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IMPACT OF *BEAUVERIA BASSIANA* AND *METARHIZIUM ANISOPLIAE* ON THE INTERACTIONS BETWEEN CUCUMBER (*CUCUMIS SATIVUS* L.), COTTON APHID (*APHIS GOSSYPII* GLOVER) AND CUCUMBER MOSAIC VIRUS (CMV).

ABSTRACT OF A DISSERTATION

For awarding of educational and scientific degree "Doctor"

Field of higher education: 6. Agrarian sciences and veterinary medicine
Professional field: 6.2. Plant Protection
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Scientific supervisor: Assoc. Prof. Dr. Slavtcho Slavov
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The dissertation is written on 160 pages and contains 7 tables and 44 figures and 13 appendices. The list of references includes 416 titles. A total of 7 conclusions were made and 4 scientific and applied contributions. The study material is outlined in 7 sections.

The dissertation defense will be held on 11 January 2023, at 14:00 in the Academic Hall "M. Dakov" at Building A of the University of Forestry, Sofia, 10 Kliment Ohridski Blvd. at an open meeting of a scientific jury approved by Order No. …….. of the Rector of the University of Forestry with the following members:

Chairman:

Prof. Dr. Rumen Ignatov Tomov – UF, Sofia.

Members:

Prof. DSc. Rossitza Borissova Batchvarova – ABI, AA, Sofia.

Prof. DSc. Ivanka Lubenova Kamenova- ABI, AA, Sofia.

Prof. Dr. Daniela Kirilova Pilarska – IBER – BAS, Sofia.

Assoc. Prof. Violeta Savova Kondakova – ABI, AA, Sofia.

The materials on the defense (dissertation, abstract, reviews and opinions) are available to those interested on the website of the University of Forestry (www.ltu.bg) and in the dean's office of AF – Sofia, 10 "Kliment Ohridski" blvd.

I. 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). 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 such as one of the most common and serious plant viruses worldwide - cucumber mosaic virus (CMV) (Blackman and Eastop, 2000) are problems that pose a serious concern about yield and quality of greenhouse crops (Hermoso de Mendoza *et al*., 2001).

A. gossypii 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. Over the years, various biological control tactics have been evaluated in many parts of the world for the management of this pest. The entomopathogenic fungi (EPF), *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metchnikov) Sorokin (Ascomycota: Hypocreales) have 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 light, 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). 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).

It has also been observed that fungal endophytes have antagonistic effects against plant viruses. For example, González-Mas *et al*. (2018) reported that endophytic *B. bassiana* colonization of melon plants confers protection against persistent (Cucurbit aphid borne yellows virus, Polerovirus) and non-persistent (Cucumber mosaic virus, Cucumovirus) plant viruses' transmission by *A. gossypii*.

Endophytes have been revealed to produce plant growth regulators, secondary metabolites, and defense compounds as a result of the widespread use of genomics, transcriptomics, proteomics, and metabolomics technologies (Chen *et al*., 2022). Metabolomics is a relatively new scientific subject that use technology advances in analytical chemistry to detect and quantify tiny chemicals naturally created by an organism. It is unknown how plant endophytes can elicit plant systemic resistance to viruses at the metabolic level. Metabolite changes have only been shown in *B. bassiana* endophytically colonized maize plants infected with *Ostrinia furnacalis* (Guenée) (Batool *et al*., 2022).

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 (3) investigate the effects of seed inoculation on the population of *A. gossypii* on cucumber (4) study the effects of entomopathogenic endophytes on the metabolomics of CMV infected cucumber plants and (5) study the effects of CMV on the metabolomics of cucumber plants.

II. PURPOSES AND TASKS

The main goal of this project is to study entomopathogenic endophytes as potential alternatives to synthetic chemical insecticides against *Aphis gossypii*, and their possible use for the eradication of this pest and subsequent transmission of many destructive viruses like cucumber mosaic virus (CMV). The current project aims to study metabolomics, a new research field, of CMV diseased cucumber plants to gain insight about the effects of applied endophytes *Beauveria bassiana* and *Metarhizium anisopliae* (EPF) on the induced defense mechanism against the virus. To fulfill these purposes the following tasks were applied in this project:

- Artificial inoculation of cucumber seeds with the biological control agents *Beauveria bassiana* and *Metarhizium anisopliae* through immersion in conidial suspensions.
- Study the endophytic ability of both applied entomopathogenic fungi through Re-isolation methodand Scanning Electron Microscope (SEM).
- Study the effect of inoculated entomopathogenic endophytes on the physiological and biochemical parameters of cucumber plants. Physiological parameters include; Germination rate, Height of the plant, Length and Number of roots, Number of leaves, flowers and fruits. Biochemical parameters include; Total Phenol Content (TPC), Chlorophylls and Carotenoids content.
- Study the effect of entomopathogenic endophytes on the population size of *A. gossypii* infesting cucumber plants.
- Mechanical inoculation of non-resistant cucumber plants and plants endophytically colonized with entomopathogenic fungi with CMV virus.
- Assessment of virus infection by Double-Antibody Sandwich Enzyme Linked Immunosorbent Assay (DAS-ELISA) and PCR test.
- Preparation of freeze-dried cucumber leave samples after methanol extraction.
- Large scale untargeted metabolome profiling of the plant samples using High Performance Chemical Isotope Labeling LC-MS.

III. MATERIALS AND METHODS

This experimental study was conducted in the laboratories of the Lebanese University- Faculty of Agricultural and Veterinary Sciences (LU-FAVS), American University of Beirut- Faculty of Agricultural and Food Sciences (AUB-FAFS) and the Lebanese Agricultural Research Institute (LARI).

3.1. Source of fungal inoculum

Lebanese strains of *Beauveria bassiana* and *Metarhizium anisolpiae* 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. In addition, fungal isolates were also grown on autoclaved rice (Ibrahim *et al*., 2015). Spores from these cultures were used for subsequent experiments.

3.2. 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´-TCCTCCGCTTATTGATATGC-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/).

3.3. Sterilization of cucumber seeds

These are the most popular among the consumers and widely grown in Lebanon, Beit Alpha F-1 hybrid and TAMARAH hybrid cucumber seeds were used in the experimental study. Among them TAMARAH hybrid variety is not resistant to CMV.

In order to remove possible epiphytic microorganisms by denaturing their DNA from the aerial surfaces, the cucumber seeds were surface sterilized with sodium hypochlorite, 70% ethanol and then rinsed with sterile distilled water (dH_2O) prior to their artificial inoculation with tested entomopathogenic fungi (EPF). 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.

3.4. Preparation of fungal inoculum and seed inoculation

Fungal inoculums were prepared from conidia grown on PDA or autoclaved rice media for different experiments. Conidia of 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 on a rotary shaker at 354 rpm for 30 min. Also, the conidia suspensions were prepared by suspending an appropriate (depending on the conidial concentration) amount of rice in 0.03% Tween 20 and shaken on an agitating shaker for 1 hour. Resultant suspensions were filtered through several layers of sterile cheesecloth under sterile conditions in order to remove any hyphal or rice fragments. Conidial concentrations were determined using a light microscope and hemocytometer (Fuches-Rosenthal), and later adjusted to 1×10^6 conidia $\cdot$ ml^{-1} (PDA) or 1×10^8 conidia $\cdot$ ml^{-1} (Rice) with sterile 0.03% Tween 20 solution. Axenic seeds were immersed in fungal conidial suspensions of *B. bassiana* and *M. anisopliae* for 2-3 h. For the control group the seeds were immersed in sterile

0.03% Tween 20 only (Fig. 1). The viability of conidia was assessed before preparation of suspensions by germinating tests on PDA medium. In all experiments, germination rates were above 95% after 24 hours at 25 ˚C. Inoculated seeds with conidia grown on PDA (Growth and *Aphis gossypii* experiments) were then sown on sterile moist filter papers and 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%). Prior to planting, the compost was analyzed for the presence of soil borne fungal communities using the soil dilution method (Matei *et al*., 2016). For other experiments (CMV), inoculated seeds with conidia grown on rice were sown on separate trays lined with sterile tissues and watered regularly with sterile distilled water for 7 days. The appeared cucumber seedlings were then transplanted each to same size pots (15 cm diameter and depth) using an autoclaved mixture of equal parts of soil (SULIFLOR PREMIUM; NPK + Trace elements), peat moss (GREENTERRA) and Cocopeat substrate, and grown under protected greenhouse located at the American University of Beirut (AUB), with controlled conditions: 25˚C temperature (D: N), a photoperiod of 16:8 hours (L: D) and 60–80% RH.

3.5. Assessment of fungal colonization

3.5.1. 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.

3.5.2. Assessment of colonization using Re-isolation method

A re-isolation method was used to confirm the endophytic colonization of plants by fungal stains using five randomly selected seedlings which were harvested from the trays (20-day-old seedlings), pots (2-week-old seedlings) and the sterile filter papers (10-day-old seedlings). Fungal

outgrowth from the plated sterilized plant samples on PDA 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 = $N_f/N_t \times 100$

where: N_f - number of sampled plant tissue showing fungal outgrowth

N_t - total number of plated plant tissue samples.

Growth-Experiment:

3.6. Effect of EPFs on seed germination

To determine the effects of seed inoculation with fungal suspensions on seed germination, the following experiment was conducted. Inoculated seeds were incubated at 25 °C under fluorescent lighting (16h Light / 8h Dark). Germination and occurrence of cotyledons were recorded over period of 10 days (every 2 days). Sterile dH_2O was periodically added to each plate to keep the filter paper moistened. Each plate was replicated 6 times with 10 seeds in each replicate and the experiment was repeated 6 times. At the same time, inoculated seeds of each treatment were cultured in 3 separate seed trays containing non-sterile compost with 40 replications for each treatment under natural fluctuating night/day temperatures (23−29 °C) and relative humidity (RH, 30−80%). There were three treatments: *B. bassiana*, *M. anisopliae* and Tween (control) treated seeds.

3.7. Effect of EPFs on seedling's growth

To determine the effects of seed inoculation with fungal suspensions on seedling growth, root and stem lengths and number of roots of the resulting seedlings of each treatment were measured after the completion of seed germination test. Endophyte re-isolation test was also done (See section 4.2.2) to check the presence of endophytes.

3.8. Effects of endophytic EPFs on physiological and biochemical parameters of cucumber plants

The 6-week-old cucumber plants transplanted from seed trays to pots 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.

3.8.1. Analysis of Total Phenolic Compounds

In order to check if fungal endophytes colonizing cucumber seedlings can alter the total content of phenolic compounds (TPCs), the Folin-Ciocalteu (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.

3.8.2. Analysis of Chlorophyll and Carotenoids Content

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: $Ca = 13.36 \times A_{665} - 5.19 \times A_{649}$ and $Cb = 27.43 \times A649 - 8.12 \times A665$, where: A_{665} = absorbance at a wavelength of 665 nm; A_{649} = 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 A_{470} = absorbance at a wavelength of 470 nm (Braniša *et al*., 2014). The test was repeated three times.

***Aphis gossypii*-Experiment:**

3.9. Effect of endophytic EPFs on the population size of *Aphis gossypii* on cucumber plants.

3.9.1. 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 3.4 section). The potted plants were regularly irrigated. Twelve plants for each treatment were used.

3.9.2. *Aphis gossypii* culture

Prior to laboratory establishment of pure *A. gossypii* culture, aphid populations collected from different plant hosts and locations in Lebanon 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.

3.9.3. Effect of endophytic EPFs on *Aphis gossypii* population size

Five-week-old plants were placed in individual clear plastic cages (20 cm diam. × 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.

Cucumber Mosaic Virus (CMV)-Experiment:

3.10. CMV infection and plant sampling

Symptomatic cucumber leaf samples were collected from different locations in Lebanon and tested for CMV presence using PCR CMV set (BIOREBA-Qualiplante) (Rizos *et al*., 1992). Positive tested samples (inoculum source) were preserved at 4 ˚C (Bos, 1977) in tightly closed containers containing calcium chloride ($CaCl_2$). Inoculum was prepared by homogenizing 1g of infected leaf tissue taken from 5 weeks-old plants in 10 mL of 50 mM phosphate buffer (pH: 7.4) using a sterile, pre-chilled and maintained on ice mortar and pestle. Prior to plant inoculation, carborundum fine powder (Fisher Scientific, Waltham, MA, USA) as an abrasive was lightly dusted on two marked leaves. The inoculum was then mechanically applied by rubbing the leaves several times from leaf base to tip using a cheesecloth (Sudhakar *et al*., 2007). In negative control (non-infected), cucumber plants were mock-inoculated with phosphate buffer only. The success of viral infection was assessed by observation of symptom development on leaves, double-antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA –BIOREBA, Switzerland) and PCR test. The RNA was isolated from cucumber leaves using CTAB method (for total RNA isolation) described by Chang *et al*. (1993) with slight modification, and the complementary DNAs (cDNAs) were synthesized using the iScript gDNA Clear cDNA Synthesis Kit (BIO-RAD, CA) by using the user's manual. A NanoDrop spectrophotometer (Thermo scientific, Waltham, MA, USA) was used

to quantify the amount of RNA, and agarose gel electrophoresis was used to access the quality of RNA. The following PCR conditions were adopted: initial denaturation step of 2 min at 95 °C followed by 35 cycles, each consisting of a 45 sec denaturation step at 95 °C, a 45 sec annealing step at 58 °C, a 1 min elongation step at 72 °C, and a final elongation step at 72 °C for 7 min. Fungal-treated and non-treated (Control) plants were assessed for endophytic presence before CMV inoculations (See section 4.2.2). All plants were grown under the above- mentioned controlled conditions in a protected green-house. For each treatment group, plants were covered with a separate net. Leaf samples were collected in ten replicates from each treatment (Fungal-treated plants, positive and negative control plants) at 3, 6, and 10 days post-virus inoculation and immediately frozen in liquid nitrogen. Samples were stored at −80 °C for further analysis.

3.11. Effects of endophytic EPFs and CMV on the metabolites produced by CMV infected cucumber plants:

3.11.1. Sample preparation

Freeze dried samples were prepared for metabolomics analysis. Samples were homogenized into fine powder using liquid nitrogen. Nine hundred µL of 80% methanol was added to 100 mg of each sample, vortexed for 90 sec, and then sonicated for 30 min at room temperature. All samples were kept at -40 °C for 1 hour. After freezing, they were vortexed again for 30 sec, incubated at room temperature for 30 min, and then centrifuged at 12,000 rpm for 15 min. The supernatants for each sample were placed into glass vials and freeze-dried (Labconco-TBA) into pellets (3 replicate pellets for each Dpi and treatment).

3.11.2. Metabolome profiling

Metabolite profiling of the plant samples was done using 2- Channel Analysis method. Data was collected using Dansyl- and DmPA- labeling Kit for each channel. Analysis were performed using IsoMS Pro 1.2.15 (NovaMT Inc., Edmonton, AB, Canada) and NovaMT Metabolite Database v2.0. Metabolomics study was performed in The Metabolomics Innovation Centre (TMIC, Vancouver, BC, Canada).

3.12. STATISTICAL ANALYSIS

All experiments were arranged in a completely 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 germination rate in addition to, fungal colonization with *M. anisopliae* and *B. bassiana* of different cucumber plant parts, the aphid population size, TPCs, chlorophyll contents and ELISA absorbance measurements were pooled together prior to one-way ANOVA analysis with the fungal strain as a main factor. 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. The heat maps were done using function heat map 2 under R program, Version 3.4. Moreover, Venn diagrams were performed using GHENT University Bioinformatics program.

IV. RESULTS

4.1. 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*.

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

4.2.1. SCANNING ELECTRON MICROSCOPE (SEM)

The efficiency of artificial inoculation of cucumber seeds was observed under scanning electron microscope (SEM). Scanning electron micrographs revealed 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. 1 D 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 1. 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* (Fig. 2).

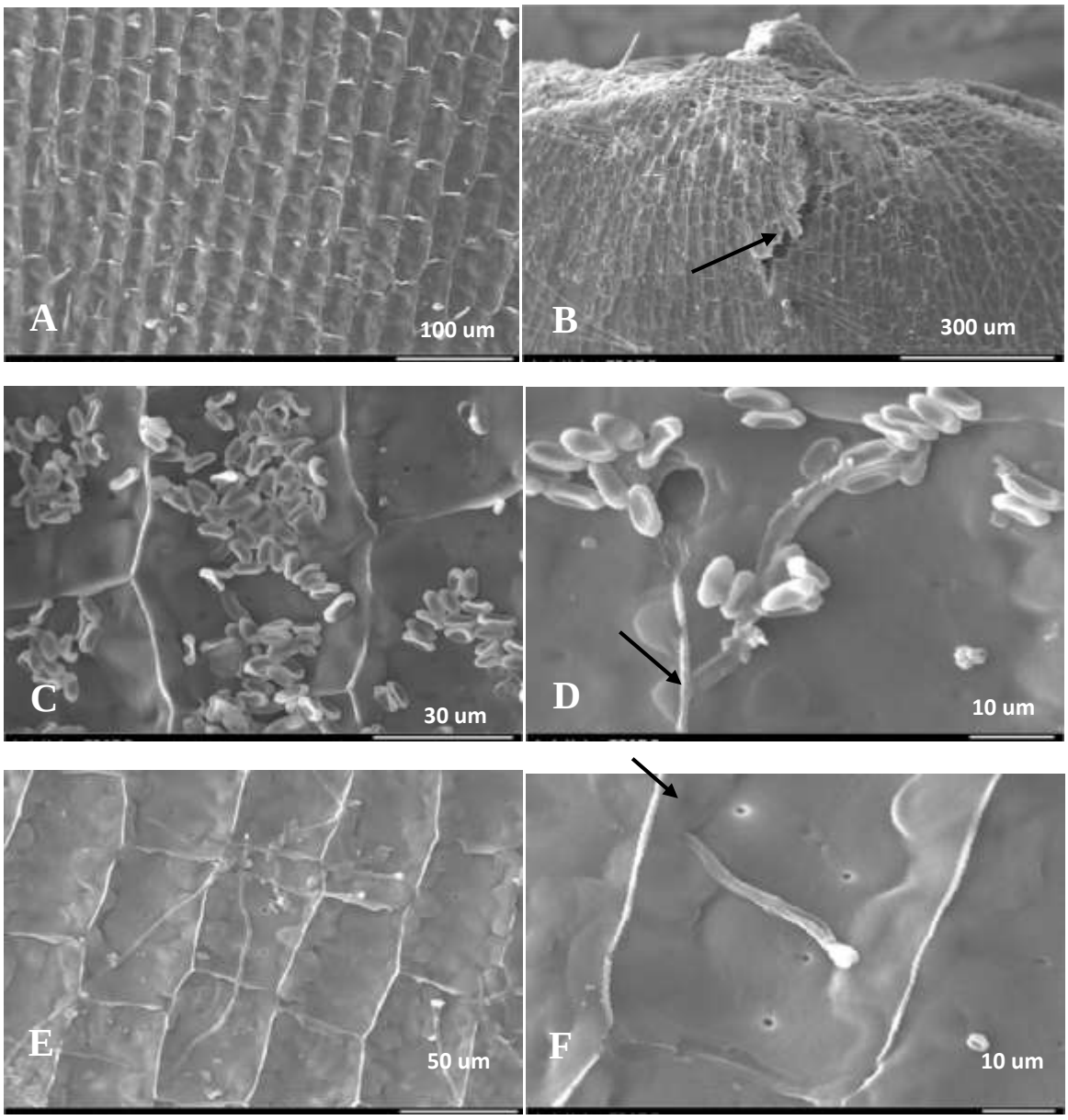

Figure 1. Scanning electron micrographs (SEM) of Tween (control), *M. anisopliae* and *B. bassiana* treated seeds 24 h post-inoculation. Micrograph A shows no fungal growth on control seed surface, whereas B shows *M. anisopliae* spores and hyphae adhered to the micropylar area showing ruptured covering 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.

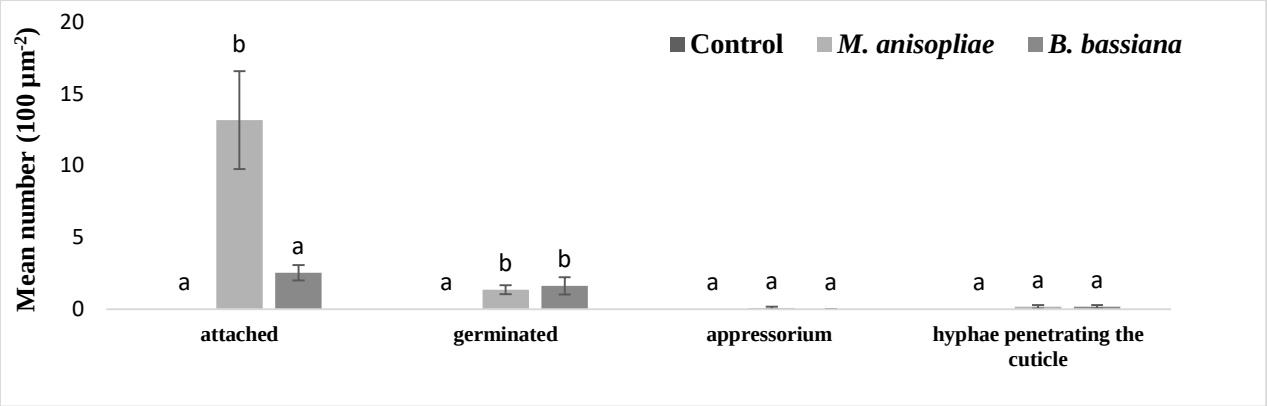

Figure 2. Mean number (100 μm^{-2}) ± SE of attached, germinated spores, appressorium and hyphae penetrating the seed epidermal cells of the treated seeds. Bars with different letters are significantly different at P = 0.05 (Duncan test, after one-way ANOVA).

4.2.2. Colonization of plant parts by *B. bassiana* and *M. anisopliae* isolates.

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 cotyledon/leaf pieces on PDA selective medium 10, 14 and 20 days post-inoculation. Results in Table 1 and Figure 3 show that both, *B. bassiana* and *M. anisopliae*, isolates have colonized cucumber seedlings 10 days post-inoculation. Significantly higher percentage recovery (100%) was observed in the cucumber plant tissues (roots, hypocotyls and cotyledons) colonized by *M. anisopliae* as compared to 58.3% (cotyledons and stems) and 50 % (roots) of *B. bassiana* endophyte. No fungal growth was recorded in control seedlings (Table 1, Fig. 3) (Growth Experiment).

Table 1. Effect of conidial seed treatments on percentage recovery of *B. bassiana* and *M. anisopliae* from different parts of cucumber seedlings grown in an incubator (25 ^{0}C), 10 days post-inoculation.

Treatment	Cotyledons (%±SE)	Stem (%±SE)	Roots (%±SE)
Control	0.0 ± 0.00 a*	0.0 ± 0.00 a	0.0 ± 0.00 a
B. bassiana	58.3 ± 0.15 b	58.3 ± 0.15 b	50.0 ± 0.15 b
M. anisopliae	100.0 ± 0.00 c	100.0 ± 0.00 c	100.0 ± 0.00 c

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

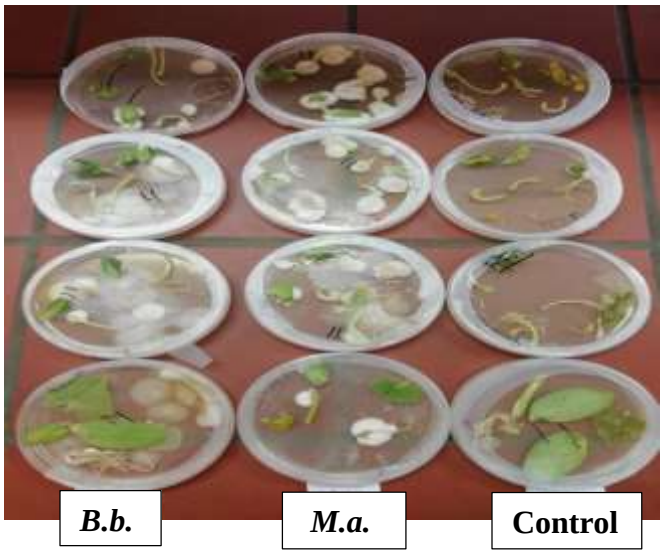

Figure 3. Re-isolation of EPFs from cucumber seedlings on selective PDA media (*B.b.*: *B. bassiana*, *M.a.*: *M. anisoplia*).

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. Results in Table 2 and Figure 3 show that cucumber seedlings grown in seed trays with peat compost have been colonized by both *B. bassiana* and *M. anisopliae* isolates 20 days post-inoculation. 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 fungi (Table 2, Fig. 3) (Growth and *Aphis gossypii* Experiments).

Table 2. Effect of conidial seed treatments on percentage recovery of *B. bassiana and M. anisopliae* from different parts of cucumber seedlings grown outdoors in non-sterilized peat moss medium, 20 days post-inoculation.

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

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

Results in Table 3 and Figure 4 show that cucumber seedlings grown in autoclaved soil pots for the CMV-experiment have been colonized by both, *B. bassiana* and *M. anisopliae*, isolates 2-week-post-inoculation. The most successful endophyte re-isolation frequency for *B. bassiana* was observed from leave tissues (100%). In addition, 40% and 60% endophyte percentage recoveries were observed in stem and root tissues, respectively, colonized by *B. bassiana*. Whereas *M. anisopliae* showed 80% percentage recovery from all seedling parts (leaves, stem and roots). None of the control plants showed signs of *M. anisopliae* or *B. bassiana* out-growth (Table 3, Fig. 4).

Table 3. Effect of conidial seed treatments on percentage recovery of *B. bassiana* and *M. anisopliae* from different parts of cucumber seedlings grown in autoclaved soil pots under controlled conditions in the green house.

Treatment	Leaves (%±SE)	Stem (%±SE)	Roots (%±SE)
Control	0.0 ± 0.00 a*	0.0 ± 0.00 a	0.0 ± 0.00 a
B. bassiana	100 ± 0.00 b	40.0 ± 0.24 bc	60.0 ± 0.24 b
M. anisopliae	80.0 ± 0.20 b	80.0 ± 0.20 c	80.0 ± 0.20 b

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

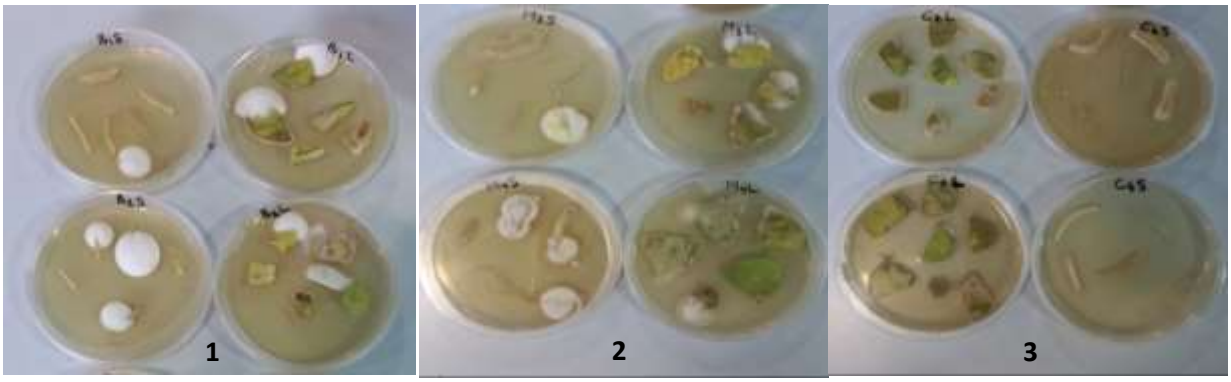

Figure 4. Re-isolation of EPFs from cucumber seedlings (1. *B. bassiana*, 2. *M. anisopliae* and 3. Control treatments).

Growth-Experiment:

4.3. The effects of entomopathogenic fungi (*B. bassiana* and *M. anisopliae*) on cucumber seed germination and seedling growth:

In the current study, seed inoculation with *B. bassiana* and *M. anisopliae* did not reduce seed germination or seedling growth, and did not result in the development of root disease. On the contrary, the results of the germination test showed that inoculating seeds with EPFs and incubating at 25 ˚C for 2 days would significantly improve germination rate by more than 26% if compared to non-inoculated seeds. After this date, the rate of germination showed no significant difference between the treatments (Table 4 and Fig. 5). Greater enhancement of germination was also observed in seeds inoculated with *B. bassiana* (72.5%) or *M. anisopliae* (47.5%) when grown under natural fluctuating night/day temperatures (23−29 °C) and relative humidity (RH, 30−80%) conditions (Table 4 and Fig. 6). In addition, seedling's roots developed from inoculated seeds grew faster than non-inoculated reaching 15 cm in length compared to 10 cm long control roots after 10 days of incubation. Moreover, seedlings from seeds inoculated with *B. bassiana* produced significantly higher number of roots, whereas seedlings inoculated with *M. anisopliae* produced the lowest number of roots per seedling. However, seed inoculations with fungal conidia had no effect on stem elongation (Figs. 7, 8).

Table 4. Germination mean (%) of inoculated and non-inoculated cucumber seeds (in an incubator 2 days post inoculation and under outdoor conditions seven days post inoculation)

Treatment	**Germination of cucumber seeds** % ± SE*	
	Germination (25 °C)	Germination (outdoor)
Control	40.0 ± 0.856^{a}	32.5 ± 0.11^{a}
M. anisopliae	66.7 ± 0.422^{b}	47.5 ± 0.05^{b}
B. bassiana	68.3 ± 0.654^{b}	72.5 ± 0.11^{c}

* Mean percentages **(**± Standard Errors, SE) within a column followed by the same letter are not significantly different according to Duncan test (P≤0.05).

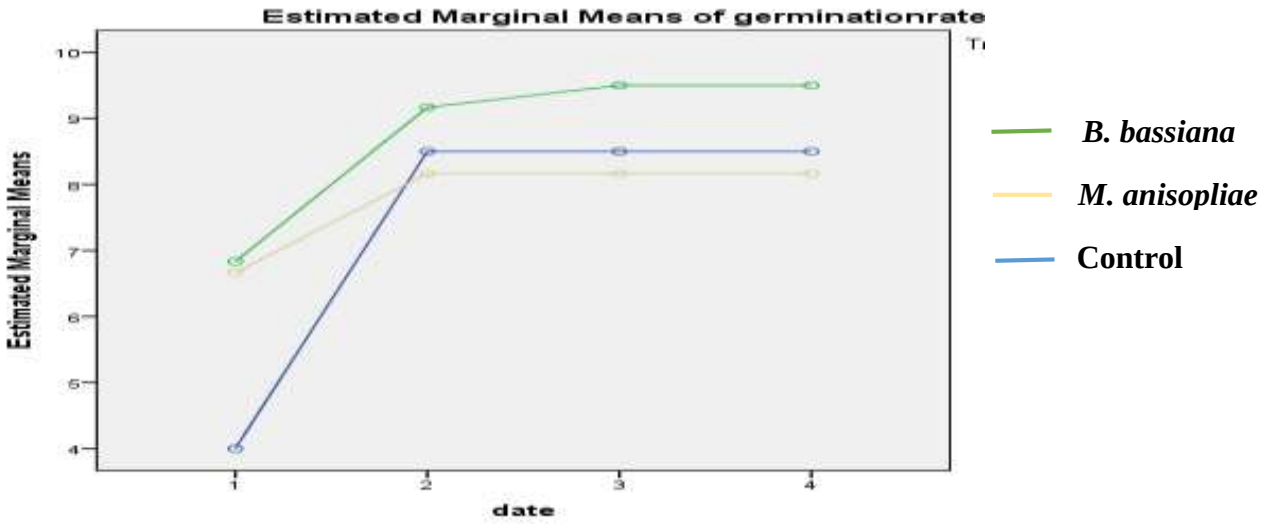

Figure 5. Germination rate of inoculated and non-inoculated seeds grown in an incubator at 25 ˚C (date 1; 2 days post-inoculation).

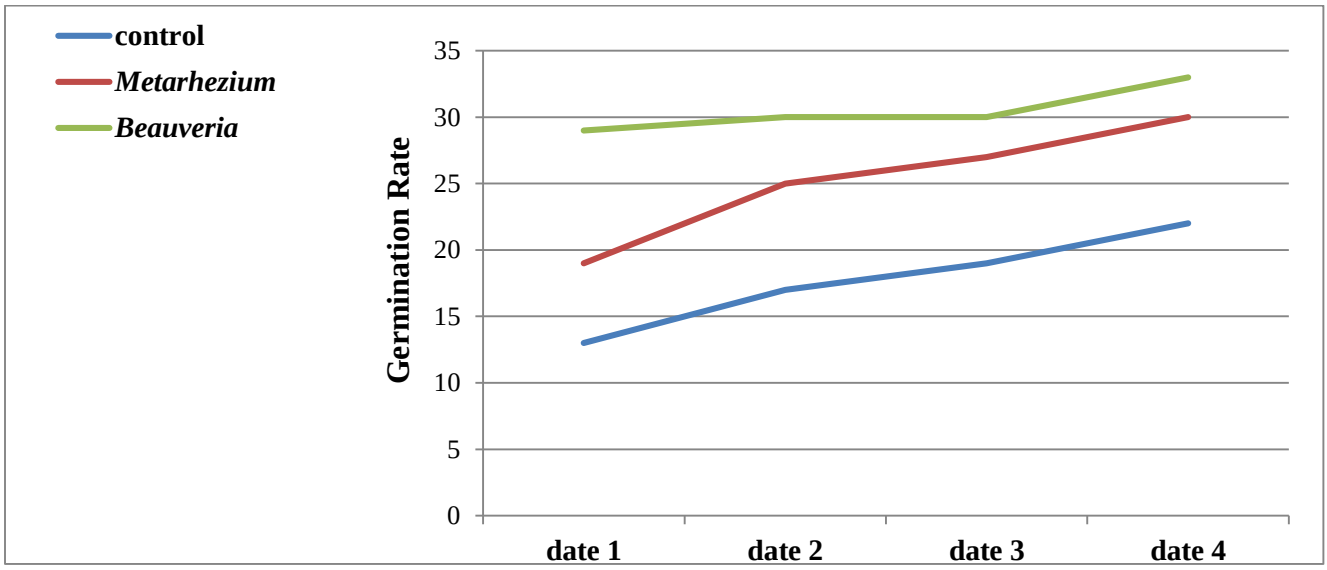

Figure 6. Germination rate of inoculated and non-inoculated cucumber seeds grown under outdoor conditions.

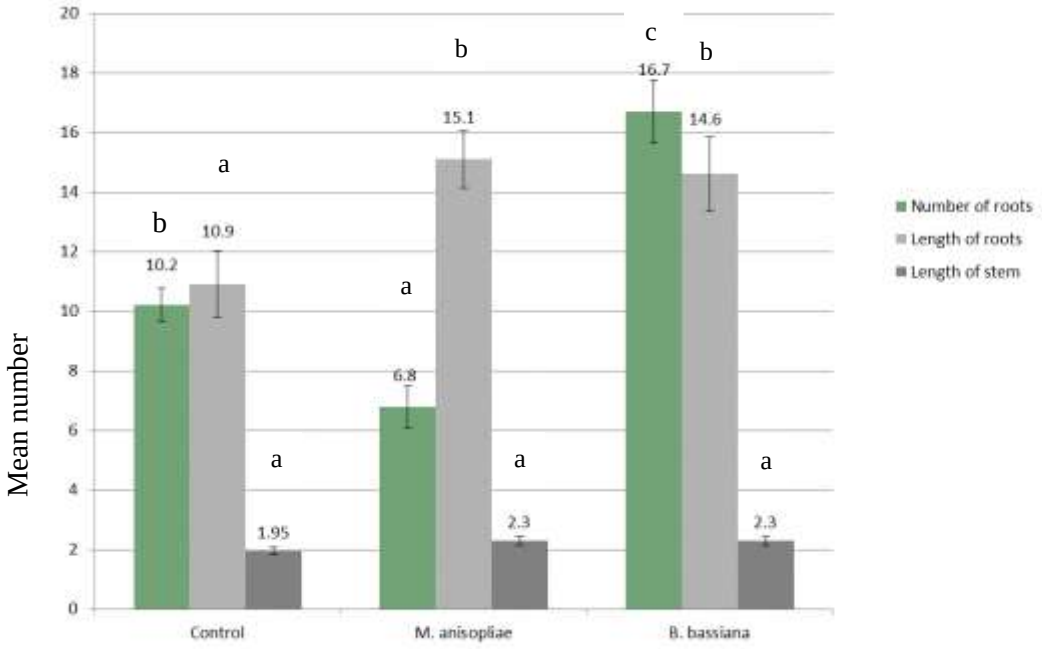

Figure 7. Physiological characteristics of roots and stems of cucumber seedlings after 10 days of incubation of inoculated and non-inoculated cucumber seeds. Same letters for each parameter indicate non-significant difference according to Duncan test (P≤0.05).

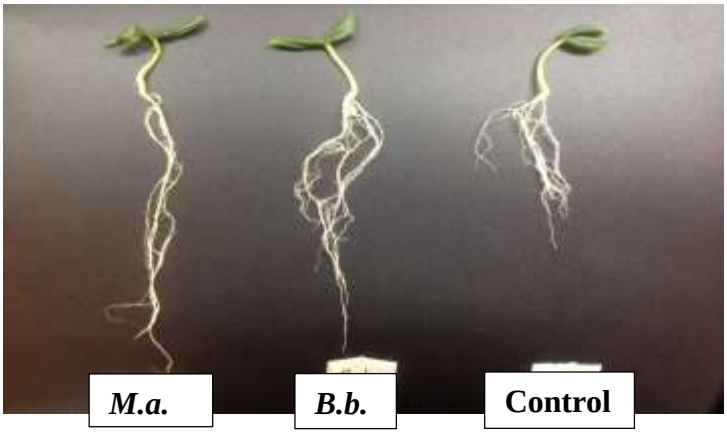

Figure 8. Development and growth of cucumber seedlings untreated and treated with EPFs (*M.a.*: *M. anisoplia*; *B.b.*: *B. bassiana*).

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

Physiological parameters

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. Plants colonized with *Beauveria* isolate produced twice as many flowers and two times more leaves than control plants, while *Metarhizium* colonized plants were almost 12 cm taller and had two more developed cucumber fruits than control plants (Fig. 9).

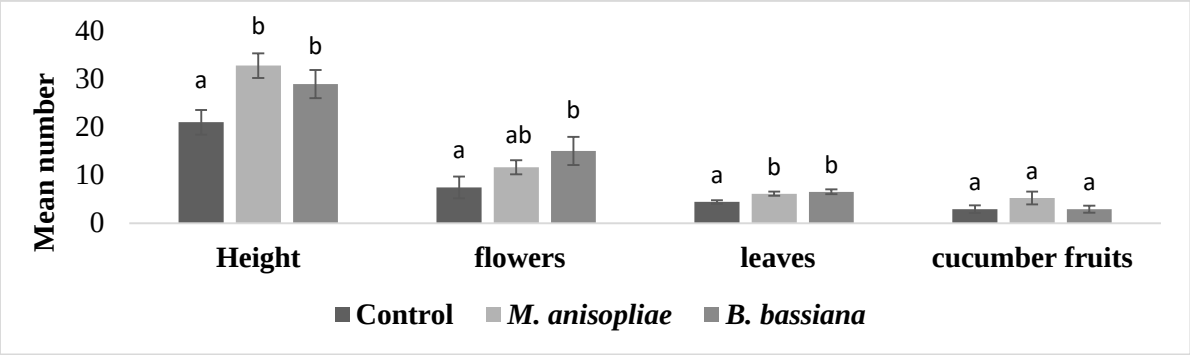

Figure 9. Mean height (cm ± SE) and the mean (± SE) number (N) of flowers, leaves and fruits of cucumber plants inoculated with *B. bassiana*, *M. anisopliae* and Tween (control). Bars with different letters are significantly different at P = 0.05 (Duncan test, after one-way ANOVA).

Biochemical parameters

The cucumber plants reacted to endophytic colonization by increased induction of soluble phenols (Table 5). 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. 10). 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 5. 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 ± 0.003a*
M. anisopliae	101.3 ± 0.003a
B. bassiana	181.8 ± 0.001b

*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).

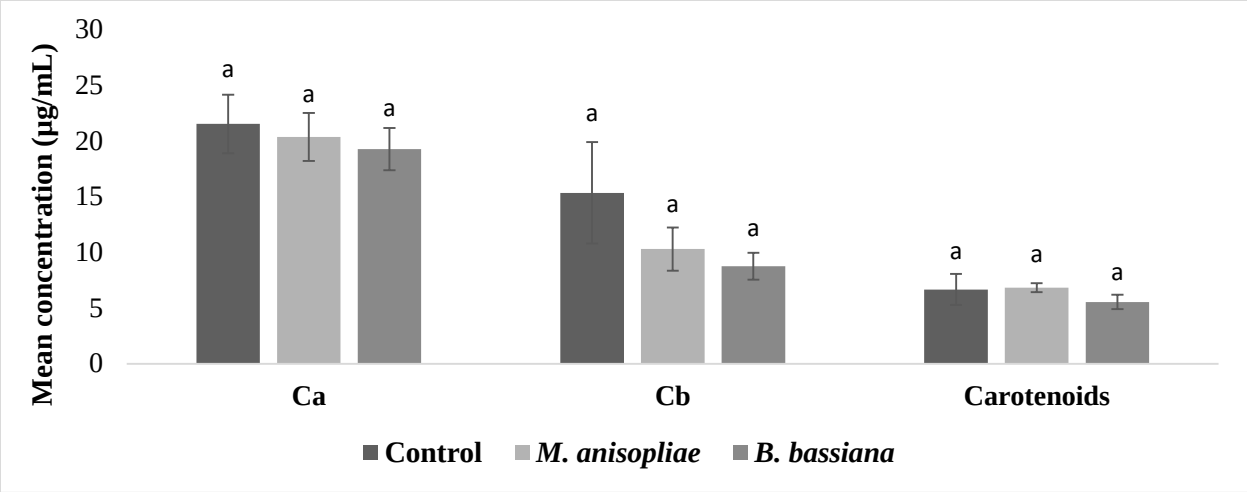

Figure 10. Effect of fungal inoculation on chlorophylls Ca and Cb and carotenoids content (mean concentration, µg · ml ± SE) in 7-week-old cucumber plants. Bars with similar letters do not differ significantly at P = 0.05 (Duncan test, after one-way ANOVA).

***Aphis gossypii*-Experiment:**

4.5. 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. 11) 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. 11) resulting in healthier/fitter cucumber plants.

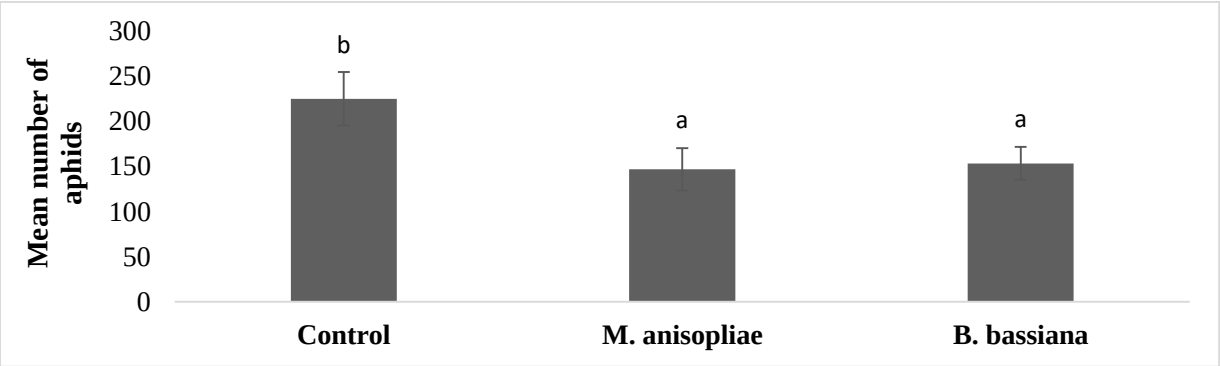

Figure 11. Mean (± SE) number of aphids per cucumber plant inoculated with *B. bassiana*, *M. anisopliae* and Tween (control). Bars with different letters are significantly different at P = 0.05 (Duncan test, after one-way ANOVA).

CMV-Experiment:

4.6. Assessment of CMV infection

All CMV-inoculated cucumber plants (fungal-treated and non-fungal-treated) showed clear mosaic and blistering symptoms on leaves 2 weeks post-inoculation. However, the negative control plants which were not inoculated with CMV or fungal endophytes showed no virus symptoms. DAS-ELISA absorbance measurements at wavelength 405 nm for all CMV- inoculated plants ((positive control plants (non-fungal-treated), *B. bassiana*- and *M. anisopliae*- treated plants)) showed that most plants tested CMV-positive at 3, 6, and 10 days post-inoculation (Dpi) of the virus. The positive control of ELISA set showed the highest absorbance measurement, while negative control plants (non-CMV-inoculated) showed the lowest absorbance measurements. There was no significant difference in the absorbance measurements between non-fungal-, *B. bassiana*- and *M. anisopliae*-treated diseased plants at different Dpi 3, 6, and 10. Furthermore, there was no significant difference in the absorbance measurements between different Dpi of the same treatment (Table 6). In addition, PCR CMV set from Bioreba, (powered by Qualiplant, France) was successfully used to identify CMV-positive plants by amplifying the expected fragment size of 870 bp. PCR results of negative control plants showed no amplification (Fig. 11). CMV-positive tested samples for each treatment were used for metabolome analysis.

Table 6. ELISA Absorbance measurements (405 nm) of CMV-inoculated plants at different Dpi.

Treatment	**Dpi 3**	**Dpi 6**	**Dpi 10**
Positive control plants	2.68 ± 0.46 [a*]	2.16 ± 0.45 [a]	1.74 ± 0.18 [a]
Metarhizium anisopliae	2.44 ± 0.36 [a]	1.9 ± 0.18 [a]	2.04 ± 0.31 [a]
Beauveria bassiana	1.86 ± 0.28 [a]	2.03 ± 0.31[a]	2.52 ± 0.62 [a]

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

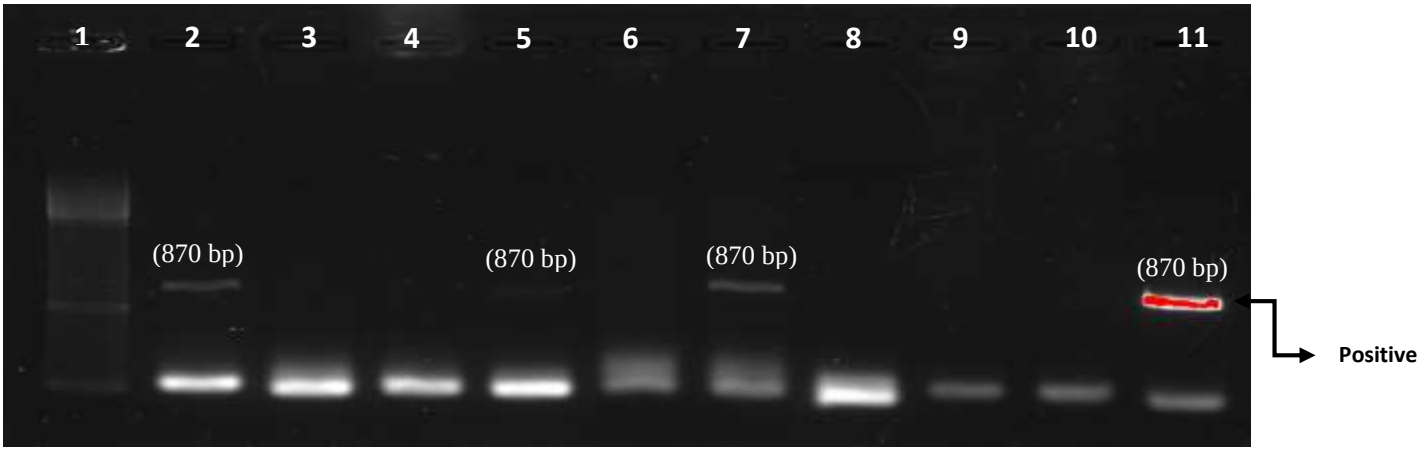

Figure 11. PCR results for some tested cucumber leaf samples (Line 1: DNA Ladder, Lines 2-10: Cucumber leaf samples and Line 11: Positive Control of PCR CMV set – 870bp).

4.7. Metabolomic adjustments triggered by *B. bassiana* and *M. anisopliae* in CMV-infected cucumber plants:

4.7.1. In comparison with non-fungal-treated diseased plants at different Dpi

Metabolome analysis comparisons between non-fungal treated and fungal treated plants (all CMV-inoculated) showed 631 metabolites that were differentially expressed. These metabolites were filtered under different pathways or functional categories. Among the differentially expressed pathways are: Amino acids and derivatives (90 metabolites) (Fig. 13), Dipeptides and tripeptides (42 metabolites), Glycine, serine and threonine metabolism (21 metabolites), Cysteine and methionine metabolism (17 metabolites), Glyoxylate and dicarboxylate metabolism (21 metabolites), Lysine degradation (13 metabolites), Arginine and proline metabolism (18 metabolites), Alanine, aspartate and glutamate metabolism (16 metabolites), Tyrosine metabolism (16 metabolites), C5- branched dibasic acid metabolism (18 metabolites), Citrate cycle (14 metabolites), Phenylpropanoid biosynthesis (13 metabolites) (Fig. 14) and Phenylalanine metabolism (12 metabolites) (Fig. 14). Some of these metabolites were significantly increased and others are significantly decreased, in which their expression differs between comparisons and treatments.

The columns in the heat maps (Figs. 12–13) indicate the comparisons between non-fungal-treated diseased cucumber plants (positive control (C)) versus fungal-treated diseased plants (MT: *Metarhizium anisopliae* treatment or BT: *Beauveria bassiana* treatment) at different days post-CMV inoculation Dpi: 3, 6, and 10 days and rows represent the expression of different metabolites

of specific pathways. The colors of heat map indicate scaled expression of metabolites in different comparisons. The color values ranging from red to yellow, while red color indicates down-regulation and yellow indicates up-regulation of metabolites. Z score is a measure of distance, in standard deviations, from the plate mean. A well with a Z score of 0 has the same raw value as the plate mean. A well with a Z score of 1.0 is exactly one standard deviation above the plate mean, and a Z score of − 0.5 is half a standard deviation below the plate mean.

(The heat maps for other above mentioned pathways are present in the complete Thesis).

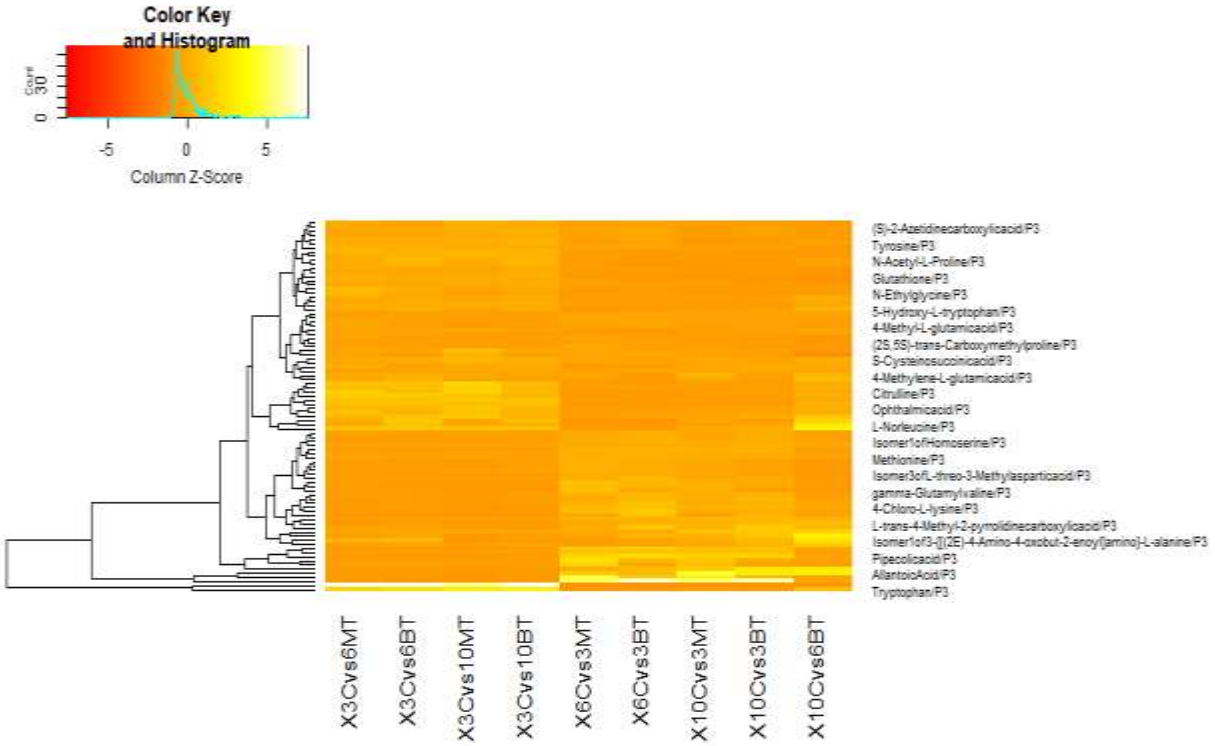

Figure 12. Heat map showing some of the differentially expressed metabolites of amino acids and derivatives pathway (P3) in different comparisons between positive control plants (C) and *Metarhizium* (MT) or *Beauveria* (BT) treatments at different Dpi (Ex: 3Cvs6M means comparison between Positive Control plants at Dpi 3 and *Metarhizium*-treated plants at Dpi 6).

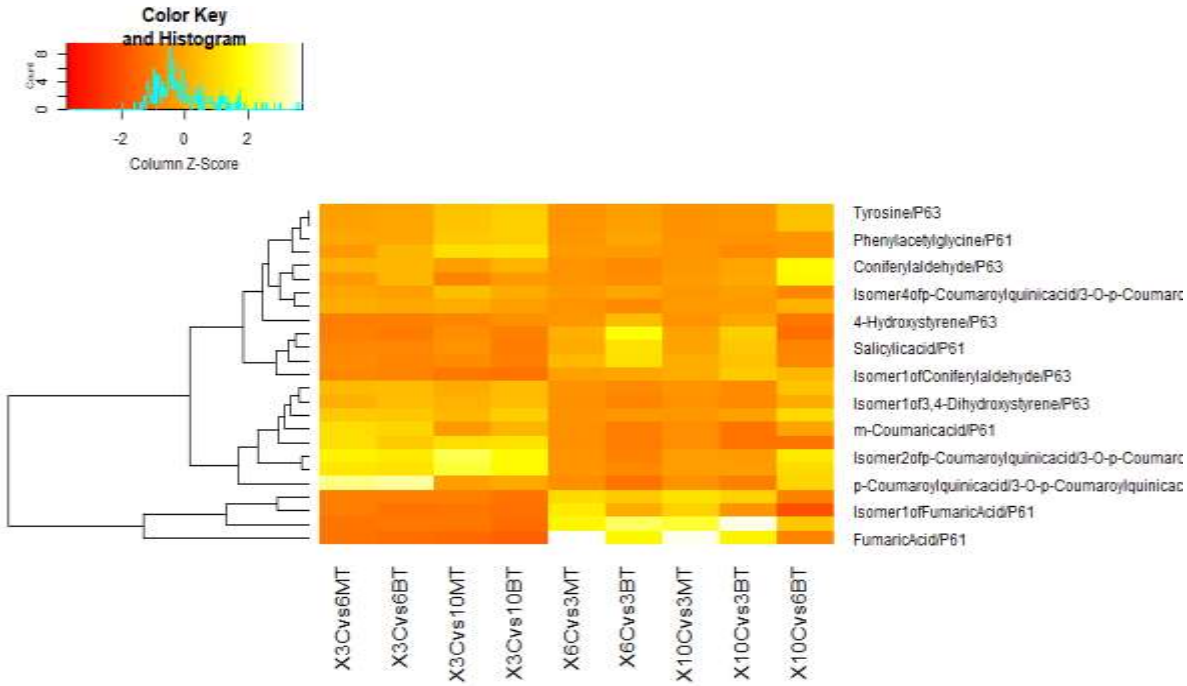

Figure 13. Heat map showing some of the differentially expressed metabolites of Phenylalanine metabolism (P61) and Phenylpropanoid biosynthesis (P63) pathways in different comparisons between positive control plants (C) and *Metarhizium* (MT) or *Beauveria* (BT) treatments at different Dpi.

4.7.2. In comparison with same fungal-treated diseased plants at different Dpi

Metabolomic analysis comparison was also performed between same fungal-treated diseased cucumber plants, but at different Dpi, in order to study the change in metabolite profile with the increase of time post-inoculation of CMV virus under the effect of fungal endophytes treatment. In addition, metabolomic analysis comparison was done between non-fungal-treated diseased cucumber plants (positive control plants) at different Dpi. The heat map in Figure 14 illustrates the significantly expressed plant metabolic pathways (*y*-axis) among the mentioned comparisons (*x*-axis) (Dpi10 *Metrahizium* vs. Dpi3 *Metrahizium*, Dpi6 *Metrahizium* vs. Dpi3 *Metrahizium*, Dpi6 *Beauveria* vs. Dpi3 *Beauveria*, Dpi10 *Beauveria* vs. Dpi3 *Beauveria*, Dpi10 Control vs. Dpi3 Control, Dpi6 Control vs. Dpi3 Control). The difference in the colors between columns in the heat map reveals the difference in the expression of metabolic pathways between the comparisons. The color scale ranges from dark blue to dark red with the increase in fold change value. So, the darker

the red color means more of an increase in metabolic pathway expression. Whereas, the darker the blue color means more of a decrease in metabolic pathway expression.

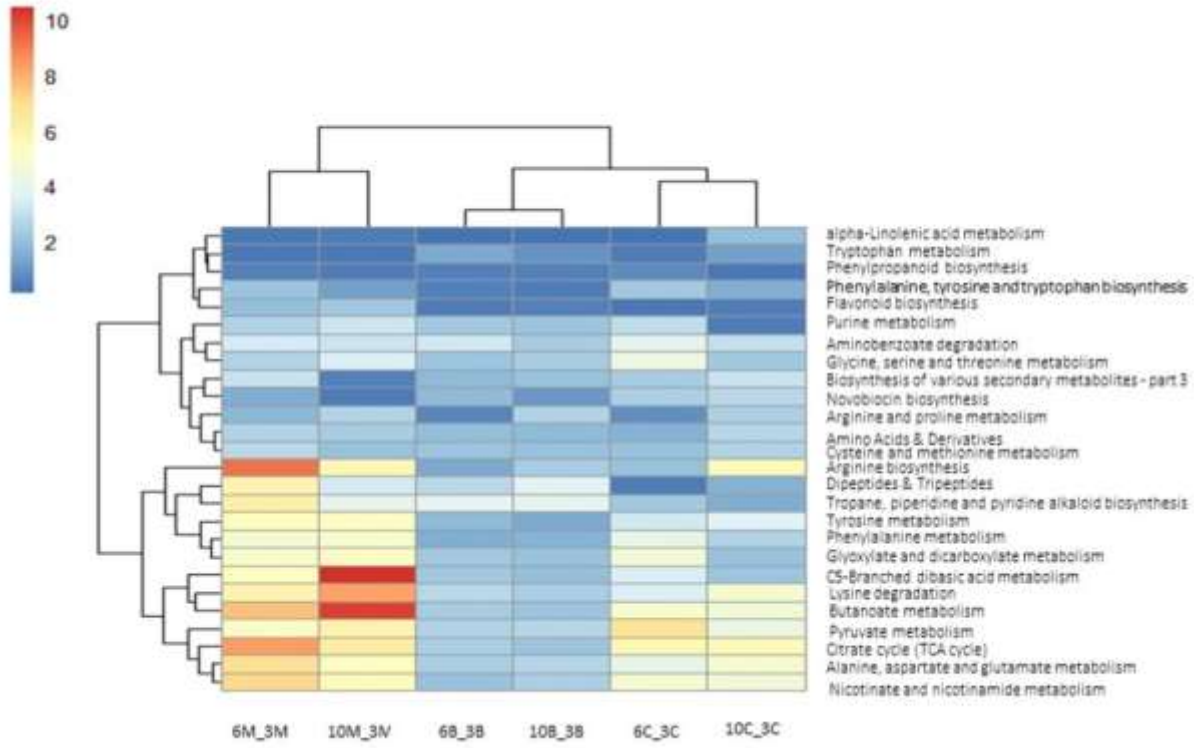

Figure 14. Heat map showing the differentially expressed plant metabolic pathways in comparisons between the same treated plants at different Dpi.

4.8. Metabolomic adjustments triggered by CMV in cucumber plants

Metabolomics analysis was achieved for positive control plants (CMV-infected) in comparison with negative control plants (non-CMV-infected) to study the effect of CMV infection on the metabolite profile of cucumber plants. The number of non-significantly expressed metabolites in CMV-infected cucumber plants is 1021, while 251 metabolites were significantly expressed, including 67 metabolites of significant increase (22 metabolites of them are of unknown pathways - NA) and 184 metabolites of significant decrease (14 metabolites of them are of unknown pathways - NA) (Fig. 15).

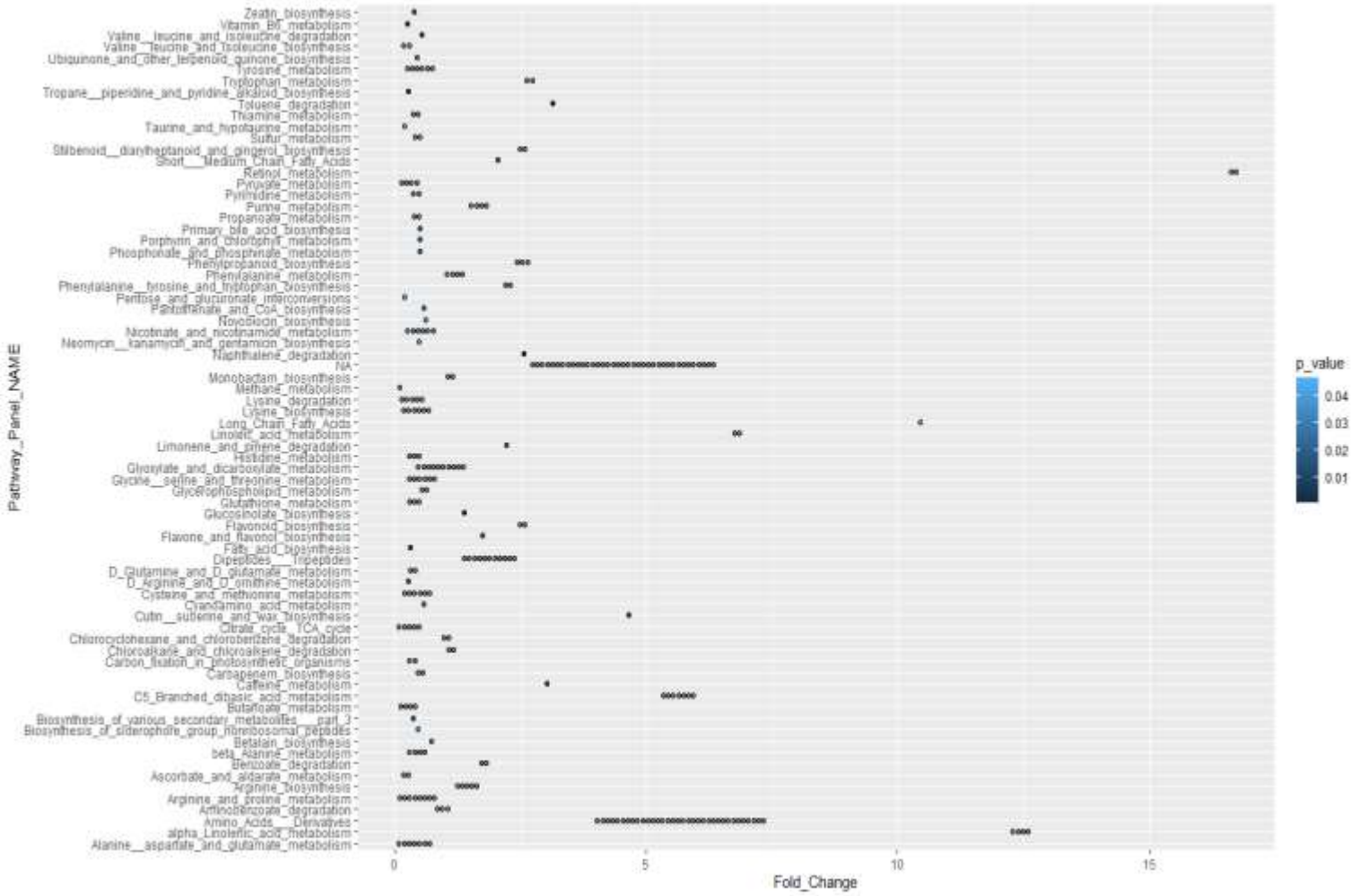

Figure 15. The number of significantly expressed metabolites for each pathway in CMV-infected cucumber plants in comparison with negative control plants (non-CMV-infected).

V. DISCUSSION

To our best knowledge, the artificial introduction of *M. anisopliae* and *B. bassiana* into the seed tissue followed by its successful establishment as an endophyte in other parts of resultant cucumber plant is demonstrated for the first time in present study. Interestingly, 79% of studies reported very variable results in the efficacy of artificial introduction of EPF into plants. There are works indicating that a leaf is a poor route of entry for EPF to colonize plants, as was demonstrated for *B. bassiana* on coffee seedlings (Posada *et al*., 2007) or on tomato plants (Qayyum *et al*., 2015). It is hypothesized that specific cuticular components might have a detrimental effect on conidium

germination, but their effects have not been tested (Posada *et al*., 2007). 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). This study proves that seed inoculation method is a very successful approach to introduction of EPF into all parts of cucumber plants. The success of internal colonization is usually determined by re-isolation of tested fungi using the culture dependent method (CDM) (McKinnon *et al*., 2017). The results of SEM observations 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. However, the same fungal isolates were detected in all plant parts obtained from cucumber seedlings cultivated on sterile substrate. 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. The application rate of conidia is also known to influence the rate of endophytic colonization (Ownley *et al*., 2008). Current data presented evidence that entomopathogenic fungi, not only had endophytic capacity, but could also act as plant growth promoters in cucumber. Entomopathogenic fungi could significantly increase the germination rate and seedling emergence of inoculated cucumber seeds under laboratory and outdoor conditions. Furthermore, seed-inoculated cucumber plants show significantly higher number and larger length of roots when compared to control plants. In addition, Colonization of cucumber plants by *B. bassiana* and *M. anisopliae* resulted in improved plant height, greater numbers of leaves and flowers without significant effects on chlorophyll synthesis. These observations prove the potential of entomopathogenic fungi in promoting plant growth and development by a symbiotic relationship that serve as extended root system possibly improving the absorption of nutrients and moisture (Sasan and Bidochka, 2012). 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)).

This study shows that endophytic presence of entomopathogenic fungi 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 endophytes compete with the phytopathogen for nutrients and niches, and they also produce substances that may disrupt the pathogen's quorum-sensing signaling (Miller and Bassler, 2001; Piewngam *et al*., 2018). Further, systemic resistance in host against pathogen is induced through the change of metabolite profile triggered by endophytes (Munir *et al*., 2020). In the current study, metabolomics was employed to gain insight into mechanisms involved in defense against CMV in endophytic *B. bassiana*- and *M. anisopliae*- treated diseased cucumber plants. Using LC- MS, we compared the metabolic profile of diseased cucumber plants fungal and non-fungal treated with endophytes. Before metabolomics analysis, the used entomopathogenic fungi were assessed for their endophytic presence in cucumber tissues. Endophytes can influence the production of specific metabolites (Chen *et al*., 2022), as evidenced by the fluctuating concentrations found in this study; this could be related to endophytic fungi's influencing mechanisms. The primary metabolism plays a major role in plant priming events by providing initiation energy and production of various vital compounds. Among others, amino acids are chief primary metabolites directly involved in plant immune responses (Mhlongo *et al*., 2020; Ting *et al*., 2020). Our data indicated increased level of various amino acids as these are building blocks of several essential secondary metabolites such as polyamines, tyramine, alkaloids, and phenylpropanoids. Amino acids present in endophyte-treated diseased cucumber plants after infection with CMV could reveal more insights about the role of amino acids in cucumber defense response against the virus. Similarly, the treatment of citrus with the endophyte *Bacillus subtilis* L1-21 has triggered the up-regulation of amino acids levels in endophyte-treated citrus after infection with the bacteria *Candidatus* Liberibacter asiaticus (Clas) (Munir *et al*., 2020).

Accumulation of organic compounds plays an important role in biotic and abiotic stresses defense (Zhang *et al*., 2020). Both entomopathogenic fungi in this study triggers the production of organic acids. A similar study (Batool *et al*., 2022) showed that *B. bassiana* inoculated maize plants under the attack of Asian corn borer (*Ostrinia furnacalis*), caused adjustments in organic acids metabolites such as succinic acid in comparison with no inoculation plants. Organic acids within cucumber plants were also higher in quantity and concentration in endophytic bacteria-applied plants (Mahmood and Kataoka, 2020).

The present metabolomics analysis revealed up regulation of metabolites within the phenylpropanoid pathway, triggered by endophytic presence in infected cucumber plants. Bajaj *et*

al. (2018) reported similar results, in which the root endophytic fungus *Piriformospora indica* induces altered phenylpropanoid and secondary metabolism in colonized soybean roots. The accumulation of metabolites belonging to organic compounds and phenylpropanoids can be a common mechanism in plant adaptation to different stresses (Batool *et al*., 2022). In addition, after treatment with the pathogen *Verticillium dahliae,* the defense response of cotton plants pre-inoculated with the biocontrol fungus *Chaetomium globosum* CEF-082 was strengthened through the differentially expressed genes (DEGs) of phenylpropanoid biosynthesis pathway (Zhang *et al*., 2020).

Although, endophyte treated plants showed higher abundance of organic acids in cucumber plants, CMV infected plants has led to the decrease of organic acids in cucumber plant. This demonstrates the positive effect of the entomopathogenic fungi on the induced defense mechanism against CMV in cucumber plants. However, CMV infection has triggered the abundance of m-Coumaric acid, p-Coumaroyl quinic acid and cis-beta-D-Glucosyl-2-hydroxycinnamic acid, which are major plant defense related phenolic compounds (Soujanya *et al*., 2021; Knollenberg *et al*., 2020). This indicates that cucumber plants produce more phenolic compounds as defense mechanism against the virus. Pérez-Clemente *et al*. 2019 have reported that Citrus tristeza virus (CTV) infection induced accumulation of amino acids and derivatives. In addition, high accumulation of amino acids and their derivatives in cucurbit chlorotic yellows virus (CCYV)-infected plants was found (Zhang *et al*., 2022). However, in our study most amino acids and derivatives detected were significantly decreased in CMV infected plants. Amino acids increase in plants promotes the growth and development of herbivore insects (Maluta *et al*., 2014, Cui *et al*., 2019), which means that CMV infection decrease the fitness of cucumber plants to its vectors. This explains the previous results for the negative effect of CMV on the development of its insect vector aphids (Mauck *et al*., 2010).

ELISA results showed no significant difference in the absorbance measurements between fungal treated and non-fungal treated diseased cucumber plants. This indicates that further research is required in order to study the effect of entomopathogenic endophytes on the titer level of CMV virus in cucumber plants.

VI. CONCLUSIONS

1. Colonization of cucumber plant tissues by both 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.

2. Colonization of cucumber plants with entomopathogenic endophytes greatly promotes plant growth and development.

3. Colonization by entomopathogenic endophytes has no effect on chlorophylls and caretonoids content of cucumber plants, but has increased the phenol content.

4. It was found that cucumber plant colonization by fungal entomopathogens is generally negative to aphids, *A. gossypii*, reducing their overall population size.

5. Cucumber plants treated with *B. bassiana* and *M. anisopliae* endophytes showed a strong metabolic response to CMV infection. In total, six hundred and thirty one metabolites were differentially expressed in endophyte treated CMV diseased cucumber plants, with highest percentage recorded for metabolites in amino acid derivatives, Dipeptides and tripeptides, Glycine, serine and threonine metabolism, Cysteine and methionine metabolism, Glyoxylate and dicarboxylate metabolism, Lysine degradation, Arginine and proline metabolism, Alanine, aspartate and glutamate metabolism, Tyrosine metabolism, C5-branched dibasic acid metabolism, Citrate cycle, Phenylpropanoid biosynthesis and Phenylalanine metabolism pathways.

6. This method of applying endophytes to plants could increase the resistance of cucumber varieties to CMV infection. Multiple metabolites such as amino acids, organic acids, and phenylpropanoids were activated in endophyte-treated diseased plants, which displayed resistance to CMV pathogen.

7. The virus CMV has triggered great changes in the metabolome profile of diseased cucumber plants. This directly demonstrates how plant respond when infected with the viral pathogen and how CMV modifies host metabolisms to enhance virus infection. CMV-infection has led to the decrease of organic acids and most amino acids and derivatives detected in cucumber plants. However, it has caused the increase of m-Coumaric acid, p-Coumaroyl quinic acid and cis-beta-D-Glucosyl-2-hydroxycinnamic acid which are major plant defense related phenolic compounds.

VII. CONTRIBUTIONS

1. This is the first study to report a significant data on the interactions between *B. bassiana*, *M. anisopliae* and cucumber plants, and thus to extend previous reports on the ability of entomopathogenic fungi to act as endophytes.
2. This study shows that endophytic application of entomopathogenic fungi to cucumber seeds can enhance plant resistance against melon aphids, *A. gossypii*. These results support previous studies where *B. bassiana* endophyte also negatively affected cotton aphid reproduction on other plants.
3. This is the first metabolomics study of entomopathogenic endophytes-treated virus diseased plants, which has shown that endophytes can be useful in the management of CMV disease by enhancing cucumber defense metabolites against the virus infection. Changes in diseased cucumber plant metabolism due to fungal endophytes treatment could give future direction for using these endophytes to gain in-depth insights about defense response to CMV pathogen. This data explains previous findings of the ability of endophytic *B. bassiana* colonization of melon plants to confer protection against CMV transmission by the vector *Aphis gossypii*.
4. Current study shows how cucumber plants respond to CMV-infection and this may provide information to seed producers and breeding for plant viral diseases control.

VIII. Conferences and Publications of the Dissertation:

Participation in conferences:

- Lebanese Association for the Advancement of Science (LAAS) International Scientific Conference at Balamand University - Lebanon (2018)/ Poster presentation.
- IX International Scientific Agriculture Symposium "AGROSYM 2018", 04 – 07 October 2018, Bosnia and Herzegovina / Oral presentation.
  *Award winning for best Oral Presentation
- Sixth CRSL Research Conference – American University of Beirut AUB (2022)/ Oral presentation.

Conference papers:

1. **Shaalan R.** and Ibrahim L., 2019. Entomopathogenic fungal endophytes: can they colonize cucumber plants? P. 853−860. In: "Book of Proceedings of the IX International Scientific Agriculture Symposium AGROSYM 2018". Bosnia and Herzegovina.

Journal papers:

1. **Shaalan R.**, Gerges E., Habib W., Ibrahim L., 2021. Endophytic colonization by *Beauveria bassiana* and *Metarhizium anisopliae* induces growth promotion effect and increases the resistance of cucumber plants against *Aphis gossypii*. Journal of Plant Protection Research. Vol. 61, No. 4: 358–370. DOI: 10.24425/jppr.2021.139244
2. **Shaalan R.**, Ibrahim L., As-sadi F., Kayal W.E., 2022. Impact of *Beauveria bassiana* and *Metarhizium anisopliae* on the metabolic interactions between cucumber (*Cucumis sativus* L.) and cucumber mosaic virus (CMV). Horticulturae, 8. (Accepted recently and will be published soon).