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ORIGINAL ARTICLE

Inoculation of cucumber plants with *Beauveria bassiana* enhances resistance to *Aphis gossypii* (Hemiptera: Aphididae) and increases aphid susceptibility to pirimicarb

Mohammad HOMAYOONZADEH[1], Mojtaba ESMAEILY[1], Khalil TALEBI[1], Hossein ALLAHYARI[1], Stuart REITZ[2] and J.P. MICHAUD[3,*]

[1] Department of Plant Protection, College of Agriculture and Natural Resources, University of Tehran, 31587-77871, Karaj, Iran; e-mails: homayoonzadeh1371@gmail.com, m_smaily@ut.ac.ir, khtalebi@ut.ac.ir, allahyar@ut.ac.ir

[2] Department of Crop and Soil Science, Oregon State University, Ontario, Oregon 97914, USA; e-mail: stuart.reitz@oregonstate.edu

[3] Department of Entomology, Agricultural Research Center-Hays, Kansas State University, Hays, KS 67601, USA; e-mail: jpmi@ksu.edu

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Abstract. The entomopathogen *Beauveria bassiana* (Bals.) Vuill. (Ascomycota: Hypocreales) can colonize plants endophytically and stimulate the production of secondary plant metabolites with anti-herbivore activities. We assayed the topical virulence of *B. bassiana* to *Aphis gossypii* Glover (Hemiptera: Aphididae), the effects of cucumber inoculation with this fungus on plant metabolites, and the physiological consequences for aphids that fed on these plants. Assays were conducted with both the commercial formulation of *B. bassiana*, 'Naturalis®-L', at the recommended concentration of 1.5 ml / L (yielding a spore concentration of 2.3×10^7 CFU per ml), and with a similar concentration of the isolated fungal strain. Topical application of 0.03 ml of solution per cm^2, or 1×10^3 CFU, caused 100% mortality to *A. gossypii* adults after seven days, whether Naturalis®-L or the isolate alone was used. The fungus grew endophytically into foliage when sprayed on cucumbers at the 2-leaf stage and concentrations of alkaloids, flavonoids, phenols, hydrogen peroxide, and total chlorophyll were higher than in control plants 28 days after inoculation. Malondialdehyde content, plant growth, and total yield were unaffected by *B. bassiana* inoculation. Aphids fed on *B. bassiana*-inoculated plants for 24 h had reduced activities of detoxifying enzymes (glutathione-*S*-transferase, carboxylesterase, and acetylcholinesterase) compared to controls. Activities of digestive enzymes, (lipase, α-amylase, α-glucosidase, and aminopeptidase) were reduced in aphids from inoculated plants, which exhibited higher activities of superoxide dismutase, ascorbate peroxidase, and phenoloxidase, but lower catalase activity. Energy reserves (lipids, protein, and glycogen) were lower in aphids from inoculated plants, and they exhibited reduced fecundity, longevity, and reproductive periods, and a 50% reduction in the LC_{50} of pirimicarb. Thus, in addition to causing direct pathogenicity, inoculation of plants with *B. bassiana* negatively impacted *A. gossypii* physiology and reproductive performance and could usefully complement other strategies for managing cotton aphids on greenhouse cucumber.

INTRODUCTION

The cotton aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae) is a polyphagous cosmopolitan pest of numerous field and greenhouse crops (Ebert & Cartwright, 1997). It has the capacity for rapid population growth, causing direct feeding damage to host plants and transmitting various plant viruses (Deguine et al., 2017). Management of cotton aphids has conventionally relied on the use of synthetic insecticides (Kandil et al., 2017), which has resulted in the aphids evolving resistance to various insecticidal modes of action, and generated a need for alternative management tactics (Wang et al., 2007; Carletto et al., 2010).

Entomopathogenic fungi can be useful tools for controlling insect pests and are usually compatible with other biologically-based tactics in integrated pest management programs (Gurulingappa et al., 2011). One of the most effective entomopathogens against *A. gossypii* is *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Hypocreales) (Loureiro & Moino, 2006). This entomopathogen can also colonize plants and proliferate as an endophyte (Klieber & Reineke, 2015; McKinnon et al., 2018). Endophytic fungi occur ubiquitously in plants, often without any adverse effects on them, and may actually improve plant tolerance of abiotic and biotic stresses (Ownley et al., 2008; Mo-

* Corresponding author; e-mail: jpmi@ksu.edu

loinyane & Nchu, 2019). For example, Gonzalez-Mas et al. (2019c) showed that *B. bassiana* growing endophytically within melon plants caused a slight increase in the fecundity of *A. gossypii* feeding on the plants, but this did not translate into aphid population growth, largely due to fungal-induced aphid mortality that ranged from 38–50%.

Endophytic entomopathogenic fungi may help protect plants against herbivores indirectly via induction of plant defenses, or directly via the production of fungal metabolites with insecticidal properties (Moloinyane & Nchu, 2019). The principal metabolites typically produced by endophytic entomopathogenic fungi within host plants include alkaloids, flavonoids, and phenolic compounds (Espinoza et al., 2019). Levels of metabolites such as hydrogen peroxide (H_2O_2), malondialdehyde (MDA) (Heidarvand & Maali-Amiri, 2013), and total chlorophyll (Croft & Chen, 2017) are often altered in plants in response to biotic and abiotic stresses and can therefore serve as indicators of plant health. Plant secondary metabolites can affect herbivore physiology via changes in levels of digestive enzymes, antioxidant enzymes (Mardani-Talaee et al., 2016), detoxifying enzymes (Carletto et al., 2010; Homayoonzadeh et al., 2020a), and energy reserves (Sinclair, 2015; Homayoonzadeh et al., 2020b). Ultimately, endophytically-induced changes in host plant physiology can alter herbivores population dynamics, creating potentially useful applications in biological pest control (Zahedi et al., 2019).

We hypothesized that inoculation with *B. bassiana* would alter the physiological status of cucumber plants, and the physiology and life history of cotton aphids that feed on these plants, resulting in reduced aphid fitness. Whereas there exists some background information on how and why endophytic *B. bassiana* might help protect cucumber plants from *A. gossypii*, little is known about how it could affect aphid susceptibility to insecticides via effects on the detoxifying enzymes that are often important in insecticide resistance. Therefore, we also examined how *B. bassiana* inoculation of cucumber plants would affect the susceptibility of *A. gossypii* to pirimicarb, an aphid-specific insecticide commonly used against this pest in greenhouses.

MATERIALS AND METHODS

Plants

Seeds of cucumber, *Cucumis sativus*, cv. Super N3 (HED, Modesto, CA, USA), were planted and grown in 15 cm (diam) plastic pots of sterilized soil (autoclaved for 20 min at 121°C and 106 kPa pressure) composed of 1 : 1 : 2 cocopeat : peat moss : perlite. Plant were grown in a greenhouse under experimental conditions of 26 ± 2°C, 16L : 8D photoperiod, 5100 lux light intensity, and 35–40% RH. Pots were irrigated to soil capacity once every three days and fertilized once every 7 days with a 1.5 g/liter solution (250 ml/plant) of 20 : 20 : 20 (N : P : K) fertilizer (Promisol, Lleida, Spain).

Aphid colony

Cotton aphids were collected in September, 2019 from cucumber gardens that had not been exposed to pesticides for more than 10 years. In order to condition the aphid colony to the cucumber cultivar, five generations of aphids were reared on seedlings of cv. Super N3 in climate-controlled growth chambers set to 25 ± 1°C, 50 ± 5% RH, and a 16L : 8D photoperiod.

Fungal formulation

Naturalis®-L (Fargro Ltd., Arundel, United Kingdom) is an oil dispersion formulation of *B. bassiana* strain ATCC 74040 that contains 2.3×10^7 CFU / ml. This formulation of *B. bassiana* was selected for testing because it is commercially available to growers of greenhouse cucumbers in Iran where it is frequently recommended for use in pest management programs. In addition, the active fungal ingredient of Naturalis®-L was isolated for use as a positive control using the method described by Rondot & Reineke (2018). An aliquot of 200 µl of the commercial formulation was plated on a solid medium consisting of 10 g soy peptone, 20 g glucose, and 18 g bacterial agar dissolved in 1000 ml sterile distilled water that had been autoclaved for 20 min at 121°C. The Petri dishes were then maintained at 24°C in the dark for eight days, whereupon conidia were harvested by gently scraping the surface of the media and suspending the scrapings in 20 ml Ringer's solution (12.5%, v/v) containing 0.02% Tween 80. The concentration of conidia was then determined using a hemocytometer and adjusted to 2×10^7 conidia / ml to be the same as in the commercial formulation treatment.

Virulence of *B. bassiana* topically applied to *A. gossypii*

In order to assay the direct pathogenicity of *B. bassiana* to *A. gossypii*, leaf discs, each 8 cm diam, were cut from newly-expanded leaves of 35 d-old cucumber plants and each placed adaxial surface upwards on moist cotton in a plastic Petri dish (9 cm diam). Seventy five adult *A. gossypii* (< 24 h since final molt) were then transferred to each leaf disc (n = 4 per treatment). Each disc was then sprayed in a Potter spray tower (Burkard Manufacturing, Rickmansworth, UK) at 69 kPa with either Naturalis®-L (1.5 ml / 1 L), or with 2 ml of the isolated *B. bassiana* fungus as a positive control, with 2 ml of deionized water serving as a negative control. This resulted in deposition of 0.03 ml of solution per cm^2, or 1×10^3 CFU of the fungus. The aphids from each replicate were then carefully transferred to a clean cucumber plant using a fine brush. Each plant (n = 4 per treatment) was then caged individually and the number of live aphids was tallied daily for the next seven days.

Inoculation of plants with *B. bassiana*

Once cucumber plants had two fully expanded true leaves, we covered the soil in each pot with aluminum foil (to prevent soil contamination and subsequent epiphytic colonization of untreated leaves) and then sprayed them with the recommended concentration of Naturalis®-L (1.5 ml / 1 L), or with isolated *B. bassiana* strain as a positive control, or with sterile distilled water as a negative control. Each treatment had three replicates consisting of three plants each. Both abaxial and adaxial leaf surfaces were sprayed to run-off with a handheld sprayer (454 Handheld Sprayer, Solo® Inc., Newport News, VA, USA) operating at a pressure of two bars.

Endophytic colonization of cucumber plants by *B. bassiana*

Cucumber plants were evaluated for endophytic colonization by *B. bassiana* at four day intervals over a total of 48 days, and only in leaves that developed after the treatment, using the methods described by Klieber & Reineke (2015). All treatments were replicated three times with three plants in each replicate. Three leaves were harvested from each plant (one each from the upper, middle, and lower plant parts) and individually surface-sterilized. Each leaf was first dipped in 0.5% NaOCl (containing 0.05% Tween 80) for 1 min, then in 70% ethanol for 30 s, followed by

two washes of 1 min each in sterile distilled water, and a final rinse in sterile distilled water. Water from the final rinse (200 µl in each of 3 replicates) was then plated on *Beauveria* selective medium (BSM) plates to verify the absence of any inoculum on the leaf surface, as per Klieber & Reineke (2015). BSM plate medium consisted of 10 g soy peptone, 20 g glucose, and 18 g bacterial agar dissolved in 1000 ml sterile distilled water and supplemented with 0.1 g/L streptomycin, 0.05 g/L tetracycline, 0.1 g/L dodine, and 0.05 g/L cyclohexamide. Surface-sterilized leaves were placed on filter paper and air-dried in a laminar flow hood, whereupon ten leaf discs (each 1 cm diam) were cut from each cucumber leaf using a sterilized cork borer. The leaf discs were then placed, adaxial surface upwards, on BSM plates. All plates were then incubated in the dark at 24°C for 20 days, at which time microscope slides of fungi growing out of the leaf discs and onto the BSM plates were prepared and examined under a binocular microscope. The presence of characteristic dense, white mycelia and clusters of conidia, matching description of Humber (1997), was considered evidence of endophytic *B. bassiana* colonization. The number of leaf discs with *B. bassiana* outgrowth was divided by the total number of leaf discs to obtain the percentage of leaf discs successfully colonized per cucumber plant.

Plant growth and yield with endophytic *B. bassiana*

In a separate experiment, cucumber plants at the 2-leaf stage (n = 3 plants per treatment), cultivated as in "Inoculation of plants with *B. bassiana*" and similarly treated with either 1.5 ml / 1 L of Naturalis®-L, with the isolated *B. bassiana* fungus, or with deionized water, were harvested regularly until the end of fruit production, whereupon plant height, stem diameter, number of nodes per plant, and total yield (kg fresh weight of fruit/plant) were all measured.

Biochemical analysis of cucumber plants

Biochemical analyses of cucumber plants were all conducted with three independent plant replicates using leaves that grew subsequent to treatment with Naturalis®-L, the isolated *B. bassiana* fungus, or with sterile water. Leaves were harvested at early flowering stages (28 d after treatment), placed in a paper bag, and dried in an oven at 35°C for seven days. The dry leaves of each plant were then ground with a mortar and pestle for two min, following the procedure of Espinoza et al. (2019).

Plant concentrations of phenols, alkaloids, and flavonoids were determined according to the methods of Espinoza et al. (2019), and concentrations of MDA, total chlorophyll and H_2O_2 were also assayed as indicators of plant health. To assay phenol concentrations, 25 µl of aqueous ethanol extracts of dried leaves were mixed with 125 µl of the Folin-Ciocalteu reagent. After five min, 100 µl of 7.5% aqueous Na_2CO_3 was added. The extracts were incubated for two hours at room temperature before the absorbance was read at 765 nm using a spectrophotometer. The standard curve was prepared using gallic acid solutions in 10% ethanol. Concentrations of alkaloids were assayed following extraction of 100 mg dried leaves in 10 ml of aqueous ethanol (60%). After centrifugation of extracts at 4000 × *g* for 10 min, two ml of supernatant and atropine standard solutions were mixed with 5 ml phosphate buffer (50 mM, pH 6.8) and 12 ml bromocresol green. Thereafter, 12 ml of chloroform was added to the extracts and standards. Absorbance was measured at 417 nm and compared with a standard curve of atropine. Flavonoid concentrations were determined from aqueous ethanol extracts of dried leaves, using quercetin in 95% ethanol as a standard. Aqueous ethanol extracts were prepared as described above and 12.5 µl of each extract was mixed with 12.5 µl of 0.1% HCl in 95% ethanol and 225 µl of 2% HCl and incubated for 30 min at room temperature. Absorbance was read at 360 nm and compared with the quercetin standard curve. MDA content, which results from lipid peroxidation, was assayed with the thiobarbituric acid test, as described by Sunkar et al. (2006). Spectrophotometric measurements were performed at 532 and 600 nm. Absorbance values at 600 nm were subtracted from those at 532 nm. MDA concentrations were calculated with a molar extinction coefficient of 155 mM^{-1} cm^{-1}. Total chlorophyll content was assayed from extracts made with aqueous acetone (80%) according to the methods of Arnon (1949), with absorbance measured spectrophotometrically at 663 nm (for chlorophyll A) and 645 nm (for chlorophyll B). H_2O_2 content was estimated according to the method of Velikova et al. (2000), which is based on potassium iodide oxidation by H_2O_2 in an acidic medium, followed by a measurement of absorbance of the reaction mixture at 390 nm.

Biochemical analysis of *A. gossypii*

Treated plants in early flowering stages (28 d post-inoculation with either Naturalis®-L or the isolated *B. bassiana* fungus, n = 3 plants per treatment) and control plants (inoculated with sterile water) were each manually infested with about 450 apterous adults of *A. gossypii*, the action threshold for this aphid on 35 d-old plants, and caged individually. Plants were treated as described above in "Inoculation of plants with *B. bassiana*". Cotton aphids were allowed access only to leaves that had fully expanded subsequent to treatment; the first two (treated) leaves were tightly covered in muslin fabric to prevent aphid access. After feeding on plants for 24 h, the aphids were collected, frozen in liquid nitrogen, and stored at –80°C.

Three independent biological replicates of *A. gossypii* were analyzed for each metabolite. Activities of detoxifying enzymes, including glutathione-*S*-transferase (GST), carboxylesterase (COE), and acetylcholinesterase (AChE), were assayed based on the methods of Kandil et al. (2017). For the GST assay, replicates consisted of 10 apterous adult *A. gossypii* from each treatment homogenized in 200 µl phosphate buffer (0.1 M, pH 6.5) and then centrifuged at 12000 × *g* for 15 min at 4°C to obtain supernatants. 100 µl samples of the supernatant were treated with 10 µl 1-chloro-2,4-dinitrobenzene (30 mM), and 10 µl GSH (50 mM). Enzyme activity was determined by continuous monitoring of changes in absorbance at 430 nm for three min at 25°C. COE activity was determined using α-naphthyl acetate (α-NA) as a substrate. For each replicate, 50 apterous *A. gossypii* adults were homogenized in 500 µl of phosphate buffer (0.1 M, pH 7.0) and centrifuged at 12000 × *g* for 15 min at 4°C to obtain the supernatant. Then, 50 µl samples of the supernatant were incubated with 50 µl α-NA (30 mM) for 15 min at 30°C. The reaction was stopped by adding 50 µl of Fast Blue RR. Absorbance was measured at 600 nm to assay the hydrolysis of α-NA and the α-naphthol standard curves were then used to calculate enzyme activity. AChE activity was determined for each replicate by homogenizing 25 apterous *A. gossypii* adults in 200 µl of 0.1 M phosphate buffer (pH 7.5) containing Triton X-100. After centrifugation of homogenized samples at 3000 × *g* for 15 min at 4°C , 25 µl samples of the supernatant were treated with 2 µl of 0.075 M acetylcholine iodide, 8 µl of 0.01 M 5,5'-dithiobis-2-nitrobenzoic acid and phosphate buffer (0.1 M, pH 7.5). Change in absorbance was measured at 405 nm over 20 min.

The energy reserves of *A. gossypii*, including protein, carbohydrate, lipid, and glycogen, were assayed according to the methods described by Foray et al. (2012). A Bradford assay was used to evaluate protein content, with absorbance read at 595 nm. Sample absorbance was compared with a bovine serum albumin standard curve. Glycogen was dissolved by addition of 20 µl of sodium sulfate solution (20%) and lipid and carbohydrate were solubi-

lized by mixing the solution with 1500 µl of a chloroform-methanol solution (1 : 2 v/v). Glycogen and carbohydrate contents were determined colorimetrically using the anthrone reagent at 630 nm with glucose as the standard. Lipid content was determined at 525 nm following addition of vanillin as a reagent and using cholesterol as the standard.

In addition, the activities of digestive enzymes including lipase, α-amylase, α-glucosidase, and aminopeptidase were analyzed according to the methods of Mardani-Talaee et al. (2016). For each replicate, 20 apterous *A. gossypii* adults were homogenized in NaCl solution (0.15 M) and centrifuged at 20000 × *g* for 5 min at 4°C. The activity of lipase was estimated using a 10 µl enzyme sample and 20 µl of p-nitrophenyl butyrate (27 mM) as substrate, both added to 50 µl of universal buffer (pH 7), mixed thoroughly, and incubated at 37°C. After one minute, 100 µl of sodium hydroxide (1 M) was added and absorbance was read at 405 nm. Activity of α-amylase was determined using starch (1%) and dinitrosalicylic acid (DNS). 20 µl of enzyme sample was incubated with 40 µl of starch solution (1%) and 80 µl of universal buffer (pH 7) for 30 min at 35°C. Then, 100 µl of DNS was added, and the tubes containing the reaction mixture were incubated for another 10 min in a boiling water bath. Finally, the absorbance of 100 µl of the reaction mixture was read at 540 nm. The activity of α-glucosidase was assayed by adding 20 µl of p-nitrophenol-α-glucopyranoside (5 mM) in 50 µl of universal buffer (pH 7). Enzyme samples (10 µl) were incubated for 10 min prior to reading absorbance at 405 nm. The activity of aminopeptidase was determined using hippuryl-L-phenylalanine as a substrate (1 mM). 20 µl of the substrate was added to 50 µl of universal buffer (20 mM, pH 7) and enzyme samples (10 µl) were incubated for 10 min at 30°C. The reaction was terminated by adding 100 µl of TCA (30%) and absorbance was read at 340 nm.

Measurements of antioxidant enzyme activities, including superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX), were conducted according to the method of Mardani-Talaee et al. (2016). For each replicate, 20 apterous *A. gossypii* adults were homogenized in NaCl solution (0.15 M) and centrifuged at 20000 × *g* for 5 min at 4°C. SOD activity was determined based on its inhibition of the reaction of nitro blue tetrazolium (NBT) with superoxide anion due to xanthine oxidation. Enzyme samples (100 µl) were added to 500 µl of reaction mixtures containing 70 µM of NBT and 125 µM of xanthine, both dissolved in phosphate buffered saline and the xanthine oxidase solution. The reaction mixture was incubated in darkness for 20 min at 25°C before activity was read at 560 nm. For CAT activity, 100 µl of enzyme sample was added into 500 µl of hydrogen peroxide (1%), incubated at 25°C for 10 min, and the activity was determined by reading the absorbance at 240 nm. For APX activity, reaction mixtures consisted of 100 µl of enzyme sample and 250 µl of 67 mM phosphate buffer (pH 7) containing 2.5 mM ascorbic acid, and 200 µl of 30 mM hydrogen peroxide. The change in absorbance at 290 nm was recorded over five min. Activity of phenoloxidase (PO) was quantified using the procedure described by Wu et al. (2015). For each replicate, 20 apterous *A. gossypii* adults were homogenized in 10 mM Tris-buffer (pH 7.4) and centrifuged at 10000 × *g* for 5 min at 4°C. Then, 25 µl aliquots of enzyme sample were added into 75 µl of 3,4-dihydroxy-L-phenylalanine and 100 µl phosphate buffer (pH 5.8), incubated at 30°C for 5 min. Activity was assayed by reading the absorbance at 490 nm.

Life history of *A. gossypii*

To determine the effects of endophytic *B. bassiana* on the life history and reproductive success of *A. gossypii*, cucumbers were treated at the two-leaf stage with either Naturalis®-L, the isolated *B. bassiana* fungus, or sterile water, and then allowed to grow for 28 days, as described above for assays of plant metabolites. The treated leaves (first two true leaves) were encased in muslin fabric to prevent aphid access. Then, approximately 450 apterous adult aphids were placed on each cucumber plant (n = 3 per treatment) and plants were caged individually.

After 24 h of feeding, all surviving aphids were transferred individually to fresh, untreated cucumber leaf discs (3.5 cm diam), one apterous female per disc, to determine their fecundity, longevity and reproductive period. This procedure standardized the feeding period and assayed the acute effects of short-term feeding exposure. Leaf discs were each isolated on 2% agar (2 cm deep) in a glass Petri dishes (8 cm diam) and replaced every 3 days. Only the adult female was transferred to the new leaf disc; its survival and the numbers of nymphs she produced were recorded daily until she died.

Susceptibility of *A. gossypii* to pirimicarb

Toxicity bioassays with pirimicarb were conducted on apterous *A. gossypii* adults that had fed on either Naturalis®L-treated, *B. bassiana* isolate-treated, or untreated cucumber plants. Plants were treated at the two-leaf stage and maintained as described above; treated leaves were encased in muslin to prevent access to aphids. Twenty eight days after treatment, approximately 450 apterous adult *A. gossypii* were transferred onto leaves of treated and control cucumber plants (n = 3 in all cases). The aphids fed for 24 h before use in bioassays.

Based on bracketing tests, five concentrations of pirimicarb (Pirimor® 50 WG, Syngenta, Switzerland), specifically 0.05, 0.1, 0.2, 0.4, and 0.8 mg a.i./L, and an untreated control (sterile distilled water) were used to estimate LC_{50} values. Previous work indicated that these concentrations should cause mortality ranging from 10%–90% (Robertson et al., 2017). According to the method of Moores et al. (1996), cucumber leaf discs (each 1 cm diam, not exposed to *B. bassiana*) were cut from each leaf using a sterilized cork borer, dipped in the appropriate pirimicarb solution for 5 s, air-dried, and then placed with the adaxial surface down on a 2% agar bed in Petri dishes (as described under "Life history of *A. gossypii*"), one per dish. Each replicate (n = 3) consisted of twenty apterous *A. gossypii* adults placed on a single leaf disc. Petri dishes were held at 25 ± 1°C, 50 ± 5% RH, and a 16L : 8D photoperiod in a growth chamber. Mortality was recorded 24 h later and probit analysis was conducted to estimate LC_{50} values.

Statistical analysis

Mortality data for *A. gossypii* following direct application of *B. bassiana* were analyzed by Chi-square, Goodness-of-fit test and Kaplan-Meier plots were generated. Data from the biochemical analysis of plants and aphids, as well as the aphid life history data, plant morphological data, and yield data were subjected to one-way ANOVA followed by Bonferroni test to separate means, after they passed tests for normality (Shapiro-Wilkes) and equality of variances (Levine's test). All analyses were performed in GraphPad Prism version 8.2.0 (GraphPad, 2019).

LC_{50} values, slopes, and 95% confidence intervals (95% CI) for the pirimicarb toxicity assays were estimated using Polo Plus version 2.0 (Polo, 2007). Significant differences between LC_{50} values were determined by logit regression to compare slopes and intercepts of logit lines, as described by Robertson et al. (2017).

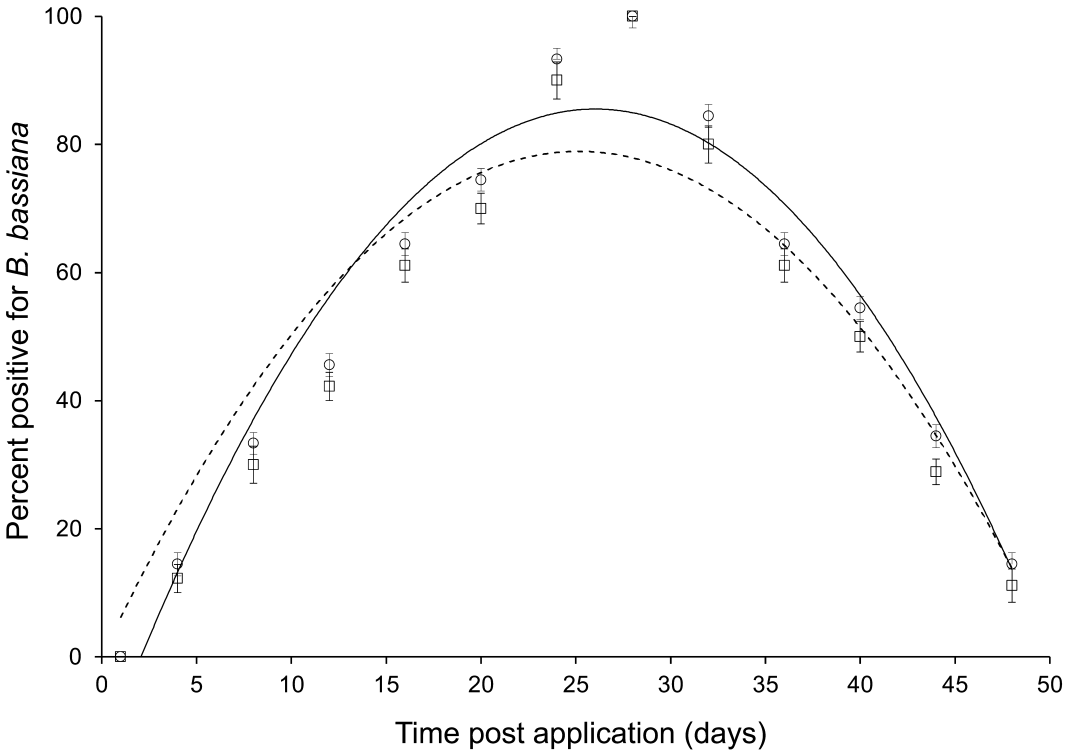

Fig. 1. Mean (± SE) percentage of cucumber leaf discs (n = 10 per day) positive for endophytic *Beauveria bassiana* when harvested various days after application of *Beauveria bassiana* to different (older) leaves as either Naturalis®-L at a concentration of 1.5 ml / L H_2O with 0.03 ml of solution deposited per cm^2, or 1×10^3 CFU per cm^2 (circles, solid line) or as the *B. bassiana* isolate alone at a concentration of 2×10^7 CFU / ml (squares, hatched line).

RESULTS

Endophytic colonization of cucumber plants by *B. bassiana*

Beauveria bassiana successfully colonized cucumber leaves that developed subsequent to the application of Naturalis®-L (1.5 ml / L), or the isolated *B. bassiana* fungus, on the first true leaves. The fungus colonized the plants progressively until all leaf discs taken from all plants inoculated with *B. bassiana* (100%) showed endophytic establishment 28 days after treatment, with the proportion positive for the fungus declining thereafter (Fig. 1), whereas leaf discs from control plants did not show any signs of fungal outgrowth (data not shown). Similarly, no fungal growth was observed on the plates receiving the final rinse water, confirming no inoculum was present on the surfaces of the leaves.

Virulence of *B. bassiana* topically applied to *A. gossypii*

Aphids treated with either Naturalis®-L or the *B. bassiana* isolate alone died at similar rates and were all dead

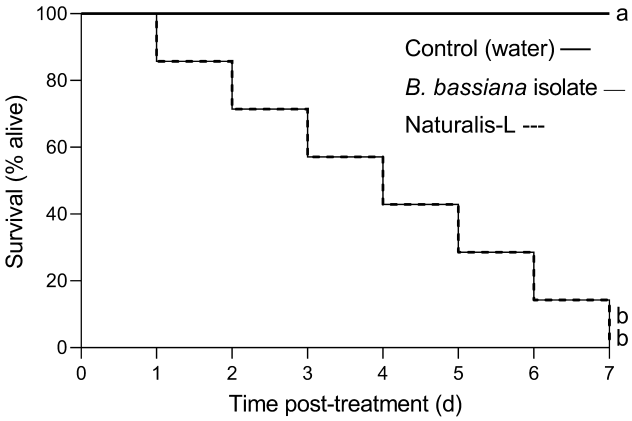

Fig. 2. Kaplan Meier plot of mortality of adult *Aphis gossypii* topically treated with either Naturalis®-L (at a concentration of 1.5 ml / L H_2O with 0.03 ml of solution deposited per cm^2, or 1×10^3 CFU per cm^2), or with the *Beauveria bassiana* isolate alone (at a concentration of 2×10^7 CFU / ml). Curves bearing different letters were significantly different at end of experiment (Control vs Naturalis®-L: $\chi^2 = 60.45$, $P < 0.001$; Control vs *B. bassiana*: $\chi^2 = 59.11$, $P < 0.001$; Naturalis®-L vs *B. bassiana*: $\chi^2 = 0.002$, $P = 0.981$).

Table 1. Mean (± SE) plant growth and yield parameters for cucumber plants (n = 3 per treatment) when treated with Naturalis®-L, the *B. bassiana* isolate alone, or water (Control) at the 2-leaf stage and then evaluated post-harvest. Values bearing different letters were significantly different within rows (ANOVA followed by Bonferroni, α = 0.05).

Variable (units)	Control	Naturalis®-L	*B. bassiana*	$F_{2,6}$	*P*
Plant height (cm)	220.0 ± 8.4[a]	235.0 ± 11.3[a]	228.0 ± 9.2[a]	29.54	0.09
Stem diameter (cm)	1.5 ± 0.12[a]	1.6 ± 0.15[a]	1.5 ± 0.11[a]	26.56	0.08
Nodes per plant (no.)	30.9 ± 2.01[a]	34.8 ± 2.14[a]	32.7 ± 2.23[a]	23.85	0.06
Yield (fresh weight in kg)	5.1 ± 0.24[a]	5.6 ± 0.28[a]	5.4 ± 0.26[a]	24.71	0.07

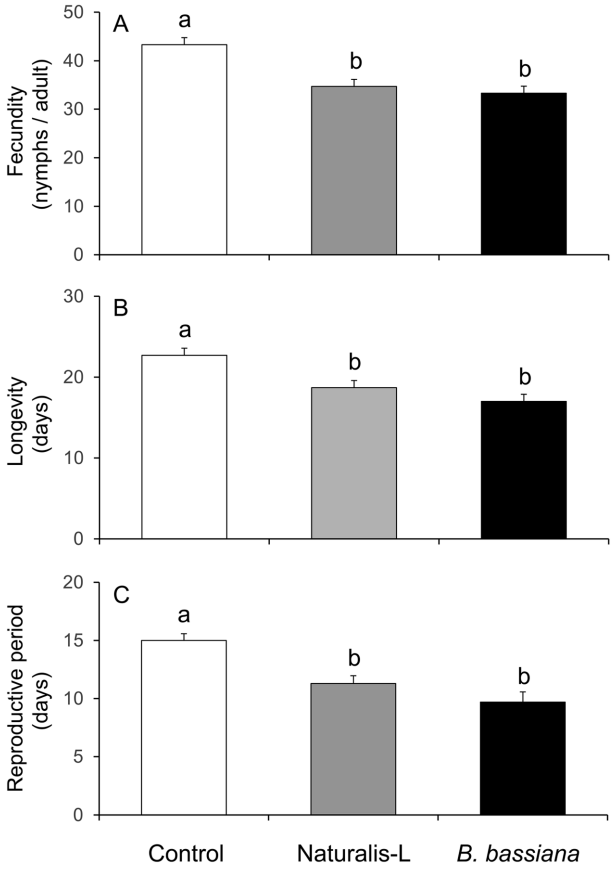

Fig. 3. Mean (± SE) fecundities, longevities, and reproductive periods of *Aphis gossypii* apterous females that fed on cucumber plants that were inoculated with either Naturalis®-L (at a concentration of 1.5 ml / L H_2O, with 0.03 ml of solution deposited per cm^2, or 1×10^3 CFU per cm^2), the *Beauveria bassiana* isolate alone (at a concentration of 2×10^7 CFU / ml), or water (Control) for 24 h before transfer to an untreated leaf disc. Values bearing different letters were significant different (ANOVA followed by Bonferroni, α = 0.05).

after seven days, whereas control aphids experienced no mortality within this time frame (Fig. 2).

Plant growth and yield with endophytic *B. bassiana*

At end of harvest, plants receiving treatment with either Naturalis®-L or the *B. bassiana* isolate did not differ significantly from control plants in any component of yield (Table 1).

Biochemical analysis of cucumber plants

Cucumber plants inoculated with either Naturalis®-L or the *B. bassiana* isolate had significantly higher levels of alkaloids, flavonoids, phenols, H_2O_2, and total chlorophyll when compared to control plants, whereas concentrations of MDA did not differ between treatment and control plants (Table 2).

Biochemical analysis of *A. gossypii*

Aphids that fed on plants inoculated with either Naturalis®-L or the *B. bassiana* isolate had significantly lower activity of COE, GST, and AChE when compared with aphids that fed on plants treated only with water (Table 3). Similarly, activities of lipase, α-amylase, α-glucosidase, aminopeptidase, and CAT were significantly lower in aphids that fed on fungus-treated plants compared to controls, whereas activities of SOD, APX, and PO were all higher.

Cotton aphids from fungus-treated plants had greater carbohydrate content compared to those that fed on controls, whereas protein content, glycogen content, and lipid content were all lower compared to controls (Table 3).

Biological traits of *A. gossypii*

Cotton aphids that fed on fungi-inoculated plants had significantly lower fecundity ($F_{2,6} = 18.56$, $P = 0.041$), reduced longevity ($F_{2,6} = 16.35$, $P = 0.020$), and shorter reproductive periods ($F_{2,6} = 14.44$, $P = 0.015$) compared to aphids that fed on control plants (Fig. 3).

Susceptibility of *A. gossypii* to pirimicarb

The LC_{50} values of pirimicarb for *A. gossypii* that fed on fungus-inoculated plants were reduced to approximately half that of control aphids (Table 4), but did not differ significantly whether Naturalis®-L or the *B. bassiana* isolate was used, indicating that endophytic colonization of cucumber plants with *B. bassiana* increased the susceptibility of *A. gossypii* to pirimicarb.

DISCUSSION

Treatment of cucumber seedlings with *B. bassiana* at the 2-leaf stage, whether using the Naturalis®-L formulation or the fungal isolate alone, resulted in endophytic fungal growth and colonization of plant tissues that expanded and developed, peaking around 28 days post-application, and declining thereafter. Endophytic fungi may eventually decline within inoculated plants in response to various factors, such as the immune responses of plant tissues, or competition with other endophytes present in the plant (Posada et al., 2007; Gurulingappa et al., 2010). Endophytic colonization of cucumber by *B. bassiana* resulted in increased

Table 2. Mean (± SE) concentrations (mg / g DW) of various metabolites in cucumber plants (n = 3 per treatment) when treated with either Naturalis®-L, the *B. bassiana* isolate alone, or water (Control), at the 2-leaf stage and then assayed in early flowering stages (28 d post-treatment). Abbreviations: Hydrogen peroxide (H_2O_2), malondialdehyde (MDA). Values bearing different letters were significantly different within rows (ANOVA followed by Bonferroni, α = 0.05).

Variable (units)	Control	Naturalis®-L	*B. bassiana*	$F_{2,6}$	*P*
Alkaloids	1.85 ± 0.04^b	2.18 ± 0.03^a	2.09 ± 0.05^a	12.63	0.01
Flavonoids	2.61 ± 0.12^b	3.12 ± 0.11^a	2.98 ± 0.10^a	13.45	0.04
Phenols	1.11 ± 0.05^b	1.48 ± 0.04^a	1.37 ± 0.06^a	14.96	0.02
Hydrogen peroxide (H_2O_2)	0.40 ± 0.01^b	0.49 ± 0.02^a	0.47 ± 0.03^a	11.63	0.03
Total chlorophyll	1.32 ± 0.04^b	1.57 ± 0.05^a	1.50 ± 0.04^a	16.89	0.01
Maldonaldehyde (MDA)	1.81 ± 0.08^a	1.85 ± 0.07^a	1.83 ± 0.06^a	21.99	0.09

Table 3. Mean (± SE) specific activities of various enzymes in *Aphis gossypii* tissues and mean concentrations of other non-enzymatic compounds, when plants (n = 3 per treatment) were treated with Naturalis®-L, the *B. bassiana* isolate alone, or water (Control) at the 2-leaf stage and assayed in early flowering stages (28 d post-treatment). Plants were infested with ca. 450 adult *A. gossypii* apterae per plant and, 24 h after treatment, aphid samples were analyzed in three replicates. Abbreviations: carboxylesterase (COE), glutathione-S-transferase (GST), acetylcholinesterase (AChE), superoxide dismutase (SOD), ascorbate peroxidase (APX), phenoloxidase (PO), catalase (CAT). Values bearing different letters were significantly different within rows (ANOVA followed by Bonferroni, α = 0.05).

Variable	Naturalis®-L	*B. bassiana*	Control	$F_{2,6}$	P
COE (μmol / min / mg protein)	0.13 ± 0.01b	0.14 ± 0.01b	0.19 ± 0.01a	18.11	0.01
GST (nmol / min / mg protein)	45.22 ± 0.86b	47.13 ± 0.75b	52.75 ± 0.91a	16.28	0.04
AChE (nmol / min / mg protein)	2.15 ± 0.02b	2.18 ± 0.03b	2.59 ± 0.04a	15.48	0.02
Lipase (μmol / min / mg protein)	54.53 ± 0.81b	56.13 ± 0.72b	59.93 ± 0.99a	13.71	0.03
α-amylase (μmol / min / mg protein)	4.46 ± 0.11b	4.60 ± 0.08b	4.98 ± 0.09a	12.36	0.01
α-glucosidase (μmol / min / mg protein)	53.00 ± 0.82b	55.13 ± 0.70b	58.98 ± 0.92a	10.89	0.02
Aminopeptidase (μmol / min / mg protein)	0.07 ± 0.01b	0.08 ± 0.01b	0.11 ± 0.01a	17.85	0.04
SOD (μmol / min / mg protein)	0.16 ± 0.01a	0.14 ± 0.01a	0.10 ± 0.01b	14.90	0.03
APX (μmol / min / mg protein)	1.16 ± 0.02a	1.14 ± 0.02a	1.01 ± 0.01b	11.23	0.01
PO (nmol / min / mg protein)	47.14 ± 0.71a	45.13 ± 0.91a	40.82 ± 0.89b	18.95	0.02
CAT (μmol / min / mg protein)	0.31 ± 0.02b	0.33 ± 0.01b	0.38 ± 0.02a	15.36	0.02
Carbohydrate (μg / insect)	13.91 ± 0.33a	13.56 ± 0.21a	12.21 ± 0.18b	16.80	0.01
Protein (μg / insect)	34.13 ± 0.46b	36.00 ± 0.31b	38.51 ± 0.52a	12.12	0.03
Glycogen (μg / insect)	7.13 ± 0.17b	8.02 ± 0.19b	9.92 ± 0.14a	13.85	0.04
Lipid (μg / insect)	5.11 ± 0.13b	5.33 ± 0.19b	5.89 ± 0.11a	10.11	0.04

levels of several metabolites within plants that have roles in plant defense against herbivores, but had no measurable effect on plant fitness, morphological parameters, or final yield. Elevated levels of these metabolites may have resulted either from endophyte-induced production by the plant (Vega, 2018), or from their production by the endophyte itself (Jaber & Ownley, 2018). Ultimately, *A. gossypii* that fed on fungus-inoculated plants showed reduced activities of detoxifying, digestive, and antioxidant enzymes, as well as reduced storage of energic compounds, all indications of physiological stress. These physiological impacts translated into reduced longevity, fecundity, and reproductive periods and rendered *A. gossypii* more susceptible to pirimicarb, a commonly used aphicide in Iranian greenhouses.

Topical application of *B. bassiana* to adult *A. gossypii*, whether as the Naturalis®-L formulation or the fungal isolate alone, resulted in significant mortality relative to controls at 24 h after treatment, and complete mortality after seven days, a significantly higher mortality than observed in control aphids. It has been previously demonstrated that applications of *B. bassiana* cause significant direct mortality to *A. gossypii* (e.g., Kang et al., 2008; Degefu et al., 2014), and any progeny produced by these aphids prior to death will be subject to the adverse impacts of endophytic *B. bassiana*.

MDA is a lipid peroxidation biomarker and a metabolite indicative of plant stress (Heidarvand & Maali-Amiri, 2013), but we found no significant difference in MDA levels between fungus-treated and untreated plants, suggesting that endophytic colonization of cucumber by *B. bassiana* did not stress the plant. On the contrary, we found evidence of physiological benefits of endophytic *B. bassiana* in treated plants in the form of higher levels of total chlorophyll. Similarly, Sanchez-Rodriguez et al. (2015) demonstrated benefits of *B. bassiana* endophytic colonization of tomato and wheat in the form of improved iron nutrition on calcareous soils associated with iron chlorosis.

Levels of alkaloids, flavonoids, phenols, and hydrogen peroxide increased in fungus-treated plants, relative to controls, by the time they reached early flowering stages. Similarly, Espinoza et al. (2019) found significant increases in the alkaloid content of chive plants, *Allium schoenoprasum*, three weeks post-inoculation with *B. bassiana*. Endophytic enhancement of such compounds has been linked to adverse impacts on various herbivorous insects (Rondot & Reineke, 2018; Vega, 2018). These secondary metabolites may be endogenously produced by endophytic fungi, sometimes using genes acquired from their host plants, or via epigenetic modification of gene expression within the plant (Meena et al., 2019). However, plant responses to endophytic *B. bassiana* can vary greatly among species and modes of inoculation (Sanchez-Rodriguez et al., 2018). For example, Moloinyane & Nchu (2019) found that *B. bassiana* inoculation of grape vines, although only 50% successful, caused elevated concentrations of calcium and magnesium in plant tissues, and enhanced the production of various anti-insect volatile compounds, without altering levels of polyphenols, flavonoids or alkaloids.

Because endophytic *B. bassiana* can alter plant physiology and biochemical composition in complex ways, its ef-

Table 4. Toxicity of pirimicarb to *A. gossypii* (n = 360 aphids per treatment) that were fed on leaves of cucumber plants inoculated either with Naturalis®-L, the *B. bassiana* isolate alone, or water (Control). LC_{50} values bearing different letters had significantly different logit lines in logit regression.

	LC_{50} (mg L^{-1})	95% CI	Slope ± SE	χ^2 (df)	Heterogeneity
Control	0.08[a]	0.06–0.11	1.96 ± 0.47	6.28 (13)	0.57
Naturalis®-L	0.04[b]	0.02–0.07	1.31 ± 0.22	4.55 (13)	0.45
B. bassiana	0.05[b]	0.03–0.08	1.39 ± 0.28	5.80 (13)	0.66

fects on insect herbivores can be equally diverse and complex (McKinnon et al., 2017). We found that the activity of at least three important detoxification enzymes, GST, COE, and AChE, were substantially reduced in cotton aphids that fed on *B. bassiana*-inoculated plants. These particular enzymes provide key mechanisms for the detoxification of plant defensive chemicals and other xenobiotics (Despres et al., 2007), while also maintaining normal physiological functions (Singh et al., 2019). GST activity is known to be inhibited by phenols (Lukasik & Golawska, 2007) and flavonoids (Yu & Abo-Elghar, 2000). Similarly, the activity of AChE can be inhibited by phenols and alkaloids (Wink et al., 1998; Rajashekar et al., 2014), and the activity of COE can be inhibited by phenols (Juntheikki & Julkunen-Tiitto, 2000) and flavonoids (Wang et al., 2016). Therefore, it is likely that the elevated levels of alkaloids, flavonoids, and phenols in *B. bassiana*-inoculated plants were at least partially responsible for the reduced activity of detoxifying enzymes in *A. gossypii* that fed on these plants.

Feeding on plants inoculated either with Naturalis®-L or the *B. bassiana* isolate also reduced the activities of digestive enzymes in *A. gossypii*. These plants had higher levels of H_2O_2 relative to control plants, which can disrupt enzyme functions in insects (Felton & Duffey, 1991), have toxicological effects on herbivore midguts (Bi & Felton, 1995), and damage the insect digestive system (War et al., 2012). Phenols can also inhibit digestive enzymes (Johnson & Felton, 2001), and the production of semiquinone in the herbivore lumen, resulting in cytotoxic effects on lumen tissues (Barbehenn et al., 2010). Thus, the decreased activities of *A. gossypii* digestive enzymes on fungus-isolated cucumber plants probably result from increases in the phenolic and H_2O_2 contents of plants induced by *B. bassiana*, and likely contributed to the lower reproductive success and fitness of aphids on these plants.

Aphids that fed on *B. bassiana*-inoculated plants had higher levels of antioxidant enzymes than those that fed on control plants. Antioxidants such as SOD can be induced by flavonoids (Ahmad & Pardini, 1990) and phenols (Lukasik, 2007), so the increased activity of SOD in *A. gossypii* on fungus-inoculated plants may be linked to the increased levels of flavonoids and phenols in these plants. SOD catalyzes the dismutation of the superoxide anion into H_2O_2. Consequently, increased SOD activity in cotton aphids may result in H_2O_2 production, a toxic free radical that is subsequently metabolized to H_2O and O_2 by the activation of CAT (Shamakhi et al., 2020). Thus, the lower activity of CAT in *A. gossypii* fed on plants inoculated with *B. bassiana* could result from the increased levels of flavonoids (Ahmad & Pardini, 1990), alkaloids (Cai et al., 2009), and phenols (Lukasik, 2007) in these plants. Reduced CAT activity would diminish the ability of *A. gossypii* to overcome further dietary oxidative stress (Felton & Duffey, 1991). Furthermore, the higher levels of phenols in fungus-treated plants could have resulted in increased APX activity in the aphids (Lukasik et al., 2009), possibly resulting in ascorbic acid deficiency, as APX oxidizes ascorbic acid to dehydro-ascorbic acid (Felton & Summers, 1993). Because ascorbic acid acts as a powerful antioxidant against dietary pro-oxidants (Mathews et al., 1997), any deficiency would cause additional physiological stress for the aphids. The increased PO activity in *A. gossypii* fed on fungus-inoculated plants could be related to the elevated levels of alkaloids (Cai et al., 2009) or phenols (Gonzalez-Santoyo & Cordoba-Aguilar, 2012) in these plants. Ingestion of these secondary metabolites from host plants tends to increase PO activity in herbivorous insects, and typically results in production of cytotoxic quinones (Sugumaran et al., 2000). Overall, our results indicate that endophytic *B. bassiana* causes oxidative stress to *A. gossypii* by disrupting antioxidative processes, which likely contributes to the observed reductions in aphid fitness.

Because stress induces compensatory changes in organismal metabolism, energy reserves often reflect overall fitness and condition (Villarroel et al., 2009, Homayoonzadeh et al., 2020b). The reduced protein content of cotton aphids fed on *B. bassiana*-inoculated plants may reflect the observed reduction in enzyme content (Neoliya et al., 2005). Reduced lipid content may reflect increased secretion of the corpus allatum hormone in response to lipid release from the fat body and other tissues (Mandal, 1982), as well as activation of the adipokinetic hormone, which increases lipolysis activity in fat body adipocytes (Patel et al., 2005). In contrast, decreased glycogen content, coupled with increased levels of carbohydrate, may reflect increased glycogenolysis, the process which converts glycogen reserves to soluble carbohydrate (Lagadic et al., 1994). Reductions in these three primary energy reserves would be expected to negatively impact aphid fitness. However, the changes we observed in *A. gossypii* nutrient reserves on fungus-inoculated plants may also reflect reduced consumption due to changes in feeding behavior. For example, Gonzalez-Mas et al. (2019b) used the electrical penetration graph technique to confirm modified *A. gossypii* feeding behavior on *B. bassiana*-colonized melon plants that resulted in reduced acquisition of two cucurbit viruses.

In this study, the longevity and reproductive success of *A. gossypii* was diminished after aphids fed on fungus-inoculated plants, whether Naturalis®-L or the *B. bassiana* isolate alone was used. Host plant antioxidant systems can directly impact herbivores, and higher levels of H_2O_2 often correlate negatively with their survival (Sohal, 1988). Strong negative correlations have been demonstrated between plant alkaloid content and herbivore growth, immature survival, reproduction, and longevity (Thakur et al., 2012). Likewise, high levels of plant phenols are also negatively correlated with the same indices of herbivore fitness (Wermelinger et al., 1991; Srjnwasaperumal et al., 1992), as are elevated levels of flavonoids (Salunke et al., 2005; Vasquez et al., 2008; Golan et al., 2017). Thus, we conclude that the altered host plant physiology induced by *B. bassiana* inoculation of cucumber plants reduced the fitness of cotton aphids and would be expected to diminish aphid abundance in the crop, above and beyond the mortality caused by direct exposure. In a natural situation, most apterous aphids will feed on a plant for much longer than

the 24 h exposure period we tested, so the chronic effects of longer term exposure would likely produce even greater negative impacts on the aphid population.

The stress caused by feeding on *B. bassiana*-inoculated plants may also enhance *A. gossypii* management by increasing their susceptibility to insecticides. Aphids that fed on fungus-inoculated plants were significantly more susceptible to pirimicarb, likely due to reduced levels of the enzymes used to detoxify this pesticide (Liang et al., 2007; Liu et al., 2015). Consequently, inoculation of cucumber plants with *B. bassiana* could potentially aid in maintaining a higher level of insecticide susceptibility within the *A. gossypii* population, thus mitigating the evolution of resistance in the aphids (Ambethgar, 2009).

We conclude that inoculation of cucumber plants with *B. bassiana* elevates levels of secondary metabolites, which alter the physiology of cotton aphids that feed on them. Therefore, endophytic *B. bassiana* has the potential to alter herbivore-plant interactions in favor of cucumber plants, and at the expense of *A. gossypii* fitness and population growth. These findings reveal that endophyte-induced changes in plant physiology hold promise as a novel management tactic for diminishing populations of *A. gossypii*, while simultaneously enhancing their susceptibility to insecticides. Further research is warranted to explore the potential impact of endophytes on other cucumber pests, and on tritropic interactions within the cucumber arthropod community, but at this point the use of Naturalis®-L, a product readily available to farmers, can be recommended on early stage cucumber plants as a viable tactic for inclusion in an integrated management program for *A. gossypii* on greenhouse cucumber.

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CONFLICTS OF INTEREST. The authors declare no conflicts of interest.

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