

## ISOLATE OF *Trichoderma brevicompactum* FOR THE CONTROL OF COCOA WITCHES' BROOM DISEASE: PRELIMINARY RESULTS

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The aim of this paper was to study in vitro and in vivo the effects of one isolate of *Trichoderma brevicompactum* as a possible biocontrol agent for witches' broom disease. The antagonist was isolated from inner trunk tissues of cocoa, after surface desinfestation with sodium hypochlorite solution, rinsed once in 70% ethanol and then twice in sterile distilled water. The isolate of *Trichoderma* produced in liquid medium metabolites capable of preventing the germination of basidiospores and the mycelial growth of *Moniliophthora perniciosa* when culture filtrate was used at 2% and 10% concentrations respectively. The production of basidiocarps was significantly reduced by one application of spore suspension ( $2 \times 10^7$  spores/ml) of *T. brevicompactum* on dried brooms. Experiments carried out in greenhouse showed that spore suspension ( $2 \times 10^7$  spores/ml) and culture filtrate at the concentration of 5% were able to reduce the incidence of witches' broom in cocoa seedlings and also the number of diseased pods in field.

**Key words:** *Moniliophthora perniciosa*, *Theobroma cacao*, antagonist, biocontrol, *Trichoderma*.

**Isolado de *Trichoderma brevicompactum* para o controle da vassoura-de-bruxa do cacaueiro: Resultados preliminares.** O objetivo do presente trabalho foi estudar *in vitro* e *in vivo* o efeito de um isolado de *Trichoderma brevicompactum* como possível agente de biocontrole da vassoura-de-bruxa do cacaueiro. O antagonista foi isolado da parte interna de um tronco de cacaueiro, depois da desinfecção dos tecidos, com hipoclorito de sódio, etanol (70%) e lavagem duas vezes com água destilada esterilizada. O fungo produziu em meio líquido metabólitos capazes de evitar a germinação de basidiósporos e o crescimento micelial de *Moniliophthora perniciosa*, quando o filtrado de cultura foi usado nas concentrações de 2% e 10%, respectivamente. A produção de basidiocarpos foi significativamente reduzida depois da aplicação da suspensão de esporos ( $2 \times 10^7$  esporos/mL) em vassoura secas. Foi verificado que a suspensão de esporos ( $2 \times 10^7$  esporos/mL) e o filtrado de cultura na concentração de 5% foram capazes de reduzir a incidência da vassoura-de-bruxa em casa de vegetação e no campo.

**Palavras-chave:** *Moniliophthora perniciosa*, *Theobroma cacao*, antagonista, controle biológico, *Trichoderma*.

## Introduction

The fungus *Moniliophthora* (= *Crinipellis*) *perniciosa* (Stahel) Aime & Phillips-Mora, the causal agent of witches' broom disease of cocoa (*Theobroma cacao* L.), is the main factor limiting cocoa production in the Americas (Baker e Holliday, 1957). Pod losses of up 90% are experienced in affected areas in Bahia and Amazon regions. Breeding for resistance, phytosanitation and the application of fungicides are still the main tools for controlling this disease (Bastos, 1996).

Intensive worldwide research during several years established biological control as a feasible alternative to the use of chemicals for the reduction of plant diseases caused by pathogenic fungi (Lewis e Papavizas, 1993). Among the most studied antagonists are species of the genus *Trichoderma*, even for plant pathogens of phylloplane, when applied as sprays. This fungus has been studied in the control of several fungi (Tronsmo e Dennis, 1977; Moretto et al., 2001; Howell, 2002) including *M. perniciosa* (Bastos, 1988; Bastos, 2000). Key strategies for managing witches' broom disease include suppression of basidiocarp production in the pathogen inoculum sources (dried brooms) and protection of flushes and fruits against infection by basidiospores of the fungus. An antagonistic fungus *Trichoderma stromaticum* has been shown to have potential to control witches' broom by reducing 99% of basidiocarp formation in brooms in contact with the soil and 56% in brooms on trees (Costa et al., 2000).

With the increasing interest in developing alternatives to chemical control with fungicides or integrated control of plant diseases, mass production of *Trichoderma* for use as bioprotectants has become a focus of industrial research and development. Besides, *Trichoderma* species are considered standard in biological studies because they are easily isolated, grow fast in several substrates, affect several pathogens, act as mycoparasites, produce antibiotics and have a system able to attack a range of plant pathogens (Well, 1986).

Epiphytic and endophytic fungi are being pursued as potential biological agents of the fungal diseases of cacao (Samuels et al., 2006). Endophytic fungi, in particular those that have coevolved with cacao or other *Theobroma* species, are being investigated for use as biocontrol agents within framework of classical biocontrol (Evans et al., 2003)

The purposes of the research reported in this paper were to evaluate one isolate of *T. brevicompactum* Kraus, Gams & Kubicek (Kraus et al., 2004) as to its ability to control *M. perniciosa* *in vitro*, on dried brooms and *in planta* under greenhouse and field conditions.

## Materials and Methods

### Organism isolate

The isolate of *T. brevicompactum* was obtained from inner healthy trunk tissues of cocoa tree, collected in CEPLAC Theobroma Germoplasm Collection at Experimental Station of Resources José Haroldo (ERJOH), in Marituba, Pará, Brazil, where witches' broom disease is endemic. For isolation of the fungus, plant tissues were surface disinfested with hypochlorite solution (3%) for 3 minutes, rinsed once in 70% ethanol and then twice in distilled sterile water. Fragments were placed onto PDA plates and incubated at 25±1 °C. The hyphal tips of mycelium that emerged from the fragments were transferred to PDA slants. The isolate was identified as *T. brevicompactum* by Dr. Jorge Teodoro de Souza of the UFRB, Cruz das Almas, BA, Brazil.

### *Trichoderma* and *Moniliophthora perniciosa* growth in dual culture

Examination of *T. brevicompactum* and *M. perniciosa* growing in dual culture was made by placing 5-mm diam. mycelial disks 4 cm apart on malt extract agar (MEA) plates. Control was also set up using the antagonist or the pathogen alone, so that growth and interaction could be precisely measured. Plates were incubated at 25±1°C in the dark and after 12 days assessment of interaction and growth of *M. perniciosa* was made by measuring colony radii at right angles. Experiments were repeated at least twice each with five replicates.

### *Trichoderma* metabolite production and its activity on germination of basidiospores and mycelial growth of *M. perniciosa*

Three disks of PDA with *Trichoderma* mycelium were placed in 250 mL- Erlenmeyer flasks containing 50 mL of potato dextrose broth (PDB) and incubated in still culture for eight days at lab conditions. The

fermented broth was filtered through filter (Whatman n°. 4) and then, through a Millipore membrane (0,22 µm).

Required concentrations of culture filtrates autoclaved and non-autoclaved (0.0, 0.1, 0.3, 0.5, 1.0, 2.0% ) were made by serial dilution with sterile distilled water. Drops (100 µL) of different concentrations of filtrates and sterile water (as control) were pipetted separately into microscope cavity slides placed in Petri dishes lined with moist filter paper. One agar block (ca 3 mm diam) containing freshly-deposited basidiospores was placed in each filtrate drop. Four replicate slides were prepared per treatment. Plates were incubated at 25°C and after 24 h one drop of cotton blue in lactophenol was placed into each cavity to stain spores and germ tubes and arrest further fungal growth. Spores were considered to have germinated when germ tube length was longer than the maximum spore dimension. Percentage germination was calculated based on the germination of 100 spores at random in four microscope fields from each cavity slide.

In addition, culture filtrate was mixed with melted MEA at concentrations of 0.0, 1.0, 2.0, 3.0, 4.0, 5.0, and 10% and this mixture was poured into Petri plates (50 mm diam) and one disk (5 mm diam) of the pathogen colony was transferred onto the centre of each plate. Plates with no filtrate served as control. Four replicates were prepared per treatment and they were incubated at 25±1 °C in the dark for seven days and then the radial growth of the mycelium was recorded.

#### **Effect of *T. brevicompactum* and its mebolite on the protection of cocoa seedlings against infection by *M. pernicioso***

The trials to test the effect of spore suspension of *T. brevicompactum* and its metabolite on the infection of cocoa seedlings by *M. pernicioso* used culture filtrates of the antagonist obtained as described above. Fermented broths both autoclaved at 120 °C and non-autoclaved were used. For production of inoculum the antagonist was grown on autoclaved rice grains in 250 mL Erlenmeyer flasks.

Seeds of cocoa cultivar PA 195, susceptible to witches' broom disease were peeled and placed on wet blotting paper in a seed germination tray. The germinated seeds were then planted singly in 9 x 25 cm conical pots containing soil and kept in a

greenhouse. When the seedlings were 40 days old, they were sprayed with culture filtrate at the concentrations of 5%, autoclaved and non-autoclaved, and spore suspension ( $2 \times 10^7$  spores/ml) of *T. brevicompactum* or water as control. The seedlings were individually sprayed with the treatments until run off, six days before inoculation with a basidiospore suspension ( $1 \times 10^5$  spores/ml) of *M. pernicioso*. The basidiospore suspension was obtained by diluting the inoculum stock suspension stored in liquid nitrogen with 0,25% water-agar. Aliquots of 30 µL of spore suspension were placed on the apical buds of the plants. Inoculations were carried out in a temperature-regulated (at approximately 25 °C) room fitted with an air humidifier to maintain RH at 100%. The estatistical design was the completely randomized blocks with 04 treatments and three replicates (10 plants/replicate). After incubation for 24 h the plants were moved to a greenhouse. The presence or lack of vegetative brooms was recorded after 45 days the inoculations and, thereafter disease incidence was determined.

#### **Effect of *T. brevicompactum* on basidiocarps production**

The experiment was conducted in "vassoureiro" (place where the brooms are hung and induced to produce basidiocarps). Hung brooms were sprayed with a spore suspension ( $2 \times 10^7$  spores/mL) and the numbers of mature basidiocarps produced on each broom were subsequently recorded. Brooms not sprayed with the antagonist were used as control. Fifty brooms were used for each treatment. The antagonist was applied once only. The brooms were then subject to a daily regime of 16 h wet and 8 h dry, when there was no rain. The number of mature basidiocarps produced was recorded twice a week for three months, starting from the week they were first found. Once mature basidiocarps had been recorded, they were removed from the brooms to avoid counting them twice.

#### **Effect of *T. brevicompactum* on control of witches' broom in the field**

The field experiment was conducted at the ERJOH in an area planted with a mixture of 13-15 year old cocoa clones susceptible to witches' broom disease. Phytosanitary pruning of all trees in the experimental area was accomplished during the dry season, prior

setting up the treatments. The trial consisted of 10 trees sprayed with spore suspension ( $2 \times 10^7$  spores/ml) as well as another 10 trees sprayed with culture filtrate (5%) compared with 10 trees untreated (control). The sprays were applied to the tree canopy at 30 days intervals between 2006 and 2007 in the months of November, December, January, and February using a motorized knapsack sprayer. Observations of diseased pods in the trees were performed, and mature and diseased pods were harvested until the end of experiment in August 2007 and, the number of dead pods were recorded.

### Statistical analysis

A linear regression analysis was used to analyse the data of effects of the culture filtrate on basidiospore germination and on mycelial growth. Data expressed in per cent of disease incidence in cocoa seedlings and diseased pods were arcsin-transformed prior to analysis of variance (ANOVA) and means compared by Tukey's test ( $P = 0.05$ ). Analysis were carried out using the statistical package SPSS version 15 (Maroco, 2003).

## Results and Discussion

In paired cultures no conspicuous visible inhibition zone was observed. *Trichoderma brevicompactum* had growth rate significantly higher than *M. perniciosa*. The antagonist overgrew the pathogen colonies, which often reached 28.7 mm diam after 12 days incubation, while the control continued growing to 68.3 mm diam.

The results on the activity of *T. brevicompactum* culture filtrates on the pathogen showed them to be highly toxic to basidiospores of *M. perniciosa* (Figure 1). No germination was recorded in the broth at concentrations above 1% after 24 h due to spore plasmolysis, compared with 100% germination in control (Figure 1a). The ability of the antagonistic metabolites to inhibit basidiospore germination is an essential requirement for protecting young tissues *in vivo*.

Similar culture filtrate (10% concentration), when incorporated into MEA plates, caused complete inhibition of the mycelial growth of *M. perniciosa* compared to the controls, MEA without filtrate, seven days after incubation (Figure 1b). In addition, the metabolites produced by the *Trichoderma* isolate did not lose their activity after autoclaving (data not shown).

*Trichoderma brevicompactum* produces various polypeptide antibiotics (peptaibiotics) which have plant-protective action against Eutype dieback and Esca disease of grapevine (Nielsen et al., 2005; Degenkols et al., 2006).

The results (Figure 2) show that autoclaved, and non-autoclaved culture filtrates and the active conidia of *T. brevicompactum* applied on cocoa seedlings were able significantly ( $P = 0.05$ ) to reduce the infection caused by *M. perniciosa*, compared with the untreated inoculated controls. It seems that the antagonist was successfully introduced into healthy tissues and suppressed witches' broom symptoms under greenhouse conditions. The reduction in the infection percentage of cocoa seedlings when the antagonistic fungus was applied six days before inoculation might be due to the

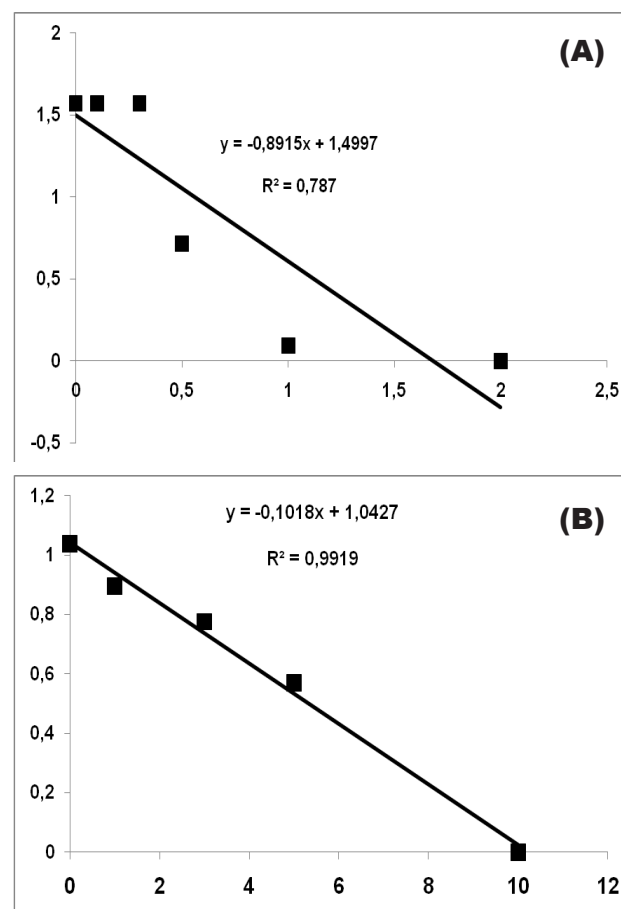


Figure 1. Inhibition of basidiospore germination (a) and mycelial growth (b) of *Moniliophthora perniciosa* by different concentrations of culture filtrate of *Trichoderma brevicompactum*.



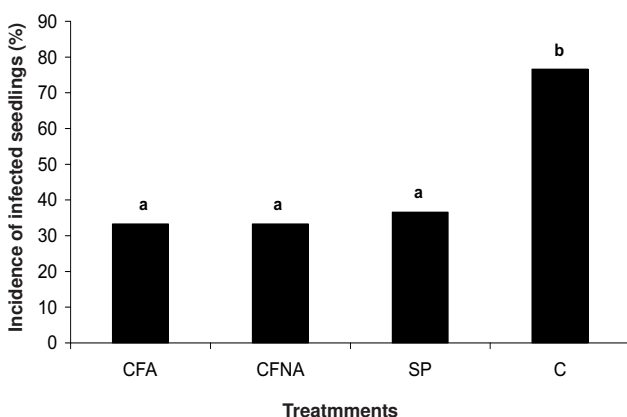


Figure 2. Effect of *Trichoderma brevicompactum* applied to cocoa seedlings 6 days before inoculation with *Moniliophthora perniciosa*. CFA= autoclaved culture filtrate (5%); CFNA = non-autoclaved culture filtrate (5%); SP= spore suspension; C= control. Columns with the same letter are not significantly ( $P = 0.05$ ).

liberation of metabolites or to the parasitism of the pathogen by the antagonist. Although it was not possible to show mycoparasitism of *T. brevicompactum* *in vitro*, trials demonstrate that this isolate has an antibiotic effect against *M. perniciosa*. Thus, it is suggested that metabolites could be produced *in planta* and they could contribute to induce resistance to witches' broom disease. *Trichoderma* strains inhibit or kill plant-pathogenic fungi through production of antifungal antibiotics and/or hydrolytic enzymes (Vizcaíno et al., 2005). Ability to promote growth and induce resistance in plants is an activity which has also been described for members of this genus (Monte, 2001).

The ability of *T. brevicompactum* to penetrate into cocoa seedlings supports previous evidence that *Trichoderma* spp. (Evans et al., 2003) isolated from the stems of wild *Theobroma* trees may invade and endophytically colonize unhardened cocoa stems and flushes. Samuels et al. (2006) showed that *T. theobromicola*, isolated as an endophyte from the trunk of a healthy cocoa tree in Amazon Peru and thereafter introduced into cocoa seedlings through shoot inoculation, was recovered from stems but not from leaves, indicating that it is an endophytic species.

Endophytic fungi cause inconspicuous infections within tissues of healthy plants for all or nearly all their life cycle (Sinclair e Cerkauskas, 1996). Endophytes, in contrast to epiphytes, are contained entirely within the substrate plant and may be either

parasitic or symbiotic. Thus, nonpathogenic endophytic organisms may play a role as biocontrol agents (Freeman e Rodriguez, 1993). Endophytic fungi can infect tissues and become established after penetration; however, infection does not imply disease symptoms expression (Sinclair e Cerkauskas, 1996). *Trichoderma brevicompactum* isolation as an endophyte and its possible contribution toward host resistance to witches' broom disease suggest that host and endophyte are in a coevolved symbiotic relationship. Therefore the antagonistic offers a strong possibility to be exploited for biological control of the destructive cocoa pathogen *M. perniciosa*.

The formation of basidiocarps on dried brooms was significantly suppressed by *T. brevicompactum* when compared with the control. The number of basidiocarps produced on the treated brooms was 232 while on the not-treated brooms (control) it was 689. There was no effect of culture filtrates when applied on dried brooms (data not showed).

The results obtained from the application of *T. brevicompactum* in the field are shown in Figure 3. Pod infection was significantly ( $P = 0.05$ ) reduced in the treatments with spores and with culture filtrates as compared to control. There was a significant reduction in the number of diseased pods by witches' broom which can be attributed to applications of spore and of metabolites of *T. brevicompactum* to the cropping areas of the trees.

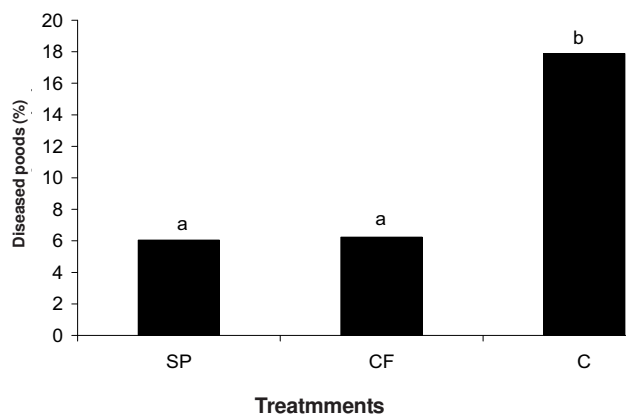


Figure 3. Effects of applications of *Trichoderma brevicompactum* on the reduction of cocoa pods affected by witches' broom disease. SP = spore suspension; CF = culture filtrate; C = control. Columns with the same letter are not significantly ( $P = 0.05$ ).

In conclusion, the isolate of *T. brevicompactum* demonstrated potential to prevent infection on plant tissues and also to reduce pathogen inoculum on dried brooms. Thus, two alternative methods of control of the witches' broom pathogen through the use of the isolates of *Trichoderma* are feasible: direct biological control, by treatment of brooms and chemical control, by treatment of healthy cocoa tissues with mycotoxin from culture filtrates. Finally, biological control of cocoa witches' broom should not be regarded as a potential panacea, but rather an additional tool that can be applied in combination with disease resistant cultivars and sanitation practices.

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