

Endophytic fungi as biocontrol agents of *Theobroma cacao* pathogens

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Abstract

Fungal endophytes isolated from healthy *Theobroma cacao* tissues were screened *in vitro* for antagonism against major pathogens of cacao. Of tested endophytic morphospecies, 40% (21/52), 65% (28/43) and 27% percent (4/15) showed *in vitro* antagonism against *Moniliophthora roreri* (frosty pod rot), *Phytophthora palmivora* (black pod rot) and *Moniliophthora perniciosa* (witches broom), respectively. The most common antagonistic mechanism was simple competition for substrate. Nonetheless, 13%, 21%, and 0% of tested morphospecies showed clear antibiosis against *M. roreri*, *P. palmivora*, and *M. perniciosa*, respectively. One isolate of *Trichoderma* was observed to be parasitic on *M. roreri*. Endophyte species that were common in the host plants under natural conditions often are good colonizers and grow fast *in vitro* whereas antibiosis producers usually appear to be relatively rare in nature, tend to grow slowly *in vitro*, and often are not good colonizers. We suggest that there is an inherent general trade-off between fast growth (high colonization) and production of chemicals that produce antibiosis reactions. Finally, field trials assessing the effects of three endophytic fungi (*Colletotrichum gloeosporioides*, *Clonostachys rosea* and *Botryosphaeria ribis*) on pod loss due to *M. roreri* and *Phytophthora* spp. were conducted at four farms in Panama. Although the overall incidence of black pod rot was very low during the tests, treatment with *C. gloeosporioides* significantly decreased pod loss due to that disease. We observed no decrease in pod loss due to frosty pod rot, but treatment with *C. rosea* reduced the incidence of cacao pods with sporulating lesions of *M. roreri* by 10%. The observed reduction in pod loss due to *Phytophthora* spp., and sporulation by *M. roreri*, supports the potential of fungal endophytes as biological control agents. Further, these studies suggest that combined information from field censuses of endophytic fungi, *in vitro* studies, and greenhouse experiments can provide useful *a priori* criteria for identifying desirable attributes for potential biocontrol agents.

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1. Introduction

Three diseases of *Theobroma cacao* L., the source of cocoa, are major biological factors that limit cocoa produc-

tion worldwide. These diseases (black pod disease, caused by *Phytophthora* spp.; frosty pod rot, caused by *Moniliophthora roreri* (Cif.) Evans et al.; and witches broom caused by *Crinipellis perniciosa* (Stahel) Singer, *Moniliophthora perniciosa sensu Aime and Phillips-Mora (2005)*) cause an annual reduction in cocoa production estimated at more than 700,000 tons of beans, corresponding to more than 700 million US dollars (Bowers et al., 2001). Traditional

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methods of chemically controlling these diseases can be expensive, ineffective, and have a negative impact on both environmental and human health. Biological control as part of integrated pest management has been suggested as the most sustainable long-term solution (Bateman, 2002). Specifically, promising results for the control of diseases of cacao have been obtained using epiphytic mycoparasitic fungi (Krauss and Soberanis, 2001; Ten Hoopen et al., 2003). Furthermore, recent evidence shows that in some cases endophytic fungi restrict cacao pathogen growth or damage *in vitro* and *in vivo* (Arnold et al., 2003; Evans et al., 2003; Mejía et al., 2003; Holmes et al., 2004, 2006; Rubini et al., 2005; Tondje et al., 2006) highlighting their status as a new source of biological control agents for combating cacao pathogens.

Endophytic fungi are taxonomically and biologically diverse but all share the character of colonizing internal plant tissues without causing apparent harm to their host (Wilson, 1995). The best understood of these are members of the Clavicipitaceae (Ascomycota), which are endophytes of some temperate grasses. In these systems, there is usually only one endophytic fungal species per host and these fungi appear to be highly coevolved with their host. Generally, these fungi are transmitted vertically (from mother to offspring through seeds, as reviewed by Clay and Schardl (2002); see also Saikkonen et al. (2004)). This transmission pattern is thought to promote beneficial relationships with the host plant (Herre et al., 1999). Nonetheless, in grasses, the net effect of endophyte associations can range from parasitic (e.g., choke disease) to strongly mutualistic (Clay and Schardl, 2002). Beneficial effects for hosts include increased drought tolerance (Arechavaleta et al., 1989), deterrence of insect herbivores (Breen, 1994; Rowan and Latch, 1994), protection against nematodes (Pedersen et al., 1988; West et al., 1988; Kimmons et al., 1990), and resistance against fungal pathogens (Gwinn and Gavin, 1992; Bonos et al., 2005; Clarke et al., 2006). The last is also true for endophytes found in some tropical grasses (Kelemu et al., 2001). Anti-pathogen protection mediated by endophytes has been observed also in nongramineous hosts. For example, endophytic fungi have been found to protect tomatoes (Hallman and Sikora, 1995) and bananas (Pocasangre et al., 2001; Sikora et al., 2008) from nematodes, and beans and barley (Boyle et al., 2001) from fungal pathogens. However, even with the accumulating evidence that endophytic fungi can reduce pathogen damage in grasses and other host plants, little is known about the generality of this role in natural systems and whether it can be exploited as a biocontrol strategy in crop protection.

Studies of endophytic fungi in *Theobroma cacao* and other dicots reveal substantial differences with the grass endophyte systems (Arnold et al., 2000; Herre et al., 2005, 2007; Van Bael et al., 2005). Specifically compared to grass endophytes, the endophytes associated with cacao and other tropical woody plants are highly diverse, horizontally transmitted (acquired from the environment), and show only some degree of host affinity (Herre et al.,

1999, 2005, 2007; Arnold et al., 2000; Van Bael et al., 2005). In cacao, leaves and fruits are endophyte-free at emergence and accumulate diverse endophytes from spore rain in the environment. Cacao tissues are heavily colonized in a short period of time (~2–3 weeks) by a group of endophyte species characterized by a few species that are consistently dominant members of the assemblage and a large number of exceedingly rare endophyte species (Arnold et al., 2003; Herre et al., 2005, 2007; Van Bael et al., 2005). In *T. cacao* and *Theobroma gileri* at least two distinctive assemblages of endophytes can be found, one assemblage in leaves (Herre et al., 2005; Van Bael et al., 2005) and a second assemblage in trunks (Evans et al., 2003; Samuels unpublished; see also Crozier et al., 2006). The endophytes found in leaves tend to be leaf- and twig-inhabiting fungi in genera such as *Colletotrichum*, *Botryosphaeria*, *Xylaria*, and *Phomopsis*, while the dominant endophytes of trunks tend to be in genera that are usually known as soil fungi (e.g., *Clonostachys* and *Trichoderma*).

These observations, jointly with *in vitro* and *in vivo* studies (Arnold et al., 2003; Evans et al., 2003; Holmes et al., 2004; Rubini et al., 2005; Tondje et al., 2006; Aneja et al., 2006; Bailey et al., 2006) suggest that different endophytic fungi associated with *T. cacao* reduce the damage associated with pathogens in a variety of different ways *in planta*. Specifically, endophytes can inhibit pathogen infection and proliferation within the host directly (e.g., via antibiosis, competition, and mycoparasitism), or indirectly via inducing resistance responses intrinsic to the host (Aneja et al., 2006; Bailey et al., 2006; S. Maximova, M. J. Guiltinan and E. A. Herre, unpublished). Correctly understanding patterns of host–endophyte ecology as well as identifying the mechanisms underlying the interactions among endophytes, pathogens, and hosts hold important implications for developing effective strategies of biocontrol (Herre et al., 2007).

This study represents a step toward understanding the ecology of endophytes as a means to develop effective biocontrol agents in *T. cacao*, with broader implications for use in other crop systems. First, we compare the interactions of endophytic fungi against *T. cacao* pathogens *in vitro*. Next we outline greenhouse studies where we compare the competitive success of different endophytic fungi in colonizing *T. cacao* tissues *in planta*. Finally, we report results from a field study following an augmentative biological control approach against *Phytophthora* spp. and *M. royeri* in farms of Bocas del Toro Province, Republic of Panama.

2. Materials and methods

2.1. Isolation of endophytes

The endophytic fungi used in this study come from the collection of the Smithsonian Tropical Research Institute Sustainable Cacao Group. This collection developed from

a survey of cacao leaves and fruits from four different sites in the Republic of Panama: Barro Colorado Island, where *T. cacao* grows as a natural part of an intact tropical forest; Nombre de Dios and Soberania National Park, where *T. cacao* grows in abandoned fields that partially or completely overgrown by tropical forest; and near Almirante, Bocas del Toro, where cacao is cultivated in commercial plantations and small farms. Fungi were isolated from healthy leaves following Arnold et al. (2000). Leaves were briefly washed in running tap water and processed as follows: 32 square pieces of 4 mm² were cut from the central part of each leaf, surface sterilized in 0.525% sodium hypochlorite for 3 min. and 70% ethanol for 2 min.; immersed in sterile water for 1 min.; and then placed on 2% malt extract agar (2% MEA). Fungi that emerged from leaf pieces were transferred to tubes containing 2% MEA for storage and classification by morphospecies. To isolate endophytes from cocoa pods, pods were washed with running tap water and then subdivided in 8 parts. Sixteen 2-mm cubes were taken from each part: eight from the exocarp and eight from the mesocarp. Surface sterilization, plating, and storage procedures were the same as for leaves.

Endophytes were classified by morphospecies as described by Arnold et al. (2000). Representative isolates of the morphospecies that were used for field and greenhouse inoculations were further delimited using DNA sequencing data from the nrDNA internal transcribed spacer regions 1 and 2 and 5.8 s gene (ca. 600 base-pairs) using primers ITS4 and ITS5 (White et al., 1990) following PCR protocols described by Rehner and Uecker (1994). Sequences were submitted to GenBank BLAST searches, and genus names were assigned based on the score and consistent similarity with the five sequences most similar to the submitted sequence. For the purpose of this work, morphospecies are considered as putative species. A subset of isolates that sporulated in culture and were used for inoculation experiments were identified to species on the basis of cultural and reproductive characteristics. This set is deposited at the USDA, Systematic Mycology and Microbiology Laboratory, Beltsville (SBML), and work is continuing on their identification.

2.2. *In vitro* tests of anti-pathogen activity

In a series of experiments, dual plate assays were conducted to evaluate the *in vitro* antagonistic activity of endophytes against three pathogens of cacao. Seventy-five endophytic fungi isolates representing 52 morphospecies were tested against *M. rozeri*; 62 isolates representing 43 morphospecies were tested against *P. palmivora* (Butl.) Butl.; and 23 isolates representing 15 morphospecies were tested against *M. pernicioso*. In many cases, multiple different isolates of the same morphospecies were tested. Because the endophyte collections were ongoing, not every endophyte morphospecies was tested against every pathogen. Pathogen isolates used in dual plate assays were isolated from cacao pods in Bocas del Toro (*M. rozeri*), Soberania

National Park (*P. palmivora*), and Nombre de Dios (*M. pernicioso*). Hyphal plugs of pathogens and endophytes were placed 4 cm apart in petri dishes containing 2% MEA. *M. rozeri* and *M. pernicioso* were plated one week earlier than the endophytes, reflecting the slow growth of these pathogens in culture. *P. palmivora* was plated concurrently with endophytes. Evaluation of interactions began 60 h after endophytes were placed into assay plates. Three types of activity were recorded: (1) Antibiosis: growth-inhibition determined by the presence of an inhibition zone; (2) competition for substrate: overgrowth of one organism by another; and (3) mycoparasitism: direct parasitism on the hyphae of the pathogen. In each case, we determined which “won” or “lost” the interaction and by which type of activity. Endophytes were considered to win if they inhibited the growth of the pathogen, showed more radial growth than the pathogen, or parasitized the pathogen. Endophytes were considered to “lose” if the pathogen “won” (showed the reverse outcome mentioned above). If endophytes and pathogens inhibited each other or showed the same amount of radial growth, the interaction was considered neutral. If different isolates of the same morphospecies interacted differently against the same pathogen in separate trials, the interaction was classified as mixed.

2.3. Spore production for inoculation experiments

A subset of endophytic fungi that won interaction trials against cacao pathogens was selected for inoculation experiments. Inocula for greenhouse experiments were produced by liquid fermentation; inocula for field experiments were produced by liquid fermentation followed by solid state fermentation. Cultures of endophytic fungi were grown for 10 days in 100-mm petri dishes with 2% MEA until they colonized the entire petri dish. Dishes were then flooded with 10 ml of sterile water and mycelia and propagules were scraped into sterile Erlenmeyer flasks containing 500 ml of 1.5% molasses yeast medium (1.5% MYM: 15 g molasses, 2.5 g yeast extract, 1 l water). This medium is a modified version of the one used by Hebbbar and Lumsden (1999). The flasks were shaken at 125 rpm and 23 °C for 10 days.

For inoculation experiments in the greenhouse, contents of flasks were filtered through a sterile net of nylon stockings to separate the mycelium from the spore suspension. Spore suspensions were concentrated by centrifugation at 6 g (IEC series 428, International Equipment Co., Nodham Heights, MA), the supernatants eliminated, and the spore pellets resuspended in 0.5% gelatin. Spore concentrations were adjusted to the rank of 10⁶–10⁷ spores per ml and sprayed onto leaves using garden sprayers.

For inoculation experiments in the field we used two solid substrates for spore production: Biodac™ (Cellulose complex mesh size 20–50 from Kadant Grantek, Inc., Green Bay, WI) and rice grains in polypropylene bags with air filters (Unicorn Imp. & Mfg. Corp., Garland TX). Bags were prepared either using 100 g of Biodac™ mixed with

50 ml of 1.5% MYM or 500 g of rice grains mixed with 200 ml of 1.5% MYM. We used each substrate in two separate experiments based on their availability. Biodac™ for the first field trial and rice for the second trial. Presterilized 1.5% MYM was added to rice or Biodac™ in bags, which were then sealed, autoclaved for 1 h, and 24 h later autoclaved again for 1 h. When bags cooled to ca. 25 °C, we inoculated them with fungi that had grown for 7 days in 1.5% MYM (liquid fermentation). We used 25 ml per 100 g (Biodac) or 100 ml per 500 g (rice) of fungi to inoculate the bags. Bags were then sealed and kept at 24 °C with a natural daylight photoperiod.

2.4. Greenhouse inoculation of seedlings

We first generated endophyte-free cacao seedlings following Arnold and Herre (2003). Cacao seeds were germinated in sterilized soil in a plastic shade house that prevented leaves from being exposed to environmental spores and water contact. Seedlings were watered without wetting aerial tissues. We inoculated three different species of endophytes to cacao leaves: *Clonostachys rosea* (Link:Fr.) Schroers et al. isolate PI004, *Botryosphaeria ribis* Grossenb. & Duggar isolate PI006, and *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. isolate 6174. These isolates were selected because they showed strong competitive ability *in vitro* against *P. palmivora* and *M. royeri*, they sporulated easily, and in the case of *C. gloeosporioides* 6174 and *B. ribis* PI006, they were common endophytes of healthy *T. cacao* growing in the Bocas region. We maintained high relative humidity inside the shade house by keeping a bed of wet towels on the bench where seedlings were located. The shade house was closed and high humidity was maintained for 48 h after inoculations.

To verify that the endophyte species could colonize leaf tissue, we treated twenty *T. cacao* seedlings with each fungus and 20 noninoculated seedlings were used as a control (total number of plants = 80). On three separate dates after inoculation, thirty-two leaf pieces of 4 mm² from three leaves per treatment were surface sterilized and plated to evaluate the percentage of fungal colonization (number of leaf pieces with mycelia growth over total leaf pieces plated per single leaf). The percent of fungal colonization per leaf was arcsine transformed and treatments were compared using separate univariate ANOVA tests on each sampling date. Differences among treatments were analyzed using Tukey's multiple comparison test.

To compare the relative colonization potential of different endophytic species *in planta*, we sprayed a mix of seven different endophytic isolates (from six species) onto cacao leaves and isolated the fungi that had colonized the leaves through time. Each endophytic isolate was grown in separate media (1.5% MYM) and spore concentrations were adjusted to 10⁵ spores per ml per species. The spore suspension from each isolate was sprayed on a plate to confirm spore viability. Then the seven spore suspensions were

combined, and the final spore concentration of the mix was 3 × 10⁶ spores per ml. The estimated percentages of spores of each of the different isolates in the mix, were *C. gloeosporioides* 5101(16%), *C. rosea* PI004 (15%), *Fusarium solani* (Mart.) Sacc. PI016 (16%) and PI20 (19%), *Fusarium decemcellulare* C. Brick PI018 (13%), *Acremonium* sp. PI23 (16%), and *Xylaria* sp. 9140 (5%). (note: the *Xylaria* was not originally collected from *T. cacao*). For this experiment, endophyte-inoculated leaves and control (noninoculated) leaves were produced in the same individual plants. During inoculations, we prevented spores from arriving on control leaves by placing a cone-shaped paper bag around the target leaves. Paper bags were removed one day after inoculations. The percent colonization of each endophyte isolate was determined on two different dates after inoculation: 14 days after inoculation (sample size $N = 6$ leaves and 192 leaf pieces of 4 mm²; and $N = 7$ and 224 leaf pieces of 4 mm² for control and inoculated leaves, respectively) and 29 days after inoculation ($N = 6$ leaves and 384 leaf pieces of 4 mm² and $N = 9$ and 560 leaf pieces of 4 mm² for control and inoculated leaves, respectively).

A second goal of the greenhouse experiments was to test whether the endophytic isolates showed any signs of being pathogenic. We observed inoculated plants to evaluate whether or not endophytes induce disease symptoms or abnormalities in leaves.

2.5. Field trials

We first conducted a preliminary trial in an abandoned cacao field in Nombre de Dios to evaluate the possibility of reintroducing *C. gloeosporioides* to cacao tissues under field conditions. Spores of *C. gloeosporioides* isolate 4467 were produced after 20 days of growth in polypropylene bags that contained Biodac™. Spores were isolated by squeezing the contents of bags through a sterile nylon stocking submerged in 10 l of 2% Tween 20. The resulting spore suspension was transferred to compression sprayers for aspersions onto target tissues. *C. gloeosporioides* isolate 4467 was applied to 9 pods ($n = 8$ trees) while 6 pods and were maintained as noninoculated controls. After 6 weeks, inoculated and noninoculated pods were sampled to measure what percentage of each pod had been colonized by the inoculated fungus.

Three species previously evaluated under greenhouse conditions were selected for the field trial in commercial plantations: *C. gloeosporioides* 6174, *B. ribis* PI006, and *C. rosea* PI004. This field trial was conducted in four farms in Bocas del Toro, Panama, following a randomized block design. In each farm a row of 20 trees per fungal endophyte treatment and a row of 20 control trees for a total of 80 experimental trees per farm were selected. Rows of trees between treatments were separated by two rows of untreated trees. Distance between trees in the farms was within 2–3 m. Target tissues for inoculation were flowers and pods. Treatments began at the peak of the flowering

season in the region (May). Before the first inoculation, we performed a phytosanitary cleaning in which all diseased pods were removed from the trees. Over 7 months, we performed monthly phytosanitary cleanings before spore applications. Each month we quantified the number of mature cacao pods that were healthy, had early stage symptoms of frosty pod (deformed fruit), late stage symptoms of frosty pod (sporulation), and/or symptoms of black pod disease.

2.6. Statistical methods: field trials

We calculated the proportions of healthy or damaged fruit by averaging the last 4 months of treatments and measurements (September–December). Because we removed all mature healthy (harvestable) and damaged fruit after each census, the fate of each fruit on each tree was counted only once in the study. Twenty-two trees did not produce any fruit (healthy or diseased) during the final 4 months of the study (September–December) and were removed from analyses. We used the logit transformation to normalize the data before proceeding with parametric tests. The four treatments were compared using a mixed model analysis of variance (PROC MIXED in SAS, 2001) where the fixed effects of treatment, farm, and their interaction were calculated. Tree nested within farm was the random factor in the model. The tests were followed up with individual comparisons of all the fixed effects, using Bonferroni adjustments to account for multiple comparisons. We present all means

as original, nontransformed values with lines drawn to represent one standard error.

3. Results

3.1. *In vitro* activity

Of the endophytic fungi morphospecies tested against three cacao pathogens, 40% (21/52), 65% (28/43), and 27% (4/15) showed *in vitro* antagonism against *M. royeri*, *P. palmivora*, and *M. perniciosa*, respectively (Table 1). Competition was the most common mode of action against pathogens and occurred for 23% (12/52), 35% (15/43), and 27% (4/15) of endophyte morphospecies tested against *M. royeri*, *P. palmivora*, and *M. perniciosa*, respectively. Antibiosis occurred for 13% (7/52) and 21% (9/43) of morphospecies challenged against *M. royeri* and *P. palmivora*, respectively. No antibiosis was observed against *M. perniciosa*. *Moniliophthora royeri* and *M. perniciosa* inhibited the growth of several endophytic morphospecies (Table 1). Only one case of mycoparasitism was observed: a *Trichoderma* sp. isolate parasitized the mycelia of *M. royeri*. Not all the same morphospecies were tested against each of the three pathogens, but 33 morphospecies were tested against both *P. palmivora* and *M. royeri*. Of these, 33% (11/33) antagonized both pathogens: four by antibiosis and eight by competition. *C. gloeosporioides* (morphospecies 1), which was used in inoculation experiments, had mixed interactions with pathogen, whereby different isolates of the same morphospecies won or lost against the

Table 1
Summary interactions of endophytic fungi and three cacao pathogens^a

| Activity | Outcome of the interaction against <i>M. royeri</i> | | | |
|--|---|------|---------|--------------------------------|
| | Win ^b | Lose | Neutral | Mixed interaction ^b |
| Competition | 12 | 6 | 2 | |
| Antibiosis | 7 | 6 | 4 | |
| Competition + antibiosis | 1 | 0 | 0 | |
| Mycoparasitism | 1 | 0 | 0 | |
| Summary of activity against <i>M. royeri</i> | 21 | 12 | 8 | 11 |
| | Outcome of the interaction against <i>P. palmivora</i> | | | |
| Competition | 15 | 1 | | |
| Antibiosis | 9 | 0 | | |
| Competition + antibiosis | 4 | 0 | | |
| Summary of activity against <i>P. palmivora</i> | 28 | 1 | 4 | 10 |
| | Outcome of the interaction against <i>M. perniciosa</i> | | | |
| Competition | 4 | 2 | 0 | |
| Antibiosis | 0 | 4 | 4 | |
| Competition + antibiosis | 0 | 0 | 0 | |
| Summary of activity against <i>M. perniciosa</i> | 4 | 6 | 4 | 1 |

^a Values indicate the number of cases in which a given outcome (“win” etc.) was observed between a given endophyte morphospecies and each of the three pathogens. The outcomes were determined 10 days after having been grown together in dual plate assays on 2% MEA. Not all endophytes were tested against all pathogens. Seventy-five endophytic fungi strains representing 52 morphospecies were tested against *M. royeri*; 62 strains representing 43 morphospecies were tested against *P. palmivora*; and 23 strains representing 15 morphospecies were tested against *M. perniciosa*.

^b Endophytes were considered to win if they inhibited the growth of the pathogen, they overgrew the pathogen, or if they parasitized the pathogen. If endophytes and pathogens were inhibiting each other or showed the same amount of radial growth, the interaction was considered neutral. If different strains of the same morphospecies interacted differently against the same pathogen in separate trials, then the interaction was classified as mixed.

same pathogen. Nonetheless, this morphospecies contained some of the most competitive isolates *in vitro* and was the best colonizer in the *in vivo* trials.

3.2. Greenhouse inoculations for seedlings

Reintroduction of endophytic *B. ribis* PI006, *C. rosea* PI004, and *C. gloeosporioides* 6174 into cacao leaves was confirmed by successful reisolations after inoculation. Sampling of inoculated leaves at 10, 25, and 33 days after inoculation showed a progressive colonization by inoculated fungi in their respective treatments, with *C. gloeosporioides* 6174 showing the highest capacity for colonization (Fig. 1 A–C). Sampling at 10 days after inoculation showed 13.54, 14.58, and 26.04% of leaf pieces sampled colonized by endophytic fungi in treatments with *B. ribis* PI006, *C. rosea* PI004, and *C. gloeosporioides* 6174 compared to 4.17% colonization in control leaves (Fig. 1A). At the same date, 10.42, 11.46, and 20.83% of leaf pieces sampled were colonized by their respective inoculum in treatments with *B. ribis* PI006, *C. rosea* PI004, and *C. gloeosporioides* 6174 (see Table 2).

Thirty-three days after inoculation 62.5%, 38.54%, and 91.67% of leaf pieces sampled were colonized in treatments with *B. ribis* PI006, *C. rosea* PI004, and *C. gloeosporioides* 6174, respectively, compared to 12.5% in control leaves (Fig. 1C). In control (not-treated, noninoculated) plants there were very few endophytes reisolated and those were not the ones used for inoculation. In the treated plants, the overall density of endophytes was higher and more than 60% of the fungi reisolated per treatment corresponded to their respective inoculum, indicating successful infection and colonization. Fig. 1 shows percentage of colonization by endophytic fungi of leaf pieces sampled overall and percent colonization by particular endophytic fungal species inoculum in each treatment at different days post inoculation.

Colonization of cacao leaves by multiple endophytes was observed when a mix of seven isolates representing six different species was used as the inoculum source. At 14 and 29 days after inoculation, 38.40% and 74.64% of leaf pieces sampled were colonized in inoculated leaves vs. 7.29% and 4.17% of leaf pieces in noninoculated leaves (Fig. 2A and B). Although the mix contained similar spore counts for the fungal isolates (with exception of *Xylaria* sp., which produced few spores), the recovery of inoculated endophytes from inoculated leaves showed a dominance of *C. gloeosporioides* 5101 over the other species. Despite the dominance of *C. gloeosporioides* 5101, 6 of 7 isolates were reisolated at 14 and 29 days after inoculation. *Xylaria* sp., which was not originally isolated from *T. cacao*, was never reisolated during this experimental period (Fig. 2A and B).

3.3. Field trials

The preliminary trial confirmed that the incidence of *C. gloeosporioides* could be increased by spraying pods in the

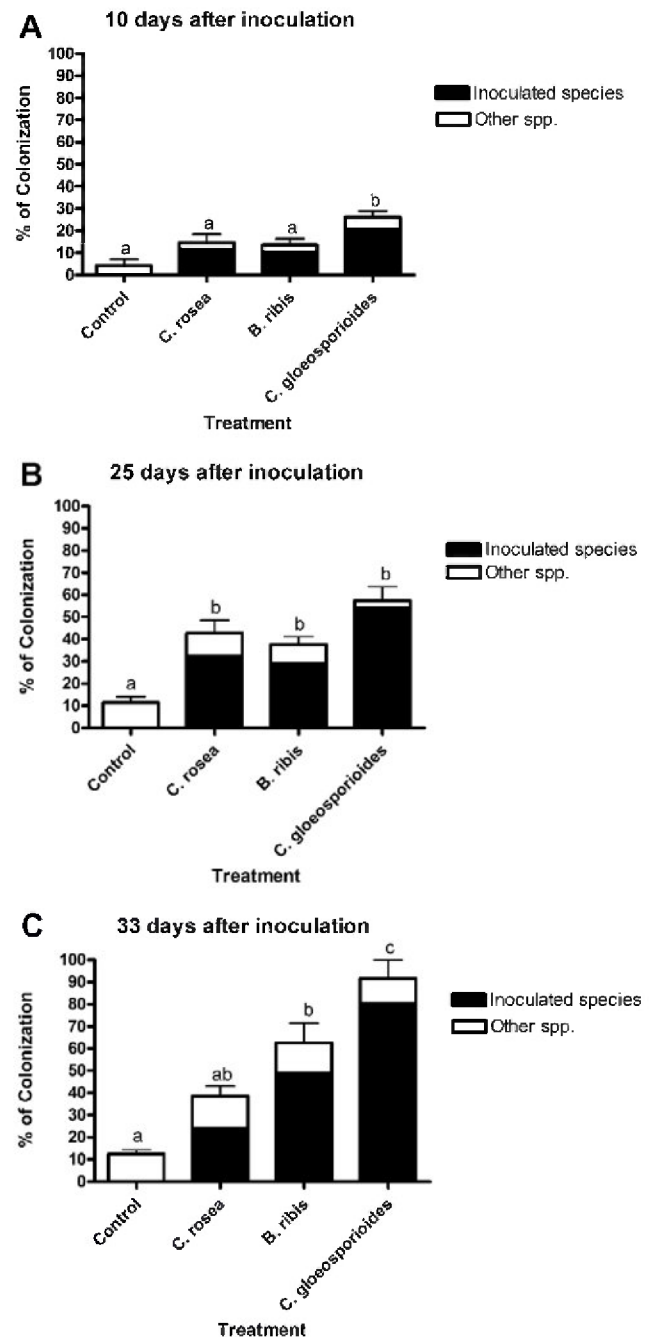


Fig. 1. Percentage of colonization of leaf segments sampled for endophytic fungi under greenhouse conditions over time. Thirty-two leaf pieces of 4 mm² from three leaves from each treatment at each date were plated to evaluate the percentage of fungal colonization. Mean (SE) percentage of colonization by endophytic fungi was significantly different among treatments at each post inoculation date: 10 days, $F_{3,8} = 6.9$, $P = 0.0128$; 25 days $F_{3,8} = 17.0$, $P = 0.0008$, 33 days $F_{3,8} = 18.1$, $P = 0.0006$. Letters indicate significant differences among treatments using Tukey's multiple comparison test.

field. Six weeks after application of endophytes, endophytes were found in 68.8% and 50.8% of sampled fruit tissue from inoculated and noninoculated pods, respectively. Within this, 41.0% and 9.1% of reisolated fungi corresponded to the inoculated isolate of *C. gloeosporioides* in

Table 2
Cacao endophytic fungi used for inoculations

| Strain | Taxonomy | Tissue of origin | Location of origin |
|-------------------|--|------------------|-----------------------|
| 5101 | <i>Colletotrichum gloeosporioides</i> (Penz.) Penz & Sacc. M1 ^a | Leaf | Barro Colorado Island |
| 4467 | <i>Colletotrichum gloeosporioides</i> (Penz.) Penz & Sacc. M1 | Pod | Nombre de Dios |
| 6174 | <i>Colletotrichum gloeosporioides</i> (Penz.) Penz & Sacc. M1 | Pod | Bocas del Toro |
| PI004 | <i>Clonostachys rosea</i> (Link:Fr) | Leaf | Bocas del Toro |
| PI006 | <i>Botryosphaeria ribis</i> Grossenb. & Duggar | Pod | Bocas delToro |
| PI016 | <i>Fusarium solani</i> (Mart) Sacc. | Pod | Barro Colorado Island |
| PI018 | <i>Fusarium decemcellulare</i> C. Brick | Pod | Bocas del Toro |
| PI020 | <i>Fusarium solani</i> (Mart) Sacc. | Pod | Bocas del Toro |
| PI023 | <i>Acremonium</i> sp. | Pod | Bocas del Toro |
| 9148 ^b | <i>Xylaria</i> sp. | Leaf | Barro Colorado Island |

^a Morphotype 1.

^b This strain was isolated from *Heisteria concinna* (Olacaceae), a naturally co-occurring species with *T. cacao* at Barro Colorado Island.

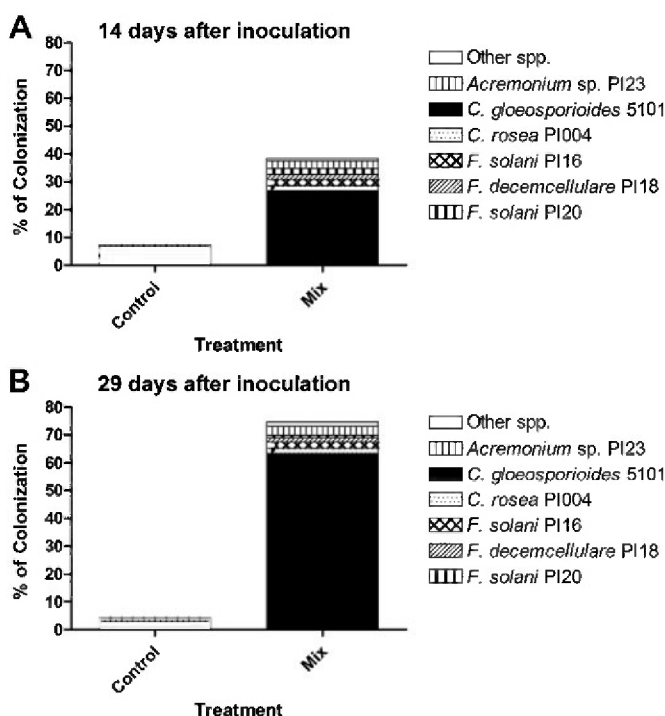


Fig. 2. *In planta* competitive ability of different endophytic fungal species measured as percentage of colonization of *T. cacao* leaf tissue. Percent colonization of leaf pieces sampled in control leaves and leaves inoculated with a mix of seven endophytic isolates. (A) 14 days after inoculation ($N = 6$ leaves and 192 leaf pieces of 4 mm²; and $N = 7$ and 224 leaf pieces of 4 mm² for control and inoculated leaves, respectively). (B) 29 days after inoculation ($N = 6$ leaves and 384 leaf pieces of 4 mm² and $N = 9$ and 560 leaf pieces of 4 mm² for control and inoculated leaves, respectively).

inoculated and noninoculated pods, respectively. Thus, the incidence of the inoculated isolate increased by four times in the 6 weeks following the single inoculation.

Cacao flowers and pods treated with *C. gloeosporioides* 6174, *B. ribis* 4467 and *C. rosea* PI004 continued their normal development without showing any evidence of being negatively affected by monthly treatments. When we examined the treatment effects on the percentage of pods with symptoms of *Phytophthora*, we observed a significant treatment by farm interaction (Treatment effect: $F_{3,204} = 0.273$,

$P = 0.044$, Farm effect: $F_{3,78} = 3.11$, $P = 0.031$, Treatment by farm effect: $F_{9,204} = 2.69$, $P = 0.005$). Specifically, the percentage of fruits infected with *Phytophthora* was reduced by *C. gloeosporioides* at 3 out of 4 farms. The other treatments did not differ from the control. However, we note the very low overall incidence of *Phytophthora* infections that we observed in all of the farms: only 20% (64/320) of the trees in the study had fruits symptomatic of *Phytophthora* infection.

None of the tested endophytes reduced the pod loss due to *M. roreri*. However, we observed a significant reduction in the percentage of pods with sporulating lesions of *M. roreri* in pods treated with *C. rosea* PI004 during the last 4 months of study (Fig. 3A). Pods treated with *C. rosea* PI004 showed 10% reduction of sporulating lesions compared to control pods but this effect was mostly due to a reduction at one farm (Fig. 3B).

4. Discussion

Recent findings have shown that endophytic fungi can help limit pathogen damage in *T. cacao* (Arnold et al., 2003; Evans et al., 2003; Mejía et al., 2003; Holmes et al., 2004, 2006; Rubini et al., 2005; Tondje et al., 2006; Pierre Roger Tonje, IRAD, Cameroon; personal communication). Our results support these findings by showing that endophytic fungi isolated from healthy leaves and pods of *T. cacao* restrict *in vitro* growth of the three most common and economically important pathogens of cacao (*P. palmivora*, *M. roreri*, and *M. pernicioso*). These suggestive *in vitro* results are further corroborated both in the greenhouse (Arnold et al., 2003; Rubini et al., 2005) and in the field (present work). Overall, these results strongly suggests that the diverse assemblage of endophyte species associated with *T. cacao* play an integral role in the resistance of their hosts to pathogen damage (Herre et al., 2007), and that endophytes can potentially be used as effective biocontrol agents.

It is clear that *in vitro* results do not necessarily translate directly to what occurs *in planta*. Nonetheless, *in vitro* studies and their results are particularly useful for identifying

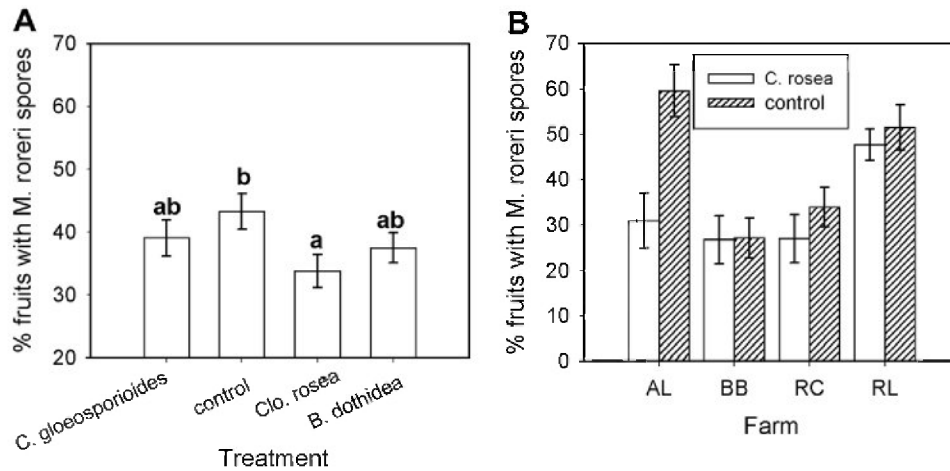


Fig. 3. (A) Mean (SE) percentage of *Theobroma cacao* fruits in which *M. roleri* sporulated after 6–9 applications of endophytic fungi in four farms of Bocas del Toro, Panama. (6174 = an isolate of *C. gloeosporioides*; PI006 = an isolate of *B. ribis*; PI004 = an isolate of *C. rosea*) Strain PI004 resulted in a significantly lower frequency of sporulation than the control (Treatment effect: $F_{3,204} = 3.37$, $P = 0.02$, farm effect: $F_{3,78} = 12.11$, $P = 0.001$, treatment by farm effect: $F_{9,204} = 2.83$, $P = 0.004$; Bonferroni adjusted $P < 0.05$). (B) Mean (SE) percentage of *T. cacao* fruits with *M. roleri* spores after 6–9 monthly applications of *C. rosea* at four farms in Bocas del Toro, Panama. Farm codes are AL, A. López, BB, B. Binns, RC, R. Castellón, RL, R. López.

likely candidates for biocontrol and for making educated guesses concerning the mechanisms by which they reduce pathogen damage. Interestingly, endophyte isolates that outcompete or displace pathogens by outgrowing them tended to be those that were commonly isolated from cacao in our field survey. Endophytes showing antibiosis tended to be slower growing and are relatively less abundant. Trial results from both MEA and for media composed of cacao leaf extract (see Arnold et al., 2003) suggest that there is a trade-off in endophytes between fast growth and the tendency to produce antibiotic chemicals (L.C. Mejía and E.A. Herre, unpublished).

In seedling bioassays, endophyte isolates (species) that showed higher colonization rates tended to be those that were more abundant in cacao tissues that we sampled in our field surveys. Generally, endophytes that were less abundant in cacao tissues in those surveys are relatively poor colonizers (Arnold et al., 2003; Van Bael et al., 2005). However, the common, good colonizers usually show less antibiosis activity than the less common, slower growing isolates, at least under the conditions we have used. This trade-off appears to have implications for biocontrol strategies. Specifically, if we choose isolates that show *in vitro* antibiosis activity against a particular pathogen, we need to recognize that effectively introducing and then keeping them inside cacao tissues may be more challenging. Indeed, even when a particular endophyte shows antibiosis against a particular pathogen, there are often major components of the natural endophytic mycoflora that are insensitive to this particular endophyte (Mejía and Herre, unpublished). If those insensitive endophytes also show a higher colonization rate, then concentrating biocontrol efforts only on endophyte isolates that show antibiosis is likely to be an ineffective strategy. Ideally, we should search for endophytes that have both relatively good colonization and growth rate combined with some

degree of antibiosis. These findings strongly suggest the importance of combining actual field data with *in vitro* testing for picking useful control agents.

Another mechanism of disease suppression by which endophytic fungi may contribute to their hosts is by inducing plants' intrinsic defense pathways. Induction of plant defense pathways upon infection by fungi may be interpreted as the recognition of endophytic fungi by the plant, followed by an induction of anti-pathogen defenses. In cacao it has been observed that endophytic *Trichoderma* are able to induce some genes implicated in plant responses to abiotic and biotic stresses (Bailey et al., 2006). Such induction of host genes also has been found for the *Colletotrichum* isolates used in this study (S. Maximova, M. J. Guiltinan, and E. A. Herre, unpublished). It is interesting to note that the disease suppression conferred by some of these endophytes in greenhouse trials appears to be relatively localized to specific endophyte-treated (or non-treated) leaves within individual host plants (Arnold et al., 2003, also see Redman et al., 1999). Ongoing research is directed at determining the relative importance of localized effects (either via direct pathogen inhibition by the endophytes (see Aneja et al., 2006) or via localized induction of host defensive pathways) and "whole plant" systemic effects in underlying the enhanced host resistance associated with endophyte colonization (see Bailey et al., 2006; Herre et al., 2007). Finally, it would indeed be interesting if a particular endophytic fungus can elicit these effective anti-pathogen defensive responses from the host without producing any obvious symptom of disease and without being negatively affected itself.

We used isolates of *C. gloeosporioides*, *B. ribis*, and *C. rosea* for field tests as biocontrol candidates. The use of *C. gloeosporioides* and *B. ribis* for field trials should not be considered risk-free because some strains of these fungi commonly occur as plant parasites (Farr et al., 1998; G.

Samuels, personal observation). However, the isolate of *C. gloeosporioides* that we selected for the field trials was the most common endophytic species found in our surveys, and it was always isolated from asymptomatic tissues of cacao. Similarly, the isolate of *B. ribis* that we used was also commonly isolated in our survey, and only found in healthy host tissues. Importantly, when the three fungi were tested in repeated seedling colonization bioassays, they colonized the tissues, were reisolated, and never showed any evidence of inducing disease symptoms in their hosts. An open possibility is that these isolates are endophytic strains specialized as nonpathogenic mutualistic endophytes on *T. cacao*, but more research is needed to determine what the genetic relationships of these apparently mutualistic isolates of *C. gloeosporioides* and *B. ribis* are to known pathogenic strains (see Freeman and Rodriguez, 1993). Further, although our isolates of *C. gloeosporioides* and *B. ribis* did not induce disease symptoms in our cacao plants, we cannot rule out the possibility that they could be pathogenic to other members of the native forest in which the cacao was cultivated. It would be a mistake to release a biocontrol agent that benefited *T. cacao*, but devastated other crops (e.g., papaya, banana, citrus, etc.). After confirming no pathogenic effects on the target host (as we had done in these studies), isolates of would-be biocontrol agents should be tested for pathogenic effects on other plant species that are part of agrosystems, polycultures, or native vegetation that is associated with the target host. Ideally, genetic comparisons of biocontrol isolates should also be made with known pathogen strains. On the other hand, *C. rosea* is not known to cause disease in any plants and, in fact, when *C. rosea* was applied in combination with phytosanitary measures and two *Trichoderma* species against multiple diseases of cacao in Costa Rica, yield was increased by 15% (Krauss and Soberanis, 2003).

As was found in previous greenhouse experiments (Arnold et al., 2003), the field test conducted in four different farms showed that pod treatment with *C. gloeosporioides* significantly reduced the proportion of pods with symptoms of black pod disease, with similar effects, albeit of different magnitudes across the farms. Importantly, during our field study, frequency of black pod disease in the fields was low, with only 20% of the trees showing diseased pods. Similar studies should be conducted under conditions of higher disease pressure.

Compared with other treatments plus controls, the treatment with *C. rosea* produced an overall reduction of 10% in the proportion of pods showing sporulation of *M. roreri*, although significant effects were confined to one farm. To our knowledge this is the first report of the application of an endophytic fungus to control some part of the life cycle of *M. roreri* under field conditions. This *C. rosea* isolate showed a moderate growth rate and some degree of antibiosis against *M. roreri* *in vitro*. The field effect of *C. rosea* is valuable for several reasons: first, a restriction on the sporulation of the pathogen can affect

the epidemiology of the disease by reducing the pathogen inoculum available to make new infections. This reduction of the sporulation of *M. roreri* was observed in a previous study of an epiphytic isolate of *C. rosea*, which was reported to be the most common epiphytic mycoparasite in cacao (Ten Hoopen et al., 2003). Nonetheless, different mechanisms appear to be operating with the different isolates: the epiphytic isolate was reported to be a mycoparasite, and the endophytic isolate presented in this study appeared to act by antibiosis. Thus, although the two biocontrol candidates have been assigned the same name, they appear to occupy two different niches. Further, endophytic *C. rosea* has been reported to control *Botrytis cinerea* in roses (Morandi et al., 2000) and antibiotic production has been reported for this species (Berry and Deacon, 1992; Hajlaoui et al., 2001). Interestingly, *C. rosea* was also found to be a common endophyte in the center of origin of *T. gileri* and *M. roreri* (Evans et al., 2003). This suggests support for the idea that more effective biological control agents are the ones with a coevolved history with the target organism (see Evans, 1999, for a classical biological control approach on cacao pathogens).

A high diversity of endophytic fungi has been found in stems, leaves, and pods of *T. cacao*, and *T. gileri* (Arnold et al., 2003; Evans et al., 2003; Rubini et al., 2005; Herre et al., 2005; Van Bael et al., 2005; Samuels unpublished). However, what their natural roles are or how they interact with the pathogens of cacao and other host plants is only beginning to be characterized and understood. We suggest that combined ecological and *in vitro* studies can help identify isolates that will prove useful as biocontrol agents.

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