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Endophytic fungal diversity in *Theobroma cacao* (cacao) and *T. grandiflorum* (cupuaçu) trees and their potential for growth promotion and biocontrol of black-pod disease

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ABSTRACT

The endophytic niches of plants are a rich source of microbes that can directly and indirectly promote plant protection, growth and development. The diversity of culturable endophytic fungi from stems and branches of *Theobroma cacao* (cacao) and *Theobroma grandiflorum* (cupuaçu) trees growing in the Amazon region of Brazil was assessed. The collection of fungal endophytic isolates obtained was applied in field experiments to evaluate their potential as biocontrol agents against *Phytophthora palmivora*, the causal agent of the black-pod rot disease of cacao, one of the most important pathogens in cocoa-producing regions worldwide. The isolated endophytic fungi from 60 traditional, farmer-planted, healthy cacao and 10 cupuaçu plants were cultured in PDA under conditions inducing sporulation. Isolates were classified based upon the morphological characteristics of their cultures and reproductive structures. Spore suspensions from a total of 103 isolates that could be classified at least up to genus level were tested against *P. palmivora* in pods attached to cacao trees in the field. Results indicated that ~70 % of isolates showed biocontrol effects to a certain extent, suggesting that culturable endophytic fungal biodiversity in this system is of a mostly mutualistic type of interaction with the host. Eight isolates from genera *Trichoderma* (reference isolate), *Pestalotiopsis*, *Curvularia*, *Tolypocladium* and *Fusarium* showed the highest level of activity against the pathogen, and were further characterized. All demonstrated their endophytic nature by colonizing axenic cacao plantlets, and confirmed their biocontrol activity on attached pods trials by showing significant decrease in disease severity in relation to the positive control. None, however, showed detectable growth-promotion effects. Aspects related to endophytic biodiversity and host–pathogen–endophyte interactions in the environment of this study were discussed on the context of developing sustainable strategies for biological control of black-pod rot of cacao.

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Introduction

Microorganisms establish symbiotic, mutualistic and other types of interactions with all animals and plants on Earth (Zilber-Rosenberg & Rosenberg 2008). Despite the fact that many fungi are plant pathogens, a large variety of fungi, including pathogenic species, live as endophytes, asymptotically within intra- and intercellular spaces of leaves and vascular tissues of roots and stems (Wilson 1995; Stone et al. 2004; Hyde & Soyong 2008). The endophytic niche is a rich source of microorganisms that can directly and indirectly promote plant growth and development (Lodewyckx et al. 2002; Barrow et al. 2008). It stands to reason that the higher the diversity of endophytes in a crop, the greater the likelihood of finding microorganisms that have salutary effects on that crop. In general, fungal endophytes are more diverse in tropical than in temperate plants (Arnold et al. 2000, 2003), likely due to the fact that tropical ecosystems harbour a higher variety of fungi (Stone et al. 2004). Since all sorts of stress-related biotic and abiotic factors are also more diverse in tropical ecosystems, this idea brings forth the concept of higher organisms being better adapted to environmental dynamics due to its specifically associated microbiota (Tadych & White 2009; Oldroyd et al. 2009).

Induction and/or synthesis of plant growth-promoting compounds (auxins, cytokinins), N₂ fixing, synthesis of enzymes/peptides that provide nutrient availability (phosphatases, siderophores, etc.), and tolerance to various types of stresses are among the direct mechanisms through which endophytes promote plant growth (reviewed by Harman 2000; Whipps 2001; Lodewyckx et al. 2002; Harman et al. 2004). On the other hand, prevention of deleterious effects by plant pathogens through biological control is considered an indirect way of promoting growth and development (Lodewyckx et al. 2002). Endophytes have a widely recognized potential as biocontrol agents (BCAs), which can exert their activity through several mechanisms (Backman & Sikora 2008; Mejía et al. 2008). For instance, endophytic fungi or bacteria can induce systemic resistance in plants against pathogens after actively penetrating and colonizing the host, promoting the synthesis of biologically active compounds or causing changes in plant morphology and/or physiology (Faeth 2002; Harman et al. 2004; Bailey et al. 2006; Melnick et al. 2008). Mycoparasitism, antibiosis, and competition are other well described mechanisms through which endophytic fungi can display biocontrol activity (Arnold et al. 2003; Harman et al. 2004; Herre et al. 2007; Bailey et al. 2008). The simultaneous occurrence of more than one of these beneficial effects has been reported for a single isolate or the same set of microbes (e.g. Raupach & Kloepper 1998; Harman et al. 2004; Vijayaragahavan & Raman 2009).

The Black-Pod Rot (BPR) is the most important disease of the cacao crop (*Theobroma cacao* L.), as it occurs in all cocoa-producing countries and worldwide losses to it can account for 20–25 % of the expected cocoa production (Bowers et al. 2001; Luz & Silva 2001; Evans 2007). This disease is caused by several species of the straminipile (formerly oomycete) genus *Phytophthora*, with damages reaching up to 90 % of pod production, depending on climatic conditions (Bowers et al. 2001). Investment on its control demands a long-term plan,

because it is very difficult to remove this pathogen from a given area, due to its ability to survive in soil and infected plant parts (Evans & Prior 1987). Chemical, genetic and cultural practices have been used to control this disease, but none has provided a sufficiently and economically efficient, and/or environmentally friendly method for desired results (Holderness 1992; Luz & Silva 2001; Ndoumbe-Nkeng et al. 2004). From an Integrated Pest Management (IPM) perspective, additional methods are certainly required and, in this context, biological control of *Phytophthora* spp. in cacao is a promising approach (Krauss & Soberanis 2002; Tondje et al. 2007; Deberdt et al. 2008; Hanada et al. 2009).

Isolation of epi/endophytic microorganisms and screening for biocontrol ability are a common strategy that relies on the available microbial biodiversity (Evans et al. 2003; Ten Hoopen et al. 2003; Rubini et al. 2005; Bailey et al. 2008), which is expected to be higher in species-rich ecosystems such as rainforests (Strobel & Daisy 2003). In addition, when plants are cultivated in areas that differ from their native habitats, endophytic diversity tends to decrease (Taylor et al. 1999) with a concomitant increase in susceptibility to diseases (Evans et al. 2003). Hence, the search for BCAs under circumstances where microbes had co-evolved with their hosts is likely to provide better results (e.g. Holmes et al. 2004; Mejía et al. 2008). Considering that the Upper Amazon region of Brazil, Bolivia, Peru and Ecuador is the centre of origin for *T. cacao* (Motamayor et al. 2002), it is not unexpected that the evolution of cacao–pathogens interactions in this region has led to co-evolution of associated endophytes that promote benefits to this crop. Since novel taxa of endophytic fungi are frequently found in important well-characterised plant species (Stone et al. 2004), as it is the case in cacao and some closely related species (Evans et al. 2003; Holmes et al. 2004, 2005; Samuels et al. 2006; Crozier et al. 2006; Hanada et al. 2008; Mejía et al. 2008), the objectives of this study were to sample and assess culturable biodiversity of fungal endophytes in two related *Theobroma* species (cacao and cupuaçu), and search for endophytes with biocontrol potential against *Phytophthora palmivora*, the most important causal agent of the BPR disease of cacao in neotropical cocoa-producing regions of Brazil.

Material and methods

Experiments were conducted on field areas and facilities at both the Instituto Nacional de Pesquisas da Amazônia (INPA, Manaus-AM) and Almirante Cacau Ltd. (Itajuípe-BA), located in the States of Amazonas and Bahia, respectively.

Theobroma spp. and isolation of fungal endophytes

Approximately 60 trees from traditional, farmer-planted (not systematically bred) populations of *Theobroma cacao* L. (cacao) and 10 of *Theobroma grandiflorum* (Willd. ex Spreng.) K. Schum (cupuaçu) from the Amazonas State of north Brazil (a few cacao plants were from the Bahia State, northeastern Brazil) were used for the isolation of fungal endophytes. The plants were chosen on the basis of four criteria: healthy appearance, overall good nutritional/physiological state, and free from any type of chemical/biological product application. Targets for

fungal isolation comprised internal tissues from branches and twigs (1.5–2.5 cm diameter), and stem bark, which were collected by using a flamed scalpel. From each plant, five ~25-mm² fragments from those tissues collectively were cut out, transferred to Petri dishes containing 20 mL potato-dextrose-agar medium (PDA – decoction of 40 g potato, 20 g dextrose and 20 g agar per liter of water) + 25 µg mL⁻¹ chloramphenicol, and incubated at 25 °C in the dark. Newly appearing mycelium was subcultured on a daily basis. These culturable endophytic fungi were labelled and stored in silica gel as described (Dhingra & Sinclair 1995). All these endophytic fungi obtained from *Theobroma* spp. comprised an assembly of isolates deposited in the microorganism collection of the Almirante Cacau Ltd., located in the Bahia State.

Identification of fungal endophytes

The isolated endophytic fungi were cultured in PDA for up to 8 weeks at 25 °C, under a 12 h/12 h dark/light cycles to induce sporulation, with daylight-type fluorescent bulbs (40 W) placed at 40 cm above the plates. Those isolates that sporulated under these conditions were classified based upon the morphological characteristics of their cultures and reproductive structures. Growth rate, mycelial texture, colony colour and sectors formation, and production of pigments were among the culture characteristics observed. Microscopic aspects of hyphae and spores were also observed, such as sizes and shapes, as well as colour of conidiophores and conidia. Semi-permanent slides with these structures were prepared with lactophenol (25 % w/v) and cotton-blue (1–5 mL of 1 % solution in 100 mL lactophenol + <20 mL glacial acetic acid) stains and observed on a light microscope; 50 measurements were randomly taken for each of the following structures: hyphae, conidia, conidiophores, conidiogenous cells and pycnidia. All characteristics assessed were used in taxonomic keys for identification purposes (Carmichael *et al.* 1980; Sutton 1980; Kiffer & Morelet 1997; Barnett & Hunter 1998).

Screening for fungal isolates with biocontrol potential

The phytopathogen *Phytophthora palmivora* strain used in these experiments (isolate '611') was kindly provided by the Centro de Pesquisas do Cacau (CEPEC/CEPLAC, Ilhéus-BA, Brazil). To obtain zoospores, this isolate was grown in Petri plates 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), at 25 °C, for 10 d in the dark and 3 d under continuous light (20-W fluorescent light bulbs set 30 cm above the plates). Afterwards, 10 mL of sterile distilled water was added to each plate, which was placed at 5 °C for 15 min, and at room temperature for 30 min. The release of zoospores was observed through a light microscope, at a 10× magnification, with their concentration being adjusted using a haemocytometer.

To provide inocula for the experiments, spore suspensions were obtained from endophytic fungi cultured for 15 d in the same conditions described above. Conidia were suspended in sterile distilled water, with their concentration adjusted with haemocytometer. The isolated fungal endophytes were evaluated in two types of field experiments regarding their biocontrol activity against BPR. First, a general selection experiment

was performed, in which each isolate, at an adjusted concentration of 5×10^6 spores mL⁻¹, was inoculated in three cacao pods from the 'SIAL 70' cultivar in the field. The pods were ~4 m old, apparently healthy, free from visible injuries or other defects, and remained attached to the plants during the experiments. Pods were sprayed to near run-off with the spores suspended in distilled water, using a 1-L sprayer-nozzle bottle. The volume of suspensions applied per pod with this procedure was 40–50 mL, considering the average surface area of cacao pods. To help with germination of spores and to prevent the applied suspensions from being washed off by rain, pods were covered with transparent polyethylene bags ~1 d before application of treatments and removed 24 h after that, to allow the trial to be conducted under natural field conditions. Seven days after endophyte applications, pods were each challenged by a suspension of 2×10^5 zoospores mL⁻¹ of *P. palmivora* which was also sprayed to near run-off. In order to avoid wash off by rain, to facilitate pathogen penetration by inducing opening of stomata, and to obtain homogeneous infection for all treatments, pods were again covered with polyethylene bags from ~1 d before until 24 h after this inoculation. Positive-control pods were inoculated only with *P. palmivora* and negative controls with sterile distilled water. After 8 d from pathogen application, disease symptoms were scored based on the following scale: '1' = no 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, coalesced or not, covering up to 25 % of the pod surface; '5' = extended/coalesced lesions and necrosis above 25 % of the pod surface. Endophytic fungi showing promise in the disease control were subjected to a second validation-type of experiment, using the same overall procedures described above, except that 20 pods per isolate were evaluated instead.

Assessment of endophytic nature of isolates and their effects on growth promotion

Isolates selected from the previous screening procedure (Table 1) had their endophytic behaviour further tested on

Table 1 – Fungal endophytes from *Theobroma* spp. selected on the basis of promising biocontrol potential against *Phytophthora palmivora*.

Host/fungi ^a	Isolate	Local of origin ^b	Source
Cacao			
<i>Pestalotiopsis</i> spp.(1)	ALF 821	Benjamin constant	Stem
<i>Curvularia</i> spp.	ALF 829	Benjamin constant	Stem
<i>Fusarium</i> spp.(1)	ALF 839	Benjamin constant	Stem
<i>Fusarium</i> spp.(2)	ALF 849	Benjamin constant	Stem
<i>Tolyposcladium</i> spp.	ALF 902	Benjamin constant	Stem
<i>Fusarium</i> spp.(3)	ALF 1587	Itacoatiara	Branch
Cupuaçu			
<i>Pestalotiopsis</i> spp.(2)	ALF 1586	Presidente Figueiredo	Branch

a Numbers between parentheses differentiate isolates from the same fungal genus/species, which were considered as different individuals because they originated from different plants.

b All municipality names listed in the Table are located in the State of Amazônia (Brazil).

cacao seedlings. A previously described *Trichoderma martiale* isolate 'ALF-247', known to have both endophytic and biocontrol properties (Hanada et al. 2008, 2009), served as a reference for this study.

Seedlings were prepared in 2.5 × 19 cm tissue-culture tubes with ~80 % of the volume filled with substrate consisting of carbonized rice husk in 35 mL of distilled water. Prior to seed planting, tubes with substrate were autoclaved twice (in 2 consecutive days) at 121 °C for 30 min. Cacao seeds were obtained from pods of SIAL 70 collected from a field area of the Almirante Cacao Ltd. (Bahia). Pod surfaces were scrubbed under running tap water with detergent and desinfested with 70 % ethanol solution in a laminar flow cabinet. Pods were then cut by sterile (flamed) retractable-blade knife to extract the seeds, which had their mucilage removed by a sterile scalpel. In order to be cultivated axenically, seeds were treated for 10 min with 2 % sodium hypochlorite, washed three times with sterile distilled water, and placed with their embryo side over the sterile substrate. Tubes were then closed with non-screwing aluminum lids (allowing gas exchange) and incubated in a growth room at 25 °C ± 2 °C.

Fungal inoculations were done 4 d after the cacao seeds were planted. Conidial suspensions were prepared from fungi grown for 10 d in PDA plates at 25 °C and 12 h/12 h dark/light cycles. A 100-µL aliquot from each 10⁷ spores mL⁻¹ suspension was applied to the radicle; same volume of sterile distilled water was applied to control seeds. A completely randomized design with 12 replicates (seedlings) per treatment was used.

Seedlings were assessed biometrically and fungi re-isolated 40 d after inoculations. The distance from roots to the cotyledons and the total seedling height were measured in millimetres. Stem diameter was measured at two points, 1 cm above and 1 cm below the cotyledons, using a calliper. Number of leaves was also annotated, considering only those that were larger than 2 cm in length. Fungal re-isolations were made from roots, stems at median and apical sections, and leaves. Five fragments of ~0.5 cm each per organ/section (4 organs × 5 fragments = 20) were collected from each of the 12 seedlings (replicates) per isolate treatment, in a total of 240 fragments. These were all desinfested with 70 % ethanol for 1 min, 2.5 % sodium hypochlorite for 3 min, and washed twice in sterile distilled water. Samples were set on PDA plates at equidistant points and incubated at 25 °C for 30 d, with 12 h/12 h dark/light cycles. Fungal growth was monitored daily and a seedling was scored positive for internal endophytic colonization on a particular organ/section when at least one out of the five fragments has shown the fungal structure typical of the inoculated isolate. The intensity of colonization per tested isolate was estimated by the proportion of five fragments from each organ, from each seedling, that showed fungal growth.

Statistics

The proportions of identifiable isolates in relation to *Mycelia sterilia* were compared between the two *Theobroma* species, with statistical significance tested by a chi-square analysis based on 2 × 2 tables, for a comparison of proportions in two independent samples (Snedecor & Cochran 1967); null hypothesis was that the proportions were the same for both species.

For the biocontrol experiment with the eight selected isolates, analysis of variance (ANOVA) was performed, using non-transformed disease scores, with normal distribution previously assessed by the Lilliefors test (Lilliefors 1967). When F-tests were significant, means comparison was performed by the Student's t-test at 5 % significance level.

To assess intensity of colonization post-inoculation, proportions of positive fragments showing fungal re-isolations (% values) were transformed by the square root of 'x + 1' (Steel & Torry 1980). Evaluations of variances homogeneity and normal distribution of data (Lilliefors test) indicated the use of non-parametric analysis of variance, which was performed by the Kruskal–Wallis test; when *H* value was statistically significant (*P* < 0.05), means were compared by the Student–Newman–Keuls test at 10 % significance level. All analyses were performed using the open-access statistical software 'BioEstat 5.0' (Ayres et al. 2007).

Results

A total of 160 culturable endophytic fungi were isolated from healthy *Theobroma* plants: 147 from *Theobroma cacao* (134 from Amazonas, 23 from Bahia) and 13 from *Theobroma grandiflorum* (cupuaçu). Under the culturing conditions used, 17 isolates lost their viability during the isolation process, five showed insufficient sporulation for an adequate identification and 35 did not sporulate, comprising a total of 57 isolates classified as '*Mycelia sterilia*'. All these non-sporulating isolates came from the Amazonas State. The majority of isolates (64.4 %) sporulated (nine from cupuaçu and 94 from cacao), which were identified on the basis of morphological characteristics and tested as potential biological control agents against *Phytophthora palmivora* (Table 2).

Members of a total of 26 different fungal genera/species were found in stem/branch tissues of *T. cacao*. *Trichoderma*, *Pestalotiopsis* and *Fusarium* were the most prevalent genera, with 19 (13.52 %), 12 (8.16 %) and 11 (7.48 %) isolates found, respectively. The presence of these genera was in agreement with results from other studies (e.g. Tejesvi et al. 2005), although the prevalences/percentages were not the same, as different tissues were used for sampling (Evans et al. 2003; Rubini et al. 2005). Eleven genera found were represented by a single isolate (Table 2). Out of the three genera most frequently found in previous endophytic surveys in cacao, i.e. *Colletotrichum*, *Fusarium* and *Xylaria* (Arnold et al. 2003), the first was underrepresented (1.38 %) and the last was not found (Table 2). For cupuaçu, only six genera were found and, considering the very small number of isolates recovered, their distribution was fairly homogeneous, varying from only one to three per identified genus. *Pestalotiopsis* was also more frequent in cupuaçu and *Asteromella* was the only genus not found in cacao (Table 2). It is noteworthy that the number of culturable fungi isolated from cacao was ~10× higher than cupuaçu, thereby reflecting the higher number of cacao plants targeted for isolations (see Methods). Nevertheless, the proportions of sporulating isolates to *Mycelia sterilia* between the two species were not different by the chi-square test (*P* > 0.10) (Table 2).

In order to verify the potential of such a collecting effort in providing fungal endophytes with control activity against

Table 2 – Prevalence of endophytic fungal species isolated from *Theobroma* spp.

Fungal species	Isolates prevalence			
	Cupuaçu		Cacao	
	No.	%	No.	%
<i>Acremonium</i> spp.	2	15.38	6	4.08
<i>Arthrinium</i> spp.	–	–	1	0.68
<i>Aspergillus</i> spp.	–	–	6	4.08
<i>Asteromella</i> spp.	1	7.69	–	–
<i>Clonostachys</i> spp.	–	–	3	2.04
<i>C. rosea</i> var. <i>catenulatum</i>	–	–	1	0.68
<i>Colletotrichum</i> ‘ <i>gloeosporioides</i> ’ ^a	–	–	2	1.36
<i>Coniothyrium</i> spp.	–	–	1	0.68
<i>Curvularia</i> spp.	–	–	6	4.08
<i>Cylindrocladium</i> spp.	–	–	1	0.68
<i>Fusarium</i> spp.	–	–	11	7.48
<i>Gliocladium viride</i>	–	–	1	0.68
<i>Lasioidiplodia theobromae</i>	2	15.38	4	2.72
<i>Myrothecium verrucaria</i>	–	–	1	0.68
<i>Paecilomyces</i> spp.	–	–	1	0.68
<i>Penicillium</i> spp.	–	–	9	6.12
<i>Pestalotiopsis</i> spp.	3	23.08	12	8.16
<i>Phoma</i> spp.	1	7.69	2	1.36
<i>Septoria</i> spp.	–	–	1	0.68
<i>Talaromyces</i> spp.	–	–	1	0.68
<i>Tolypocladium</i> spp.	–	–	3	2.04
<i>Trichoderma</i> spp.	–	–	9	6.12
<i>Trichoderma asperellum</i>	–	–	2	1.36
<i>T. martiale</i> ^b	–	–	1	0.68
<i>T. stromaticum</i>	–	–	6	4.08
<i>T. virans</i>	–	–	1	0.68
<i>Verticillium</i> spp.	–	–	2	1.36
Sub-total (103)	9	69.23	94	63.95
<i>Mycelia sterilia</i> (57)	4	30.77	53	36.05
Total (160)	13	100	147	100

a Species related to *C. gloeosporioides*, a likely temperate species *sensu stricto* (Rojas *et al.* 2010; G.J. Samuels, pers. comm.)
b Reference isolate previously described by Hanada *et al.* (2008, 2009).

P. palmivora, all sporulating isolates that could be identified at least to genus level (103 total, Table 2) were tested in a biocontrol field experiment in cacao pods (Fig 1; Table 3). A histogram showing the frequency of isolates per disease-score category indicated a Gaussian-like distribution slightly skewed towards higher disease scores. However, the highest frequency of isolates corresponded to an average disease score of 3.33, which is below the average score obtained for the positive control inoculated only with the pathogen (4.0). From this general screening experiment, 74 out of the 103 isolates tested (71.8 %) showed some degree of reduction in the disease severity (Fig 1). However, no particular genus/species could be considered specifically more or less effective in terms of biocontrol ability, i.e. varying degrees of activity were observed for all taxa with more than two isolates identified, as the corresponding isolates were well distributed among disease categories (Table 3). A total of eight isolates from the genera *Fusarium* (ALF-839, -849, -1587), *Pestalotiopsis* (ALF-821, -1586), *Curvularia* (-829), *Tolypocladium* (-902) and *Trichoderma* (-247) showed the strongest effect on the control of

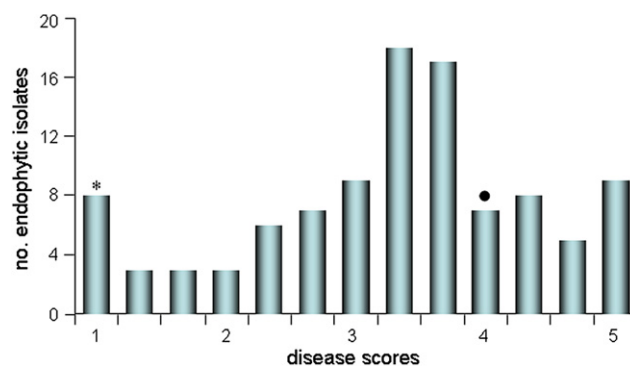


Fig 1 – Frequency distribution of 103 endophytic fungal isolates in relation to their biocontrol capability in a first-tier screening procedure. Each isolate was classified on the basis of the average disease severity of three cacao pods challenged with *Phytophthora palmivora*. Disease scores 1–5 in the x-axis are described in Materials and methods. Water was used as negative control and inoculation only with *P. palmivora* as the positive control, with ‘1’ (*) and ‘4’ (●), respectively, as the corresponding disease scores. Star indicates the class of biocontrol activity whose isolates were further studied regarding confirmation of endophytic behaviour, growth promotion and biocontrol effects.

P. palmivora, with no disease symptoms being detected on their pods (Fig 1; Tables 1 and 3). These results were the same as those obtained for the negative control sprayed only with water. Based on this stringent criterion, these isolates were further studied regarding their endophytic behaviour, growth promotion and biocontrol potential. The *Trichoderma martiale* ALF-247 has been recently characterized in various aspects related to its potential as biological control agent (Hanada *et al.* 2008, 2009) and was considered here as a reference for comparisons among isolates.

With an increase to 20 in the number of tested pods per isolate, a similar biocontrol experiment was performed with the eight isolates that showed the strongest effect against *P. palmivora*, which included the reference *T. martiale* ALF-247 (Fig 2). The severity of disease after application of the selected endophytes was significantly reduced ($P < 0.05$) for all isolates in relation to positive control, in which only the pathogen was applied to the pods. Although the reference isolate showed the largest reduction in disease, all other isolates did not behave significantly differently, based on the t-test (Fig 2).

The potential BCAs studied in the biocontrol experiment reported above were further investigated on cacao seedlings cultivated axenically, regarding their endophytic behaviour and effects on growth promotion. The endophytic recovery of inoculated isolates was assessed in different parts of the seedlings. Most isolates were recovered from roots in at least 10 (83.3 %), and from median section of stems in at least 6 (50 %) out of the 12 plants inoculated (Table 4). Contrariwise, a very poor recovery was observed in leaves and apical section of stems, with only the reference *T. martiale* isolate being recovered from the former in three plants (25 %), and only ALF-1587 from the latter in two plants (16.7 %). ALF-902 was not recovered from any organ/plant assessed (Table 4). In general,

Table 3 – Number of isolates from endophytic fungi^a showing different levels of activity against *Phytophthora palmivora*, based on four groups of disease scores^b.

Fungal species	Disease scores			
	1.0–2.0	2.1–3.0	3.1–4.0	4.1–5.0
<i>Acremonium</i> spp.	1	1	5	1
<i>Arthrinium</i> spp.	–	–	–	1
<i>Aspergillus</i> spp.	–	–	4	2
<i>Asteromella</i> spp.	–	1	–	–
<i>Clonostachys</i> spp.	–	–	2	1
<i>C. rosea</i> var. <i>catenulatum</i>	–	–	1	–
<i>Colletotrichum</i>	–	1	1	–
' <i>gloeosporioides</i> ' ^c	–	–	–	–
<i>Coniothyrium</i> spp.	–	1	–	–
<i>Curvularia</i> spp.	1	3	2	–
<i>Cylindrocladium</i> spp.	–	–	1	–
<i>Fusarium</i> spp.	4	2	4	1
<i>G. viride</i>	–	–	1	–
<i>Lasiodiplodia theobromae</i>	–	–	4	2
<i>Myrothecium verrucaria</i>	–	–	1	–
<i>Paecilomyces</i> spp.	–	–	–	1
<i>Penicillium</i> spp.	–	4	2	3
<i>Pestalotiopsis</i> spp.	4	1	6	4
<i>Phoma</i> spp.	1	–	–	2
<i>Septoria</i> spp.	–	–	–	1
<i>Talaromyces</i> spp.	–	–	–	1
<i>Tolypocladium</i> spp.	1	–	1	1
<i>Trichoderma</i> spp.	1	4	4	–
<i>T. asperellum</i>	–	1	1	–
<i>T. martiale</i>	1	–	–	–
<i>T. stromaticum</i>	2	3	–	1
<i>T. virens</i>	–	–	1	–
<i>Verticillium</i> spp.	1	–	1	–
Total (103)	17 (16.5 %)	22 (21.4 %)	42 (40.7 %)	22 (21.4 %)

a Included genera/species isolated from both *Theobroma* species altogether.

b Disease scores as described in details in Materials and methods.

c See Table 2.

based on the average proportion of fragments per organ/plant showing recovery of the inoculated endophyte, the intensity of colonization was significantly different among isolates for roots, but not for the median section of stems (Table 4). We also tested the effects of these isolates on growth promotion of the corresponding inoculated seedlings. No significantly different growth-promotion effects were observed for any of the tested isolates, considering all the biometric parameters assessed for the control plants. Taking all isolates and control together, the ranges of average measures were 14.7–17.3 cm for plant height up to cotyledons, 23.0–26.6 cm for total plant height, 2.5–2.8 and 3.3–3.5 mm for stem diameters above and below cotyledons, respectively, 5.2–6.0 for number of leaves, and 0.17–0.21 g for roots dry weight, with coefficients of variation (CV) of 18.9, 13.4, 10.8, 7.6, 22.8 and 25.65 %, respectively.

Discussion

The plants from tropical regions, in general, tend to harbour a larger diversity of endophytic microorganisms than those from temperate areas (Strobel & Daisy 2003). This provides

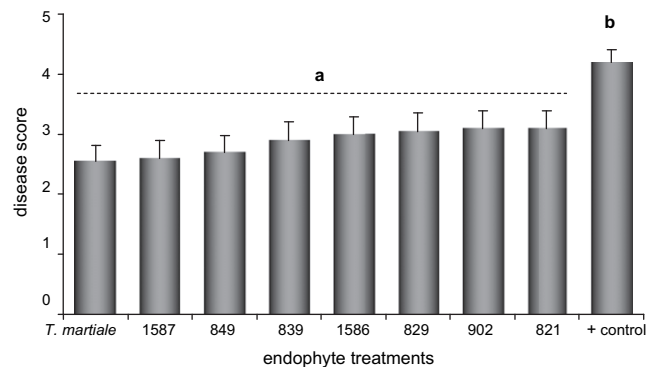


Fig 2 – Levels of black-pod disease after inoculation with endophytic fungi (from the 'ALF-' series) selected on the basis of activity against *Phytophthora palmivora* (see Table 1). The disease scores for each treatment are the average of 20 pods. Statistical significance is indicated by letters on top of the bars; treatments with the same letter were not significantly different by the t-test ($P < 0.05$).

a special opportunity for exploring biological features of agricultural (productivity) and environmental (sustainability) interests, such as the search for BCAs against *Phytophthora palmivora*, the major pathogen causing BPR disease of cacao in Amazon and Atlantic rainforests of Brazil. The diversity of endophytic fungi found in this study for species of *Theobroma* (cacao and cupuaçu) was not unexpected (Strobel & Daisy 2003; Arnold et al. 2003; Rubini et al. 2005), as well as the possibility of employing such endophytes as BCAs against cacao diseases (Crozier et al. 2006; Mejía et al. 2008; Hanada et al. 2009). Nevertheless, four aspects taken together differentiate our study from others: (i) focus only on culturable/sporulating fungi from stem and branches, (ii) focus on *in vivo* (field) screening for biocontrol activity, (iii) inclusion of cupuaçu as a potential source of BCAs, and (iv) the specific geographic regions for search (Amazon) and testing (Bahia) of isolates identified.

To explore the microbial diversity towards a specific function in agricultural settings, culturability and sporulation *in vitro* for the isolates are required, since mass production and formulation for large-scale applications depend on those traits. The methodology we used, though, certainly limited the number of fungi that could be isolated, such as those that preferably inhabit different tissues, or that grow on specific media and/or conditions, and those of biotrophic species, which do not grow in culture media. Hence, considering a rough estimation that only 0.1–5 % of microbes on Earth are culturable (Bull 2004), and that we recovered >100 culturable/sporulating isolates in cacao and cupuaçu from Amazon region, within a total of 27 genera (Table 2), the total endophytic diversity for *Theobroma* in this region (accessible through molecular methods in a similar collecting effort), should range from 2×10^3 to 1×10^5 possible isolates, with 540– 2.7×10^4 different genera/species to be found. These results and biodiversity perspectives are in agreement with information currently available (for example, see Stone et al. 2004; Gazis & Chaverri 2010). Obviously, a significant proportion of such diversity represents novel, yet undescribed species of microorganisms, representing a rich source of natural

Table 4 – Percentage of axenic cacao seedlings with fungal endophytes recovered 40 d after inoculation^a.

ALF-	Seedlings/fragments (%)							
	Roots ^b		Stem				Leaves	
			Median		Apical			
247	100	85.0 a	83.3	48.3	–	–	25	13.3
821	83.3	50.0 c	50	18.3	–	–	–	–
829	91.7	63.3 bc	66.7	36.7	–	–	–	–
839	100	68.3 bc	66.7	30.0	–	–	–	–
849	100	75.0 ab	66.7	33.3	–	–	–	–
902	–	–	–	–	–	–	–	–
1587	100	76.7 ab	91.7	45.0	16.7	5.0	–	–
1586	100	63.3 bc	66.7	30.0	–	–	–	–

a A total of 12 axenically cultivated seedlings were inoculated per each of the eight isolates (see Table 1). From each seedling, 0.5-cm fragments from the roots, stem (median and apical sections), and leaves (five fragments each organ/section) were cut, superficially desinfested and plated to allow endophytic fungal growth. For each organ/section, % values on the left columns indicate proportion of 12 plants that scored positive (at least 1 out of 5 fragments showing the characteristic mycelium of each isolate); on the right columns, % values indicate average proportion of five fragments per organ/plant/isolate in which fungal growth was observed (intensity of colonization estimate). Non-inoculated control seedlings were free from endophytic fungi.

b Values (%) of the right columns (intensity of colonization) were transformed by the square root of 'x + 1' prior to statistical analysis. Statistics was performed by a non-parametric analysis of variance (Kruskal–Wallis test, $P < 0.05$) on a per organ/section basis (apical stem and leaves were not tested); H value was not significant for median stem. Means with same letters are not significantly different by the Student–Newman–Keuls test ($P < 0.1$).

bioactive compounds potentially useful for pharmaceutical industries and agriculture (Hawksworth 2001; Schulz et al. 2002; Daniel 2004; Zhang et al. 2005). Among several possible reasons to explain a high endophytic diversity in the cocoa-producing region of Amazon, one is the typical low-technology, subsistence-farming type of agroforestry systems, which may be favouring less disturbed (and so, more diverse) ecosystems that would, in turn, favour more microorganisms. Another possibility is that high levels of endophytic diversity may be related to the pressure of phytopathogens. This would induce the plants to rely part of their defences on anti-fungal substances produced by beneficial endophytes (Redman et al. 2001), or even on other effects, such as vegetative growth promotion, increased photosynthetic, nutrition and water efficiency, abiotic/biotic stresses tolerance, and mycoparasitism, competition, or induced/acquired systemic resistance to pathogens (Harman et al. 2004; Bailey et al. 2008; Barrow et al. 2008; Vijayaragahavan & Raman 2009).

The most frequent taxa obtained in this work were cited in previous studies on endophytes isolated from cacao leaves in Panama (Arnold et al. 2003) and from branch cortex and leaves in Bahia (Rubini et al. 2005). However, more than 50 % of fungi reported here had not yet been identified as cacao endophytes. Some have been identified as endophytes in other plants, such as *Coniothyrium* in *Picea abies* L. Karst. (Barklund & Kowalski 1996); *Asteromella* in *Quercus emoryi* (Faeth & Hammon 1997); *Colletotrichum*, *Fusarium*, *Pestalotiopsis*, *Phomopsis* and *Phoma* in

banana trees (Brown et al. 1998; Pereira et al. 1999); and *Aspergillus*, *Fusarium*, *Gliocladium*, *Phoma*, *Phomopsis*, *Phyllosticta* and *Trichoderma* in medicinal plants from China (Huang et al. 2008) and India (Verma et al. 2007). It was somewhat surprising that *Xylaria* spp. was not found, as it might be expected based on previous surveys (Arnold et al. 2003), as well as on its potential function in wood trees decay (Pereira et al. 1999; Osés et al. 2008). Overall, these results confirm a tendency of recent surveys in which new genera not previously reported in the corresponding species of study are shown (Verma et al. 2007; Huang et al. 2008). As we further discuss below, based on the idea of linking fungal biodiversity with the myriad of important and useful functions they can provide (e.g. Tejesvi et al. 2005; Verma et al. 2007; Hanada et al. 2009), the terminology 'genetic resources collection' has been properly proposed instead of 'culture collections', aiming at changing the management and funding perspectives for these microbial resources (Hawksworth 2004).

In general, there are changes in endophytic communities, both in abundance and species composition, depending on the location, source tissue for isolation and conditions around the plant (e.g. Tejesvi et al. 2005; Verma et al. 2007; Gazis & Chaverri 2010). This is likely related to host variations in chemical composition and physiological state (Espinosa-Garcia & Langenheim 1991), as well as to environmental conditions (Lappalainen et al. 1999) and temporal dynamics (Kinkel 1991). Interestingly, various studies have indicated that an endophytic microbiota is generally composed of two major types of taxa: those that are found consistently associated with a given plant species, and those that are incidentally identified (Stone et al. 2004). Vascular plants provide a unique ecological niche for several communities of cryptic symbionts that often contribute to multiple benefits, such that biochemical and genetic studies have revealed a reprogramming of plant cells to adjust to the endosymbionts requirements/characteristics (Barrow et al. 2008; Oldroyd et al. 2009). This concept fits well into the 'holobiont/hologenome' theory of evolution (Zilber-Rosenberg & Rosenberg 2008), leading to the notion that extinction of yet unknown endemic plant species from heavily deforested areas shall also take away the whole suite of associated microorganisms and potential beneficial products they can make, thereby further increasing the harsh effects of biodiversity losses (Strobel & Daisy 2003).

More than 70 % of fungal endophytes isolated in this study were capable of reducing severity of BPR disease on cacao, under field conditions. This suggests the existence of a mostly mutualistic interaction host–endophytes in the culturable fungal diversity observed in cacao and cupuaçu trees, although it is not possible to address its cost/benefit relationship at this point. Moreover, these results indicate that the search for potential BCAs in the endophytic community of the target plant, and in the geographic location considered to be the centre of origin and dispersion of the *T. cacao* plant (Motamayor et al. 2002), was indeed a valid and fruitful strategy (Holmes et al. 2004; Mejía et al. 2008). Nevertheless, independently from the mechanism through which endophytes can be beneficial, a prolonged survival inside the plant is required for these effects to be sustained longer, for which appropriate environmental and biological conditions are needed (Elad & Kirshner 1992; Hallmann et al. 1997;

Lodewyckx et al. 2002). Presumably, a higher survival ability of an endophyte under a wider array of conditions tends to increase its likelihood of success in competition with other microorganisms of the phyllosphere and/or endosphere, obviously interfering with the final endomicrobiota composition and/or the beneficial effects to the host (e.g. Krauss et al. 2006; Herre et al. 2007; Szczech 2008). Considering that the lack of knowledge about ecological requirements for survival and adequate colonization of plants by BCAs has contributed to a lesser efficiency in their practical use (Jacobsen & Backman 1993), one ought to understand the factors involved in maintaining longer periods of survival and higher population densities for the BCAs inside the plants. All these aspects are certainly to be considered for selection and further exploration of the potential BCAs identified in this study.

Considering the approximately same level of biocontrol that all seven selected isolates and the reference one presented (Fig 2), further work aiming at technological development of formulations and application strategies is surely warranted (e.g. Hanada et al. 2009). Although previous work has reported potential use of isolates from the genus *Fusarium* as potential BCAs against plant pathogens (e.g. Benhamou et al. 2002), the widespread existence of *Fusarium* diseases in tropical, perennial crops requires caution in this approach (Ploetz 2006; Rojas et al. 2010). By the same token, several other reports on the other three genera showing activity against *P. palmivora* (*Pestalotiopsis*, *Curvularia* and *Tolyposcladium* – Table 1) has shown relevance of strains/species as both plant pathogens or beneficial microbes. In fact, it is acknowledged that harmless/beneficial endophytes in trees can become pathogens depending on other frequently unknown inducing factors (Kogel et al. 2006; Sieber 2007). Alternatively, because mature fruits are likely no longer under the protection of the systemic resistance of the plant, physiological changes in the fruit (such as reactive oxygen species, increased sugar levels, etc.) may favour an asymptomatic endophytic fungus to become an aggressive rot-causing microbe (G.J. Samuels, pers. comm.). For instance, the *Colletotrichum* cf. *gloeosporioides* isolates of this work are very likely different species from the epitype, temperate, pathogenic strain, belonging to a distinct phylogenetic clade of mostly endophytic species (Rojas et al. 2010); they, however, bear the potential to cause post-harvest fruit rot.

Further taxonomic characterization and more detailed studies on the interaction of the seven isolates with host are certainly required. In addition, the development of effective biocontrol strategies also depends upon understanding the interaction host–endophyte–pathogen, which is related to the confirmation of endophytic behaviour, colonization pattern and type of transmission (Herre et al. 2007). Our results suggest that the mode of endophytic transmission in *T. cacao* is horizontal, as non-inoculated axenic plants, used as controls for the colonization experiment (Table 4), showed no signs of fungal endophytes carried through the seeds. This is in agreement with Gazis & Chaverri (2010) who worked with rubber tree, as well as with Arnold et al. (2003), who also concluded that horizontal transmission is operating in cacao, by showing that less than 1 % of cacao plantlets grown under controlled conditions were infected by fungi and that under non-controlled conditions, young leaves were free from endophytes, which were ubiquitous in older leaves.

The eight most promising isolates for biocontrol of *P. palmivora* demonstrated adequate levels of colonization (Table 4). The cultivation of axenic plants also demonstrated that endophytic fungi have particular modes of colonization. Considering that inoculation was performed on roots, this organ and the stem showed a higher recovery of endophytes, but not with the same intensity (Table 4). This was not unexpected as our endophytes were isolated from stem/branches (see Methods). In addition, a sampling bias has likely occurred, with a higher recovery of non-systemic, localized isolates, since endophytic fungi in trees tend to be more restricted to the points of infection (Stone et al. 2004), although evidence exists for endophytic fungal colonization in woods through the xylem sap (Oses et al. 2008). Similar results were previously reported for the reference isolate ALF-247 (Hanada et al. 2008), as well as by Huang et al. (2008), who have shown tissue specificity for endophytic fungi. A possible explanation for this phenomenon was earlier advanced by Espinosa-Garcia & Langenheim (1991), who concluded that different chemical and physiological states of plant organs can determine the spatial distribution of fungal endophytes.

In conclusion, our results indicated that *Theobroma* trees (cacao and cupuaçu) harbour a large diversity of fungal endophytes, as a consequence of being a tropical, perennial species. In this sense, the high amount of culturable isolates from stem/branches (~72 %) showing some degree of control of *P. palmivora* (Fig 1; Table 3), with a significant but not so strong antagonistic activity (Fig 2), might suggest that the microbial community dynamics inside the plant is an important disease-suppressing mechanism under play (Streets-Bautista et al. 2008; Barrow et al. 2008). The approach of aiming at the isolation and biocontrol characterization of only culturable endophytes was effective in identifying potential BCAs against *Phytophthora* diseases. Furthermore, it provided the means to further study several aspects of host–endophyte interactions that can be manipulated towards devising efficient and environmentally sustainable strategies/products to achieve beneficial effects for healthier and productive cacao plants.

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REFERENCES

- Arnold AE, Mejía LC, Kyllö D, Rojas EI, Maynard Z, Robbins N, Herre EA, 2003. Fungal endophytes limit pathogen damage in a tropical tree. *Proceedings of the National Academy of Sciences of the United States of America* 100: 15649–15654.

- Arnold AE, Maynard Z, Gilbert GS, Coley PD, Kursar TA, 2000. Are tropical fungal endophytes hyperdiverse? *Ecology Letters* 3: 267–274.
- Ayres M, Ayres Jr M, Ayres DL, dos Santos AAS, 2007. BioEstat – Aplicações estatísticas nas áreas de ciências biomédicas. Fundação Mamirauá, Belém-PA, Brazil, Available from: <http://www.mamiraua.org.br>.
- Backman PA, Sikora RA, 2008. Endophytes: an emerging tool for biological control. *Biological Control* 46: 1–3.
- Bailey B, Bae H, Strem M, Roberts D, Thomas S, Crozier J, Samuels GJ, Choi I-K, Holmes KA, 2006. Fungal and plant gene expression during the colonization of cacao seedlings by endophytic isolates of four *Trichoderma* species. *Planta* 224: 1449–1464.
- Bailey BA, Bae H, Strem MD, Crozier J, Thomas SE, Samuels GJ, Vinyard BT, Holmes KA, 2008. Antibiosis, mycoparasitism, and colonization success for endophytic *Trichoderma* isolates with biological control potential in *Theobroma cacao*. *Biological Control* 46: 24–35.
- Barklund P, Kowalski T, 1996. Endophytes fungi in branches of Norway spruce with particular reference to *Trybliidiopsis pinastri*. *Canadian Journal of Botany* 74: 673–678.
- Barnett HL, Hunter BB, 1998. *Illustrated Genera of Imperfect Fungi*. APS Press, St. Paul, USA, 218 pp.
- Barrow JR, Lucero ME, Reyes-Vera I, Havstad KM, 2008. Do symbiotic microbes have a role in plant evolution, performance and response to stress? *Communicative & Integrative Biology* 1: 69–73.
- Benhamou N, Garand C, Goulet A, 2002. Ability of nonpathogenic *Fusarium oxysporum* strain Fo47 to induce resistance against *Pythium ulimum* infection in cucumber. *Applied and Environmental Microbiology* 68: 4044–4060.
- Bowers JH, Bailey BA, Hebbar PK, Sanogo S, Lumsden RD, 2001. The impact of plant disease on world chocolate production. *Plant Health Progress*. <http://www.apsnet.org/online/feature/cacao> [accessed 17.02.06].
- Brown KB, Hyde KD, Guest DI, 1998. Preliminary studies on endophytic fungal communities of *Musa acuminata* species complex in Hong Kong and Australia. *Fungal Diversity* 1: 27–51.
- Bull AT, 2004. How to look, where to look. In: Bull AT (ed), *Microbial Diversity and Bioprospecting*. ASM Press, Washington D.C., pp. 71–79.
- Carmichael JW, Kendrick WB, Connors IL, Sigler L, 1980. *Genera of Hiphomycetes*. The University of Alberta Press, Edmonton, p. 386.
- Crozier J, Thomas SE, Aime MC, Evans HC, Holmes KA, 2006. Molecular characterization of fungal endophytic morphospecies isolated from stems and pods of *Theobroma cacao*. *Plant Pathology* 55: 783–791.
- Daniel R, 2004. The soil metagenome – a rich resource for the discovery of novel natural products. *Current Opinion in Biotechnology* 15: 199–204.
- Deberdt P, Mfegue CV, Tondje PR, Bon MC, Ducamp M, Hurard C, Begoude BAD, Ndoumbe-Nkeng M, Hebbar PK, Gilas C, 2008. Impact of environmental factors, chemical fungicide and biological control on cacao pod production dynamics and black pod disease (*Phytophthora megakarya*) in Cameroon. *Biological Control* 44: 149–159.
- Dhingra OD, Sinclair JB, 1995. *Basic Plant Pathology Methods*. Lewis Publishers, Boca Raton, FL, p. 435.
- Elad Y, Kirshner B, 1992. Establishment of an active *Trichoderma* population in the phylloplane and its effect on grey mould (*Botrytis cinerea*). *Phytoparasitica* 20: 137S–141S.
- Espinosa-Garcia FJ, Langenheim JH, 1991. Effect of some essential oil phenotypes from coastal redwood on growth of its predominant endophytic fungus, *Pleuroplaconema* sp. *Journal of Chemical Ecology* 17: 1837–1857.
- Evans HC, 2007. Cacao diseases – the trilogy revisited. *Phytopathology* 97: 1640–1643.
- Evans H, Holmes K, Thomas S, 2003. Endophytes and mycoparasites associated with an indigenous forest tree, *Theobroma gileri*, in Ecuador and a preliminary assessment of their potential as biocontrol agents of cocoa diseases. *Mycological Progress* 2: 149–160.
- Evans HC, Prior C, 1987. Cocoa pod disease: causal agents and control. *Outlook on Agriculture* 16: 35–41.
- Faeth SH, Hammon KE, 1997. Fungal endophytes in oak trees: experimental analyses of interactions with leafminers. *Ecology* 78: 820–827.
- Faeth SH, 2002. Are endophytic fungi defensive plant mutualists? *Oikos* 98: 25–36.
- Gazis R, Chaverri P, 2010. Diversity of fungal endophytes in leaves and stems of wild rubber trees (*Hevea brasiliensis*) in Peru. *Fungal Ecology* 3: 240–254.
- Hallmann J, Quadt-Hallmann A, Mahaffee WF, Kloepper JW, 1997. Bacterial endophytes in agricultural crops. *Canadian Journal of Microbiology* 43: 895–914.
- Hanada RE, Souza JT, Pomella AWV, Hebbar KP, Pereira JO, Ismael A, Samuels GJ, 2008. *Trichoderma martiale* sp. nov., a new endophyte from sapwood of *Theobroma cacao* with a potential for biological control. *Mycological Research* 112: 1335–1343.
- Hanada RE, Pomella AWV, Soberanis W, Loguercio LL, Pereira JO, 2009. Biocontrol potential of *Trichoderma martiale* against black-pod disease (*Phytophthora palmivora*) of cacao. *Biological Control* 50: 143–149.
- Harman GE, 2000. Myths and dogmas of biocontrol – changes in perceptions derived from research on *Trichoderma harzianum* T-22. *Plant Disease* 84: 377–393.
- Harman GE, Howell CR, Viterbo A, Chet I, Lorito M, 2004. *Trichoderma* species – opportunistic, avirulent plant symbionts. *Nature Reviews Microbiology* 2: 43–56.
- Hawksworth DL, 2001. The fungal dimension of biodiversity: the 1.5 million species estimate revisited. *Mycological Research* 105: 1422–1432.
- Hawksworth DL, 2004. Fungal diversity and its implications for genetic resource collections. *Studies in Mycology* 50: 9–18.
- Herre EA, Mejía LC, Kyllö DA, Rojas E, Maynard Z, Butler A, Van Bael SA, 2007. Ecological implications of anti-pathogen effects of tropical fungal endophytes and mycorrhizae. *Ecology* 88: 550–558.
- Holderness M, 1992. Comparison of metalaxyl/cuprous oxide sprays and potassium phosphonate as sprays and trunk injections for control of *Phytophthora palmivora* pod rot and canker of cocoa. *Crop Protection* 11: 141–147.
- Holmes KA, Arroyo C, Bateman RB, Brown NA, Evans HC, Garcia J, Hidalgo E, Krauss U, Samuels GJ, Schroers H-J, Thomas SE, 2005. *Trichoderma ovalisporum*, a novel biocontrol agent of frosty pod rot (*Moniliophthora roreri*) of cocoa (*Theobroma cacao*): from discovery to field. In: Sorvari S, Toldi O (eds), *Proceedings of the 1st International Conference on Plant Microbe Interactions: endophytes and biocontrol agents*. Saariselka, Lapland, Finland, pp. 54–65.
- Holmes KA, Schroers H-J, Thomas SE, Evans HC, Samuels GJ, 2004. Taxonomy and biocontrol potential of a new species of *Trichoderma* from the Amazon basin of South America. *Mycological Progress* 3: 199–210.
- Huang WY, Cai YZ, Hyde KD, Corke H, Sun M, 2008. Biodiversity of endophytic fungi associated with 29 traditional Chinese medicinal plants. *Fungal Diversity* 33: 61–75.
- Hyde KD, Soyong K, 2008. The fungal endophyte dilemma. *Fungal Diversity* 33: 163–173.
- Jacobsen BJ, Backman PA, 1993. Biological and cultural plant disease controls: alternatives and supplements to chemicals in IPM systems. *Plant Disease* 77: 311–315.
- Kinkel L, 1991. Fungal community dynamics. In: Andrews JH, Hirano SS (eds), *Microbial Ecology of Leaves* Springer-Verlag, New York, pp. 253–270.
- Kiffer E, Morelet M, 1997. *The Deuteromycetes. Mitosporic Fungi: classification and generic keys*. Science Publishers Inc, Enfield, USA.

- Kogel K-H, Franken P, Hückelhoven R, 2006. Endophyte or parasite – what decides? *Current Opinion in Plant Biology* 9: 358–363.
- Krauss U, Soberanis W, 2002. Effect of fertilization and biocontrol application frequency on cocoa pod diseases. *Biological Control* 24: 82–89.
- Krauss U, Ten Hoopen GM, Hidalgo E, Martínez A, Stirrup T, Arroyo C, García J, Palacios M, 2006. The effect of cane molasses amendment on biocontrol of frosty pod rot (*Moniliophthora roreri*) and black pod (*Phytophthora* spp.) of cocoa (*Theobroma cacao*) in Panama. *Biological Control* 39: 232–239.
- Lappalainen JH, Koricheva J, Helander ML, Haukioja E, 1999. Densities of endophytic fungi and performance of leafminers (Lepidoptera: Eriocraniidae) on birch along a pollution gradient. *Environmental Pollution* 104: 99–105.
- Lilliefors H, 1967. On the Kolmogorov–Smirnov test for normality with mean and variance unknown. *Journal of the American Statistical Association* 62: 399–402.
- Lodewyckx C, Vangronsveld J, Porteous F, Moore E, Taghavi S, Mezgey M, Van der Lelie D, 2002. Endophytic bacteria and their potential applications. *Critical Reviews in Plant Sciences* 21: 583–606.
- Luz EDMN, Silva SDVM, 2001. Podridão-parda dos frutos, cancro e outras doenças causadas por *Phytophthora* no cacau. In: Luz EDMN, Santos AFDos, Matsuoka K, Bezerra JL (eds), *Doenças causadas por Phytophthora no Brasil* Livraria Rural, Campinas-SP, pp. 175–265 [portuguese].
- Mejía LC, Rojas EI, Maynard Z, Van Bael S, Arnold AE, Hebbar P, Samuels GJ, Robbins N, Herre EA, 2008. Endophytic fungi as biocontrol agents of *Theobroma cacao* pathogens. *Biological Control* 46: 4–14.
- Melnick RL, Zidack NK, Bailey BA, Maximova SN, Gultinan M, Backman PA, 2008. Bacterial endophytes: *Bacillus* spp. from annual crops as potential biological control agents of black pod rot of cacao. *Biological Control* 46: 46–56.
- Motamayor JC, Risterucci AM, Lopez PA, Ortiz CF, Moreno A, Lanaud C, 2002. Cacao domestication I: the origin of the cacao cultivated by the Mayas. *Heredity* 89: 380–386.
- Ndoumbe-Nkeng M, Cilas C, Nyemb E, Nyasse S, Bieysse D, Flori A, Sache I, 2004. Impact of removing diseased pods on cocoa black pod caused by *Phytophthora megakarya* and on cocoa production in Cameroon. *Crop Protection* 23: 415–424.
- Oldroyd GED, Harrison MJ, Paszkowski U, 2009. Reprogramming plant cells for endosymbiosis. *Science* 324: 753–754.
- Oses R, Valenzuela S, Freer J, Sanfuentes E, Rodriguez J, 2008. Fungal endophytes in xylem of healthy Chilean trees and their possible role in early wood decay. *Fungal Diversity* 33: 77–86.
- Pereira JO, Carneiro Vieira ML, Azevedo JL, 1999. Endophytic fungi from *Musa acuminata* and their reintroduction into axenic plants. *World Journal of Microbiology & Biotechnology* 15: 37–40.
- Ploetz RC, 2006. *Fusarium*-induced diseases of tropical, perennial crops. *Phytopathology* 96: 648–652.
- Raupach GS, Klopper JW, 1998. Mixtures of plant growth-promoting rhizobacteria enhance biological control of multiple cucumber pathogens. *Phytopathology* 88: 1158–1164.
- Redman RS, Dunigan DD, Rodriguez RJ, 2001. Fungal symbiosis from mutualism to parasitism: who controls the outcome, host or invader? *New Phytologist* 151: 705–716.
- Rojas EI, Rehner SA, Samuels GJ, Van Bael S, Herre EA, Cannon PF, Chen R, Pang J, Wang R-W, Zhang Y, Peng Y-Q, Sha T, 2010. *Colletotrichum gloeosporioides* s.l. associated with *Theobroma cacao* and other plants in Panama: multilocus phylogenies distinguish host-associated pathogens from asymptomatic endophytes. *Mycologia* doi:10.3852/09-244.
- Rubini MR, Silva-Ribeiro RT, Pomella AWW, Maki CS, Araújo WL, Santos DR, Azevedo JL, 2005. Diversity of endophytic fungal community of cacao (*Theobroma cacao* L.) and biological control of *Crinipellis pernicioso*, causal agent of witches' broom disease. *International Journal of Biological Sciences* 1: 24–33.
- Samuels GJ, Suarez C, Solis K, Holmes KA, Thomas SE, Ismaiel A, Evans HC, 2006. *Trichoderma theobromicola* and *T. paucisporum*: two new species from South America. *Mycological Research* 110: 381–392.
- Schulz B, Boyle C, Draeger S, Römmert AK, 2002. Endophytic fungi: a source of novel biologically active secondary metabolites. *Mycological Research* 106: 996–1004.
- Sieber TN, 2007. Endophytic fungi in forest trees: are they mutualists? *Fungal Biology Reviews* 21: 75–89.
- Snedecor GW, Cochran WG, 1967. *Statistical Methods*, 6th edn. Iowa State University Press, Ames, USA, p. 593.
- Steel RG, Torry JH, 1980. *Principles and Procedures of Statistics*, 2nd edn. McGraw-Hill Book Co., NY, USA.
- Stone JK, Polishook JD, White JFF, 2004. Endophytic fungi. In: Mueller GM, Bills GF, Foster MS (eds), *Biodiversity of Fungi: inventory and monitoring methods* Academic Press, Burlington, pp. 241–270.
- Streets-Bautista J, Garcia-Espinosa R, Perez-Moreno J, Zavaleta-Mejía E, Montes-Belmont R, Ferrera-Cerrato R, 2008. Induction of a suppressive soil phytopathogens – a holistic approach to biological control. *Interciencia* 33: 96–102 [spanish].
- Strobel G, Daisy B, 2003. Bioprospecting for microbial endophytes and their natural products. *Microbiology and Molecular Biology Reviews* 67: 491–502.
- Sutton BC, 1980. *The Coelomycetes. Fungi Imperfecti with Pycnidia, Acervuli and Stromata*. Commonwealth Mycological Institute, Kew, UK, p. 696.
- Szczecz M, 2008. Mixtures of microorganisms in biocontrol. In: Kim M-B (ed), *Progress in Environmental Microbiology*. Nova Science Publishers, New York, pp. 69–110.
- Tadych M, White JF, 2009. Endophytic microbes. In: Schaechter M (ed), *Encyclopedia of Microbiology*. Academic Press, Oxford, pp. 431–442.
- Taylor JE, Hyde KD, Jones BG, 1999. Endophytic fungi associated with the temperate palm, *Trachycarpus fortunei*, within and outside its natural geographic range. *New Phytologist* 142: 335–346.
- Tejesvi MV, Mahesh B, Nalini M, Prakash H, Kini K, Subbiah V, Shetty H, 2005. Endophytic fungal assemblages from inner bark and twig of *Terminalia arjuna* W. & A. (Combretaceae). *World Journal of Microbiology & Biotechnology* 21: 1535–1540.
- Ten Hoopen GMT, Rees R, Aisa P, Stirrup T, Krauss U, 2003. Population dynamics of epiphytic mycoparasites of the genera *Clonostachys* and *Fusarium* for the biocontrol of black pod (*Phytophthora palmivora*) and (*Moniliophthora roreri*) on cocoa (*Theobroma cacao*). *Mycological Research* 107: 587–596.
- Tondje PR, Roberts DP, Bon MC, Widmer T, Samuels GJ, Ismaiel A, Begoude AD, Tchana T, Nyemb-Tshomb E, Ndoumbe-Nkeng M, Bateman R, Fontem D, Hebbar KP, 2007. Isolation and identification of mycoparasitic isolates of *Trichoderma asperellum* with potential for suppression of black pod disease of cacao in Cameroon. *Biological Control* 43: 202–212.
- Verma VC, Gond SK, Kumar A, Kharwar RN, Strobel G, 2007. The endophytic mycoflora of bark, leaf, and stems tissues of *Azadirachta indica* A. Juss (Neem) from Varanasi (India). *Microbial Ecology* 54: 119–125.
- Vijayaraghavan K, Raman S, 2009. Synergistic effect of beneficial rhizosphere microflora in biocontrol and plant growth promotion. *Journal of Basic Microbiology* 49: 158–164.
- Whipps JM, 2001. Microbial interactions and biocontrol in the rhizosphere. *Journal of Experimental Botany* 52: 487–511.
- Wilson D, 1995. Endophyte – the evolution of a term, and clarification of its use and definition. *Oikos* 73: 274–276.
- Zhang L, An R, Wang J, Sun N, Zhang S, Hu J, Kuai J, 2005. Exploring novel bioactive compounds from marine microbes. *Current Opinion in Microbiology* 8: 276–281.
- Zilber-Rosenberg I, Rosenberg E, 2008. Role of microorganisms in the evolution of animals and plants: the hologenome theory of evolution. *FEMS Microbiology Reviews* 32: 723–735.