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Selection of *Trichoderma stromaticum* isolates for efficient biological control of witches' broom disease in cacao

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ABSTRACT

Witches' broom is the most devastating disease of cacao in Brazil, and losses to it entail serious socio-economical and environmental problems. Biological control of the causal agent *Moniliophthora perniciosa* (*Mp*) using the antagonistic fungus *Trichoderma stromaticum* (*Ts*) is promising, although the identification of superior isolates is necessary. Here, we report a study on the selection of more effective *Ts* isolates based on field experiments. Sixty-three *Ts* isolates from a local collection were applied on brooms and placed under typical conditions of shaded-cacao plantations in southeastern Bahia State (Brazil), during two periods of three months each. The percentages of *Ts* sporulation and incidence and severity of *Mp* were the parameters used for biocontrol assessments. The results from both experiments were very distinct, indicating a high phenotypic variation in this collection and suggesting a significant effect of the environment in the *Ts*–*Mp* interaction. *Ts*-sporulation rates were negatively correlated with the presence of *Mp* in the brooms and a number of isolates reduced *Mp* incidence more efficiently than the reference isolate. Contrasting isolates in their efficiency of reducing *Mp* incidence were selected and further tested in four subsequent field trials for validation purposes. The results partially confirmed their biocontrol phenotypes but also suggested isolate-specific responses to environmental variations. Inhibition of *Mp*-basidiospore germination by total protein secreted in culture supernatants of *Ts* isolates correlated well with field results and revealed a potentially useful procedure for pre-screening of large collections towards selection of better biological control isolates. The characteristics and efficiency of the method as a reliable protocol for identification of superior BCAs in the witches' broom—cacao pathosystem is discussed.

1. Introduction

The witches' broom of cacao, caused by the fungus *Moniliophthora perniciosa* (Stahel) Aime & Phillips-Mora (ex *Crinipellis*—Aime and Philips-Mora, 2005), has become the most devastating disease on this crop in the Brazilian States of Amazonas, Bahia, and Espírito Santo. For the last 17 years, losses in cacao production in Bahia have averaged 60%, leading to severe economical, social and environmental problems (Donald, 2004; Griffith et al., 2003; Anderbrhan et al., 1999; Pereira et al., 1996). *M. perniciosa* (*Mp*) is a hemibiotrophic pathogen, whose basidiospores infect plant meristematic tissues and develop intercellularly, causing loss of apical dominance, hypertrophy, and hyperplasia (biotrophic phase); later, tissue necrosis of the broom is induced, whose exploitation (saprophytic phase) leads to formation of basidiocarps, the source of the infectious propagules (Silva et al., 2002; Griffith et al., 2003; Meinhardt et al., 2008).

Phytosanitation and protective fungicide applications are common practices to control *Mp* (Purdy and Schmidt, 1996; Costa et al., 2006). The use of copper-based fungicides has shown to be economically nonviable and technically ineffective in some cases (e.g. Krauss and Soberanis, 2002), whereas systemic fungicides are not routinely used in cacao farming, due to high costs and risks of contaminating cacao beans and the environment (Meinhardt et al., 2008). The replacement of diseased trees with genetically resistant clonal varieties is considered the most viable alternative (Lopes et al., 2003). However, this results in a delay for production to restart due to the long life cycle of cacao, and the possibility of

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resistance breakdown threatens the long-term sustainability of this approach. Integrated pest management (IPM) is, therefore, a complementary and necessary strategy (Costa et al., 2006).

Biological control is an important component of IPM programs as it helps both in reducing diseases to economically viable levels and decreasing the use of chemical fungicides (Krauss and Soberanis, 2001; Bajwa and Kogan, 2004). Increasing public concerns about pesticide residues in food products and the degrading effects of fungicides on soil biota that negatively impacts soil health (Van-Zwieten, 2004; Van-Zwieten et al., 2004), indicate that an environmentally sustainable method for disease control in cacao is necessary. In areas of organic certified production of cocoa with restrictions to use of chemicals, biologically based management appears to be one of the few viable approaches (Krauss et al., 2006). Antagonistic fungi such as *Trichoderma* spp. have shown significant success against plant diseases in several agricultural systems (reviewed by Benitez et al., 2004; Kubicek et al., 2001; Hjeljord and Tronsmo, 1998). Studies on the potential of *Trichoderma*-based biological control against pod diseases in cocoa-producing regions of Peru (Krauss and Soberanis, 2002), Cameroon (Tondje et al., 2007; Deberdt et al., 2008) and Brazil (Pomella et al., 2007; Hanada et al., 2008, 2009) indicate promise for this approach. The most likely mode of action of *Trichoderma* in these cases is parasitism of the pathogen. Significant diversity in species of *Trichoderma* has been described (Druzhinina and Kubicek, 2005; Samuels et al., 2006), thereby providing the possibility of finding distinct biocontrol mechanisms and host specificities (Hjeljord and Tronsmo, 1998; Lorito, 1998). In addition, antagonists naturally found in the areas of application tend to be more easily and widely acceptable by the public, with potentially faster benefits.

Trichoderma stromaticum Samuels & Pardo-Schultheiss (Samuels et al., 2000) is a mycoparasite of *Mp* that is promising as a biological control agent (BCA) for the witches' broom disease of cacao, as it colonizes the necrotic tissue of the plant and prevents basidiocarp production by the pathogen (Pomella et al., 2007; Hjorth et al., 2003; Bastos, 1996, 2000; Costa et al., 1996). However, the isolate currently used in the cacao-growing area of southeastern Bahia was introduced from the Amazon region and its field performance is greatly influenced by environmental factors (Santos, 2005; Sanogo et al., 2002). *T. stromaticum* (*Ts*) isolates are naturally widespread in Bahia and comprise two distinct genetic groups, I and II, with the latter showing a higher level of variability within the group, based on AFLP analysis (De Souza et al., 2006). Moreover, isolates from group II tend to persist longer as endophytes in cacao trees than those of group I (De Souza et al., 2008). Since the teleomorphic form of the genus, *Hypocrea stromatica* (Bezerra et al., 2003), was also identified in this cacao region, genotypic variability of *Ts* is thereby expected to be found. Hence, the search for locally adapted isolates that may be more efficient as BCAs against *Mp* is likely to be worthwhile (Costa et al., 2006). In addition, the ideal situation of achieving a "biological broom pruning" by treating the cacao canopy with *Ts* would avoid the need to remove dead brooms, one the most expensive practices on cocoa farming for control of witches' broom (Pomella et al., 2007).

Due to the complexity of variables involved in the phenotype of a BCA (Rajkumar et al., 2005; Elad, 2003; Hermosa et al., 2000; Knudsen et al., 1997), field experiments are more likely to render a methodology capable of selecting superior isolates because all genetic and environmental factors are considered in an integrative manner. Nevertheless, this strategy might be hampered if an excessive number of isolates are to be screened, due to potential space or time constraints, or resource limitation. In this study, 63 *Ts* isolates were assessed for their antagonistic capabilities against the witches' broom pathogen, under usual field conditions of cacao farming in southeastern Bahia. The objectives were to establish a reliable strategy for identifying and selecting superior strains for improvement of this method for disease control. Additional studies were also performed, to investigate the potential of using *in vitro* methods for a first round of isolates selection, prior to screening experiments in the field.

2. Materials and methods

2.1. Isolates and culture conditions

Sixty-three isolates of *T. stromaticum* (*Ts*) were provided by Almirante Cacau Ltda (Itajuípe-BA, Brazil) for use in this study. They were previously collected in the tropical cacao-growing region of southeastern Bahia (Brazil) and classified into genetic groups I and II based on AFLP analysis (De Souza et al., 2006), as shown in Table 1. The abbreviations 'GI' and 'GII' are used throughout this text. Of the 63 isolates available, 54 were from GI and 9 from GII. An isolate designated 'TVC' (AM7, GII) was kindly provided by the Centro de Pesquisas do Cacau (CEPEC/CEPLAC). Because this isolate has been made available to producers since 1999 as the basis of the product 'Tricovab' for biological control of *M. perniciosa* (*Mp*), it was used as the *Ts* isolate of reference.

Pieces of 0.25-cm^2 filter paper containing spores from *Ts* isolates stored at 4 °C (Dhingra and Sinclair, 1985) were placed in Petri dishes containing potato-dextrose-agar medium for incubation at room temperature (~25 °C) for 14 days until sporulation (Sanogo et al., 2002). The conidia that formed were collected and suspended in distilled water, and their concentrations adjusted with a haemocytometer to 10^7 spores.mL^{-1}. These suspensions were applied to the dead *Mp*-infected cacao-stem segments (brooms) used for the biocontrol field experiments.

For the experiments involving extraction of total secreted protein, the *Ts* spore-containing filter papers were transferred into liquid TLE medium (0.1% Bacto peptone, 0.03% urea, 0.2% KH_2PO_4, 0.14% $(NH_4)_2SO_4$, 0.03% $MgSO_4$, 0.03% $CaCl_2$, and 0.1% trace elements, composed of 2.5% citric acid, 2.5% $ZnSO_4$, 0.5% $Fe(NH_4)_2(SO_4)_2.6H_2O$, 0.125% $CuSO_4.5H_2O$, 0.025% $MnSO_4$, 0.025% $NaNO_3$, 0.025% H_3BO_3). Two alternative carbon sources, 0.5% glucose or 0.5% dried mycelia of *Mp* + plus 0.03% glucose, were used. *Ts* isolates were cultured in 1-L flasks, containing 500 mL of medium, under 110 rpm constant agitation for 6 days at 25 °C, with the supernatants being processed afterwards as described below.

2.2. Broom collection and preparation

Brooms of several lengths, averaging 0.75 cm in diameter, were collected from plants located in areas heavily infested with *Mp*. To select those for use in the experiments, brooms were tested for the presence of the pathogen and for incidental, spontaneous *Ts* colonization. Segments of 2 cm in length were cut from the ends of each broom and placed inside humid chambers of small plastic bags containing wet filter paper. These segments were incubated at 25 °C for 7 days; appearance of the characteristic white, cotton-like mycelia, and white-to-green sporulation were the indicators of *Mp* and *Ts*, respectively. Brooms were standardized to lengths of 20 cm and only those containing *Mp* and free of *Ts* were used.

2.3. Biological control field experiments

Two screening experiments were conducted with the 63-isolates collection, the first extending from 9 May until 14 August, 2003, and the second from 31 July until 6 October, 2003. Rainfall and temperature data were collected from the station '591' at the Almirante Cacau Farm. For these field trials, the 63 *Ts* isolates and the control treatment without *Ts* were applied on the 20