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Effect of different bacterial treatment strategies on the damping-off disease control in cotton seedlings

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Bacillus halotolerans that was isolated from *Gossypium barbadense* and molecularly identified using 16S rRNA gene sequencing analysis was tested for its antifungal potential in cotton seedlings infected with the phytopathogenic fungus *Rhizoctonia solani*. The antifungal activity of the rhizobacterial strain was studied under greenhouse conditions. In soil contaminated with *R. solani*, the bacterial strain was applied in three replicates as seed presoaking, seed coating, and soil drench. Its efficacy was evaluated based on plant growth and disease suppression. According to our findings, seed coating and soil drench treatments were more effective than seed presoaking. Significant improvements are shown, including 26, 34, and 40% increase in seed emergence for seed presoaking, soil drenching, and seed coating, respectively. Comparing the treated plants to the uninoculated ones, the fresh and dry weights of the soil drench group increased significantly by 21.6 and 23.4%, respectively, in contrast to 45.1 and 33.7% for the seed presoaking group as well as 51.3 and 47.87% for the seed coating group. Furthermore, it appeared that the treated seedlings' levels of the enzymatic and nonenzymatic antioxidants, glutathione reductase, superoxide dismutase, ascorbate peroxidase, catalase, glutathione, and ascorbic acid, were positively inducted specifically for the presoaking group. The total protein and carbohydrate contents of the different treatments varied greatly with the highest levels in the soil drench group, while the highest levels of total phosphorus and chlorophyll (a) were observed in the seed coating group. GC-MS analysis showed that the bacterial supernatant included several antifungal bioactive compounds, including diethyl phthalate and 2,4-di*tert*-butyl phenol as major compounds.

Keywords: *Bacillus halotolerans*, antifungal, *Rhizoctonia solani*, antioxidants, GC-MS

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INTRODUCTION

Cotton is an essential global crop due to the high economic value of its fiber and seed oil (Sherzad & Canming, 2020). Often referred to as "The White Gold," it is the most valuable cash crop in many countries and serves as the primary raw material for the growing textile industry (Müller, 2019). However, controlling *Rhizoctonia solani*, a highly aggressive soil-borne pathogen, remains a significant challenge (Selim et al., 2017). This pathogen does not produce asexual spores; instead, it survives in the soil as sclerotia. In many cotton fields in Egypt, *R. solani* is the leading cause of the cotton seedling damping-off disease (Mikhail et al., 2010).

Plant microbiome plays a crucial role in promoting plant growth and health without harming the environment, making it an essential part of plant-microbe interactions (Andrade et al., 2023). Various microorganisms, such as endophytic, rhizospheric, and mycorrhizal organisms, establish mutualistic relationships with plants. These interactions have gained significant attention from the scientific community due to their proven benefits (Silva et al., 2020). Numerous studies have demonstrated that these microbial symbioses provide microorganisms with nutrients and protection while enhancing the plant's physiology, fitness, and metabolite profile (Eid et al., 2021; Latif et al., 2021; Abd El-Megeed and Mohiy, 2022; Zaghloul et al., 2024).

Biological control of soil-borne pathogens and use of biofertilizers have attracted substantial global interest in recent years. Previous studies have highlighted the critical role of *Pseudomonas* spp. and other Gram-negative bacteria in biocontrol and induction of systemic resistance (ISR) (Zohora et al., 2016). Additionally, earlier research has shown that *Bacillus* species can effectively manage various plant pathogens (Collins & Jacobsen, 2003). *Bacillus* species, particularly *B. subtilis* and its closely related strains, have been extensively studied due to their disease-controlling capabilities (Wang et al., 2018). In a study by Khiyami et al. (2014), twenty *Bacillus* isolates from cotton waste and livestock manure composts were tested for their ability to antagonize *Phytophthora capsici*, *R. solani* AG-4, *Fusarium oxysporum*, and *Sclerotinia sclerotiorum* in vitro. These bacteria have been shown to release chemicals that damage the pathogen's cellulose, protein, hemicellulose, or DNA (Zohora et al., 2016). The current study indicates the specific responses by which *Bacillus halotolerans* C3 suppresses *Rhizoctonia solani*, the bioactive compounds it produces, and its potential as a biocontrol agent for the cotton damping-off disease.

Bacterial biocontrol agents are commonly used to manage fungal diseases in plants, which can devastate entire crops even with small amounts of the pathogen in the soil. These bacterial chemicals, such as hydrogen cyanide, ammonia, and antibiotics like 2,4-diacetylphloroglucinol, affect fungal cell membrane integrity and secondary metabolite production, such as mycotoxins and proteases, which are crucial for fungal virulence (Stepanov et al., 2024) and also influence the expression of genes related to cell membrane formation, as seen in their interaction with *Penicillium* species, leading to structural damage in fungal hyphae (Sindhu et al., 2016). Pyrrolnitrin interrupts the terminal electron transport system, leading to impaired cellular respiration and also hampers the synthesis of DNA, RNA, and proteins, affecting fungal growth and reproduction (Leonardo & de Oliveira Freire, 2016). Pyoluteorin disrupts key biological processes in fungi, including DNA and protein synthesis (Quan et al., 2011). These biocontrol agents provide numerous benefits by promoting plant growth and assisting in disease management (Zohora et al., 2016). Certain bioactive compounds produced by bacteria possess antifungal and antimicrobial properties, making them highly effective as biocontrol agents (Hong & Park, 2016).

This study aims to evaluate the antagonistic activity of the rhizobacterium *Bacillus halotolerans* C3 to suppress the cotton damping-off disease caused by *R. solani* under greenhouse conditions, identify the bioactive compounds produced by this strain, and demonstrate the impact of various bacterial seed inoculation techniques on defense responses of the infected plant.

MATERIALS AND METHODS

Soil Sampling and Bacterial Isolation

Rhizosphere soil samples of 3-month-old *Gossypium barbadense* L. plants were collected from El-Azab, Fayoum, Egypt ("29°15'07.9"N 30°50'42.4"E). Soil characteristics are summarized in Table 1. Sampling was carried out in April 2022, peak growing season for *Gossypium barbadense* L. Samples of soil were collected within 20 to 30 cm. Individual samples, such as roots and soil aggregates, were put in sterilized plastic bags, kept at 4ºC, and then sent right away to the laboratory. Nine bacterial isolates were collected. Following being aseptically transferred, the antagonistic activity was tested to isolate the bacteria that have the highest antagonistic effect on potato dextrose agar (PDA) supplemented with fungal spores of *Rhizoctonia solani* (Mahdi et al., 2020).

Bacterial Characterization and Identification

Gram staining of the bacterial isolate was carried out according to Kristensen et al. (2023). Biochemical characterization of the bacterial isolate was performed using VITEK II automated system (Funke et al., 1998). A 12 x 75 mm clear plastic (polystyrene) test tube was inoculated with the proper number of colonies from a pure culture using a sterile swab. The microorganism was then suspended in 3.0 mL of saline that had been sterilized. The turbidity is adjusted to 0.5 using a DensiChek turbidity meter. The Gram-positive identity cards were infected with microbe samples using a combined vacuum system. The Armed Forces Chemical Warfare Institute (AFCWI) then used 16S rRNA sequencing technology to identify the chosen bacterial isolate. Using universal primers that target conserved regions of bacterial DNA, the 16S rRNA gene was amplified by PCR. The reverse primer (1492R: 5'-TACGGYTACCTTGTTACGACTT-3') and the forward primer (27F: 5'-AGAGTTTGATCMTGGCTCAG-3') were used. 1 µL of each primer, 1 µL of template DNA (~50 ng), 12.5 µL of 2× PCR master mix (Thermo Fisher Scientific or equivalent), and nuclease-free water were added to each 25 µL PCR reaction mixture to complete the final volume. 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 1 minute, and final extension at 72°C for 5 minutes comprised the thermal cycling conditions. The initial denaturation lasted 5 minutes at 95°C. After being electrophoresed on a 1.5% agarose gel, the PCR products were stained with ethidium bromide and examined under a UV lamp. About 1,500 bp of amplified fragments were prepared for sequencing (Mularoni et al., 2023). The sequence analysis was then carried out using the NCBI database (http://www.ncbi.nlm.nih.gov) and the sequence alignment program BLASTn. To obtain an accession number, its sequence was submitted to GenBank database.

Table 1. Physiochemical properties of the soil sample

Site	El-Azab, Fayoum, Egypt 29°15'07.9"N 30°50'42.4"E
Crop	Cotton
Type	Clay
pH	7.3
E.C mS/m	510
Organic matter %	30.34%
Total organic carbon %	17.6
Total Mg (meq./100 g)	3.6
Total Ca (meq./100 g)	8.4
Available phosphorus (mg/kg)	2.674
Total nitrogen (mg-N/g soil)	0.97

Fungal Strain

Rhizoctonia solani used in this study was obtained from the Plant Pathology Department, Faculty of Agriculture, Fayoum University, Egypt.

Antagonistic Activity Test

The antagonistic activity of the bacterial isolates was tested on a potato dextrose agar (PDA) medium by the plate diffusion assay (Jalaluldeen et al., 2014) with some minor modifications by placing an inoculum of the rhizobacterial isolate in a line against an agar disc with mycelium near the edge of a PDA plate. Plates were incubated at 28ºC and checked for inhibition. Negative isolates, *Escherichia coli* or *Pseudomonas putida*, were used as a control.

The percentage inhibition of diameter growth (PIDG) was determined according to the following equation:

$$\text{PIDG } (\%) = \frac{\text{Diameter of control} - \text{Diameter of sample}}{\text{Diameter of control}} \times 100.$$

The highly antagonistic isolate (designated as C3) was selected for further work.

Assay of Phosphate-Solubilizing Ability

On phosphate agar medium plates, the phosphate solubilization of bacteria was determined according to Mahdi et al. (2020). Plates were incubated at 28°C and monitored daily for the development of transparent halos, which signify the ability to solubilize P. *Pseudomonas fluorescens* NCIMB 11764 was used as a control (no solubilization ability). In liquid culture, fifty millimeters of mineral salt medium (MSM) was injected with 500 µL of the bacterial strains at the same initial concentration of roughly $1x10^8$ CFU/mL, wherein the quantitively measurement of phosphate solubilization in broth was carried out as described by Selvakumar et al. (2009). The activity of acid phosphatase was determined according to Pereira & Castro (2014) using *p*-nitrophenyl phosphate. The released yellow color of *p*-nitrophenol was estimated spectrometrically at 405 nm. The specific activity is expressed in moles of *p*-nitrophenol released per minute per milligram of protein.

Determination of NaCl Stress Tolerance and Bacterial Growth Optimization

Two hundred mL flasks were filled with about 50 mL of MSM medium (Tao et al., 2019) containing NaCl concentrations of 0, 50, 100, 200, and 300 mM. 500 µL of the same concentration of bacterial inoculum was incubated at 28°C. Using spectrometry, growth was measured at OD 600 nm. According to Ji et al. (2024), the effect of some factors such as temperature and pH on bacterial growth was investigated, and the optical density at 600 nm was measured using a UV-VIS spectrophotometer to track bacterial growth. The bacterial culture was allowed to grow at 20°C, 30°C, and 40°C with shaking for 48 hrs. The impact of pH levels on bacterial growth was simultaneously examined at three different pH levels (5, 7, and 9).

Greenhouse Experiment

Preparation of Bacteria-Treated Seeds: Nutrient broth medium was inoculated with a loopful of bacterial isolate C3, which was then incubated at 28ºC in a rotating shaker operating at 150 rpm. The cultures were centrifuged for 10 minutes at 10,000 rpm after 48 hours of incubation, and the bacterial pellets were resuspended at a cell number of 9×10^8 CFU/mL. Next, as a soil drench treatment, cotton seeds were planted in pots with a 25 cm diameter, and 5 mL of the bacterial suspension was added to the pot's surface (Selim et al., 2017). At the same time, seed coating was prepared by mixing 400 mL of bacterial suspensions, one kg of the sterilized talc powder, and 15 g calcium carbonate under sterilized conditions (Basheer et al., 2019). The seeds were surface-sterilized and coated with this formulation (20 g/kg of seeds). After that, the seeds were allowed to air-dry for 24 hours in a sterilized environment. Also, presoaked seeds were prepared by soaking surface-sterilized seeds in a bacterial suspension (9×10^8 CFU/mL) for 4 hours under sterilized conditions before planting (Kharat et al., 2022).

Preparation of Fungal Inoculum: According to Khiyami et al. (2014), The bottles' contents were autoclaved for thirty minutes. Fungi grew on substrates that were prepared in 500 mL glass bottles with 50 g of sorghum grains and 50 mL of water in each bottle. A two-week-old sorghum culture was used to extract the fungal inoculum, which was then placed into bottles and allowed to colonize the grains. The soil used in this study was autoclaved clay loam. Each fungus inoculum was added to batches of soil at a rate of 1 g/kg of soil. The infested soils were poured into clay pots with a diameter of 10 cm. The Egyptian Ministry of Agriculture, Giza, graciously provided seeds of the cotton plant cultivar Giza 90 (*Gossypium barbadense* L.). In each pot, twenty seeds were planted at a depth of 10 mm. For every treatment, three replicate pots were utilized, and the soil was kept at field capacity through frequent watering. For three weeks, the pots were dispersed at random throughout a greenhouse that was kept at 25°C.

Bacterial strain (*Bacillus halotolerans* C3) was applied as a soil drench, presoaked seeds, and seeds coating to *R*. *solani* infested soil to study its effect on cotton seedlings, in the presence of control-contained plant seedlings at normal conditions without bacteria and fungus.

Emergence and Disease Severity: Each treatment's percentage of seed emergence was determined by comparing it to the corresponding control (Kelly H, 2018). For each treatment, the percentage of seedling survival was computed in relation to the corresponding control. After 21 days of planting, the severity of the disease was estimated according to Selim et al. (2017) with minor modification. On a scale of 1 to 4, "1" denotes less than 2% root discoloration, "2" denotes 2–10% root discoloration with a few pinpoint lesions, "3" denotes 11–50% root discoloration with a clear necrotic lesion, and "4" denotes more than 50% root discoloration (dead plant).

Assessment of Preemergence and Postemergence Damping Off: To evaluate the impact of different treatments of C3 isolate on disease control, preemergence damping-off percentage was calculated as the proportion of seeds that failed to emerge relative to the total number of seeds sown, following the method described by Mc Laren & Rijkenberg (1989) and Atta et al. (2022). Postemergence percentage was determined based on the number of emerged seedlings with necrotic symptoms relative to the total number of germinated seedlings according to Mc Laren & Rijkenberg (1989) and Larkin & Fravel (1998). Seedlings were monitored daily; the emergence rate was recorded until no further changes were observed.

Plant Growth Parameters: Following careful removal from the soil and multiple distilled water washes, the cotton plant seedlings were measured for root and shoot lengths after 21-day experimental period. The fresh and dry weights were measured simultaneously (Selim et al., 2017). Some biochemical analysis was carried out, including total carbohydrate (Abbott et al., 1995), total protein (Bonjoch & Tamayo, 2001), and chlorophyll content (Dixit et al., 2023). In order to estimate the total phosphorus of the seedlings, the digested plant tissues were estimated using the molybdenum method (Khan, 1992).

Determination of Metabolites and Antioxidant Enzymes: The plant seedlings were homogenized in 20 mL of 0.1 M sodium phosphate buffer (pH 6.5) and centrifuged for 15 minutes at 4°C at 16,000 rpm. The activity of the ascorbate peroxidase (APX) assay was evaluated according to Yoshimura et al. (2000). In order to measure the catalase activity, 40 µL of enzyme extract was added to 9.96 mL of recently prepared H_2O_2 phosphate buffer according to Kong et al. (1999). Glutathione reductase (GR) activity was assayed by measuring the rise in absorbance when 5,5-dithio-2-nitrobenzoic acid (DTNB) and oxidized glutathione (GSSG) were mixed (Upton et al., 2009). The nitroblue tetrazolium (NBT) assay was used to measure the activity of superoxide dismutase (SOD) at 560 nm according to Gupta et al. (1993).

To investigate some metabolites such as ascorbic acid, a one-gram seedling sample was homogenized in 10 mL of distilled water and centrifuged at 16,000 rpm for 15 min at 4°C according to Loeffler & Ponting (1942). Glutathione (GSH) content was determined by the florescence spectroscopy method (Zhang & Ying, 2008). Zieslin and Ben-Zaken's (1993) estimation of the total phenolic content was used. Ten milliliters of 80% methanol were used to homogenize one gram of root tissue. The absorption of the developed blue color was measured spectrophotometrically at 725 nm after 1 mL of the methanol extract was combined with 250 µL of Folin–Ciocalteu reagent (1 N) and 5 mL of distilled water. The catechol equivalent per gram of tissue weight was used to express the total phenol content.

GC-MS Analysis of Bacterial Secondary Metabolites

After centrifuging 20 milliliters of each 48-hour bacterial culture at 3000 g for 10 minutes, the supernatant was extracted using 60 milliliters of ethyl acetate in three stages using a separating funnel. Each step's ethyl acetate phase was gathered and centrifuged for 10 minutes at 3000 g. An anhydrous sodium sulfate (1 g) was then added. One milliliter of methanol was added after the mixture had been dried out at 45°C using a rotary evaporator (Selim et al., 2017). To determine which substances are present in the bacterial exudates, gas chromatography-mass spectrometry (GC-MS) (model: QP2010 Ultra, Shimadzu Corporation, Kyoto, Japan) was used to analyze the secondary metabolites produced by the bacteria. Active chemical compounds were evaluated by comparing their mass spectra and retention periods with those found in the databases of the WILEY library and the National Institute of Standards and Technology (NIST), which are integrated into the GC/MS instrument.

Statistical Analysis

Statistical analysis was performed using R software (RCoreTeam, 2023). A *P* value < 0.05 is considered statistically significant.

RESULTS

Antagonistic activity

Nine rhizosphere bacterial isolates were obtained from the rhizosphere cotton crop plants. Their antagonistic effect was screened against *R. solani* on PDA agar plates. Based on the appearance of surrounding clearing zones, isolate C3 exhibited the highest antifungal effect against *R. solani* (Table 2). Figure 1. shows the antagonistic effect of C3 towards *R. solani.* The percentage inhibition of diameter growth (PIDG%) varied significantly among the bacterial isolates (Table 2), as indicated by the Kruskal-Wallis rank sum test (*P* = 0.002). Isolate C3 exhibited the highest inhibitory activity (47.03 ± 0.70%), significantly outperforming all other isolates.

Phosphate solubilization ability

Our isolate, C3, was tested for P solubilization after being spot inoculated on MSM agar plates with $CaHPO_4$ as the only phosphate source. Based on the appearance of surrounding solubilization halos, the isolation exhibited high phosphate solubilization activity (Figure 2). Also, to quantify P solubilization, the isolate was grown in liquid MS broth supplemented with $CaHPO_4$ for 4 days. The C3 isolate showed high capacities to solubilize inorganic $CaHPO_4$ (77.59 ± 12.20 µg/mL) and organic *para*-nitrophenyl phosphate (16.56 ± 6.10 mM) (Table 3). The values represent means of replicates (n = 3) ± standard deviations. Wilcoxon rank sum test was used to compare the optical density and concentration of phosphate solubilization with significant P value < 0.01 indicating the ability of phosphate-solubilizing rhizobacterial C3 to solubilize organic and inorganic insoluble phosphate. The different letters in superscript (a, b) indicate the statistically significant difference at P value < 0.05.

Optimization of bacterial growth

The results in Figure 3 indicate significant differences of bacterial growth and concentration of phosphate solubilization through different pHs, temperatures, and salinity concentrations suggesting that the optimum pH for growth and phosphate solubilization is pH=7. Also, the optimum temperature for phosphate solubilization is 30°C and for growth 40°C. Under salinity stress, growth and phosphate solubilization decrease.

Bacterial characterization and identification

The growth of the bacterial isolate on solid media was used to establish its morphology and Gram staining

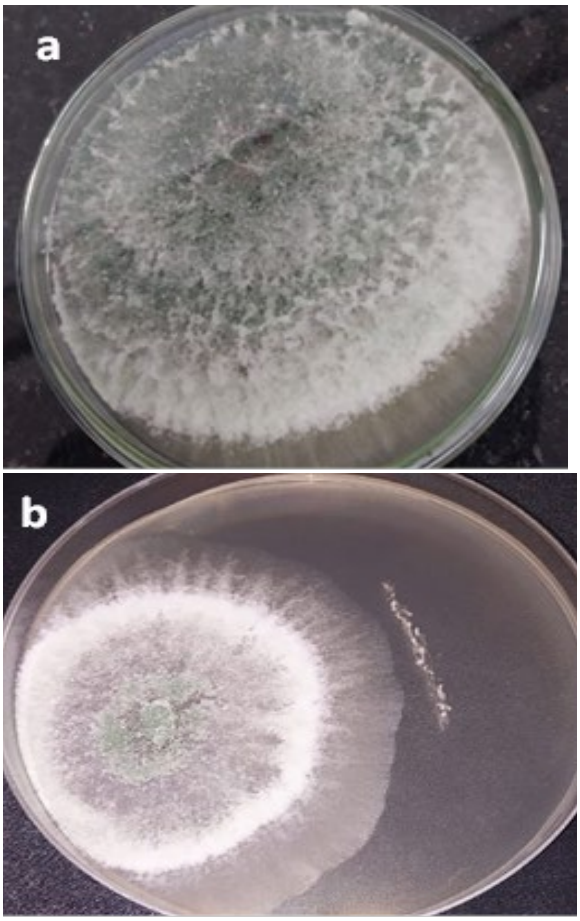


Figure 1. a) *Rhizoctonia solani* on PDA plate inoculated with reference strain and b) antagonistic effect between C3 isolated from cotton and *R. solani* showing clear zone and little fungal growth indicating an antifungal activity.

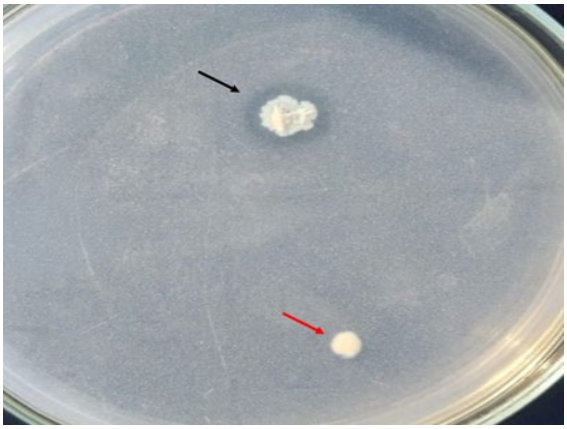


Figure 2. Phosphate dissolution halo zone around the phosphate-solubilizing bacterial colony C3 (black arrow). The red arrow indicates the non-solubilizing reference colony.

Table 2. Percentage inhibition of diameter growth in *R. solani* using nine bacterial isolates from cotton.

Isolate	C1	C2	C3	C4	C5	C6	C7	C8	C9	*P* value*
PIDG (%)	(9.88 ± 1.63)^{a}	(7.66 ± 0.96)^{a}	(47.03 ± 0.70)^{c}	(6.91 ± 1.63)^{a}	(11.41 ± 0.53)^{b}	(6.74 ± 1.13)^{a}	(20.34 ± 0.75)^{b}	(3.23 ± 0.81)^{a}	(14.30 ± 0.91)^{b}	0.002
Values between brackets are represented as median ± IQR										
*Kruskal-Wallis rank sum test										

Table 3. Phosphate solubilization of inorganic and organic insoluble phosphate by selected phosphate-solubilizing bacteria (PSB) isolate C3.

Group	Inorganic phosphate ($CaHPO_4$)		Organic phosphate (*p*-Nitro-phenylphosphate) Conc. (mM)
	OD 600 nm	Conc. (µg/mL)	
Bacterial isolate C3	(0.80 ± 0.10)^{a}	(77.59 ± 12.20)^{a}	(16.56 ± 6.10)^{a}
Control	0 ^{b}	0 ^{b}	0 ^{b}
P value*	0.007**	0.007**	0.007**
Values between brackets are represented as median ± IQR			
*Wilcoxon rank sum test			

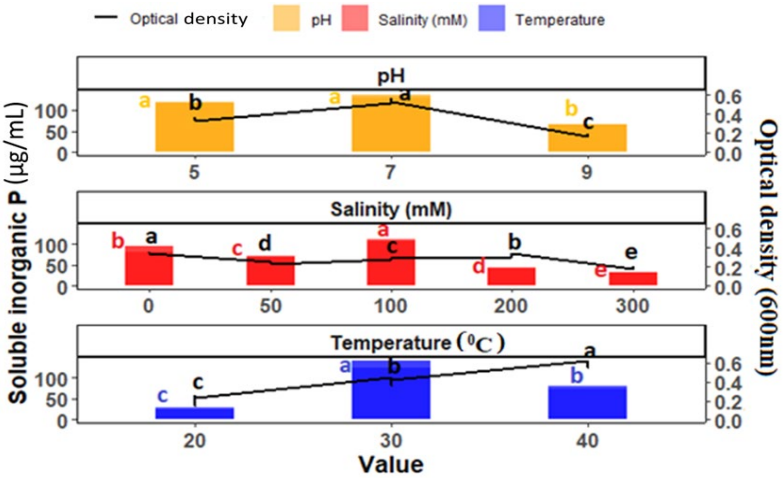


Figure 3. Optimum pH, temperature, and salinity for phosphate solubilization and for bacterial growth (optical density) of selected PSB (phosphate-solubilizing bacteria) in the presence of 8 g/L $CaHPO_4$ in mineral salt medium (MSM). The different letters indicate significant difference at *P* value < 0.05.

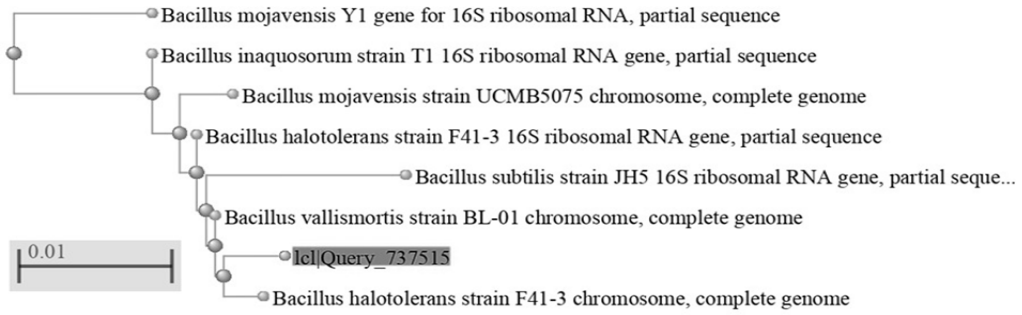


Figure 4. The location of the plant growth-promoting rhizobacterial strain isolated from cotton soil within the related genera is displayed by a neighbor-joining phylogenetic tree based on the 16S rRNA gene sequence.

and other biochemical assays were used to examine the bacteria under a microscope. Our isolate was Gram-positive and bacilli. Supplementary Table 1 displayed the bacterial characteristics. PCR was used to separate and amplify the C3 isolate's genomic DNA. After being amplified, the 16S rDNA gene segments in the sample were sequenced. C3 isolate revealed the closest similarity with *Bacillus* at the genus level and *halotolerans* at the species level (Figure 4). The bacterial isolate C3 was identified as *Bacillus halotolerans* with maximum homology of 99.41%. The 16S rDNA gene sequences of the bacterial strains were deposited in GenBank database under accession number PP907831.1.

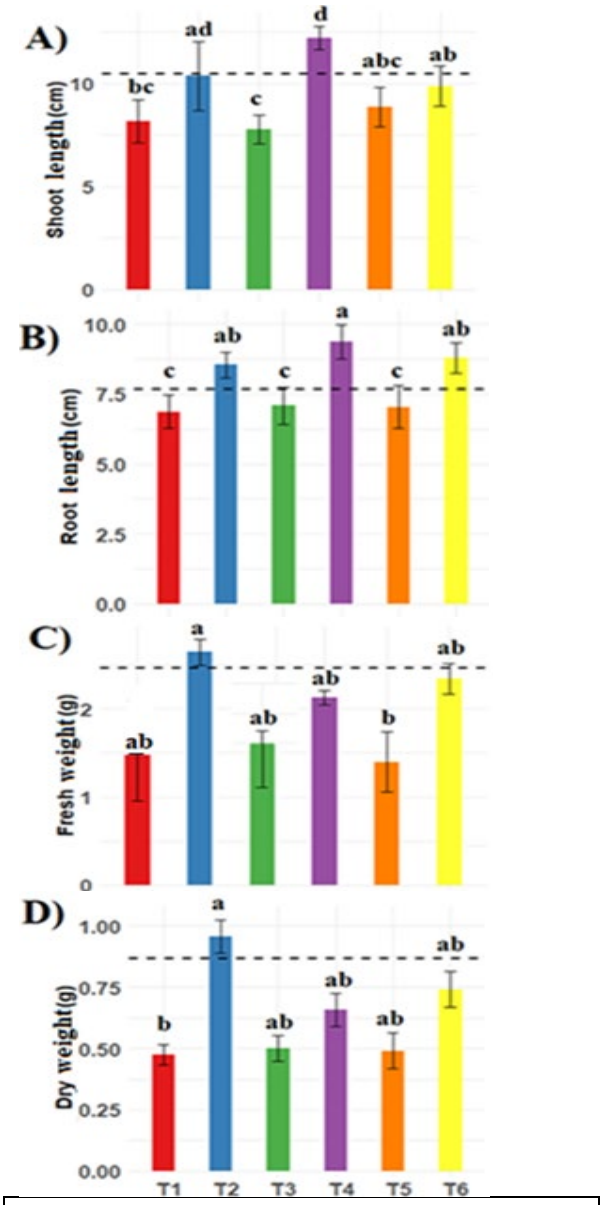
Seedling Experiment

Effect of *B. halotolerans* C3 rhizobacteria on seed emergence: The treatments of cotton seeds with the rhizobacterial *B. halotolerans* C3 significantly increased seed emergence (Table 4). The percentage of seed emergence was significantly affected by the different treatments applied. The control group exhibited the highest seed emergence percentage (93.0±3.0%), followed by the soil drench treatment (87.0±3.0%) and the seed coating treatment (80.0±3.0%). In contrast, treatments involving pathogens alone resulted in lower seed emergence percentages. The treatments that included beneficial bacteria were associated with improved seed emergence rates, suggesting a protective effect against pathogen-induced stress. The disease severity scores varied significantly among the treatments (Table 4), as evidenced by the Kruskal-Wallis rank sum test (P=0.007). The control group consistently exhibited the lowest disease severity (1.00±0.00). Similarly, T4 and T6 also demonstrated low severity scores, indicating their effectiveness in mitigating disease impact. In contrast, treatments T1, T3, and T5 recorded the highest severity scores (4.00±0.00, 4.00±0.50, and 4.00±0.50, respectively). T2 showed intermediate severity (2.00±0.50), suggesting partial control of disease symptoms. These findings highlight the differential efficacy of treatments in reducing disease severity.

Effect of *B. halotolerans* C3 on preemergence and postemergence damping-off disease: The results presented in Table 5 demonstrate that the application of *Bacillus halotolerans* C3 significantly reduced preemergence and postemergence damping-off in cotton seedlings infected with *Rhizoctonia solani* (P = 0.004 and P = 0.005, respectively). For preemergence damping-off, treatments with *B. halotolerans* C3 consistently exhibited lower damping-off rates compared to their untreated counterparts. The lowest incidence was observed in the soil drench treatment with *B. halotolerans* C3 (13±3), followed by seed coating (20±3) and seed presoaking (27±3). Similarly, for postemergence damping off, the presence of *B. halotolerans* C3 led to a notable reduction in disease incidence. The soil drench treatment with *B. halotolerans* C3 exhibited the lowest damping-off rate (23±5), while the highest was observed in the seed coating treatment without *B. halotolerans* C3 (57±8). Also, the survival rate was assessed by calculating the difference between groups with and without bacterial treatment. For preemergence damping off, the increase in survival due to bacterial treatment was 40% in the seed coating method, 34% in the soil drench method, and 26% in the seed presoaking method. Similarly, for postemergence damping-off, survival rates increased by 32%, 34%, and 23% for the seed coating, soil drench, and seed presoaking, respectively. These results highlight the biocontrol potential of *B. halotolerans C3* in mitigating *R. solani*-induced damping-off.

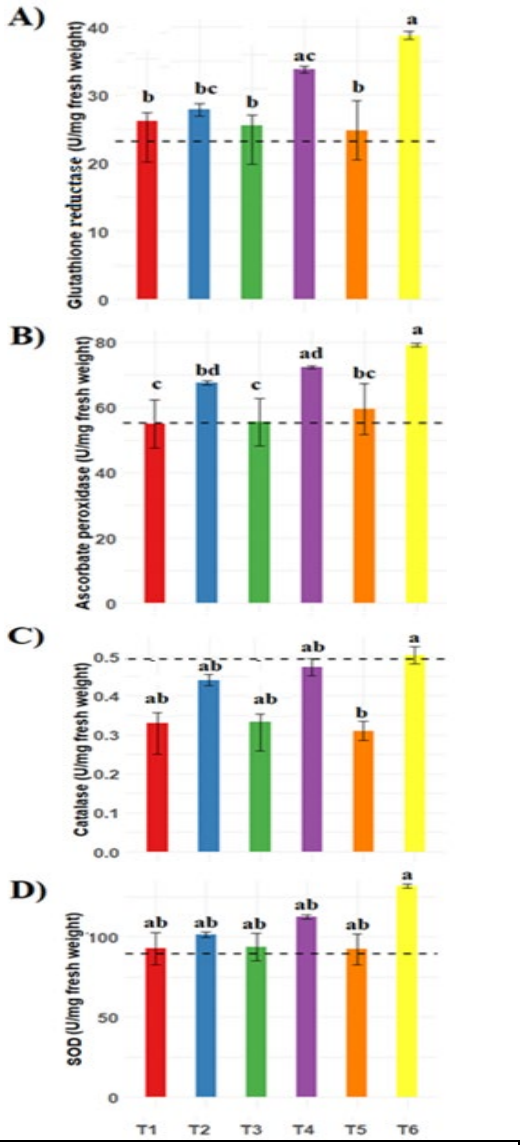
Effect of PGP rhizobacterial treatment on seedling growth parameters: Data in Figure 5 demonstrates the differences in shoot and root lengths as well as fresh and dry weights across the different treatment groups. Shoot length was significantly affected by the different treatments, with the soil drench group exhibiting the longest shoots (12.21±0.40), compared to the negative control (10.47±0.30) and the seed coating group (10.37±1.16). The soil drench infected group had the shortest shoot length, equal to 7.77±0.49. Root length differences were even more striking, as the soil drench group demonstrated the longest root length (9.37±0.43), while the seed coating infected group had the shortest length (6.86±0.41). Fresh weight recorded the highest value (2.65±0.11) for seed coating, compared to infected seeds that had the lowest value (1.29±0.32). Similarly, dry weight measurements followed a similar trend, with the seed coating group measurements of 0.94±0.05 compared to 0.49±0.03 for the non-bacterially treated infected group.

Effect of PGP rhizobacterial treatment on enzymatic and nonenzymatic antioxidants: Significant variations in enzyme activity were noted among the various treatment groups in this investigation (Figure 6). The soil drench infected group recorded the lowest levels of catalase, superoxide dismutase (SOD), ascorbate peroxidase, and total phenolic content, while the presoaking (pathogen + bacteria) group consistently displayed the highest levels. The presoaking infected group had the lowest measurement of glutathione reductase activity, whereas the soil drench group had the highest. Furthermore, the levels of ascorbic acid and glutathione were considerably higher in the seed coating treatment than in the soil drench group (Table 6). These findings demonstrated the potential of bacterial treatments to improve plant stress response mechanisms under pathogenic exposure by highlighting the notable effects of different treatments on antioxidant enzyme activities and phenolic content.



Dashed line refers to control in normal conditions (without *R. solani* and *B. halotolerans* C3)
(T1) Seed coating without *B. halotolerans* C3
(T2) Seed coating with *B. halotolerans* C3 in in *R. solani* infested soil
(T3) Soil drenches without *B. halotolerans* C3 in R. solani infested soil
(T4) Soil drench with *B. halotolerans* C3 in R. solani infested soil
(TS) Seed presoaking without *B. halotolerans* C3 in R. solani infested soil
(T6) Seed presoaking with *B. halotolerans* C3 in *R. solanit* infested soil

Figure 5. Effect of rhizobacterial *B. halotolerans* C3 on the growth parameters of cotton seedlings in *R. solani* infested soil where (A) represents shoot length, (B) represents root length, (C) represents fresh weight, and (D) represents dry weight. The values are means of three replicates ± standard error. One-way ANOVA was used to compare different treatments in case of normally distributed data while the Kruskal-Wallis rank sum test was used in case of not normally distributed data. Different letters refer to a significant difference.



Dashed line refers to control in normal conditions (without *R. solani* and *B. halotolerans* C3)
(T1) Seed coating without *B. halotolerans* C3
(T2) Seed coating with *B. halotolerans* C3 in in *R. solani* infested soil
(T3) Soil drenches without *B. halotolerans* C3 in R. solani infested soil
(T4) Soil drench with *B. halotolerans* C3 in R. solani infested soil
(TS) Seed presoaking without *B. halotolerans* C3 in R. solani infested soil
(T6) Seed presoaking with *B. halotolerans* C3 in *R. solanit* infested soil

Figure 6. Effect of rhizobacterial *B. halotolerans* C3 on the antioxidant enzymes of cotton seedlings in *R. solani* infested soil, wherein (A) represents glutathione reductase activity, (B) represents ascorbate peroxidase activity, (C) represents catalase activity, and (D) represents superoxide dismutase (SOD) activity. The values are means of three replicates ± standard error. One-way ANOVA was used to compare different treatments in case of normally distributed data while Kruskal-Wallis rank sum test was used in case of not normally distributed data. Different letters refer to significant differences.

Table 5. Effect of *B. halotolerans* C3 treatments on the preemergence and postemergence damping-off disease.

Treatments	Control (healthy soil)	Seed coating		Soil drench		Seed presoaking		*P* value
		Without *B. halotolerans*	With *B. halotolerans*	Without *B. halotolerans*	With *B. halotolerans*	Without *B. halotolerans*	With *B. halotolerans*	
Preemergence damping off (%)	$(0 \pm 0)^{e}$	$(60 \pm 3)^{a}$	$(20 \pm 3)^{c}$	$(47 \pm 7)^{b}$	$(13 \pm 3)^{d}$	$(53 \pm 7)^{a}$	$(27 \pm 3)^{c}$	0.004
Postemergence damping-off (%)	$(0 \pm 0)^{d}$	$(57 \pm 8)^{a}$	$(25 \pm 6)^{c}$	$(57 \pm 3)^{a}$	$(23 \pm 5)^{c}$	$(63 \pm 5)^{a}$	$(40 \pm 5)^{b}$	0.005
Values between brackets are represented as median ± IQR								
*Kruskal-Wallis rank sum test								

Table 6. Effect of *B. halotolerans* C3 on nonenzymatic antioxidants and total phenolic content of cotton seedlings in *R. solani* infested soil.

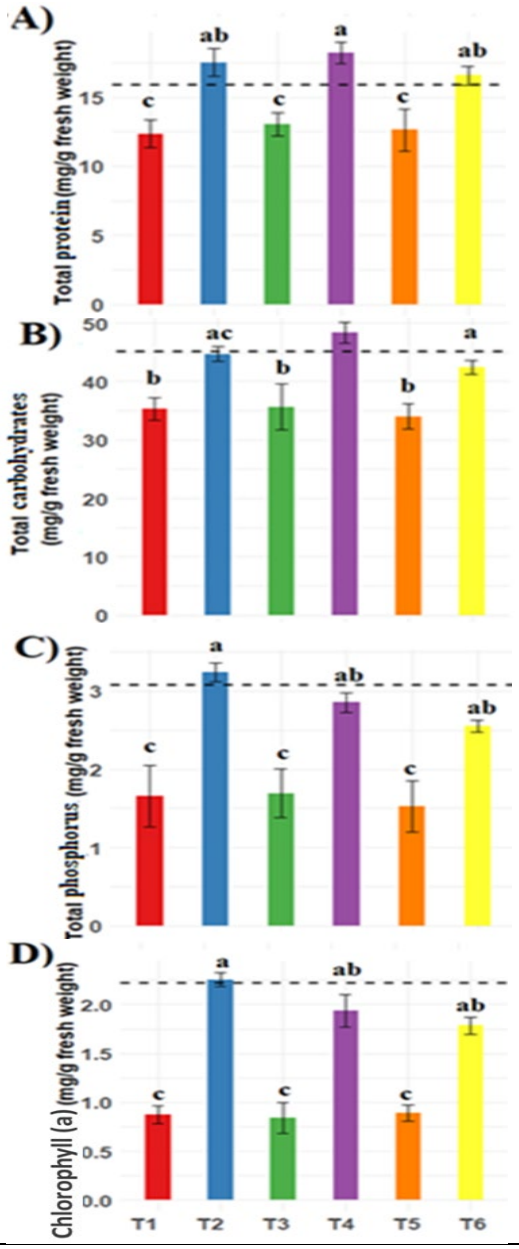
Treatments	Control, N = 3	Seed coating		Soil drench		Seed presoaking		*P* value
		Without *B. halotolerans* C3	With *B. halotolerans* C3	Without *B. halotolerans* C3	With *B. halotolerans* C3	Without *B. halotolerans* C3	With *B. halotolerans* C3	
Glutathione (mg/g)	$(2.14 \pm 0.02)^{a}$	$(1.57 \pm 0.08)^{b}$	$(2.24 \pm 0.02)^{a}$	$(1.67 \pm 0.19)^{b}$	$(2.03 \pm 0.02)^{a}$	$(1.70 \pm 0.16)^{b}$	$(1.98 \pm 0.01^{]a}$	< 0.001
Ascorbic acid (mg/g)	$(2.22\pm 0.01)^{ab}$	$(1.66 \pm 0.45)^{b}$	$(2.46 \pm 0.01)^{a}$	$(1.99 \pm 0.14)^{ab}$	$(2.34 \pm 0.01)^{ab}$	$(1.75 \pm 0.53)^{ab}$	$(2.12 \pm 0.01)^{ab}$	0.0209
Total phenolic content (mg/g dry weight)	$(127.43\pm 7.05)^{d}$	$(102.16 \pm 2.85)^{e}$	$(180.63 \pm 10.10)^{b}$	$(103.49 \pm 6.05)^{e}$	$(150.17 \pm 9.25)^{c}$	$(105.51 \pm 5.05)^{e}$	$(212.13 \pm 7.48)^{a}$	< 0.001
Values between brackets are represented as mean ± SD or median ± IQR								
*Kruskal-Wallis rank sum test One-way ANOVA								

Table 7. GC-MS analysis of the secondary metabolites of the rhizophytic bacterial strain: *Bacillus halotolerans* C3

Compound	Peak area (%)	RT (min)	M. wt. (g/mol)	M. formula	Activity
2,4-Di-*tert*-butylphenol	12.29	3.708	206.33	$C_{14}H_{22}O$	Antifungal (Fan et al., 2023)
Arsenous acid, tris(trimethylsilyl) ester	4.65	5.117	342.49	$C_9H_{27}AsSi_3O_3$	Antimicrobial (Manikandan et al., 2019)
Carbonic acid, tridecyl 2,2,2-trichloroethyl ester	1.76	6.920	333.7	$C_{16}H_{29}Cl_3O_3$	Antifungal (Ugbogu et al., 2019)
Carvacrol	0.91	7.021	150.2	$C_{13}H_{22}OSi$	Antifungal (Numpaque et al., 2011)
Cycloheptasiloxane, tetradecamethyl-	2.78	8.137	519.0776	$C_{14}H_{42}O_7Si$	Antifungal (Nguyen & Cao, 2022)
Heptasiloxane	1.54	9.026	292.59	O6Si7	Antifungal (Nguyen & Cao, 2022)
Fumaric acid	5.70	9.747	116.07	$C_4H_4O_4$	Antimicrobial (Unver, 2024)
Indole-2-one	0.66	9.848	131.13	C_8H_5NO	Antifungal (Akhaja & Raval, 2011)
Diethyl phthalate	43.08	9.924	222.24	$C_{12}H_{14}O_4$	Antifungal (Raman & Parameswari, 2007)
Tetradecyl trifluoroacetate	7.16	12.465	310.39	$C_{16}H_{29}F_3O_2$	Antiviral (Boadu et al., 2024)
Benz[b]-1,4-oxazepine-4(5*H*)-thione	0.74	12.549	207.29	$C_{11}H_{13}NOS$	Antifungal (Wasihun et al., 2023)
2-Ethylacridine	1.10	12.885	207.27	$C_{15}H_{13}N$	Antifungal (Muthukrishnan et al., 2022)
N-Methyl-1-adamantaneacetamide	1.75	14.219	207.31	$C_{13}H_{21}NO$	Antifungal (Chellappandian et al., 2021)
1-Methyl-2-phenylindole	0.46	14.672	207.27	$C_{15}H_{13}N$	Antifungal (Sharma & Singh, 2023)
Tris(*tert*-butyldimethylsilyloxy)arsane	1.02	14.823	468.7262	$C_{18}H_{45}AsO_3Si_3$	Antioxidant (Kalaivani et al., 2023)
Dichloroacetic acid	6.04	14.965	128.94	$C_2H_2Cl_2O_2$	Antifungal (Sharma & Singh, 2023)
Diethyl bis(trimethylsilyl) ester	0.51	16.593	296.58	$C_{10}H_{28}O_4Si_3$	Antioxidant (Momin and Thomas, 2020)
5-Eicosene	3.90	17.272	280.5	$C_{20}H_{40}$	Antifungal (Naragani et al., 2016)
Cyclotrisiloxane	3.11	19.428	138.30	$H_6O_3Si_3$	Antifungal (Helal et al., 2019)
Octasiloxane	0.83	19.772	336.68	O_7Si_8	Antifungal (Kasim Mohamed et al., 2022)

RT: retention time, peak area % represents the concentration, M. wt.: molecular weight, M. formula: molecular formula.

Effect of Rhizobacterial Treatment on Total Proteins, Carbohydrates, Total Phosphorus, and Chlorophyll (a): Distinct differences were observed in total protein, total carbohydrates, total phosphorus, and chlorophyll (a) contents across the various treatment groups (Figure 7). Total protein and carbohydrate contents varied significantly among the treatments, with the soil drench group exhibiting the highest levels, followed closely by the seed coating group. In contrast, the soil drenched group had the lowest contents. Total phosphorus and chlorophyll (a) contents were the highest in the seed coating group compared to the presoaking infected group, which had the lowest levels.



Dashed line refers to control in normal conditions (without *R. solani* and *B. halotolerans* C3)
(T1) Seed coating without *B. halotolerans* C3
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(T4) Soil drench with *B. halotolerans* C3 in R. solani infested soil
(TS) Seed presoaking without *B. halotolerans* C3 in R. solani infested soil
(T6) Seed presoaking with *B. halotolerans* C3 in *R. solanit* infested soil

Figure 7. Effect of rhizobacterial *B. halotolerans* C3 on (A) total protein (mg/g), (B) total carbohydrates (mg/g), (C) total phosphorus (mg/g), and (D) chlorophyll (a) (mg/g). The values are means of three replicates ± standard error. One-way ANOVA was used to compare different treatments in case of normally distributed data while the Kruskal-Wallis rank sum test was used in case of not normally distributed data. Different letters refer to significant differences.

GC-MS Profile of the Bacterial Supernatant: GC-MS analysis of *Bacillus halotolerans C3* filtrate revealed the presence of 20 different metabolite components (Table 7), including diethyl phthalate with 43.08 % and 2,4-di-*tert*-butylphenol with 12.29% as major compounds.

DISCUSSION

Cotton production is severely affected by *Rhizoctonia solani* (*R. solani*), which causes the damping-off disease (Selim et al., 2017), a persistent challenge in regions such as Jammu and Kashmir, Punjab, Haryana, Rajasthan, and Western Uttar Pradesh. Economically significant cotton species are particularly vulnerable, with symptoms progressing from plant wilting to round diseased patches and reddened leaves (Monga D & Sheo Raj, 2018). The first obvious sign of this disease is the wilting of the entire plant. Later, this wilting develops into round diseased patches, which lead to the leaves eventually turning red. It has been noted that antagonistic rhizobacteria, like *Bacillus subtilis* and *Pseudomonas fluorescens*, were successful in controlling the pathogen's colony growth (Salaheddin K, 2010; Samaneh Samavat et al., 2014). The results of the present study showed that cotton seedlings were treated with the rhizobacterial strain whereas the application of *Bacillus halotolerans* C3 as soil drench was the most effective treatment. It has been observed that certain strains of *Pseudomonas fluorescens* and *Bacillus subtilis*, as well as botanicals such as *Azadirachta indica*, *Lantana camera*, *Calotropis procera*, *Ocimum sanctum*, *Allium cepa*, and *Allium sativum*, considerably inhibit the pathogenic fungus's mycelial growth (Prasad et al., 2017). *Erwinia persicinus, Bacillus pumilus*, and *Exiguobacterium acetylicum* were the most successful as biological control agents (Ahmad et al., 2023). *Pseudomonas strain* Os17, which was isolated from the rice rhizosphere and is a member of the *P. protegens* subgroup, inhibits the root rot and the damping-off that *P. ultimum* causes in cucumbers (Höfte, 2021).

Cotton seedlings treated with *Bacillus halotolerans* C3 showed substantial improvements in growth parameters compared to both the untreated control and *R. solani*-infected seedlings. Root and shoot lengths were significantly enhanced, consistent with findings by Akhtar et al. (2023), where *Lactobacillus* sp. and *Weissella* sp. were shown to promote similar growth improvements. This increase in growth may be attributed to the ability of the biocontrol agent to produce growth-promoting hormones or chemicals

that facilitate root elongation. Moreover, the treated plants exhibited higher fresh and dry weights, corroborating observations by Srivastava et al. (2016), who reported similar outcomes in rice plants treated with *Bacillus amyloliquefaciens* under stress. This increase in biomass suggests enhanced nutrient uptake and metabolic activity stimulated by *Bacillus halotolerans* C3. In terms of photosynthetic efficiency, chlorophyll (a) content was significantly higher in *Bacillus halotolerans* C3-treated plants. This is consistent with Srivastava et al. (2016), who reported elevated chlorophyll levels in rice plants treated with *Trichoderma* biofertilizer. The enhanced chlorophyll content reflects improved carbon assimilation and plant vitality induced by bacterial treatment. Conversely, fungal infection by *R. solani* resulted in a reduction in chlorophyll levels, likely due to the degradation of cell walls and pectin desertification caused by the pathogen, as described by Bellincampi et al. (2014). Also total carbohydrate content was significantly higher in *Bacillus halotolerans* C3-treated plants, this is consistent with Chen et al. (2016) who reported that *Bacillus amyloliquefaciens* SQR9 improved maize seedlings' resistance to salt stress (100 mM NaCl), and bacterial inoculation raised the amounts of chlorophyll and total soluble sugar.

They are also involved in a variety of processes, including cell differentiation, cell growth/division, senescence and sulphate transport regulation, xenobiotic detoxification, metabolite conjugation, enzymatic activity regulation, protein and nucleotide synthesis, phytochelatins, and the expression of stress-responsive genes (Rajput et al., 2021).

The GC-MS analysis of *Bacillus halotolerans* C3 exudates identified several bioactive compounds, notably diethyl phthalate, 2,4-di-*tert*-butyl phenol, thymol, and carvacrol, all recognized for their potent antifungal properties. Thymol and carvacrol are natural isopropyl cresols known to exhibit significant antifungal activities (Sharifzadeh et al., 2021). Thymol has demonstrated efficacy against a range of fungal pathogens, including fluconazole-resistant *Candida* species. Studies have shown that thymol exhibits in vitro antifungal activity against fluconazole-resistant and fluconazole-susceptible clinical isolates of some microbial species (Biernasiuk & Malm, 2023). Carvacrol has been shown to induce apoptosis in *Candida albicans*, suggesting its potential as an antifungal agent (Niu et al., 2020). In comparison to conventional antifungal agents, thymol and carvacrol have been found to exhibit synergistic effects when combined with azole antifungals like fluconazole. This

Biochemical responses related to plant defense were also significantly enhanced. Activities of key antioxidants, such as ascorbate peroxidase, catalase, superoxide dismutase (SOD), glutathione reductase, glutathione, ascorbic acid, and total phenolics, were markedly increased in plants treated with *Bacillus halotolerans* C3. These results align with previous studies (Akhtar et al., 2023; Srivastava et al., 2016), who observed heightened antioxidant enzyme activity in plants treated with biocontrol agents under stress. These enzymes play a critical role in scavenging reactive oxygen species (ROS), which are commonly generated under biotic and abiotic stresses, thus protecting plants from oxidative damage (Gusain, 2015). Under stress, SOD dismutates O_2 to produce H_2O_2, which CAT then transforms into water and molecular oxygen (O_2). Oxidized glutathione (GSSG; dimeric) is reduced to reduced glutathione (GSH; monomeric) by glutathione reductase, and APX uses ascorbate as a specific electron donor to scavenge H_2O_2 to water (Rajput et al., 2021). By regulating cellular–subcellular processes like mitosis, cell elongation, senescence, and cell death, these enzymes not only shield different cell components from harm but also play a significant role in plant growth and development.

combination inhibits the overexpression of efflux-pump genes in *Candida albicans*, enhancing antifungal efficacy (Mina, 2023). These findings suggest that the presence of diethyl phthalate, 2,4-di-*tert*-butyl phenol, thymol, and carvacrol in the exudates of *Bacillus halotolerans* C3 may contribute significantly to its antifungal activity, potentially offering advantages over traditional antifungal agents.

The results of this study evaluate the effectiveness of *Bacillus halotolerans* C3 as a biocontrol agent against *R. solani* in cotton plants. The significant improvements observed in growth parameters (e.g., root and shoot lengths, as well as fresh and dry weights), elevated activity of antioxidant enzymes, and the production of antifungal compounds all support the hypothesis that this bacterial isolate enhances plant resistance to pathogen-induced stress. These findings not only demonstrate the efficacy of *Bacillus halotolerans* C3 in mitigating biotic stress but also highlight its potential role in improving plant growth under fungal infection conditions. Furthermore, the GC-MS analysis underscores the role of bioactive compounds such as diethyl phthalate, 2,4-di-*tert*-butyl phenol, thymol, and carvacrol in inhibiting *R. solani* growth, providing

additional evidence of the mechanisms underlying the effectiveness of this biocontrol agent. It is recommended to apply *Bacillus halotolerans* C3 in enhancing crop resistance to *Rhizoctonia solani* in field trials and improving phosphorus availability in soil under natural conditions.

CONCLUSION

According to the current study, *Bacillus halotolerans* C3 showed the greatest inhibition of *R. solani* growth. The highest growth was observed in the germination percentage of seedlings biocontrolled with bacteria, particularly for the soil drench and presoaking groups. Maximum increase was observed in cotton seedlings' fresh and dry weights. The treatment of the fungus-infected plant with *Bacillus halotolerans* C3 exhibited an increase in carbohydrates, proteins, and phosphorus contents. Elevated levels of enzymatic and nonenzymatic antioxidants CAT, SOD, APX, GR, glutathione, ascorbic acid, and total phenolic content have been detected. *Bacillus halotolerans* C3 showed enhanced antifungal activity and protection of cotton against *R. solani*. Therefore, it is possible to propose this bacterium as a possible biocontrol agent.

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