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Biocontrol potential of *Trichoderma martiale* against the black-pod disease (*Phytophthora palmivora*) of cacao

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ABSTRACT

The Black-Pod Rot of cacao (*Theobroma cacao*) caused by *Phytophthora* species is one of the most important diseases affecting this crop worldwide, with average annual losses above 30%. The aim of this work was to assess the potential for the endophytic *Trichoderma martiale* strain ALF 247 to control *Phytophthora palmivora*. When ALF 247 was applied at concentrations ranging from 1×10^4 to 5×10^7 conidia per milliliter, the disease severity decreased proportionally. Addition of vegetable oil and/or sucrose in the formulations did not affect the biocontrol results. Fungicides such as copper hydroxide and fosetyl-Al had no effect on conidial germination of this *T. martiale* strain, with the germination percentage maintained above 90%. Once sprayed, the population of *T. martiale* tended to decrease progressively on the surface of cacao pods (~30–40 days post-application), with a concomitant increase in the severity of pod disease. Production of 1.02×10^8 conidia g^{-1} was obtained after culturing ALF 247 on solid substrate (rice grains) supplemented with calcium carbonate. The results indicate a clear-cut potential of the *T. martiale* ALF 247 to be used for control of Black-Pod Rot of cacao, although further studies are required to render this isolate technically and economically efficient as a biocontrol agent on agronomic scale.

1. Introduction

Black-Pod Rot (BPR), caused by several species of the straminipile (formerly oomycete) genus *Phytophthora*, is the main disease affecting the cacao crop (*Theobroma cacao* L.) worldwide (Brassier and Griffin, 1979; Oliveira and Luz, 2005). It can cause annual losses of up to 90% of pod production, depending on the environmental conditions (Nyassé et al., 1995; Bowers et al., 2001). In southeastern Bahia, the major cocoa-producing region of Brazil, *Phytophthora palmivora* (Butler) Butler is the predominant species causing BPR. Phytosanitation, chemical fungicides and genetically resistant varieties are the main methods of controlling pod infection by *Phytophthora* spp. However, chemical control frequently requires several applications of sprays and is often not efficient (Holderness, 1992). The removal of diseased pods has shown some efficiency in reducing secondary inoculum (Ndoumbe-Nkeng et al., 2004), however, it is labor intensive, expensive and economically viable only if market prices of cocoa are high. Although genetic resistance is the most cost-effective control measure, a standardized methodology to evaluate cacao germplasm for resistance against *Phytophthora* spp. is lacking in several cocoa-producing countries (Luz and Silva, 2001).

In this context and from the perspective of Integrated Pest Management (IPM), biological control is an additional method that can help in reducing the disease to economically viable levels, with a concomitant decrease in the use of chemicals (Krauss and Soberanis, 2001; Bajwa and Kogan, 2004). Studies on the potential of *Trichoderma*-based biological control against *P. palmivora* in Peru (Krauss and Soberanis, 2002) and the aggressive *Phytophthora megakarya* Brasier & Griffin in cocoa-producing regions of Cameroon (Tondje et al., 2007; Deberdt et al., 2008) indicate promise for this approach. The most likely mode of action of the *Trichoderma* in these cases is parasitism on the pathogen. A second mode of biocontrol action is stimulation of the resistance reaction in the host to parasites (Harman et al., 2004; Arnold et al., 2003; Bailey et al., 2006). Hanada et al. (2004) carried out a screening procedure in cacao and cupuaçu trees [*Theobroma grandiflorum* (Wild. ex Spring) Schumann] of Amazon and Bahia states (Brazil) for endophytic fungi suitable for the biocontrol of BPR. From this work strain ALF 247 was considered the most promising antagonist for the control of *P. palmivora* among the screened strains. Strain ALF

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247 was isolated as an endophyte from sapwood of the trunk of a cacao tree and was described as a new species, *Trichoderma martiale* Samuels, sp. nov., by Hanada et al. (2008).

Based on increasing public concern about pesticide residues in food products, as well as about soil-degrading effects of fungicides, the availability of a sustainable, environmentally friendly method for disease control in cacao crop is highly desirable. In areas of organic certified production of cocoa where chemicals are restricted, biocontrol appears to be one of the few viable approaches (Krauss et al., 2006). Hanada et al. (2008) provided preliminary evidence for the biological control of *P. palmivora* by *T. martiale* in field assays, where artificially inoculated cacao pods showed a reduced severity of black-pod symptoms. In addition, the endophyte could be recovered from pods and other parts of the plants up to 3–4 months after application, although at low levels. Because no other strain or formulation is currently available for biological control of this disease under the tropical conditions of southeastern Bahia, the objective of this work was to further investigate the potential of *T. martiale* strain ALF 247 as a biocontrol agent (BCA) against *P. palmivora*. Different inoculum concentrations, medium compositions, sensitivity to contact and systemic fungicides, and methods for mass production of conidia were evaluated. The residual biocontrol effects on the pods surface after application were also assessed.

2. Materials and methods

2.1. Culture conditions and spore viability assessments

The endophytic *T. martiale* strain ALF 247 was collected from stems of healthy cacao plants in the municipality of Inema (Bahia, Brazil). This strain was grown in Petri dishes containing potato–dextrose–agar (PDA – decoction of 40 g potato, 20 g dextrose and 20 g agar per liter of water) and cultured for 7 days at 25 °C under a 12 h photoperiod to produce conidia for all inoculation experiments. For quality control of inocula, viability was assessed by applying 100 μL aliquots of spore suspensions in Petri dishes containing PDA and incubating for 16 h at 25 °C. The percentage of germination of a sample was determined by examining at least 100 conidia under a light microscope; only spore suspensions with more than 90% germination were used for all experiments of this study. *P. palmivora* strain '611,' from a collection maintained at the Cacao Research Center (CEPEC/CEPLAC, Ilhéus-BA, Brazil), was used in all experiments. This strain was grown 10 days in the dark and 3 days under near-UV light in Petri dishes containing T3 medium (decoction broth from 20 g of carrots, 45 g tomato extract, 3 g calcium carbonate and 15 g agar in 1 L distilled water). Formation and collection of zoospores were achieved after adding 10 mL of cold (4 °C) sterile distilled water in each plate and incubating at 4 °C for 15 min, followed by 30 min at room temperature.

2.2. Biocontrol effect of different spore concentrations

Conidial suspensions of ALF 247 were adjusted to concentrations of 1×10^4, 1×10^5, 1×10^6, 1×10^7 and 5×10^7 conidia mL^{-1} in distilled water and sprayed to near run-off on cacao pods in the field, using a 1-L sprayer-nozzle bottle. Considering the average surface area of cacao pods, the volume of suspensions applied per pod with this procedure were 40–50 mL. Four month-old pods in trees of the cultivar 'SIAL 70' showing no evidence of injury or disease were used for each suspension tested. To facilitate humidity for spores germination and help prevent the applied suspensions to be washed off by frequent rains in the area, pods were covered with transparent plastic bags. These bags were applied ~1 day before application of treatments and removed 24 h after that, thereby allowing the experiment to be conducted under natural field conditions. Seven days after the BCA applications, each pod was challenged by a suspension of 2×10^5 zoospores mL^{-1} of *P. palmivora* which was also sprayed to near run-off. To prevent wash off by rain, to induce stomata opening that facilitate pathogen penetration, and to obtain a homogeneous infection for all inoculated treatments, pods were again covered with plastic bags from ~1 day before until 24 h after this inoculation, being removed afterwards. After 13 days, disease symptoms were scored based upon the following scale: '1' = no sign of disease symptoms; '2' = restricted lesions of approximately 2 mm in diameter or 5 mm in length; '3' = expanding lesions with diameters from 0.2 to 2 cm; '4' = lesions and/or necrosis of several sizes, covering up to 25% of the pod surface; '5' = extended lesions and necrosis over 25% of the pod surface. The whole experiment was repeated 1 month later. The experiments were conducted on a completely randomized design, with each endophyte concentration (treatment) being applied on 10 pods (replicates). For the analysis of variance, the severity score was converted to their square root and the means were compared by the Fisher's Least Significant Difference (LSD) test. A logarithmic regression analysis was performed based on a linear scale for the disease scores (y axis) and a logarithmic scale for the spore concentrations (x axis), using the data analysis tool of the Microsoft® Office Excel 2003.

2.3. Formulation assessments

Suspensions of 1×10^7 conidia mL^{-1} of *T. martiale* ALF 247 were sprayed to near run-off on field pods under the following formulation treatments (i) ALF 247 + 2% emulsified vegetable oil derived from soybean (Nortox); (ii) ALF 247 + 2% sucrose; (iii) ALF 247 + 2% sucrose + 2% Nortox; (iv) ALF 247 isolate only; (v) 2% Nortox + 2% sucrose; (vi) 2% Nortox; (vii) 2% sucrose. These amendments were tested not only due to their generally low costs for obtainment, but also because their effectiveness both in UV protection (oil) and in supplying nutrients for the spores (sucrose) were previously demonstrated in the formulation of *Trichoderma stromaticum* Samuels & Pardo-Schultheiss that has been used for biocontrol of the witches' broom disease [*Moniliophthora perniciosa* (Stahel) Aime & Phillips-Mora] in cacao (Pomella et al., 2007). Again, pods were kept in plastic bags from ~1 day before up to 24 h after treatments application, as described. Seven days after application of these treatments, the pods were challenged with *P. palmivora* suspensions of 2×10^5 zoospores mL^{-1}, being covered with plastic bags in the same way described above. Four month-old healthy and undamaged pods in trees of the SIAL 70 cultivar were used for each formulation tested. Positive and negative control formulations used only water with and without pathogen, respectively. The whole experiment was performed twice in a completely randomized design, with 10 pods (replicates) per formulation in each trial. The disease was scored based on the same scale described above and the values were converted to their square root. Means were compared by the Scott–Knott test at a 5% significance level.

2.4. Evaluation of mass production of spores

Conidia of ALF 247 were produced in large quantities in plastic bags containing 100 g of autoclaved rice grains, plus 30% of distilled water. Conidial production was assessed in these rice cultures (controls), testing three additional treatments: rice + 0.45% calcium carbonate, rice + 0.6% urea, and rice + 0.225% calcium carbonate + 0.3% urea. For each of these four substrate treatments, rice cultures were initiated with either mycelium + conidial plugs (10 culture discs of 5-mm diameter each, from PDA plates previously grown at 25 °C for 7 days – see above), or spore suspensions

(5 mL of 4.5×10^7 conidia mL^{-1}), or pre-colonized rice (5 g of rice already colonized with 5×10^7 conidia g^{-1}) as the inoculum sources. The amended substrates were placed in 2 kg polyethylene bags and autoclaved twice for 30 min at 121 °C on alternate days. The bags were tied with a string and kept at 25 °C in the dark. Two days after inoculation, the bags were untied to allow oxygenation. Data were collected after 6 days of incubation by transferring 1 g of colonized rice to a screw-cap tube, adding 10 mL of distilled water with a drop of Tween 80, vortexing for 1 min and counting conidia concentrations in a hemacytometer. To check for spore viability levels (quality control), 100 μL of these suspensions were spread into PDA and allowed to incubate for 16 h at room temperature (~25 °C). A minimum of 100 conidia were counted at a light microscope to assess germination percentage. The experiment was performed in a completely randomized design, with 12 treatments (4 substrates × 3 types of inoculum) and 3 replicates each, in a total of 36 bags. The means were compared by the Scott–Knott test. The whole experiment was repeated twice.

2.5. Evaluation of conidium sensitivity to fungicides

Copper hydroxide is a generic contact fungicide that has been widely used against pod diseases in cacao farming of Bahia, whereas fosetyl-Al is a systemic fungicide specific against species from the Oomycota phylum, such as *Phytophthora* spp. The effects of copper hydroxide and fosetyl-Al (Aliette®, Aventis Crop Sci.) fungicides on conidial germination were assessed by incubating 10^6 ALF 247 conidia mL^{-1} directly into fungicide suspensions, containing copper hydroxide (1.5% active ingredient) or fosetyl-Al (0.4% active ingredient), at 25 °C for 30, 60, 90, 120 and 180 min. Conidial suspension in sterile distilled water was used as control. After each time period, an aliquot of 100 μL of each suspension was spread onto Petri dishes containing PDA and incubated at 25 °C for 16 h. The percentage of germination was assessed by counting ~200 conidia of each sample using a light microscope. Two of such experiments were set in a completely randomized design with 15 treatments (3 fungicide suspensions × 5 culture times) and three replicates per treatment in each trial. Germination percentages were converted to their square root for the analysis of variance (ANOVA).

2.6. Residual biocontrol effects and survival

A suspension of 5×10^7 *T. martiale* ALF 247 conidia mL^{-1} containing 2% vegetable oil + 2% sucrose was applied to 138 pods in a cacao plantation never sprayed with *Trichoderma* spp. After 1, 5, 10, 20, 30 and 40 days from the *T. martiale* inoculation, 20 pods per time interval were sprayed to run off with a *P. palmivora* suspension of 2×10^5 zoospores mL^{-1}, totaling 120 pods. In addition, three further pods per time interval were not challenged by the pathogen to check for survival of the inoculated BCA (18 pods total). As controls for disease incidence, 20 pods per time interval were inoculated only with the pathogen (positive control) and 20 only with water (negative), in a total of 240 pods. Inoculation procedures and disease evaluation were as described above. Remaining viable conidia of *T. martiale* were checked on three unchallenged pods per time point, by excising 10 randomly distributed discs of 2.5-cm diameter from each pod surface and immersing all in 25 mL of sterilized distilled water with two drops of Tween-20. The spore counts were expressed per cm^2, considering a total surface area of 49.1 cm^2 [$=10 \times 3.1416 \times (1.25\ cm)^2$] for the discs. A 10-fold serial dilution procedure up to 10^{-7} was carried out, transferring 100 μL of each dilution step to three replicates of Petri dishes containing PDA with 25 μg mL^{-1} chloramphenicol. Plates were incubated at 25 °C under a 12 h photoperiod and the number of colonies was counted after 98 h. The average values for the three pods in each time point were plotted.

3. Results

In order to verify the effect of a range of inoculum concentrations in the control of Black-Pod Rot (BPR) of cacao, five different concentrations of ALF 247 conidia were tested against *P. palmivora*. Based on a logarithmic regression analysis (Fig. 1), increasing inoculum concentrations ranging from 1×10^4 to 5×10^7 conidia mL^{-1} showed a progressive decrease in disease severity ($R^2 = 0.8978$). Analysis of variance indicated the regression was statistically significant ($P = 0.0143$). Lower concentrations of ALF 247 conidia were less effective in controlling the disease, with the severity score of the lowest concentration not differing from the control, based on the LSD test at 5% significance (Fig. 1). These results provided a quantitative assessment that confirmed the preliminary biocontrol trends found in our previous study (Hanada et al., 2008).

As a key aspect to be considered in the establishment of useful formulations for field applications, different compositions of the antagonist's conidium suspension were assessed. Sucrose and vegetable oil, either alone or combined, were tested as amendments in the formulation of ALF 247. The results demonstrated a lack of effect for these compounds on either the pathogen or the antagonist (Fig. 2). Considering the control treatments without antagonist, no difference in symptoms was found among the amendments. A significant reduction in the disease scores was achieved for all formulations that included the antagonist, again with no difference among amendment treatments. Negative control pods sprayed with water and not challenged with *P. palmivora* showed no symptoms of disease.

Urea and $CaCO_3$ were added to rice in an effort to increase large-scale production of ALF 247 conidia on solid-substrate fermentation; in addition, three forms of starting inoculum was used for initiating mass production of conidia (Tables 1 and 2). Analysis of variance for conidial production showed a significant effect for the inoculum type and substrate treatments, as well as for their interaction, based on the corresponding values obtained for the

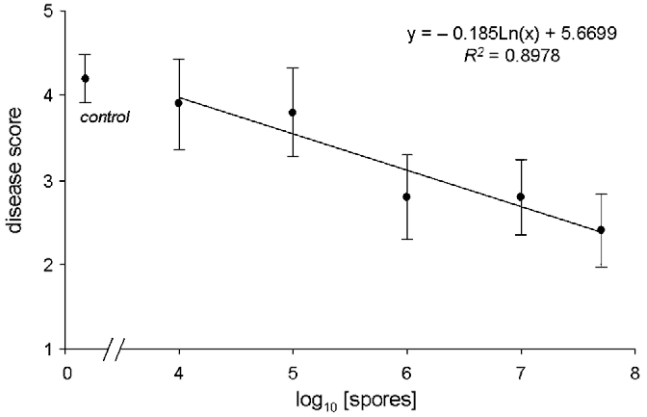

Fig. 1. Logarithmic Regression analysis of *T. martiale* ALF 247 inoculum concentration and *P. palmivora* disease severity. ALF 247 conidia (at indicated concentrations) from 7-day PDA cultures and *P. palmivora* (at 2×10^5 zoospores mL^{-1}) from 13-day T3-medium cultures were suspended in distilled water and applied to pods to near run-off, with a 7-day interval between applications (Section 2). Water was used as control. Scores are 1, no symptoms; 2, lesions of 2-mm diameter or 5-mm length; 3, expanding lesions of 2- to 20-mm diameter; 4, lesions and/or necrosis up to 25% of the pod surface; 5, extended lesions and necrosis over 25% of pod surface. ANOVA for the regression indicated statistical significance ($P < 0.05$). The coefficient of variation (CV) was 24.8%. Each value and bars are the average + std error of 10 pods from a single experiment. The experiment was repeated once in the same overall conditions, showing similar results.

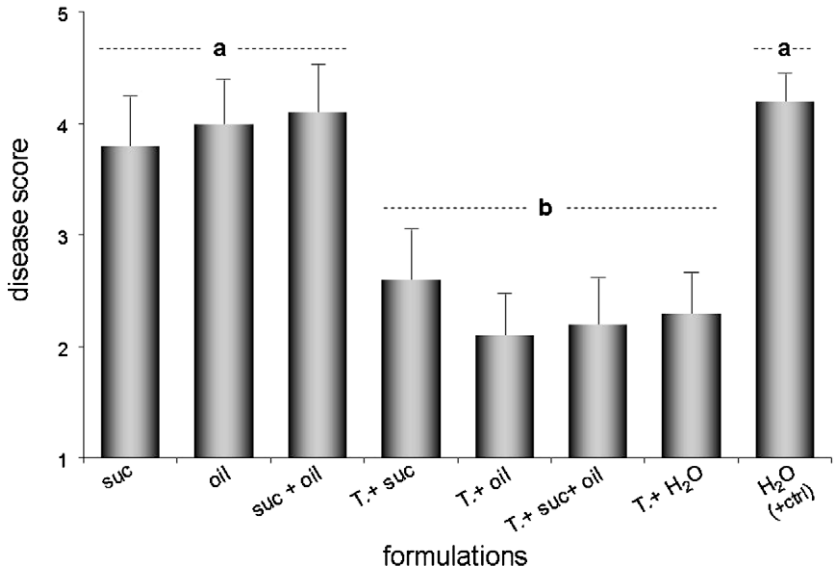

Fig. 2. Severity of *P. palmivora* disease after application of different biocontrol formulation treatments. 'T.' = *T. martiale* ALF 247 inoculum, 'suc' = 2% sucrose, 'oil' = 2% soybean vegetable oil (Nortox). Suspensions of 1×10^7 conidia mL^{-1} of ALF 247 for the different formulations and of 2×10^5 zoospores mL^{-1} of *P. palmivora* were sprayed on the pods to near run-off. For the positive control formulation, only water was sprayed to pods prior to the pathogen challenge. Disease scores are described in the legend of Fig. 1. Letters 'a' and 'b' indicate the results are significantly different by the Scott–Knott test ($P < 0.05$). The coefficient of variation (CV) was 23.2%. Average + std error of 10 pods from a single experiment are shown. This experiment was repeated once in the same overall conditions, showing similar results.

Table 1
Analysis of variance for conidia production of the ALF 247 isolate, based on three forms of inoculation and grown in rice supplemented with different nutrients.

Source variation[a]	df	MS	*F*	
Inoculum type	2	123.200	400.39	*
Substrate	3	9.463	30.75	*
Inoc. type × substrate	6	7.850	25.51	*
Residue	22	0.308		
Total	35			

[a] 'inoculum types' were (i) pre-colonized rice, (ii) spore suspensions and (iii) mycelium + conidial plugs; 'substrate' treatments for each inoculum type were addition of (i) $CaCO_3$, (ii) urea, (iii) combination of both and (iv) absence of both (controls). df, degrees of freedom; MS, mean square.
* $P < 0.01$.

Table 2
Conidia production of the ALF 247 isolate in rice supplemented with different nutrients.

Nutrients added	Inoculum type	Spore production[a] (107 conidia g^{-1} of rice)
$CaCO_3$	Mycelium + conidial plugs	***10.17*** a
$CaCO_3$ + urea	Mycelium + conidial plugs	8.50 b
Urea	Mycelium + conidial plugs	8.60 b
Control	Mycelium + conidial plugs	7.20 c
$CaCO_3$	Spore suspension	5.53 d
$CaCO_3$ + urea	Spore suspension	***9.77*** a
Urea	Spore suspension	6.50 c
Control	Spore suspension	4.30 e
$CaCO_3$	Pre-colonized rice	4.10 e
$CaCO_3$ + urea	Pre-colonized rice	1.70 f
Urea	Pre-colonized rice	1.53 f
Control	Pre-colonized rice	1.97 f

[a] Means followed by the same letters are not significantly different according to Scott–Knott test ($P \geqslant 0.05$). The coefficient of variation (CV) was 8.8%.

F test (Table 1). As determined by the Tukey test, the three methods of inoculation were significantly different from each other ($P < 0.001$), with mycelium + conidial plugs being best, followed by spore suspensions and pre-colonized rice as the poorest. Among the substrate treatments, the presence of $CaCO_3$, either with or without urea, significantly increased the conidial production in relation to urea alone or to water control, which were also statistically different from each other. Based on the significant interaction found between inoculum type and substrate, all treatments were taken together and assessed by Scott–Knott test ($P < 0.05$). Cultures started with mycelium + conidial plugs and grown on rice with $CaCO_3$, and those starting with spore suspensions and grown on rice with $CaCO_3$ + urea showed significantly higher production of conidia (Table 2). When pre-colonized rice was the starting material, conidial production was decreased by ~10-fold (ca. 1.5–1.9×10^7 conidia g^{-1}). For these treatments, however, the conidial production more than doubled (4.1×10^7 conidia g^{-1}) with the addition of $CaCO_3$ to the substrate (Table 2).

Germination of ALF 247 conidia was tested in the presence of copper hydroxide (1.5% active ingredient) and fosetyl-Al (0.4% active ingredient) solutions, in order to assess compatibility of conidia with those two commonly used contact and systemic fungicides, respectively. Under the concentrations and incubation periods used in this study (up to 3 h), the average conidial germination varied from 91.3% to 94.7%, with no statistical difference found among all treatments, including control (*F* test, $P > 0.05$). These results presented strong evidence that conidial germination of ALF 247 was not affected by these two most commonly used fungicides.

The residual biocontrol effects of ALF 247 were also investigated under field conditions. After specific time intervals from the moment of application of the antagonist, cacao pods were challenged by the zoospores of the pathogen and disease levels were evaluated (Fig. 3). The lowest disease scores occurred during the first 10 days after inoculation with *T. martiale*. Afterwards, disease increased slowly but steadily, reaching severity levels similar to the positive control when pods were challenged at 40 days of application of ALF 247. The amount of viable spores of the antagonist remaining on the pod surface was greatly reduced after 10 days, with only traces found at the end of the experiment (Fig. 3).

4. Discussion

A previous study demonstrated a clear possibility for *T. martiale* ALF 247, an endophytic species of cacao sapwood (Hanada et al.,

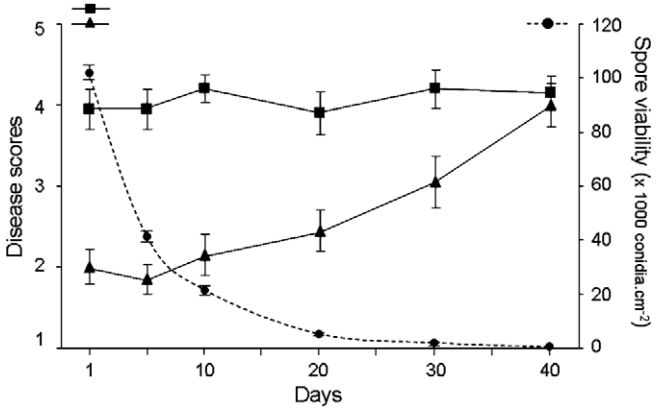

Fig. 3. Residual biocontrol effect of the *T. martiale* ALF 247 isolate on pods challenged by *P. palmivora* and temporal profile of conidia survival. The number of days after ALF 247 inoculation is shown on *x*-axis; on each day indicated, a group of 20 *T. martiale*-inoculated pods were sprayed to run off with a *P. palmivora* suspension of 2×10^5 zoospores mL^{-1} and a group of three unchallenged pods were collected for assessment of ALF 247 survival. For disease severity (*y*-axis on left), average + std error of scores for 20 pods are shown; '■' = only *P. palmivora* (control), '▲' = ALF 247 + *P. palmivora*; disease scores are described on the legend of Fig. 1. For survival (*y*-axis on right), the average ± std error of ALF 247 spore viability counts for three pods ('●') are shown. Negative control pods treated with water and not challenged by the pathogen showed no symptoms of disease (not shown).

2008), to serve as a biocontrol agent (BCA) against *P. palmivora*. With the aim of developing a biological control strategy for Black-Pod Rot (BPR) disease in southeastern Bahia (Brazil), we extended the observations of Hanada et al. (2008) by addressing aspects related to application, formulation, mass production of spores and residual biocontrol effects for this potential BCA.

The progressive reduction in disease severity by higher inoculum concentrations of the antagonist (Fig. 1) is not unexpected as high initial inoculum enables full colonization of the pod by the *Trichoderma*, thereby improving its biocontrol efficacy. Since the cacao tree presents a complex endophytic (Arnold et al., 2003; Crozier et al., 2006) and epiphytic (Ten Hoopen et al., 2003) microbiota, lower initial amounts of the antagonist on the other hand are likely more susceptible to competition effects by other microorganisms. Our results are in agreement with Morandi et al. (2003), who reported the use of higher concentrations of the BCA *Clonostachys rosea* (Link) Schroers, Samuels, Seifert & Gams for control of *Botrytis cinerea* Pers., the causal agent of the gray-mold disease in rose. It is noteworthy, though, that the highest inoculum concentration tested for the ALF 247 (5×10^7 conidia ml^{-1}) did not eliminate the disease symptoms (Hanada et al., 2008; this study). In terms of developing a proper biocontrol strategy, this suggests that more frequent applications, or higher concentrations of the antagonist may be necessary. Combined application with certain fungicides may be also an alternative that warrants further investigation.

Because the definition of adequate doses and methods of application on a case-by-case basis are required for biocontrol products (Melo, 1996), studies on different formulations are relevant to help optimizing their practical use on the crop system of interest. Finding appropriate formulations for field use requires investigation into the effects of the spore suspension composition both on the antagonist and the pathogen (Harman, 1991; Krauss et al., 2006). This relates to improving the ability of the BCA to survive in adverse environmental conditions, not only by overcoming higher populations of the pathogen and/or the competing phylloplane mycoflora, but also by enduring the lack of available nutrients. Hidalgo et al. (2003) has shown significant interaction between application techniques and an oil adjuvant in the final biocontrol results of *C. rosea* against pod diseases in cacao. Amendments that also provide carbon sources (such as sucrose) have the potential of interfering with the viability, germination and establishment of the antagonist, the pathogen, or other microorganisms of the phyllosphere and/or endosphere (Hjeljord et al., 2000; Krauss et al., 2006). By occupying the same ecological niche, some of these microbes can serve as further aids in the biocontrol of the target disease by means of competition (exclusion mechanism) antibiosis, mycoparasitism or induction of plant resistance (Arnold et al., 2003; Crozier et al., 2006; Herre et al., 2007; Mejía et al., 2008). As a result of the influence of formulation amendments, a possibly confounding effect of other actively plant-colonizing endophytes that can outcompete the pathogen and/or the applied BCA must be, therefore, taken into account. Potential effects of the tested amendments on the pathogen and/or on the native microbiota was likely not detected or not relevant for the pathosystem under study. The sucrose and/or vegetable oil amendments tested in this study did not alter the biocontrol effect of ALF 247 in field trials (Fig. 2), suggesting that simple formulations of this antagonist will provide adequate control of BPR. Lack of amendment effects on biocontrol preparations has also been reported in other systems (e.g., Guetsky et al., 2002). Interesting lines of investigation to optimize ALF 247-based biocontrol strategies include the assessment of potential effects by endophytic competitors, their interaction with formulation amendments, and responses to a wider range of environmental conditions that exert a strong interference on biocontrol systems (e.g., Hjeljord et al., 2000; Inglis et al., 2001; Loguercio et al., 2009).

The practical use of a BCA in agriculture depends upon a technical package that assures cost-effectiveness in crop production (Whipps and Lumsden, 2001). This includes an inexpensive and efficient procedure for large-scale production of the chosen BCA (Jacobsen and Backman, 1993), as well as formulation, storage, delivery and application characteristics that permit full expression of its biocontrol potential (Becker and Schwinn, 1993; Harman, 1996). To optimize solid-state fermentation processes, it is desirable to identify components whose addition on a given substrate is capable of enhancing mass production of spores without increasing the costs/benefits relationship (Krishna, 2005; Bhargav et al., 2008). Rice is a common substrate for mass production of BCAs in Brazil (Faria and Magalhães, 2001), is efficient for conidia production, is inexpensive and easily found in local markets, and can be obtained from the rice processing industry. It has also been successfully used in the manufacturing of the *Trichoderma*-based 'Tricovab' (CEPLAC, Bahia), a biocontrol product against the Witches' Broom Disease of cacao (Pomella et al., 2007). Studies on other solid-state fermentation systems have shown that calcium salts improve sporulation and production of certain enzymes (Chiu et al., 1998; Saxena et al., 2001; Wuyep et al., 2003), likely due to effects on pH and mineral nutrition (Horst et al., 2005; Krishna, 2005). Calcium in formulations also tends to improve shelf life and activity of the antagonist after application (Spadaro and Gullino, 2004). In the conditions of this study, a positive and significant effect of $CaCO_3$ on the increase of conidial production for ALF 247 was observed, followed by an also significant effect of urea, though to a lesser extent (Table 2). The roles of urea as a pH-controlling compound and as a nitrogen source that tends to induce conidiation in fungal species are known (Krishna, 2005). The results also showed that the type of inoculum used for fermentation on this solid substrate was critical to maximize yield of conidia (Table 2). Higher levels of conidia production were obtained when mycelium was used as the starting propagules. This may be explained by the tendency of vegetative inoculum to display lag times shorter than those of conidia (Krishna, 2005), thereby allowing a faster colonization of the substrate. The significant interaction found between inoculum type and substrates helps to explain the lack of statistical difference between the two best treatments in terms of conidial

production, which included two types of inoculum (plugs and spore suspensions) and two additive treatments ($CaCO_3$ alone or in conjunction with urea); additionally, this interaction would also explain why the additive treatments gave different results, depending the inoculum type (Table 2). The excellent conidial production obtained with low-cost solid substrates suggests that ALF 247 is prone to a cost-effective large-scale production, a topic that merits further study.

The possibility of combining biocontrol agents and chemical fungicides is important for the IPM approach, because the levels of disease suppression given by combined applications are typically equal or superior to the use of BCA alone (e.g., Hidalgo et al., 2003; Deberdt et al., 2008). When disease pressure increases or an outbreak occurs, maximal levels of protection are needed, such that chemical fungicides tend to exhibit an efficient short-term control effect, which can be later sustained by the BCA (Harman, 1996; Whipps and Lumsden, 2001; Backman and Sikora, 2008). Effective combinations of chemicals and BCAs in IPM programs tend to reduce the negative impact on the environment by lowering the number and/or amounts of chemical applications (Melo, 1998), which is particularly important in the context of cacao production (Tondje et al., 2007; Deberdt et al., 2008). Conidia of ALF 247 were compatible with two common fungicides used against pod diseases; there was no decrease in conidia germination 3 h after mixing conidia with the fungicide, which is more than the time required in average to mix and disperse the fungus/pesticide mixture on the field. This suggests that combined application strategies are promising for the control of *P. palmivora* from an IPM perspective.

Effectiveness of a BCA is related to its potential to proliferate and survive on the plants for a prolonged period after application, mainly on tissues that are usually prone to infection by the target pathogen (Elad, 1990). ALF 247 is an endophyte that colonizes the whole plant body after application (Hanada et al., 2008). Although the number of remaining viable conidia on the pod surface sharply decreased after 5–10 days, biocontrol effects of *T. martiale* in the testing pods could be observed up to 30 days (Fig. 3). Despite that only traces of the antagonist were found at 40 days, previous results have indicated that ALF 247 can be recovered from pods up to 3 months after its application (Hanada et al., 2008). However, the assessment method on that study was based on the percentage of pod-surface discs in which growth and sporulation of *T. martiale* was detected, rather than upon spore viability on the pod surface (Fig. 3). Thus, these apparently contradictory results can be reconciled by suggesting that sprayed *T. martiale* actively colonizes pod tissues during the first 20–30 days but, after 35–40 days, colonization likely stabilizes at levels that are not sufficient to provide control against *P. palmivora*. This may be especially true if one considers that the amount of zoospores used to infect the pods in the present study was far higher than those expected in the field, under common inoculum circumstances of a disease-affected crop. Considering usual inoculum pressures of *P. palmivora* on infested areas and exploring the endophytic nature of *T. martiale*, further experiments are required to address a potential disease reduction after a single or more applications of the BCA. Under conditions of antagonistic interaction, the period of survival (in terms of conidial viability) of *T. martiale* ALF 247 on the surface of cacao pods and the level of residual disease control achieved up to 30 days post-application can be considered as reasonably appropriate for biological control purposes. Based on the fungicide-compatibility trends observed, this antagonist could be applied every 3 or 4 weeks to increase its efficacy, which is a common frequency of fungicide applications for managing BPR. Alternatively, protection schemes that alternate preventive fungicide applications with antagonist sprays can also be developed. Our results clearly indicate that the *T. martiale* ALF 247 has a true potential to become an effective BCA against BPR of cacao, caused by *P. palmivora*, with further studies being required to start developing a commercially applicable, environmental-friendly biofungicide for the cacao crop in southeastern Bahia.

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