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Inoculation of coffee plants with the fungal entomopathogen *Beauveria bassiana* (Ascomycota: Hypocreales)

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ABSTRACT

The entomopathogenic fungus *Beauveria bassiana* was established in coffee seedlings after fungal spore suspensions were applied as foliar sprays, stem injections, or soil drenches. Direct injection yielded the highest post-inoculation recovery of endophytic *B. bassiana*. Establishment, based on percent recovery of *B. bassiana*, decreased as time post-inoculation increased in all treatments. Several other endophytes were isolated from the seedlings and could have negatively influenced establishment of *B. bassiana*. The recovery of *B. bassiana* from sites distant from the point of inoculation indicates that the fungus has the potential to move throughout the plant.

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Introduction

The coffee berry borer *Hypothenemus hampei* (*Coleoptera: Curculionidae*) is the most important coffee pest worldwide (Le Pelley 1968). Adult females deposit their eggs in galleries inside the coffee berry, with larval feeding on the seeds, reducing yields as well as quality. Pest management strategies against the coffee berry borer have met limited success mainly due to the cryptic life-cycle of the insect inside the berry. These strategies have included the use of chemical insecticides (Mansingh 1991; Carrillo *et al*. 1989; Godoy *et al*. 1984; Villalba *et al*. 1995),

the application of entomopathogenic fungi (Posada 1998; de la Rosa *et al*. 2000; Haraprasad *et al*. 2001; Posada *et al*. 2004), the release of parasitoids (Aristizabal *et al*. 2004; Quintero *et al*. 1998), and cultural practices emphasizing frequent harvest of ripe and infested berries in order to interrupt the life cycle (Bustillo *et al*. 1998).

Another possible pest management alternative involves the inoculation and endophytic establishment in coffee plants with the entomopathogenic fungus *Beauveria bassiana* (*Ascomycota*: *Hypocreales*). *B. bassiana* has been reported as an endophyte in maize (Vakili 1990; Bing & Lewis 1991, 1992a, 1992b;

Lomer *et al.* 1997; Cherry *et al.* 1999, 2004; Wagner & Lewis 2000; Arnold & Lewis 2005), tomato (Leckie 2002), potato, cotton, cocklebur, and jimsonweed (Jones 1994), *Theobroma gileri* (Evans *et al.* 2003), in the bark of *Carpinus caroliniana* (Bills & Polishook 1991), in seeds and needles of *Pinus monticola* (Ganley & Newcombe 2005), in opium poppies (Quesada-Moraga *et al.* 2006), and in coffee berries from Colombia (Vega *et al.* unpubl.). In maize, endophytic *B. bassiana* was reported as a mortality agent against the European corn borer *Ostrinia nubinalis* (Bing & Lewis 1991; Arnold & Lewis 2005).

Recently, positive *B. bassiana* detection was attained in *in vitro* grown cocoa (Posada & Vega 2006a) and coffee seedlings (Posada & Vega 2006b) inoculated on the radicle and evaluated 30 and 60 d post-inoculation. The objective of this study was to evaluate whether *B. bassiana* can be established as an endophyte in coffee seedlings using different fungal isolates and inoculation methods. Our ultimate goal is to establish *B. bassiana* as an endophyte in the coffee berry, which is the target site for the coffee berry borer.

Materials and methods

Isolates

Four *Beauveria bassiana* isolates were used in this study. Three were obtained from the ARS Collection of Entomopathogen Fungal Cultures (ARSEF; http://www.ars.usda.gov/is/np/systematics/fungibact.htm): ARSEF 5486 from Ivory Coast; ARSEF 2687 from Kenya; and ARSEF 1480 from Brazil. These were previously isolated from the coffee berry borer and selected for this study from a screening of single spore isolates (Posada & Vega 2005). The fourth isolate was a multispore isolate obtained as an endophyte from healthy coffee berries from Colombia and identified as CS16-1 (Insect Biocontrol Laboratory Culture Collection = IBL03032). Isolates ARSEF 5486, ARSEF 1040, and CS16-1 (IBL03032) were previously determined to belong to AFNEO_1, a clade of generalist entomopathogens that is widely established throughout the neotropics and in coffee plantations in western equatorial Africa (Rehner *et al.* 2006). ARSEF 2687 has been determined to have affinities to the clade Asia 4 (Rehner *et al.* 2006), however, this isolate is the only member of this lineage that has been examined to date, thus its phylogenetic position and status within the *B. bassiana* complex is unresolved (Rehner unpublished).

The isolates were cultured on yeast malt agar (YMA; Sigma Aldrich, St Louis, MO) to which 0.1 % stock antibiotics was added. The stock antibiotic solution consisted of 0.02 g each of tetracycline, streptomycin and penicillin, dissolved in 10 ml sterile, distilled water, filter-sterilized through a 0.2 μm filter (Nalgene Disposable Filterware, Nalge Nunc International Rochester, NY); from this, 1 ml was added per litre of medium. The cultures were approximately one-month old when used, and a germination test was conducted before inoculating the plants. For the test, a low concentration of conidia was suspended in 0.1 % Triton X-100 (Sigma Aldrich) and from this stock a 15 μl aliquot was plated on 2.5 % Noble agar (Becton Dickinson, Sparks, MD). The aliquot was spread with a sterile glass rod onto the medium surface and incubated in the dark at $25 \pm 2\,°C$ for 24 h after which 25 % lactophenol

blue solution (Fluka Chemie, Buchs, Switzerland) was added to stop germination, and three groups of 100 conidia selected at random were assessed for germination (Vélez *et al.* 1997; Goettel & Inglis 1997).

Coffee plants

The coffee seedlings used for inoculation were obtained from The Behnke Nurseries, Beltsville, MD. Seedlings were transplanted to sterile potting media, and at the time of inoculation, had on average eight pairs of leaves and were *ca* 20 cm tall.

Inoculation methods

Three inoculation methods were tested: foliar spray, stem injection, and soil drench. For each isolate a spore stock suspension was prepared in 0.1 % Triton X-100, and the spore concentration was adjusted to 1×10^8 conidia ml^{-1}. The foliar spray inoculation method was performed with a hand sprayer (Spraymaster Consolidated Plastics, Twinsburg, OH), and an average of 5 ml per plant was applied. The spray was directed mainly to the leaves but also incidentally coated the stems. To avoid conidial runoff to the soil, the top of each pot was covered with aluminum foil. For stem injection inoculations, a hole was made on the stem using a 21 G sterile disposable hypodermic needle to facilitate injection of 1 μl conidial suspension with a Hamilton syringe (Hamilton, Reno, NV). For the drench inoculation method, 10 ml of the spore suspension was applied to the surface of the soil in each pot. Controls received sterile 0.1 % Triton X-100 applied in the same way as each treatment mentioned above.

After spraying, each plant was covered with a plastic bag for 24 h to maintain a high level of humidity. For the plants that were treated with either drench or injection no coverage was needed as the fungus was applied on the soil or inside the plant, where humidity is naturally high. The experiment was run for a total of eight months, and the recovery of *B. bassiana* and other endophytic fungi and bacteria was evaluated by culture methods at two, four, six, and eight months post-inoculation.

Experimental design

The experiment was run as a completely randomized design where the treatments were four *Beauveria bassiana* isolates, three methods of inoculation (foliar spray, stem injection, soil drench), and the corresponding controls. After inoculation, the pots containing the plants were randomized and kept in plant growth chambers (Model E-36L, Percival Scientific, Boone, IA) at $22 \pm 2\,°C$ and 12:12 photoperiod. The plants were fertilized monthly using 25 ml Miracle-Gro (Scotts Miracle-Gro Products, Marysville, OH) plus chelated iron 10 % (Becker Underwood, Ames, IA), and they were watered every 2 d.

Evaluation for presence of Beauveria bassiana

For each evaluation at two, four, six, and eight months post-inoculation, three separate plants per treatment per isolate were used. The pots containing coffee plants were moved from the growth chambers to the laboratory, where the plants

were uprooted and individually washed out with running tap water. From each plant, two leaves, two pieces of stem and two pieces of root were surface sterilized in a 0.5 % sodium hypochlorite suspension for 2 min, 70 % ethanol for 2 min, and rinsed in sterile autoclaved water and dried on sterile towel paper. The final rinse water was plated to determine whether the sterilization process was successful in eliminating epiphytic microorganisms. The leaves were randomly selected from the middle section of the seedling, i.e. no leaves were selected from the apical area or from the basal area. Similarly, two parts of the stem were sampled, one towards the middle of the plant and the second one closer to the soil surface. As for roots, the tap root was sampled by dividing it in two parts, but the root tips were not sampled.

Tissues were cut using a sterile scalpel and forceps into pieces averaging $2 \times 3 \text{ mm}^2$ and six pieces were plated on two 60 mm diam Petri dishes containing YMA plus three antibiotics and kept at $22 \pm 3 \,^\circ\text{C}$. Two hundred and thirty-four Petri dishes were plated for each evaluation period containing a total of 1404 subsamples of plant tissues. A total of 156 plants and 5616 tissue subsamples were evaluated during the course of the experiment.

The results are summarized in Tables 1–3 and are expressed as the percentage of plants positive for the presence of *Beauveria bassiana* after inoculation. The proportion of coffee plant tissues on which *B. bassiana* was found was also evaluated. At the time of the second evaluation the incidence of *B. bassiana* had dropped sharply, and it was decided to increase the number of tissue samples per plant in the remaining two evaluations. For the eight-month post-inoculation evaluation,

in addition to the regular YMA plus antibiotic media, two additional replicate dishes per treatment containing a selective growth-suppressing medium that favours *B. bassiana* (Chase *et al.* 1986) were used to reduce competition of other endophytic fungi likely to out-compete *B. bassiana* during re-isolation. Endophytic fungi other than *B. bassiana* isolated two and four months post-inoculation were sequenced (see below). The diversity of fungi was estimated using the Shannon index (Fowler *et al.* 1998; Magurran 2004).

Inoculation of coffee seeds

An experiment was conducted to evaluate the feasibility of inoculating coffee seeds (Colombia variety) with *Beauveria bassiana* conidia. Coffee seeds were soaked for 24 h in a conidial suspension at a final concentration of 1×10^8 conidia ml^{-1} prepared from each of the four isolates mentioned above. The control was soaked in a sterile solution of 0.1 % Triton X-100.

Table 2 – Number of fungal and bacterial isolates obtained as endophytes from coffee plants inoculated with *Beauveria bassiana* throughout four evaluations

Evaluation	Total	Fungi		Bacteria	
		n	%	N	%
Two months	1073	632	58.9	441	41.1
Four months	876	249	28.4	627	71.7
Six months	718	652	90.8	66	9.2
Eight months	1001	847	84.6	154	15.4

Table 1 – Incidence of four *Beauveria bassiana* isolates in coffee plants two, four, and six months post-inoculation using spray, injections, or drenches

Isolate	n[a]	Drench						Injection						Spraying						Total	
		Leaves		Stems		Roots		Leaves		Stems		Roots		Leaves		Stems		Roots			
		+	%	+	%	+	%	+	%	+	%	+	%	+	%	+	%	+	%	+	%
Two months																					
1480	3	0	0	0	0	1	33.3	0	0	3	100	0	0	0	0	0	0	0	0	4	44.4
CS16-1	3	0	0	0	0	1	33.3	0	0	1	33.3	0	0	0	0	0	0	0	0	2	22.2
5486	3	0	0	1	33.3	0	0	0	0	1	33.3	0	0	0	0	0	0	0	0	2	22.2
2687	3	0	0	0	0	0	0	0	0	2	66.7	0	0	1	33.3	0	0	0	0	3	33.3
Total		0	0	1	8.3	2	16.7	0	0	7	58.3	0	0	1	8.3	0	0	0	0	11	30.6
Four months																					
1480	3	0	0	0	0	0	0	0	0	2	66.7	0	0	0	0	0	0	0	0	2	22.2
CS16-1	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5486	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2687	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total		0	0	0	0	0	0	0	0	2	16.7	0	0	0	0	0	0	0	0	2	5.5
Six months																					
1480	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CS16-1	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5486	3	0	0	0	0	0	0	0	0	0	0	1	33.3	0	0	0	0	0	0	1	11.1
2687	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total		0	0	0	0	0	0	0	0	0	0	1	8.3	0	0	0	0	0	0	1	2.7

Beauveria bassiana was not detected eight months post-inoculation.
a There were three replicates for each isolate by treatment combination: three different plants were used, and within each plant, leaves, stems and roots were assessed for *B. bassiana* presence.

Table 3 – Endophytic fungi isolated from coffee plants two and four months post-inoculation with *Beauveria bassiana*

Endophyte	GenBank no.	Two months post-inoculation									Four months post-inoculation								
		n	Total		Leaves		Stems		Roots		n	Total		Leaves		Stems		Roots	
			n	%	n	%	n	%	n	%		n	%	n	%	n	%	n	%
Alternaria sp.	DQ682562	632	1	0.2	1	0.2	0	0.0	0	0.0	0	0	0.0	0	0	0	0	0	0
Arthopyrenaceae	DQ682563	0	0	0.0	0	0.0	0	0.0	0	0.0	249	3	1.2	0	0.0	3	1.2	0	0.0
Aspergillus tamari	DQ682564	0	0	0.0	0	0.0	0	0.0	0	0.0	249	1	0.4	0	0.0	0	0.0	1	0.4
A. westerdijkiae	DQ682565	632	1	0.2	0	0.0	0	0.0	1	0.2	0	0	0.0	0	0	0	0	0	0
Beauveria bassiana	DQ682566	632	14	2.2	1	0.2	8	1.3	5	0.8	249	8	3.2	0	0	8	3.2	0	0
Bionectriaceae	DQ682567	632	1	0.2	1	0.2	0	0.0	0	0.0	249	8	3.2	0	0	7	2.8	1	0.4
Chaetomium sp.	DQ682568	632	2	0.3	2	0.3	0	0.0	0	0.0	0	0	0.0	0	0	0	0	0	0
Cladosporium cf. *sphaerospermum*	DQ682569	0	0	0.0	0	0.0	0	0.0	0	0.0	249	6	2.4	6	2.4	0	0.0	0	0.0
Cladosporium sp.	DQ682570	0	0	0.0	0	0.0	0	0.0	0	0.0	249	1	0.4	0	0.0	1	0.4	0	0.0
Claviciptaceae	DQ682571	0	0	0.0	0	0.0	0	0.0	0	0.0	249	6	2.4	1	0.4	0	0.0	5	2.0
Colletotrichum gloeosporoides complex	DQ682572	632	12	1.9	0	0.0	11	1.7	1	0.2	0	0	0.0	0	0	0	0	0	0
Cylindrocarpon sp.	DQ682573	632	51	8.1	0	0.0	4	0.6	47	7.4	249	20	8.0	0	0	0	0	20	8
Exobasidiomycetidae	DQ682574	632	1	0.2	1	0.2	0	0.0	0	0.0	0	0	0.0	0	0	0	0	0	0
Exophiala sp.	DQ682575	632	2	0.3	0	0.0	0	0.0	2	0.3	0	0	0.0	0	0	0	0	0	0
Fusarium cf. *oxysporum* f. sp. *vasinfectum*	DQ682576	632	174	27.5	0	0.0	73	11.6	101	16.0	249	24	9.6	1	0.4	6	2.4	17	6.8
F. oxysporum complex (1)	DQ682577	632	5	0.8	0	0.0	5	0.8	0	0.0	249	10	4.0	0	0.0	2	0.8	8	3.2
F. oxysporum complex (2)	DQ682579	632	8	1.3	0	0.0	1	0.2	7	1.1	249	2	0.8	0	0	2	0.8	0	0
Fusarium sp. (1)	DQ682581	632	41	6.5	0	0.0	40	6.3	1	0.2	0	0	0.0	0	0	0	0	0	0
Fusarium sp. (2)	DQ682582	632	56	8.9	1	0.2	26	4.1	29	4.6	0	0	0.0	0	0	0	0	0	0
Fusarium sp. (Lateritium clade 1)	DQ682580	632	4	0.6	0	0.0	0	0.0	4	0.6	0	0	0.0	0	0	0	0	0	0
Hypocrea sp. (VI Lutea clade)	DQ682583	632	5	0.8	0	0.0	1	0.2	4	0.6	0	0	0.0	0	0	0	0	0	0
Hypocreales sp. (1)	DQ682584	632	5	0.8	5	0.8	0	0.0	0	0.0	0	0	0.0	0	0	0	0	0	0
Hypocreales sp. (2)	DQ682585	0	0	0.0	0	0.0	0	0.0	0	0.0	249	1	0.4	0	0.0	0	0.0	1	0.4
Macrophomina sp.	DQ682587	632	4	0.6	1	0.2	3	0.5	0	0.0	0	0	0.0	0	0	0	0	0	0
Paecilomyces sp.	DQ682588	632	4	0.6	0	0.0	1	0.2	3	0.5	249	4	1.6	0	0	4	1.6	0	0
Penicillium citrinum	DQ682589	632	194	30.7	8	1.3	156	24.7	30	4.7	249	80	32.1	0	0	62	24.9	18	7.2
P. brevicompactum	DQ682592	632	6	0.9	0	0.0	0	0.0	6	0.9	0	0	0.0	0	0	0	0	0	0
P. cecidicola	N/A	632	4	0.6	0	0.0	4	0.6	0	0.0	0	0	0.0	0	0	0	0	0	0
P. glabrum	DQ682590	632	1	0.2	0	0.0	0	0.0	1	0.2	249	1	0.4	0	0	0	0	1	0.4
P. janthinellum	DQ682591	0	0	0.0	0	0.0	0	0.0	0	0.0	249	8	3.2	0	0.0	0	0.0	8	3.2
Penicillium sp. near *daleae*	DQ682593	0	0	0.0	0	0.0	0	0.0	0	0.0	249	8	3.2	0	0	2	0.8	6	2.4
P. steckii	DQ682594	632	1	0.2	0	0.0	0	0.0	1	0.2	249	1	0.4	0	0	0	0	1	0.4
P. toxicarium	DQ682595	632	1	0.2	0	0.0	1	0.2	0	0.0	0	0	0.0	0	0	0	0	0	0
Phyllachoraceae	DQ682596	0	0	0.0	0	0.0	0	0.0	0	0.0	249	3	1.2	0	0.0	0	0.0	3	1.2
Plectosphaerella sp.	DQ682597	0	0	0.0	0	0.0	0	0.0	0	0.0	249	1	0.4	0	0.0	0	0.0	1	0.4
Pleosporales sp.	DQ682598	632	3	0.5	1	0.2	0	0.0	2	0.3	0	0	0.0	0	0	0	0	0	0
Pseudallescheria cf. *boydii*	DQ682599	632	3	0.5	0	0.0	0	0.0	3	0.5	0	0	0.0	0	0	0	0	0	0
Rhizopycnis sp.	DQ682600	0	0	0.0	0	0.0	0	0.0	0	0.0	249	3	1.2	0	0.0	0	0.0	3	1.2
Trichoderma sp.	DQ682604	632	1	0.2	0	0.0	1	0.2	0	0.0	249	30	6.4	0	0	1	1.6	29	4.8
T. hamatum	DQ682602	632	19	3.0	0	0.0	5	0.8	14	2.2	249	18	7.2	0	0.0	4	1.6	14	5.6
T. harzianum	DQ682603	0	0	0.0	0	0.0	0	0.0	0	0.0	249	2	0.8	0	0.0	0	0.0	2	0.8
Not identified		632	8	1.3	0	0.0	0	0.0	8	1.3	0	0	0.0	0	0.0	0	0.0	0	0.0

Percentages are based on 632 positive fungal isolations two months post-inoculation and 249 four months post-inoculation.

After soaking, seeds were germinated on a tray containing moistened sterile sand and covered with a black plastic bag to maintain humidity. The sand was watered every 2 d. After emergence of the cotyledon, each seedling was planted individually in a pot with a mixture of sterile potting media and sand. Evaluations were performed two, four, and six months post-planting, and each time two plants were sampled per isolate. Leaf, roots, and stem tissues were surface sterilized and plated as previously described.

Fungal and bacterial endophytes

The number of fungal and bacterial endophytes present in the tissues sampled was assessed at each evaluation period. Fungi other than *Beauveria bassiana* isolated from plants two and four months post-inoculation were sequenced (described below) and compared with records deposited in GenBank. All fungal isolates have been deposited at the Insect Biocontrol Laboratory (USDA, ARS) fungal collection. Bacteria were classified based on colony shape and colour as morpho-species.

Fungal endophyte DNA extraction

Fungal isolates were individually grown on potato dextrose broth (Becton Dickinson, Sparks, MD) at 25 °C and 150 rev min^{-1} in an Innova 4000 Incubator Shaker (New Brunswick Scientific, Edison, NJ). At 8 d, the fungal mass was transferred to 50 ml sterile tubes, centrifuged for 10 min at 10,000 rev min^{-1}, and the supernatant discarded. This was followed by washing with sterile, distilled water and centrifuging again for 10 min at 10,000 rev min^{-1}. The fungal mass was removed from the sterile tube in the laminar flow hood, dried on sterile filter paper, transferred to 1.5 ml sterile Eppendorf tubes, and lyophilized. Samples were stored at −80 °C.

For DNA extraction, approximately 50 mg lyophilized mycelium was placed in a 2 ml microcentrifuge tube with *ca* 0.2 ml 1.0 mm diam zirconia-glass beads (BIOSPEC, Bartlesville, OK). The mycelium was crushed with a plastic pestle and further ground in a FastPrep-120 sample grinder (Q-BIOgene, Irvine, CA) for 3 s at the maximum speed setting of 6. The powdered mycelium was suspended in 700 μl detergent solution (2 M NaCl, 0.4 % (w/v) deoxycholic acid–sodium salt, 1 % (w/v) polyoxyethylene 20 cetyl ether) and then agitated for 14 s in the Fast-Prep at maximum speed. Vials were incubated 5 min at 55 °C in a heat block and then centrifuged at 10,600 rev min^{-1} for 5 min followed by emulsion with 700 μl chloroform–isopropyl and centrifugation at 10,600 rev min^{-1} for 5 min. The aqueous phase was transferred to a clean tube to which an equal volume of 6 M guanidinium thiocyanate was added. Fifteen microlitres of silica powder was suspended in the solution by gently mixing for 5 min at room temperature, followed by 3 s centrifugation, after which the supernatant was discarded. The glass powder was rinsed twice by suspending in 750 μl ethanol buffer (10 mM Tris–HCl, pH 8, 0.1 mM EDTA, 50 % ethanol) with a disposable transfer pipette, then collected by centrifugation, and the supernatant discarded; the glass powder pellet was dried on a heat block at 55 °C for 5–10 min. The glass powder was rehydrated with 105 μl ultra-pure water and the genomic DNA eluted from the silica matrix by incubating on a heat block at 55 °C for 5–10 min. After vortexing and centrifugation, 100 μl of the aqueous supernatant was transferred to a clean tube.

Fungal endophyte DNA sequencing and analyses

The ITS region of the nu-rDNA repeat was sequenced for each isolate; primers ITS1-F (fungal-specific) (Gardes & Bruns 1993) and ITS4 (White *et al.* 1990) were used for both amplification and sequencing. PCRs were performed in 25 μl reaction volumes with 12.5 μl PCR Master Mix (Promega, Madison, WI), 1.25 μl each of 10 μM primers, and 10 μl of diluted (10- to 100-fold) DNA template. Amplification was achieved with an initial denaturation step of 5 min at 94 °C; 35 cycles of 30 s at 94 °C, 45 s at 50 °C, and 45 s at 72 °C; and a final extension of 7 min at 72 °C. The PCR products were cleaned with Montage PCR Centrifugal Filter Devices (Millipore, Billerica, MD) according to the manufacturer's protocol. Cleaned PCR products were sequenced with BigDye Terminator sequencing enzyme version 3.1 (Applied Biosystems, Foster City, CA) in the reaction: 2 μl of diluted BigDye in a 1:3 dilution of BigDye:dilution buffer (400 mM Tris pH 8, 10 mM MgCl$_2$); 0.3 μl of 10 μM primer; 10–20 ng cleaned PCR template; and water to a 5 μl total reaction volume. Cycle sequencing parameters consisted of a 2 min denaturation step at 94 °C, then 35 cycles of 94 °C for 39 s, 50 °C for 15 s, and 60 °C for 4 min. Sequencing reactions were cleaned by ethanol precipitation and sequenced on an ABI 3100 Genetic Analyser (Applied Biosystems). Sequencing reactions were edited and contiguous sequences for each isolate were assembled in Sequencher version 4.1.4 (Gene Codes, Ann Arbor, MI).

Initial identification of isolates was obtained by BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST/). Refinements on species identification were performed for various groups: *Colletotrichum* was aligned using a *Colleotrichum* sequence database at the USDA, ARS Systematic Botany and Mycology Laboratory (Beltsville, MD); *Trichoderma/Hypocrea* species were identified using TrichOKey (http://www.isth.info/tools/molkey/index.php?do=unset; Druzhinina *et al.* 2006). For any ITS sequence leading to *Fusarium*, an EF1-α sequence was obtained using primers developed by Rehner & Buckley (2005) followed by on-line sequence comparisons against the *Fusarium* Database (http://fusarium.cbio.psu.edu/). *Penicillium* and *Aspergillus* species were further identified using unpublished sequences from ex-type isolates (S.W. Peterson). Multilocus DNA sequence studies with genealogical concordance analysis (Taylor *et al.* 2000) identify ITS genotypes included in particular species, but such studies are published only for some species in these genera. For those species where multilocus studies are not available, exact ITS sequence matches to type sequences were considered reliable identification and differences of one to two bases were probable identifications. Larger differences were considered to be non-identifications. DNA sequences have been deposited in GenBank (Table 3).

Results

Conidial viability of the four *Beauveria bassiana* isolates was 97.4 ± 2.5 % for ARSEF 1480 (Brazil); 94.6 ± 4.0 for ARSEF 5486

(Ivory Coast); 92.2 ± 3.9 % for the endophytic isolate CS16-1 (Colombia); and 89.7 ± 2.5 % for ARSEF 2687 (Kenya). The water used to rinse the coffee tissues after surface sterilization did not yield any fungi.

Recovery of Beauveria bassiana *as an endophyte*

All fungal inoculation methods were effective in introducing *Beauveria bassiana* into the plant, although at different levels of efficiency (Table 1). For example, seven out of 12 injected plants (58.3 %) yielded positive detection at two months, in contrast to three out of 12 (25 %) for the drenched plants, and one out of 12 (8.3 %) for the sprayed plants. At four months post-inoculation, *B. bassiana* was detected in only two plants out of 12 (16.7 %) in the injected treatment, and at six months it was only detected in one plant out of 12 in the injected treatment (8.3 %). *B. bassiana* was not detected in any of the treatments eight months post-inoculation. *B. bassiana* was not isolated from any of the control plants.

When the data are combined for all inoculation methods, the total percent of plants that tested positive for *B. bassiana* was 30.6 % at two months, 5.5 % at four months, 2.7 % at six months, and 0 % at eight months (Table 1). At two months, isolate 1480 was detected in four plants out of nine (44.4 %), isolates CS16-1 and 5486 in two out of nine plants (22.2 %), and isolate 2687 in three out of nine plants (33.3 %). At four months and six months post-inoculation, only isolate 1480 was detected in two and one plant, respectively.

B. bassiana was isolated from leaves, stems, and roots (Table 1). In the first evaluation, *B. bassiana* was found in the stems of eight plants, in the root of two plants, and on the leaves of one plant. At four months, *B. bassiana* was only isolated from two stems, and at six months from just one root. *B. bassiana* was not isolated from any of the tissues sampled eight months post-inoculation.

ARSEF 2687 (Kenya) was the only isolate that colonized the plant through the leaves (Table 1), while with the CS16-1 from Colombia, 1480 from Brazil and 5486 from Ivory Coast colonized through drenching. All isolates colonized the plant *via* injection.

Despite the addition of two replicates per each tissue and the use of selective medium, it was not possible to recover *B. bassiana* eight months post-inoculation. Plants that were positive for *B. bassiana* inoculation did not present any physical symptoms of damage that might otherwise indicate that the fungus was deleterious to the plant.

Inoculation of coffee seeds

Beauveria bassiana recovery in seeds that had been soaked in conidial suspensions was positive only two months post-inoculation and for only one isolate. ARSEF 5486 (Ivory Coast) was recovered from the stem of one seedling. This result indicates that *B. bassiana* rarely established endophytic associations with the host using this inoculation method.

Fungal and bacterial endophytes

Throughout all the evaluations, various fungal and bacterial endophytes were recovered from the tissues sampled. The highest number of endophytic fungi was obtained two months post-inoculation, followed by eight months post-inoculation (Table 2). The lowest number was obtained six months post-inoculation. Fungi isolated two and four months post-inoculation were identified using morphological and molecular criteria and are presented in Table 3. At two months post-inoculation, 632 isolates were recovered as endophytes from which 35 species were identified, whereas at four months post-inoculation, 249 endophytic fungi were isolated and identified as belonging to 24 species. The two most frequently isolated fungal species two months post-inoculation were *Penicillium citrinum* and *Fusarium* cf. *oxysporum* f. sp. *vasinfectum*, whereas at four months post-inoculation the most abundant species recovered were *Penicilium citrinum*, *Trichoderma* sp., and *Fusarium* cf. *oxysporum* f. sp. *vasinfectum*. The number of endophytic fungi isolated in the first and second evaluation exhibited great variation; however, the species diversity using the Shannon index was very similar, at 2.14 and 2.42, respectively.

The lowest incidence of endophytic fungi over all treatments was obtained from the leaves (Table 3). At two months post-inoculation, the highest incidence of endophyte recovery was in the stems followed by the roots, whereas at four months post-inoculation the highest incidence was in the roots followed by the stems (Table 3). At two months post-inoculation, only three species, *B. bassiana*, *Fusarium* sp. (2), and *P. citrinum*, occurred in leaves, stems and roots, whereas at four months post-inoculation only one species, *F.* cf. *oxysporum* f. sp. *vasinfectum*, was recovered from all three tissues. Stems and leaves shared only one species (*Macrophomina* sp.) two months post-inoculation. Stems and roots shared ten species two months post-inoculation and six species four months post-inoculation (Table 3). Leaves and roots did not share any species in both evaluations.

Altogether, the most frequently isolated species was *P. citrinum*, found in all plant tissues in the first evaluation and in the stem and roots of the second evaluation. *B. bassiana* showed the same pattern as *P. citrinum*, whereas *F.* cf. *oxysporum* f. sp. *vasinfectum* was present in the stem and roots of the plants of the first evaluation and in all tissues of the second evaluation. *Trichoderma hamatum* and *Trichoderma* sp. were present only in the stem and roots in both evaluations.

Bacteria were identified only as morpho-species, except for *Chromobacterium* sp., which was identified based on morphological comparisons with a previously identified isolate (Vega *et al.* 2005). The highest number of bacteria ($n = 627$) was recovered four months post-inoculation, followed by two months post-inoculation ($n = 441$; Table 2). The lowest number ($n = 66$) was observed six months post-inoculation (Table 2). Seven bacterial morpho-species were found two months post-inoculation and six four months post-inoculation. *Chromobacterium* sp. was the second most abundant species (20.2 %) two months post-inoculation, and the third most abundant (19.5 %) four months post-inoculation.

Discussion

Beauveria bassiana has been established as an endophyte in various plants using different inoculation methods, e.g., in

potatoes after foliar spray applications (Jones 1994); in maize plants after stem injections (Bing & Lewis 1991, 1992a, 1992b) or after granular applications to the whorl (Lewis & Bing 1991; Bing & Lewis 1991, 1992a); in maize leaves after applying a 20 µl conidia suspension to the abaxial side of leaves (Wagner & Lewis 2000); in tomatoes after coating seeds with a *B. bassiana* conidia (Leckie 2002; Ownley *et al.* 2004); in opium poppies after spraying leaves or coating seeds with *B. bassiana* spore suspensions (Quesada-Moraga *et al.* 2006), and in cocoa (Posada & Vega 2005) and coffee seedlings (Posada & Vega 2006b) after placing spore suspensions on the radicle. Another mode of entry for *B. bassiana* could be through wounds caused by the coffee berry borer. This might explain our finding of *B. bassiana* as an endophyte in Colombia (Vega *et al.*, unpubl.) in plots where there was a high level of coffee berry borer infestation as well as a high *B. bassiana* infection rate in the insects.

In contrast to the successful inoculation of leaves mentioned above, in the present study, only one of four *B. bassiana* isolates was recovered from sprayed coffee leaves, thus suggesting that the leaf is a poor route of entry for the fungus to colonize the coffee plant. The low recovery of *B. bassiana* from leaves could be due to specific cuticular components on the leaf and the lack of stomata on the adaxial side. It is possible that the main components on the leaf cuticle, waxes and cutin (Martin 1964), might have a detrimental effect on *B. bassiana* conidium germination. Over 70 % of the total coffee leaf waxes are comprised of free primary alcohols, free acids, and alkanes (Stocker & Wanner 1975), while more than 60 % of the cutin is composed of long-chain fatty acids, such as dihydroxy-hexadecanoic acids (Holloway *et al.* 1972). Both the wax and cutin confer hydrophobic properties, thus making it harder for the fungal spore suspensions to adhere to the coffee leaves, which also lack hairs that could help retain the sprayed spore suspension. Cuticular waxes extracted from coffee leaves and berries have been shown to have a toxic effect on spores of *Colletotrichum coffeanum*, the causal agent of coffee berry disease (Steiner 1972; Lampard & Carter 1973; Vargas 1977). However, the effects of *Coffea* cuticular waxes on *B. bassiana* conidia has not been tested. The waxy coating could also prevent infection by fungi; Heather (1967) found increased infection by *Phaeoseptoria eucalypti* when the waxy leaf coating in *Eucalyptus bicostata* leaves was removed.

The low recovery of *B. bassiana* from leaves might have also been influenced by the few natural openings in the adaxial side of the leaf, which is where the spray is deposited. Stomata in coffee are found in the abaxial side (Dedecca 1957) and are recognized in many plants as a route for infection for pathogenic bacteria and fungi (Agrios 2005), including the coffee leaf rust, *Hemileia vastatrix* (Silva *et al.* 2002; Coutinho *et al.* 1993). Domatia are also located in the abaxial side (Dedecca 1957; Wintgens 2004) and thus foliar sprays containing fungal suspensions are not likely to reach these natural cavities on the leaf. Another possibility for the lack of infection *via* the leaves could be the production of phytoalexins in response to fungal challenges (Rodrigues *et al.* 1975; Medeiros & Rodrigues 1977).

The highest recovery of *B. bassiana* was from the stems of plants injected with conidia suspensions. This method of inoculation circumvents the physical barrier of the plant cuticle. A similar method has been successfully used in grasses,

where fungal endophyte mycelium is inserted between the shoot and root sections (Johnson-Cicalese *et al.* 2000) or at the junction of the coleoptile and mesocotyl (Latch & Christensen 1985).

Santamaría & Bayman (2005) have reported *Xylaria*, *Colletotrichum*, *Guignardia*, *Botryosphaeria* and *Pestalotia* as coffee leaf endophytes in Puerto Rico. Of these, we only detected *Colletotrichum* in the seedlings used in our study. The reduction in *B. bassiana* recovery as post-inoculation time increased could be due in part to competition with other endophytes present in the coffee plant (Table 3). Even though the presence of these endophytic fungi might pose a constraint for the establishment of a specific fungal endophyte (e.g. *B. bassiana*), there are no published reports to support or to reject this hypothesis (but see Freeman & Rodriguez (1993), Redman *et al.* (1999) and Arnold *et al.* (2003)). In addition to the endophytes reported in this study, we have isolated and sequenced over 170 different endophytes in coffee plants collected from the field in Hawaii, Mexico, Puerto Rico, and Colombia (Vega *et al.*, unpubl.). A large number of fungal genera, including many of the endophytes we have found in coffee, are known to be involved in the production of metabolites active against fungi, bacteria, and insects (Vega *et al.*, unpubl., and references therein).

It is possible that the presence of *B. bassiana* in inoculated *Coffea* seedlings was underestimated, as it is slow growing on the media we used as compared with the rapidly growing species *Penicillium* and *Fusarium*, which were frequently isolated as endophytes in this study. These latter fungi rapidly covered the entire surface of isolation plates, thus making it more difficult to observe and to isolate outgrowths of other fungi. The use of *in planta* molecular detection techniques, which we are developing for future endophyte studies, should result in a more precise estimate of *B. bassiana* establishment as an endophyte.

One important finding is that *B. bassiana* was isolated from plants at locations distant from the point of inoculation. Thus, *B. bassiana* is capable of movement within the plant. In one plant inoculated by injection in the stem with ARSEF 2687 (Kenya), the fungus was subsequently recovered from the stem and roots of the plant. Similarly, in one plant inoculated with ARSEF 5486 (Ivory Coast) *via* stem injection, the fungus was recovered from the roots. *B. bassiana* was recovered from the stem of another plant inoculated by soil drenching with ARSEF 5486 (Ivory Coast). Our attempts to visualize fungal growth in coffee tissues using various dyes have not been successful. This might be due to the selection of tissues that did not harbour *B. bassiana*, considering the low establishment rates we recorded in the study. In contrast, visualization of endophytes in other plants is relatively easy (Clark *et al.* 1983; Saha *et al.* 1988; Hinton & Bacon 1985; Bacon & White 1994; Menéndez *et al.* 1997; Christensen *et al.* 2001; Barrow & Aaltonen 2004). Wagner & Lewis (2000) observed *B. bassiana* hyphal growth in the xylem elements using light and electron microscopy; conidia were never detected in their examinations.

The results indicate that the four *B. bassiana* isolates used in this study have different capabilities for infection and colonization of coffee plant tissues even though there were not clear tendencies between isolates in their ability to persist as endophytes. ARSEF 1480 (Brazil) could be detected in the

injected plants four months post-inoculation, in contrast to all the other isolates, none of which was detectable at four months. Strain suitability for endophytism is perhaps an aspect that needs to be taken into account when evaluating candidates for plant inoculation. The failure to establish endophytic infections may thus be due to innate characteristics of the isolate being used.

The low *B. bassiana* establishment in sprayed plants is unfortunate. Of all the inoculation methods tested, foliar sprays are the easiest to accomplish in the field. Even though stem injection resulted in highest post-inoculation recovery of *B. bassiana* of the three methods compared, stem inoculation might pose some difficulties in implementation unless it can be done with seedlings, although persistence of *B. bassiana* would be questionable due to other endophytes occurring in the plant. Future work will determine whether *B. bassiana* can be established in coffee berries after applying spore suspensions on flowers and in young coffee berries in the field.

Acknowledgements

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