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Rhizobacteria from Iraq: A Novel Biocontrol Approach for Tomato Root Rot Disease

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ABSTRACT

Background: Tomato production in Iraq is subject to significant decline due to contamination of agricultural field soils with soil-borne pathogenic fungi, particularly *Rhizoctonia solani*. Rhizobacteria are the best alternative to chemical fungicides in controlling the disease.

Methods: Dual culture approach was employed to assess the antifungal activity of 324 rhizobacterial isolates against *R. solani in vitro*. Superior antigenic isolates were identified using 16S rRNA, gyrA, gyrB and rpoB gene sequencing. Physiological characteristics such as hydrogen cyanide, protease, chitinase, siderophore and nitrogen fixation of the antigenic isolates were screened.

Result: A total of 324 rhizobacterial isolates were extracted from rhizosphere samples, with four isolates demonstrating strong antifungal activity above 66% against *R. solani*. The isolates were identified as *Leclercia adecarboxylata* DKS3, *Bacillus halotolerans* DMC8, *Bacillus subtilis* NAS1 and *Paenibacillus polymyxa* TRS4. These isolates demonstrated notable biocontrol and plant growth-promoting traits *in vitro*, including nitrogen fixation and the generation of protease, chitinase, hydrogen cyanide and siderophores, as well as the solubilization of potassium and phosphate. These attributes are essential for enhancing plant growth and resilience to pathogenic stress. This research highlights the ability of these rhizobacteria to function as effective biocontrol agents and promoters of plant growth in sustainable agriculture.

Key words: Biological control, Rhizobacteria, *Rhizoctonia solani*, Tomato.

INTRODUCTION

Root rot infections are highly significant and devastating plant diseases with a worldwide impact, they may result in a reduction in agricultural yield and quality between 10% and 90%, root rot diseases are caused by several fungal infections, including *Rhizoctonia solani* Kuhn (Agrios, 2005). This fungus is a pathogen that afflicts several plant families and is accountable for inducing significant diseases in more than 142 plant species across 125 genera, including tomatoes (Kareem and Hassan, 2013). *R. solani* infects plants at various development stages, resulting in substantial crop yield losses (Bhamra *et al.,* 2022). It can infect seeds in the soil, seedlings prior to or following emergence, roots and numerous aerial plant structures, including pods, fruits, leaves and stems (Agrios, 2005). The tomato (*Solanum lycopersicum*) is extensively cultivated and widely consumed globally. FAOSTAT (2024) reports that tomato output in Iraq decreased from 1,321,000 tons in 2001 to 630,180 tons in 2022. Therefore, there is a necessity for alternative and sustainable methods to address root rot disease that are safe and effective, as well as ecologically friendly (Kaya *et al.,* 2020). One ecofriendly strategy utilized is the application of rhizobacteria for biological control (Abdelghany *et al.,* 2024). Rhizobacteria can suppress the proliferation of root rot pathogens through various mechanisms, including resource and space competition (Lavanya *et al.,* 2023; Hussein, 2024), antibiotic production (Saeed *et al.,* 2021), secretion of lytic enzymes (Santoyo *et al.,* 2021) and the induction of systemic resistance in plants (Pieterse *et al.,* 2001). Rhizobacteria can enhance plant growth through many mechanisms, including nitrogen fixation, phosphate solubilization, phytohormone synthesis and siderophore production (Chauhan *et al.,* 2014; Hussein *et al.,* 2025). The aim of this study was to isolate and identify effective, environmentally friendly rhizobacterial isolates for controlling tomato root rot disease.

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MATERIALS AND METHODS

Isolation of local rhizobacteria

Rhizobacteria were extracted from the roots of numerous plant species, including herbs, crops and trees, in many

places in Iraq throughout 2022. Rhizosphere soil samples were cultivated and purified on sterilized Nutrient Agar using the protocol of Sadiqi *et al.* (2022), after dilution to 10^{-7} as per the technique of Hussein and Al Zubidy (2019). Purified isolates were supplemented in sterilized Nutrient Broth medium combined with a 20% glycerol solution, stored at -20°C.

Evaluation of antifungal activity

The fungal pathogen *R. solani* was isolated from diseased tomato roots in a prior investigation (Hussein *et al.,* 2022). The dual culture approach was employed to assess the antifungal activity of 324 rhizobacterial isolates *in vitro*, following the methodology of Hussein *et al.* (2024). The experiment included three duplicates and was conducted twice. The inhibition zone was quantified using the subsequent formula:

$$\text{Inhibition (\%)} = \frac{\text{Pathogen growth on control plates} - \text{Pathogen growth on dual culture plates}}{\text{Pathogen growth on control plates}} \times 100$$

Evaluation of the physiological properties of rhizobacteria

Physiological characteristics of the four antagonistic rhizobacterial isolates exhibiting significant antagonistic activity was assessed *in vitro*. The capacity for protease synthesis was evaluated by cultivating the isolates on skimmed milk agar, following the methodology of Krechel *et al.* (2022). The capacity for chitinase production was assessed using the method of Gamal-Eldin *et al.* (2008) by culturing isolates on chitinase medium supplemented with colloidal chitin. The presence of a clear zone (halo) around the colonies signified a positive reaction for protease and chitinase. The capacity for hydrogen cyanide (HCN) production was evaluated by culturing rhizobacterial isolates on King's B medium. Filter paper saturated with picric acid and Na_2CO_3 was positioned within the upper lid of Petri dishes, with a color change of the filter paper from yellow to brown signifying a positive reaction (Reetha *et al.,* 2014). The capacity for siderophore synthesis was assessed using Chrome Azurol S Agar (CAS) for Gram-negative isolates and O-CAS for Gram-positive isolates, following the methodology of Perez-Miranda *et al.* (2007). A yellow-orange halo surrounding the bacterial colonies was interpreted as a positive result. The capacity of insoluble phosphate and potassium solubility was assessed by culturing rhizobacterial isolates on Pikovskaya's Agar and Aleksandrov's Agar media, following the methodologies of Hussein *et al.* (2024). The presence of a halo zone around the bacterial colony indicated that the isolates exhibited positive activity for both phosphorus and potassium solubilization. The nitrogen-fixing capability of the isolates was evaluated by their growth on N-free Jensen's medium, cultured and incubated at 30±2°C for 48 hours. The entire experiment was conducted in duplicate and performed twice.

Identification of antagonistic rhizobacterial isolates

Four rhizobacterial isolates were identified using four pairs of primers for 16S rRNA, gyrA, gyrB and rpoB (Table 1). Each tube of the amplification mixture comprised two microliters of DNA template, one microliter of each primer and twenty-one microliters of Master Mix (Promega, USA). The amplification protocol comprised an initial denaturation of five minutes at 95°C, followed by thirty cycles of denaturation at 95°C for thirty seconds, annealing for thirty seconds at 60°C and 65°C for the 16S rRNA and gyrB reactions, respectively, one minute of extension at 72°C and a final extension of seven minutes at 72°C (Yamamoto and Harayama, 1995; dos Santos *et al.,* 2019). The PCR profile for the gyrA gene included an initial denaturation at 94°C for 2 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 51°C for 45 seconds and extension for 60 seconds, concluding with a final extension at 68°C for 10 minutes (De Clerck *et al.,* 2004). The PCR profile for the rpoB gene included an initial denaturation at 94°C for 5 minutes, succeeded by 25 cycles comprising denaturation at 94°C for 30 seconds, annealing at 50°C for 1.5 minutes and extension at 72°C for 1.5 minutes. A concluding extension phase at 72°C for 10 minutes was subsequently executed (Dahllo *et al.,* 2000). The measured DNA concentration ranged from 23 to 26 ng/µl. Agarose gel electrophoresis (1.5%) was employed to confirm the presence of amplification subsequent to PCR

Table 1: Primers properties.

Gene	Primer name	Sequence 5′-3′	Annealing temp. (°C)	Product size (bp)	Reference
16S rRNA	27F	AGAGTTTGATCCTGGCTCAG	60	1500	(Santos *et al.,* 2019)
	1492R	TACGGTTACCTTGTTACGACTT			
gyrB	UP-1	GAAGTCATCATGACCGTTCTG	65	1200	(Yamamoto and Harayama, 1995)
	UP-2r	CAYGCNGGNGGNAARTTYGA			
		AGCAGGGTACGGATGTGCGAGCCR			
		TCNACRTCNGCRTCNGTCAT			
gyrA	gyrA-f	CAGTCAGGAAATGCGTACGTCCTT	51	373	(De Clerck *et al.,* 2004)
	gyrA-r	CAAGGTAATGCTCCAGGCATTGCT			
rpoB	rpoB1698f	AACATCGGTTTGATCAAC	50	1025	(Dahllo *et al.,* 2000)
	rpoB2041r	GTTGCATGTTGGTACCCAT			

amplification. Sanger sequencing of the purified amplicons was conducted with an automated DNA sequencer from Macrogen Corporation in South Korea. Subsequently analyzed with the Basic Local Alignment Search Tool (BLAST), which identifies regions of local similarity between sequences, utilizing closely related culture sequences acquired from the National Center for Biotechnology Information (NCBI) database. The GenBank nucleotide sequence database comprises the gene sequences identified in this investigation.

RESULTS AND DISCUSSION

Rhizobacteria isolation

The research discovered 324 rhizobacterial isolates by *in vitro* cultivation on Nutrient Agar. The findings indicate a substantial and diverse array of microbial species linked to the rhizosphere of plants in Iraq. Understanding this spectrum of variances is crucial for several agricultural and environmental applications, including promoting plant growth, mitigating diseases and maintaining soil health.

Antifungal assay

The observed inhibition rates of 324 rhizobacterial isolates against *R. solani* ranged from 0% to 100%. The extensive range of inhibition underscores the variability in the antifungal activity of the examined rhizobacterial isolates. Four isolates exhibited robust antifungal activity, above the 66% inhibition threshold (Table 2). This indicates that although a significant proportion of the isolates have antifungal activity, only a limited number are particularly efficient in inhibiting fungal growth (Fig 1).

Physiological properties of rhizobacterial isolates

The results in (Table 3) showed that the four rhizobacterial isolates conducted a positive reaction in the production of HCN, siderophores, protease and chitinase enzymes and a positive ability to solubilize insoluble phosphorus and potassium. Also they demonstrated the capacity to fix nitrogen when grown positively on nitrogen-free Jensen's medium, except for the isolate DKS3, which did not show a positive reaction in the HCN and chitinase production. Hydrogen cyanide is a secondary metabolite that is produced by rhizobacteria and is essential for inhibiting the growth of pathogenic fungi in the rhizosphere, one possible main mechanism for rhizobacteria's antifungal action is their synthesis of HCN (Voisard *et al*., 1989). One important mechanism in the inhibition of pathogenic fungi is the generation of protease enzymes by species of rhizobacteria, which plays a major role in their antagonistic characteristics because they break down the structural proteins in fungal cell walls, protease enzymes that hydrolyze proteins, play crucial roles in microbial defense tactics by preventing fungal development (Santoyo *et al.,* 2021). An enzyme called chitinase degrades chitin, which is an essential structural element of fungal cell walls (Veliz *et al.,* 2017). Fungal cell wall integrity is compromised by this enzymatic breakdown, which results in cell lysis and inhibits the initiation and spread of fungal infections (Edreva, 2005). All of the isolates in this investigation had qualitative phosphate and potassium solubilizing activity, as evidenced by the formation of a halo surrounding the bacterial colony on Pikovskaya's agar medium and Aleksandrov's agar respectively. Phosphorus and Potassium is an essential mineral for the growth of plants, but its availability in the soil is frequently restricted because it exists in forms that cannot be dissolved. Phosphate-solubilizing bacteria (PSB) can transform insoluble forms of phosphorus into soluble ones, so enabling plants to access it (Richardson and Simpson, 2011). Potassium can enhance the tolerance of plants to cold, drought and stress, as well as stimulate the process of photosynthesis (Chen *et al.,* 2022). The orange halo surrounding the bacterial colonies indicates that all of the isolates were able to generate siderophores (Table 3). In their assessment of 30 rhizobacterial isolates most of them belong to *Bacillus* genus for their ability to promote plant development *in vitro*, Bhattacharyya *et al.* (2020) found that every isolate was positive for siderophore, IAA generation

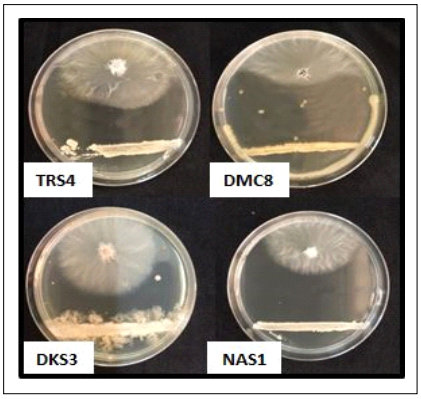


Fig 1: Dual culture interaction between rhizobacterial isolates and *R. solani.*

Table 2: Antifungal activity of rhizobacterial isolates against *R. solani.*

Isolates code	Cultivar origin	Cultivar location	Inhibition efficacy (%)
DKS3	Tomato (*Solanum lycopersicum*)	Diyala, Khanaqin	66.67^{d}
DMC8	Sweet orange tree (*Citrus* × *sinensis*)	Diyala, Moqdadiah	100.00^{a}
NAS1	Common sowthistle (*Sonchus oleraceus*)	Najaf, Abasia	87.46^{b}
TRS4	Tomato (*S. lycopersicum*)	Thi Qar, Rifai	80.00^{c}

Different letters within each column represent significant difference (P<0.01) as determined by least significant difference (LSD).

and phosphate solubilization activity (Alwan *et al.,* 2019; Qaisy *et al.,* 2016; Hussin *et al.,* 2018). All of the rhizobacterial isolates examined in this study demonstrated the capacity to fix nitrogen when grown on nitrogen-free Jensen's medium (Table 3). One essential mechanism for converting atmospheric nitrogen into a form that plants can use is biological nitrogen fixation, the nitrogenase enzyme, which is present in nitrogen-fixing bacteria, mediates this process (Mia *et al.,* 2013).

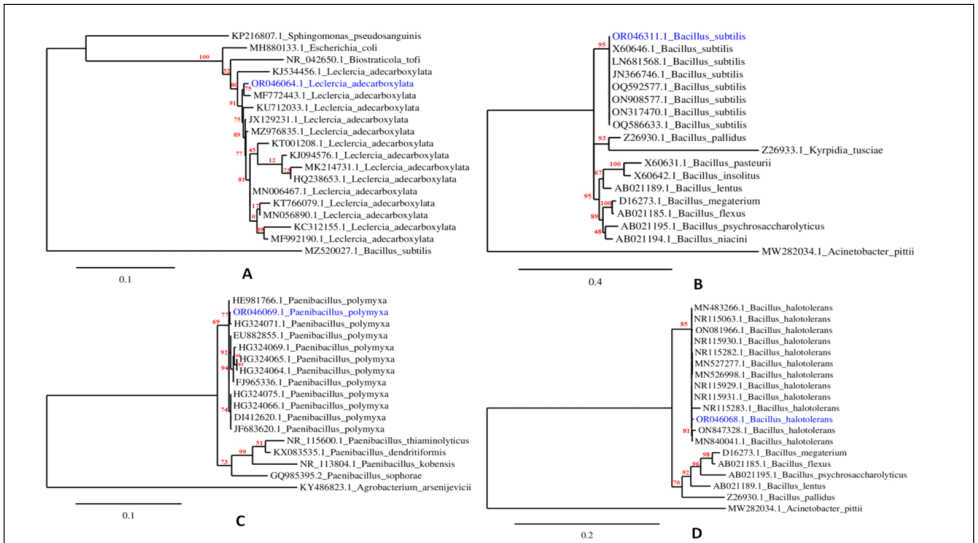
Identification of rhizobacteria

The identification of microorganisms is a fundamental aspect of microbial ecology and biotechnology. The four hostile rhizobacterial isolates were identified using the 16S rRNA gene (Table 4). This approach facilitates the characterisation of diverse microorganisms and the understanding of their functional roles in the rhizosphere. The 16S rRNA analysis identified the bacterial species *Leclercia adecarboxylata* DKS3, *Bacillus halotolerans* DMC8, *Bacillus subtilis* NAS1 and *Paenibacillus polymyxa* TRS4 (Table 4). *L. adecarboxylata* DKS3 and *B. subtilis* NAS1 were effectively validated using gyrB gene primers. *B. halotolerans* DMC8 and *B. subtilis* NAS1 were verified using gyrA gene primers, whereas TRS4 was successfully confirmed using rpoB gene primers (Table 4). The discrepancies in the confirmation results of bacterial isolates utilizing various housekeeping genes (gyrB, gyrA and rpoB) can be ascribed to the variability in gene conservation and specificity across bacterial species, whereas the 16S rRNA gene is universally conserved and the gyrB, gyrA and rpoB genes exhibit greater sequence divergence. Primer mismatches or the absence/truncation of genes in specific isolates might impede amplification. Moreover, PCR settings may not be ideal for all isolates, resulting in inconsistent diagnosis. One dependable marker for phylogenetic investigations is the 16S rRNA gene, which is a highly conserved area in bacterial genomes. Fig 2 shows that the four bacterial isolates' 16S rRNA gene sequences are substantially similar to those of their registered type strain counterparts, indicating tight evolutionary ties. The convergence rates range from 75 to 99%. The gradual evolution and conservation of the 16S rRNA gene across bacterial species account for the high degree of similarity. A component of the DNA gyrase enzyme, which is involved in DNA replication, is encoded by the gyrB gene. Its decreased conservation compared to

Table 3: Physiological characterization of rhizobacterial isolates.

Isolate code	HCN production	Protease production	Chitinase production	Phosphate production	Potassium production	Siderophore production	Nitrogen fixation
DKS3	-	+	-	+	+	+	+
DMC8	+	+	+	+	+	+	+
NAS1	+	+	+	+	+	+	+
TRS4	+	+	+	+	+	+	+

Data are presented as mean of triplicates, (+) indicates a positive interaction, (-) indicates a negative reaction.



(A), *B. subtilis* NAS1 (Accession No. OR046311.1) (B), *P. polymyxa* TRS4 (Accession No. OR046069.1) (C), *B. halotolerans* DMC8 (Accession No. OR046068.1) (D). Numbers at nodes indicate levels of bootstrap support (%) based on 1000 resampled datasets.

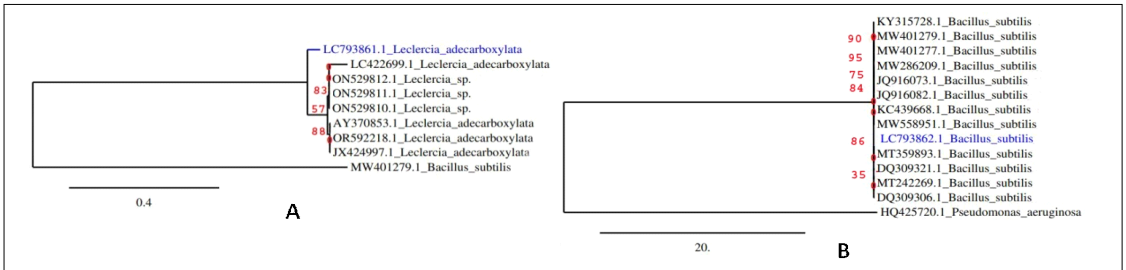
Fig 2: Maximum likelihood tree based on the 16S rRNA sequences, *L. adecarboxylata* DKS3 (Accession No. OR046064.1).

the 16S rRNA gene may explain why convergence rates are lower. Isolates *L. adecarboxylata* DKS3 and *B. subtilis* NAS1 show high levels of similarity despite minor evolutionary divergence with their normal strain counterparts, with convergence rates of 57% and 86%, respectively (Fig 3). Compared to the 16S rRNA gene, the convergence percentages for the gyrB gene are lower because of the diversity in this gene that allows for discrimination between closely related bacteria. Another part of DNA gyrase, the gyrA gene, may be used to differentiate between related bacterial strains because of its variability. Fig 4 shows that *B. subtilis* NAS1 and *B. halotolerans* DMC8 isolates are somewhat similar with their normal strain counterparts, with convergence rates of 79% and 67%, respectively. This shows that while these isolates have a shared ancestor with others, there is evolutionary divergence reflected in the discrepancies in the gyrA gene sequences. Because of its greater precision than the 16S rRNA gene, the rpoB gene-which encodes the beta subunit of RNA polymerase-is used as a molecular marker for bacterial identification. Fig 5 shows

Table 4: Identification of antagonistic rhizobacterial isolates.

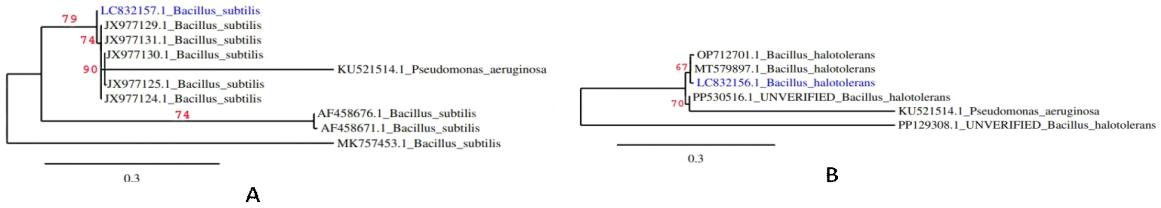
Bacterial code	16S rRNA	gyrB	gyrA	rpoB
DKS3	*Leclercia adecarboxylata*	*L. adecarboxylata*	-	-
DMC8	*Bacillus halotolerans*	-	*B. halotolerans*	-
NAS1	*Bacillus subtilis*	*B. subtilis*	*B. subtilis*	-
TRS4	*Paenibacillus polymyxa*	-	-	*P. polymyxa*

(-) No reaction resulted.



(A), *B. subtilis* NAS1 (Accession No. LC793862.1) (B). Numbers at nodes indicate levels of bootstrap support (%) based on 1000 resampled datasets.

Fig 3: Maximum likelihood tree based on the GyrB sequences, *L. adecarboxylata* DKS3 (Accession No. LC793861.1).



(A), *B. halotolerans* DMC8 (Accession No. LC832156.1) (B). Numbers at nodes indicate levels of bootstrap support (%) based on 1000 resampled datasets.

Fig 4: Maximum likelihood tree based on the GyrA sequences, *B. subtilis* NAS1 (Accession No. LC832157.1).

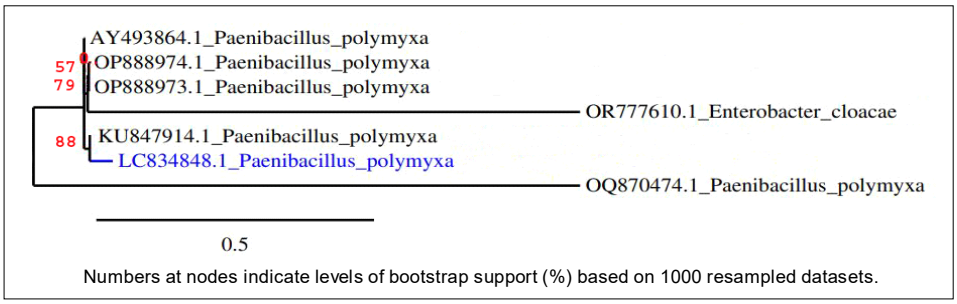


Fig 5: Maximum likelihood tree based on the rpoB sequences, *P. polymyxa* TRS4 (Accession No. LC834848.1).

that the *P. polymyxa* TRS4 isolate is quite close to its normal strain counterparts, with a convergence rate of 88%. This strong converging trend indicates that the rpoB gene sequences are highly conserved in this family, which makes it a solid phylogenetic marker.

Genes with varied rates of evolution and degrees of conservation have variable convergence percentages; this is evident when looking at the 16S rRNA, gyrB, gyrA and rpoB genes. Due to its high degree of conservation, the 16S rRNA gene exhibits greater convergence rates, suggesting tight evolutionary ties. On the other hand, the less conserved gyrB and gyrA genes have lower convergence rates, demonstrating their usefulness in differentiating closely related strains. In the middle ground, the highly-resolved rpoB gene offers trustworthy phylogenetic information. The sequencing data acquired and analyzed in this study are accessible at NCBI with accession numbers OR046068.1, OR046311.1, OR046064.1, OR046069.1, LC793862.1, LC793861.1, LC832156.1, LC832157.1 and LC834848.1.

CONCLUSION

The extensive study conducted on rhizobacterial isolates across various climatic zones in Iraq has provided substantial insights into their potential as biocontrol agents and growth promoters for tomato plants, irrespective of normal conditions or stress induced by *R. solani*, the pathogen responsible for tomato root rot disease. Among the 324 rhizobacterial strains isolated from several plant species, four isolates exhibited significant antifungal activity against *R. solani*, surpassing a 66% inhibition rate. The four isolates shown positive activity in protease, chitinase, hydrogen cyanide, siderophore production and nitrogen fixation. Furthermore, the capacity to solubilize insoluble phosphate and potassium. The four antagonistic isolates are the bacterial species *B. halotolerans* DMC8, *B. subtilis* NAS1, *P. polymyxa* TRS4 and *L. adecarboxylata* DKS3, discovered using 16S rRNA gene sequencing and validated by gyrA, gyrB and rpoB gene sequencing. This work underscores the significant potential of rhizobacterial isolates as biocontrol agents and biofertilizers, promoting plant growth in sustainable agriculture. These findings establish a basis for future field investigations focused on improving crop yield and soil health under various environmental circumstances.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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