Bulgarian Journal of Agricultural Science, 27 (Suppl. 1) 2021
Agricultural Academy

Endophytic colonization of *Solanaceae* family plants by fungal entomopathogen *Beauveria bassiana* strain 339 to control Colorado potato beetle (*Leptinotarsa decemlineata* Say)

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Abstract

Petkova M., Spasova-Apostolova V., Masheva, V., Atanasova, D. & Tahsin, N. (2021). Endophytic colonization of *Solanaceae* family plants by fungal entomopathogen *Beauveria bassiana* strain 339 to control Colorado potato beetle (*Leptinotarsa decemlineata* Say). *Bulg. J. Agric. Sci., 27 (Suppl. 1),* 143–149

In the current study, molecular identification of *Beauveria bassiana* isolate 339 was done by partial sequence analysis of ITS1-5.8-ITS2 region of the nuclear ribosomal DNA with universal primers. Biochemical characterization was conducted by Biolog FF MicroPlate. The efficiency of the endophytic colonization of *B. bassiana* strain 339 was studied by two methods of inoculation – leaf spraying and soil drench in tobacco, tomatoes, eggplant and pepper. The distribution of the fungus within the plant parts was established by incubation of explants on a selective nutrient medium. Colonization of the stems, roots and leaves of the plants from *B. bassiana* strain 339 was reported on the 7th, 14th and 28th day after inoculation. The results showed that *B. bassiana* strain 339 endophytically colonized various tissues in the studied plants of the *Solanaceae* family within 28 days after inoculation. The second objective of the study focused on the efficacy of *B. bassiana* strain 339 against adults and larvae of the Colorado potato beetle (*Leptinotarsa decemlineata* Say) in the laboratory experiment. There was a gradual increase in the entomopathogenic effect on the 7th day after treatment, when 90% mortality of larvae and 40% mortality of adult insects were reported. These studies show the possibility of expanding the use of the entomopathogenic fungus *B. bassiana* to control pests of plants of the family *Solanaceae*.

Keywords: Beauveria bassiana; endophytic colonization; *Solanaceae* family; *Leptinotarsa decemlineata*

Introduction

The *Solanaceae* family includes 90 genera and more than 2000 species, many of which are of great economic importance such as food and medicinal plants. Among the most important members of the *Solanaceae* family are eggplant (*Solanum melongena* L.); tomatoes (*Lycopersicon esculentum* Mill); pepper (various species of Capsicum), potatoes (*Solanum tuberosum* L.), and tobacco (*Nicoti-*

ana tabacum L. and *Nicotiana rustica* L.) (Shah et al., 2013).

Changes in climatic conditions confront modern selection with various and multifaceted problems related to plant protection and the provision of organic products. There is a growing interest in the production of organic production of tobacco and vegetable crops (McNeil et al., 2010). All this requires adaptation of organic production in plant breeding programs and ensuring environmentally friendly plant protection. In recent years, the production of organic products has increasingly relied on knowledge of natural mechanisms, through the unique metabolic properties of some microorganisms to ensure effective and environmentally friendly plant protection applications.

Fungi are ubiquitous and hold unique biochemical pathways to assimilate a vast array of substrates and produce unique secondary metabolites, some of which are well-known pharmaceuticals (Keller et al., 2005). The morphological and molecular features of fungi are common tools for identification of *Beauveria*. The Biolog FF MicroPlate, based on the company's Phenotype Array Technology, was also introduced for rapid identification and characterization of filamentous fungi (FF MicroPlate™ Instruction for Use). The FF MicroPlate contains 95 discrete substrates that are utilized differently by different species leading to a distinct substrate utilization and growth fingerprint (Rice & Currah, 2005; Buyer et al., 2001).

B. bassiana is a suitable candidate for biological control, as it has the ability on the one hand to colonize various plant tissues and on the other to control certain insects and pathogenic fungi (Ownley et al., 2008). Entomopathogenic fungi infect their hosts through the external cuticle and are pathogenic to both soft- and hard-bodied insects, as well as a range of other arthropods including acari (Singh, 2006). The duration and level of colonization for the different parts of the plant depends on the species and the used inoculation technique (Petkova et al., 2020). Among the most common enemies of great economic importance in the production of potatoes, eggplants and tomatoes are the Colorado potato beetle. In the production of potatoes, the damage from it amounts to an average of 20-30% (Mateeva-Radeva, 1997), and an eggplant and direct varieties of tomatoes (up to the flowering phase) of 50-60%.

However, the study of the effectiveness of biological control agents is a long and multi-stage process involving molecular identification, biochemical screening, an examination of endophytic and entomopathogenic activity. In the current experiment, Biolog FF MicroPlate was used for rapid identification based on substrate utilization, growth, secondary metabolite and antimicrobial profiles of *Beauveria bassiana* strain 339. The study aimed to determine the ability of the entomopathogenic fungus to colonize tobacco, tomatoes, peppers and eggplants and to assess its potential in the production of biologically clean products.

Materials and Methods

Plant material

Five different plants of *Solanaceae* family were used in the current study– eggplant, tomatoes, pepper, potatoes, and tobacco. The plant materials were grown in the in Tobacco and Tobacco Products Institute – Markovo. The seeds were surface sterilized with 70% ethanol for 1 min, hypochlorite and sterile dH_2O in a ratio (1:1) and washed three times with sterile dH_2O for 5 min, 10 min and 15 min. The seeds were left for a night to dry. Sowing was done in four terrines with a pre-autoclaved mixture of 10 L peat and 1.5 L perlite. The seeds in the terrines were watered with 25 mL of spring water depending on the needs of the seedlings. In order demonstrate the ability of *B. bassiana* srain 339 to colonize plants of the *Solanaceae* family, two inoculation techniques were applied in the laboratory experiments – soil drench and leaf spaying.

Potato potted plants were used to assess the entomopathogenic activity of *B. bassiana* 339 against the Colorado potato beetle in the department of Entomology of Agricultural University – Plovdiv.

Collection of Colorado potato beetles

Adults and larvae of the Colorado potato beetle were collected from a 2 hectares potato crop located in the Proslav district of Plovdiv.

Inoculum preparation

The fungal isolates were provided by prof. Slavimira Draganova Agricultural Academy – Bulgaria, Institute of Soil Science, Agrotechnologies and Plant Protection (ISSAPP). Strain 339 of *Beauveria bassiana* Bals was isolated from the larvae of the green apple aphid (*Aphis pomi* De Geer, genus *Homoptera*, family *Aphidaea*). Fungal cultures, started from dry conidia, were grown on Sabouraud's dextrose agar in the

dark at 27°C. After the treatment of Colorado potato beetle with strain 339 of *B. bassiana* in the laboratory, the fungus was re-isolated from the adults.

Identification of the fungal isolate using the BIOLOG system

FF MicroPlate Pre-made FF MicroPlates (catalog #1006) containing different carbon and nitrogen sources were purchased from Biolog (21124 Cabot Blvd, Hayward, CA) and stored at 4 °C until needed. The Biolog FF plates for filamentous fungi (FF) rely on the iodonitrophenyltetrazolium redox dye, which can detect respiration (NADH formation) on sole carbon sources. 95 wells contain the substrate and the dye, while the control well (the first top left on the plate) contains the dye alone (FF MicroPlate™ Instruction for Use). Results are reported between 24 and 196 hours after inoculation with a pre-prepared mold sponge isolate. A computer system compares the metabolic profile of each strain with its database and identifies the species by color change (Bochner, 1989; Bochner, 2003).

For this test, the fungal isolate was incubated on YMA agar for 7 days, after which a plaque inoculum was prepared by transfer to inoculation media and inoculated on FF plate. An important point is the determination of cell density by turbidimeter, which for fungi is 75%. The data is read with MicroStation Reader. Biolog Software is used to analyze the obtained data. The similarity index must be > 0.50 in order for the results to be considered acceptable.

Data from the Excel sheet was imported into Spotfire software (Spotfire Inc, Somerville, MA) and then analyzed for growth pattern, substrate utilization for the target secondary metabolites produced.

Molecular identification

DNA was isolated with HiPurA™ Fungal DNA Purification Kit (HiMedia, India). The control of purity and concentrations of genomic DNA was conducted by electrophoresis in an agarose gel. ITS1-5.8-ITS2 region of the nuclear ribosomal DNA was amplified with ITS1 and ITS4 universal primers (White et al., 1990). PCR analysis was performed in 20 µl reaction final volumes containing 1 µl (30-50 ng) of DNA and 2 µL 10 × reaction PCR buffer mixtures, containing 200 nM solution of dNTPs, 5 µM $MgCl_2$, 1 µl of 10 µM of both primers and 0.25 µl of 5 U/µl of Red-Taq DNA polymerase (Canvax, Spain).

The amplification reaction conditions consisted of 2 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 55°C and 2 min at 72°C with a final extension of 5 min at 72°C on Thermal Cycler (Techne, Cole-Parmer, UK). Expected amplicons were excised from the gel and purified with a Gel isolation kit (Exgene Cells SV, mini, Gene All, U.K.). PCR products were separated on 1% agarose gel stained with Safe View (NBS Biologicals, UK) at 100 V for 50 minutes using a VWR Mini. The resulting sequence was analyzed with BLAST software and compared with nucleotide sequences in the gene bank database ncbi.nlm.nih.gov). For the alignment analysis, MEGA 7 program has been applied the with two – parameter model (Kumar et al., 2001) to calculate the base composition, Kimura two-parameter distance and the ratio of sequence divergence. For phylogenetic analysis, we used MEGA 7 (neighbor-joining method) to study the relationship of different species to construct the phylogenetic tree.

Conidial suspension

Conidia were obtained from cultures grown on YEA after incubation for 10 days at 25°C in darkness. Conidia were harvested with glass cell scrapers and placed in test tubes containing 0.01% (v/v) Tween 80 (polyoxyethylene sorbitan monolaurate) (Merck® KGaA, USA). Suspensions were vortexed for 2 min, filtered through four layers of sterile cotton, and adjusted to 1×10^4 conidia ml^{-1} (Gurulingappa et al., 2010) after cell counting by Burger camera. Conidial viability was assessed before every experiment (Goettel & Inglis, 1997). This germination test was repeated for each stock suspension to maintain the constancy of the viability assessments. In all cases, the average viability of the conidia was over 89.52% for isolate 339 of *B. bassiana*.

Inoculation techniques

The treatment was carried out in two ways, soil and foliar. A total of 100 plants were used and from each plant 20 accessions were treated by the two inoculation methods-soil drench and foliar spray. Soil inoculation was performed with 10 mL of conidia suspension with a concentration of 1×10^4, which are placed in the soil as close as possible to the roots of plants.

Endophyte evaluation

On the 7th, 14th and 28th day after inoculation, samples of treated plants were taken to detect the presence of *B. bassiana* by inoculation of leaf, stem and root explants on YEA medium. One control plant was also taken from each plant of the *Solanaceae* family (4 plants in total). The plants were removed from the soil and washed with dH_2O. Prior to the introduction of the explants *in vitro*, superficial sterilization of the leaves, stems, and roots was performed according to Petkova et al. (2020). Isolation frequency (IF) of the *Beauveria* strain 339 was calculated using the following formula:

Isolation frequency (IF) = Ni/ Nt × 100

A)

Result
Comment
Notice

Species ID: Beauveria bassiana (Bals.) Vuillemin BGB

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.997	0.671	5.013	FF	Beauveria bassiana (Bals.) Vuillemin BGB
2	0.003	0.001	7.034	FF	Beauveria bassiana (Bals.) Vuillemin BGA
3	0.000	0.000	9.954	FF	Engyodontium album (Limber) deHoog
4	0.000	0.000	13.193	FF	Penicillium chermesinum Biourge

B)

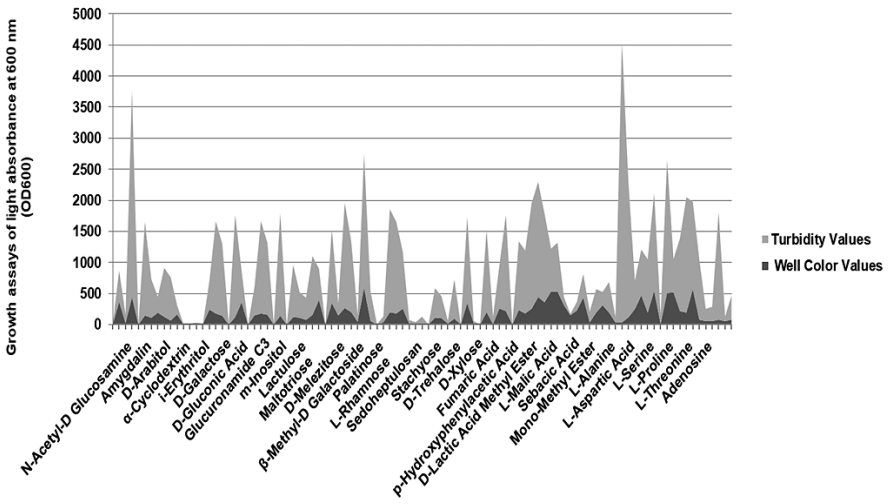

Fig. 1. (*A*.) Identification of *B. bassiana* strain 339 with the Biolog FF plates for filamentous fungi after 5 days inoculation at 27°C. (*B*.) Correlation between the growth and metabolism of 95 different carbon and nitrogen substrates of *B. bassiana* strain 339 analyzed on the 7th day after inoculation with MicroStation Biolog.

where Ni is the number of segments from which fungi have been isolated; and Nt is the total number of segments (Russo et al., 2015).

Evaluation of B. bassiana 339 as an entomopathogenic agent in potatoes

On pre-grown potato plants in pots, 20 adults and 20 larvae of four Colorado beetle larvae were colonized, after which they were sprayed with 10 mL solution of the fungal suspension of 1×10^4 spores and 0.01% Tween 80 as an emulsifier. Three replicates were used for each isolate and the control was treated with water. To assess the efficacy of the *B. bassiana* 339 foliar sprays, the mortality of adults and Colorado potato beetle larvae on the 1st, 3rd, 5th and 7th day after an infestation of leaf – treated potatoes were reported. The efficacy was calculated by the formula of Henderson & Tilton (1955).

Results and Discussion

Identifications by Biolog system were accepted as correct if the similarity index of the genus and species name was 0.750 or greater at 24 h or 0.500 or greater at 48 h. The identifications were accepted if the assigned identity met or exceeded 92% probability. In addition to meeting these manufacturers' criteria, sample identities were accepted as correct if the sample organism was listed in the system's database (Klingler, 1992).

Strain 339 was identified with a high probability of 0.997 and a similarity higher than 0.5 (0.671) at 168 hours. Isolate 339 was identified as the insect pathogenic fungus *B. bassiana* (Bals. Vuillemin) (Figure 1A).

The Biolog system is now widely used for providing information on the global phenotypes and specific nutrient utilization pattern of fungal isolates on 95 low molecular weight carbon sources (Rice & Currah, 2005), The *B. bassiana* 339 showed a metabolic profile displaying of the 96 substrates in the FF plates, 47 of which inducing a faster or/and greater respiration and growth.

These included N-acetyl-D-glucosamine, m-erythritol, D-melezitose, D-sorbotol, N-acetyl-L-glutamic acid, L-phenylalanine, adenosine. C-sources with intermediate fungi growth-promoting are D-arabitol, β-methyl-D-glucoside, stachyose, L-asparagine, L-aspartic acid, L-glutamic acid. Several substrates which showed low growth-promoting activities on *B. bassiana* strain 339 are D-arabinose, arbutin, D-cellobi-

ose, α-D-lactose, ρ-hydrohyphenilacetic acid and uridine on the Figure 1 B.

The identification of naturally isolated representatives of *B. bassiana* by classical methods with a microscope is time-consuming and not as effective as the methods of molecular biology (Biswas et al., 2015). The *B. bassiana* isolates used in the present study was characterized by sequencing and subsequent BLAST analysis. Based on 18S gene sequences isolate was identified as *B. bassiana*. Comparing the 18S rDNA sequences obtained in this paper to other species retrieved from GenBank, the genetic distance reflect that strain 339 had closer genetic relationship with an 18S rDNA of *B. bassiana* accession number AB576868.1 (Mukawa, 2011). A phylogenetic tree presented in Figure 2 was built by using the Neighbor-Joining method (Saitou & Nei, 1987). Strains of *B. bassiana 538, 730, and Beauveria brogniartii 646* were used in previous experiments for evaluation of endophytic colonization of tobacco (Petkova et al., 2020), showed high level of similarity with strain 339 of *B. bassiana* used in the current study.

In the current paper endophytic colonization of tomatoes, tobacco, peppers and eggplant plants by *B. bassiana* 339 was evaluated according to the inoculation technique (soil drench and leaf spraying) on 7th, 14th and 28th day post-inoculation. A total of 100 plants and different plant organs were examined. The fungus was never recorded in the control plants. It was successfully established as endophyte in four plants by using two different inoculation methods.

Using the direct root inoculation technique, twice the rate of root colonization on day 14 was observed compared to infection on day 7 on all *Solanaceae* plants. Similar to a 2017 study that found that the highest rate of colonization in tomatoes was reported 7 days after inoculation (Allegrucci et al., 2017) in the present study in tomatoes, colonization of stems was highest on 7th day and decreases gradually over time. On the first week 45% colonization of plants was reported. In comparison, on 14th day and 28th day 33.33% colonization was detected after incubation of explants on YMA medium (Figure 3).

In tobacco and eggplant, there is a tendency to gradually increase the percentage of colonization of roots, stems and leaves up to 14 days, then decreases up to 28 days. The lowest percentage is in the colonization of the upper leaves up to 14 days from 16 to 25.50%. The results obtained with tobacco were similar to previous studies conducted with tobacco, where the highest percentage of colonization was found on 21th day after inoculation (Petkova et al., 2020).

In contrast to the results of Russo et al. (2015), the highest degree of tobacco colonization was achieved on 7th day. A possible explanation was the activity of these fungal isolates and the climatic conditions in the country, as in both studies of tobacco in Bulgaria with different results increase in the percentage of

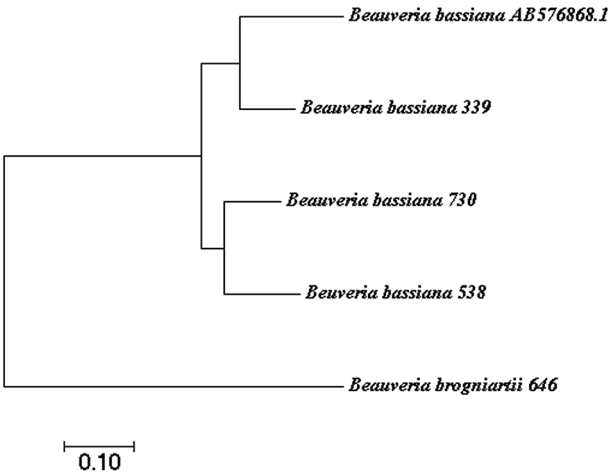

Fig. 2. A phylogenetic tree obtained after sequence analysis of *B. bassiana* strain 339. The evolutionary tree was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 1.27 is show. Branch lengths are proportional to the number of nucleotide differences. The marker bar denotes relative branch length. The analysis involved 5 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 272 positions in the final dataset.

colonization, the highest percentage of colonization from the 14th to the 21st day and a gradual decrease to 28th day.

The lowest percentages were recorded of potato root explants (66.50-83.33%) on the first week of soil treatment with *B. bassiana* 339 (Figure 3 A).

The highest degree of colonization was reported in the second experimental week 65.65-83.30% and decreased in the fourth week after inoculation to 66.66% (Figure 3 B).

The only exception was pepper, in which the percentage remains high around 83.30% for to 28th day post inoculation (Figure 3C).

We have further analyzed the efficacy of strain 339 on adults and larvae of the Colorado potato beetle (Figure 4A).

The results of the experiments show that 24 hours after treatment, no Colorado potato beetle mortality was reported. On third day after treatment, 40% larval mortality and 10% adult mortality were reported, and the efficacy of the isolate was doubled to 80% larval mortality and 20% adult mortality on 5th day after treatment. On the 7th day the highest efficiency of the preparation was reported, as the mortality increased to 40%, but the mortality of the larvae on the 7th day increased by only 10% and reached 90% (Figures 4B and 4C).

The higher efficiency of *B. bassiana* strain 339 against larvae than in adult individuals is impressive, which can be ex-

plained by the weaker chitinase activity against shells of the body of adult individuals.

Wagner and Lewis reported in 2000, that *B. bassiana* hyphae have been detected in the vascular elements of the xylem of corn and theoretically could move throughout the plant via the interconnecting xylem tissues to colonize the entire plant. Current results, confirmed that *B. bassiana* strain 339 colonized all tested plant tissues and is not a plant pathogen.

The results unequivocally show that *B. bassiana* 339 has the potential to be used and applied in organic farming after the necessary analyzes. The future study maybe focuses on the potential endophytic of entomopathogenic fungi and their impact on plant physiology and the Colorado potato beetle population under field conditions.

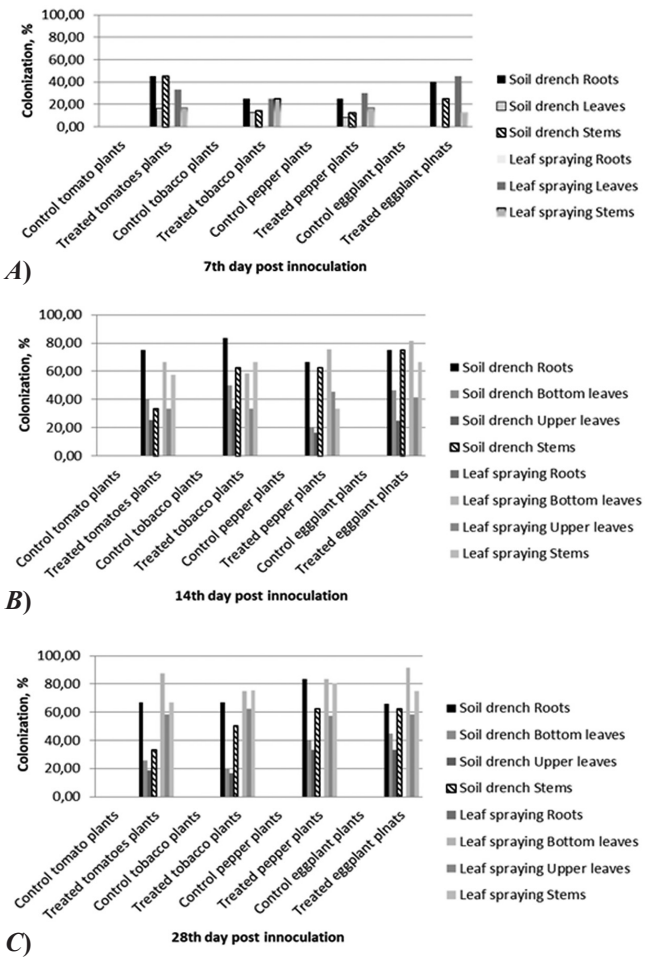

Fig. 3. Recover percentage of *B. bassiana* 339 colonization of tomato, tobacco, pepper and eggplant plants by root drench and foliar spraying inoculation methods on (*A*) on 7th, (*B*) on 14th and (*C*) on 28th day post inoculation.

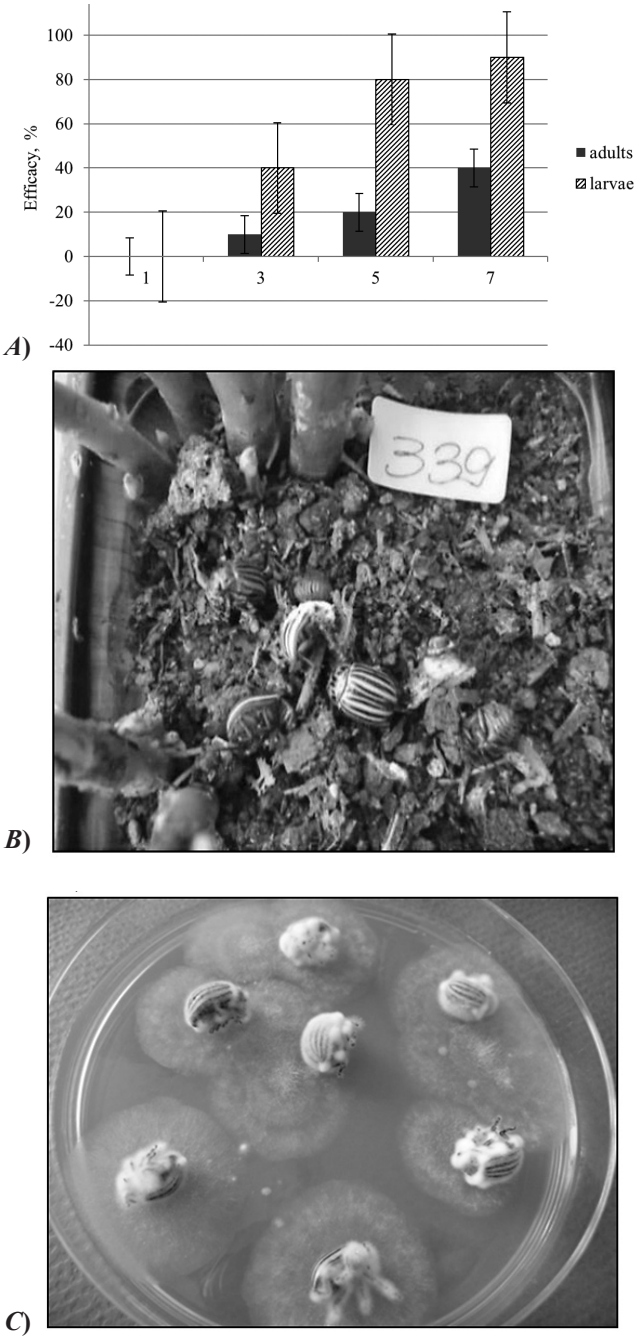

Fig. 4. (*A*) Efficacy of *Beauveria bassiana* 339 foliar spray against Colorado potato beetle (*Leptinotarsa decemlineata* Say).
(*B*) Dark lesions on the insect body indicate symptoms of *Beauveria bassiana* infection.
(*C*) Transfer of Colorado potato beetle adults with *Beauveria bassiana* sporulation on the YMA medium.

Conclusions

B. bassiana 339 shows an endophytic nature to all studied members of the family *Solanaceae* (tobacco, pepper, eggplant and tomato).

In the two applied techniques, colonization of different parts of the plants is observed.

No significant difference in colonization efficiency was observed when applying the 2 different inoculation techniques in the aboveground parts of the plant.

In both techniques, the percentage of colonization of the lower leaf is always higher than the percentage of colonizer of the upper leaf. The colonization of plants does not affect the normal physiological development of tobacco, pepper, eggplant and tomatoes.

B. bassiana 339 is effective against the Colorado potato beetle with higher efficacy against larvae than adults.

Acknowledgments

The research presented in this paper was financially supported by Centre of Research, Technology Transfer and Protection of Intellectual Iroperty Rights at the Agricultural University of Plovdiv by the project 01-2020.

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Received: May, 19, 2021; *Accepted:* June, 25, 2021; *Published:* September, 2021