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RESEARCH ARTICLE
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Bacillus Thuringiensis Pb-3-1 Strain of Complex Action against *Phytonomus Variabillis* and *Fusarium* of Alfalfa and Profile Its Voc

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ABSTRACT

A novel bacterial strain, PB-3-1, was isolated from the rhizosphere soil of alfalfa (Medicago sativa) and identified as Bacillus thuringiensis based on morphological, physiological, and 16S rRNA gene sequence analysis. The objective of this study was to evaluate the insecticidal, antifungal, and plant growth-promoting properties of the PB-3-1 strain and to characterize its volatile organic compounds (VOCs). In laboratory bioassays, treatment with PB-3-1 culture broth resulted in 50% mortality of *Phytonomus variabillis* larvae by day 10. Antifungal activity was demonstrated against phytopathogenic fungi of the genus Fusarium, with inhibition zones measuring 41.3 ± 2.1mm for *F. oxysporum* and 32.0 ± 2.0mm for *F. solani*. A phytotoxicity assay on alfalfa seedlings revealed that PB-3-1 was non-toxic and significantly stimulated plant growth. At a concentration of 10^5CFU/mL, the culture broth enhanced root and stem elongation by 42% and 68%, respectively, compared to the control. VOC profiling using GC–MS identified 15 components in the culture broth, dominated by pyrazine derivatives, including pyrazine, 2,5-dimethyl- (75.36%), and others. The total pyrazine content constituted 80.15% of the detected VOCs, suggesting their key role in the strain's biological activity. These findings indicate that B. thuringiensis PB-3-1 exhibits strong biocontrol potential against *P. variabillis* and Fusarium spp., while also promoting alfalfa growth, highlighting its value as a multifunctional biological agent in sustainable crop protection.

Keywords: Alfalfa; *Phytonomus variabillis*; Fusarium; *Bacillus thuringiensis*; VOCs; Pyrazine

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INTRODUCTION

Alfalfa (Medicago sativa) is one of the most important agricultural crops, providing livestock with high-protein feed and participating in maintaining a high level of soil fertility. Pests and diseases cause significant damage to biocenoses that have developed over the period of many years of alfalfa growing in one place, especially in single-species crops. The most common pests of alfalfa are the alfalfa leaf weevil, the root nodule weevil, the alfalfa bug, the alfalfa thick-legged moth, aphids, etc. (Atanasova & Semerdjieva, 2010; Esenbekova et al., 2019). The most harmful of these is the alfalfa weevil *Phytonomus variabillis*, which damages the first cut of alfalfa in the second and third years of life. On alfalfa in the 3rd-4th years of life, up to 100% of plants can be damaged and 60-75% of inflorescences can be destroyed.

The number of *Phytonomus* on alfalfa plants in the amount of 6-8 pieces reduces the height of plants by 29-41%, and the weight of seeds by 78-93%. Severe infestation of alfalfa with larvae leads to the death of plants. In addition to the negative impact on seed yield, damage to alfalfa by *Phytonomus* larvae also leads to a deterioration in the forage quality of plants. In addition, in alfalfa with severe damage by *Phytonomus*, the fat content decreases by 1.6% and protein by 7% (Baltayev & Boltayev, 2021). *Fusarium* is the main disease of alfalfa caused by phytopathogenic fungi. In the form of mycelium, it can be both on the surface and inside the seeds, and infected seeds contribute to the transmission of the disease over long distances. Throughout the growing season, *Fusarium* can infect the host at different growth stages and re-infect

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the field many times (Wang et al., 2023, 2024). In addition, fungi of the genus *Fusarium* produce mycotoxins, contaminate seeds and can cause diseases in humans and animals (Ferrigo et al., 2016; Munkvold et al., 2021).

Farmers often resort to chemical pesticides to control pests and diseases. However, their use has become excessive over time. Visible negative impacts on the environment, the emergence of increasing numbers of pesticide-resistant pathogens, and unforeseen consequences for human and animal health have been calling for a shift to new environmentally friendly alternatives to chemical pesticides for some time (Mobin & Usmani, 2017; Rani et al., 2021). In this regard, biological methods are the safest and most effective, including the use of preparations based on microorganisms. The harmlessness of bacterial biopreparations for plants allows them to be used in any phase of plant vegetation. The use of microbial preparations is accompanied by an increase in the volume of the biotic environment and stabilization of biocenotic connections in agrocenoses (Thomas et al., 2020). This is the fundamental ecological difference between biological preparations and chemical ones.

In recent years, bacteria of the genus Bacillus have become promising objects for agrobiotechnology, having shown their effectiveness as antagonists of a wide range of pests and phytopathogens on many agricultural crops (Mishra & Ashok Kumar, 2012; Vitullo et al., 2012; Villarreal-Delgado et al., 2018; Öğütçü et al., 2020). Members of the genus Bacillus are known to produce a wide range of antimicrobial peptides such as Gramicidin, Bacitracin A, Polymyxin B, Mersacidin, Sublancin, etc. (Jung et al., 2008; Stoica et al., 2019) and there are reports regarding the antagonistic effects of bacteria as a result of their production of volatile and non-volatile compounds (Wu et al., 2019; Shemshura et al., 2020; Weisskopf et al., 2021; Dos Santos et al., 2023). The bacterium *B. thuringiensis* is of the greatest practical importance in the fight against insect pests; it forms the basis of the modern industry for the production of bacterial insecticides. B. thuringiensis unites varieties of spore-forming bacteria that produce special entomocidal toxins that are highly active against insects (Sánchez–Yáñez et al., 2022). There are also reports of antifungal activity of *B. thuringiensis* against phytopathogenic fungi in soybean seeds (Reyes-Ramírez et al., 2004); against powdery mildew of barley and cucumber (Choi et al., 2007); against verticillium wilt of tomatoes (Hollensteiner et al., 2017); against phytopathogenic fungi of the genera *Phytophthora* and *Fusarium* (Kamenek et al., 2012). In addition, antifungal activity of B. thuringiensis VOCs has been reported (He et al., 2020; Kloser et al., 2024). In this regard, it is of interest to study the complex action of *B. thuringiensis* in relation to the alfalfa weevil and phytopathogenic fungi of the genus *Fusarium* and to determine the profile of its volatile organic compounds.

In light of the growing interest in environmentally friendly and sustainable approaches to managing pests and diseases, the current study was created to investigate the biocontrol potential of a newly isolated strain of *Bacillus thuringiensis*, known as PB-3-1, from the alfalfa rhizosphere. The objective of this work is to describe the multifunctional activity of this strain and evaluate its suitability for biological protection of alfalfa crops. The primary objective of this study lies in the isolation and identification of the PB-3-1 strain by applying morphological, physiological, and molecular-genetic characteristics to validate its taxonomic classification as *Bacillus thuringiensis*. The study also aims to assess the insecticidal activity of the PB-3-1 strain on *Phytonomus variabillis*, a major insect pest of alfalfa. Additionally, the study aims to examine the potential antifungal effect of the PB-3-1 strain on phytopathogenic fungi of the genus Fusarium, *F. oxysporum* and *F. solani*, which cause major diseases of alfalfa root and vascular wilt. In addition to its potential insecticidal and fungicidal effects, the study aims to determine the strain safety and overall influence on plant health and growth parameters by further examining the phytotoxicity and potential plant-growing effect of the PB-3-1 strain by comprehensively analyzing the impact of the strain at the early growing stages of alfalfa seedlings. Furthermore, it is necessary to profile the secondary metabolites produced by the PB-3-1 strain by analyzing the volatile organic compounds (VOCs), especially pyrazine derivatives, which may be involved in its biological activities such as biocontrol or plant signaling.By precisely identifying and measuring the predominant VOCs in the PB-3-1 train of *Bacillus thuringiensis*, the study aims to highlight the chemical framework responsible for the observed bioactivity. Collectively, these objectives aim to establish *B. thuringiensis* PB-3-1 as a promising candidate for integrated pest and disease management strategies in alfalfa cultivation, contributing to the development of sustainable agricultural practices.

MATERIALS & METHODS

Isolation of Bacterial Isolate

The bacterial isolate PB-3-1 was obtained in 2023 from rhizospheric soil samples of *Medicago sativa* (variety Semirechenskaya) in the Kyzylorda region of Kazakhstan. Soil suspensions were prepared in 0.85% saline, heat-treated at 75°C for 20min to eliminate non-spore-forming bacteria, and serially diluted up to 10^{-7}. Aliquots (0.2mL) were plated onto fish peptone agar (FPA) and incubated at 28°C for 24 hours. Morphologically distinct colonies were purified and the isolate with insecticidal and antifungal potential was designated PB-3-1. The strain was maintained on RPA slants at 4°C (Hassan et al., 2021).

Phenotypic and Molecular Identification

Phenotypic characteristics were assessed following *Bergey's Manual of Determinative Bacteriology* (Holt, 1994). Colony morphology, Gram staining, and spore formation were recorded. Biochemical tests included catalase activity, starch hydrolysis, gelatin liquefaction, urea decomposition, and utilization of glucose, sucrose, and nitrogen sources. Molecular identification was performed by amplification of the 16S rRNA gene using primers 27F and 1492R. Sequencing was done with the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) on a 3500 DNA Analyzer. BLAST searches were conducted against

GenBank (NCBI), and phylogenetic analysis was performed in MEGA6 using the ClustalW algorithm (Sanger et al., 1977).

Insecticidal Activity against *Phytonomus variabillis*

Second instar larvae of *P. variabillis* were collected from alfalfa fields in the Kyzylorda region. The PB-3-1 strain was cultured for 72 h in a liquid medium (corn flour 20g/L, fodder yeast 30g/L, $(NH_4)_2HPO_4$ 5 g/L, $MgSO_4 \cdot 7H_2O$ 0.3 g/L, $CaCl_2 \cdot 2H_2O$ 0.2g/L; pH 7.5) at 200 rpm and 28°C. The broth was diluted to 10^8CFU/mL.

Alfalfa leaves were immersed in the broth, dried, and provided to larvae in Petri dishes (15 larvae per replicate, 3 replicates). The control group was fed leaves dipped in sterile broth. Mortality was recorded over 10 days, and corrected mortality was calculated using Abbott's formula.

Antifungal Activity against *Fusarium* spp.

Antifungal activity was tested using the dual culture method (Balouiri et al., 2016). Spore suspensions of *F. oxysporum* and *F. solani* (10^7 CFU/mL), previously isolated from alfalfa rhizosphere (Shemshura et al., 2025) and were spread onto PDA plates. Wells (8 mm diameter) were filled with 100µL of PB-3-1 broth (10^8 CFU/mL). Plates were incubated at 25°C for 5 days. Inhibition zones were measured. Negative controls used sterile broth.

Phytotoxicity and Growth Promotion in Alfalfa Seedlings

Alfalfa seeds (Semirechenskaya) were surface-sterilized (50% ethanol, 5min), rinsed thrice with distilled water, and soaked in PB-3-1 broth at concentrations of 10^8 and 10^5CFU/mL for 4h. Seeds (n=50 per group) were placed on moistened 12 × 42cm strips of filter paper, rolled, and incubated vertically in 100mL water at 28°C for 3 days. Root and stem lengths of 3-day-old seedlings were measured and compared to the control group, which was treated with sterile broth (Klykov et al., 2023).

VOC Profiling by GC–MS

Volatile metabolites of PB-3-1 were analyzed by solid-phase microextraction–gas chromatography–mass spectrometry (SPME–GC–MS) (Sadanov et al., 2024). Broth (5 mL) was incubated with DVB/CAR/PDMS fiber for 30min at 40°C. Desorption was carried out at 260°C for 3min. GC-MS analysis used an Agilent 7890 GC with 5977A MS and a DB-35MS column (30m × 0.25mm × 0.25µm). The temperature program ranged from 40°C (3min hold) to 300°C (5min hold) at 5°C/min. Total run time: 60min. Detection was performed in SCAN mode (m/z 34–750). Compounds were identified via Wiley 7th and NIST'08 libraries. Peak areas were normalized to determine relative percentages. Blank controls included empty fiber, sterile media, and clean vials.

Statistical Analysis

All experiments were conducted in triplicate. Results are presented as mean ± standard deviation. Data were analyzed using one-way ANOVA with Tukey's post-hoc test in STATISTICA 10. Statistical significance was accepted at $P<0.05$. This applied to mortality rates, inhibition zone diameters, and seedling growth parameters.

RESULTS AND DISCUSSION

Isolation of Bacteria; Morphological, Physiological and Biochemical Characteristics

Isolate PB-3-1 was isolated from soil samples taken from the rhizosphere of the Semirechenskaya alfalfa variety using the method described by Hassan et al. (2021).

Morphological and microscopic characteristics of isolate PB-3-1. Bacterial colonies on the FPA (fish-peptone agar) nutrient medium are flat, pasty, matte, with jagged edges, beige in color, 2-6 mm in diameter (Fig. 1a). Gram-positive spore-forming rods, cylindrical in shape, located singly, in pairs or chains (Fig. 1b). Forms heat-resistant spores and 2 types of crystals, rectangular and bipyramidal, with clear edges: endo- and exotoxins. The results indicate morphological uniformity and a pure culture free from contamination. The observed morphological features in the study agree generally with the established characteristics of *Bacillus thuringiensis*. It also aligns with the studies of Muniz et al. (2025) and Handayani et al. (2025), who reported on the larvicidal activity of B. thuringiensis strains and the presence of crystalline and cytolytic proteins crystals which are discernible tell signs of the bacterial strain. Physiological and biochemical characteristics of isolate PB-3-1. Catalase isolate is positive. Liquefies gelatin, peptonizes milk, hydrolyzes starch, decomposes urea. Does not form hydrogen sulfide and indole. Relation to carbon sources: assimilates glucose and sucrose with the formation of acid without gas. Does not assimilate arabinose, dulcitol, lactose, cellobiose, maltose. Relation to nitrogen sources: assimilates peptone and ammonium forms of nitrogen.

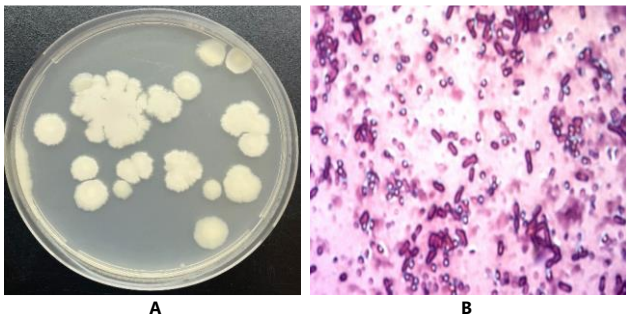


Fig. 1: Morphological and microscopic structure of isolate PB-3-1; a - colonies of isolate PB-3-1 on FPA; c - cells under a microscope x 1000.

Oxygen tolerance: Aerobic. Temperature tolerance:minimum – 10^0C-15^0C; optimum – 28^0C-30^0C; maximum – 40-45℃ Molecular genetic identification. Isolate PB-3-1 was identified using 16S rRNA gene sequencing and genetic markers. The results are presented in Fig. 2. It can be seen from Fig. 2, NR 114581.1:44-779 *Bacillus thuringiensis* strain ATCC 10792 was 100.00%. Thus, the isolate was identified as *B. thuringiensis* strain PB-3-1. The physiological and biochemical characteristics are in line with the general characteristics of *B. thuringiensis*, catalase reaction, gelatin liquefaction due to protease production, urease, amylase and proteolytic activities. A distinguishing biochemical feature of the Isolate PB-3-1 lies in its carbon-source profile and its inability to assimilate arabinose, dulcitol, lactose,

cellobiose, maltose. This feature could be as a result of the lack of certain catabolic pathways or a metabolical streamline, that favors specific substrates such as glucose and sucrose. Further research is needed to understand the reason for this specificity.

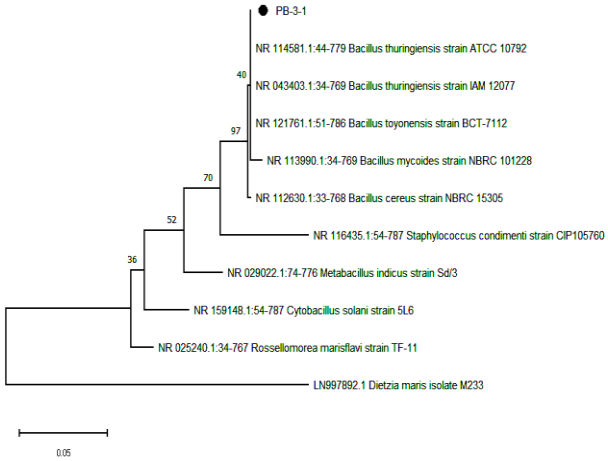


Fig. 2: Identification of isolate PB-3-1 using 16S rRNA gene sequencing.

Study of Insecticidal Activity of Strain PB-3-1

In integrated pest management (IPM) programs, biological methods are an important tool for reducing the number of insect pests withminimal risks to humans and the environment, this is further reinforced by the studies of Hajjar et al. (2023) and Galli et al. (2024), who reported on the application of the toxins from B. thuringiensis to inhibit the larvae of rice-stray borers. These studies also concluded that the inclusion of biocontrol methods into IPM programs was crucial since they allow for the incorporation of other methods, maximize efficiency and also lessen the dependence of chemically synthesized products. In this regard, a laboratory study was conducted on the insecticidal activity of the culture broth of the PB-3-1 strain, obtained by culturing it on a nutrient medium with corn flour ($1.9x10^9$ CFU/ml. For the study, the culture broth was diluted with warm tap water to a concentration of 10^8 CFU/ml.).

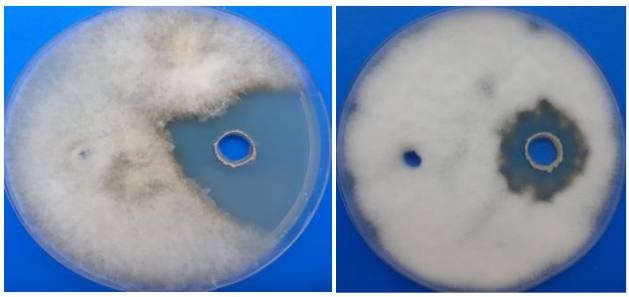
According to the results of the study, the mortality rate of insects on the third day was 32.6±3.21%, on the fifth - 39.6±1.52; on the seventh - 45.6±0.57%, on the tenth - 50.0±1%. In the control, where a nutrient medium without bacteria was used, the insect larvae remained active until the end of the study (Table 1). The increasing mortality rate shows the progressive effects of applying the PB-3-1 strain in insecticidal activity and its potential to be used in biocontrol programs. In comparison with other studies such as Elsharkawy et al. (2022), we observe the use of a higher concentration, longer testing duration and an increase in mortality rate with an increase in concentration. Therefore, future research focused on understanding the effect of increased concentrations of the PB-3-1 strain and longer experimental durations is recommended to address this limitation.

Antifungal Activity of the Strain PB-3-1

The results of the antifungal action of the culture broth of strain PB-3-1 ($1.9 x10^8$ CFU/ml) showed a high level of inhibition of phytopathogens *F. oxosporum* and *F. solani*. Thus, the diameter of the growth inhibition zones of *F. oxosporum* and *F. solani* were 41.3±2.1 mm and 32.0±2.0 mm, respectively. In the control, where the nutrient medium without bacteria was used, the inhibition zone was absent (Fig. 3). According to Oktarina et al. (2024), *B. thuringiensis* isolates are generally less potent in terms of antifungal activity compared to other Bacillus species such as *B. amyloliquefaciens* and *B. subtilis*. The results from this study indicate that PB-3-1 strain displays potent antifungal activities, thereby increasing its value as a biocontrol agent. Future research is recommended to understand its effects on other fungi species, actions under different concentration levels and application period.

Table 1: Insecticidal activity of strain PB-3-1 against *Phytonomus variabillis* larvae at stage 2 of age in laboratory conditions

Experience options	CFU/mL	Insect mortality,%			
		3 days	5 days	7 days	10 days
PB-3-1	$1.9 x10^8$	32.6±3.21	39.6±1.52	45.6±0.57	50.0±1
Control nutrient medium without bacteria	-	0	0	0	0



F. oxysporum (41,3±2,1) *F. solani* (32,0±2,0)

Fig. 3: Antifungal activity of strain PB-3-1 against fungi of the genus *Fusarium* on the right - well with PB-3-1; on the left - well with nutrient medium without bacteria (control).

Study of Phytotoxicity of Strain PB-3-1 to Alfalfa Plants

In our study, we investigated the effect of bacterial broth of strain PB-3-1 on the linear growth of alfalfa seedlings, the seeds of which were treated with bacterial broth in concentrations of 10^8 cfu/mL and 10^5 cfu/mL. At a culture broth concentration of 10^8 cfu/mL, the biometric parameters of the seedlings in the experiment and in the control differed little, so a slight stimulation of root growth by 6% and stem by 8% was noted compared to the control. The greatest increase in the linear length of alfalfa seedlings was noted in the variant with a culture broth concentration of 10^5 cfu/mL, at which the root length exceeded the control by 42%, and the stem length by 68% (Table 2). Thus, treatment with bacterial broth of strain PB-3-1 at a culture broth concentration of 10^5 cfu/mL showed a significant ability to stimulate the growth of both root and stem of alfalfa seedlings. The results align with the studies of Al-Shammari et al. (2024) and Pérez-Leal et al. (2025), who explored on the effects of B. thuringiensis as seed treatments. These studies reported an increase in growth characters, chlorophyll content, branch numbers, productivity and effectiveness in drought-stressed plants. Despite the positive results, more research is needed to understand this biostimulation mechanism.

Chromatographic Analysis of Metabolites in the Culture Broth of Strain PB-3-1

The results obtained from the analysis of the component composition of substances secreted by the PB-3-1 strain showed that the culture broth contains 15 components, the main one of which is a pyrazine derivative (Pyrazine, 2,5-dimethyl-) - 75.36%. Also found here were: (2-Aziridinylethyl)amine (5.0%); Oxime-, methoxy-phenyl- (4.26%); 3-Octanone- (3.2%); Pyrazine, trimethyl- (2.78%); Pyrazine, methyl- (1.56%); Carbon dioxide -1.54%; 1,2-Benzenediamine, 4-methyl-1.34% and other components, the content of which is less than 1% (Table 3). The total content of various pyrazine derivatives (Pyrazine, methyl-, Pyrazine, 2,5-dimethyl-, Pyrazine, trimethyl- and Pyrazine, 2-methyl-5-(1-propenyl)-, (Z)-) in the VOCs of strain PB-3-1 was 80.15%.

Table 2: Biometric parameters of 3-day-old alfalfa seedlings after treatment of alfalfa seeds with PB-3-1 culture broth

Experience options	CFU/ml	Root		Stem	
		Length, cm	%	Length, cm	%
	10^8	3.2±0.43	106	0.88±0.1	108
	10^5	4.26±0.73	142	1.36±0.11	168
Control nutrient medium - without bacteria		3.0±1.0	100	0.81±0.17	100

Table 3: Component composition of the PB-3-1 strain culture broth, identified by the chromatographic method of analysis

No.	Holding time,min	Compounds	Probability of identification, %	Percentage
1	3.336	Carbon dioxide	98	1.54±0.16
2	3.492	(2-Aziridinylethyl)amine	87	5.0±0.12
3	13.465	Oxime-, methoxy-phenyl-_	80	4.26±0.13
4	14.160	Pyrazine, methyl-	79	1.56±0.24
5	14.652	2-Heptanone	84	0.73±0.09
6	17.120	Pyrazine, 2,5-dimethyl-	85	75.36±0.64
7	17.889	3-Octanone	94	3.2±0.49
8	18.171	2-Octanone	79	0.94±0.09
9	20.136	1,2-Benzenediamine, 4-methyl-	77	1.34±0.07
10	20.380	Pyrazine, trimethyl-	87	2.78±0.24
11	23.237	Benzeneacetaldehyde	88	0.51±0.06
12	23.840	Pyrazine, 2-methyl-5-(1-propenyl)-, (Z)-	70	0.45±003
13	23.904	Acetophenone	92	0.95±0.01
14	29.776	2-Piperidinone	85	0.59±0.02
15	38.984	Isopropyl myristate	88	0.85±0.09

Pyrazines and their derivatives with antibacterial activity were identified in the VOC profile of B. megaterium (Grahovac et al., 2023). Antifungal activity was found in pyrazine derivatives of Lactobacillus bacteria (Li et al., 2012; Elamin et al., 2021). Huang et al. (2025) reported that B. velezensis can produce pyrazine VOCs and chemically synthesized pyrazine VOCs can also inhibit the growth of F. graminearum. Also Rajini et al. (2011) noted the antimicrobial and insecticidal activities of pyrazine derivatives in their work. In addition, the growth-promoting activity of pyrazines has been established (Heenan-Daly et al., 2021; Wang et al., 2023). Some authors noted that bacterial pyrazines could be used as ingredients in pesticides and insecticides (Moore et al., 1990; Dickschat et al., 2007). The component 2-Heptanone is found in the VOCs of microorganisms, including B. thuringiensis, it belongs to the aliphatic ketones and is known as an insect repellent pheromone (Saccà et al., 2021; Chandrasekaran et al., 2023). The component 3-Octanone is mentioned in the scientific literature as an insecticide for the control of garden snail (Yavasoglu et al., 2023). Acetophenone for the control of rice weevil Sitophilus oryzae was reported by Ramadan et al. (2024), and de Aguiar et al. (2016) reported the antifungal activity of Acetophenone. Thus, it can be assumed that the pyrazines present in the VOC composition are one of the components that determine the antifungal and insecticidal activity of the PB-3-1 strain. Such components as 3-Octanone, 2-Heptanone and Acetophenone can have a synergistic effect and enhance both the insecticidal and antifungal activity of the PB-3-1 strain.

Another limitation to this study is the lack of in vivo/field trials. The results of the study have not been validated in actual field settings; and are purely based on laboratory experiments. The effectiveness of Bacillus thuringiensis PB-3-1 may be strongly impacted by environmental variables like temperature, humidity, and soil microbiota. Its consistency and practical applicability in agricultural settings are still unknown in the absence of in vivo or field trials. Also, the possibility of developed resistance by insects to *B. thuringiensis* strains should be considered when implementing the PB-3-1 strain into IPM programs, to prevent this, rotation with other biocontrol agents, use of multi-toxin strains, and field monitoring for resistance should be implemented (Su, 2022; Afzal et al., 2024).

Conclusion

Laboratory evaluations demonstrated that the novel strain *Bacillus thuringiensis* PB-3-1 exhibits significant insecticidal and antifungal potential without causing phytotoxic effects. The culture broth (10^8 CFU/mL), obtained through deep cultivation on a corn flour-based medium, induced 50% mortality in *Phytonomus variabillis* larvae within 10 days of feeding on treated foliage. Furthermore, seed treatment with 10^5 CFU/mL culture broth markedly enhanced alfalfa growth, stimulating root and shoot development by 42% and 68%, respectively, compared to the control. Analysis of volatile organic compounds (VOCs) revealed that pyrazines, accounting for 80.15% of the VOC profile, likely play a key role in the observed antifungal and insecticidal effects, while compounds such as 3-octanone, 2-heptanone, and acetophenone may act synergistically to enhance bioactivity. Recent evidence highlights microbial VOCs as promising agents for suppressing pathogens and promoting plant health, offering sustainable alternatives to chemical pesticides and fertilizers. Overall, these findings suggest that *B. thuringiensis* PB-3-1 holds strong potential as a multifunctional biocontrol agent against *P. variabillis*, *Fusarium oxysporum*, and *F. solani* in alfalfa cultivation.

DECLARATIONS

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Conflict of Interest: None.

Data Availability: Data will be available at request.

Ethics Statement: This article does not involve research with human participants or vertebrate animals. The experiments were conducted with microbial cultures, insect larvae, and fungal isolates under controlled laboratory conditions, following standard biosafety procedures.

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