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Beauveria bassiana (balsamo) vuillemin as an endophyte in tissue culture banana (*Musa* spp.)

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Abstract

Beauveria bassiana is considered a virulent pathogen against the banana weevil *Cosmopolites sordidus*. However, current field application techniques for effective control against this pest remain a limitation and an alternative method for effective field application needs to be investigated. Three screenhouse experiments were conducted to determine the ability of *B. bassiana* to form an endophytic relationship with tissue culture banana (*Musa* spp.) plants and to evaluate the plants for possible harmful effects resulting from this relationship. Three Ugandan strains of *B. bassiana* (G41, S204 and WA) were applied by dipping the roots and rhizome in a conidial suspension, by injecting a conidial suspension into the plant rhizome and by growing the plants in sterile soil mixed with *B. bassiana*-colonized rice substrate. Four weeks after inoculation, plant growth parameters were determined and plant tissue colonization assessed through re-isolation of *B. bassiana*. All *B. bassiana* strains were able to colonize banana plant roots, rhizomes and pseudostem bases. Dipping plants in a conidial suspension achieved the highest colonization with no negative effect on plant growth or survival. *Beauveria bassiana* strain G41 was the best colonizer (up to 68%, 79% and 41% in roots, rhizome and pseudostem base, respectively) when plants were dipped. This study demonstrated that, depending on strain and inoculation method, *B. bassiana* can form an endophytic relationship with tissue culture banana plants, causing no harmful effects and might provide an alternative method for biological control of *C. sordidus*.

Keywords: Banana; Banana weevil; *Beauveria bassiana*; *Cosmopolites sordidus*; Endophyte; Microbial control; *Musa*; Tissue culture

1. Introduction

The East African highland banana (*Musa* spp., genome group AAA-EA) is an important food and cash crop in the Great Lakes Region of Eastern Africa. However, banana production is threatened by the banana weevil, *Cosmopolites sordidus* (Coleoptera: Curculionidae). This pest has been a principal factor in the decline and disappearance of highland banana from its traditional growing areas in central Uganda and western Tanzania (Gold et al., 1999; Mbwana and Rukazambuga, 1999). Banana weevil larvae tunnel in the rhizome and pseudostem, damaging the vascular system, interfering with nutrient uptake, and reducing plant stability. Yield losses, attributable to snapping, toppling, reduced bunch weights and disappearance of banana mats that fail to produce suckers, can exceed 50% (Rukazambuga et al., 1998; Gold et al., 2004).

The biology and integrated pest management of the banana weevil have been reviewed by Gold et al. (2001). Adults have a long lifespan, low fecundity, hydrotropism, nocturnal activity, limited dispersal and slow population growth. They are most commonly in close association with banana mats and crop residues. Eggs are laid in the rhizome or lower pseudostem. Damage to the rhizome central cylinder appears to have the greatest effect on yield

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(Gold et al., 2005). Control strategies directed at banana weevil immatures within the plant are likely to be more beneficial than those targeting adults.

Beauveria bassiana offers a promising option for the management of the banana weevil. Strains virulent against the banana weevil have been identified in East Africa (Nankinga, 1994), West Africa (Godonou et al., 2000) and Latin America (reviewed by Gold et al., 2001). For example, laboratory bioassays using different *B. bassiana* strains originating from insect cadavers and soil samples in Uganda resulted in adult banana weevil mortalities of up to 100% (Nankinga, 1994). In laboratory bioassays conducted by Godonou et al. (2000), *B. bassiana* strain IMI330194 was identified as a possible control agent for *C. sordidus* based on its virulence and its potential for mass production.

Although virulent *B. bassiana* strains have been identified against the banana weevil, field studies have yielded suboptimal results. Whereas the damaging larval stages are protected within the plant rhizome, field applications have targeted banana weevil adults. Nankinga (1999) applied 500 g of maize bran containing *B. bassiana* conidia to the topsoil around banana mats. Four weeks after *B. bassiana* application, 48% of adult banana weevils in treated plots were infected, while 20% of adults were infected five months after application. In field experiments performed by Godonou et al. (2000), *B. bassiana* was applied as either an oil palm kernel cake-based formulation or as a conidial powder to planting holes and suckers. The cake-based formulation caused 42% mortality among adult banana weevils, but only 6% of adults were infected by applications of conidial powder. These and other studies suggest that field performance of *B. bassiana* against banana weevils may be constrained by abiotic factors (Hallsworth and Magan, 1999; Nankinga, 1999; Bruck and Lewis, 2002). In addition, the high cost of inoculum production and delivery is a serious bottleneck precluding delivery to farmers (Nankinga, 1999; Gold et al., 2001). Hence, there is need to develop an efficient and cost-effective delivery system for *B. bassiana* against *C. sordidus* larvae in East Africa.

It is now known that *B. bassiana* can form an endophytic association with maize (*Zea mays*) (Bing and Lewis, 1991, 1992; Cherry et al., 1999; Lewis et al., 2001) and coffee (*Coffea arabica*) (Posada and Vega, 2005). For example, Bing and Lewis (1991) demonstrated that *B. bassiana* strain ARSEF 3113 could invade the maize plant via the epidermis, persist during the entire growing season in the plant and reduce tunneling by *Ostrinia nubilalis* (Lepidoptera: Pyralidae). The fungus was recovered from the plants several weeks after application and subsequently demonstrated no loss of virulence to *O. nubilalis* after direct application (Bing and Lewis, 1992). Studies in West Africa also demonstrated that local strains of *B. bassiana* can exist endophytically within maize and provide protection against *Sesamia calamistis* (Lepidoptera: Noctuidae) (Cherry et al., 1999).

Whether *B. bassiana* can exist as an endophyte in the banana plant is currently unknown. Other fungal species, such as non-pathogenic *Fusarium oxysporum* (Ascomycota: Hypocreales) have been reported to be naturally associated with banana plants as endophytes (Griesbach, 2000). These endophytes act as antagonists against pests and diseases and also confer resistance to the plant against abiotic stresses (Dubois et al., 2006b). Research is currently focusing at reintroducing these naturally occurring endophytes into banana plants with commercial tissue culture producers, before the plants are sold to farmers (Dubois et al., 2006a). The use of *B. bassiana* as an artificial endophyte in banana would potentially solve the constraints limiting its field application. Endophytic *B. bassiana* would not be exposed to abiotic factors and would require little inoculum, drastically reducing its application costs. Furthermore, once established as an endophyte, *B. bassiana* might offer the most suitable protection against the damaging larvae in the rhizome and pseudostem. The current study was conducted to determine if East African strains of *B. bassiana* can be established as an endophyte in banana and what effects endophytic *B. bassiana* might have on plant growth.

2. Materials and methods

2.1. Experimental site and design

Three screenhouse experiments were conducted at the International Institute of Tropical Agriculture (IITA) in Namulonge, Uganda. Namulonge is 28 km northeast of Kampala, Uganda (0°32′N, 32°35′E), 1260 m.a.s.l., with an average temperature of 22 °C and relative humidity of 65%. In all experiments, three *B. bassiana* strains (G41, S204 and WA) were inoculated in tissue culture banana plants (cv. Kibuzi, AAA-EA) using three different methods: (1) root and rhizome dip in a conidial suspension, (2) injection of a conidial suspension into the plant, and (3) use of a solid substrate inoculum. Ten plants were used per strain × inoculation method combination. In addition, 10 plants each were injected with water or treated with a fungus-free solid substrate. The 10 plants that were dipped in fungus-free water (representing the control treatment of the dip inoculation method) were considered controls for establishing normal plant growth. Keeping banana tissue culture plants in water is standard procedure during their postflask management (Vuylsteke and Talengera, 1998). The 12 treatments were organized as a completely randomized design.

In experiment 1, high conidial dosages were used (300 ml of 1.5×10^{10} conidia/ml for the root and rhizome dip method, 2 ml of 10^8 conidia/ml for the conidial injection method and 1.6% (w/v) for the solid substrate method). In experiments 2 and 3, the inoculum concentration was maintained for the root and rhizome dip method but reduced for the conidial injection method (to 1 ml of

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10^8 conidia/ml) and the solid substrate method (to 1% (w/v)). Experiment 3 acted as a repeat of experiment 2.

2.2. Fungal strains

Three Ugandan *B. bassiana* strains (G41, S204 and WA) were selected on the basis of high virulence (92–97% mortality) against *C. sordidus*, high sporulation (4.49×10^{10}–1.16×10^{11} conidia/ml) and origin of isolation (G41 and S204 from soil in banana plantation fields and WA from *C. sordidus*) (Nankinga, 1994, 1999). The strains were obtained from the Ugandan National Banana Research Programme at the Kawanda Agricultural Research Institute, where they had been stored on silica gel at 21–24 °C and a relative humidity of 55–78%. Silica gel containing conidia and mycelium was sprinkled on Sabouraud dextrose agar medium supplemented with yeast extract (SDAY) (10 g peptone, 20 g dextrose, 5 g yeast extract and 15 g agar/l distilled water) and containing antibiotics (0.1 g penicillin, 0.2 g streptomycin and 0.05 g chlortetracycline/l SDAY) in 55 mm diameter Petri dishes. Three Petri dishes were used per strain. The Petri dishes containing the fungi were incubated for three weeks in the laboratory (22–30 °C and a photoperiod of ~12:12 h).

2.3. Tissue culture plants

Plants were propagated in vitro using a shoot tip culture protocol for banana multiplication (Vuylsteke, 1998). Young suckers were selected from healthy and true-to-type mother plants. Outer leaf sheaths, leaf bases and rhizome tissues were trimmed off each sucker until a 2–4 cm^3 cube enclosing the shoot apex was obtained. The cubes were soaked in a solution of 15% (v/v) NaOCl and 0.5 µl/ml Tween 20 for 15 min, a solution of 70% (v/v) EtOH for 5 min, and a a solution of 15% (v/v) NaOCl and 0.5 µl/ml Tween 20 for 15 min, respectively. Finally, cubes were rinsed thrice with sterile deionized water. In a laminar flow cabinet, cubes were further reduced to 1 cm^3. Each cube was bisected into two equal parts and each part inoculated in 18 ml sterile multiplication medium (MM) in 250 ml glass containers. The MM medium contained Murashige and Skoog (1962) mineral salt medium modified by Vuylsteke (1998) by reducing $MnSO_4$ from 22.3 to 16.9 mg/l, and supplemented with 30 g sucrose, 40 mg thiamine-HCl, 200 mg glycine, 50 mg pyridoxine-HCl, 50 mg nicotinic acid, 1 g ascorbic acid, 5 mg benzylaminopurine and 2.3 g phytagel/l distilled water. The cultures were incubated at 27 °C and a photoperiod of 16:8 h. After eight weeks, newly sprouted adventitious buds induced from meristematic tissue were separated and subcultured on fresh MM. After three months, shoots were transferred singly to 100 ml rooting medium in 25×150 mm culture test tubes. Ingredients for the rooting medium were identical as those of the MM after omission of benzylaminopurine and amendment with 0.1 mg naphthalene acetic acid/l sterile distilled water. Four weeks after root development, plants were removed from the rooting medium, and their roots and rhizomes washed with tap water. Each plant was transferred singly to sterile nutrient solution containing 1 g/l Poly-Feed (Haifa Chemicals, Haifa, Israel) in 250 ml sterile tap water in 300 ml plastic cups. A sponge wrapped around the pseudostem base provided support when plants were placed in the nutrient solution through a hole made in the lid. The plants were grown in a humidity chamber for four weeks at 19–32 °C under natural light conditions. The humidity chamber was constructed using a wooden frame ($2 \times 1 \times 3$ m) that was completely covered with a transparent polythene sheet. The nutrient solution was changed weekly.

2.4. Inoculum preparation

After three weeks, for each strain, conidia were gently scraped from the three Petri dishes containing *B. bassiana* cultures and suspended in 20 ml sterile water containing 0.01% Tween 80. The conidial suspension was equally transferred with a sterile pasture pipette onto SDAY in 90 mm diameter Petri dishes, using 25 Petri dishes per strain. The Petri dishes were incubated for three weeks in the laboratory. Petri dish lids were removed and the cultures were air-dried overnight in a laminar airflow cabinet. To prevent cross-contamination, each fungal strain was dried and harvested separately. Conidia were harvested by gently scraping them off the surface of the dried medium onto a 200 mm diameter sieve (150 µm pore size) using a sterile scalpel blade. The conidial powder was forcefully collected into a sterile 250 mm diameter container through vigorous rubbing of the conidia and mycelium against the sieve mesh. The conidial powder was weighed and dried overnight in a 6 dm^3 desiccator. Conidial concentration for each strain was determined by dissolving 0.1 g conidial powder in 10 ml sterile deionized water containing 0.01% Tween 80 in a sterile 20 ml bottle. After vortexing for 1 min, serial dilutions of $\times 0.1$ and $\times 0.01$ were made, and the conidial concentration determined using an improved Neubauer haemocytometer. Conidial concentrations for each strain were: $120.4 \pm 3.5 \times 10^9$ conidia/g (G41; mean ± standard error), $49.9 \pm 0.7 \times 10^9$ conidia/g (S204) and $44.9 \pm 0.7 \times 10^9$ conidia/g (WA) for experiment 1; $3190.8 \pm 6.6 \times 10^9$ conidia/g (G41), $515.6 \pm 18.2 \times 10^9$ conidia/g (S204) and $12.6 \pm 0.9 \times 10^9$ conidia/g (WA) for experiment 2; $305.5 \pm 41.2 \times 10^9$ conidia/g (G41), $422.5 \pm 25.7 \times 10^9$ conidia/g (S204) and $50.5 \pm 5.5 \times 10^9$ conidia/g (WA) for experiment 3.

For each strain, conidial suspensions were made by adding 0.15–0.75 g conidial powder (depending on conidial concentration of the strains) in 300 ml sterile water containing 0.01% Tween 80 in a sterile 500 ml bottle. Conidial concentration was adjusted to 1.5×10^{10} conidia/ml, yielding the dip suspension. Inoculum preparation for the injection suspension followed the same procedure except that final conidial concentration was adjusted to 1×10^8 conidia/ml.

A solid substrate inoculum was prepared by boiling 1 kg of washed milled rice (*Oryza sativa*) grains in 300 ml tap

water until all the water was absorbed by the seeds (Nankinga, 1994). For each strain, 1 kg of the substrate was weighed and put in five 500 ml Erlenmeyer flasks in equal aliquots of 200 g, sterilized and cooled for 24 h prior to inoculation. For each strain, a 10 ml suspension of 10^8 conidia/ml was prepared and added in equal aliquots to the five Erlenmeyer flasks. Five Erlenmeyer flasks containing sterile rice substrate were inoculated with 2 ml each of sterile water containing 0.01% Tween 80 and acted as controls. Erlenmeyer flasks were incubated in the laboratory for three weeks and shaken daily. On the day of plant inoculation, the rice substrate from each flask was pooled among Erlenmeyer flasks and thoroughly mixed, and the conidial concentration was determined by vortexing 10 g of the solid substrate for 1 min in 100 ml of sterile water containing 0.01% Tween 80. Conidial concentrations for each strain were: $1.23 \pm 0.03 \times 10^9$ conidia/g (G41), $2.79 \pm 0.03 \times 10^9$ conidia/g (S204) and $0.51 \pm 0.01 \times 10^9$ conidia/g (WA) for experiment 1; $0.27 \pm 0.01 \times 10^9$ conidia/g (G41), $5.11 \pm 0.12 \times 10^9$ conidia/g (S204) and $0.38 \pm 0.02 \times 10^9$ conidia/g (WA) for experiment 2; $0.11 \pm 0.03 \times 10^9$ conidia/g (G41), $8.05 \pm 0.06 \times 10^9$ conidia/g (S204) and $0.08 \pm 0.02 \times 10^9$ conidia/g (WA) for experiment 3.

2.5. Plant inoculation

Banana tissue culture plants were inoculated with *B. bassiana* strains using three different methods: (1) root and rhizome dip in a conidial suspension, (2) injection of a conidial suspension into the plant and (3) use of a solid substrate inoculum.

2.5.1. Root and rhizome dip

Plants were removed from the nutrient solution and their root tips broken at 20–50 cm depending on the length of the root. The roots and rhizomes were dipped in 300 ml of the 1.5×10^{10} conidia/ml suspension for 90 min. Control plants were dipped in 300 ml sterile water containing 0.01% Tween 80. Subsequently, plants were planted in 3 l polythene bags containing steam-sterilized loamy forest soil.

2.5.2. Injection

Plants were removed from the nutrient solution and their rhizomes injected with 2 ml (experiment 1) or 1 ml (experiments 2 and 3) of the 10^8 conidia/ml suspensions using 1 ml insulin injection needles (Becton Dickinson, Dublin, Ireland). Fungus-free plants were injected with 2 ml (experiment 1) or 1 ml (experiments 2 and 3) of sterile water containing 0.01% Tween 80. Plants were subsequently planted in 3 l black polythene bags containing steam-sterilized soil.

2.5.3. Solid substrate

For each plant, 1.6% (w/v) (50 g:3000 ml) (experiment 1) or 1.0% (w/v) (30 g:3000 ml) (experiments 2 and 3) of rice substrate containing *B. bassiana* was mixed evenly with 3 l steam-sterilized soil. The soil-rice substrate mixture was dispensed into 3 l black polythene potting bags. Control plants were planted in steam-sterilized soil mixed with 50 g (experiment 1) or 30 g (experiments 2 and 3) sterile rice grains.

2.6. Collection of data

Plants were kept in a screenhouse (~26 °C and natural light conditions of ~12:12 h) and watered daily. Four weeks after inoculation, plant growth parameters were determined. Plant height (the distance from the base of the plant to the youngest leaf axil), number of fully developed leaves, and width (widest part of the lamina) and length (the distance from the leaf apex to the leaf stalk) of the youngest leaf were recorded. Number of fully developed leaves, and width and length of the youngest leaf, are indications of photosynthetic capacity and disease incidence, respectively (Carlier et al., 2002). The plants were then removed from the soil, and their roots and rhizomes thoroughly washed under running tap water to remove soil. Pseudostems were cut off (about 2 cm above the pseudostem base) from the rhizomes using a sterile blade. After determining fresh shoot weight (pseudostem together with leaves), the shoots were dried in a hot air oven at 60 °C for 48 h and dry shoot weight was recorded.

For each plant, the roots were pared from the rhizome and three live roots were randomly selected for re-isolation. The selected roots, together with the rhizome containing the pseudostem base, were sterilized in a laminar airflow cabinet by dipping in 5% NaOCl containing 0.05% Tween 80 followed by dipping in 75% EtOH for 1 min. The plant parts were rinsed thrice in sterile deionized water. The rhizome was cut off from the pseudostem base and six cubes (0.2–0.4 cm^3) were obtained from both the rhizome and the pseudostem base using a sterile scalpel blade. Six root pieces (0.4–0.5 cm long) were cut from each sampled root using a sterile scalpel blade. The six pieces for each sampled plant part were placed singly on SDAY in 55 mm diameter Petri dishes. The medium was supplemented with antibiotics (0.1 g penicillin, 0.2 g streptomycin sulphate, 0.25 g chloramphenicol and 0.05 g chlortetracycline/l SDAY) to prevent bacterial contamination, and 0.75 mg/l 50% (w/w) benomyl (Benlate, Dupont, Wilmington, USA) to eliminate other fungi.

The Petri dishes were incubated for two weeks in the laboratory, after which all plant pieces were visually examined for fungal outgrowth. A fungal colony was characterized as *B. bassiana* based on white dense mycelia, becoming cream to pale yellow at the edge (Humber, 1997). In all cases where there was contamination or potential confusion with other fungal taxa, both mycelium and conidia were removed using a sterile needle and mounted in a drop of water on a microscope slide. The mounted slide was examined microscopically for characteristic *B. bassiana* features (globose conidia and zigzag-shaped conidiophores) (Humber, 1997). Percentage colonization was calculated

as number of pieces exhibiting *B. bassiana* outgrowth per total number of pieces.

2.7. Statistical analysis

Plant height, leaf length and width, and fresh and dry shoot weight were analyzed using analysis of variance (ANOVA). Prior to analysis, data were tested for normality and homogeneity of variance. Fresh shoot weight was square-root transformed to obtain a normally distributed data set with homogenous variance among treatments. If different, treatment means were separated using Tukey's studentized range test and groups of treatment means compared using linear orthogonal contrasts. Since most plants inoculated with the solid substrate in experiment 1 died (see Section 3), this treatment was not included in the ANOVA analysis. Plant survival was analyzed using logistic regression in experiments 1 and 3. If differences among treatments were detected, likelihood ratio tests were performed to separate treatment means. Number of leaves was modeled as an underdispersed Poisson distribution. In all the experiments, percentage colonization was analyzed using logistic regression. Alpha-levels for pairwise mean comparisons were adjusted according to the Dunn–Sidak correction to obtain overall α-levels of 0.05. In experiment 1, since most plants inoculated with the solid substrate died (see Section 3), this treatment was not included in the analyses of percentage colonization (Ury, 1976; SAS Institute, 1989; Sokal and Rohlf, 1995).

3. Results

3.1. Beauveria bassiana colonization

In all experiments, *B. bassiana* was reisolated from nearly all plants that had been inoculated with the fungus (experiment 1: 95% of plants; experiment 2: 91% of plants; experiment 3: 90% of plants). *Beauveria bassiana* was not isolated from any of the control plants. Colonization was significantly different among inoculation methods (experiment 1: $\chi^2 = 66.39$, df = 1, $P < 0.0001$; experiment 2: $\chi^2 = 87.10$, df = 2, $P < 0.0001$; experiment 3: $\chi^2 = 27.41$, df = 2, $P < 0.0001$) (Fig. 1). In experiment 1, percentage colonization by *B. bassiana* was significantly higher for plants dipped in a conidial suspension compared to plants injected with a conidial suspension (likelihood ratio test, $P < 0.016$). In experiment 2, percentage *B. bassiana* colonization for plants injected with a conidial suspension and those dipped in a conidial suspension were significantly higher than percentage colonization in plants grown in soil mixed with solid substrate inoculum (likelihood ratio test, $P < 0.016$). In experiment 3, plants dipped in a conidial suspension had significantly higher percentage colonization than those injected with a conidial suspension, which in turn had significantly higher percentage colonization than plants grown in soil mixed with solid substrate inoculum (likelihood ratio test, $P < 0.016$).

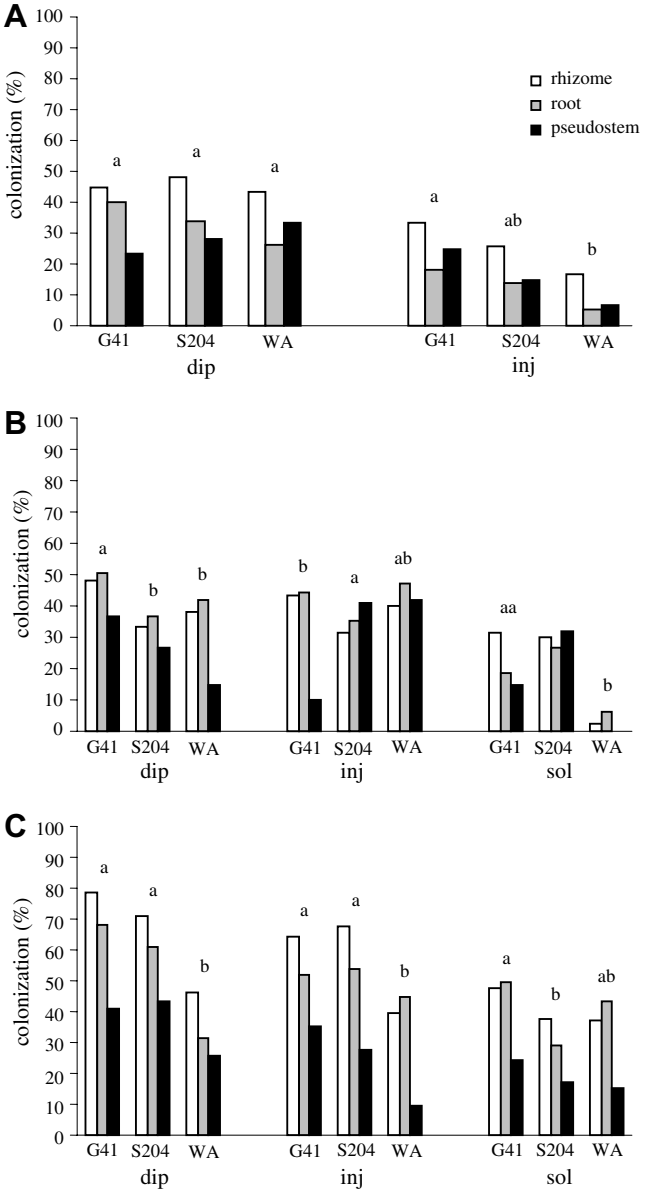

Fig. 1. Percentage colonization of tissue culture banana plant parts (rhizome, roots and pseudostem bases) (cv. Kibuzi, AAA-EA) by *Beauveria bassiana* strains G41, S204 and WA. Dip: root and rhizome dip inoculation method; inj: conidial injection method; sol: solid substrate inoculation method. A, B and C represent percentage colonization in experiments 1, 2 and 3, respectively. Within each inoculation method, likelihood ratio tests were performed for colonization among strains (after pooling colonization among plant parts for each strain). Sets of bars representing a particular strain across plant parts are not significantly different from each other when labeled with the same letter ($P > 0.05$). Sample size = 60 (rhizome and pseudostem base) and 180 (roots) samples/treatment.

Percentage *B. bassiana* colonization also varied significantly among the three strains (experiment 1: $\chi^2 = 16.63$, df = 2, $P = 0.0002$; experiment 2: $\chi^2 = 37.30$, df = 2, $P < 0.0001$; experiment 3: $\chi^2 = 29.16$, df = 2, $P < 0.0001$) (Fig. 1). Moreover, there was an interaction between *B. bassiana* strain and inoculation method in all the experiments (experiment 1: $\chi^2 = 11.34$, df = 2, $P = 0.0034$;

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experiment 2: $\chi^2 = 50.82$, df = 4, $P < 0.0001$; experiment 3: $\chi^2 = 13.96$, df = 4, $P = 0.0074$). In experiment 1, only plants injected with a conidial suspension showed significant difference in colonization among strains, with colonization by strain G41 being significantly higher than that by strain WA. In experiment 2, strain G41 colonized plants better than strains S204 and WA in plants dipped in a conidial suspension. However, when injected, strain S204 colonized plants better than strain G41. In experiment 3, strains S204 and G41 colonized plants better than strain WA when dipped or injected.

Percentage *B. bassiana* colonization varied significantly among plant parts (experiment 1: $\chi^2 = 27.64$, df = 2, $P < 0.0001$; experiment 2: $\chi^2 = 15.98$, df = 2, $P = 0.0003$; experiment 3: $\chi^2 = 69.65$, df = 2, $P < 0.0001$). In experiment 1, percentage rhizome colonization was significantly higher than percentage pseudostem base colonization but not percentage root colonization (likelihood ratio test, $P < 0.016$). In experiment 2, percentage rhizome and root colonization were significantly higher than percentage pseudostem base colonization (likelihood ratio test, $P < 0.016$). Similarly, in experiment 3, percentage rhizome and root colonization were significantly higher than percentage pseudostem base colonization (likelihood ratio test, $P < 0.016$).

3.2. Plant survival and growth

In experiment 1, plant survival was significantly affected by inoculation method ($\chi^2 = 68.81$, df = 2, $P < 0.0001$). Most plants (71%) inoculated with a solid substrate died, whereas those dipped or injected with a conidial suspension all survived. Plant survival was not influenced by whether or not the inoculum contained *B. bassiana* ($\chi^2 = 3.65$, df = 3, $P = 0.30$). All plant growth parameters (plant height, leaf length and width, fresh and dry shoot weight, and number of standing leaves) were significantly higher for plants dipped in a conidial suspension than for those injected with a conidial suspension ($F \geqslant 39.74$ or $\chi^2 = 5.83$, df = 1, $P \leqslant 0.016$) (Table 1a). Within plants that were dipped, there was no difference among any of the plant growth parameters between those that were dipped in a *B. bassiana* suspension or those that were dipped in a control ($F \leqslant 1.48$ or $\chi^2 = 3.98$, df = 3, $P \geqslant 0.24$).

In experiment 2, plant survival was not affected and, with the exception of one dead plant inoculated using a solid substrate, all plants survived. With the exception of number of leaves ($\chi^2 = 0.29$, df = 2, $P = 0.86$), all other plant growth parameters varied among inoculation methods ($F \geqslant 4.43$, df = 2, $P \leqslant 0.014$) (Table 1b). Plant height, leaf length and width, and fresh and dry shoot weight of plants dipped in a conidial suspension were significantly higher than those of plants injected with a conidial suspension. Only leaf length and leaf width of plants dipped in a conidial suspension was significantly higher than leaf length and width of plants inoculated using a solid substrate. None of the plant growth parameters was influenced by whether or not the inoculum contained *B. bassiana* ($F \leqslant 1.13$ or $\chi^2 = 0.060$, df = 3, $P \geqslant 0.34$).

In experiment 3, plant survival was significantly affected by the inoculation method ($\chi^2 = 9.71$, df = 2, $P = 0.0078$). Using a solid substrate, 33% of the plants died, whereas 93% of plants dipped or injected with conidial suspension survived. All plant growth parameters varied among inoculation methods ($F \geqslant 3.25$ or $\chi^2 = 8.14$, df = 2, $P \leqslant 0.043$) (Table 1c). Plant height, leaf length and width, and fresh and dry shoot weight of plants dipped in a conidial suspension were significantly higher than those of plants injected with a conidial suspension. Among the surviving plants grown in soil mixed with solid substrate inoculum, plant height and leaf length showed no significant difference from those dipped in conidial suspension. However, leaf width, number of standing leaves, and fresh and dry shoot weight of plants dipped in a conidial suspension were significantly higher than those inoculated using a solid substrate. With the exception of leaf width ($F \leqslant 3.40$, df = 3, $P \geqslant 0.021$) and number of standing leaves ($F \leqslant 9.36$, df = 3, $P \geqslant 0.025$), all the plant growth parameters were not influenced by whether or not the inoculum contained *B. bassiana* ($\chi^2 \leqslant 2.24$, df = 3, $P \geqslant 0.12$). Compared with control plants, only plants inoculated with strain WA had a significantly lower leaf length and number of standing leaves (likelihood ratio test, $P < 0.016$).

4. Discussion

Four weeks after inoculation, *B. bassiana* was successfully reisolated from the interior of roots, rhizomes and pseudostem bases, clearly demonstrating that *B. bassiana* was able to establish an endophytic relationship with tissue culture banana plants. The use of *B. bassiana* as a banana endophyte might solve the constraints limiting its field application: endophytic *B. bassiana* would not be exposed to abiotic factors and would require little inoculum, drastically reducing its application costs. Equally important, application of *B. bassiana* as an endophyte is the only technique that allows the entomopathogen to target the damaging larvae inside the banana plant, rather than the adults, and might therefore be more effective.

Beauveria bassiana colonization differed among plant parts (roots, rhizomes and pseudostem bases). Roots and rhizomes were colonized to a higher extent than pseudostem bases. The reason for higher colonization in roots and rhizomes are unclear, but could be caused by different microbial and physiological conditions present among the plant parts. Many endophytic fungi show a certain degree of tissue specificity because they are adapted to particular conditions present in a given organ (Carroll et al., 1977; Bertoni and Cabral, 1988; Fisher et al., 1991).

Colonization in plants dipped in a conidial suspension was higher than in those that were inoculated using a solid substrate. Presumably, for plants dipped in a conidial suspension, the roots and rhizome provided the highest surface area for *B. bassiana* conidia attachment. For plants

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Table 1
Effects of *Beauveria bassiana* application methods (root and rhizome dip, injection and solid substrate) on growth of tissue culture banana plants (cv. Kibuzi, AAA-EA) four weeks after inoculation

Inoculation method[a]	Growth parameter					
	Plant height (cm)	Leaf length (cm)	Leaf width (cm)	Fresh weight (g)	Dry weight (g)	Number of leaves
Experiment 1						
Root and rhizome dip						
Average	9.1 ± 0.3a	18.9 ± 0.4a	8.7 ± 0.2a	13.2 ± 0.6a	1.4 ± 0.1a	5.6 ± 0.1a
B. bassiana	9.4 ± 0.3	18.8 ± 0.4	8.9 ± 0.2	13.8 ± 0.7	1.5 ± 0.1	5.7 ± 0.1
Control	8.0 ± 0.6	19.3 ± 0.9	8.3 ± 0.4	11.5 ± 1.1	1.3 ± 0.1	5.6 ± 0.2
Conidial injection						
Average	4.5 ± 0.3b	11.2 ± 0.7b	4.8 ± 0.3b	11.2 ± 0.6b	0.7 ± 0.1b	5.2 ± 0.2b
B. bassiana	4.3 ± 0.3	11.3 ± 0.7	4.9 ± 0.3	6.5 ± 0.7	0.7 ± 0.1	5.1 ± 0.2
Control	5.3 ± 0.6	10.7 ± 1.5	4.7 ± 0.7	6.9 ± 0.9	0.8 ± 0.1	5.4 ± 0.3
Experiment 2						
Root and rhizome dip						
Average	12.7 ± 0.6a	23.1 ± 0.5a	11.8 ± 0.2a	23.4 ± 1.1a	1.8 ± 0.1a	4.5 ± 0.1a
B. bassiana	12.5 ± 0.6	23.1 ± 0.6	11.9 ± 0.3	23.7 ± 1.3	1.9 ± 0.1	4.4 ± 0.1
Control	13.3 ± 1.5	23.3 ± 1.0	11.6 ± 0.3	24.4 ± 1.9	1.8 ± 0.2	4.9 ± 0.2
Conidial injection						
Average	10.1 ± 0.5b	16.3 ± 0.8 c	8.1 ± 0.4b	19.2 ± 1.2b	1.3 ± 0.1b	4.6 ± 0.2a
B. bassiana	9.6 ± 0.6	15.7 ± 0.9	7.8 ± 0.4	18.5 ± 1.4	1.2 ± 0.1	4.5 ± 0.2
Control	11.4 ± 1.0	18.1 ± 1.3	9.0 ± 0.4	21.5 ± 2.4	1.6 ± 0.1	4.9 ± 0.3
Solid substrate						
Average	12.8 ± 0.5a	20.9 ± 0.5b	10.6 ± 0.3b	20.2 ± 1.2ab	1.5 ± 0.1ab	4.5 ± 0.2a
B. bassiana	13.2 ± 0.6	21.5 ± 0.5	10.8 ± 0.3	19.8 ± 1.0	1.6 ± 0.1	4.7 ± 0.2
Control	11.6 ± 1.4	19.8 ± 1.3	10.0 ± 0.9	21.3 ± 3.8	1.3 ± 0.3	3.8 ± 0.4
Experiment 3						
Root and rhizome dip						
Average	10.3 ± 0.7a	21.1 ± 0.9a	9.4 ± 0.7a	22.4 ± 1.9a	2.3 ± 0.2a	4.0 ± 0.2a
B. bassiana	10.4 ± 0.9	20.4 ± 1.0	9.2 ± 0.8	22.4 ± 2.3	2.3 ± 0.3	3.8 ± 0.2
Control	10.3 ± 1.1	23.0 ± 1.5	9.8 ± 1.1	22.1 ± 3.5	2.3 ± 0.4	4.6 ± 0.2
Conidial injection						
Average	7.9 ± 0.7b	16.6 ± 0.9b	7.1 ± 0.5b	15.6 ± 1.8b	1.6 ± 0.2b	4.0 ± 0.2a
B. bassiana	7.2 ± 0.7	15.8 ± 1.1	6.4 ± 0.3	14.1 ± 1.9	1.4 ± 0.2	3.7 ± 0.3
Control	9.8 ± 1.9	18.9 ± 1.6	9.1 ± 1.4	20.0 ± 4.2	2.1 ± 0.4	4.0 ± 0.2
Solid substrate						
Average	8.5 ± 0.7ab	18.2 ± 0.9ab	7.4 ± 0.5b	10.8 ± 1.8b	1.2 ± 0.2b	3.3 ± 0.4b
B. bassiana	8.0 ± 0.8	17.6 ± 1.1	7.0 ± 0.5	9.3 ± 1.8	1.0 ± 0.1	3.2 ± 0.4
Control	9.7 ± 1.4	19.3 ± 1.7	8.4 ± 0.9	15.2 ± 4.5	1.7 ± 0.5	3.4 ± 0.6

Plant height represents the distance from the soil level to the youngest leaf axil, leaf length represents distance from the leaf apex to the leaf stalk of the youngest leaf, leaf width represents width at the widest part of the lamina of the youngest leaf, fresh weight represents fresh shoot (pseudostem together with leaves) weight and dry weight represents dry shoot weight. Within each of the inoculation methods, '*B. bassiana*' represents averaged growth parameters of plants inoculated with one of three strains (G41, S204 or WA); 'control' represents growth parameters of plants inoculated with water or treated with a fungus-feee solid substrate; 'average' represents average growth parameters. For each of the inoculation methods, means followed by the same letter within columns are not significantly different ($P > 0.05$). Sample size = 40 plants/treatment.

[a] Root and rhizome dip, plants dipped in 300 ml conidial suspension containing 1.5×10^{10} conidia/ml; injection, inoculation with 2 ml (experiment 1) or 1 ml (experiments 2 and 3) conidial suspension containing 1×10^{8} conidia/ml; solid substrate, plants grown in sterile soil mixed with 1.6% (w/v) (experiment 1) or 1.0% (experiments 2 and 3) solid substrate inoculum.

grown in soil mixed with a solid substrate, there is a possibility that the roots and rhizome were unable to come in contact with the conidia. Also, plants were watered daily and this might have led to vertical loss of conidia through water filtration, reducing their chances of uptake by the roots. Hyphal penetration of *B. bassiana* from the banana plant surfaces into the tissues seemed not to be a limiting factor, because colonization in plants dipped in a conidial suspension was equal to or higher than colonization in those injected with a conidial suspension.

Beauveria bassiana strains G41 and S204, both originally isolated from soil, colonized plant tissues better than strain WA, originally isolated from a banana weevil cadaver. Based on the origin of the strains and the results obtained in this study, we hypothesize that *B. bassiana* strain WA is less adapted to a saprophytic lifestyle which is needed to endophytically colonize banana tissue culture plants. This hypothesis was further confirmed by a slower growth rate of *B. bassiana* strain WA on the different liquid and solid media in the laboratory prior to inoculation. Differences between *B. bassiana* strains G41 and S204 were less noted, but *B. bassiana* strain G41 colonized plants to an equal or higher extent than *B. bassiana* strain S204 when plants were dipped in a conidial suspension.

In experiments 1, plants died after inoculation using a solid substrate. Interestingly, it did not matter whether or not the inoculum contained *B. bassiana*. Plant mortality was avoided in experiment 2 after reduction of the inoculum concentration from 1.6% to 1.0% (w/v), indicating that for a solid substrate inoculation method, plant survival depends on the quantity of solid substrate inoculum used. However, although the same quantity of solid substrate inoculum was used in experiments 2 and 3, mortality among plants inoculated with a solid substrate was noted in experiment 3. In experiment 3, some plants showed symptoms of wilting and vascular bundle discoloration, which are associated with Fusarium vascular wilt caused by *Fusarium oxysporum* (Agrios, 1997). These observations were not made in experiment 2. Reduced plant fitness due to systemic Fusarium wilt infection thus might have influenced plant survivability in experiment 2.

Plant growth depended on inoculation method. Interestingly again, it did not matter whether or not the inoculum contained *B. bassiana*. Injecting plants with a volume of 1 or 2 ml reduced plant growth. Using the solid substrate inoculation method, plant death could be lowered by reducing the inoculum concentration from 1.6% to 1.0% (w/v), but even at the low inoculum concentration of 1.0% (w/v), plant growth was negatively affected. Dipping the plants in a suspension containing *B. bassiana* conidia did not affect plant growth. In maize, Lewis et al. (2001) also found no differences in plant growth between *B. bassiana*-treated and control plants when plants were dipped in a conidial suspension.

The results of this research indicate that *B. bassiana* can form an endophytic relationship with tissue culture banana plants. Based on *B. bassiana* colonization, and plant survival and growth, dipping plants in a conidial suspension was the best method for delivery of *B. bassiana* into tissue culture banana plants, since colonization was highest and no adverse plant effects were noted. If the dip inoculation method is used, *B. bassiana* strain G41 appears to achieve the highest percentage colonization. This study provides a foundation on which further investigations can be based. In this study, colonization was assessed after four weeks in one banana cultivar. Nothing is known about the colonization and persistence of endophytic *B. bassiana* in other banana cultivars and after longer periods of time. Concurrently, virulence of endophytic *B. bassiana* against the banana weevil needs to be investigated.

5. Uncited reference

Schuster et al. (1995).

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