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Comparison of the laccases, molecular marker proteins, and induction of pycnidia by three species of botryosphaeriaceous fungi

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Three species of botryosphaeriaceous fungi, *Botryosphaeria* sp. isolate MAMB-5, *Botryosphaeria ribis* and *Lasiodiplodia theobromae*, were compared for the production of pycnidia and laccases. Laccases were produced both intra- and extra-cellularly when the fungi were cultivated on basal medium in the presence and absence of veratryl alcohol, with *Botryosphaeria* sp. MAMB-5 showing the highest enzyme titres. Electrophoretic examination of intracellular marker proteins (esterases and phosphatases) and laccases indicated that the three species were genetically distinctly different, although the laccase zymograms for the three fungi showed similarity. The production of pycnidia occurred under continuous lighting at 28°C, but conditions differed among the three fungal species. Production could be induced on artificial media (potato-dextrose and oat agar) under stress-induced conditions where the mycelium was stimulated by physical abrasion, and in the case of *Botryosphaeria* sp. isolate MAMB-5 on eucalypt woodchips. Evidence is presented that veratryl alcohol facilitated the secretion of intracellular-localised laccases into the extracellular medium.

Key Words——*Botryosphaeria* species; laccases; *Lasiodiplodia theobromae*; pycnidium formation; veratryl alcohol.

Fungi belonging to the genus *Botryosphaeria* Ces. & De Not., and especially *Botryosphaeria ribis* Grossenb. & Duggar and *B. dothidea* (Moug.) Ces. & De Not. (Yuan and Mohammed, 1999), are well recognised as plant pathogens causing the production of stem cankers in eucalypts and melaleuca species (Rayachhetry et al., 1999) and dieback diseases in various fruit trees and woody ornamental plants (Ramos et al., 1997). *Botryosphaeria* species also infest preharvest fruits, causing storage rots that result in significant economic losses to the fruit industry (Biggs, 1995). *Lasiodiplodia theobromae* (Pat.) Griffiths & Maubl. (Hanlin and Menezes, 1996; Jacobs and Rehner, 1998), frequently described taxonomically as an anamorph of *Botryosphaeria*, or as the teleomorph, *Botryosphaeria rhodina* (Berk. & M. A. Curtis) Arx (Jacobs and Rehner, 1998), and belonging to the group of botryosphaeriaceous fungi, is the causal agent of many storage rots in fruits and tubers, and a serious pathogen of many agricultural and horticultural crops (Gupta et al., 1999).

A species of *Botryosphaeria* MAMB-5 (Barbosa et al., 1995) has been isolated from a stem canker on an eucalypt tree and shown to be ligninolytic and an active producer of the enzyme laccase (Barbosa et al., 1996), which was strongly induced by 3,4-dimethoxybenzyl (veratryl) alcohol (Vasconcelos et al., 2000; Dekker and Barbosa, 2001). *Botryosphaeria* sp. has potential for application in the bioremediation of aromatic compounds as it was capable of growing on a wide range of aromatic and phenolic compounds structurally-related to lignin, and in each case growth resulted in the induction of laccases (Dekker, unpublished results). Laccases (*p*-diphenol:dioxygen oxidoreductases, EC 1.10.3.2) constitute a family of multi-copper oxidases (Messerschmidt, 1997) widely distributed among fungi (Gianfreda et al., 1999), which are involved in the reduction of oxygen to water concomitantly with a one-electron oxidation of various phenolic and non-phenolic reducing substrates (Gianfreda et al., 1999). Laccases have been implicated in pathogenesis (Williamson, 1997; Gianfreda et al., 1999), degradation of lignin (Eggert et al., 1996a; Srebotnik and Hammel, 2000), lignin biosynthesis in plants (O'Malley et al., 1993), and in the bioremediation of aromatic compounds (Johannes and Majcherczyk, 2000).

The aim of the work reported herein was to examine the production and properties of the laccases produced by *Botryosphaeria* sp. (isolate MAMB-5), and the induction of pycnidia, and to compare these profiles with two other species of the genus, *B. ribis* and *L. theobromae*. Also examined were the electrophoretic profiles of the

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intracellular laccases and the marker proteins, esterases and phosphatases. On the basis of our findings we communicate that the three fungi were distinctly different genetically, although their laccases appeared similar. Evidence is also presented that the laccase inducer, veratryl alcohol, facilitated the secretion of intracellular-localised laccases into the extracellular medium.

Materials and Methods

Microorganism and cultivation *Botryosphaeria ribis* (Silveira et al., 1996), isolated from *Eucalyptus citriodora* in São Paulo (Brazil), and *L. theobromae* (Silveira et al., 1996), isolated from currupixá wood in Belém (Brazil), were obtained from Dr. T. L. Krugner, Dept. of Phytopathology, Escola Superior de Agronomia Luiz de Queiroz, Universidade de São Paulo, Piracicaba, SP, Brazil. *Botryosphaeria* sp. (isolate MAMB-5) was isolated from a stem canker on a eucalypt tree (Barbosa et al., 1995). All three fungal species were maintained, through regular 3-monthly transfer, at 4°C on potato dextrose agar (PDA, Oxoid) slants. Pycnidia production by all three fungal species was evaluated at 28°C under continuous fluorescent lighting for 2 wk on PDA, glucose-minimal salts-agar (Vogel, 1956), and 5% (w/v) oat-agar medium, and on twice-autoclaved (121°C for 20 min) *E. citriodora* woodchips in the absence of nutrient medium.

Inoculum was prepared by growing the three fungal species on agar plates containing minimal salts medium (Vogel, 1956), agar (2% w/v), and glucose (1% w/v) at 28°C until covered by a profuse mycelial mat (96 h). Three 0.7-cm diam plugs were removed from the growing edge on the fungal-colonised agar plates for inoculation of each flask. The three fungal species were grown in submerged liquid culture in 125-ml baffled Erlenmeyer flasks (4 baffles/flask) containing 25 ml of basal medium (Vogel minimum salts medium and glucose (1% w/v) as the carbon source, pH adjusted to 6.0). In cultures grown on basal medium containing veratryl alcohol, flasks contained 30.4 mM veratryl alcohol; the concentration at which maximal laccase was produced in *Botryosphaeria* sp. MAMB-5 (Vasconcelos et al., 2000). All cultures were incubated at 28±2°C on a rotary shaker at 180 rpm, and were grown in replicates of four. The results reported represent average values±SD.

Preparation of crude enzyme extracts Cell-free culture fluid was obtained after removal of the mycelium by centrifugation (30 min at 1250×*g*) and used as the source of extracellular enzyme. The mycelium recovered was washed three times with water, filtered, and ground under liquid nitrogen. The ground material was extracted with 0.6 M Tris-HCl buffer, pH 6.8 (1 ml g mycelium^{-1}), and the intracellular fluid recovered by centrifugation (5000×*g* for 20 min), stored at −20°C, and used in electrophoresis.

Enzyme assays Laccase activity was measured against two putative substrates, 2,2^1-azino-bis(3-ethyl-benzthiazoline-6-sulfonic acid) (ABTS, Sigma), and 2,6-dimethoxyphenol (DMP, Fluka Chemika AG, Switzerland). Activity towards ABTS (laccase PPO-I) was determined at pH 3.0 and 50°C (Barbosa et al., 1996), while activity against DMP (laccase PPO-II) was measured at pH 6.5 and 45°C (Vasconcelos et al., 2000). Laccase activity was expressed in units as μmol oxidised product formed min^{-1} ml^{-1} of enzyme solution for each substrate. All enzyme assays were performed in duplicate, and results represent mean values±SD as indicated by the error bars.

Polyacrylamide gel electrophoresis (PAGE) Electrophoresis was performed on polyacrylamide gels under non-denaturing conditions at alkaline pH by the procedure of Laemmli (1970) using crude cell-free (extracellular and intracellular) enzyme preparations. The separating and stacking gels contained 10% and 4% (w/v) acrylamide, respectively. The buffer solution used for the separating gel contained 40 mM Tris-HCl (pH 8.9), and that for the stacking gel, 10 mM Tris-HCl (pH 6.8). The electrode buffer contained 50 mM Tris-glycine (pH 8.9). Developed gels were stained for protein (Coomassie Brilliant Blue), esterases (α- and β-naphthyl-acetates (Sigma) in 50 mM Tris-HCl buffer (pH 7.1), Shaw and Prasad, 1970), phosphatases (α- and β-naphthyl-phosphates in 50 mM acetate (pH 5.5) and 50 mM Tris (pH 8.5) buffers, respectively; Brune et al., 1998), and laccases (50 mM ABTS and 20 mM DMP in McIlvaine's citrate-phosphate buffers, pH 3.0 (120 mM) and 6.5 (170 mM), respectively).

Characterisation of crude enzyme preparations Studies on the physicochemical properties of laccases in the crude enzyme preparations from the three fungal species included: pH optima (conducted over a pH range of 2.6–7.0 using McIlvaine's citrate-phosphate buffer system at 50°C), temperature optima (over a temperature range of 27–70°C at the optimum pH of each enzyme preparation), and the laccase activity-time relationship (over an interval of 60 min at the optimum pH and temperature for each enzyme preparation).

Results and Discussion

Laccase production and properties Three phytopathogenic botryosphaeriaceous fungi, *Botryosphaeria* sp. MAMB-5, *B. ribis* and *L. theobromae*, were examined to compare the production of laccases in both un-induced and veratryl alcohol-induced cultures. The profiles for the production of extracellular laccases by the three fungal species are shown in Fig. 1, and were similar for each of the species examined, although the levels of laccases produced differed significantly. *Botryosphaeria* sp. MAMB-5 produced the highest laccase titres towards the putative substrates, ABTS and DMP. The different laccase titres in un-induced cultures may have been due to the different nutrient requirements of each fungal species. Veratryl alcohol stimulated the extracellular production of laccase of both activity types (PPO-I and PPO-II, Fig. 1) for all three fungal species, but the high concentration employed (30.4 mM, optimised for *Botryosphaeria* sp. MAMB-5, Vasconcelos et al., 2000) may also have influenced the levels of laccase produced by

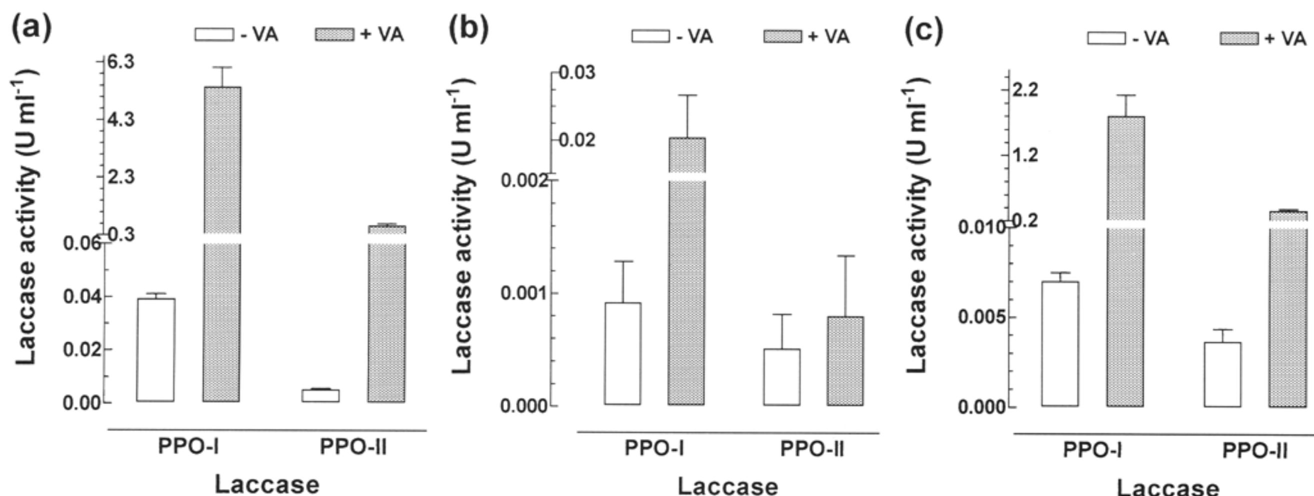

Fig 1. Extracellular laccase production by three species of botryosphaeriaceous fungi grown on basal medium in the presence and absence of veratryl alcohol. (a) *Botryosphaeria* sp. isolate MAMB-5; (b) *Botryosphaeria ribis* (10 replicate experiments); and (c) *Lasiodiplodia theobromae*. Values shown are means ± SD. PPO-I and PPO-II represent laccase activities towards ABTS and DMP substrates, respectively. + VA and − VA denote the presence and absence of veratryl alcohol, respectively.

the two other fungal species. This is because concentrations >30 mM veratryl alcohol became toxic to *Botryosphaeria* sp. MAMB-5 which could, nevertheless, tolerate concentrations up to 100 mM before growth was severely arrested (Dekker and Barbosa, 2001). *Botryosphaeria ribis* produced rather low levels of laccases of the PPO-II type, which could be induced above constitutive levels in the presence of veratryl alcohol (Fig. 1b). *Botryosphaeria* sp. MAMB-5 is unusual in that, as an ascomycete, it required rather high concentrations of veratryl alcohol to induce laccases above constitutive levels (Dekker and Barbosa, 2001). By contrast, basidiomycetes generally produced laccases when induced on low concentrations (1–4 mM) of veratryl alcohol (Eggert et al., 1996b), or not at all (Palmieri et al., 2000; Pointing et al., 2000;).

The physicochemical properties showed that the crude extracellular laccase activities produced by the three fungal species had similar pH (3.0 and 6.5) and temperature optima (50 °C and 45 °C) for PPO-I and PPO-II, respectively, in both veratryl alcohol-induced and uninduced fungal grown cultures. The enzyme activity-time relationship for the laccases produced by the three fungi when induced under both conditions showed a linear response over the first 10 min for both types of laccase activities.

Production of pycnidia and pycnidiospores Light was required for pycnidium formation in all cases where pycnidia were produced. When pycnidia were formed, the fungi colonised the agar medium and eucalypt woodchips with hyphae that interweaved to form a tight network in which a spherical mass formed that developed into the pycnidium fruiting body (primordium). The pycnidia were black, separated and globose in shape with diameters of 2–4 mm, and a mature pycnidium was observed in section under the microscope to contain black-pigmented pycnidiospores.

PDA did not support the production of pycnidia by all three fungal species, except under conditions where the mycelium was stimulated by physical abrasion, and then only for *B. ribis* and *L. theobromae* (Table 1). Physical abrasion of the mycelium of 10 day-grown cultures, such as produced through vigorous scraping with a spatula, induced a stressed condition which caused pycnidium formation in the fungi. All three fungal species failed to produce pycnidia when plated on glucose-minimal salts-agar, and only *B. ribis* and *L. theobromae* produced pycnidia following stress-induced stimulation, while *Botryosphaeria* sp. MAMB-5 failed to produce pycnidia under these conditions (Table 1). Citric acid has been reported to have a toxic effect on the germination of pycnidiospores in *L. theobromae* (Aderiye et al., 1998), and may have had a similar effect on pycnidium formation, since citric acid was a component in the minimal salts medium used. Oat-agar medium supported pycnidium formation when *B. ribis* and *L. theobromae* were cultivated under continuous light, and appeared to be the best artificial medium for effective pycnidium production by the three fungal species when stimulated under the conditions indicated in Table 1. Pycnidium formation in *Botryosphaeria* sp. MAMB-5 could not be triggered by lowering the temperature, which is a common feature for formation of fruiting bodies in basidiomycetes (Kües and Liu, 2000). Pycnidia, however, could easily be produced after 10 d under continuous light when *Botryosphaeria* sp. MAMB-5 was grown on eucalypt woodchips, the natural host for this pathogen.

Molecular characterisation of the two *Botryosphaeria* species and *Lasiodiplodia theobromae* An examination of the electrophoretic pattern of intracellular proteins stained with Coomassie Brilliant Blue showed that they were distinctly different for all three sets of fungi examined (data not presented), indicating that the three species were genetically unrelated. In *Botryosphaeria* sp.

Table 1. Production of pycnidia by two species of *Botryosphaeria* and *Lasiodiplodia theobromae* grown on different media at 28 °C under the conditions outlined below.

Conditions of growth[b]	Botryosphaeriaceous fungi[a]		
	Botryosphaeria sp. MAMB-5	*Botryosphaeria ribis*	*Lasiodiplodia theobromae*
PDA medium			
1	−	−	−
2	−	−	−
3	−	−	−
4	−	+	+
Oat-agar medium			
1	−	−	−
2	−	+	+
3	−	−	−
4	+++	++	+++
5	−	nt	nt
6	−	nt	nt
Glucose-minimal-salts-agar medium			
1	−	−	−
2	−	−	−
3	−	−	−
4	−	+	+
5	−	nt	nt
6	−	nt	nt
Eucalypt woodchips			
7	++++	nt	nt

a) +, ++, +++, ++++, relative degree of pycnidia production; −, no pycnidia production; nt, not tested

b) Growth: (1) in darkness; (2) under continuous fluorescent light; and physical abrasion by vigorously scraping the surface of 10 day-old mycelium with a spatula, and then allowing further growth for 2 wk under fluorescent light under: (3) a layer of added sterile water; (4) continuous fluorescent light; and at reduced temperatures: (5) 4 °C for 24 h; and (6) 0 °C for 6 h; followed by restoring the temperature to 28 °C. *Botryosphaeria* sp. MAMB-5 grown on (7) eucalypt woodchips under continuous fluorescent light at 28 °C (10 d).

MAMB-5, veratryl alcohol stimulated the synthesis of extracellular proteins (unpublished data), and it likewise stimulated the synthesis of intracellular proteins in all three fungal species examined, as judged by the intensity of protein staining with Coomassie Blue on PAGE.

All three fungal species produced intracellular α- (black staining) and β- (red staining) esterases, which in the case of *Botryosphaeria* sp. MAMB-5 and *B. ribis* were less heavily stained (low enzymatic activity) when the fungi were cultivated in the presence of veratryl alcohol (Fig. 2a). By contrast, *L. theobromae* esterase bands were more heavily stained (high esterase activity), and additional esterase bands appeared on PAGE when this fungus was cultivated on basal media containing veratryl alcohol. The results clearly show that there were distinct differences in the esterases produced by the three fungal species, indicating that they were genetically different. This observation was further corroborated by the activity stains for phosphatases. Only alkaline phosphatases were detectable and acid phosphatases failed to stain, probably due to low enzyme activity. Only one red-staining alkaline phosphatase band appeared on PAGE for each fungal species examined, which in *Botryosphaeria* sp. MAMB-5 and *L. theobromae* was more heavily stained (more enzymatic activity) when the cultures were grown in the presence of veratryl alcohol (Fig. 2b). In *B. ribis*, however, cultures grown in the absence of veratryl alcohol produced more alkaline phosphatase. In each case, the alkaline phosphatases migrated differently on PAGE, indicating that each protein was different, and unrelated to those in the other fungal species examined. The different zymogram patterns obtained for intracellular proteins, and the two genetic molecular markers (esterase and alkaline phosphatase) used to differentiate species of the same genus, have demonstrated that *Botryosphaeria* sp. isolate MAMB-5 was distinctly different genetically from the other two fungal species examined.

Zymogram staining for laccases with DMP revealed that all three fungi produced intracellular laccases of the PPO-II type which were electrophoretically similar, i.e., migrating to the same positions on the gels. The intensity of enzyme staining for each of the three fungal species showed that the intracellular levels of laccases in uninduced cultures were much higher than in veratryl alcohol-induced cultures. This finding was consistent with observations that extracellular laccase titres were generally higher when the fungi were cultured on basal media in the presence of veratryl alcohol (Fig. 1). The conclusion drawn from the zymogram observations was that veratryl alcohol facilitated the secretion of intracellular laccase into the extracellular medium, perhaps through some change in membrane structure or property, for all three fungal species examined. Electrophoretograms stained for laccases using ABTS revealed that laccase of the PPO-I type was also produced intracellularly.

In conclusion, the findings of this work demonstrated that *Botryosphaeria* sp. MAMB-5 was genetically unrelated to *B. ribis* and *L. theobromae*, although the laccases produced were electrophoretically similar and shared similar physical properties. The induction pattern of extracellular laccase titres by veratryl alcohol was also similar. Evidence was also presented that veratryl alcohol facilitated the secretion of intracellular laccase, probably through some change in membrane structure or property.

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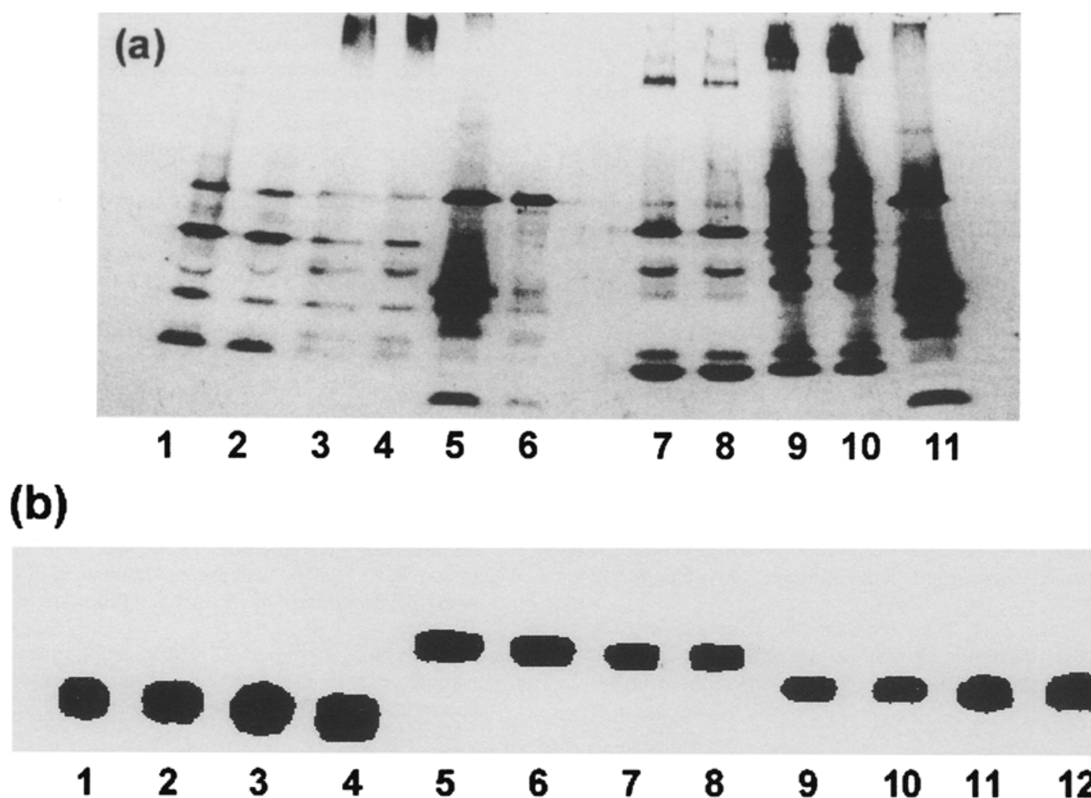

Fig 2. Electrophoretograms of intracellular molecular marker proteins of two species of *Botryosphaeria* and *Lasiodiplodia theobromae*. (a) Esterases stained with α- and β-naphthyl-acetates (lanes 1 to 4 represent *Botryosphaeria* sp. MAMB-5; lanes 5, 6 and 11, *B. ribis*; and lanes 7 to 10, *L. theobromae*. Lanes 3, 4, 6, 9 and 10 represent fungal cultures grown in the presence of veratryl alcohol), and (b) Alkaline phosphatases stained with β-naphthyl-phosphate (lanes 1 to 4 represent *Botryosphaeria* sp. MAMB-5; lanes 5 to 8, *B. ribis*; and lanes 9 to 12, *L. theobromae*. Lanes 3, 4; 7, 8; and 11, 12 represent cultures grown in the presence of veratryl alcohol). The gels were photographed and the photos scanned using a Genius-EP Scanner. Corel PhotoHouse software was used to lighten the background in (a), and to crop the darkened background around the staining bands in (b). Microsoft Windows Paint software was used to add labeling.

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