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New *Beauveria bassiana* Strains from Kyrgyzstan with Endophytic and Insecticidal Activities

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Abstract

Beauveria bassiana (Ascomycota: Cordycipitaceae) is an entomopathogenic fungus used as an ecofriendly insecticide to infect arthropods. *B. bassiana* also possesses endophytic activity to contribute to plant growth. This study aimed to evaluate the potential insecticidal and endophytic activity of native *B. bassiana* isolates. The nymphal and adult stages of the apple tree aphid (*Aphis pomi*) and whitefly (*Trialeurodes vaporariorum*) were used as targets in bioinsecticidal experiments, and vegetables (beans, tomatoes and cucumbers) were used as targets in biostimulant experiments. The endophytic activity of the *B. bassiana* strains was assessed after inoculation them to the crop seeds and plants via soil drenching, foliar spraying and seed immersion. In bean plants, seed immersion was the most effective application method. Soil drenching was more effective in the cucumber and tomato plants. The results of *in vitro* bioassay tests against pests have revealed the LC_{50} and LT_{50} values of *B. bassiana* isolate Col-2. The LC_{50} of this isolate for *A. pomi* adults and nymphs was 2.5×10^6 conidia/mL^{-1}; for *T. vaporariorum*, it was lower 1.8×10^6 conidia/mL^{-1}. Such mortality occurred after 55.49 h. in *A. pomi* adults and nymphs (LT_{50}), after 62.3 h. in *T. vaporariorum* (LT_{50}).

Keywords

Entomopathogenic Fungus, Bioinsecticide, Bioinoculant, Biocontrol

1. Introduction

The excessive use of chemical fertilizers has led to several ecological and environmental problems, such as soil pollution and degradation and reductions in

beneficial soil organisms. In recent years, there has been a growing trend of using biological agents as an alternative to chemically synthesized pesticides in global markets [1] [2]. Currently, many microorganisms are used as biopesticides. From this group, entomopathogenic fungi play an important role and have been widely studied [2] [3]. *Beauveria bassiana* (Ascomycota: Cordycipitaceae) is an entomopathogenic fungus that is used as an ecofriendly insecticide due to its ability to infect and kill arthropods. It causes mycosis in about 700 species of harmful insects from various orders, including *Lepidoptera*, *Hemiptera*, *Hymenoptera*, *Coleoptera* and *Diptera* [4]-[7]. Like other entomopathogenic fungi, *B. bassiana* produces conidia that penetrate the cuticle of host insects and mites [8] [9]. Hence, *B. bassiana* may have an advantage over other biological agents (*i.e.*, bacteria and viruses) for some applications as a contact biopesticide. Because of its ability to rapidly produce infectious conidia when incubated on inexpensive nutrient media, there is significant interest in the commercial mass production of highly effective bioproducts with broad ranges based on *B. bassiana* [10]. Another important property of this fungus has been identified: the ability to suppress plant pathogens [11]-[16]. In addition, *B. bassiana* possesses endophytic activity, and this may be able to contribute to plant growth. *B. bassiana* species have been found to be able to colonise plants and exist as endophytes [17]-[23]. Endophytes are plant-associated microorganisms that spend part of their life cycle inside the plant without causing harm or disease to the host [24] [25]. They are physiologically dependent on host plants, receiving nutrition and protection from the host [26]. The endophytic activity of *B. bassiana* has been well-proven by many studies, although its application as a bioinoculant in modern agricultural systems is relatively under-explored [19] [27] [28]. For example, the study demonstrated the process of endophytic colonization, in which *B. bassiana* colonizes the interior of cowpea (*Vigna unguiculata*) seeds. It also showed the positive effects on plant growth in both laboratory and field conditions, as well as their increased resistance to insect attacks [29]. The results of the study have practical implications. In laboratory conditions, significant differences were observed between inoculated and control plants in terms of plant height and root dry weight. In the field, treated plants showed reduced susceptibility to pests such as *Aphis craccivora* (Koch, 1854), *Liriomyza* sp., and *Crinocerus sanctus* (Fabricius, 1775), indicating the potential of *B. bassiana* in pest management.

The main abiotic factors influencing the effectiveness and sustainability of *B. bassiana* are temperature, relative humidity, soil water content, and pH regimes. Conidia survival in nonsterile soil that was amended with carbon sources, nitrogen sources, or combinations of carbon and nitrogen was significantly decreased, and loss was often complete in less than 22 days, highlighting the urgent need to address this issue. In contrast, sterile soil treated in the same manner showed dramatic increases in number, demonstrating that *B. bassiana* is capable of growth in sterile soil [30].

It's important to recognize that the effectiveness and sustainability of *B.*

bassiana in field conditions hinge on the control of abiotic factors, such as desiccation and ultraviolet radiation. The authors also discovered that *B. bassiana* is highly sensitive to UV radiation, a factor that could potentially reduce the fungus's effectiveness in plants [31].

Enhanced virulence through genetic engineering of *B. bassiana* blastospores against a wide range of arthropod hosts and its resistance to environmental stressors. To achieve this, the protoplasts of *B. bassiana* were transformed with a constitutively expressed endogenous gene encoding chitinase (BbChit1) [32]-[34].

To improve contact of the fungus with the insect cuticle, some studies have used diatomaceous earth in polyvinyl alcohol to immobilize conidia of *B. bassiana* and algal shells present in diatomaceous earth have been shown to cause damage to the cuticle of *G. mellonella*; the effectiveness of the fungus was increased and guaranteed [35]. Novel research has been undertaken to identify the most effective method for propagating *B. bassiana* in the development of biopesticides. The efficiency of blastospores formed during submerged cultivation was compared with aerial conidia produced on a solid medium. In general, blastospores and submerged conidia demonstrated a faster insect-killing rate than aerial conidia against the cotton boll weevil *Anthonomus grandis* and the fall armyworm *Spodoptera frugiperda* [36].

Many biological agents are developed in public sector laboratories. They come with additional costs and restrictions, and economic assessments must be carried out early in the development process for farmers to adopt them [37]. However, biological control is primarily funded by governments, which require evidence of its return on investment. Cost/benefit analyses can also compare biological control to other management methods. Seeking advice from economists throughout a biological control program should be as routine as consulting statisticians [38].

This is despite the fact that it is often necessary to obtain and use local isolates to meet regulatory and environmental requirements for field applications of such bioagents. In addition, for the successful application of an entomopathogenic fungus and its integration into plant protection programs as an endophyte and a long-term preventive measure against insect pests, it is essential to critically examine the potential inoculation methods and optimize the colonization of the host plants [16]. Therefore, there is an urgent need to identify *B. bassiana* strains that possess both high bioinsecticidal and growth-stimulating activities for the development of biological products based on this fungal species.

Aphis pomi (Aphididae) commonly known as the apple aphid is an economically important sucking pest that annually causes significant damage to horticulture and fruit nurseries in the country. Especially in the southern regions, this pest forms several generations, damaging especially young trees of apple, pear and other rosaceous plants. To combat these annoying insects, gardeners use high concentrations of chemical insecticides, which are not desirable for the environment and the emergence of resistant pest races.

Trialeurodes vaporariorum (Aleyrodidae), commonly known as the greenhouse whitefly, causes significant damage in greenhouses and open fields in the country. Nymphal stages and adults of the pest damage many fruit, vegetable and ornamental crops, in addition, they spread viral plant diseases and create conditions for the development and spread of bacterial and fungal pathogens on crops. In the country, effective control is achieved using selective chemical insecticides; an annual increase in the population of this pest is noted, especially in greenhouse farms that grow tomatoes and cucumbers, as well as flowers.

The aims of the present study were: i) to evaluate the effect of different inoculation methods on the ability of Kyrgyz *B. bassiana* isolates to colonize bean, tomato and cucumber plants under laboratory conditions, ii) to determine the persistence of the fungus inside the plant tissues and iii) to determine the effect of the fungus on the growth potential of the plant organs. In addition, we aimed to evaluate the insecticidal potential of native *B. bassiana* isolates against nymphal and adult stages of the apple tree aphid (*Aphis pomi*) and whitefly (*Trialeurodes vaporariorum*) *in vitro* and *in vivo*.

2. Materials and Methods

2.1. *Fungal isolates*

The local native Col-2, VT and 12K strains of *B. bassiana* were isolated from soil and dead insects found in the Chui region of Kyrgyzstan (42˚56˚17; 74˚34˚8). The isolates were identified morphologically as *B. bassiana* based on the work of [39] [40] and [41]. Cultures of the above strains were stored at 4˚C on Czapek' agar medium (Sigma-Aldrich, USA) (20 g sucrose, 2 g $NaNO_3$, 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCL, 0.01 g $FeSO_4$, 20 g agar, 1000 mL distilled water).

2.2. *Fungal biomass* Production

Low-cost, solid nutrient media consisting of barley, wheat, millet and rice groats were used to produce biomass of each *B. bassiana* strain. The groats were sterilized in an autoclave and dried in an oven at 120˚C - 140˚C for additional sterilization. The groats were distributed into sterile dishes (5 - 10 cm layer) after adjusting pH values (5.5 - 5.6) and 70% humidity by adding sterilized salt solution. The dishes with groats were inoculated with the *B. bassiana* strains that had been previously grown in modified Czapek's liquid medium (15 g fodder yeast, 3 g sucrose, 3 g $(NH_4)_2SO_4$, 0.1 g KH_2PO_4, 0.05 g $MgSO_4$, 0.05 g KCl, 0.002 g $ZnSO_4$, 0.003 g KI, traces of $FeSO_4$, 100 mL water) on a shaker (Shaker-incubator ES-20/8, BIOSAN, USA) within 48 h. After incubation, a suspension was prepared from growing fungi conidia in distilled water, and 0.01% Tween 80, vortexed and the concentration of conidia was counted using a Neubauer hemacytometer. Inoculants containing 1×10^9 spores/mL were prepared and used to inoculate the cereals groats. The suspensions were mixed well to ensure the uniform distribution of the *B. bassiana* spores across all the layers of the nutrient medium. The dishes were incubated at room temperature (25˚C) and with natural night and daylight. Each type

of groats for the production of fungi biomass was tested in three replicates. After the 15-day incubation on the barley, wheat, millet and rice groats, a spore suspension was prepared from the dried biomass. For this, 10 mL of distilled water containing 1% Tween 80 was added to 1 g of biomass. Then, each conidial suspension was transferred into a 50 mL tube and mixed for 3 min at 1500 rpm to separate the conidia and mycelium from each other. The conidial concentration (conidia/g biomass) was determined by first counting the number of conidia (using phase contrast microscopy and a Neubauer chamber) and using the following formula: $N = a \cdot 1000K/h \cdot S$ (where N = number of conidia in 1 g of biomass, a = average number of spores in one cell of the chamber, K = dilution number, h = chamber depth and S = area of one cell of the chamber) [41]. Conidia viability was assessed using the germination assessment method [40]. The percentage of germination was determined by randomly counting at least 300 spores for each plate. A conidium was considered to have germinated when it had a germ tube that was at least as long as the smallest diameter of the conidium.

2.3. Plant Seeds and Sowing Conditions

Lopatka variety bean plants (*Phaseolus vulgaris*), Linda variety tomato plants (*Solanum lycopersicum*) and Curling variety cucumber plants (*Cucumis sativus*) were used in the experiments. Plant seeds were soaked in 0.5% sodium hypochlorite for 2 min and in 70% ethanol for 2 min. They were then washed three times with sterile distilled water. Sterile soil was mixed with perlite (1:1) and placed in pots. After sowing, the seeds were grown in a greenhouse with a temperature of 25˚C and a photoperiod of 16 h of light and 8 h of darkness.

2.4. Bioinoculation of Crops with *B. bassiana* Strains

The endophytic activity of the *B. bassiana* strains was evaluated by bioinoculating crops in three ways: seed immersion (soaking seeds for 2 h in a 1×10^6 spores/mL suspension of *B. bassiana strains),* soil drenching (watering with a 1×10^6 spores/mL suspension of *B. bassiana* strains around the root system, 50 mL/per plant) and foliar spraying (spraying the surface of the leaves and stems with a 1×10^6 spores/mL suspension of *B. bassiana* strains). Foliar spraying was performed manually, and 5 mL of suspension was sprayed on each plant. To ensure that the suspensions did not drip from the leaves and enter the soil, each pot was covered with aluminium foil. The fungal suspension was sprayed on the plants three times for one month. The soil drenching was applied three times for one month. Ten plants of each variety were cultivated for each treatment, and five replicates were used for each sampling date. Control pots received sterile water containing 0.01% Tween 80 in place of suspensions. The endophytic activity of the *B. bassiana* strains was assessed 60 days after last application of fungal suspension by three ways: seed immersion, soil drenching and foliar spraying. Experimental plants were kept at 25˚C temperature, 60% - 70% humidity and exposed to light for no more than 16 hours a day.

2.5. Assessment of the Endophytic Colonisation by the *B. bassiana* Strains

Analyses were performed according to the method of Barta [42] to determine the endophytic colonization activity of the *B. bassiana* strains in plants grown in laboratory conditions. When the plants reached the stem elongation stage 60 days after the last application of fungal suspension were used for the assessment of endophytic activity. The experimental plants had new cells at the tips of growing shoots, and the side shoots constantly appeared next to the main shoot. Data were collected and analyzed from groups of 10 plants inoculated with each variant. Various parts of the plants (*i.e.*, the leaves, stems and roots) were washed thoroughly with tap water. Their surfaces were disinfected by washing in 2.5% sodium hypochlorite solution for 3 min, then in 70% ethanol for 1 min, and rinsed three times in sterile water for 2 min. They were then dried on sterile filter paper, stored at -20˚C in sterile bags. From the last volume of rinsing water, 100 µL were sown on Potato Dextrose Agar (PDA) for two weeks. To avoid bacterial pollution 10 µg/mL of kanamycin was added to the medium. If the surface disinfection was successful (*i.e.*, there was no fungal colony growth), then the plant parts were cut into small pieces (4 × 4 mm each). Six pieces from each plant organ were placed on PDA and incubated, and the grown colonies were studied morphologically under a microscope. The *B. bassiana* colonization was calculated using the following formula: % colonization = (number of plant pieces showing fungal colony growth/total number of plant pieces) × 100 [43] [44]. The ability of the studied *B. bassiana* strains to induce systematic growth in non-inoculated symptoms observed in the inoculated plants compared to the control plants were recorded.

2.6. Assessment of *in Vitro* Insecticidal Activity against the Apple Tree Aphid (*A. pomi*)

B. bassiana isolates' conidia produced on the grain bran, harvested after 15 d cultivation and used for inoculum to infect the pests. The mycelium-containing dry biomass of the strains was suspended with adding 0.01% Tween 80 and vortexed. For bioassays, fungal conidial suspensions were adjusted to 1×10^6; 1×10^7 and 1×10^8 conidia/mL using a hemocytometer. The apple leaves were washed with sterile distilled water. Ten wingless females and ten second-stage nymphs of *A. pomi* were released on one leaf surface of host plants and directly sprayed with different concentrations (above mentioned) of *B. bassiana* conidia suspensions. Spraying was carried out using a Potter -Spray-Tower (Burkard Manufacturing Company Limited, England), so that each leaf received 2 ml of suspension. Three leaves in triplicate for each dose, in total, the complete experiment was repeated 3 times. Insects sprayed with warm sterile water with 0.01% Tween 80 were used as a control. Observations for infected insects have lasted within seven days at 25˚C ± 2˚C. The development of mycosis on the experimental and control insects and the humidity in the Petri dishes were monitored daily. To create moist conditions for the germination of the fungal conidia, the filter paper under the leaves was sprayed

with sterile water as the humidity decreased. The infected and dead insects were observed under a microscope every day.

2.7. Molecular Identification of Fungal Isolates

Each isolate was grown in a YE (yeast extract) liquid media for 48 h at 28˚C and the resulting mycelia were harvested by filtration. For DNA extraction, 0.5 g of frozen mycelia was ground to a fine powder in liquid nitrogen, and immediately transferred to a 2-ml microcentrifuge tube with 800 µL of DNA extraction buffer (100 mM Tris-HCl, pH 8.0, 25 mM EDTA, 1% SDS, and 25 mM NaCl) and placed at 65˚C for 20 min. The suspension was deproteinized once by extraction with an equal volume of phenol and once with an equal volume of chloroform- isoamyl alcohol (24:1). Genomic DNA was precipitated by adding two volumes of ice-cold ethanol and 10% (v/v) 3 M NaCl. The precipitate was collected by centrifugation, washed with 70% ethanol, then dried and resuspended in TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).

The ITS1 and ITS4 DNA fragments of select fungal isolates were amplified by PCR using the primer pairs ITS1 (CTTGGTCATTTAGAGGAAGTAA) and ITS4 (CAGGAGACTTGTACACGGTCCAG) and genomic DNA as the template as described by White *et al.* [45], using the following conditions: initial denaturation step of 2 min at 95˚C followed by 35 cycles, each consisting of a 45 s denaturation step at 95˚C, a 45 s annealing step at 52˚C, and a1 min extension step at 68˚C, and then followed by a final extension step for 5 min at 68˚C.

An approximately 1.200 bp fragment of the EF1-α gene region was amplified using following primers: EF1T (5'-ATGGGTAAGGARGACAAGAC-3' and 1567R (5 ACHGTRCCRATACCACCSATCTT-3' as described by Rehner and Buckley [46]. PCR conditions were adapted essentially as described by Rehner and Buckley [46].

Amplification products were purified using Montage PCR Cleanup Filter Plates (Millipore). Sequencing reactions were conducted by Starseq (Mainz, Germany). Phylogenetic analysis was performed in MEGA 7 [47] using the Maximum Likelihood method with a General Time Reversible model. The complete deletion option was used, and the level of bootstrap support was calculated from 1000 replicates.

Data Collection and Statistical Analysis. The virulence of each fungal isolate was estimated by calculation of the median lethal time (LT_{50}) and the median lethal dose (LD_{50}) by Probit statistical analyses using the STATISTICAR 6.0 software (Stat Soft Inc). Mortality data obtained from the study were normalized using an arc-sine square root transformation and subjected to an analysis of variance (ANOVA). Untransformed means are presented here P Values: (P) of ≤0.05; $P < 0.01$; $P < 0.001$ were accepted as statistically significant (the STATISTICAR 6.0 software).

To determine whether there were any differences among the fungal strains in terms of their endophytic activities multiple mean comparisons were made using Tukey's honestly significant difference (HSD) *test* when statistical differences were found between data sets ($P \leq 0.05$).

3. Results

3.1. Mass Production of Fungal Isolates in Laboratory Conditions

To grow the *B. bassiana* strains in laboratory conditions and determine their entomopathogenic and endophytic activities, it was necessary to choose low-cost nutrient media that meet the physiological requirements of these fungal isolates. We used low-cost, solid nutrient media for their cultivation that consisted of barley, wheat, millet and rice groats. Colonization of the solid media by fungal mycelia was seen from the third day. All tested strains grew well on wheat and barley, completely covering the medium and forming dense mycelia (Figure 1(a), Figure 1(b) and Figure 2(a)). However, the millet and rice groat media were not completely covered with fungal mycelia. To select the most suitable growth medium for producing inexpensive bioproducts based on *B. bassiana*, the conidial titer of each fungal biomass grown on the various cereal groats was determine. It was found that conidial formation varied among the different strains and depended on the medium used. High conidial titers were recorded when the VT (25×10^9 spores/g) and Col-2 (23×10^9 spores/g) strains were grown on wheat groats. When grown on barley groats, the VT strain produced 23×10^9 spores/g, and the 12 K strain produced 24×10^9 spores/g. On millet groats, the VT strain formed 17×10^9 spores/g, the Col-2 strain formed 18×10^9 spores/g and the 12 K strain formed 14×10^9 spores/g. The lowest conidial titers were recorded when the Col-2 strain was grown on rice groats (8×10^9 spores/g) and the 12 K strain was grown on wheat groats (10×10^9 spores/g) (Figure 2(b)). Thus, based on these results, we selected two medium compositions—wheat and barley groats—to produce biomass from the *B. bassiana* strains for use in bioproducts. The resultant biomass material was dried in a drying oven at 30˚C - 31˚C and subsequently packed in waterproof paper bags that were labelled with the product's weight.

3.2. Assessment of Plant Growth after Bioinoculation with *B. bassiana* Strains: Foliar Spraying

It was found that the growth-stimulating effect of the *B. bassiana* strains on the different vegetable crops varied with the bioinoculation method. When the *B. bassiana*

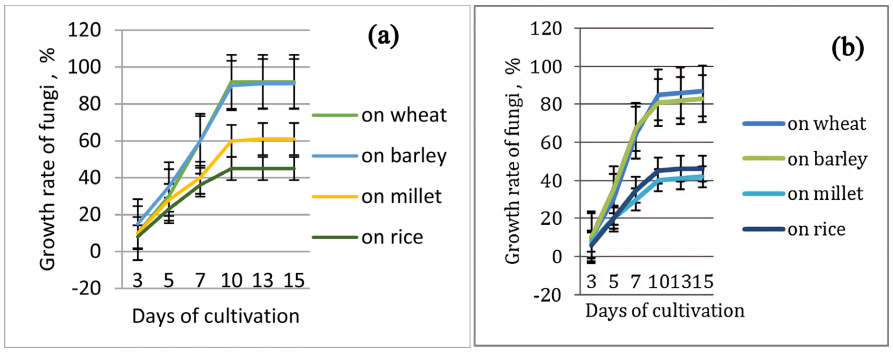

Figure 1. Growth intensity of *Beauveria bassiana* VT (a) and 12 K (b) strains on cereal groats during 15 days of surface cultivation. Values are given as mean ± SD (n = 3) and significantly different when $P \leq 0.05$.

strain suspensions were delivered by foliar spraying, the VT strain exhibited the greatest effect on the growth and development of tomato plants. In this case, the stems of the experimental plants were 4.0 - 4.5 ± 0.71 cm longer than those of the control plants. The stems of the experimental plants sprayed with the Col-2 and 12 K strain suspensions were 2.5 - 3.0 ± 0.45 cm longer than those of the control plants (Figure 3).

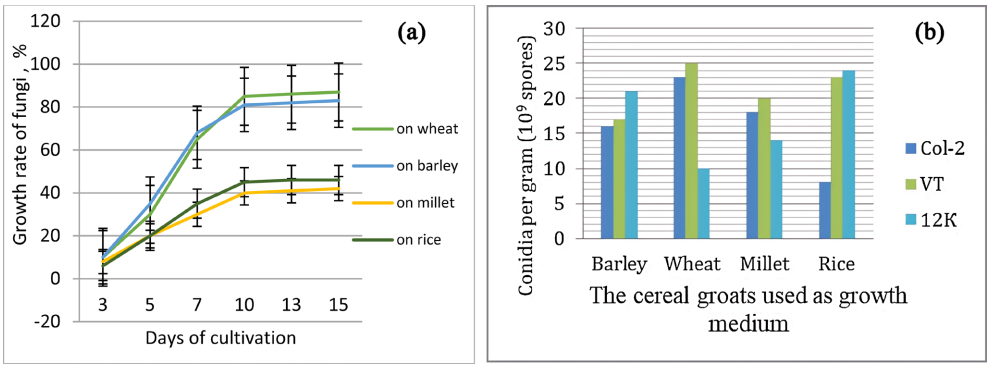

Figure 2. (a) Growth intensity of *Beauveria bassiana* Col-2 strain on cereal groats during 15 days of surface cultivation. (b) Number of fungal conidia per 1 g of dry bioproduct produced by the different fungal strains grown on different types of cereal groats. Values are given as mean ± SD (n = 3) and significantly different when $P \leq 0.05$.

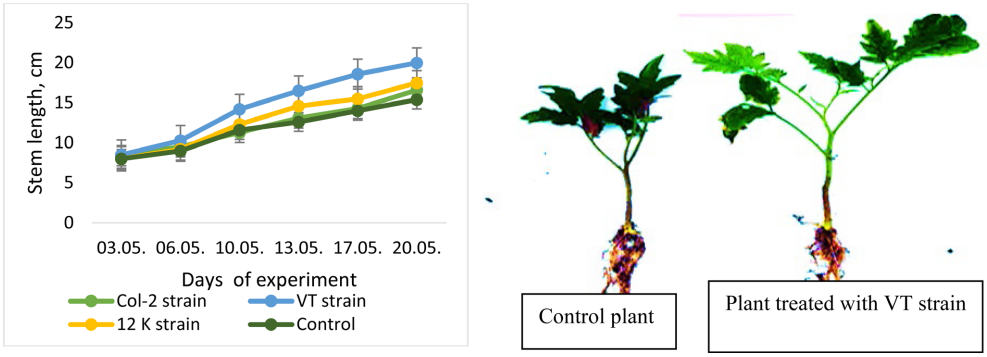

Figure 3. Effect of *Beauveria bassiana* strain suspensions delivered by foliar spraying on the growth of tomato seedlings. Values are given as mean ± SD (n = 3) and significantly different when $P \leq 0.05$.

The fungal strains had less effect on the growth of cucumber plants compared to tomato plants. Here, the 12 K strain was found to have the greatest effect on plant growth. Plants sprayed with the 12 K strain grew 2.0 ± 0.31 cm taller than the control plants. Plants sprayed with the Col-2 and VT strains grew only 1.0 ± 0.15 cm taller than the control plants (Figure 4).

The most potent growth stimulating effect exhibited by these *B. bassiana* strains was observed in the bean plants. Three days after spraying, the experimental plants were 5.5 - 7.0 ± 0.90 cm taller than the control plants, and after seven days, they were 10.0 ± 0.95 cm taller. Spraying with the VT and 12 K strain suspensions resulted in 9.0 ± 0.73 cm and 5.5 ± 0.71 cm of additional growth, respectively, compared to that of the control plants (Figure 5).

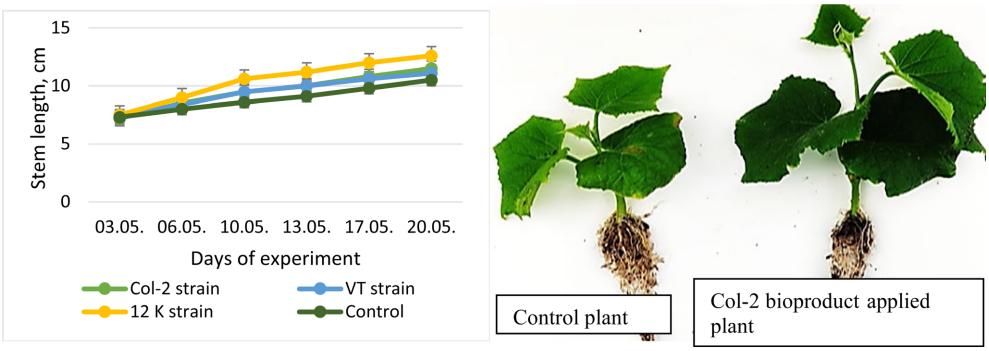

Figure 4. Effect of *Beauveria bassiana* strain suspensions delivered by foliar spraying on the growth of cucumber s' seedlings. Values are given as mean ± SD, n = 3, significantly different at $P \leq 0.05$.

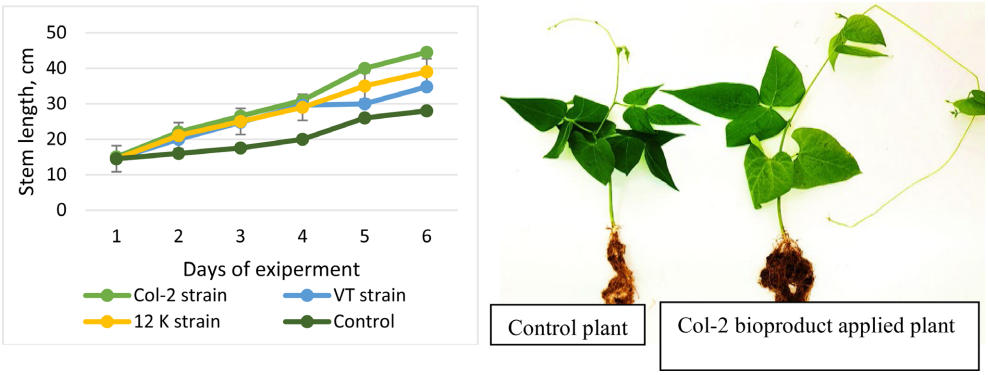

Figure 5. Effect of *Beauveria bassiana* strain suspensions delivered by foliar spraying on the growth of been seedlings. Values are given as mean ± SD, n = 3, significantly different at $P \leq 0.05$.

3.3. Assessment of Plant Growth after Bioinoculation with *B. bassiana* Strains: Seed Immersion

Tomato, cucumber and bean seeds were soaked for 2 h in suspensions of the *B. bassiana* strains. The effect on tomato plant growth was observed only after the appearance of several true leaves, seven days after the start of the experiment. Unlike the other strains, the 12 K strain showed promising results, producing experimental plants that were 3 ± 0.21 cm taller than the control plants. Immersing seeds in suspensions of the VT and Col-2 strains resulted in minimal shoot growth in the experimental group (1.5 ± 0.11 cm).

Slight differences between the experimental and control plants were also noted in the cucumber plants. When seeds were soaked in suspensions of the Col-2 and 12 K strains, the experimental plants were 1.8 - 2.5 ± 0.12 cm taller than the control plants, whereas when seeds were soaked in a suspension of the VT strain, the resultant plants were 1.5 ± 0.09 cm taller than the control plants. A similar effect was observed when bean seeds were soaked in suspensions of all tested strains. The difference in height between the control and experimental plants was almost 2.0 ± 0.19 cm.

Hence, it was concluded that *B. bassiana* has endophytic activity and can affect plant growth. Our findings show that the level of plant growth in the tested vegetable crops depends on the bioinoculation method used to deliver the *B. bassiana*

strains. Soil drenching produced the highest level of growth in the tomato and cucumber plants, whereas all the tested bioinoculation methods produced the same high levels of growth in the bean plants (Figure 6(a), Figure 6(b) and Figure 7).

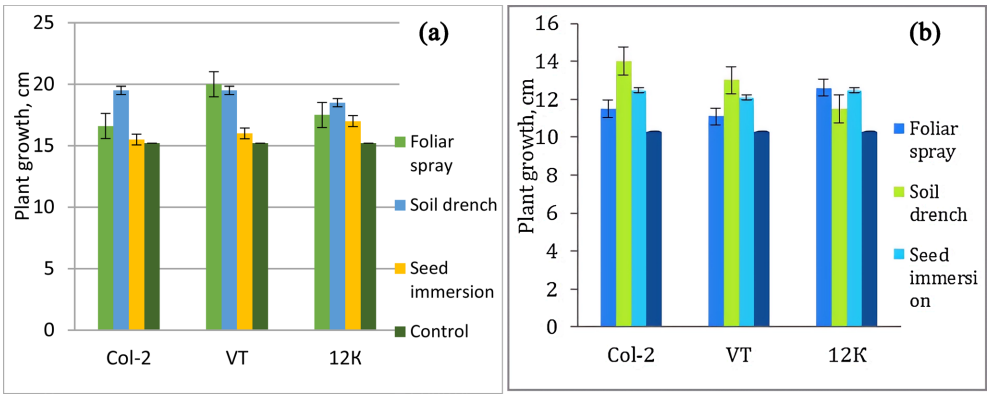

Figure 6. (a) The effect of the *Beauveria bassiana* strains on the growth of tomato seedlings; (b) The effect of the *Beauveria bassiana* strains on the growth of cucumber seedlings. The strains were applied in three different ways (foliar spraying, soil drenching or see immersion). Values are given as mean ± SD (n = 3) and significantly different when $P \leq 0.05$.

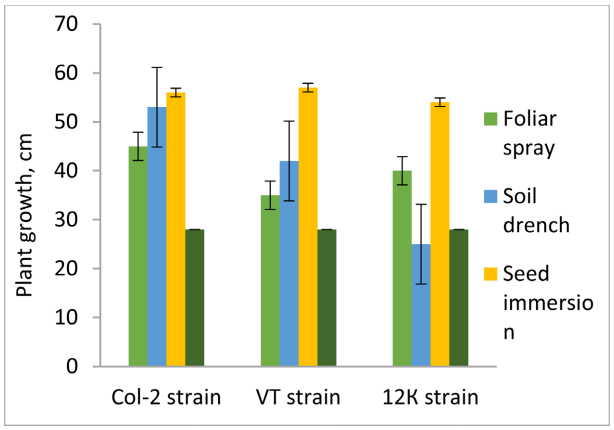

Figure 7. The effect of the *Beauveria bassiana* strains on the growth of bean seedlings. The strains were applied in three different ways (foliar spraying, soil drenching or see immersion). Values are given as mean ± SD (n = 3) and significantly different when $P \leq 0.05$.

Using the soil drenching method to apply the *B. bassiana* strains showed promising results for tomatoes: all three tested strains impacted plant growth. When foliar spraying was used, the VT strain showed a strong effect, the 12 K strain showed a moderate effect and the Col-2 strain had a weak effect. In contrast, the seed immersion method proved ineffective compared to soil drenching and foliar spraying.

In terms of cucumber plant growth, soil drenching was found to be the most effective delivery method for maximising Col-2 and VT strain activity. All three strains had almost equal effects on plant growth when the seed immersion method was used. Foliar spraying resulted in only the 12 K strain having an effect on plant

growth; the other two strains had no effect.

Regarding the growth of the bean plants, seed immersion proved to be the most effective method.

Seeds immersed in suspensions of all three strains produced plants that were 6 - 10 cm taller than those exposed to the strains via foliar spraying and soil drenching. The Col-2 strain showed excellent results in terms of effect on plant growth when delivered via all tested bioinoculation methods.

3.4. Endophytic Colonization: Presence and Persistence of Endophytic *B. bassiana* Strains in Vegetable Crops

In the tested tomato plants, application of the fungal strains via soil drenching resulted in a high level of root colonization by the endophytic *B. bassiana* strains—up to 73.0% ± 0.15%. Stem and leaf colonization levels were also significant (67.0% ± 0.2% and 53.0% ± 0.2%, respectively). When the strains were applied via foliar spraying, root colonization reached 55.0% ± 0.2%. When the seeds were immersed in the various *B. bassiana* suspensions, colonization was low: up to 32% ± 0.2% in the roots, 21.0% ± 0.2% in the stem and 17.0% ± 0.2% in the leaf tissue (Figure 8(a)).

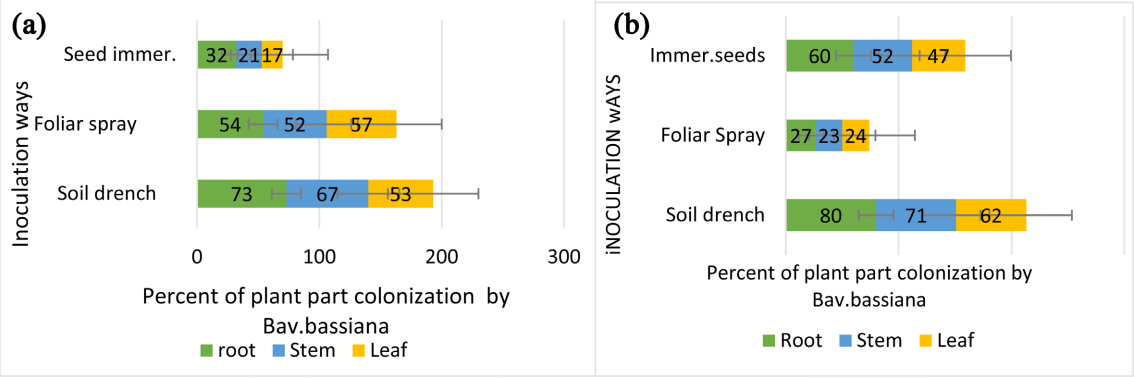

Figure 8. Colonization of tomato (a) and cucumber plant organs (b) by *Beauveria bassiana* strains delivered via three different methods. Values are given as mean ± SD, n = 3, significantly different at $P \leq 0.05$.

In the tested cucumber plants, colonization reached almost 80% ± 0.2% when the Col-2 and VT strains were delivered via soil drenching. When seeds were immersed in and leaves were sprayed with *B. bassiana* strain suspensions, colonization reached 60% ± 0.2% and 27% ± 0.2%, respectively (Figure 8(b)).

When bean seeds were immersed in the *B. bassiana* strain suspensions, high levels of colonization were observed, reaching almost 85% ± 0.2%. The other inoculation methods also resulted in significant colonization. Soil drenching resulted in up to 62% ± 0.2% of root samples, 53.0% ± 0.2% of stem samples and 47% ± 0.2% of leaf samples being colonized (Figure 9). Sixty days after injection, fungal colonies were isolated from plant stems, leaves and roots and their morphological features were studied, proving that they belong to *Beauveria bassiana* (Figure 10).

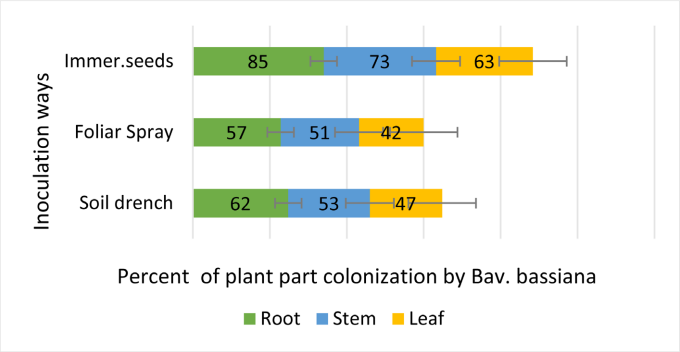

Figure 9. Colonization of beans plant organs by *Beauveria bassiana* strains delivered via three different methods. Values are given as mean ± SD, n = 3, significantly different at $P \leq 0.05$.

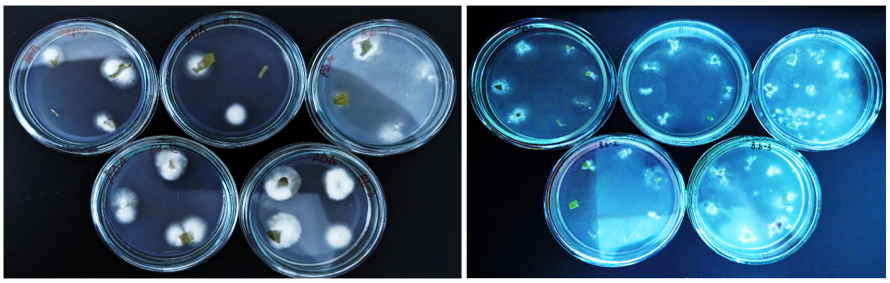

Figure 10. (a) The colonies of *Beauveria bassiana* grown from leaves on PDA media; foliar spray inoculation method. (b) The colonies of *Beauveria bassiana* grown from stems on PDA media; Soil inoculation method.

3.5. Insecticidal Activity of *B. bassiana* Strains against *A. pomi*

Dried formulation with high content of tested isolates conidia was used for assessment of their insecticidal activity against adults and nymphs of *A. pomi* using the doses 1×10^6; 1×10^7 and 1×10^8 conidia/mL. As a result, the tested *B. bassiana* strains have shown varying biological activity. At a dose of 1×10^6 conidia/mL, the mortality of adults and nymphs of the pest did not exceed 40.2% ± 0.95%, when using a dose of 1×10^7 conidia/mL, mortality increased to 63.7% ± 0.95%. The Col-2 strain was effective at all doses, especially at a dose of 1×10^8 conidia/mL, mortality reached 76.7% ± 0.95% within 5 days, and the death rate of control individuals was up to 12.0% ± 0.95% (**Figure 11**). The fungus mycelium began attaching to the covers of adults and nymphs in 48 h (**Figure 12**). Complete coverage of the insects with a white felt mycelium occurred after five days. It should be noted that the humidity has a direct impact on the development of the entomopathogenic fungus, since the experiments resulted in mortality when the dishes were kept in conditions with a humidity of 80% - 85%, at a temperature of 25˚C ± 2˚C.

3.6. Insecticidal Activity of *B. bassiana* Strains against *T. vaporariorum*

The *B. bassiana* strains grown on the media composed of wheat and barley groats

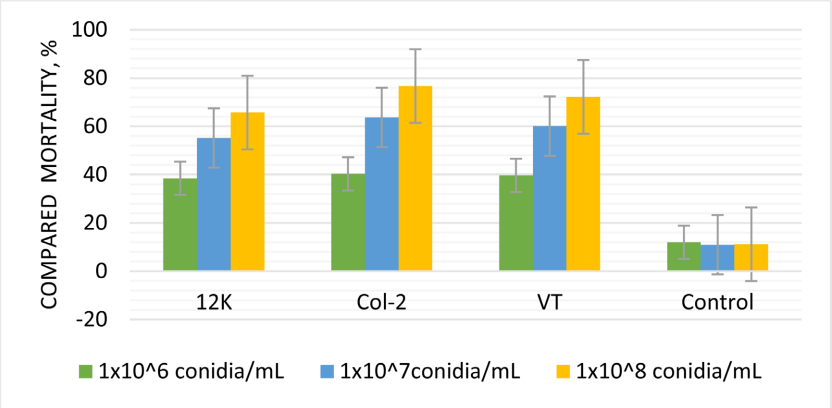

Figure 11. Insecticidal activity of the *Beauveria bassiana* strains against apple aphids' (*A. poma*) nymphs and adults by contact infection (1×10^6; 1×10^7 and 1×10^8 conidia/mL). Values are given as mean ± SD (n = 3) and significantly different when $P \leq 0.05$.

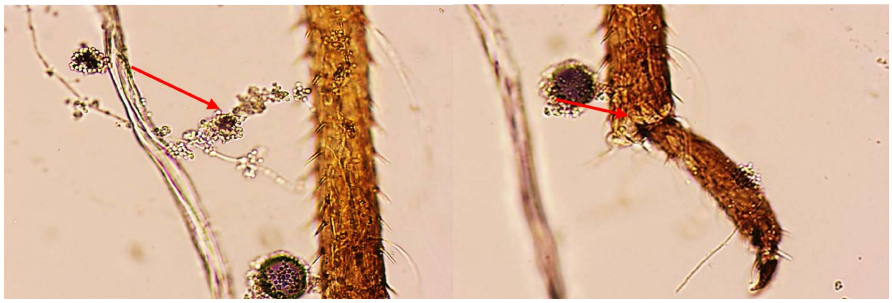

Figure 12. The hyphal and conidial germination of *Bav. bassina* attached to legs of *A. poma* adults. Images were produced using × 40 stereo microscopy.

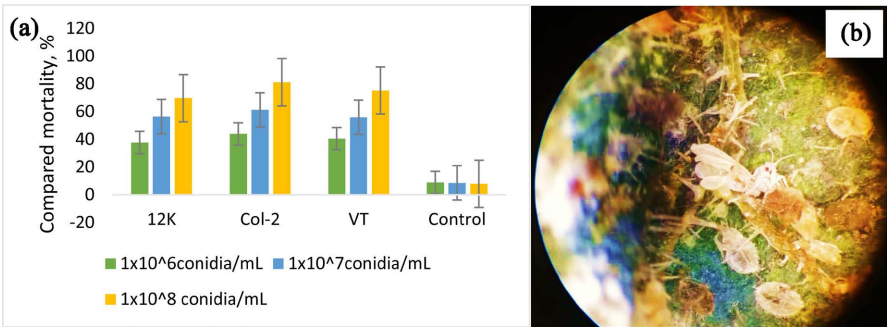

Figure 13. (a) Insecticidal activity of the *Beauveria bassiana* strains against whitefly' (*Trialeurodes vaporariorum*) nymphs and adults. Values are given as mean ± SD (n = 3) and significantly different when $P \leq 0.05$. (b) Mycosis observed in whitefly' nymphs and adults exposed to the *Beauveria bassiana* strains in 48 h.

showed significant insecticidal activity against whitefly adults and nymphs within five days. Exposure of the whitefly adults and nymphs to 1×10^6 conidia/mL dose of the 12 K, Col-2 and VT strains resulted in mortality of 37.7% ± 0.95%, 43.9% ± 0.95% and 40.5% ± 0.95% respectively ($P \leq 0.05$). The mortality recorded for the whitefly adults and nymphs was higher by using 1×10^7 conidia/mL dose of the 12 K, Col-2 and VT strains: 56.4% ± 0.95%, 61.3% ± 0.95%, 55.9% ± 0.95%

respectively ($P \leq 0.05$). The mortality of tested insects reached to 69.7% ± 0.95%, 81.2% ± 0.95% and 75.2% ± 0.95% respectively ($P \leq 0.05$) of the 12 K, Col-2 and VT strains at the dose 1×10^8 conidia/mL. Of all the tested strains, the Col-2 strain demonstrated the highest levels of insecticidal activity against both stages of the pest (**Figure 13(a)**). The results *in vitro* bioassay tests against sucking pests have revealed the LC_{50} and LT_{50} values of *B. bassiana* isolate Col-2. The dose that caused 50% mortality in the population of used pests was different. The LC_{50} of this isolate for *A. pomi* adults and nymphs was 2.5×10^6 conidia\mL^{-1}; for *T. vaporariorum* it was lower - 1.8×10^6 conidia\mL^{-1}. Such mortality occurred after 55.49 hours in *A. pomi* adults and nymphs (LT_{50}), after 62.3 hours in *T. vaporariorum* (LT_{50}). These data indicate that sucking pests have shown susceptibility to infection caused by *B. bassiana* isolate Col-2.

Complete coverage of the insects with a white felt mycelium occurred after five days (**Figure 13(b)**). The death rate of control individuals was up to 8.9% ± 0.95%. It should be noted that the humidity has a direct impact on the development of the entomopathogenic fungus, since the experiments resulted in mortality when the dishes were kept in conditions with a humidity of 80% - 85%, at a temperature of 25˚C ± 2˚C.

The results *in vitro* bioassay tests against sucking pests have revealed the LC_{50} and LT_{50} values of *B. bassiana* isolate Col-2. The dose that caused 50% mortality in the population of used pests was different. The LC_{50} of this isolate for *A. pomi* adults and nymphs was 2.5×10^6 conidia\mL^{-1}; for *T. vaporariorum* it was lower - 1.8×10^6 conidia/mL^{-1}. Such mortality occurred after 55.49 hours in *A. pomi* adults and nymphs (LT_{50}), after 62.3 hours in *T. vaporariorum* (LT_{50}). These data indicate that sucking pests have shown susceptibility to infection caused by *B. bassiana* isolate Col-2. LC_{50} and LT_{50} values and 95% confidence limits (CL) expressed as conidia ($\times 10^4$) (Proc Probit (SPSS). LT_{50} values for mortality were estimated by analysis (Proc Probit (SPSS) data for >72 hours for *A. pomi* and *T. vaporariorum*, corresponding to the last day of incubation in bioassay > 5 days.

3.7. Molecular Characterization of Fungal Isolates; Sequencing of 18S rDNA, ITS1-5.8S-ITS2, and EF1-α and EF1-α Genomic Loci

Selected fungal isolates were subjected to DNA sequencing for identification. The ITS1 and ITS4 DNA fragments of select fungal isolates were amplified by PCR using the primer pairs ITS1(CTTGGTCATTTAGAGGAAGTAA) and ITS4 (CAGGAGACTTGTACACGGTCCAG) and genomic DNA as the template. Based on their morphological characteristics, including the production of ellipsoidal conidia, and molecular characteristics (ITS, partial 18S [SSU rDNA] and EF1-α sequences), the isolates were identified as *B. bassiana* belonging to Clade E from Asia (**Figure 14**).

4. Discussion

Studies emphasize that biological agents are highly effective, with their main

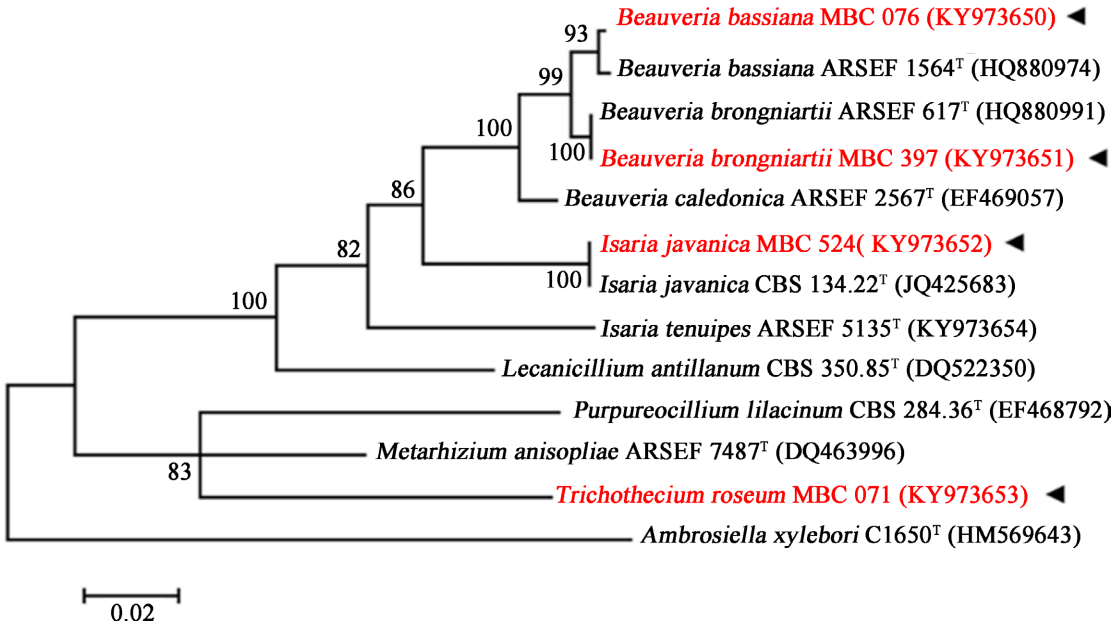

Figure 14. Phylogenetic position of the isolate Col-2 strains (KY973650) within *Beauveria* genus based on ITS1-5.8S-ITS2 sequence, among other closely related *Beauveria bassiana* species from the database.

advantages being lower negative impacts on human health and the environment. They have low toxicity; biocontrol also has very short or no pre-harvest intervals. The biological agents as natural products degrade quickly without disturbing biodiversity or ecosystems. Compared to chemicals, the critical advantage of biocontrol agents is that they slow down pest resistance. Some biocontrol agents can actively seek out pests. A significant advantage of biocontrol is its compatibility with environmental and health regulations; they also align with integrated pest management (IPM) and organic certification schemes. Moreover, they can support biodynamic agriculture [48]-[51].

The endophytic and insecticidal activities of entomopathogenic fungi allow them to be used as agents in long-term preventive measures against pests and diseases. In recent years, many studies have identified *B. bassiana* strains with several beneficial properties [9] [16] [21] [22] [29]. Such isolates can potentially be incorporated into an industrial formula with insecticidal and growth-promoting effects. Combining several entities with valuable properties into one biological product is a rational, economically sound and ecofriendly way to protect plants from pests and diseases. In this study, we examined the potential of endophytic and entomopathogenic fungi as biocontrol agents and plant growth promoters, as well as the impact of various artificial inoculation methods on their endophytic colonization of host plants. More specifically, we evaluated the endophytic and insecticidal activity of newly isolated, naturally occurring *B. bassiana* strains.

The first step was to develop a low-cost growth medium that can support the generation of high conidial titres and be used in adverse field conditions. Several low-cost nutrient formulations were tested, and the findings indicate that media

composed of wheat and barley groats support the generation of biomass with sufficiently high conidial titres for the production of an industrial formula. Other studies have also reported abundant growth of *B. bassiana* colonies in food industry waste [42]-[54].

Next, the endophytic activity of the newly isolated *B. bassiana* strains was assessed using three important vegetable crops: beans, tomatoes and cucumbers. The strains were delivered to the seeds and plants using three different inoculation methods to optimize the delivery of the fungi and their effects and to identify the factors that limit the colonization of host plants by *B. bassiana*. The results show that the most suitable method for the delivery of *B. bassiana* varies among plant species; therefore, it is necessary to determine the most appropriate method for each individual species, such as leaf spraying, soil impregnation or seed inoculation.

The colonization of the plant organs by the *B. bassiana* strains across the plant species and depended on the inoculation method. However, considerable colonization of all tested organs (*i.e.*, roots, stem and leaves) was observed in the bean plants regardless of the type of inoculation. Successful colonization by entomopathogenic fungi delivered via different methods has been observed in several plant types [20] [55]-[58]. María *et al.* [59] found that colonisation by *B. bassiana* delivered via leaf spraying peaked in soybean seedlings at 24% after seven days. They also reported that root immersion resulted in peak colonisation 14 days post-inoculation (10.5%) and that seed inoculation resulted in peak colonisation at seven days (14%).

In another study, *B. bassiana* strains were more successful colonizers than *Metharisium* strains, showing 100%, 90% and 45% recovery from leaves, stem and roots, respectively, seven days after inoculation [60]. In the same study, the ability of the entomopathogenic fungi to move through plant tissues was demonstrated, entering through the roots, stems and leaves. Leaf aspersion has been reported as the most efficient technique for inoculating different fungal strains into soybean plants [24]. However, others have mostly reisolated entomopathogenic fungi from roots and in lower proportions from leaves [61] [62]. In our study, high colonisation levels were observed when tomato plants were inoculated with fungal strains via soil drenching. Foliar spraying resulted in low levels of colonisation. Other studies have shown that selected *Bav. bassiana* strains can colonise tomato plants as endophytes and control two critical disease agents (*Botrytis cinerea* and *Alternaria alternata*) and the pest aphid *Macrosiphum euphorbiae* [63]. Similarly, soil drenching led to the highest levels of colonisation in cucumber plants. The observed colonisation of different plant organs indicated that these fungi may move systemically throughout the plant [23] [58] [64]. Early studies in this area reported that *B. bassiana* can colonise the spaces between parenchymal cells and can move within the plant and colonise untreated tissues [65] [66].

The apple tree aphid (*A. pomi*) and whitefly (*T. vaporariorum*) are significant agricultural pests responsible for yield losses across several crops, including fruits and vegetables. In this study, we examined the potential of the same three local

entomopathogenic *B. bassiana* isolates tested for endophytic activity for the management of these pests in Kyrgyzstan. For this part of the study, *B. bassiana* conidial suspensions were prepared and sprayed onto the pests. Other studies have also reported that *B. bassiana* can persist inside different cucumber tissues for up to 90 days after inoculation [54]. Soil drenching led to the highest recovery rates, while foliar spraying led to the lowest recovery rates [54]. Others have also achieved high mortality rates by spraying *B. bassiana* onto pest species, namely *Lipaphis erysimi* (Kalt.) (Hemiptera: Aphididae) [67]. Many studies have confirmed that *B. bassiana* is an aphid pathogen [22] [68] [69]. Asi *et al.* [70] reported high mortality rates in the cabbage aphid (*Brevicoryne brassicae* L.) as a result of increasing the concentration of *B. bassiana* conidia and exposure time. It has also been found that foliar spraying and foliar spraying with root impregnation reduces the fecundity of pests such as *Eurygaster integriceps* [71] and *Aphis gossypii* Glover [22]. Furthermore, other studies have shown that endo endophytic entomopathogens can increase plant viability exposure to abiotic and biotic stress [31] [49] [70] [72] [73] [74].

5. Conclusion

Our findings show that *B. bassiana* can promote plant growth, support the growth of taller plants, improve seed germination and kill pests. Hence, this study confirms the potential of the tested *B. bassiana* strains as biological pest control agents and bioinoculants that increase the growth and development of plant seedlings. These findings can be used to develop bioagents, which will reduce the indiscriminate use of chemical insecticides on crops and thereby protect the environment and its vital natural components from excessive pesticide pollution. Therefore, we recommend incorporating a formulation of the tested *B. bassiana* isolates into integrated pest management (IPM) programmes to improve the growth of vegetable crops and control economically important sucking pests, such as aphids and whiteflies, in greenhouse and open field conditions in Kyrgyzstan.

Authors' Contributions

Conceptualization and design were done by Tinatin Doolotkeldieva. Sample preparation, data collection, and analysis were performed by Elita Ismailova.

The first draft of the manuscript was written by Elita Ismailova and revised by Tinatin Doolotkeldieva. All authors read and approved the final manuscript.

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Ethical Approvals

This experiment does not involve the use of animal or human subjects.

Data Availability

The authors confirmed that, the data supporting the finding of the present study are available within the article.

Use of Artificial Intelligence (AI)-Assisted Technology

The authors confirm that there was no use of artificial intelligence (AI)-assisted technology for assisting in the writing or editing of the manuscript and no images were manipulated using AI.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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