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# Isolation, Identification and Determination of Biocontrol Activities of Rhizobacteria against Cown Rot Disease Agent *Rhizoctonia solani* AG 2-2 in Sugar Beet (*Beta vulgaris L.*)

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## ABSTRACT
This study aims to investigate the biocontrol potential of antagonistic and plant growth-promoting rhizobacteria (PGPR) isolated from plant roots and soils against the crown rot pathogen *Rhizoctonia solani* AG 2-2 in sugar beet (*Beta vulgaris* L.) tubers. A total of 219 soil samples were collected from 20 provinces to obtain putative bacterial PGPR isolates that could potentially be used as biological control agents (BCAs). A total of 752 bacterial isolates were obtained, and there *in vitro* antifungal activity against *R. solani* AG-2.2 was tested in double culture experiments. The result was that 117 PGPR bacterial isolates exhibited antibiotic properties that inhibited mycelial growth of the fungal pathogen. These isolates were also characterized for their antagonistic and plant growth-promoting properties, including phosphorus solubility, hydrogen cyanide (HCN) activity, siderophore production, ACC deaminase activity, and indole-3-acetic acid (IAA) production. The 12 best PGPR isolates were then subjected to an *in vivo* pot test using a weighted scoring system. The isolate *Bacillus subtilis* 119.2 was identified as the most effective isolate that completely inhibited the outbreak of the disease caused by *R. solani* AG-2.2 in sugar beet. Considering the results of the *in vitro* and *in vivo* studies, the *B. subtilis* 119.2 isolate showed its potential as an environmentally friendly biological control agent against *R. solani* in sugar beet. Overall, *B. subtilis* 119.2 is a PGPR isolate with potential for use as a biofertilizer or biofungicide.

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# Şeker Pancarında (*Beta vulgaris* L.) Taç Çürüklüğü Hastalık Etmeni *Rhizoctonia solani* AG 2-2'ye Karşı Kökbakterilerinin İzolasyonu, Tanılanması ve Biyokontrol Etkinliklerinin Belirlenmesi

## ÖZET
Bu çalışmanın amacı, şeker pancarı (*Beta vulgaris* L.) yumrularında sorun taç çürüklüğü hastalık etmeni *Rhizoctonia solani* AG 2-2'ye karşı bitki kök ve topraklardan izole edilmiş potansiyel antagonistik (BCAs) ve bitki gelişimini teşvik eden kökbakterilerinin (PGPR) hastalık etmenine karşı biyokontrol potansiyelini araştırmaktır. Potansiyel biyolojik kontrol etmeni bakteri izolatlarını elde etmek için 20 ilden toplam 219 toprak örneği toplanmıştır. Bu örneklerden 752 bakteri izolatı elde edilmiş ve bu bakterilerin, *R. solani* AG-2.2'ye karşı *in vitro* antifungal etkinlikleri, ikili kültür denemeleri ile test edilmiştir. Sonuçta, 117 bakteri izolatının misel gelişimini engelleyen antibiyosis özelliği gösterdiği belirlenmiştir. Bu izolatların antibiyosis etkinliklerinin yanısıra, fosfor çözünürlüğü, hidrojen siyanür (HCN) aktivitesi, siderofor üretimi, ACC deaminaz aktivitesi ve indol-3-asetik asit (IAA) üretimi gibi bitki gelişimini teşvik edici özellikleri karakterize edilmiştir. Test edilen PGPR izolatlar arasında, en iyi 12 bakteri, tartılı derecelendirme sistemi kullanılarak *in vivo* saksı testlerine tabi tutulmuştur. *Bacillus subtilis* 119.2 izolatı, şeker pancarında *R. solani* AG-2.2' kaynaklı hastalık çıkışının engellenmesinde %100 oranla en etkili izolat olarak belirlenmiştir. Hem *in vitro* hem de *in vivo* çalışma sonuçları göz önünde bulundurulduğunda, *B. subtilis* 119.2 izolatının şeker pancarı tarımında

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*Bacillus*
Antagonist
Bitki Gelişimini Teşvik Etme

*R. solani*'ye karşı çevre dostu biyolojik mücadele etmeni olarak potansiyelinin bulunduğunu göstermiştir. Tüm sonuçlar birlikte değerlendirildiğinde *B. subtilis* 119.2 biyolojik gübre veya biyolojik fungisit olarak kullanım potansiyeline sahip PGPR izolatıdır.

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## INTRODUCTION

Sugar beet (*Beta vulgaris* L. var. Saccharifera) is a key industrial crop that contributes significantly to worldwide sugar production. Turkey ranks as the 5th largest producer of sugar from sugar beet, contributing significantly to national sugar demand (Şahin, 2022). Root rot diseases, primarily caused by the fungus Rhizoctonia, lead to substantial yield losses and negatively impact sugar beet productivity in major production areas worldwide. Among the most aggressive strains are *Rhizoctonia solani* AG-2-2 IIIB, AG-2-2 IV, and AG-4, which are particularly destructive to sugar beet, causing severe root necrosis, stunted growth, and up to 50-70% yield loss in infected fields under favorable environmental conditions (Şahiner, 2020; Barreto et al., 2020). Cultural and chemical control strategies are utilized to combat the disease; however, developing environmentally friendly agricultural methods is critical. The use of resistant cultivars is beneficial in fighting this disease, though certain limitations exist. Consequently, the agricultural industry should minimize its use of chemicals and implement biological management approaches.

*Rhizoctonia* root rot has a substantial economic impact, leading to more than 20% yield reduction in over 200 crops annually. A significant portion of the damage (30–60%) is often asymptomatic until severe crop loss becomes evident across the field (Barreto et al., 2020). The disease first appears on older leaf stalks that touch the soil. These symptoms appear as black lesions at the base of the plant's root and crown, as well as on the leaf stalk. The earliest indicators on the stem are localized, black, ring-like lesions that eventually expand into a ladder pattern. Over time, wilting and death develop in the field (Scholten et al., 2001; Avan & Katırcıoğlu, 2019; Liu et al., 2019; Barreto et al., 2020). Considering the disease is both soil-borne and seed-borne, it poses greater difficulty to manage than the majority of diseases. Cultural strategies, including inoculum source sanitation, certified seed usage, soil sanitation, rotation, harvest timing, and soil sterilization, are crucial for disease control (Aydın, 2008; Lehtonen, 2009; Tsror, 2010; Khedher et al., 2015). To change microbial communities in the soil, monoculture should be avoided and three-year or longer rotations implemented (Tsror, 2010). The toxic compounds produced by certain bacteria can either limit or enhance the number of microorganisms competing with the pathogen, inhibiting disease spread (Larkin & Brewer, 2020). Despite using host resistance cultivars, there are certain drawbacks (Holmquist, 2018). Sustainable agricultural approaches for combating *Rhizoctonia solani* remain limited and primarily rely on chemical management. Flutolanil, pencycuron, azoxystrobin, captan, fludioxonil, iprodione, mancozeb, thiophanate-methyl, and thiabendazole are the chemicals utilized in this control (Strausbaugh et al., 2011; Bienkowski, 2012; Kankam et al., 2021). However, attitudes regarding pesticide use in agriculture differ due to resistance problems, pollution of the environment, and worries about human health (Kara et al., 2024). Furthermore, excessive chemical use on agricultural soils can deplete beneficial microbial flora and eliminate natural disease control agents. As an alternative to chemical control, biological control seeks to reduce the use of chemicals in agriculture. This technique encompasses naturally occurring or antagonist-induced mechanisms that reduce pathogen levels, such as antibiosis, mycoparasitism, induced resistance, and promoting plant growth (Ji et al., 2013; Kotan, 2020; Hough, 2021).

Numerous studies have demonstrated the potential of PGPRs and fungal antagonists in the biological control of *Rhizoctonia solani*. For instance, Mahmoud (2016) reported that four *Bacillus subtilis* isolates, three *Trichoderma harzianum isolates*, and three *T. hamatum* isolates significantly inhibited the mycelial growth of *R. solani* under *in vitro* conditions and also exhibited effective control in greenhouse trials. Similarly, Karimi et al. (2016) evaluated bacterial isolates from sugar beet rhizosphere and apple and walnut trees against *R. solani* AG-4 and AG-2-2. Among these, *Bacillus amyloliquefaciens* SB14 showed the highest antagonistic activity, suppressing *R. solani* AG-4 and AG-2-2 by 58% and 52.5%, respectively, under *in vivo* conditions. These findings emphasize the importance of host-specific and rhizosphere-adapted bacterial strains in achieving effective biocontrol outcomes.

A comprehensive literature review revealed a limited number of studies investigating alternative control methods against soil-borne pathogens, as opposed to chemical treatments (Kara et al., 2024). Increased interest in sustainable agriculture has resulted in the use of Plant Growth-Promoting Rhizobacteria (PGPR) to improve soil

fertility and crop yield (Glick, 2014). PGPRs are beneficial microorganisms found on plant roots that promote growth and enhance plant health. Plant roots exude various organic compounds such as organic acids, sugars, and amino acids. These compounds serve as both nutrient sources and chemical signals for soil-dwelling microorganisms. As a result, they attract microbes capable of metabolizing these substances, thereby promoting the formation of a rich microbial population in the rhizosphere (Vacheron et al., 2013). They additionally increase plant nutrient intake and protect against infections via mechanisms such as atmospheric nitrogen fixation, phosphate solubilization, phytohormone production, and siderophore synthesis (Ji et al., 2013; Selva Kumar et al., 2013; Dinu et al., 2019).

Understanding how PGPRs work is critical for discovering biocontrol agents for biological control. *Bacillus* bacteria are commonly used for controlling soil-borne plant diseases (Soylu et al., 2020; Soylu et al., 2021). Plant Growth-Promoting Rhizobacteria (PGPR) support plant growth through enhanced nutrient uptake (e.g., nitrogen fixation, phosphate solubilization, siderophore-mediated iron acquisition) and phytohormone modulation. They also improve stress tolerance via ACC deaminase activity (Duman & Soylu, 2019; Aktan & Soylu, 2020). Notably, *Bacillus* species contribute to biocontrol by producing diverse antimicrobial metabolites, including lipopeptides (surfactin, iturin, fengycin) and polyketides, which suppress various phytopathogens (Wang et al., 2024). Furthermore, *Bacillus* and *Paenibacillus* species can colonize plants endophytically and produce biofilms, which enhance their biocontrol capabilities. *Bacillus* species are becoming increasingly popular in agricultural systems due to their resistance to UV light and drought, as well as the convenience of commercial formulation (Ji et al., 2013; Selva Kumar et al., 2013; Karimi et al., 2016; Dinu et al., 2019; Kara et al., 2020).

The use of biological control agents against soil-borne plant pathogens such as *Rhizoctonia solani* has gained increasing attention due to their potential to offer eco-friendly plant protection approaches. Although several studies have demonstrated the antagonistic effects of biological control agents on *R. solani*, further screening of diverse bacterial isolates is essential to expand the current knowledge base and enhance their practical application in sustainable agriculture. Therefore, this study aims to investigate the antifungal activity of bacterial isolates against *R. solani* AG 2-2 through dual culture assays and to evaluate their plant growth-promoting traits, including phosphate solubilization, siderophore production, indole-3-acetic acid (IAA) production, hydrogen cyanide (HCN) production, and ACC deaminase activity. These traits may contribute to the suppression of fungal growth and the improvement of plant health, representing a promising step toward eco-friendly and sustainable disease management strategies.

## MATERIAL and METHOD

### Fungal Disease Agent

The pathogen *Rhizoctonia solani* AG 2-2 strain used in this study was collected from an *R. solani*-infected sugar beet field in Konya Province, Turkey. The strain was obtained from the culture collection of the Selcuk University Faculty of Agriculture Mycology Laboratory. Salman and Boyraz (2023) previously investigated the pathogenicity and molecular characterization of this strain. The *R. solani* AG 2-2 isolate was preserved at +4°C in potato dextrose agar (PDA) and grain growth media, as described by Carling and Sumner (1992). The strain was deposited under the accession number as OQ526202.1 (https://www.ncbi.nlm.nih.gov/nuccore/OQ526202.1).

### Isolation and Long-Term Preservation of Rhizospheric Bacteria

Rhizospheric bacteria were isolated from soil samples collected between June and September 2020–2021 from the rhizospheres of 40 different plant species (e.g., *Phaseolus vulgaris*, *Beta vulgaris*, *Solanum tuberosum*, *Zea mays*, *Solanum lycopersicum*, *Cicer arietinum*, *Triticum aestivum*, *Capsicum annuum*, *Cucurbita pepo*, *Helianthus annuus*, etc.) cultivated under diverse climatic and ecological conditions. The approach described by Küsek (2007) was used for bacterial isolation. Briefly, 10 g of soil was weighed into sterilized Erlenmeyer flasks and supplemented with 90 ml of sterile distilled water, then shaken for 30 minutes. This initial sample was diluted until it reached $10^6$ CFU/mL. A sterile glass rod spreader was used to inoculate 100 µl of the prepared suspension onto the nutrient agar media (NA). After inoculation, plates were incubated at 25±1°C for 24-48 hours to promote bacterial growth. Following incubation, specific colonies with distinct morphological traits were chosen, streaked onto fresh nutrient agar plates using a sterile loop, and then cultured at 27°C for 24-48 hours. The pure culture isolates were subsequently stored in 30% glycerol at -20°C for long-term preservation.

### Determination of Antagonistic Effects of Bacterial Isolates Against Rhizoctonia solani AG 2-2

The antifungal activity of all bacterial isolates was tested *in vitro* using the dual culture technique. The isolated antagonist bacteria's effectiveness against *R. solani* was tested using antibiotic-free concentrated potato dextrose agar (CPDA) medium at 45 g/l. To obtain new cultures of *R. solani* isolates, one barley grain was transferred to

PDA media and grown for seven days at 25°C. A 5 mm-diameter agar disc was taken from this growing culture and placed in the center of 9 cm-diameter petri dishes with CPDA. Around the pathogen, a circle with a 30 mm diameter was streaked using a loop full of bacteria sourced from 24–48-hour-old bacterial cultures grown on nutrient agar (NA) medium. A 30 mm diameter circle was streaked around the pathogen with bacteria from 24-48-hour-old bacterial cultures produced on NA. The Petri dishes were subsequently kept at 27°C for 7 days. In the control petri dishes, only the pathogen was inoculated, and no bacterial strains were added. After 7 days of incubation, the mycelial development of *R. solani* in both treatment and control petri dishes was evaluated. The percentage of fungal inhibition was calculated by comparing the mycelial growth in the treatment plates (with bacteria) to the control plates (without bacteria). Antifungal activity was determined by measuring the inhibition of mycelial growth by bacteria, using the formula below:

% Inhibition= (1-(Mycelial growth of treatment/Mycelial growth of control group)) x 100 formula was used (Tariq et al., 2010).

Bacterial isolates that demonstrated 50% or greater efficacy against *R. solani* AG 2-2, as indicated by the aforementioned formula, were further evaluated for PGP activities.

### Identification of Antagonistic Bacteria via Maldit of Biotyping

Bacterial isolates exhibiting substantial antagonistic activity were identified by using MALDI-TOF biotyping before being tested for PGP activities (Kara & Soylu, 2022). To do this, bacteria selected as possible biocontrol agents were streaked onto NA media and sent to a private laboratory (Hatay Plant Health Clinic Application and Research Center) for testing.

### Determination of PGP Activities of Potential Biocontrol Bacteria Showing Antibiosis Activity

### Determination of Phosphate Solubilizing Activity of Bacterial Isolates

To assess phosphatase activity, Pikovskaya medium, as described by Pikovskaya (1948), was used. The medium was composed of glucose (10g), $Ca_3(PO_4)_2$ (5g), $(NH_4)2SO_4$ (0.5g), NaCl (0.2g), $MgSO_47H_2O$ (0.1g), KCl (0.2g), yeast extract (0.5g), $MnSO_4H_2O$ (0.002g), $FeSO_47H_2O$ (0.002g), agar (15g), and distilled water (1000ml) with a pH of 7.0±0.2. The medium was prepped and sterilized at 121°C for 20 minutes.

Bacterial cultures that were 24-48 hours old were used to prepare a solution with a concentration of $10^8$ CFU/ml. Next, 10 µl of the solution was placed in the center of the Pikovskaya medium. The experiment included three replications for each isolate. Petri dishes were then incubated at 27°C for three days. Bacteria that can solubilize phosphorus could create a clear halo zone in the petri dish. Bacterial isolates that formed a clear zone were considered positive for phosphatase activity, and the activity was measured by the ratio (R), calculated as the clear halo diameter divided by the colony diameter (R = halo diameter/colony diameter) (Qingwei et al., 2023).

### Determination of Indole-3-acetic acid (IAA) Activity of Bacterial Isolates

L-tryptophan (Sigma-Aldrich) was used as a precursor at a concentration of 5 mM (1 g/L) in Luria-Bertani (LB) medium to assess IAA production. Bacterial cell density was adjusted to ~$10^8$ CFU/mL after 48h of growth in LB medium before transfer to tryptophan-supplemented LB. A 2% (v/v) inoculum was added to 5 mL of tryptophan-supplemented LB medium in 50 mL Erlenmeyer flasks. Cultures were incubated at 30°C with 200 rpm shaking for 72 hours, with uninoculated medium serving as a negative control. After incubation, cultures were centrifuged at 10,000 × g for 10 min. For colorimetric analysis, 1 mL of supernatant was mixed with 40 µL of 85% orthophosphoric acid and 4 mL of Salkowski reagent (0.5 M $FeCl_3$ in 35% $HClO_4$, prepared fresh daily). The reaction mixture was incubated in complete darkness at 25±2°C for 30 minutes, during which a pink-red color developed proportional to IAA concentration. Absorbance was measured at 535 nm against a reagent blank. A standard curve was generated using pure IAA (0-50 µg/mL) with each experiment, showing excellent linearity ($R^2$>0.995). This approach was modified from IAA assays described by many researchers (Gordon & Weber, 1951; Ambrosini & Passaglia, 2017). To determine the amount of IAA, standard IAA solutions were prepared at concentrations of 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 ppm, and absorbance was measured at the same wavelength using a spectrophotometer.

### Determination of Siderophore Activity of Bacterial Isolates

Bacteria were assessed for siderophore synthesis using a modified Blue-CAS Agar medium (Schwyn & Neilands, 1987; Kaya Özdoğan, 2020). Blue CAS Agar was prepared in four steps. Initially, 60.5 mg of Chrome Azurol S was dissolved in 50 mL of distilled water. $FeCl_3.6H_2O$ (13.5 milligrams) and HCl (41 µl) were dissolved in 50 ml of distilled water, and 10 ml of the solution was used. In the third step, 72.9 mg of HDTMA was dissolved in 40 mL

of water and added to the first and second processes. The final solution was sterilized at 121°C for 15 minutes. In the last step, 18 g of NA was dissolved in 900 mL of distilled water, and the pH was set to 6.8. The preparation process was completed with an autoclave at 121°C for 15 minutes.

In 9-cm-diameter petri dishes with Blue-CAS Agar medium, 10 µl of a $10^8$ CFU/ml solution was dropped at four equal places. The Petri dishes were then kept at 24°C for seven days. Bacteria capable of synthesizing siderophores chelated the iron in the medium, changing its color from blue to yellow. As a result, the existence of a yellow or orange zone around bacterial colonies showed good siderophore production (Klement, 1990). The diameter of the yellow zones generated by the bacteria was measured in millimeters, and the average diameter was calculated.

### Determination of HCN (Hydrogen Cyanide) Activity of Bacterial Isolates

The hydrogen cyanide (HCN) activity of biocontrol agent bacteria was determined using Tryptic Soy Agar Medium (TSA; Merck Milipore, 40 g/l). Bacteria aged 24 to 48 hours were streaked onto TSA medium and incubated at 28°C for three days. Sterile Whatman No. 1 papers were cut to suit the Petri dish lids and immersed in a picric acid-sodium carbonate solution (0.5 g picric acid, 2 g $Na_2CO_3$, 100 mL distilled water) (Gupta & Pandey, 2019). To avoid contact with bacterial colonies, forceps were used to carefully lay the solution-soaked filter papers on the inner surface of the Petri dish lid. The Petri plates were then parafilm-sealed and incubated for another 3-5 days at 28°C. The color change from yellow to brown to orange on the filter paper indicated cyanide production (Bakker & Schippers, 1987). *Pseudomonas koronensis* was used as a positive control in this experiment.

### Determination of Amylocyclopropane Carboxylate Deaminase (ACCD) Activity of Bacterial Isolates

The ACC-deaminase production activity of bacterial biocontrol agents was evaluated using the Dworkin Foster Salt Medium (DF), adapted from the method described by Penrose and Glick (2003). The DF medium was composed of the following: 2g glucose, 2g gluconic acid, 4g $K_2HPO_4$, 6g $Na_2HPO_4$, 0.2g $MgSO_4.7H_2O$, 2g citric acid, 18g bacto agar, 100 µl 1a solution (0.1 g $FeSO_4.7H_2O$, 10 ml distilled water), 100 µl 2b solution (0.01g $H_3BO_3$, 0.011 g $MnSO_4H_2O$, 0.12 g $ZnSO_47H_2O$, 0.008 g $CuSO_45H_2O$), 1000 ml distilled water. The pH was adjusted to 7.2 with 5 M KOH. The prepared DF medium was added to Petri dishes and was allowed to solidify (Dworkin & Foster, 1958). Next, use a Drigalski spatula to spread 50 µl of 0.5 M ACC on the surface of the Petri dishes. The Petri dishes' lids were left open in the laminar cabinet until they dried. Each bacterial isolate was plated on DF medium without adding ACC (which served as a nitrogen-free source). Petri dishes lacking ACC were utilized as a negative control. Positive control was also added to the DF medium in the form of $(NH_4)2SO_4$ (2 g/L). A $10^8$ cfu/ml suspension was prepared from bacterial colonies that had been incubated for 24 to 48 hours. Bacterial suspension (10 µl) was plated onto DF medium with ACC and into Petri dishes for negative and positive controls. The Petri dishes were then incubated at 27°C for 48-72 hours, and the bacterial isolates were monitored for colony.

### Molecular Characterization of Potential Biocontrol Agents

Candidate bacterial isolates were streaked on NA medium and incubated at 28°C for 48 hours. DNA was extracted from a single colony grown on the medium using the EurX GeneMATRIX Bacterial and Yeast DNA Isolation Kit (Poland). DNA isolation was performed in accordance with the manufacturer's instructions. The purity and concentration of extracted DNA were determined with Nanodrop equipment (Thermo Scientific Nanodrop 2000, USA). The isolated DNA was kept at -20°C until the polymerase chain reaction (PCR) tests were performed.

In PCR experiments, universal primers 27F-1492R (27F: 5'-AGA GTT TGA TCM TGG CTC AG-3', 1492R: 5'-ACG GCT ACC TTG TTA CGA CTT-3') (Weisburg et al., 1991) were used to amplify the 16S rRNA target gene area. PCR amplifications were performed in a final volume of 50 µl reaction mixture, consisting of 1 µl DNA (approximately 10 ng) template, 5 µl PCR buffer (10x), 4 µl dNTPs (4 mmol/L), 3 µl $MgCl_2$ (25 mmol/L), 1 µl of each primer (1 mmol/L), 0.5 µl Taq Polymerase (1.25 U) (FIREPol® DNA Polymerase), and 34.5 µl double-distilled water.

The PCR schedule included one cycle of initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 45 seconds, annealing at 57°C for 45 seconds, and extension at 72°C for 60 seconds, with a final extension cycle of 72°C for 5 minutes. The temperature subsequently lowered to 4°C to complete the PCR. The PCR amplification results were electrophoresed on a 1.5% agarose gel prepared with 1xTAE buffer at 100 volts for 90 minutes, then visualized under UV light using ethidium bromide. A single-step PCR process was performed to amplify a region of approximately 1470 base pairs. Following PCR, a single band was observed on the agarose gel, showing the success of the process. Sanger sequencing was performed using an ABI 3730XL Sanger Sequencing Device (Applied Biosystems, Foster City, CA) and a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA).

The sequence results of 12 bacterial isolates were compared to other bacterial DNA sequences at the National Center for Biotechnology Information (NCBI). Partial 16S rDNA sequences were deposited in GenBank and given accession numbers, as shown in. In addition, a phylogenetic tree based on maximum likelihood analysis of 16S rRNA gene nucleotide sequences of bacterial isolates were created using the Tamura Nei Model with a two-parameter model in MEGA X 11 (Fig 1).

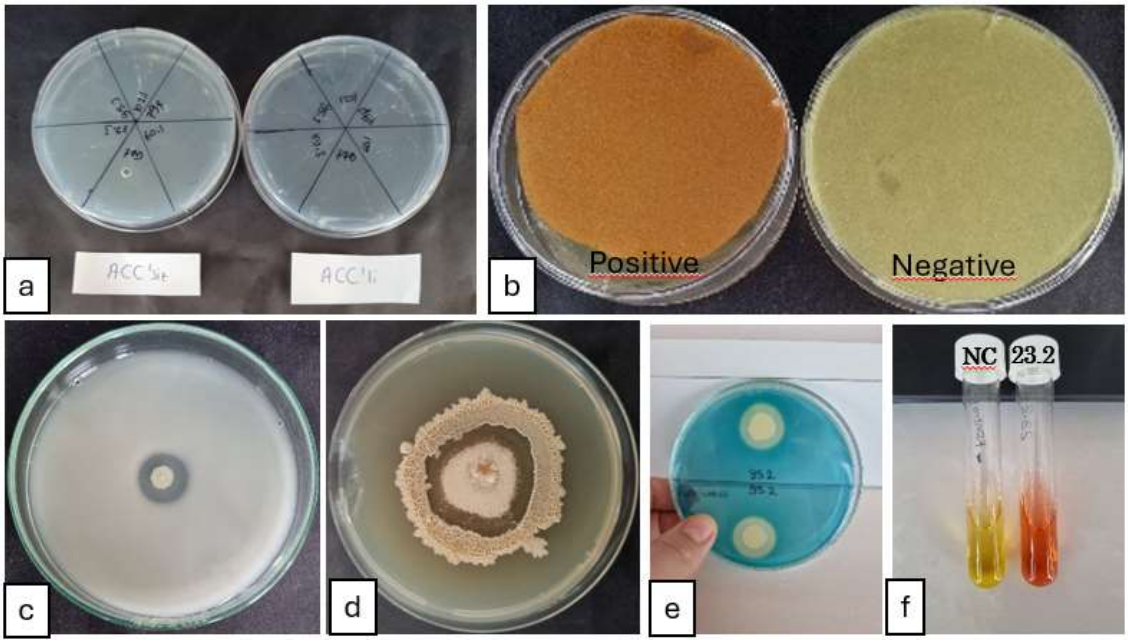


Figure 1. Tests to determine the PGP activities of BCA isolates: a-ACC deaminase test, b-HCN test, c-Phosphate solubility test, d-Dual culture test, e-Siderophore production test on Modified Blue-CAS Agar, f-IAA production (pink color development) by bacterial strain in a test tube.

*Şekil 1. Bakteriyel biyolojik mücadele etmeni izolatların PGP aktivitelerini belirlemek için yapılan testler a-ACC deaminaz testi b-HCN testi c-Fosfat çözünürlük testi d-Çift kültür testi e-Modifiye Mavi-CAS Agar üzerinde siderofor üretimi testi f-Test tüpünde bakteri suşu tarafından IAA üretimi (pembe renk gelişimi).*

## Selection of Bacteria Used in *In vivo* Experiments According to the Weighted Grading Method

Several biochemical tests were conducted to evaluate the antifungal activity of the obtained bacterial isolates against *Rhizoctonia solani* and to identify the most effective biocontrol agent for use in *in vivo* studies. The score distribution of this system, also known as the weighted rating method, is shown in Table 1. Following positive test results, the isolates with the highest overall scores were chosen for *in vivo* testing. The scoring of bacterial isolates was performed based on their plant growth-promoting and biocontrol traits, with specific criteria for each characteristic. Isolates positive for both HCN production and phosphate solubilization were awarded 10 points, while IAA production was scored as 5 points (7–21 ppm), 10 points (22–35 ppm), or 15 points (>35 ppm). In antagonistic activity assays, isolates showing 50–70% inhibition received 15 points, whereas those with >70% inhibition were assigned 25 points. Additionally, ACC deaminase-positive isolates were granted 20 points, and siderophore production was evaluated based on inhibition zone diameters, with above-average producers scoring 20 points and below-average producers receiving 10 points (Koçak & Salman, 2023; Salman, 2023).

Table 1. Scores of *in vitro* tests used in the weighted grading method
*Çizelge 1. Ağırlıklı derecelendirme yönteminde kullanılan in vitro testler*

| *In Vitro* Tests | Score Given to Test Results |
|---|---|
| Indole Acetic Acid (IAA) Production Test | 15 |
| Phosphate (P) Solubility Test | 10 |
| Siderophore Secretion Test | 20 |
| ACC deaminase Activity Test | 20 |
| Antagonistic Effect to *R. solani* | 25 |
| HCN Production | 10 |
| Total | 100 |

### Preparation of Pathogen Inoculum, Bacterial Suspension, and Soil Mixture for Pot Trials

In this experiment, 1-liter pots were used. The potting soil was prepared using a 2:1:1 ratio of peat, soil, and perlite and sterilized at 121°C for 60 minutes. The inoculum of *Rhizoctonia solani* was prepared using a barley culture medium. An agar plate from a fresh *R. solani* AG 2-2 culture, incubated at 25°C for 7 days, was transferred to barley culture medium in test tubes and incubated at 25°C for 3 weeks. To initiate infection, a single barley grain colonized by the pathogen was placed next to each seed.

*Bacillus* spp. Isolates were cultured on NA medium at 27 ± 1°C for two days. After incubation, the bacterial cultures were harvested by adding 15 ml of sterile distilled water, followed by spreading the bacterial suspension across the surface of the Petri dish using a sterile loop. The bacterial density was adjusted to $10^8$ CFU/ml, measured using a spectrophotometer at $OD_{660}$nm= 0.15. To enhance viscosity, 1.5% carboxymethyl cellulose (CMC) was incorporated into the bacterial suspension.

### Pot Experiment

The study used the sugar beet variety 'Lider', which was developed by Beta Ziraat Company and is widely cultivated in Türkiye. The experiment consisted of three replications for each bacterial isolate, with three seeds sown per pot. To ensure aseptic conditions, untreated seeds were surface sterilized with a 2% sodium hypochlorite (NaOCl) solution, followed by rinsing with sterile distilled water. Sterilized seeds were pre-germinated in water agar (10g/l) at 25°C for 4-5 days. In the positive control group, one infected barley grain was placed next to the germinating seeds. In the negative control pots, a sterilized barley grain was placed. Bacterial application was performed by immersing germinated seeds in 15 mL of bacterial suspension ($10^8$ CFU/mL) for 15 minutes. The seeds were then placed on sterile filter papers and allowed to air dry. After immersion, the seeds were placed on sterile filter papers and allowed to air dry under aseptic conditions. Each treated seed was then planted in a pot, accompanied by one barley grain pre-inoculated with the pathogen, and covered with soil. The 15 mL suspension was equally divided among three pots to ensure uniform distribution (Carling, 1996; Buhur, 2014; Basbagci, 2019).

To assess the efficacy of This bacterial isolates, a commercial *Bacillus subtilis* strain MBI 600 preparation (Subtilex®, Bioglobal) was included in the study. The commercial product was applied using the same method as for the isolates, starting from a Petri plate culture. Following the application, the pots were placed in a climate chamber at 25°C and 65% humidity for one month, alternating between 12 hours of light and 12 hours of darkness to promote plant growth. The plants were then uprooted, properly washed with tap water, and disease severity was assessed on a scale of 0 to 4 based on Muyolo's (1993) criteria. The average disease severity for each repeat was calculated using Towsend & Heuberger's (1943) formula (Table 2). The formula used to calculate the percentage efficacy of bacterial applications is:

Efficacy (%) = ((PC-BA)/PC) x 100.

Accordingly:

PC: Average disease severity (percentage of positive control)

BA: Disease severity (%) for each group where bacterial application was performed.

*Table 2. The descriptive scale is used to evaluate disease severity.*
*Çizelge 2. Hastalığın şiddetini değerlendirmek için kullanılan ölçek.*

| Scale Value | Disease Severity Description |
|---|---|
| 0 | Healthy seedling |
| 1 | Small brown superficial lesions on roots or stems |
| 2 | Deep and broad lesions on roots or stems; limited root development |
| 3 | Severe root rot, severe lesions encircling the main root or stem, and much shorter root length |
| 4 | Dead Plant |

The Disease Severity Index is calculated using the following formula, based on Townsend (1943):

% Disease Severity Index = [Σ(nxv)/(ZxN)] x100

Accordingly;

n: Number of plants in the disease scale

v: Numerical value of disease score

Z: Highest score number.

N: Total number of plants

### Statistical Analyses

The data obtained from the evaluations were compared using the Duncan Multiple Comparison test in SPSS 17.0 statistical software (SPSS Inc, Chicago, IL, USA) at a significance level of $p<0.05$.

## RESULTS and DISCUSSIONS

### Identification and Antagonistic Potentilas of Bacterial Isolate

Between 2020 and 2021, 219 soil samples were collected from 20 locations in Turkey, reflecting distinct ecological characteristics, in order to collect biocontrol bacteria. Isolation techniques obtained 752 bacterial isolates from these soil samples. In dual culture investigations, 752 bacteria were tested against *R. solani* AG-2-2. Among these, 117 bacterial isolates suppressed fungal mycelial growth to varying degrees.

Bacterial isolates displaying antagonistic activities were identified using MALDI-TOF biotyping in Hatay Mustafa Kemal University, Plant Health and Clinic Research Center, according to the method described previously (Kara & Soylu, 2022). Consequently, 94% of the bacteria were identified as *Bacillus*. These findings, combined with the tests done, suggest that *Bacillus* is a promising biocontrol agent. MALDI-TOF biotyping identified 29 *Bacillus mojavensis*, 18 *B. atrophaeus*, 9 *B. cereus*, 28 *B. pumilus*, 5 *B. subtilis*, 3 *B. altitudinis*, 13 *B. amyloliquefaciens*, 1 *Cronobacter*, 1 *B. thuringiensis*, 2 *Enterobacter cloacae*, 2 *Leclercia adecarboxylata*, 2 *B. simplex*, 1 *B. megaterium*, 2 *B. mycoides*, and 1 *Flavobacterium anhuiense*. Among these bacteria, *B. mojavensis* 95.2 and *B. mojavensis* 168.2 totally suppressed the pathogen's mycelial growth (Fig 2).

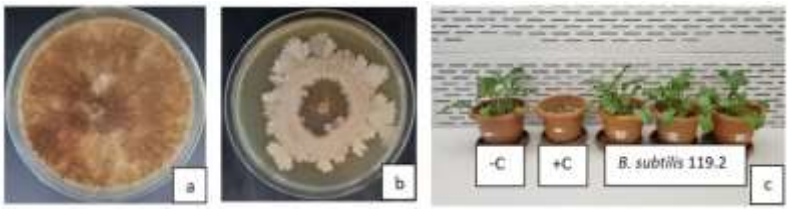


Figure 2. The use of image representations of *Bacillus subtilis* isolates' *in vitro* efficacy against *R. solani* AG-2-2 sugar beet isolate includes a- Control, b- Dual culture of *R. solani* and *Bacillus subtilis* 119.2, and c- *B. subtilis* 119.2 efficacy against *R. solani* under *in vivo* conditions (NC: Negative Control, PC: Positive Control).

*Şekil 2. Bacillus subtilis izolatlarının şeker pancarı R. solani AG-2-2 izolatına karşı in vitro etkinliğinin görsel gösterimi a- Kontrol, b- R. solani ve Bacillus subtilis 119.2'nin ikili kültürü, c- B. subtilis 119.2'nin in vivo koşullarda R. solani'ye karşı etkinliği (NK: Negatif Kontrol, PK: Pozitif Kontrol).*

### Determination of PGP Activities of Bacterial Isolate

In This study, 78 of the 117 bacteria tested for phosphate solubility produced a clear zone on Pikovskaya agar media and were classified as positive. Thus, approximately 67% of the screened bacteria exhibited phosphate solubilization activity. Among the bacterial isolates, *Enterobacter hormaechei* 77D (R: 3.2), *Bacillus siamensis* 216.1 (R: 2.29), *B. atrophaeus* 205.4 (R: 1.66), and *B. mojavensis* 164.4 (R: 1.58) were identified as the bacteria with the highest phosphate solubilization capacity. The ratio R values of the other isolates were below 1.

In This investigation, 76 of the 117 bacteria tested positive for the capacity to change the color of Blue CAS Agar media (to yellowish orange), showing that they can synthesize siderophores. The zone diameter created by these 76 bacteria as a result of iron chelation ranged from 1 to 33.5 mm. *Bacillus pumilus* 145.3 had the greatest zone diameter, measuring 33.5 mm. *B. pumilus* came in second at 56.3 (33 mm), followed by *B. mojavensis* at 168.2 (30 mm). *B. safensis* (1), *B. atrophaeus (2)*, *B. mojavensis* (2), *B. pumilus* (1), *B. subtilis* (1), *B. siamensis* (2), and *B. toyonensis* 187.1 (1) all produced siderophores.

Bacterial IAA levels were determined using the formula presented in the standard curve graph and data from spectrophotometric measurements. The measurements revealed that *Bacillus atrophaeus* 23.2 produced the highest level of IAA (50.3 ppm), followed by *Enterobacter hormaechei* 77 D (39 ppm), *E. cloacae* 139.4 (32.3 ppm), and *Leclercia adecarboxylata* 118.5 (31.6 ppm).

Of the 117 bacteria collected, 22 isolates showed positive ACC deaminase activity. These 22 bacterial isolates are from the species *Bacillus amyloliquefaciens*, *Enterobacter hormaechei, B. mojavensis, B. cereus, B. simplex, B. pumilus, B. atrophaeus, B. subtilis, B. safensis*, and *B. siamensis*.

The test showed that six bacterial isolates had positive hydrogen cyanide activity. Positive bacteria include *Bacillus mojavensis* 30.2, *B. subtilis* 35.2, *B. mojavensis* 103.1, *B. mojavensis* 163.6, *B. mojavensis* 168.2, and *B. amyloliquefaciens* 209.2b.

## Molecular Characterization of Bacteria Selected for *In Vivo* Studies

Bacterial PGP activities were assessed using a weighted scoring system (Table 1), and the bacteria with the highest scores were chosen for use *in vivo* efficacy experiments against *R. solani*. Bacterial isolates having the highest ratings under the scoring system were chosen for inclusion *in vivo* experiments. Despite the lack of beneficial results in the current dual culture assays, the *B. toyonensis* 187.1 isolate was selected for *in vivo* testing based on earlier laboratory experiments, in which it showed inhibitory effects against the *R. solani* AG-3 isolate.

After the tests to evaluate bacterial PGP activities were completed and graded, the bacteria with the highest scores were chosen for use *in vivo* efficacy experiments against *R. solani*. Among the bacteria that were analyzed using the scoring system, those with the highest scores were chosen for *in vivo* investigations. Despite the lack of effective results in dual culture experiments, the *B. toyonensis* 187.1 isolate was chosen for *in vivo* testing due to its efficacy against several *R. solani* isolates in earlier laboratory studies. Table 5 shows the molecular characterisation of the 12 possible biocontrol agents employed *in vivo* research (Fig 3).

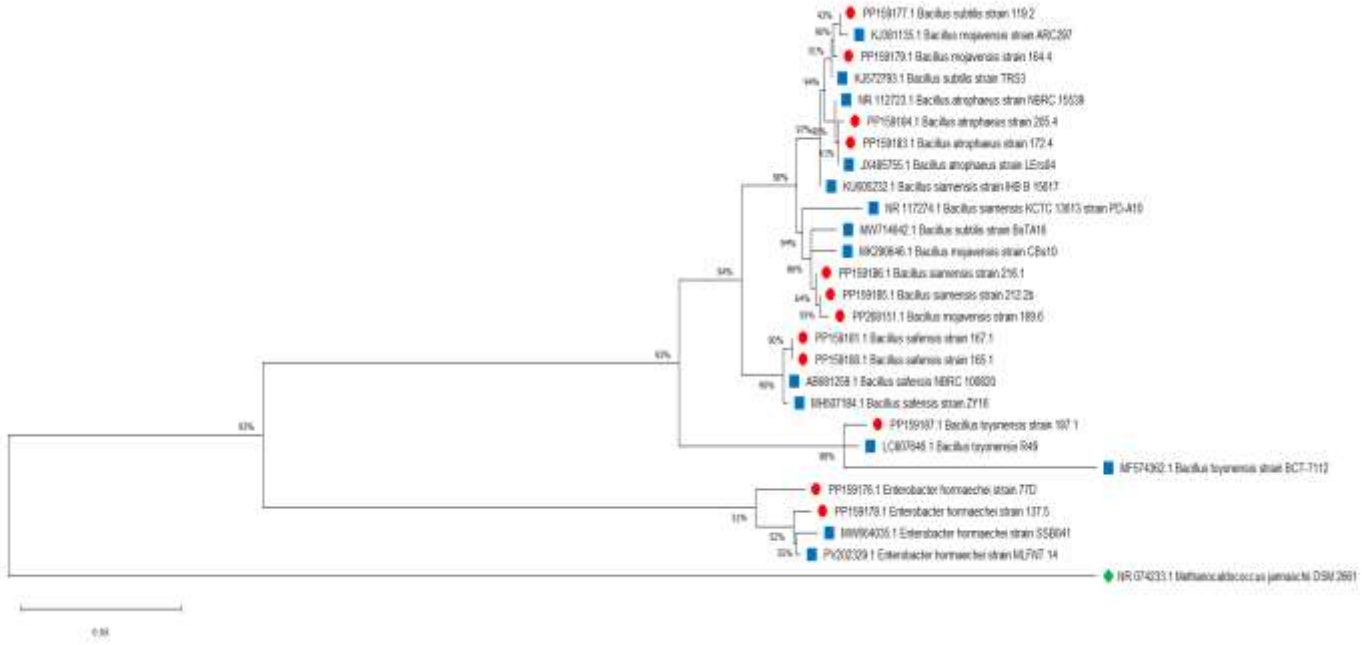


Figure 3. Neighbor-joining tree based on the Maximum Likelihood method and Tamura-Nei model of the nucleotide sequences of the 16S rRNA sequence of bacterial isolates selected for *in vivo*. The scale bar represents 0.05 nucleotide substitutions per nucleotide position.

*Şekil 3. In vivo için seçilen bakteri izolatlarının 16S rRNA dizisinin nükleotid dizilerinin Maksimum Olasılık yöntemi ve Tamura-Nei modeline dayalı komşu birleştirme ağacı. Ölçek çubuğu, nükleotid pozisyonu başına 0,05 nükleotid ikamesini temsil eder.*

In this study, ten bacterial isolates were evaluated based on their 16S rDNA gene sequences and showed approximately 100% similarity to *Bacillus* spp., while two isolates exhibited close phylogenetic relationships with *Enterobacter* species. The sequence of *Methanocaldococcus jannaschii* DSM 2621 (NR_074233.1) was used as an outgroup in the phylogenetic analysis. Figure 3 presents the phylogenetic tree constructed using the Neighbor-Joining method based on the 16S rRNA gene sequences obtained in this study, along with reference sequences retrieved from the NCBI database. Upon closer examination of the representative isolates marked with red circles on the tree, it was observed that *Bacillus subtilis* isolate PP159177.1 (strain 119.2) clustered within the same clade as the reference strains KJ381135.1 (*B. mojavensis* ARC297) and PP159179.1 (*B. mojavensis* strain 164.4) and was also closely related to KJ572793.1 (*B. subtilis* strain TRS3). Similarly, *Bacillus atrophaeus* isolates PP159184.1 (strain 205.4) and PP159183.1 (strain 172.4) formed a monophyletic group together with the reference strains JX485755.1 (*B. atrophaeus* strain LErs04) and NR_112723.1 (*B. atrophaeus* strain NBRC 15539). *Bacillus siamensis* isolates PP159186.1 (strain 216.1) and PP159185.1 (strain 212.2b) clustered with MK290646.1 (*B. mojavensis* strain CBs10) and were placed within the same main clade as *B. subtilis* MW714642.1 and *B. siamensis* NR_117274.1, indicating a close genetic relationship among *B. subtilis*, *B. siamensis*, and *B. mojavensis* species. On the other hand, *Bacillus safensis* isolates PP159181.1 (strain 167.1) and PP159180.1 (strain 165.1), along with

the reference strains AB681259.1 (*B. safensis* NBRC 100820) and MH507184.1 (*B. safensis* ZY16), formed a strongly supported monophyletic group. The *Bacillus toyonensis* isolate PP159187.1 (strain 187.1) showed high similarity to the reference strain LC807646.1 (*B. toyonensis* R49), and together they formed a sister group to the *B. safensis* clade. In contrast, MF574362.1 (*B. toyonensis* strain BCT-7112) was placed distantly from the other two *B. toyonensis* isolates. The *Enterobacter hormaechei* isolates PP159176.1 (strain 777D) and PP159178.1 (strain 137.5) formed a separate monophyletic group along with the reference strains MW664035.1 (strain SSB041) and PV202329.1 (strain MLFNT14), clearly diverging from the *Bacillus* species.

### *In Vivo* Efficacy of *Bacillus* spp. Isolates Against *Rhizoctonia solani* AG 2-2 Sugar Beet Isolate

*In vivo* studies were carried out to determine the efficiency of the selected 12 bacterial isolates against damping-off caused by *R. solani* AG 2-2 in sugar beet. When compared to the control group, seedlings treated with four antagonist bacterial isolates had significantly higher pre-emergence mortality rates due to damping-off (100%). *Bacillus subtilis* 119.2 was found to minimize damping-off entirely when compared to a control group treated with *R. solani*. Six isolates (164.4, 165.1, 167.1, 172.4, 205.4, and 212.2b) demonstrated a beneficial effect, however, at a modest level, as compared to the control group (Table 3).

Table 3. Effectiveness of *Bacillus* spp. against *R. solani* AG-2-2 sugar beet isolate *in vivo*.
*Çizelge 3. Bacillus spp.'nin şeker pancarı R. solani AG-2-2 izolatına karşı in vivo etkinliği.*

| Treatments | Disease Incidence (%) | Disease Severity (%) | Control Efficacy (%) |
|---|---|---|---|
| 77 D+*R. solani* | 100.00±0.00$^{a}$ | 100.00±0.00$^{a}$ | 0.00±0.00$^{d}$ |
| 119.2+*R. solani* | 0.00±0.00$^{d}$ | 0.00±0.00$^{d}$ | 100.00±0.00$^{a}$ |
| 137.5+*R. solani* | 100.00±0.00$^{a}$ | 100.00±0.00$^{a}$ | 0.00±0.00$^{d}$ |
| 164.4+*R. solani* | 89.00±1.00$^{b}$ | 89.00±1.00$^{b}$ | 11.00±1.00$^{c}$ |
| 165.1+*R. solani* | 89.00±3.61$^{b}$ | 89.00±3.61$^{b}$ | 11.00±2.65$^{c}$ |
| 167.1+*R. solani* | 89.00±3.61$^{b}$ | 89.00±3.61$^{b}$ | 11.00±4.36$^{c}$ |
| 169.6+*R. solani* | 100.00±0.00$^{a}$ | 100.00±0.00$^{a}$ | 0.00±0.00$^{d}$ |
| 172.4+*R. solani* | 78.00±4.36$^{c}$ | 78.00±4.36$^{c}$ | 22.00±2.65$^{b}$ |
| 187.1+*R. solani* | 78.00±8.72$^{c}$ | 78.00±8.72$^{c}$ | 22.00±2.65$^{b}$ |
| 205.4+*R. solani* | 89.00±4.00$^{b}$ | 89.00±4.00$^{b}$ | 11.00±2.65$^{c}$ |
| 212.2b+*R. solani* | 89.00±2.65$^{b}$ | 89.00±2.65$^{b}$ | 11.00±1.73$^{c}$ |
| 216.1+*R. solani* | 100.00±0.00$^{a}$ | 100.00±0.00$^{a}$ | 0.00±0.00$^{d}$ |
| Subtilex | 89.00±1.73$^{b}$ | 89.00±1.73$^{b}$ | 11.00±6.08$^{c}$ |
| Control (*R. solani* alone) | 100.00±0.00$^{a}$ | 100.00±0.00$^{a}$ | 0.00±0.00$^{d}$ |
| Control (no *R. solani*) | 0.00±0.00$^{d}$ | 0.00±0.00$^{d}$ | 100.00±0.00$^{a}$ |

P<0.05 (No statistically significant difference exists between the means represented by the same letter in the same column)

The gathered data were assessed using a weighted ranking system, and the results showed that 12 bacterial isolates had significant levels of antifungal activity against the pathogen (Table 4,5).

*Rhizoctonia solani* is a pathogen that causes root rot and wilting symptoms in sugar beet production. Its soil- and seed-borne nature complicates the management process (Zhao et al., 2019). To prevent root rot infections, producers have become excessively dependent on chemical treatments. However, the fungicides utilized cause harm to other microorganisms in addition to the intended diseases (Webb et al., 2015). A rise in consumer demand for pesticide-free produce has resulted in a decrease in chemical use and an increase in interest in the use of naturally beneficial bacteria known as biocontrol agents (Karimi et al., 2016). In this context, this work investigates the biological control capability of 12 antagonistic bacteria isolated from rhizosphere soil against *R. solani* AG 2-2 in sugar beet plants. Previous research on the biological control of *R. solani* applying antagonistic bacteria has shown comparable findings (Farhaoui et al., 2022; Farhaoui et al., 2023).

Rhizobacteria enhance plant growth and disease resistance through multiple mechanisms, such as nitrogen fixation, phosphate solubilization, phytohormone production, and siderophore synthesis (Ji et al., 2013; Selva Kumar et al., 2013; Dinu et al., 2019). Certain *Bacillus* species are especially effective at controlling diseases caused by soil-borne plant pathogens that live in soil. These *Bacillus* species not only promote plant development, but they additionally produce antibiotic-like compounds or activate plant host defenses before pathogen infection, hence decreasing illness (Dinu et al., 2019). In this work, 752 bacterial isolates from soil samples collected under different ecological settings were tested for inhibitory activity against *R. solani* AG 2-2 using a dual culture assay.

Table 4. Evaluation of bacterial isolates based on PGP and *in vitro* bioactivity tests using a weighted ranking system

*Çizelge 4. PGP ve ağırlıklı sıralama sistemi kullanılarak yapılan in vitro biyoaktivite testlerine dayalı bakteriyel izolatların değerlendirilmesi*

| | Species Name/Isolate Code | HCN test* | Phosphate Solubilization* | IAA* | IAA Concentration (ppm) | Dual Culture* | Inhibition (%) | ACC Deaminase Test* | The Siderophore Production Test* | The diameter of siderophore producing bacteria zone (mm) | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | *Enterobacter hormaechei* 77D | - | 10 | 15 | 39.0 | 25 | %77 | 20 | - | - | 66,6 |
| 2 | *Bacillus subtilis* 119.2 | - | 10 | 5 | 8.6 | 25 | %82 | - | 20 | 11.5 | 60 |
| 3 | *Enterobacter hormaechei* 137.5 | - | - | 10 | 10.4 | 25 | %91 | 20 | - | - | 51,6 |
| 4 | *Bacillus mojavensis* 164.4 | - | 10 | 10 | 10.2 | 25 | %72 | 20 | 10 | 4 | 71,6 |
| 5 | *Bacillus safensis* 165.1 | - | 10 | 5 | 7.2 | 25 | %80 | 20 | 10 | 3 | 70 |
| 6 | *Bacillus pumilus* 167.1 | - | 10 | 5 | 9.4 | 25 | %78 | 20 | 10 | 2.5 | 70 |
| 7 | *Bacillus mojavensis* 169.6 | - | 10 | 5 | 8.0 | 25 | %79 | 20 | 20 | 11 | 80 |
| 8 | *Bacillus atrophaeus* 172.4 | - | 10 | 5 | 7.7 | 25 | %79 | 20 | 10 | 4.5 | 70 |
| 9 | *Bacillus toyonensis* 187.1 | - | 10 | 10 | 10.1 | - | - | 20 | 20 | 7 | 60 |
| 10 | *Bacillus atrophaeus* 205.4 | - | 10 | 5 | 9.4 | 25 | %71 | - | 20 | 8 | 66,6 |
| 11 | *Bacillus siamensis* 212.2b | - | 10 | 5 | 7.1 | 25 | %76 | 20 | 10 | 3.5 | 70 |
| 12 | *Bacillus siamensis* 216.1 | - | 10 | 10 | 12 | 25 | %98 | 20 | 20 | 12.5 | 85 |

*Weighted rating parameters

Table 5. Location and host plants of bacterial isolates tested *in vivo*

*Çizelge 5. İn vivo test edilen bakteri izolatlarının konumu ve izole edildikleri konukçu bitkileri*

| Bacterial Isolates | MALDITOF Biyotipleme sonuçları | Blast Research Results | Accession Number | Isolation Region | Plants |
|---|---|---|---|---|---|
| 77D | *Bacillus atrophaeus* | *Enterobacter hormaechei* | PP159176 | Urla-İzmir | *Capsicum annum L.* |
| 119.2 | *Bacillus subtilis* | *Bacillus subtilis* | PP159177 | Çumra-Konya | *Beta vulgaris var. saccharifera* |
| 137.5 | *Bacillus cereus* | *Enterobacter hormaechei* | PP159178 | Sarayönü-Konya | *Solanum tuberosum L.* |
| 164.4 | *Bacillus mojavensis* | *Bacillus mojavensis* | PP159179 | Kaymaklı-Nevşehir | *Cicer arietinum L.* |
| 165.1 | *Bacillus pumilus* | *Bacillus safensis* | PP159180 | Söke-Aydın | *Gossypium hirsutum L.* |
| 167.1 | *Bacillus pumilus* | *Bacillus safensis* | PP159181 | Söke-Aydın | *Gossypium hirsutum L.* |
| 169.6 | *Bacillus amyloliquefaciens* | *Bacillus mojavensis* | PP268151 | Söke-Aydın | *Gossypium hirsutum L.* |
| 172.4 | *Bacillus atrophaeus* | *Bacillus atrophaeus* | PP159183 | Söke-Aydın | *Gossypium hirsutum L.* |
| 205.4 | *Bacillus atrophaeus* | *Bacillus atrophaeus* | PP159184 | Derbent-Konya | *Phaseolus vulgaris L.* |
| 212.2b | *Bacillus subtilis* | *Bacillus siamensis* | PP159185 | Silifke-Mersin | *Salsola cali L.* |
| 216.1 | *Bacillus amyloliquefaciens* | *Bacillus siamensis* | PP159186 | Silifke-Mersin | *Xanthium strumarium L.* |

The findings revealed that 117 bacterial isolates were effective against the severe *R. solani* AG 2-2 isolate, with inhibition rates of 50% or above. MALDI-TOF MS analysis revealed that 94% of these bacteria belonged to the *Bacillus* genus. Similarly, *Bacillus* spp. was also reported as dominant antagonists against several fungal pathogens in another studies (Soylu et al., 2020; Gümüş & Soylu, 2024), supporting their potential role in disease suppression. Moreover, *Bacillus* spp. has been shown to lessen *Rhizoctonia* disease pressure by increasing sucrose content (Misra et al., 2023). Furthermore, earlier study suggests that *Enterobacter cloacae* is an efficient biocontrol agent against soil-borne infections (Abdelijalil et al., 2021; Kumar et al., 2022). These findings highlight the possible use of antagonistic bacteria like *Bacillus* and *Enterobacter* as biological control agents for sugar beet diseases.

The 117 bacterial isolates identified as successful using dual culture assays were evaluated for PGP activity to identify the best of them. These activities consisted of phosphate solubilization, HCN production, siderophore production, ACC deaminase activity, and IAA production. *Pseudomonas, Bacillus, Rhizobium, Burkholderia, Achromobacter, Agrobacterium, Micrococcus, Aerobacter, Flavobacterium*, and *Erwinia* can solubilize insoluble inorganic phosphates by producing organic acids that reduce the pH. However, these phosphate-solubilizing bacteria are not found in significant numbers in the rhizosphere. Increasing the number of these bacteria in soil can improve plant growth (Rodríguez & Fraga, 1999; Billah et al., 2019). In this investigation, 78 of 117 bacterial isolates solubilized phosphate. Among these, 73 isolates belong to various *Bacillus* species. *Bacillus* species have developed a variety of strategies to promote plant development. *Bacillus megaterium, B. subtilis, B. pumilus, B. polymyxa, B. sphaericus,* and *B. thuringiensis* are examples of bacteria that may solubilize inorganic phosphates or mineralize organic phosphorus. This study found that, in addition to *Bacillus* species, *Leclercia adecarboxylata* (1 isolate), *Enterobacter cloacae* (3 isolates), and *Cronobacter* sp. (1 isolate) can solubilize phosphate. Previous investigations have also reported phosphate solubilization capability (Bektaş, 2021; Chen et al., 2023; Schmid et al., 2009).

Furthermore, certain bacteria produce hydrogen cyanide (HCN), which inhibits pathogenic fungus growth. Fluorescent *Pseudomonas* species, *Aeromonas, Chromobacterium, Burkholderia, Rhizobium*, and *Cyanobacteria* are typical cyanogens. Aside from its role in biological regulation, HCN helps to chelate metal ions and indirectly improves phosphate uptake (Sehrawat et al., 2022). *Bacillus mojavensis* (30.2, 103.1, 163.6, 168.2), *B. subtilis* (35.2), and *B. amyloliquefaciens* ssp. *plantarum* (209.2b) are among the *Bacillus* species found to be HCN-positive in this study. Recent research produced similar results, demonstrating that *Bacillus* species can produce HCN. *Bacillus* species isolated from the rhizospheres of tomato and bean plants have been shown to produce HCN (Agrawal & Agrawal, 2013; Sendi et al., 2020; Soylu et al., 2021). Several studies on *Bacillus* isolates from various plants, such as *B. megaterium* from sugarcane, *B. megaterium* TV-91C from cabbage, *B. subtilis, B. amyloliquefaciens,* and *B. cereus* from tomato, and *Bacillus* sp. from cucumber, have confirmed the HCN activity of these bacteria (Sundara et al., 2002; Almaghrabi et al., 2013; Turan et al., 2014). Furthermore, some PGPR bacteria can use low-molecular-weight siderophores to convert ferric iron ($Fe^{3+}$) into a form that can be used by the bacteria, particularly in iron-competitive situations. These bacteria's siderophores have several beneficial impacts on plants, including phytopathogenic microbe control, iron provision, and salt tolerance enhancement (Güney, 2014). In this study, 76 of the isolated rhizosphere bacteria tested positive for siderophore synthesis. *In vivo* studies revealed that all nine *Bacillus* isolates chosen from the 117 bacteria generated siderophores. These findings support *Bacillus'* ability to produce considerable amounts of siderophores. Similar research supports these conclusions. Güney (2014) researched and improved siderophore synthesis in *Bacillus cereus* DSM 4312. Ghazy and El-Nahrawy (2021) identified siderophores in *B. subtilis, B. circulans, B. coagulans, B. licheniformis, P. fluorescens*, and *P. koreensis*. PGPR bacteria with ACC deaminase activity (e.g., *Bacillus, Pseudomonas*, and *Rhizobium* spp.) enhance plant stress tolerance under drought, salinity, and heavy metal exposure (Çakmakçı, 2009; Zahir et al., 2009). Gupta and Pandey (2019) further demonstrated that such bacteria improve disease resistance. This experiments revealed that 22 bacterial isolates have ACC deaminase activity. Gupta and Pandey (2019) detected rhizobacteria with ACC deaminase activity in the garlic rhizosphere (*Allium sativum*). Two of the six isolates (*Aneurinibacillus aneurinilyticus* and *Paenibacillus* sp.) can convert ACC to α-ketobutyrate. In *in vitro*, these isolates displayed tolerance to salt and drought stress, and when evaluated *in vivo*, they promoted bean seedling growth by increasing root length, root weight, shoot length, root biomass, and chlorophyll content. In research of endophytic bacteria, Maheshwari et al. (2020) found that *Bacillus mojavensis* PJN13 had significant levels of ACC deaminase activity. Various rhizobacteria produce indole acetic acid (IAA), which has a substantial impact on plant growth. Plant-synthesised IAA stimulates cell division, elongation, seed germination, xylem and root development, lateral root formation, photosynthesis, pigment formation, metabolite biosynthesis, and stress resistance. IAA generated by PGPR increases root surface area and length, allowing for greater nutrient uptake and plant growth, hence increasing disease resistance (Ahemad & Kibret, 2014). In this study, all bacterial isolates produced IAA at various levels. Similarly, several rhizospheric bacteria, such as *Azospirillum, Enterobacter,*

*Azotobacter, Klebsiella, Alcaligenes faecalis, Azoarcus* sp., *Serratia* sp., *Sulphurbacter*, *Enterobacter* sp., *Bacillus* sp., *Pseudomonas* sp., and *Rhizobium*, are known to form IAA. *Bacillus megaterium, B. thuringiensis, B. pumilus, B. weihenstephanensis, B. cereus, B. toyonensis*, and *B. subtilis* are known to produce considerable amounts of IAA (Akköprü, 2012; Özdal et al., 2016; Wagi & Ahmed, 2019; Aktan & Soylu, 2020; Widawati, 2020). Rhizobacteria not only promote plant growth but also act as biocontrol agents, activating resistance mechanisms in plants and thereby protecting them from disease (Guo et al., 2015; Khoso et al., 2023). In this work, we used a weighted grading method to assess the antifungal and PGP activities of rhizobacteria isolated. *In vivo* experiments were carried out using the top 12 bacterial isolates based on their efficacy. Other studies have used similar grading systems to find the best bacterial candidates (El-Sayed et al., 2014; Babier & Akköprü, 2020). The 12 chosen bacterial isolates were identified using partial 16S rDNA sequencing data. *Bacillus mojavensis* 77D, *B. mojavensis* 164.4, *B. subtilis* 119.2, *B. siamensis* 212.2b, *Enterobacter hormaechei* 137.5, *B. safensis* 165.1, *B. safensis* 167.1, *B. mojavensis* 169.6, *B. toyonensis* 187.1, *B. siamensis* 216.1, *B. atrophaeus* 172.4, and *B. atrophaeus* 205.4 have been identified. These microorganisms, which belong to the Bacillaceae and Enterobacteriaceae families, have previously been documented in various studies for their effectiveness against *R. solani* in different plant species (Schmiedeknecht et al., 1998; Moussa, 2002; Elkahoui et al., 2012; Dönmez et al., 2015; Khedher et al., 2015; Mahmoud, 2016; Dinu et al., 2019; Farhaoui, 2023).

Other bacterial isolates were found to be ineffective or minimally beneficial, with 22% efficacy. Comparably, Karimi et al. (2016) tested the efficacy of eight bacteria against *R. solani* AG-2-2 in greenhouse conditions. The researchers showed that *B. amyloliquefaciens* SB14, *B. pumilus* SB6, *B. siamensis* AP2, and *B. siamensis* AP were more effective against the disease, with SB14 being the most effective isolate. SB14 indicated 58% efficiency against *R. solani* AG-4 and 52.5% against *R. solani* AG-2-2. Further investigations found bacterial biocontrol agents to be less efficient against *R. solani* isolated from sugar beets. Aksoy (2019) performed pot tests to determine the efficacy of six *Bacillus* spp. and six *Pseudomonas* spp. isolates that had previously shown high efficacy *in vitro* and found that *Bacillus* sp. isolated from Yenice 1.1 was the most effective bacterium, with a 16.38% efficacy. *Bacillus velezensis* SS2 and FB1 were also found to be more than 50% effective against AG 2-2 in sugar beets *in vivo*. *Bacillus subtilis* 119.2, isolated from the rhizosphere of sugar beets, was found to be highly efficient against *R. solani* AG 2-2. Root exudates are known to differ depending on plant species, cultivar characteristics, growth stages, and biotic and abiotic stress factors. As a result, rhizobacteria that prefer various substrates colonise distinct rhizospheres (Molefe et al., 2023). This findings highlight the potential of rhizosphere-derived *Bacillus* and *Enterobacter* strains as biocontrol agents against *R. solani* in sugar beet. Future studies should explore field applications and synergistic interactions with other PGPs to optimize sustainable disease management.

## CONCLUSION

In this present study, 752 bacterial isolates were tested against *Rhizoctonia solani* AG 2-2 using the dual culture method, and bacteria that showed antifungal activity were evaluated based on their biochemical features. The gathered data were assessed using a weighted ranking system, and the results showed that 12 bacterial isolates had significant levels of antifungal activity against the pathogen. Furthermore, based on their biochemical features, these isolates were expected to have a beneficial impact on plant growth. *In vivo* experiments found *Bacillus subtilis* 119.2 to be an efficient bio-agent against *R. solani* AG 2-2 in sugar beet (*B. vulgaris*). *B. subtilis* 119.2 isolate demonstrated 100% effectiveness, the greatest antifungal activity among the studied bacteria. These results suggest that *B. subtilis* 119.2 is a promising candidate for the biological control of plant diseases. This study's findings show that *B. subtilis* 119.2 has the potential to be formulated as a biofertilizer or biopreparation, providing an environmentally acceptable solution. As a result, it is advised that this biocontrol agent be evaluated in the field and then developed into a highly effective and environmentally friendly biofungicide or biofertilizer. The use of such biocontrol agents as an alternative to chemical pesticides can improve agricultural sustainability while reducing environmental effects.

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### Author's Contributions

The authors declare that they have contributed equally to the article.

### 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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