Evaluating the Effect of *Beauveria bassiana* on Secondary Metabolite Contents and Green Peach Aphid (*Myzus persicae*) Infestation Level on Lettuce (*Lactuca sativa* L.)

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Abstract— Endophytic fungus play a crucial role in protecting food crops against phytophagous insects through endophytism. In this greenhouse study, two sets of potted lettuce plants were inoculated with one of four fungal conidial concentrations: 0, 1×10^6, 1×10^7, and 1×10^8 conidia mL^{-1}. The first set of plants was used to test the effect of *B. bassiana* inoculation on aphid infestation levels on lettuce in meshed boxes. The second set of plants was used for assessing the effects of fungal inoculation on secondary metabolite contents. The results showed that the fungus did not significantly ($P > 0.05$) affect insect infestation levels. However, total polyphenol contents varied significantly with conidial concentrations. The GC-MS analysis detected a wide range of volatile compounds, with two well-known insect repellents, 3-octanol and 2,4-di-tert-butyl-phenol, occurring at significantly ($P < 0.01$) higher concentrations in the fungus-treated plants than the control plants. In conclusion, *B. bassiana* inoculation significantly enhanced polyphenol content and the quantities of some volatile compounds.

Keywords— *Beauveria bassiana*; lettuce; *Myzus persicae;* secondary metabolites; insect infestation

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I. Introduction

Green peach aphid (*Myzus persicae*) is among the most devastating pests of agriculture and horticulture crops [1]- [3]. The financial loss caused by this pest is estimated at several billion US dollars per annum [4]. They transmit some highly infectious viral agents to plants. These include potato virus y (PVY), mosaic virus, and beet western yellows virus (BWYV) [5]-[7]. Furthermore, high sums of money are spent on their control [8], which has primarily relied on the use of synthetic insecticides.

Chemical-based synthetic insecticides, such as organophosphates, carbamates, pyrethroids, and neonicotinoids, are still widely employed for the control of aphids [9], [10]. However, despite advances made in developing new synthetic insecticides, it is hard to achieve sustainable control of green peach aphids. Insecticide resistance and toxicity have been empirically linked to synthetic insecticides [11], [12].

Consequently, interest in bio-rational control methods, which are considered to be environmentally friendly, is on the rise. Biological control agents, such as fungi, have shown good prospects in managing insect pest populations under field and greenhouse conditions [13]. However, inconsistent stability and efficacy under adverse environmental conditions have negatively affected their widespread use [14]. Despite these setbacks, entomopathogenic fungi are quite versatile; they have specific characteristics that can be exploited to enhance their efficacy against aphids [15]. Some entomopathogenic fungi are endophytic, living symbiotically in the tissues of plants without causing visible symptoms nor damage to the host plants [16]. *B. bassiana* and *Clonostachys spp.* can successfully colonize plant tissues, offering protection against insects and pathogens, and enhancing plant growth [[17]-[20]. Previously chemical analysis of plant tissues colonized by endophytic fungi revealed that fungi enhance secondary metabolite contents in plants [21]-[23]. Ren and Dai [24] demonstrated that inoculating plants with fungus enhanced

Jasmonic acid (JA) and increased the production of volatile oil compounds in plant hosts. *B. bassiana* and *M*etarhizium *robertsii* can produce volatile organic compounds (VOC) that are insecticidal or repellent [25]. Nitrosoamide, produced by *Muscodor* spp., is a classic example of a fungal volatile compound that is detrimental to insects [25]. Recently, Moloinyane and Nchu [26] found a higher number of volatile compounds, including insect repellent, naphthalene, in fungus-treated grapevines than in the control plants. *Beauveria* and *Metarhizium* Other well-known repellents produced by the endophytic fungi *Beauveria* and *Metarhizium* are 1- octen- 3- ol, 3- octanone, and 1- octene [27]. Some species of the genus *Beauveria* produce toxic metabolites that reduce insects' survival or delay pest reproduction [28]. Hence, endophytic fungi are enticing biocontrol agents [29]. We hypothesised that inoculating lettuce seedlings with conidia of *B. bassiana* will enhance bioactive secondary metabolite contents in plants and reduce aphid infestations of the treated plants.

This paper's objectives were to assess the effects of *B. bassiana* inoculation on secondary metabolites contents of lettuce as well as to assess the effect of *B. bassiana* inoculation on *M. persicae* infestation level lettuce plants in a greenhouse.

II. Materials And Methods

A. Fungus preparation

An existing *B. bassiana* strain (SM 3) that was previously isolated from a vineyard and identified molecularly by [26] was used in this study. The fungus was cultured on a selective medium: Half-strength (19.5 g/ 1000 mL) of Potato Dextrose Agar (PDA) (Sigma-Aldrich PTY. LTD., South Africa), 0.04 g streptomycin, and 0.02 g ampicillin sodium salt. The PDA was prepared in 9 cm- and 14 cm-diameter Petri dishes. Fungal cultures were incubated for three weeks at 25 ± 2 ^{o}C in the darkness. The mature conidia of *B. bassiana* were harvested using a sterile spatula and transferred into a 50 mL centrifuge tube containing 30 mL distilled water. The tube was capped and shaken for 3 min and mixed vigorously for two minutes using a vortex mixer (MI0101002D Vortex Mixer) at 3000 rpm to homogenize the conidial suspension. The homogenous conidial suspension was transferred into 1000 mL bottles containing 500 mL of distilled water and 0.05% Tween 80 (Polysorbate, Sigma-Aldrich, South Africa). The conidia concentration was determined using a haemocytometer (Bright-Line, Sigma-Aldrich, South Africa) and observed with a light microscope at 400 x magnification to determine the required concentration of (0, 1×10^6, $1 \times 10^{7,}$ and 1×10^8 conidia mL^{-1}). Germination percentage was assessed on 100 spore count at 40 x magnification (Latifian and Rad, 2012). Each plate was replicated four times, and over 90 % germination was observed.

B. Plants

Two-week-old lettuce (*Lactuca sativa* L.; cultivar Green Oak) purchased from Stodels Nurseries (Pty) Ltd in Bellville, Western Cape Province, South Africa were used in this study. Plants were maintained at the Cape Peninsula University of Technology's greenhouse in Bellville, South Africa, under the following conditions: At 25 ± 2 ^{o}C, 60-80% RH, and 14/10 natural light/ dark regime.

C. Aphid rearing

Green peach aphid (*M. persicae* Sulzer [Homoptera: Aphididae]) were reared on Lettuce (*Lactuca sativa* L.; cultivar Green Oak), in the greenhouse of the Cape Peninsula University of Technology, Bellville campus. The aphids were reared under the following controlled conditions: 60– 65% RH, 26 ± 2 ^{o}C, and 12:12 light: dark (L: D) photoperiod.

D. Greenhouse study

Research design/greenhouse experiment

For the greenhouse study, two sets of potted lettuce plants were allocated to one of four treatment groups in a completely randomized design. Plants in each treatment group were exposed to one of four fungal conidial concentrations: 0 (control), 1×10^6, $1 \times 10^{7,}$ and 1×10^8 conidia mL^{-1} (Figure 1). The first set of plants was used to study the effect of fungal inoculation on insect infestation. The plants were confined in meshed boxes (with a mesh size of 0.6 mm) to prevent insects from moving between treatments. The second set of plants was not infested with insects, but rather it was used for assessing the effects of fungal inoculation on secondary metabolite contents. The greenhouse conditions were: temperature 27 ± 3 ^{o}C, 70 ± 3 % relative humidity, and the average light intensity was 31.77 kilolux. Fourteen-day-old lettuce seedlings were transferred into 15 cm pots containing a substrate mix of 25 % silica sand, 25 % coco peat, 25 % perlite, and 25 % vermiculite. Plants were fed using a hydroponics fertilizer Nutrifeed® (Starke Ayres Pty. Ltd., South Africa). The nutrient contents of the fertilizer were: P (27 mg kg^{-1}), N (65 mg kg^{-1}), Ca (70 mg kg^{-1}), K (130 mg kg^{-1}), Cu (20 mg kg^{-1}), Mo (10 mg kg^{-1}), Fe (1500 mg kg^{-1}), Mg (22 mg kg^{-1}), S (75 mg kg^{-1}), B (240 mg kg^{-1}), Mn (240 mg kg^{-1}), and Zn (240 mg kg^{-1}). The fertilizer was mixed with sterile distilled water at a concentration of 10 g/ 5000 mL, and 200 mL was supplied to each plant once a week by drenching. Additionally, each plant was drenched with distilled water twice a week.

Fig. 1 Potted lettuce plants exposed to four conidial concentrations of *Beauveria bassiana* (the yellow tag is the control treatment, blue tag is 1x 10^6 conidial mL^{-1}, red tag 1x 10^7 conidial mL^{-1} and green tag is 1x 10^8 conidial mL^{-1} (A) and meshed boxes containing aphid-infested lettuce plants that were exposed to control or fungal treatment (B).

Insect infestation

After 35 days, the plants were infested with ten adult female aphids using a camel hairbrush, and the number of infested plants was counted on the fifth day using handheld magnifying lenses to check the number of aphid adults and the number of nymphs per plant. In each meshed box with a size of 0.6 mm, there were five plants and 10 adults per plant of *M. persicae*. The effect of fungal inoculation on insect infestations was assessed.

F. Secondary metabolites

Extraction of plant materials

At the end of the greenhouse experiments, plants that showed successful fungal colonization were randomly selected for the analysis of secondary metabolite contents. The successful fungal colonization of the tissues was determined using the method described in Moloinyane and Nchu [26]. Briefly, After 21 days post-treatment, fresh leaves were picked-off plants and taken to the laboratory to assess fungal colonization. Leaf sections were surfaced sterilized in the following sequence: 0.5 % of sodium hypochlorite for two minutes, 70 % ethanol for two minutes, and then rinsed with sterile distilled water for 1 min. The sterilized leaf sections were placed on selective solid agar plates made up of half-strength potatoes dextrose agar (PDA) (19.5 g/1000 mL of sterile water containing 0.04 g streptomycin, and 0.02 g ampicillin sodium salt) and were incubated at 25 ± 2 ^{o}C. Plants were oven-dried at 35 °C for 168 hours and were ground into plastic bags. For each treatment, three replicates were prepared. 0.1 g of each of the powdered materials from each replicate was transferred into separate centrifuge tubes. The samples were extracted with 25 mL of 60% ethanol and placed inside the incubator for 24 hours.

Analysis of secondary metabolites on leaves of inoculated plants

Total alkaloids: The spectroscopic method was used to determine total alkaloids in the plant [30]. Briefly, 0.1 g of powdered lettuce leaves were extracted with 25 mL of 60 % ethanol and 40 % of sterile distilled water for 24 hours in total darkness, centrifuged (4000 x g for 10 min), and the supernatant was used in the assay. Subsequently, two millimetres of the extract supernatant and atropine standard solutions were mixed with 12 mL bromocresol green solution and 5 mL sodium phosphate buffer. A volume of 12 mL of chloroform was added to the above-mentioned solution, and the solution was mixed using a vortex mixer. The spectrometric absorbance at 417 nm and a standard curve of atropine was used to determine the concentration of mg atropine equivalent per g dry weight (mg AE/g DW) in the sample.

Total polyphenol: The Folin-Ciocalteu method was used to determine the total polyphenol content of the crude extracts of leaves [31], [32]. A volume of 25 µL of the crude extract sample was mixed with 125 µL Folin-Ciocalteu reagent (diluted 1:10 with distilled water) (Merck, South Africa). 100 µL (7.5 %) aqueous sodium carbonate (Na_2CO_3) (Sigma-Aldrich, South Africa) was added to each well after 5 min followed by the absorbance reading of the solution in the microplates. The results are expressed as mg gallic acid equivalents per gram dry weight (mg GAE/g DW).

Total flavonol: The flavonol content was determined using the protocol described by [34]. Quercetin standard concentrations of 0, 5, 10, 20, 40, and 80 mg/L in 95 % ethanol (Sigma-Aldrich, South Africa) were used. A volume of 12.5 µL of the crude sample extracts was mixed with 12.5 µL 0.1 % hydrochloric acid (HCl) (Merck, South Africa) in 95 % ethanol in the sample wells and then incubated for 30 min at room temperature. The results were expressed as mg quercetin equivalent per g dry weight (mg QE/g DW).

G. GC-MS analysis

Sample preparation

Twelve potted plants, three from each fungal treatment were used for this analysis. Only plants that showed fungal colonization among the fungus-treated plants were used for GC-MS analysis.

GC-MS Analysis

The GC-MS method described by [26] was adopted for this study. The whole leaves from the fresh lettuce plants were removed and freeze-dried at -80 ^{0}C (overnight). The leaves were then crushed in liquid nitrogen and transferred 1 g of the crushed leaves into a solid-phase microextraction (SPME) vial, and then transferred 2 mL of 12 % ethanol solution (v/v) at pg 3.5 and 3 mL of 20 % NaCl in the vial. The samples were mixed vigorously using a vortex mixer and finally analyzed the headspace of the samples using a Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) SPME fibre (gray).

Chromatographic separation

The volatile compounds were identified and separated from the lettuce plants using a gas chromatograph (6890N, Agilent Technologies Network) coupled to an inert Xl EI/CI Mass selective detector (model 5975B, Agilent Technologies Inc., Palo Alto CA). We used the protocol described in [26]. The GC-MS system used was combined with a CTC Analytics PAL autosampler. The volatiles were separated on a polar ZB-WAX (30 m, 0.25 mm ID, 0.25 µm film thickness) Zebron 7HG-G007-11 capillary column. Helium was used as the carrier gas. The flow rate of the helium was maintained at 1 mL/min. The injector temperature was 250 °C, with a split ratio of 5:1 and oven temperature was timed at 35 °C for 6 min, at a rate of 3 °C/min to 70 °C for 5 min, then at 4 °C/min to 120 °C for 1 min, and finally increased to 240 °C at a rate of 20 °C/min and maintained for 2.89 min. The mass selective detector was oporared in full scan mode while maintaining the source, quad, and transfer temperatures at 230 °C, 150 °C, and 250 °C, respectively. The electron impact mode at ionization energy of the mass spectrometer was run below 70 eV, scanning from 35 to 500 *m/z*. Relative ratios were used to estimate quantities of volatiles, and they were determined using the expression (peak area/IS peak area) × IS concentration (IS = internal standard) and. A cut-off match quality of at least 90% was used for organic volatile compound identification.

G. Statistical analysis

Count data for insect infestation was arcsin square root transformed, and then analyzed using one-way ANOVA. The post hoc Turkey HSD was performed to separate the different means. The data were analyzed using Statistica (TIBCO Statistica® 13.3.0 Dell Inc., USA).

III. Results

A. Effect of fungus on secondary metabolites

Generally, there was a significant difference (DF = 3.8; F = 15.518; P < 0.001) among treatments for the total polyphenol contents in plants. The highest total polyphenol content was recorded among plants treated with the highest conidial concentration (1×10^8 conidia mL^{-1})(Table 1). The fungus had no effect on total flavonols (mg/QE/g) at (DF = 3.8; F = 3.68; P > 0.05); however, 1×10^8 conidia mL^{-1} showed the best result (7.46 ± 0.68 mg QE/g) and 1x 10^6 conidia mL^{-1} showed the lowest value (45 ± 0.59 mg QE/g) (TABLE I). Alkaloids were not detected in the lettuce plants in this study.

TABLE I. EFFECT OF *B. BASSIANA* INOCULATION ON SECONDARY METABOLITES OF *LACTUCA SATIVA* ON DIFFERENT TREATMENTS (N=12)

Treatments	**Polyphenols (*Mean ± SE mg GAE/g)**	**Flavonols (*Mean ± SE mg QE/g)**	**Total alkaloids**
Control	65.93 ± 4.22a	7.11 ± 0.63a	ND
1×10^6 conidia mL^{-1}	34.98 ± 0.27b	4.45 ± 0.59a	ND
1×10^7 conidia mL^{-1}	43.21 ± 7.30 b	5.94 ± 0.89a	ND
1×10^8 conidia mL^{-1}	71.54 ± 2.94a	7.46 ± 0.68a	ND

The same lowercase letters in the same column indicate that means ± SE are not significantly different using the Tukey HSD test at P = 0.05 level of significance. ND denotes not detected

B. Infestation level of aphid in the greenhouse

The inoculation of the plant with *B. bassiana* did not significantly affect immature aphids infestation (x^2 = 1.49; DF = 3; P = 0.68). Similar results were obtained for the immature and the adult aphids (x^2 =7.56; DF =3; P =0.60). Nevertheless, the highest fungal concentration (1×10^8 conidia mL^{-1}) showed lower infestation by adult aphids, 29.40 ± 0.68 (Table 2). Generally, the fungus-inoculated plants showed lower infestation by immature aphids compared to control (TABLE II).

TABLE II. EFFECT OF ENDOPHYTIC FUNGUS (*B. BASSIANA*) ON THE INFESTATION LEVEL OF APHIDS (*MYZUS PERSICAE*) (MEAN ± SE NUMBER OF INSECTS PER PLANT) IN THE GREENHOUSE

Treatments	**Immature aphids**	**Adults aphids**
Control	51.20 ± 1.71a	33.40 ± 1.21a
1×10^6 conidia mL^{-1}	48.00 ± 1.10a	32.00 ± 1.30a
1×10^7 conidia mL^{-1}	47.40 ± 1.25a	30.80 ± 1.36a
1×10^8 conidia mL^{-1}	48.00 ± 0. 89a	29.40 ± 0.68a

The same lowercase letters in the same column indicate that means ± SE are not significantly different using the Tukey HSD test at P = 0.05 level of significance.

C. GC-MS analysis of volatile compounds

In the current study, diverse volatile compounds were identified using the GC-MS analyses as shown in the supplementary table (TABLE 1). Some well-known insect repellents and semiochemicals, such as limonene, dodacane, hexadene, benzaldehyde, hexadene, beta-cyclocitral, aromadendrene, and hexadecenal were detected in plants from all the treatments. Interestingly, the quantities of 3-octanol ($DF = 3.8$; $F = 18.94$; $P < 0.01$) and 2,4-di-tert-butyl-phenol ($DF = 3.8$; $F = 27.53$; $P < 0.01$) were significantly higher in fungus-treated plants.

VI. Discussion

Inoculation of lettuce with *B. bassiana* conidia had varied influences on secondary metabolites. The total polyphenol content was significantly influenced by *B. bassiana* exposure, while the total flavonol content was not affected. It is worth mentioning that the total polyphenol content was higher in the plants inoculated with the highest conidial concentration of *B. bassiana*. Similar findings were obtained in a study conducted by [35], which focused on the effect of the *B. bassiana* (using the same fungal strain that we used in this study) on the secondary metabolite contents of chives. Previously, [36] reported that some endophytic fungal strains could increase the synthesis of secondary metabolites including flavanoids in host plants. The role of flavonoids in plants has been studied extensively in plant resistance against phytophagous insects [37], [38].

Despite the detected positive effect of fungus inoculation on the yield of some secondary metabolites, the number of adults and immature insects foraging on the lettuce plants was not affected by *B. bassiana* treatment. Three volatile compounds were found in the fungus-treated plants at significantly higher levels ($P < 0.05$) following the GC-MS analysis. Among these volatile compounds is 3-octanol, a well-known insect repellent [39]. The other volatile compound that was significantly correlated with fungal treatment was 2,4-di-tert-butyl-phenol. Nevertheless, the higher quantities of these two volatile compounds did not translate into a reduction in the insect infestations on the lettuce plants. This result is different from that of [40], which showed a reduction in aphid population and delayed fecundity on plants that were inoculated with endophytic fungus *B. bassiana*. The repellent response of insects may vary with the insect species, the concentration of a volatile, and the combination of volatile constituents. These findings highlight the complex relationship between secondary metabolites on insect herbivory and foraging.

Studies that focus on the effect of fungal endophytism on lettuce are scarce. The results obtained in this study provide insights on the effects of *B. bassiana* inoculation on secondary metabolite production and aphid infestation on lettuce plants as well as the complex yet intriguing endophytic fungus-lettuce-aphid relationship. It would be interesting to study the long-term sublethal effects of the fungus on the foraging aphids in a future study.

V. Conclusion

Generally, *B. bassiana* inoculation significantly ($P < 0.05$) affected total polyphenol content. The highest total polyphenol content occurred in extracts from plants that were treated with the highest conidial concentration (1×10^8 conidia mL^{-1}). This study also revealed that lettuce plants that were exposed to lower concentrations of *B. bassiana* (1×10^6 and $1x10^7$ conidia mL^{-1}) had lower mean total polyphenol contents than the control plants and plants that were exposed to the highest conidial concentration (1×10^8 conidia mL^{-1}). Further investigations on the effects of conidial concentrations on plant secondary metabolite levels are important in indentifying optimum conidial concentrations for inoculating plants. While the effect of fungal treatments on the number of volatile compounds was not significant, it had a positive influence ($DF = 3.8$; $P < 0.01$) on the quantities of two well-known anti-insect compounds (3-octanol and 2,4-di-tert-butyl-phenol). The study also revealed that the inoculation of plants with conidia of endophytic entomopathogens fungi may not always lead to a reduction in insect infestations. This study provides some insights into the endophytic *B. bassiana*-lettuce-aphid relationship and recommends further studies on the anti-insect compounds induced or produced by endophytic entomopathogenic fungal strains in a fungus-plant-insect relationship.

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