MYCORRHIZAL POTENTIAL OF *BEAUVERIA BASSIANA* ON THE GROWTH AND DEVELOPMENT OF LETTUCE (*LACTUCA SATIVA*)

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Abstract. The microfungus *Beauveria bassiana* is known primarily as an insecticidal agent, but recent studies suggest that it can form a mycorrhiza-like interaction with its plant host. While such interactions have been confirmed in several crop species, research on lettuce remains limited. The aim of this study was to evaluate the potential mycorrhiza-like interaction of an autochthonous *B. bassiana* isolate on the growth and development of lettuce (*Lactuca sativa*) under greenhouse conditions. Repeated applications of a *B. bassiana* conidia suspension were performed at the 2–3 leaf phenological stage (BBCH 12–13) by watering the plants in the root zone over a five-week period. To quantify the potential of *B. bassiana* to promote lettuce growth through such an interaction, the biomass, rosette area, and root area of each plant were measured. The results show a significant increase in root area (by 31.6% and 30.5%) in plants treated with the conidia suspension compared to the control, when treatments were applied weekly or biweekly. Additionally, significantly higher plant biomass (by 16.7% and 20%) was recorded in all treatment variants relative to the control. These findings indicate that *B. bassiana* is capable of establishing a mycorrhiza-like relationship with lettuce. However, further research under in vivo conditions is needed to better elucidate the mechanisms underlying this interaction.
Keywords: *Beauveria bassiana, mycorrhiza, endophyte, in vivo, spore suspension, lettuce*

Introduction

A new paradigm in agriculture focuses on beneficial soil microorganisms because of their positive effects on plants. Fungi that have the potential to establish mycorrhizal or "mycorrhizal-like" interactions with the roots of host plants are receiving increasing attention worldwide. By forming this type of mutualistic symbiosis, fungi facilitate nutrient uptake by the host plant through an extensive network of hyphae that extend from the colonized root into the surrounding soil and function as a supplementary absorption system (Avio et al., 2018). This symbiotic relationship provides numerous benefits to plants, including growth promotion and increased resistance to biotic and abiotic stresses, with consequent modulation of antioxidant enzyme activity and the biosynthesis of secondary metabolites (phytochemicals) such as polyphenols, anthocyanins, phytoestrogens and carotenoids, which are important for human health as well (Rillig et al., 2016; Avio et al., 2018).

Current studies in the field of phytomedicine (Dara et al., 2016; Kramski et al., 2023) show that entomopathogenic fungi can also develop a "mycorrhizal-like interaction" with plants, leading to increased plant resistance to diseases and pest attacks. It is known that entomopathogenic fungi of the genus *Beauveria* (Vuillemin) play an important role in controlling insect populations and are increasingly used for their biological control. However, members of this genus also have the ability to colonize a wide range of plant hosts as endophytes without causing disease, while simultaneously being capable of infecting insects (McKinnon et al., 2018). The species *Beauveria*

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bassiana Bals.-Vuill. (Ascomycota: Hypocreales) is a cosmopolitan fungus transmitted through the soil, where it comes into contact with plants and establishes colonization of the plant host. Several studies (Dara, 2015, 2016; Dara et al., 2019; Kramski et al., 2023) suggest that this microfungus has the ability to establish a "mycorrhizal-like interaction" and thereby promote the growth and development of colonized plants. The potential of *B. bassiana* to promote plant growth has been confirmed in strawberry (Dara, 2013, 2016), bean (Dash et al., 2018), tomato (Nishi et al., 2021), cabbage (Heviefo et al., 2017), cucumber (Rajab et al., 2020), barley, wheat and corn (Zitlalpopoca-Hernandez et al., 2017), and grapevine (Moloinyane and Nchu, 2019), but studies on this activity of *B. bassiana* in lettuce are scarce (Macuphe et al., 2021).

Lettuce (*Lactuca sativa* L.) is a leafy vegetable from the *Asteraceae* family with high nutritional value for human health. The leaves of this annual plant are a rich source of fiber and bioactive compounds. They are mainly consumed fresh, although in some regions of the world the stems are also prepared as part of cooked dishes (Ríos-Castillo et al., 2023; Siswanto et al., 2024). Lettuce is one of the most widely cultivated crops globally, and many varieties are suitable for protected winter cultivation due to their ability to develop rosettes of acceptable quality and mass under low-light and low-temperature conditions (Siswanto et al., 2024). The use of microfungi that promote the growth and development of lettuce reduces the need for chemical fertilizers and consequently decreases the negative impact on the environment and human health (Macuphe et al., 2021).

In recent years, several studies have further highlighted the multifunctional role of *B. bassiana* as an endophyte and plant biostimulant in various crops. Proteomic and physiological analyses in tomato demonstrated that endophytic colonization by *B. bassiana* modulates growth- and defense-related pathways, enhances biomass accumulation and increases resistance to *Botrytis cinerea* (Proietti et al., 2023; Russo et al., 2023). Endophytic strains have also been reported to promote drought tolerance and early flowering in maize (Kuzhuppillymyal-Prabhakarankutty et al., 2020), as well as to enhance drought tolerance in tomato through a "water spender" physiological strategy associated with improved water relations, antioxidant activity and up-regulation of stress-related genes (Guo et al., 2024). Moreover, in rice and oilseed rape, *B. bassiana* has been shown to colonize internal tissues, suppress sheath blight disease and induce the biosynthesis of flavonoids and other secondary metabolites while simultaneously promoting plant growth (Deb et al., 2022; Muola et al., 2024). These findings are consistent with recent reviews emphasizing the potential of endophytic entomopathogenic fungi as promising tools for simultaneous plant growth promotion and biocontrol in sustainable agriculture (Ahsan et al., 2024).

Based on this, the aim of the present study was to test the mycorrhizal potential of the *B. bassiana* isolate on the growth and development of lettuce under greenhouse conditions. The tested hypothesis was that *B. bassiana* would significantly enhance the growth and development of lettuce in a greenhouse environment.

Materials and methods

In vitro experiment

Origin and cultivation of Beauveria bassiana

For the purpose of conducting the research, the *B. bassiana* isolate used in this study was obtained from a dead adult of the potato beetle (*Leptinotarsa decemlineata* Say).

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The isolate was molecularly identified to the species level using a conventional PCR method (according to Gebremariam et al., 2021), with sequencing performed at Macrogen Europe (Amsterdam, The Netherlands).

The microfungus was grown on potato dextrose agar (PDA, Sigma Aldrich) and incubated in a climate chamber at 24°C in the dark. These growth conditions were selected because PDA and incubation at approximately 24°C in darkness represent standard and optimal laboratory conditions for culturing *B. bassiana*, supporting consistent mycelial growth and conidia production (Dash et al., 2018; Gebremariam et al., 2021; Nishi et al., 2021).

The isolate is maintained in the collection of the Department of Phytopathology at the University of Zagreb, Faculty of Agriculture.

Preparation of spore suspension

The isolate of *B. bassiana* was grown on PDA and incubated at 24°C in the dark for 21 days to stimulate sporulation. After 21 days, conidia were collected by adding a solution of sterile distilled water (10 ml) and surfactant (0.1% Tween-80, Sigma Aldrich) to remove the water surface tension. The submerged conidia were gently collected with a sterile rubber brush. The solution was filtered to remove mycelial fragments, and the conidia suspension was collected in a sterile bottle. The spore concentration in the liquid inoculum was determined by serial dilution and counting of spores in the suspensions using a Neubauer hemocytometer under a light microscope to achieve a spore concentration of 1×10^7 spores/ml. The suspension (9.6×10^7 spores/ml) was stored at 4°C until use.

In order to check the viability and germination of spores, 5 µl of spore suspension was applied to a sterile microscope slide with PDA, and germination was determined after 24 h of incubation in a climate chamber. Spores were considered to have germinated if the germ tube of the conidia was longer than its diameter. Based on this, a suspension with conidia that germinated more than 90% was used in the experiment (Zemek et al., 2021).

Lettuce sowing

In order to carry out the sowing of lettuce plants (*Lactuca sativa* L.) under greenhouse conditions, seeds of the cultivar Ostralie RZ (Kadmo d.o.o., Zagreb, Croatia) were used. The seeds were sown in November 2024 according to the manufacturer’s recommendations. The cultivar is intended for protected cultivation, with a recommended planting spacing of 35 × 35 cm, and the producer indicates that sowing for greenhouse production may be carried out during September, October, November, and December, with an additional optimal sowing window in January and February.

During the experiment, plants were grown under natural daylight conditions, with a day/night regime typical for the season. The average afternoon temperature in the greenhouse ranged between 15°C and 18°C, while morning temperatures were approximately 10°C.

Seeds were sown into Potgrond H substrate (Metković, Croatia) placed in containers inside the greenhouse. A total of 32 plants were grown and used in the study once they developed 2–3 true leaves (BBCH 12–13). Seedlings were watered as needed.

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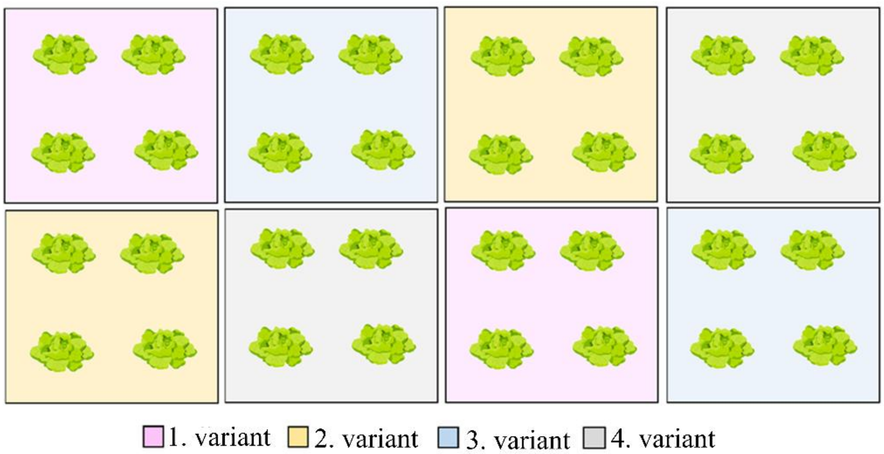
Greenhouse experiment

The study was conducted in a greenhouse at OPG Šutalo (Metković, Croatia) according to the modified method of Staffa et al. (2020). Lettuce seedlings were initially grown in containers until they reached the 2–3 true leaf stage (BBCH 12–13). After this stage, a total of 32 seedlings were transplanted directly into soil, where the experimental treatments were applied.

Multiple applications of the conidia suspension (50 ml per plant) were carried out by watering the root zone of lettuce plants over a period of five weeks (*Fig. 1*). The first application of the conidia suspension (9.6×10^7 spores/ml) was performed immediately after the seedlings reached BBCH 12–13.

The experiment was set up in a randomized block design with four treatment variants, each comprising eight replicates. Each individual plant was considered one replicate, as plants were grown separately in soil following transplantation. Treatment variants included plants receiving applications of *B. bassiana* conidia suspension, while the control group consisted of plants watered with sterile distilled water containing 0.1% Tween-80.



		Applications by week				
		1.	**2.**	**3.**	**4.**	**5.**
Test (plant + *B. bassiana*)	var. 1.	+	-	-	-	-
	var. 2.	+	-	+	-	+
	var. 3.	+	+	+	+	+
Control (plant + dH_2O)	var. 4.	-	-	-	-	-

Figure 1. *Schematic representation of the in vivo experiment: multiple applications of the suspension (+) over 5 weeks*

Data analysis

Lettuce harvest was carried out 10 weeks after the experiment was established. Whole plants (including roots and rosettes) were collected, and the effect of the potential mycorrhiza was assessed by measuring the rosette and root area and by weighing the plant biomass. The rosette and root areas were measured by placing the rosettes and roots

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individually on a white background and photographing them (with the camera lens positioned perpendicular to the plant at a height of 70 cm). The photographs were then processed using the ImageJ software according to Martinko et al. (2022).

Statistical analysis

The results of the *B. bassiana* promotion effect are presented as means with standard deviations (SD). The obtained data were statistically analyzed using a one-way ANOVA followed by Tukey's test ($p < 0.05$) in the SPSS software (IBM SPSS Statistics, version 15.0, Chicago, IL, USA).

Re-isolation of Beauveria bassiana from lettuce roots

Re-isolation of *B. bassiana* from the roots of the tested plants was carried out in the 10th week of the experiment, i.e., five weeks after the last application, according to the modified method of Staffa et al. (2020). The collected roots (n = 32) were gently rinsed with tap water and then placed on sterile paper in a laminar flow hood. After being cut into smaller fragments, the roots were surface-sterilized with 70% ethanol for one minute, rinsed twice with sterile distilled water, and subsequently placed (according to the treatment variants) onto the surface of PDA in Petri dishes.

The inoculated plant material was incubated in a climate chamber at 25°C in the dark. The presence or absence of *B. bassiana* growth was recorded after seven days. The presence of *B. bassiana* in at least one portion of the root was considered evidence of plant colonization. Colonization data were expressed as percentage colonization ([number of colonized plants/number of replications] × 100). Microscopic analysis was performed to morphologically confirm the presence of *Beauveria* species.

Results

Effect of application on lettuce root growth

In all test variants, a numerical increase in the root area of lettuce was recorded compared to plants from the control variant. Application of the conidia suspension once a week (variant 3) and every other week (variant 2) resulted in increases of 30.5% and 31.6%, respectively, while a single application (variant 1) led to an increase of 23.7% compared to the control plants (*Table 1; Fig. 2*). However, although variants 2 and 3 showed numerically higher root area values, these differences were not statistically significant when compared with variant 1 or with the control group, according to Tukey's test ($p > 0.05$).

Table 1. *Effect of repeated application of Beauveria bassiana conidia suspension on lettuce root growth after 10 weeks*

Variants	1.	2.	3.	4.
Number of applications/5 weeks	1/5 *B.b.* + plant	3/5 *B.b.* + plant	5/5 *B. b.* + plant	dH_2O + plant
Root area $\bar{x}$ (cm^2) ± SD	30.4 ± 6.6 ab	33.9 ± 5.5 b	33.4 ± 4.6 b	23.2 ± 4.7 a
Root area (%)	**23.7**	**31.6**	**30.5**	**0**

Different letters indicate a statistically significant difference between the mean values ($\bar{x}$) of the variants in the experiment (Tukey test, $p < 0.05$), SD – standard deviation

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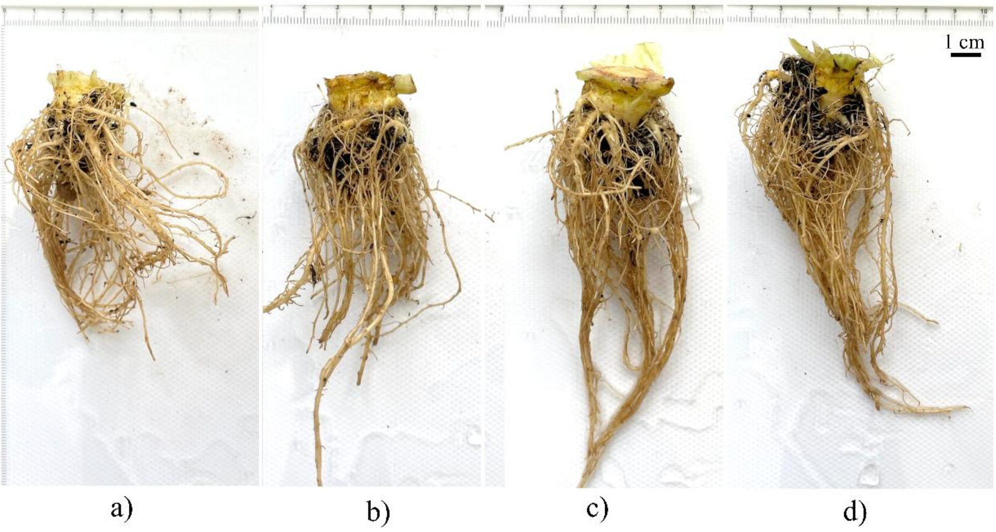

Figure 2. *Effect of repeated applications of Beauveria bassiana conidia suspension on lettuce root growth after 10 weeks: (a) control, (b) one application, (c) three applications, (d) five applications*

Effect of application on lettuce rosette growth

In all test variants, a larger area of lettuce rosette was recorded compared to the control group. Application of the conidia suspension carried out only once within five weeks (1st variant) led to an increase in the rosette area of treated lettuce plants by 5.5%, while applications carried out every other week (2nd variant; 3/5) and once a week (3rd variant; 5/5) resulted in increases of 12.6% and 14.7%, respectively, compared to the control (*Table 2; Fig. 3*). The mean values of lettuce rosette area in all test variants (1st, 2nd and 3rd) were not significantly higher compared to the mean values of control plants (Tukey test, $p > 0.05$).

Table 2. *Effect of repeated application of Beauveria bassiana conidia suspension on lettuce rosette growth after 10 weeks*

Variants	1.	2.	3.	4.
Number of applications/5 weeks	1/5 *B.b.* + plant	3/5 *B.b.* + plant	5/5 *B. b.* + plant	dH_2O + plant
Rosette area $\bar{x}$ (cm^2) ± SD	798.8 ± 92.3 a	863.2 ± 64.5 a	884.4 ± 94.7 a	754.8 ± 90.3 a
Rosette area (%)	**5.5**	**12.6**	**14.7**	**0**

Different letters indicate a statistically significant difference between the mean values ($\bar{x}$) of the variants in the experiment (Tukey test, $p < 0.05$), SD – standard deviation

Effect of application on lettuce biomass

In all test variants, a higher biomass of lettuce plants was recorded compared to the control group. Application of the conidia suspension once a week (variant 3) and every other week (variant 2) within a five-week period resulted in a 20% increase in plant biomass, while a single application (variant 1) led to an increase of 16.7% compared to the control plants (*Table 3*).

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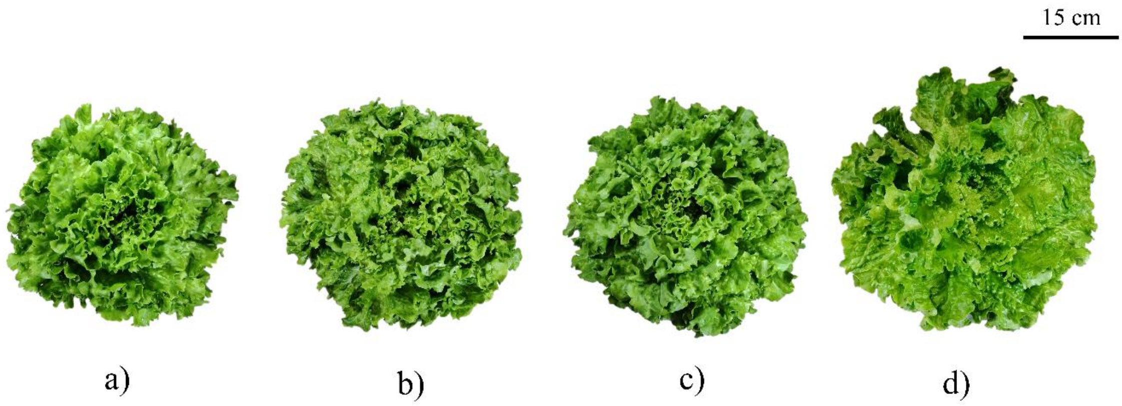

Figure 3. *Effect of multiple applications of Beauveria bassiana conidia suspension on lettuce rosette growth after 10 weeks: (a) control, (b) one application, (c) three applications, (d) five applications*

Table 3. *Effect of repeated application of Beauveria bassiana conidia suspension on lettuce weight after 10 weeks*

Variants	**1.**	**2.**	**3.**	**4.**
Number of applications/5 weeks	1/5 *B.b.* + plant	3/5 *B.b.* + plant	5/5 *B. b.* + plant	dH_2O + plant
biomass $\bar{x}$ (kg) ± SD	0.31 ± 0.33 b	0.31 ± 0.31 b	0.31 ± 0.43 b	0.25 ± 0.53a
biomass (%)	**16.7**	**20**	**20**	**0**

Different letters indicate a statistically significant difference between the mean values ($\bar{x}$) of the variants in the experiment (Tukey test, $p < 0.05$), SD – standard deviation

The mean biomass values of all treatment variants (variants 1, 2 and 3) were significantly higher than those of the control group, according to Tukey's test ($p < 0.05$). No significant differences were observed among the three treatment variants, as all were assigned to the same statistical group (letter b).

Re-isolation and microscopic analysis of lettuce root samples

Re-isolation of root samples from treated lettuce plants, performed 10 weeks after the start of the experiment and five weeks after the last application, confirmed the presence of the microfungus *B. bassiana* in root samples from the 2nd and 3rd experimental variants (*Fig. 4; Table 4*).

Table 4. *Re-isolation of the microfungus Beauveria bassiana from lettuce root samples after 10 weeks, depending on application frequency*

Variants	**1.**	**2.**	**3.**	**4.**
Number of applications/5 weeks	1/5 *B.b.* + plant	3/5 *B.b.* + plant	5/5 *B. b.* + plant	dH_2O + plant
Number of samples (root + *B.b.*)/8 plants	0	3	5	0
Number of colonized plants (%)	**0**	**37.5**	**62.5**	**0**

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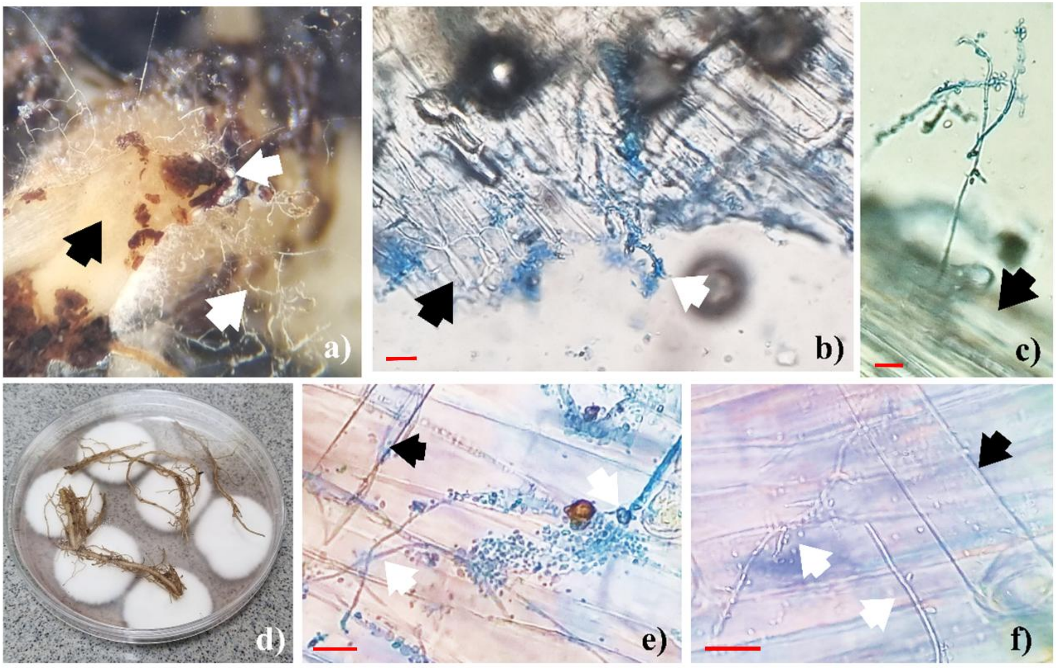

Figure 4. *Colonization and presence of Beauveria bassiana in the roots of treated lettuce plants (black arrows – root; white arrows – Beauveria bassiana): (a) mycelium on lettuce roots (stereomicroscopy, 20–40×); (b, c) conidiophores on root sections (microscopy, 400×); (d) re-isolation after five weeks; (e, f) spores and conidiophores on root tissue and root hairs (microscopy, 400×). Scale bars: 20 µm (b, c, e, f)*

Re-isolation of the microfungus was not confirmed in root samples from the 1st variant. Microscopic analysis, based on morphological identification keys, confirmed that the observed mycelium and associated structures belonged to the genus *Beauveria.*

Discussion

As demonstrated in the results, all treatments involving the application of *B. bassiana* conidia suspension led to an increase in root area, rosette area, and total biomass of lettuce plants compared to the control. The increase in root area was most pronounced in variants with weekly and biweekly applications (by 31.6% and 30.5%, respectively), whereas the single-application treatment resulted in a smaller, non-significant increase relative to the control (*Table 1)*. Regarding rosette development, all treated variants showed a larger rosette area than the control, although the increase was not statistically significant (*Table 2*). Biomass accumulation, however, was significantly higher in all treated variants, with increases ranging from 16.7% to 20% relative to the untreated control (*Table 3*). Re-isolation confirmed the presence of *B. bassiana* in lettuce roots five weeks after the last application in variants receiving multiple treatments, while colonization was not detected in the single-application variant at week 10 (*Table 4*).

These results are consistent with several recent studies demonstrating the growth-promoting effects of endophytic entomopathogenic fungi. For instance, Liu et al. (2022) reported successful colonization of maize by *B. bassiana* and *Metarhizium anisopliae*, resulting in increased shoot height, root length and biomass. Similarly, Tall and Meyling (2018) observed enhanced root length and above-ground biomass in maize

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seedlings derived from *B. bassiana*-treated seeds. Canassa et al. (2019) noted that seed inoculation of common bean with *B. bassiana* improved plant height, leaf number and root weight, supporting the notion that this microfungus stimulates both below- and above-ground plant development. In contrast, Dara (2016) reported no significant improvement in strawberry growth following *B. bassiana* application. However, since re-isolation data were not provided, successful colonization cannot be confirmed, which may explain the absence of a growth-promoting effect. In cucumber, *B. bassiana* was found to colonize plant tissues for up to 90 days post-inoculation, regardless of the method used (Rajab et al., 2020). The results of Heviefo et al. (2017) further support the ability of this fungus to colonize cabbage, although colonization varied across plant parts and did not enhance growth parameters. More recently, several new studies have provided additional evidence supporting the growth-promoting activity of *B. bassiana*. Bamisile et al. (2023) demonstrated that endophytic *B. bassiana* significantly increased biomass, nutrient uptake, chlorophyll content, and photosynthetic efficiency in tomato, aligning with the increases in biomass observed in the present study. Likewise, Khun et al. (2022) reported that endophytic colonization by *B. bassiana* in pepper enhanced root growth and physiological performance, consistent with our finding of improved root system development. Furthermore, Lopez and Sword (2021) showed that *B. bassiana* enhances plant growth and confers stress tolerance in cotton, reinforcing the multifunctional role of endophytic entomopathogenic fungi as plant biostimulants. Taken together, the variability among published results can be attributed to differences in fungal isolates, plant species, inoculation methods, and environmental conditions, as previously emphasized by Vidal and Jaber (2015).

The results of the present study contribute to the growing body of evidence indicating that *B. bassiana* is capable of establishing a mycorrhiza-like endophytic association with lettuce, resulting in enhanced plant growth, particularly in terms of root development and biomass accumulation.

Conclusion

Based on the results, it can be concluded that repeated application of a conidia suspension of the *B. bassiana* isolate significantly increases root growth and biomass of lettuce under in vivo conditions, while the increase in rosette area is not significant. The *B. bassiana* isolate, applied as a spore suspension to the root zone, successfully colonizes lettuce and remains detectable in the plant for up to five weeks after the last application, as confirmed by microscopic analysis and re-isolation. More precisely, *B. bassiana* was not detected in lettuce roots in the 10th week of the experiment following only a single application.

Taken together, these findings indicate that the entomopathogenic/endophytic microfungus *B. bassiana* exhibited a “mycorrhiza-like interaction” with treated lettuce plants, stimulating their growth and development through a significant increase in biomass and root area under greenhouse conditions. The confirmed biological activity of this multifunctional microfungus suggests promising potential for its increased use in plant production; however, additional research under in vivo conditions is still required.

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Conflict of interests. The authors declare no competing interests.

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