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Research Article

Biological control of pathogenic fungi using *Pseudomonas brassicacearum* isolated from *Aronia* × *prunifolia* (Marshall) Rehder roots

Luay B. Mustafa[1,2*], **Ahmed Al-Bayati**[1,2], **Dunya Albayati**[3], **İbrahim Özkoç**[1]

[1]Department of Biology, College of Sciences, University of Ondokuz Mayis, Samsun, Türkiye
[2]Department of Biology, College of Education for Pure Science, University of Tikrit, Tikrit, Iraq
[3]General Directorate of Educational Salah Al-Din Province, Iraqi Ministry of Education, Tikrit, Iraq

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Abstract: Endophytic bacteria, which are the subject of this study, serve as natural antifungal agents in the struggle against fungal infections, offering an eco-friendly alternative to chemical fungicides. So, it was aimed to determine the antifungal capacities of endophytic bacteria from *Aronia ×prunifolia* roots in the study. 25 endophytic bacteria were isolated, and their ability to act as biocontrol agents was evaluated by measuring fungal growth inhibition and chemical properties. Later, bacteria that showed a positive effect were identified through 16S gene sequencing. The results showed that the LB2 bacteria had the greatest ability to inhibit the selected fungi and the biochemical tests showed that the bacteria were Gram-negative, did not form spores, their colonies were well defined, and they could break down starch and gelatin, which was later diagnosed as *Pseudomonas brassicacearum* according to phylogenetic relationships. This study is the first report on which *P. brassicacearum* was isolated from *A. ×prunifolia* roots for the first time. These findings contribute to our understanding of the potential of endophytic bacteria, particularly *P. brassicacearum*, as natural antifungal agents in plant and human protection, offering a promising and sustainable approach to combat fungal infections while reducing the use of chemical fungicides.

1. INTRODUCTION

The fungal kingdom is believed to have 1.5 million species on our planet; of around 100,000 known fungal species, 400 have been recognized as pathogens to humans, animals and plants. Synthetic substances, such as antifungal medicines and fungicides, are commonly used to avoid the detrimental effects of fungus on human health and agriculture (Alsohiby *et al*., 2016; Yang *et al*., 2016). Isolating novel molecules from biological resources has attracted much interest due to the demand for safe and efficient antifungal treatments.

To maximize the likelihood of finding new antifungals, there is a continuous global search for new bacterial populations since the synthesis of antifungal metabolites in bacteria is highly dependent on strain and species. Recently, the use of microorganisms as a source of bioactive chemicals has attracted the attention of researchers (Anand *et al*., 2023). In environments that support the growth of a mixed bacterial and fungus flora, antifungal activity is a relatively

*CONTACT: Luau B. MUSTAFA ✉ luayburhan25@gmail.com Department of Biology, Faculty Of Sciences, University of Ondokuz Mayis, Samsun, Türkiye

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common characteristic of bacteria that offers an ecological benefit (Santra & Banerjee, 2023). Endophytic bacteria can be isolated from plant tissues infected on their surface or from inside plants without endangering the host plants (Naranjo *et al*., 2023).

The potential of endophytic bacteria as biological control agents is quite promising. Berg and Hallmann (2006) found that a significant portion of the naturally occurring endophytic bacteria in plant roots had the ability to fight against fungal infections. This finding has implications for developing therapeutic antifungal drugs and creating plant protection chemicals (Boonman *et al*., 2023). A single plant may have many distinct bacterial endophyte species (Zinniel *et al*., 2002). Recent studies on plant endophytic bacteria have focused on their roles in plant nutrition (Cipriano *et al*., 2021; Rana *et al*., 2021; Adeleke & Babalola, 2022); pollutant catabolism (Siciliano *et al*., 2001); stress or defense responses (Cho *et al*., 2002); and invading pathogens (Sturz, 1999). Many endophytes have antifungal activity against fungi such as *Fusarium oxysporum* and *Rhizoctonia solani* on cotton (Chen *et al*., 1995), *Verticillium dahliae* and *Rhizoctonia solani* on potato (Berg *et al*., 2005), *Sclerotium rolfsii* on beans (Mahaffee & Kloepper, 1997), *Verticillium longisporum* on *Brassica napus (*Granér *et al*., 2003), and *Rhizoctonia solani*, *Fusarium oxysporum*, and *Phythium ultimunn* in balloon flower (Cho *et al*., 2002).

Aronia ×prunifolia (purple chokeberry) is native to North America and may be grown well in Europe and Asia (Szopa *et al*., 2017; Kulling & Rawel, 2008). Thus far, fruit extracts have been the subject of phytochemical studies that have revealed the presence of flavonoids, anthocyanins, proanthocyanidins, polyphenols, and hydroxycinnamic acids (Celka & Szkudlarz, 2010; Taheri *et al*., 2013; Szopa *et al*., 2017). Endophytic gram-negative bacteria like *Pseudomonas* sp. have been extensively studied as biological control agents in terms of the synthesis of antibiotic metabolites (Nielsen *et al*., 2002). Endophytic pseudomonads quickly and violently infiltrate the root system, preventing harmful bacteria from growing, promoting plant development, and increasing agricultural output (Singh *et al*., 2021).

This is the first report to determine the capacity of endophytic bacteria from *A. ×prunifolia* root to suppress pathogenic fungi. This study aimed to assess the antagonistic endophyte *Pseudomonas brassicacearum*'s capacity to control and produce bioactive extracellular compounds against the phytopathogenic *R. solani* and the two clinically important *T. rubrum* and *F. solani*.

2. MATERIAL and METHODS

2.1. Plant Material

The *A. ×prunifolia* plant was obtained from the Department of Biology, Faculty of Science, Ondokuz Mayis University, Samsun, Turkey.

2.2. Fungal Culture and Growth Maintenance

The virulent strains of *R. solani*, *T. rubrum* and *F. solani* responsible for human and plant diseases, were used. The strains were obtained from Ondokuz Mayis University, Department of Biology, Samsun, Türkiye. On potato dextrose agar (PDA) plates, the fungi were cultivated and incubated at 27 ± 2 °C for five days. For later usage, the fungal cultures were kept in PDA slants at 4 °C.

2.3. Endophytic Bacterial Isolation, Purification, and Culturing

To eliminate soil and dust, the root samples were washed in running tap water and then washed again with double-distilled water before processing. Surface sterilization was then carried out using laminar airflow. Surface sterilization is a vital step for getting rid of surface germs. Plant tissue was washed with 70% ethanol for 5 minutes, then with 2% sodium hypochlorite for 10 minutes, and then three times with sterile water. After being cleaned, plant samples were immersed for 15 minutes in a 10% sodium hydrogen carbonate ($NaHCO_3$) solution to disrupt and prevent endophytic fungal development (Cao *et al*., 2005). Afterward, tiny pieces of root

(0.5-1.0 cm) were cut, dried aseptically, and then plated on Nutrient agar (NA) which was then incubated at 27 ± 2 °C for 48 h for maximum recovery of bacterial colonies. Uncut, surface-disinfected root sections and uninfected parts were used as controls and incubated on the same medium. There was no growth on the control plate, there was no epiphytic contamination. To obtain bacterial isolates, physically different bacterial colonies on agar plates were chosen and repeatedly streaked after 48 hours. All purified bacterial isolates were kept at 4 °C after being subcultured on NA slants.

2.4. Phenotypic Identification

The biochemical and physiological characterization of selected bacteria was done using Berge's Manual of Systematic Bacteriology (Safaa & Qaysi, 2016). In addition to biochemical tests, phenotypic characteristics such as form, size, margin, surface, elevation, color, pigmentation, and Gram staining were used to define bacterial isolates. Known biochemical and physiological procedures, including the catalase test (3% H_2O_2), the oxidase reaction (Kovacs method), and the production of diffusible pigments, were used for the latter. Starch hydrolysis was finished by culture isolation on Starch nitrate agar media, and carbohydrate fermentation was carried out, in addition, using a medium containing specific carbohydrate sources (sucrose, glucose, lactose, mannitol, maltose, and rhamnose). After 24 hours of incubation at 37 °C, the plate was covered with iodine, and a definite zone enclosing the growth of colonies on the medium plate was found. When performing gelatin hydrolysis utilizing nutritional agar puncture tubes, liquefaction of the gelatin was seen after being inoculated with test bacteria (Sneath, 1992).

2.5. Molecular Identification

With slight modifications, Pascual's (2000) CTAB (cetyltrimethyl ammonium bromide) technique was used to extract DNA from fungal mycelia. The isolated samples' bacterial DNA was amplified using universal primers for 16S rDNA. According to Heuer *et al.* (1997), the study's primer sequences for amplification of the 16S rDNA gene were 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1525R (5′-AAGGAGGTGATCCAGCC-3′). PCR amplification reactions were performed in a 50 µL reaction containing 1µL genomic DNA (1 ng µL-1), 1 µL (2.5 mM) dNTP mix (Sigma), 0.25 µL Taq DNA polymerase (5 U/µL) (Promega, Go-TaqFlexi DNA Polymerase), 1 µL each of primers (25 pmoles), 10 µL 5 × PCR buffer supplied by manufacturer (Promega, Go-Taq Green Buffer) and 3 µL MgCl2 (1.5 mM) (Sigma) and 32.75µL sterile ddH20. An initial denaturation step of 94 °C for 3 min was followed by 30 cycles of 94 °C for 1 min, 49 °C for 2 min, 72 °C for 3 min, and finally an extension step of 72 °C for 7 min during the PCR amplification (Salazar *et al*., 1999).

The 16S rDNA region's PCR products were sequenced by Macrogen (Macrogen Inc., Seoul, Republic of Korea). The program BioEdit version 7.2.5 was used to create a consensus sequence (Hall, 1999). Using the BLAST program, the consensus sequences for the 16S rDNA region were compared to the sequence data in GenBank (National Center for Biotechnology Information). To distinguish between different bacterial species and genera, we employed 97 to 100% sequence identity. The BLAST tool on the NCBI website (http://www.ncbi.nlm.nih.gov/) was used to analyze and identify each isolate's nucleotide sequences of the 16S rDNA gene. Using MEGA software version 11.0, the alignments were examined to create a phylogenetic tree and assess relationships between the sequences using the neighbor-joining approach (Tamura *et al*., 2013; Saitou & Nei, 1987).

2.6. Antagonistic Action in Vitro Screening

2.6.1. *Dual culture method*

In a PDA medium using a dual-culture approach, bacterial isolate was evaluated for in vitro biocontrol efficacy toward *R. solani*, *T. rubrum*, and *F. solani*. On one side of the PDA medium-filled plate (9 cm in diameter), a mycelial disc (5 mm in diameter) from a culture that had been growing for seven days was put. Thereafter, an endophytic bacteria isolate was streaked on the plate's other side. As a control, plates were infected only with the pathogen. The plates were

incubated for 7 days at 25 ± 2 °C with 12 hr of light and 12 hr of darkness. Fungal colonies' diameters were measured (Moreira *et al*., 2014). Then, the formula provided by Trivedi (2008) was used to compute the percentage of growth inhibition of the tested fungi: growth inhibition (%) = (R1 − R2/R1)×100, where R1 represents the radial growth of the control and R2 represents the radial growth of the fungus in dual culture.

2.6.2. *Effect of endophytic bacterium on fungal biomass*

The approach provided by Kim (2005) was modified to investigate the impact of endophytic bacteria on the biomass of fungus. The chosen endophytic bacteria were grown in NB media and incubated for 72 hours at 26 ± 2 °C on a rotary shaker. After that, it was filtered through a 0.22 m Millipore filter and centrifuged at 10,000 rpm for 15 minutes. 10% (v/v) of the strain's cell-free culture filtrates were added to the potato dextrose broth (PDB) medium in an Erlenmeyer flask (100 mL). One 5-mm-diameter plug of a mycelial disk from a 7-day-old culture of fungi was used to inoculate the Erlenmeyer flask, which was then incubated at 25 ± 2 °C for 14 days while being shaken at 150 rpm.

Cultures of the fungus grown without bacteria culture filtrates were used as the control. By the preweighed filter paper, 48-hour-grown dual cultures of the fungus and bacteria or the control culture (without bacteria) were passed to determine the differences in the dry weights between the two (Whatman No. 1). The filter sheets were weighed after drying for 24 hours at 70 °C. Using the formula (w1-w2/w1) 100, the weight loss of the test fungus was estimated as a percentage. where w1 (control value) is the weight of the test fungus in flasks devoid of bacteria (i.e., control flasks) and w2 is the weight of the fungus in sets that were contaminated with bacteria.

3. RESULTS

3.1. Isolation and Antagonistic Activity

The isolation of bacterial endophytes was carried out using fresh ***A. ×prunifolia*** roots. Surface sterilization was an essential step in eliminating epiphytic microorganisms from sample explants, and it worked well in our study because there was no growth on the control plate. Based on morphological traits, 25 different endophytic bacterial strains were isolated from the healthy roots and purified before being kept at 4 °C (Figure 1).

Figure 1. Growth of endophytic bacteria from pieces of *A. ×prunifolia* root on NA medium.

The isolates were evaluated their potential for serving as effective biological control agents using the dual-culture and Cell Free Supernatant (CFS) approach; these isolates were tested for their antagonistic activity against *F. solani*, *T. rubrum*, and *R. solani*. Among the isolates, strain LB2 demonstrated the most significant antagonistic activity against the target pathogens. The inhibition rates were 68.21±0.84, 39.35±0.59, and 48.35±0.28%, respectively (Table 1, Figure 2).

Table 1. After 7 days, LB2 strain in dual culture (live cells) inhibited the development of fungi's mycelia in in vitro testing.

Pathogenic fungi	Zone of inhibition (%)
Fusarium solani	68.21±0.84
Trichophyton rubrum	39.35±0.59
Rhizoctonia solani	48.35±0.28

Note: The assay was performed in triplicate and results are the mean of three values ± Standard Deviation.

Likewise, CFS treatment has also been shown to reduce the weight of tested pathogenic fungi. *F. solani*, *T. rubrum* and *R. solani* all exhibited substantial reductions in mycelial mass, with values of 34.53±0.22%, 30.18±0.73%, and 26.15±0.91%, respectively (Table 2). This result suggests that strain LB2 holds promising potential as a valuable biological control agent in managing the target pathogens.

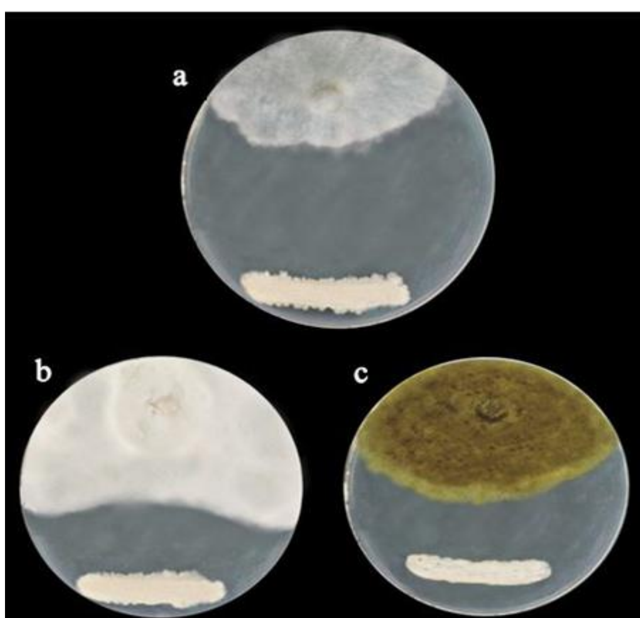

Figure 2. In vitro antagonistic activities of endophytic LB2 isolated from *A.* ×*prunifolia* roots against pathogenic fungi (a) *F. solani*, (b) against *T. rubrum*, (c) Antagonist activity against *R. solani*.

Table 2. After being incubated for 7 days at room temperature, LB2 (Cell-free culture filtrates) reduced the percentage of biomass of pathogenic fungus in PDB broth.

Pathogenic fungi	Fungal mycelia mass reduction (%)
Fusarium solani	34.53±0.22
Trichophyton rubrum	30.18±0.73
Rhizoctonia solani	26.15±0.91

Note: The assay was performed in triplicate and results are the mean of three values ± Standard Deviation.

3.2. Biochemical Characterization

The biochemical characteristics of the isolated bacterium play a crucial role in understanding its taxonomy, metabolic profile and potential applications. The biochemical analysis results are summarized in Table 3.

It was carried out based on the various tests. The bacterium LB2 exhibits several distinctive characteristics as revealed by Gram testing and various biochemical assays. It was Gram-negative, forming well-defined and even colonies on agar plates. LB2 lacks spore formation, suggesting alternative survival mechanisms. It ferments lactose and grows in the presence of bile salts and crystal violet, distinguishing it from non-lactose-fermenting Gram-negative

bacteria. It does not possess hemolytic factors, indicating non-pathogenic and non-hemolytic attributes. LB2 lacks urease, which is relevant in medical and environmental contexts. It can break down starch, potentially aiding in nutrient utilization and biotechnological applications. The presence of cytochrome c oxidase suggests it can use oxygen in aerobic respiration. It has catalase, which is important for handling reactive oxygen species. LB2 can break down gelatin, potentially relevant in nutrient cycling and bioremediation. The presence of tryptophanase is significant for identification. Fluorescence requires further investigation for source and significance. LB2 can metabolize specific organic compounds like Tween 40 and α-keto-butyric acid, exhibiting metabolic versatility. It can ferment various sugars and acetoacetic acid, enhancing its metabolic diversity for various applications. A few biochemical and physiological test findings are displayed in Figure 3.

Table 3. The isolate's biochemical and physiological testing.

Test	Resut
Gram staining	- / rod-shaped
Colony texture	Smooth
Spore	-
Growth on MacConkey	+
Blood Hemolysis	-
Urea hydrolysis	-
Starch hydrolysis	+
Oxidase	+
Catalase	+
Motility	+
Gelatin hydrolysis	+
Indole	+
Fluorescence	+
Tween 40	+
α-keto-butyric acid	+
Glucose	+
Lactose	+
Mannitol	+
Maltose	+
Acetoacetic acid	+

3.3. Identification of Bacterial Endophyte

The selected bacteria were identified by analyzing their 16S rRNA gene sequences. These associated bacteria displayed a complete match, showing 100% homology, with the gene sequences of *P. brassicacearum* available in the NCBI GenBank database. To further explore the evolutionary relationships among these bacteria, the top-scoring sequences were retrieved from the database and aligned with the 16S rRNA sequences of the endophytes to construct a phylogenetic tree.

This analysis was performed using Mega XI software, see Figure 4, and the resulting phylogram clearly distinguished between gram-positive and gram-negative endophytic bacteria. Specifically, all *P. brassicacearum* strains were found to cluster together as a distinct group (Cluster Ia) with a high bootstrap value of 100%. Notably, *Pseudomonas putida* was positioned as the outgroup in the constructed phylogenetic tree. This analysis sheds light on the evolutionary relationships and genetic diversity among the endophytic bacteria associated with *Aronia* roots.

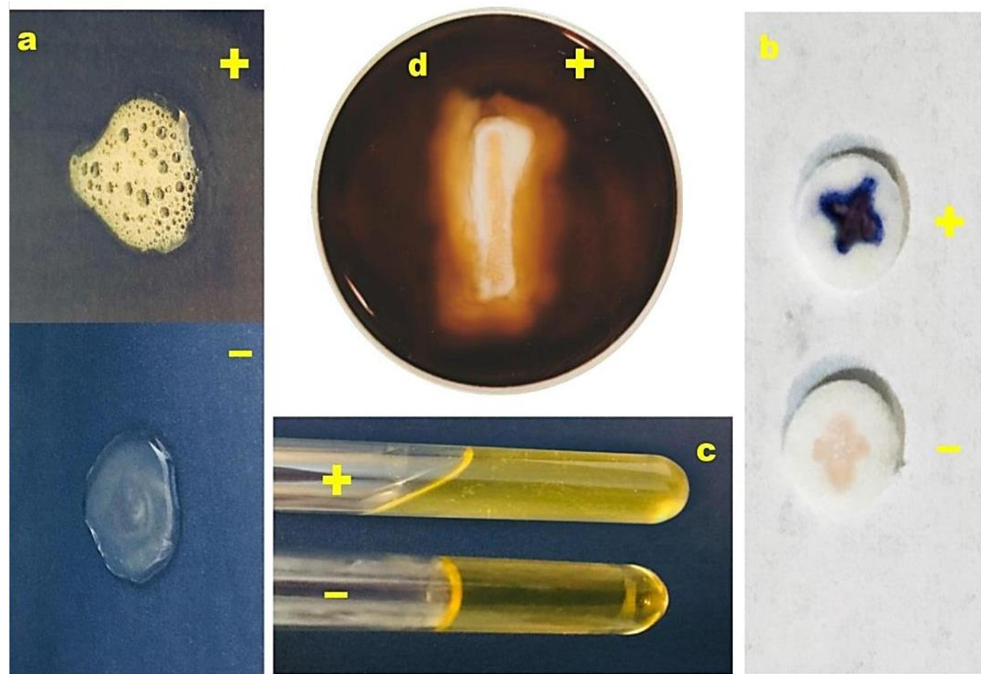

Figure 3. Biochemical and physiological tests a- Catalase test b- Oxidase test c- Gelatin hydrolysis test d- Starch hydrolysis on starch agar.

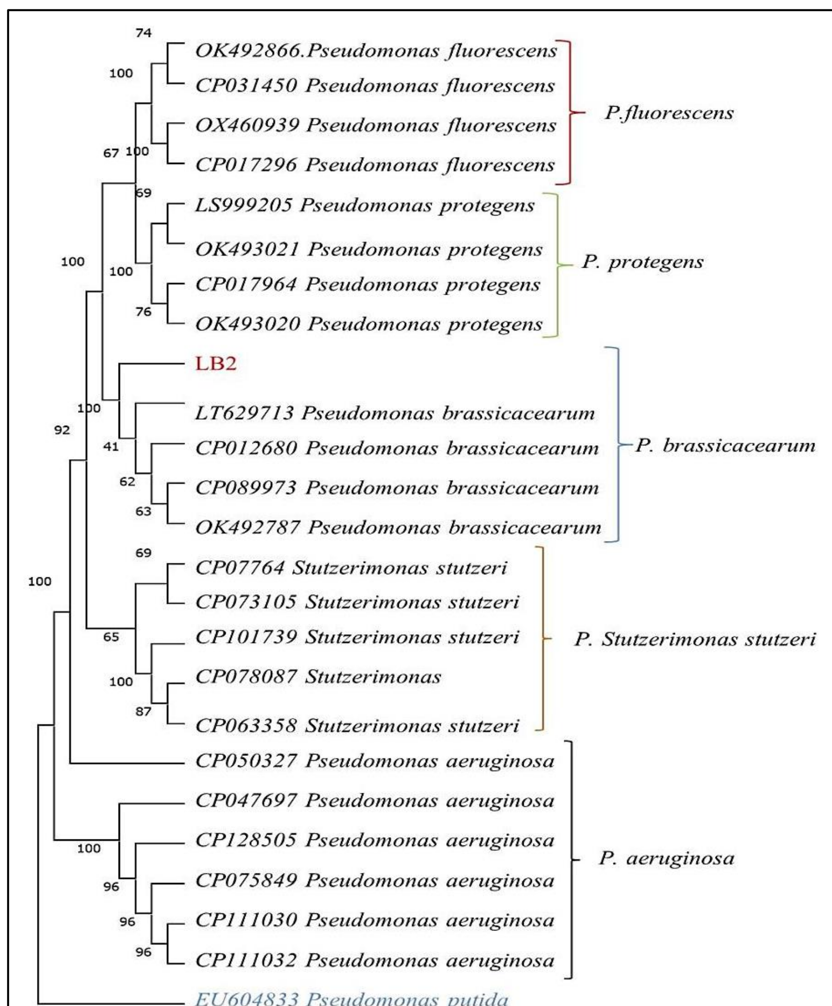

Figure 4. Neighbor-joining phylogenetic tree based on 16S rDNA gene sequences illustrating connections between isolate LB2 (in red hue) and many other *Pseudomonas* sp. 1000 bootstrap replicates were used to create the tree in MEGA 11.0 using the neighbor-method.

4. DISCUSSION and CONCLUSION

A. prunifolia, also known as purple chokeberry, is a type of berry that is rich in phenolic compounds and antioxidants (Szopa *et al*., 2017; Kim *et al*., 2021). *Aronia* has been cultivated in Turkey, and as its output rises, the *Aronia* market is thought to be expanding (Akdemir *et al*., 2023). According to studies by Szopa *et al*. (2018), the *Aronia* plant contains a variety of medicinally significant compounds, such as chlorogenic acid, rosmarinic acid, and neochlorogenic acid that developed in the shoot and callus cultures of *A. prunifolia*.

Endophytic bacteria living in plants are important for maintaining plant health because they produce a variety of advantageous metabolites (Köberl *et al*., 2013; Musa *et al*., 2020). These molecules are excellent sources of biological activity. As previously indicated, biologically active chemicals produced in *A. ×prunifolia* significantly impact endophytic microorganisms that live inside plant tissue and their physiological processes (Köberl *et al*., 2013). So, these compounds can affect many biological processes.

In our study, strain LB2, isolated from the roots of *A. prunifolia*, was selected because it gave the highest inhibitory ability compared to the rest of the isolated strains. It was later characterized as *P. brassicacearum*. Many earlier investigations (Achouak *et al*., 2000; Ross *et al*., 2000; Chung *et al*., 2008; Bahmani *et al*., 2021) have already identified *P. brassicacearum* from other plants except *A. ×prunifolia* as endophytic bacteria. According to recent research, endophytic bacteria are an efficient biocontrol agent for various plant and human infections. Pseudomonas strains that have been isolated from various plant samples have demonstrated excellent effectiveness as plant-growth-promoting and biocontrol agents as well as makers of antimicrobial substances, antibiotics, enzymes, and volatile chemicals (Weller, 2007; Khan *et al*., 2022; Mustafa *et al*., 2024). In both greenhouse and field experiments, *P. brassicacearum* strain has been shown to be an effective biocontrol agent for preventing illness brought on by the plant-pathogenic fungus *Sclerotinia sclerotiorum* (Bhaskar *et al*., 2005; Berry *et al*., 2010). This bacterium generates a wide range of extracellular metabolites, such as oxidizing enzymes, hydrogen cyanide (HCN), and a brand-new lipopeptide known as sclerosin (Berry *et al*., 2010; Crawford *et al*., 2011). *Gaeumannomyces graminis* var. *tritici*, also known as the take-all fungus, is a destructive root disease that affects wheat; numerous investigations have demonstrated that *P. brassicacearum* isolates may stop this fungus from growing and may be useful as a biological control agent for plant diseases (Ross *et al*., 2000; Fromin *et al*., 2001).

In our study, diffusible metabolites and cell-free culture filtrates of *P. brassicacearum* strains have shown high inhibitory action against *R. solani*, *T. rubrum*, and *F. solani*. Many authors have shown that *P. brassicacearum* can stop the growth of fungal pathogens (Chung *et al*., 2008; Laveilhé *et al*., 2022). Studies on non-pathogenic microorganisms with antagonistic potentials have stimulated the search for an environmentally acceptable method of disease control. (Caulier *et al*., 2018). *Pseudomonas* spp. are significant organisms since they aggressively colonize different crops and exhibit a wide range of antagonistic activity against soil- and seed-borne diseases (Wang *et al*., 2018).

One of the most important assays for in vitro first antagonistic compound screening is the dual culture assay (Islam *et al*., 2018). A bacterial strain's antagonistic effects are frequently demonstrated by the development of inhibition zones between bacterial and fungal colonies (Ji *et al*., 2014) or by measuring the percentage of control mycelial growth that is inhibited by the bacterial colonies (Lee *et al*., 2017). According to an earlier study, Pseudomonas species compete for resources and habitats, synthesizing siderophores, secreting lytic enzymes, and inducing systemic resistance to plant diseases (Kang *et al*., 2015). On the other hand, broth-based treatments in vitro dual culture may be a superior way to assess the antagonistic potential of the bioagents because the broth media are favorable environments from all potentially interacting locations for the antagonisms (Trivedi *et al*., 2008). Many different gram-negative bacteria have been used in the treatment of fungal infections. These bacteria function as antifungal agents by synthesizing siderophores, salicylic acid, antibiotics, and volatile

byproducts such as hydrogen cyanide (Manwar *et al*., 2004; Afsharmanesh *et al*., 2006; Correa *et al*., 2022; Shahid *et al*., 2022).

In this study, it was determined that the isolate *P. brassicacearum* LB2 reduced the biomass of all studied fungal organisms by producing diffusible chemicals in broth media. However, the extracellular filtrates obtained from *P. brassicacearum* have presented low antifungal activity compared to dual culture assay. The Gram-negative bacteria isolated in this study likely generate antifungal volatile chemicals or enzymatic activity that are lost during the extraction of the filtrates. Therefore, it may be inferred that the compounds that cause the inhibition must exist in the presence of bacteria and may be linked to the live bacterial properties. The findings reported here suggest that *P. brassicacearum* may be transformed into a biocontrol-friendly product. In fact, endophytic fungi within the host plants are a flexible reservoir of numerous bioactive metabolites and may be used in contemporary agriculture, industry and medicine.

The findings of this study highlight the significance of endophytic bacteria, particularly *P. brassicacearum*, isolated from *A. ×prunifolia* roots as natural antifungal allies in the ongoing struggle against plant fungal infections. Their multifaceted mechanisms of action, including resource competition, antibiosis, induced systemic resistance, and mycoparasitism, offer a sustainable and environmentally responsible approach to protecting plants. Due to the search for alternatives to chemical fungicides, this research will reinforce the importance of eco-friendly alternatives in plant protection and medical practices.

This study may also pave the way to benefit from this endophyte in different areas. So, this contribution highlights their potential as potent biocontrol agents and opens new avenues for harnessing their capabilities in agricultural and horticultural practices.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Luay B. Mustafa: Design, Experimental Studies, Writing the Original Draft, and Manuscript Review. **Ahmed İ. N. Al-Bayati**: Analysis, Manuscript Checking, and Correction. **Dunya Albayati**: Literature Survey, Data Collection, and Data Editing. **İbrahim Özkoç**: Supervision, Validation, and Editing.

Orcid

Luay B. Mustafa https://orcid.org/0000-0001-8197-4357

Ahmed İsmael Naqee Al-Bayati https://orcid.org/0000-0001-5411-7448

Dunya Albayati https://orcid.org/0009-0006-9152-2323

İbrahim Özkoç https://orcid.org/0000-0001-8179-0961

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