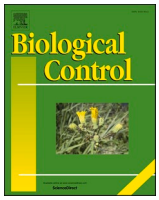
Biological Control 206 (2025) 105789

Contents lists available at ScienceDirect

Biological Control

journal homepage: www.elsevier.com/locate/ybcon



Versatile role of *Bacillus velezensis*: Biocontrol of *Fusarium poae* and wireworms and barley plant growth promotion

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HIGHLIGHTS

- Biocontrol on *Fusaium poae* and *Agriotes lineatus* has not been fully studied yet.
- This is the first report of *B. velezensis* for barley protection and plant growth.
- *Bacillus velezensis* BHC 5.6 had antifungal and insecticidal effect to these pests.
- *B. velezensis* BHC 5.6 had PGP traits and increased barley yield in pot experiment.
- This new strain could contribute to food safety in sustainable agriculture.

ARTICLE INFO

Keywords:
Bacillus velezensis
Antifungal activity
Pest biocontrol
Agriotes lineatus larvae
Insecticidal toxins
Sustainable agriculture

ABSTRACT

The major losses in cereal production are most commonly caused by phytopathogenic fungi and insect larvae, indicating the need for sustainable pest control management. Although bacterial bioinoculants offer an eco-friendly alternative for plant protection and growth promotion (PGP), their effects on *Fusaium poae* and *Agriotes lineatus* larvae have not been comprehensively studied yet. To find an effective biological control agent against these pests, *Bacillus* strains were isolated from soil and tested for PGP and biocontrol traits, including the presence of antibiotic and toxin-coding genes. Out of eleven strains, *B. velezensis* BHC 5.6 showed a wide range of PGP and biocontrol abilities, while the presence of *fenD, bmyB, srfAA, spaS* genes was also detected. Only two strains, *P. megaterium* BHC 5.5 and B. *velezensis* BHC 5.6, showed antifungal effect against *F. poae* with inhibition percentage of 62% and 67%, respectively. The highest insecticidal effect against wireworms was recorded for *B. velezensis* BHC 5.6 (56.67%) and *B. safensis* BHC 11.4 (43.33%). The PGP activity of *B. velezensis* BHC 5.6 was also confirmed in a pot experiment, where an increment of barley yield was recorded both for infected (17.09%) and uninfected barley seeds (10.12%). This is the first time demonstrating that the *B. velezensis* BHC 5.6 could be used for integrated pest management of *F. poae* and *A. lineatus* larvae in barley and for plant growth promoting. Therefore, the implementation of this strain could contribute to the food safety in sustainable agricultural practices.

1. Introduction

Cereals have immense economic and nutritional importance worldwide, as the most of the world's population depends on cereal-based foods. Apart from the challenges of high food demand, modern agriculture is commonly affected by the climate change. Climate change-induced weather disruptions can increase crop vulnerability to biotic stress, posing a significant threat to sustainable crop production. These weather conditions modify the spatial and temporal distribution, abundance, lifecycle, and population dynamics of pests and pathogens. In this way crop yield and quality become severely compromised (Subedi et al., 2023; Lahlali et al., 2024). *Fusarium poae* and *Agriotes lineatus* are increasingly recognized as major implications for food security, particularly under the pressures of climate change.

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https://doi.org/10.1016/j.biocontrol.2025.105789
Received 25 February 2025; Received in revised form 9 April 2025; Accepted 6 May 2025
Available online 13 May 2025

Fusarium head blight (FHB) is one of the major diseases affecting the most regions worldwide and causing devastating economic damage of small-grain cereals (Dinolfo et al., 2012). This disease causes both quantitative and qualitative losses including a reduction in yield, kernel weight and germination index of seeds (Martínez et al., 2019). The main causative agents of FHB are fungi belonging to *Fusarium* genus (such as: *F. graminearum, F. poae, F. culmorum,* and *F. avenaceum*). Although *F. poae* is often considered a minor FHB-inducing pathogen, its ability to produce a diverse range of mycotoxins, highlights its potential risk to food safety and crop health. Thus, among FHB-causing species, *F. poae* is the least studied one. In addition to plant diseases caused by plant-pathogenic fungi, the production of small-grain cereals is often affected by soil-borne pests, primarily wireworms. Wireworms *Agriotes lineatus* L. (Coleoptera: Elateridae), are recognized as widely distributed soil pests that affect a variety of economically significant crops globally (including cereals) (Barsics et al., 2013; Furlan et al., 2021; Kozina et al., 2013). The main mode of action of wireworms is regarded to their feeding on the germinating cereal seeds, roots and belowground plant organs. In this manner, wireworms compromise plants directly or by creating wounds that are ideal for pathogenic fungi and other disease-causing organisms (Traugott et al., 2013). Wireworms can be found in the almost all soil types, but their current presence in the soil could be influenced by various environmental factors (Kozina et al., 2013). However, due to the changing lifecycle, soil presence and seasonal movements of wireworms, as well as due to the low efficiency of commercially available insecticides, this group stands out as one of the most demanding to control (Nikoukar and Rashed, 2022). Despite the urgency of addressing these pests, research on integrated biocontrol strategies that simultaneously target both *F. poae* and *A. lineatus* remains extremely limited. Most studies focus on single-target biocontrol approaches, leaving a critical gap in the development of biological solutions for pest and disease management (Nikoukar and Rashed, 2022; Zanon et al., 2024). Additionally, there are many studies regarding the use of *Bacillus* strains in biocontrol of FHB, but there is a very limited number of researches about *F. poae*, as the emerging FHB agent (Stenglein, 2009; Schöneberg et al., 2018). Therefore, this study aimed to assess the dual antagonistic effect of novel *Bacillus* strains against *Fusarium poae* and *Agriotes lineatus* larvae. It examined the identification of new bacterial and fungal isolates, the presence of antibiotic-related (*fenD, bmyB, ituC, srfAA, bacA, spaS*) and toxin-producing *(cry11, cry1B*) genes, as well as biocontrol and plant growth-promoting traits of bacterial strains. Additionally, the ability of *B. velezensis* to enhance barley growth was tested under semi-controlled conditions.

2. Materials and methods

2.1. Soil sampling and analysis

2.1.1. Soil sampling

The soil was sampled from three fields in Serbia located in Futog (sample 5) and Čenta (samples 10 and 11). The fields were selected due to the intensive agricultural production, primarily of various types of cereals, where an increased number of wireworms has been observed in recent years. Soil samples were taken from the rhizosphere of wheat (sample 5), soybean (sample 10) and corn (sample 11). The soil samples for further bacterial isolation were placed in plastic sterile bags and transferred to the laboratory in a portable refrigerator (4 °C). For physico-chemical analysis, the soil was sampled and air-dried in the laboratory, crushed and passed through a sieve ($\leq$ 2 mm) prior to the analyses in accordance with the SRPS ISO 11464:2004.

2.1.2. Physico-chemical analysis of soil

Soil acidity and the carbonate content in the soils samples were determined based on the standard procedures (SRPS ISO 10390:2007; SRPS ISO 10693:2005, respectively). Plant – available phosphorus and potassium were analyzed by Al-method: K_2O by flame emission photometry and P_2O_5 by spectrophotometer (Egnér, et al., 1960). The soil organic matter (SOM) content was determined based on the Kotzmann method (JDPZ, 1966). Mechanical soil analysis was performed by combination of sieving and the pipette method – modified International B method (Hadžić et al., 1997). Soil texture classes were determined according to International Union of Soil Science textural triangle (IUSS) (Moeys, 2024).

2.2. Isolation and identification of bacteria

2.2.1. Isolation of bacteria

For the isolation of bacteria, 10 g of each fresh soil sample was dissolved in 100 mL of sterilized distilled water and suspended on a rotary shaker (15 min, 150 rpm) to obtain a soil extract. The dilutions of soil extracts (10^{-6}) were spread onto plates containing nutrient agar (NA) and incubated (28 °C, 48 h). *Bacillus*-like colonies were re-streaked several times on NA in order to obtain single colonies. The obtained pure cultures of bacteria were transferred on the inclined NA and stored in refrigerator at 4 °C until the moment of use.

2.2.2. Molecular identification of bacterial isolates

Isolation of the total bacterial DNA for all molecular analyses was performed according to the CTAB protocol (Dimkić et al., 2013). Identification of bacterial isolates was done based on the 16S RNA gene sequences obtained after amplification of DNA with primers P0 (5′-GAGAGTTTGATCCTGGCTCAG-3′) and P6 (5′-CTACGGCTACCTTGTTACGA-3′) by using the following PCR temperature profile: initial denaturation on 95 °C for 90 s, followed by 35 cycles consisted of 95 °C for 30 s, 30 s at 60 °C for the first five cycles, 55 °C for the next five cycles, 50 °C for the last 25 cycles and 72 °C for 4 min, and finally incubated at 72 °C for 10 min and 60 °C for 10 min. (Oro et al., 2020). For the identification of bacterial isolates, the Neighbour-Joining tree was constructed based on the sequences of 15 comparative bacterial species from the NCBI. The tree was rooted by *Methanobacterium espanolae*. In order to achieve better resolution of species identification, additional identification of isolates was performed based on the sequences of *tuf* gene (elongation factor Tu) using tufGPF (5′-ACGTTGACTGCCCAGGACAC-3′) and tufGPR (5′-ATACCAGTTACGTCAGT TGTACGGA-3′) primers and the following PCR protocol: initial denaturation step at 95 °C for 8 min, followed with 35 cycles (30 s of denaturation at 95 °C, annealing at 55 °C for 1 min and 30 s of extension at 72 °C) and final extension at 72 °C for 10 min (Draganić et al., 2017). Both PCR reaction mixtures consisted of 25 μL of PCR TaqNova-RED Master Mix (2X, Blirt), 0.5 μL of forward and reverse primers (10 μM), 1 μL of sample DNA, and PCR-grade water to a total volume of 50 μL.

The obtained PCR products were purified and sequenced by the commercial services of Macrogen Europe (Amsterdam, the Netherlands). Gene sequences of 16S rRNA and *tuf* were deposited to the National Center for Biotechnology Information database (NCBI) to obtain accession numbers (Table S1) and the identification of bacterial isolates was done based on the comparison to the NCBI base.

2.3. Isolation and identification of a plant pathogenic fungus

Plant pathogenic fungus was isolated from the commercially obtained barley seeds after seed germination in Petri dishes with Jensen agar. Most of the germinated seeds were unable to continue seedling growth due to the development of a fungal infection. The fungus which appeared at the seed germination stage (coded as BHC.FP) was subsequently transferred to potato dextrose agar (PDA) medium and maintained on slanted PDA at 4 °C in a refrigerator until further use. For fungal identification, molecular analysis was conducted based on the amplification of Internal Transcribed Spacer region. ITS1 (5′TCCGTAGGTGAACCTGCGG 3′) and ITS4 (5′TCCTCCGCTTATTG ATATGC 3′) DNA primers were used, as well the following PCR temperature profile: initial denaturation at 95 °C for 5 min, followed by 30

cycles of denaturation at 94 °C for 40 s, annealing at 58 °C for 40 s and extension at 72 °C for 40 s, with a final extension at 72 °C for 5 min (Zarrin et al., 2016). The PCR products were purified and sequenced by the commercial services of Macrogen Europe (Amsterdam, the Netherlands). The obtained sequence was deposited in the NCBI database in order to obtain accession number. For the identification of fungus to the species level the Neighbour-Joining tree was constructed based on the sequences of nine comparative *Fusarium* species from the NCBI. The tree was rooted by *Monilinia laxa*.

2.4. Collecting the larvae of pests

During spring of 2024, wireworm larvae were collected using a combination of soil sampling around plant roots and traps baited with germinating cereal seed, as described by Morales-Rodriguez et al. (2017). Collected material was examined in the laboratory, where wireworm larvae were isolated and reared under laboratory conditions until they were used in biological tests.

2.5. Biocontrol traits of bacterial strains

The bacterial traits which are significant for their biocontrol potential, such as the production of hydrogen cyanide (HCN), hydrolytic enzymes, as well as the detection of antibiotic and toxin − coding genes were accessed.

2.5.1. Hydrogen cyanide and hydrolytic enzymes production

Hydrogen cyanide production was tested using Cyantesmo paper. Plates with NA were streaked with bacterial strains in which Cyantesmo was placed on the inner top of the Petri dish. Plates were incubated (28 °C, 5 days) and the change of Cyantesmo paper colour to dark blue was considered a positive result.

The production of hydrolytic enzymes (amylase, cellulase, pectinase, protease and lipase) was evaluated qualitatively. Bacterial strains were spot-inoculated on the medium supplemented with starch, carboxymethyl cellulose or pectin as described in Mihajlovski et al. (2015), on Skim milk agar for protease and on NA amended with Tween 80 for lipase (Figueira et al., 2019). Results were obtained after 5 days of incubation at 28 °C.

2.5.2. Detection of antibiotic- and toxin-producing genes

Bacterial strains were screened for the presence of the following genes associated with the biosynthesis of different lipopeptides which could be significant in biocontrol: *srfAA* (surfactin), *bacA* (bacylisin), *fenD* (fengycin), *bmyB* (bacyllomicin), *spaS* (subtilin) and *ituC* (iturin). Amplifications were done based on the PCR temperature profiles described by Mora et al. (2011). Further, the presence of genes coding for the production of crystal proteins (*cry11* and *cry1B*) which could contribute to the insecticidal activity of strains was evaluated based on the methods described by Jain et al. (2017) and Thammasittirong and Attathom (2008). The obtained PCR products were checked for the presence of bands on the expected position in relation to the 100–10000 bp DNA ladder.

2.6. Plant growth promoting traits of bacterial strains

The traits of bacterial strains which are significant for their plant growth potential, such as the production of siderophores, indole-3-acetic acid (IAA) and the ability of strains to solubilise inorganic phosphates were determined. The production of siderophores was determined by inoculating bacteria on CAS agar by following a method described by Milagres et al. (1999). The ability of bacteria to solubilise inorganic phosphates was evaluated on Pikovskaya medium (PVK) after 7 days of inoculation at 28 °C (Rokhbakhsh-Zamin et al., 2011). The zones around the bacterial growth were measured in mm both for siderophore production and phosphate solubilization ability. The concentration of IAA produced by bacterial strains in nutrient broth enriched with tryptophan (2 mg mL^{-1}) was determined spectrophotometrically based on the IAA standard curve and by using Salkowski reagent (Gordon and Weber, 1951).

2.7. Biocontrol potential of bacterial strains

2.7.1. Insecticidal effect of bacterial strains

To evaluate the insecticidal activity of *Bacillus* strains against wireworm larvae, barley seeds were soaked in a bacterial suspension (50 mL, 10^9 CFU mL^{-1}) for 5 min, while control seeds were soaked in sterile distilled water for the same duration. Subsequently, 100 seeds per treatment were placed in approximately 180 mm-diameter Petri dishes including sterile soil and allowed to germinate for 3 days. Following germination, ten *A. lineatus* larvae were introduced into each Petri dish. All treatments were replicated three times. Larval mortality was monitored daily for 10 days, and mortality data were calculated using Abbott's formula (1925).

2.7.2. Antifungal effect of bacterial strains

The biocontrol potential of bacterial strains against plant-pathogenic *F. poae* was evaluated *in vitro* on PDA medium. A disk of fresh *F. poae* mycelia was placed in centre of Petri dish, 20 μL of bacterial cultures were inoculated near the edge of Petri dish in four replicates and then incubated (28 °C, 7 days). For the fungal control sample no bacterial inoculation was applied. All tests were done in triplicates. After incubation, the diameter of the fungal growth with and without bacterial inoculation was measured (mm) and the inhibition of mycelial growth was determined.

2.8. Evaluation of PGP ability of Bacillus velezensis

2.8.1. Pot experiment under semi-controlled conditions

The pot experiment was performed from May – July 2024 in order to evaluate the plant growth promoting effects of *B. velezensis* BHC 5.6 on barley growth. Seeds of barley were inoculated by submersion in BHC 5.6 culture or *F. poae* suspension under agitation on a rotary shaker (5 min, 160 rpm) and air-dried (for bacterial treatments and infected control treatments, respectively). For BHC 5.6 + BHC.FP treatment, seed were infected by *F. poae* BHC.FP and inoculated by *B. velezensis* BHC 5.6. Non-inoculated seeds were used as a control sample (control). For the pot experiment, the soil sample BHC 5 was used, and the experiment was set-up in the following way: pots were filled with 300 g of gravel and 700 g of soil, inoculated seeds were placed in pots and covered by a thin layer of soil, in triplicates. The experiment was kept in a greenhouse (semi-controlled conditions: average temperature: 24 °C, average humidity: 65 %, exposed to a natural daylight) in a randomised system, watered as needed, and the plant material was harvested after 2 months of planting.

2.8.2. Chemical analysis of plant material

After harvest, the plant material was dried until the constant mass was achieved, and the shoot dry weight was measured. In addition, total content of elements (Ca, K, Mg, P) in the plant material samples was determined following a sample preparation procedure: samples were air-dried, grounded with a grinding mill, and then dried at 105 °C for 2 h (Miller, 1998). After preparation, the samples were digested with concentrated nitric acid (HNO_3) and 30 % hydrogen peroxide (H_2O_2) (Jones and Case, 1990). Elemental analysis was then performed using the THERMO iCAP 6300 Duo Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES).

2.9. Accession numbers

DNA sequence for fungal ITS region has been deposited in NCBI database under the following accession number: PV077297, while the accession numbers for bacterial 16S rRNA and *tuf* gene are provided in

the Table S1.

2.10. Statistical analysis

For plant parameters (yield, Ca, K, Mg, P) and plant growth-promoting (PGP) traits (siderophore production, phosphate solubilization, IAA production), data were analyzed using one-way ANOVA followed by Duncan's multiple range test. For the insectical activity, two-way ANOVA, with Duncan's multiple range test as a post-hoc analysis, was used to determine the significance of differences in the average efficiency of individual treatments between the fourth and tenth day, as well as the significance of differences in the average efficiencies between the fourth and tenth day for each treatment. Data are presented as mean ± standard deviation (SD).

3. Results

3.1. Physico-chemical analysis of soil

The results of the soil analysis are summarized in the Tables 1 and S2. Based on the pH values measured in 1 M KCl (Table 1), soil samples BHC 5 and BHC 10 had an alkaline reaction, while a neutral reaction was recorded in BHC 11. Samples BHC 10 and BHC 11 were classified as low-carbonate soils with medium potassium availability, whereas BHC 5 was categorized as a medium-carbonate soil with high content of plant-available potassium and the lowest organic matter content. The content of plant-available phosphorus varied and phosphorus availability was low (BHC 5), medium (BHC 10), and high (BHC 11). The mechanical composition of the analysed soils demonstrated substantial variability (Table S2). Based on the composition of mechanical elements, the soils BHC 5, BHC 10 and BHC 11 were classified as sandy loam, clay loam, and light clay textural classes, respectively.

3.2. Identification of Bacillus spp. and plant pathogenic fungus

Based on the analysis of the 16S rRNA (Fig. S1) and *tuf* gene (Table 2) DNA sequences, the isolates were identified as follows: *B. altitudinis* BHC 5.1; *P. megaterium* BHC 5.2; *L. capsici* BHC 5.4; *P. megaterium* BHC 5.5; *B. velezensis* BHC 5.6; *P. megaterium* BHC 10.1; *B. pumilus* BHC 10.2; *P. megaterium* BHC 11.1; *Peribacillus frigoritolerans* BHC 11.2; *B. cereus* BHC 11.3 and *B. safensis* BHC 11.4. The sequences of the eleven tested isolates were deposited to the NCBI database under the accession numbers both 16S rRNA and *tuf* (Table S1).

After the initiation of the barely seed germination, the emerged fungus was isolated (Fig. S2). It was observed that the germination of infected seeds was highly impaired.

Based on the analysis of the obtained DNA sequence of the ITS region, the isolated fungus was identified as *F. poae* BHC.FP (Fig. 1). The obtained sequence was deposited in the NCBI under the accession number: PV077297.

Table 1
The basic chemical analysis of the soil samples.

Soil parameters	Soil Samples		
	BHC 5	BHC 10	BHC 11
pH (KCl)	8.11	7.29	6.95
PH (H20)	8.70	7.79	7.70
$CaCO_3$ (%)	7.02	4.68	0.26
SOM (%)	1.20	1.69	3.76
P_2O_5 (mg 100 g^{-1})	13.1	23.8	60.4
K_2O (mg 100 g^{-1})	36.5	20.9	15.5

SOM – soil organic matter.

Table 2
Identification of the tested isolates based on partial sequences of 16S rRNA and *tuf* genes according to NCBI BLASTn analysis.

Bacterial isolate	Identity 16S rRNA	Identity *tuf*	Species
BHC 5.1	99.88 %	100 %	*Bacillus altitudinis*
BHC 5.2	99.74 %	100 %	*Priestia megaterium*
BHC 5.4	99.16 %	100 %	*Lysinibacillus capsici*
BHC 5.5	99.69 %	100 %	*Priestia megaterium*
BHC 5.6	99.88 %	99.85 %	*Bacillus velezensis*
BHC 10.1	99.65 %	99.86 %	*Priestia megaterium*
BHC 10.2	100 %	99.85 %	*Bacillus pumilus*
BHC 11.1	99.77 %	99.86 %	*Priestia megaterium*
BHC 11.2	99.76 %	99.86 %	*Peribacillus frigoritolerans*
BHC 11.3	99.87 %	99.86 %	*Bacillus cereus*
BHC 11.4	99.88 %	100 %	*Bacillus safensis*

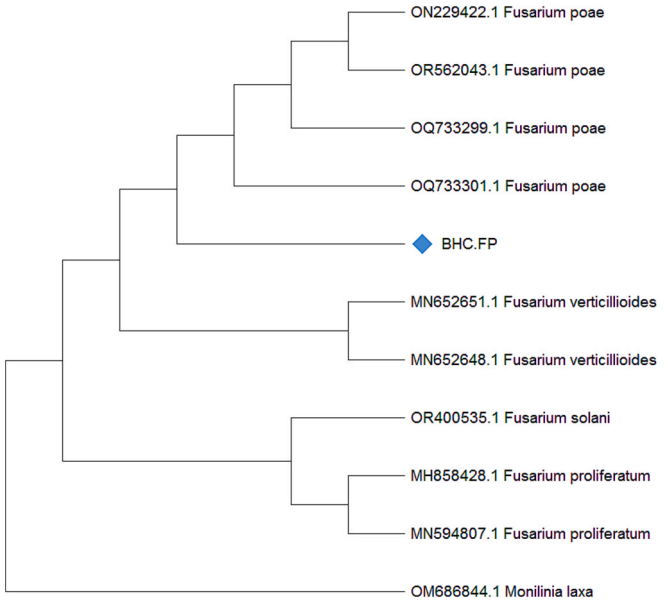

Fig. 1. Neighbour-joining phylogenetic tree based on the sequences of fungal ITS region for isolate from this study (marked with blue rectangle) and 9 comparative *Fusarium* species. The tree was rooted with *Monilinia laxa.* (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Biocontrol and plant growth promoting properties of Bacillus spp.

The results obtained from qualitative and quantitative tests for the traits of plant growth-promoting and biocontrol of new *Bacillus* strains are presented in Fig. 2 and Table 3.

Bacillus velezensis BHC 5.6 showed a wide range of plant growth promoting and biocontrol abilities, except HCN production. Among tested strains, only *B. velezensis* BHC 5.6 was able to solubilize phosphate. None of the eleven strains were capable to produce HCN. Out of the tested strains, only four strains (*B. altitudinis* BHC 5.1, *L. capsici* BHC 5.4, *B. velezensis* BHC 5.6 and *B. safensis* BHC 11.4) were able to produce lipase. Cellulase and protease production was confirmed in the same strains except for *B. altitudinis* BHC 5.1, *L. capsici* BHC 5.4, *B. pumilus* BHC 10.2 and *B. safensis* BHC 11.4. *Priestia megaterium* BHC 5.5, *B. velezensis* BHC 5.6, *B. pumilus* BHC 10.2 and *B. cereus* BHC 11.3 have shown the ability to produce amylase. Among the tested *Bacillus* strains, the highest siderophore producer was strain *B. velezensis* BHC 5.6, followed by strains *B. altitudinis* BHC 5.1 and *P. megaterium* BHC 5.2. *Priestia megaterium* BHC 10.1, *B. cereus* BHC 11.3, *B. altitudinis* BHC 5.1,

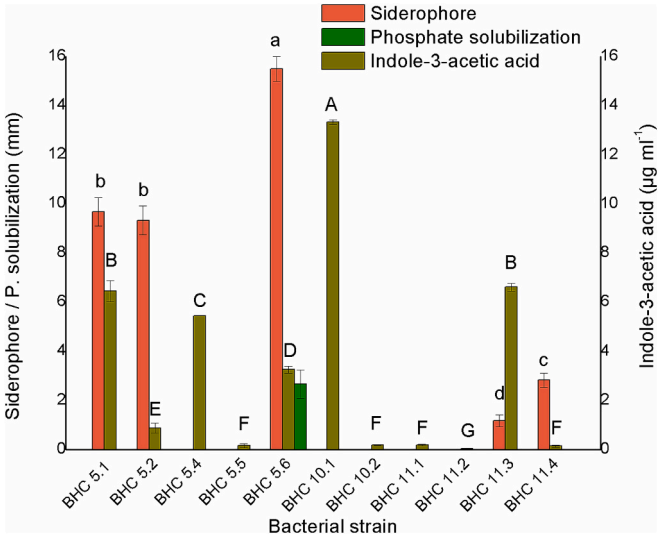

Fig. 2. Plant growth promoting traits of the tested bacterial strains. Data were expressed as mean values ± SE, a-d (for siderophore production) and A-G (for indole-3-acetic acid production): Means in a column followed by the same superscript letters are not significantly different according to Duncan's multiple range test ($P \leq 0.01$).

Table 3
Biocontrol traits of the tested bacterial strains.

Bacterial strains	Biocontrol traits					
	Amylase	*Cellulase*	*Pectinase*	*Protease*	*Lipase*	*HCN*
B. altitudinis BHC 5.1	–	–	+	–	+	–
P. megaterium BHC 5.2	–	+++	+	++	–	–
L. capsici BHC 5.4	–	–	–	–	+	–
P. megaterium BHC 5.5	+	++	++	++	–	–
B. velezensis BHC 5.6	++	+++	+++	+++	+++	–
P. megaterium BHC 10.1	–	+++	++	++	–	–
B. pumilus BHC 10.2	+	–	–	–	–	–
P. megaterium BHC 11.1	–	++	+	++	–	–
P. frigoritolerans BHC 11.2	–	++	–	++	–	–
B. cereus BHC 11.3	+	++	+	++	–	–
B. safensis BHC 11.4	–	–	–	–	++	–

– no production; + low production; ++ moderate production; +++ high production. – no production (no halo zone); + low production (halo zone radius up to 5 mm); ++ moderate production (halo zone radius up to 10 mm); +++ high production (halo zone radius above 10 mm).

L. capsici BHC 5.4 and *B. velezensis* BHC 5.6 were distinguished as IAA producers.

3.4. Biocontrol potential of bacterial strains

Out of all bacterial strains tested against *F. poae,* only *P. megaterium* BHC 5.5 and *B. velezensis* BHC 5.6 showed the ability to suppress the mycelial growth on PDA medium (Fig. 3). The inhibition of *F. poae* mycelial growth induced by these strains was 67.62 %±3.60 and 62.86 %±5.71 for *P. megaterium* BHC 5.5 and *B. velezensis* BHC 5.6, respectively.

In the first 24–48 h, only individual mortality of wireworms was recorded, which is why Fig. 4 shows mortality after four days and at the end of the treatment (after 10 days). After four days, an efficiency of 23.33 % were recorded in treatments with strains *B. velezensis* BHC 5.6 and *B. cereus* BHC 11.3, while an efficiency of 16.67 % were recorded with strains *L. capsici* BHC 5.4 and *B. safensis* BHC 11.4. After ten days, the highest efficiency (56.67 %) was recorded in the treatment with strain *B. velezensis* BHC 5.6. This strain stood out as the most effective treatment, exhibiting the highest mortality rates at both four and ten days. The mortality nearly doubled (day four to ten), suggesting a strong and sustained effect. Subsequently, it was followed by treatments with strains *B. safensis* BHC 11.4 (43.33 %) and *B. cereus* BHC 11.3 (40 %). These strains also showed high mortality, although slightly lower than BHC *B. velezensis* 5.6. It followed a similar pattern of increased mortality from day four to ten. The lowest efficiency was recorded in the treatment with strain *P. megaterium* BHC 5.2 (6.67 %). By comparing the average efficiencies between the fourth and tenth days for each treatment individually, post-hoc analysis showed that for all treatments the average efficiency was higher on the tenth day than on the fourth day.

3.4.1. Detection of antibiotic- and toxin-producing genes

Based on the results of PCR test, the presence of antibiotic-producing genes was detected only for *B. velezensis* BHC 5.6 (Table S3). Interestingly, out of six tested genes, the presence of the following four genes was detected for this strain: fengycin, bacyllomicin, surfactin and subtilin. The presence of bacyllomicin and itruin was not detected in tested strains. Regarding the detection of toxin-producing genes, the presence of crystal protein (*cry1B*) was detected only for the strain BHC 11.4, while the presence of crystal protein (*cry11*) was not detected in the tested strains.

3.5. Pot experiment

The results of pot experiment indicated that the infection of barley by *F. poae* BHC.FP decreased the plant yield up to 16.03 %, in comparison to the control (Table 4). Further, inoculation of barley seeds by *B. velezensis* BHC 5.6 increased the yield up to 10.12 % in comparison to the control, while the increase of yield for up to 17.09 % was recorded for *F. poae* BHC.FP + *B. velezensis* BHC 5.6, in comparison to the infected control. The concentration of Ca remained stable across treatments, with slight increases in the infected control (*F. poae*) and *B. velezensis* BHC 5.6-treated plants. A slight reduction in K was observed in *B. velezensis* BHC 5.6-treated plants, but values remained within a biologically acceptable range. Inoculation of barley with *B. velezensis* BHC 5.6 led to a notable increase in Mg levels compared to other treatments, while the levels remained consistent across all treatments, indicating that *B. velezensis* BHC 5.6 did not adversely affect phosphorus uptake. However, the content of Ca, K, Mg and P was in the range of values for this plant species, and the bacterial inoculation/fungal infection did not affect the nutrient composition.

4. Discussion

Biological control based on the use of microorganisms and their metabolites against various pests and diseases is an important alternative in contemporary agriculture. In addition, the need for resilient crops to cope with pests and diseases gains importance in a changing climate. Biological inoculants may be highly useful in organic farms where synthetic pesticides cannot be applied due to certification requirements and/or consumer expectations. On the other hand, microbial inoculants can also be used for pre-inoculation of cereal seeds or as a protection of grains during storage (Wachowska et al., 2013; Zanon et al., 2024). Bacteria of the *Bacillus* genus are the most studied and the most commonly used microorganisms as biocontrol agents, due to their ability to produce a wide range of bioactive compounds with the potential to inhibit the growth of phytopathogenic fungal species and

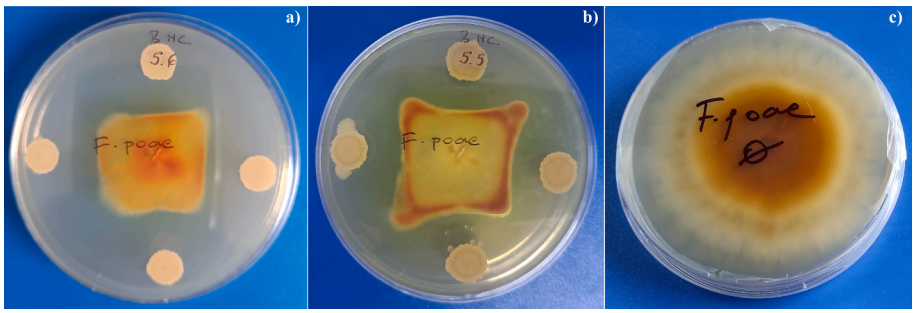

Fig. 3. Suppression of *Fusarium poae* mycelial growth by *P. megaterium* strain BHC 5.5 (a) and *B. velezensis* strain BHC 5.6 (b), *F. poae* BHC.FP control (c).

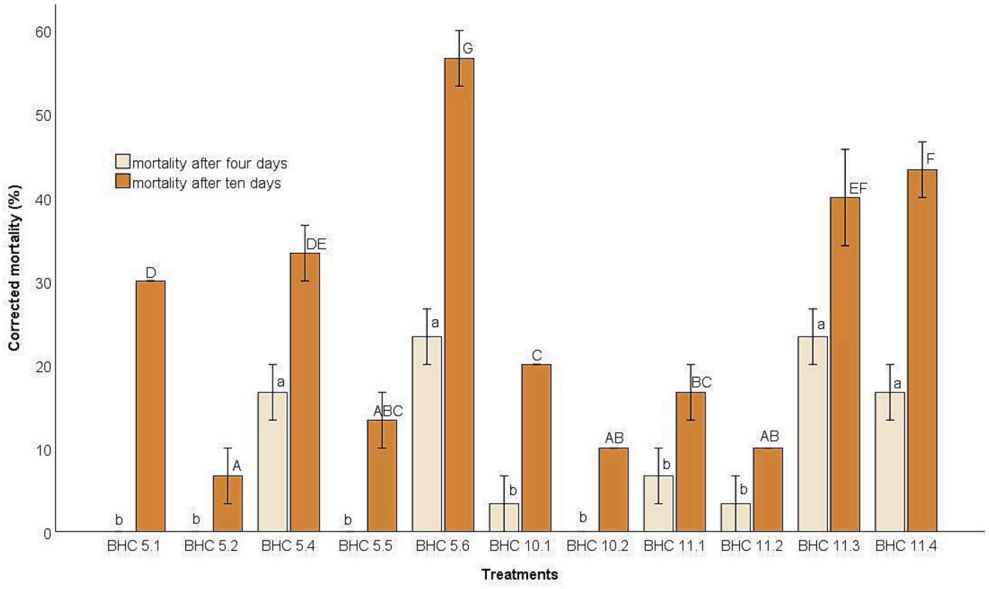

Fig. 4. Mortality (%) of *Agriotes lineatus* larvae induced by bacterial strains. Two-way ANOVA (Post hoc: Duncan) was applied to determine the significance of the differences between the average efficacy of individual treatments on the fourth and tenth day. Data were expressed as mean values ($P < 0.01$) ± SE. Lowercase and uppercase letters indicated the significance of differences between individual treatments on the fourth and tenth days, respectively.

Table 4
Effects of *B. velezenisis* BHC 5.6 inoculation and *F. poae* BHC.FP infection on plant parameters.

Plant parameter	Treatment			
	Control	Infected control	BHC 5.6	BHC.FP + BHC 5.6
Yield (g pot^{-1})	2.37 ± 0.13^{b}	1.99 ± 0.04^{c}	2.61 ± 0.14^{a}	2.33 ± 0.05^{c}
Ca (mg kg^{-1})	6193 ± 38.6^{b}	6709 ± 27.8^{a}	6577 ± 33.5^{a}	6132 ± 130.4^{b}
K (mg kg^{-1})	62064 ± 88.1^{b}	62490 ± 126.1^{a}	56759 ± 111.2^{c}	62788 ± 297.3^{a}
Mg (mg kg^{-1})	2365 ± 16.0^{c}	2641 ± 38.9^{b}	3052 ± 2.5^{a}	2312 ± 20.1^{d}
P (mg kg^{-1})	5128 ± 7.5^{c}	5216 ± 9.0^{a}	4831 ± 21.3^{d}	5141 ± 6.3^{b}

a-d: Means in a column followed by the same superscript letters are not significantly different according to Duncan's multiple range test ($P \leq 0.01$). Control – non-inoculated nor infected seeds; Infected control – seeds infected with *F. poae* BHC.FP; BHC 5.6 – seeds inoculated with *B. velezensis* BHC 5.6; BHC.FP + BHC 5.6 – seeds inoculated with *B. velezensis* BHC 5.6 and infected with *F. poae* BHC.FP.

reduce the abundance of insect larvae (entomopathogenic activity) (Zanon et al., 2024).

Cereals, including barley, are susceptible to Fusarium Head Blight (FHB) whose occurrence has been associated with over seventeen species of *Fusarium* species. Although *F. poae* is often considered as a minor pathogen among FHB- causing species, its ability to produce a diverse range of mycotoxins highlights its potential risk. Alaniz-Zanon et al. (2025) demonstrated that *F. poae* accounted for 12 % of all *Fusarium* species isolated from barley grains, and linked its presence to specific regions in Argentina. It was also demonstrated that the prevalence of this fungi could be determined by environmental factors of the examined region. Besides its molecular identification, *F. poae* BHC.FP isolated in this study showed specific morphology on PDA medium, including mycelium density and specific colour (Stenglein, 2009). In addition, the germination of infected seeds was severely impaired as a result of *F. poae* infection, indicating the significance of this plant pathogen. In general, there is a lack of research on the suppression of *F. poae* growth by bacteria. Zanon et al. (2024) were the first ones to publish the antifungal activity of *B. velezensis* species against *F. poae* species. *Bacillus velezensis* showed efficacy against two strains of *F. poae* (58.4 % and 31.6 % of reduction) (Zanon et al., 2024). The efficiency of *B. velezensis* BHC 5.6 against *F. poae* BHC.FP in this research was higher, with a fungal growth inhibition of 67.62 %. In our previous research, *Bacillus* spp. isolates BHC 4.5, BHC 4.7, BHC 2.4 and BHC 2.3 showed antifungal activity again *F. poae* with inhibition rate of 20 %, 15 %, 12 % and 7 %, respectively (Jovković et al., 2024). Inhibition of *F. poae* mycelial growth by other *Bacillus* species was rarely studied. Antifungal activities of *Bacillus* strains against *F. poae* were reported by two groups of authors.

Illueca et al. (2021) showed potential of *B. licheniformis B. megaterium*, *B. subtilis*, *B. amyloliquefaciens* and *B. thuringiensis* to inhibit mycelia growth of *F. poae* by 16.9 %, 11.4 %, 25.2 %, 22.2 % and 18.5 %, respectively. *Bacillus inaquosorum* and *B. nakamurai* were capable to reduce *F. poae* growth by 32.8 % and 14.5 % (Zanon et al., 2024). *B. velezensis* BHC 5.6 showed the greatest inhibition of fungal growth of all the *Bacillus* strains reported in the literature. Moreover, suppression of *F. poae* mycelial growth was confirmed by the use of other bacterial species such as *Rhodococcus sp.*, *Azotobacter nigricans*, *Streptomyces rimosus* and *Cryptococcus carnescens* (Nagaraja et al., 2016; Podgórska-Kryszczuk et al., 2022; Tan et al., 2021). The present work provides valuable data on the biocontrol of *F. poae* including the first report of *P. megaterium* BHC 5.5 as a biocontrol agent against *F. poae*. The *P. megaterium* species has been previously studied in biocontrol of various phytopathogenic fungi, including *Fusarium* species, but to the best of our knowledge its activity against *F. poae* has not been studied (Saleh et al., 2021; Yang et al., 2022; Fu et al., 2015). The suppression of mycelial growth by *P. megaterium* BHC 5.5 was slightly lower than *B. velezensis* 5.6 (BHC 5.5: 62 %). Both of the most effective strains were isolated from the same alkaline soil classified as Sandy loam (rhizosphere of wheat).

In the biocontrol of *Fusarium*-induced plant diseases, lipopeptides produced by *Bacillus* species play a crucial role in the pathogen suppression (Khan et al., 2017). Lipopeptides, such as fengycin, surfactin, bacillomycin D, and iturin A exhibit potent antifungal activity by interacting with fungal cell membranes, leading to membrane disruption and loss of cellular integrity. These lipopeptides bind to membrane phospholipids, forming pores that increase membrane permeability, resulting in ion leakage, cellular component release, and ultimately, cell death (Zhang et al., 2010; Schulz et al., 2009). Additionally, these lipopeptides induce oxidative stress and interfere with fungal processes such as spore germination, hyphal growth, and nutrient absorption, making them effective in controlling fungal pathogens like *F. poae* through multiple modes of action (Liu et al., 2013; Li et al., 2017). Vitullo et al. (2012) highlighted the antifungal role of surfactin from *B. amyloliquefaciens* against *F. oxysporum*. Blacutt et al. (2016) demonstrated that surfactin and fengycin produced by *B. mojavensis* had the ability to inhibit *F. verticillioides*. Li et al. (2013) recorded an increase in fengycin and bacillomycin production when *B. amyloliquefaciens* was exposed to *F. oxysporum*, while as a response to *F. solani* the surfactin production increased. Zihalirwa Kulimushi et al. (2017) detected increased iturin and fengycin levels in *B. subtilis* as a response to *Fusarium* co-inoculation. In this research, for the strain with the highest antagonistic activity against *F. poae* (*B. velezensis* BHC 5.6), the presence of *fenD*, *bmyB*, *srfAA* and *spaS* genes was detected. The presence of these bacterial genes could be responsible for the strong biocontrol potential of BHC 5.6. Similarly, Palazzini et al. (2016) detected the presence surfactin and fengycin in the *B. velezensis*. Théatre et al. (2021) also suggested that some of the *Bacillus* − produced lipopeptides, such as surfactin and fengycin could have a synergistic effect in controlling fungal pathogens. On the other hand, hydrolytic enzymes produced by rhizospheric bacteria have significant importance in the biocontrol of various *Fusarium* species (Abed et al., 2016; Sindhu et al., 2017). Pectinases, cellulases, amylases and chitinases-producing bacteria, including phosphate solubilizing organisms, have the ability to engage with pathogens by hydrolyzing various polysaccharides and thus play a key role in the direct suppression of plant pathogens (Zaidi et al., 2014; Dukare and Paul, 2021). *Bacillus velezensis* BHC 5.6, which showed the strongest antifungal activity against *F. poae*, is simultaneously the only phosphate-solubilising strain among the tested strains and a good producer of cellulase, pectinase, and amylase.

Bacillus velezensis has been identified as an agent with the ability to promote the growth of variety of plant species under semi-controlled and field conditions (Bai et al., 2023; Jang et al., 2023). In addition, *B. velezensis* produces plant growth-promoting compounds such as auxin, 1-aminocyclopropane-1-carboxylate deaminase and iron chelators (Zhong et al., 2024). Chebotar et al. (2021) showed that *B. velezensis* isolated from the rhizosphere of wheat could enhance plant growth, most probably due to the presence of gene clusters for indole-3-acetic acid synthesis. It has been previously shown that siderophores produced by *B. velezensis* could support the overall plant growth that by enhancing the bacterial iron uptake with iron-siderophore complex (Zhong et al., 2024). In a pot experiment conducted by Shi et al. (2022) it was demonstrated that IAA–producing and phosphate-solubilising *B. velezensis* strains increased the growth parameters of *Prunus davidiana*. *Bacillus velezensis* BHC 5.6 was the only tested strain capable of producing all three key plant growth-promoting compounds: phosphate-solubilizing compounds, indole-3-acetic acid (IAA), and siderophores. Previous studies have demonstrated that *B. velezensis* strains significantly enhance plant growth through these mechanisms (Afzal et al., 2023; Mosela et al., 2022). The concurrent production of these metabolites by *B. velezensis* BHC 5.6 underscores its potential as a multifunctional biofertilizer, supporting findings that such traits synergistically promote plant development (Afzal et al., 2023). The ability of *B. velezensis* BHC 5.6 to enhance barely growth under semi-controlled conditions could be due to its PGP traits, such as siderophores and IAA production, as well as its ability to solubilise inorganic phosphates. Further, *B. velezensis* BHC 5.6 improved the growth of infected barely in the pot experiment, suggesting its additional role in the promotion of plant growth in the presence of fungal infection. The nutritional composition of plants (both infected by *F. poae* BHC.FP and inoculated by *B. velezensis* BHC 5.6) had optimal values, indicating that nutritional composition of plants was not affected by *F. poae* infection (Birsin et al., 2010).

Wireworms are a significant challenge in sustainable agricultural production due to their multi-year life cycle in the soil, broad host range, and the difficulty in controlling them with conventional methods (Vernon and Van Herk, 2013). They are recognized as economically significant pests of cereal crops globally. In Europe and North America, wireworm infestations have led to losses estimated in the tens of millions of dollars annually, with wheat, barley, and maize among the most affected crops (Parker and Howard, 2001; Vernon et al., 2009). Economic thresholds are low, as even moderate wireworm densities (1–2 larvae m^{-2}) can significantly impair crop performance, particularly under conservation tillage systems that favor wireworm survival. Their subterranean feeding on seeds and seedlings leads to reduced plant stands, stunted growth and reduced tillering, further diminishing grain yield and ultimately impacting profitability (Furlan et al., 2017; Parker and Howard, 2001). Traditional control strategies face limitations, highlighting the need for innovative approaches (Parker and Howard, 2001), and while the use of soil bacteria with insecticidal activity against *A. lineatus* larvae has not been thoroughly investigated, there is a generally limited number of studies on the biological control of wireworms (Poggi et al., 2021; Nikoukar and Rashed, 2022). Given the long history of *Bacillus* strains being used to control various pests, these microorganisms represent promising biological agents and potential solutions for wireworm control, offering diverse mechanisms of action, including the production of insecticidal toxins and the potential to induce plant resistance. Among *Bacillus* species, *B. thuringiensis* has been the most studied one active against larval stages of different insect orders (Mampallil et al., 2017; Kumar et al., 2021), and to the best of our knowledge, the biocontrol activity of *B. velezensis* and *B. safensis* against wireworms has not yet been reported in the literature. Danismazoglu et al. (2012) isolated bacteria from *A. lineatus* and tested them against this pest, where the larval ranged from 16 % to 83 %, while the highest wireworms mortality (100 %) was recorded for *A. gandavensis, B. thuringiensis and P. plecoglossicida* ten days after treatment (Danismazoglu et al., 2012). In addition, the insecticidal effect of specific bacterial strains is thought to result from the action of bacterial lytic enzymes and toxins involved in the lysis of epithelial cells in the midgut of insects (Sindhu et al., 2017). The highest insecticidal activity was confirmed for *B. velezensis* BHC 5.6 of 56.67 % ten days after treatment which also

showed the greatest ability to produce proteases and lipases. On the other hand, for the *B. safensis* BHC 11.4, the second most effective strain in the biocontrol of wireworms (43.33 %), the *cry1b* gene was detected, while this strain also had the ability to produce lipase. Several studies have shown that the proteases and lipases produced by *Bacillus* species could have synergistic insecticidal effect on insect larvae (Rakshiya et al., 2016; Ruiu, 2020; Ajuna et al., 2023). Furthermore, the wide range of Cry toxins, with Cry1 being the most studied in *B. thuringiensis*, was mainly active against Coleoptera, Lepidoptera and Diptera, (Khan et al., 2022). Cry toxins, particularly Cry1B, produced by *Bt* strains, have shown promising insecticidal activity against coleopteran larvae, including wireworms. Cry1B exerts its effect by binding to specific receptors in the midgut epithelial cells of the target insect. This interaction leads to the formation of pores in the gut lining, disrupting ion flow and causing cell lysis. The result is gut paralysis, septicemia, and eventual insect death (Bravo et al., 2007). Cry1B is particularly effective against insects with alkaline gut pH, which facilitates toxin solubilization and activation. In addition, proteolytic and lipolytic enzymes also play a crucial role in the insecticidal arsenal of microbial biocontrol agents. Proteases degrade essential gut proteins, disrupting digestion and nutrient absorption. They may also interfere with the insect's immune response by degrading immune-related peptides and proteins. Lipases, on the other hand, break down lipid membranes of cells, contributing to tissue damage and cytotoxicity. In combination, these enzymes enhance the efficacy of *Bt* toxins or other bioinsecticides by weakening the insect's physiological defence and promoting systemic collapse (Ramzi et al., 2013; Singh and Singh, 2020).

Although *B. velezensis* BHC 5.6 has demonstrated potential to suppress the growth of *F. poae* and increase wireworm mortality under laboratory conditions, its efficacy still needs to be verified under field conditions. In order to confidently claim that a bio-inoculant based on this strain could be beneficial in agricultural practice, further research is required to assess its effectiveness. Soil conditions such as extreme pH values, soil temperature influenced by climatic conditions at a given location, as well as the native soil microbiota at the site of inoculant application, could potentially affect the performance of the bacterial strain under real-world conditions (Saad et al., 2020). Given that the effective strains identified in this study belong to the genus *Bacillus*, it is likely that it could survive and remain effective even in extreme environments, considering the well-documented sporulation capacity of *Bacillus* species (Valencia-Marin et al., 2024). Prior to the commercialization of such a bio-inoculant, it would be necessary to develop an optimal formulation for its application. Since liquid bio-inoculants based on *Bacillus* species have proven to be among the most commonly used in agriculture and their production process is considered highly cost-effective, the development of such a formulation, along with confirmation of its efficacy under realistic environmental conditions, would be essential.

5. Conclusion

In conclusion, this study highlights the potential of *Bacillus*-based biocontrol agents, particularly *Bacillus velezensis* BHC 5.6, as an effective solution for integrated pest management in cereal crops. To the best of our knowledge this is the first report of *Bacillus velzensis* showing both antifungal effect against *Fusarium poae* and insecticidal effect against wireworms (*Agriotes lineatus* larvae) in barley. *Bacillus velezensis* BHC 5.6 had both antifungal and insecticidal effect against *F. poae* and wireworms above 65 % and 55 %, respectively. The detection of key antibiotic-producing genes further supports its potential as a biofungicide. Notably, this strain also contributed to increased barley yield in both infected and uninfected conditions, making it a promising candidate for sustainable pest management strategies. Besides dual biocontrol effectives of *B. velezensis* BHC 5.6, this is the first time demonstrating the antagonistic effect of *P. megaterium* against *F. poae*. This research indicates the need for further research on *Bacillus*-based bio-inoculants, with a focus on *B. velezensis* species, in pest control of both *F. poae* and wireworms in cereals.

CRediT authorship contribution statement

Magdalena Knežević: Writing – review & editing, Writing – original draft, Methodology, Data curation, Conceptualization. **Marina Dervišević:** Writing – original draft, Methodology, Formal analysis, Data curation. **Marina Jovković:** Formal analysis. **Galina Jevđenović:** Formal analysis. **Jelena Maksimović:** Formal analysis. **Aneta Buntić:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization.

Funding

This research was funded by the Ministry of Science, Technological Development and Innovations of the Republic of Serbia, contract Nos. 451-03-136/2025-03/200011, 451-03-136/2025-03/200214 and 451-03-136/2025-03/200135 and by the Science Fund of the Republic of Serbia, GRANT No. 10815, The necessity of healthy crops: Development of a multifunctional bacterial inoculant for the biological protection of cereals – BioHealCrop.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2025.105789.

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