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ORIGINAL ARTICLE

Endophytic colonization by *Beauveria bassiana* and *Metarhizium anisopliae* induces growth promotion effect and increases the resistance of cucumber plants against *Aphis gossypii*

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Abstract

The entomopathogenic fungi (EPF) are characterized as fungi with various functions and numerous mechanisms of action. The ability to establish themselves as beneficial endophytes provides a sound ground for their exploitation in crop production and protection. The purpose of this study was to evaluate the entomopathogenic strains of *Beauveria bassiana* and *Mertarhizium anisopliae* for their potential to colonize cucumber plants under natural environmental conditions in non-sterile substrate. Seed submersion in conidial suspension resulted in systemic colonization of cucumber plants 28 days post-inoculation. Scanning electron microscope micrographs demonstrated that conidia of both fungal genera have adhered, germinated and directly penetrated seed epidermal cells 24 hr post-submersion. Treated with EPF cucumber seeds resulted seedlings tissues of which contained a significantly higher amount of total phenolic compounds and unchanged amounts of chlorophylls. There was a significant negative effect of endophytic colonization on the *Aphis gossypii* population size after 5 days of exposure as well as a positive effect on cucumber growth and development 7 weeks post-inoculation. We suggest that reduction of *A. gossypii* population on mature *Cucumis sativus* plants is caused via an endophyte-triggered improvement of plant's physiological parameters such as enhanced plant growth with subsequent increase in plant resistance through augmented production of phenolic compounds.

Keywords: aphid biocontrol, entomopathogenic fungi as endophytes, growth promoters, phenolic compounds

Introduction

Cucumber, *Cucumis sativus* L. (Cucurbitaceae), is an important cash crop cultivated around the world, contributing to the total income and living standards of many societies through increased foreign exchange (Vandre 2013). The popularity of its fruit rests not only on its availability but also on its essential nutrients (e.g., vitamins and minerals) and other beneficial substances (e.g., soluble fibers and antioxidants). Increasing demand and sustainable availability of this commodity depend on its enhanced productivity. Since cucumber is one of the most important greenhouse crops, its intensive production is more susceptible to the rapid spread of problematic pests such as cotton aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae). The damages caused by sucking the sap of its host plants, honeydew production, and by the transmission of plant viruses (Blackman and Eastop 2000) are problems that pose a serious concern about yield and quality of greenhouse crops (Hermoso de Mendoza *et al.* 2001). This pest is mainly controlled by chemical insecticides. However,

persistent use of these insecticides has resulted in the development of resistance by the pest (Shi *et al.* 2012), in the decrease of its natural enemy populations (Godfrey *et al.* 2000), in the resurgence of secondary pests, as well as in posing environmental and economic hazards. These problems have brought about the need to introduce other sustainable, effective, and safe alternative control strategies against *A. gossypii*.

Over the years, various biological control tactics have been evaluated in many parts of the world for the management of this pest. The most commonly used approach is augmentation of the seven-spot beetle, *Coccinella septempunctata* L. (Coleoptera: Coccinellidae) in cotton fields (Omkar 2004) and the aphid parasitoid, *Aphidius colemani* Viereck (Hymenoptera: Braconidae) in greenhouses (Gissella *et al.* 2006). However, entomopathogenic fungi (EPF), *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metchnikov) Sorokin (Ascomycota: Hypocreales) have also been shown to be promising alternatives. They are currently being developed, registered, marketed and used as biopesticides against many insect species (Lacey 2016) including aphids (Ibrahim *et al.* 2015). These and many other fungal entomopathogens have been inundated as foliar and/or soil applications to manage agricultural insect pests more effectively (Skinner *et al.* 2014). Their effectiveness, however, is limited by adverse environmental conditions such as UV, temperatures and low humidity (Wraight *et al.* 2007; Vega *et al.* 2012). Therefore, this method relies on the direct action of the released agent rather than on secondary effects on successive pest generations (Vincent *et al.* 2007).

As a result, recent research is aimed at introducing fungal entomopathogens as endophytes that allow colonization of internal plant tissues without causing apparent harm to the host (Wilson 1995). There is a general opinion that entomopathogenic endophytes, while internally protected, could cope better with negative environmental factors (Vega *et al.* 2009). Although the ecological function of endophytic EPFs remains largely unknown, some studies have implicated them in plant growth as probiotics (Dora 2013), herbivore and plant disease resistance (Vega *et al.* 2008), increased stress tolerance of plants to abiotic factors (Rodriguez *et al.* 2009) and bioremediation of heavy metals (Bajan *et al.* 1998). Some of the fungi have been reported as naturally occurring endophytes while others have been introduced into plants using different techniques (Vega *et al.* 2008; Bamisile *et al.* 2018). Among the most studied fungal endophytes are the grass endophytes in the genus *Neotyphodium* (Ascomycota) which has shown different levels of activity against grass herbivores including grubs (Saikkonen *et al.* 2006). However, recent findings show that EPFs could be successfully explored as endophytes for the management of many insect pests (Vidal and Jaber 2015) including aphids (Castillo-Lopez *et al.* 2014). The endophytic colonization of *B. bassiana* and *M. anisopliae* in cucumber plants was achieved through artificial inoculation of seeds (Shaalan and Ibrahim 2019). *B. bassiana* has been reported as an endophyte in various crop plants, but its endophytic activities against *A. gossypii* have been studied only on cotton *Gossypium hirsutum* L. (Malvales: Malvaceae), pumpkin, *Curcubita pepo* (Cucurbitaceae) (Gurulingappa *et al.* 2011; Castillo-Lopez *et al.* 2014), and melon, *Cucumis melo* (Cucurbitaceae) (González-Mas *et al.* 2019). A significant reduction in the reproduction rate of *A. gossypii* and populations of bean aphid, *Aphis fabae* Scopoli (Hemiptera: Aphididae) and pea aphid, *Acyrthosiphon pisum* Harris (Hemiptera: Aphididae) was reported on *B. bassiana* colonized cotton plants and fava bean, *Vicia faba* L. (Fabales: Fabaceae), respectively (Akello and Sikora 2012; Castillo-Lopez *et al.* 2014). However, *M. anisopliae* had no effect on bean aphids. On the other hand, population sizes of soybean aphid, *Aphis glycines* Matsumura increased on soybean plants colonized by *M. brunneum* but did not change in similar treatments with *B. bassiana* (Clifton *et al.* 2018). Such discrepancy suggests that endophytic EPF colonizing the plant material may induce or suppress plant systemic resistance to insect attack by altering a plant's kairomones (plant signaling molecules) or secondary plant metabolites (e.g., terpenoids) (Vega 2018). The abilities of endophytes to prime plant defense mechanisms with chemical or biological inducers against future insect attack depend on microbial, biochemical and physiological conditions of plant material (Jaber and Enkerli 2016), microbial and plant host genotypes, time of the analysis and their interactions (Pańka *et al.* 2013). To date, there is no information in the literature on the endophytic effects of *M. anisopliae* on *A. gossypii* in cucumber production.

Therefore, the aims of the present study were to: (1) evaluate the ability of *B. bassiana* and *M. anisopliae* to establish themselves in cucumber plants through seed inoculation; (2) determine if EPF colonization modifies the physiological and biochemical status of cucumber plants and (3) investigate the effects of seed inoculation on the population of *A. gossypii* on cucumber.

Materials and Methods

Source of fungal inoculum

Two strains, *B. bassiana* and *M. anisopliae*, isolated from fallow soil (Ibrahim *et al.* 2011) were used in this study. Long term storage of the isolates was achieved by freezing conidia in 35% w/w glycerol at –80°C. When prepared, frozen conidia were re-hydrated by suspending in a small volume of sterile 0.03% Tween

20 solution, placed on potato dextrose agar (PDA) and incubated at 23 ± 2°C in the dark for 14 days (Shaalan and Ibrahim 2019).

Molecular identification of *B. bassiana* and *M. anisopliae*

Mycelial plugs from 2-day-old single spore cultures of *B. bassiana* and *M. anisopliae* were placed on malt extract agar (MEA) overlaid with a disc of sterilized cellophane and incubated at 21 ± 1°C in the dark for 2 days. The resultant mycelia were reduced to powder using iron balls, liquid nitrogen and tissue lyser Qiagen (Hilden, Germany) for subsequent DNA extraction using the CTAB method (Lee *et al.* 1988; Wu *et al.* 2001). DNA from both isolates were purified with 1% RNAase A (10 mg · ml^{-1}), and precipitated with 5 M ammonium acetate and absolute ethanol. A NanoDrop spectrophotometer (Shimadzu, Japan) was used to quantify the amount of purified DNA required for amplification. The internally transcribed spacer (ITS) region was amplified using ITS4 (5′-TCC TCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAG TAAAAGTCGTAACAAGG-3′) primers (White *et al.* 1990) using Taq PCR Master Mix (Qiagen, USA). The specific mixtures of this reaction contained 1 X ready Master Mix, 3 mM $MgCl_2$, 0.2 µM of each primer, and 25 ng of template DNA in 25 µl. The following PCR conditions were adopted: initial denaturation step of 5 min at 95°C followed by 25 cycles, each consisting of a 1 min denaturation step at 95°C, a 1 min annealing step at 58°C, and a 1 min extension step at 72°C, and a final extension step at 72°C for 7 min. The polymerase chain reaction (PCR) products were purified using QIAquick PCR amplification kit (Qiagen) and sequenced using Sanger sequencing at the Medical Genetics Unit of Saint Joseph University of Beirut (USJ). For species identification, sequences were compared with the available sequences in the NCBI BLAST database. The chromatographs were viewed and edited as needed using Chromas software (https://chromas.software.informer.com/download/).

Sterilization of cucumber seeds

Variety Beit Alpha hybrid cucumber seeds were used in this experiment. In order to remove possible epiphytic microorganisms by denaturing their DNA from the aerial surfaces, the cucumber seeds were surface sterilized prior to their artificial inoculation. Seeds were soaked in sterile distilled water (dH_2O) for 15 min, and then surface-sterilized by immersion in 3% sodium hypochlorite for 2 min with subsequent rinsing with sterile dH_2O and additional immersion in 70% ethanol for 2 min, followed by 3 rinses with sterile dH_2O. The axenically treated seeds were stored overnight at 5°C for synchronization of seed germination and growth. To assess the effectiveness of the sterilization method, 20 randomly selected seeds from the seeds used in the screening test were placed onto PDA, incubated at 25°C in darkness and then examined after 3, 5 and 10 days. The disinfection was considered successful when no fungal growth was observed on the PDA plates (Shaalan and Ibrahim 2019).

Preparation of fungal inoculum and seed inoculation

Conidia from 14-day-old cultures grown on PDA were harvested by scraping the agar surface with a sterile spatula, suspended in 0.03% Tween 20 and shaken at 354 rpm for 30 min. Resultant suspensions were filtered through sterile cheesecloth under sterile conditions in order to remove any hyphal fragments. Conidial concentrations were determined using a light microscope and hemocytometer (Fuches-Rosenthal), and later adjusted to 1 × 10^6 conidia · ml^{-1} with sterile 0.03% Tween 20 solution. Axenic seeds were immersed in fungal conidial suspensions of *B. bassiana* and *M. anisopliae* for 2 h. Preliminary tests indicated that a conidial concentration of 10^6 conidia · ml^{-1} and a 2 h immersion time were the optimal parameters for the most successful colonization of cucumber seeds and plantlets tested by *B. bassiana* and *M. anisopliae* isolates (Shaalan and Ibrahim 2019). Inoculated seeds were then sown on seed trays containing non-sterile compost supplemented with N 150 mg · l^{-1}, P_2O_5 185 mg · l^{-1}, and K_2O 250 mg · l^{-1} (pH (H_2O) 5.2–6.0, neutral peat, MIKSKAAR) and grown under natural fluctuating night/day temperatures (23–29°C) and relative humidity (RH, 30–80%). For the control group the seeds were immersed in sterile 0.03% Tween 20 only. Prior to planting, the compost was analyzed for the presence of soilborne fungal communities using the soil dilution method (Ratna Kumar *et al.* 2015; Umboh *et al.* 2016) with slight modification. For this purpose, 10 g of used peat were resuspended in 100 ml of sterile water. The resultant stock suspension was then shaken for 1 h at 200 rpm. Tenfold diluted (10^{-1}) samples were plated on 1/4-strength PDA media containing 20 mg · l^{-1} amoxicilline, 2 ml · l^{-1} Tween 20, and additional agar of 1.5% (w/v) final concentration. The test was replicated three times. The plates were incubated for 2 weeks at 25°C, and the fungal colonies which developed were then isolated further to be observed under a microscope and identified.

Assessment of fungal colonization

Assessment of inoculated seeds using scanning electron microscope (SEM)

Inoculated seeds were incubated at room temperature under sterile conditions for 24 h and then fixed in glutaraldehyde, dehydrated through an alcohol-

acetone series, dried in a critical-point drying apparatus, mounted on stubs in different positions and coated with gold in a gold metallization with a Cressington 108 auto sputter coater (Pathan *et al.* 2010). The specimens were observed and photographed with SEM (Seron AlS2100, Korea). There were 11 seeds examined for each treatment and the numbers of attached and germinated spores, and spores producing an appressorium or penetrating the seed coat were recorded on a randomly chosen surface area (100 μm^{-2}) of observation on each seed.

Assessment of colonization using re-isolation

Five randomly selected 4-week-old seedlings (3-5 true leaf stage) were harvested from the trays, cleaned and surface-sterilized. First, the seedlings were immersed in 0.5% sodium hypochlorite for 2 min, and then in 70% ethanol for 30 s, followed by three rinses in sterile dH_2O. Disinfected seedlings were air-dried in a laminar flow hood for 15 min, the root, stem and leaf parts were cut using a sterile scalpel. The obtained plant materials were placed on PDA selective media amended with antibiotics (20 mg · l^{-1} Amoxicilline), incubated at 25°C for 4 weeks and inspected regularly to observe fungal outgrowth. The success of the disinfection process was assessed by plating 0.5 ml of residual rinse water on PDA and by making imprints of surface sterilized plant tissue. Fungal outgrowth from the plated plant samples were identified as *B. bassiana* or *M. anisopliae* based on differential growth on selective media, colony morphology, and microscopic examination of conidia (Humber 1997). Percent colonization of different seedling parts by the respective inoculated fungus was calculated following the Petrini and Fisher (1987) formula: % colonization = number of sampled plant tissue showing fungal outgrowth divided by the total number of plated plant tissue samples ×100. The test was repeated three times.

The effects of endophytic EPFs on the population size of *Aphis gossypii* on cucumber plants.

Propagation of cucumber plants

Cucumber seedlings at 1–2 true leaf stage were transplanted from seed trays into plastic pots (10 cm diameter and 15 cm depth) containing unsterilized compost and propagated for 5 weeks under the same conditions as described previously (see 2.4 section). The potted plants were regularly irrigated. Fifteen plants for each treatment were used.

Aphis gossypii culture

Prior to laboratory establishment of pure *A. gossypii* culture, aphid populations collected from different plant hosts and locations were identified using morphological and molecular techniques. Primary morphological identifications were carried out according to Stoetzel *et al.* (1996) and Blackman (2010). For molecular identification, a single aphid body from each sample was used and total DNA was extracted according to the CTAB method (Gawel and Jarret 1991). Two sets of species-specific primers for *A. gossypii* (KBR(AG)-F-5'-TCTTCTCTTAGAATTT TAATCCGATTA-3' and KBR(AG)-R-5'-AAGAAT AGGGTCTCCCCCACCT-3' (Rebijith *et al.* 2012)) and for *Myzus persicae* (KBR(MP)-F-5'-TCATCACT TAGAATCTTAATTCGTCTT-3' and KBR(MP)-R-5'- TGGTATTATATTTAAGATTGTACAAATA-3' (Rebijith *et al.* 2012)) were used in PCR reactions with genomic DNA as the template using the following conditions: initial denaturation step of 4 min at 94°C, followed by 35 cycles, each consisting of a 30 s denaturation step at 94°C, a 45 s annealing step at 64°C, and a 40 s extension step at 72°C, with a final extension step for 20 min at 72°C (Matallanas *et al.* 2013). PCR products were run on 1.2% agarose gel, stained with UView loading dye (Bio-Rad, USA) and visualized under UV lights by the Molecular imager system Gel Doc 1000 (Bio-Rad) using ImageLab software (Bio-Rad). Colony individuals that were identified as *A. gossypii* were used for laboratory rearing and maintained on healthy cucumber plants grown in pots and in wooden cages (70 cm × 50 cm × 50 cm) at 21 ± 5°C. Adults of this colony were used in the bioassays.

Effect of endophytic EPFs on *Aphis gossypii* population size

Five-week-old plants were placed in individual clear plastic cages (20 cm dim. × 30 cm high) and 10 *A. gossypii* adults were introduced to a middle leaf of each experimental plant. Cages were covered with lids and sealed with no-see-um mesh to prevent aphids from escaping. There were 36 caged plants (12 replicate plants for each treatment) randomly distributed and maintained under natural conditions (25–30°C and 30–80% RH).

After 5 days of exposure to the aphids, tested plants were observed and the total number of aphids (adults + nymphs) on each plant was recorded using a magnifier. This experiment was repeated twice.

The effects of endophytic EPFs on physiological and biochemical parameters of cucumber plants

The 6-week-old cucumber plants were examined, the number of fully developed leaves and flowers was measured and counted for each tested plant. One week later, the number of small cucumber fruits formed, and the height of each plant were also recorded.

Analysis of total phenolic compounds

Some studies have reported that grass endophytes indirectly influence the defense reaction of the host plant (Pańka *et al.* 2013) by involving e.g., synthesis of phenolic compounds. Phenolic compounds are the main class of secondary metabolites in plants and are divided into phenolic acids and polyphenols. They perform a variety of functions, including acting as antioxidants (Grace and Logan 2000). They may exist in free, soluble conjugated (acid and alkaline hydrolysable) and insoluble-bound forms (Perea-Domínguez *et al.* 2018). Cucumber plant is rich in polyphenolic compounds such as p-coumaric, caffeic, and ferulic acids and p-coumaric acid methyl ester (Daayf *et al.* 2000). The Folin-Ciocalteu (F-C) assay provides a convenient, rapid and simple estimation of the content of total phenolics and other oxidation substrates in plant extracts. This assay also provides a high-throughput tool for screening the response of plants to biotic factors and environmental stress. In this study, to see if fungal endophytes colonizing cucumber seedlings can alter the total content of phenolic compounds (*TPC*s), the F-C procedure for plant material (Ainsworth and Gillespie 2007) with caffeic acid (CA) as a standard was applied.

The *TPC* for each sample was determined using formula: $TPC = C \times V/M$, where: *TPC* is the total phenolic content ($mg \cdot g^{-1}$) of the extracts as CA equivalents, *C* is the concentration of CA established from the calibration curve ($mg \cdot ml^{-1}$), *V* is the volume of the extract solution (ml) and *M* is the weight of the sample (g). The assay consisted of one randomly sampled leaf from 4-week-old cucumber plants with three plants for each treatment. The assay was repeated three times.

Analysis of chlorophyll and carotenoids content

Chlorophylls and carotenoids are abundant pigments directly influencing cucumber growth through photosynthetic activities. In cucumber, chlorophylls are synthesized in green tissues, whereas carotenoids are synthesized in the leaves, flowers and fruits. In the cucumber leaf tissues carotenoids (mainly α-carotene and β-carotene) act as photoprotectors. The presence of the β-carotene and xanthophylls confer the characteristic yellow coloration to the flowers and to the fruit's meso- and endocarps (Hui Song *et al.* 2010). To determine the effects of fungal colonization on the accumulation of total chlorophyll (chlorophyll a and b) and carotenoids content in 7-week-old cucumber seedlings, leaves (0.5 g) were randomly sampled from three replicate plants of each treatment, chopped and immediately homogenized in 90% ethanol using mortar and pestle with gradual addition of 17.5 ml of ethanol. The homogenate was centrifuged at 1,500 rpm for 15 min. The absorbance of chlorophyll and carotenoid extracts was read after 90% aqueous ethanol was used as the blank, to zero the instrument initially and after every wavelength resetting. Chlorophyll content was determined according to the method described by Barnes *et al.* (1992). Chlorophyll a and b absorbencies were measured at 664 and 649 nm, respectively, with a spectrophotometer (Thermospectronic) and calculated for chlorophyll concentration in fresh weight for three replicates. Formulas for chlorophyll content were as follows: $Ca = 13.36 \times A665 - 5.19 \times A649$ and $Cb = 27.43 \times A649 - 8.12 \times A665$, where: A665 = absorbance at a wavelength of 665 nm; A649 = absorbance at a wavelength of 649 nm. Carotenoids (mixture of α-carotenes and β-carotenes) absorbance was measured at 470 nm and carotenoid concentration was determined using the following formula: $C = (1{,}000A_{470} - 2.13\,Ca - 97.63\,Cb) / 209$, where A470 = absorbance at a wavelength of 470 nm (Braniša *et al.* 2014). The test was repeated three times.

Statistical analysis

All experiments were arranged in a complete randomized design. All data sets were analyzed with the statistical program IBM SPSS Statistics for Windows, Version 23.0 (2015) using one-way ANOVA after checking the assumptions for normality and the homogeneity of variance (Levene's test). Data sets from repeated tests for percentage values of fungal colonization with *M. anisopliae* and *B. bassiana* of different cucumber plant parts in addition to the aphid population size, TPCs, and chlorophyll contents were pooled together prior to one-way ANOVA analysis with the fungal strain as a main factor. A plant's physiological parameters were subjected to one-way ANOVA with the fungal strain as a main factor. When a significant *F* test was obtained at $p = 0.05$, separation of treatment means was performed using Duncan test.

Results

Molecular identification of microorganisms and aphids

Total DNA extracted from both fungal strains was PCR amplified by ITS4 and ITS5 primers. The amplification of the ITS region resulted in a single product (~ 600 bp) for both isolates. The ITS sequences of both isolates (BbL1, MaL1) were deposited in GenBank with the accession numbers. MT533246 and MT533250, respectively. The BLAST analysis of the ITS sequences of BbL1, MaL1 revealed 100% similarity with *B. bassiana* (accession No. KC753391) and 99.46% similarity with *M. anisopliae* (accession No. MG786739) in the NCBI database, respectively. This molecular identification confirmed the phenotypic identification of the fungal isolates used in this study.

Primer sets KBR (AG)-F and KBR (AG)-R successfully identified *A. gossypii* sp. by amplifying the expected fragment size of 600 bp in two samples only. None of the tested aphids was identified as *Myzus persicae*.

Assessment of colonization of *Beauveria bassiana* and *Metarhizium anisopliae* in cucumber plants

The efficiency of artificial inoculation of cucumber seeds was observed under scanning electron micrographs (SEM). Scanning electron micrographs revealed that seeds of Beit Alpha hybrid were emarginate and elliptical with pointed ends. The seed sculpture of the studied taxa (*C. sativus*) showed that surfaces were smooth and covered with pits (hollows). The outer epidermal cells were of regular rectangular shapes separated with cell boundaries. Moreover, these micrographs showed that inoculated seed surfaces were covered with germinated and non-geminated conidia. All germinated spores were observed at different stages of development while their germ tubes spread over the seed's surfaces at different distances. Direct hyphal penetration through the epidermal cell wall was also observed (Fig. 1D

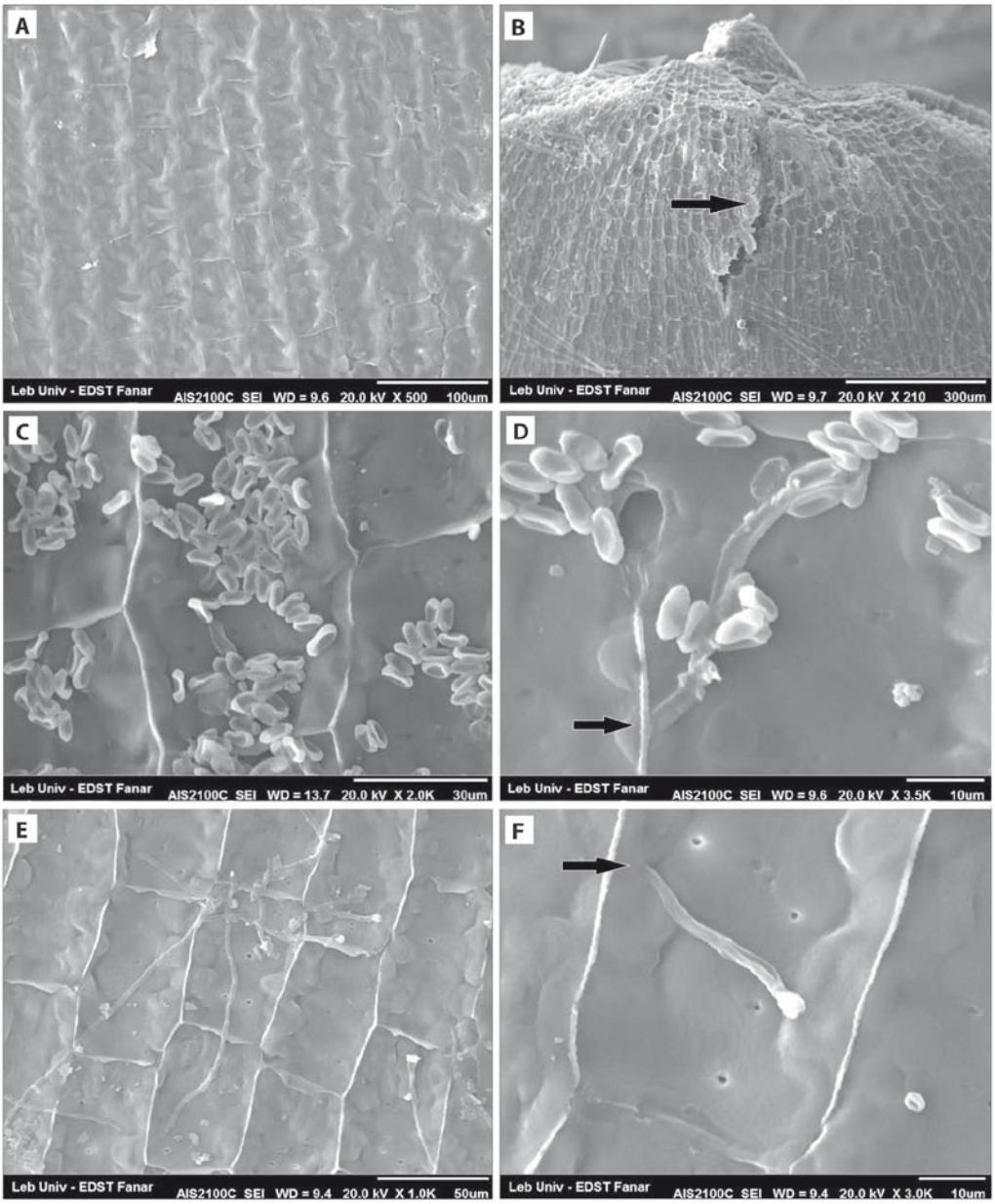

Fig. 1. Scanning electron micrographs (SEM) of Tween (control), *M. anisopliae* and *B. bassiana* treated seeds 24 h post-inoculation. Micrograph A shows no fungal growth on control seed surface, whereas B shows *M. anisopliae* spores and hyphae adhered to the micropylar area showing ruptured covering layers (black arrows) and emergence of the radicle. Micrographs C and D of *M. anisopliae* treated seeds show attached conidia and conidial hyphae penetrating across cell boundary (black arrow), respectively. Micrographs E and F of *B. bassiana* treated seeds show hyphae growing on seed surface and directly penetrating the epidermal cell wall (black arrow), respectively

and F (black arrows) demonstrating the evidence of applied EPF becoming endophytic. The results of the number of attached conidia, germinated conidia, appressorium producing conidia and hyphae penetrating the seed cuticle are presented in Figure 2. No conidia were found on the surfaces of non-treated control seeds (Fig. 1A). The number of spores attached to the surface of *M. anisopliae* treated seeds was significantly higher (13.2 conidia 100 µm^{-2}; $p < 0.0001$) than that on *B. bassiana* (2.5 conidia 100 µm^{-2}) treated seeds (Fig. 1C and E). Moreover, a greater number of *M. anisopliae* spores adhered to the micropylar area of the examined seeds showing ruptured covering layers and emergence of radicles (Fig. 1B) than to the other parts of the same seed. In contrast, a more uniformed distribution of spores on cucumber seed surfaces was recorded on *B. bassiana* inoculated (Fig. 1E). However, not all attached spores germinated after 24 h and there was no difference in the number of germinated spores ($p = 0.014$), either penetrating ($p = 0.342$) or appressorium producing spores ($p = 0.38$) on the surfaces of treated seeds (Fig. 2). Moreover, approximately 11% and 13% of hyphae developed by *B. bassiana* and *M. anisopliae*, respectively, entered the seed through direct penetration. Appressoria were produced by 6.6% of the *Metarhizium* spores. There was no appressorium formation observed for *B. bassiana*.

All the fungal populations found in the peat compost were dominated by Ascomycetes, particularly by *Aspergillus*, *Penicillium*, and *Sclerotium* spp. The second most abundant group was the Mesomycetozoa (Protists), Basidiomycetes, and by a few unidentified yeasts. There were no *Metarhizium* or *Beauveria* spp. detected in the compost used for planting. Artificial inoculation of cucumber seeds carried out by their direct submersion in conidial suspensions provided successful colonization of plant parts with tested *B. bassiana* and *M. anisopliae* isolates. The presence of fungi in the internal tissues was confirmed by their recovery from roots, stems and leaf pieces on PDA selective medium. Results in Table 1 show that both, *B. bassiana* and *M. anisopliae,* isolates colonized cucumber seedlings. The most successful endophyte re-isolation frequency was observed from root tissues for both isolates (100%) ($p < 0.0001$). In addition, slightly higher percentage recoveries (50%, $p = 0.1$ and 25%, $p = 0.405$) were observed in stem and leaf tissues, respectively, colonized by *M. anisopliae*. However, no fungal recovery was achieved from stem and leaf tissues of *B. bassiana* inoculated plants. None of the leaf discs obtained from control plants showed signs of *M. anisopliae* or *B. bassiana* outgrowth, however, the control plants were observed to be colonized by naturally occurring endophytes such as *Penicillium*, *Aspergillus* as well as by other unidentified fungi.

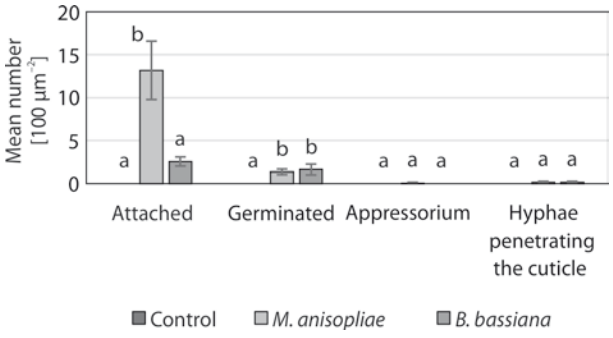

Fig. 2. Mean number (100 µm^{-2}) ± SE of attached, germinated spores, appressorium and hyphae penetrating the seed epidermal cells of the treated seeds. Bars with different letters are significantly different at $p = 0.05$ (Duncan test, after one-way ANOVA)

The effects of endophytic EPFs on the population size of *Aphis gossypii* on cucumber plants

In general, there was a significant negative effect of cucumber plant colonization with both fungal entomopathogens on the population size of *A. gossypii* 5 days after exposure. The data (Fig. 3) show that the number of aphids in both treatments was significantly lower than that in the control plants ($p < 0.05$).

The aphid population size on *Metarhizium* colonized plants was reduced by almost 80 aphids per plant (35%) and on *Beauveria* colonized plants by 72 aphids (32%) (Fig. 3) resulting in healthier/fitter cucumber plants.

Table 1. Effects of conidial seed treatments on percentage recovery of *B. bassiana and M. anisopliae* from different parts of cucumber plants cultured outdoors in non-sterilized peat moss

Treatment	Leaves (% ± SE)	Stem (% ± SE)	Roots (% ± SE)
Control	0.00 ± 0.00 a*	0.00 ± 0.00 a	0.00 ± 0.00 a
B. bassiana	0.00 ± 0.00 a	0.00 ± 0.00 a	100 ± 0.00 b
M. anisopliae	25 ± 0.25 a	50 ± 0.28 a	100 ± 0.00 b

Medium 4 weeks post-inoculation

*means (%± SE) within a column followed by the same letter are not significantly different at $p = 0.05$ (Duncan test, after one-way ANOVA)

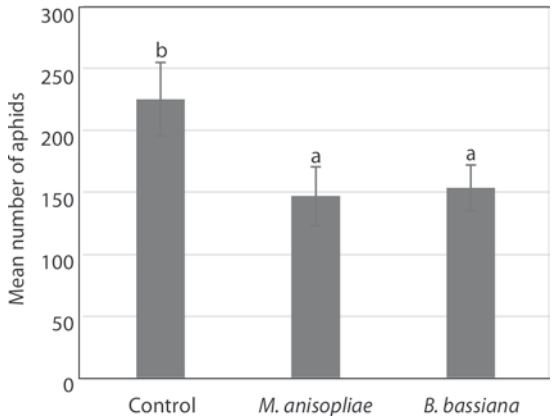

Fig. 3. Mean (± SE) number of aphids per cucumber plant inoculated with *B. bassiana*, *M. anisopliae* and Tween (control). Bars with different letters are significantly different at $p = 0.05$ (Duncan test, after one-way ANOVA)

The effects of endophytic EPFs on physiological and biochemical parameters of cucumber plants

Physiological parameters

The number of fully developed leaves and flowers was counted for each replicate and treatment 6 weeks post-inoculation. The number of small cucumber fruits and the plant's height were recorded for each potted plant 7 weeks post inoculation. The results show that there was a significant difference in the number of flowers ($p = 0.05$), leaves ($p < 0.005$) and the height of the plants ($p < 0.01$) colonized with tested fungal endophytes compared to uncolonized control plants (Fig. 4). On one hand, plants colonized with *Beauveria* isolate produced twice as many flowers and two times more leaves than control plants (Fig. 3). On the other hand, *Metarhizium* colonized plants where almost 12 cm taller and had two more developed cucumber fruits than control plants (Fig. 4).

Biochemical parameters

To see if the suggestion of Pańka *et al.* (2013) that endophytes could indirectly influence the defense reaction in the host plant by synthesizing more phenolic compounds than plants without endophytes, the total phenolic content was determined for 2-week-old seedlings. The results presented in Table 2 show that cucumber plants reacted to endophytic colonization by increased induction of soluble phenols. Thus, plants grown from treated seeds with *B. bassiana* accumulated a significantly ($p < 0.0001$) higher amount of TPC (182 mg · g^{-1}) than those plants treated with *M. anisopliae* (101 mg · g^{-1}) or Tween (95 mg · g^{-1}).

However, analyses of Ca, Cb and carotenoid content in the leaf blades of 7-week-old cucumber plants showed no effect of endophytic EPFs on measured parameters (Fig. 5). The leaf extracts from colonized and uncolonized cucumber tissues contained similar amounts of both chlorophylls ($p = 0.785$ and $p = 0.325$, respectively) and carotenoids ($p = 0.593$).

Table 2. Effects of endophytic entomophatogenic fungi (EPFs) on total phenolic compounds in 4-week-old plants grown from seeds treated with *B. bassiana*, *M. anisopliae* and Tween (control)

Treatment	Total phenol content [μg · g^{-1}]
Control	94.5 ± 0.003 a*
M. anisopliae	101.3 ± 0.003 a
B. bassiana	181.8 ± 0.001 b

*means (μg · g^{-1} ± SE) within a column followed by the same letter are not significantly different at $p = 0.05$ (Duncan test, after one-way ANOVA)

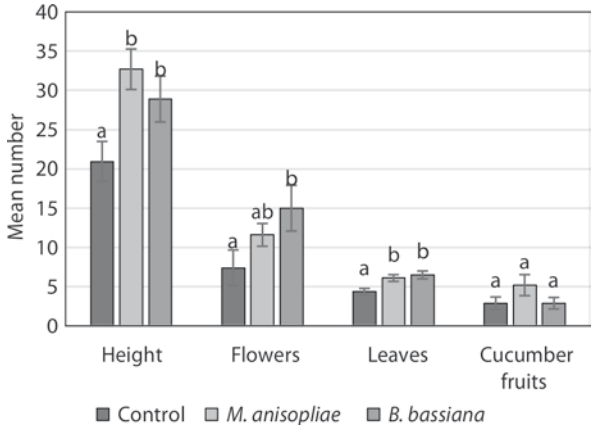

Fig. 4. Mean height (cm ± SE) and the mean (± SE) number (N) of flowers, leaves and fruits of cucumber plants inoculated with *B. bassiana*, *M. anisopliae* and Tween (control). Bars with different letters are significantly different at $p = 0.05$ (Duncan test, after one-way ANOVA)

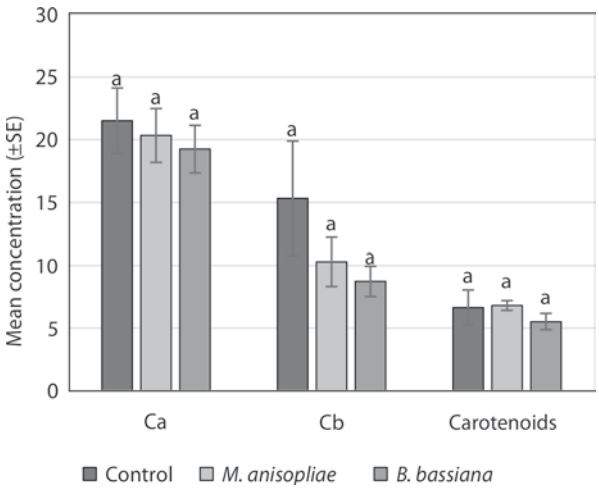

Fig. 5. Effect of fungal inoculation on chlorophylls Ca and Cb and carotenoids content (mean concentration, μg · ml ± SE) in 7-week-old cucumber plants. Bars with similar letters do not differ significantly at $p = 0.05$ (Duncan test, after one-way ANOVA)

Discussion

The main aims of this study were to determine if *M. anisopliae* and *B. bassiana* can establish an endophytic relationship with cucumber plants under naturally fluctuating conditions in non-sterile peat compost, and if these fungi can act as plant growth promoters, or if inoculation of seeds with EPF affects the aphid, *A. gossypii*. Seed inoculation was shown to be very successful for EPF colonizing many plant tissues (Kabaluk and Ericsson 2007; Ownley *et al.* 2008; Powell *et al.* 2009; Akello and Sikora 2012; Akutse *et al.* 2013; Lopez *et al.* 2014; Russo *et al.* 2015) including cucumber seedlings (Shaalan and Ibrahim 2019; Rajab *et al.* 2020). The success of internal colonization is usually determined by re-isolation of tested fungi using the culture dependent method (CDM) (McKinnon *et al.* 2017). However, some other techniques such as molecular identification and visualization of endophytes within plant tissues using dyes, histological techniques, immunofluorescence, light and electron microscopy have also been shown to be reliable for endophyte detection (McKinnon *et al.* 2017; Ullrich *et al.* 2017; Vega 2018).

Seed submersion used in the current study resulted in the attachment of conidia to seed surfaces, their germination, hyphal growth across seed surfaces and direct penetration through epidermal cell walls 24 h post inoculation. Similarly, germinated conidia of *B. bassiana* produced hyphae that penetrated the leaf cuticle directly or grew through stomates into the corn leaf (Wagner and Lewis 2000). When a spore lands on the plant surface, it perceives the immediate environment, the topography of the host cuticle and nutrient availability, which are the essential factors that trigger spore attachment and stimulate germination (Tucker and Talbot 2001). Indeed, submerging cucumber seeds in spore suspension prompted an imbibition of water, which, in turn, caused a rupture of seed covering layers exposing micropylar endosperms and emerging radicles. Therefore, a germinating cucumber seed was likely to provide a perfect humid environment and accessible nutrients for conidia to adhere to and germinate. It is interesting that while *M. anisopliae* conidia in this study preferred to aggregate and attach near the micropylar area of radicle emergence, conidia of *B. bassiana* spread homogeneously across the entire seed surface. A previous study indicated that in the environment *Metarhizium* spp. appear to be restricted to the rhizosphere (Behie *et al.* 2015). Also, initial stages of plant-*Metarhizium* interaction through seed treatment would involve extensive rhizoplane colonization followed by endophytic growth (Barelli *et al.* 2018). The results of SEM observations also revealed that around 13% of germinated spores of both spp. penetrated seeds through epidermal cells directly without using epidermal pits. This rate of initial seed colonization, perhaps, was enough to set off a mechanism for systemic colonization of the whole plant (Wagner and Lewis 2000).

Indeed, the fungal recovery from 4-week-old cucumber seedlings grown on non-sterile compost was achieved for both entomopathogens. *Beauveria bassiana* was isolated only from roots whereas *M. anisopliae* was isolated from roots, stems and leaves. In the previous study, however, the same fungal isolates were detected in all plant parts obtained from 10-day-old cucumber seedlings cultivated on sterile substrate (Shaalan and Ibrahim 2019). Likewise, in the study of Tefera and Vidal (2009), colonization was not recorded in stems and leaves of *Beauveria* seed-treated seedlings grown on non-sterile soil but occurred in vermiculite and sterile soil. The fungistatic effects of soil (Lingg and Donaldson 1981), biotic antagonism (Pereira *et al.* 1993) or microbial competition in the compost may have prevented or delayed *B. bassiana* from reaching the stem and leaves. On the other hand, the initial low number of attached spores (Fig. 2) as well as the transient nature of *B. bassiana* (Russo *et al.* 2015) could explain negative endophytism with cucumber stems and leaves. The application rate of conidia is also known to influence the rate of endophytic colonization (Ownley *et al.* 2008).

In addition, this study not only reports the development of entomopathogenic fungi introduced endophytically but also shows that endophytic presence in cucumber plant tissues can increase the resistance against melon aphids, *A. gossypii*. The results of the present work support previous studies where *B. bassiana* endophyte also negatively affected cotton aphid reproduction (Castillo-Lopez *et al.* 2014; González-Mas *et al.* 2019), but are in opposition to endophytic *B. bassiana* effects on other aphid species (Clifton *et al.* 2018; Jensen *et al.* 2019). The mechanisms by which herbivores can be negatively affected by clavicipitaceous obligate endophytes have been studied in a few different grass species and can vary from antixenosis and/or antibiosis mediated by constitutive production and or induction of secondary compounds produced by the plant (Clay *et al.* 1993; Clay 1996; Carriere *et al.* 1998) or secondary metabolites produced by the endophytes themselves (Jaber and Vidal 2010; Gurunlingappa *et al.* 2010; Saari *et al.* 2010; Vega 2018). Another hypothesis for the mechanism by which endophytes can negatively affect herbivores is based on the idea that endophytes can alter the phytosterol profiles of plants and compete with insects for these compounds which are essential for their development (Dugassa-Gobena *et al.* 1996; Raps and Vidal 1998). It was also

suggested that plant responses to endophyte invasion may involve e.g., synthesis of phenolic compounds (Pańka *et al.* 2013). The presence of *Neotyphodium lolii* endophyte increased significantly the production of total phenolics in all ryegrass genotypes. In general, phenolic compounds and some reactive oxygen species are synthesized in plants partly as a response to ecological and physiological pressures (Ibrahim *et al.* 2001; Chung *et al.* 2003; Schulz and Boyle 2005; Diaz *et al.* 2010) and possess anti-herbivore properties (Fürstenberg-Hägg *et al.* 2013; Vega 2018). Total phenolic content analysis showed that *B. bassiana* and *M. anisopliae* endophytes colonizing 4-week-old cucumber seedlings influenced the defense reaction in the cucumber plant by synthesizing more phenolic compounds than seedlings without an endophyte. Such a response could have rendered plants more capable of self-defense against initial attack by *A. gossypii*. The mechanisms of plant defense responses to fungal colonization could be species-dependent, meaning that the same plants could react differently to different fungal endophytes. When the roots-inoculated tomato plants with *B. bassiana* and *Trichoderma koningiopsis* were analyzed by GC-MS and compared to non-inoculated plants, it was found that all successfully colonized roots, stems and leaf tissues contained modified tomato volatile organic compounds (VOCs) with significant differences between isolates (Pineda *et al.* 2010). However, the profiles of cucumber VOCs were not determined in colonized or non-colonized tissues for this study, and the hypothesis of modifications to the chemical signals produced by the plant (Vega 2018; Gange *et al.* 2019) remains to be tested as an explanation of the data in the current study.

Recent reviews by McKinnon *et al.* (2017), Jaber and Enkerli (2017) and Vega (2018) presented examples of many studies reporting the effects of endophytism on plant growth and development. It was evident that some plants were unresponsive (neutral) to endophytic colonization. Some responded with a positive outcome (e.g., improved growth (Vega *et al.* 2009; Liao *et al.* 2014; Jaber and Enkerli 2016) and increased yields (Maniania *et al.* 2003; Kabaluk and Ericsson 2007)), while a very few exhibited a negative response (e.g., alleviated iron chlorosis symptoms (Sánchez-Rodríguez *et al.* 2015)). Colonization of cucumber plants by *B. bassiana* and *M. anisopliae* in our study resulted in improved plant growth, greater numbers of leaves and flowers without significant effects on chlorophyll synthesis. It is possible that since seed treatment with these two isolates can significantly improve seed germination rates and strengthen root systems of resultant seedlings (Shaalan and Ibrahim 2019), this may explain the subsequent improved plant growth. In addition, the ability of entomopathogenic endophytes to produce plant growth regulators (e.g., indole-3acetic acid (IAA)) (Liao *et al.* 2017) as well as to improve nutrient absorption (Sasan and Bidochka 2012) may indicate a high potential of the isolates used in this study to promote plant growth (Vega *et al.* 2009).

Conclusions

Colonization of cucumber plant tissues by entomopathogenic endophytes, *B. bassiana* and *M. anisopliae,* began with direct hyphal penetration of the seed surface. Conidia of *M. anisopliae* preferred the micropylar area of cucumber seeds for their initial attachment and penetration, while conidia of *B. bassiana* spread continually across the entire seed surface. Colonization of cucumber plants occurred under natural environmental conditions and on a non-sterile substrate, greatly promoting plant growth and development. Here, we showed that cucumber plant colonization by fungal entomopathogens is generally negative to aphids, *A. gossypii*, reducing their overall population size. Activation of multiple mechanisms related to plant-mediated systemic resistance could supress the invasion of piercing-sucking insects and, thus, improve quality and quantity of agricultural crops. Since cucumber growers regularly purchase plantlets from plant nurseries, it would be viable to commercialize the production of *B. bassiana* and *M. anisopliae* pre-colonized plants.

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