



Antibiosis, mycoparasitism, and colonization success for endophytic *Trichoderma* isolates with biological control potential in *Theobroma cacao*

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Abstract

Theobroma cacao (cacao) suffers severe yield losses in many major production areas due to fungus-induced diseases. Cacao supports a complex endophytic microbial community that offers candidates for biocontrol of cacao diseases. Endophytic isolates of *Trichoderma* species were isolated from the live sapwood of trunks of *Theobroma* species, pods of *Theobroma* species, and a liana (*Banisteriopsis caapi*). Fifteen isolates of *Trichoderma*, potentially representing seven species, were selected for characterization of the influence of seedling inoculation on the establishment of endophytic growth in cacao seedlings. An isolate of *Colletotrichum gloeosporioides* was also included. The isolates studied *in vitro* varied in their abilities to produce metabolites inhibitory to *Moniliophthora roreri* and in their abilities to parasitize *M. roreri* cultures. The five inoculation methods used were: (1) inoculation of germinating seed on agar plates; (2) plate inoculation followed by planting in sterile soil; (3) planting sterile seed in pre-inoculated soil; (4) inoculation of emerged seedlings at the soil surface; and (5) inoculation of emerged seedlings between the cotyledon and stem. All the isolates studied were able to colonize *Theobroma cacao* seedlings, but isolates DIS 110a (*Trichoderma* cf. *harzianum*), DIS 219b (*T. hamatum*), DIS 219f (*T. harzianum*), and TA (*T. asperellum*) were the most efficient across inoculation methods. These same isolates also caused moderate to severe discoloration of roots of cacao seedlings germinated on water agar plates. Isolates DIS 173a (*T. spirale*), DIS 185c (*T. stromaticum*), and Col (*Colletotrichum gloeosporioides*) were inefficient colonizers of cacao. Most of the isolates studied were able to establish an endophytic relationship with cacao by colonizing the above ground portions of the cacao seedling, and exploitation of this characteristic could lead to the development of novel biocontrol strategies for control of cacao diseases.

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1. Introduction

Theobroma cacao (cacao), the source of chocolate, is grown in many tropical environments (Wood and Lass, 2001). Several fungal pathogens attack cacao and cause

severe yield losses in many production areas (Wood and Lass, 2001; Bowers et al., 2001). The major diseases of cacao include black pod (causal agent *Phytophthora* species), witches' broom (causal agent *Crinipellis pernicioso* = *Moniliophthora pernicioso* according to Aime and Phillips-Mora 2005), and frosty pod (causal agent *M. roreri*) (Wood and Lass, 2001; Bowers et al., 2001). These pathogens are capable of causing complete yield loss. Small

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farmers typically produce cacao and the cost can limit the use of chemical and cultural disease management methods (Purdy and Schmidt, 1996; Adejumo, 2005). We are considering using endophytes as a durable, plant-based, biocontrol alternative for management of cacao diseases that presently cannot be controlled with standard integrated approaches.

Theobroma cacao (cacao) grows in the wild as a forest understorey tree in many tropical regions of Central and South America (Wood and Lass, 2001). These forests are some of the most diverse ecosystems in the world. Several studies of the endophytic and epiphytic fungi associated with cacao have been carried out (Arnold et al., 2003; Arnold and Herre, 2003; Rubini et al., 2005; Evans et al., 2003), but still, it is likely that only a small part of the vast microbial diversity associated with cacao has been described. These endophytic and epiphytic microbes offer unique candidates for biocontrol of cacao diseases.

Trichoderma species are typically considered soilborne organisms associated with the roots of plants and are commonly considered for their potential to control plant disease in what can be a close association with many aspects typical of endophytic associations (Harman et al., 2004). We have obtained a diverse collection of more than 100 *Trichoderma* isolates. Most were isolated from live sapwood immediately below the bark of trunks of wild and cultivated *Theobroma cacao* and other *Theobroma* species (Evans et al., 2003), although representatives isolated from pods and other tissues were also included. The approach for making the collection was to look for endophytes co-evolved with the pathogens of cacao and its relatives in the upper Amazon region and Chocó phytogeographic region (Evans et al., 2003; Holmes et al., 2004). Only a small number of *Trichoderma* isolates from the collection have been studied in any detail for their biocontrol ability (Holmes et al., 2004, Holmes et al., 2005; Samuels et al., 2006a,b). In addition very little is known about the nature of the *Trichoderma*/cacao interaction. We plan to screen this diverse collection for their endophytic and biocontrol abilities.

The primary objective of the research presented here was to characterize the influence of seedling inoculation method on cacao seedling colonization by selected isolates of *Trichoderma*. Fifteen *Trichoderma* isolates, potentially representing 7 species from the *Trichoderma* collection, were evaluated (Table 1). Detailed studies were carried out on a subset of 9 *Trichoderma* isolates. Additional studies were carried out characterizing the abilities of the *Trichoderma* isolates studied to produce antimicrobial metabolites and to parasitize *M. royeri*.

2. Materials and methods

2.1. Soluble inhibitory metabolite production

The conidia of 14 *Trichoderma* isolates (Table 1) were harvested from two-week-old cultures grown at 25 °C on

20% potato dextrose agar, composed of 4.8 g potato dextrose broth powder (Sigma, Dorset, United Kingdom), 12 g technical agar No. 3 (Oxoid, Cambridge, United Kingdom) per liter of distilled water to promote sporulation. Conidia were harvested from the PDA plates by placing 5 ml of 0.05% Tween 80 (Polyoxyethylene Sorbitan Monooleate, Sigma) on the plate surface and agitating the surface with a sterile glass rod. The concentrated spore suspensions were collected using a sterile syringe and filtered through sterile glass wool to remove mycelium and the concentration of the suspension was adjusted with the use of a haemocytometer and a series of dilutions with 0.05% Tween 80. Isolates TA and Col were not available for the antibiosis studies. Three flasks containing 150 ml of 3% oxoid malt extract broth (ME) and three containing minimal salts broth (MIN; Srinivasan et al., 1992) were each inoculated with 1 ml of a 1×10^6 conidia suspension of the endophyte and incubated in an orbital incubator at 25 °C and 110 rpm for 7 days. The mycelia were removed by filtration after 7 days of growth, and the filtrate sterilized by passing through a 0.22 µm membrane disposable filter unit (Millipore). The sterile culture filtrate was then stored at –20 °C before use. Before being incorporated into the medium, the sterile filtrate was placed in a 90 °C water bath for 2 h, after which it was added to an equal volume of the corresponding strengthened agar, MEA or MIN (3% agar No. 3) and poured into Petri dishes. The plates were inoculated centrally with a 4 mm plug of *M. royeri* from the growing edge of a 7-day-old colony. Controls were prepared insert by replacing the fungal filtrate with the corresponding uninoculated broth. Three replicate plates were used for each test and all plates were incubated at 25 °C. Inhibition of mycelial growth of *M. royeri* was recorded as the difference between mean radial growth in the presence and absence of the fungal filtrate.

2.2. Mycoparasite screening

Fourteen *Trichoderma* isolates (Table 1) were screened for mycoparasitic ability using a pre-colonized plate method as previously described (Evans et al., 2003; Holmes et al., 2004). Isolates TA and Col were not available for the mycoparasitism studies. A 2.5 × 0.5 cm strip of inoculum excised from a freshly sporulating colony of the *Trichoderma* species was placed at one edge of a 9 cm diameter PDA plate wholly pre-colonized by an isolate of *M. royeri*. Five replicate plates were prepared. Over a 5-week incubation period, at 25 °C in the dark, a total of 15 samples from each replicate were removed weekly with a 5 mm cork borer starting at the *M. royeri* inoculum. These were plated out onto 20% PDA and incubated at 25 °C under black light (near UV) and observed over 14 d for the growth of the *Trichoderma* sp. or *M. royeri*. The percentage colonization was determined. This was carried out for 5 weeks or until complete colonization by the *Trichoderma* isolate had occurred.

Table 1
Sources of fungal isolates

Isolates# (DIS)	<i>Trichoderma</i> Sp.	Source of isolate
67a	<i>T. harzianum</i>	<i>Theobroma bicolor</i> stem in Napo Province, Ecuador
70a	<i>T. ovalisporum</i>	<i>Banisteriopsis caapi</i> broom in Sucumbios Province, Ecuador
110a	<i>T. cf. harzianum</i>	<i>Theobroma cacao</i> stem in Napo Province, Ecuador
169c	<i>T. harzianum</i>	<i>Theobroma speciosum</i> stem in Belem, Para, Brazil
172ai	<i>T. TKON 21</i>	<i>Theobroma grandiflorum</i> stem in Belem, Para, Brazil
172h	<i>T. ovalisporum</i>	<i>T. grandiflorum</i> stem in Belem, Para, Brazil
173a	<i>T. cf. spirale</i>	<i>Theobroma</i> stem species in Belem, Para, Brazil
173f	<i>T. harzianum</i>	<i>Theobroma</i> stem species in Belem, Para, Brazil
173k	<i>T. strigosum</i>	<i>Theobroma</i> stem species in Belem, Para, Brazil
185c	<i>T. stromaticum</i>	<i>T. cacao</i> stem in Medici Landia, Para, Brazil
203c	<i>T. ovalisporum</i>	<i>T. speciosum</i> stem in Rio Xingu, Brazil
217a	<i>T. harzianum</i>	<i>Theobroma gileri</i> stem in Esmeraldas Province, Ecuador
219b	<i>T. hamatum</i>	<i>T. gileri</i> pod in Esmeraldas Province, Ecuador
219f	<i>T. harzianum</i>	<i>T. gileri</i> pod in Esmeraldas Province, Ecuador
TA	<i>T. asperellum</i>	<i>T. cacao</i> stem in Beltsville, MD, USA
Col	<i>C. gleosporioides</i>	<i>T. cacao</i> stem in Beltsville, MD, USA

2.3. Production of fungal inoculum for seedling inoculations

Fifteen *Trichoderma* isolates, plus an isolate of *Colletotrichum gloeosporioides*, were included in this study (Table 1). The fungal isolates were grown on cornmeal dextrose agar (CDA) plates, 1.7% w/v Difco cornmeal agar (DIFCO Laboratories, Detroit, MI) plus 20% w/v dextrose, in Petri dishes in an incubator at 23 °C for 5 days before use.

2.4. Sterile cacao seedling production

Open pollinated seeds of *Theobroma cacao* variety comun (Lower Amazon Amelonado type) were collected from established plantings at the Almirante Cacau, Inc. farm (Itabuna, Bahia, Brazil) and shipped to the Sustainable Perennial Crops Laboratory in Beltsville, MD for use. The seed coat was removed from the seeds, and the seeds were surface sterilized by soaking in 14% sodium hypochlorite for 3 min followed by three washes in sterile distilled water. The seed coat was removed to eliminate the associated fleshy pulp and facilitate surface sterilization. Three sterile seeds were placed side-by-side on 1.5% water agar in 100 mm diameter Petri dishes and wrapped tightly with Parafilm. The seeds were pregerminated under fluorescent lights at 23 °C.

2.5. Seedling inoculation methods

The colonization of cacao seedlings was characterized using five different methods.

2.5.1. Method 1

After pregerminating for 4 days, two 0.6-cm agar plugs, of one *Trichoderma* isolate, were placed on the water agar surface near the emerging roots. The fungi were allowed to grow out of the agar plug, through the water agar and onto the cacao seedlings. The seedlings were rated for root dis-

coloration after 6 days of colonization using a scale from 0 (no discoloration) to 4 (severe browning).

2.5.2. Method 2

After colonization on plates for 6 days, using the techniques outlined in Method 1, the germinating seeds were planted in 3 cm of sterile soil-less mix (80 g of 2:2:1, sand:Perlite:Promix) in double magenta boxes (20 cm high and approx. 6.5 cm square, Magenta Corp., Chicago, IL). Twenty milliliters of sterile distilled water was added to the dry, soil-less mix after planting and seedlings were grown on fluorescent light benches at 23 °C for 2 weeks.

2.5.3. Method 3

A colonized agar plug was transferred to the soil surface of magenta boxes and 20 mL of sterile distilled water was added. The magenta boxes were incubated at 23 °C for 10 days before a sterile pregerminated seed (3 days on water agar) was planted in each box. The seedlings were grown 3 weeks after inoculation before being dissected.

2.5.4. Methods 4 and 5

Methods 4 and 5 were similar in all aspects except for the point of inoculation. Pregerminated cacao seeds were planted in soil-less mix in magenta boxes with the addition of sterile distilled water (20 mL). Once the seedlings had emerged, a colonized agar plug was transferred to the soil surface next to the stem (Method 4, bottom) or between the seedling cotyledons (Method 5, top). The seedlings were grown 2 weeks after inoculation before being dissected for Methods 4 and 5.

2.6. Determination of seedling colonization

For each method, cacao seedlings were dissected and 1-cm tissue sections were plated on CDA. For Method 1, sections of roots, stems, cotyledons, and plumules were surface sterilized as previously described for the

cacao seed and plated on CDA. For Method 2, sections of root tips, mature primary roots, stems, bark, xylem, cotyledons, plumules, and leaves were plated on CDA after surface sterilization. The bark and xylem were separated by splitting the bark down to the woody xylem and peeling off the bark before surface sterilization of both tissues. Methods 3–5 were handled similar to Method 2 but without the bark and xylem samples. For mature root, stem, xylem, bark, cotyledons, and leaves, two sections were plated from each seedling. For root tips and plumules, one section was plated. All the plated plant sections were incubated on the lab bench (23 °C) for 5–7 days until the *Trichoderma* isolates grew out of the cacao tissue sections and were counted as positive or negative for colonization. For each method, at least 4 replicates (seedlings) were inoculated with each isolate. Experiments were carried out in sets of 2 or more replicates using seeds from separate shipments.

2.7. Colonization index

A colonization rating index (CI) was determined for each seedling in order to evaluate the endophytic abilities of each isolate over all tissues. For Method 1, the CI formula gave positive plumule colonization, a value of 2 versus a maximum value of 1 for cotyledon, stem and root tissues. Plumule colonization was given the higher value due to our interest in colonization of meristematic tissues. For Method 1 the maximum CI for a seedling was 5. For the remaining methods, plumule and root tip (meristematic

tissues) were given a maximum value of 2 for positive colonization and leaf, stem, root, and cotyledon tissues were given a maximum value of 1. The maximum CI for Methods 2–5 was 8.

2.8. Statistical analysis

The data were expressed as percent growth inhibition for the antibiosis study and % plate colonization for the mycoparasitism studies. The seedling colonization study data were presented as percent tissue colonization or used the colonization index (CI). Mixed model ANOVA was fit to the data for each tissue and method using $\alpha = 0.05$ level of significance. Means separations among isolates were obtained using the Tukey–Kramers (SAS Institute Inc. 2005) adjustment to protect against false positives (i.e., inflated Type I error). Heterogeneous within-isolate variances were modeled using the GROUP = option (Littell et al., 2006) of the REPEATED statement in SAS/STAT[®] Proc MIXED (SAS Institute Inc., 2005). Diagnostic statistics were calculated on each model's residuals to confirm goodness-of-fit.

Summary tables are provided indicating the endophytic colonization efficiency (80%) for each tissue and method combination across all fungal isolates (Table 2) and the endophytic colonization efficiency (80%) for each isolate and tissue combination across methods of inoculation (Table 3). The value of 80% colonization is related directly to the percentage of tissue pieces colonized (data not shown).

Table 2
Summary of endophytic colonization efficiency for each tissue and method combination across all fungal isolates

Method	Root tip	Mature root	Stem	Cotyledons	Plumule	Leaf
M1	nd	6	6	7	3	nd
M2	8	10	10	10	9	6
M3	5	8	7	8	4	0
M4	2	7	7	0	0	1
M5	1	7	4	8	6	2
Sum	16/40	38/50	34/50	33/50	22/50	9/40

The numbers represents the number of isolates with at least 80% efficiency at colonization of a tissue for a specific method. The sum for each tissue over all method and isolate combinations where a tissue was at least 80% colonized is also presented. The table only includes data on the 10 isolates included in all 5 methods of inoculation.

Table 3
Summary of endophytic colonization efficiency for each isolate and tissue combination across methods of inoculation

Tissue	70a	110a	172ai	172h	173a	185c	219b	219f	TA	Col
Root tip	2	3	2	1	0	0	3	3	1	1
Mature root	4	5	5	5	2	1	5	5	5	1
Stem	5	4	4	3	2	2	4	4	5	1
Cotyledons	4	4	4	3	3	1	4	4	4	2
Plumule	1	4	2	2	1	0	4	3	4	1
Leaf	0	1	1	1	0	0	1	3	2	0
Sum	16/28	21/28	18/28	15/28	8/28	4/28	21/28	22/28	21/28	6/28

The numbers represent the number of methods in which the colonization of a tissue by a specific isolate was at least 80%. The sum for each isolate over all method and tissue combination where isolates colonized at least 80% of the tissue segments is also presented.

3. Results

3.1. Soluble inhibitory metabolite production and mycoparasitism

The isolates studied varied in their abilities to produce metabolites that inhibited growth of *M. roreri* (Fig. 1A and B). Culture filtrates from isolates DIS 172ai (*T. sp.* TKON 21) and DIS 203c (*T. ovalisporum*) produced in MIN media completely inhibited growth of *M. roreri* when incorporated into MIN agar. Isolates DIS 173a (*T. cf. spirale*), DIS 185c (*T. stromaticum*), and DIS 219f (*T. harzianum*) inhibited *M. roreri* growth 48%, 50%, and 47%, respectively, on MIN agar. Culture filtrates from isolates DIS 172ai (*T. sp.* TKON 21) and DIS 203c (*T. ovalisporum*) produced in MEA media also strongly inhibited growth of *M. roreri*. For most isolates, culture filtrates were less inhibitory when produced and incorporated into MEA media. This demonstrates the importance of growth substrates in the production of soluble inhibitory metabolite. Exceptions were culture filtrates from isolates DIS 172h, DIS 173k, DIS 217a, and DIS 219b.

Some of the *Trichoderma* isolates were aggressive mycoparasites which completely colonized the *M. roreri* cultures to the point that the pathogen could not be recovered (Fig. 1C). Isolates DIS 67a, DIS 70a, DIS 110a, DIS 169c, DIS 219b, and DIS 219f completely colonized the pseudostroma of *M. roreri* on pre-colonized plates after 5 weeks. Isolates DIS 172ai, DIS 172h, DIS 173k, and DIS 203c were poor mycoparasites of *M. roreri*, eliminating the pathogen on 20% or less of the agar plugs from pre-colonized plates after 5 weeks.

3.2. Seedling colonization studies

For Method 1, germinated cacao seedlings were inoculated on water agar plates. Six days after inoculation, the root discoloration ratings varied from 0.1 for uninoculated seedlings to 3.3 for isolate DIS 169c, 4.0 being a maximum (Fig. 2A). Isolates DIS 70a, DIS 172h, DIS 173a, DIS 185c, DIS 203c, and Col had ratings of 2.0 or below. Isolates DIS 67a, DIS 169c, DIS 172ai, DIS 173f, DIS 173k, DIS 217a, DIS 219b, and DIS 219f had ratings of 3.0 or above.

The colonization index was developed as a general assessment of the abilities of a *Trichoderma* isolate to establish an endophytic relationship with cacao seedlings. A CI greater than 0 by definition indicates the isolate was living inside the plant tissue and is therefore an endophyte of cacao. Isolates with high CI values within an experiment are expected to be efficient in establishing the endophytic relationship with cacao. Using Method 1, isolates DIS 173f and TA completely colonized the cacao seedlings, resulting in CI values of 5 (Fig. 2B). A CI of 5 means 28 of 28 tissue segments including 4 tissues (8 root, 8 stem, 8 cotyledon, and 4 plumule segments) were colonized for each of these isolates using Method 1. Isolates DIS 110a,

DIS 173k, and DIS 219b had near maximum CI values of 4.9. DIS 185c and Col were the poorest colonizers using Method 1 with CI values less than 3 (Fig. 2B). Only 10 of the 16 isolates studied using Method 1 were included in Methods 2–5. Of these 10, mature roots, plumules, stems, and cotyledons were at least 80% colonized by 6, 3, 6, and 7 isolates, respectively (Table 2). All isolates, other than isolate DIS 185c, could be re-isolated from all plant tissues at least once using Method 1 for inoculation. Isolate DIS 185c was re-isolated from all tissues but the plumule.

In Method 2, seedlings inoculated on agar plates were transferred to soil after 6 days of incubation. The seedlings were dissected 2 weeks after transplanting to soil in magenta boxes. Isolates DIS 172h, DIS 219b, DIS 219f, and TA heavily colonized all the seedling tissues sampled using Method 2, resulting in CI values of the maximum 8 (Fig. 3A). With Methods 2–5 a CI of 8 indicates 10 of 10 tissue segments including 6 tissues (2 root, 2 stem, 2 cotyledon, 2 leaf, 1 root tip, and 1 plumule segments) were colonized for each of these isolates for each replicate seedling. Isolates DIS 173a, and DIS 185c were poor colonizers of cacao seedlings using this method, having CI values less than 6. Cacao root tips were heavily colonized using this method with only isolates DIS 173a and DIS 185c failing to colonize all root pieces (Table 2). Mature roots, stems, and cotyledons were heavily colonized by all isolates (Table 2). Plumules were heavily colonized by 9 isolates (Table 2), but DIS 185c was not re-isolated from plumules after inoculation using Method 2. Leaves were less colonized (Table 2), although 4 isolates (DIS 172h, DIS 219b, DIS 219f, and TA) completely colonized all the leaf pieces sampled using Method 2. Similar to the results with stems, the bark of cacao seedlings was heavily colonized by all the *Trichoderma* isolates studied (Fig. 3B). The xylem, on the other hand, was heavily colonized (>80%) by isolates DIS 219b and DIS 219f only (Fig. 3C). Isolates DIS 172h, DIS 173a, DIS 185c and Col were poor colonizers of the cacao xylem (Fig. 3C).

Using Method 3, seeds were planted into soil preinoculated for 10 days by the *Trichoderma* isolates. The seedlings were grown for 3 weeks before being dissected. Isolates DIS 110a, DIS 219b, and DIS 219f had CI values near the maximum of 8 using Method 3 (Fig. 4) and DIS 185c and Col had CI values less than 4. When seeds were planted in preinoculated soil, five isolates were efficient colonizers of root tips (Table 2). 8 isolates heavily colonized mature root pieces, 7 isolates heavily colonized cacao stems, and 8 isolates heavily colonized cotyledons (Table 2). All isolates except for DIS 185c were isolated at least once from plumules of seedlings planted in preinoculated soil. Plumules were heavily colonized by 4 isolates using Method 3 (Table 2). Leaves were poorly colonized by all isolates and isolates DIS 185c and Col were not isolated from leaves when seedlings were inoculated using Method 3 (Table 2).

Isolate DIS 219b had a CI value of 7.76 (maximum CI = 8) when seedlings were inoculated at the base of the

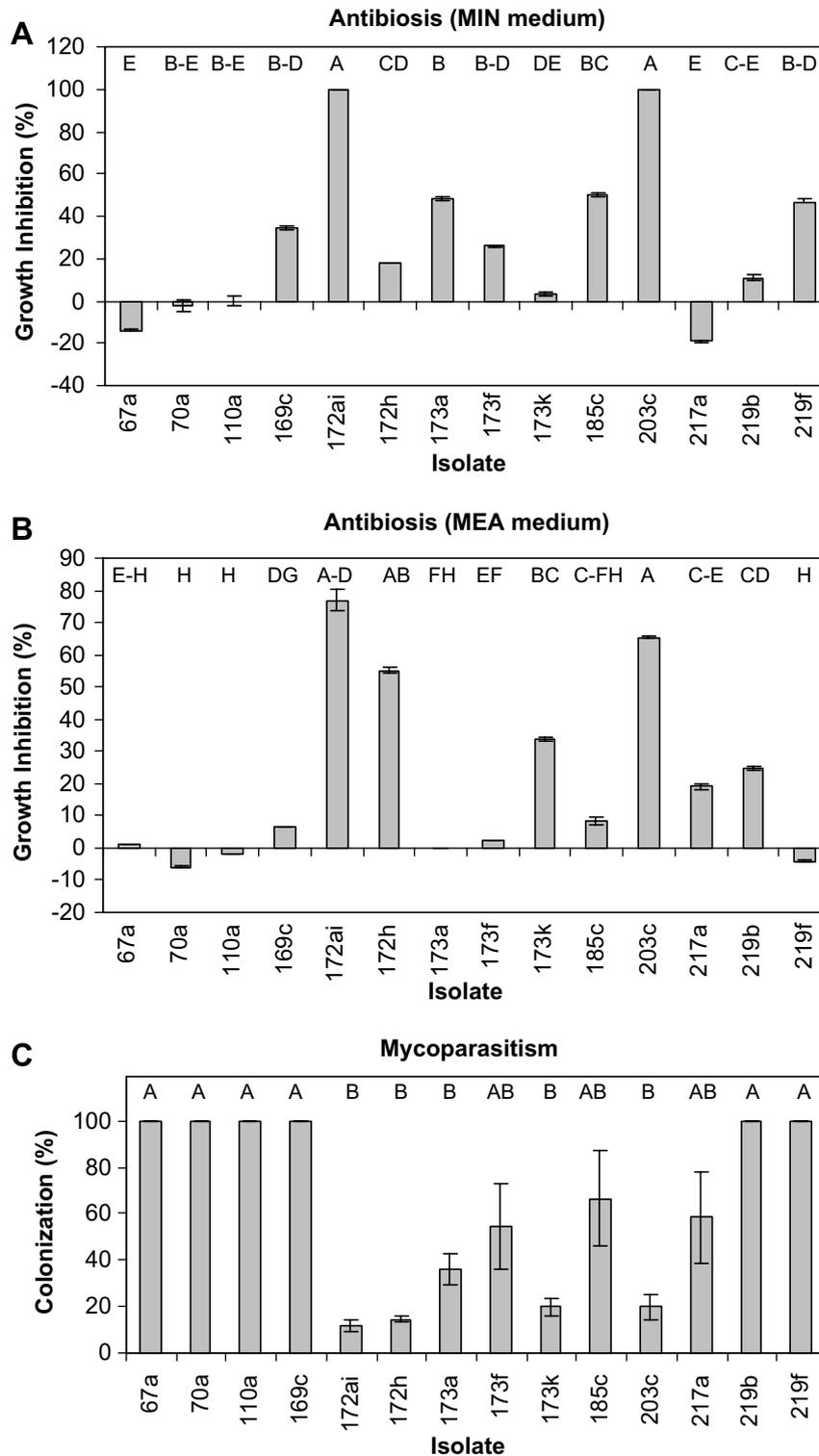


Fig. 1. Antibiosis and mycoparasitism against *Moniliophthora roreri* by endophytic *Trichoderma* isolates. Studies of antibiosis and mycoparasitism were carried out as described in the materials and methods. For antibiosis studies the means represent percent inhibition of growth of *M. roreri* on plates including filtrates from *Trichoderma* isolates grown on MIN (A) or MEA (B) medium. For the mycoparasitism (C) studies the means represent the percentage of *M. roreri* precolonized plates where the *Trichoderma* isolate was reisolated and *M. roreri* was eliminated. The means separations as indicated by letters above bars were accomplished by the Tukey–Kramers adjustment as described in the text and error bars (\pm one standard error) are also presented. Means with non-overlapping letters indicate statistical significant differences ($p < 0.05$). The dashes between letters indicate the range of letters is included.

stem (Method 4, Fig. 5A) and DIS 219f had a CI value of the maximum 8 when seedlings were inoculated between

the cotyledons (Method 5, Fig. 5B). Isolates DIS 185c and Col had the lowest CI values (CIs less than 2) for both

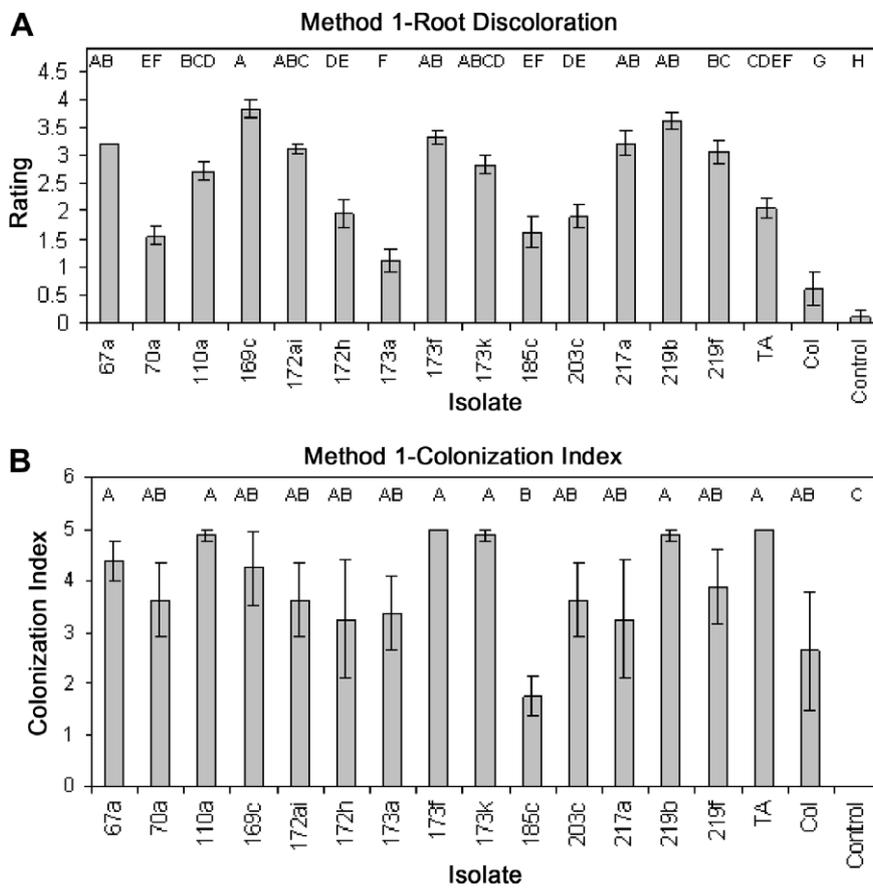


Fig. 2. Root discoloration and endophytic seedling colonization of cacao by *Trichoderma* isolates after inoculation of cacao seedlings on agar plates (Method 1). Sterile cacao seeds were pregerminated 4 days on water agar plates and then inoculated with agar plugs from *Trichoderma* cultures. Six days after inoculation, the seedlings were rated for root discoloration (A) using a scale from 0 (no discoloration) to 4 (severe browning). Rating for 18 individual seedlings (18 replicates) inoculated with each isolate were included. A subset of 4 seedlings (4 replicates) were dissected into roots, stems, cotyledons, and plumules and surface sterilized sections were plated on CDA plates. Colonization was verified 5–7 days later and the colonization index (B) was determined using weighted values of 1 for positive root, stem, and cotyledon colonization, and 2 for positive plumule colonization. The means separations as indicated by letters above bars were accomplished by the Tukey–Kramers adjustment as described in the text and error bars (\pm one standard error) are also presented. Means with non-overlapping letters indicate statistical significant differences ($p < 0.05$).

Method 4 and Method 5. In general terms, Methods 4 and 5 performed as expected (Table 2). Inoculations of seedlings at the stem base resulted in slightly better colonization of the lower portions of the plant (root tips, roots, and stems), and inoculation at the cotyledon stem juncture resulted in greater colonization of the upper portions of the plant (cotyledons, plumules, and leaves).

Isolates DIS 110a (*T. harzianum*), DIS 219b (*T. hamatum*), DIS 219f (*T. harzianum*) and TA (*T. asperellum*) were the most consistent colonizers when data from Methods 2–5 were combined to calculate CI (Fig. 6). These four isolates colonized at least 80% of the tissue section samples in at least 21 of 28 method and tissue combinations studied (Table 3). Isolates DIS 173a (*T. cf. spirale*), DIS 185c (*T. stromaticum*), and Col (*Colletotrichum gleosporioides*) were generally poor endophytic colonizers of cacao tissues, having a CI of less than 4 using data from Methods 2–4 (Fig. 6). These four isolates colonized at least 80% of the tissue section samples in no more than 8 of 28 method and tissue combinations studied (Table 3).

4. Discussion

Several *Trichoderma* species have been extensively studied for their biocontrol potential (Harman, 2000; Howell, 2003; Harman et al., 2004). *Trichoderma harzianum* isolate T-22 is marketed for the control of many different diseases in many different crops (Harman et al., 2004). T-22 is typically applied as a seed treatment or soil amendment but has also been used for foliar applications to control diseases of apple, grape, and other fruit crops. *T. harzianum* isolate T39 has been extensively studied for control of gray mold on grapes using foliar applications (Elad, 1994; O'Neill, 1996). *T. stromaticum* (Samuels et al., 2000) is a mycoparasite of *C. perniciosa* and is being used in the field for control of witches' broom (Bastos, 1996a,b). Isolates of *T. harzianum*, *T. hamatum*, *T. asperellum*, as well as other species of *Trichoderma* have been shown effective in inducing resistance to disease in many plant species (Howell, 2003; Yedidia et al., 2003; Khan et al., 2004; Harman et al., 2004; Horst et al., 2005). Although *Trichoderma*

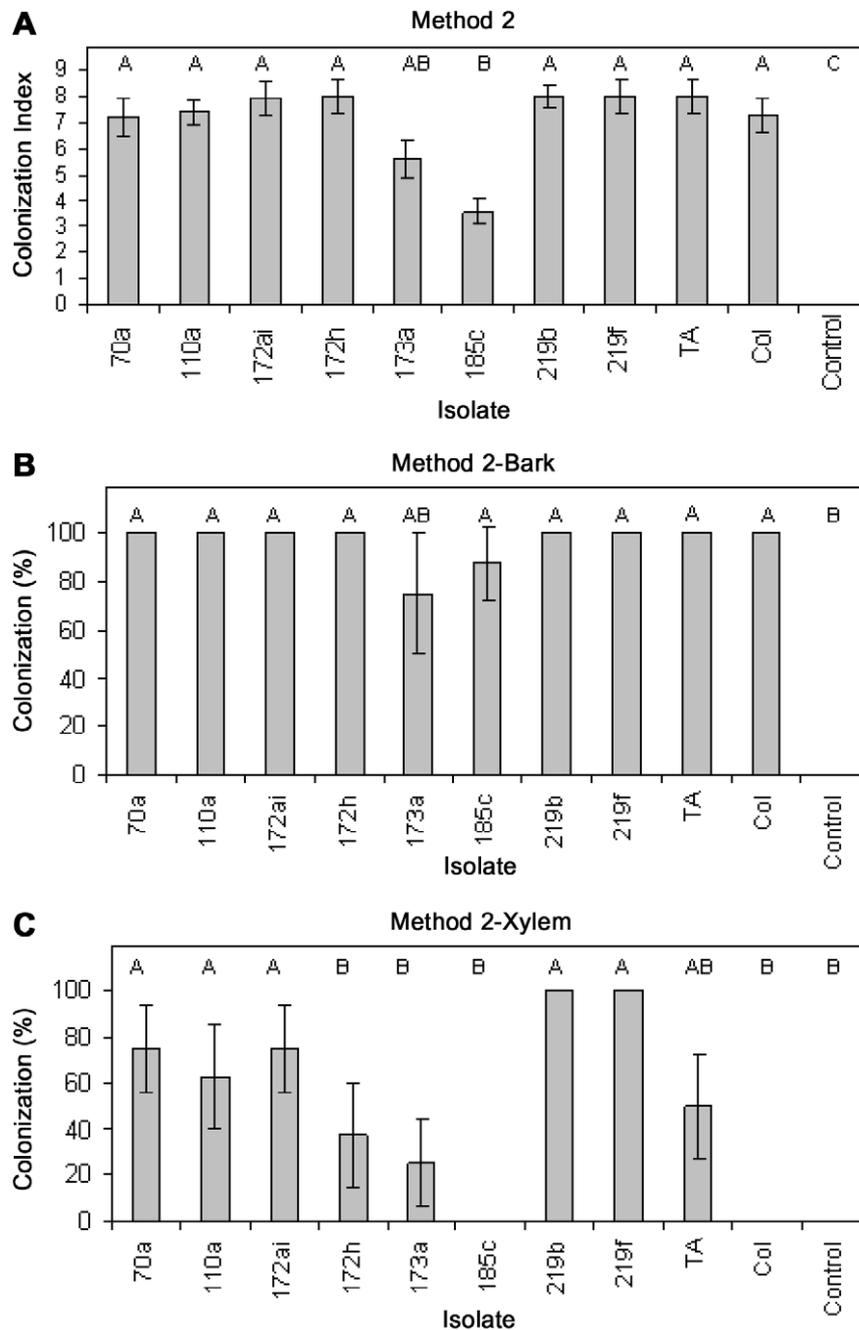


Fig. 3. Endophytic seedling colonization of cacao by *Trichoderma* isolates after inoculation on agar plates followed by planting in sterile soil-less mix (Method 2). Sterile cacao seeds were pregerminated 4 days on water agar plates and then inoculated with agar plugs from *Trichoderma* cultures. Six days after inoculation, the seedlings were transplanted to sterile soil-less mix and grown for two weeks before being dissected into root tips, roots, stems, cotyledons, plumules, leaf, bark, and xylem. Surface sterilized sections of each tissue were plated on CDA plates. Colonization was verified 5–7 days later and the colonization index (A) was determined using weighted values of 1 for positive root, stem, cotyledon, and leaf colonization, and 2 for positive plumule and root tip colonization. The percent colonization is also presented for bark (B) and xylem (C). Five seedlings (5 replicates) inoculated with each isolate were dissected. The means separations as indicated by letters above bars were accomplished by the Tukey–Kramers adjustment as described in the text and error bars (+/– one standard error) are also presented. Means with non-overlapping letters indicate statistical significant differences ($p < 0.05$).

has been extensively studied for its biocontrol potential, the endophytic ability of *Trichoderma* has only recently received attention (Harman et al., 2004). The concept of *Trichoderma* as an endophyte of above ground plant tissues has received even less attention (Evans et al., 2003; Holmes et al., 2004, 2006; Bailey et al., 2006).

Trichoderma species are typically considered soil and root colonizers (Howell, 2003; Harman et al., 2004; Samuels, 2006). Several of the *Trichoderma* isolates studied here were efficient endophytic colonizers of the above ground portions of the cacao seedling when inoculations were made away from the soil surface between the cotyledons

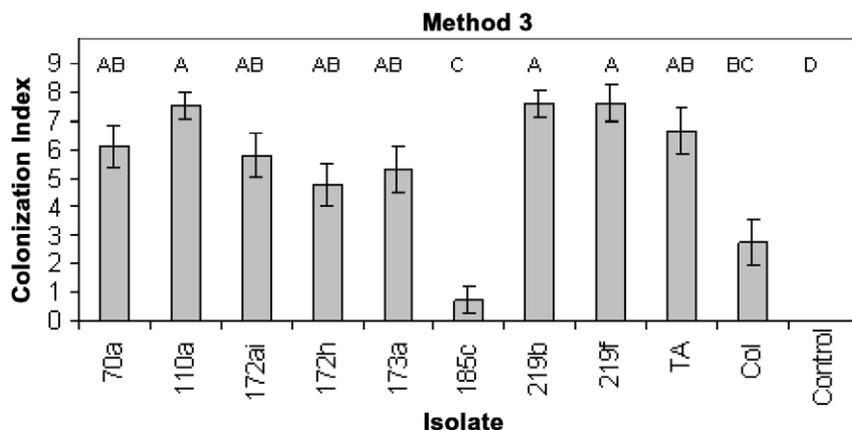


Fig. 4. Endophytic seedling colonization of cacao by *Trichoderma* isolates after planting of seed in pre-inoculated soil (Method 3). Sterile cacao seeds were germinated on water agar and grown in soil-less mix pre-inoculated with *Trichoderma* isolates (10 days). Three weeks after planting, the resulting seedlings were dissected into root tips, roots, stems, cotyledons, plumules, and leaf. Surface sterilized sections of each tissue were plated on CDA plates. Colonization was verified 5–7 days later and the colonization index was determined using weighted values of 1 for positive root, stem, and cotyledon colonization, and 2 for positive plumule and root tip colonization. Five seedlings (5 replicates) inoculated with each isolate were dissected. The means separations as indicated by letters above bars were accomplished by the Tukey–Kramers adjustment as described in the text and error bars (\pm one standard error) are also presented. Means with non-overlapping letters indicate statistical significant differences ($p < 0.05$).

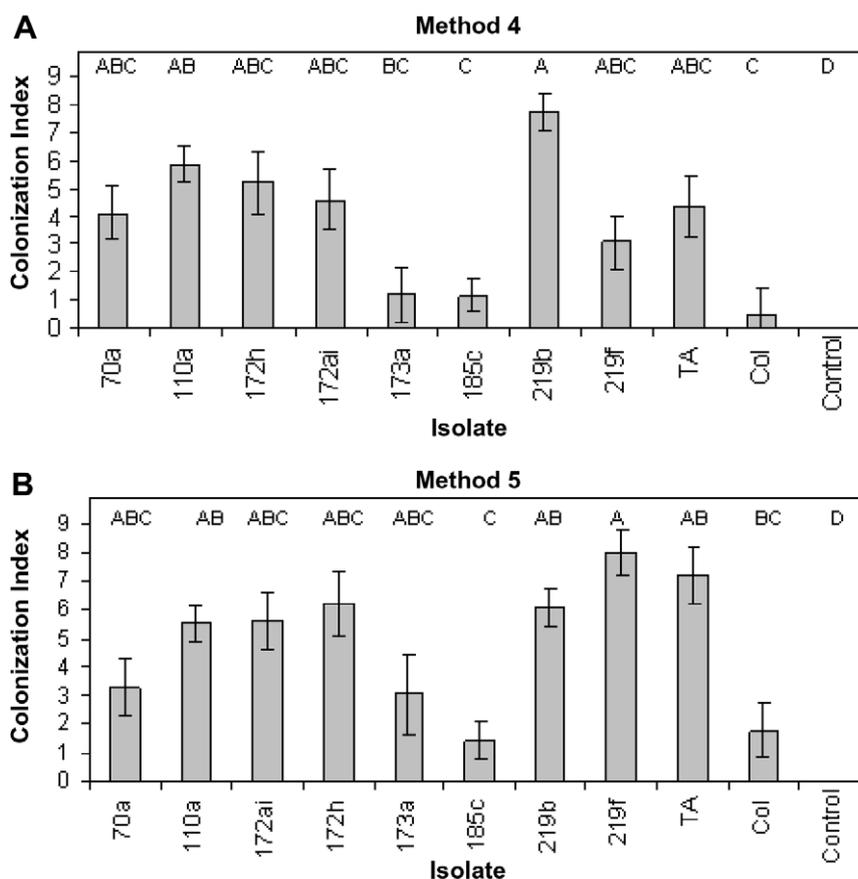


Fig. 5. Endophytic seedling colonization of cacao by *Trichoderma* isolates using two methods: (A) Method 4, inoculating seedlings at the stem base or (B) Method 5, inoculating seedlings between the cotyledons. Sterile cacao seeds were germinated on water agar and grown in sterile soil-less mix until the seedlings emerged. An agar plug carrying a *Trichoderma* isolate was placed either at the base of the stem on the soil surface or between the cotyledons of the seedlings and seedlings were grown for two weeks allowing colonization. The seedlings were dissected into root tips, roots, (C) stems, cotyledons, plumules, and leaf. Surface sterilized sections of each tissue were plated on CDA plates. Colonization was verified 5–7 days later and the Colonization Index was determined using weighted values of 1 for positive root, stem, and cotyledon colonization, and 2 for positive plumule and root tip colonization. Four seedlings (4 replicates) inoculated with each isolate were dissected for each method. The means separations as indicated by letters above bars were accomplished by the Tukey–Kramers adjustment as described in the text and error bars (\pm one standard error) are also presented. Means with non-overlapping letters indicate statistical significant differences ($p < 0.05$).

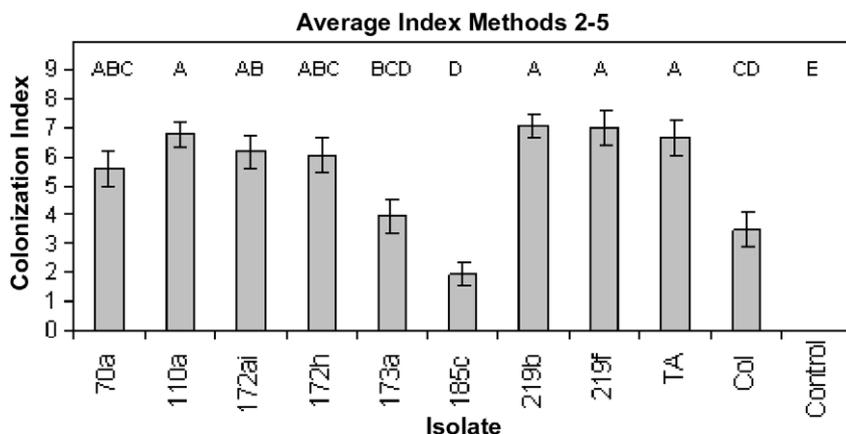


Fig. 6. Combined colonization index using combined data from Methods 2–5. Colonization index was determined for each seedling using weighted values of 1 for positive root, stem, and cotyledon colonization, and 2 for positive plumule and root tip colonization. The resulting CI values for all seedlings and fungi combinations using Methods 2–5 were analyzed together disregarding method of inoculation. The means separations as indicated by letters above bars were accomplished by the Tukey–Kramers adjustment as described in the text and error bars (\pm one standard error) are also presented. Means with non-overlapping letters indicate statistical significant differences ($p < 0.05$).

and stem using method 5 (Fig. 5). These isolates not only colonized the tissues in the area of inoculation but also colonized distant tissues including root tips, roots, stems, plumules, and leaves (Table 2). It is this ability to colonize above-ground plant parts (Elad, 1994; O’Neill, 1996; Wilson, 1997) that is of key interest in control of cacao diseases. Lewis et al. (2001) characterized the progressive endophytic colonization of corn by *Beauveria bassiana*. Although colonization of corn by *B. bassiana* increased over time, colonization was not complete in this annual crop, even after seed inoculation. Because conditions on the plant surface are typically harsh compared to soil, establishing long lasting endophytic associations in the above-ground parts of the cacao tree may contribute to limiting the number of applications required in a season. Harman and Björkman (1998) were able to identify efficient root colonizing *Trichoderma* isolates by careful screening. It may be possible to identify efficient colonizers of specific cacao tissues using the methods described here.

When considered over all tissue and isolate combinations, mature roots, stems, and cotyledons were most heavily endophytically colonized by the isolates studied (Table 2). The stem tissues studied were maturing and contained a central xylem core. Although not examined in detail, we were able to isolate *Trichoderma* from the xylem of cacao seedling stems. To reach the xylem, the *Trichoderma* isolates had to penetrate the immature bark, a relatively thick tissue. Under most conditions studied, *Trichoderma* isolates are thought to penetrate only a few cells into the roots (Harman et al., 2004). Isolates DIS 70a, DIS 110a, DIS 172ai, DIS 219b, DIS 219f, and TA were all isolated from 50% or more of the xylem pieces plated on cornmeal agar plates. These 6 *Trichoderma* isolates were collected from divergent plant species using several isolation methods (Table 1). DIS 110a and DIS 172ai were isolated from live sapwood immediately below the bark of trunks of *Theobroma cacao* and *T. grandiflorum*, respectively (Evans

et al., 2003). DIS 70a was isolated from a *M. pernicioso* infected broom on *Banisteriopsis caapi*, a woody liana (Holmes et al., 2004). DIS 219b and DIS 219f were isolated from pods of *Theobroma gileri*, while TA was isolated from greenhouse grown trees in Beltsville, Maryland. The data indicate the general aggressiveness of the isolate in colonizing cacao tissues may be more critical to the isolate’s ability to colonize woody tissues versus the source and method of the original isolation. The isolate of *C. gleosporioides* (Col) included in these studies was less efficient in colonizing cacao tissues than many of the *Trichoderma* isolates studied. This may have been influenced by the methods of inoculation used. Extensive studies have been carried out detailing the interactions between cacao and leaf endophytes, including isolates of *Colletotrichum* (Arnold et al. 2003; Arnold and Herre, 2003).

Cacao seedlings can be precolonized with *Trichoderma* before planting and it is likely these methods of inoculation could be adapted to other cacao propagation methods (Wood and Lass, 2001; Maximova et al., 2002). Considering the endophytic nature of the *Trichoderma* isolates being studied, it is reasonable to ask if *Trichoderma* isolates could potentially colonize a cacao tree from a single inoculation point. Results presented here suggest that colonization of cacao by *Trichoderma* isolates is dependent upon both time and opportunity. The observed failure of *Trichoderma* isolates to always completely colonize growing points would likely limit the colonization of subsequent plant growth and over time minimize the presence of the endophytic *Trichoderma*. In assembling the *Trichoderma* collection, multiple *Trichoderma* species were often isolated from different parts of the same tree (isolates with the same numbers as with DIS 219b and DIS 219f came from the same tree). Our methods used surface sterilized seedlings and sterilized soil, and other microbes were rarely observed on the dissected tissues. Competition between the many different endophytic and epiphytic microbes found on

cacao trees (Arnold et al., 2003; Arnold and Herre, 2003; Rubini et al., 2005) would likely further limit the ability of a single *Trichoderma* isolate to maintain complete colonization of a cacao tree.

Isolates DIS 110a (*T. harzianum*), DIS 219b (*T. hamatum*), DIS 219f (*T. harzianum*) and TA (*T. asperellum*) were the most consistent colonizers when all the inoculation methods are considered (Fig. 6, Table 3). These isolates caused moderate to severe discoloration (discoloration ratings of 2.0–3.6) of seedling roots when seedlings were inoculated on water agar plates. The two *T. ovalisporum* isolates DIS 70a and DIS 172h (Holmes et al., 2004), and *Trichoderma* TKON 21 (DIS 172ai), a new species (Samuels et al., 2006a), were moderately efficient at colonizing cacao seedlings (Fig. 6, Table 3) and had root discoloration ratings of 1.5, 1.9, and 3.1, respectively. Isolates DIS 173a (*T. spirale*), DIS 185c (*T. stromaticum*), and Col (*C. gloeosporioides*) were the poorest colonizers of cacao seedlings (Fig. 6, Table 3) and caused very little root discoloration during colonization (discoloration ratings of 0.6–1.6). Endophytic colonization of cacao seedlings did not necessarily result in significant root discoloration. It is unclear what changes are occurring in roots during colonization by *Trichoderma* species that result in their discoloration. Some *T. virens* isolates induce synthesis of terpenoid compounds in cotton roots (Howell et al., 2000) that have been correlated with the *Trichoderma* isolate's ability to protect cotton seedlings against *Rhizoctonia solani*. At a minimum, the discoloration indicates an active response of the cacao seedlings to the presence of the specific *Trichoderma* strains. Recent studies of the molecular interactions between *Trichoderma* species and cacao (Bailey et al., 2006) verify that gene expression in cacao seedling is altered during colonization by *Trichoderma*. These same studies demonstrated that *Trichoderma* gene expression is altered during colonization of cacao seedlings.

Antibiosis, the production of antimicrobial compounds, and mycoparasitism, the feeding on a fungus by another organism, are mechanisms whereby *Trichoderma* species provide protection to plants against plant pathogens (Chet et al., 1998; Howell, 1998, 2003; Harman et al., 2004). The isolates studied here varied greatly in their abilities to produce compounds inhibitory to *M. roreri* and in their abilities to parasitize *M. roreri*. The *T. harzianum* isolates DIS 67a, DIS 110a, DIS 169c, DIS 173f, DIS 217a, and DIS 219f displayed a range of biological activities, but the *T. harzianum* isolates tended to have moderate to low antibiosis activities (Fig. 1), moderate to high mycoparasitism activities (Fig. 1), and moderate to high endophytic abilities (Fig. 2). Isolates DIS 172ai and DIS 203c completely inhibited growth of *M. roreri* in the antibiosis screens but were poor mycoparasites. It is unclear what compounds are being produced by these isolates that are inhibitory to *M. roreri* although many antimicrobial compounds have been identified as being produced by other *Trichoderma* isolates (Howell, 1998, 2003). The most efficient colonizers of cacao seedlings (Fig. 6, Table 3) were not superior for

both antibiosis and mycoparasitism (Fig. 1) which suggests that the ability to colonize cacao seedlings is not critically linked to either antibiosis ability or mycoparasitism. Additional isolates will need to be studied if conclusions concerning correlations between these activities and *Trichoderma* species are to be drawn.

For many crops, disease control is required for a season with significant periods of fallow or dormancy. Cacao is a perennial crop with trees lasting many years. In cacao, the annual harvest can vary from a single major harvest period per year, two major harvests per year, or continual harvests throughout the year (Wood and Lass, 2001). Protection may be required for the whole year adding an extra burden to achieving successful biocontrol. The identification of *Trichoderma* isolates adapted to survival on the above ground portions of cacao is a first step in the development of a biocontrol strategy for control of cacao diseases. Although diseases caused by *Phytophthora* sp. (black pod) and *M. perniciosa* (witches' broom) damage leaves, stems, and meristems, frosty pod caused by *M. roreri* only damages pods. How *Trichoderma* sp. will grow and proliferate on pod surfaces during the long growing season is the subject of future studies. Using the described screens it may be possible to identify *Trichoderma* isolates that are efficient colonizers of cacao tissues and are both superior producers of antimicrobial compounds and have superior mycoparasitic abilities. Additional methods may allow identification of *Trichoderma* isolates that induce resistance to disease in cacao. The selection of *Trichoderma* isolates with good endophytic ability, in addition to other attributes such as mycoparasitism, antibiosis, and/or induced resistance could greatly improve the possibilities of developing functional biocontrol strategies for cacao diseases.

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