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Control of Chickpea Root Rot Disease by Rhizobacteria in a Greenhouse

Safaa N. Hussein[1]

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ABSTRACT

Background: Chickpeas are a prevalent and significant crop in the agricultural sectors of northern Iraq. In recent years, production has significantly declined due to diseases affecting chickpea plants. Investigating the pathogenic fungi responsible for these diseases is essential to restore production levels to their prior and improved states.

Methods: Samples of diseased chickpea plants were collected from fields in the Erbil, Sulaymaniyah and Salah al-Din governorates in northern Iraq. Isolated associated fungi led to the identification of the dominant pathogenic species. A pathogenicity test was performed on the fungal isolates, showing the most aggressive strain for subsequent antagonism testing against three rhizobacterial isolates: *Azotobacter chroococcum*, *Bacillus cereus* and *B. pumilus*. This testing was conducted both *in vitro* and in a greenhouse setting, using single treatments and combinations.

Result: The results showed that the pathogenic fungus *Rhizoctonia solani* was predominant, with the strain Srs-21 exhibiting the highest virulence *in vitro*. The three rhizobacterial isolates, *A. chroococcum*, *B. cereus* and *B. pumilus*, showed high efficacy in inhibiting the pathogenic fungus *in vitro* on potato dextrose agar (PDA) medium. In the greenhouse, the triple bacterial interaction treatment showed a seed germination rate of 100% and diminished disease incidence and severity to 0%, in contrast to the positive control treatment, which recorded 90% and 70%, respectively. It also showed the highest rate of rise in the dry weight of plants, signifying enhanced growth metrics.

Key words: Biological control, Rhizobacteria, *Rhizoctonia solani*, Root rot.

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is recognized as an important leguminous crop on a global scale. This legume is recognized as the most productive in South Asia and is the third most produced globally, trailing only beans and peas (Gaur and Gowda, 2005). In 2003, Iraq's total production was documented at 104,000 metric tons, followed by a steady decrease that culminated in an average of 203 metric tons by 2019 (FAOSTAT, 2021). The chickpea crop encounters several pathogens, including *Fusarium solani*, *F. oxysporum*, *Macrophomina phaseolina* and *Rhizoctonia solani* (Kuhn), which are considered as major factors in seed rot, damping off in seedlings and the deterioration of roots and crowns (Agrios, 2005; Hussein *et al*., 2025c). *Rhizoctonia solani* is a facultative parasitic fungus categorized within the phylum Basidiomycota, subphylum Agaricomycotina, class Agaricomycetes, order Cantharellales and family Ceratobasidiaceae. The sexual basidiospores are generally generated on infected plants; nonetheless, the fungus primarily reproduces in its sterile anamorph stage (Alexopoulos *et al*. 1996; Agrios, 2005). This stage is marked by the emergence of rapidly expanding, brown-hued hyphae with significant diameters of 15-20 µm. These structures display distinct constrictions at their points of emergence and exhibit the formation of transverse septa in the branches close to the emergence area, characterized by double holes and multiple nuclei. The development of barrel cells or irregular shapes is a key feature, which amalgamate to form a mass known as sclerotia. This sclerotium functions as a mechanism for the fungus to endure adverse environmental conditions (Blazier and Conway, 2004; Howard *et al*., 2007). The fungus affects the plant during different growth stages, infecting seeds in the soil, which results in seed and seedling rot, leading to damping off both prior to and following emergence and impacting the roots and crown of the plant (Rivera *et al*. 2004; Agrios, 2005). There is an increasing focus on the use of environmentally friendly beneficial microorganisms for managing plant diseases (Hussein *et al*., 2025b). The rhizosphere-living bacteria that are antagonistic represent a significant and effective alternative approach for managing diseases in chickpea. Rhizosphere-living bacteria function as biocontrol agents through various mechanisms, including the production of antibiotics, siderophores and enzymes, which effectively

[1]Department of Environmental Engineering, Mustansiriyah University, College of Engineering, Iraq.

Corresponding Author: Safaa N. Hussein, Department of Environmental Engineering, Mustansiriyah University, College of Engineering, Iraq.
Email: safaahussein1979@uomustansiriyah.edu.iq
ORCID: 0000-0003-2934-6788

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inhibit the proliferation of pathogenic microbes (Bhargavi *et al*., 2024). This study focused on identifying the fungus that causes rot root disease in chickpeas in northern Iraq, while also assessing various rhizobacterial isolates for their potential as biocontrol agents against the pathogen.

MATERIALS AND METHODS

Isolation and identification of the causal agents of the disease

Samples were collected from chickpea plants showing signs of root rot disease, which included yellowing and wilting leaves, along with rotting that displayed brown to black discoloration in the roots and crown region (Fig 1). The samples were collected from the fields in the Erbil, Sulaymaniyah and Salah al-Din governorates. Root samples underwent a washing procedure using running water for a duration of 15 minutes, after which they were cut into small segments of 0.5 cm in size. The segments underwent surface sterilization with a 2% sodium hypochlorite solution for 2 minutes, followed by two rinses with sterile distilled water and were subsequently dried using sterile filter paper in a laminar flow cabinet. Four samples were positioned on PDA medium within 9 cm petri dishes. The medium underwent sterilization in an autoclave at a temperature of 121°C, maintaining a pressure of 1.5 kg/cm^2 for a duration of 20 minutes. The dishes were subsequently incubated at 25±1°C for a duration of 7 days. The fungal isolates were subjected to examination with a light microscope, resulting in their identification at both the genus and species levels through morphological characteristics and alignment with established taxonomic keys (Parmeter and Whitney, 1970; Ellis, 1971; Booth, 1977; Domsch *et al*., 1980; Klich, 2002; McClenny, 2005). The proportion of appearance and frequency of the fungal isolates has been calculated utilizing the formulas established by Hussein (2024).

$$\text{Appearance (\%)} = \frac{\text{Count of genus and species occurrences in samples}}{\text{Total samples}} \times 100$$

$$\text{Frequency (\%)} = \frac{\text{Count of plant pieces exhibiting fungal presence in the dishes}}{\text{Total count of pieces utilized in the sample}} \times 100$$

Anastomosis interactions test of *R. solani* isolates

The number of nuclei per cell of *R. solani* isolates was counted by growing the isolates on the PDA and staining the hyphae with 1ug/ml of 42 -6 diamidino-2-phenylindole, petri plates examined under a fluorescent microscope (Gondal *et al*., 2019). Nine isolates of *R. solani* belong to nine AGs provided by Microbiology laboratory/ University of Mustansiriyah, used as tester strains of the respective AGs, the fifty-seven unknown strain of *R. solani* were paired with tester strains, by placing an agar disc (5 mm) of the hyphal growth of unknown isolate at the one side of sterile glass slide covered with a layer of the 1.5% water agar and at the other side of the slide a similar disc of the tester strain and incubated for 72 hours at a 25°C, the hyphal interactions between tester strain and unknown strain was determined according to the method of Gondal *et al*. (2019).

Pathogenicity testing assay

The pathogenicity of fifty- seven isolates of *R. solani* was evaluated on a sterile PDA. Plates were inoculated at the center with a 0.5 cm diameter disc of *R. solani* isolates that had been cultivated on PDA culture medium for seven days. The dishes were incubated at a temperature of 25 ±1°C for a duration of two days. Each plate was then sown with 10 seeds of the Marrakechy cultivar of chickpeas, which had undergone surface sterilization using a 2% sodium hypochlorite solution for two minutes, followed by two rinses with sterile distilled water. The seeds were then dried using sterile filter paper and arranged in a circular pattern near the edge of the plate. Four replications were conducted for each treatment, including the control treatment of seedling only without the fungus. The dishes were incubated at a temperature of 25±1°C for a duration of 14 days, under a lighting schedule of 12 hours per day. The findings were documented and the germination percentage was determined using the equation provided by Hussein (2019).

$$\text{Germination (\%)} = \frac{\text{Count of seeds that have germinated}}{\text{Total number of seeds used}} \times 100$$

Antagonistic activity *in vitro*

Evaluation of antagonism activity of three rhizobacterial isolates of *A. chroococcum*, *B. cereus* and *B. pumilus* which optioned from Mustansiriyah University, laboratory of microbiology, against the most virulent isolate of *R. solani* (Srs-21), these rhizobacterial isolates isolated from the rhizosphere of healthy chickpea plants in Iraq (Hussein, 2022). On the PDA a 0.5 cm diameter disc of the fungal isolate placed at the edge of the petri plate (about 2 cm out of the edge) and in the opposite side a loop full of each bacterial isolate 10^9 CFU/ml placed individually, plates incubated at 25±1 C° for 7 days, each treatment repeated 4 times, antagonism activity calculated using the following formula:

$$\text{Antagonistic activity (\%)} = \frac{b}{b + f} \times 100$$

Where:

b = The distance between the bacterial line towards the fungal growth.

f = The distance of fungal growth towards the bacterial line.

Greenhouse evaluation

Inoculum of the fungal pathogen Srs-21 were prepared by growing it on the seeds of local millet, by adding 5 discs (0.5 cm diameter) of the pathogen in the culture medium (PDA) of 5 days old to a 250 ml glass beaker containing autoclaved millet seeds mixed with 10 ml of distilled and sterile water and incubated for 14 days. The inoculum was added to mixture of sterilized soil and compost in 1 kg plastic pots at a ratio of 1% (w/w). Each pot irrigated with 10 ml of the rhizobacterial inoculum of *A. chroococcum*, *B. cereus* and *B. pumilus* which were grown on the nutrient broth with concentration of 10^9 CFU/ml added in the same time of the seed planting, each treatment repeated 4 times and control treatments irrigated with distilled sterile water. Five seeds of chickpea (Marrakechy cultivar) were planted in each pot surface sterilized with 1% sodium hypochlorite solution. Pots were distributed according to a completely randomized design (CRD) and watered carefully. The percentage of germination was calculated after 14 days and the disease incidence was calculated after 40 days of planting and the disease severity index was measured according to the disease rating scale and the formulas described by Hussein (2022). The dry weight of plants was also calculated to measure the effect of bacterial isolates on plant growth parameters.

$$\text{Seed germination (\%)} = \frac{\text{Count of seed germinated}}{\text{Total seed germinated}} \times 100$$

$$\text{Disease severity index (\%)} = \frac{\text{Count of infected plants} \times \text{their infected degree}}{\text{Total tested plants} \times \text{higher infected degree}} \times 100$$

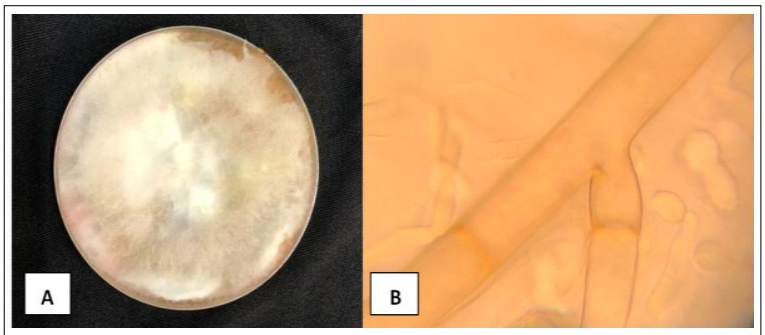
$$\text{Disease incidence (\%)} = \frac{\text{Count of infected plants}}{\text{Total count of plants assessed}} \times 100$$

RESULTS AND DISCUSSION

Fungi associated with infected chickpea plants

The findings from the isolation and diagnosis showed that *R. solani* was the most prevalent fungus, appearing in 77% of the samples, with a frequency percentage of 59.5% (Table 1). All *R. solani* isolates exhibited fundamental characteristics, including branching near the terminal septum of cells within the mycelium, marked by a narrowing at the starting point of the branches. Additionally, barriers appeared near the starting point of the branches, along with

Fig 1: Symptoms of rot roots on infected chickpea plants.



The fugal colony on the PDA medium (a) Fungal hypha branch at nearly right angles with constriction near the branching area and a transverse septum under light micorscope (b)

Fig 2: Morphological characteristics of *R. solani.*

barriers featuring double holes (Fig 2). The coloration of the fungal colonies transitioned from white initially to light brown, then deep brown (Fig 2). All isolates produced sclerotia that diversified in size and form, with diameters ranging from 1-3 mm and with thick brown walls. Some isolates additionally produced barrel cells of varying sizes, consistent with the characteristics described by Parmeter and Whitney (1970). These results align with the findings of Ganeshamoorthi and Dubey (2013), who isolated 50 isolates of R. solani from chickpea fields across 10 various states within India. Hussein (2022) demonstrated that within the soil-borne pathogenic fungi collected from infected chickpea plants in Iraq, *R. solani* emerged as the most prevalent, with an appearance rate of 85.5% and a frequency of 61.2%.

Anastomosis group test

The findings displayed in Table 2 regarding hyphal dyeing with DAPI and microscopic examinations of *R. solani* isolates indicated that all isolates were multinucleate. The interactions of hyphal anastomosis among the 57 isolates of *R. solani* and the tester strains of the respective AGs revealed that 40% of the isolates were classified under AG 4, which was the most prevalent. In contrast, 12%, 30%, 9%, 5% and 4% of the isolates were categorized into AG 1, AG 3, AG 5, AG 7 and AG 10, respectively (Fig 3). Hussein (2022) discovered that 58% of the *R. solani* isolates isolated from infected chickpea plants in the Nineveh and Duhok governorates in northern Iraq were classified within the AG3 group and 3% belong to AG4.

Pathogenicity assay

The findings from the pathogenicity test indicated that the germination rate varied from 0% to 65%, in contrast to the control group, which exhibited a 100% germination rate

Table 1: Fungal isolates associated with the diseased chickpea plants.

Fungus	Appearance (%)	Frequency (%)
Alternaria alternata (Fres.) Keissler	11.8	3.3
Aspergillus niger Van Tieghem	9.7	6.2
Aspergillus sp.	16.9	9.5
Cladosporium cladosporioides (Fresen.) G.A. de Vries	9.3	5.5
Fusarium oxysporum Schlesht	12.4	8.8
Fusarium solani (Mart.) Sacc.	18.5	11.8
Penicillium sp.	28.6	17.6
Rhizoctonia solani (Kuhn)	77.0	59.5
Rhizopus sp.	22.0	17.4
Ulocladium atrum Preuss	8.5	4.3

Table 2: Pathogenic test of *R. solani* isolates using chickpea seeds.

Isolate code	Location	AG group	Germination (%)	Isolate code	Location	AG group	Germination (%)
Control			32.5	Srs-08	Sulaymaniyah	AG 4	52.5
Ers-01	Erbil	AG 1	52.5	Srs-09	Sulaymaniyah	AG 7	40.0
Ers-02	Erbil	AG 4	27.5	Srs-10	Sulaymaniyah	AG 4	32.5
Ers-03	Erbil	AG 3	40.0	Srs-11	Sulaymaniyah	AG 4	32.5
Ers-04	Erbil	AG 5	30.0	Srs-12	Sulaymaniyah	AG 7	17.5
Ers-05	Erbil	AG 3	32.5	Srs-13	Sulaymaniyah	AG 3	22.5
Ers-06	Erbil	AG 4	60.0	Srs-14	Sulaymaniyah	AG 4	40.0
Ers-07	Erbil	AG 4	52.5	Srs-15	Sulaymaniyah	AG 3	52.5
Ers-08	Erbil	AG 4	25.0	Srs-16	Sulaymaniyah	AG 4	30.0
Ers-09	Erbil	AG 4	30.0	Srs-17	Sulaymaniyah	AG 1	52.5
Ers-10	Erbil	AG 4	42.5	Srs-18	Sulaymaniyah	AG 1	52.5
Ers-11	Erbil	AG 3	60.0	Srs-19	Sulaymaniyah	AG 3	40.0
Ers-12	Erbil	AG 1	65.0	Srs-20	Sulaymaniyah	AG 7	42.5
Ers-13	Erbil	AG 3	40.0	Srs-21	Sulaymaniyah	AG 4	0.0
Ers-14	Erbil	AG 4	35.0	Srs-22	Sulaymaniyah	AG 10	40.0
Ers-15	Erbil	AG 3	42.5	Srs-23	Sulaymaniyah	AG 3	52.5
Ers-16	Erbil	AG 3	60.0	Srs-24	Sulaymaniyah	AG 4	60.0
Ers-17	Erbil	AG 4	42.2	Srs-25	Sulaymaniyah	AG 3	42.5
Ers-18	Erbil	AG 3	52.5	Srs-26	Sulaymaniyah	AG 1	22.5
Ers-19	Erbil	AG 3	47.5	Srs-27	Sulaymaniyah	AG 3	35.0
Ers-20	Erbil	AG 4	65.0	Drs-01	Sulaymaniyah	AG 1	27.5
Ers-21	Erbil	AG 3	40.0	Drs-02	Sulaymaniyah	AG 3	60.0
Srs-01	Erbil	AG 3	57.5	Drs-03	Sulaymaniyah	AG 4	15.0
Srs-02	Erbil	AG 5	60.0	Drs-04	Sulaymaniyah	AG 5	42.5
Srs-03	Erbil	AG 5	52.5	Drs-05	Sulaymaniyah	AG 10	47.5

(Table 2). The Srs-21 isolate, classified as AG3 from the Sulaymaniyah governorate, demonstrated a complete inhibition of seed germination, achieving 0% germination and outperforming all other isolates in this regard (Fig 4). The variation in the pathogenic ability of these fungal isolates can be attributed to genetic differences among individuals of the same species, the response of the plant host varies depending on the isolates, which is also attributed to genetic variation, particularly since they are sourced from various regions.

Antagonistic activity *in vitro*

The findings from the antagonism test involving three rhizobacterial isolates against the fungal pathogen *R. solani* (Srs-21 isolate) indicated that the *B. pumilus* isolate demonstrated the highest level of antagonistic activity at 95.0%. This was followed by *B. cereus*, which showed an antagonism activity of 91.4%, while *A. chroococcum* exhibited a lower activity of 66.4% (Table 3) (Fig 5).

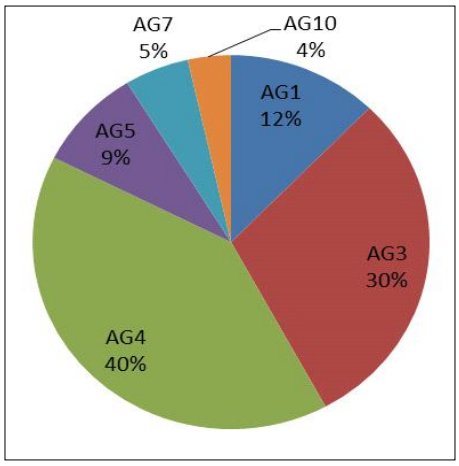


Fig 3: Distribution of *R. solani* isolates categorized by anastomosis groups.

Fig 4: Pathogenicity test of *R. solani* isolates *in vitro.*

Table 3: Antagonistic activity of the rhizobacterial isolates.

Treatment	Antagonistic activity (%)
Srs-21 × *A. chroococcum*	66.4c
Srs-21 × *B. cereus*	91.4b
Srs-21 × *B. pumilus*	95.0a

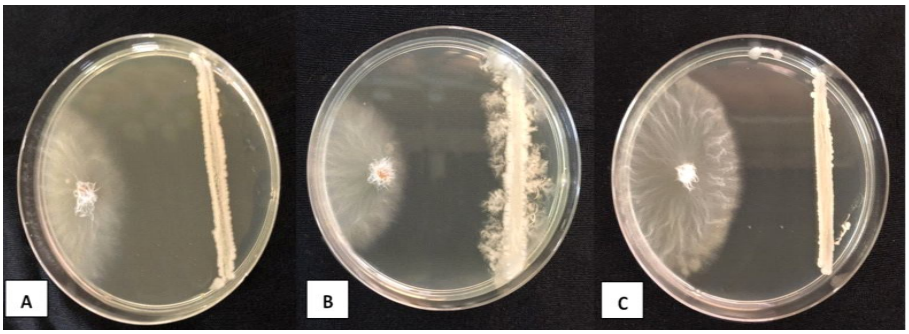
LSD (5%) = 1.7.

Greenhouse assay

The findings indicated that individual, dual and triple treatments of the rhizobacterial isolates enhanced seed germination percentages, ranging from 80% to 100%, in contrast to the positive control treatment, which recorded 70% (Table 4). The combination treatment of *A. chroococcum*, *B. cereus* and *B. pumilus* proved a substantial efficacy in diminishing disease incidence and severity to 0%, in contrast to the positive control treatment, which recorded 90.0% and 70.0%, respectively (Table 4). A combination treatment of *B. cereus* and *B. pumilus* resulted in disease incidence and severity rates of 35% and 28%, respectively. Regarding individual treatments, while they resulted in a 70-80% reduction in disease incidence, they did not significantly decrease disease severity, which ranged from 61-65%. The triple treatment resulted in the most significant increase in the average dry weight of chickpea plants, reaching 15 g/plant, in contrast to the positive and negative control treatments, which recorded 3.8 and 12.8 g/plant, respectively. Bacteria in the rhizosphere of plants safeguard the roots by inhibiting fungal growth and invasion into the root cells and the adjacent stalk tissues near the soil surfaces (Hussein and Ibrahim, 2018). The inhibitory action of *A. chroococcum* on *R. solani* growth may be attributed to its capacity to synthesize metabolites and organic chemicals, including stearic acid, enzymes, antibiotics and hydrogen cyanide (Hussein *et al*., 2025a). The results aligned with those reported by Hussein (2019), which illustrated the efficacy of *B. pumilus* in managing cucumber root rot disease induced by *F. solani*. Aljuboori *et al*. (2022) illustrated that the *Rhizoctonia solani - Meloidogyne javanica* complex disease affecting chickpea roots can be biologically managed using a rhizobacterial isolate of *Pseudomonas* sp., which also enhanced both the dry and wet weight of the plants, significantly elevating the activity of polyphenol oxidase and peroxidase enzymes, as well as the total phenolic content. Agarwal *et al*. (2017) shown that *B. pumilus* significantly suppressed the development of *R. solani* and *F. oxysporum* through the synthesis of chitinolytic enzymes and the generation of antibiotics. These results align with the findings of Zarrin *et al*. (2009), who revealed the capacity of *A. chroococcum* to prevent the development of several pathogenic fungus, including *R. solani*. Al Isawy (2010) demonstrated the efficacy of *A. chroococcum* as a means of biological management in suppressing the growth of *R. solani* on PDA medium. Hidayah and Yulianti (2015) discovered that *B. cereus* reduced growth of *R. solani* and *Sclerotium rolfsii in vitro* by 68.9% and 33%, respectively, on PDA medium. Additionally, *B. cereus*, obtained from the rhizosphere of mustard plants, prevented the growth of *R. solani*, *Pythium ultimum* and *S. rolfsii* (Pleban *et al*., 1995). Huang *et al*. (2012) demonstrated that *B. pumilus* altered the fungal hyphae of *R. solani in*

Table 4: Biological control of chickpea root rot disease in greenhouse.

Treatment	Germination (%)	Disease incidence (%)	Disease severity index (%)	Dry weight (g/plant)
Negative control (Plants alone)	100.0a	0.0a	0.0a	12.8b
Positive control (Srs-21 alone)	70.0e	90.0f	70.0f	3.8e
A. chroococcum (Ac alone)	100.0a	0.0a	0.0a	14.5a
B. cereus (Bc alone)	100.0a	0.0a	0.0a	12.9b
B. pumilus (Bp alone)	100.0a	0.0a	0.0a	13.3b
Srs-21 × Ac	95.0b	80.0e	65.0ef	6.8d
Srs-21 × Bc	80.0d	70.0d	61.0e	4.4e
Srs-21 × Bp	85.0c	72.0d	64.0ef	4.0e
Srs-21 × Ac × Bc	95.0b	55.5c	47.3d	9.3c
Srs-21 × Ac × Bp	100.0a	50.0c	38.5c	10.2c
Srs-21 × Bc × Bp	100.0a	35.0b	28.4b	13.3b
Srs-21 × Ac × Bc × Bp	100.0a	0.0a	0.0a	15.0a
LSD (5%)	3.2	5.8	7.3	0.9



Srs-21 x *B. cereus* (A), Srs-21 x *B. pumilus* (b), Srs-21 x *A. chroococcum* (C)

Fig 5: Antagonism activities of rhizobacterial isolates against *R. solani.*

vitro, resulting in the increase of cytoplasmic vacuoles and cytoplasmic exudation within the fungal cells.

CONCLUSION

In vitro experiments demonstrated that the three bacterial isolates of *A. chroococcum*, *B. cereus* and *B. pumilus*, obtained from the rhizosphere of healthy chickpea plants, exhibited superior capabilities in inhibiting the growth of the highly aggressive isolate of the pathogenic fungus *Rhizoctonia solani* (Srs-21) on the PDA culture medium. In controlled greenhouse conditions, the integration of the three bacterial isolates yielded the most favorable outcome in enhancing the germination rate of chickpea seeds, while also decreasing both the incidence and severity of the disease, alongside promoting the growth parameters of the plants as measured by dry weight.

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Conflict of interest

The author declares that they have no conflicts of interest.

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