ADVANCES IN UNDERSTANDING THE ABILITY OF SAPSTAINING FUNGI TO PRODUCE CELL WALL-DEGRADING ENZYMES

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ABSTRACT

The ability of selected sapstaining fungi to produce the enzymes cellulase, xylanase, mannanase, pectinase, and amylase was investigated *in vitro*. While all test fungi secreted low amounts of xylanase (up to 1.64 μmoles/min/ml) and pectinase (up to 0.11 μmoles/min/ml) into the growth medium, extracellular cellulase was not detected. Furthermore, mannanase was produced only by *Ophiostoma piceae* (Münch) Syd. & P. Syd. (0.29 μmoles/min/ml). To the best of our knowledge, this is the first report of mannanase activity for any *Ophiostoma* sp. Amylase activity was higher than xylanase, mannanase, and pectinase activities for all test fungi. This confirms that sapstaining fungi preferentially metabolize readily accessible, nonstructural wood components, such as starch. Possible roles of the enzymes detected are discussed.

Keywords: Sapstaining fungi, *Ophiostoma*, *Sphaeropsis sapinea*, *Pinus radiata*, cellulase, xylanase, mannanase, pectinase, amylase.

INTRODUCTION

It is well known that sapstaining fungi produce extracellular enzymes to utilize nonstructural components of sapwood, namely sugars, proteins, and extractives (Abraham et al. 1993, 1998; Brush et al. 1994; Breuil and Huang 1994; Breuil et al. 1995; Gao and Breuil 1995; Abraham and Breuil 1996; Gao and Breuil 1998). However, quantitative information on the ability of sapstaining fungi to produce cell wall-degrading enzymes is limited. *Botryodiplodia theobromae*, a sapstaining fungus common in the tropics, produces extracellular cellulase, xylanase, and pectinase (Umezurike 1969, 1970a, b, 1971; Seehann and Tabirih 1983) and causes cavities in the wood cell walls indicative of soft-rot (Krapivina 1960). Recent observations suggest that this fungus can also cause erosion of wood cell walls (Florence et al. 2002). Causative agents of Dutch elm disease, *O. ulmi* and *O. novo-ulmi*,

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TABLE 1. *List of sapstaining and decay fungi used.*

Species	Isolate #	Origin
Ophiostoma floccosum Math.-Käärik	138	Riverhead Forest, NI
O. floccosum Math.-Käärik	148	Hanmer Springs, SI
O. piceae (Münch) Syd. & P. Syd.	170	Greymouth, SI
O. piceae (Münch) Syd. & P. Syd.	272	Rotorua, NI
O. pluriannulatum (Hedgc.) Syd. & P. Syd.	151	Abel Tasman National Park, SI
O. ips (Rumbold) Nannf.	308	Waipa sawmill, Rotorua, NI
O. ips (Rumbold) Nannf.	294	Nelson, SI
Leptographium procerum (W. B. Kendr.) M. J. Wingf.	1852	Whitford Forest, NI
Sphaeropsis sapinea (Fr.) Dyko & B. Sutton	4	Dome State Forest, NI (pine cone)
S. sapinea (Fr.) Dyko & B. Sutton	35	Kinleith Forest, NI (live tree)
Gloeophyllum trabeum (Pers.) Murrill	BAM Ebw. 109	*Forest Research*, Rotorua
Schizophyllum commune (L.) Fr.	3	HortResearch, Hamilton

Abbreviations: NI = North Island; SI = South Island.

produce cellulase and pectinase (Beckman 1956; Biehn and Dimond 1971; Elgersma 1976; Svaldi and Elgersma 1982). However, Binz (1996) was unable to detect cellulase using the same fungi. Carboxymethylcellulase, xylanase, ß-galactosidase, mannanase, polygalacturonase, and laccase were detected in *Aureobasidium pullulans* (Deshpande et al. 1992), while *Ceratocystis minor* produces carboxymethylcellulase, polygalacturonase, pectinesterase, and laccase (Rösch et al. 1969). Laccase has also been detected in other sapstaining fungi (Rösch and Liese 1971). These contradictory results on enzyme production of sapstaining fungi may reflect differences in the sensitivity of the assay methods used, in the substrates employed, and in the variability between strains of one species. Highly sensitive assays for the determination of carbohydrate degrading enzymes are based on the detection of reducing sugars (Miller 1959; Lever 1973).

Sapstaining fungi colonize wood tissues by spreading from cell to cell primarily through pits, and in some instances also by penetrating cell walls (Liese and Hartmann-Fahnenbrock 1953; Liese and Schmid 1961, 1964; Eaton and Hale 1993). Liese (1970) proposed that sapstaining fungi penetrate cell walls using a combination of localized enzymatic action at the tip of the fungal transpressorium and hydrostatic pressure; however, to date this question still remains unresolved. Recently, Florence et al. (2002) suggested that enzymes rather than hydrostatic pressure may be involved in the breakdown of pit membranes in rubberwood by *B. theobromae*.

The principal objective of this study was to detect and quantify the secretion of cell wall-degrading enzymes, specifically cellulase, xylanase, mannanase, and pectinase *in vitro*, using sapstaining fungi isolated in New Zealand. Futhermore, production of amylase for utilising amylose, a nonstructural wood component, was determined.

MATERIALS AND METHODS

Fungal isolates and culture conditions

All sapstaining fungi used in this study were isolated in New Zealand between 1996 and 2000 and belong to the mycological culture collection at the University of Waikato, Hamilton (Table 1). The white-rot fungus *Schizophyllum commune* and the brown-rot fungus *Gloeophyllum trabeum* were included to serve as positive controls (Table 1).

Ophiostoma spp. cultures were maintained on petri dishes containing 2 g yeast extract (Becton Dickinson and Company, U.S.), 15 g malt extract (Difco, U.S.), 18 g agar (Germantown, New Zealand), 200 mg chloramphenicol (Sigma, U.S.), 100 mg streptomycin sulphate (Sigma, U.S.) and 400 mg cycloheximide (Sigma, U.S.) per one liter of water. *Ophiostoma* species are cycloheximide-resistant (Harrington 1981). *Sphaeropsis sapinea* and *S.*

commune were maintained on the same medium, but cycloheximide was omitted. *Gloeophyllum trabeum* was grown on 2% malt agar. Liquid starter cultures were prepared by transferring a mycelial plug (1-cm diameter) taken from the edge of an actively growing colony into universal flasks containing 10 ml yeast-malt extract broth (2 g yeast extract, 15 g malt extract and one liter of water; medium I). After inoculation, starter cultures were incubated on a rotary shaker set at 120 rpm and 25°C for 24 h.

Determination of enzyme activities in culture supernatant

Starter cultures were inoculated into sterile medium II (80 ml in 250-ml flasks) consisting of 0.9 g NaCl, 0.2 g $MgCl_2 \cdot 6H_2O$, 1.5 g K_2HPO_4, 0.75 g KH_2PO_4, 0.9 g NH_4Cl, 0.05 g $CaCl_2 \cdot 2H_2O$, 0.05 g yeast extract, 0.1 g tryptone, 5 g of carboxymethylcellulose (CMC; medium viscosity; Sigma) or larchwood xylan (Sigma) or locust bean gum (Sigma) or citrus fruit pectin (Sigma) in one liter of distilled, deionized water. Pectin was moistened with ethanol prior to dissolving in water. For the detection of amylase, 10 ml of the starter cultures were inoculated into medium III (80 ml in 250-ml flasks) consisting of 0.5 g $(NH_4)_2SO_4$, 1 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2$, 0.5 g yeast extract, 10 g potato starch (Sigma) and one liter of water. Duplicate flasks were used for each fungal isolate. The cultures were incubated on a rotary shaker set at 120 rpm and 25°C for up to 11 days (sapstaining species) or 26 days (decay fungi). Two ml of the cultures were harvested at defined time periods, spun down in an Eppendorf benchtop centrifuge at 13,000 rpm, and the supernatant was stored at −20°C until required.

Enzyme activities were spectrophotometrically determined by measuring the reducing sugars released upon reaction with a supplied substrate. For the detection of cellulase activity, *p*-hydroxybenzoic acid hydrazide (PAHBAH, Sigma) was used (Lever 1973). The reaction mixture of the cellulase assay consisted of 300 µl substrate and supernatant in different dilutions to bring the total volume to 500 µl in a 1.5-ml centrifuge tube. CMC was used in two concentrations (0.2% and 1%) in 100 mM MOPS (3-[N-Morpholino]propane-sulfonic acid) buffer at pH 6 and at pH 7. A commercial cellulase from *Trichoderma viride* (Sigma) was included to serve as a positive control in the cellulase assays.

Xylanase, mannanase, pectinase, and amylase activities were measured using dinitrosalicylic acid (DNSA) reagent (Miller 1959). For the xylanase assays, the reaction mixture consisted of 300 µl larchwood xylan (1.33% in McIlvaine citrate phosphate buffer at pH 4.6; Binz 1996), 180 µl McIlvaine citrate phosphate buffer, and 20 µl supernatant in a 1.5-ml centrifuge tube. To detect mannanase activity, 300 µl locust bean gum (0.2% in McIlvaine citrate phosphate buffer at pH 4.6), 100 µl buffer and 100 µl supernatant were used. The xylan or locust bean gum was dissolved in buffer, heated to boiling point, cooled; and centrifuged at 10,000 rpm and 4°C for 15 min, only the supernatant was used in the assay. In the pectinase assays, the standard reaction mixture consisted of 300 µl polygalacturonic acid (0.33% in McIlvaine citrate phosphate buffer at pH 4), 100 µl buffer and 100 µl supernatant. For the determination of amylase activity, 300 µl potato starch (1% in 100 mM MOPS buffer at pH 7), 100 µl sterile distilled water and 100 µl supernatant were used.

Standards in all assays were prepared according to Bailey et al. (1992). The standard was either D-glucose, D-xylose, mannose, or galacturonic acid (all from Sigma), depending on the assay. All samples, controls, and standards were analyzed in duplicates. Absorbance was read at 420 nm on a spectrophotometer (Pharmacia Biotech Ultrospech 3000) for the CMC-samples and at 575 nm for the xylan-, mannan- and amylose-samples. Enzyme activity was expressed as µmoles of glucose, xylose, mannose, or galacturonic acid equiva-

lents released per minute per ml at a defined temperature.

Determination of cell-bound pectinase activity

Starter cultures of *O. ips* #294 were grown for 36 h and transferred into medium II (80 ml in 250-ml flasks) containing pectin as the carbon source. After 3 and 6 days of incubation, 3 ml of culture were harvested from triplicate flasks and frozen immediately. The remaining 74 ml of each culture were transferred into sterile 250-ml tubes and spun in a Beckman centrifuge for 15 min at 4°C and 20,000 rpm. The supernatants were decanted and the mycelial pellets resuspended in assay buffer (McIlvaine citrate phosphate buffer, pH 4). The washed pellets were spun for 30 min at 4°C and 20,000 rpm, and the washing step was repeated once. Three ml of the supernatants were harvested and the fungal pellets resuspended. The original supernatant and the supernatant after washing and centrifuging were used separately in the DNSA assays. In order to determine cell-bound enzyme activity, the mycelial pellet suspension was ground using a commercial power drill and subsequently used in the DNSA assay. In all assays, the reaction mixture consisted of 50 μl supernatant, 150 μl buffer (McIlvaine citrate phosphate buffer, pH 4) and 300 μl substrate (0.33% polygalacturonic acid in buffer) and was incubated at 30°C for 60 min.

Determination of fungal biomass

Biomass was determined after the last culture supernatants were harvested and used to correlate enzyme activities and fungal growth. Cultures of a known volume were filtered under vacuum through predried and weighed filter-papers which were subsequently dried to constant weight in an oven at 80°C.

Ultrafiltration of crude enzyme solutions

Enzyme supernatants harvested at the end of the cultivation were concentrated five-fold by ultrafiltration using Centricon-10 concentrators (Amicon, Beverly, U.S.A.) with a molecular weight cut-off of 10,000 daltons.

Protein determination

Total extracellular protein of selected cultures grown in larchwood xylan medium was determined using a commercial protein assay kit (Bio-Rad Laboratories) based on the method of Bradford (1976). Bovine serum albumin (Sigma) was employed as the standard using concentrations between 0 and 0.2 mg/ml. All concentrations were tested in duplicate.

RESULTS AND DISCUSSION

None of the sapstaining fungi tested showed any extracellular cellulase activity under the various test conditions employed in the present study (Table 2). Extracellular cellulase, however, was produced by the two positive controls, *G. trabeum* and *S. commune,* thus validating the methodology used for cellulase detection. Cellulolytic activity has been reported for some sapstaining fungi, namely *A. pullulans* (Greaves and Savory 1965; Rösch et al. 1969), *C. coerulescens* (King and Eggins 1973), *C. piceae* (Nilsson 1973), *C. minor* (Rösch et al. 1969), *C. pilifera* (King and Eggins 1973) and *B. theobromae* (Umezurike 1969). *Aureobasidium pullulans* and *Sclerophoma pityophila* have been shown to rapidly penetrate membrane filters of regenerated cellulose of very small pore dimensions (i.e., 0.2-μm pore diameter), suggesting that these fungal species are secreting cellulolytic enzymes (Bardage and Daniel 1997). Binz and Canevascini (1996) found no cellulolytic activity for *O. ulmi* contrary to the results of earlier work using this fungus (Beckman 1956; Elgersma 1976). The results obtained in the present study are supported by experiments on enzyme activities of staining fungi in the presence or absence of bioprotectants (Giron et al. 1998). Giron et al. (1998) found very little glucosidase activity in bamboo strips exposed to a mixture of the staining fungi *Alternaria alternata, Ophiostoma piliferum,* and *Phialocephela dimorphospora.* Glucosidase is re-

TABLE 2. *Average extracellular enzyme activities of sapstaining and decay fungi after 184 h incubation in liquid culture (n = 2).*

Isolate	Maximal activity (μmoles/min/ml) during incubation*					
	Cellulase	Xylanase	Mannanase	Pectinase[1]	Pectinase[2]	Amylase
O. floccosum #138	0	0.28	0	0.02	0.03	0.44
O. floccosum #148	0	0.37	0	n.d.	n.d.	0.24
O. piceae #272	0	0.08	0.04	0.03	0.02	0.25
O. piceae #170	0	0.17	0.29	n.d.	n.d.	0.25
O. ips #308	0	0.04	0	0.04	0.11	0.43
O. pluriannulatum #151	0	0.51	0	0.04	n.d.	0.46
L. procerum #1852	0	0.06	0	0.04	n.d.	0.26
S. sapinea #4	0	1.59	0.06	0.03	0.04	0.39
S. sapinea #35	0	1.64	0.01	0.03	0.04	0.46
S. commune	1.31	0.31	0.19	0.05	0.04	0.31
G. trabeum	0.95	0.10	0.02	0.42	0.11	0.24

* Activities per g of fungal biomass were calculated for individual enzymes (see Tables 3 and 4) but cannot be compared between different enzymes because biomass was determined after different incubation times for the individual enzymes.
[1] on pectin.
[2] on polygalacturonic acid.
n.d. = not determined.

quired for complete hydrolysis of cellulose and hydrolyzes cellobiose and other water-soluble cellodextrins to glucose (Eriksson et al. 1990).

It is unlikely that the sapstaining fungi tested in this study produce extracellular cellulase because a variety of assay parameters were considered, such as substrate concentration, incubation time, and type of buffer. However, it may be possible that cellulase associated with the sapstaining fungi investigated here is tenaciously bound to a hyphal sheath and thus not released into the medium. This aspect may warrant further research.

While some xylanase was produced by all of the sapstaining and decay fungi tested, highest activities were determined for the two isolates of *S. sapinea* (Table 3; Fig. 1). Xylanase activities determined for *S. sapinea* were comparable to values reported for *O. ulmi* by Binz (1996), showing a xylanase activity of 1.42 μmol/min/ml on birch wood xylan 62 h after inoculation. *Ophiostoma ips* showed the least xylanase activity but also produced mainly blastospores and the least biomass of all isolates tested when grown in liquid xylan medium. Assuming that cell wall-degrading enzymes of *Ophiostoma* species are preferential-

TABLE 3. *Average xylanase activity, fungal biomass, and final pH of cultures after 184 h incubation in media supplemented with larchwood xylan (n = 2 for sapstaining fungi and n = 3 for decay fungi).*

Isolate	Xylanase act. (μmoles/min/ml)	Xylanase act. after 5-fold conc. (μmoles/min/ml)	Fungal biomass (g)	Xylanase act. (μmoles/min/ml/g)	pH
O. floccosum #138	0.28	1.66	0.12	2.33	6.4
O. floccosum #148	0.33	2.70	0.12	2.75	6.2
O. piceae #272	0.08	0.70	0.15	0.53	6.3
O. piceae #170	0.17	1.18	0.14	1.21	6.3
O. ips #308	0.03	0.04	0.05	0.60	6.7
O. pluriannulatum #151	0.51	2.81	0.13	3.92	6.6
L. procerum #1852	0.04	0.05	0.11	0.36	6.7
S. sapinea #4	1.59	3.67	0.14	11.36	6.2
S. sapinea #35	1.64	3.72	0.17	9.65	6.1
S. commune	0.31	1.91	0.15	2.07	6.2
G. trabeum	0.10	0.64	0.08	1.25	4.6

Abbreviations: act. = activity; conc. = concentration.

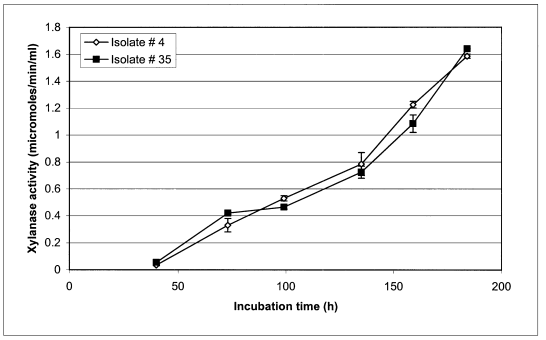

FIG. 1. Xylanase activities of *S. sapinea* #4 and #35 grown in liquid medium supplemented with larchwood xylan. Bars represent the range of values obtained for duplicate cultures.

ly secreted in the mycelial stage (Binz 1996), *O. ips* might produce more xylanase under culture conditions that induce mycelial growth. There was also very little xylanase activity in the supernatant of *Leptographium procerum* culture, despite the fact that this fungus grew well in xylan medium (Table 3). Xylanase activity in this species may be bound to the hyphal sheath; however, cell-bound xylanase activity was not detected in a related species, *O. ulmi.* (Binz personal communication). The possibility that enzyme activity of *Ophiostoma* species is cell-bound has been tested with regard to pectinase in *O. ips* #294, as discussed later.

Several of the sapstaining isolates tested in the present study demonstrated higher xylanase activity than the decay fungi *G. trabeum* and *S. commune.* This may be attributed to the fact that the culture conditions for the sapstaining and decay fungi tested were identical and that a considerable amount of variation can be expected even when comparing enzyme activities of different strains within a single fungal species (Eriksson et al. 1990). It is difficult to produce culture and assay conditions that accommodate sapstaining as well as decay fungi since both have very different physiological requirements. The possibility that synergistic action of various cellulolytic and hemicellulolytic enzymes contributes to overall loss of structural wood integrity by decay fungi *in vivo* also has to be considered.

With the exception of *G. trabeum,* pH of the growth medium did not change significantly during incubation (Table 3; original pH was 6.7). The effect of pH on xylanase secretion was not tested, but it has been shown that xylanase activity and biomass production on xylan by *O. ulmi* and *O. novo-ulmi* at pH 4 were lower than those obtained at pH 5.5 and 7.0 (Binz 1996).

In general, total extracellular protein concentration in the supernatants harvested after 184 h incubation in xylan medium was less than 20 μg/ml. This value is similar to that obtained for *O. ulmi* (17 μg/ml; Binz personal communication).

Although mannan is the main constituent of hemicelluloses in softwoods (Fengel and Wegener 1989), it is significant that mannanase activity on galactoglucomannan (locust bean gum) was detected in only one sapstaining species, namely *O. piceae* (activity of the crude supernatant ≤0.3 μmoles/min/ml; Table 4). To our knowledge, this is the first report of quantified mannanase activity for an *Ophiostoma* species. There is little information available on production of mannanase by sapstaining fungi. Mannanase activity has been reported for *Aureobasidium pullulans* (Berndt and Liese 1971; Kremnický et al. 1996; Kremnický and Biely 1997). A possible reason for not detecting mannanase activity in cultures other than *O. piceae* in the present study is that the physiological conditions of cultivation were not suitable for enzyme production. Locust bean gum was the substrate of choice because it has been successfully used for the detection of mannanase in yeasts and yeast-like microorganisms (Kremnický et al. 1996). It may also be possible that mannanase activity in sapstaining fungi is cell-bound, i.e., attached to the hyphal wall or sheath; however, mannanases are usually secreted into the medium in which the microorganism is cultivated (Eriksson et al. 1990).

Mannanase may be involved in the pathogenesis of certain sapstaining fungi, but this

TABLE 4. *Mannanase activity, fungal biomass, and final pH of cultures after 184 h incubation in media supplemented with locust bean gum. Values represent the average of two (sapstaining species) and three (decay fungi) flasks.*

Isolate	Mannanase activity (μmoles/min/ml)	Mannanase act. after 5-fold conc. (μmoles/min/ml)	Fungal biomass (g)	Mannanase activity (μmoles/min/ml/g)	pH
O. floccosum #138	0	0	0.03	0	6.8
O. floccosum #148	0	0	0.11	0	6.8
O. piceae #272	0.04	0.50	0.06	0.67	6.5
O. piceae #170	0.29	0.75	0.17	1.71	6.2
O. ips #308	0	0	0.08	0	6.7
O. pluriannulatum #151	0	0	0.09	0	6.8
L. procerum #1852	0	0	0.08	0	6.6
S. sapinea #4	0.06	0.09	0.22	0.27	6.4
S. sapinea #35	0.01	0.03	0.16	0.06	6.8
S. commune	0.19	n.d.	0.26	0.73	6.3
G. trabeum	0.02	n.d.	0.12	0.17	4.1

Abbreviations: act. = activity; conc. = concentration; n.d. = not determined.

remains purely speculative. *Ophiostoma piceae*, which is saprophytic but has also been described as weakly parasitic (Brasier and Kirk 1993), was the only fungal species in the present study showing mannanase activity. The fact that none of the other sapstaining species tested showed mannanase activity under the conditions tested does not exclude the possibility that these species do secrete mannanase.

The results obtained in this study suggest that although galactoglucomannans are the major hemicelluloses in softwoods (Fengel and Wegener 1989), the arabino-4-methylglucuronoxylans are preferentially used by sapstaining fungi when growing in radiata pine.

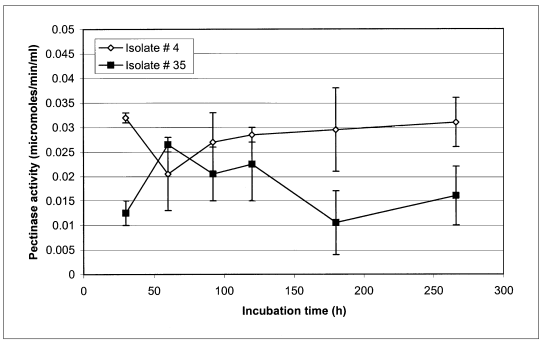

FIG. 2. Pectinase activity of *S. sapinea* #4 and #35 grown in liquid medium supplemented with pectin. Bars represent the range of values obtained for duplicate cultures.

This may be attributed to the accessibility of the xylans in the S_3-layer of the softwood cell wall, as compared to other regions of the cell wall (Sjöström 1992).

All isolates tested produced low amounts of pectinase in a growth medium supplemented with pectin or polygalacturonic acid (Table 2; see Fig. 2 for pectinase activity in *S. sapinea* isolates #4 and #35). Maximal pectinase activity (0.04 μmoles/min/ml) in pectin medium was determined for *O. ips* #308, *O. pluriannulatum* #151, and *L. procerum* #1852. Fungal biomass and pectinase activity were not significantly different when polygalacturonic acid or pectin was used as a substrate, except for *O. ips* #308 which showed almost three times as much activity on polygalacturonic acid (see Table 2). Binz (1996) measured polygalacturonase activities secreted by isolates of the pathogenic sapstaining fungi *O. ulmi* (0.12 μmoles/min/ml) and *O. novo-ulmi* (0.11 μmoles/min/ml) grown on pectin. Results obtained in the present study indicate that *O. ips* may have the potential to secrete similar amounts of polygalacturonase. According to Harrington (1993), *O. ips* has been reported to be a substantial pathogen (Basham 1970; Himelick 1982; Raffa and Smalley 1988), but it does not appear to be particularly pathogenic in artificial inoculations (Rane and Tattar 1987; Parmeter et al. 1989). Pectic enzymes play an essential role in phytopathogenesis and can elicit

TABLE 5. *Average pectinase activities of* O. ips *#294 determined in original supernatant, in supernatant after washing and in the mycelial pellet (n = 3).*

	Pectinase activity (μmoles/min/ml)			Fungal biomass (g) in 90 ml culture
	Original supernatant	Supernatant after washing	Mycelial pellet	
After 3 days	0.21	0	0.04	0.20
After 6 days	0.17	0	0	0.17

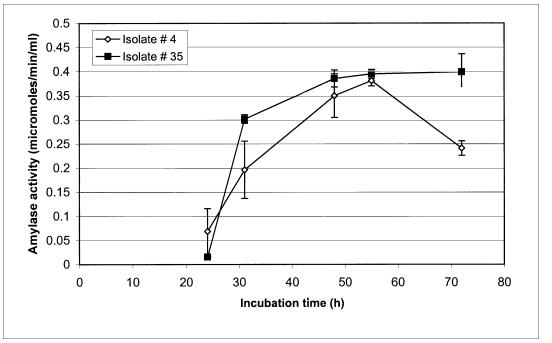

FIG. 3. Amylase activities of *S. sapinea* #4 and #35 grown in liquid medium supplemented with potato starch. Bars represent the range of values obtained for duplicate cultures.

a cascade of defence reactions in plant tissues challenged by invading fungi (Jarvis 1984). The involvement of pectinases in the pathogenesis of the Dutch elm disease was demonstrated by Gagnon (1967), who showed that pectins were the main constituents of the plugging material in the vessels of infected elm trees. The action of pectinases on vessel pits allows the flow of protoplasm from adjoining parenchyma cells into the vessels, which contributes to vessel plugging (Gagnon 1967).

In this study, we found no evidence that pectinase activity of *O. ips* #294 was bound to the fungal cell wall or associated with the extracellular fungal sheath. Enzyme activity of the original supernatant was up to 0.21 μmoles/min/ml, whereas activity of the fungal pellet was not more than 0.04 μmoles/min/ml. This showed that *O. ips* #294 secreted pectinase into the growth medium (Table 5).

There are few studies of pectinolytic enzymes in sapstaining or decay fungi. This may be because the amount of pectin in wood is rather low (less than 4%; Fengel and Wegener 1989) as compared to other cell-wall constituents, and because the primary focus has been on understanding the degradation of major components of lignified cell walls (Green and Clausen 1999). However, pectin degradation in the tori of pit membranes appears to be a key step in wood colonization by decay fungi (Green et al. 1996; Green and Clausen 1999). Pectin degradation may also be important for sapstaining fungi since all are capable of growing through pit membranes although some can also grow through cell walls (Eaton and Hale 1993). It is likely that small amounts of pectinase secreted by sapstaining fungi *in vivo* are sufficient to facilitate the penetration of pit membranes. In future research, immunolabelling techniques (Blanchette et al. 1989; Daniel et al. 1989, 1996; Green et al. 1992; Hoffert et al. 1995) may be useful for obtaining information on the localization of pit membrane-degrading enzymes produced by sapstaining fungi in the fungal hyphae as well as in the host cells.

Amylase activity of sapstaining fungi was determined for comparative purposes since amylase is not a cell wall-degrading enzyme. Amylase activity was highest between 48 and 55 h of incubation, depending on the fungal isolate (see Fig. 3 for *S. sapinea* #4 and #35). Maximal amylase activities of supernatants harvested during 60 h incubation were between 0.24 μmoles/min/ml and 0.46 μmoles/min/ml.

Amylase activities of *S. commune* and *G. trabeum* were comparable to results obtained for the sapstaining fungi. Amylase activity of the decay and sapstaining fungi was most significant after 55 h incubation and decreased afterwards (see Fig. 3 for results obtained with two isolates of *S. sapinea*). The amounts of amylase produced by the sapstaining fungi tested were higher than the amounts of xylanase, mannanase and pectinase. This confirms that sapstaining fungi preferentially use nutrients like starch that are easily accessible in wood, as has been demonstrated by King and

Eggins (1973), Nilsson (1974), Umezurike (1969), Tabirih and Seehann (1984), and Encinas and Daniel (1999).

The results obtained in the present study support the findings from previous toughness and weight loss tests (Schirp et al. 2003). None of the sapstaining fungi tested caused toughness and weight loss in radiata pine, with the exception of *O. ips* isolate #308, which reduced toughness by 18%, though only in one out of four experiments (Schirp et al. 2003).

CONCLUSIONS

Structural wood components are not the main nutrient source for sapstaining fungi; however, all isolates tested in the present study were shown to secrete low amounts of xylanase and pectinase. No extracellular cellulase was detected. Mannanase activity was demonstrated only for *O. piceae*. Pectinase may assist the fungal penetration of pit membranes and may also play a role in pathogenicity of certain sapstaining fungi.

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