Research article

Harnessing rhizobacteria: Isolation, identification, and antifungal potential against soil pathogens

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ABSTRACT

Rhizobacteria play a crucial role in plant health by providing natural antagonism against soil-borne fungi. The use of rhizobacteria has been viewed as an alternative to the use of chemicals that could be useful for the integrated management of plant diseases and also increase yield in an environmentally friendly manner. However, there is limited understanding of the specific mechanisms by which rhizobacteria inhibit these pathogens and the diversity of rhizobacterial species involved. This study aims to isolate, identify, and characterize rhizobacteria with antagonistic activities against soil-borne fungi. Laboratory tests were carried out on isolated rhizobacteria to evaluate their inhibitory activity against *Rhizoctonia solani*, *Pythium aphanidermatum* and *Macrophomina phaseolina*. The selected bacteria were identified using the Vitek 2 compact system and 16S rRNA genes. Experiments were carried out to evaluate the plant growth promotion and biocontrol ability of these selected isolates. Out of 324 rhizobacteria isolates obtained from various plant species, twelve were chosen due to their strong (>50 %) wide-ranging antifungal activity against three significant phytopathogenic fungi species. According to the identification results, they belong to the following species: *Aeribacillus pallidus* ECC4, *Alloiococcus otitis* BRE6, *Aneurinibacillus thermoaerophilus* ECL1, *A. thermoaerophilus* SDV1, *Bacillus halotolerans* DMC8, *B. megaterium* SKE2, *B. megaterium* TNK1, *B. subtilis* NAS1, *Enterobacter cloacae complex* BZD3, *Leclercia adecarboxylata* DKS3, *Paenibacillus polymyxa* TRS4, and *Staphylococcus lentus* BZD2. Eleven isolates produced protease, six isolates produced chitinase, and seven isolates were highly effective in producing hydrogen cyanide. Ten isolates could fix nitrogen, while all isolates could produce potassium, indole-3-acetic acid, siderophore, and ammonia. These findings enhance our understanding of rhizobacterial biodiversity and their potential as biocontrol agents in sustainable agriculture.

1. Introduction

Plant diseases are responsible for 20–60 % of Iraq's agricultural losses [45]. Rhizobacteria are soil bacteria that inhabit plant roots and affect plant health and growth. They can affect plants in a beneficial or detrimental way, depending on how they interact with the plant and its environment [30]. Rhizobacteria known as plant growth-promoting rhizobacteria (PGPR) are a particular kind of rhizobacteria that aid in the development of plants by producing siderophores, phytohormone synthesis, nitrogen fixation, phosphate and

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potassium solubilization, among other activities [35]. Rhizobacteria known as biological control agents (BCAs) decrease plant diseases by lytic enzymes, competition, antibiosis, and induced systemic resistance, among other mechanisms [10]. Rhizobacteria with dual purposes are able to carry out BCA and PGPR activities [48]. Rhizobacteria are thought to be effective alternatives to conventional pesticides and fertilizers, which have a number of drawbacks and restrictions, such as high cost, environmental pollution, risks to human health, pest resistance, pest resurgence, and off-target effects [30]. The most prevalent and varied group of plant diseases are fungi, which may infect roots, stems, leaves, flowers, fruits, and seeds in all kinds of plants. Additionally, a variety of plant diseases, such as blights, powdery mildews, wilts, and root rots, can be brought on by them [21]. Root rot disease is one of the most prevalent and dangerous fungal diseases in plants [16]. Root rot infections are caused by a wide range of fungal species, including *P. aphanidermatum*, *M. phaseolina*, and *R. solani*, which have a wide host range and may grow in a variety of soil types and conditions [21]. According to Sallam et al. [6], rhizobacteria that promote plant growth have proven to be useful in agricultural environments as biofertilizers and biocontrol agents for a range of phytopathogens. In particular, they found that in a greenhouse setting, the *E. cloacae* isolate might boost maize (*Zea mays*) immune response against *Fusarium oxysporum* infection. *In vitro* fungal growth of *R. solani* was strongly inhibited by 9 out of 28 rhizobacterial isolates that Tariq et al. [32] isolated from the rhizosphere of potato (*Solanum tuberosum* L.) plants. Strains of *B. subtilis* and *E. cloacae* with high protease activity significantly reduced the prevalence of *R. bataticola* by dissolving the fungal cell walls [11]. Thirty rhizobacterial isolates, the majority of which are from the *Bacillus* genus, were found by Bhattacharyya et al. [13] to have the capacity to stimulate plant development through the production of siderophores, the synthesis of IAA, and phosphate solubilization activity. The soil bacteria *B. halotolerans* was demonstrated by Rana et al. [3] to be an efficient biocontrol agent against a variety of phytopathogenic fungi, such as *M. phaseolina*, *R. solani*, *P. aphanidermatum*, and *Sclerotinia sclerotiorum*. *Bacillus* species that generate proteases have the ability to effectively suppress root rot pathogens such as *R. solani*, as demonstrated by Jamali et al. [24]. El-Kazzaz et al. [33] reported that the fungal development of *F. oxysporum* and *R. solani* was greatly inhibited *in vitro* by the rhizobacterial isolate of *P. polymyxa*. According to Jana et al. [46], *Pantoa* sp. was the most successful in generating ammonia and phosphate and potassium solubilization. This study hypothesizes that rhizobacteria isolated from Iraqi soils exhibit significant antagonistic activity against root rot pathogens and possess plant growth-promoting properties. Despite the extensive research on PGPR and BCAs, there is still a need to better understand the specific mechanisms by which these rhizobacteria promote plant growth and control pathogens. This research aims to fill the knowledge gap by isolating and identifying rhizobacteria from Iraqi soils, which have not been extensively studied. This study helps to reduce the usage of chemical pesticides and fertilizers and establish sustainable agriculture practices by describing their dual roles as PGPR and BCAs. The primary objective of this study is to isolate and identify rhizobacteria from soil collected in Iraq that demonstrate strong antagonistic activity against serious root rot pathogens and significant capacities to promote plant growth and development. This research seeks to enhance our understanding of the biodiversity and functional capabilities of rhizobacteria in Iraqi soils, contributing to more sustainable and effective agricultural practices.
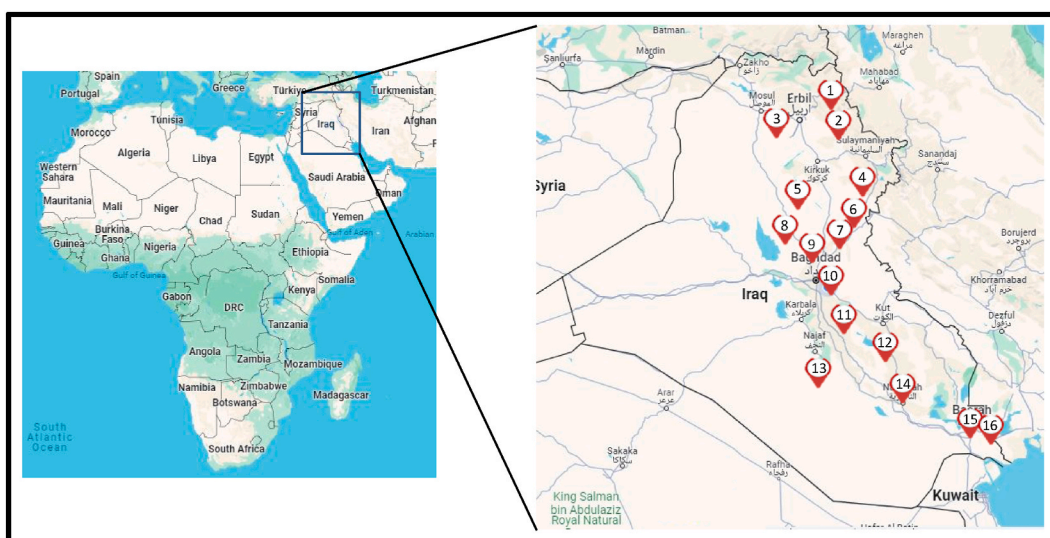

Fig. 1. Geographic distribution of soil samples from the rhizosphere in Iraq.
a.Erbil, Choman, b.Sulimaniah Dukan, 3.Erbil, Makhmur, 4.Sulimaniah, Kalar, 5.Salah Al Din, Samarra, 6.Diyala, Khanaqin, 7.Diyala, Miqdadiyah, 8.Salah Al Din, Dejail, 9.Baghdad, Rashdiya, 10.Baghdad, Madain, 11.Najaf, Al Abassiya, 12.Thi Qar, Rifai, 13.Najaf, Mishkhab, 14.Thi Qar, Nasiriyah, 15.Basra, Zubayr, 16.Basra, Abu Al Khaseeb

2. Materials and methods

2.1. Collection of samples

Samples were taken from the rhizosphere, the soil that adheres firmly to the roots of various plant species, such as trees, crops, and herbs, and which is distinguished by superior root and vegetative development. Between August and October of 2022, samples were gathered from 16 areas spread among 8 governorates in northern, central, and southern Iraq (Baghdad, Diyala, Salah Al Din, Erbil, Sulaymaniyah, Basra, Najaf, and Thi Qar) (Fig. 1). At the time of sampling, the plants were identified by the use of the botany scientific application of (PictureThis) technology (Glority Global Group Ltd., Hangzhou, Zhejiang, China).

2.2. Rhizobacteria isolation and purification

Rhizobacteria were isolated and purified using Moradi et al.'s [31] methodology. Rhizospheric soil was randomly selected from eight governorates in northern, central, and southern Iraq, including 80 different plant samples. Before being sent to the lab for processing, samples were promptly cooled and kept in labeled propylene bags. Using a brush, soil from the rhizosphere was meticulously removed from root surfaces in the lab. Sub-samples were then obtained by completely homogenizing the soil samples and passing them through a 2-mm sieve. The soil sample was serially diluted with concentrations ranging from 10^{-1} to 10^{-7} g of soil in order to identify bacterial strains. Each of the two dilutions, 10^{-5} and 10^{-7}, included a 100 µl aliquot that was equally distributed among four nutritional agar (NA) plates (Condalab, Torrejon de Ardoz, Madrid, Spain) in triplicate. After 48 h of incubation at 37 °C, the plates were checked for bacterial growth. To obtain single, pure colonies, morphologically distinct bacterial colonies were chosen and re-spread onto new NA plates. The purified were temporarily preserved in sterilized nutrient broth (NB) (Condalab, Torrejon de Ardoz, Madrid, Spain), evenly mixed with 20 % glycerol solution, at −20 °C [44].

2.3. Investigating in vitro the biocontrol properties of rhizobacterial isolates

2.3.1. Antagonism assay
The involvement of antagonism as a mode of action of the BCA was determined using a dual culture plate test. The antagonistic interaction was evaluated by observing the presence of an inhibitory zone between the bacterial colonies and the fungal mycelia on an agar surface. The antagonistic effects of 324 rhizobacterial isolates against *R. solani*, *P. aphanidermatum*, and *M. phaseolina* were tested *in vitro* using the method described by Singh et al. [38]. The isolates were obtained from the University of Baghdad, College of Agriculture, Department of Plant Protection, and the Ministry of Sciences and Technology, Agricultural Research Department. The PDA medium was prepared using the dual culture approach. A 5 mm segment of each fungal mycelia plug was extracted from an agar plate using a cork borer. The plugs were then put 2 cm away from the edge of a PDA agar plate. Similarly, each bacterial isolate was streaked separately on the other side of the medium, also 2 cm away from the border. The PDA plate with paired samples was incubated at a temperature of 28 ± 2 °C for duration of 7 days. Plates that contained solely fungal pathogens were used as a control. Three replicates were used in the experiment, and it was repeated twice. The plates were assessed for any potential antagonistic interaction between the rhizobacterial isolates and the fungal isolates by measuring the inhibition zone created between them using the following formula:

$$I(\%) = [(C - T) / C] x 100$$

Where I is the percentage of mycelial growth inhibition, C is the pathogen growth on control plates, and T is the pathogen growth on dual culture plates.

2.3.2. Test for bacterial strain cross-streak
Twelve antagonistic rhizobacterial isolates were prepared using Santiago et al.'s [15] method. The isolates were streaked perpendicularly on freshly prepared Nutrient Agar after being grown in Nutrient Agar at 30 °C for 24 h. After letting the first strain develop for 3 days at 30 °C, the second strain was streaked at an approximate angle of 90° heading outward from the first strain's emerging colonies. The experiment was carried out in duplicate with three repetitions, with the second colony being cultured at 30 °C for a further three days. Following this, photographic documentation of the agar plates was collected to record the intersecting zones of matched strains.

2.3.3. Production of hydrogen cyanide (HCN)
Using sterile filter paper soaked in a solution of 2.5 g of picric acid, 12.5 g of Na_2CO_3, and 1000 mL of distilled water, the HCN production of 12 antagonistic isolates was assessed qualitatively and placed inside the Petri plate's upper lid. On King's B medium (HiMedia Laboratories Ltd., Mumbai, Maharashtra, India), which had been modified with glycine at 4.4 g/l, the antagonistic bacteria were streaked. The plates were incubated for 48 h at 28 °C while being covered with parafilm. The experiment was carried out in duplicate with three repetitions. It was noted that the filter paper's color changed from yellow to light brown, brown, or reddish-brown [9].

2.3.4. Protease production

The twelve antagonistic isolates were cultivated on skimmed milk agar plates made using Krechel et al.'s [1] methodology. After three days of incubation at 30 °C, the formation of a clear zone (halo) surrounding the colonies on the plates indicated the presence of protease activity, which is indicative of the bacteria hydrolyzing proteins. The experiment was carried out in duplicate with three repetitions.

2.3.5. Chitinase production

The method of Frandberg and Schnurer [19], as described by Gamal-Eldin et al. [23], was used to qualitatively assess chitinase production. It involved spot-testing isolates on a chitinase medium (HiMedia Laboratories Ltd., Mumbai, Maharashtra, India) amended with colloidal chitin (Biosynth, Switzerland). After incubating the plates at 30 °C for 5 days, the development of a clear zone (halo) surrounding the colony was deemed positive for chitinase production. The experiment was carried out in duplicate with three replicates.

2.4. Identification antagonistic bacteria

Twelve antagonistic rhizobacterial isolates were prepared on a fresh culture plate for identification using the Vitek 2 compact system technique (BioMerieux, France). Four isolates were not identified by Vitek 2, so they were identified by polymerase chain reaction (PCR) amplification of the 16S rRNA gene.

2.4.1. Isolation of DNA

Using a bacterial genomic DNA isolation kit (ABIOpure, USA), genomic DNA was extracted from pure bacterial colonies overnight, following the manufacturer's recommendations. 1 ml of the culture was centrifuged for 2 min at 13000 rpm, and the supernatant was discarded, after adding 100 μl of lysozyme solution and 100 μl of nuclease-free water to the pellet, vortexing was performed. The samples were then incubated for 30 min at 37 °C in the water bath. Following the incubation period, samples were centrifuged for 2 min at 13000 rpm, and the supernatant was discarded. To facilitate protein digestion and cell lysis, 200 μl of Buffer BL and 20 μl of Proteinase K solution (20 mg/ml) were added to the sample. The tube was then well mixed with a vortex and heated at 56 °C for 30 min. It was then incubated for 30 min at 70 °C to promote additional lysis. After adding 200 μl of 100 % ethanol to the sample, it was thoroughly mixed with a pulse-vortex technique. Each combination was carefully transferred to the tiny column and centrifuged for 1 min at 6000 rpm. Then, a new collecting tube was employed in its place. A new collection tube was added after the tiny column was filled with 600 μl of Buffer BW and centrifuged at 10,000 rpm for 1 min. After adding 700 μl of Buffer TW, the mixture was centrifuged for 1 min at 10,000 rpm. The pass-through was disposed of, and the mini-column was then placed back into the collecting tube. The mini-column was placed into a fresh 1.5 ml tube after being centrifuged for 1 min at the highest speed at 13,000 rpm to remove any leftover wash buffer. After centrifuging the mixture for 5 min at 5000 rpm and incubating it for 1 min at room temperature, 100 μl of Buffer AE was added. The extracted DNA concentration was measured using a Quantus Fluorometer to determine if the samples were suitable for use in further processes. A microliter of DNA was combined with 199 μl of Quantifluor dye that had been diluted. The concentration of DNA was found after incubation for 5 min at room temperature.

2.4.2. PCR amplification of 16S rRNA

According to dos Santos et al. [25], the amplified 16S rRNA was prepared using universal primers (27F forward 5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492R reverse 5′-TACGGTTACCTTGTTACGACTT-3′). One tube of the amplification mixture contained 2 μL of the DNA template, 1 μL of each primer, and 21 μL of the Master Mix (Promega, USA). Thermo Fischer Scientific, USA's Thermal Cycler was used for the amplification process. The amplification profile included 5 min of initial denaturation at 95 °C, thirty cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, 1 min of extension at 72 °C, and a final 7 min of extension at 72 °C. Agarose gel (1.5 %) electrophoresis was used to verify the existence of amplification following PCR amplification. Sanger sequencing of the purified amplicons was performed using an automated DNA sequencer from Macrogen Corporation in Korea. Further examined using the Basic Local Alignment Search Tool (BLAST), which locates areas of local similarity between sequences, using closet culture sequences that were obtained from the National Center for Biotechnology Information (NCBI) database. The GenBank nucleotide sequence database contains the 16S rRNA gene sequences found during this study.

2.5. Morphological and physiological characteristics of the rhizobacterial isolates

The morphological characteristics of the twelve antagonistic isolates, such as their colony boundary, color, and form, as well as their Gram staining, were examined according to Kloepper et al. [28]. Tests for spore formation were conducted using the Schaeffer-Fulton technique, as reported by Oktari et al. [2]. The bacterial isolates' motility was assessed using the Collee et al. [27] methodology.

The durability of bacterial isolates at several temperatures was assessed in Nutrient Broth injected with 50 μl of bacterial cultures (10^9 cfu ml-1). The cultures were incubated at 15 °C, 25 °C, 35 °C, 45 °C, and 50 °C. The optical density (O.D.) was determined at 420 nm using a UV–Vis spectrophotometer (Shimadzu, Japan) after 48 h. The experiment conducted in three replicates and repeated twice.

To assess the growth of bacteria at different pH levels, 50 μl of bacterial cultures (10^9 cfu ml-1) were used to inoculate nutritive broth maintained at different pH values (4, 5, 6, 7, 8, 9) using either HCl or 1N NaOH. O.D. was measured at 420 nm in a UV–Vis spectrophotometer 48 h later. The experiment conducted in three replicates and repeated twice.

2.6. Evaluating the efficiency of rhizobacterial isolates in promoting plant growth in vitro

2.6.1. Assessment of the insoluble phosphate's solubility

On Pikovskaya's Agar (0.5 g Yeast extract; 10 g Dextrose; 5 g $Ca_3(PO_4)_2$; 0.5 g $(NH_4)_2SO_4$; 0.2 g KCl; 0.5 g $MgSO_4.7H_2O$; 0.0001 g $MnSO_4$; 0.0001 g $FeSO_4$ and 15.0 g Agar), the isolates were qualitatively screened for their ability to solubilize phosphates. A total of 31.3 g of the medium was suspended in 1000 ml distilled water and sterilized by autoclaving at 121 °C for 15 min. The isolates were then streaked individually and incubated for 72 h at 30 ± 2 °C. A halo zone surrounding the bacterial colony suggested that the isolates were positive [36]. By adding (0.5 ml of 10^9 CFU ml-1) of pure bacterial suspension to each 250 ml flask containing 100 ml of Pikovskaya's broth enriched with tri-calcium phosphate as an insoluble phosphate source, these isolates' phosphate solubilization potential was quantitatively estimated [36]. Uninoculated flasks with the same volume of the medium were used as the control. Following a 7-day incubation period at 30 °C and 150 rpm, the cultures were centrifuged for 25 min at 1000 rpm. The Phosphate Colorimetric Assay Kit (Sigma-Aldrich, St., Louis, MO, USA) was used to measure the soluble P content using the supernatant. The phosphate standards for colorimetric detection were prepared according to the manufacturer's instructions.

Samples are measured directly by filling wells with 150 μl of sample and then adding distilled water to get a final volume of 200 μl.

The assay reaction was carried out by filling each well with 30 ml of the phosphate reagent, mixing thoroughly with a horizontal shaker, and then allowing the plate to sit at room temperature for 30 min while it was covered to keep out light. A SmartReader 96 Microplate Absorbance Reader (Accuris Instruments, A Division of Benchmark Scientific Inc., Hercules, NJ, USA) was used to read the microplate at 650 nm time.

The concentration of phosphate in the test samples is calculated as follows: samples diluted to 10^{-5} in Tris Buffer to ensure your readings will fall within the standard values. When performing calculations, average the three readings for each standard and sample performed, and subtract the mean absorbance value of the blank standard (0 blank) from all readings and standard curve readings plotted. The experiment was conducted twice and the concentration of phosphate in the test samples is calculated based on the following formula:

Phosphate Concentration = (B/V) *D

Where B is the sample's phosphate content, as determined by the standard curve (nmol). V is the initial volume of sample (μl) introduced to the reaction well. D is the factor of sample dilution.

2.6.2. Assessment of potassium's insoluble solubility

The isolates were subjected to a potassium solubilization screen on Aleksandrov's Agar. A halo zone surrounding the bacterial colony indicated that the isolates were positive, as demonstrated by the presence of the zone surrounding the colony after autoclaving 29.6 g of the medium suspended in 1000 ml distilled water at 121 °C for 15 min, followed by streaking and incubating at 30 ± 2 °C for 72 h [22].

By adding 0.5 ml of 10^9 CFU ml-1 of pure bacterial suspension to each 250 ml flask containing 50 ml of Aleksandrov's broth enriched with K-feldspar powder, these isolates' potassium solubilization potential was quantitatively estimated in Aleksandrov's medium. The flask was then incubated for 7 days at 30 °C in an incubator, with an uninoculated Aleksandrov solution serving as a control. Following incubation, 5 ml of broth was centrifuged for 10 min at 8000 rpm. Using a UV–Vis spectrophotometer (Shimadzu, Japan), the optical density was measured at 600 nm. Two runs of the experiment were conducted, with an average of three readings calculated. A standard graph of KCl acquired in the range of 0–1000 μg ml-1 was used to assess the concentration of potassium generated by cultures [20].

2.6.3. Estimation of indole-3-acetic acid synthesis

The bacterial isolates were cultured in a nutritional broth that had been added with 0.5 % (w/v) ʟ-tryptophan (L-Trp). The broths with and without tryptophan were used as a reference. The cultures were incubated for seven days at 30 ± 2 °C with continuous shaking at 150 rpm. The supernatant was collected in a new, sterile tube after the nutrient broth culture was centrifuged for 10 min at 10,000 rpm. 25 mints were stored in the dark at room temperature after 2 ml of the supernatant and 2 ml of Salkowski reagent (50 ml of 35 % $HClO_4$ and 1 ml of 0.5 M $FeCl_3$) were combined in a sterile tube. The emergence of the pink hue signifies the creation of IAA. Utilizing a UV–Vis spectrophotometer (Shimadzu, Japan), absorbance was measured at 530 nm. Two runs of the experiment were conducted, with an average of three readings calculated, and the quantity of IAA generated was quantified and compared with the IAA standard curve (0–100 μg ml–1) [7,36].

2.6.4. Estimation of ammonia production

The ability of each bacterial isolate to produce ammonia was examined. Peptone water was used for the quantitative determination of ammonia generation, and it was shaken continuously at 150 rpm for 24 h at 30 ± 2 °C. 5 % Nessler's reagent (100 g Mercury(II) Iodide; 70 g Potassium Iodide; 160 g Sodium Hydroxide in 1L distilled water), manufactured by Panreac Quimica S.L.U., Madrid, Spain, was added to cell-free supernatants of peptone water, and an uninoculated nutrient broth containing Nessler's reagent served as a control. According to qualitative testing, color changes in the supernatant from pale to deep yellow or yellowish orange were noted for positive isolates [41]. The experiment was carried out in triplicate and repeated twice, with absorbance being measured at 450 nm and the quantity of ammonia generated being quantified using the ammonium chloride standard curve of values in the range of 0–1000 μg ml-1.

2.6.5. Estimation of siderophore production

The method of Alexander and Zubrer [18] that was described by Louden et al. [12] was utilized to estimate siderophore production for gram-negative bacterial isolates using a chrome azurol S agar (CAS) plate. For gram-positive bacterial isolates, a modified method known as the O-CAS method was employed, as reported by Perez-Miranda et al. [43]. 5 μl of bacterial suspension (10^9 cfu ml-1), was added to the middle of each Petri dish. For four days, the dishes were incubated at 26 °C. The experiment was carried out in triplicate and repeated twice, and the creation of a yellow-orange halo surrounding the bacterial colonies was subsequently assessed as a positive reaction.

2.6.6. Evaluation of the ability to fix nitrogen

According to Kumar et al. [42], Triplicate runs of the experiment were conducted twice, and the bacterial isolates' qualitative nitrogen-fixing ability was assessed based on their capacity to grow on N-free Jensen's media by culture and incubating them at 30 ± 2 °C for 48 h.

2.7. Statistical analysis

Completely randomized designs (CRD) were used for the experiments, and GenStat Discovery Edition 10 software (VSN International Ltd., Rothamsted Experimental Station, UK) was used to analyze the data after an ANOVA. The Least Significant Differences (LSD) was used to compare means at the 0.05 probability level.

3. Results and discussion

3.1. Culturable bacteria in the plant rhizosphere

Three hundred twenty-four rhizobacteria isolates were recovered through *in vitro* culturing of rhizosphere samples on NA obtained from 80 plant samples belonging to 66 plant species randomly collected from weeds, crops and trees in eight northern, central, and southern governorates in Iraq (Table 1). Of these, 116 came from the semiarid/steppe regions of Iraq (Salah Aldeen, Baghdad and Thi Qar), 81 from an arid region (Najaf and Basra) and 127 from the country's Mediterranean climate zones (Erbil, Sulimaniah and Diyala)

Table 1
The origin sources of rhizobacterial isolates.

| #	Plant name	Scientific name	No. of isolates	#	Plant name	Scientific name	No. of isolates
1	Horse mint	*Mentha longifolia*	4	34	Brant's oak tree	*Quercus brantii*	4
2	Zucchini	*Cucurbita pepo*	5	35	Panic veldtgrass	*Ehrharta erecta*	8
3	Hairy Willowherb	*Epilobium hirsutum*	11	36	Tumbleweed	*Amaranthus albus*	6
4	Eggplant	*Solanum melongena*	9	37	European heliotrope	*Heliotropium europaeum*	4
5	Sidra tree	*Frangula alnus*	6	38	Watermelon	*Citrullus lanatus*	3
6	Wall barley	*Hordeum murinum*	5	39	Chinese hibiscus	*Hibiscus rosa-sinensis*	5
7	Caspian manna	*Alhagi maurorum*	2	40	Pennyroyal	*Mentha pulegium*	6
8	Spiny sowthistle	*Sonchus asper*	4	41	Common Sunflower	*Helianthus annuus*	5
9	London rocket	*Sisymbrium irio*	8	42	Cucumber	*Cucumis sativus*	8
10	Dwarf nettle	*Urtica urens*	4	43	Paperflower	*Bougainvillea glabra*	5
11	Fig tree	*Ficus carica*	7	44	Florida hopbush	*Dodonaea viscosa*	7
12	Bermuda grass	*Cynodon dactylon*	5	45	Eucalyptus tree	*Eucalyptus globulus*	5
13	Tomato	*Solanum lycopersicum*	8	46	Common cocklebur	*Xanthium orientale*	2
14	Horseweed	*Erigeron canadensis*	6	47	Oleander	*Nerium oleander*	4
15	White leadtree	*Leucaena leucocephala*	3	48	Bitter orange	*Citrus aurantium*	5
16	Pomegranate tree	*Punica granatum*	8	49	Apricot tree	*Prunus armeniaca*	2
17	Cheeseweed	*Malva parviflora*	5	50	China rose	*Rosa chinensis*	4
18	Sweet orange tree	*Citrus × sinensis*	8	51	Olive tree	*Olea europaea*	11
19	Scotch pine tree	*Pinus sylvestris*	4	52	Common myrtle	*Myrtus communis*	8
20	Petty spurge	*Euphorbia peplus*	4	53	Tamarisk	*Tamarix ramosissima*	3
21	Chaste tree	*Vitex agnus-castus*	7	54	Common lantana	*Lantana camara*	2
22	Bitter orange tree	*Citrus × aurantium*	4	55	Alfalfa	*Medicago sativa*	3
23	White mulberry	*Morus alba*	6	56	Natal plum	*Carissa macrocarpa*	2
24	Rye	*Secale cereale*	3	57	Multiflora rose	*Rosa multiflora*	7
25	Common pear tree	*Pyrus communis*	5	58	Common sowthistle	*Sonchus oleraceus*	2
26	Common mugwort	*Artemisia vulgaris*	3	59	Prickly lettuce	*Lactuca serriola*	5
27	Italian thistle	*Carduus pycnocephalus*	5	60	Common wild oat	*Avena fatua*	3
28	Potato	*Solanum tuberosum*	4	61	Bush seepweed	*Suaeda niger*	6
29	Dyer's litmus	*Chrozophora tinctoria*	5	62	Cheatgrass	*Bromus tectorum*	2
30	Willowleaf lettuce	*Lactuca saligna*	3	63	Chamiso	*Atriplex canescens*	6
31	Nettle-leaved goosefoot	*Chenopodiastrum murale*	3	64	Persian Clover	*Trifolium resupinatum*	3
32	Common rush	*Juncus effusus*	5	65	Crown of thorns	*Koeberlinia spinosa*	4
33	Giant reed	*Arundo donax*	2	66	Potato tree	*Solanum erianthum*	3
34	Horse mint	*Mentha longifolia*	4				
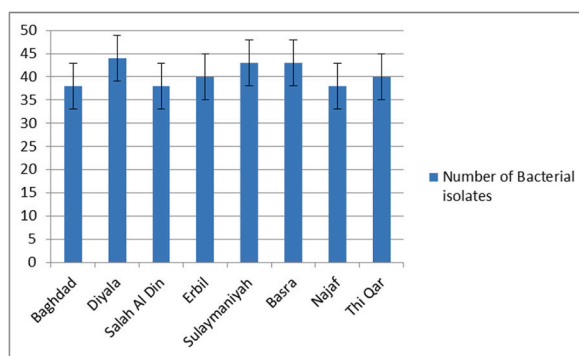
Fig. 2. Geographical distribution of rhizobacterial isolates in the Iraqi governorates.

(Fig. 2).

3.2. Examining bacterial isolates in vitro for biocontrol properties

3.2.1. Antifungal activity of rhizobacterial isolates

The results of dual culture assay (Table 2) carried out *in vitro* against the phytopathogenic fungi *R. solani* (Rs), *M. phaseolina* (Mp), and *P. aphanidermatum* (Pa), showed different degrees of inhibition, ranging from 0 % to a notable maximum inhibition of 97.99 %. Only 12 of the 324 rhizobacteria isolates showed strong antifungal activity, while they could suppress fungal growth by more than 50 % (between 57.62 and 97.99 %) (Fig. 3a–h). The DMC8 isolate from the sweet orange tree (*Citrus* × *sinensis*) of Diyala governorate was superior and showed an inhibition activity of 97.99 % (Fig. 3a). BZD2 from the Florida hopbush (*Dodonaea viscosa*) of Basra governorate (Fig. 3b) and NAS1 from the common sowthistle (*Sonchus oleraceus*) of Najaf governorate (Fig. 3h) both showed 93.65 % and 87.62 %, respectively, and 312 of the 324 rhizobacteria isolates had antifungal activity of less than 50 %. Geographically, an average antifungal activity of 20.19 % was recorded in rhizobacteria isolates from Sulimania governorate, followed by Thi Qar governorate with 18.03 % and lower activity in isolates from Najaf governorate which was 10.67 %.

3.2.2. Cross-streak test between rhizobacterial strains

Twelve hostile bacteria were examined on agar plates. Each isolate was co-cultured with others on the same plate, and each showed no symptoms of growth inhibition at the center where the two strains crossed. This suggested that the isolates were a compatible combination and that they coexisted on the roots of plants (Fig. 4).

The sequence was shown by arrows on each co-inoculated strain, which was streaked perpendicularly.

3.2.3. Hydrogen cyanide (HCN) production

Seven of the twelve hostile rhizobacterial isolates (BRE6, DMC8, SKE2, BZD3, NAS1, TRS4, and TNK1) produced HCN, according to a qualitative study of HCN production. This is displayed in Table 3. This might be one of the explanations for these isolates' capacity to prevent the formation of pathogenic fungi (Fig. 5a).

3.2.4. Production of proteases

Except for the BRE6 isolate, all antagonistic rhizobacterial isolates were found to be capable of producing the protease enzyme

Table 2
Antifungal capacity of rhizobacterial isolates against phytopathogenic fungi.

| Bacterial isolates code	Cultivar origin	Cultivar location	Inhibition efficacy (%)			
			R. solani	*M. phaseolina*	*P. aphanidermatum*	Average
BRE6	Hairy Willowherb	Baghdad, Rashdiah	60.00	54.76	58.10	57.62
DKS3	Tomato	Diyala, Khanaqin	66.67	60.00	60.00	62.22
DMC8	Sweet orange tree	Diyala, Moqdadiah	100.00	93.97	100.00	97.99
SDV1	Chaste tree	Salah Al Din, Dijeel	64.29	67.62	65.71	65.87
ECC4	Italian thistle	Erbil, Chomn	70.16	71.75	70.79	70.90
ECL1	Willowleaf lettuce	Erbil, Chomn	76.86	76.51	75.40	76.26
SKE2	Horseweed	Sulimania, Kalar	79.20	78.57	77.30	78.36
BZD2	Florida hopbush	Basra, Zubir	96.67	91.59	92.70	93.65
BZD3	Florida hopbush	Basra, Zubir	66.19	66.83	68.57	67.19
NAS1	Common sowthistle	Najaf, Abasia	87.46	88.57	86.83	87.62
TRS4	Tomato	Thi Qar, Rifai	80.00	71.43	77.14	76.19
TNK1	Crown of thorns	Thi Qar, Nasria	86.03	83.81	84.29	84.71
LSD (5 %)	Between Fungal isolates = 0.05, Between Bacterial isolates = 0.58, Interaction between fungal and bacterial isolates = 1.00					
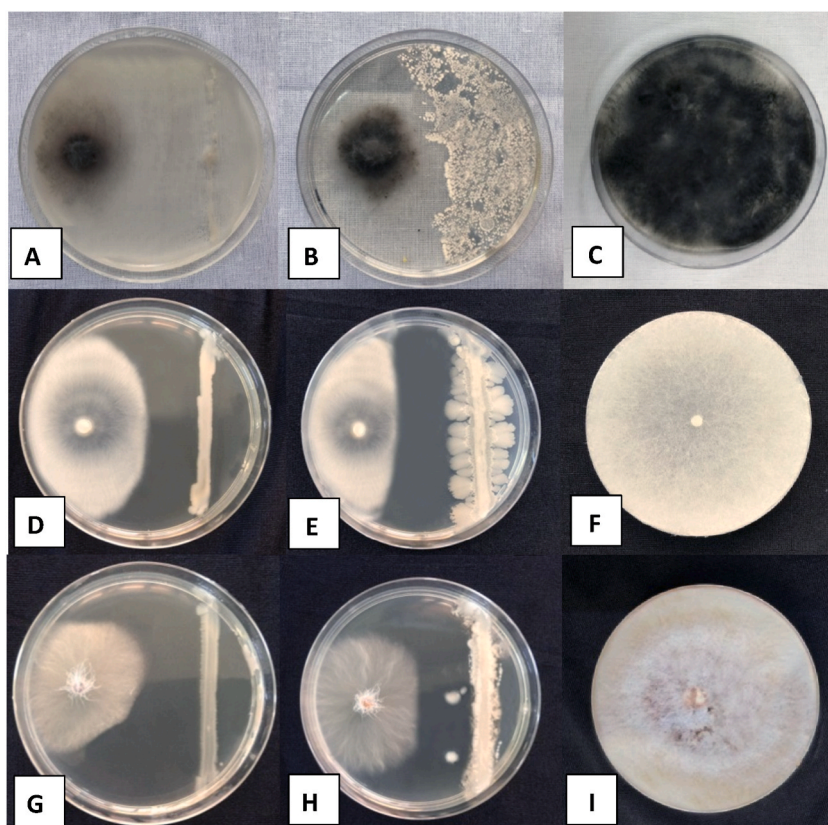
Fig. 3. Rhizobacterial isolates' antagonistic activity toward phytopathogenic fungi of *R. solani* (Rs), *M. phaseolina* (Mp), and *P. aphanidermatum* (Pa) A.Mp x DMC8, B. Mp x BZD2, C. Mp (Control), D. Pa x DKS3, E. Pa x ECL1, F. Pa (Control), G. Rs x BZD3, H. Rs x NAS1, I. Rs (Control).

(Table 3), indicating the high effectiveness of the isolates in preventing the growth of pathogenic fungi *in vitro* experiments (Fig. 5b).

3.2.5. Chitinase synthesis

The 12 rhizobacterial isolates varied in their capacity to produce chitinase; only 6 isolates (Table 3) had a positive response on the plate. These isolates were DMC8, ECC4, SKE2, NAS1, TRS4, and TNK1. The ability of rhizobacteria to produce this enzyme is an additional trait that makes them effective in preventing the formation of harmful fungi (Fig. 5c).

3.3. Antagonistic rhizobacteria identification

Eight of the twelve rhizobacterial isolates were successfully identified at the species level listed in Table 4 using the Vitek 2 compact system technology. The bacterial species shown in Table 4 and Fig. 6 were discovered when the 16S rRNA gene was used in the PCR process to identify the remaining 4 isolates (DKS3, DMC8, NAS1, and TRS4). The sequencing data obtained and processed in this work are available at NCBI (https://www.ncbi.nlm.nih.gov/) with accession numbers OR046068.1, OR046311.1, OR046064.1, and OR046069.1.

3.4. Morphological and physiological characteristics of the bacterial isolates

Phenotypic examination of the 12 antagonistic rhizobacterial isolates revealed that 10 isolates were positive for Gram stain, except two isolates of *L. adecarboxylata* DKS3 and *E. cloacae* complex BZD3, which were negative for Gram stain (Table 5 and Fig. 7). In addition, the results of the phenotypic examination also showed that all bacterial cells were rod-shaped, except for the *A. otitis* BRE6 and *S. lentus* BZD2 isolates, which had a spherical shape (Fig. 7). The bacterial colonies were all circular in shape but differed in color, elevation, and margin, as shown in Table 5.

The sporulation ability of the rhizobacterial isolates was examined and the results showed that eight isolates of *A. otitis* BRE6, *L. adecarboxylata* DKS3, *S. lentus* BZD2, and *E. cloacae* complex BZD3 produced spores, and four isolates did not form spores (Table 6). Furthermore, all isolates showed motility ability, except two isolates each of *A. otitis* BRE6 and *S. lentus* BZD2, which did not show motility ability.

In response to different temperatures, the rhizobacterial isolates exhibited a wide temperature range of growth (25 °C–45 °C), with
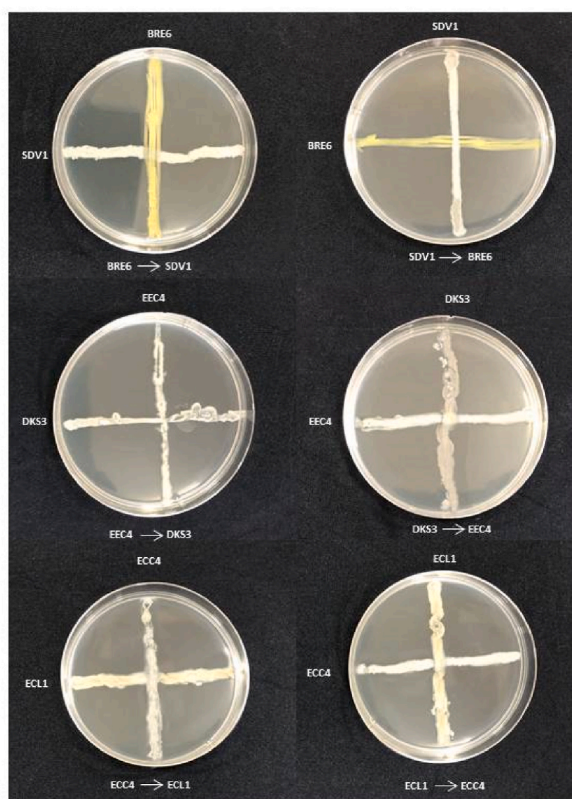
Fig. 4. Cross-streak test between isolates of rhizobacteria co-inoculated together.

Table 3
Biocontrol characteristics of the antagonistic rhizobacterial isolates.

Bacterial isolate code	HCN production	Protease production	Chitinase production
BRE6	+	−	−
DKS3	−	+	−
DMC8	+	+	+
SDV1	−	+	−
ECC4	−	+	+
ECL1	−	+	−
SKE2	+	+	+
BZD2	−	+	−
BZD3	+	+	−
NAS1	+	+	+
TRS4	+	+	+
TNK1	+	+	+

(+) indicates a positive interaction, (−) indicates a negative reaction.

the lowest growth observed at 15 °C, as confirmed by agar medium analysis (Fig. 8). The most optimal growth temperature was 35 °C, and maximum growth was often observed between 25 and 45 °C (Table 6). The results show that three isolates, *A. thermoaerophilus* ECL1, *A. thermoaerophilus* SDV1, and *A. pallidus* ECC4, belong to the group of thermophilic bacteria since they have extraordinary resistance to high temperatures (50 °C) and no growth at 15 °C (Fig. 8).

When grown at various pH levels, our studies confirmed that rhizobacterial isolates can thrive in both acidic and alkaline soils over a wide pH range (Fig. 9). The greatest growth of all isolates was observed at pH 7, followed by pH 8 (Table 6). The majority of bacterial isolates showed the least growth at pH 4. The results also showed that *L. adecarboxylata* DKS3 had the greatest growth at all pH settings, followed by *B. halotolerans* DMC8 and *A. pallidus* ECC4. As Fig. 9 shows, the density of bacterial growth decreases at pH values below or above 6 and 8.
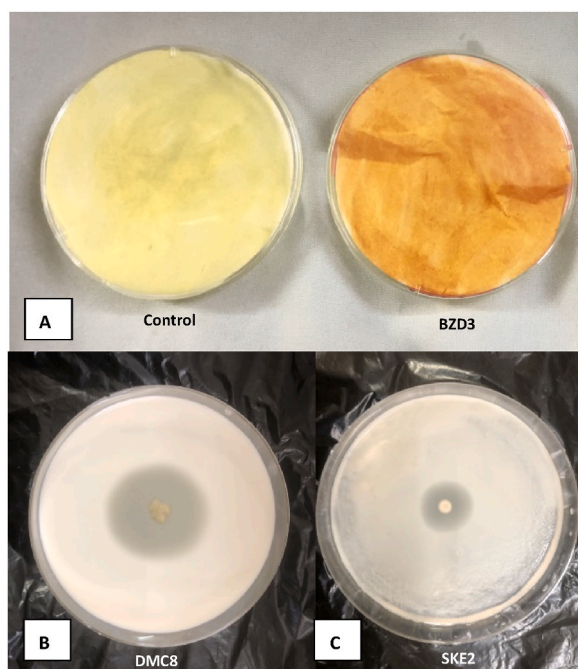
Fig. 5. The antagonistic rhizobacterial isolates' biocontrol properties
A.HCN production, B. Protease production, C. Chitinase production.

Table 4
Identification of antagonistic rhizobacterial isolates.

#	Bacterial isolate code	Bacterial species identified using Vitek 2 compact system	Bacterial species identified using 16S rRNA amplification
1	BRE6	*Alloiococcus otitis*	
2	DKS3	Unidentified organism	*Leclercia adecarboxylata*
3	DMC8	Unidentified organism	*Bacillus halotolerans*
4	SDV1	*Aneurinibacillus thermoaerophilus*	
5	ECC4	*Aeribacillus pallidus*	
6	ECL1	*Aneurinibacillus thermoaerophilus*	
7	SKE2	*Bacillus megaterium*	
8	BZD2	*Staphylococcus lentus*	
9	BZD3	*Enterobacter cloacae complex*	
10	NAS1	Unidentified organism	*Bacillus subtilis*
11	TRS4	Unidentified organism	*Paenibacillus polymyxa*
12	TNK1	*Bacillus megaterium*	

3.5. Plant growth promoting traits of rhizobacterial isolates in vitro

In these tests, twelve different species of antagonistic rhizobacteria were tested based on their PGP characteristics, such as their ability to fix nitrogen, produce IAA, ammonia and siderophores, as well as solubilize potassium and phosphate. Their capacity for many features that promote plant development varied, according to the data.

The phosphate solubilization activity of all 12 isolates was qualitatively defined (Fig. 10a) by the presence of a halo around the bacterial colony on Pikovskaya's agar medium. However, quantitatively, there were significant differences amongst all isolates (Table 7), with *E. cloacae complex* BZD3 having the highest amount of soluble P at 628.99 µgml-1, followed by *B. halotolerans* DMC8 and *B. megaterium* TNK1 with respective values of 554.14 and 497.61 µgml-1. *A. otitis* BRE6 recorded the lowest amount of soluble P of 97.92 µgml-1.

Twelve isolates were examined on Aleksandrov's agar for their ability to solubilize potassium. The results showed a clear halo zone around the bacterial colony (Fig. 10b). In terms of quantitative K solubilization values, *B. subtilis* NAS1 isolates had the highest value of 642.79 µg ml-1, followed by *B. megaterium* TNK1 and *E. cloacae* complex BZD3 isolates, with 550.86 and 540.15 µg ml-1, respectively (Table 7). *S. lentus* BZD2 and *A. otitis* BRE6 isolates showed the lowest K solubilization values, 56.26 and 89.98 µg ml-1, respectively.

While there are quantitative differences among all isolates, *B. subtilis* isolate NAS1 recorded the highest IAA production of 43.81 µg ml-1, followed by *E. cloacae complex* BZD3 with 38.15 µg ml-1 (Table 7). At the same time, *A. thermoaerophilus* SDV1 recorded the lowest IAA production value of 6.87 µg ml-1. The bacterial isolates mixed with Salkowski reagent showed the development of a pink
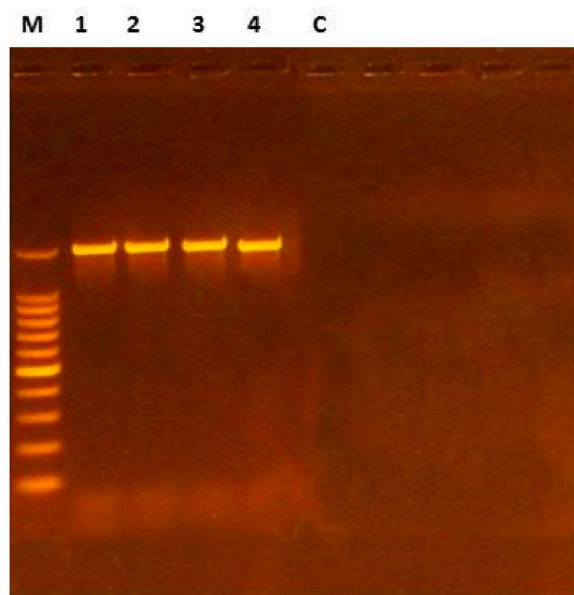
Fig. 6. 16S rRNA gene amplification of rhizobacterial isolates was fractionated on 1.5 % agarose gel electrophoresis and labeled with an Eth.Br. M: 100 bp ladder marker. C: Control. Lanes 1–4, which correspond to *Bacillus subtilis*, *B. halotolerans*, *L. adecarboxylata*, and *P. polymyxa*, respectively resemble 1500bp PCR products.

Table 5
Morphological characteristics of Rhizobacterial isolates.

Isolate name	Cell morphology		Colony morphology			
	Gram stain	Shape	Form	Elevation	Margin	Color
A. otitis BRE6	+	Spherical	Circular	Slightly convex	Entire	Pale yellow
L. adecarboxylata DKS3	–	Rod	Circular	Flat	Entire	Creamy-white
B. halotolerans DMC8	+	Rod	Circular	Flat	Curled	Milky-white
A. thermoaerophilus SDV1	+	Rod	Circular	Flat	Entire	Creamy-white
A.pallidus ECC4	+	Rod	Circular	Flat	Entire	Light beige
A. thermoaerophilus ECL1	+	Rod	Circular	Flat	Entire	Creamy-white
B. megaterium SKE2	+	Rod	Circular	Convex	Curled	Milky-white
S. lentus BZD2	+	Spherical	Circular	Flat	Entire	Creamy-white
E. cloacae complex BZD3	–	Rod	Circular	Flat	Entire	Creamy-white
B. subtilis NAS1	+	Rod	Circular	Convex	Undulate	Creamy-white
P. polymyxa TRS4	+	Rod	Circular	Convex	Curled	Creamy-white
B. megaterium TNK1	+	Rod	Circular	Convex	Curled	Creamy-white

color, indicating IAA production (Fig. 10d).

In the inoculated NB amended with Nessler's reagent, the color of all isolates changed from pale to yellow and deep yellow in the ammonia production test (Fig. 10e). Quantitatively, all isolates differ significantly from each other, except the *B. megaterium* SKE2 and *B. subtilis* NAS1 isolates, which showed 957.27 and 956.14 μg ml-1, respectively (Table 7). The DMC8 isolate of *B. halotolerans* performed better, displaying 997.29 μg ml-1, while *S. lentus* BZD2 isolate recorded 403.03 μg ml-1 for leas ammonia production.

All twelve isolates demonstrated the ability to produce siderophore, while they presented an orange halo around the bacterial colonies (Fig. 10c). Additionally, all the rhizobacteria isolates showed nitrogen-fixing ability except for *A. otitis* BRE6 and *A. pallidus* ECC4, which did not grow on the N-free Jensen's medium (Table 7).

4. Discussion

Recently, developing environmentally friendly processes based on biological sources has proven to be a viable technique for environmental protection [40]. Overall, the results of this study, which included *in vitro* culturing rhizosphere samples, provided insightful information on the variety and possible antifungal properties of rhizobacterial isolates from different plant species present throughout Iraq. The discovery of 324 rhizobacterial isolates from 80 plant samples, representing 66 different plant species, demonstrates the abundance of microbial communities associated with weeds, crops, and trees. These knowledgeable microorganisms successfully colonize all biological niches in the root zone of the plant [5].
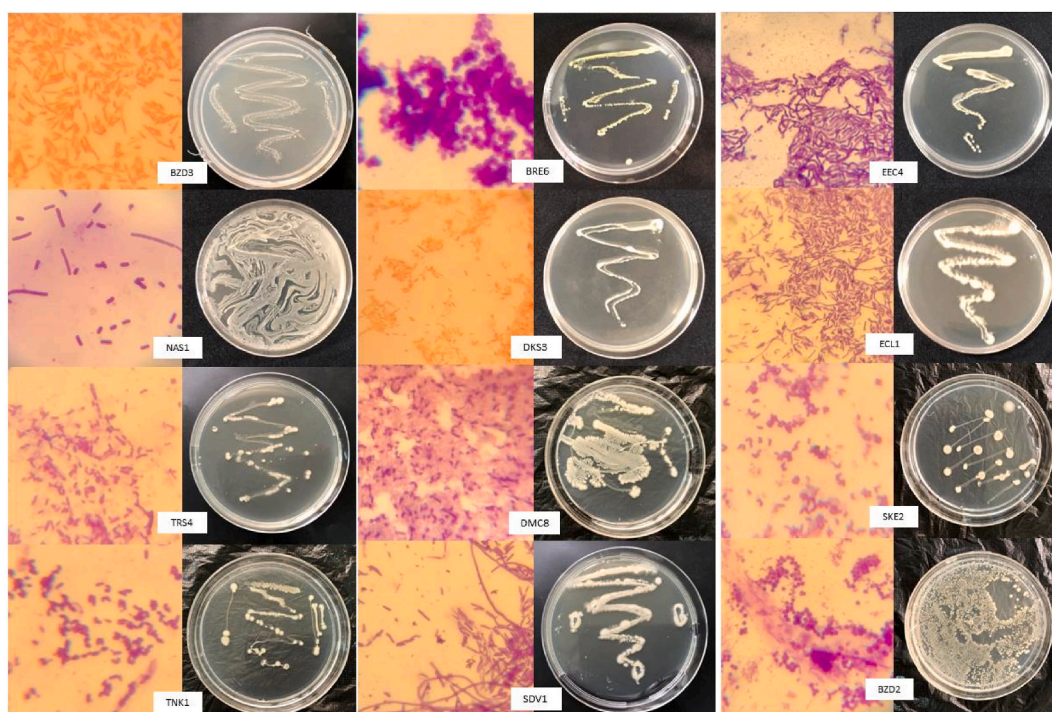
Fig. 7. Morphology characteristics of the antagonistic rhizobacterial isolates.

Table 6
Physiological characterization of bacterial isolates.

Isolate name	Sporulation	Motility	Optimum temperature °C	Optimum pH
A. otitis BRE6	−	−	35	8
L. adecarboxylata DKS3	−	+	35	7
B. halotolerans DMC8	+	+	35	7
A. thermoaerophilus SDV1	+	+	50	8
A.pallidus ECC4	+	+	50	7
A. thermoaerophilus ECL1	+	+	50	8
B. megaterium SKE2	+	+	35	8
S. lentus BZD2	−	−	35	7
E. cloacae complex BZD3	−	+	25	7
B. subtilis NAS1	+	+	35	7
P. polymyxa TRS4	+	+	35	7
B. megaterium TNK1	+	+	25	8

(+) indicates a positive interaction, (−) indicates a negative reaction.

The distribution of isolates across governorates showed numerous patterns, with the majority originating from Mediterranean climate areas, semiarid/steppe regions, and arid zones. The Tigris and Euphrates rivers are important for habitat development as they provide water for agriculture and support a variety of ecosystems. The average annual precipitation is about 250 mm in the north, while in the south it is only about 100 mm or less [39].

Twelve isolates showed antifungal activity in the dual culture experiment against phytopathogenic fungi; among these, the DMC8 isolate from a sweet orange tree in Diyala governorate showed an impressive inhibitory activity of 97.99 %. Antifungal activity varied by region, with Sulimania governorate having the highest average, followed by Thi Qar and Najaf. It was clear that different plants and locations have different populations of rhizobacteria [5]. Co-culture investigations demonstrating no growth inhibition at the crossing of strains offered insights into the compatibility and coexistence of isolates on plant roots, as demonstrated by the analysis of hostile bacteria on agar plates. These results identify the isolates with potent antifungal properties and highlight the importance of considering both geographical and ecological aspects in such studies, expanding our understanding of the potential biocontrol agents in the rhizosphere.

Seven of the twelve antagonistic rhizobacterial isolates (BRE6, DMC8, SKE2, BZD3, NAS1, TRS4, and TNK1) were found to be capable of producing hydrogen cyanide (HCN), which may have contributed to their ability to inhibit the growth of plant pathogenic fungi. Since bacterial HCN effectively combats fungal infections by suppressing cytochrome *c* oxidase and severely damaging the
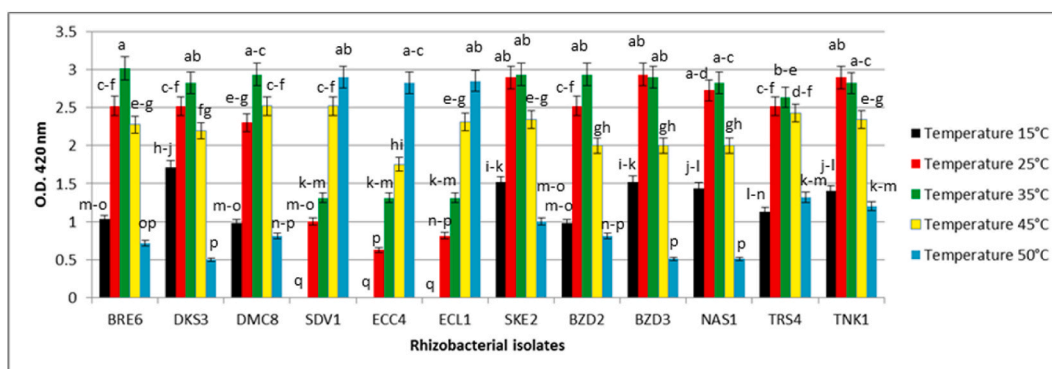
Fig. 8. Temperature-dependent growth of rhizobacterial isolates
Same letters within each column represent non-significant difference (P < 0.05) as determined by least significant difference (LSD).
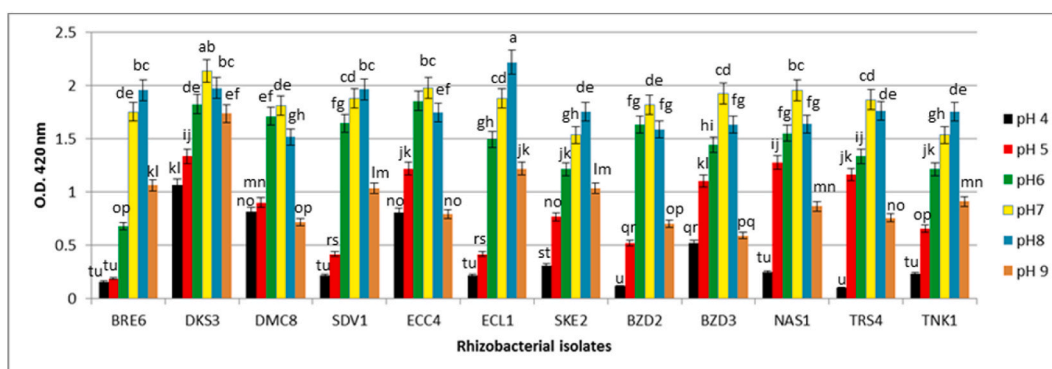

Fig. 9. Growth of isolated rhizobacteria at various pH levels
Same letters within each column represent non-significant difference (P < 0.05) as determined by least significant difference (LSD).

fungal metabolic processes, this secondary metabolite may contribute to the biocontrol properties of these isolates [40].

Furthermore, examination of the enzymatic activity of the isolates demonstrated that all isolates showed evidence of protease enzyme production except for isolate BRE6. This enzymatic property probably plays an essential role in the ability of the isolates to effectively inhibit the development of pathogenic fungi *in vitro* tests. HCN and protease activities have been reported to limit the growth of phytopathogens [47,50]. PGPR produces proteases and other hydrolytic enzymes that kill microbes and stop the growth of harmful pathogens in the rhizosphere. This makes the root system of the plants healthier [37]. Only six of the isolates had the ability to produce chitinase, and this property was thought to increase their potential for biological control of phytopathogens. Chitinolytic enzymes, found in many species of bacteria, break down chitin into carbon and nitrogen which can be used as an energy source. Chitinases are enzymes that catalyze the hydrolysis of beta-1,4-N-acetyl-glucosamine bonds in chitin polymers [29].

The diverse microbial composition of hostile rhizobacterial isolates was discovered by the identification of bacterial species using the Vitek 2 compact system and PCR targeting the 16S rRNA gene. Phenotypic examination revealed differences in Gram staining, cell morphology, colony characteristics, sporulation ability and motility among isolates.

The investigation also evaluated the responses of the isolates to different temperatures and demonstrated their adaptability to a wide range of temperatures, with 35 °C being the best growth temperature. Notably, some isolates showed an exceptional ability to withstand high temperatures, leading to their classification as thermophilic bacteria. The ideal pH range of the isolates was proven to be between 7 and 8. Plant growth promoting (PGP) features were evaluated for each isolate and the results demonstrated the potential advantages of these traits on plant growth and health.

Investigations were conducted on the solubilization of phosphate and potassium, the synthesis of indole-3-acetic acid (IAA), ammonia, and siderophore, and the capacity to fix nitrogen. The findings showed that the isolates differed significantly in the quantitative elements of these features. For instance, *B. subtilis* NAS1 had the highest potassium solubilization value, but the *E. cloacae* complex BZD3 showed higher phosphate solubilization. According to Richardson et al. [8], phosphate solubilization activity increases plant absorption of phosphate by solubilizing insoluble phosphate in the soil. The differences in siderophore, ammonia, and IAA production highlighted the different PGP capacities of the rhizobacterial isolates. While IAA is a plant hormone that promotes root growth, siderophores are iron chelators that boost the efficiency of iron absorption in plants [4,14].

According to Vacheron et al. [26], microbe-mediated IAA synthesis in the rhizosphere could increase root length and lateral root development, which would increase plant uptake of water and nutrients. Bacterial ammonia contributes significantly to the
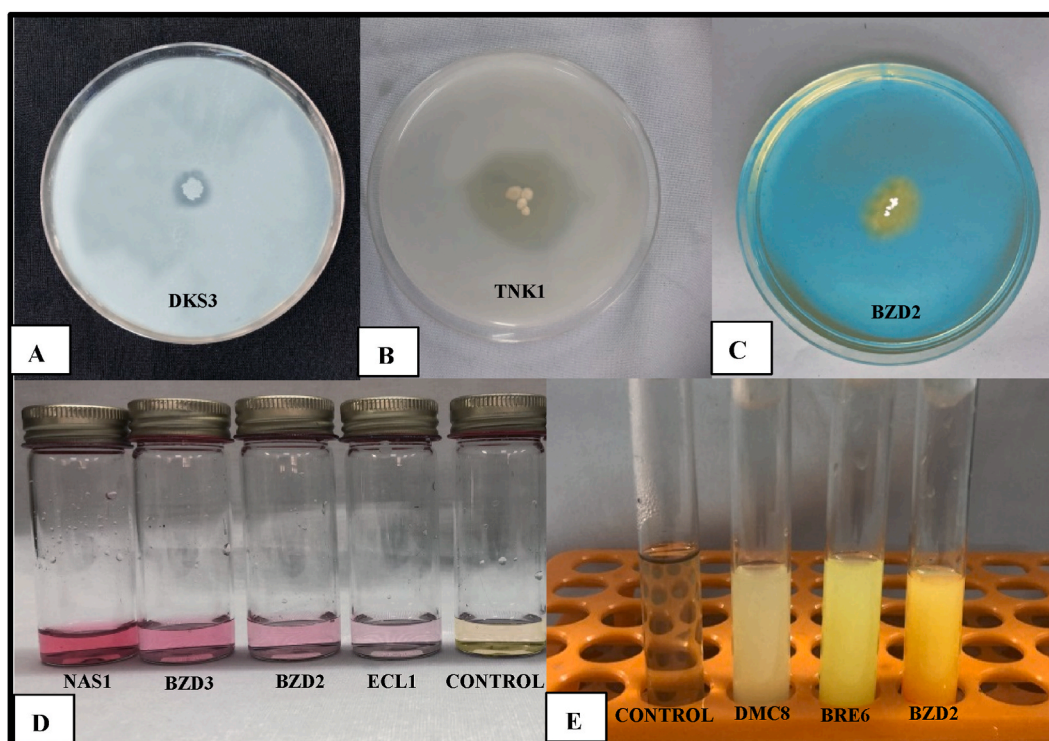
Fig. 10. Plant growth promoting potential of rhizobacterial isolates
A.Phosphate solubilization activity, B. Potassium solubilization activity, C. Siderophore production, D. IAA production, E. Ammonia production.

Table 7
PGP traits of antagonistic rhizobacterial isolates.

Isolate name	Phosphate (µg ml-1)	Potassium (µg ml-1)	IAA (µg ml-1)	Ammonia (µg ml-1)	Siderophore production	Nitrogen fixation
A. otitis BRE6	97.92 ± 0.180	89.98 ± 0.000	7.39 ± 0.100	635.74 ± 0.000	+	−
L. adecarboxylata DKS3	241.49 ± 0.000	171.08 ± 0.198	11.77 ± 0.005	527.86 ± 0.078	+	+
B. halotolerans DMC8	554.14 ± 0.312	422.81 ± 0.000	19.31 ± 0.000	997.29 ± 0.000	+	+
A. thermoaerophilus SDV1	160.70 ± 0.477	258.02 ± 0.141	6.87 ± 0.013	514.39 ± 0.117	+	+
A.pallidus ECC4	147.79 ± 0.000	197.99 ± 0.000	10.66 ± 0.000	662.72 ± 0.000	+	−
A. thermoaerophilus ECL1	172.78 ± 0.000	290.88 ± 0.028	7.19 ± 0.000	430.01 ± 0.000	+	+
B. megaterium SKE2	494.48 ± 0.000	520.56 ± 0.000	18.39 ± 0.017	957.27 ± 1.947	+	+
S. lentus BZD2	382.04 ± 0.000	56.26 ± 0.000	25.94 ± 0.013	403.03 ± 0.000	+	+
E. cloacae complex BZD3	628.99 ± 0.361	540.15 ± 0.049	38.15 ± 0.024	848.26 ± 0.078	+	+
B. subtilis NAS1	460.13 ± 0.000	642.79 ± 0.085	43.81 ± 0.000	956.14 ± 0.000	+	+
P. polymyxa TRS4	394.54 ± 0.000	193.10 ± 0.000	15.77 ± 0.017	976.38 ± 0.000	+	+
B. megaterium TNK1	497.61 ± 0.000	550.86 ± 0.000	12.27 ± 0.000	942.65 ± 0.000	+	+
LSD (0.05)	0.47	0.18	0.07	1.29		

Data are presented as mean of triplicates ± standard deviation, (+) indicates a positive interaction, (−) indicates a negative reaction.

development of shoots and roots. Moreover, ammonia promotes plant growth by forming several nitrogen-containing biomolecules [34]. Oosterhuis et al. [17] and Xu et al. [49] say that K turns on and controls the ATPase in the plasma membrane to produce acid, which then causes the cell wall to activate hydrolase. Overall, these findings contribute to a comprehensive understanding of the multifaceted characteristics of the rhizobacterial community and their potential applications in sustainable agriculture.

The study on rhizobacterial diversity and antifungal properties in Iraq provides a comprehensive understanding of rhizobacterial diversity and their potential in sustainable agriculture. It identifies isolates with significant biocontrol potential, such as the DMC8 isolate with 97.99 % inhibition efficacy. Advanced techniques for species identification and plant growth-promoting traits enhance the study's relevance. The detailed analysis of enzymatic activities and adaptability of isolates to different environmental conditions further enhances understanding. However, the study's *in vitro* conditions may not fully replicate natural soil interactions. Despite these limitations, the research offers valuable insights for future studies.

5. Conclusion

This study effectively obtained and described 324 strains of rhizobacteria from the rhizospheres of 80 plant samples among 66 plant species in eight governorates of Iraq, which encompass Mediterranean, semiarid/steppe, and arid climatic zones. Remarkably, out of all the isolates, 12 of them shown substantial antifungal activity against fungi that harm plants, including phytopathogenic fungi. Among these isolates, the DMC8 isolate obtained from a sweet orange tree in Diyala governorate showed the greatest level of inhibitory efficiency, reaching an impressive 97.99 %.

The research emphasized the enzymatic capacities of these isolates, indicating that all isolates, with the exception of BRE6, exhibited protease synthesis. Additionally, six isolates shown the ability to create chitinase. These enzymatic activities are expected to contribute to their potential for biocontrol. The investigation also found some isolates that have the ability to produce hydrogen cyanide (HCN), which enhances their antifungal activities. Notably, species such as *B. halotolerans* DMC8 and *B. subtilis* NAS1 exhibited significant biocontrol and plant growth-promoting characteristics. Phenotypic tests revealed a wide range of morphological and physiological characteristics in these isolates, such as their ability to tolerate different temperatures and pH levels, their capacity for sporulation, and their ability to move, which demonstrates their ability to adapt to different environmental circumstances.

The isolates exhibited notable skills in promoting plant development, including the solubilization of phosphate and potassium, formation of indole-3-acetic acid (IAA), ammonia, siderophores, and nitrogen fixation. *E. cloacae* complex BZD3 shown exceptional ability to solubilize phosphate, whereas *B. subtilis* NAS1 displayed the best proficiency in solubilizing potassium.

Overall, this study highlights the considerable capacity of rhizobacterial isolates to serve as biocontrol agents and stimulate plant development in sustainable agriculture. These findings establish a basis for future *in vivo* investigations and practical implementations focused on improving crop yield and soil health under various environmental circumstances.

Data availability statement

The authors declare that the data supporting the findings of this study are available within the paper, a request for more detailed data should be sent to the corresponding authors with the permission of all authors.

CRediT authorship contribution statement

Safaa N. Hussein: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Naser Safaie:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Masoud Shams-bakhsh:** Writing – review & editing, Validation. **Hurria H. Al-Juboory:** Writing – review & editing, Validation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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