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FULL PAPER

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Inoculation and colonization of coffee seedlings (*Coffea arabica* L.) with the fungal entomopathogen *Beauveria bassiana* (Ascomycota: Hypocreales)

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Abstract The fungal entomopathogen *Beauveria bassiana* became established as an endophyte in coffee seedlings grown in vitro and inoculated with *B. bassiana* suspensions in the radicle. The fungus was recovered as an endophyte 30 and 60 days postinoculation, from stems, leaves, and roots, and at 60 days postinoculation one of the isolates was also recovered as an epiphyte. *Fusarium* sp., *Rhodotorula* sp., and four bacterial morpho-species were also detected, indicating these were present as endophytes in the seed.

Key words *Beauveria bassiana* · Biological control · *Coffea* · Coffee · Endophyte

Introduction

The most important insect pest attacking coffee (*Coffea arabica* L. and *Coffea canephora* Pierre ex. Froehner) throughout the world is the coffee berry borer *Hypothenemus hampei* Ferrari (Coleoptera: Curculionidae) (Le Pelley 1968). Female coffee berry borers tunnel into the coffee berry and lay 60–100 eggs; upon hatching, the larvae feed on the endosperm, thus greatly reducing yields and quality, and frequently causing premature abscission of the berry (Cárdenas and Posada 2001). Larvae pupate inside the berry, where sibling mating occurs; thus, once females emerge from the berry they are already inseminated (Cárdenas and Posada 2001). The life cycle lasts on average 27 days, and up to 150 adults have been recorded in one berry (Muñoz 1989; Bustillo et al. 1998).

The small size of the insect and the development of all insect stages inside the berry make control extremely difficult. Pest management strategies against the coffee berry borer have focused mainly on the use of chemical insecticides, an expensive strategy with adverse environmental and human health effects. In addition, the insect has developed resistance to endosulfan, one of the most commonly used insecticides (Brun et al. 1989; Villalba et al. 1995). Thus, there is a need for effective pest management methods that can be used in an integrated pest management program against the coffee berry borer (Villalba et al. 1995; Bustillo et al. 1998; Posada et al. 2004). Cultural control practices are very effective (Bustillo et al. 1998; Cárdenas and Posada 2001) and could be complemented with the use of traps to capture and monitor populations (González and Dufour 2000; Cárdenas 2000). Entomopathogenic fungi have been widely studied and methods for their production and application in coffee agroecosystems have been developed (Antia et al. 1992; Posada 1998; Haraprasad et al. 2001; Posada et al. 2004); the same applies to coffee berry borer parasitoids (Quintero et al. 1998; Portilla 1999; Aristizábal et al. 2004).

One novel pest management mechanism against the coffee berry borer involves the inoculation of coffee plants with the fungal entomopathogen *Beauveria bassiana* (Bals. -Criv.) Vuill. (Ascomycota: Hypocreales) in an attempt to determine if the fungus can become established as a fungal endophyte (Arnold and Lewis 2005). The ultimate goal is to determine whether coffee plants inoculated with *B. bassiana* in the seedling stage can sustain the fungus in the field until the production of the coffee berries, and more importantly, whether *B. bassiana* can be detected in the berries, where, ideally, it would help control the insect.

Beauveria bassiana has been reported as an endophyte in maize (Bing and Lewis 1991; Arnold and Lewis 2005), potatoes, jimsonweed, cotton, and cocklebur (Jones 1994), tomatoes (Leckie 2002), the bark of *Carpinus carolinata* Walter (Bills and Polishook 1991), *Theobroma cacao* (Posada and Vega 2005), and *Theobroma gileri* (Evans et al. 2003). Recently, we have isolated *B. bassiana* as an endophyte in coffee plants in Colombia, and preliminary results in our laboratory demonstrated that it is possible to inoculate coffee plants with *B. bassiana* using injections, sprays,

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and soil drenching (Posada and Vega, unpublished data). The objective of this study was to determine whether it would be possible to inoculate coffee seedlings with *B. bassiana*.

Materials and methods

Coffea arabica var. Colombia seeds were surface sterilized by dipping in 0.5% sodium hypochlorite for 2 min and 95% ethanol for 2 min, and then rinsed in sterile distilled water three times. The seeds were placed on moistened sterile filter paper placed inside 100 × 15 mm petri dishes. The dishes were sealed with parafilm and kept in a growth chamber at 25° ± 2.0°C with a 12:12 photoperiod.

When the seeds had germinated (about 22 days) and the roots were approximately 1.5–2.0 cm long, they were transferred to a laminar flow hood for inoculation with two *B. bassiana* isolates: (1) Ivory Coast 5486 (IC-5486), isolated from the coffee berry borer; and (2) an endophytic strain obtained from coffee berries collected in Colombia (CS16-1). Both isolates were retrieved from single-spore stocks stored in 10% glycerol and grown in yeast maltose agar (YMA; Sigma-Aldrich, St. Louis, MO, USA) to which a 0.1% stock antibiotic solution was added. The antibiotic stock consisted of 0.02 g of each of three antibiotics (tetracycline, streptomycin, and penicillin) dissolved in 10 ml sterile distilled water, followed by filter sterilization through a 0.2-µm filter (Nalgene Disposable Filterware; Nalge Nunc International, Rochester, NY, USA); from this, 1 ml was added to each liter of media. Spores were less than 30 days old when used. Before the inoculation, spore germination was determined in 2.5% noble agar (BD Difco Agar, Noble; Becton Dickinson, Franklin Lakes, NJ, USA) as follows: a 15-µl aliquot from a 10^{-3} dilution was plated on the noble agar surface, spread with a sterile glass rod, and subsequently incubated at 25° ± 2.0°C. After 24 h, three groups of 100 spores were counted and the germination was expressed as percentage.

To inoculate each seedling, 100 µl of a 1×10^7 spores ml^{-1} suspension of *B. bassiana* was placed on the main radicle using a pipette. The seeds were then placed inside a 100 × 15 mm sterile petri dish and allowed to dry in the laminar flow hood before transferring to sterile 25 × 250 mm test tubes (Kimble Glass, Vineland, NJ, USA) containing 20 ml sterile 10% water agar (Difco Bacto Agar; Becton Dickinson). Using sterile tweezers, the germinated seeds were inserted individually in each tube, ensuring that the radicle was inserted in the agar. Tubes were capped with sterile sponges, overlaid with sterile aluminum foil, and kept in the laboratory at about 25° ± 2.0°C and approximately a 12:12 photoperiod.

Evaluations to assess *B. bassiana* colonization of coffee tissues were conducted 30 and 60 days after inoculating the radicle. Twelve plants were used for each evaluation: four plants for each *B. bassiana* isolate and four for the control, whose radicles were inoculated with 100 µl sterile water. At all sampling times, the control was processed first, followed by CS16-1 and then IC-5486. Tissues (roots, stems, leaves) were disinfected in 0.5% sodium hypochlorite for 2 min and 70% ethanol for 2 min, and then rinsed with sterile distilled water (Arnold et al. 2001). The tissues were dried on sterile towel paper and, after cutting off the edges to remove the dead tissue originating from the disinfection process, they were cut into 4- to 9-mm^2 pieces, placing six pieces of tissue in each of two petri dishes containing YMA plus three antibiotics (as previously described). Tissues were examined 4 days later, and any fungal growth was isolated into individual plates containing YMA plus antibiotics for subsequent identification.

To evaluate the quality of the surface sterilization method, 10 ml of the water used to rinse the tissues after surface sterilization were taken, and 15-µl aliquots of a 10^{-2} dilution were plated on YMA plus three antibiotics (as previously described) and spread using a sterile glass rod. For each suspension, two petri dishes were plated, and after incubation for 4 days at 25° ± 2.0°C, colonies were counted and expressed as colony-forming units (CFU) per milliliter (ml).

To assess for the presence of epiphytes at each evaluation period, leaves, stems and roots were cut from the seedling and placed inside a 50-ml sterile vial containing 20 ml sterile water plus 0.1% Triton X-100 (Sigma Chemical, St. Louis, MO, USA). These suspensions were diluted to 10^{-4} aliquots, and 15 µl was plated on YMA plus three antibiotics (as previously described) and spread with a sterile glass rod. For each suspension corresponding to one plant, two replicates were prepared and kept in the laboratory at about 25° ± 2.0°C and approximately a 12:12 photoperiod. Four days after inoculation, the colonies were counted and expressed as CFU/ml.

The experiment was organized as a completely randomized design with a factorial arrangement. The factors were the isolates (CS16-1, IC-5486, and the control), two times of evaluation (30 and 60 days postinoculation), and three types of tissues sampled (leaves, stems, and roots). Thus, at each evaluation time, 432 subsamples of tissues were plated, based on the combination of two isolates and one control by four plants per treatment by three tissues (leaves, stems, and roots) by two replicates for each tissue and 6 subsamples plated on each petri dish. The data obtained from the evaluation were expressed as colonization frequency using the formula of Fisher and Petrini (1987) [colonization frequency = 100 × (segments colonized by a single endophyte/total number of segments)]. The data were square root transformed and analyzed using analysis of variance (ANOVA) (SAS Institute 1998).

Results

Spore germination for the two *B. bassiana* isolates used was >85%. Neither *B. bassiana* nor any other microorganism was ever observed growing on the water agar medium used to grow the seedlings. The evaluation of the water used to rinse tissues after sterilization yielded no microorganisms in

Table 1. Colony-forming units (CFUs) ml^{-1} of *Beauveria bassiana*, *Fusarium* sp., *Rhodotorula* sp., and three bacterial morpho-species isolated as epiphytes from coffee seedlings inoculated with *B. bassiana*

	n	Treatment					
		Bb CS16-1		Bb IC-5486		Control	
		Days postinoculation		Days postinoculation		Days postinoculation	
		30	60	30	60	30	60
B. bassiana	8	0	0	0	2.5×10^4	0	0
Fusarium sp.	8	4.0×10^5	1.7×10^6	8.3×10^5	7.0×10^5	1.1×10^6	1.8×10^6
Rhodotorula sp.	8	0	0	0	2.0×10^5	0	0
Bacterial morpho-species 1	8	0	1.3×10^5	1.8×10^5	4.3×10^5	5.0×10^4	5.3×10^5
Bacterial morpho-species 2	8	2.5×10^4	0	0	0	0	0
Bacterial morpho-species 3	8	2.5×10^4	7.5×10^4	0	0	0	0

Data presented are for 30 and 60 days postinoculation of the radicles with *B. bassiana* CS16-1 or *B. bassiana* IC-5486

both evaluations; thus, any ensuing fungal growth had to originate from internal tissues, i.e., as endophytes.

The water used to wash tissues to determine if epiphytes were present revealed the presence of *B. bassiana*, *Fusarium* sp., *Rhodotorula* sp., and three bacterial morpho-species (Table 1). *Beauveria bassiana* was not recovered as an epiphyte from the controls nor from plants treated with isolate CS16-1 (Table 1). *Fusarium* was present in all treatments, and the highest level was found on plants that were not inoculated with *B. bassiana* (Table 1). *Rhodotorula* sp. was found as an epiphyte 60 days postinoculation only on plants treated with *B. bassiana* isolate IC-5486. Bacterial morpho-species 1 was present 30 and 60 days postinoculation on plants treated with *B. bassiana* isolate IC-5486 and in the control and at 60 days postinoculation in plants treated with *B. bassiana* isolate CS16-1 (Table 1). Bacterial morpho-species 2 and 3 were only found on plants treated with *B. bassiana* isolate CS16-1.

The presence of *B. bassiana* as an epiphyte demonstrates that it was able to move through the coffee tissues, entering through the radicle and emerging through the cotyledons, stems, leaves, and roots (Fig. 1A–D). Further confirmation of this finding was supported by observations on four spare plants from the original treatments that were kept in the laboratory for 1 year. *B. bassiana* was observed on leaves, stem, and roots, and plating revealed that the fungus was viable (Fig. 1E,F).

From the 432 subsamples plated 30 days postinoculation, 266 (61.6%) yielded endophytic fungi, bacteria, or yeast in contrast to 210 (48.6%) 60 days postinoculation. In addition to *B. bassiana*, a *Fusarium* species was recovered; this fungus appears to be a coffee endophyte possibly transmitted through the seed. Four unidentified bacteria were found 30 days postinoculation and two at 60 days postinoculation.

At 30 days postinoculation, *B. bassiana* was isolated from 25% of the seedlings ($n = 12$) for both treatments (CS16-1 and IC-5486). At 60 days postinoculation, *B. bassiana* was isolated from 25% of the seedlings inoculated with CS16-1 and 50% of the seedlings inoculated with IC-5486. *Beauveria bassiana* was never isolated from the controls.

Analysis of the colonization frequency for all pooled tissues by treatment and evaluation period shows that *B. bassiana* was present in all the evaluations. At 30 days postinoculation, isolate CS16-1 was recovered from 0.69% ± 0.69% (mean ± standard error) of the tissues sampled and isolate IC-5486 from 1.38% ± 1.38%. At 60 days postinoculation, recovery for both isolates increased to 1.38% ± 0.96% for CS16-1 and 4.86% ± 2.92% for IC-5486. The statistical analysis revealed no significant differences for the interaction between isolates and evaluation ($df = 2, 143$, $F = 0.76$, $P < 0.4676$).

Analyzing the combination of factors isolates by tissues showed that *B. bassiana* was present in stems, leaves, and roots. CS16-1 was isolated from roots (2.08% ± 1.42%) and stems (1.04% ± 1.04%) whereas IC-5486 was isolated from leaves (3.12% ± 2.27%) and roots (6.25% ± 4.27%). The statistical analysis revealed no significant differences for the interaction between isolates and evaluation ($df = 4, 143$, $F = 1.12$, $P < 0.3512$).

Analysis of the combination of factors isolates by evaluation by tissues showed that CS16-1 was present at 30 days postinoculation only in the roots (2.08% ± 2.08%) whereas IC-5486 was present only on the leaves (4.16% ± 4.16%) (Table 1). At 60 days postinoculation, CS16-1 was isolated from roots (2.08% ± 2.08%) and from stems (2.08% ± 2.08%) whereas IC-5486 was isolated from leaves (2.08% ± 2.08%) and roots (12.50% ± 8.18%) (Table 2). The statistical analysis revealed no significant differences for the interaction between isolates, evaluation and tissues ($df = 4, 143$, $F = 2.00$, $P < 0.0985$).

For *Fusarium*, the analysis of the combination of factors isolates by evaluation by tissues showed that there were no significant differences ($df = 17, 126$, $F = 4.4$, $P > 0.05$). *Fusarium* was present in all the tissues and showed a tendency to reach higher values on the control than on the tissues that were colonized by *B. bassiana* (see Table 2). It also showed a tendency to decrease from 30 to 60 days postinoculation in all the tissues of the plants treated with IC-5486, whereas in the plants treated with CS16-1 only the leaves presented a slight decrease at 60 days postinoculation and the stems and roots showed an increase (Table 2). In the controls, the presence of *Fusarium* showed a high variation, and it decreased sharply in leaves and stems 60 days postinoculation, while roots had the higher incidence at both 30 and 60 days postinoculation

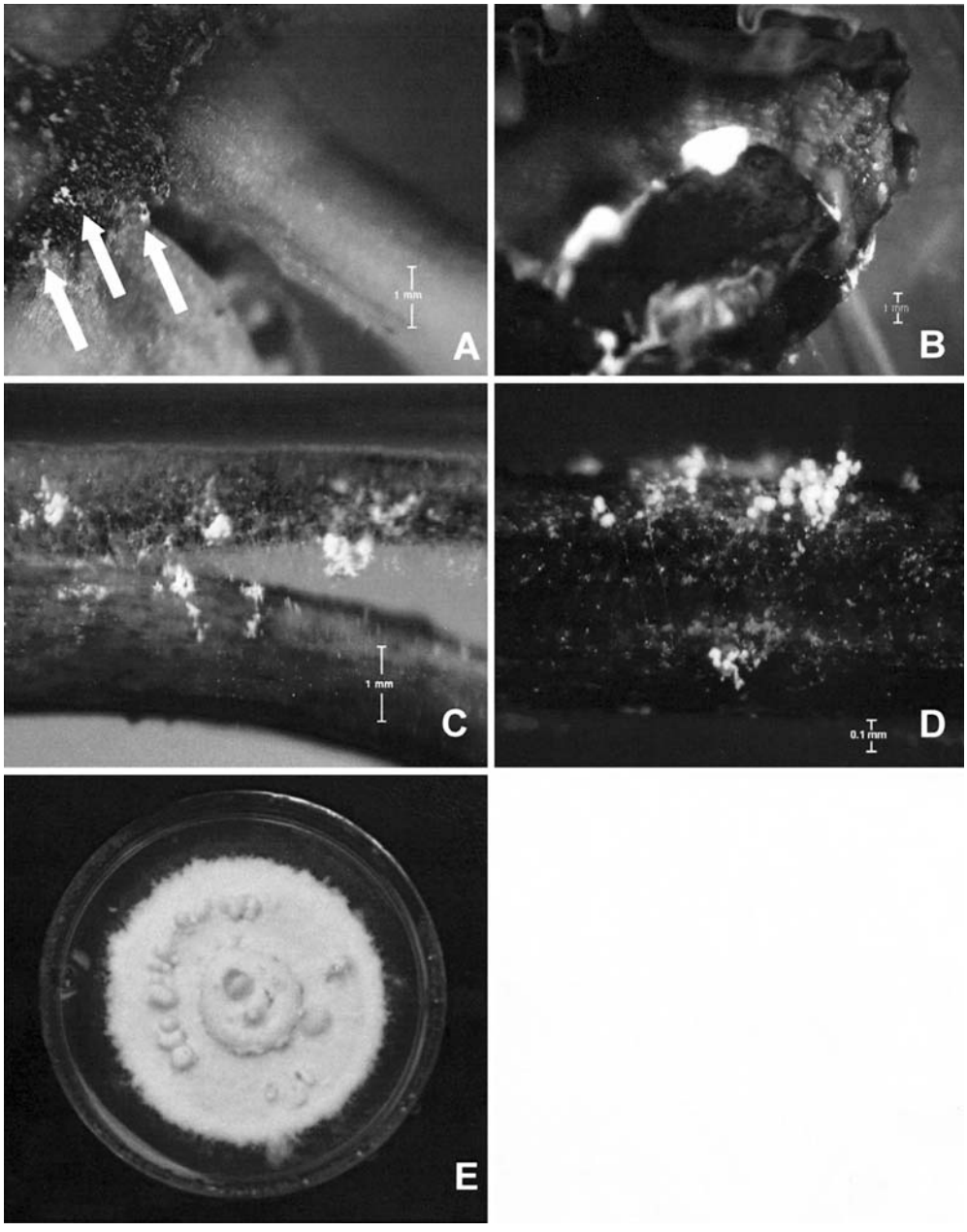

Fig. 1. *Beauveria bassiana* growing as an epiphyte on leaves (**A**; *arrows* indicate areas with *B. bassiana* growth), cotyledons (**B**), stems (**C**), and roots (**D**) of coffee seedling after inoculation in the radicles. **E** *B. bassiana* culture (in 60 × 15 mm petri dish) from material isolated from aerial plant parts

(Table 2). The highest incidence for *Fusarium* was 79.2% ± 11.3% in root tissues inoculated with CS16-1 at 60 days postinoculation followed by root tissues in the control (75.0% ± 9.5%) also 60 days postinoculation (see Table 2).

Based on visual characteristics, four types of endophytic bacterial morpho-species were isolated (four at 30 days postinoculation and two at 60 days postinoculation). Bacterial morpho-species 1 and 2 were recovered in both evaluations. Bacterial morpho-species 1 showed a higher incidence on the plants inoculated with *B. bassiana* when compared to the control (Table 2). It was present in all the tissues inoculated with CS16-1 and IC-5486, and for plants inoculated with CS16-1 the highest values were found on the roots, followed by the stems and the leaves (see Table 2). The statistical analysis of the combination of factors isolates by evaluation by tissues showed that there were significant differences (df = 4, 143, F = 3.27, P > 0.0138). Bacterial morpho-species 2 showed a similar incidence on the coffee tissues as bacterial morpho-species 1, but the percentages were higher (see Table 2). The statistical analysis of the combination of factors isolates by evaluation and tissues showed that there were significant differences (df = 4, 143, F = 3.89, P > 0.0051). The bacteria were present in all tissues, and both *B. bassiana* and *Fusarium* sp. were isolated growing together with bacteria.

Table 2. Percent colonization (±SE) for *B. bassiana*, *Fusarium* sp., and bacterial morpho-species 1 and 2 in leaves, stems, and roots of coffee seedlings inoculated with *B. bassiana* CS16-1 or *B. bassiana* IC-5486, 30 and 60 days postinoculation of the radicles

Microbes detected	Tissue	Treatment					
		Bb CS16-1		Bb IC-5486		Control	
		Colonization (%)		Colonization (%)		Colonization (%)	
		30 days[a]	60 days	30 days	60 days	30 days	60 days
B. bassiana	Leaves	0	0	4.2 ± 4.2	2.1 ± 2.1	0	0
	Stems	0	2.1 ± 2.1	0	0	0	0
	Roots	2.1 ± 2.1	2.1 ± 2.1	0	12.5 ± 8.2	0	0
Fusarium sp.	Leaves	43.8 ± 11.3	41.7 ± 14.4	27.1 ± 7.0	25.0 ± 7.7	50.0 ± 11.8	8.3 ± 5.5
	Stems	2.1 ± 2.1	18.8 ± 12.4	12.5 ± 10.3	2.1 ± 2.1	54.2 ± 17.5	16.8 ± 6.3
	Roots	52.1 ± 11.1	79.2 ± 11.3	68.8 ± 8.6	56.3 ± 17.2	70.8 ± 5.2	75.0 ± 9.5
Bacterial morpho-species 1	Leaves	4.2 ± 4.2	0	2.1 ± 2.1	2.1 ± 2.1	0	4.2 ± 2.7
	Stems	6.3 ± 3.1	0	2.1 ± 2.1	4.2 ± 4.2	0	0
	Roots	0	37.5 ± 18.3	4.2 ± 4.2	0	0	0
Bacterial morpho-species 2	Leaves	8.3 ± 5.5	10.4 ± 7.0	0	2.1 ± 2.1	0	0
	Stems	4.2 ± 4.2	12.5 ± 6.1	0	20.8 ± 10.8	0	8.3 ± 3.2
	Roots	0	43.8 ± 17.5	8.3 ± 5.5	6.3 ± 4.4	0	0

Means are based on eight replicates
[a] Days postinoculation

Discussion

The recovery of *B. bassiana* from coffee tissues indicates that this fungal entomopathogen can become established as a coffee endophyte after radicles of seedlings are inoculated with a *B. bassiana* spore suspension. Our results also show that *B. bassiana* can move throughout internal plant tissues based on recovery from stems, leaves, and roots.

Our *B. bassiana* recovery results differ from those obtained with inoculated cocoa seedlings (Posada and Vega 2005). At 60 days postinoculation, *B. bassiana* was isolated from 25% of the coffee seedlings inoculated with CS16-1 and 50% of the seedlings inoculated with IC-5486. In contrast, at 60 days postinoculation with the same *B. bassiana* isolates, more than 80% of cacao seedlings were positive for *B. bassiana* (Posada and Vega 2005). Observations on a 1-year-old cohort of coffee seedlings from the same experiment reported in this paper, indicating *B. bassiana* was present in all the plants, suggests that sampling times longer than 30 or 60 days might be necessary to detect *B. bassiana*.

The detection of *Fusarium* sp. and four bacterial morpho-species indicates that they must be coffee endophytes. Fungal and bacterial endophytes in the seeds have been reported in many different plants (Vega et al. 2005). The data indicate that there was a high incidence of *Fusarium* sp. in the control and that bacteria were also present. It was interesting to observe that plants challenged with *B. bassiana* had a lower overall incidence of *Fusarium* sp. whereas the opposite was found with bacterial morpho-species 1 and 2, which were more abundant in the seedlings inoculated with *B. bassiana*. The presence of *B. bassiana* in planta might be somewhat antagonistic to *Fusarium* sp. whereas the bacterial morpho-species seem to benefit from the presence of *B. bassiana*. In contrast, when both fungi were developing from tissues in vitro, *Fusarium* sp. overgrew *B. bassiana* and dominated the plate. In the 1-year-old cohort of coffee seedlings from the same group that *B. bassiana* colonized completely, it was observed that once they had died, *Fusarium* sp. appeared and covered all the tissues and *B. bassiana* was barely discernible.

The lack of any detrimental symptoms on the coffee seedlings indicates that they are not adversely affected by serving as a niche for *B. bassiana*. The finding that *B. bassiana* becomes an epiphyte points to the possibility that the fungus might sporulate from the plant surface once it has been translocated through internal tissues, thus serving as a constant source of fungal inoculum in the coffee agroecosystem.

Our results show that *B. bassiana* can become established in coffee seedlings as an endophyte. Future studies will use molecular techniques to detect *B. bassiana* in planta to reduce time, labor, and supplies required with the current method, which involved sampling 432 subsamples at each evaluation period. Development of an in planta molecular detection technique for *B. bassiana* will also reduce problems faced when classical isolation in petri dishes is used, which results in the isolation of other endophytic fungi that might underestimate the presence of *B. bassiana*, as is the case when *Fusarium* sp., a faster grower than *B. bassiana*, takes over the entire plate. Further studies are needed to determine whether *B. bassiana* will reach the berries, and if so, whether it will kill the coffee berry borer.

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