



Identification of Bacterial Isolates with Antagonistic Activity Against *Pseudomonas syringae* pv. *atrofaciens*, the Causal Agent of Basal Glume Rot in Wheat, and Their Plant Growth-Promoting Traits

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ABSTRACT

In this study, bacterial isolates exhibiting antagonistic properties against the seed-borne pathogen *Pseudomonas syringae* pv. *atrofaciens* in wheat were investigated. Among a total of 298 bacterial isolates obtained from wheat seed samples, 19 candidate antagonistic isolates were selected based on their pectolytic activity and hypersensitivity test results. Molecular identification revealed that these isolates belonged to the genera *Pseudomonas*, *Bacillus*, *Paenibacillus*, *Citrobacter*, and *Staphylococcus*. In vitro antagonistic tests showed that *Pseudomonas japonica* isolates W57.4 and W57.5, along with *Paenibacillus polymyxa* isolate W58.1, exhibited the highest inhibitory effects. These isolates also demonstrated multiple plant growth-promoting traits such as protease enzyme production, phosphate solubilization, siderophore synthesis, ammonia production, and indole-3-acetic acid (IAA) production. Notably, *P. japonica* isolates (W57.4, W57.5) exhibited strong siderophore production and phosphate solubilization, while *P. polymyxa* (W58.1) showed high protease activity. These multifunctional traits indicate the potential of these isolates to be used as biological control agents against seed-borne pathogens. As the first study in Türkiye focusing on the biological control of seed-borne bacterial pathogens causing basal glume rot in wheat, this research provides a significant contribution to the development of environmentally friendly and effective alternative management strategies.

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Buğday Kavuz Dibi Çürüklüğü Hastalığına Neden Olan *Pseudomonas syringae* pv. *atrofaciens*'e Karşı Antagonist Etkili ve Bitki Gelişimini Teşvik Edici Bakteri İzolatlarının Belirlenmesi

ÖZET

Bu çalışmada, buğdayda tohum kaynaklı hastalık etmeni *Pseudomonas syringae* pv. *atrofaciens*'e karşı antagonistik özellik gösteren bakteri izolatları araştırılmıştır. Buğday tohum örneklerinden izole edilen toplam 298 bakteri izolatı arasından pektolitik aktivite ve hipersensitivite testi sonuçlarına göre 19 aday antagonistik izolat seçilmiştir. Moleküler tanımlama ile bu izolatların *Pseudomonas*, *Bacillus*, *Paenibacillus*, *Citrobacter* ve *Staphylococcus* cinslerine ait olduğu belirlendi. *In vitro* antagonistik testlerde, *Pseudomonas japonica* izolatları W57.4 ve W57.5 ile *Paenibacillus polymyxa* izolatı W58.1 en yüksek inhibitör etkiyi göstermiştir. Bu izolatlar ayrıca proteaz enzimi üretimi, fosfat çözünürlüğü, siderofor sentezi, amonyak ve indol-3-asetik asit (IAA) üretimi gibi çoklu bitki gelişimini destekleyen özellikler sergilemiştir. Özellikle *P. japonica* izolatları (W57.4, W57.5) güçlü siderofor üretimi ve fosfat çözünürlüğü gösterirken, *P. polymyxa* (W58.1) yüksek proteaz aktivitesiyle dikkat çekmiştir. Bu çok işlevli özellikler, söz konusu izolatların tohum kaynaklı patojenlerin kontrolünde biyolojik mücadele ajanı olarak kullanılma potansiyelini ortaya koymaktadır. Türkiye'de buğdayda tohum kaynaklı bakteriyel patojen olan kavuz dibi çürüklüğünün biyolojik kontrolüne yönelik gerçekleştirilen ilk çalışma

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olması bakımından, bu araştırma çevre dostu ve etkili alternatif mücadele yöntemlerinin geliştirilmesine önemli bir katkı sunmaktadır.

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INTRODUCTION

Cereals are among the most strategically important agricultural commodities worldwide, and wheat (*Triticum aestivum* L.) stands out as one of the most significant species due to its high adaptability, extensive cultivation area, and critical role in human nutrition. Approximately 48% of global cereal production is attributed to wheat, which supplies around 21% of the daily caloric intake and 20% of the protein requirement (Rajaram, 2010).

Türkiye contributes about 3% of global wheat production and ranks among the top ten wheat-producing countries, particularly in the production of both bread and durum wheat. During the 2020–2021 period, more than 770 million tons of wheat were produced globally on approximately 220 million hectares of land. In Türkiye, wheat is cultivated on about 8.3 million hectares, with an annual production averaging around 22 million tons (Anonymous, 2023). The Central Anatolia Region of Türkiye represents the most significant wheat-producing area, where approximately 35% of total agricultural land is devoted to wheat cultivation (Pekcan et al., 2006).

However, wheat production is severely threatened by both abiotic and biotic stress factors. Yield losses caused by diseases and pests can reach up to 20%, with approximately 21.5% of these losses attributed to plant diseases (Savary et al., 2019). In addition to fungal pathogens, bacterial diseases also result in significant economic losses in wheat cultivation. The most common bacterial pathogens that attack wheat are reported as *Xanthomonas translucens* (Bragard et al., 1997) and *Pseudomonas syringae* pathovars (Tambong, 2022).

P. syringae, a Gram-negative bacterium, possesses a broad host range and is capable of causing disease in numerous plant species (Young, 2010). One of its pathovars, *P. syringae* pv. *atrofaciens* (McCulloch, 1920), is responsible for basal glume rot disease in wheat. It was first identified in the United States and has since been reported in many other countries (Zaharieva, 1995; Maraite & Weyns, 1997).

P. syringae pv. *atrofaciens* is an important bacterial pathogen capable of infecting a wide range of cereal crops, primarily wheat, as well as barley, rye, oats, and durum wheat (Alexandrova et al., 1995; Matveeva et al., 2003; Slovareva, 2020). In addition, it has been isolated from several wild grass species, including foxtail (*Alopecurus* spp.), wild oats (*Avena fatua*), oat grass, barley grass, and *Cynodon dactylon* (Taghavi & Keshavarz, 2003). The bacterium has also been reported to exhibit pathogenicity on lilac and shows a high degree of similarity to *P. syringae* pv. *syringae* (Iacobellis et al., 1997).

This pathogen has been reported in various countries. In Russia, it has been identified as a significant causal agent of basal glume rot (Matveeva et al., 2003), and extensive studies have also been conducted in Ukraine (Pasichnyk, 2000; Butsenko and Pasichnyk, 2018). In Bulgaria, research has focused on virulence variability and race differentiation (Vassilev et al., 1996). In Germany, the disease has been found to be widespread, although associated with minor economic losses (Toben et al., 1991). In Belgium, it has been occasionally observed causing noticeable symptoms in the cultivar Fidel, while in Italy, it has been isolated from various cereals, especially durum wheat (Alexandrova et al., 1995). In Iran, it was reported for the first time in wheat (Kazempour et al., 2010). Furthermore, the pathogen is listed as a quarantine organism in Mexico and Egypt (Slovareva, 2020).

The epidemiology of the basal glume rot disease is closely linked to environmental conditions. The disease typically appears during the heading stage, initially manifesting as dark green to brown lesions at the base of immature glumes. These lesions gradually expand and darken, turning black as the infection progresses toward the grain, ultimately causing significant losses in both quality and yield (Toben et al., 1989; Slovareva, 2020). Strains of the pathogen can be highly epidemic due to their ability to spread over long distances through airborne transmission (Alexandrova et al., 1995; Tambong, 2022). Moreover, epiphytic populations may serve as primary sources of infection under favorable environmental conditions. The bacterium is also seed-borne and can persist latently as an endophyte within the seed (Taghavi & Keshavarz, 2003). As the infection progresses, it interferes with grain filling, ultimately reducing yield. A study conducted in Lithuania reported yield losses ranging from 5% to 50% depending on the severity of infection (Butsenko et al., 2020).

Research on bacterial diseases of cereals in Türkiye remains quite limited, and studies specifically focusing on *P. syringae* pv. *atrofaciens*, the causal agent of basal glume rot in wheat, is almost nonexistent. A comprehensive

study recently conducted by Aydın (2025) addressed the isolation, pathogenicity, and molecular characterization of *P. syringae* pv. *atrofaciens* in wheat-growing areas of Yozgat Province. In that study, fluorescent *Pseudomonas* colonies isolated on King's B medium from wheat seeds were tested for pathogenicity through artificial inoculation on wheat leaves. The pathogenic isolates were classified as Group 1a *P. syringae* based on LOPAT tests. Molecular characterization using MALDI-TOF MS and *rpoD* gene sequencing revealed high similarity to the reference strain *P. syringae* pv. *atrofaciens* CFBP2213 PT. Furthermore, BOX-PCR analysis distinguished the isolates into two different genotypes. All isolates were confirmed to be pathogenic and exhibited characteristic traits specific to the pathotype at both biochemical and molecular levels. This study represents the first detailed report confirming the presence of *P. syringae* pv. *atrofaciens* in wheat production areas of Türkiye.

Maraite et al. (2007) reported that, like many other crops, wheat is affected by various bacterial diseases that hinder optimal leaf development, reduce yield, and impose restrictions on international grain trade. However, compared to fungal diseases such as rusts, *Helminthosporium*, and *Septoria* leaf blotches, farmers generally pay less attention to the regular monitoring and management of bacterial diseases. One of the main reasons for this is that symptoms caused by bacterial infections are often confused with those resulting from physiological disorders or environmental stress conditions. Moreover, bacterial epidemics typically require a specific combination of climatic factors to develop, which leads to considerable variability in disease occurrence across years, regions, and even continents. Variations in cultivar susceptibility and seed-borne infection levels further contribute to this inconsistency. Maraite et al. (2007) emphasized that chemical control alone is insufficient for effective disease management; thus, the use of disease-free seed material and the cultivation of resistant cultivars should be adopted as the primary strategies for controlling bacterial diseases.

Due to the adverse effects of chemical pesticides on human health and the environment (such as phytotoxicity, residue accumulation, resistance development, and ecosystem disruption), biological control management has gained increasing importance within the scope of sustainable agricultural practices (Bale et al., 2008; Tariq et al., 2020). Biological control methods involving beneficial microorganisms have emerged as a promising alternative to chemical applications in the management of bacterial diseases in plants (Varhan& Bozkurt, 2021). In particular, antagonistic bacteria that establish symbiotic or commensal relationships with plants are considered ideal beneficial agents, owing to their potential to suppress pathogens and promote plant growth through mechanisms such as indole-3-acetic acid (IAA) production, siderophore synthesis, phosphate solubilization, ammonia and protease production (Schulz & Boyle, 2006; Hazarika et al., 2021; Shreshtha et al., 2025).

Apart from antibiotics and copper agents, no registered bactericides are currently available for controlling many of the members of *P. syringae*. However, the frequent use of these antibiotics and copper compounds has accelerated the development of resistance in the pathogen and led to significant environmental and animal health concerns (Zhao et al., 2024). Despite the economic and phytopathological significance of *P. syringae* pv. *atrofaciens*, no biological control studies targeting this pathogen have been conducted within the country to date. To address this critical research gap, the present study aimed to isolate and characterize antagonistic bacterial strains with the potential to suppress the pathogen and to investigate their in vitro biocontrol mechanisms.

MATERIAL and METHODS

Sampling from Wheat Production Areas

In 2023, a total of 200 wheat seed samples were collected from wheat production areas located in Yozgat province and its districts. The samples were placed in dry, clean, and sterile polyethylene bags and transported to the laboratory for further analysis.

Pathogen Culture Used in the Study

The pathogenic bacterial culture used in this study, *Pseudomonas syringae* pv. *atrofaciens* isolate W49.6 was isolated from wheat seeds collected in Yozgat province. The identification of the isolate was performed using pathogenicity testing, MALDI-TOF MS analysis, and phylogenetic analysis based on the *rpoD* gene (Aydın, 2025).

Bacterial Isolation

To isolate antagonistic bacteria from wheat seeds, the seeds were subjected to surface sterilization. They were first rinsed with sterile distilled water, then treated with 70% ethanol for 30 seconds, followed by 1% sodium hypochlorite (NaOCl) solution for 150 seconds. After sterilization, the seeds were rinsed three to four times with sterile distilled water to remove any chemical residues.

Subsequently, 10 g of the seed sample was placed into sterile Falcon tubes, and 30 ml of physiological saline solution (0.85% NaCl) was added. The tubes were incubated at room temperature, at 200 rpm for 4 hours in a

shaking incubator. Then, 100 µl of the resulting suspension was spread onto King's B Agar (KB) medium (López et al., 2018).

The same suspension was also subjected to serial dilutions ranging from 10^{-2} to 10^{-6} and spread onto KB media using a sterile glass rod. The inoculated Petri dishes were incubated at 25 °C for 48 hours. Bacterial colonies that developed were examined based on their morphological characteristics, and those with distinct morphologies were isolated and purified. The purified isolates were stored at -20 °C in cryovials containing 40% sterile glycerol for further analyses (Aktan & Soylu, 2020; Soylu et al., 2022).

Selection of Bacterial Isolates

Purified bacterial isolates were subjected to two different bioassays to determine their potential pathogenicity: pectolytic activity on potato slices and the hypersensitive reaction (HR) on tobacco leaves. These tests were performed to assess whether the isolates posed a risk of causing disease in plants. Isolates that gave positive results in either test were considered to have pathogenic potential and were therefore excluded from further evaluation as antagonistic candidates (Duman & Soylu, 2019; Yörük & Mirik, 2021; Varhan & Bozkurt, 2021).

Molecular Identification of Bacterial Isolates

For the molecular identification of candidate isolates, the 16S rRNA gene region was amplified using the primers 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492r (5'-TACGGTTACCTTGTTACGACTT-3'). A single colony was picked with a sterile pipette tip and directly transferred into a 25 µl PCR tube containing 12.5 µl of 2x master mix (MyTaq Mix, Biorline, UK), 1 µl of forward primer, 1 µl of reverse primer, 1 µl of DMSO, and 9.5 µl of sterile water. PCR amplification was performed in a Bio-Rad T100 thermal cycler using a touch-down protocol as follows: Initial denaturation at 95 °C for 4 minutes, 10 cycles of 94 °C for 30 seconds (denaturation), 65 °C for 30 seconds (annealing, decreasing by 1 °C per cycle down to 56 °C), and 72 °C for 1 minute (extension), followed by 24 cycles at a constant annealing temperature of 56 °C with the same parameters. A final extension was applied at 72 °C for 5 minutes (Aksoy et al., 2017).

PCR products were separated using 1.5% agarose gel electrophoresis, stained with ethidium bromide in 1x TAE buffer at 100 V for 1 hour. Product sizes were compared against a Hyperladder 1 kb DNA marker (Biorline, UK) and analyzed using a gel documentation system. Forward and reverse sequences were processed and assembled using Chromas Pro (v1.7.6) to generate consensus sequences of the 16S rRNA gene. The resulting partial nucleotide sequences were compared against the NCBI GenBank database using BLASTn for taxonomic identification.

Determination of Antagonistic Activity of Bacterial Isolates

The antagonistic effects of the candidate antagonist bacterial isolates against *Pseudomonas syringae* pv. *atropaciens* isolate W49.6 was evaluated using the dual culture method (Varhan & Bozkurt, 2021). From each isolate's 24-hour fresh culture, inoculations were performed at four equidistant points on 9 cm diameter Petri dishes containing KB media. Plates were incubated at 25 °C for 48 hours. After incubation, a pathogen suspension of *P. syringae* pv. *atropaciens* at a concentration of 10^8 cfu/ml (prepared from a 24-hour culture) was evenly sprayed onto the surface using a sterile sprayer. The Petri dishes were then incubated again at 25 °C for an additional 48 hours. Each test was conducted in six replicates and repeated twice independently. At the end of the incubation period, inhibition zones caused by suppression of pathogen growth on the KB medium were observed. For each isolate, bacterial colony diameter (BC) and the inhibition zone diameter (IZ) surrounding the colony were measured. The Antagonistic Index (Ant-Index = IZ / BC) was calculated and used to evaluate the antagonistic potential of each isolate against the pathogen (Ullah et al., 2017).

Determination of *In Vitro* Plant Growth-Promoting Mechanisms of Bacterial Isolates

Protease Enzyme Production Potential

The protease enzyme production potential of candidate antagonist bacterial isolates was evaluated on Luria-Bertani Agar (SMLBA) medium supplemented with 2% skimmed milk powder. From each isolate's two-day-old fresh culture, bacterial inoculum was taken using a sterile toothpick and inoculated at the center of Petri dishes containing SMLBA. The plates were incubated at 25 °C for 48 hours. At the end of the incubation, the formation of a clear zone around the bacterial colony was considered a positive indicator of protease enzyme production. For each isolate, the Protease Production Index (P-index = clear zone diameter / colony diameter) was calculated to quantify proteolytic activity (Perneel et al., 2007). The experiment was conducted in six replicates and repeated twice independently.

Determination of Phosphate Solubilization Potential

The phosphate solubilization potential of the candidate antagonist bacterial isolates was evaluated on Pikovskaya Agar (PVK) medium supplemented with tricalcium phosphate (Kumar et al., 2012). Bacterial inoculum from two-day-old fresh cultures was taken using a sterile toothpick and spot-inoculated onto the surface of PVK medium. The inoculated Petri dishes were incubated at 25 °C for five days.

At the end of the incubation period, the appearance of a clear halo around the bacterial colony was considered a positive indicator of phosphate-solubilizing ability. For each isolate, the Phosphate Solubilization Index (F-index = halo diameter/colony diameter) was calculated. The experiment was conducted in six replicates and repeated twice independently.

Determination of Siderophore Production Potential

The siderophore production potential of the candidate bacterial isolates was assessed using the Chrome Azurol S (CAS) agar medium described by Schwyn & Neilands (1987). Bacterial inoculum from two-day-old fresh cultures was spot-inoculated onto the surface of CAS medium using a sterile toothpick. The inoculated Petri dishes were incubated at 25 °C for five days.

From the second day of incubation, the appearance of yellow-orange halos around the bacterial colonies was considered a positive indicator of siderophore production. For each isolate, the Siderophore Production Index (S-index = halo diameter/colony diameter) was calculated. The experiment was conducted in six replicates and repeated twice independently.

Determination of Ammonia (NH₃) Production Potential

The ammonia production potential of candidate bacterial isolates was determined according to the method described by Cappuccino & Sherman (1992). Inoculum from two-day-old fresh cultures was transferred into glass tubes containing 5 ml of sterile peptone water using a sterile toothpick. The inoculated tubes were incubated at 25 °C and shaken at 150 rpm for four days.

At the end of the incubation period, 250 µl of Nessler's reagent was added to each tube. A color change of the medium from pale yellow to brown or dark yellow was considered a positive indicator of ammonia production. The experiment was carried out in six replicates and two independent runs for each isolate.

Determination of Indole-3-Acetic Acid (IAA) Production Potential

The indole-3-acetic acid (IAA) production potential of candidate antagonistic bacterial isolates was determined according to the method described by Glickman & Dessaux (1995). For this purpose, 500 µl of bacterial suspension with a concentration of 10⁸ cfu/ml, prepared from two-day-old fresh cultures, was added to glass tubes containing 5 ml of sterile LB broth supplemented with 0.5% L-tryptophan. The tubes were incubated at 30 °C and 200 rpm in an orbital shaker incubator for 48 hours.

After incubation, 2 ml of bacterial suspension was transferred into sterile Eppendorf tubes and centrifuged at 5000 rpm for 30 minutes at 4 °C to remove solid particles. Then, 1 ml of the clear supernatant was mixed with 2 ml of Salkowski reagent in clean glass tubes and incubated at 30 °C in the dark for 25 minutes. A color change from yellow to reddish-pink was considered a positive indicator of IAA production.

The amount of IAA produced was quantitatively measured using a UV-vis spectrophotometer (Perkin Elmer, Lambda 25, USA) at 535 nm wavelength (Patten & Glick, 2002). Absorbance values were compared to a standard calibration curve prepared with pure IAA (Merck, Darmstadt, Germany), and the results were expressed in ppm (µg/ml).

Statistical Analysis of *In Vitro* Experiments

All *in vitro* experiments were conducted using a completely randomized design (CRD), with data subjected to rigorous statistical analysis. Raw inhibition data were analysed using SPSS Statistics 17.0. One-way ANOVA was performed to assess treatment effects. Post-hoc comparisons were done via Tukey's HSD Test, significance threshold was set at $P \leq 0.05$.

RESULTS and DISCUSSIONS

Isolation and Pre-Selection of Candidate Antagonistic Bacterial Isolates

A total of 298 bacterial isolates were purified based on distinct colony morphologies observed after 48 hours of incubation at 25 °C on KB media. These purified isolates were subjected to pectolytic activity and hypersensitive response (HR) assays for the preliminary screening of candidate antagonists. In the pectolytic activity test, isolates

were inoculated onto potato slices, and the presence of soft rot symptoms around the inoculation point within 24–48 hours was considered positive for pectolytic activity. Similarly, for the HR assay, isolates were infiltrated into tobacco leaves, and the development of tissue collapse and necrotic lesions at the inoculation site within 48–72 hours was interpreted as a positive hypersensitive reaction.

Isolates showing positive reactions in either of these assays were excluded from further analyses due to their potential pathogenic characteristics (Duman & Soylu, 2019; Yörük & Mirik, 2021; Varhan & Bozkurt, 2021). As a result of this preliminary screening, 19 bacterial isolates were selected for use in tests.

Identification of Candidate Antagonistic Bacterial Isolates

To determine the molecular identity of the candidate antagonistic bacterial isolates, sequence chromatograms were first edited using ChromasPro software to obtain clean and reliable nucleotide sequences. These sequences were then analyzed using the BLASTn (Basic Local Alignment Search Tool for nucleotides) algorithm available through the NCBI database in order to identify each isolate's taxonomic position at the species level. Similarity analysis based on the 16S rDNA gene region revealed that the isolates shared 98–100% sequence similarity with reference strains deposited in the GenBank database (Table 1).

Table 1. Isolates Identified by BLASTn Analysis Based on the 16S rDNA Gene Region
 Çizelge 1. 16S rDNA Gen Bölgesi Bazında BLASTn Analizi ile Tanımlanan İzolatlar

No	İsolate	Species	Similarity (%)
1	W10.2	<i>Pseudomonas marginalis</i>	99.6
2	W16.4	<i>P. fluorescens</i>	99.2
3	W18.4	<i>Bacillus megaterium</i>	98.8
4	W22.5	<i>B. thuringiensis</i>	99.9
5	W25.2	<i>Citrobacter freundii</i>	99.7
6	W26.1	<i>B. mycooides</i>	99.5
7	W28.2	<i>B. cereus</i>	99.6
8	W29.3	<i>B. cereus</i>	99.9
9	W32.2	<i>B. thuringiensis</i>	100
10	W32.3	<i>B. mycooides</i>	99.8
11	W32.4	<i>B. cereus</i>	98.9
12	W34.1	<i>B. mycooides</i>	99.7
13	W34.2	<i>B. cereus</i>	99.1
14	W43.1	<i>B. thuringiensis</i> serovar <i>israelensis</i>	99.5
15	W44.1	<i>Staphylococcus xylosus</i>	98.9
16	W48.1	<i>B. mycooides</i>	99.4
17	W57.4	<i>P. japonica</i>	100
18	W57.5	<i>P. japonica</i>	100
19	W58.1	<i>Paenibacillus polymyxa</i>	99.3

According to Panpatte et al. (2016), fluorescent pseudomonads are highly suitable as biological control agents due to their abundance in natural soils and plant rhizospheres, along with their ability to metabolize various plant-derived exudates. Moreover, these bacteria possess key adaptive traits such as motility, prototrophy, adherence to soil particles and the rhizoplane, as well as the capacity to synthesize antibiotics and hydrolytic enzymes. Importantly, *Pseudomonas* spp. also display plant growth-promoting characteristics, including nitrogen fixation, phosphate solubilization, iron chelation, and the production of phytohormones. Consistent with these traits, isolates W10.2, W16.4, W57.4, and W57.5 showed 99–100% similarity to species within the genus *Pseudomonas*, with W57.4 and W57.5 being 100% identical to *P. japonica*.

In parallel, Gram-positive Plant Growth Promoting Rhizobacteria (PGPR)—particularly species of *Paenibacillus*, *Bacillus*, and *Streptomyces*—have demonstrated superior effectiveness compared to non-endospore-forming strains, largely due to their resilience against environmental stressors such as heat, desiccation, radiation, and toxic chemicals. *P. polymyxa*, for example, is widely recognized for its capacity to produce a broad spectrum of metabolites and traits that contribute to plant growth, biofertilization, biocontrol, and abiotic stress tolerance (Zhang et al., 2018; Singh & Wesemael, 2022). Supporting this, isolates W18.4, W29.3, W28.2, and W32.4 were identified as closely related (98.5–100%) to *B. cereus* and *B. megaterium*. Similarly, isolates W34.1, W26.1, W32.3, and W48.1 exhibited 99.5–100% similarity to *B. mycooides*, while W22.5, W32.2, and W43.1 were matched (99–100%) with *B. thuringiensis* and its subspecies, including *B. thuringiensis* serovar *israelensis*.

Further analysis revealed that isolate W25.2 had 99.7% sequence similarity with *Citrobacter freundii*, a species previously reported to possess both PGPR traits and antagonistic activity against plant pathogens (Messiha et al., 2019; Harvianti & Kasiamdari, 2021). Likewise, isolate W44.1 was identified as *Staphylococcus xylosus* (98.9–99.6% similarity), a bacterium known for its in vitro antagonistic activity and its ability to synthesize phytohormones such as ethylene, auxins, and cytokinins. Through these mechanisms, *S. xylosus* can enhance plant growth and activate systemic resistance against a range of diseases (Adame-García et al., 2016; Beitsayahi et al., 2025). Finally, isolate W58.1 showed 99.3% similarity to *P. polymyxa*, further emphasizing the potential of these endospore-forming bacteria as multifunctional PGPR candidates.

Determination of *in vitro* Antagonistic Effects of Candidate Antagonist Bacterial Isolates

To assess the *in vitro* antagonistic effects of the candidate antagonist isolates, the inhibition zones they formed against the pathogen bacterial culture were evaluated. The results revealed statistically significant differences among the isolates according to Tukey's HSD Test ($F=2810.13$; $df=19,120$; $\eta^2=0.998$; $P\leq 0.05$). Based on the observations, a total of nine bacterial isolates exhibited antagonistic activity (Figure 1).

Many strains of *P. fluorescens* are known to promote plant growth and reduce the severity of various plant diseases. Therefore, it is considered a potential biopesticide for augmentative biological control of numerous agriculturally and horticulturally significant diseases. In support of this, a study reported by Vidhyasekaran et al. (2001) demonstrated that when rice seeds were treated with a formulation of *P. fluorescens* Pf1 before sowing, at sowing, and again 30 days after sowing, the seedlings developed resistance to *Xanthomonas oryzae* pv. *oryzae*, and disease incidence was reduced significantly from 6.8% to 1.2%. (Ganeshan & Kumar, 2005). The largest inhibition zone (3.9 cm) was observed in *P. fluorescens* W16.4, which had a colony diameter of 2.87 cm. However, this isolate had the lowest antagonistic index value (1.34) among all tested isolates ($P\leq 0.05$). In contrast, *P. japonica* W57.5 produced a 3.58 cm inhibition zone despite having a colony diameter of only 0.5 cm, resulting in the highest antagonistic index value of 7.12 ($P\leq 0.05$). Similarly, *P. japonica* W57.4 exhibited strong antagonistic activity with a 3.38 cm inhibition zone and an index value of 6.72. *P. japonica* is a newly identified bacterial species, originally isolated from activated sludge, and recognized for its remarkable ability to degrade environmentally persistent alkylphenols. In addition, it belongs to the group of soil-dwelling degrading bacteria, contributing to the natural breakdown of organic pollutants in the environment (Pungrasmi et al., 2008; Iswanto et al., 2019).



Figure 1. Dual-culture assays demonstrating bacterial growth inhibition potential of different antagonist bacterial isolates against *P. syringae* pv. *atrofaciens* isolate W49.6

Şekil 1. *P. syringae* pv. *atrofaciens* izolatı W49.6'ya karşı farklı antagonist bakteri izolatlarının büyüme engelleme potansiyelini gösteren ikili kültür testleri

It is known that *P. polymyxa* is an endospore-forming bacterium that could colonize a range of ecological niches. It is commonly found in agricultural soils, especially in close association with plants, and has been isolated from diverse geographic locations. Also, *P. polymyxa* is renowned for its ability to act as a biocontrol agent against a wide array of plant pathogens (Padda et al., 2017; Hossain et al., 2023). *P. polymyxa* isolate W58.1 exhibited high antagonistic activity with a zone diameter of 1.93 cm and an antagonistic index value of 6.79 ($P\leq 0.05$). *P. marginalis* isolate W10.2 had a zone diameter of 2.45 cm and an index value of 2.34, placing it statistically in the moderate

group. Isolates W44.1 (0.98 cm), W18.4 (1.2 cm), W26.1 (2.0 cm), and W25.2 (1.43 cm), which exhibited relatively lower zone diameters, showed lower antagonistic index values of 1.79, 1.69, 1.69, and 1.45, respectively. These results suggest that *P. japonica* W57.5, *P. japonica* W57.4, and *P. polymyxa* W58.1 have high antagonistic potential and could be considered as promising candidates for use as biocontrol agents (Table 2).

Table 2. Potential antagonistic activity of bacterial isolates against *Pseudomonas syringae* pv. *atrofaciens* their biocontrol mechanisms

Çizelge 2. *Pseudomonas syringae* pv. *atrofaciens*'e karşı bakteri izolatlarının potansiyel antagonistik aktiviteleri ve biyokontrol mekanizmaları.

No	Isolate	Species	Ant-ind	Pro-ind	P-ind	Sid-İnd	NH ₃	IAA (µg/ml)
1	W10.2	<i>Pseudomanas marginales</i>	2.34 ± 0.06 ^d	1.87 ± 0.08 ^b	1.4±0.066 ^c	0.00± 0.00 ^b	+	0.00± 0.00 ^e
2	W16.4	<i>P. floresans</i>	1.34 ± 0.03	0.00± 0.00 ^e	0.00± 0.00 ^d	0.00± 0.00 ^b	++	0.00± 0.00 ^e
3	W18.4	<i>Bacillus megaterium</i>	1.69 ± 0.16 ^e	1.87 ± 0.19 ^b	0.00± 0.00 ^d	0.00± 0.00 ^b	-	0.00± 0.00 ^e
4	W22.5	<i>B. thuringiensis</i>	0.00± 0.00 ^g	0.00± 0.00 ^e	0.00± 0.00 ^d	0.00± 0.00 ^b	++	0.00± 0.00 ^e
5	W25.2	<i>Citrobacter freundii</i>	1.45 ± 0.09 ^f	0.00± 0.00 ^e	0.00± 0.00 ^d	0.00± 0.00 ^b	+	0.00± 0.00 ^e
6	W26.1	<i>B. mycooides</i>	1.69 ± 0.04 ^e	0.00± 0.00 ^e	0.00± 0.00 ^d	0.00± 0.00 ^b	+	0.00± 0.00 ^e
7	W28.2	<i>B. cereus</i>	0.00± 0.00 ^g	1.16 ± 0.58 ^d	0.00± 0.00 ^d	0.00± 0.00 ^b	++	0.00± 0.00 ^e
8	W29.3	<i>B. cereus</i>	0.00± 0.00 ^g	1.52 ± 0.04 ^{bcd}	0.00± 0.00 ^d	0.00± 0.00 ^b	++	10.08± 1.56 ^e
9	W32.2	<i>B. thuringiensis</i>	0.00± 0.00 ^g	1.50 ± 0.00 ^{bcd}	0.00± 0.00 ^d	0.00± 0.00 ^b	++	0.00± 0.00 ^e
10	W32.3	<i>B. mycooides</i>	0.00± 0.00 ^g	1.61 ± 0.03 ^{bc}	0.00± 0.00 ^d	0.00± 0.00 ^b	++	0.00± 0.00 ^e
11	W32.4	<i>B. cereus</i>	0.00± 0.00 ^g	1.22 ± 0.60 ^{ed}	0.00± 0.00 ^d	0.00± 0.00 ^b	++	0.00± 0.00 ^e
12	W34.1	<i>B. mycooides</i>	0.00± 0.00 ^g	0.00± 0.00 ^e	1.81±0.137 ^b	0.00± 0.00 ^b	-	28.52± 1.42 ^a
13	W34.2	<i>B. cereus</i>	0.00± 0.00 ^g	0.00± 0.00 ^e	0.00± 0.00 ^d	0.00± 0.00 ^b	++	0.00± 0.00 ^e
14	W43.1	<i>B. thuringiensis</i> serovar <i>israelensis</i>	0.00± 0.00 ^g	0.00± 0.00 ^e	0.00± 0.00 ^d	0.00± 0.00 ^b	+	0.00± 0.00 ^e
15	W44.1	<i>Staphylococcus xylosus</i>	1.79 ± 0.20	0.00± 0.00 ^e	0.00± 0.00 ^d	0.00± 0.00 ^b	-	0.00± 0.00 ^e
16	W48.1	<i>B. mycooides</i>	0.00± 0.00 ^g	0.00± 0.00 ^e	0.00± 0.00 ^d	0.00± 0.00 ^b	++	0.00± 0.00 ^e
17	W57.4	<i>P. japonica</i>	6,72 ± 0,29 ^b	0.00± 0,00 ^e	1.76±0.14 ^b	4.13±0.16 ^a	++	19.40± 0,21 ^b
18	W57.5	<i>P. japonica</i>	7,12 ± 0,20 ^a	0.00± 0,00 ^e	2.46±0.051 ^a	4.08±0.11 ^a	++	17.89± 0.81 ^c
19	W58.1	<i>Paenibacillus polymyxa</i>	6,79 ± 0,12 ^c	3,20 ± 0,19 ^a	0.00± 0,00 ^d	0.00± 0,00 ^b	-	14.42 ± 1.22 ^d
	Control		-	-	-	-	-	-

Ant-ind: Antagonistic activity index; **Pro-İnd:** Protease activity index; **PS-ind:** phosphate solubilisation index Phosphate solubilization index; **Sid-İnd:** Siderophore production index; **NH₃:** Ammonia production; **IAA:** Indole-3-acetic acid; - : No color change (negative result); + : Low production (weak positive); ++ : Moderate production; +++ : High production (intense color change)

^aWithin a given column, mean values ± SD followed by the same lowercase letter are not statistically significant (Tukey's HSD test, P≤0.05).

Determination of Protease Enzyme Production Potential of Bacterial Isolates

The protease enzyme production capacities of bacterial isolates were evaluated on milk agar medium (Figure 2A). Statistically significant differences were observed among the isolates in terms of their protease production capacity according to Tukey's HSD Test (F=139,90; df=19,118; η² =0.964; P≤0.05). As reported strains of *P. polymyxa* have the ability to produce a wide range of hydrolytic enzymes, which play a crucial role in the suppression of plant pathogens and contribute significantly to biological control strategies in agriculture (Raza et al., 2008). In parallel with these, the highest enzyme index (PI) was calculated as 3.2 for *P. polymyxa* isolate W58.1 (P≤0.05). Despite having a small colony diameter (0.4 cm), this isolate formed a wide clearance zone (1.27 cm), indicating a high level of protease enzyme production.

P. marginalis W10.2 (1.86) and *B. megaterium* W18.4 (1.87) isolates showed similar enzyme index values, both exhibiting moderate proteolytic activity. *B. mycooides* W32.3 (1.60), *B. cereus* W29.3 (1.52), *B. cereus* W28.2 (1.16), and *B. cereus* W32.4 (1.12) had lower enzyme indices, indicating significantly lower proteolytic enzyme production capacities compared to the others (P≥0.05). The lowest enzyme index was calculated as 1.29 for *B. megaterium* W18.4, placing it statistically in the weakest group (P≤0.05). These findings highlight *P. polymyxa* W58.1 and *P. marginalis* W10.2 as promising candidates for enzyme production or biological control applications due to their high proteolytic activity (Table 2).

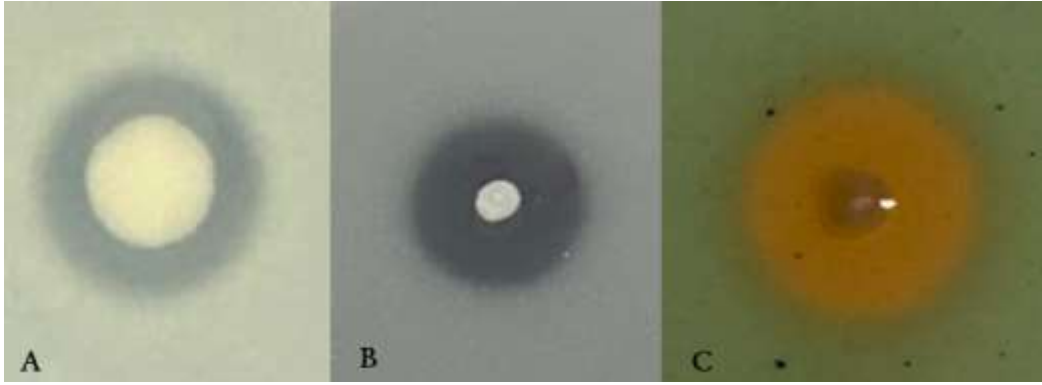


Figure 2. (A) Protease enzymes activity on SMLBA medium. (B) Phosphate solubilization on PVK medium (C) Siderophore production on CAS agar medium.

Şekil 2. (A) SMLBA besiyerinde proteaz enzimleri aktivitesi. (B) PVK besi yerinde fosfat çözünürlüğü (C) CAS agar besiyerinde siderofor üretimi.

Determination of Phosphate Solubilization Potential of Bacterial Isolates

Phosphorus deficiency limits key plant functions, but certain bacteria—such as *Bacillus*, *Azotobacter*, *Pseudomonas*, and *Rhizobium*—are known for their strong phosphate-solubilizing abilities and are widely used as bioinoculants in agriculture today (Alaylar et al., 2019). The phosphate solubilization zone diameters, colony diameters, and the resulting phosphate solubilization indices of bacterial isolates on PVK Agar were evaluated (Figure 2B). Statistically significant differences were observed among the isolates in their phosphate solubilization abilities according to Tukey's HSD Test ($F=1614,58$; $df=19,119$; $\eta^2=0,997$; $P\leq 0,05$). The highest phosphate solubilization index was recorded for *P. japonica* isolate W57.5, with a value of 2.46 ($P\leq 0,05$).

Similarly, *P. japonica* isolate W57.4 exhibited a high index value as well. Despite having relatively small colony diameters, these isolates produced wide solubilization zones, indicating strong phosphate solubilizing capacity (Cherchali et al., 2019) ($P\geq 0,05$). *B. mycoides* isolate W34.1 achieved an index value of 1.77, with a colony diameter of 0.43 cm and a zone diameter of 0.76 cm. The lowest phosphate solubilization index was observed in *P. marginalis* isolate W10.2 (1.39), which was statistically classified in a distinct group ($P\leq 0,05$).

Determination of Siderophore Production Potential of Candidate Bacterial Isolates

Iron is an essential micronutrient involved in chlorophyll synthesis, redox reactions, and various physiological processes. However, its availability is limited in alkaline soils. In response to iron deficiency, microorganisms produce siderophores—small, high-affinity iron-chelating compounds such as catecholates, hydroxamates, and carboxylates. These molecules enhance iron uptake and support plant growth and productivity (Eshaghi et al., 2019). The siderophore production capacities of bacterial isolates were evaluated on Blue-CAS Agar medium (Figure 2C). Following incubation of spot-inoculated petri dishes, the siderophore index (S-Index) was calculated by dividing the diameter of the yellow or orange halo around the bacterial colonies by the colony diameter. Based on the results, only *P. japonica* W57.4 and W57.5 isolates were found to have siderophore-producing ability, and there was no statistically significant difference between them ($F= 4605,09$, $df=19,119$; $\eta^2 = 0,99$; $P\geq 0,05$) (Table 2). Eshaghi et al. (2019) reported that several siderophore-producing bacterial strains were isolated, among which *P. japonica* strains significantly enhanced maize yield. In a greenhouse experiment, inoculation with F21A and F37 isolates notably increased plant height, as well as the fresh and dry weight of corn, compared to the control. These strains show strong potential as bio-fertilizers for Fe-deficient soils. León et al. (2024) indicated that *P. japonica* strains are capable of nitrogen fixation and siderophore production. Their inoculation into sorghum crops has been shown to enhance grain yield, plant height, panicle length, stem diameter, and above-ground dry weight.

Determination of Ammonia (NH₃) Production Potential of Candidate Bacterial Isolates

Based on the observations, 15 isolates produced ammonia at varying levels in the peptone-containing medium by developing of from pale yellow to brown or dark yellow color. Among the antagonistic strains, *P. japonica* stood out with its high ammonia production capacity. This finding suggests that high-level ammonia synthesis may be one of the key mechanisms contributing to their biocontrol potential. The dual role of these isolates—both suppressing pathogens and releasing growth-influencing volatile compounds—highlights their promise as multifunctional agents in sustainable plant protection strategies.

Determination of Indole-3-Acetic Acid (IAA) Production Potential of Candidate Bacterial Isolates

Among the most important bacterial groups, *Bacillus* strains are known for their high ability to produce indole-3-acetic acid (IAA) and are also known to promote the growth of rice plants compared to low IAA-producing strains. Significant differences in IAA production were observed among the isolates according to Tukey's HSD Test ($F=1819150.47$; $df=19,111$; $\eta^2=1$; $P\leq 0.05$). The highest IAA production was recorded in isolate W34.1 (28.52 $\mu\text{g/mL}$), identified as *B. mycoides*. *P. japonica* W57.4 (19.40 $\mu\text{g/mL}$), *P. polymyxa* W58.1 (14.42 $\mu\text{g/mL}$), and *B. cereus* W29.3 (10.08 $\mu\text{g/mL}$) also exhibited substantial IAA production. In contrast, most of the remaining isolates did not produce detectable levels of IAA ($P\leq 0.05$). *P. polymyxa* has demonstrated strong potential in suppressing a wide range of plant pathogens, thanks to its stress tolerance and plant growth-promoting traits. Its IAA-producing strains support the formation of a stable root system by stimulating secondary root development—an effect that has been tested and validated on wheat plants (Li et al., 2023). These findings indicate significant variation in IAA production capacity among the tested isolates. Almoneafy et al. (2014) reported that *Bacillus* strains with strong potential to promote plant growth also exhibited significant antibacterial activity against *Ralstonia solanacearum*, indicating their dual function in enhancing tomato growth and controlling bacterial wilt.

CONCLUSION

In this study, *Pseudomonas japonica* and *Paenibacillus polymyxa* strains were identified as promising biocontrol agents against *P. syringae* pv. *atrofaciens* based on their strong antagonistic activity and multiple plant growth-promoting (PGP) traits, including IAA production, nitrogen fixation, and siderophore synthesis. These features not only support their potential to suppress seed-borne pathogens but also enhance early plant development, making them suitable candidates for sustainable disease management practices in wheat cultivation.

To date, no study in Türkiye has focused on the biological control of seed-borne pathogens in wheat using antagonistic bacteria. Thus, this study represents the first step toward filling this gap by investigating native bacterial isolates as biocontrol agents. Isolates of this study may offer effective and environmentally friendly alternatives to chemical bactericides in future field applications for wheat.

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Author's Contributions

The contribution of the authors is equal.

Statement of Conflict of Interest

Authors have declared no conflict of interest.

Ethics approval and consent to participate

This article lacks any study related to human or animal participants performed by any of the authors.

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