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Involvement of Hydrogen Peroxide in Wood Decay by Brown-Rot and White-Rot Fungi

by

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Summary

To gain further understanding of the role of H_2O_2 in wood degradation by brown- and white-rot fungi, we studied the following: (a) extracellular H_2O_2 production, (b) effect of various hydroxyl radical (·OH) and singlet O_2 ($^1O_2$) quenching agents on wood and cellulose degradation, (c) intracellular H_2O_2 production and catalase activity, and (d) cytochemical localization of H_2O_2 with diaminobenzidine (DAB) during wood degradation.

Extracellular H_2O_2 detection varied with the growth media and chromogen. The chromogen 2,2'-azino-di-(3-ethyl benzthiazoline-6-sulphonic acid) (ABTS) was more sensitive than o-dianisidine. Extracellular H_2O_2 was not detected in half of the brown-rot fungi. One white-rot fungus did not produce detectable amounts of H_2O_2. ·OH and $^1O_2$ quenching agents generally did not inhibit decay of wood or decomposition of cellulose by either brown- or white-rot fungi.

DAB did not detect the presence of H_2O_2 within or outside cells of the . brown-rot fungus, Poria placenta. Nor was H_2O_2-generating activity detected in sonicated extracts of this fungus. With the white-rot fungus, Coriolus versicolor, H_2O_2 occurred predominantly in the periplasmic space, but also in the cytoplasm and hyphal sheath. Sonicates of C. versicolor contained H_2O_2-generating activity. These observations provide, further support for H_2O_2 involvement in degradation of wood by white-rot fungi, but raise doubts concerning its participation in wood degradation by brown-rot fungi.

Keywords: White-rot, brown-rot, wood decay, hydrogen peroxide, lignin degradation, cellulose degradation

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Introduction

Evidence from a number of studies suggests that oxidants such as hydrogen peroxide (H_2O_2), superoxide ion ($O_2^{\cdot-}$), hydroxyl radical (·OH) or singlet oxygen ($^1O_2$) are involved in wood degradation by both brown-rot and white-rot fungi (Bes et al., 1983; Forney et al., 1982a,b; Crawford and Crawford, 1984). Of these oxidants, ·OH is the most powerful and thus most likely to be a degrading agent. It is formed from H_2O_2 by reaction with $O_2^{\cdot-}$ or with Fe^{+2}. These oxidants are small enough to penetrate the microstructure of the wood cel1 wall and predispose cell wall constituents to wall-degrading enzymes secreted by the fungi. Ultrastructural studies suggest that both types of fungi produce small nonenzyme degrading agents capable of penetrating and diffusing throughout the cell wall of wood (Liese, 1970; Highley et al., 1983, 1984).

Numerous studies have proposed that the ·OH radical derived from H_2O_2 is utilized by white-rot fungi to degrade lignin (Crawford and Crawford, 1984). Evidence for ·OH involvement in lignin degradation comes primarily from scavenger studies which are inconclusive because of questions of specificity (Crawford and Crawford, 1984). However, Faison and Kirk (1983) found that production of ·OH did not correlate with ligninolytic activity of Phanerochaete chrysosporium. They propose that H_2O_2 plays an indirect role in lignin degradation. Recently Tien and Kirk (1984) isolated a H_2O_2-requiring oxygenase from P. chrysosporium that breaks down a portion of the lignin molecule. Forney et al. (1982a) demonstrated by cytochemical staining of cells with diaminobenzidine (DAB) that H_2O_2 production is localized in the periplasmic space of cells from ligninolytic cultures of P. chrysosporium but not in cells from nonligninolytic cultures. Thus the evidence for H_2O_2 involvement in lignin degradation by the white-rot fungus P. chrysosporium, is conclusive but there is little evidence to support involvement of other 0_2 species (Crawford and Crawford, 1984).

The participation of extracellularly produced radicals has been postulated by Ericksson (1981) as possibly involved in the primary attack on crystalline cellulose by white-rot fungi. Ultrastructural observation of cellulose decomposition by several white-rot fungi showed that the fungi produced a cellulose degrading system capable of diffusing throughout cellulose fibers suggesting that a small nonenzyme night be involved (Highley et al., 1984).

The nature of cellulose degradation by brown-rot fungi also suggests that a small nonenzyme may be involved, at least initially, in the decomposition of cellulose (Koenigs, 1972, 1974; Highley, 1973). Circumstantial evidence suggests that the small nonenzyme may be ·OH generated from H_2O_2. However, several ·OH scavengers did not inhibit cellulose or wood degradation by the brown-rot fungus, Poria placenta, and extracellular H_2O_2 was not detected in filtrates of several brown-rot fungi (Highley, 1982). Alcohol oxidase is reported in several brown-rot fungi and generates H_2O_2 but it is an intra-cellular enzyme. Bringer et al. (1980), using DAB, demonstrated H_2O_2 production by alcohol oxidase in microbodies of the brown-rot fungus, Poria contiqua (= Phellinus contiquus). However, to degrade cellulose in wood, H_2O_2 or other activated oxygen species must be released from hyphal cells into the wood cell wall.

The purpose of this study is to (a) examine further the production of extra-cellular H_2O_2 by several brown- and white-rot fungi, (b) determine the effect of various radical quenching agents on wood and cellulose degradation, (c) measure H_2O_2-generating activity and catalase activity in hyphae of the brown-rot fungus, Poria placenta, and the white-rot fungus, Coriolus versicolor, and (d) use the DAB reaction to cytochemically identify and localize H_2O_2 in P. placenta and C. versicolor during degradation of wood.

Method

H_2O_2 production

The white-rot and brown-rot fungi tested for H_2O_2 formation are listed in tables 1 and 2. Fifteen g of agar were added to 900 ml of a basal salts medium containing 10 g cellobiose. The basal salts medium contained 2 g NH_4NO_3, 2 g KH_2PO_4, 0.5 g $MgSO_4$ $7H_2O$, 0.1 g $CaCl_2$, 1 mg thiamine and minor elements (Highley, 1973). After autoclaving at 121°C for 15 min, the medium was cooled to 50°C. Horseradish peroxidase (20 mg, Sigma, type II) and 300 mg of 2,2'-azino-di-(3-ethyl benzthiazoline-6-sulphonic acid) (ABTS) or o-dianisidine were dissolved in 100 ml of distilled H_2O, filter-sterilized and added aseptically to the agar medium. Three-ml portions were poured into presterilized test tubes. To determine the effect of wood cell wall polymers on H_2O_2 formation, xylan (Sigma), cellulose (Hercules, cotton Type A-600) or hemlock sawdust were layered onto the agar surface. Nitrogen source and concentration also were varied: (a) 0.02 percent NH_4NO_3, (b) 0.02 percent and 0.2 percent casein amino acids (Difco), (c) 0.2 percent yeast nitrogen base (Difco), and (d) 0.2 percent malt extract.

Measure of alcohol oxidase and catalase

P. placenta and C. versicolor were grown according to Bringer et al. (1980) in 250-ml Erlenmeyer flasks containing 25 ml of basal medium (Highley, 1983), 0.4 percent (W/V) yeast extract, and 1 percent (W/V) malt extract. Flasks were inoculated with a washed mycelial suspension and incubated on a shaker for 3 weeks.

Mycelium was harvested by filtration over a Buchner funnel and washed with 50 mM phosphate buffer, pH 7.5. The washed mycelium was suspended in 10 ml of the same buffer and sonicated three times for 2 min on a Braun sonicator. The crude extract was centrifuged (10,000 rpm, 30 min) and the supernatant used as the cell-free extract. A portion of the cell-free extract was dialyzed prior to enzyme analysis. Alcohol oxidase activity was measured by determination of H_2O_2 production with a methanol-peroxidase-dianisidine reagent (Janssen and Ruelius, 1968). Catalase was assayed by the decrease in absorbance at 240 nm caused by decomposition of H_2O_2 (Bringer et al., 1980).

Effect of radical quenching agents on degradation of cellulose and wood

To measure the effect of materials interfering with the H_2O_2 /radical system, weighted cellulose (Hercules, cotton Type A-600) or blocks of southern pine and sweetgum (1/4 x 3/4 x 3/4 in., small dimension in grain direction) were impregnated under vacuum with sterile solutions of the test compounds (Tables 4 and 5). The standard ASTM D 2017 soil-block method (ASTM, 1971) was used to test the ability of the quenching agents to prevent degradation by brown- and white-rot fungi (Tables 4 and 5). Following incubation, test samples were removed from bottles, conditioned at 27°C and 70 percent humidity, and weighed. Weight loss was used as an estimate of decay susceptibility.

Cytochemical localization of H_2O_2

Hemlock sawdust (Tsuga heterophylla (Raf.) Sarg.) was degraded by the white-rot fungus, Coriolus versicolor (L:Fr) [Mad-697], 2nd the brown-rot fungus, Poria placenta (Fr.) Cke. [Mad-698], in chambers patterned after the standard ASTM soil-block procedure (ASTM, 1971). Samples were removed for cytochemical staining and electron microscopy after 6 weeks incubation.

Localization of hydrogen peroxide production by C. versicolor and P. placenta was achieved by cytochemical staining with 3,3'DAB tetrahydrochloride (Aldrich). Sawdust samples were fixed in glutaraldehyde-formaldehyde mixture in 0.1 M phosphate buffer (pH 7.0) and treated with DAB according to Forney et al. (1982a). Controls were treated with DAB plus 3-amino, 1,2,4-triazole (aminotriazole) (Sigma Chemical Company).

Samples from both treatments were postfixed in 1.33 percent OsO_4 in 0.1 M s-collidine buffer, washed in buffer and stained with 1.5 percent uranyl acetate in 30 percent ethanol. They were dehydrated in acetone series and propylene oxide and embedded in araldite, epon, and dodecenyl succinic acid anhydride mixture following the procedure of Mollenhauer (1964). Embedded samples were polymerized at 70°C and sectioned with a diamond knife. Sections were viewed in transmission electron microscope RCA-EMU 3D, using 50 kv.

Results

Extracellular H_2O_2 formation

The ABTS-peroxidase medium detected H_2O_2 production by more fungi than did the o-dianisidine-peroxidase medium (Table 1). Müller found this medium more sensitive than benzidine in detection of H_2O_2. However, even with ABTS only 3 out of 14 brown-rot fungi produced detectable H_2O_2. H_2O_2 was detected in four out of six white-rot fungi. Addition of xylan, cellulose or wood to the medium did not change the results. With variation in nitrogen source and concentration, H_2O_2 was detected in several additional fungi (Table 2). H_2O_2 was detected in all but one of the white-rot fungi but was still not detected in half of the brown-rot fungi.

Alcohol oxidase and catalase in sonicated mycelial extracts

Extracts from sonicated hyphae of C. versicolor contained H_2O_2-generating oxidase activity while hyphae of P. placenta did not (Table 3). H_2O_2 was produced by C. versicolor with both dialyzed and undialyzed extracts whether or not methanol was added. Thus, we conclude that C. versicolor extracts contain H_2O_2-generating activity, but we do not know the source of the H_2O_2. Catalase activity was detected in sonicates of both fungi (Table 3).

Quenching agents

Decay of wood by the white- and brown-rot fungi was not significantly reduced in the presence of *OH or $^1O_2$ quenching agents (Table 4). In some instances decomposition of cellulose was decreased with quenching agents, but no quenching agent inhibited all of the brown-rot or all of the white-rot fungi (Table 5).

Cytochemical localization of H_2O_2

In electron micrographs, we found oxidized DAB deposits in hyphae of the white-rot fungus C. versicolor, but not in hyphae of the brown-rot fungus,

Table 1 Comparison of chromogens in detection of H_2O_2 by white-rot and brown-rot fungi

Fungus	Coloration of medium[1]	
	ABTS	o-dianisidine
BROWN-ROT		
Phellinus contiquus (Pers.:Fr.)	+++	+++
Leucogyrophana arizonica Ginns	-	-
L. olivascens (Berk. and Curt) Ginns and Weres.	-	-
Coniophora puteana (Schum.:Fr.) Karst.	-	-
Poria placenta (Fr.) Cke	-	-
Gloeophyllum trabeum (Pers. ex. Fr.) Murr.	-	-
Lentinus lepideus Fr.	-	-
P. carbonica Overh.	++	-
P. xantha Cke.	-	-
Antrodia serialis Fr.	-	-
Fibroporia vaillantii (DC:Fr.) Parm	+	-
Serpula incrassata (Berk. and Curt) Donk	-	-
Latiporus sulphureus (Bull.:Fr.) Bond and Sing.	-	-
Fomitopsis meliae (Underw.) Gilbn.	-	-
WHITE-ROT		
Phanerochaete chrysosporium Burds.	-	-
Ganoderma applanatum (Fr.)	+	+
Xylobolus frustulatus (Pers.:Fr.)	-	-
Coriolus versicolor (L:Fr.)	++	++
Ph. ferrugineofuscus (Karst.) Bourd	+++	+++
Spongipellis unicolor (Schw.) Murr.	+++	+++

[1]No coloration, -
Slight coloration, +
Moderate coloration, ++
Heavy coloration, +++

Table 2 Effect of nitrogen source and concentration on H_2O_2 formation by white-rot and brown-rot fungi

Fungus	Coloration of medium[1]					
	Nitrogen source[2]					
	1	2	3	4	5	6
BROWN-ROT						
Ph. contiquus	+++	+++	+++	+++	+++	+++
L. arizonica	-	-	-	-	-	-
L. olivascens	-	-	-	-	-	+++
C. puteana	-	-	-	-	-	+
P. placenta	-	-	-	-	-	-
G. trabeum	-	-	-	-	-	-
L. lepideus	-	-	-	-	-	-
P. carbonica	++	+	+	++	++	++
P. xantha	-	+	-	-	++	-
A. serialis	-	-	-	-	-	-
F. vaillantii	+	++	++	++	++	-
S. incrassata	-	-	-	-	-	-
L. sulfureus	-	++	-	++	++	+
P. meliae	-	-	-	-	-	-
WHITE-ROT						
P. chrysosporium	-	-	+	-	-	+
G. applanatum	+	+	+	+	+	++
X. frustulatus	-	-	-	-	-	-
Ph. ferrigineofuscus	+++	+++	+++	+++	+++	++
S. unicolor	+++	+++	+++	+++	+++	++
C. versicolor	++	+	+	+	+	++

[1]No coloration, -
Slight coloration, +
Moderate coloration, ++
Heavy coloration, +++

[2]1. 0.2% NH_4NO_3
2. 0.02% NH_4NO_3
3. 0.2% casein amino acids
4. 0.02% casein amino acids
5. 0.02% yeast nitrogen base
6. 0.2% malt extract

Table 3 H_2O_2–producing oxidase and catalase activities of cell–free extracts from Poria placenta and Coriolus versicolor

Fungus	H_2O_2–producing oxidase[1]				Catalase[2]
	Dialyzed		Undialyzed		
	Methanol	Nonmethanol	Methanol	Nomethanol	
Coriolus versicolor	0.023	0.029	0.029	0.031	0.320
Poria placenta	0	0	0	0	0.275

[1]Increase in absorbance/min/ml filtrate at 430 nm.
[2]Decrease in absorbance/min/0.1 ml filtrate at 240 nm.

Table 4 Effect of ·OH and $^1O_2$ quenching agents on decay of wood by brown–rot and white–rot fungi

Treatment	Weight loss by[1]			
	Brown–rot		White–rot	
	Poria placenta	Gloeophyllum trabeum	Phanerochaete chrysosporium	Ganoderma applanatum
	%			
Dimethyl sulfoxide (1%)	57.0	59.2	76.0	84.6
u–Tocopherol (1%)	56.9	63.0	74.6	89.7
Diethylenetriamine–penta–acetic acid (0.1%)	50.0	42.8	78.0	83.6
Bathophenanthroline (0.1%)	44.6	66.7	84.1	84.0
Histidine (1%)	42.0	53.0	66.0	87.0
p-Nitrosodimethylaniline (0.1%)	66.5	54.0	72.8	80.8
Desferrioxamine (0.1%)	50.0	45.6	79.4	91.5
Control	55.8	53.5	67.6	91.3

[1]Blocks removed at 8 wk. Pine decayed by brown–rotters and sweetgum by white–rotters

Table 5 Effect of ·OH and $^1O_2$ quenching agents on decomposition of cellulose by brown-rot and white-rot fungi

Treatment	Weight loss by					
	Brown-rot[1]			White-rot[2]		
	Poria placenta	Gloeophyllum trabeum	L. olivascens	Phanerochaete chrysosporium	Ganoderma applanatum	Coriolus versicolor
	%					
Dimethyl sulfoxide (1%)	80	54	3	100	100	100
a-Tocophcnol (1%)	24	84	10	70	100	100
Diethylenetriamine-penta-acetic acid (0.1%)	40	0	14	100	100	100
Bathophenanthroline (0.1%)	50	36	0	84	100	80
Histidine (1%)	0	40	10	100	100	100
p-Nitrosodimethylaniline (0.1%)	80	64	16	100	100	100
Desferrioxamine (0.1%)	40	46	10	100	100	82
2,5-dimethylfuran	20	53	15	--	--	--
t-butylphenelate	67	23	13	--	--	--
Sodium benzoate	65	80	20	--	--	--
Control	43	60	21	100	100	100

[1]Exposed 16 wk.
[2]Exposed 12 wk.

P. placenta. The oxidized dark DAB deposits, in the form of vesicles, within the hyphae of C. versicolor were aligned near the wall in a periplasmic space (Fig. 1), as observed by Forney et al. (1982a) with P. chrysosporium cells grown on low-nitrogen medium. In a cell at right in—Fig. 1, the section went through the tip of-the cell and through the vesicle with the dark contents (as visualized from a longitudinal section in Fig. 2, at top). As judged from Fig. 2, the dark DAB deposits, which appear as vesicles in cross sections (Fig. 1), may actually be larger areas in periplasmic space filled with these deposits.

The cells, which have the vesicles with dark DAB deposits, have very thick walls (Fig. 1), and we think that they represent spores. They may correspond to the cells of P. chrysosporium cultured on low-nitrogen medium (Forneyet --al., 1982a). We did Lot observe these vesicles in the cells with thinner walls (Fig. 3). The vesicles also were not present in the thick-walled cells treated with aminotriazole, a catalase inhibitor (Fig. 4). L. J. Forney et al. (1982a) found similar inhibition of the DAB staining reaction by aminotriazole in cells of the white-rot fungus, P. chrysosporium. Thus, as in P. chrysosporium, the electron-dense regions observed in our micrographs must be due to the peroxidative oxidation of DAB by catalase.

In addition to the vesicles with dark DAB deposits, C. versicolor has abundant particles in the mature cells (Fig. 5) and in the periplasmic space (Figs. 6, 7 arrows). These particles were found also in the hyphal sheath (Figs. 8, 9). They appear morphologically different from the vesicles. But they accumulate in the periplasmic space and respond to the DAB staining technique indicating the presence of H_2O_2. The particles were not observed in aminotriazole-treated samples. At this stage, we do not know whether functionally they correspond to the vesicles with dark DAB deposits, although their location in periplasmic space and their absence in aminotriazole-treated samples suggests the two are the same.

We did not find oxidized DAB deposits in the brown-rot fungus, P. placenta (Fig. 10). The cells often contained many vesicles in the periplasmic space, but they did not have dark contents (Fig. 11 arrow). As mentioned, H_2O_2-generating oxidase activity also was not present in sonicated extracts. S. Bringer et al. (1980) report peroxidative oxidation of DAB by catalase in microbodies of the brown-rot fungus, Poria contigua. Source of the H_2O_2 was attributed to alcohol oxidase which was present in cell-free extracts of this fungus.

Discussion

Evidence from this study suggests that H_2O_2 or other reduced oxygen species are not involved in wood degradation by brown-rot fungi. We failed to detect extracellular H_2O_2 production by several brown-rot fungi grown on various media. The cytochemical reagent, DAB, did not detect the presence of H_2O_2

within or outside cells of the brown-rot fungus Poria placenta during degradation of hemlock. Nor was H_2O_2-generating activity detected in sonicated extracts of this fungus. Finally, ·OH and $^1O_2$ quenching agents in most instances were not inhibitory to wood decay or cellulose decomposition.

Although the evidence in this study questions the involvement of extracellular H_2O_2 in cellulose degradation by brown-rot fungi, the nature of cellulose breakdown by brown-rot fungi seems to rule out cellulose degradation by the conventional synergistic action of exo- and endo-cellulases (Cowling and Brown, 1969). Exo-cellulase activity is not present in filtrates of many brown-rot fungi (Highley, 1973). Furthermore, brown-rot fungi drastically reduce the degree of polymerization of cellulose at very low weight loss, and the decomposition products are readily soluble in alkali (Cowling, 1961; Highley, 1977). Thus, these fungi must produce a depolymerizing agent which penetrates the cellulose structure and breaks down the glucosidic bonds. To accomplish this, a depolymerizing agent smaller than any known cellulase would be required. No known isolated cellulase has been able to duplicate the effect of brown-rot fungi on cellulose. Thus, brown-rot fungi apparently employ an alternative mechanism of cellulose breakdown employing a small depolymerizing agent. Although we failed to provide supportive evidence of hydroxyl radical involvement, other types of radicals may be produced that oxidatively degrade cellulose. In a previous study (Highley, 1977) cellulose degraded by the brown-rot fungus, P. placenta, had oxidized characteristics. An extracellular matrix or hyphal sheath which in a previous study was observed always in contact with cellulose fibers may also play a role in degradation. Hyphal sheaths have been proposed to facilitate cellulolytic action by aiding transport of the degrading agents to the substrate and the uptake of degradation products of cell wall dissolution (Jutte and Sachs, 1970; Leightley and Eaton, 1980). Sheaths also could function to protect degrading agents from destruction or perhaps serve as a medium for chemical reactions that produce degrading agents.

Our observations provide further support that H_2O_2 is involved in degradation of wood by white-rot fungi. Extracellular H_2O_2 was not detected in the white-rot fungus, Xylobulus frustulatus, but H_2O_2 production in some white-rot fungi may be very low under atmospheric oxygen. Faison and Kirk (1983), for example, found barely detectable extracellular H_2O_2 production by the white-rot fungus, Phanerochaete chrysosporium, when grown at atmospheric oxygen, but with increased oxygen, H_2O_2 production was substantially increased.

During degradation of wood by the white-rot fungus, Coriolus versicolor, H_2O_2 occurred predominantly in the periplasmic space, but also in the cytoplasm and hyphal sheath. Forney et al. (1982a) found H_2O_2 in the periplasmic space in cells of P. chrysosporium cultured on low-nitrogen medium but not in this area in cells grown on a high-nitrogen medium.. The only nitrogen available in our tests was that provided by the wood and soil, which is supposedly low. The exogenous souce of nutrients, particularly nitrogen concentration, therefore,

may be critical in dictating the location of H_2O_2. Kirk et al. (1978) and Forney et al. (1982) found that low-nitrogen grown cells of P. chrysosporium had high ligninolytic activity while those on high-nitrogen medium were low in ligninolytic activity. From these observations, Forney et al. (1982a) propose that H_2O_2 production activity associated with lignin degradation is localized in the subcellular structure located in periplasmic space of cells of ligninolytic cultures. With H_2O_2 production activity located outside the cytoplasmic membrane, they propose that H_2O_2 could readily diffuse from cells where it could undergo reductive cleavage to produce ·OH which may be involved in lignin degradation. Kirk et -- al. (1985) and Faison and Kirk (1983), however, provide evidence that ·OH is not involved in lignin degradation and suggest that the role of H_2O_2 in lignin degradation by P. chrysosporium is indirect. We also found in this study that ·OH and $^1O_2$ quenching agents did not inhibit cellulose or wood decomposition by several white-rot fungi. Recently, Tien and Kirk (1984) found an extracellular oxygenase from P. chrysosporium requiring H_2O_2 that degrades a portion of the lignin molecule. Thus, H_2O_2 apparently is involved in lignin degradation by white-rot fungi but its role is indirect. Also white-rot fungi evidently do not employ reduced oxygen species to degrade cellulose.

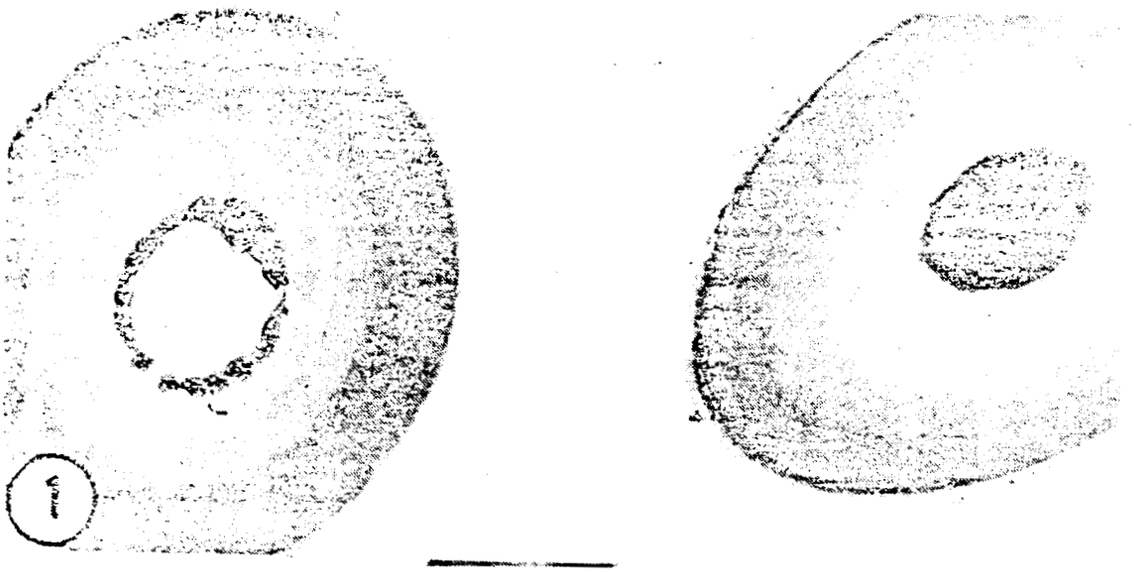


Fig. 1 C. versicolor. Thick-walled spore (at left) with oxidized DAB stained vesicles aligned near the wall in the periplasmic space. In the cell at right, the section went through the vesicle with dark contents. 54,560 x

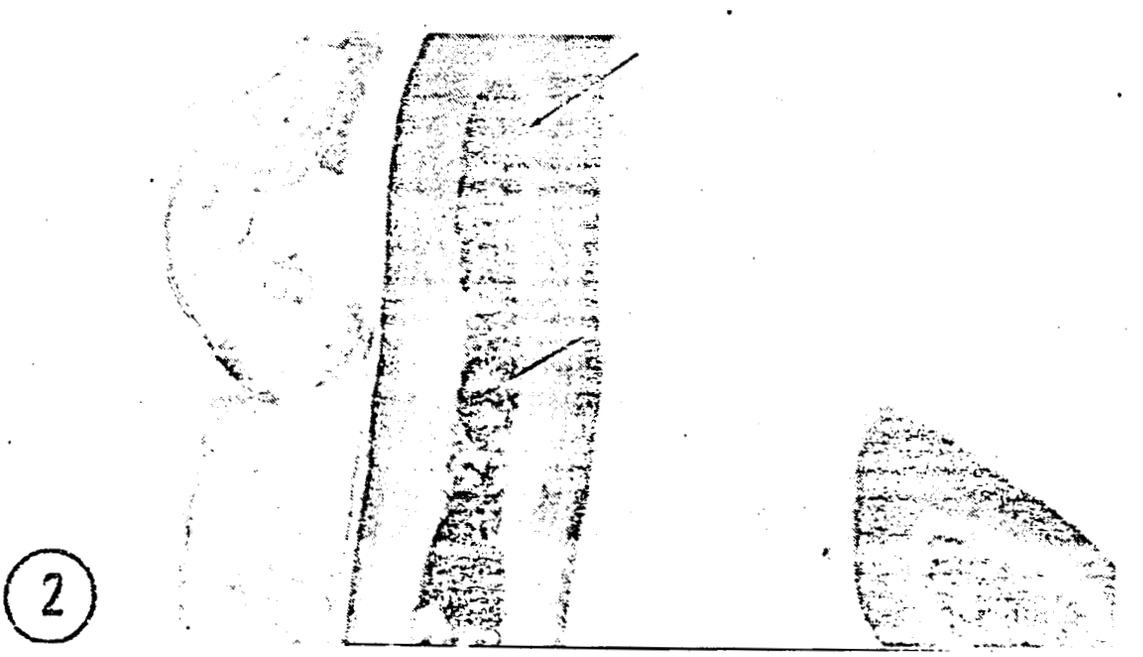


Fig. 2 C. versicolor. Longitudinal section of hypha showing large areas of oxidized DAB stained deposits (arrows). 17,000x

Fig. 3 C. versicolor. Thin-walled cells in sample treated with DAB have no oxidized DAB deposits. 17,000x

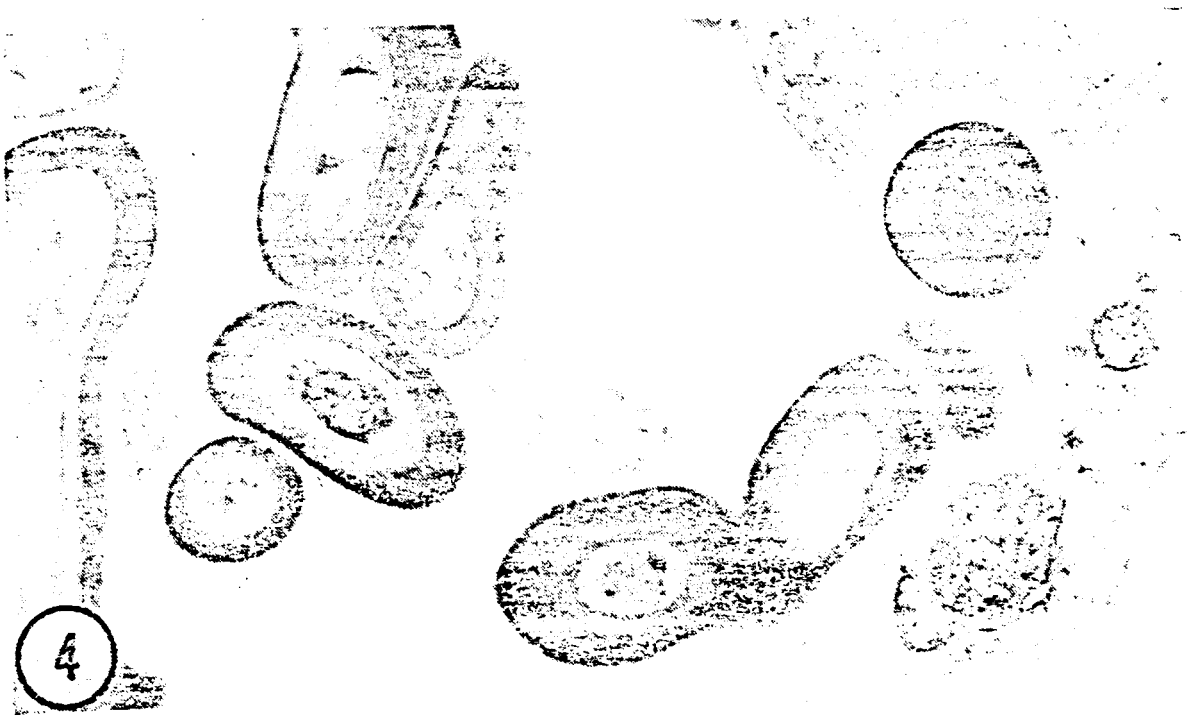


Fig. 4 C. versicolor. Thick-walled cells in sample treated with DAB and aminotriazole do not contain oxidized DAB deposits. 17,000x

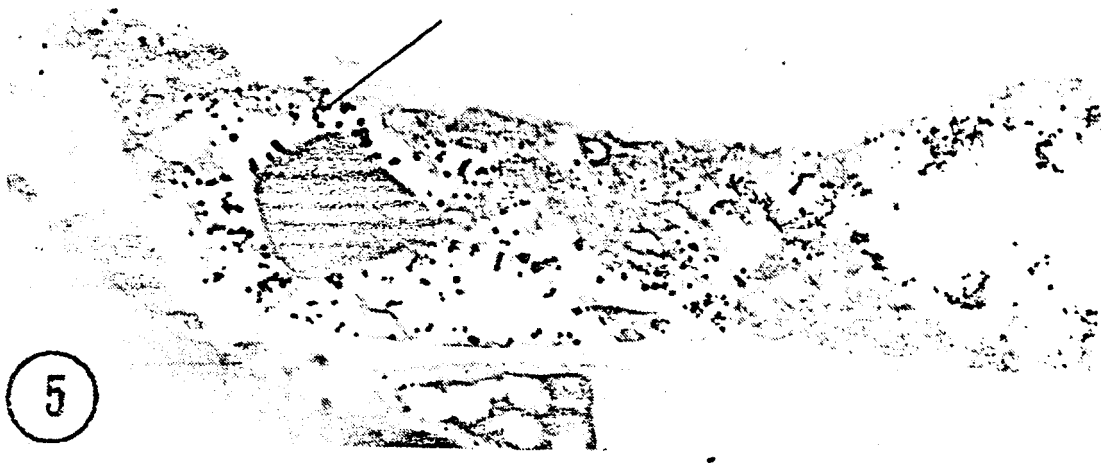
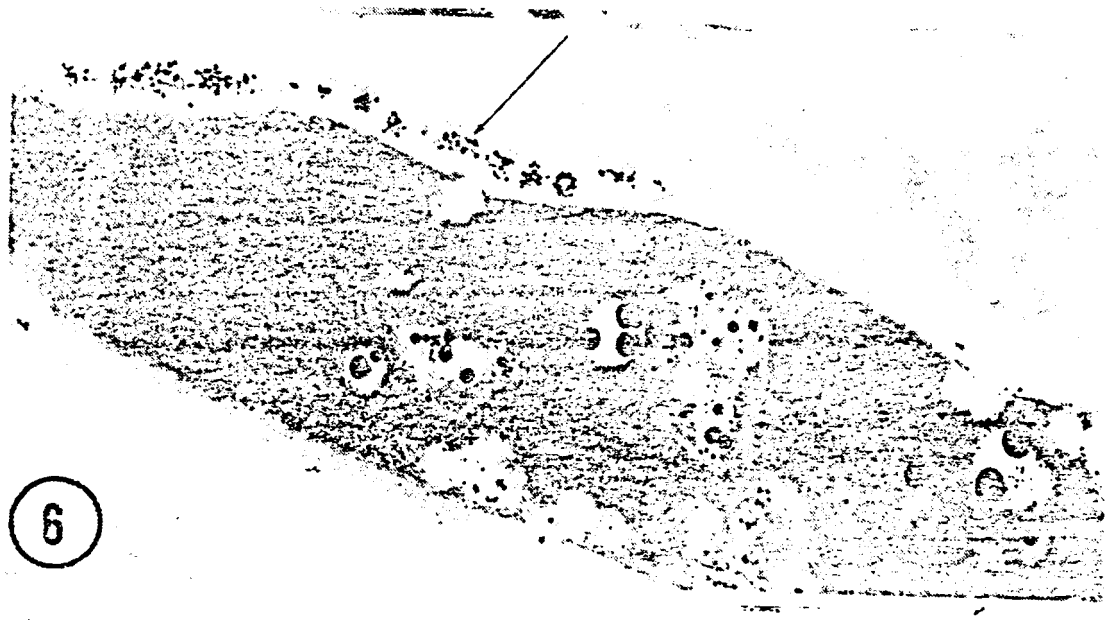
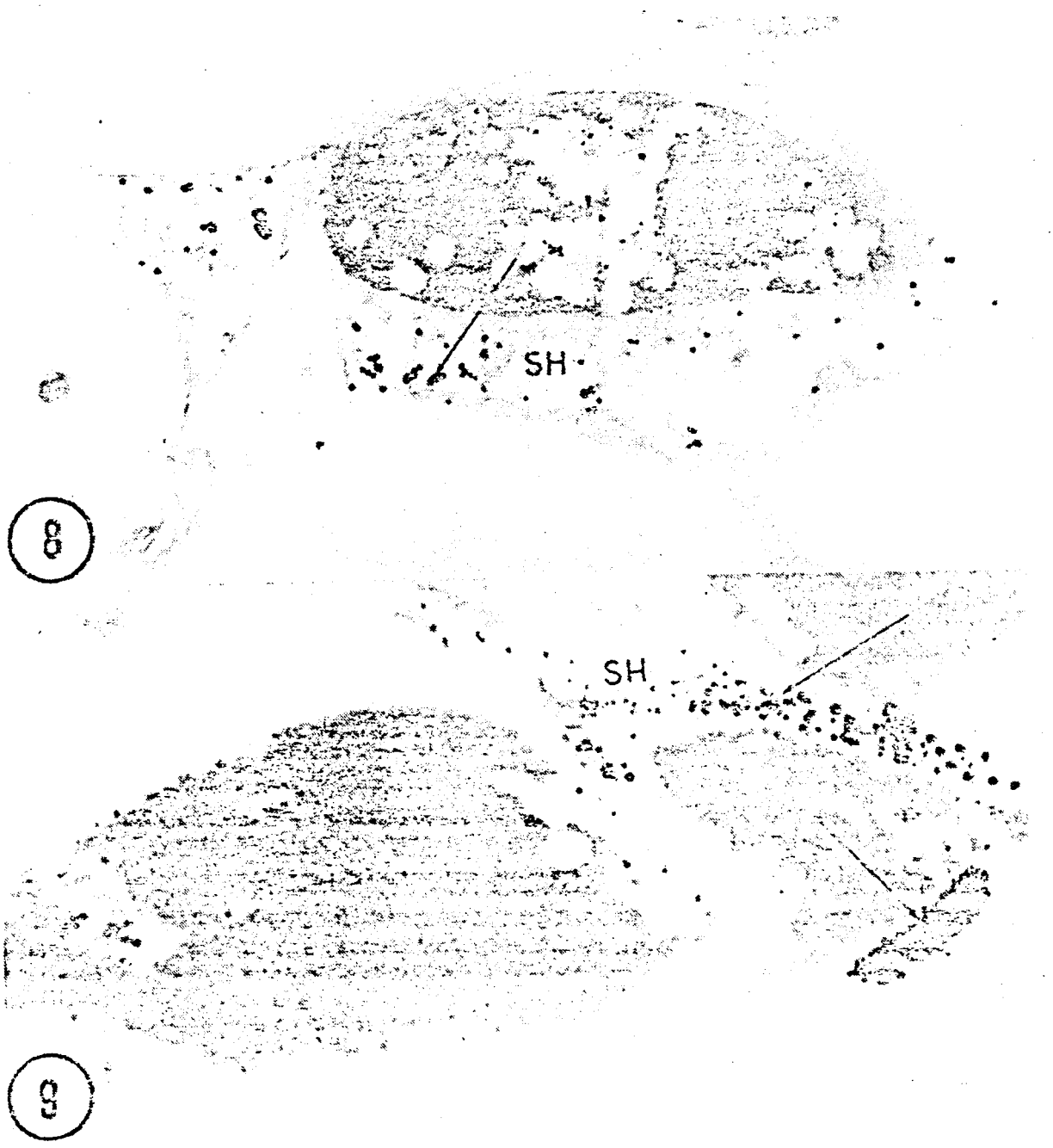


Fig. 5 C. versicolor. Hypha treated with DAB shows oxidized DAB stained particles–(arrow) throughout cytoplasm. 20,400x



Figs. 6 and 7 C. versicolor. Oxidized DAB particles present in the periplasmic space (arrows). 33,480x and 30,600x



Figs. 8 and 9 C. versicolor. Oxidized DAB particles (arrow) in hyphal sheath (SH). 39,950x and 25,500x

Fig. 10 P. placenta. Hyphae (H) in samples treated with DAB have no oxidized DAB deposits. 12,000x

Fig. 11 P. placenta. Hyphae treated with DAB and aminotriazole contain many vesicles in periplasmic space (arrow) but they do not have dark deposits. 12,000x

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