Immuno-scanning electron microscopic localization of extracellular wood-degrading enzymes within the fibrillar sheath of the brown-rot fungus *Postia placenta* [1]

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Extracelhdar wood-degrading enzymes of the brown-rot fungus *Postia placenta* were localized using colloidal gold labeled monoclinal antibodies to the ß-1 ,4-xylanase (32 to 36 kDa) fraction of *P. placenta. Postia placenta was* grown from agar onto glass cover slips, immunolabeled with or without prior fixation, and examined by scanning electron microscopy. Enzymes were localized on the hyphal surface and on the clumped fibrillar elements mycofibrils of the hyphal sheath following fixation with glutaraldehyde. If fixation was omitted, labeling was diffuse and not localized on individual or clumped mycofibrils. We conclude that extracellular decay enzymes are weakly bound (noncovalently) to, but not identical with, the linear mycofibrillar elements of the hyphal sheath. The linear structural elements of the hyphal sheath may play an important role in transport and presentation of wood-degrading enzymes during the decay process.

Key words: brown-rot fungi, enzymes, mycofibrils, hyphal sheath, immunolabeling, monoclinal antibodies, colloidal gold, scanning electron microscopy.

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Des enzymes extracellulaires qui dégradent le bois produits par *Postia placenta,* un agent de la pourriture brune, ont été localisées à l'aide d'anticorps monoclonaux marqués à l'or colloidal dirigés contre la fraction ß-1, 4-xylanase (32 à 36 kDa) de *P. placenta.* Le champignon cultivé sur gélose fixée à des lamelles de verre a été marqué avec l'anticorps avec ou sans fixation préalable et examiné en microscopic électronique à balayage. Après fixation au glutaraldéhyde, les enzymes ont été locahsées à la surface de hyphes et sur les éléments fibrillaires groupés (mycofibrilles) de la gaine des hyphes. En l'absence de fixation, le marquage s'est révélé diffus et non localisé sur les micofibrilles individuelles ou groupées. La conclusion dégagée est que les enzymes extracellulaires qui causent la pourriture ne seraient que faiblement liées (de façon non covalente), mais non identiques aux éléments micofibrillaires linéaires de la gaine des hyphes. Ces éléments structuraux linéaires pourraient jouer un rôle important dans le transport et la présentation des enzymes qui dégradent le bois au cours du processus de la pourriture.

Mots clés : champignons de la pourriture brune, enzymes, mycofibrilles, gaine hyphale, immunomarquage, anticorps monoclonaux, or colloïdal, microscopic électronique à balayage.

[Traduit par la rédaction]

Introduction

The mechanisms by which wood-decay fungi depolymerize or modify the hemicellulose, cellulose, and lignin components of the woody substrate have not yet been elucidated. One perplexing aspect of existent studies is the relative inability of cell-free extracts containing wood-decay enzymes to effect penetration or solubilization of intact wood without pretreatment with acid or alkali (Grethlein 1985; Bech-Anderson 1987; Highley 1987; Murmanis and Highley 1987; Enoki *et al.* 1988, 1990; Ucar 1990). A conspicuous ultrastructural feature of these fungi is the extensive hyphal sheath that covers the S_3 layer of the wood cell wall. This sheath appears to penetrate all wood cell wall layers and to form the functional interface between the fungal hyphae and the wood (Murmanis *et al.* 1984; Highley and Murmanis 1985; Eriksson 1988; Green *et al.* 1989b; Ruel *et al.* 1990; Reul and Joseleau 1991). Brown-rot decay of wood has been shown to occur large distances from hyphae and is characterized by early removal of hemicellulose. Proposed functions for the extracellular sheath include *(i)* modification of the extracellular ionic environment and pH, *(ii)* recognition of and adhesion to the substrate, *(iii)* concentration, storage, and transport of decay agents, *(iv)* protection against dehydration and environmental injury, *(v)* conditioning of the woody substrate to degradation, *(vi)* storage of nutrients, especially glucose, *(vii)* regulation of the decay process, and *(viii)* increased surface area for aerobic respiration (Highley 1987; Eriksson 1988; Ruel *et al.* 1990).

The ultrastructure of the extracellular fibrillar sheath of fungi has been described using electron microscopy (Murmanis *et al.* 1984; Benhamou and Ouellette 1987 *a,* 1987 *b).* Considerable effort has been made to localize sites of extracellular enzymes and fungal metabolizes by using polyclonal antibodies (MAbs) and postembedding immunogold labeling for transmission electron microscopy (TEM). The lignin peroxidases of the white-rot fungus *Phanerochaete*

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chrysosporium Burds. Have been the most extensively studied system, using these methods (Garcia *et al.* 1987; Srebotnik *et al.* 1988a, 1988b; Blanchette *et al.* 1989; Daniel *et al.* 1989, 1990; Ruel *et al.* 1990). Few studies have tried to localize the extracelluar metabolizes of the brown-rot fungi (Goodell *et al.* 1988; Blanchette *et al.* 1989; Kim *et al.* 1990; Srebotnik and Messner 1991). Even fewer studies have taken advantage of the technology of MAbs for immunogold localization studies in TEM (Benharnou *et al.* 1985; Blanchette *et al.* 1989).

The ultrastructure and extent of the hyphal sheath of filamentous fungi have also been extensively described by scanning electron microscopy (SEM) (Eriksson *et al.* 1980; Palmer *et al. 1983a, 1983b;* Benhamou *et al.* 1986; Day *et al.* 1986; Mouzouras *et al.* 1986; Tsuneda *et al.* 1987, 1989; Gardiner and Day 1988; Green *et al.* 1989b, 1991). In spite of advances in appropriate technology (DeHarven *et al.* 1984; Hodges *et al.* 1987; Helinski *et al.* 1990), immunogold labeling of the surface of fungal hyphae using SEM is rare (Day *et al.* 1986).

This paper represents our attempts to localize extracellular wood-degrading enzymes on the surface of hyphae and extracellular sheaths of the brown-rot fungus *Postia placenta* (Fr.) M. Lars. et Lomb. grown on glass cover slips, using MAbs produced to the ß-1, 4-xylanase fraction from decayed wood blocks as previously described (Clausen *et al. 1992a)* using immunogold SEM. The results suggest that these enzymes are localized on fibrillar elements of the sheath structure, on the hyphal surface, and within the soluble sheath matrix.

Materials and methods

Fungi and culture conditions

Postia placenta (Fr.) M. Lars. et Lomb. (isolate MAD-698) was grown onto sterile 12-mm glass cover slips (Assistant, West Germany) placed on the surface of 2% (w/v) malt agar plates. Cover slips were removed when about 50% covered (about 1-2 weeks) and were processed for SEM.

Monoclinal antibodies and colloidal gold labeling

Hybridoma clones were produced to the xylanase fraction of *P. placenta grown* on southern yellow pine blocks and fractionated by column chromatography on FG-55 as previously described (Green *et al. 1989a).* Two clones, F1 and C9, were selected for immunolabeling studies from Western blot analyses (CIausen *et al.* 1992a). Colloidal gold beads (~30 nm) were prepared by the reduction of 4% $HAuCl_4$, using the method of Park *et al.* (1987). Clones C9 and F1 were coupled directly to Au_{30} colloidal gold beads (pH 7.0) by the method of Simmons and Albrecht (1989).

Immunogold specimen preparation

Cover slips were washed in 0.1 M sodium acetate buffer (pH 4.5) or Triton X-100 for 15 min to remove excess glucan and fixed in 1% glutaraldehyde and 4% paraformaldehyde in 0.1 cacodylate buffer (PH 7.2) supplemented with either *(i)* 0.5% $CaC1_2$ + 0.05% digitonin or *(ii)* aqueous ruthenium red (500 parts per million) for 15 min and washed in phosphate-buffered saline (pH 7.0) as previously described (Green *et al.* 1991). Colloidal gold labeled MAbs were applied directly to the washed surface of cover slips and incubated in a moist chamber for 1-2 h at room temperature. Cover slips were washed by dipping in distilled water to remove unattached beads, frozen in liquid nitrogen slush (LN_2), dehydrated by lyophilization, and air dried. Specimens were sputter coated with gold and examined in a Hitachi S530 scanning electron microscope at an accelerating voltage of 10-25 kV. Localization of colloidal gold label was enhanced by decreasing the working distance to 5 mm and by adjustment of illumination controls to maximum contrast and minimum brightness.

Results

Localization of enzymes

Preliminary fixation experiments designed to solubilize excess glucan and to visualize fibrillar elements of the hyphal sheath on the hyphae on glass cover slips are shown in Figs. 1 and 2. Localization of enzymes on the surface of aggregated fibrillar elements of the sheath following fixation is shown in Figs. 3-6. The ultrafine mycofibrillar (~ 10 to 50 nm) elements and best revealed embedded in the sheath matrix following solubilization of the sheath by 0.1% Triton X-100 and acetate buffer without fixation prior to immunogold labeling with MAb C9 gold complex (Fig. 7).

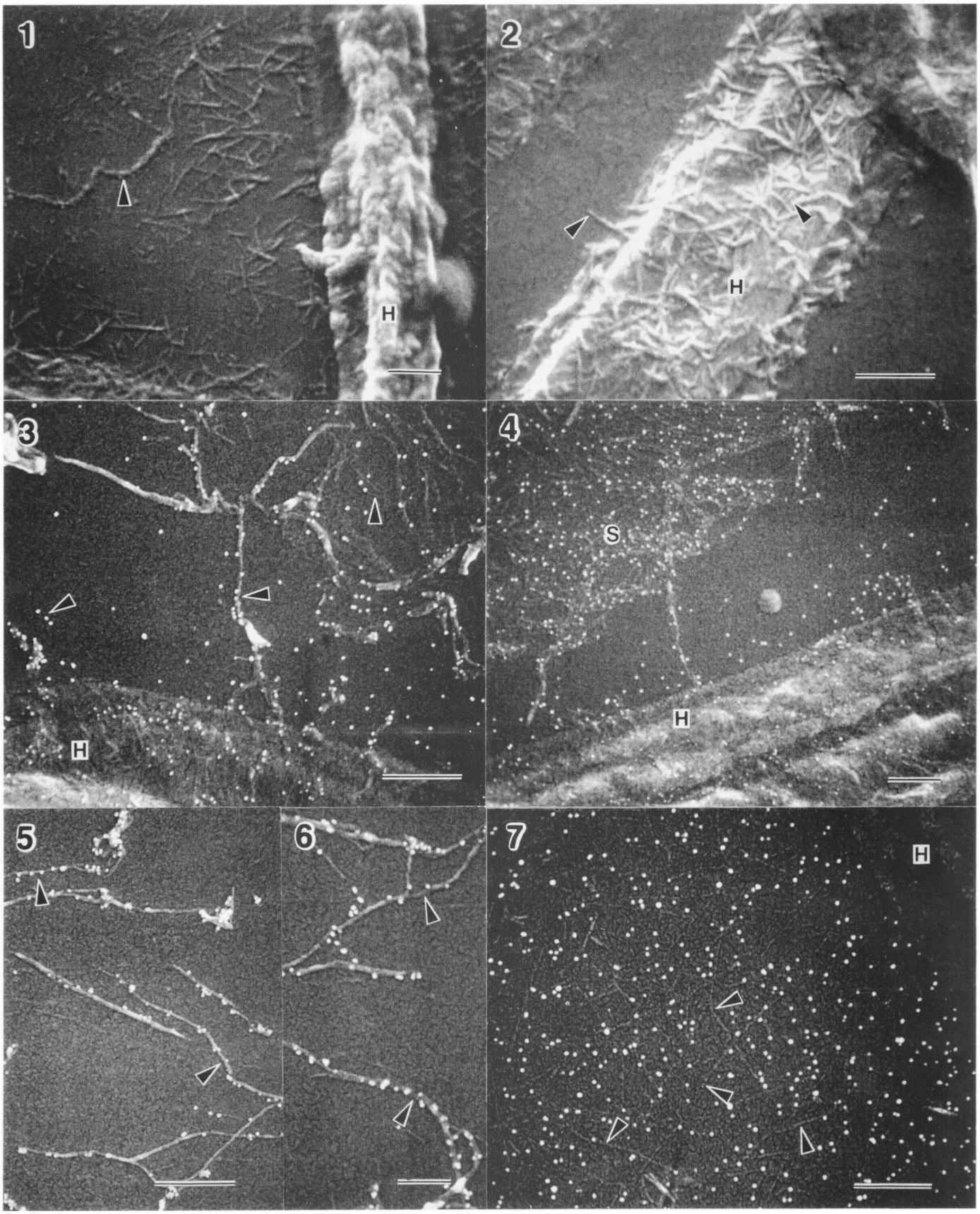
Specificity of immunolabel

Blocking experiments with homologous (MAb C9, Figs. 8 and 9) and heterologous (MAb F1, Figs. 10 and 11) labeled MAbs prior to specific binding of MAb C9 - gold complex diminishes the role of nonspecific binding of MAbs and confirms the independent nature of the antigenic epitopes of MAbs C9 and F1, respectively. Fewer binding sites, revealed by lower bead density, are exposed for binding by MAb F1 - gold complex than by MAb C9 - gold complex (Figs. 12 and 13). However, beads are localized on clumped fibrillar sheath elements (Fig. 12).

Discussion

This paper presents results that demonstrate the immunogold localization by SEM of extracellular wood-degrading enzymes on hyphae, mycofibrils, and within the' 'fluid" or soluble sheath matrix of *P. placenta* grown on glass cover slips. Enzymes were localized using colloidal gold labeled MAbs specific for extracellular wood-degrading enzymes of *P. placenta* isolated from wood. The xylanase fraction was selected for study because hemicellulose degradation is reported to be an early event preceding cellulose depolymerization during brown-rot decay (Wiley *et al.* 1968; Ruel *et al.* 1981; Highley 1987; Donaldson 1988).

Interpretation of these results is partially dependent upon the specificity of the MAbs produced. MAbs F1 and C9 were produced to the extracellular xylanase fraction of *P. placenta* grown on wood and characterized by ELISA, Western blot, and MAb affinity chromatography (Clausen *et al.* 1992a). Both F1 and C9 exhibited specific binding to two bands on Western blot at 32 and 36 kDa. Enzyme assays of eluted proteins from affinity columns of F1 and C9 contained activity for xylanase, laminarinase, carboxymethylcellulase, and mannanase. We conclude that laminarinase (~ 55 kDa) activity represents nonspecific column binding because *(i)* activity was approximately 10% total. *(ii)* the 55-kDa band was not observed in Western blots, and *(iii)* MAbs did not bind to laminarinase fractions by direct ELISA (Clausen *et al. 1992b).* Our previous estimation (Green *et al. 1989a)* of molecular size for xylanase (42 kDa) appears to be reduced by more complete solubilization in Triton X-100 and reduction in carbohydrate moieties as reported by others (Gallagher and Evans 1990). The size, degree of glycosylation, configuration of decay agents, and degree of porosity have important implications for the mechanism of transport and penetration of enzymes into the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woody substrate during the decay process *in situ* (Cooper 1983; Keen *et al.* 1984; Daniel *et al.* 1989; Srebotnik and Messner 1991; Flournoy *et al.* 1991).

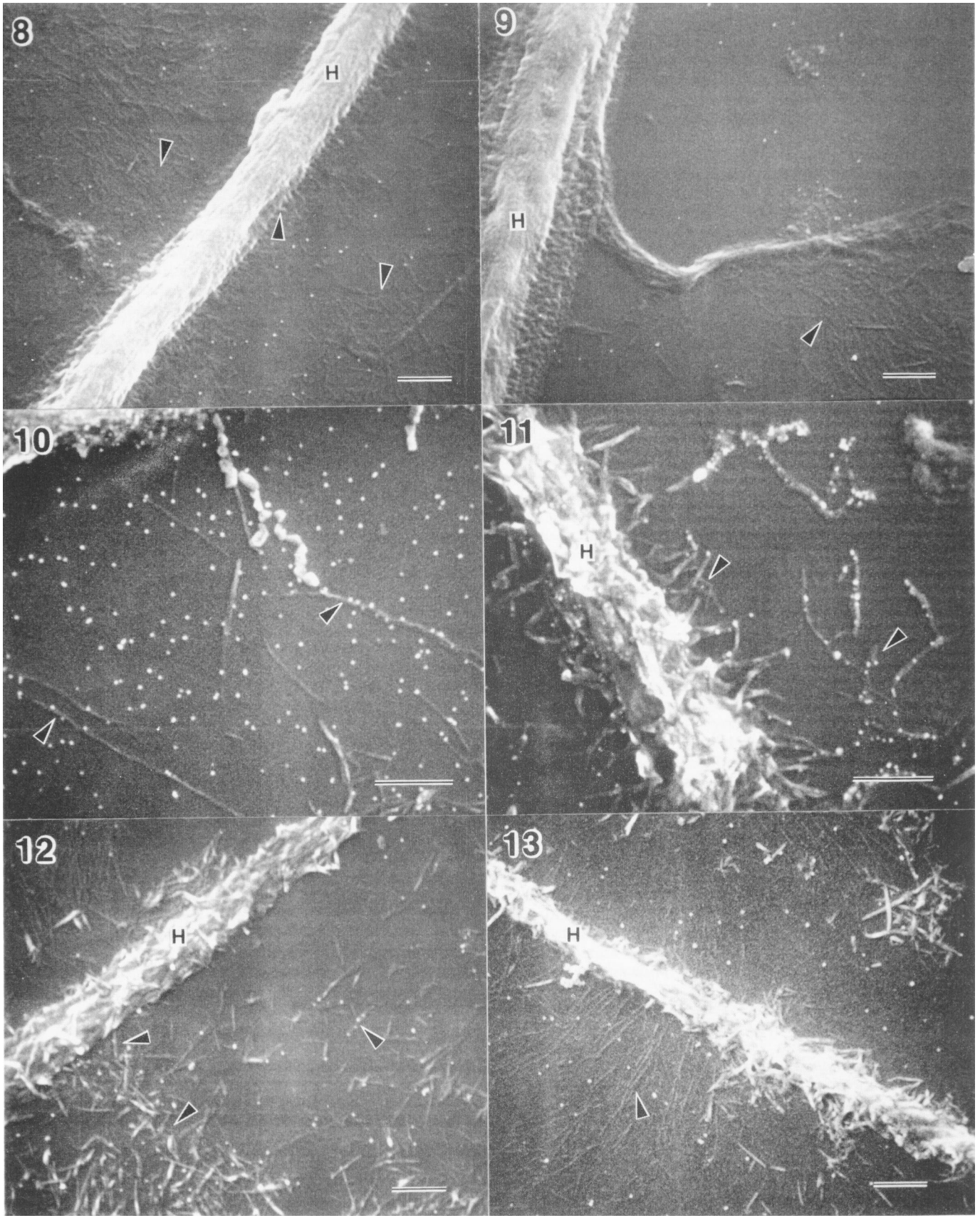
The size estimates of mycofibrils and fungal fimbriae are close to those reported for fungal cellulases (6 to 20 nm in diameter) (Grethlein 1985). In a separate paper, we have confirmed the existence of small (10 to 30 nm) linear extensions of the hyphal cell wall of *P. placenta* grown on glass cover slips and wood (Larsen and Green 1992). Similar structures were described for wood-decay fungi in 1962 by Liese and Schmidt (reviewed by Liese, 1970) and were called mycofibrils. Day *et al.* (1986) and Gardiner and Day (1985, 1988) described similar structures in *Ustilago* sp. and called them fungal fimbriae, which are analogous to bacterial fimbriae (pili). The latter have been extensively characterized as proteinaceous filaments of polymerized 74-kDa protein subunits, 6 to 10 nm in diameter and up to 20 nm long. The biochemical nature of the mycofibrils of *P. placenta is* not known. Similar fibrillar elements, usually chitin and ß-1,3 glucan, occur in hyphal cell walls of basidiomycetes and, cellulose (ß-1,4 glucan), in some ascomycetes (Benhamou 1989). Wang and Bartnicki-Garcia (1976) reported the synthesis of ß-1,3 glucan mycofibrils with cell-free extracts of *Phytopthora cinnamomi* Rands. Day *et al.* (1986) and Svircev *et al. (1986)* have immunogold labeled the fungal fimbriae of *Ustilago violacea* and other fungi, using polyclonal antibodies to the 74-kDa fimbrial protein and indirect immunogold labeling. Our results suggest that the linear structures visualized by SEM are not simply strings of decay enzymes. Ruel and Joseleau (1991) have reported that the fibrillar elements of the white-rot decay fungus *Phanerochaete chrysosporium, as* observed by TEM, are dehydrated, cross-linked carbohydrates (glucan). Glutaraldehyde would not be expected to fix carbohydrates (Simkins 1972). However, Day *et al.* (1986) concluded that fungal fimbriae are electron lucent in thin section, a result of either their small size or poor staining characteristics (hydrophobicity). Further characterization of mycofibrils as either protein or carbohydrate will require treatment with specific enzymes or production of specific MAbs to mycofibrils if, indeed, mycofibrils are protein.

Localization of wood-degrading enzymes on the mycofibrillar elements of the sheath matrix in this study was dependent upon glutaraldehyde fixation. Immunogold labeling in the absence of fixation resulted in a diffuse pattern of labeling (Fig. 7), which suggests that these enzymes are localized within the fluid or glucan matrix of the sheath. The linearity of MAb - colloidal gold labeling after fixation supports an association of extracellular decay enzymes with the mycofibrillar structures of the sheath *in situ.* However, results without fixation suggest a weak association easily disrupted by buffers required for immunogold labeling. As a result of possible precipitation of extracellular proteins onto mycofibrils during fixation with glutaraldehyde, precise localization of wood-degrading enzymes within the hyphal sheath was only partially confirmed. Similarly, Dickerson and Baker (1979) reported noncovalent binding of enzymes to the extracellular carbohydrate matrix (glucan) of *Sphacelia* SP. These results contribute to a more cautious interpretation of the effects of fixation and dehydration on hyphal sheath structures as related to protocols used commonly for TEM and SEM. Our preparative methodology reflects the partial volubility of the sheath in distilled water and buffers and is similar to the methods we use to remove decay enzymes from wood (Green *et al. 1989a).* Similar methods of removing or reducing the sheath matrix, including organic solvents, have been reported (Gardiner and Day 1988; Green *et al.* 1991).

Recent publications exploring extracellular localization of wood-decay enzymes and fungal metabolizes within the hyphal sheath using immunogold labeling and TEM are similar to the results reported here. Daniel *et al.* (1989) localized lignin peroxidase in association with the cell membrane, fungal cell wall, and extracellular slime matrix of *Phanerochaete chrysosporium,* using polyclonal antibodies. Reul *et al.* (1990) confirmed that the extracellular sheath of *Phanerochaete chrysosporium* bridges the space between the mycelium and the wood cell wall as a discrete structure that functions as a receptor for retention of extracellular enzymes. Gallagher and Evans (1990) reported immobilization of ß-glucosidase within the glucan matrix surrounding the hyphae of the white-rot fungus *Trametes versicolor* (L. : Fr.) Pil. Kim *et al.* (1990) demonstrated immunogold labeling of extracellular metabolizes of *P. placenta* in the slime matrix and S_3 layer of the wood cell wall. Taken in concert with this paper, those reports underscore the potential complexity of the hyphal sheath matrix in delivery and regulation of decay agents. Confirmation of linear binding of decay enzymes to mycofibrils should serve to dispel the notion of "simple diffusion" as the mechanism for delivery of decay enzymes to the woody substrate. In addition, detection of extracellular laminarinase in the wood extracts of *P. placenta* would support the hypothesis that glucanases play an important function in brown-rot decay similar to that proposed for the white-rot decay by *Phanerochaete chrysosporium* (Eriksson 1988; Ruel and Joseleau 1991).

In summary, extracellular wood-degrading enzymes of *P. placenta* have been localized on linear elements in the glutaraldehyde-fixed hyphal sheath that resemble previously described fungal sheath structures called mycofibrils. We conjecture that decay enzymes or agents, especially low molecular weight hemicellulases, may associate and migrate in concert with the formation of mycofibrillar sheath elements as an integral step in their presentation to woody substrates during incipient decay. Confirmation of this hypothesis will require isolation and characterization of mycofibrils and immunocytochemical localization in the hyphal sheath on wood.

FIGS. 1–7. Scanning electron micrographs of *P. placenta* on glass cover slips. Figs. 1 and 2. Samples fixed with glutaraldehyde and 1% ruthenium red and air dried. Hyphal sheath filaments can be seen in the sheath matrix (arrowheads) and on the hyphae (H). Figs. 3 to 6. Samples fixed with glutaraldehyde–paraformaldehyde, digitonin, and calcium chloride following acetate buffer wash. Enzymes localized with MAb C9 – gold complex appear on aggregated filaments and sheath matrix (S) (Fig. 4). Individual and cabled mycofibrils with bound MAb – colloidal gold (Figs. 5 and 6). Fig. 7. Sample washed in Triton X-100 without fixation; labeled with MAb C9 – gold complex. Note unclumped fibrillar elements (mycofibrils) and diffuse labeling on background sheath matrix. Figs. 1–5 and 7. Scale bars represent 1 μm. Fig. 6. Scale bar represents 0.5 μm.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FIGS. 8–13. Scanning electron micrographs of *P. placenta* on glass cover slips. Figs. 8 and 9. Samples fixed with glutaraldehyde – paraformaldehyde, digitonin, and calcium chloride; blocked with MAb C9, immunolabeled with MAb C9 – gold complex and dehydrated liquid nitrogen slush. Note very low beading density. Figs. 10 and 11. Sample fixed as above, blocked with MAb F1, and immunolabeled with MAb C9 – gold as in Figs. 8 and 9. Sheath matrix (arrowheads) (Fig. 10), hyphae (H), and clumped sheath matrix (Fig. 11, arrowheads) are gold bead labeled. Figs. 12 and 13. Sample fixed as above, immunolabeled with MAb F1 – gold complex. Note linearity of gold label (Fig. 12) and low bead density (Fig. 13). Scale bars represent 1 μm.

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