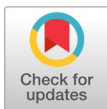


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Evaluation of Drought-Tolerant Epiphytic Bacteria for Plant Growth Promotion

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ABSTRACT

Background. Drought is the leading environmental factor affecting the yield of crops, such as *Zea mays*. The current study aimed to explore the potential of epiphytic bacteria isolated from the Cholistan Desert to enhance *Zea mays* growth and drought tolerance.

Methods. For this purpose, 27 epiphytic bacterial strains were isolated from *Aerva javanica*; screened for drought tolerance potential using different concentrations of PEG-6000; and tested for PGP ((Plant growth promoting) characteristics. Based on the screening, six best epiphytic bacterial strains were selected and a plant-microbe-interaction study was conducted in four treatment groups. *Zea mays* seeds were treated with water, PEG, selected bacterial strains, and a combination of selected bacterial strains along with 15% PEG. After 30 days of germination, the biochemical characteristics (chlorophyll content, relative water content, soluble protein content, and auxin content) were checked for each group.

Results. The results showed an increase in chlorophyll content, relative water content, soluble proteins, and auxin content in plants treated with drought-tolerant epiphytic bacterial strains in combination with 15% PEG. Among the tested strains, the 2 most efficient bacterial strains were identified as *Bacillus rugosus* under the accession number PP556211.1 and *Enterobacter cloacae* under the accession number MT186255.1. It was observed that under PEG-6000, *Enterobacter cloacae* produced the highest amount of Exopolysaccharides, followed by *Bacillus rugosus*.

Conclusion. These epiphytic strains, identified as *Enterobacter cloacae* and *Bacillus rugosus* with drought tolerance potential and Plant Growth Promotion (PGP) characteristics, can be used as an effective, sustainable agricultural approach to enhance crop resilience, improve plant growth parameters, physiological responses, and biochemical characteristics in water-deficient conditions.

Keywords: drought-tolerance, epiphytic bacteria, growth promotion, sustainable agriculture, *Zea mays*

Highlights

- Epiphytic bacteria isolated from drought-inflicted regions, such as the Cholistan Desert, have drought-tolerance potential.
- Drought-tolerant epiphytic bacteria with plant growth-promoting characteristics can increase plant yield in drought conditions.
- *Bacillus rugosus* and *Enterobacter cloacae* increased maize production in water-deficient conditions.

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1. INTRODUCTION

Climate change is the most prevalent global challenge, causing inconsistent weather patterns, longer drought spells, heatwaves, storms, as well as floods and hurricanes. These conditions are a threat to the world's food security. Among all the above-mentioned threats, drought is a climatic event that is getting worse due to human activities [1]. The United Nations (UN) states that drylands have increased by more than 29% since 2000 [2]. This has severe consequences for agriculture and food security, as seen by the numerous crop failures, killed livestock, and skyrocketing food prices. Plants as well as microbes are affected by drought. Drought affects plants or crops by disrupting their vital functions. However, being one of the most commonly consumed crops across the world, *Zea mays* are especially sensitive to drought stress. Drought stress affects growth, development, and hence the productivity of the maize crops [3]. This is upsetting, especially because the world's demand for cereal foods for humans and animals has been projected to double by the years 2025 and 2050 [4].

To combat these effects of drought, desert plants have developed morphological, physiological, and biochemical mechanisms of drought tolerance. For instance, most desert plants have large root systems [5], thick leaves, and stems to store water and an extensive number of molecular events that maximize stress-signaling, antioxidant defense, and osmotic adjustment genes. Moreover, the microbes of the desert ecosystem, including epiphytic bacteria, also contain several survival strategies to cope with drought stress in these regions [6]. These mechanisms include osmotic adjustment strategies, accumulation of compatible solutes to maintain cell turgor, and

protection of cellular machinery under water stress. Moreover, some bacteria and fungi can form highly resistant spores that allow them to survive extreme dehydration [7].

One of the most drought-inflicted regions is the Cholistan Desert, which stretches across the southeastern region of Pakistan, bordering the Thar Desert of India. High temperatures exceeding 50°C during summer, a rainfall of less than 200mm per year, and recurrent droughts are characteristics of the Cholistan Desert [8]. Still, agriculture is one of the main sources of income in the Cholistan area, whereby local people depend on animal husbandry and rainfed farming [9]. The Cholistan Desert also harbors a diverse array of plant species that have evolved to thrive in this arid environment. The desert is rich in plant species, such as *Aerva persica*, *Sesuvium sesuvioides*, *Caralluma edulis*, and *Haloxylon recurvum* [10]. However, drought stress is becoming a major constraint for these plants; thus, drought tolerance has indeed become a priority.

There is scarce literature available on the Cholistan Desert microbial diversity. However, the current study reported diverse and comparatively dense Cholistan microbiota [11]. This region is reported to host several epiphytic bacteria with some features of osmotic stress tolerance and PGP [6]. By investigating deeper into this region, researchers may engage a 'gold mine' of unknown microbes with drought tolerance mechanisms [12]. Although specific studies on the drought adaptation strategies of Cholistan Desert microbes are limited, research on similar arid environments suggests potential mechanisms. Literature review shows plants belonging to arid ecosystems, such as the Cholistan desert contain diverse

beneficial microbes that are advantageous to plant health and stress tolerance [6].

While the role of rhizosphere and endophytic microbes in plant drought tolerance has been relatively well-studied, epiphytes remain an under-explored frontier [13,14]. This study, however, aimed to evaluate the drought tolerance of epiphytic bacteria isolated from the plant *Aerva javanica* of the Cholistan Desert. Previous studies regarding the epiphytic bacterial diversity of desert plants, such as *Prosopis juliflora* have been conducted but not on *Aerva javanica* [15]. The usage of epiphytic bacteria from other desert plants has been proven beneficial in increasing crop productivity under stress [16]. Improvement of drought tolerance in maize is important to increase yield and food production for the world's growing population, especially for the arid and semi-arid areas that are affected by climate change. The purpose of this research was to identify and characterize epiphytic bacterial strains of *Aerva Javanica* capable of tolerating PEG-6000-induced drought stress and having plant-growth-promoting characteristics. Moreover, the study also evaluated the potential of these strains to improve physiological and biochemical responses of *Zea mays* seedlings under PEG-associated osmotic stress.

2. METHODOLOGY

2.1. Isolation and Characterization of Epiphytic Bacterial Strains

A plant sample of *Aerva javanica* was aseptically collected near the Chanan Peer region, Latitude (28.97696° or 28° 58' 37" north), Longitude (71.6657° or 71° 39' 57" east) of the Cholistan Desert, Bahawalpur, Pakistan. This plant sample was put in aseptic plastic bags in cold containers and transported to Punjab University, Lahore, within 24 hrs of time. Then, dilutions of 1g

of the root and leaf samples were made and spread on LB and EMB agar plates. After incubation, a diversity of epiphytic bacterial strains appeared on both EMB and LB agar plates. From these epiphytic bacteria, 27 strains were purified for future screening. Colony morphology of 27 selected epiphytic bacterial strains was determined by assessing different parameters of colonies, such as shape, size, margins, elevation, and colour. Cell morphology of purified epiphytic bacterial strains was determined using gram staining and spore staining. All the bacterial strains were biochemically characterized based on catalase, oxidase, starch hydrolysis, MR, VP, and citrate tests [17].

2.2. Anti-bacterial Activity Evaluation of Epiphytic Bacterial Strains

The antibacterial activity of 27 selected epiphytic bacterial strains was assessed using the well-diffusion method against *Bacillus sp.* (KC881030) and *E. coli* (ATCC259223) [18]. The 24-hour old cultures of *Bacillus* and *E. coli* were swabbed and spread onto the surface of the agar plates. 10 µg/ml of Ampicillin was used as the positive control, and simple broth was used as the negative control.

2.3. PGP Characterization of Epiphytic Bacterial Strains

Assessment of plant growth-promoting characteristics of 27 selected epiphytic bacterial strains was done by conducting the following tests:

2.3.1. Phosphate Solubilization.

Phosphate solubilizing ability was measured as described in an earlier study [19]. A total of 27 selected epiphytic bacterial strains were grown on Pikovskaya's agar and incubated at 28°C for a week. After the incubation period, the plates were observed for the presence of

clear halos around the bacterial colonies.

2.3.2. HCN Production. For the HCN production test [20], 27 bacterial strains were streaked on the LB agar plates supplemented with 4.4 g/L glycine. Then, the filter paper dipped in the solution of 5% picric acid and 2% sodium carbonate was placed over the lids and incubated for five days at 37°C. The appearance of a reddish-brown colour was checked for HCN production.

2.3.3. Ammonia Production. Selected epiphytic bacterial strains were incubated in test tubes containing 5 mL peptone broth and incubated at 37°C for 48 hours. Subsequently, after the incubation period, to each tube, 0.5 ml of Nessler's reagent was added. Yellow to brown coloration associated with the production of ammonia was assessed [21].

2.3.4. IAA Production. The production of indole-3-acetic acid (IAA) by selected bacterial strains was determined [22]. A total of 27 strains were inoculated onto a medium with 0.5% L-tryptophan. After incubation for 24 hours at 37°C, 0.2ml of Salkowski's reagent was added to the medium. The colour change to pink was observed as a positive indicator of IAA production.

2.3.5. Salinity Stress Tolerance. LB agar medium containing different concentrations of NaCl (1%, 4%, 7%) was prepared and poured into Petri plates following a modified version of the procedure used in a previous study [23]. Then, each bacterial strain was streaked on each concentration and incubated for 48 hours at 37°C. After 48 hrs, the growth of the strains was observed.

2.4. Screening for Drought Tolerance Potential

The drought tolerance potential of the

selected epiphytic bacterial strains was assessed using a modified version of the procedure used in a previous study [24]. Before the experiment, PEG-6000 was sterilized using an autoclaved Swinix filter assembly. LB broth test tubes were prepared with varying concentrations of PEG-6000, including 5% PEG, 10% PEG, 15% PEG, and without PEG. The bacterial strains were inoculated into each of these test tubes, and the optical densities were taken at 0hrs, 24hrs, and 48hrs of incubation at 600nm. Then, the growth curve of bacterial strains was studied.

Six epiphytic bacterial strains with the best-observed characteristics were chosen for further testing (Supplementary Table 3).

2.5. Antibiotic Resistance Profiling of Epiphytic Bacterial Strains

The disc diffusion method was used in accordance with a modified version, as conducted in a previous study [25]. Firstly, a Petri plate containing MH agar was prepared and then spread with bacterial suspension. Five antibiotic discs: Rifampicin (5 µg), Neomycin (30 µg), Streptomycin (10 µg), Chloramphenicol (30 µg), and Gentamicin (10 µg) were placed on the agar surface. The plates were then incubated at 37 °C overnight. After incubation, the diameter of the inhibition zones around each of the antibiotic discs was measured in millimetres.

2.6. Heavy Metal Resistance Profiling of Epiphytic Bacterial Strains

Heavy metal resistance was determined per an altered version as used in a previous study [25]. Six selected epiphytic bacterial LB agar plates were prepared with the addition of heavy metal salts: NiCl₂, PbCl₂, ZnCl₂, CuSO₄, and HgSO₄ at 250 µg/ml, 500 µg/ml, and 1000 µg/ml. Then, each bacterial strain was

streaked on the plates using an inoculating loop. The plates were then incubated at 37 °C for the next 24 hours.

2.7. EPS Production by Epiphytic Bacterial Strains

The evaluation of exo-polysaccharides production by six selected epiphytic bacteria involved growing the strains in LB broth with and without 15% polyethylene glycol (PEG) for seven days. After the incubation period, the cultures were centrifuged at 6000 rpm for 12 minutes to obtain the supernatant. The supernatant from each culture was mixed with chilled ethanol in a 1:2 ratio and incubated overnight at 4°C. The next day, the supernatant and ethanol solution were transferred into Falcon tubes and centrifuged again to sediment the EPS Pellets. The precipitate was sedimented at the bottom of the Falcon tubes, and the supernatant was discarded. The amount of EPS produced by each strain was calculated [26].

2.8. Plant-microbe Interaction Study (PMI)

A total of 180 corn seeds of *Triticum aestivum* were surface-sterilised by washing in a 0.1% mercuric chloride (HgCl₂) solution for 10-15 minutes. Subsequently, the seeds were washed gently in sterile-distilled water three times [27]. Four treatment groups were established. Control group W = treated only with distilled water. The Control Group P = treated with 15% PEG. The experimental group I = treated with six strains of bacteria, including LE2, RE1, LL2, RL1, RL3, and RL7, grown in a simple broth. Experimental group II = treated with the same six bacterial strains plus 15% PEG solution. The seeds were kept in each of these solutions for 30-minutes. The treated seeds were sown in 18 pots, each filled with

fertile soil. The pots were divided into four groups corresponding to the treatment conditions: three pots for PEG-treated seeds, three for water-treated seeds, six for bacterial-treated seeds, and six for bacterial plus PEG-treated seeds. Equal amount of water was provided to all pots to ensure consistent moisture levels. The harvesting was done after 30 days of growth.

2.9. Estimation of Plant Biochemical Parameters

2.9.1. Chlorophyll Content. The amount of chlorophyll in the leaves of plants was estimated in accordance with a modified procedure as used in a previous study [28]. Leaf samples from each treatment group were weighed and then crushed with 80% acetone. The mixture was filtered out, and the extract volume was made up to 10ml. The absorbance of the extract was measured at 645 nm and 663 nm using a spectrophotometer. Then, the chlorophyll content was calculated using the following formula:

$$\frac{8.2 \times \text{Absorbance at 663nm} + 20.2 \times \text{Absorbance at 645nm}}{\text{Dry weight of leaves}}$$

2.9.2. Relative Water Content (RWC). RWC was determined following a modified version used in a previous study [29]. Fresh leaf samples were collected and weighed to obtain the fresh weight (FW). The leaves were soaked in distilled water for 4 hours and the turgid weight was recorded. The leaves were then dried in an oven at 80°C overnight, and the dry weight (DW) was recorded. The RWC was then calculated using the following formula:

$$\frac{\text{FW} - \text{DW}}{\text{TW} - \text{DW}}$$

2.9.3. Soluble Protein Content. Fresh leaf samples (1 g) were crushed with 4 ml of phosphate buffer (1:4 ratio) following the modified version of the procedure used

in previous research [30]. The homogenate was centrifuged at 14,000 rpm for 10 minutes. 0.1 ml of the supernatant was transferred to test tubes. 2 ml of Folin's reagent mixture was added to each test tube and incubated for 15 minutes. 0.2 ml of Folin-Ciocalteu's phenol reagent was added and incubated for another 15 minutes. The absorbance was measured at 750 nm to determine the protein content.

2.9.4. Auxin Content. About 2 ml of ethyl ether was added to 1g of plant leaf sample from each treatment group in a test tube following the modified procedure of auxin estimation used in a previous study [31]. The samples were incubated at 4°C for one day. Then, the ethyl ether layer was transferred to another test tube, and 2 ml of sodium bicarbonate solution was added to it and shaken vigorously. The plant material was extracted again by the addition of 1 ml of ether, and the supernatant thereby obtained was transferred to another test tube. To each test tube, 1 ml of 5% NaHCO₃ solution was added, and the contents of each test tube were mixed well. The bicarbonate layer was then mixed with 6 N HCl until the pH reached 3. Then, 1 ml of ethyl ether was pipetted into each test tube, and the inorganic layer was discarded. 2 ml of Salkowski's reagent was added to the organic layer. 1 ml of ethyl ether was mixed with 2 ml of Salkowski's reagent to prepare a blank. After the preparation of the test tubes, they were left for 30 minutes in the dark for the colour to develop. The OD was taken at 535 nm. Levels of auxin were estimated with the aid of a standard curve obtained from known concentrations of auxin.

2.10. FTIR Analysis of Epiphytic Bacterial Strains

The two epiphytic bacterial strains considered best PGP and drought-tolerant,

LE2 and RE1, were grown with/without PEG for 24h. Then, after centrifugation at 6000 rpm for 10 minutes, a big pellet was obtained, the supernatant was discarded, pellet was taken to a reliable laboratory that had an FTIR analysis facility in accordance with a slightly modified procedure used in a previous study [32].

2.11. Identification of Epiphytic Bacteria by 16S Ribotyping

LE2 and RE1 were cultured and forwarded to a professional molecular biology laboratory for 16S rRNA gene sequencing. Subsequently, the obtained sequences were aligned with the homologous reference sequences with the help of the PMIC database using the BLAST tool incorporated into it.

3. RESULTS

3.1. Isolation and Characterization of Epiphytic Bacterial Strains

In total, 27 colonies of epiphytic bacteria were purified using the streak plate method. A total of 26% of the epiphytic bacterial colonies were large, 33% were medium, and 41% were small in size. A total of 15% of the epiphytic bacterial isolates were Gram-ve and 85% were Gram+ve. In addition, 7% of the epiphytic bacterial isolates were Cocci and 93% were rods. Out of the rods, 2 of the strains formed chains. The observation of spore staining slides under a microscope revealed that out of 27 epiphytic bacterial strains, 56% of the isolates were spore formers, and 44% of the epiphytic bacterial isolates were non-spore formers. All of the epiphytic bacterial strains were catalase-positive. As for the presence of oxidase enzymes, about 52% of the epiphytic bacteria were oxidase positive, and 48% showed negative results. Moreover, about 19% of the epiphytic bacterial strains were non-starch

hydrolysers, and the remaining 81% were positive for starch hydrolysis. The results of the MR test showed that all of the epiphytic bacterial strains were negative for the MR test. Furthermore, it was observed that 81% of the bacterial isolates were negative for the VP test, and 29% showed positive results. A positive result for citrate utilization was shown by 74% of the strains. While, 26% of the strains were citrate-negative.

3.2. Anti-bacterial Activity of Epiphytic Bacterial Strains

Approximately, 67% of the strains showed antimicrobial activity against *Bacillus*, while 33% of the strains showed no anti-microbial activity against *Bacillus*. However, only 22% of the epiphytic bacterial strains showed antimicrobial activity against *E. coli*, and 88% showed no zone of inhibition, hence no anti-microbial activity against *E. coli* was observed (Supplementary Table 1).

3.3. PGP Characterization

All the epiphytic bacterial strains showed no phosphate solubilizing ability; hence, no halo zones were observed around them. As for HCN production, only two strains, RE1 and LL2, showed positive results, making 7% of the results positive. However, the other 25 strains (93%) showed no change in filter paper colour and hence were negative for HCN production. Only 4 strains (15%), namely RL3, RL7, LE2, and RE1, showed a positive reaction on the addition of Nessler's reagent. The remaining 23 strains (85%) showed no change in colour and hence were non-producers of Ammonia. Only 2 strains, RL1 and RE1, showed positive reactions, producing a pink colour on the addition of Salkowski's reagent for IAA production. The other 25 strains showed no change in colour on the addition of the reagent and

hence were rendered non-producers of IAA (Supplementary Table 2). It was also observed that osmotic stress did not reduce the growth rate of these bacterial isolates. All of the epiphytic bacterial isolates showed positive results for salinity stress tolerance.

3.4. Drought Tolerance Potential

It was observed that all of the strains showed significant growth under PEG-induced drought stress (Figure1). At 0 hours, there was no significant difference in the growth of epiphytic bacterial isolates. However, when given time to grow at 24hrs, the bacteria showed more growth under stress conditions, specifically at 10% PEG. Moreover, at 48 hours of incubation, a further increase in growth was observed, and RE1, LL2, RL1, LE2, RL3, and RL7 showed more growth under PEG-15% than all other strains. However, overall, all the strains showed peak growth under PEG-10% even after 48 hours of incubation time. Six epiphytic bacterial strains, LE2, RE1, LL2, RL1, RL3, and RL7, were selected based on their positive characteristics for PGP, PEG at 15%, antibacterial activity, and osmotic stress tolerance. Six strains were selected based on these characteristics (Supplementary Table 3).

3.5. Antibiotic and Heavy Metal Resistance

It was observed that all six strains were susceptible to the used antibiotics except for RE1, which was resistant to Rifampicin only. LE2 also showed a zone of inhibition only against Streptomycin (Supplementary Table 4). Other strains were inhibited by all the antibiotics. As for heavy metal resistance, almost all the strains exhibited significant resistance to heavy metals.

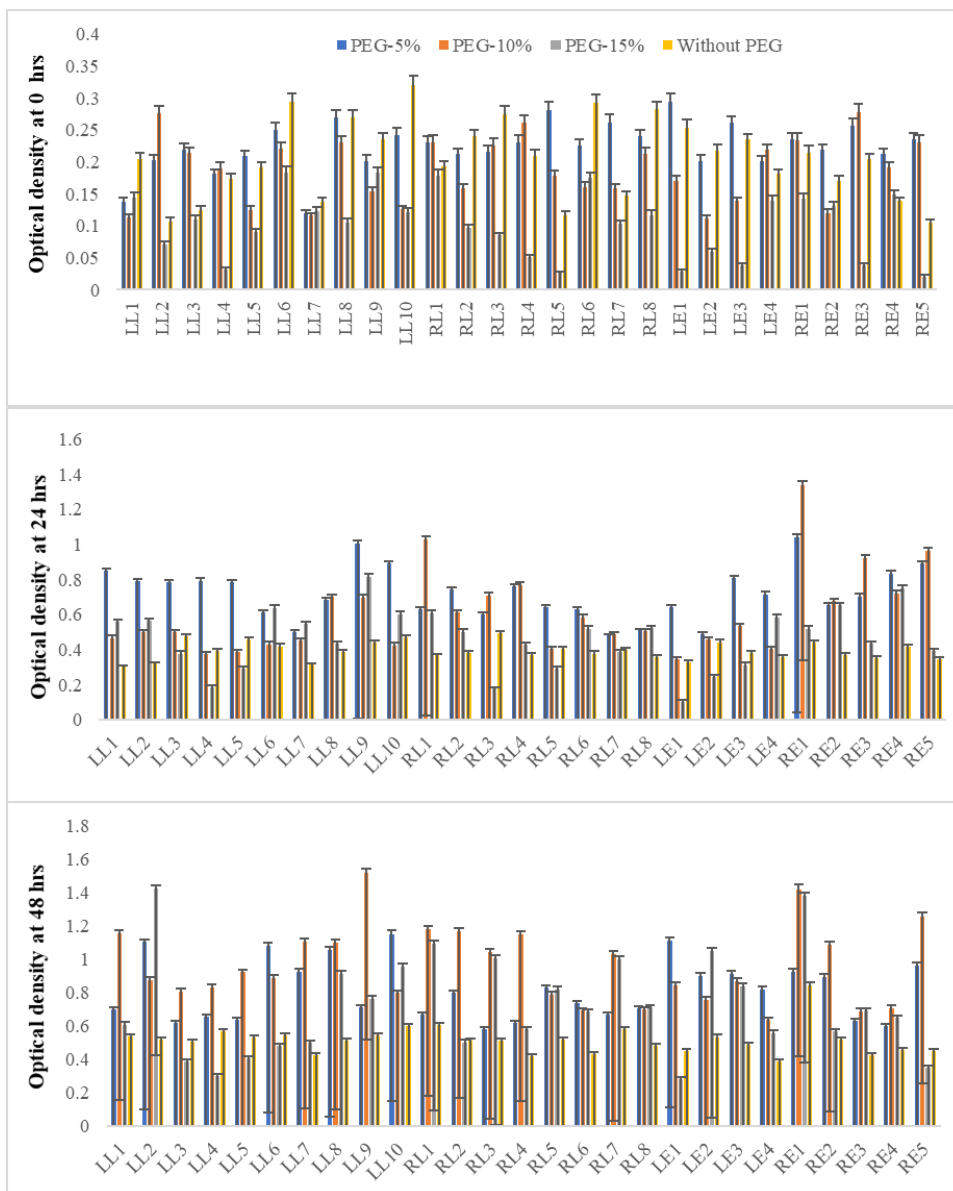


Figure 1. Growth of Epiphytic Bacterial Strains under Different Concentrations of PEG at 0hrs, 24hrs, and 48hrs

- RE1 (Resistant to NiCl₂ at 250 mg/ml, PbCl₂ at 250 and 500 mg/ml, ZnCl₂ and CuSO₄ at all concentrations, HgSO₄ at 250 and 500 mg/ml)
- LE2 (Resistant to PbCl₂ at 250 and 500 mg/ml, ZnCl₂ and CuSO₄ at all concentrations, HgSO₄ at 250 and 500 mg/ml)

- RL7 (Resistant to PbCl₂ at all concentrations, ZnCl₂ at 250 and 500 mg/ml, CuSO₄ and HgSO₄ at 250 and 500 mg/ml)
- RL3 (Resistant to NiCl₂ at 250 mg/ml, PbCl₂ at 250 mg/ml, ZnCl₂ at all concentrations, CuSO₄ at 250 and 500 mg/ml, HgSO₄ at 250 and 500 mg/ml)
- LL2 (Resistant to NiCl₂ at 250 mg/ml, PbCl₂ at 250 and 500 mg/ml, ZnCl₂ at all concentrations, CuSO₄ at 250 and 500 mg/ml, HgSO₄ at 250 mg/ml)
- RL1 (Resistant to NiCl₂ at 250 mg/ml, ZnCl₂ and CuSO₄ at 250 mg/ml, HgSO₄ at 250 mg/ml)

The order of most resistant epiphytic bacterial isolates to least resistant is as follows:

RE1 > LE2 > RL7 > RL3 > LL2 > RL1

3.6. Exo-Polysaccharide Production

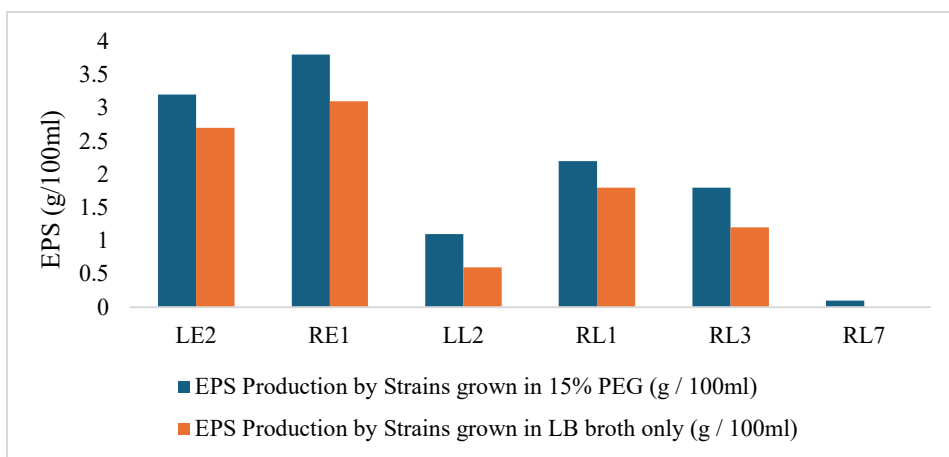


Figure 2. EPS Production by Six Selected Epiphytic Strains with and Without Stress.

It was observed that almost all the strains grown with PEG produced higher EPS than the strains grown simply in LB broth. Only one strain, RL7, did not show any variation in EPS production with or without PEG (Figure2).

3.7. Plant-microbial Interaction Study

Treatment group II (bacteria + PEG treated seeds) (LE2P, RE1P, LL2P, RL1P, RL3P, and RL7P) showed a higher percentage germination, a greater number of leaves, more root length, more shoot length, and higher seedling length overall. However, Control Group P demonstrated

the lowest percentage of germination, number of leaves, shoot length, root length, and seedling length than Control Group W. Moreover, among treatment group I, it was observed that LE2, RE1, and RL3 showed a higher percentage of germination than other epiphytic bacterial isolates. LE2 and RE1 strains had a greater number of roots than other epiphytic bacteria. However, treatment groups LE2P and RE1P showed more roots than the LE2 and RE1 treatment groups. LE2 and RE1 enhanced root length and the length was further increased when they were combined with PEG, that is, LE2P and RE1P. Seedlings treated with

LE2 and RE1 had a length and it further increased when it was combined with PEG, which is LE2P and RE1P (Supplementary Table 5).

3.8. Plant Biochemical Parameters

3.8.1. Chlorophyll Content.

Treatment Group II (LE2P, RE1P, LL2P, RL1P, RL3P, and RL7P) developed more chlorophyll than the Control Groups (W and P) and less than Treatment Group I (LE2, RE1, LL2, RL1, RL3, and RL7). Furthermore, the chlorophyll content was lower in Control Group P than in Control Group W. Among the bacterial isolates, both LE2 and RE1 enhanced the chlorophyll content of the seedlings, and it was further enhanced when combined with PEG LE2P, RE1P, respectively (Supplementary Table 6).

3.8.2. Relative Water Content (RWC). Treatment Group II (LE2P, RE1P, LL2P, RL1P, RL3P, and RL7P) had more RWC than the Control Groups (W and P) and Treatment Group I (LE2, RE1, LL2, RL1, RL3, and RL7). Moreover, the Control Group P had a lower RWC than the

Control Group W. Among epiphytic bacterial isolates, LE2 and RE1-treated seedlings had higher RWC, which even increased when they were combined with PEG, that is, LE2P and RE1P (Supplementary Table 6).

3.8.3. Soluble Protein Content.

However, there was a general increase in the soluble protein content when moved from Treatment Group I (LE2, RE1, LL2, RL1, RL3, and RL7) to Treatment Group II (LE2P, RE1P, LL2P, RL1P, RL3P, and RL7P) (Supplementary Table 6).

3.8.4. Auxin Content. Treatment Group II (LE2P, RE1P, LL2P, RL1P, RL3P, and RL7P) had more auxin content than the Control Groups (W and P) and Treatment Group I (LE2, RE1, LL2, RL1, RL3, and RL7). Moreover, the Control Group P had less auxin content than the Control Group W. Among epiphytic bacterial isolates, LE2 and RE1-treated seedlings had more auxin content, which even increased when they were combined with PEG, that is, LE2P and RE1P (Supplementary Table 6).

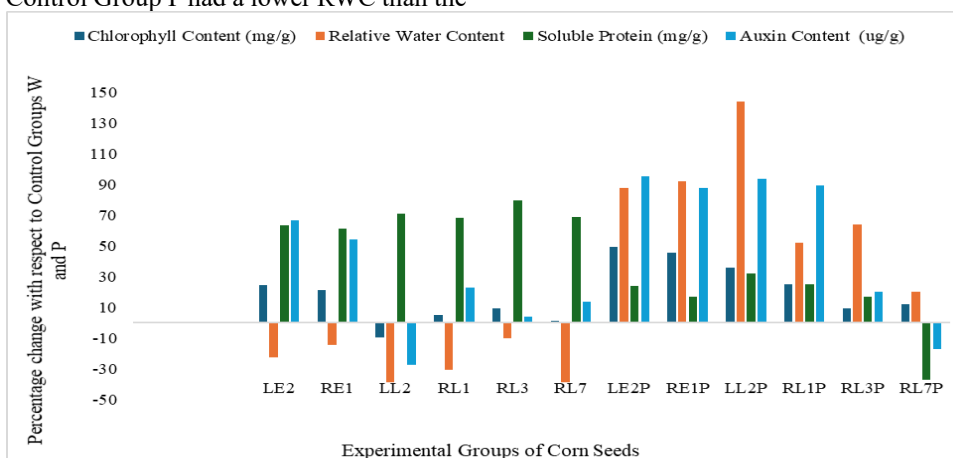


Figure 3. Percentage Change of Plant Biochemical Characters of Experimental Groups (RE1, LE2, LL2, RL1, RL3, RL7 and RE1P, LE2P, LL2P, RL1P, RL3P, RL7P) w.r.t the Control Groups W and P.

3.9. FTIR Analysis of Epiphytic Bacterial Strains

The strains LE2 and RE1 that significantly contributed to PGP with and without PEG were selected for FTIR analysis. FTIR analysis was done with and without PEG to compare the effect of PEG on these bacterial strains. FTIR analysis of RE2 and RE2P showed variable trends of % transmittance (Figure 4). At some intervals of wavelength, the transmittance was the same, and at various intervals, the transmittance of RE2P was less. At the

wavelength/cm of 875, 1080, 1400-1460, 3250, and 3350, the transmittance of RE1P was less at functional groups C=O inorganic carbonate, CO carbohydrate, C=O inorganic carbonate, NH₂ amino acidic group, and OH carbohydrates proteins and polyphenols, respectively. However, at the wavelength/cm of 1510, 1540, and 2850-2950, the transmittance of RE1 was lower for functional groups, polyphenol skeletal aromatic, CN amide II band, and CH and CH₂ stretching aliphatic groups.

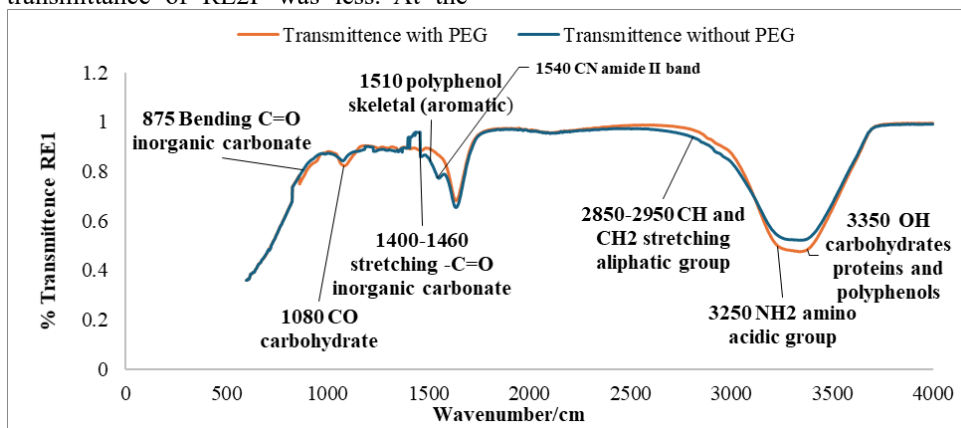


Figure 4. Comparison of the FTIR Spectra of RE1 (*Enterobacter cloacae*) with and without stress

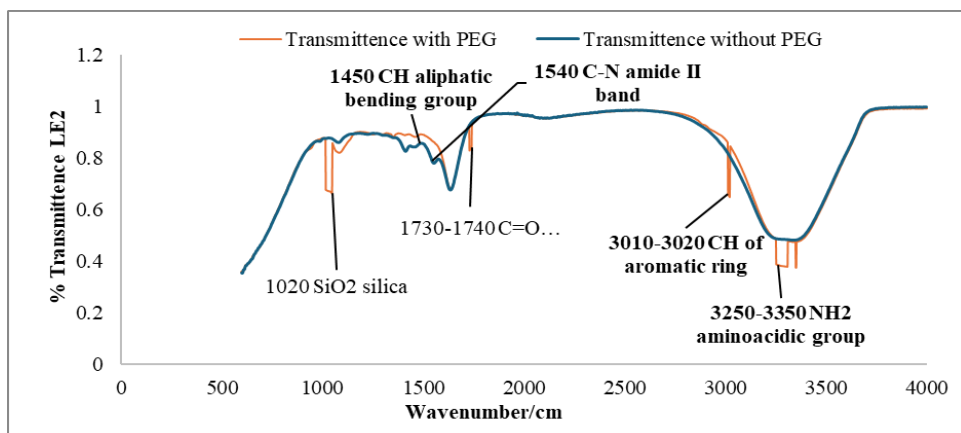


Figure 5. Comparison of the FTIR Spectra of LE2 (*Bacillus rugosus*) with and without stress

Moreover, the FTIR analysis of LE2 and LE2P showed that the overall transmittance of LE2 without PEG was more than that of LE2P, which had PEG (Figure 5). Moreover, at certain intervals of wavelength, an obvious deviation was shown in transmittance (%) values, making the transmittance of LE2P very low. It was observed that the deviation in transmittance (%) was at functional groups SiO₂ silica, CH aliphatic bending group, CN amide II band, C=O ester fatty acid group, CH of aromatic ring, and NH₂ amino acidic group at wavenumber/cm 1020,1450,1540, 1730-1740, 3010-3020, and 3250-3350, respectively.

3.10. 16S Ribotyping of Epiphytic Bacterial Strains

The 2 selected strains, LE2 and RE1, were sent for 16S ribotyping. The results of 16S ribotyping showed that RE1 was *Enterobacter cloacae* (Supplementary Figure1) and LE2 was *Bacillus rugosus* (Supplementary Figure2). In Blast analysis, 99% similarity was identified with *Enterobacter cloacae* under the accession number MT186255.1. The phylogenetic tree of the LE2 query indicated that it belonged to the *Bacillus* genus and had the closest phylogenetic relationship with *Bacillus rugosus*. In Blast analysis, 97% similarity was found with *Bacillus rugosus* under the accession number PP556211.1 (Supplementary Table 7).

4. DISCUSSION

The isolation of epiphytic bacterial strains was done using leaf and root samples of *Aerva javanica* (Desert Cotton), as these parts of plants, such as roots and leaves, are excellent reservoirs of epiphytic bacterial species [33]. Most of the epiphytic bacterial isolates were rods, as rod-shaped bacteria have a larger surface area to volume ratio for efficient nutrient uptake,

specifically in harsh environments, such as the Cholistan Desert [34]. More than half of the rods were spore formers, as the spores help them survive harsh environmental conditions, such as extreme heat or lack of nutrients [35].

All the epiphytic bacterial strains were catalase positive, which corresponds to the fact that catalase protects bacteria from oxidative damage by breaking down hydrogen peroxide, aiding their survival in extreme conditions [36]. More than half of the epiphytic bacterial strains had oxidase enzymes [37]. Oxidase enzymes in bacteria play a crucial role in the electron transport chain for energy production and help manage oxidative stress. It was found that about 81% of the epiphytic bacterial strains were positive for starch hydrolysis. Earlier research shows that starch-hydrolysing enzymes, such as amylases and glucoamylases are commonly found in extremophilic archaea and bacteria. These enzymes remain active in extreme temperature and pH conditions without denaturation [38]. About 74% of the epiphytic bacterial strains have shown citrate utilisation as citric acid metabolism produces intermediates, such as oxaloacetate and pyruvate which help in PH and ion balance, along with energy production [39].

Anti-bacterial activity was also exhibited by the selected epiphytic bacterial strains (Supplementary Table 1), as previous research using bacteria isolated from the desert of Saudi Arabia also reported the anti-bacterial activity exhibited by those strains [40]. The overall PGP potential of selected epiphytic bacterial strains was observed in six strains, LE2, RE1, LL2, RL1, RL3, and RL7, making 16% of the total strains beneficial for PGP (Supplementary Table 2). A similar study also found PGP strains while

investigating the epiphytic bacteria isolated from the desert of Saudi Arabia [16].

These bacteria, being the inhabitants of the Cholistan Desert, showed enhanced growth in water-scarce conditions (Figure 1). These results suggested that, like other xero-tolerant bacteria in deserts, these strains also utilize dormancy, sporulation, EPS secretion, and compatible solute accumulation to tolerate drought [41], ensuring DNA and protein stability under low water activity. Moreover, the tolerance of PEG-6000 is also reported by other studies investigating the drought-tolerant bacterial strains isolated from harsh soils. After confirming the drought tolerance potential of selected epiphytic bacterial strains, six strains (LE2, RE1, LL2, RL1, RL3, RL7) were further shortlisted based on positive PGP characters and highest absorbance values (LE2=1.053, RE1=1.380, LL2=1.423, RL1=1.094, RL3=1.009, RL7=1.003) at 15% PEG after 48hrs of incubation (Supplementary Table 3). The 15% PEG-6000 concentration was chosen for further screening as it was the highest concentration tested at which measurable growth was observed by the selected bacterial isolates. This created a relatively harsh osmotic-stress environment to distinguish drought-tolerant isolates. Research suggests that bacteria produce exopolysaccharides as a mechanism for drought tolerance [42].

Antibiotic resistance was shown by RE1 and LE2 against Rifampicin and Streptomycin, respectively (Supplementary Table 4), and heavy metal resistance was shown by almost all the strains. Some other studies also suggest the heavy metal resistance of PGP bacteria and the use of these bacteria to enhance the growth of plants [43]. The plant-microbe interaction study showed that the efficiency of six epiphytic bacterial strains (LE2, RE1, LL2,

RL1, RL3, and RL7) improved drought stress in *Zea mays* (Supplementary Table 5). As these six selected epiphytic bacterial strains showed positive PGP and PEG enhanced their growth, when the seedlings were treated with bacteria along with PEG, their growth increased. The findings of this study imply that the combination of bacterial inoculation and PEG treatment can improve drought tolerance. This accredits similar studies that show how the use of beneficial bacteria can enhance plant stress tolerance [44].

Testing of plant biochemical parameters (chlorophyll content, relative water content, soluble protein, and auxin content) showed an overall enhanced photosynthetic efficiency in seedlings treated with Treatment Groups LE2P and RE1P (Supplementary Table 6). These exhibited the highest chlorophyll content, indirectly found to increase the rate of photosynthesis. The peak in auxin levels in the LE2P-treated plants gives credence to the excellent growth-promoting activity, as supported by other research on auxin, which underpins drought tolerance [45].

In the FTIR analysis of RE2 and RE2P, the intervals where the transmittance of RE2P is less indicate the fact that the absorbance of RE2P is greater; hence, RE2 grows more under stressful conditions. The deviation in the transmittance (%) values of RE2 and RE2P is due to the attachment of PEG with respective functional groups at those intervals (Figure 4). Moreover, the FTIR analysis of LE2 and LE2P showed that the overall transmittance (%) of LE2 without PEG was greater than with PEG (LE2P). This means that the attachment of PEG with respective functional groups increased the absorbance of LE2, increasing its growth under drought stress (Figure 4). According to this research, the RE1 and LE2 strains showed drought

tolerance potential as well as plant growth promotion characteristics. However, similar studies have reported the PGP characteristics of (RE1) *Enterobacter cloacae* [46]. However, studies on PGP and drought tolerance potential of (LE2) *Bacillus rugosus* have not been found. However, similar studies for *Bacillus* species investigating their PGP characters have reported PGP-positive results for *Bacillus* species [47,48]. These bacterial strains have the potential for use in sustainable agricultural practices.

4.1. Conclusion

Water deficit conditions compromise global food security, particularly for crops, such as *Zea mays* (maize), a staple food. The exploration of modern and ecological practices, including the use of PGP bacteria, is especially important in combating the effects caused by a drought period. In a holistic method of biochemical analysis, growth curve study, PGP characterization, antibiotic and heavy metal resistance profiling, exopolysaccharide synthesis, and plant-microbe interaction study, this research brought out innovative information on the process of bacterially-induced drought tolerance. Moreover, this research explored those epiphytic bacteria isolated from drought-inflicted regions, such as the Cholistan Desert, have drought-tolerance potential. It has been discovered that the two identified strains, *Bacillus rugosus* and *Enterobacter cloacae*, can act as plant growth promoters, especially in water-deficient conditions. Moreover, these have the potential to improve growth parameters, physiological responses, and biochemical characteristics of maize in the arid and semi-arid zones. Therefore, there is a need to explore the diversity and characteristics of bacteria from harsh environments, such as deserts, to harness their potential for the benefit of humankind.

Using such bacteria with drought tolerance and PGP characters may serve as a biological method for tackling drought stress in staple crops.

Author Contribution

Ayesha Faisal: investigation, data curation, formal analysis, validation, visualization, writing – original draft. **Nazia Jamil:** resources, project administration. **Rida Batool:** conceptualization, methodology, supervision, writing – review & editing.

Conflict of Interest

The authors have no financial or non-financial conflict of interest in the subject matter or materials discussed in the manuscript.

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The data supporting the findings of this study is available within the manuscript and in the supplementary folder.

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Annexure

Supplementary Table 1. Anti-Bacterial Activity of Selected Epiphytic Bacterial Strains

Sr.no	Strain	Anti-bacterial Activity of Epiphytic Bacterial strains					
		Zone of Inhibition					
		<i>Bacillus</i>			<i>E. coli</i>		
		Ampicillin 10ug/ml	Control	Test Organism	Ampicillin 10ug/ml	Control	Test Organism
1.	LL1	0.3mm		0.3mm	0.2mm		
2.	LL2	0.3mm			0.2mm		0.3mm
3.	LL3	0.3mm			0.2mm		0.3mm
4.	LL4	0.3mm		0.9mm	0.2mm		0.4mm
5.	LL5	0.3mm			0.3mm		
6.	LL6	0.3mm		0.5mm	0.3mm		
7.	LL7	0.3mm		0.3mm	0.3mm		
8.	LL8	0.3mm		0.3mm	0.3mm		
9.	LL9	0.2mm			0.2mm		
10.	LL10	0.2mm		0.2mm	0.2mm		
11.	RL1	0.4mm			0.2mm		
12.	RL2	0.4mm		0.5mm	0.2mm		
13.	RL3	0.4mm		0.6mm	0.2mm		
14.	RL4	0.4mm			0.2mm		
15.	RL5	0.3mm		0.4mm	0.3mm		0.4mm
16.	RL6	0.3mm		0.4mm	0.3mm		0.3mm
17.	RL7	0.3mm		0.4mm	0.3mm		
18.	RL8	0.3mm		0.3mm	0.3mm		
19.	LE1	0.4mm		0.4mm	0.3mm		
20.	LE2	0.4mm		0.7mm	0.3mm		
21.	LE3	0.4mm		0.4mm	0.3mm		
22.	LE4	0.4mm		0.3mm	0.3mm		0.3mm
23.	RE1	0.4mm			0.3mm		
24.	RE2	0.4mm		0.5mm	0.3mm		
25.	RE3	0.4mm		0.3mm	0.3mm		
26.	RE4	0.2mm			0.2mm		
27.	RE5	0.2mm			0.2mm		

Supplementary Table 2. Plant Growth *Promotion Tests* of Selected Epiphytic Bacterial Strains

Sr.no.	Strain	Plant Growth Promotion Tests				
		Phosphate Solubilization	HCN Production	Ammonia Production	Indole Acetic Acid production	Protease activity
1.	LL1					
2.	LL2		+			

Plant Growth Promotion Tests						
Sr.no.	Strain	Phosphate Solubilization	HCN Production	Ammonia Production	Indole Acetic Acid production	Protease activity
3.	LL3	-	-	-	-	-
4.	LL4	-	-	-	-	-
5.	LL5	-	-	-	-	-
6.	LL6	-	-	-	-	-
7.	LL7	-	-	-	-	-
8.	LL8	-	-	-	-	-
9.	LL9	-	-	-	-	-
10.	LL10	-	-	-	-	-
11.	RL1	-	-	-	+	-
12.	RL2	-	-	-	-	-
13.	RL3	-	-	+	-	-
14.	RL4	-	-	-	-	-
15.	RL5	-	-	-	-	-
16.	RL6	-	-	-	-	-
17.	RL7	-	-	+	-	-
18.	RL8	-	-	-	-	-
19.	LE1	-	-	-	-	-
20.	LE2	-	-	+	-	-
21.	LE3	-	-	-	-	-
22.	LE4	-	-	-	-	-
23.	RE1	-	+	+	+	-
24.	RE2	-	-	-	-	-
25.	RE3	-	-	-	-	-
26.	RE4	-	-	-	-	-
27.	RE5	-	-	-	-	-

Keywords: + = *positive*, - = *negative*

Supplementary Table 3. Selection Criteria of The Six Selected Strains

Characteristics of Selected Bacterial Strains						
Strain	HCN Production	Ammonia Production	IAA Production	48h OD in 15% PEG	Antimicrobial Activity	
					<i>Bacillus</i>	<i>E. coli</i>
LE2	-	+	-	1.053	0.7mm	-
RE1	+	+	+	1.380	-	-
LL2	+	-	-	1.423	-	0.3mm
RL1	-	-	+	1.094	-	-
RL3	-	+	-	1.009	0.6mm	-
RL7	-	+	-	1.003	0.4mm	-

Supplementary Table 4. Antibiotic Resistance of Six Selected Epiphytic Bacterial Strains

Strain	Zone of Inhibition (mm)				
	Rifampicin	Neomycin	Streptomycin	Chloramphenicol	Gentamicin
	(5ug) R5	(30ug) N30	(10ug) S10	(30ug) C30	(10ug) CN10
LE2	13	19	1.7	25	22
RE1		15	18	20	20
LL2	14	18	21	19	22
RL1	12	14	14	25	11
RL3	16	18	25	28	24
RL7	17	20	12	26	25

Supplementary Table 5. Plant Growth Parameters of all Treatment Groups in Plant-Microbe Interaction Study

Sr.no.	Treatment Groups	Seed Treatments	Percentage Germination (%)	No. of leaves	Number of Roots	Root Length (Gusain et al.)	Shoot Length (Gusain et al.)	Seedling Length (Gusain et al.)
1.	Control Groups	W	90 ± 0.42	5±0.18	16±0.30	29.71±0.45	39.9±0.27	69.5±0.35
2.		P	80 ± 0.25	4±0.12	14±0.22	25.90±0.33	35.3±0.26	61.2±0.47
3.	Experiment Group 1	LE2	90 ± 0.34	5±0.24	15±0.41	29.9±0.14	31.7±0.29	61.7±0.39
4.		RE1	90 ± 0.48	6±0.11	16±0.37	39.9±0.19	35.6±0.50	74.9±0.42
5.		LL2	80 ± 0.26	4±0.31	14±0.10	24.8±0.44	26.2±0.17	51.1±0.28
6.		RL1	80 ± 0.13	4±0.36	12±0.45	23.1±0.23	43.9±0.32	67.0±0.14
7.		RL3	90 ± 0.17	4±0.21	12±0.49	25.6±0.12	33.02±0.39	58.7±0.3
8.		RL7	90 ± 0.33	4±0.50	13±0.25	16.8±0.18	20.3±0.22	37.1±0.47
9.		LE2P	100 ± 0.47	6±0.29	17±0.38	33.3±0.16	47.7±0.11	84.1±0.49
10.	Experiment Group 2	RE1P	100±0.41	7±0.13	18±0.27	42.1±0.22	38.9±0.48	81.0±0.40
11.		LL2P	80 ± 0.30	5±0.16	11±0.50	15.2±0.15	34.5±0.45	49.8±0.38
12.		RL1P	90 ± 0.19	5±0.43	12±0.20	25.4±0.28	37.1±0.12	62.5±0.33
13.		RL3P	100 ± 0.24	5±0.35	15±0.49	25.4±0.11	33.3±0.46	58.7±0.21
14.		RL7P	80 ± 0.38	6±0.42	14±0.34	24.9±0.12	30.5±0.23	55.4±0.19

The mean of triplicates ± Standard error of the mean.

Supplementary Table 6. Plant Biochemical Parameters of all Treatment Groups in Plant-Microbe Interaction Study

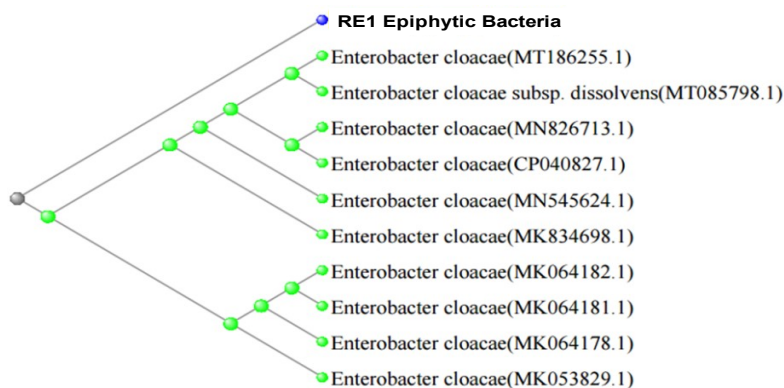
Sr.no.	Seed Treatments	Chlorophyll Content (mg/g)	Relative Water Content	Soluble Protein (mg/g)	Auxin Content (ug/g)
1.	Water	9.30±0.16	0.49±0.13	157.504±0.45	145±0.18
2.	PEG	8.70±0.34	0.25±0.27	236.256±0.30	123±0.24
3.	LE2	11.16±0.63	0.38±0.22	257.6±0.18	242±0.12
4.	RE1	11.26±0.44	0.42±0.49	254.28±0.32	224±0.38
5.	LL2	9.06±0.12	0.30±0.32	269.37±0.39	105±0.2
6.	RL1	9.77±0.11	0.34±0.12	264.96±0.17	178±0.19
7.	RL3	10.19±0.35	0.44±0.38	282.62±0.29	151±0.49
8.	RL7	9.42±0.24	0.30±0.25	265.67±0.47	165±0.32
9.	LE2P	13.0±0.45	0.47±0.16	292.56±0.11	240±0.46

Sr.no.	Seed Treatments	Chlorophyll Content (mg/g)	Relative Water Content	Soluble Protein (mg/g)	Auxin Content (ug/g)
10.	RE1P	12.67±0.22	0.48±0.41	276.37±0.13	231±0.17
11.	LL2P	11.84±0.31	0.61±0.27	312.43±0.28	238±0.44
12.	RL1P	10.89±0.15	0.38±0.19	294.78±0.46	233±0.50
13.	RL3P	9.53±0.10	0.41±0.16	276.74±0.21	148±0.24
14.	RL7P	9.74±0.37	0.30±0.25	147.94±0.34	102±0.47

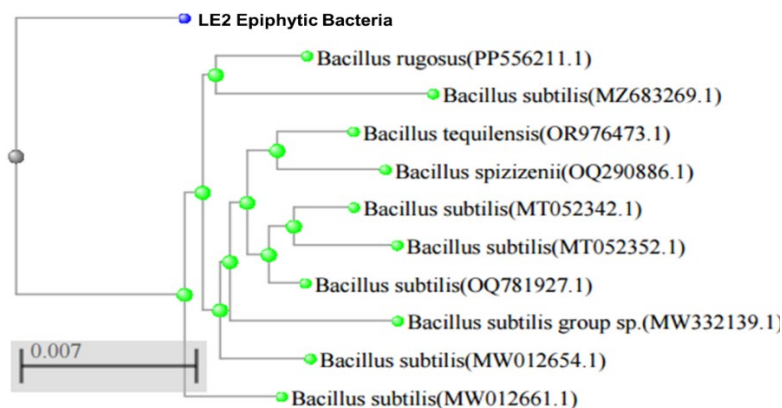
The Mean of Triplicates ± Standard Error

Supplementary Table 7. Summarised Results of 16S rRNA ribotyping of LE2 and RE1

Sr. no.	Strain	bps	Identity	Accession no.
1.	RE1	1214	<i>Enterobacter cloacae</i>	MT186255.1
2.	LE2	1230	<i>Bacillus rugosus</i>	PP556211.1



Supplementary Figure 1. Phylogenetic Tree of RE1 Epiphytic Bacterial Strain (Enterobacter Cloacae)



Supplementary Figure 2. Phylogenetic Tree of LE2 Epiphytic Bacterial Strain (Bacillus rugosus)