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Biological Control xxx (2008) xxx–xxx
Biological Control
www.elsevier.com/locate/ybcon

Colonization of cacao seedlings by *Trichoderma stromaticum*, a mycoparasite of the witches' broom pathogen, and its influence on plant growth and resistance

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Received 20 July 2007; accepted 15 January 2008

Abstract

Trichoderma stromaticum, a mycoparasite of the cacao witches' broom pathogen *Moniliophthora perniciosa*, is being used in Bahia, Brazil to control the disease under field conditions. The endophytic potential of this biocontrol agent was studied in both shoot and root tissues of sterile and nonsterile cacao and bean seedlings. Agar plate and light and electron microscopy studies showed that *T. stromaticum* is able to colonize extensively both cacao and bean plants grown under sterile conditions. However, colonization was lower when plants were grown under nonsterile conditions, especially in the shoot tissues. Recovery of *T. stromaticum* from field-grown trees showed that isolates belonging to genetic group II are more persistent (>120 days) as endophytes than isolates from group I. Endophytic colonization of cacao plants by *T. stromaticum* did not result in plant growth promotion nor induced resistance against *M. perniciosa* on seedlings that had been treated 30 days prior to application of the pathogen. These results were confirmed by Northern blot studies, where the fungus was unable to alter the expression of selected genes involved in plant defense such as *ChiB*, a putative class VII chitinase, *Glu-1*, a putative endo-1,4-β-glucanase, *Caf-1*, a putative caffeine synthase, and *Per-1* apoplastic quiacol peroxidase, genes involved in the regulation of plant growth *TcORFX-1*(*fw2.2*-like) and *TcLhca-1*(photosystem I 24 kDa protein) involved in energy production. This study indicates that induced resistance and growth promotion are not responsible for the activity of *T. stromaticum* in the biocontrol of the witches' broom pathogen.

Keywords: *Theobroma cacao*; *Trichoderma stromaticum*; Witches' broom disease; Biological control; Endophytes; Colonization

1. Introduction

Trichoderma stromaticum is a biological control agent of *Moniliophthora* (ex *Crinipellis*) *perniciosa* (Aime and Phillips-Mora, 2005), the causal agent of witches' broom disease of cacao (*Theobroma cacao*). This biocontrol agent is being used on an experimental basis in Brazil since 1999 as part of the integrated management of cacao (Samuels et al., 2000; Sanogo et al., 2002; De Souza et al., 2006).

The witches' broom disease has been responsible for the rapid decline in cacao production in Bahia, Brazil, by almost 75% since its introduction (Pereira et al., 1990). The pathogen is able to infect all meristematic tissues of the plant, including vegetative flushes, flower cushions,

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doi:10.1016/j.biocontrol.2008.01.010

single flowers, and pods. On vegetative flushes and cushion flowers, it causes symptoms known as "brooms", which are hyperplasic and hypertrophic branches caused by the hormonal imbalances induced by the pathogen. Infected flowers may result in parthenocarpic strawberry- and/or banana-shaped pods whereas infection of developed pods show necrotic lesions on the surface and rotten beans in the interior (Silva et al., 2002). Basidiospores produced inside the mushroom-like basidiocarps formed on the surface of all dead, infected tissues are the only infective propagule that occurs in nature (Silva et al., 2002).

Current integrated management recommendations for withes' broom include the use of copper fungicides, removal of diseased tissues (phytosanitation), grafting susceptible trees with resistant genotypes, and in a few cases biological control. Since 1999, CEPLAC, the institute responsible for cacao research and extension in Brazil, has been producing on a pilot scale a product known as 'Tricovab', based on a conidial preparation of *T. stromaticum* grown on rice grains (De Souza et al., 2006). *T. stromaticum* was shown to be an efficient mycoparasite of *M. perniciosa* (Bastos, 1996; Costa et al., 1996). Dead brooms and old infected pods are colonized by *T. stromaticum* that parasitizes the mycelia of *M. perniciosa* causing an acceleration in the decomposition of the dead infected tissues, leading to a reduction on basidiocarp production, and consequently on the basidiospore inoculum levels (Hjorth et al., 2003).

Diversity studies in Bahia State have shown the existence of two genetic groups among the isolates of *T. stromaticum*: the naturally occurring 'group I' and the 'group II', which are similar to the strain introduced from the Amazonian region of Brazil to compose the product 'Tricovab' (De Souza et al., 2006).

Some species in the genus *Trichoderma* are able to colonize plant roots and induce growth and resistance to diseases (Harman, 2000; Holmes et al., 2004; Yedidia et al., 2000). Earlier studies have shown that *T. stromaticum* was found as an endophyte in cacao, cupuassu (*T. grandiflorum*) and the closely related genus *Herrania* (De Souza et al., 2006). In this study, we adopt the term endophyte in its broader meaning: 'fungi that grow inside their host plants without causing visible symptoms'. Latent infection (Sinclair and Cerkauskas, 1996) by pathogens is not considered as endophytic colonization here. *T. stromaticum* is only found in Latin America, in close association with *Theobroma* and *Herrania*, suggesting that it probably co-evolved with the pathogen, *M. perniciosa*, as a mycoparasite or with the host plant as an endophyte (Hjorth et al., 2003). This intimate relationship with cacao suggests that, besides its action as a mycoparasite, *T. stromaticum* could also be exploited to induce resistance to diseases and to promote plant growth.

This research was conducted to investigate the potential of *T. stromaticum* as an endophyte of cacao and its possible activity as an inducer of resistance against *M. perniciosa* and as a promoter of plant growth. Experiments were conducted on sterile and nonsterile environments to study the ability of *T. stromaticum* to endophytically colonize cacao. Bean seedlings were used for comparison purposes. Field inoculations were carried out to study the recovery of *T. stromaticum* from adult cacao trees. Electron microscopy and culture on agar media were used to verify endophytic colonization. Resistance to *M. perniciosa* was studied by inoculating the pathogen and the mycoparasite on cacao seedlings in greenhouse experiments. Finally, Northern blot studies were conducted to verify the expression of selected genes involved in plant defense, gene regulation, and energy production.

2. Materials and methods

2.1. Microorganisms and inoculum production

Isolates AM13, BA17, and BA47 (group I), AM7 and BA66 (group II) of *T. stromaticum* were used in this study. Isolate AM13 was obtained from a dead broom collected in Paraiso, Colombia; AM7 was obtained from a dead cacao broom in Belém, Pará State, Brazil; BA17 from dead fallen broom in Ituberá, Bahia State; BA47 from a dead pod in Uruçuca, Bahia State and BA66 was isolated from inside the trunk of a cupuassu tree in Itajuípe, Bahia State. For spore production, *T. stromaticum* strains were grown on corn meal agar medium (CMA) (Difco, Detroit, MI) for 4 days at 25 °C. Spores were scraped from the plates and the concentration was adjusted to 10^7 spores per ml with the use of a hemocytometer. All isolates were stored in 10% glycerol at −80 °C. For every experiment, fresh plates were obtained by culturing the isolates from the −80 °C stocks. *M. perniciosa* isolate ALF 42, obtained from green, infected branches at the municipality of Itajuípe, Bahia State, was used in the induction of resistance assays. Basidiospores of *M. perniciosa* were produced, collected, and stored according to Macagnan et al. (2005). Briefly, the fungus was cultivated on PDA for 10 days under laboratory conditions. Mycelium plugs were then transferred to petri dishes containing a sterile substrate composed of a homogeneous mixture of finely ground dry witches' brooms (37%), oat flour (10%), $CaSO_4$ (1.5%), and water (51.5%). Petri dishes were kept in an incubator at 25 °C for 15 days. Fully colonized substrate was placed in a glass chamber ($1.0 \times 0.5 \times 0.5$ m) submitted to a watering regime of 1 l per day and a photoperiod of 8 h light and 16 h dark. After 15 days of incubation, the water supply was interrupted for 4 days to induce basidiocarp formation. The basidiocarps produced were collected, disinfested with streptomycin sulfate (150 μg per ml), washed with sterile distilled water, and had their caps fixed with vaseline to the upper part of petri dishes. The lower part of the cap remained free for the discharge of basidiospores, which were collected in a solution containing glycerol (16%) and 2-(*N*-morpholine) ethane sulfonic (MES) acid (0.195%) with shaking for a period of 18 h under laboratory conditions. Basidiospore suspensions were kept in liquid nitro-

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gen until use. Before use, the concentration of the basidiospore suspensions was adjusted to 5×10^5 basidiospores per ml.

2.2. Seed treatment and plant growth

Open-pollinated seeds of *Theobroma cacao* variety comum (Lower Amazon Amelonado type) were collected from established plantations at the Almirante Cacau, Inc., farm, Itabuna, Bahia State. Seeds were removed from the pods and peeled under sterile conditions; surface sterilized by soaking in 1% sodium hypochlorite for 5 min, then in 70% ethanol for 5 min and finally rinsed three times with sterile water. The seeds were air dried for 15 min in a sterile chamber and transferred to petri dishes containing water–agar. After 7 days of incubation at room temperature, when the radicles of the seeds were approximately 2 cm long, 50 μl of spore suspensions of the *T. stromaticum* isolates was deposited on the radicle of each seed. Control seeds were inoculated with 50 μl of sterile water. Petri dishes containing fungi- and/or bacteria-contaminated seeds were discarded. Bean seeds (variety Contender, Ferry-Morse Seed Co., Fulton, KY) were disinfested, incubated and treated as described for the cacao seeds, except that the incubation period was 4 days. Treated cacao and bean seeds were transferred either to sterile test tubes (~200 ml) containing approximately 30 ml of 0.5% water–agar (sterile system) or to nonsterile 3-kg-pots filled with a mixture of sand, perlite, and potting soil (1:2:1) (nonsterile system). Pots were kept inside glass boxes and both pots and tubes were maintained under fluorescent light at 25 °C in the laboratory. Plants growing in test tubes were not watered nor fertilized while the ones growing in pots were fertilized with 10 ml of Hoagland's solution every 15 days and water was given as needed. Unless otherwise indicated, all plants used in this study were grown as described above.

2.3. Endophytic colonization experiments

To quantify the endophytic colonization of different *T. stromaticum* isolates, five cacao and bean plants treated with each of the isolates used in this study were removed from the sterile test tubes or from the pots one month after the inoculations. Cacao and bean plants were divided into roots, crown, stem, cotyledon, leaves, and shoot tips. Cotyledons of bean plants were not evaluated in the nonsterile system because they detached early and were decomposed by saprotrophs. Each of these parts was disinfested according to the methodology described by Petrini et al. (1989) and then subdivided into 3–15 pieces to estimate the percentage of colonization, plated onto 1/5 strength PDA and incubated at room temperature. The efficiency of this method of disinfestation is well established in the literature (Petrini et al., 1989; Schulz et al., 1993; Arnold et al., 2000, 2001). The number of fragments colonized by each *T. stromaticum* isolate was recorded after 5 days, when colonies of the fungus were easily recognized by their typical sporulation on plates. The experiment was installed in a completely randomized design with five treatments (four *T. stromaticum* isolates and one water control), five replicates of one plant each and the whole experiment was done twice.

2.4. Light and electron microscopy studies

Roots and cotyledons of cacao seedlings uninoculated (control) and inoculated with strains AM7 and BA66 and grown under sterile conditions were collected 1 month after the inoculation and used in the light and electron microscopy studies. Roots were examined by transmission (TEM) and scanning (SEM) electron microscopy. For the light microscopy observations, roots and cotyledons of sterile plants were cut with a sharp blade, stained with 1% aqueous solution of toluidine blue, mounted on glass slides and observed microscopically. For the SEM, sterile seedlings were removed from the tubes using a large forceps and excess agar was carefully removed using a scalpel blade. All samples were placed on the surface of a copper specimen holder ($16 \times 29 \times 1.5$ mm). A small amount of the cryo-adhesive 'Tissue Tek' was applied to the mounting surface of the specimen holder prior to placement of the samples. Holders containing specimen materials were rapidly placed on the surface of a square brass metal tube pre-cooled in liquid nitrogen (−196 °C) within a styrofoam box. This process of contact freeze immobilization was performed on all samples used in the experiment. Numbered, frozen sample holders were placed into the same square brass tubing for storage in a liquid nitrogen storage dewar until removal for observation. Samples were transferred into a modified specimen carrier and transferred to an Oxford CT1500 HF cryo preparation system attached to a Hitachi S-4100 scanning electron microscope. Here the sample temperature was raised to −90 °C for 10 min to etch surface water from the sample surface. The sample was then cooled to below −120 °C and coated with 5–10 nm of platinum metal, using a magnetron sputter coater, to render the surface electrically conductive and yield more secondary electrons when scanned with the electron beam during observation in the SEM. Samples were transferred to the cold stage in the SEM at −170 °C and observed with an electron beam accelerating voltage of 2 kV. Micrographs were recorded on Polaroid Type 55P/N film. Some samples were returned to the cryopreparation system after observation and fractured at −170 °C, (in vacuo), using a cold scalpel blade (−196 °C), to visualize internal interactions of fungal hyphae with cacao cells. For the TEM observations, tissues from cacao plants inoculated as described above and control plants were fixed for 2 h at room temperature by immersion in 3% glutaraldehyde–0.05 M sodium cacodylate buffer, pH 7.0. They were then placed into a refrigerator at 4 °C overnight. This was followed by washing 6 times over 1 h with the above-stated buffer, post fixed in 2% buffered osmium tetroxide for 2 h,

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dehydrated in 100% ethanol and infiltrated with Spurrs low-viscosity embedding resin. Thin (10 nm) gold sections of the tissue were cut on a Riechert/AO Ultracut microtome with a Diatome diamond knife and mounted onto 200 mesh Ni grids. They were stained with 4% uranyl acetate and 3% lead citrate and viewed in an H-7000 Hitachi TEM at 75 kV. Micrographs were recorded on a digital camera.

2.5. Plant growth promotion experiments

The height of cacao seedlings growing in sterile tubes and on pots treated as described above (seed treatment and plant growth) was measured one month after the inoculations. The experiment was installed in a completely randomized design with five treatments (four *T. stromaticum* isolates and one water control), five replicates of one cacao seedling and was repeated at least three times.

2.6. Induction of resistance against M. perniciosa

Cacao seedlings were grown on test tubes filled with approximately 20 g of sterile carbonized rice husks. After 22 days, 10 ml of Hoagland's solution was supplied every 15 days and water was given as needed. One month after the *T. stromaticum* treatments, seedlings were kept in a humid chamber for 24 h before and 48 h after inoculation with 70 µl of basidiospore suspension of *M. perniciosa* on the shoot tips. Controls were inoculated either with the pathogen alone or with sterile distilled water. Evaluation was done 60 days after pathogen inoculation by recording the incidence of witches' broom disease. The disease is easily recognized by the hyperplasia and hypertrophy on the apex of the seedlings. The experiment was installed in a randomized block design with six treatments (four *T. stromaticum* isolates, *M. perniciosa* only and water control), five replicates of four plants each, and was done three times.

2.7. Recovery of T. stromaticum from field plants

A field plantation established at Almirante Cacau farm, Itajuípe, Bahia, Brazil containing 6-year-old cacao trees (clone TSH 1188) was used in these experiments. Each treatment consisted of spraying 10 trees separately with 300 ml of spore suspensions containing 10^7 conidia per ml of each of the *T. stromaticum* isolates. The experiment was installed in a completely randomized design with 10 replicates of one tree each and five treatments—four *T. stromaticum* isolates: AM17 and BA47 (group I); AM7 and BA66 (group II) (De Souza et al., 2006), and the control. Isolate AM13 was not used in these experiments to avoid the introduction of a foreign isolate in the field plantations of Bahia State. Control plants were sprayed with 300 ml of sterile distilled water. Trees sprayed with isolates from groups I and II were separated by at least 500 m to avoid contamination. Thirty days after spraying, eight small pieces of wood, ca. 25 mm^2 were removed from the trunk of each tree with a sterile scalpel according to the method described by Evans et al. (2003). The wood pieces were collected randomly from the trunk of the trees at heights above 1 m. The pieces of wood were placed in petri plates containing 20 ml of 1/5 potato dextrose agar acidified with acetic acid (pH 5.0) and incubated at 25 °C. The number of wood pieces colonized by *T. stromaticum* was recorded 5 days later. The isolation was repeated on the same trees 120 days after spraying. The experiment was done twice and analyzed separately.

2.8. RNA isolation and Northern blot experiments

Leaves were on three developmental stages, young red (2–6 cm long and pliable), immature green (6–15 cm long and pliable), and mature green (6–15 cm long and rigid), were collected from uninoculated seedlings (control) or seedlings inoculated with isolates AM7 and BA66 of *T. stromaticum* as described in seed treatment and plant growth. Leaves were collected after one month of growth under sterile and nonsterile conditions. RNA was isolated as described by Bailey et al. (2005). Ten micrograms (1 µg per µl) of total RNA was mixed with an equal volume of glyoxal/DMSO load dye (NorthernMax-Gly™ load dye, Ambion Inc., Austin, TX), denatured at 50 °C for 30 min, and separated by electrophoresis at 70 V for 1.5 h on a 1.3% agarose gel containing 300 mM Bis–Tris (bis[2-hydroxyethyl]iminotris[hydroxymethyl]methane, 100 mM Piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) [PIPES], and 10 mM EDTA, at pH 8.0 (1× BPTE). RNA was attached to Zeta-Probe®GT membrane (Bio-Rad Laboratories, Hercules, CA) by upward transfer in 10× SSC buffer. After cross-linking RNA to the membrane (UV Stratalinker 8600, Stratagene, La Jolla, CA), membranes were air-dried and stored at −20 °C. Six cacao cDNA fragments were cloned and used as probes in Northern blot experiments. These clones were selected to represent genes potentially involved in plant defense (*Caf-1*, *ChiB*, and *Per-1*), growth, and energy production (*ORFX-1*, *Glu-1*, and *Lhca-1*). Probes *ChiB*, a putative Class VII chitinase and *Glu-1*, a putative endo-1,4-β-glucanase, were isolated by suppression subtractive hybridization and reverse transcriptase-polymerase chain reaction, respectively (De Mayolo, 2003). *Caf-1*, a putative caffeine synthase, was isolated by reverse transcriptase-polymerase chain reaction. *ORFX-1* (*fw2.2*-like protein regulating cell number), *Per-1* (apoplastic quiacol peroxidase), and *Lhca-1* (photosystem I 24 kDa protein) were isolated from cacao leaves based on differential display expression in response to abiotic stresses (Bailey et al., 2005). The cDNA probes were purified using the GENECLEAN *Turbo* for PCR Kit (Q-BIO gene, Carlsbad, CA) following manufacturer's directions. For each probe, 35 ng of cDNA was labeled with [α-^{32}P] dCTP using a Random Primed DNA Labeling Kit™ (Roche Diagnostics, Inc., Indianapolis, IN), and gel-filtered (Edge Gel Filtration Cartridges™, Edge BioSystems, Gaithers-

burg, MD). Blots were prehybridized in ExpressHyb™ solution (BD Biosciences Clontech, Palo Alto, CA) at 68 °C for 1 h. Radioactively labeled cDNA probes were denatured at 95–100 °C for 5 min. The probe was added to 15 ml of fresh ExpressHyb™ solution, and blots were incubated with continuous shaking at 68 °C for 2 h. Blots were washed according to the ExpressHyb™ protocol, covered in plastic wrap, and exposed to a storage phosphor screen (Molecular Dynamics/Amersham Biosciences, Piscataway, NJ), and imaged at 200 μm resolution on a Typhoon 8600 Variable Mode Imager. Following imaging, the replicate blots were washed by placing in 10 mM Tris–HCL, pH 7.5, 1 mM EDTA, 1% SDS and micro-waving on high for 10 min, scanned to verify that no probe remained, and probed a second time with radioactively labeled 28S ribosomal cDNA probe.

2.9. Statistical analyses

Experimental data obtained for colonization, growth promotion and induction of resistance were subjected to normality test and two-way ANOVA (SAS Institute Inc., Cary, NC). Comparison of the means was done by Tukey's studentized range test. To compare the average colonization percentages between sterile and nonsterile conditions, for each cacao and bean seedling parts and to analyze the data sets from the induction of resistance experiments, the one-way nonparametric ANOVA of Kruskal–Wallis with comparison of means by the Wilcoxon's two sample test (SAS Institute) was employed. All experiments were analyzed separately.

3. Results

3.1. Colonization of cacao seedlings by T. stromaticum

There was no significant difference among the *T. stromaticum* isolates with regard to the percentage of colonization of the various plant parts of cacao and bean seedlings ($P < 0.05$) within each of the seedling assays studied, sterile or nonsterile. When sterile and nonsterile growing systems are compared with each other within each of the plant species, there were clear differences in colonization of all plant parts ($P < 0.05$), except for roots (Fig. 1). In the sterile system, *T. stromaticum* was able to endophytically colonize all parts of cacao and bean seedlings (Fig. 1A). The percentage of colonization was higher in parts closer to the point where the spore suspension was originally applied (roots, cotyledons, and crown) and lower in parts away from the application point (leaves, stem, and shoot tips). In the nonsterile system, the overall percentage of colonization was lower than in the sterile system (Fig. 1A). In this system *T. stromaticum* was not detected on leaves and shoot tips of both cacao and bean seedlings (Fig. 1). The fungus was never found in uninoculated control plants. Microorganisms other than *T. stromaticum* (fungi and bacteria) were found in all parts of cacao and bean seedlings grown under nonsterile conditions, but not on seedlings growing under sterile conditions.

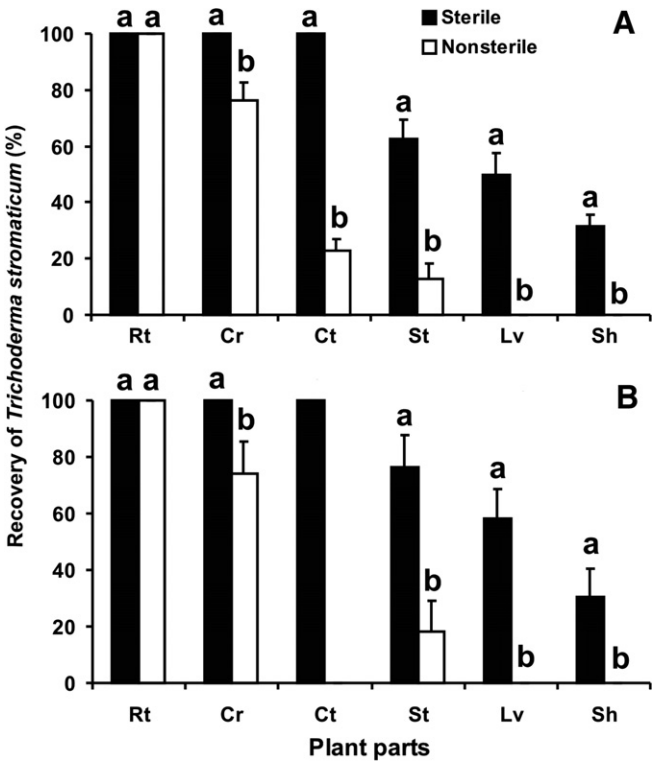

Fig. 1. Endophytic colonization of cacao (A) and bean (B) seedlings by *Trichoderma stromaticum* under sterile and nonsterile conditions. Seeds were treated with four different isolates of *T. stromaticum* and grown for one month under sterile and nonsterile conditions. Seedlings were harvested and divided into the following parts: Rt (roots), Cr (crown), Ct (cotyledons), St (stem), Lv (leaves), and Sh (shoot tip). Bean cotyledons were not evaluated under nonsterile conditions. Each plant part was disinfested, subdivided into 3–15 pieces and plated onto 1/5 PDA. The number of colonized pieces within each plant part was scored after 5 days of incubation. The data presented correspond to the average percentage of recovery of the four isolates of *T. stromaticum* from the different parts of the seedlings. Bars with the same letter above indicate that means are not significantly different according to Wilcoxon's two sample test ($P = 0.05$). Comparisons were done within each plant part and between sterile and nonsterile conditions. Error bars represent the standard error of the means.

3.2. Light and electron microscopy studies

These studies were carried out to confirm the presence of *T. stromaticum* in the interior of cacao tissues. Roots and cotyledons of seedlings grown under sterile conditions were examined by light microscopy, and roots were examined by transmission (TEM) and scanning (SEM) electron microscopy. *T. stromaticum* was found growing between the cacao cells in all tissues examined by light (Fig. 2A–C) and electron microscopy (Fig. 2D–H). Light, SEM, and TEM observations showed that *T. stromaticum* was located in the superficial layers of the inoculated cacao roots, not being observed beyond the outer cortex (data not shown). On the other hand, the fungus was widely distributed in the

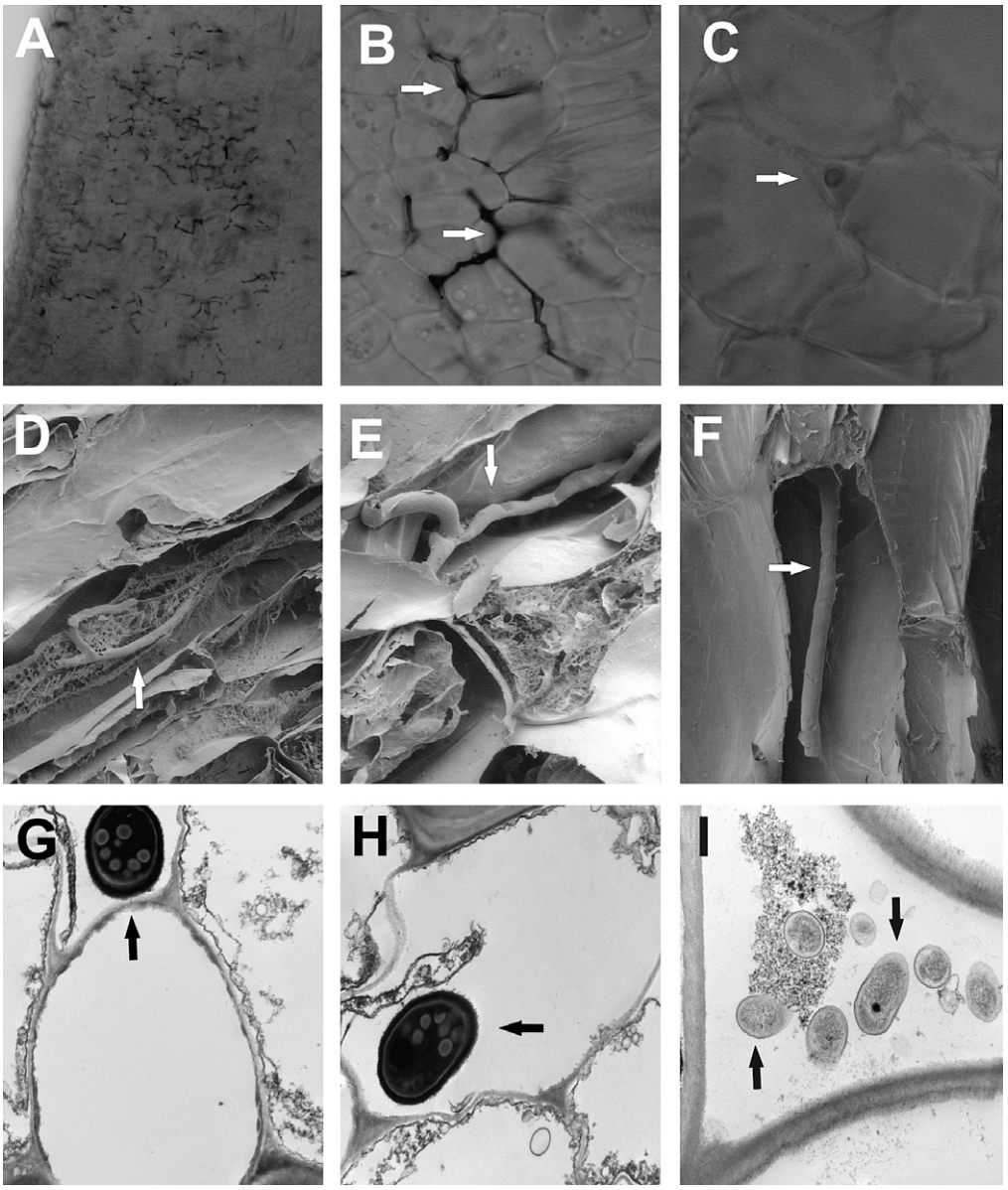

Fig. 2. Endophytic colonization cacao seedlings by *Trichoderma stromaticum*. Light microscopy photographs of cotyledons (A and B) and roots (C) colonized by *T. stromaticum*. Magnifications are 10× (A), 40× (B), and 100× (C). Scanning electron micrographs of colonized roots (D–F). Magnifications are 500× (D), 900× (E), and 1000× (F). Transmission electron micrographs of colonized roots. Magnifications are 10,000× (G and H) and 30,000× (I). Arrows indicate the mycelium of the fungus (A–H) and bacterial cells (I).

cotyledons (Fig. 2A and B). No fungi were observed growing in the interior of uninoculated seedling tissues (data not shown). However, bacterial cells were occasionally observed in some of the root samples (Fig. 2I). These bacteria were not able to grow on the medium used to isolate *T. stromaticum* from seedlings grown under sterile environment.

3.3. Cacao growth promotion

The results of these experiments showed no significant differences among the isolates ($P > 0.05$) and between the control and inoculated seedlings ($P > 0.05$) with respect to plant height within both sterile and nonsterile systems (data not shown). The average height of control and inoculated cacao seedlings was 16.92 cm and 16.5 cm, respectively, for the sterile system and 19.6 and 20.8 cm for the nonsterile system. The other two experiments had similar results. These results showed that *T. stromaticum* was not able to promote growth of cacao seedlings.

3.4. Induction of resistance against M. perniciosa

According to the results obtained, there was no significant difference in the percentage of diseased cacao seedlings treated with different *T. stromaticum* isolates and between the treated and untreated seedlings ($P = 0.78$; data not shown). Percentage of diseased seedlings was 60% for the

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control (only *M. perniciosa*) and the average was 46.3% for the *T. stromaticum*-treated seedlings. No symptoms of the disease were observed on untreated seedlings. Another repeated experiment showed similar results (data not shown). These results demonstrated that inoculation of cacao seedlings with *T. stromaticum* 30 days before the inoculation of *M. perniciosa* was not able to induce resistance to the pathogen.

3.5. Recovery of *T. stromaticum* from field trees

Isolations done 30 days after spraying revealed that an average of 80%, 50%, 90%, and 70% of the cacao trees were colonized by strains BA17, BA47, AM7, and BA66, respectively. After 120 days, colonization by strains BA17, BA47, AM7, and BA66 was 0%, 0%, 20%, and 10% of the sprayed trees, respectively. No *T. stromaticum* was ever found colonizing control trees. Results of a repeated experiment were similar. These results (data not shown) indicate that strains from genetic group II (AM7 and BA66) were more persistent in the field and could be recovered from inside sprayed trees 4 months after the treatment. Strains from genetic group I (BA17 and BA47) could not be recovered 120 days after spraying.

3.6. Influence of *T. stromaticum* in the expression of selected cacao genes

To further characterize the interaction between *T. stromaticum* and cacao plants, the influence of isolates AM7 and BA66 of *T. stromaticum* on the expression of six genes was studied. The genes studied and their putative function were: *ChiB*, a putative class VII chitinase, *Glu-1*, a putative endo-1,4-β-glucanase, *Caf-1*, a putative caffeine synthase, and *Per-1* (apoplastic quiacol peroxidase) are potentially involved in plant defense; *ORFX-1*(*fw2.2*-like) involved in the regulation of cell number (growth), and *Lhca-1*(photosystem I 24 kDa protein), involved in energy production. Our results showed no difference between the two isolates of *T. stromaticum* or between the control and the *T. stromaticum* isolates with respect to the expression of all six genes (Fig. 3). There were also no differences in gene expression among the different developmental stages of the leaves in both sterile and nonsterile systems (data not shown).

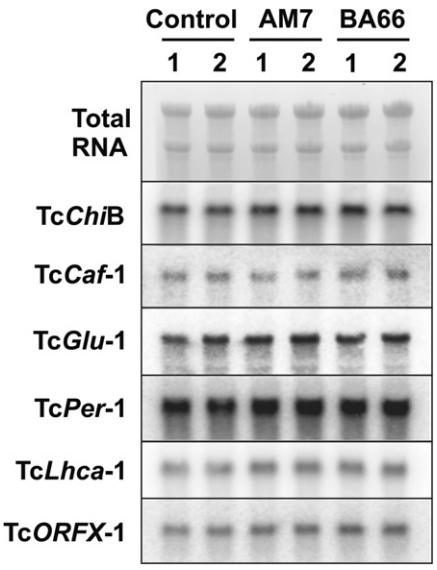

Fig. 3. Influence of *Trichoderma stromaticum* on the expression of cacao genes on young red leaves grown under sterile conditions. Cacao leaves were collected from 1-month-old seedlings inoculated with isolates AM7 and BA77 of *T. stromaticum* or sprayed with water (control). The expression of the different genes was evaluated by Northern blots employing radioactive probes. The expression of the following cacao genes was evaluated: ChiB (chitinase), Caf-1 (caffeine synthase), Glu-1 (endo-1,4-β-glucanase), Per-1 (peroxidase), Lhca-1 (photosystem I protein), and ORFX-1 (a *fw2.2*-like protein). Results are shown in duplicate for each isolate of *T. stromaticum*.

4. Discussion

Trichoderma stromaticum is currently being used in the cacao plantations of Bahia State, Brazil as a biocontrol agent of witches' broom disease. This mycoparasite is occasionally found in the field as an endophyte in association with the cortex of cacao trunks. The experiments reported here were performed to gain more insight on the significance of the endophytic activity of *T. stromaticum* in cacao. Our results showed that this fungus colonized endophytically cacao seedlings but was unable to induce plant growth and resistance to *M. perniciosa*.

Colonization studies showed that *T. stromaticum* isolates were able to endophytically colonize cacao and bean seedlings under sterile conditions better than under nonsterile conditions (Fig. 1). These results were expected once plants growing under sterile conditions are free of, or have smaller populations of microbial competitors. Although the isolates used in this study belong to both genetic group I (AM13 and BA47) and group II (BA66 and AM7), they had similar colonization levels when it was analyzed 30 days after the inoculations. Field experiments also confirmed that strains from both genetic groups showed similar colonization levels 30 days after spraying adult trees in a commercial plantation. However, when endophytic colonization was evaluated 120 days after spraying, only isolates from genetic group II (BA17 and BA47) could be recovered, indicating that these isolates persist longer as endophytes. Other observations support these results: (a) only isolates from group II were ever found endophytically in cacao and other closely related species in nature (De Souza et al., 2006); (b) in other experiments we found that *T. stromaticum* isolate BA29, which belongs to group II and is closely related to AM7 and BA66 (both used in this study), was recovered from approximately 5% of the cacao trees that had been sprayed with this isolate four months after the applications; c) extensive surveys on more than 1000 cacao trees in Bahia State failed to recover endophytic

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strains from group I in areas with natural occurrence of this group in high densities over brooms on the ground (De Souza, J.T. and Pomella, A.W.V., unpublished data). The reasons for the longer persistence of group II isolates as endophytes are currently unknown, but are probably related to the genetic differences existent between the groups (De Souza et al., 2006).

Despite being a species that supposedly coevolved with cacao (Hjorth et al., 2003), *T. stromaticum* is also able to colonize bean seedlings at similar levels (Figs. 1 and 2) indicating, as expected, a lack of host specificity under controlled conditions. Under natural conditions, the occurrence of *T. stromaticum* in association with plants other than cacao and related species is not expected because of its close association with cacao. Although able to colonize cacao and bean seedlings, *T. stromaticum* seems to have a poor competitive ability to colonize endophytically the aerial parts of the seedling. This is corroborated by the dramatic decrease, as shown in this study, in the recovery of *T. stromaticum* from seedlings grown under nonsterile environment as compared to the ones grown under sterile environment and from plant parts away from the point of inoculation (Fig. 1). These data are in part in agreement with the ones obtained by other authors who pointed out the poor competitive ability of most *Trichoderma* species in soil environments (Papavizas, 1981, 1985; Chao et al., 1986; Ahmad and Baker, 1987). Competition with fungi and bacteria from the soil that invaded the cacao seedlings are probably responsible for the lower colonization of cacao seedlings grown under nonsterile conditions as compared to the ones grown under sterile conditions.

The lack of induction in cacao growth and resistance to *M. perniciosa* by *T. stromaticum* is supported by our molecular analyses that showed no influence of the biocontrol agent on the expression of cacao genes putatively involved in disease resistance and plant growth (Fig. 3). The phenomenon of plant growth apparently does not occur in all *Trichoderma* plant interactions and is induced by a limited number of *Trichoderma* species, including *T. viride*, *T. harzianum*, and *T. pseudokoningii* (Chang et al., 1986; Windham et al., 1986; Zheng and Shetty, 2000; Harman, 2000; Yedidia et al., 1999, 2001). These *Trichoderma* species promoted growth on cucumber, corn, poinsettia, geranium, petunia, and pea.

Resistance against plant pathogens may be localized or systemic (van Loon et al., 1998). Systemic resistance may occur by two mechanisms: systemic acquired resistance (SAR) and induced systemic resistance (ISR). SAR is induced by several agents, including certain chemicals, pathogens and avirulent forms of pathogens (Durrant and Dong, 2004). This mechanism involves the accumulation of salicylic acid (SA) and pathogenesis-related proteins (PRs) (Ryals et al., 1996). ISR is induced by nonpathogenic bacteria and SA and PR proteins are not involved (van Loon et al., 1998). A recent report have shown that *M. perniciosa* itself is capable of producing SA, both in *in-vitro* and in *in-vivo* (Chaves and Gianfagna, 2006). The role of SA in the pathogen infection, subsequent disease progression, and tissue necrosis is still unknown.

Compelling evidence accumulated and indicates that metabolic changes induced by some *Trichoderma* species are responsible for the increased systemic resistance to pathogens in plants. In the case of the resistance induced by *Trichoderma* species, PR proteins seem to be involved. Increased activity of the PR proteins chitinase, peroxidase, glucanase, and cellulase is pointed out as responsible for the induction of defense responses in cucumber by *T. harzianum* (Yedidia et al., 1999, 2000). These increases initiated between 48 and 72 h and decreased after 120 h post-inoculation of *T. harzianum* on cucumber. In our studies, due to the fact that our inoculations were done on seeds and cacao is a perennial tree, the transcription of putative defense response- (chitinase, glucanase, caffeine synthase, and peroxidase) and growth-related genes (cell number regulator and photosystem I) was assessed in leaves collected 30 days after the inoculation of *T. stromaticum* on cacao seeds. This might explain the lack of increase in the amount of transcripts of these genes. Further studies are required to assess the expression of these and other genes at different time intervals after the inoculation of cacao seedlings with *T. stromaticum*. We are initiating studies on the pattern of gene expression with real-time quantitative polymerase chain reaction (Q-PCR) to assess the expression of cacao and *T. stromaticum* genes during colonization. Preliminary results showed 6- and 2.3-fold inductions for two unknown cacao genes upon colonization with *T. stromaticum* and slight (~1.0-fold) inductions of a apoplastic quiacol peroxidase-like protein and a chitinase Tc*Chi*B, these last two putatively involved in plant defense. These studies were conducted with samples obtained from 1-week-old seedlings colonized by isolate AM7 of *T. stromaticum* (Bae, H. and Bailey, B.A., unpublished data). These levels of gene expression are in the same range of expression showed by four other *Trichoderma* species associated with cacao (Bailey et al., 2006).

Arbuscular mycorrhizal fungi form symbiotic associations with a wide range of plants including cacao and induce local and systemic resistance against some pathogens through gene expression or through direct anti-fungal effect (Graham, 2001). Studies on these associations demonstrated that the expression of plant genes related to resistance is weak upon mycorrhizal colonization and, at later stages, colonization even suppresses plant defense related genes (Blee and Anderson, 2000). It is possible that the levels of resistance induced by *T. stromaticum* were not high enough to block the infection of the witches' broom pathogen. On the other hand, SAR may not provide resistance to *M. perniciosa* in cacao. It is well established that SAR is not effective against all pathogens of a given host plant. For example, in tobacco, this mechanism confers protection to seven out of nine pathogens (Ryals et al., 1996).

Field trials in Bahia have shown increased pod production and reduced flower cushion brooms in trees sprayed with *T. stromaticum* group II formulations (Hebbar,

P.K., unpublished data). *Trichoderma* species such as *T. harzianum*, as well as cacao-associated *T. stromaticum*, *T. paucisporum*, and *T. theobromicola* are able to produce several anti-fungal compounds that can inhibit, at low concentrations, both *M. perniciosa* and *M. roreri* (Aneja et al., 2006). This would partly explain the activity of *T. stromaticum* against the *M. perniciosa* in the field. Additionally, the ability of *T. stromaticum* to colonize root tissue endophytically is worth investigating further because there are no remedies against important diseases such as *Ceratocystis cacaofunesta* that causes wilting and eventual death of mature cacao trees in Brazilian plantations (Baker et al., 2003; Engelbrecht and Harrington, 2005).

In conclusion, this study shows that *T. stromaticum*, although able to colonize cacao is unable to induce growth and resistance to *M. perniciosa* when applied to seeds 30 days before the inoculation of the pathogen. *T. stromaticum* isolates belonging to genetic group II persist longer than isolates from group I as endophytes in adult trees in the field. This work provides preliminary evidence that growth promotion and induction of resistance are not involved in the biocontrol activity of *T. stromaticum* against the witches' broom pathogen.

Acknowledgments

The authors thank Aguinaldo Guimarães, Alício Costa, Mary Strem, and Christie-Sue Decker for technical assistance. USDA-ARS and Mars Inc. for financial support.

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