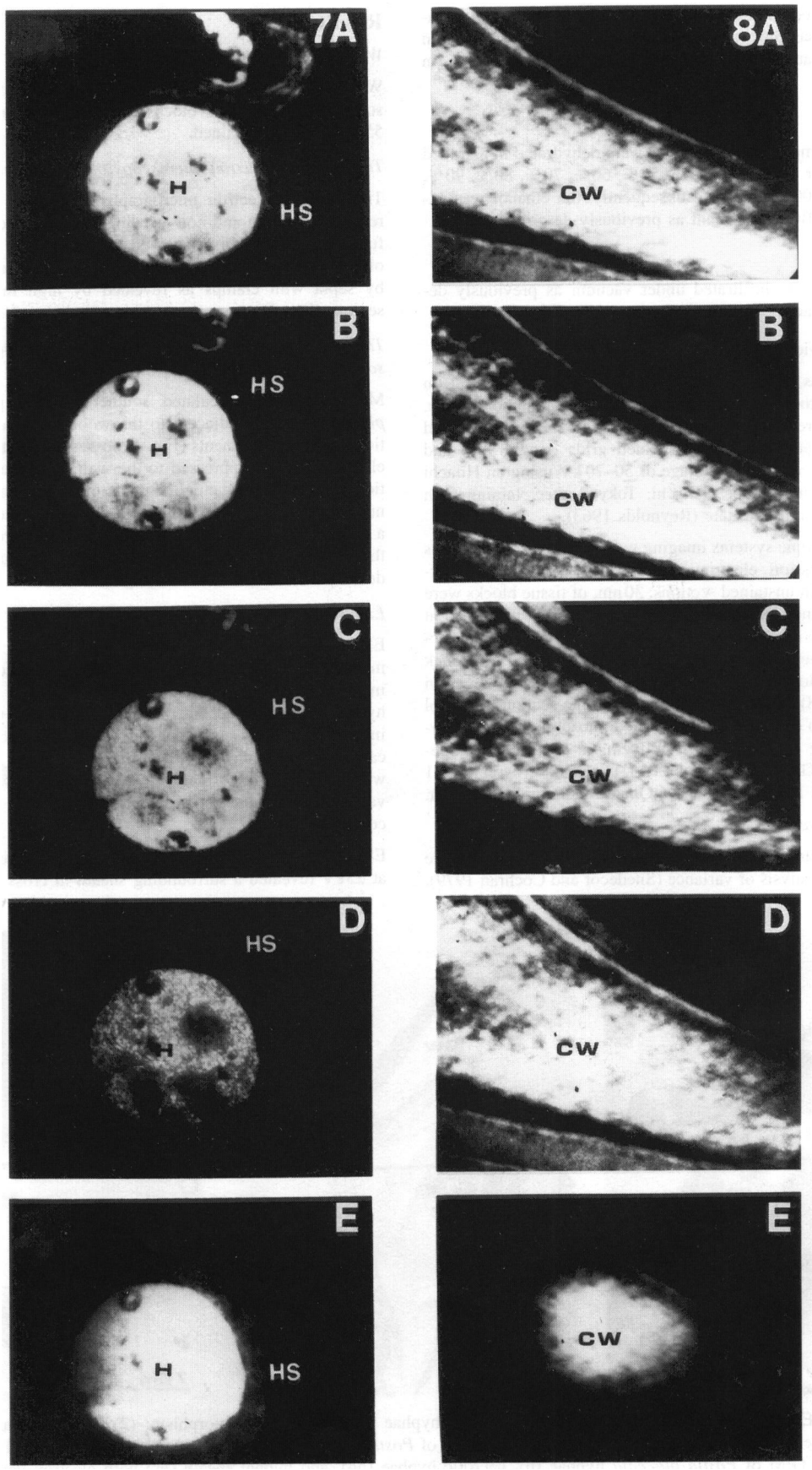
Electron systems imaging

Electron microscopic systems imaging coupled with the employment of a charge-coupled device (ccd) yielded element-related images at 25, 59, 110, 222, and 710eV energy losses for fungal hyphae, uninoculated and inoculated southern yellow pine. The images brightness corresponded qualitatively to the amounts of carbon (25eV), phosphorus (110eV), and iron (222 and 710eVs) within the samples. Imaging was performed at 59eV since this value did not represent an elemental edge and thus served as a control.

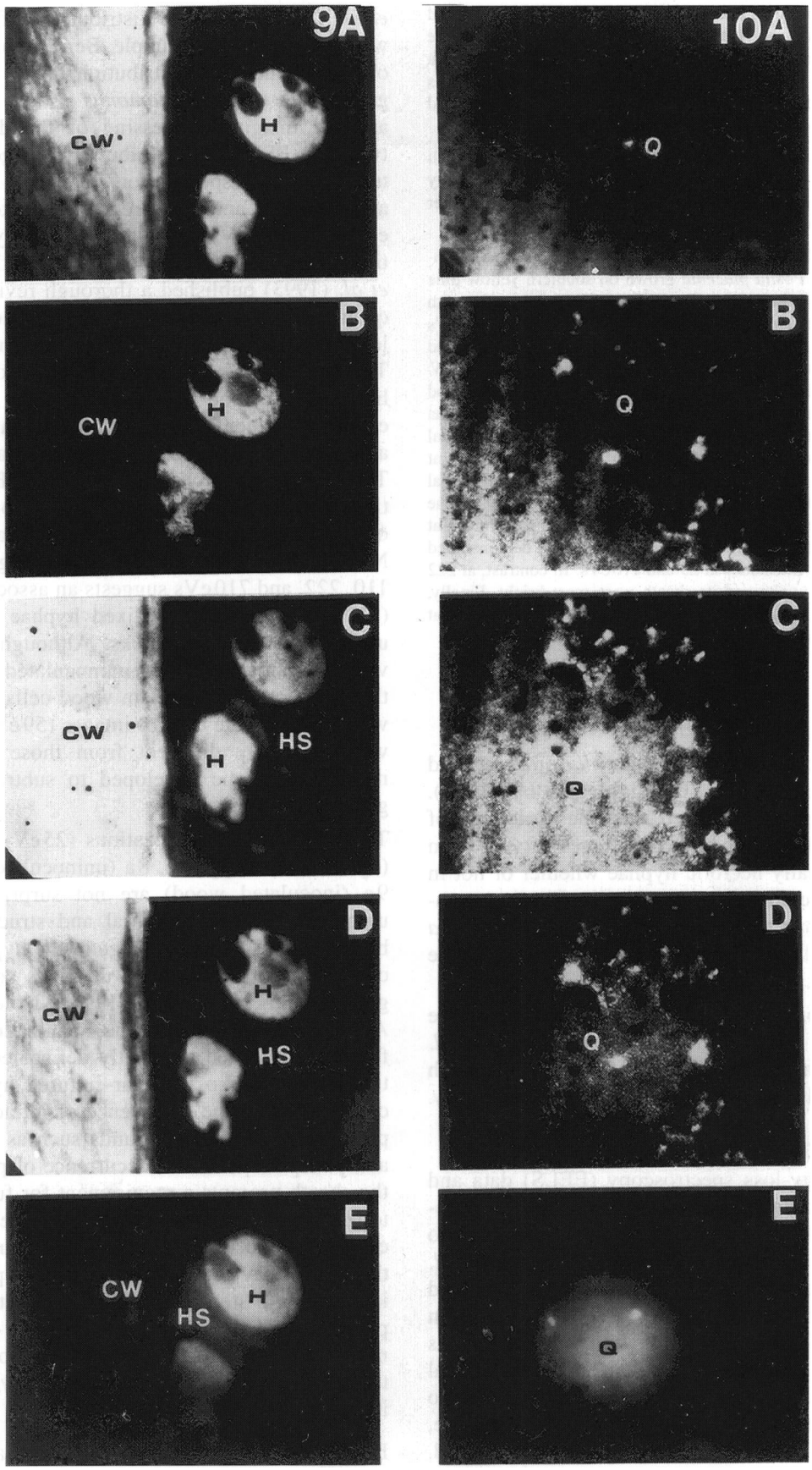
ESI of *Postia placenta* Hyphae – Imaging of agar-cultured hyphae at 25eV revealed a surrounding sheath in cross section (Fig. 7a).



Figs. 1–6. (1) TEM of several agar-cultured *Postia placenta* hyphae (h) revealing polymorphism; (2) TEM of agar-cultured *Postia placena* hyphae exhibiting septum (s) with clamp (c); (3) TEM of *Postia placenta* hyphae (h) adjacent to the cell wall (CW) of southern yellow pine; (4) TEM of *Postia placenta* hyphae (h), necrotic hyphae (nh), and hyphal sheath (hs) with electron dense particles in 3 week inoculated southern yellow pine; (5) TEM of southern yellow pine cell wall after 3 weeks of degradation by *Postia placenta,* zone of separation (zs); (6) TEM of southern yellow pine with *Postia placenta* hyphae (h) within cell lumen (1) after 3 weeks incubation, cell wall (CW)



Figs. 7–8. (7) ESI of *Postia placenta* hyphae (h) and hyphal sheath (hs), 25 **(a),** 50 **(b),** 110 **(c),** 222 **(d),** and 710 **(e)** eV energy losses: **(8)** ESI of southern yellow pine cell wall (cw), **25 (a),** 50 **(b),** 110 **(c),** 272 **(d)** and 710 **(e)** eV energy losses.



Figs. 9-10. (9) ESI of *Postia placenta* hyphae (h) and hyphal sheath (hs) on southern yellow pine cell wall (cw), 25 **(a),** 59 **(b),** 110 **(c),** 222 **(d)** and 710 **(e)** eV energy losses; **(10)** ESl of Quetol embedding resin at 25 **(a),** 59 **(b),** 110 **(c),** 222 **(d)** and 710 **(e)** eV energy losses.

At 59eV, the same hypha and sheath were slightly less defined (Fig. 7b). However, at 110eV, the fungal hypha appeared more brightly delineated and the sheath less so (Fig. 7c). At 222eV, a less bright, but well defined hypha with a faint sheath was observed (Fig. 7d). Finally, bright illumination of the hypha and a well defined sheath were noted at 710eV (Fig. 7e).

ES I of Southern Yellow Pine – Southern yellow pine wood cell walls revealed approximately the same brightness at all energy loss levels (Fig. 8 a–d) except at 710eV where there was either little brightness or definition to the wood cell wall (Fig. 8e).

ESI of *Postia plancenta* -Inoculated Southern Yellow Pine – Imaging at 25eV of *Postia placenta* grown on southern yellow pine revealed brightness in the wood cell wall and fungal hypha with sheath, but limited definition in either (Fig. 9a). At 59 and 110eVs (Fig. 9b and c), the same definition in the wood cell wall and fungal hypha was apparent but with less brightness than at 25eV. However, at 222eV (an indication of iron) the brightest and most well-delineated point was that corresponding to the hypha (Fig. 9d). The brightness was qualitatively deeper in the fungal hypha and correspondingly less bright in the wood cell wall at 710eV (Fig. 9e). Further examination of the wood cell wall-fungal interface (on a different sample) revealed approximately the same qualitative data as in the previous series, but lacking an image at 59eV. That is, both the wood cell wall and hypha demonstrated similar brightness intensities at 25 and 110eVs. In contrast, at 222 and 710eVs, the hypha and its sheath were most bright. Finally, background illumination appeared greatest at 110eV and lowest at 25eV for quetol resin alone (Fig. 10a–e).

Discussion

Transmission electron microscopy

An ultrastructural analysis of *P. placenta* decayed wood has been reported by Highley *et al.* (1983). These investigators reported the fine structures of hyphae of various developmental stages varying from young to partially necrotic hyphae whether or not in contact with cellulosic microfibrils. Figure 1 confirms these investigators' findings that *P. placenta* hyphae are polymorphic possessing an extension, the hyphal sheath.

The TEMS presented in Figures 3–6 support the findings of a previous investigation (Green *et al.* 1989) regarding the association of the hyphal sheath with the wood surface during brown-rot wood decay.

Electron systems imaging

Electron energy-loss spectroscopy (EELS) data and those acquired by electron dispersive x-ray microanalysis (EDAX) can add chemical information to static electron microscopical images (de Bruijn *et al.* 1993). Furthermore, the acquisition of digitized electron spectroscopic images (ESI) from ultrathin sectioned cells and tissues allows the simultaneous morphometric and chemical analysis of biological materials. Therefore, attempts were carried out to obtain ESI of Fe, C, and P from fungal hyphae, uninoculated wood, and hyphal infected wood. Although the ESI images did not demonstrate background levels above or below the specific elemental ionization edges (Ahn and Krivanek 1983; Beckers *et al.* 1993) qualitative distributions of C, P, and Fe were observed. For example, Benchimol *et al.* (1993) obtained a net Ca^{++} distribution image in the hydrogenosomes of *Tritrichomonas foetus* by computer assisted imaging processing of the difference between an ESI taken just above the edge of the electron absorption specific for Ca^{++} ($Ca_{1,2,3}$ edge 346eV) at 3 = 360eV and a reference ESI taken below the edge (E = 320eV), e.g., an image carrying information about background levels. Recently, de Bruijn *et al.* (1993) published a thorough review regarding quantitation of elements revealed by electron energy loss spectroscopy and electron x-ray probe analysis. This review indicates that quantitation of ESI images has lagged behind that of x-ray probe analysis. For example, elemental standards for the latter are available but are in the developmental stages for ESI. Thus, future research could repeat the ESI investigations performed herein but include co-embeddment of elemental standards once commercially available. Nevertheless, comparison of ESI images at 25, 59, 110, 222, and 710eVs suggests an association of iron (710eV) with aldehyde-fixed hyphae grown either upon agar or wood blocks. Although Fe was not visualized in cell walls of uninoculated wood blocks, this element was in certain wood cells of inoculated wood blocks. The control images (59eV and Quetol) were markedly different from those for iron but methods must be developed to subtract out background brightness.

The ESI carbon visualizations (25eV) in Figure 7a (agar-cultured hyphae), 8a (uninoculated wood) and 9a (inoculated wood) are not surprising as C is ubiquitous in non-structural and structural organic bio-molecules. An ESI image of the hyphal sheath is consistent with the reports that it is composed of a glucan matrix (Green *et al.* 1991; Nicole *et al.* 1994). As for the ESI phosphorus Vocalizations (110eV), figure 7e revealed a brightly delineated hypha with the sheath less so for agar-cultured hypha. This is consistent with the occurrence of intracellular phosphorus containing compounds such as nucleic acids and phospholipids. The occurrence of phosphorus in the sheath prompts a requirement for further characterization of the sheath which has been reported to contain extracellular membranous structures. Thus, the sheath may be comprised partly of phospholipids in addition to sterols. The ESI localizations of phosphorus in uninoculated and inoculated cell walls are not readily explainable. However, phosphorus containing compounds could adhere to xylem elements in cell walls during xylem ontogeny.

Figures 7e and 9e reveal brightness at 710eV (an indication of iron) in agar – and wood – cultured hyphae and their associated sheaths and to some extent in certain wood cell walls from hyphal infected

wood. Because the ESI samples were aldehyde-fixed, dehydrated and infiltrated, the detected iron would have to be bound (complexed) or "trapped" as elemental iron would be "washed out" during sample preparation. In this connection, Goodell and Jellison (1990) and Jellison *et al.* (1990, 1991) reported that high affinity iron-chelators (siderophores) from brown-rot fungi may be involved in wood decay. Earlier, Fekete *et al.* (1989) detected extracellular chelators in liquid and solid cultures of decay fungi. At least one of these iron-containing compounds appears to be an iron-associated glycopeptide of MW 1600–2000 (Enoki *et al.* 1992). This compound seems to possess H_2O_2-dependent 2-keto-4-thiomethylbutyric acid (KTBA)-oxidizing activity (Chandhoke *et al.* 1991) but whether this activity is related to cellulose depolymerization is unknown.

However, a recent report by Enoki *et al.* (1992) indicated that the *Gloeophyllum trabeum* glycopeptide was extracellular, absorbed one equivalent of Fe II and could both produce H_2O_2 via an O_2 radical anion and reduce H_2O_2 to OH. The latter may mediate cellulose depolymerization (Highley *et al.* 1994). As for the source of the Fe^{2+}, Schmidt *et al.* (1981) proposed that oxalic acid, which lowers the pH of wood (Green *et al.* 1992) reduces Fe^{3+} to Fe^{2+} and recently Lu *et al* (1994) suggested the same as well as other functions for low-molecular weight chelators isolated from *G. trabeum*.

The observed association of iron with the hyphal sheath is significant since H_2O_2, an important component of Fe^{2+}/H_2O_2 Fenton chemistry may be generated in close proximity to its site of action, the hyphal sheath (Highley *et al.* 1994).

In contrast to the very limited use of ESI in wood decay research (present investigation first report), SEM, TEM, and STEM coupled to EDAX have been extensively employed to assess the ultrastructural localization of lignin across wood cell walls (Saka *et al.* 1978; Saka and Thomas 1982; Saka and Goring 1985; Westermark *et al.* 1986; Saka 1992) and to ascertain the elemental composition of inorganic substances, e.g. MnO_2, calcium oxalate as well as P, S, Fe, K, Mn and Mg associated with wood during fungal decay (Eriksson *et al.* 1990).

Although more quantitative than ESI at the moment, a limitation of the EDAX (EDAX International) system is that at least 10^{-18}g of the element of interest is required for detection ruling out the possibility of distinguishing Fe^{2+} from Fe^{3+} involved in Fenton reactions. These considerations and others have been thoroughly described by Daniel (1994). Therefore, it may be of value to compare ESI and EDAX upon the same sample to determine whether similar localization of Fe can be obtained by the two methods.

In summary, comparative ESI imaging at 25, 59, 100, 222 and 710eVs revealed an association of Fe with *Postia placenta* hyphae and their sheaths as well as certain fungal-infected wood cell walls. Future research will be concerned with refining the technique to yield valence state. This research will be coupled to an investigation concerned with detecting, quantifying and localizing H_2O_2 by different assay procedures for agar and wood cultured hyphae harvested at regular time periods during a time course.

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