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Establishment of the fungal entomopathogen *Beauveria bassiana* as an endophyte in sugarcane, *Saccharum officinarum*

Trust Kasambala Donga [a, b], Fernando E. Vega [c], Ingeborg Klingen [d, *]

[a] Department of Plant Sciences, Norwegian University of Life Sciences (NMBU), Campus ÅS, Universitetstunet 3, 1433, Ås, Norway
[b] Lilongwe University of Agriculture and Natural Resources (LUANAR), P.O. Box 219, Lilongwe, Malawi
[c] Sustainable Perennial Crops Laboratory, United States Department of Agriculture (USDA), Agricultural Research Service, Beltsville, MD, 20705, USA
[d] Division for Biotechnology and Plant Health, Norwegian Institute of Bioeconomy Research (NIBIO), Høgskoleveien 7, 1431, Ås, Norway

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ABSTRACT

We investigated the ability of the fungal entomopathogen *Beauveria bassiana* strain GHA to endophytically colonize sugarcane (*Saccharum officinarum*) and its impact on plant growth. We used foliar spray, stem injection, and soil drench inoculation methods. All three inoculation methods resulted in *B. bassiana* colonizing sugarcane tissues. Extent of fungal colonization differed significantly with inoculation method ($\chi^2 = 20.112$, d. f. = 2, $p < 0.001$), and stem injection showed the highest colonization level followed by foliar spray and root drench. Extent of fungal colonization differed significantly with plant part ($\chi^2 = 33.072$, d. f. = 5, $p < 0.001$); stem injection resulted in *B. bassiana* colonization of the stem and to some extent leaves; foliar spray resulted in colonization of leaves and to some extent, the stem; and soil drench resulted in colonization of roots and to some extent the stem. Irrespective of inoculation method, *B. bassiana* colonization was 2.8 times lower at 14–16 d post inoculation (DPI) than at 7–10 DPI ($p = 0.020$). Spraying leaves and drenching the soil with *B. bassiana* significantly ($p = 0.01$) enhanced numbers of sett roots. This study demonstrates for the first time that *B. bassiana* can endophytically colonize sugarcane plants and enhance the root sett and it provides a starting point for exploring the use of this fungus as an endophyte in management of sugarcane pests.

1. Introduction

Sugarcane (*Saccharum officinarum*; Poaceae) is one of the world's most valuable crops. Although sugarcane originated in Polynesia, it is grown in approximately 120 tropical and subtropical countries with a global production of about 1.89 billion tonnes of crushed sugarcane in 2016 (FAOSTAT, 2018). The sugarcane ecosystem (phytobiome) comprises numerous weeds, arthropods and more than 50 plant pathogens (Ferreira and Comstock, 1993; Verma, 2004; Leach et al., 2017). Arthropod pests associated with the crop worldwide include complexes of stalk feeders, sap sucking insects (e.g., aphids, thrips, mealybugs), root feeders (e.g., white grubs, stemborers), and spider mites (Dittrich et al., 2005; Barker et al., 2006; Leslie, 2008, 2009; Goebel and Sallam, 2011; Goble et al., 2014; SASRI, 2014; Bharu, 2015).

The main arthropod pests infesting sugarcane in Africa include stemborers (*Chilo* and *Sesamia* spp.), black maize beetles (*Heteronychus* spp.), thrips (*Fulmekiola serrata*), scale insects (*Aulacaspis tegalensis*), mealybugs (*Saccharicoccus sacchari*) and spider mites (*Tetranychus urticae*) (Smith-Meyer, 1974; Conlong, 2001, 2008; Nuessly, 2014; SASRI, 2014; Language, 2015). The sugarcane yellow aphid (*Sipha flava*) was first recorded in southern Africa in 2013 (Conlong and Way, 2014; Way et al., 2014). Management of all these pests currently relies on cultural methods, host plant resistance, chemical insecticide application, and biological control focusing on use of insect predators and parasitoids (Akbar et al., 2010; Goebel et al., 2010; Bowling et al., 2016). Chemical insecticides provide rapid and effective control of many pests and reduce labour costs associated with mechanical pest removal. However, health and environmental problems, the development of insecticide resistance, and cost, limit their use (WHO, 2014; Kasambala Donga and Eklo, 2018). Host plant resistance may contribute to reduced pesticide load in the environment, but it might not be long lasting or practical in instances of a new virulent pest species (Humphries et al., 2010). Biological control agents are usually compatible with other pest control methods and are central in integrated pest

* Corresponding author.
E-mail address: ingeborg.klingen@nibio.no (I. Klingen).

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management (IPM) programs of many crops.

Fungal entomopathogens belonging to the order Hypocreales (Ascomycota) or to the phylum Entomophthoramycota have been reported to protect plants from insect pests (Pell et al., 2009; Vega et al., 2012). Fungi in the Entomophthoromycota are generally associated with natural epizootics on foliar insect hosts and are mostly used in conservation biological control (Ekesi et al., 2005; Baverstock et al., 2008; Pell et al., 2009). The major disadvantage with Entomophthoromycota is that they are mainly biotrophic with a close association with their insect or mite host and many cannot be mass-produced on artificial media (Jaronski and Jackson, 2012). On the other hand, hypocrealean fungi such as *Beauveria* and *Metarhizium* are hemibiotrophic, cosmopolitan and ubiquitous in the soil but do not commonly cause natural, large-scale epizootics on foliar insects in annual crops (Pell et al., 2009; Jaronski, 2010). For instance, in a survey of natural enemies of *Chilo sacchariphagus* in sugarcane plantations in Moçambique, Conlong and Geobel (2002) found *Beauveria bassiana* infesting only three cadavers of *C. sacchariphagus* larvae. Hypocrealean fungi are traditionally employed in both inundation and inoculation biological control (Maniania et al., 2001; Meyling and Eilenberg, 2007; Remadevi et al., 2010; Klingen et al., 2014). Currently, large-scale inundation and inoculative biological control is being practiced in many countries including Austria, Brazil and South Africa (Lacey et al., 2015).

There is growing evidence that fungal entomopathogens occur naturally or can be established artificially as endophytes in various crop plants and that such establishment might adversely affect insect pests (Vega, 2008, 2018; Vega et al., 2009; Quesada-Moraga et al., 2014a; Greenfield et al., 2016). *Beauveria bassiana* artificially introduced as an endophyte in cotton (*Gossypium hirsutum*) negatively affected cotton aphid reproduction (Castillo Lopez et al., 2014) and endophytic *B. bassiana* in maize (*Zea mays*) resulted in all-season suppression of the European corn borer, *Ostrinia nubilalis* (Bing and Lewis, 1992a; 1992b). In banana (*Musa* spp.), endophytic *B. bassiana* significantly reduced damage caused by larvae of *Cosmopolites sordidus* by 42–87% depending on the plant tissue (Akello et al., 2007).

Several approaches have been used in establishing *B. bassiana* as an endophyte in target plants. Lewis and Bing (1991), Bing and Lewis (1992a; 1992b) and Wagner and Lewis (2000) successfully established *B. bassiana* as an endophyte in maize using foliar application at the two-leaf or whorl stage. *Beauveria bassiana* was also established as an endophyte in cocoa (*Theobroma cacao*; Posada and Vega, 2005) and coffee (*Coffea arabica*; Posada and Vega, 2006) by inoculating the main radicle of seedlings. Posada et al. (2007) also established *B. bassiana* in coffee seedlings using stem injections, foliar sprays, and soil drenches, with highest endophytic recovery obtained in plants whose stems had been injected with a *B. bassiana* spore suspension. Tefera and Vidal (2009) reported that *B. bassiana* could be established as an endophyte in different sorghum (*Sorghum bicolor*) tissues through seed dressing, foliar sprays, and soil inoculation, with foliar sprays being the best method. Brownbridge et al. (2012) introduced *B. bassiana* into pine seedlings (*Pinus radiata*) using seed coating and root dipping. Quesada-Moraga et al. (2014b) established *B. bassiana* as an endophyte in opium poppy (*Papaver somniferum*) tissue via seed soaking and found that *B. bassiana* was vertically transmitted via seeds from endophytically colonized maternal plants. Evaluating the potential of an entomopathogenic fungal species to establish as an endophyte in a given plant species is the first step in the process of determining whether this fungus might protect the plant from insect pests or mites. The most common method for evaluating endophytic establishment is the fragment plating method (Torres et al., 2011). This method involves the elimination of epiphytes, by surface sterilizing plant tissue sections, and plating the sterilized sections on selective growth media (Vega, 2018). Post-inoculation time for performing this step varies. Ten days were enough to confirm that *B. bassiana* could establish endophytically in artichoke, *Cynara scolymus* (Guesmi-Jouini et al., 2014). Greenfield et al. (2016) evaluated *B. bassiana* endophytic colonization of cassava (*Manihot esculenta*) at 7–9 and 47–49 d. Renuka et al. (2016) traced post-inoculation persistence of *B. bassiana* in maize (*Z. mays*) for 90 d.

Information on the ability of *B. bassiana* to endophytically colonize sugarcane and the effects of *B. bassiana* on sugarcane plant growth is not available. We report that *B. bassiana* can become established as an endophyte in sugarcane using foliar spray, stem injection and soil drench and that endophytism with *B. bassiana* resulted in enhanced sugarcane plant growth.

2. Materials and methods

2.1. Treatments, study location, and experimental design

The experiment was conducted in a greenhouse at the ILLOVO Malawi sugarcane quarantine facility at Bvumbwe Agricultural Research Station, Thyolo District, Malawi (15°55′27.1″S 35°04′12.5″E, 1174 m a.s.l). The experiment was set up as a completely randomized design with subsampling, and treatments consisted of three different fungal inoculation methods (foliar spray, stem injection, soil drench) and the control. The experiment was repeated four times. Each replicate had 36 plants: 9 foliarly-sprayed plants, 9 stem-injected plants, 9 soil-drenched plants, and 9 control plants. Therefore, the experiment consisted of 144 plants. Destructive sampling of plant tissue (leaves, stems, roots) to evaluate endophytic colonization by *B. bassiana* was done 7 and 14 d post-inoculation (DPI). For method, see below. Evaluation of plant growth was done 16 DPI.

2.2. Plants

The sugarcane variety MN1 was used. This is a commonly grown variety in Malawi (Kasambala Donga and Eklo, 2018). Sugarcane stems free from pests and diseases were collected from 7 to 10-month-old irrigated seedcane growing at the ILLOVO Nchalo Sugar Estate (Chikwawa District, Malawi). The stems were cut into smaller sections approximately 13.5 cm long. Each of these sections had two buds. These stem cuttings are referred to as 2-bud cane-setts (Fig. 1A). To prevent ratoon stunting disease and other bacterial sugarcane pathogens, cane-setts are routinely dipped in 50 °C water for 2 h. This treatment could have negative effects on germination (McFarlane, 2013); therefore, surface sterilization in alcohol and sodium hypochlorite was used as described below. Two-bud cane-setts were washed for 1 min in running tap water to remove any debris before surface sterilizing by immersing for 3 min in 1% sodium hypochlorite followed by 1 min in 70% ethanol (Parsa et al., 2013; McKinnon et al., 2016). The tissues were then rinsed in sterile distilled water three times. The sterilized plant tissues were dried on sterile filter paper for 30 min before plating. Effectiveness of the sterilization process was evaluated by plating 100 μl of the last rinse water on Sabouraud dextrose agar (SDA) and incubating the plate for 10 d at 25 °C. Imprints of sterilized plant tissue were also prepared to ensure that the sterilization was successful. This was done by momentarily placing and pressing a surface sterilized plant tissue on SDA and incubating the plate for 10 d at 25 °C.

Two surface sterilized two-bud cane-setts were horizontally planted in each 10 L plastic bucket (height 235 mm, upper diameter 265 mm, lower diameter 170 mm) containing a steam-sterilized mixture (2:1:1) of sandy loam soil, bagasse and sand from the

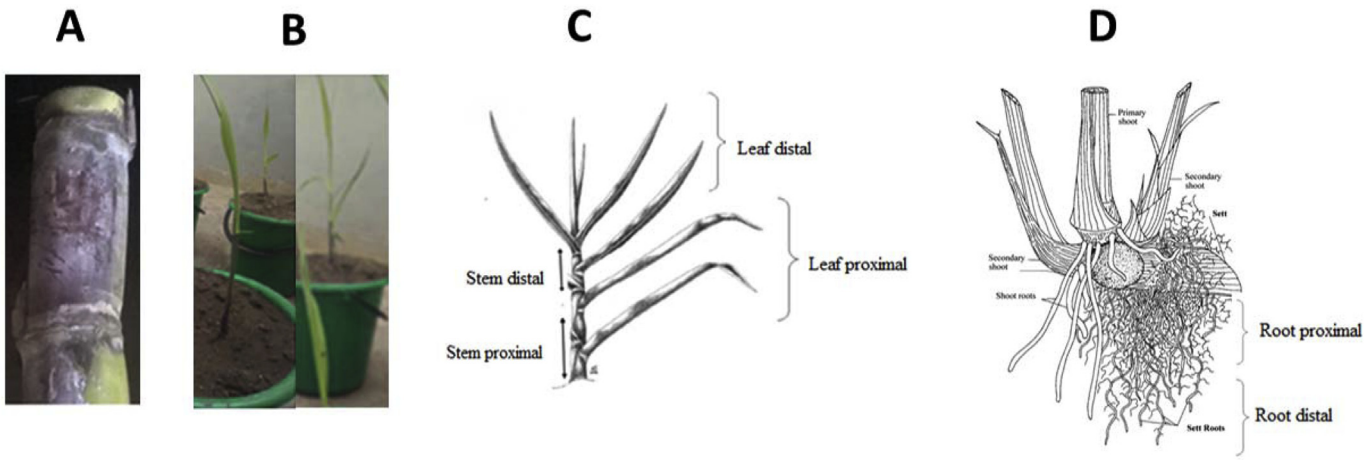

Fig. 1. Sugarcane stem cutting with two buds (two-bud cane-sett) used in propagating sugarcane in this study (A). Sugarcane plants growing in 10 L plastic buckets (B). Definition of proximal and distal in reference to sugarcane leaves, stems and roots used in this paper (C, D; photo credits: Blackburn, 1984).

ILLOVO Nchalo Sugar Estate (Fig. 1B). The sterilization of the soil involved leading steam from a 210 L metal drum into a perforated hosepipe under a heavy-duty PVC black sheet secured at the edges by heavy stones. The temperature inside the PVC sheets was maintained at 92–95 °C for 5 h. The soil was cooled for 24 h before planting. Diammonium phosphate (25 g) was mixed with the soil mixture to provide phosphate in each 10 L plastic bucket. The soil was moistened using sterile distilled water 24 h before planting. After germination, buckets were thinned by discarding cane-setts with poorly growing shoots; therefore, each plastic bucket had only one two-bud sett with one or two healthy shoots. Plants were watered with sterile distilled water as required. The plastic buckets were kept in a greenhouse for 14 d after planting.

2.3. Fungal strain

A commercial strain of *B. bassiana* (GHA) formulated as BotaniGard® ES was used (Laverlam International Corporation, Butte, MT). The strain was chosen based on its registered use against aphids and sugarcane borers. To generate the stock inoculum, one inoculating loop of liquid emulsifiable suspension was suspended in 1 ml of a 0.1% sterile water solution of Tween 80 (Sigma-Aldrich, St. Louis, MO) and vigorously hand-shaken for 30 s. From the suspension, 100 µl was plated on SDA and incubated for 24 h at 25 ± 5 °C. A single germinating conidium was transferred to a 90 mm diameter Petri dish containing SDA mixed with a 0.1% stock solution of antibiotics to inhibit bacterial growth (Posada and Vega, 2005). The antibiotic stock consisted of 0.2 g of each of three antibiotics (chloramphenicol, penicillin and tetracycline) dissolved in 10 ml sterile distilled water, followed by filter sterilization through a 0.2 mm filter. From this, 1 ml was added to each liter of medium. The fungus was grown in the dark at 25 ± 5 °C until it covered the entire plate.

The fungus was then harvested by scraping it off the SDA using a sterile spatula and suspending it in 10 ml sterile 0.1% Tween 80 and vigorously hand-shaking for one min. The suspension was filtered through sterile cheesecloth to remove hyphae and to obtain the stock suspension. An improved Neubauer haemocytometer was used to estimate the spore concentration of the stock suspension. Sterile distilled water was used to adjust the stock concentration to a final concentration of 1×10^8 conidia ml^{-1}. Conidial viability was assessed just after harvest and prior to inoculation of plants by plating 100 µl of 1.0×10^8 conidia ml^{-1} on SDA and incubating at 25 ± 5 °C for 24 h. Three random groups of 100 spores were examined using a stereoscope to estimate percent germination. A conidium was considered germinated when a visible germ tube longer than half the diameter of the conidium was observed. Conidial germination was >90% and was considered acceptable for use in the experiments. The stock suspension was stored in sterile 300 ml glass bottles in darkness at 4 °C for 24 h before use.

2.4. Plant inoculation

Plants were watered to saturation using sterile distilled water 24 h before inoculations. Seven days after the emergence of the primary shoot, the plants were inoculated with *B. bassiana*. Three different inoculation methods were used: foliar spray application; stem injection and soil drench. For inoculation by foliar spray, plants were sprayed in a separate room to prevent accidental inoculation of the other treatments via spray droplets. A manual atomizer was used to apply 100 ml inoculum (1×10^8 conidia ml^{-1}) onto the sugarcane leaves. The top of the plastic bucket was covered with aluminum foil to avoid conidial runoff to the soil. After spraying, the plants were covered with a plastic bag for 24 h to maintain a high level of humidity to facilitate fungal germination and plant colonization (Parsa et al., 2013) before being returned to the experimental blocks in the greenhouse. For inoculation by stem injection, a hole was made on the primary shoot using a 5 ml sterile disposable insulin hypodermic needle to facilitate injection of 1 ml of conidial suspension (Akello et al., 2007; Posada et al., 2007). For inoculation by soil drench, 100 ml of inoculum was applied to the soil surface in close proximity with the root area. Control plants of all three treatments were treated with sterile water with 0.1% Tween 80.

2.5. Sampling for endophytic colonization

The first sampling was done 7 DPI. Due to problems with availability of a consistent power supply throughout the experiments, collection, surface sterilization and plating of plants samples onto Petri dishes was done on four consecutive days for the first sampling. The second sampling was done 14 DPI and took three consecutive days to process. At each sampling time, 3 foliarly-sprayed plants, 3 stem-injected plants, 3 soil-drenched plants, and 3 control plants were carefully uprooted (from each replicate) to avoid damage to roots using a sterilized garden spade and placed in plastic bags. The garden spade was dipped in 70% alcohol after

each plant was uprooted. The plants were then transferred to the laboratory for examination of endophytic colonization by *B. bassiana*. The base of the plant was washed under running tap water to remove debris and soil while carefully avoiding destruction of root tissue. After washing, leaves were processed first followed by roots, and lastly the stems.

The endophytic colonization evaluation method outlined by Greenfield et al. (2016) was followed. Leaves (60 mm), stems and roots sections were surface sterilized (McKinnon et al., 2016) as described above. The outer edges of the tissues were dissected and discarded. Each trimmed sample was cut into six sections, averaging 6 × 6 mm for leaves and 6 mm long for stems and roots and plated on a 90 mm Petri dish with SDA supplemented with antibiotics (as described above). The Petri dish was sealed with parafilm and incubated in the dark at 25 ± 5 °C. The last rinse water was changed after processing each block of a given treatment. Before discarding the final rinsing water, a 100 µl sample was plated on SDA and incubated for 10 d at 25 ± 5 °C to assess sterilization success. Imprints as described above were also done to assess sterilization success. The plates were inspected for fungal growth every 2–3 d for 20 d. If fungal growth was detected, the corresponding samples were discarded. No fungal growth on the medium used for the imprint indicated that sterilization was successful. Each plant yielded six plates, two per plant part divided into proximal and distal parts as described in Fig. 1C, D.

2.6. Growth of *B. bassiana*-treated sugarcane plants

The following sugarcane growth parameters were determined 16 DPI: number of healthy green leaves; sett roots and shoot roots; plant height; length of longest root (the distance in cm from plant base to the tip of the root); length of newly emerged leaves (the distance in cm from stem/leaf joint blade to the tip of the leaf); and wet and dry biomass. Plant height was measured from the soil surface to the tip of the stem. Dry weight was determined after oven-drying whole plant samples at 50 °C for 72 h (Greenfield et al., 2016).

2.7. Data analysis

Colonization was considered as the number of tissue parts showing *B. bassiana* growth in each Petri dish. We modelled the number of tissue part inoculated using a negative binomial regression model. The model was chosen because overdispersion was observed under Poisson distribution (sample mean of the outcome = 0.71, variance = 2.07, variance/mean ratio = 2.92). Inoculation method, sampling time and plant part were the predictors in the model. We included an interaction term of treatment sampling time (time 1 = 7–10 DPI; time 2 = 14–16 DPI) and plant part (plant part 1 = leaf distal; plant part 2 = leaf proximal; plant part 3 = stem distal; plant part 4 = stem proximal; plant part 5 = root distal; plant part 6 = root proximal) inoculated was used to test if colonization in the different plant tissues differed with time. All plant growth data was subjected to general linear model multivariate procedures. Prior to analyses number of bud roots data were subjected to $\log_{10+1}$ transformation because positive skewness was observed. Tukey HSD test ($p = 0.05$) was used to separate significant means. Model estimation and multivariate analysis were performed in SPSS software version 24 (IBM® Corp. 2016).

3. Results

3.1. Evaluation of endophytic colonization

All three inoculation methods resulted in *B. bassiana* becoming established as an endophyte in sugarcane tissues. Fungal colonization levels differed significantly with inoculation method ($\chi^2 = 20.112$, d. f. = 2, $p < 0.001$), sampling time ($\chi^2 = 11.187$, d. f. = 1, $p < 0.001$) and plant part ($\chi^2 = 33.072$, d. f. = 5, $p < 0.001$). Foliar spray resulted in successful colonization of leaves and stems but not roots (Fig. 2). When using foliar sprays, the highest mean number of leaves colonized by *B. bassiana* was recorded at 7–10 DPI in distal leaves (2.6 ± 0.05) and at 14–16 DPI in distal parts of the stem (2.44 ± 0.97). These were significantly ($p < 0.001$) higher than that in proximal leaf and stem (Fig. 2). *Beauveria bassiana* colonization of leaf tissues significantly ($p < 0.001$) decreased between 7-10 and 14–16 DPI (Fig. 2).

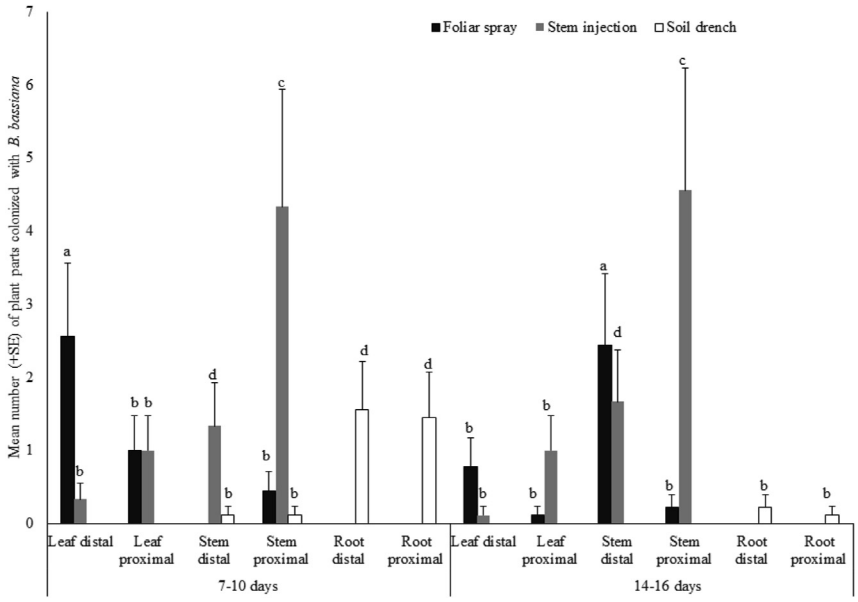

Fig. 2. Mean number (±SE) of plant part pieces with *B. bassiana* isolate GHA recovered from sugarcane leaves, stems, and roots 7 and 14 d post-inoculation (DPI) following foliar spray (black), stem injection (grey), or soil drench (white). Different letters above columns indicates statistical difference using negative binomial regression ($p = 0.05$).

Stem injections led to *B. bassiana* colonizing stems and leaves but not roots (Fig. 2), and colonization was significantly higher in proximal parts of stems at both 7–10 and 14–16 DPI (4.6 ± 0.05) compared to distal stems (1.67 ± 0.70) (Fig. 2). *Beauveria bassiana* also colonized leaves following stem injection but at significant ($p < 0.001$) lower levels than that in stems. *Beauveria bassiana* recovery in stems and leaves did not change over time (Fig. 2).

Soil drench inoculation resulted in successful colonization of roots, and there was no significant ($p = 0.05$) difference in the colonization of proximal and distal roots. The highest root colonization (1.6 ± 0.05) was recorded 7–10 DPI and it was significantly ($p = 0.01$) higher than at 14–16 DPI. *Beauveria bassiana* was also detected in stems following soil drenches only at 7–10 DPI (Fig. 2).

Based on the negative linear regression analysis and irrespective of inoculation method, *B. bassiana* colonization was 2.8 times lower at 14–16 DPI than at 7–10 DPI ($p = 0.020$). Based on the same analysis, expectations of *B. bassiana* colonization level of sugarcane was higher than the observed *B. bassiana* colonization level for all factors tested (inoculation methods, plant parts, time).

Beauveria bassiana was never recovered from control plants. *Penicillium* and *Aspergillus* were the only other fungi isolated from plants receiving stem injections and foliar sprays.

3.2. Growth of *B. bassiana*-treated sugarcane plants

Plant growth data indicate that inoculation method affected plant height ($F = 3.985$; $df = 3$; $p = 0.013$), number of sett roots ($F = 6.762$; $df = 3$; $p = 0.01$) and fresh weight ($F = 6.430$; $df = 3$; $p = 0.011$). Plants in the foliar spray and soil drench treatments developed more sett roots than plants in the stem injection and control treatments (Table 1). The length of leaves and height of plants that had received stem injections or a soil drench were not significantly different from each other (Table 1). None of the plants showed any signs of disease.

4. Discussion

This study has demonstrated for the first time the ability of *B. bassiana* to endophytically colonize sugarcane roots, stems and leaves following foliar spray, stem injection and soil drenching. Our results agree with Behie et al. (2015) who found that, unlike *Metarhizium* spp., *B. bassiana* does not display preferential tissue colonization. In addition, *B. bassiana* recovery was significantly higher in plants inoculated via foliar sprays and stems injections than soil drenching. Several papers have reported similar results (Quesada-Moraga et al., 2007; Tefera and Vidal, 2009; Guesmi-Jouini et al., 2014; Russo et al., 2015; Jaber and Enkerli, 2017). In a study involving coffee plants, soil drenching was a more effective way of introducing *B. bassiana* as an endophyte than foliar sprays (Posada et al., 2007). One possible explanation for this finding is that the leaf might be a poor route of entry for *B. bassiana* due to the absence of stomata on the adaxial (upper) surface and the presence of substances/structures on the leaf surface that may have negatively affected germination of conidia (Posada et al., 2007). In contrast to coffee plants, sugarcane has stomata on both sides of the leaf (Ferreira et al., 2007). Considering that spray droplets from foliar spray application may not totally cover the abaxial leaf surface, the adaxial stomata are probably an important route of entry for the *B. bassiana* germination tube in sugarcane. However, the germinating conidium has to overcome a cuticular wax layer that may completely cover the sugarcane plant stomata (Ferreira et al., 2007). Use of stem injection as an inoculation method bypasses these physical hurdles.

Drenching the soil with *B. bassiana* did result in root colonization. *Beauveria bassiana* persistence in root tissue did not result in systemic colonization of other sugarcane tissues, as has been reported for banana (Akello et al., 2007), sorghum (Tefera and Vidal, 2009), and red campion (*Silene dioica*; Yan et al., 2015). There was no statistical difference in *B. bassiana* establishment in distal and proximal part of the roots. This is in contrast with what Greenfield et al. (2016) reported for cassava roots. The following explanation could account for this difference. During the first weeks of sugarcane germination, the root system is comprised chiefly of thin, hairy and highly branched sett roots arising from the root band and thick, fleshy, and less branched shoot roots (Smith et al., 2005). These roots are concentrated in the top 20 cm of soil (Blackburn, 1984). Using a pot experiment, Kim et al. (2010) found that within 18 d of soil inoculation, *B. bassiana* strain GHA growth was concentrated in the upper soil surface. In our study, both the proximal and distal portions of the roots were concentrated in the upper soil surface. In addition, we could not attribute the reason for the poor establishment of *B. bassiana* in roots following soil drenches to the presence of *B. bassiana* antagonists in the soil as suggested by Tefera and Vidal (2009), since the soil used in our experiment had been sterilized. Furthermore, no other endophytes were isolated from roots of plants inoculated by soil drenching. Lastly, *B. bassiana* has been reported to have lower soil persistence when applied as unformulated conidia using the soil drench method (Vänninen et al., 2000).

Overall, the incidence rate of *B. bassiana* colonization of sugarcane decreased over time and significantly differed among sugarcane tissues irrespective of inoculation method. This observation is similar to previous findings in other crops such as maize (Renuka et al., 2016), crested wheat grass (*Agropyron cristatum*) (Inglis et al., 1993) and iceberg lettuce (*Lactuca sativa* cv. Mirette) (Shrestha et al., 2015). Dilution of initial fungal inoculum due to rapid plant growth (Inyang et al., 1998) may account for the low persistence of *B. bassiana*. We would expect *B. bassiana* persistence to be very low as the plant ages. Therefore, multiple applications may be required to ensure persistence in the first 5 months when the plant is established.

Recovery of *B. bassiana* from the distal part of leaves, stems, and roots following foliar sprays, stem injections, and soil drenches indicate that *B. bassiana* was capable of some movement within the plant, as already reported for maize (Bing and Lewis, 1991, 1992a; Wagner and Lewis, 2000), coffee (Posada et al., 2007), tomato (*Solanum lycopersicum*) (Klieber and Reineke, 2015) and pine trees (*P. radiata*) (Lefort et al., 2016). Yan et al. (2015) found that fungal

Table 1
Effects of *B. bassiana* strain GHA inoculation method (foliar spray, stem injection and soil drench) on mean (±SE) plant height, leaf length, number and length of sett and shoot roots, and fresh and dry weight 16 days post-inoculation (DPI).

Inoculation method	Plant height (cm)	Leaf length (cm)	# sett roots	# shoot roots	Sett roots length (cm)	Shoot root length (cm)	Fresh weight (g)	Dry weight (cm)
Foliar spray	24.9 ± 1.1b	94.3 ± 5.5b	36.7 ± 2.3b	2.2 ± 0.6a	15.2 ± 1.3a	6.0 ± 1.3a	23.4 ± 2.0b	2.8 ± 0.2a
Stem injection	20.7 ± 1.0a	66.3 ± 5.5a	26.4 ± 2.0a	1.1 ± 0.6a	15.6 ± 1.1a	1.7 ± 1.1a	10.7 ± 1.8a	2.0 ± 0.2a
Soil drench	24.0 ± 1.1ab	79.5 ± 5.5ab	36.3 ± 2.1b	0.7 ± 0.6a	13.9 ± 1.2a	2.3 ± 1.2a	15.9 ± 1.9ab	2.1 ± 0.2a
Control	25.1 ± 1.1b	82.5 ± 5.5ab	28.1 ± 2.0a	2.0 ± 0.6a	14.0 ± 1.1a	2.9 ± 1.1a	16.0 ± 1.8ab	2.2 ± 0.2a

Different letters following means in the same column indicate statistical difference using Tukey HSD test ($p = 0.05$).

endophytes displayed very limited systematic growth within plants; the inoculated fungal endophyte remained localized in the plant part that had received the initial fungal treatment. This seems to be the case with sugarcane, where the level of *B. bassiana* recovered was significantly higher in the plant part that received the initial fungal inoculum. In maize, however, mycelial growth in xylem vessels was the main mechanism in which the fungus applied on the leaves colonized the stem (Wagner and Lewis, 2000; Cherry et al., 2004).

It is important to note that fungal entomopathogen endophytism might induce plant responses that might have an effect on the plant, insects and/or plant pathogens (Cory and Hoover, 2006; Gomez-Vidal et al., 2006; Cory and Ericsson, 2010; Yan et al., 2015). If compounds involved in host plant resistance are induced, systematic colonization over long periods by an entomopathogenic fungus in a given plant tissue may not be essential for detrimental effects on insect pests. For instance, terpenoids are an integral part of the plant chemical defense system (Singh and Sharma, 2015). Shrivastava et al. (2015) found that *B. bassiana*-inoculated tomato leaves significantly altered the plants' terpenoid chemistry (α-phellandrene, δ-2-carene, sabinene, and α-humulene) and a monoterpene (myrcene) was detected in *B. bassiana*-treated but not in control plants. Similarly, Gan et al. (2017) found that the concentration of carbon was significantly higher in roots of *B. bassiana*-treated tall fescue plants (*Festuca arundinacea*) compared to control plants. Therefore, even though the fungus might not be detected, induced plant responses might still be present. The effect of endophytic *B. bassiana* on sugarcane biochemistry and the possible interaction with other beneficial endophytes that colonize sugarcane (Rodrigues et al., 2016; Jaber and Ownley, 2017) needs further investigation.

Inoculation method, inoculum concentration and host plant properties are important factors in evaluation of the effect of fungal endophytes as plant growth promoters (Jaber and Enkerli, 2017). Several studies have reported enhanced plant growth following *B. bassiana* inoculation via foliar spray, soil drench, or seed immersion (Reddy et al., 2009; Gurulingappa et al., 2010; Lopez and Sword, 2015; Jaber and Enkerli, 2017). In our study, spraying the leaves and drenching the soil with *B. bassiana* did result in enhanced plant growth (number of sett roots). Sett roots play a significant role in the establishment of the sugarcane plant. In addition, growth of the primary shoot is significantly affected by the growth and functionality of the sett root system (Pankhurst et al., 2004; Blair and Stirling, 2006). It will be worth investigating whether promotion of sett roots through foliar sprays and soil inoculation could confer inoculated plants an advantage to better withstand abiotic stresses such as drought during the germination phase especially in this era of changing climate and extreme weather variability. In terms of plant height, plants that had received stem injection were significantly shorter than control plants. Stem injection involved wounding of the stalk and this action could have affected plant health (Akello et al., 2007; Doccola and Wild, 2012). However, according to Yan et al. (2015), an introduced fungal inoculum may interact with the host plant defense system. The results of this interaction could be beneficial or detrimental. For instance, *B. bassiana* inoculated into tall fescue negatively affected the ability of the plant to regrow after root herbivory infestation (Gan et al., 2017). In faba beans (*Vicia faba*), inoculating the plants with *B. bassiana* did not result in consistent growth promotion (Jaber and Enkerli, 2017). In-depth studies aimed at elucidating the mechanism responsible for enhanced plant growth need to be conducted.

Foliar spray for endophytic establishment of *B. bassiana* could have a potential in sugarcane IPM programs since *B. bassiana* is already known to be effective against arthropod pests that infest sugarcane (Cherry et al., 2004; Tefera and Pringle, 2004; Goble et al., 2012; Wu et al., 2014). In addition, the *B. bassiana* strain used in this study is commercially available and can be sprayed using conventional farm equipment (Legaspi et al., 2000), which would facilitate its use in sugarcane plantations. Future studies will focus on determining *B. bassiana* endophytism effects on sugarcane insect pests, interaction with host-plant's endophytes and elucidating the mechanism responsible for enhanced plant growth.

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