



Determination of the *in vitro* biocontrol potential and mechanisms of action of rhizobacterial antagonist isolates from sugar beet plants against *Fusarium culmorum*

Şeker pancarı bitkilerinden izole edilen antagonist kökbakteri izolatlarının *Fusarium culmorum*'a karşı *in vitro* biyolojik mücadele potansiyelleri ve etki mekanizmalarının belirlenmesi

Murat ÖZTÜRK¹ , Soner SOYLU^{2*} , Şeval KOÇ³ , Emin AYDIN⁴ , Sevim ATMACA⁵

^{1,5}Yozgat Bozok University, Agriculture Faculty, Department of Plant Protection, 66900 Yozgat, Türkiye

²Hatay Mustafa Kemal University, Agriculture Faculty, Department of Plant Protection, 31034 Hatay, Türkiye

³Yozgat Bozok University, Institute of Graduate, Department of Agriculture Sciences, 66900 Yozgat, Türkiye

⁴Yozgat Bozok University, Institute of Graduate, Department of Plant Protection, 66900 Yozgat, Türkiye

¹<https://orcid.org/0000-0002-9677-3651>, ²<https://orcid.org/0000-0003-1002-8958>, ³<https://orcid.org/0009-0005-5892-0517>, ⁴<https://orcid.org/0000-0002-7853-8885>, ⁵<https://orcid.org/0000-0001-8568-3469>

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*Address for Correspondence:

Soner SOYLU

e-mail:

soylu@mku.edu.tr

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ABSTRACT

Soil-borne fungal pathogens of the *Fusarium* species cause significant yield and quality losses in economically important crops. *Fusarium culmorum* (W.G. Smith) Sacc. is a common pathogen associated with crown rot, root rot, and head blight in cereals. Biological control agent (BCA) antagonistic bacterial isolates are a sustainable approach to managing soil-borne pathogens. This study aimed to evaluate the *in vitro* antifungal potential of rhizobacterial isolates obtained from healthy sugar beet plants against *F. culmorum*. The *in vitro* biological control potential of 11 isolates, among 43 potential bacterial isolates obtained from the rhizosphere of sugar beets grown in ten different fields in Yozgat, Turkey, was evaluated in detail. The average inhibition rate of mycelium growth of the fungal pathogen by the 11 selected representative bacterial isolates was 40.45%. The most effective isolates were *Paenibacillus polymyxa* 14/7 (74.14%), followed by *Bacillus mojavensis* 45/2 (71.75%) and *Bacillus thuringiensis israelensis* 51/3 (68.58%). The antagonistic activity of the most effective isolates was associated with a combination of mechanisms, particularly iron competition via siderophores, protease activity causing cell wall degradation, and volatile antifungal ammonia production, rather than a single factor. This study demonstrated that root-associated bacteria isolated from healthy sugar beet plants possess significant *in vitro* antagonistic potential against *F. culmorum*. The *in vitro* results suggest that *Paenibacillus polymyxa* 14/7, *Bacillus mojavensis* 45/2, and *Bacillus thuringiensis israelensis* 51/3 are the most promising candidates for the development of biological control formulations against *F. culmorum* in wheat.

Key Words: Antagonistic bacteria, biological control, wheat, *Fusarium culmorum*, soil-borne

ÖZ

Solgunluk ve kök çürüklüğü hastalıklarına neden olan toprak kökenli fungal hastalık etmeni *Fusarium* türleri ekonomik öneme sahip kültür bitkilerinde ciddi verim ve kalite kayıplarına neden olur. *Fusarium culmorum* (W.G. Smith) Sacc., tahıllarda *Fusarium* taç çürüklüğü, kök çürüklüğü ve başak yanıklığı ile ilişkili yaygın bir patojendir. Sağlıklı bitkilerden elde edilen antagonistik bakteriler kullanılarak yapılan biyolojik mücadele, topraktan bulaşan patojenler ile mücadelede sürdürülebilir bir yöntemdir. Bu çalışma,



sağlıklı şeker pancarı bitkilerinden elde edilen kök bakterisi (rizobakteriyel) izolatların *Fusarium culmorum*'a karşı *in vitro* antifungal potansiyellerini belirlemek amacıyla yapılmıştır. Türkiye'nin Yozgat ilindeki 10 farklı tarlasında yetişen şeker pancarı rizosferlerinden elde edilen 43 potansiyel bakteri izolatı arasından seçilen 11 izolatın *in vitro* biyolojik mücadele potansiyeli ayrıntılı olarak değerlendirilmiştir. Temsili seçilen 11 bakteri izolatının fungal etmenin misel gelişimini ortalama engelleme oranı %40,45 olarak belirlenmiştir. Misel gelişimini engelleyen en etkili antagonist bakteri izolatı, %74,14 engelleme oranı ile *Paenibacillus polymyxa* 14/7 olup, bu izolatı %71,75 engelleme oranı ile *Bacillus mojavensis* 45/2 ve %68,58 engelleme oranı ile *Bacillus thuringiensis israelensis* 51/3 izolatları izlemiştir. En etkili bakteri izolatlarının antagonistik etkinliği, tek bir faktörden ziyade, siderofor aracılı demir rekabeti, hücre duvarı bozunmasına neden olan proteaz aktivitesi ve uçucu antifungal amonyak üretimi gibi bir dizi mekanizmanın birleşimi ile bağlantılı olduğu belirlenmiştir. Bu çalışma, sağlıklı şeker pancarı bitkilerinden elde edilen kökbakterilerinin *F. culmorum*'a karşı önemli *in vitro* antagonistik potansiyele sahip olduğunu göstermiştir. *In vitro* çalışma sonuçları *Paenibacillus polymyxa* 14/7, *Bacillus mojavensis* 45/2 ve *Bacillus thuringiensis israelensis* 51/3 izolatlarının buğdayda *F. culmorum*'a karşı biyolojik mücadele formülasyonları geliştirmek için en uygun adaylar olduğunu göstermiştir.

Anahtar Kelimeler: Antagonist bakteri, biyolojik mücadele, buğday, *Fusarium culmorum*, toprak kökenli

Introduction

Wheat (*Triticum aestivum* L.), one of the world's oldest domesticated cereals, was first cultivated in the Fertile Crescent around 10,000 years ago. Today, it remains a staple food crop and plays a critical role in global food security. According to the Food and Agriculture Organization of the United Nations (FAO, 2023), global wheat production reached approximately 785 million tonnes in 2023, cultivated on around 220 million hectares. The top three wheat-producing countries were China, India, and Russia. In Turkey, wheat production is of strategic agricultural importance. In 2023, Turkey produced about 20.5 million tonnes of wheat on approximately 6.8 million hectares (TUIK, 2023). The main wheat-growing regions in Turkey are Central Anatolia, Southeastern Anatolia, and Marmara, with the highest production in the provinces of Konya, Ankara, and Diyarbakır.

Wheat production is limited by various biotic stressors, including fungal, viral, and bacterial pathogens, as well as competitive weeds. Fungal diseases, in particular, cause substantial yield losses worldwide, affecting roots, stems, leaves, and grains. Among soil-borne fungal pathogens, species in the genera *Fusarium*, *Rhizoctonia*, *Pythium*, and *Gaeumannomyces* are responsible for significant root and crown diseases, leading to reduced plant vigour, yield, and grain quality. These pathogens thrive in diverse agro-ecological zones and are often difficult to manage due to their persistence in soil and crop residues.

Root and crown rot diseases caused by soil-borne fungi are among the most destructive threats to wheat production. *Fusarium culmorum* (W.G. Smith) Sacc. is a prevalent pathogen associated with *Fusarium* crown rot, root rot, and head blight in cereals. This fungus not only causes direct tissue damage but also produces trichothecene mycotoxins, such as deoxynivalenol, which pose serious risks to human and animal health. Recent studies continue to highlight the economic impact of *F. culmorum* in major wheat-growing regions worldwide (Shakhnazarova et al., 2023; Kouki et al., 2023). In Turkey, *F. culmorum* is frequently reported in wheat-growing areas, causing significant yield reductions under favourable environmental conditions, such as high soil moisture and moderate temperatures (Imriz et al., 2020).

The management of *F. culmorum* has relied heavily on chemical fungicides. However, chemical control presents several challenges, including environmental pollution, residual toxicity in grains, and the emergence of fungicide-resistant pathogen strains. Prolonged use of chemicals also disrupts soil microbial balance and poses risks to non-target organisms. These drawbacks highlight the urgent need for sustainable and environmentally friendly disease management strategies. In this context, biological control using antagonistic microorganisms has received considerable attention as a viable alternative.

Biological control using plant growth-promoting rhizobacteria (PGPR) and antagonistic bacteria

offers a sustainable method to suppress soil-borne pathogens (Kumar et al., 2012; Figueroa-López et al., 2016; Comby et al., 2017; Bozkurt & Soylu, 2019; Soylu et al., 2020). In Turkey, several studies over the past decade have demonstrated the potential of native bacterial isolates in controlling *F. culmorum*. For example, Imriz et al. (2020) reported that *Bacillus* spp. strains with ACC-deaminase activity reduced *F. culmorum* infections in wheat by up to 81.25% under controlled conditions. Similarly, Senol et al. (2014) purified chitinase enzymes from *Bacillus subtilis* TV-125, which showed significant antifungal activity against *F. culmorum*. Other studies have highlighted the role of *Pseudomonas fluorescens* in inducing systemic resistance in barley and wheat against *F. culmorum* (Roberti et al., 2000; Strunnikova et al., 2015; Ridout et al., 2019; Podgorska-Kryszczuk et al., 2022; Shakhnazarova et al., 2023). Additionally, research by Kouki et al. (2023) demonstrated the effectiveness of *Bacillus subtilis* and *B. amyloliquefaciens* in reducing disease severity and enhancing growth in Tunisian wheat cultivars. These findings collectively highlight the potential of bacterial antagonists as biocontrol agents (BCAs) in integrated disease management systems (Stein, 2005). Furthermore, combining biological control agents with organic amendments, as demonstrated in chrysanthemum (Zhao et al., 2016) and watermelon (Ling et al., 2010), may enhance efficacy and sustainability.

This study aims to evaluate the *in vitro* antagonistic potential of rhizobacterial isolates obtained from healthy sugar beet plants against *Fusarium culmorum*, the causal agent of wheat root rot. Using dual-culture assays, we assessed the ability of selected bacterial isolates to inhibit mycelial growth of the pathogen. Furthermore, we characterized key mechanisms involved in antifungal activity, such as the production of siderophore, the lytic enzyme protease, hydrogen cyanide and ammonia. The findings of this study are expected to contribute to the development of effective and sustainable biocontrol strategies for managing *F. culmorum* in wheat production systems.

Material and Methods

Materials

The *Fusarium culmorum* isolate used in this study was kindly provided by Prof. Dr Soner AKGÜL from Çukurova University, Faculty of Agriculture, Department of Plant Protection. Antagonistic rhizobacterial isolates were obtained from the root regions of healthy sugar beet plants cultivated in various districts of Yozgat province, Turkey. All culture media used in the study, including King's B Agar (KB), Luria Bertani Broth (LB), Nutrient Agar (NA), Tryptic Soybean Agar (TSA), and Potato Dextrose Agar (PDA), were purchased commercially (Merck, Darmstadt, Germany). Media for specific mechanism assays were prepared by supplementing with the relevant chemicals. Other materials included standard laboratory equipment, glassware, plasticware, and chemicals available in the Plant Protection Department laboratories.

Methods

Isolation of Bacterial Isolates

Epiphytic and endophytic bacteria were isolated from root samples of healthy sugar beet plants following the procedures of Aktan and Soylu (2020). Briefly, for endophytic isolation, root segments were surface-sterilized sequentially with 70% ethanol and 2% sodium hypochlorite (NaOCl), then rinsed with sterile distilled water. The sterilised tissues were macerated in sterile 50 mM MgCl₂ buffer. The resulting suspension was serially diluted and spread onto KB and TSA agar plates. For epiphytic bacteria, root fragments were shaken directly in MgCl₂ buffer without surface sterilization, and the suspension was diluted and plated. All plates were incubated at 26°C for 48 hours. Morphologically distinct colonies were purified and stored on TSA slants at +4°C or in 40% glycerol at -80°C for long-term preservation.

Screening of Bacterial Isolates for Pathogenicity

All putative bacterial isolates were screened to confirm they were not plant pathogens by using two standard assays.

Hypersensitive Response (HR) Test on Tobacco

Bacterial suspensions (10^8 CFU/mL in 50 mM MgCl_2) were prepared from 48-hour-old bacterial cultures. Suspensions (50 μL) were injected into the interveinal areas of leaves of 8-week-old tobacco plants (*Nicotiana tabacum*). *Pseudomonas syringae* pv. *phaseolicola* Pph12 was used as a positive control. Reactions were observed after 24–48 hours (Lelliot & Stead, 1987).

Potato Slice Soft Rot Test

Surface-sterilised potato tubers were sliced aseptically. Fresh bacterial colonies from HR-negative isolates were inoculated into wells made in the potato slices. Inoculated slices were placed in humid chambers and incubated at 26°C for 48 hours (Lelliot & Stead, 1987). *Pectobacterium carotovorum* subsp. *carotovorum* was used as a positive control. The presence of maceration and soft rot at the inoculation site was evaluated.

Isolates that did not cause HR on tobacco leaves or soft rot on potato slices were further tested for human pathogenicity by assessing their ability to grow at 37°C on TSA for 48–72 hours. Isolates showing no growth at 37°C were considered non-human pathogens. Following these comprehensive tests, bacterial isolates were confirmed as non-pathogenic to both plants and humans and were selected for subsequent antagonism assays.

In vitro Antagonistic Activity by Dual Culture Assay

The *in vitro* antagonistic potential of the putative bacterial isolates against *F. culmorum* was assessed using a dual culture assay on PDA plates (90 mm diameter), following the method described by Soylu et al., (2021). Briefly, a bacterial streak (4 cm long) was made on one side of the PDA plate and incubated at 25°C for 48 hours. A mycelial plug (6 mm diameter) from a 5-day-old culture of *F. culmorum* was then placed 4 cm from the bacterial streak. Control plates contained only the fungal plug. All plates were incubated at 25°C until the mycelium fully covered the control plates (3–5 days).

The inhibition of mycelial growth was calculated using Abbott's formula:

$$\text{Inhibition of Mycelial Growth (IMG) \%} = [(Mc -$$

Mt) / Mc] $\times$ 100, where Mc is the mycelial growth radius (mm) in the control plate and Mt is the mycelial growth radius towards the bacterium in the dual culture plate.

Based on the IMG percentage, isolates were categorized into four groups: (i) ineffective (0%), (ii) weakly inhibitory (0.5–24.9%), (iii) moderately inhibitory (25.0–49.9%), and (iv) strongly inhibitory ($\geq 50.0\%$) (Gümüş & Soylu, 2024). Each combination was tested in three replicate plates, and the entire experiment was repeated twice. Isolates showing no inhibition were excluded.

Identification of Bacterial Isolates

The selected bacterial isolates were identified to species level using Matrix-Assisted Laser Desorption/Ionisation Time-of-Flight Mass Spectrometry (MALDI-TOF MS) according to a standard protocol (Aktan & Soylu, 2020; Ferreira et al., 2011). Briefly, a small amount of bacterial biomass from a pure 48-hour culture was processed with ethanol, formic acid, and acetonitrile. The extracted supernatant (1 μL) was spotted in duplicate onto a target plate, overlaid with 1 μL of HCCA matrix (α -Cyano-4-hydroxycinnamic acid), and allowed to dry. Protein mass spectra were acquired using a Microflex LT MALDI-TOF MS (Bruker Daltonics, Germany) and analysed with Biotyper 3.0 software by comparison with reference spectra in the library.

Characterization of Antagonistic Mechanisms

The identified bacterial isolates were tested to produce metabolites involved in antagonism.

Siderophore production was tested on Chrome Azurol S (CAS) agar plates (Schwyn & Neilands, 1987). The formation of an orange-yellow halo around the bacterial colony after 3–5 days of incubation at 26°C indicated siderophore production.

Protease activity was assessed on Skimmed Milk LB Agar (SMLBA) (Perneel et al., 2007). A clear zone (halo) around the colony after 3–5 days at 26°C indicated proteolytic activity.

Ammonia (NH_3) production was detected using Nessler's reagent (Cappuccino & Sherman, 1992). Bacterial isolates were grown in peptone water for 4 days at 26°C. After centrifugation, 50 μL of

Nessler's reagent was added to the supernatant. A colour change from pale yellow to brownish-yellow indicated ammonia production.

Hydrogen cyanide (HCN) production was assessed by the method of Bakker and Schippers (1987). Bacteria were streaked on TSA supplemented with 4.4 g/L glycine. A filter paper soaked in a picric acid–sodium carbonate solution was placed in the lid. After sealing and incubating at 26°C for 3–5 days, a colour change of the filter paper from yellow to orange-brown indicated HCN production.

Qualitative and Semi-Quantitative Assay Scoring Criteria

To ensure objectivity, reproducibility, and precise interpretation of the qualitative and semi-quantitative assays, the observed results were scored based on explicitly defined criteria. The production of siderophores and extracellular proteases was evaluated using the Chrome Azurol S (CAS) agar plate method and skim milk agar plates, respectively. After incubation, the diameter of the clear halo zone around the bacterial colony was measured and scored as follows: (-): No inhibition/clear zone observed; ($\pm$): A clear zone with a diameter between 1 and 5 mm; (+): A clear zone with a diameter between 6 and 10 mm. (++) : A clear zone with a diameter greater than 10 mm

Ammonia production was assessed in peptone water using Nessler's reagent, while HCN production was evaluated on glycine-supplemented agar using picric acid-soaked filter paper. The intensity of the colorimetric reaction was scored according to the following benchmarks: (-): No color change observed; ($\pm$): A trace or faint color change, indicating minimal production; (+): A low but distinct color change, indicating moderate production; (++) : An intense and immediate color change, indicating high production (Gümüş & Soylu, 2024).

All mechanism assays were performed with three replications per isolate and repeated twice.

Experimental Design and Statistical Analysis

Both *in vitro* antagonism and mechanism studies were arranged in a completely randomised design. All experiments were conducted with three

replicates and repeated at least twice. Data for mycelial growth inhibition, efficacy indices, and other quantitative measurements were subjected to one-way analysis of variance (ANOVA) using SPSS Statistics software (Version 17.0). Mean separations were performed using Duncan's Multiple Range Test at a significance level of $P \leq 0.05$.

Results and Discussion

Isolation and Preliminary Screening of Bacterial Isolates

Bacterial isolates putatively considered as biocontrol agents (BCAs) were collected from the root regions of healthy sugar beet plants grown in various districts of Yozgat province, Turkey. A total of 122 bacterial isolates were initially obtained. Of these, 75 representative candidate isolates were selected for further screening to confirm they were not plant or human pathogens. All 75 isolates underwent two standard assays: the hypersensitive response (HR) test on tobacco leaves and a soft rot assay on potato slices. Sixty-two isolates that did not induce HR or soft rot were further tested for human pathogenicity by assessing their ability to grow on Tryptic Soy Agar (TSA) at 37°C for 48–72 hours. Isolates showing no growth at 37°C were considered non-pathogenic to humans. Following these tests, 43 candidate bacterial isolates were confirmed as non-pathogenic and selected for subsequent antagonism assays.

Identification of Bacterial Isolates

The antagonistic activity of the 43 bacterial isolates against the mycelial growth of *F. culmorum* was evaluated using dual culture tests on Potato Dextrose Agar (PDA) medium (Soylu et al., 2021). Isolates showing no inhibition were excluded from further study. Based on their inhibition potential, 11 representative isolates were selected for identification using the MALDI-TOF MS method. The identification results for the 11 selected bacterial isolates are presented in Table 1. The isolates belonged to the genera *Paenibacillus*, *Bacillus*, *Priestia*, *Enterobacter*,

Pantoea, and *Microbacterium*. Six isolates were Gram-positive and five were Gram-negative. The isolates originated from various plant parts, including roots, stems, leaves, and fruit. The

MALDI-TOF similarity indices (SI) ranged from 2.15 to 2.33, indicating reliable species-level identification (Figure 1).

Table 1. Identification results and general characteristics of bacterial isolates obtained from sugar beet plants.

Bacterial isolates	Gram Reaction*	MALDI SI
<i>Paenibacillus polymyxa</i> 14/7	+	2.34
<i>Bacillus mojavensis</i> 45/2	+	2.18
<i>Bacillus thuringiensis israelensis</i> 51/3	+	2.27
<i>Priestia megaterium</i> 76/2	+	2.17
<i>Paenibacillus amylolyticus</i> 64/2	+	2.21
<i>Enterobacter asburiae</i> 58/3	-	2.22
<i>Pantoea agglomerans</i> 67/3	-	2.15
<i>Priestia megaterium</i> 98/5	+	2.25
<i>Enterobacter asburiae</i> 76/4	-	2.18
<i>Paenibacillus amylolyticus</i> 113/5	+	2.17
<i>Microbacterium phyllosphaerae</i> 115/2	+	2.33

* -: Gram negative; +: Gram positive; SI: Similarity indices

Rank (Quality)	Matched Pattern	Score Value	NCBI Identifier
1 (+++)	<i>Paenibacillus polymyxa</i> DSM 36T DSM_2	2.343	1406
2 (+)	<i>Paenibacillus polymyxa</i> DSM 36T DSM	1.767	1406
3 (-)	<i>Paenibacillus jamilae</i> DSM 13815T DSM	1.626	114136

Figure 1. Identification of the most efficient representative bacterial isolate *Paenibacillus polymyxa* 14/7 by MALDI-TOF MS based on the protein spectra in the MALDI-TOF/MS library

Antagonistic Activity Against *Fusarium culmorum*

The antagonistic effects of the 11 identified bacterial isolates on the mycelial growth of *F. culmorum* are shown in Table 2 and Figure 2. The mean inhibition percentage for all 11 isolates was 40.45%. Five isolates (*Paenibacillus polymyxa* 14/7, *Bacillus mojavensis* 45/2, *Bacillus thuringiensis israelensis* 51/3, *Priestia megaterium* 76/2, and *Paenibacillus amylolyticus* 64/2) showed strong inhibitory activity, with the inhibition of mycelial growth (IMG)% values equal to or greater than 50.0%.

The inhibitory activity of antagonist bacterial

isolates against the mycelial growth of *Fusarium culmorum* was evaluated using one-way analysis of variance (ANOVA). The results revealed highly significant differences among bacterial treatments ($F_{11,60} = 799.72, p < 0.001$). Effect size analysis using eta-squared (η^2) demonstrated that 99.32% of the total variance in mycelial growth inhibition was explained by differences among bacterial isolates ($\eta^2 = 0.9932$). This very large effect size indicates that the antagonistic performance varied markedly among isolates and that bacterial identity was the primary determinant of *F. culmorum* growth suppression under *in vitro* conditions.

Table 2. Antagonistic effects of bacterial isolates on the inhibition (%) of mycelial growth (mm)^a of *Fusarium culmorum* in *in vitro* dual culture tests.

Treatment	<i>F. culmorum</i>	
	MG (mm)	IMG (%)
Control	60.00 ^g	-
<i>Paenibacillus polymyxa</i> 14/7	15.52 ^a	74.14
<i>Bacillus mojavensis</i> 45/2	16.95 ^a	71.75
<i>Bacillus thuringiensis israelensis</i> 51/3	18.85 ^b	68.58
<i>Priestia megaterium</i> 76/2	28.80 ^c	52.00
<i>Paenibacillus amylolyticus</i> 64/2	29.35 ^c	51.08
<i>Enterobacter asburiae</i> 58/3	43.50 ^d	27.50
<i>Pantoea agglomerans</i> 67/3	44.70 ^d	25.50
<i>Priestia megaterium</i> 98/5	48.02 ^e	19.97
<i>Enterobacter asburiae</i> 76/4	48.10 ^e	19.83
<i>Paenibacillus amylolyticus</i> 113/5	48.92 ^{ef}	18.47
<i>Microbacterium phyllosphaerae</i> 115/2	50.35 ^f	16.08

MG: Mycelial Growth; IMG: Inhibition of Mycelial Growth

^a Different letter following mean values within the same column indicate statistically significant differences among treatments (Duncan's Multiple Range Test, $P \leq 0.05$).

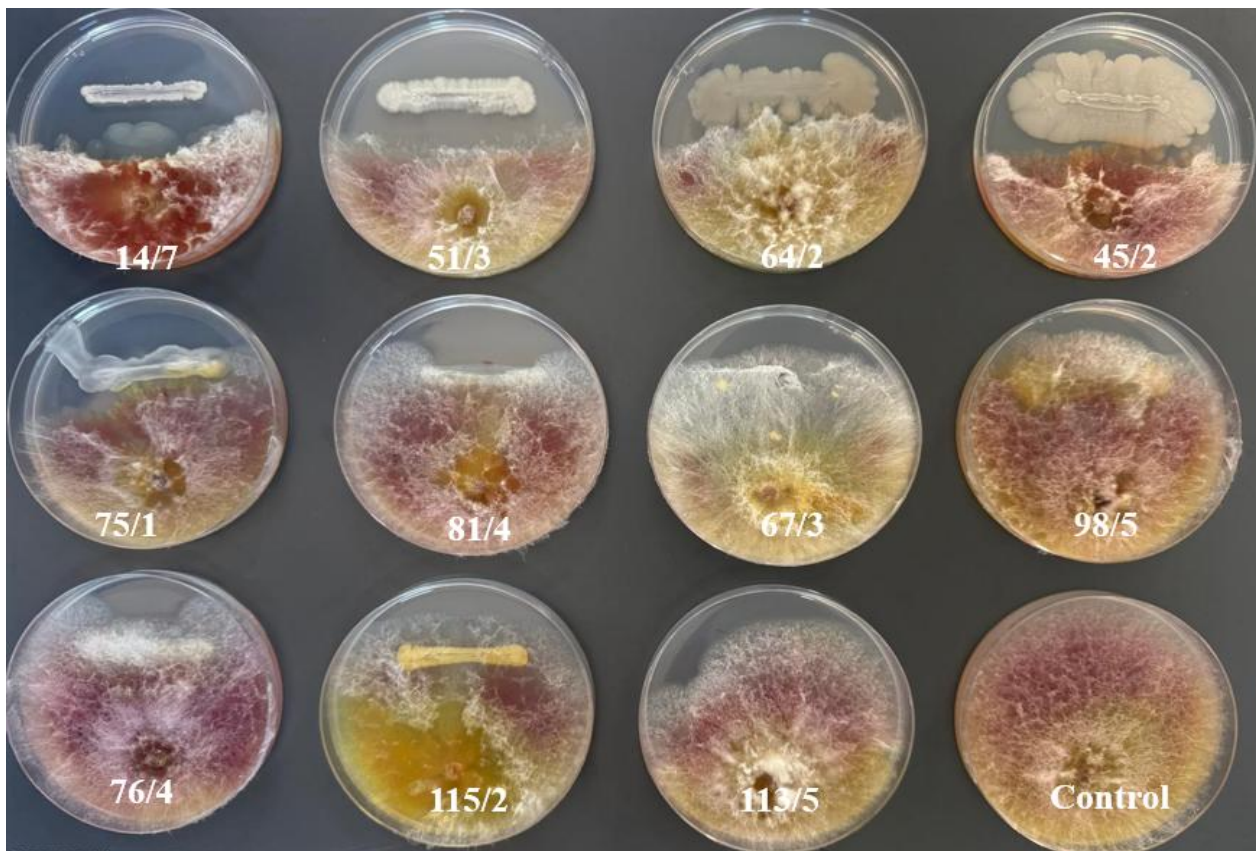


Figure 2. The antagonistic effects of bacterial isolates on the inhibition of mycelial growth of *Fusarium culmorum* in *in vitro* dual culture tests.

The most effective isolate was *Paenibacillus polymyxa* 14/7, which showed 74.14% inhibition, followed by *Bacillus mojavensis* 45/2 (71.75%) and *Bacillus thuringiensis israelensis* 51/3 (68.58%). Statistical analysis (Duncan's test, $P < 0.05$) revealed significant differences in antagonistic efficacy among the isolates. The top three performers (all Gram-positive *Bacillus* spp.) were grouped in statistically superior categories. In

contrast, isolates such as *Priestia megaterium* 98/5, *Enterobacter asburiae* 76/4, *Paenibacillus amylolyticus* 113/5, and *Microbacterium phyllosphaerae* 115/2 showed the lowest inhibitory activity, with IMG% values below 20%, forming statistically distinct groups.

Potential Biocontrol Mechanisms

The *in vitro* potential of the bacterial isolates to produce various biocontrol-related metabolites is

presented in Table 3. The results are expressed using a defined scoring system to ensure clarity and reproducibility. For siderophore and protease assays, scores correspond to the diameter of the hydrolysis halo: (-): No inhibition/clear zone observed; ($\pm$): A clear zone with a diameter between 1 and 5 mm; (+): A clear zone with a diameter between 6 and 10 mm. (++) : A clear zone with a diameter greater than 10 mm. For NH_3 and HCN assays, scores reflect the intensity of the colorimetric reaction: (-) none, ($\pm$) trace or faint, (+) moderate, (++) intense.

The top three antagonistic isolates (*P. polymyxa* 14/7, *B. mojavensis* 45/2, and *B. thuringiensis israelensis* 51/3) consistently showed moderate (+) to high (++) siderophore and protease activities, and high ammonia (NH_3) production. None of the tested isolates produced hydrogen cyanide (HCN). These results suggest that the antagonistic activity of the most effective isolates may be linked to a combination of mechanisms, particularly siderophore-mediated iron competition, protease activity for cell wall degradation, and ammonia production, rather than a single factor.

Table 3. Biocontrol mechanisms of antagonistic bacterial isolates involved in inhibiting the mycelial growth of *F. culmorum*

Bacterial isolate	Siderofofor	Protease	NH_3	HCN
<i>Paenibacillus polymyxa</i> 14/7	++	++	++	-
<i>Bacillus mojavensis</i> 45/2	++	+	++	-
<i>Bacillus thuringiensis israelensis</i> 51/3	+	++	++	-
<i>Priestia megaterium</i> 76/2	+	+	+	-
<i>Paenibacillus amylolyticus</i> 64/2	+	+	++	-
<i>Enterobacter asburiae</i> 58/3	++	++	++	-
<i>Pantoea agglomerans</i> 67/3	+	++	$\pm$	-
<i>Priestia megaterium</i> 98/5	+	++	++	-
<i>Enterobacter asburiae</i> 76/4	++	+	+	-
<i>Paenibacillus amylolyticus</i> 113/5	+	+	+	-
<i>Microbacterium phyllosphaerae</i> 115/2	+	+	$\pm$	-

For siderophore and protease production, ($\pm$): A clear zone with a diameter between 1 and 5 mm; (+): A clear zone with a diameter between 6 and 10 mm. (++) : A clear zone with a diameter greater than 10 mm. For NH_3 and HCN production, (-): No color change observed; ($\pm$): A trace or faint color change, indicating minimal production; (+): A moderate color change, indicating moderate production; (++) : An intense and immediate color change, indicating high production.

This study demonstrated that root-associated bacteria from healthy sugar beet plants possess significant *in vitro* antagonistic potential against *F. culmorum*. The extremely high eta-squared (η^2) value obtained in this study highlights the strong and isolate-dependent inhibitory effects of antagonist bacteria against *F. culmorum*. The finding that more than 99% of the variation in mycelial growth inhibition was attributable to bacterial isolate differences underscores the critical role of specific microbial traits in biocontrol efficacy. Such pronounced effects are commonly associated with mechanisms including the production of antifungal secondary metabolites, secretion of hydrolytic enzymes (e.g., protease, chitinases and glucanases), antifungal compounds, siderophores, HCN, ammonia, competition for

nutrients and space, and the emission of volatile organic compounds (Sülü et al., 2016; Kuştoğan & Soylu, 2025). The strong isolate-specific responses observed here suggest that not all bacterial antagonists are equally effective against *F. culmorum*, emphasizing the importance of targeted screening and selection of high-performing strains. Similar large effect sizes have been reported in *in vitro* biocontrol assays where potent bacterial isolates exerted near-complete suppression of *Fusarium* species, supporting the reliability of the present findings.

The utilization of MALDI-TOF mass spectrometry for the identification of antagonistic bacterial isolates has been established as a reliable analytical approach, gaining prominence as an alternative to chromatographic techniques and

molecular methods dependent on DNA analysis (Panda et al., 2013). Empirical studies further indicate that this technology demonstrates superior accuracy in speciating bacterial strains when compared to conventional diagnostic methodologies (Uysal et al., 2019; Soylu et al., 2021; Bozkurt et al., 2023; Soylu et al., 2024). The most effective isolates belonged to the Gram-positive genera *Paenibacillus* and *Bacillus*, consistent with their well-documented role in biocontrol (Xu et al., 2018). Notably, *Paenibacillus polymyxa* 14/7 (74.14% inhibition), *Bacillus mojavensis* 45/2 (71.75%), and *Bacillus thuringiensis israelensis* 51/3 (68.58%) showed the highest activity. These findings are in agreement with numerous studies in the literature, which consistently report the efficacy of *Bacillus* and *Paenibacillus* species in suppressing various *Fusarium* pathogens.

The strong antagonism of *Paenibacillus polymyxa* observed in this study is supported by multiple reports. For example, *P. polymyxa* was effective against *Fusarium graminearum* in wheat, with biofilm polysaccharides, particularly D-glucuronic acid, playing a critical role in antagonism (Timmusk et al., 2019). Similarly, *P. polymyxa* reduced disease severity and deoxynivalenol (DON) production in wheat heads (He et al., 2009) and controlled *Fusarium* wilt in watermelon when applied as a bioorganic fertiliser (Ling et al., 2010). Another study on cucumber *Fusarium* wilt also identified *P. polymyxa* as an effective antagonist (Tan et al., 2024). The consistency across these studies underscores the robustness of *P. polymyxa* as a biocontrol agent (BCA) against multiple *Fusarium* species.

Bacillus species, particularly *B. mojavensis* and *B. thuringiensis*, also demonstrated high antagonistic activity in this study. This is consistent with findings by Bacon and Hinton (2002), who noted that *B. mojavensis* exhibits endophytic colonisation and antagonism against *Fusarium moniliforme*. *B. thuringiensis* has been reported to produce lipopeptide biosurfactants active against *F. oxysporum* (Deepak & Jayapradha, 2015) and to harbour genes for antimicrobial metabolites

(Adeniji et al., 2021). Other studies have shown *B. amyloliquefaciens* and *B. subtilis* to be effective BCAs against *F. culmorum* in wheat, enhancing plant growth and inducing systemic resistance (Kouki et al., 2023; Zalila-Kolsi et al., 2016). Bacon et al. (2012) characterised endophytic *B. mojavensis* strains and their production of surfactin isomers, which contribute to antifungal activity.

The mechanisms identified in our study such as siderophore production, protease activity, and ammonia production, are frequently reported in the literature (Duman & Soylu, 2019; Kara et al., 2020; Gümüş et al., 2025). The mechanistic profile of these top performers, characterized by simultaneous production of siderophores, proteases, and ammonia, suggests a multifactorial mode of action. For example, siderophore-mediated iron competition and protease activity have been observed in *Bacillus* and *Pseudomonas* strains effective against *Fusarium* spp. (Khalil et al., 2021; Kara & Soylu, 2022; Gümüş & Soylu, 2024). Similarly, *Pseudomonas fluorescens* reduced *F. culmorum* virulence in barley, potentially through induced systemic resistance and toxin detoxification (Shakhnazarova et al., 2023). In contrast, hydrogen cyanide (HCN) production, which was absent in our isolates, has been reported in some *Pseudomonas* and *Bacillus* strains as an antagonistic mechanism against soil-borne fungal pathogens (Adeniji et al., 2020; Gümüş & Soylu, 2024; Tangül & Soylu, 2025). *Bacillus thuringiensis* is known primarily as an insecticide, but certain strains also display antifungal properties. Kim et al. (2004) purified a lipopeptide from *B. thuringiensis* CMB26 with antimicrobial activity. Our isolate, *B. thuringiensis israelensis* 51/3, showed 68.58% inhibition, suggesting it may produce similar antifungal metabolites, thereby expanding its utility beyond insect control.

Not all strains of the same species showed consistent efficacy. For example, while *Priestia megaterium* 76/2 in our study showed moderate inhibition (52.0%), another study reported *P. megaterium* TSA-10E with up to 63.21% inhibition

against *F. oxysporum* (Zhu et al., 2025). This variability may be due to strain-specific traits, differences in experimental conditions, or pathogen isolates. Similarly, *Enterobacter asburiae* and *Pantoea agglomerans* in our study showed lower inhibition ($\leq 27.50\%$), whereas other studies have reported *Pantoea* and *Enterobacter* as effective antagonists against citrus fungal pathogens (Gümüş & Soylu, 2024). These discrepancies highlight the importance of screening multiple strains and considering ecological and genetic diversity.

Conclusion

The consistent identification of *Bacillus* and *Paenibacillus* species across various crops and geographic regions supports their potential as broad-spectrum biological control agents. The results of this *in vitro* study are promising and indicate that *Paenibacillus polymyxa* 14/7, *Bacillus mojavensis* 45/2, and *Bacillus thuringiensis israelensis* 51/3 are strong candidates for developing biocontrol formulations against *F. culmorum* in wheat. Future research should focus on elucidating the molecular basis of antagonism, optimising formulation and application methods, and evaluating multi-strain consortia for integrated disease management.

Author's Contributions

The contribution of the authors is equal. The final version of the manuscript was reviewed and approved by all authors.

Statement of conflict of interest

The authors declare that they have no conflict of interest.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Studies in humans and animals

This work does not involve the study of humans

and animals.

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