Isolation of Actinomycetes and Endospore-forming Bacteria from the Cacao Pod Surface and Their Antagonistic Activity against the Witches' Broom and Black Pod Pathogens

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In this study, actinomycetes and endospore-forming bacteria were isolated from the surface of cacao pods. The activity of these microorganisms against *Crinipellis perniciosa* and *Phytophthora palmivora*, causal agents of witches' broom and black pod diseases of cacao, respectively, was investigated. A total of 336 isolates of actinomycetes and endospore-forming bacteria were tested on a detached pod assay against *C. perniciosa*. The screening procedure used proved to be fast and inexpensive, allowing the selection of five actinomycetes as the most promising isolates for the biocontrol of *C. perniciosa*. Under laboratory conditions the actinomycetes were able to inhibit 100% of *C. perniciosa* basidiospore germination. However, under field conditions the selected actinomycetes were unable to protect cacao pods against both pathogens. In these experiments, inhibition of *C. perniciosa* ranged from 6% to 21% in relation to the control, whereas there was no inhibition of black pod caused by *P. palmivora*. Formulations need to be improved in order to enhance the activity of the actinomycetes against cacao pathogens in the field. Molecular identification of the selected isolates showed that they are species of the genus *Streptomyces*.
KEY WORDS: *Theobroma cacao;* biological control; actinomycetes; endospore-forming bacteria; *Streptomyces* sp.; *Crinipellis perniciosa; Phytophthora palmivora.*

INTRODUCTION

Cacao (*Theobroma cacao*) is widely cultivated in the tropics of South and Central America, Asia, and Africa. In Brazil, witches' broom disease caused by the basidiomycete *Crinipellis perniciosa* is the most limiting factor for cacao cultivation (26). The pathogen is able to infect all meristematic parts of cacao plants, including vegetative buds, flower cushions and pods (26). In the southern region of Bahia State, Brazil, losses up to 70% were inflicted by the disease following its outbreak in 1989 (2). More resistant genotypes are being developed and this control strategy is expected to reduce the severity of the disease (34). The control of witches' broom disease with fungicides is not always efficient and economically feasible (24). Biological control is being investigated in several countries with different microorganisms obtained from various parts of cacao trees (4,5). The most promising results obtained to date were with the use of *Trichoderma stromaticum*, a

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mycoparasite of *C. perniciosa*, that causes a reduction in the production of the basidiocarps and consequently in the number of basidiospores, which are the infective structures of the pathogen (5). However, this mycoparasite does not protect the plant against new infections. In this context, it is important to develop strategies to protect the susceptible tissues of the plant against the pathogen.

Black pod, caused by *Phytophthora* spp., is an economically serious problem in all areas of the world where cacao is grown. In Brazil, losses due to black pod vary between 20% and 30% (26). There are few sources of resistance and the situation is especially complicated because there are four species of *Phytophthora* associated with this disease (26). Chemical control with copper-based fungicides presents good results, but is not always economically feasible (10). The biological control of this disease has received little attention and most attempts have been made with antagonistic fungi (22).

The phylloplane, aerial plant surfaces, is inhabited by numerous and diverse microbes (21). In this complex microbiological habitat, the most diverse interactions among microorganisms occur. It is possible to find microorganisms adapted to these habitats and with notable antagonistic activity against pathogens (7). Besides being microbiologically rich, the phylloplane is extremely inhospitable because of its large variations in temperature, light, water, and nutrients (3). To survive in these environments bacteria have adopted two strategies: resistance and escape. The first requires the capacity to tolerate extreme conditions by the production of resistance structures and certain compounds. The second considers the ability of the bacteria to explore sites less prone to environmental stresses (6). When these epiphytes are considered for biological control, their population densities must be high enough to exert an antagonistic effect against the pathogen. They also need to maintain their population in time, which is achieved through resistance structures that allow their survival under adverse conditions. Bacteria belonging to the *Bacillus* group are able to produce structures called endospores, which are considered to be among the most resistant biological structures known (27). The mechanisms involved in the biological control by these bacteria include antibiosis (13), competition for space, water and nutrients, and the activation of a plant's defense responses (33).

Actinomycetes constitute a morphologically diverse group, distinguished from other Gram-positive bacteria by their filamentous growth and GC-rich DNA (23). Although actinomycetes are present in both terrestrial and aquatic environments (16), the great majority of the research on biological control focuses on the isolation and use of these organisms on the rhizosphere and rhizoplane (16). Several actinomycetes are producers of antimicrobial compounds able to inhibit the growth of other bacteria, fungi, and protozoa (11). For this reason they are being studied with the objective of obtaining new antibiotics (27). These organisms have a great capacity to survive in adverse environments. It was shown that the ability to accumulate high endogenous concentrations of trehalose is related to the capacity of these organisms to resist dry conditions due to the preservation of membrane integrity by this substance (9).

Although it is assumed that a high population of microorganisms is associated with all parts of cacao plants, there is little or no information available on the microorganisms obtained from the pod surface with respect to biological control. In the present research the objectives were to isolate and select endospore-forming bacteria, including the ones from the *Bacillus* group and actinomycetes from the surface of cacao pods. We focused on the ability of these bacteria to protect susceptible pods against *C. perniciosa*. A detached

pod assay was employed in the selection of microorganisms with the potential to control witches' broom and the ones selected as the most promising were tested against black pod. The selected microorganisms were identified by partial sequencing of the 16s rDNA.

MATERIALS AND METHODS

Sampling, isolation, and cultivation of the biocontrol agents Cacao pods in different development stages and from different genotypes were collected from farms in the south of Bahia State, Brazil. The method of Földes *et al.* (13) was adopted for isolation of endospore-forming bacteria. In short, 3-cm^2 fragments from the surface of the pods were transferred to 15 ml of Finley and Fields' liquid medium to induce the formation of endospores. This medium contained meat extract (3 g l^{-1}), peptone (1.9 g l^{-1}), and $MnSO_4$ (0.03 g l^{-1}) adjusted to pH 7 (12). After 3 days of incubation at 25°C with shaking, a 500-μl aliquot of the medium was heated at 80°C for 10 min to inactivate the vegetative cells. The sample was spread on solid agar at 15 g l^{-1} Finley and Fields' medium and incubated for 48 h at 25°C. Individualized colonies with different morphological characteristics were selected, transferred and maintained on petri dishes containing the same medium. For the isolation of actinomycetes, the technique of Hirsch and Christensen (19) was used. Pod samples were exposed to 70°C for 72 h and extracted with saline solution (NaCl 0.85%) amended with Tween 80 (0.05 ml l^{-1}) for 24 h. A 15-ml aliquot of the extract was passed through a 0.45-mm filter and the membranes were transferred to soil extract medium containing cyclohexamide (10 mg ml^{-1}) and incubated at 25°C for 5 days. Soil extract medium (31) is composed of 100 ml l^{-1} of soil infusion supernatant (200 g soil l^{-1}, boiled for 5 min, and decanted for 15 min), glucose (1 g l^{-1}), K_2HPO_4 (0.5 g l^{-1}), KNO_3 (0.1 g l^{-1}), agar (15 g l^{-1}), pH 7. After the incubation, membranes were removed and the petri dishes incubated for 5 additional days. Actinomycete-like colonies were purified and maintained on the same medium. All strains were stored at –80°C in tryptic soy broth amended with glycerol to a final concentration of 10% (v/v).

Pathogen cultivation and inoculum production Isolate ALF 42 of *C. perniciosa*, obtained from green infected branches in Itajuipe, Bahia State, Brazil, was used in all assays. Basidiospore production was based on a modified methodology proposed by Griffith and Hedger (17) and Niella *et al.* (29). 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 brooms, which are hypertrophic, and hyperplasic cacao branches resulted from the pathogen's colonization (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 × 0.5 × 0.5m) submitted to a watering regime of 1 l min^{-1} day^{-1} and a photoperiod of 8:16 L:D. After 15 days of incubation, the water supply was interrupted for 4 days to induce basidiocarp formation. The basidiocarps produced were collected, surface-sterilized with streptomycin sulfate (150 μg ml^{-1}), 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 sulphonic (MES) acid (0.195%) with shaking for a period of 18 h under laboratory conditions (15). Basidiospore suspensions were kept in liquid nitrogen until use. *Phytophthora palmivora* isolate ALF 625, obtained from a green infected pod collected in Itajuipe, was cultivated

on a medium composed of the extract obtained by boiling 25 g of carrots, tomato extract (45 g l^{-1}), $CaCO_3$ (3 g l^{-1}) and agar (15 g l^{-1}). For zoospore production, *P. palmivora* was cultivated for 7 days in the dark and 3 days under fluorescent light (40 w). To each plate, 10 ml of cold water was added; plates were incubated for 15 min in a refrigerator and for 20 min at room temperature. Zoospores were collected and the concentration adjusted to 10^5 zoospores ml^{-1} (25).

Inoculum of the biocontrol agents For the laboratory experiments on the selection of the most promising biocontrol agents, inoculum of endospore-forming bacteria was produced by cultivating the isolates for 24 h on Finley and Fields' medium and the actinomycetes for 5 days on soil extract medium (31). The propagules used were vegetative cells for endospore-forming bacteria and spores for actinomycetes. The vegetative cells or spores were suspended in saline solution (NaCl 0.85%) amended with Tween 80 (0.05 ml l^{-1}) and the concentration was adjusted with a spectrophotometer to A_{540}=0.8. In the experiments on the effect of the type of propagule on the germination of *C. perniciosa* basidiospores, spores of the actinomycetes were produced as described above for the laboratory experiments.

Vegetative cells and the supernatant were produced by cultivating the actinomycetes in a modified TSB medium, containing peptone (12.5 g l^{-1}) and NaCl (5 g l^{-1}). After incubation at 25°C for 48 h under shaking, vegetative cells were separated from the supernatant by centrifuging at 10,000 × *g* for 15 min. The spores produced on soil extract medium and vegetative cells were suspended in saline solution and their concentration was adjusted as described above. For the experiments on the effect of the formulations on the germination of *C. perniciosa* basidiospores, the actinomycetes were cultivated as described above for obtaining vegetative cells. The suspensions were added to each of the five following formulations: (i) yeast-sucrose, composed of 20% of fresh yeast (Burns Philp, Pederneiras, SP, Brazil) autoclaved for 30 min at 120°C, 0.01% of xanthan gum (Tec Pharma, São Paulo, Brazil), 0.1% of sucrose, 10% of Hoagland's solution and 0.1% of Tween 80; (ii) colloidal chitin 0.03% plus Break-thru®0.03% (Goldschmidt Chemical Corp., Hopewell, VA, USA); (iii) peptone 1% plus OPPA®2% (Petrobras, Macae, RJ, Brazil); (iv) sucrose 2% plus Break-thru®0.05%; and (v) meat extract 2% (Knorr, Pouso Alegre, MG, Brazil) plus Break-thru®0.05%. The final concentration of all formulations was adjusted to A_{540}= 0.8. For the field experiments on the activity of the actinomycetes against *P. palmivora*, the five isolates were cultivated and the inoculum was prepared as described for the experiments on the effect of the formulations. The isolates were applied with the following formulations: saline solution; yeast-sucrose; and meat extract. Controls were treated with the formulations without the microorganisms.

Selection of the biocontrol agents under laboratory conditions For the selection of the most effective isolates, 20- to 30-day-old pods of cacao genotype TSH 516 were collected, and washed with tap water and distilled water. The suspensions of each isolate were applied by atomization on the surface of one pod until the suspension was close to runoff. The pods were kept in a humid chamber for 24 h for endospore-forming bacteria and 48 h for actinomycetes. After the incubation, the pods were brushed with a suspension of *C. perniciosa* containing 10^6 basidiospores ml^{-1} and again incubated in a humid chamber for 3 h. Pods were air-dried and transparent nail polish was applied on portions of their surface. From the covered portions, three sections (3 × 15 mm) were removed from each

pod and transferred to glass slides, stained with cotton blue, and 50 basidiospores were counted, registering the percentage of germinated basidiospores in relation to the control. Isolates that inhibited more than 50% of the basidiospore germination were re-applied on three detached pods. Application of the biocontrol agents, *C. perniciosa,* and evaluation were done as described above. The screening experiment was conducted once.

Effect of the type of propagule on the activity of the actinomycetes Because the production of spores was slow and cumbersome, the effect of other types of propagules on the germination of *C. perniciosa* basidiospores was studied. Spores, vegetative cells and the supernatant of the five most promising isolates of actinomycetes (Ac4, Ac19, Ac 26, Ac 68 and Ac 79) selected in the initial bioassays were atomized on three detached pods obtained from the genotype TSH 516. After the application of the actinomycetes, the pods were incubated at 25°C for 48 h. The application of *C. perniciosa* inoculum and evaluation were done as described for the screening experiments. The treatments were the different inoculum types of each of the five selected isolates of actinomycetes suspended in saline solution. The experiment was repeated twice.

Effect of the formulations The effect of five formulations (see: Inoculum of the Biocontrol Agents section) was tested for the five most promising isolates of actinomycetes. The assays were done on 20- to 30-day-old pods from genotype TSH 516 and ICS 1. The treatments were five formulations and the five selected actinomycetes. Each treatment was applied on three pods in the field. Pods were maintained in humid chambers consisting of plastic bags for 24 h after the application of the actinomycetes. The controls were each isolate of actinomycete without the formulations and the formulations without the actinomycetes. Seventy-two hours after the application, pods were detached from the trees, brought to the laboratory, basidiospores of *C. perniciosa* were applied, and the germination was evaluated as described for the screening experiments. The experiment was repeated twice.

Activity against *Phytophthora palmivora* The five most promising isolates were tested against the development of black pod caused by *P. palmivora* in field experiments. Each treatment (see: Inoculum of the Biocontrol Agents section) was applied to seven pods. After application of the treatments, the pods were kept in humid chambers for 24 h; 72 h later they were sprayed with a zoospore suspension until close to runoff. After inoculation of the pathogen, pods were again kept in a humid chamber for 24 h and disease development was evaluated after 7 days according to the following index: 1= no symptoms; 2= diameter of lesions <2 mm; 3= lesions with a diameter between 2 mm and 2 cm; 4= pods with up to 25% of the surface lesioned; 5= pods with >25% of the surface lesioned (18). The experiment was conducted twice.

Statistical analyses Data from the laboratory experiments were analyzed in a randomized design. The experiments on the effect of different types of propagules on the germination of *C. perniciosa* basidiospores were analyzed in a factorial scheme, with five isolates and three types of propagules. The experiments on the effect of the formulations were analyzed in a factorial scheme, with five isolates and five formulations. ANOVA was conducted with the software SAEG version 8.0 (32).

Molecular identification of the actinomycetes The five most promising isolates were identified by sequencing a fragment of the rDNA gene. Genomic DNA was extracted from

3- to 4-day-old cultures according to Hopwood *et al.* (20). The amplification was done with primers p27f and p1401r, which are homologous to the conserved regions of bacterial 16S rDNA (1). The PCR products were purified by using a purification column (GFX PCR DNA and Gel Band purification kit, Amersham Biosciences, Piscataway, NJ, USA). Sequencing was done directly by using the amplification primers on a MegaBACE 1,000 (Amersham Biosciences) and the DyeDeoxy terminator cycle sequencing kit (Amersham Biosciences) according to the manufacturer's instructions. Sequences were aligned with Clustal W1.8 (35) and compared with the ones available in public databases.

RESULTS

Collection of microorganisms A total of 63 pods were collected from farms located in five municipalities in the south of Bahia State, Brazil. From these samples, 203 isolates of endospore-forming bacteria and 94 isolates of actinomycetes were obtained. To this collection were added 17 isolates of endospore-forming bacteria selected by Macagnan in a M.Sc. research project and 22 actinomycete isolates obtained from soil samples collected in South Bahia by Dr. Prakash Hebbar (Masterfoods USA). A total of 336 isolates, 220 of endospore-forming bacteria and 116 of actinomycetes, were tested.

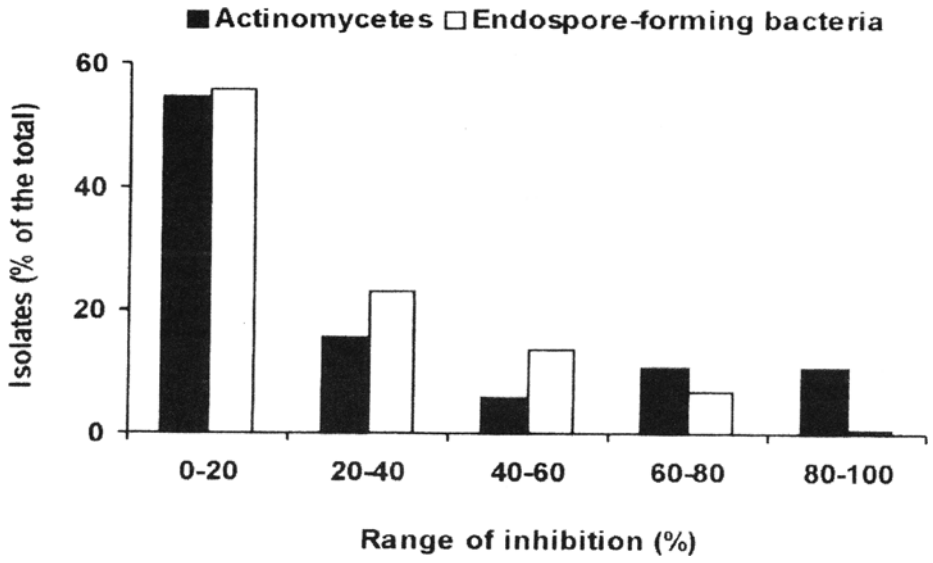

Fig. 1. Activity of endospore-forming bacteria and actinomycetes in inhibiting the germination of *Crinipellis perniciosa* basidiospores.

Selection of the most promising isolates The data from the bioassay on detached pods showed that 7% of the endospore-forming bacteria were able to inhibit the germination of *C. perniciosa* basidiospores at levels of 60% to 100%, whereas 21% of the actinomycetes were inhibitory at these levels. When the inhibition classes >80% are considered, 0.50% of the endospore-forming bacteria and 11% of the actinomycetes were effective (Fig. 1).

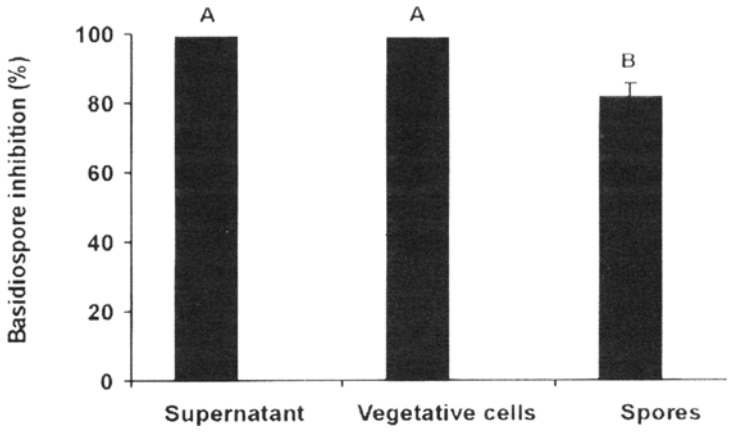

Fig. 2. Inhibition of *Crinipellis perniciosa* basidiospore germination on the surface of detached cacao pods. Treatments with the same letter do not differ significantly according to Tukey's studentized range test (P=0.05). Error bar represents the S.E.M.

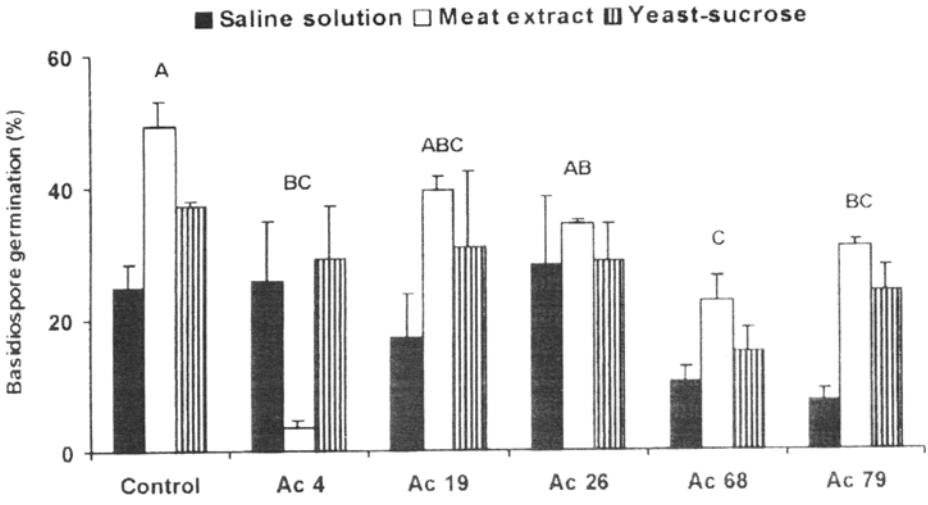

Fig. 3. Germination of basidiospores of *Crinipellis perniciosa* on cacao pod surface. Pods were treated in the field with five actinomycetes suspended in saline solution, meat extract, or yeast-sucrose. Means with a common letter do not differ significantly according to Tukey's studentized range test (P=0.05). Error bars represent the S.E.M.

Eighteen isolates were selected based on their capacity to inhibit >50% of the germination of *C. perniciosa* basidiospores on the pod surface (data not shown). Of these isolates, 13 were actinomycetes and five were endospore-forming bacteria. The 18 pre-selected isolates were re-evaluated on the pod surface and isolates Ac 4, Ac 19, Ac 26, Ac 68, and Ac 79 were selected as being the most promising ones based on their inhibitory effects on

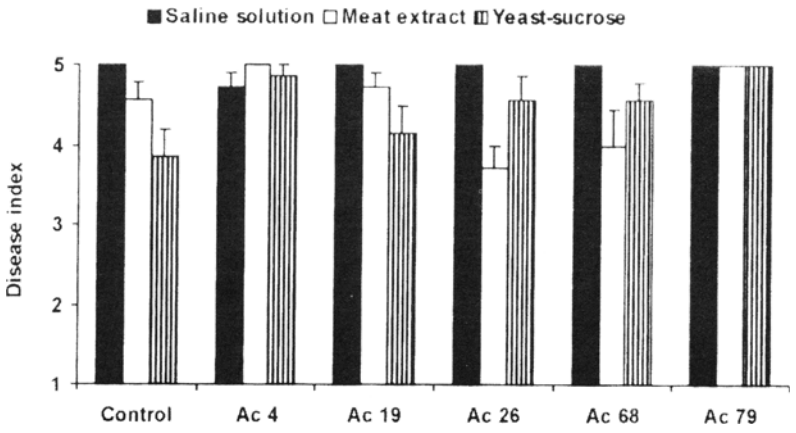

Fig. 4. Effect of actinomycetes on the severity of black pod caused by *Phytophthora palmivora* under field conditions. Disease severity was determined according to an index, where 1 represents no disease and 5 represents >25% of the pod surface with black pod symptoms. Error bars represent the S.E.M.

basidiospore germination, fast growth and abundant sporulation in the soil extract medium.

Molecular identification The five most promising isolates were identified by sequencing a portion of the 16S rDNA gene and comparing it with sequences of the same gene from public databases. Nucleotide sequences were deposited with GenBank under the accession numbers DQ066885 to DQ066889. All isolates were classified as *Streptomyces* spp.

Effect of type of inoculum on inhibition of *C. perniciosa* germination In these experiments, different kinds of inoculum, including supernatant, vegetative cells and spores, were used on detached pods. The results did not show any difference in the ability of the five most promising isolates to inhibit *C. perniciosa*. All isolates tested inhibited approximately 100% of the basidiospore germination. However, the inoculum types differed in their activity against basidiospore germination. Vegetative cells and the supernatant had similar inhibitory activity, whereas spores were significantly less active (Fig. 2). On the basis of these results, all other experiments were done with vegetative cells produced in modified TSB medium.

Inhibition of *C. perniciosa* germination Several experiments with cacao genotype TSH 516 were done to test the effect of the five most promising isolates and the formulations on the germination of *C. perniciosa* basidiospores. The first experiments showed that meat extract and yeast-sucrose provided the highest levels of inhibition of basidiospore germination (data not shown), and in further experiments only these formulations were used. Saline solution was used for comparison purposes. In general, the formulation based on meat extract showed the highest inhibition of basidiospore germination (Fig. 3). The isolates tested inhibited from 70% to 85% of the germination of basidiospores, whereas the control inhibited an average of 60% (Fig. 3). Statistical analyses showed no interaction between formulations and biocontrol agents ($P>0.05$). Combination of isolates Ac 79 and Ac 26 did not result in increased inhibition of basidiospore germination (data not

shown). Experiments done on cacao pods from genotypes TSH516 and ICS1 did not show differences in the inhibition of basidiospore germination (data not shown), indicating that the cacao genotype did not influence the activity of the actinomycetes.

Assays against *P. palmivora* In field experiments against *P. palmivora*, none of the isolates or formulations was able to inhibit black pod at acceptable levels (Fig. 4). The average of the disease indexes obtained for all isolates tested was higher than 4.

DISCUSSION

Cacao is an economically, socially and environmentally important crop for South Bahia State, Brazil. Diseases, especially witches' broom and black pod, are responsible for losses of up to 70% in this area. Biological control is an attractive method to manage these diseases. In this work we isolated actinomycetes and endospore-forming bacteria from the surface of cacao pods and studied their activity against two pathogens. The most common application of actinomycetes and endospore-forming bacteria in biological control is in the rhizoplane and plant rhizosphere (33), with some reports of actinomycetes as endophytes (8). In this work we attempted to find potential biocontrol agents on the cacao pod surface, because direct damage is caused by pathogens in this part of the plant.

The selection procedure used in this work enabled simple and fast identification of the five most promising isolates among the 336 tested for the biocontrol of witches' broom disease of cacao. All isolates selected as promising for biocontrol were obtained from the pod surface. This procedure was revealed to be very efficient for the system studied; *C. perniciosa* only shows external symptoms on pods a minimum of 2 months after the inoculation (Pomella, A.W.V., unpublished data), whereas with this methodology results were obtained within 48 h. This assay also eliminated the environmental conditions that can affect *C. perniciosa* basidiospore germination and penetration. The screening procedure is less expensive and less time-consuming than the ones done under field conditions.

The results showed a clear superiority of the actinomycetes in the inhibition of germination of *C. perniciosa* basidiospores in comparison with the endospore-forming bacteria (Fig. 1). Partial sequencing of the 16S rDNA allowed the identification of all the selected actinomycetes as *Streptomyces* spp. However, identification of the isolates at the species level was not possible because of the low resolving power of the 16S rDNA gene to distinguish closely related species (14). Sequencing of other genes will be necessary to identify these bacteria at the species level.

Actinomycetes are known as producers of antimicrobial compounds that can be used in agriculture, medicine and research (27). In particular, the genus *Streptomyces* is very important in the production of these compounds. Approximately 50% of the isolates from this genus are producers of some kind of antibiotic and up to now, more than 500 different antibiotics have been shown to be produced by actinomycetes. For example, streptomycin and cyclohexamide are both produced by *Streptomyces griseus*, kanamycin by *S. kanamyceticus*, tetracycline by *S. aureofaciens*, nystatin by *S. noursei*, and chloramphenicol by *S. venezuelae* (27). It is possible that the isolates selected in this work were antibiotic producers. The supernatant and the vegetative cells were equally efficient in inhibiting the germination of the basidiospores (Fig. 2), which indicates that the presence of living cells is not required for inhibition. The higher activity of vegetative cells in comparison with spores can be explained by the fact that spores constitute survival structures and the production of antimicrobial compounds is done by vegetative cells

(28). Furthermore, when spores are used as inoculum, they need to germinate to start the production of antimicrobial compounds, whereas vegetative cells are already active. Vegetative cells were more inhibitory and easier to obtain than spores. Whereas vegetative cells were obtained in 48 h, spores were obtained only in 7 days.

This work showed that cacao pods harbor actinomycetes that have the potential to be explored for biocontrol. One of the remaining limitations of biocontrol is the reproduction of laboratory experiments in the field. Although the five selected actinomycete isolates were able to inhibit 100% of the germination of *C. perniciosa* in the laboratory (Figs. 1 and 2), under field conditions the inhibition ranged from 6% to 21% (data not shown). Experiments on black pod showed no effect of the actinomycetes on the control of this disease. Other results from our laboratory showed that disease indices lower than 2.5 indicate that the treatment under study is effective against black pod (Pomella, A.W.V., unpublished data). The formulations may have been responsible for the poor activity of the actinomycetes. It is still difficult to provide the ideal formulation for the activity of the microorganisms in the field. In fact, according to Paau *et al.* (30), the use of incorrect formulations is one of the main causes of inconsistency of biocontrol in the field. It is also possible that the poor effects of the actinomycetes against the cacao pathogens under field conditions are due to varying environmental conditions and competition with the indigenous microflora of the pod surface. Further studies of the population dynamics of these antagonists on the pod surface are necessary to elucidate their fate under field conditions.

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