

GENETIC DIVERSITY AMONG *BOTRYOSPHAERIA* ISOLATES AND THEIR CORRELATION WITH CELL WALL-LYTIC ENZYME PRODUCTION

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ABSTRACT

Nine isolates of *Botryosphaeria* spp. were evaluated for their growth and the production of cell wall-lytic enzymes (laccase, pectinase and β -1,3-glucanase) when grown on basal medium in the absence and presence of the laccase inducer, veratryl alcohol (VA). The genetic relationship among the nine isolates collected from different host plants was determined by RAPD analyses. ITS sequence analysis showed eight closely related isolates classified as *Botryosphaeria rhodina*, and one isolate classified as *Botryosphaeria ribis*. RAPD analysis resolved the isolates into three main clusters based upon levels of laccase and β -1,3-glucanase activity. There appears to be no correlation between pectinase production and genetic diversity among the nine isolates. However, the strain characterized as *B. ribis*, positioned out of the main cluster, was found to be the highest producer of pectinases in the presence of VA.

Key words: *Botryosphaeria* isolates; ITS and RAPD; Laccase; Pectinase; β -1,3-Glucanase

INTRODUCTION

Fungi belonging to the genus *Botryosphaeria* (Botryosphaeriaceae, Dothideomycetes, Ascomycota) are plurivorous phytopathogens colonising a wide range of host plants of agricultural, forestry, ecological and economic importance, causing diseases, and are widely distributed worldwide in temperate and tropical climatic regions. As plant pathogens, the *Botryosphaeria* species primarily direct their attack on the plant cell wall by producing key enzymes associated with its hydrolysis (9).

An isolate of *Botryosphaeria* (MAMB 05) characterised as *B. rhodina* has been extensively studied in our laboratory regarding its production of laccases and exopolysaccharides. As a ligninolytic ascomycete, this fungal isolate produced only one kind of polyphenol oxidase, a laccase (*p*-diphenol:dioxygen oxidoreductase, EC 1.10.3.2) (4) whose production could be enhanced above constitutive levels in the presence of the inducer, veratryl alcohol (VA) (8). Laccases are multi-copper oxidases widely distributed among fungi associated with wood-

decay, basidiomycetes and ascomycetes alike, and are involved in plant pathogenesis (16). *B. rhodina* isolate MAMB 05 also produced the cell wall-lytic enzymes, pectinase (2) and β -1,3-glucanase (11), recognised as playing a role in pathogenesis in plants and fungi, as virulence factors.

Identification of species in the genus *Botryosphaeria* is complicated on several aspects. Morphological diversity among teleomorphs is often insufficient to allow identification at the species level, and species identification is based upon morphological characteristics of the anamorphs; the most common form of *Botryosphaeria* (10). Sequencing of the ITS region of rDNA has been extensively used in inter- as well as intra- specific comparisons of fungi (7). Sequence analysis of the ITS region alone (15), or in combination with sequences from others regions (22), have made a significant contribution to resolving taxonomic problems in the genus *Botryosphaeria*, and have been used to study relationships among species and to distinguish closely-related *Botryosphaeria* spp. (3).

Random amplified polymorphic DNA (RAPD) has been used extensively as genetic markers in different fungal species aimed

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at establishing a correlation between genetic diversity, and strain identification and differentiation (14). Consequently, RAPD has been used to demonstrate relationships among pathogenicity in *Botryosphaeriaceae* strains from different geographical locations (25).

A wide variety of commercial fruits and vegetables are subject to attack by pathogenic microorganisms including *Botryosphaeria* species that cause rots and spoilage. A characteristic of microbial spoilage of fruit is usually indicative of pectinolytic activity (20) as fruits are rich in pectins. Several *Botryosphaeria* isolates obtained from rotting fruit and vegetables and identified by morphological characteristics as *Lasiodiplodia theobromae* (the anamorphic form of *B. rhodina*), and other plant hosts (*B. rhodina* and *B. ribis*), were evaluated for their genetic diversity using RAPDs. In addition, the cell wall-lytic enzymes (laccase, pectinase and β -1,3-glucanase) were examined when the fungal isolates were grown under similar physiological conditions in attempts to determine a relationship between the genetic diversity of these pathogenic isolates and "pathogenicity-related" enzyme production.

MATERIALS AND METHODS

Botryosphaeria isolates

Botryosphaeria rhodina (isolate MAMB 05) was obtained from a stem canker on an eucalypt tree (4). *B. ribis* (EC 01) was isolated from *Eucalyptus citriodora*, and *L. theobromae* (MC 01) from currupixá wood (*Micropholis* spp.) (21). Six isolates of fungi morphologically identified as *L. theobromae* from different fruits and vegetables were obtained from Pernambuco (Brazil): MMLR (orange, *Citrus* sp.); MMGR (graviola *Annona muricata*); MMPI (pinha, *A. squamosa*); MMBJ (eggplant, *Solanum* sp.); MMMFR and MMMFO (mango, *Mangifera* sp., fruit and leaf, respectively).

Culture conditions

Fungal isolates were maintained through periodic transfer on potato-dextrose-agar at 4°C. Inoculum was prepared by growing the *Botryosphaeria* isolates on glucose-agar plates (8). Fungal cultures were grown in submerged liquid cultivation at 28°C and 180 rpm for 4.5 d in baffled Erlenmeyer flasks containing basal medium (Vogel minimal salts medium (29) and glucose (10 g/l) as carbon source, pH 6.0) (8). In the laccase induction experiments, veratryl alcohol was added to the basal medium prior to inoculation at a concentration of 30.4 mM (27). All experiments were carried out in replicates of 4, and the results represent the mean value \pm SD.

Species identification based on sequencing of the ITS1-5.8S-ITS2 regions of rDNA

Fungal genomic DNA was extracted according to the protocol of Bogo *et al.* (6). DNA quantification was performed

according to Sambrook and Russel (19). PCR reactions were carried out in a final volume of 25 μ l containing 5 - 25 ng of template; 10 mM Tris-HCl (pH 9.0); 50 mM KCl; 2 mM MgCl₂; 0.2 mM of each dNTP; 0.6 μ M of each primer; and 1 U of *Taq* DNA polymerase (Invitrogen, Brazil). DNA primers were used in the forward, ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and reverse, ITS4 (5'-TCCTCCGCTTATTGATATGC-3') directions (30). The amplifications were performed in a Thermocycler (PTC 100. MJ Research, Waltham, MA, USA) and programmed for an initial denaturation of 4 min at 92°C, followed by 35 cycles of 1 min at 95°C, 30 sec at 57°C and 1 min at 72°C, and a final extension of 5 min at 72°C. The amplified product was precipitated with 7.5 M ammonium acetate (0.1 volume) and absolute ethanol (3 volumes), and recovered in 10 μ l of ultrapure water.

Precipitated PCR fragments were sequenced in the forward and reverse directions with a MegaBace 1000 Sequencer (Amersham Biosciences, Piscataway, NJ, USA) using primers ITS1 and ITS4. Analyses of sequence quality and the construction of the consensus sequence were performed using Phred/Phrap/Consed software (12), and compared to sequences in the GenBank database using BLASTn as a search tool (1). The criterion used for species identification was based on the best score verified. Alignments were performed using BioEdit v 5.0.9 (13) and Clustal X (26). Sequences were manually adjusted to facilitate the analysis.

RAPD analysis

DNA was used as template for RAPD-PCR using the primers OPW2, OPW4, OPW5, OPW12, OPW14, OPX9, OPX13, OPX14 (Operon Technologies, Alameda, USA). PCR reactions were performed in a final volume of 10 μ l containing 1-10 ng of genomic DNA, 0.5 μ M of primer, 0.1 mM dNTPs (Eppendorf, Hamburg, Germany), 1.5 mM MgCl₂ and 1 U of *Taq* DNA polymerase (Invitrogen, Brazil). Reactions were done in a T1 Thermocycler (Biometra, Göttingen, Germany) set to the following conditions: 92°C for 3 min; 40 cycles each of 40 s at 92°C, 1.5 min at 40°C and 5 min at 72°C, followed by a final amplification period at 72°C for 7 min. At least two separate amplifications were conducted for each fungal isolate. Amplicons were separated by electrophoresis in 2% agarose gels (19). Gels were stained with ethidium bromide. The Jaccard similarity index (23) was used to construct a pairwise similarity matrix, which was used to perform hierarchical cluster analyses based upon the unweighted pairgroup method with arithmetic mean (UPGMA) using NTSYS software version 1.30 (18).

Analytical techniques

Extracellular fluid (ECF) was obtained after removal of the mycelium by centrifugation (30 min at 1250 x g) and used as the source of enzyme. Laccase activity was assayed against ABTS (Sigma; 2,2'-azino-bis(3-ethyl-benzthiazoline-6-sulfonic acid)) at pH 3.0 and 50°C (4), and expressed in units as μ mol oxidized

product formed/min/ml of enzyme. Pectinase and β -1,3-glucanase activity were determined using citrus pectin (Sigma; 10 g/l) and laminarin (Sigma; 4 g/l) as respective substrates at pH 5.0 and 50°C, and measured the reducing sugars produced (24). The unit of pectinase and β -1,3-glucanase activity are defined as the number of μ mol reducing sugars produced/min/ml under the standard assay conditions.

RESULTS AND DISCUSSION

Optimal fungal growth was previously established with *B. rhodina* (isolate MAMB 05) under non-induced (basal medium, no inducer) and induced (VA) conditions (27), and was used to grow the *Botryosphaeria* isolates in this study. All *Botryosphaeria* isolates grew on basal medium producing similar amounts of mycelial biomass.

PCR consistently produced a single amplification product of approximately 510 bp. It was possible to determine a 505 bp length sequence corresponding to the 18S partial sequence, the ITS1, 5.8S and ITS2 complete sequence, and the 28S partial sequence. Comparison of the sequence of *Botryosphaeria* isolate MAMB 05 with sequences deposited in GenBank showed a score between 914 and 971, with 99% similarity to *B. rhodina* and its anamorph, *L. theobromae*. The comparison of the sequence data strongly suggested that *Botryosphaeria* isolate MAMB 05 extensively studied by our group was *B. rhodina*. Isolates morphologically classified as *L. theobromae* were confirmed as *B. rhodina* by ITS comparison with sequences in GenBank. Similarly, ITS analysis also confirmed *B. ribis*.

The comparative analysis of the ITS1-5.8S-ITS2 sequences among all of the *B. rhodina* isolates showed high nucleotide similarity among them when compared to that identified as *B. ribis*. An intra-specific variation of 1.2% was observed between isolate MAMB 05 and all of the isolates from the fruits and vegetables examined, especially positioned at the 18S and ITS1 region. The majority of substitutions (83.33%) was observed for isolate MMMFO (leaf of mango tree), and only one substitution was observed for the sequence from isolate MMBJ (eggplant). The nucleotide changes comprised one transition at the 18S fragment, three at the ITS1 and two transversions at the 18S and ITS1, respectively.

The inter-specific variation was from 16.67% with 16 transitions (one at the 5.8S), 19 transversions distributed over the ITS1, ITS2 and 28S fragments, and two small deletions (two nucleotides each) at the ITS1 (all *B. rhodina* isolates) and the ITS2 (only *B. ribis* EC 01). Two important deletions, of approximately 24 and 17 bp, were observed between positions 67/91 and 98/113, respectively. This deletion was observed in all of the *B. rhodina* isolates and was found to be conserved in all of the *B. rhodina* and *L. theobromae* sequences deposited in GenBank, but were not observed in the Botryosphaeriaceae family (data not shown). This deletion should be considered an

important characteristic in discriminating the *B. rhodina* isolates from the other *Botryosphaeria* species.

Laccase production commenced by 48 h when the C source (glucose) was near-depleted from the culture medium and was accompanied by a fall in pH to 4.2 (8). Results for laccase production by all of the *Botryosphaeria* isolates examined are shown in Fig. 1a. Basal laccase levels (no inducer), albeit low, were highest for isolate MAMB 05 followed by MMLR (orange) and MC 01 (currupixa wood). *B. ribis* was a poor producer of laccase as confirmed in an earlier study (28). Under induced conditions, laccase activity could be increased up to 60-fold by the addition of VA to the basal medium with highest enzyme titres produced by isolate MMLR, which was some 1.5-fold higher than isolates MAMB 05 and MC 01 (Fig. 1a).

Previous studies (2) demonstrated that isolate MAMB 05 was pectinolytic and produced pectinase when grown on glucose as sole carbon source. The pectinolytic nature of other *Botryosphaeria* species was first reported in *B. ribis* (17). In the present study, all *Botryosphaeria* isolates grown on basal medium produced pectinase constitutively (Fig. 1b) indicating that all of the isolates were pectinolytic. In the presence of VA, the pectinase titres decreased except for *B. ribis*, which increased 3-fold under the conditions of cultivation. The decreased enzyme activities were due to VA repressing the synthesis of pectinase (2,9). In the case of *B. ribis* it appears, in contrast to the *B. rhodina* isolates studied, where the cell wall-degrading enzymes were repressed by VA that VA appeared to de-repress the synthesis of pectinase. How this occurs is not yet understood,

B. rhodina isolate MAMB 05 was previously demonstrated to produce β -1,3-glucanase when cultured on the substrate botryosphaeran (5), but also produced β -1,3-glucanase when grown on basal medium (11). In the present work, all of the *Botryosphaeria* isolates examined produced β -1,3-glucanase with highest enzyme titres produced by *B. ribis* (Fig. 1c). All *Botryosphaeria* isolates with the exception of MMMFR (mango fruit) and MMMFO produced higher β -1,3-glucanase levels than isolate MAMB 05. When the *Botryosphaeria* isolates were cultured under conditions for inducing laccase, the β -1,3-glucanase levels decreased for all isolates (MMMFO was an exception), in agreement with previous findings that VA repressed the synthesis of polysaccharide-degrading enzymes (9).

Ninety-one loci were observed showing 81.95% polymorphism. Although isolate MAMB 05 showed low variation at the ITS sequence compared to the other *B. rhodina* isolates, at the RAPD clustering, however, it was positioned out of the main group, showing 25% genetic similarity with the other *B. rhodina* isolates (Fig. 2). Comparison of the RAPD profiles with the ITS sequences suggests that the isolates examined can have a common genetic basis, with low variation at conserved regions, in spite of the high variability observed by RAPD analysis. ITS and RAPD analyses did not show any

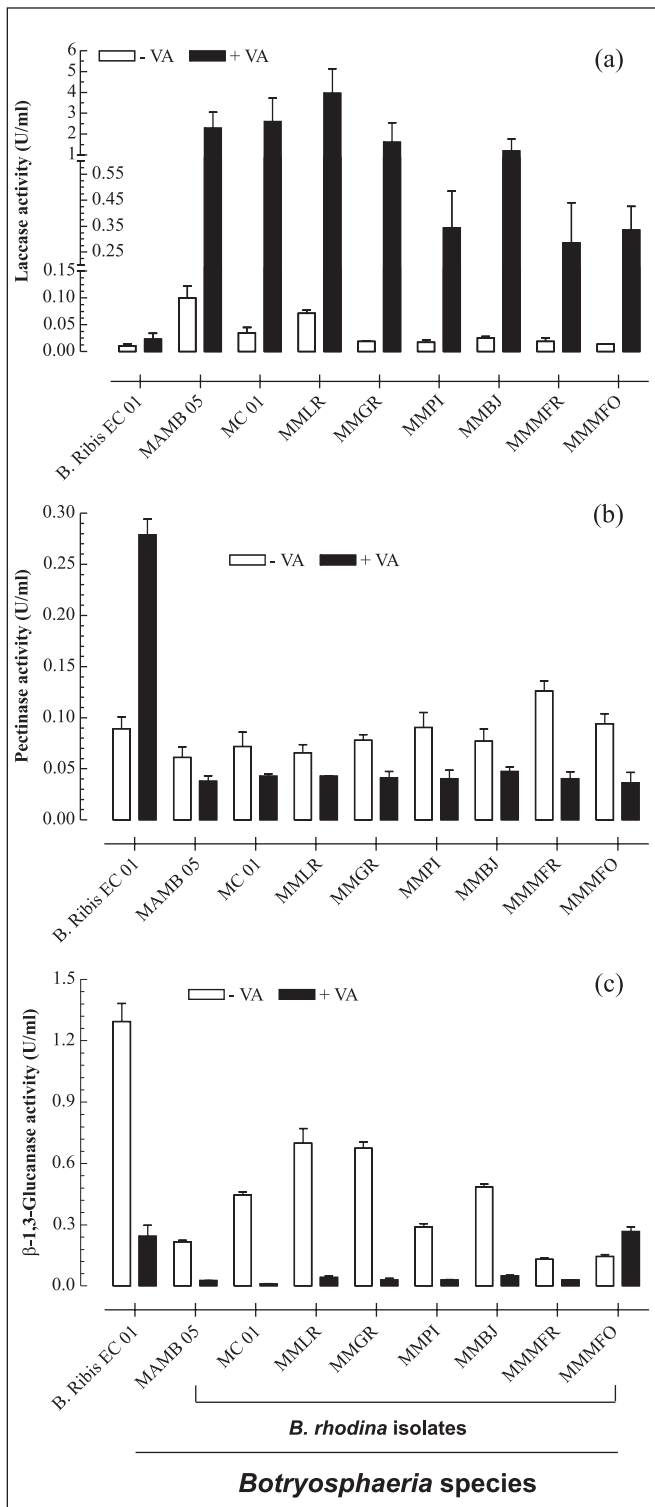


Figure 1. Comparison of the production of cell wall-lytic enzymes: (a) laccase, (b) pectinase and (c) β -1,3-glucanase by *Botryosphaeria* isolates grown on basal medium in the absence and presence of the laccase inducer, VA.

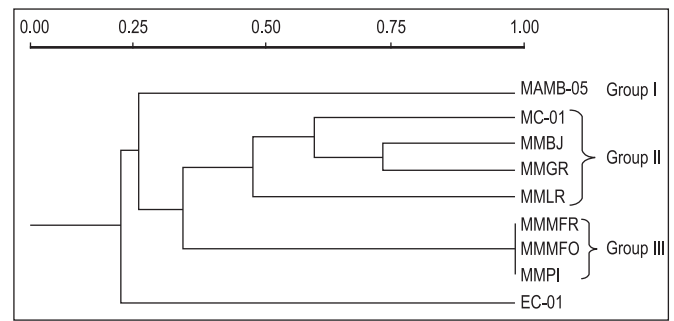


Figure 2. Genetic relationships among the nine isolates of *Botryosphaeria* based on UPGMA clustering of matrix obtained by the Jaccard similarity coefficient.

correlation with plant host origin and the genetic diversity of the isolates, an observation consistent with that described by Zhou and Stanosz (31). Three clear RAPD groups comprising the *B. rhodina* isolates can be observed (Fig. 2). Group-I, isolate MAMB 05 alone; Group-II, with the highest degree of genetic variation, comprised isolates MC 01, MMBJ, MMGR and MMLR; and Group-III, without variation despite having used 8 primers and comprised isolates MMMFR, MMMFO and MMPI (pinha). Comparison of the RAPD clustering and the enzyme production profiles shows a strong association between the groups and the production of laccase and β -1,3-glucanase. Groups I and II showed high levels of production of laccase ranging from 0.02 to 0.06 U/ml (non-induced) and 1.04 to 5.30 U/ml (induced), as well as greater levels of β -1,3-glucanase (from 0.45 to 0.70 U/ml), while Group-III showed significant lower production levels of these enzymes.

B. ribis strain EC 01 was positioned at an external branch of the tree (Fig. 2) with genetic similarity of 15% of the *B. rhodina* isolates group as expected. At the enzyme production levels, *B. ribis* demonstrated a very distinct pattern from the *B. rhodina* isolates, showing a basal level of synthesis of the inducible laccases. The levels of the β -1,3-glucanase in the absence of VA was higher than that observed for all of the *B. rhodina* isolates. The reason for the differences in the production of inducible laccase, as well as β -1,3-glucanase without VA, when compared to the other isolates, have yet to be clarified. In contrast to the laccases and β -1,3-glucanases, the pectinase production profiles did not show any correlation with genetic diversity. No apparent association could be observed between the groups and the levels of pectinase produced. No correlation could be established with the RAPD tree topology and the plant hosts of the fungal strains. According to Zhou and Stanosz (31), host types or their taxonomic relationships are not necessarily indicative of close relationships among different *Botryosphaeria* species and associated anamorphic fungi. The reason for this observation is still not clear.

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RESUMO

Diversidade genética entre isolados de *Botryosphaeria* e correlação com a produção de enzimas líticas da parede celular

Nove isolados de *Botryosphaeria* spp foram avaliados quanto ao crescimento e produção de enzimas líticas da parede celular (lacase, pectinase e β -1,3-glucanase) quando cultivados em meio basal na ausência e presença do indutor de lacase álcool veratrílico (VA). As relações genéticas entre os nove isolados coletados de diferentes plantas hospedeiras foram determinadas por RAPD. A análise das seqüências de nucleotídeos da região ITS mostrou oito isolados estreitamente relacionados, os quais foram classificados como *Botryosphaeria rhodina* e um isolado como *Botryosphaeria ribis*. A análise por RAPD agrupou os isolados em três grupos principais condizentes com os níveis de atividades de lacase e β -1,3-glucanase. Nenhuma correlação foi detectada entre a produção de pectinase e a diversidade genética nos nove isolados. Entretanto, a linhagem caracterizada como *B. ribis*, posicionada fora dos grupos principais, se mostrou maior produtora de pectinase na presença de álcool veratrílico.

Palavras chave: *Botryosphaeria*, ITS e RAPD, Lacase, Pectinase, β -1,3-Glucanase

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