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Endophytic and pathogenic isolates of the cacao fungal pathogen *Moniliophthora perniciosa* (Tricholomataceae) are indistinguishable based on genetic and physiological analysis

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ABSTRACT. We evaluated the genetic and physiological variability of *Moniliophthora perniciosa* obtained from healthy and diseased branches of cacao (*Theobroma cacao*) plants. The diversity of the isolates was evaluated by RAPD technique and by studies of virulence and exoenzyme production. The genetic variability of endophytic and pathogenic *M. perniciosa* was evaluated in association with pathogenicity assays. RAPD analysis showed eight genetic groups, which were not related to plant disease status (healthy versus diseased branches). Isolates from cacao were included in three groups, excluding isolates from other host plants. Pathogenicity and enzyme analysis showed that the virulence of the isolates is not

related to exoenzyme production. This is the first evidence that *M. perniciosa* colonizes healthy parenchymatic tissues, showing that endophytic behavior may occur in this species.

Key words: Cacao; Genetic variability; RAPD; Witches' broom; Exoenzymes

INTRODUCTION

The basidiomycete *Moniliophthora perniciosa* (formerly *Crinipellis perniciosa* (Stahel) Singer) is the causal agent of witches' broom disease of cacao (*Theobroma cacao* L.). Witches' broom is one of the main limiting factors for cacao production in South America and Caribbean islands, and is considered to be one of the most important pathogens for the crop (Griffith et al., 1994). The infection process occurs after basidiospore germination and penetration of the germ tube into meristematic tissues and pods (Orchard et al., 1994), starting the biotrophic stage of colonization (Muse et al., 1996). This phase is characterized by intercellular growth, thick and monokariotic hyphae without clamp connections (Calle et al., 1982; Silva and Matsuoka, 1999). In the next phase, the saprophytic stage, necrosis and intracellular colonization by dikaryotic hyphae occur (Muse et al., 1996).

The determination of the genetic variability of the pathogen is an important aspect for the selection of resistant genotypes in breeding programs. The characterization of different *M. perniciosa* isolates has been done by testing the pathogenicity with artificial inoculation (Bastos et al., 1988; Wheeler and Mepsted, 1988), somatic incompatibility assays and by assessing the biochemistry profile during the saprophytic phase (Griffith and Hedger, 1994). Molecular markers, including random amplified polymorphic DNA (RAPD), have also been used in studies of genetic diversity of plant-associated fungi (Anderbrhan and Furtek, 1994; Yamada et al., 1998; Anderbrhan et al., 1999; Sebastianes et al., 2007).

Endophytic fungi colonize their hosts without causing any external disease symptoms (Carroll, 1988), except when the host is under stress conditions. Studies on microorganisms from tropical plant species have recently become more frequent, since these fungi and bacteria are now studied for biological control and production of compounds with pharmacological properties (Araújo et al., 2008). Studies on the cacao endophytic community are recent, but practical aspects have been already evaluated (Arnold et al., 2003; Rubini et al., 2005). On the other hand, more studies on witches' broom disease and endophytic community interaction should be carried out.

Hypovirulent fungal strains can also be used on the biocontrol of plant diseases, as reported by Manandhar et al. (1998), when a hypovirulent *Pyricularia oryzae* isolate was able to reduce significantly neck blast caused by virulent strain under field conditions. Because of the pathological importance of *M. perniciosa*, the genetic (based on RAPD markers) and physiological (based on pathogenicity and extracellular enzymatic activity) analyses were used to distinguish isolates obtained from healthy and diseased cacao tissues. Therefore, the aim of the present study was to isolate *M. perniciosa* from healthy cacao tissues, and evaluate the pathogenicity of this isolate. This approach was used to

evaluate the presence of avirulent genotypes in healthy plants, which could be used in cross-inoculation for disease control, or to determine if the resistant plants could be used as a reservoir for pathogenic strains.

MATERIAL AND METHODS

Isolation of *M. perniciosa* from cacao

The *M. perniciosa* strains evaluated for this study were obtained from freshly cut healthy and infected cacao branches collected from different 20- to 40-year-old trees, located in Uruçuca and Lomanto Júnior, Bahia, Brazil. Fragments (12 cm) of cacao branches were surface disinfected according to Araújo et al. (2001). After disinfection, the bark was removed and branches were cut, and pieces (1 cm) were placed onto PDA (potato dextrose agar, Merck) medium containing tetracycline (50 μg/mL) and incubated for 5-20 days at 28°C. Colonies, from plant fragments, morphologically similar to *M. perniciosa*, were selected for further analysis. These isolates were identified based on hyphal morphology and rDNA sequencing. Isolates from other Brazilian cacao regions and also from different hosts were kindly supplied by cacao researchers and included in the present study for comparison (Table 1). For isolation of *M. perniciosa* from healthy tissues, fresh branches were collected from completely asymptomatic plants.

Pathogenicity assay

Pathogenicity of the *M. perniciosa* isolates (5, 6, 8, 9, 10, 31, 79, and FA284) was evaluated. The isolate FA42 was used as the positive control due to the high virulence exhibited in previous trials. Mycelial plugs from *M. perniciosa* cultures on PDA plates were transferred to Petri dishes filled with a sterile substrate for *M. perniciosa* basidiocarp development (517 g ground dry brooms, 21 g Ca_2SO_4, 138 g oat flour in 700 mL water). After substrate colonization, the plates were transferred to humid chambers at 28°C. Formation of basidiocarps commenced after 30 days. Spore suspensions were prepared according to Frias et al. (1995), where fresh basidiocarps were stuck on the inside of the lid of a Petri dish and basidiospores were collected in a 16% (v/v) glycerol solution.

Forty-five-day-old seedlings of the susceptible variety "common cacao" were inoculated by deposition of 50 μL of a 5 x 10^5 basidiospores/mL suspension on the apical active meristematic tissue. After inoculation, the seedlings were maintained in humid chambers for 48 h and then placed in the greenhouse. Evaluation was done after 60 days of the recording by checking the disease incidence. The experiment was designed in randomized blocks with three replicates, where each replicate consisted of five seedlings. Plants inoculated with sterile distilled water were used as controls. The presence or absence of diseased seedling in each replicate was counted and the frequency was calculated.

Extracellular enzyme production

All strains reported in Table 1 were evaluated for enzymatic activity on solid medium. Mycelial plugs (8 mm), from young colony (approximately 5 days old) borders, were

individually transferred to minimal medium - MM (Pontecorvo et al., 1953), without glucose, supplemented with different carbon sources according to the exoenzyme activity evaluation. All plates were incubated for 11 days at 28°C, when the halo/colony ratio was measured. The strains were evaluated using 3 replicates started at different times.

Lipase activity was evaluated on MM supplemented with Tween® 20 (1% - v/v). After fungal growth the culture was incubated at 4°C for 24 h and lipase production was measured by halo formation around the fungal colony. Amylase production was evaluated on MM supplemented with 1% (w/v) soluble starch. After fungal growth, 10 mL iodine solution (10 g iodine, 6 g potassium iodide dissolved in 20 mL water and the volume completed to 100 mL with ethanol) was added on the colony and the enzyme production was characterized by halo formation. Pectinase activity was determined on modified MM (7 g/L K_2HPO_4, 2 g/L KH_2PO_4, 1 g/L $(NH_4)_2SO_4$, 0.5 g/L $MgSO_4.7H_2O$, 10 g/L citric pectin, pH 6.8). The halo was measured after adding a 1% (w/v) solution of hexadecyltrimethylammonium bromide. The endoglucanase activity was evaluated after fungal growth on MM supplemented with carboxymethylcellulose. Congo red solution (0.1% - w/v) was added to the colony and then rinsed with 4 M NaCl. Finally, the growth of *M. perniciosa* strains on MM supplemented with 1% (w/v) Sigmacel indicated the production of endo- and exoglucanases.

RAPD analysis

Total DNA was extracted according to Raeder and Broda (1985). RAPD analysis was carried out in 25-µL reactions, containing 10 ng template DNA, 2.5 mM each of dNTP (Invitrogen), 5 mM $MgCl_2$, 2.5 U Taq DNA polymerase (Invitrogen), and 0.4 µM primer (OPA07, OPA16, OPA20, OPAX10, OPAX03, OPAX20, OPC08, OPP16, or OPX17; Operon Technologies, USA) in 10 mM Tris-HCl, pH 8.4, containing 10 mM KCl. The amplification conditions were 5 min at 94°C, followed by 40 cycles of 1 min at 92°C, 1 min at 35°C, 3 min at 72°C with a final extension at 72°C for 5 min. Polymerase chain reaction (PCR) products were analyzed on 1.4% agarose gel. Negative controls contained water instead of DNA.

Data analysis

The Dunnett test (P = 0.01) was used for statistical analysis and data comparisons were made using the strains FA42 as control. A dendrogram based on simple matching was constructed using unweighted pair group method with averages (UPGMA) cluster analysis. A consensus tree was obtained using the Winboot software (Yap and Nelson, 1996) with the bootstrap replicate number test set at 1000.

RESULTS

Isolation and pathogenicity of *C. perniciosa*

Two strains of *M. perniciosa* (31 and 54) isolated from healthy tissues of highly resistant and asymptomatic plants were considered to be endophytes; 11 strains (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, and 79) were obtained from diseased tissues of susceptible plants. Although the strains 31 and 54 were obtained from healthy plants, they were able to in-

duce witches' broom symptoms in inoculated seedlings at the same frequency as strains obtained from diseased branches. After 15 days a typical swelling at the apical meristem of the infected plants was observed. The disease frequency ranged from 53.3 to 100% for isolates 10 and FA42, respectively. The endophytic isolate 31 induced disease symptoms in 60% of the inoculated seedlings.

RAPD analysis

RAPD analysis of 35 *M. perniciosa* isolates (Table 1), using nine primers, generated 57 polymorphic bands that ranged from 600 to 3000 bp. These bands were used to construct a dendrogram (Figure 1). Eight major groups were identified (Figure 1 and Table 1); the cacao strain was included in groups G1, G2 and G3, while strains from other plant hosts were included in other groups. Endophytes were included in G2, but were indistinguishable from the pathogenic strains. Strains 1, 2, 3, 4, 5, and 6 were obtained from the same green broom and were arranged in the same group, as expected. They were also grouped together with the pathogenic isolates FA154 and FA152, isolated from cacao in Rondônia, a Brazilian State located 2000 km from Bahia.

Table 1. Description of *Moniliophthora perniciosa* strains used in the present study.

Strains	Isolation site	Host plant[§]	RAPD	Extracellular enzymes				
			group	Amyl	Pect	Egl	Lip	SiC
FA42	Lomanto Junior/BA	Cacao	G2	-	-	-	-	-
1, 5, 6, FA23	Uruçuca/BA	Cacao	G1	B	B	B	B	C
2	Uruçuca/BA	Cacao	G1	B	A	B	B	B
3	Uruçuca/BA	Cacao	G1	B	B	B	B	B
4	Uruçuca/BA	Cacao	G1	B	B	B	B	B
FA154	Ariquemes /RO	Cacao	G1	A	B	B	B	B
FA27, FA282	Lomanto Junior/BA	Cacao	G1	B	B	B	B	C
FA278	Lomanto Junior/BA	Cacao	G1	B	C	B	B	B
FA279	Uruçuca/BA	Cacao	G1	B	B	B	B	C
FA283	Lomanto Junior/BA	Cacao	G1	B	B	A	B	C
FA286	Inema/BA	Cacao	G1	A	B	A	B	C
FA287	Inema/BA	Cacao	G1	B	B	B	B	B
FA292	Itapé/BA	Cacao	G1	B	B	B	B	C
FA35	Belém/PA	Cacao	G1	B	B	B	B	B
FA152	Ouro Preto/RO	Cacao	G1	A	B	B	B	C
10	Lomanto Junior/BA	Cacao	G2	B	C	B	B	A
31	Uruçuca/BA	Cacao	G2	A	B	B	B	B
54	Uruçuca/BA	Cacao	G2	A	B	A	B	C
7	Lomanto Junior/BA	Cacao	G2	B	B	B	B	B
79	Uruçuca/BA	Cacao	G2	A	B	B	B	B
8, 9	Lomanto Junior/BA	Cacao	G2	A	A	B	B	C
FA276	Lomanto Junior/BA	Cacao	G2	B	A	B	B	A
FA281	Aiquara/BA	Cacao	G2	B	C	B	B	A
FA284	Barra do Rocha/BA	Cacao	G2	B	B	B	B	B
11	Rondônia	Cacao	G3	B	B	B	B	C
12	Unknown	*Solanum* sp	G6	A	A	B	B	C
13	Unknown	*S. licocarpum*	G4	B	B	B	B	C
FA104	Viçosa/MG	*S. cernuun*	G5	B	C	B	B	C
FA136	Ecuador	Liana	G7	B	C	B	B	C
322	Ecuador	Liana	G8	B	C	A	B	B

[§]All strains were isolated from symptomatic branches, except strains 31 and 54, which were isolated from healthy branches, obtained from resistant asymptomatic plants. Amyl = amylase; Pect = pectinase; Egl = endoglucanases; Lip = lipases; SiC = production of endo- and exoglucanases; A = halo/colony (H/C) ratio higher than the strain FA42; B = H/C similar to the strains FA42; C = H/C lower than the strains FA42 (Dunnett test, $P > 0.01$).

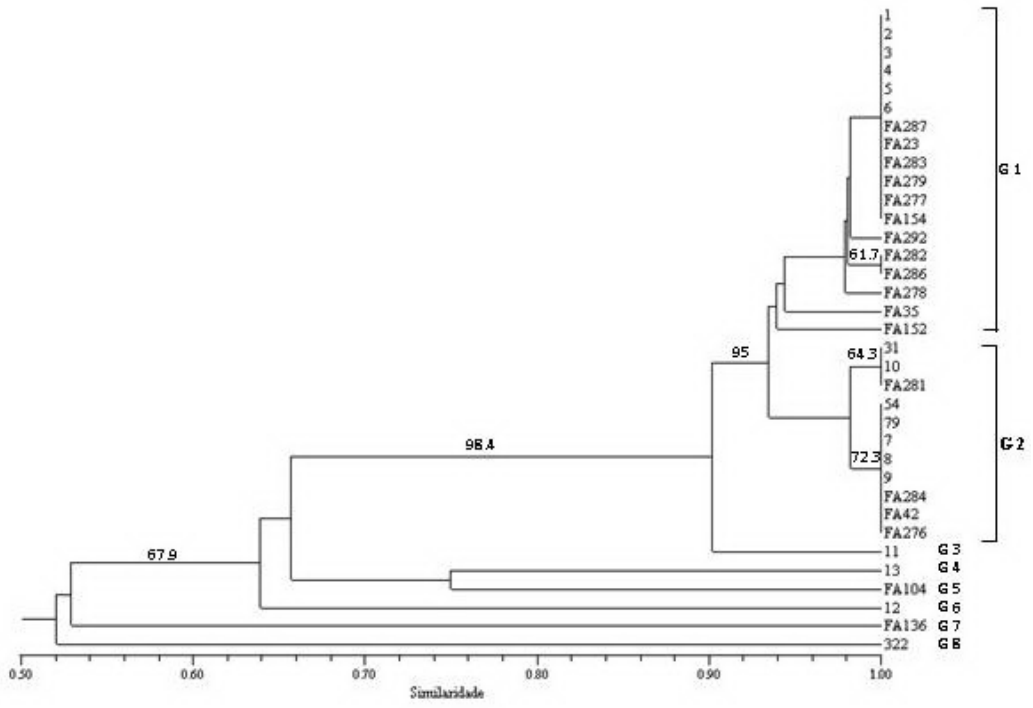

Figure 1. Dendrogram showing genetic relationships among *Moniliophthora perniciosa* isolates based on RAPD analysis. Bootstrap values (based on 1000 bootstrap samples) are placed on the tree.

Extracellular enzyme production

Lipase production by the strains evaluated did not differ from that of control strain FA42 (Table 1). Production of extracellular pectinases, amylase, exo- and endoglucanases allowed differentiation of the assayed isolates. However, this differentiation was not related to pathogenic/endophytic behavior (Table 1). The isolates 10, FA104, FA136, FA278, and FA281 did not produce detectable pectinases, while the isolates 54, FA283, FA286, and 322 showed higher production of endoglucanase than the isolate FA42 (Table 1). Isolates obtained from the same infected sample (1, 2, 3, 4, 5, and 6) showed different enzymatic activity. The endophytic isolate 31 was distinguished from FA42 only based on amylase production, while the endophytic isolate 54 was distinguished by amylase and exo- and endoglucanase.

DISCUSSION

Endophytic and pathogenic strains of *M. perniciosa* were characterized by RAPD banding patterns and were compared for their pathogenicity in cacao and their production of exoenzymes. Although the isolates 1, 2, 3, 4, 5, and 6, obtained from the same infected branch, showed the same RAPD profile, the exoenzyme production differentiated these

isolates, indicating that genetically different strains may colonize the same plant tissue despite having different virulence expressions. Similar results were obtained by Araújo et al. (2001) demonstrating that more than one strain of the same species of bacterium could be endophytic within an individual citrus plant. Anderbrhan and Furtek (1994) found that several basidiospores could share the same infection site in the field and, by RAPD analysis, a genetic heterogeneity was found in one monosporic culture, when compared with seven others, isolated from the same basidiocarp produced on *Solanum rugosum*. The presence of more than one genotype of *M. perniciosa* colonizing the same tissue can allow hyphal anastomosis leading to further heterokaryosis and eventually increasing genetic diversity in basidiospore generations (Wheeler and Mepsted, 1988).

M. perniciosa was isolated from healthy plant tissues. This is the first report of asymptomatic colonization of cacao tissues by *M. perniciosa* apart from apical meristems and buds. Because bark was removed from branches and the buds were removed, it is unlikely that the endophytic strains arose from meristematic tissues, where *M. perniciosa* may remain in latent infection. This suggests that the endophytic asymptomatic infection of cacao trees by this pathogen may be from common plant-pathogenic *Colletotrichum* species that are able to asymptomatically colonize plants and express nonpathogenic life-styles, conferring disease resistance, drought tolerance, and/or plant growth promotion (Redman et al., 2001).

RAPD analysis, pathogenicity tests and enzyme production did not reveal any genetic differences between endophytic and pathogenic isolates. In fact, the ability to induce disease symptoms may be under the control of a few genes, which could not be detected by these analyses. Freeman and Rodriguez (1993) observed that a mutation at a single locus could transform a virulent strain of *Colletotrichum magna* into an avirulent strain that was able to colonize its host endophytically; moreover, prior infection with the mutant protected plants against the wild type. The isolate 31 was virulent when inoculated onto seedlings, but this result may be explained by the high concentration of the spore in the inoculum suspension, since in the field the disease pressure would not reach that level. Mutualistic effects, such as induction of resistance will be further evaluated for endophytic strains 31 and 54.

The isolate 31 was virulent when inoculated on seedlings, but this result may be explained by the high concentration of the spore suspension inoculated, since in the field the disease pressure would not reach that level. Mutualistic effects, like resistance induction, have to be further evaluated for isolates 31 and 54. Sinclair and Cerkauskas (1996) clearly distinguished endophytic colonization and latent infection, based on the harmful effect on the host caused by pathogens. Latent infection is the state in which a host is infected with a pathogen but does not show symptoms and persists until signs or symptoms are prompted to appear by environmental or nutritional conditions or by the state of maturity of the host or pathogen (Agrios, 1988). The situation presented here indicates that the isolates of *M. perniciosa* obtained from a resistant host may be a pathogenic genotype that is able to colonize the resistant plant in latent state. Further investigation needs to be done to clarify if it will complete its life cycle after the plant dies, behaving like *M. perniciosa* L-type, which produces basidiocarp only on dead Liana-infected tissue (Evans HC, personal communication). In light of this, our future study will be directed at analyzing the nature of these isolates and the mechanisms involved in the endophytic colonization of cacao by *M. perniciosa*.

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