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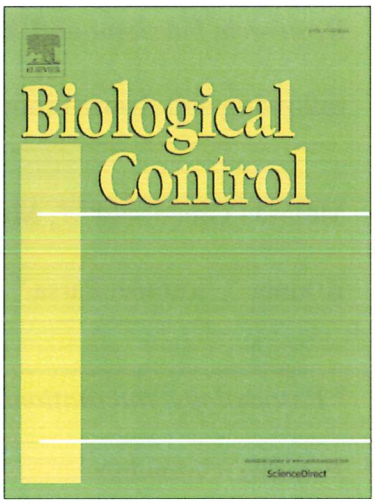

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Beauveria bassiana and *Metarhizium anisopliae* endophytically colonize cassava roots following soil drench inoculation

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Abstract

We investigated the fungal entomopathogens *Beauveria bassiana* and *Metarhizium anisopliae* to determine if endophytic colonization could be achieved in cassava. An inoculation method based on drenching the soil around cassava stem cuttings using conidial suspensions resulted in endophytic colonization of cassava roots by both entomopathogens, though neither was found in the leaves or stems of the treated cassava plants. Both fungal entomopathogens were detected more often in the proximal end of the root than in the distal end. Colonization levels of *B. bassiana* were higher when plants were sampled at 7-9 days post-inoculation (84%) compared to 47-49 days post-inoculation (40%). In contrast, the colonization levels of *M. anisopliae* remained constant from 7-9 days post-inoculation (80%) to 47-49 days post-inoculation (80%), which suggests *M. anisopliae* is better able to persist in the soil, or as an endophyte in cassava roots over time. Differences in colonization success and plant growth were found among the fungal entomopathogen treatments.

Keywords:

Beauveria, Cassava, Endophyte, Fungal entomopathogen, *Metarhizium*

1. Introduction

Cassava (*Manihot esculenta* Crantz; Malpighiales: Euphorbiaceae) is the third most important food crop in the tropics after rice and maize, and is a staple food for at least 700 million people in Africa, Latin America, Asia and the Pacific Islands (Fauquet and Tohme, 2004). Cassava tolerates nutrient-poor soils and drought and is an important crop for food security and generation of income in developing countries, where it is grown mostly by smallholder farmers (Bellotti et al., 2012; Cock, 2011). The main agricultural product is the edible root, which is high in carbohydrates, but the leaves may also be eaten as a source of protein (Gleadow et al., 2009). Various parts of cassava are also used to feed livestock, and in some countries cassava starch is an important product for the paper, textiles and food processing industries (Nassar and Ortiz, 2007). Cassava is attacked by at least 200 species of arthropod pests known to cause root yield losses, including whiteflies, mealybugs, mites, hornworm, thrips and burrower bugs (Bellotti et al., 2012).

Integrated pest management (IPM) of arthropod pests in cassava includes cultural practices, host plant resistance and biological control (Bellotti et al., 2005, 2012). Cultural practices include varietal mixtures, intercropping, and the treatment of stem cuttings to ensure pest-free planting material (Bellotti et al., 2012). Host plant resistance offers an economic and environmentally sound approach but many traditional farmers use varietal mixtures in cultural practice, and it can be difficult to implement host plant resistance across multiple cassava varieties (Bellotti et al., 2012; Bellotti and Arias, 2001). Chemical pesticides can be effective for controlling many arthropod pests but are prohibitively expensive for traditional smallholder farmers, can have adverse effects on human health and the environment, and are often incompatible with IPM schemes because they can disrupt control by natural enemies (Bellotti et

al., 2005; Holguín and Bellotti, 2004). Biological control using natural enemies of arthropod pests, such as predators, parasitoids and fungal entomopathogens provides an important component in IPM schemes for cassava because of its compatibility with other strategies (Bellotti et al., 2012).

Fungal entomopathogens, including *Beauveria bassiana s. l.* (Balsamo-Crivelli) Vuillemin and *Metarhizium anisopliae* (Metchnikoff) Sorokin (Ascomycota: Hypocreales), have been tested as biological control agents in laboratory and greenhouse trials against many cassava pests (Alean et al., 2004; Amnuaykanjanasin et al., 2013; Barreto et al., 2004; Jaramillo and Borgemeister, 2006; Jaramillo et al., 2005). However, when fungi are sprayed onto plants, pests can be difficult to target because of their location on the underside of leaves, such as the whitefly *Aleurotrachelus socialis* Bondar (Hemiptera: Alelyrodidae) or because they are subterranean, such as the burrower bug *Cyrtomenus bergi* Froeschner (Hemiptera: Cydnidae). The efficacy of fungal entomopathogens is also limited by abiotic factors (e.g., UV radiation, temperature, low humidity) that reduce viability of fungal conidia (Vega et al., 2012). An alternative application method is to inoculate plants with fungal entomopathogens that become established as endophytes, thereby possibly providing the plant with protection against pests from within, lowering the volume of inoculum required, and protecting the fungus against abiotic factors.

Fungal endophytes are commonly defined as fungi that live for all or part of their life cycle asymptomatically inside healthy plant tissues without causing disease (Petrini, 1991; Wilson, 1995; Hyde and Soytong, 2008). Colonization by fungal endophytes may be systemic (Gurulingappa et al., 2010; Quesada-Moraga et al., 2006), localized in plant parts (Wearn et al., 2012; Yan et al., 2015) or partitioned within plant parts (Behie et al., 2015; Zambell and White, 2014). Fungal endophytes fulfill a variety of roles comprising symbiotic and ecological functions

(Rodriguez et al., 2009) that may benefit plants including improved plant growth, protection against plant pathogens (Ownley et al., 2008) and reduction of herbivory (Akello and Sikora, 2012). *Beauveria bassiana* has been found naturally as an endophyte in several plant species and has been artificially introduced into many others (Vega, 2008). Artificial introduction of *B. bassiana* as an endophyte has been successful in maize (Bing and Lewis, 1991), cacao (Posada and Vega, 2005), date palm (Gómez-Vidal et al., 2006), coffee (Posada and Vega, 2006), banana (Akello et al., 2008), radiata pine (Brownbridge et al., 2012), fava beans (Akello and Sikora, 2012), opium poppy (Quesada-Moraga et al., 2014), cotton (Gurulingappa et al., 2010; Lopez and Sword, 2015; Ownley et al., 2008), the common bean (Parsa et al., 2013), and tomato (Ownley et al., 2008). *Metarhizium* species are less well known as endophytes but have been successfully introduced into tomato (Garcia et al., 2011), fava bean (Akello and Sikora, 2012), oilseed rape (Batta, 2013), and haricot bean (Behie et al., 2015; Sasan and Bidochka, 2012). Two unidentified *Metarhizium* species and *Metarhizium anisopliae* have been found naturally as endophytes in roots of wall barley (*Hordeum murinum* L.) (Murphy et al., 2015).

As part of a study investigating the potential use of *B. bassiana* and *M. anisopliae* to control whiteflies (*A. socialis*) in cassava, we conducted greenhouse experiments to determine (1) if *B. bassiana* and *M. anisopliae* can be established as systemic or localized endophytes in cassava after soil inoculation, and (2) if inoculation with these entomopathogens affects plant growth.

2. Material and methods

2.1 Cassava plants

Cassava plants (CMC-40 variety) grown at the International Center for Tropical Agriculture (CIAT, Cali, Colombia) were used as a source of stem cuttings for all experiments. Stems of approximately 1 m length and 25 mm diameter were harvested from 9-10 month-old cassava plants the day before inoculations. On the day of inoculation, the 1 m stems were cut into smaller "cuttings" of approximately 200 mm in length, each with 7-9 buds. The cuttings were planted in steam-sterilized loam soil (approximately 20% clay, 50% sand and 30% silt) in disinfected pots (height 140 mm, lower diameter 105 mm, upper diameter 148 mm with approximately 1.5 kg of soil per pot) and maintained in a greenhouse with daily average (mean ± SEM) temperature of 27.5°C ± 0.1°C and relative humidity of 66% ± 0.3%. Each plant was fertilized with 50 mL of NPK 15:15:15 (4g/L) 13 days after the cuttings were planted in the pots. The plants were watered as needed during experiments.

2.2 Fungal inoculum

The fungal inoculum was prepared following protocols modified from Parsa et al. (2013). Ten isolates/strains were used in the experiments, including five *B. bassiana* and five *M. anisopliae*. For *B. bassiana*, two isolates (CIAT 359 and CIAT 405) were obtained from the fungal entomopathogens collection at CIAT and three strains were obtained from commercially available products in Colombia, known as Beauveriplant® WP (Sanoplant, Palmira, Colombia), Bovetrópico® WP (Soluciones Microbianas del Trópico Ltda., Chinchiná, Colombia) and Micosis® WP (Bio-Protección, Chinchiná, Colombia). For *M. anisopliae*, three isolates (CIAT 001, CIAT 014A and CIAT 053) were obtained from the CIAT collection and two strains were obtained from commercially available products known as Metarhiplant® WP (Sanoplant, Palmira, Colombia) and BioMa® (Bio-Protección, Chinchiná, Colombia). Cultures of *B. bassiana*

and *M. anisopliae* were grown on 75% potato dextrose agar (PDA) and oatmeal agar (Difco™, Becton, Dickinson and Company, Sparks, MD) respectively, in 100 x 15 mm Petri dishes and incubated at 25±2°C with a photoperiod of 12:12 hours. The cultures were allowed to grow for 14-18 days, after which conidia were harvested by scraping the surface of the agar with a sterile spatula, and rinsing the surface of the agar with sterile distilled water containing 0.1% Triton X-100. The suspensions were then filtered to remove mycelium and agar debris. Conidial concentrations were determined using an improved Neubauer haemocytometer and the suspensions were adjusted to 1 x 10^8 conidia mL^{-1} in sterile distilled water containing 0.1% Triton X-100 to make up the required volume of inoculum for each isolate. For all experiments, conidial viability of each isolate was evaluated by taking a 100 µL sample of each inoculum, spreading it on PDA, incubating, and assessing germination 24 hours later. The percentage germination of conidia was determined from 100 randomly selected conidia under a light microscope. Conidia were deemed to have germinated if hyphae were visible or the germ tube was at least twice the length of the conidia. The average of three replicate counts was calculated for each isolate.

2.3 Screening experiments

2.3.1 Inoculation

Two screening experiments were conducted in a greenhouse to evaluate the ability of the ten isolates/strains to endophytically colonize cassava plants; one with the five *B. bassiana* isolates/strains and one with the five *M. anisopliae* isolates/strains. Each treatment consisted of 12 cassava plants, which were grown and prepared for inoculation according to the methods outlined above at 2.1. Each cassava plant root area was drenched with 100 mL of inoculum,

applied to the soil surface around the base of the plant 14-15 days after the cassava cuttings were planted in the pots. At this time, the buds on the cutting of the cassava plants had already produced roots and shoots. Control plant pots were inoculated with 100 mL of sterile distilled water containing 0.1% Triton X-100. The plants were arranged in the greenhouse in a randomized block design with 12 blocks, each block containing six plants (five treatment plants and one control). A root drench was chosen as the best inoculation method after concluding a pilot study comparing root drench to immersion of cuttings. The immersion method involved the immersion of 200 mm long cassava stem cuttings in fungal inoculum for up to 2 hours prior to planting the cuttings. However, this method resulted in only one root subsequently being colonized by *B. bassiana* and no roots colonized by *M. anisopliae* (unpubl. data).

2.3.2 Endophyte evaluation

Due to the large number of plant samples that needed to be surface-sterilized and plated onto Petri dishes, processing for the endophyte evaluation required three consecutive days; therefore, six of the 12 blocks were evaluated for endophytic colonization by the fungal entomopathogens 7-9 days post-inoculation and the remaining six blocks were evaluated 47-49 days post-inoculation. For the first evaluation, the two longest bud roots were removed from each plant and gently washed under running tap water for approximately 30 seconds to remove soil particles. From each of these roots, two 60 mm pieces were taken from both the proximal and distal ends of each root (i.e., there were four 60 mm pieces of root for each plant). The root pieces were pre-washed in 0.05% Triton X-100 for three minutes and then surface-sterilized by immersing in 0.5% NaOCl (diluted in 0.05% Triton X-100) for three minutes, ethanol (70%) for one minute and then rinsing three times in sterile distilled water for 15 seconds each rinse. The

bulk surface-sterilization system described by Greenfield et al. (2015) was used in all surface-sterilizations. To confirm that surface-sterilization was effective, eight root pieces were randomly selected from each block (with each block containing 24 root pieces in total) to make imprints on 75% PDA (in 100 mm x 15 mm Petri dishes) by gently pressing the root piece onto the surface of the agar (Schulz et al., 1998). Each root piece was then dissected into three 8 mm length sections (discarding the ends) and placed onto individual 60 mm x 15 mm Petri dishes containing PDA (75%) supplemented with antibiotics (0.1 g penicillin, 0.2 g streptomycin and 0.05 g tetracycline/L). All Petri dishes were incubated at 25±2°C in darkness and were inspected for 30 days for the presence of *B. bassiana* or *M. anisopliae*. Other fungal endophytes were also recorded and assigned morphotype codes. The proportion of root parts colonized was calculated for each plant as the number of root sections exhibiting fungal growth divided by the total number of root sections plated. The imprints were also incubated and monitored for at least 14 days for presence of fungi and if any fungi were found on an imprint, the corresponding block was discarded from the dataset.

At the 47-49 days post-inoculation evaluation, leaves and stems were evaluated as well as the roots for the presence of fungal endophytes. The roots were sampled in the same manner as described above. For the leaves, the second or third leaf (fully emerged) from the top (a young leaf) and the third leaf from the base of the plant (an old leaf) were removed from the plant. For each of these leaves, the longest lobule of the leaf was cut and trimmed to a length of 60 mm from where the leaf attaches to the petiole to the distal end of the leaf. A stem was removed from the plant and two pieces were cut from this stem; one from the top of the plant (a young stem) and one from the base of the plant where it arises from the cutting (an old stem). These stem pieces were trimmed to a length of 60 mm. Surface-sterilization proceeded as above, however

the timings for leaves and stems were different from the timings used for the roots. For both leaves and stems, sterilization timings were one minute in 0.5% NaOCl, 30 seconds in ethanol (70%) followed by three rinses in sterile distilled water (15 seconds each). Imprints of leaves and stems were made as described above for roots. Leaves were dissected and six square sections (8 mm^2) were cut from the lobule along the mid vein. Stems were dissected into approximately 8-10 mm lengths (discarding the ends). The leaf and stem sections were placed onto PDA (75%) with antibiotics (as above). Petri dishes were incubated and inspected for 30 days for the presence of *B. bassiana* or *M. anisopliae*. Other fungal endophytes were also recorded and assigned morphotype codes. The proportion of root, leaf and stem parts colonized was calculated as the number of sections exhibiting fungal outgrowth divided by the total number of sections plated.

2.4 Additional colonization experiments

Additional experiments were conducted with four of the best performing isolates/strains from the screening experiments (two *B. bassiana* and two *M. anisopliae*), which were selected based on the highest levels of colonization in cassava roots and the percentage of total cassava plants successfully colonized. Twelve blocks of cassava plants were inoculated in the same manner as described above for the screening experiments. Six blocks, each with five plants (the four treatment plants and one control) were evaluated for endophytic colonization by the fungi 7-9 days post-inoculation and six blocks were evaluated for endophytic colonization 47-49 days post-inoculation. The methodology for evaluating endophytic colonization was identical to that described above for the screening experiments including surface-sterilization, except for the manner in which the imprints were made after surface-sterilization. In these experiments, to confirm the effectiveness of our surface-sterilization technique, root, leaf and stem imprints were

made on PDA for every individual piece of root, leaf and stem. If any fungi grew on an imprint, that individual piece of root, leaf or stem was removed from the dataset (instead of the entire block being removed).

2.5 Plant growth and fungal treatment differences

Various plant growth measurements were obtained from the cassava plants in both screening experiments and one of the additional (best performing isolate/strains) experiments. Aboveground measurements (stem length, number of stems, and chlorophyll content) were taken 46 days post-inoculation, i.e., the day before the plants were destroyed for the second sampling time 47-49 days post inoculation. Stem length was measured from the point of origin of the longest stem on the cutting to the tip of that stem. Leaf chlorophyll content was determined with a SPAD 502 Plus Chlorophyll Meter (Minolta Co., Ltd., Osaka, Japan). The longest lobule of the most recent fully expanded leaf was used for obtaining the SPAD value, which included an average of three measurements along the lamina of the lobule. The root measurements (dry root weight and root length) were taken over three days from 47-49 days post-inoculation because they had to be measured after plants were removed from the pots. On the day of destructive sampling, the roots of each plant were collected by sieving and washed under running tap water and then dried in an oven for 72 h at 50°C to obtain the dry root weight for each plant. The root weight was the entire root mass in the pot (minus the 2 x 60 mm pieces of root taken to evaluate endophytic colonization). The root length was the entire length of a root from where the root attached to the cassava stem cutting to the distal tip of the root (of the two longest roots).

2.6 Data analysis

248 The proportion of roots colonized by the various fungal treatments from the two screening
249 experiments and the additional experiments were analyzed using logistic regression with random
250 effects using the R package lme4 (Bates et al., 2015), with fungal treatment, sampling date, root
251 part, and other endophyte presence as fixed effects and block nested in experiment as random
252 effects. The control plant data was not included in these analyses. A means comparison, using
253 the glht function in the R package multcomp (Hothorn et al., 2008) was used to separate the
254 differences in proportional colonization among treatments. This method adjusts *p*-values for the
255 number of comparisons made. A similar analysis was done using other endophyte presence as
256 the dependent variable.

257 A preliminary analysis suggested that several measured plant growth variables might show
258 differences among fungal treatments, but only the proportion of plants with roots colonized were
259 statistically significant. Therefore we decided to create composite scores, which are often helpful
260 in this type of analysis. The composite score can be considered a latent variable (a proxy for an
261 unobservable dependent variable) and is a weighted linear function of the measured variables,
262 first screened to remove those with little information value (Table 1) based on canonical (linear)
263 discriminant analysis (Kramer et al., 2009). This method finds the optimal weighting for each
264 measured variable's contribution to the composite score. For these data, two orthogonal latent
265 variables (LDA1 and LDA2) appeared adequate to describe differences in fungal treatments.

266 We did two analyses, using each latent variable as the dependent variable. A mixed model
267 was estimated using the lmer function of the R package lme4 (Bates et al., 2015), with fungal
268 treatment as a fixed effect and block nested in experiment as random effects. We used the glht
269 function in the R package multcomp (Hothorn et al., 2008), based on a multivariate *t* distribution,

to do all pairwise comparisons (method = "Tukey") of fungal treatments for each of the two composite scores.

3. Results

3.1 Screening experiments

Conidial viability was >90% for all of the *B. bassiana* and *M. anisopliae* isolates/strains in the screening experiments, except for Beauveriplant® WP, which was approximately 70% at the time of the inoculations. It is unknown why germination of Beauveriplant® WP conidia was lower for the two screenings. All of the fungal isolates/strains in both screening experiments successfully colonized at least some cassava plant roots. At 7-9 days post-inoculation, 84% of all *B. bassiana* treated plants were colonized (including 100% of plants treated with Beauveriplant® WP and 80% treated with Bovetrópico® WP, Micosis® WP, CIAT 359 and CIAT 405), and 80% of all *M. anisopliae* treated plants were colonized (including 100% of plants treated with CIAT 014A, 75% with Metarhiplant® WP, BioMa® and CIAT 053, and 50% with CIAT 001). At 47-49 days post-inoculation, 40% of *B. bassiana* treated plants were colonized (including 67% of plants treated with Beauveriplant® WP and CIAT 359, 33% treated with Bovetrópico® WP and CIAT 405 and 0% for Micosis), and 80% of *M. anisopliae* treated plants were colonized (including 100% of plants treated with CIAT 014A, CIAT 053 and BioMa®, 80% treated with Metarhiplant® WP, and 20% with CIAT 001). *Beauveria bassiana* and *M. anisopliae* were not found in the control plants or on any of the imprint plates. However, three imprint plates did have another (unidentified) fungus and therefore those blocks of data were removed from the analysis (one block for the *B. bassiana* screening and two blocks for the *M. anisopliae*

292 screening). Neither *B. bassiana* nor *M. anisopliae* were found as endophytes in any leaf or stem
293 samples.

294 Colonization levels differed significantly between some treatments in each of the screening
295 experiments, namely Beauveriplant® WP and Micosis® WP for the *B. bassiana* screening (χ^2 =
296 12.97, df = 4, p<0.01) (Fig. 1) and CIAT 001 and CIAT 053 in the *M. anisopliae* screening (χ^2 =
297 13.32, df = 4, p<0.01) (Fig. 2). The highest percentage root colonization was observed for plants
298 inoculated with the *B. bassiana* strain Beauveriplant® WP (Fig. 1) and the *M. anisopliae* isolate
299 CIAT 053 (Fig. 2). Colonization levels were higher in the proximal portion of the root than in the
300 distal portion in both the *B. bassiana* screening (Fig 1; χ^2 = 42.20, df = 1, p<0.001) and the *M.*
301 *anisopliae* screening (Fig. 2; χ^2 = 28.23, df = 1, p<0.001). In the *B. bassiana* screening,
302 colonization levels were lower at 47-49 days post-inoculation compared to 7-9 days post-
303 inoculation (Fig. 1; χ^2 = 16.22, df = 1, p<0.001). In the *M. anisopliae* screening, the level of
304 colonization did not vary between 7-9 days post-inoculation and 47-49 days post-inoculation
305 (Fig. 2; χ^2 = 0.11, df = 1, p=0.743).

306 Colonization levels by other endophytes were higher at 47-49 days post-inoculation than at
307 7-9 days post-inoculation for both the *B. bassiana* screening (χ^2 = 64.32, df = 1, p<0.001) and
308 the *M. anisopliae* screening (χ^2 = 22.31, df = 1, p<0.001). In the *B. bassiana* screening, there was
309 no difference in the level of colonization by other endophytes between the proximal and distal
310 portions. In the *M. anisopliae* screening, colonization levels by other endophytes were higher in
311 the proximal portion than in the distal portion of the roots (χ^2 = 51.62, df = 1, p<0.001). The
312 presence of other endophytes in cassava roots lowered the probability of isolating either *B.*
313 *bassiana* (χ^2 = 25.92, df = 1, p<0.001) or *M. anisopliae* (χ^2 = 62.62, df = 1, p<0.001) in the
314 screening experiments.

315

316 *3.2 Additional colonization experiments*

317 The four isolates/strains selected from the screening experiments for additional
318 experimentation were CIAT 359 and Beauveriplant® WP from the *B. bassiana* screening and
319 CIAT 014A and Metarhiplant® WP from the *M. anisopliae* screening. Metarhiplant® WP and
320 CIAT 014A were chosen based on the 7-9 day post-inoculation evaluation. Conidial viability
321 was >90% for all of the isolates/strains at the time of inoculation. All four isolates successfully
322 colonized cassava plant roots with approximately 78% of *B. bassiana* treated plants and 62% of
323 *M. anisopliae* treated plants colonized 7-9 days post-inoculation. Approximately 35% of *B.*
324 *bassiana* treated plants and 67% of *M. anisopliae* treated plants were colonized 47-49 days post-
325 inoculation. *Beauveria bassiana* and *M. anisopliae* were not found in any control plants. Ten
326 root imprints (from a total of 408 imprints) had fungal growth; one contained *B. bassiana* and
327 one contained *M. anisopliae*; the rest were unidentified fungi. The root pieces that corresponded
328 to these 10 imprints were removed from the dataset. *Beauveria bassiana* and *M. anisopliae* were
329 not found in any leaf or stem samples at any time.

330 Colonization levels did not differ significantly between the four fungal isolates/strains in
331 this experiment. Colonization levels were higher in the proximal portion of the root than in the
332 distal portion of the roots for all four *B. bassiana* and *M. anisopliae* isolates/strains ($\chi^2 = 50.49$,
333 $df = 1$, $p<0.001$). For the two *B. bassiana* isolates/strains, colonization levels were higher at 7-9
334 days post-inoculation compared to 47-49 days post-inoculation ($\chi^2 = 9.23$, $df = 1$, $p<0.01$) and
335 for the two *M. anisopliae* isolates/strains, the levels of colonization were higher at 47-49 days
336 post-inoculation compared to 7-9 days post-inoculation ($\chi^2 = 8.15$, $df = 1$, $p<0.01$). The levels of
337 colonization by other endophytes did not differ significantly between the two evaluation days

(i.e., 7-9 and 47-49 days post-inoculation). The presence of other endophytes lowered the probability of isolating both *B. bassiana* and *M. anisopliae* ($x^2 = 34.74$, df = 1, $p<0.001$).

3.3 Plant growth and fungal treatment differences

We interpreted the first latent variable (1st LDA; Table 1) as colonization success. The largest contributor to it is the degree of fungal colonization of roots (this is simply the presence/absence sum for each root examined for a plant), with a smaller contribution from stem length and the number of roots sampled. We interpreted the second latent variable (2nd LDA; Table 1) as plant growth. The largest contributor to this variable is stem length, followed by root length and root weight, with a negative contribution from leaf chlorophyll content.

The results of multiple mean comparisons of the fungal treatments are shown in Table 2. The upper right triangle gives *p* values for comparisons for the first dependent latent variable (LDA1) and the lower left gives *p* values for comparisons on the second dependent latent variable (LDA2). Nine contrasts on colonization success (Fig. 3, top panel) were significant with Micosis® WP and control (similar to each other) vs Beauveriplant® WP, Metarhiplant® WP, CIAT 014A, and CIAT 053 (latter four similar to each other). Five contrasts on plant growth (Fig. 3, bottom panel) were significant with BioMa® vs CIAT 359, Beauveriplant® WP and Micosis (latter three similar to each other), and CIAT 359 vs CIAT 001 and CIAT 014A (latter two similar to each other). Metarhiplant® WP vs BioMa® was not significant (p=0.0503).

4. Discussion

We have demonstrated for the first time that *B. bassiana* and *M. anisopliae* can endophytically colonize cassava roots. The soil drench inoculation method led to colonization of

361 cassava roots by *B. bassiana* and *M. anisopliae* for up to seven weeks after inoculation. This
362 suggests that successful endophytic colonization by *B. bassiana* and *M. anisopliae* can be
363 achieved in actively growing roots of cassava. Colonization of internal plant tissues of many
364 crops has been achieved with *B. bassiana* (Vega et al., 2008; see Introduction) and with
365 *Metarhizium* species (Akello and Sikora, 2012; Batta, 2013, Behie et al., 2015) suggesting that
366 these entomopathogens have the potential to colonize many different plant species.

367 We reisolated *B. bassiana* and *M. anisopliae* from surface-sterilized roots of cassava plants
368 but never from the leaves or stems of those plants. This indicates that the fungi were not systemic
369 within the plant, but rather remained localized in the roots. This localization is in contrast to
370 other studies that have found *B. bassiana* can establish as an endophyte throughout the entire
371 plant, particularly after seed inoculation (Akutse et al., 2013; Ownley et al., 2008; Quesada-
372 Moraga et al., 2009). However, for *M. anisopliae,* our results are not surprising given that
373 species of *Metarhizium* are more often reported as endophytes of roots and not the upper parts of
374 plants (Akello and Sikora, 2012; Behie et al., 2015; Murphy et al., 2015). We do not know if
375 systemic endophytic colonization by entomopathogens would be important in cassava for
376 protecting the plant against pests of the leaves, such as the whitefly *A. socialis*. The mechanisms
377 involved in the control of arthropod pests and diseases using endophytes include antagonism,
378 induction of plant host defenses, host plant tolerance, or a combination of these (Ownley et al.,
379 2010; Gómez-Vidal et al., 2009; Porras-Alfaro and Bayman, 2011). If host plant defense is
380 induced post-inoculation with a fungal entomopathogenic endophyte, it may not be necessary for
381 the fungus to be systemic (Jaber and Vidal, 2010). In the present study, an attempt to investigate
382 the effects of the fungal treatments on resistance by cassava to *A. socialis* was made, but large
383 variability in the results among replicates precluded learning whether the various endophytic

384 fungal entomopathogens differentially affected resistance by cassava against insects (unpubl.
385 data).

386 There are several possible explanations for the lack of systemic colonization by our
387 isolates/strains. Firstly, some studies have shown that colonization by the applied fungus is more
388 likely in the plant part that was in direct contact with the inoculum and less likely or not at all in
389 plant parts distant to the application site (Akello et al., 2007, 2009; Tefera and Vidal, 2009). This
390 would explain why our soil drench inoculation resulted in colonization only in the roots. This is
391 supported by several surveys that have suggested there is a lack of evidence for systemic growth
392 by fungal endophytes from one plant tissue type to another (Wearn et al., 2012; Yan et al., 2015).
393 Secondly, competition with other endophytes is likely to be important. Indeed, approximately 40
394 other morphospecies were recovered from the surface-sterilized root samples in our study and
395 our analyses showed that the probability of finding *B. bassiana* and *M. anisopliae* was reduced
396 significantly when other endophytes were present. We do not know if these other endophytes
397 originated from the environment in which the plants were growing or from the stem cutting
398 itself. The stem cutting may contain a store of fungal and bacterial endophytes that originate
399 from the parent plant and these could compete with *B. bassiana* and *M. anisopliae* inside the
400 plant. A study investigating the endophyte community within the stem cutting and how these
401 other endophytes interact with *B. bassiana* and *M. anisopliae* would be useful.

402 Colonization by *B. bassiana* and *M. anisopliae* was higher in the proximal portion of the
403 root than in the distal portion across all of our experiments. It is unknown if conidia of these
404 fungi were concentrated in the upper soil strata, where the proximal end of the root is located but
405 this is one hypothesis that could explain our results (Kim et al., 2010; Storey and Gardner 1988).
406 Future studies could evaluate the presence of fungal entomopathogens inoculated into the soil in

the different soil strata to determine if conidia adhere to soil particles in the upper soil layer around cassava stem cuttings. Another hypothesis is that the proximal end of the root provides different conditions that influence colonization. For example, in some cassava varieties, the proximal end of cassava root has been found to contain higher levels of cyanogenic glycosides than the distal end (Cooke, 1978). We do not know if this is the case in the cassava CMC-40 variety used in our experiments, which is low in cyanogenic glycosides overall, but it shows that conditions can be different across the longitudinal gradient of cassava roots.

Beauveria bassiana and *M. anisopliae* persisted in cassava roots for up to seven weeks in all of the experiments. *Metarhizium anisopliae* colonization levels remained relatively constant over time (in the screening experiment) and increased in time (for the additional experiment) between the two sampling dates, whereas *B. bassiana* colonization levels decreased by half between sampling dates in all experiments. This is not surprising given previous studies have shown *M. anisopliae* is rhizosphere competent (Bruck, 2005, 2010; Hu and St. Leger, 2002; St. Leger, 2008) and persists well in the soil environment, but *B. bassiana* does not persist as well (Lingg and Donaldson, 1981; Vänninen et al., 2000). Further, *B. bassiana* is more commonly found aboveground whereas *M. anisopliae* is very common belowground (Meyling et al., 2011). At the same time, the presence of other endophytes increased over time (screening experiments) or remained constant (additional experiment) and, as mentioned above, for those plants that had other endophytes, the probability of finding either *B. bassiana* or *M. anisopliae* was reduced. This indicates that the presence of other endophytes in cassava roots influenced the levels of colonization by both *B. bassiana* and *M. anisopliae*. The reason that the level of colonization by *M. anisopliae* remained constant despite an increase in other endophytes is likely to be related to its competence in the rhizosphere. There was opportunity for other endophytes to increase over

time because at both sampling times, some cassava root pieces were not colonized by any fungi. In other words, colonization by other endophytes could increase over time despite the level of colonization by *M. anisopliae* being maintained and this is evident because there were less un-colonized root pieces overall at the second sampling time.

Using the composite scores we have shown clear differences in the fungal entomopathogen isolates/strains in terms of colonization success and plant growth. These results show that the commercial products are not equivalent, with one of them (Micosis® WP) being no different than the control on the first composite score. Two of the entomopathogens (CIAT 053 and Beauveriplant® WP) were among the best performing for colonization success and two (CIAT 359 and Beauveriplant® WP) were among the best for plant growth. Only one of the 10 fungal entomopathogens (Beauveriplant® WP) resulted in both higher colonization success and plant growth, indicating it would be a good candidate for further studies. The negative loading for chlorophyll was unexpected; it could be a result of increased plant resource allocation to stem and roots, consequently not producing as much chlorophyll.

Our results support previous studies where plant growth promotion has been reported for *B. bassiana* (Lopez and Sword, 2015) and *M. anisopliae* (Kabaluk and Ericsson, 2007). Future research could allow inoculated cassava plants to grow for a longer period of time to investigate the influence of entomopathogens as endophytes on plant growth and whether root growth in particular increases, which might be beneficial for increasing root yield.

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637

638 **Figure Legends**

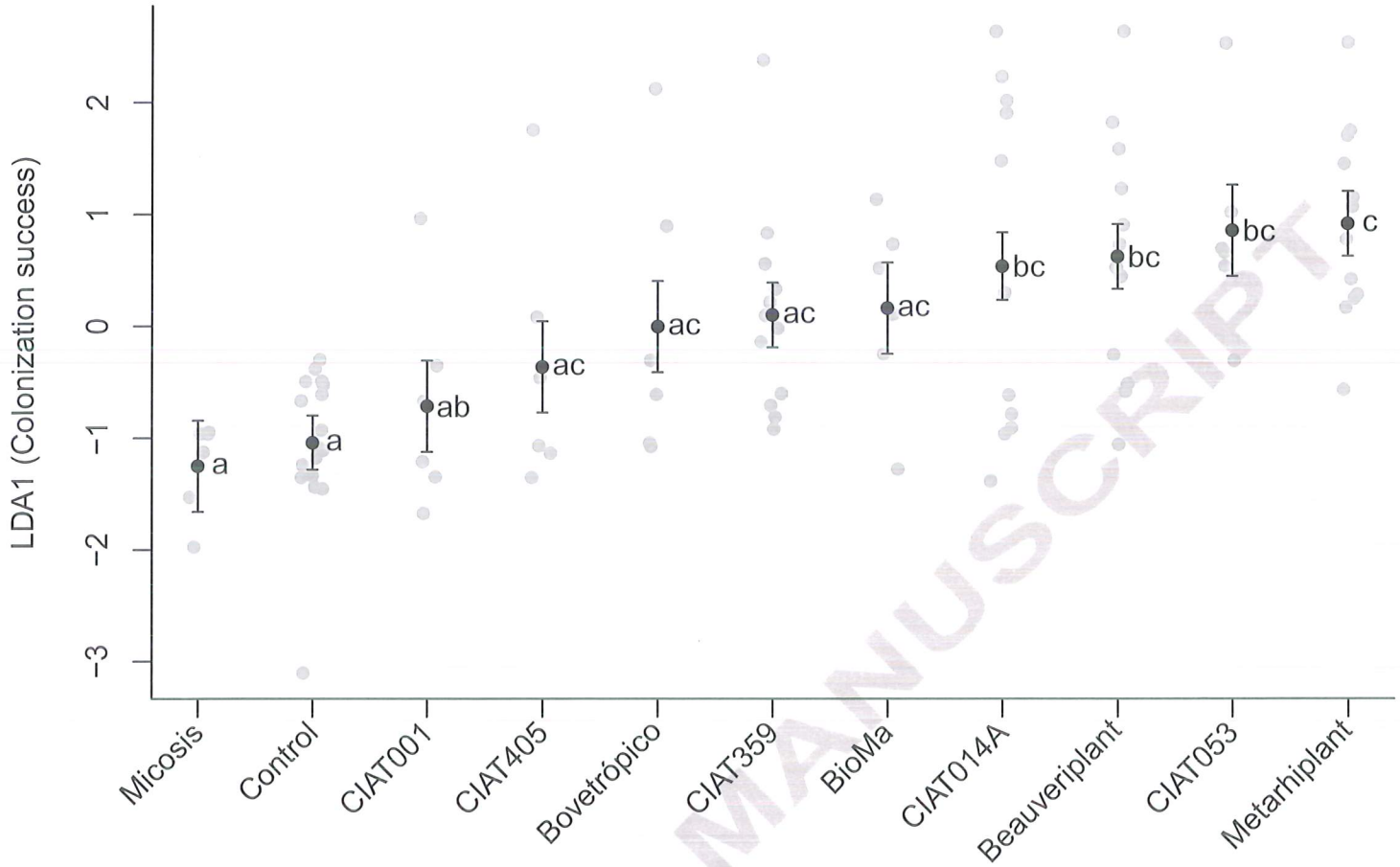
639 **Figure 1.** Proportion of root part colonized 7-9 and 47-49 days post-inoculation with five
640 different *B. bassiana* isolates/strains. Root colonization levels differed significantly between
641 Beauveriplant® WP and Micosis® WP and the highest percentage root colonization was observed
642 for plants inoculated with Beauveriplant® WP. Colonization levels were higher in the proximal
643 portion of the root than in the distal portion of the root and were lower at 47-49 days post-
644 inoculation compared to 7-9 days post-inoculation. See Results section for details. The same
645 letter underneath treatment names indicates that the means are not significantly different using
646 Tukey's procedure (family-wise error rate = 0.05).

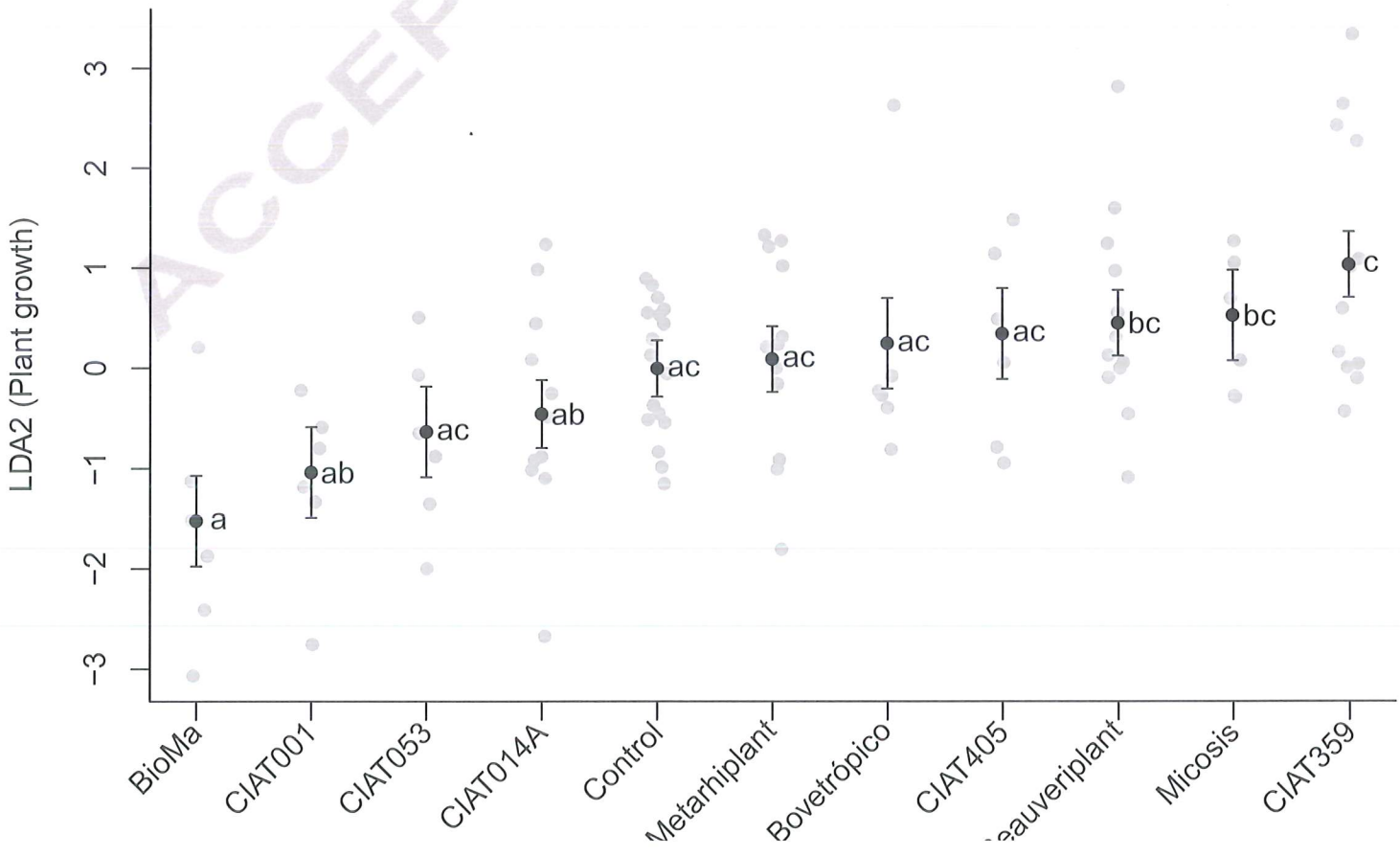
647

648 **Figure 2.** Proportion of root part colonized 7-9 and 47-49 days post-inoculation with five
649 different *M. anisopliae* isolates/strains. Root colonization levels differed significantly between
650 CIAT 001 and CIAT 053 and the highest percentage root colonization was observed for plants
651 inoculated with CIAT 053. Colonization levels were higher in the proximal portion of the root
652 than in the distal portion of the roots. The level of colonization did not vary between 7-9 days
653 post-inoculation and 47-49 days post-inoculation. See Results section for details. The same letter

654 underneath treatment names indicates that the means are not significantly different using Tukey's
655 procedure (family-wise error rate = 0.05).
656
657 **Figure 3.** Results for the 10 fungal entomopathogens and control on the two latent axes
658 (composite scores). LDA1 (top panel) is interpreted as colonization success, with larger numbers
659 indicating higher success. LDA2 (bottom panel) is interpreted as plant growth, with larger
660 numbers indicating better plant growth. Gray dots are values for each plant on their respective
661 axes. Black dots are mixed model estimates (composite score, either LDA1 or LDA2, is the
662 dependent variable) for each entomopathogen, with +/- one standard error of the model estimate
663 given by the vertical lines. Means separation letters follow each mean. Note that the
664 entomopathogen order differs between panels.
665
666 **Table Legends**
667 **Table 1.** Linear discriminant analysis (LDA) weights (loadings) for variables including plant
668 growth measurements and degree of colonization by fungi. These were used to create the
669 composite scores used for statistical comparisons of the treatment fungi. Variables were scaled to
670 mean = 0, standard deviation = 1 prior to calculating weights.
671
672 **Table 2.** *p*-values for *a posteriori* pairwise comparisons of composite scores. Composite scores
673 were created using predictions from the first two dimensions of a linear discriminant analysis
674 using six cassava plant health measurements (root dry weight, root length, number of roots
675 sampled, stem length, number of stems on plant, leaf chlorophyll content) and degree of

676 colonization of cassava roots by treatment fungi. The upper right portion of the table is for LDA1
677 and the lower left portion LDA2.
678
679
680





681

Variables	Weight (1st LDA)	Weight (2nd LDA)
Root (dry weight total)	-0.265	0.267
Root length	0.223	0.337
Number of roots sampled	0.323	-0.129
Leaf chlorophyll content (SPAD value)	0.029	-0.480
Stem length	0.411	0.956
Number of stems on plant	-0.122	0.152
Degree of fungal colonization of roots	1.129	-0.032

682

683

684

685 Table 1

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688

Treatment	Beauveri plant	Bovetr opic	Mico sis	CIAT 359	CIAT 405	Bio Ma	Metarhi plant	CIAT 001	CIAT 0 14A	CIAT 053	Cont rol
Beauveri plant (Bb)		0.98	<0.0 1*	0.97	0.66	1.00	1.00	0.21	1.00	1.00	<0.0 1*
Bovetropic (Bb)	1.00		0.52	1.00	1.00	1.00	0.75	0.98	0.99	0.92	0.51
Micosis (Bb)	1.00	1.00		0.19	0.90	0.33	<0.01*	1.00	0.02*	0.01*	1.00
CIAT 359 (Bb)	0.93	0.89	1.00		1.00	1.00	0.64	0.86	1.00	0.91	0.08
CIAT 405 (Bb)	1.00	1.00	1.00	0.95		1.00	0.26	1.00	0.80	0.56	0.94
BioMa (Ma)	0.01*	0.14	0.04 *	<0.01 *	0.10		0.91	0.91	1.00	0.98	0.28
Metarhiplant (Ma)	1.00	1.00	1.00	0.47	1.00	0.05		0.042*	1.00	1.00	<0.0 1*
CIAT 001 (Ma)	0.16	0.60	0.29	<0.01 *	0.49	1.00	0.46		0.32	0.18	1.00
CIAT 014A (Ma)	0.57	0.97	0.77	0.02*	0.93	0.57	0.96	0.99		1.00	<0.0 1*
CIAT 053 (Ma)	0.63	0.94	0.74	0.07	0.89	0.89	0.93	1.00	1.00		<0.0 1*
Control	0.98	1.00	0.99	0.17	1.00	0.06	1.00	0.54	0.98	0.97	

689
690 Bb = *Beauveria bassiana*
691 Ma = *Metarhizium anisopliae*

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694 Table 2

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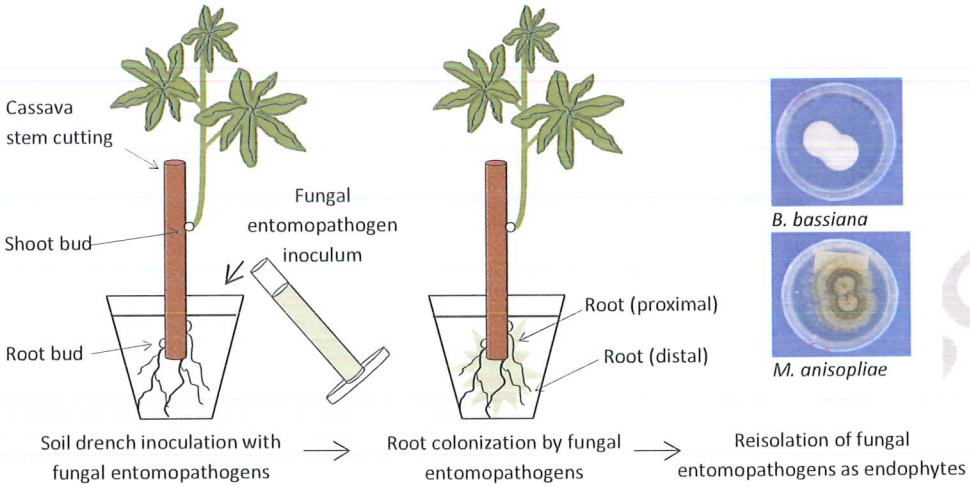

Graphical abstract

- First time *B. bassiana* and *M. anisopliae* introduced as endophytes in cassava roots
- *Beauveria bassiana* and *M. anisopliae* colonized cassava roots for up to 7 weeks
- Colonization of cassava roots by *M. anisopliae* remained relatively constant over time
- Colonization of cassava roots by *B. bassiana* declined over time
- Colonization levels were higher in the proximal end than in the distal end of the cassava root