horticulturae

MDPI

Article

Exploring the Identity and Properties of Two *Bacilli* Strains and their Potential to Alleviate Drought and Heavy Metal Stress

Aruna Kumari Andy [1,2], Vishnu D. Rajput [3], Marina Burachevskaya [4] and Vinod Singh Gour [2,*]

[1] Directorate of Research, Sam Higginbottom University of Agriculture, Technology and Sciences, Allahabad 211007, India
[2] Amity Institute of Biotechnology, Amity University Rajasthan, Jaipur 303002, India
[3] Academy of Biology and Biotechnology, Southern Federal University, 344006 Rostov-on-Don, Russia
[4] Soil Chemistry and Ecology Laboratory, Faculty of Natural Sciences, Tula State Lev Tolstoy Pedagogical University, Lenin Avenue, 125, 300026 Tula, Russia
* Correspondence: vinodsingh2010@gmail.com

Abstract: Naturally available plant growth-promoting rhizobacteria (PGPR) have 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase enzymes, and are capable of processing the plant-borne ACC by converting it into α-ketobutyrate and ammonia. Thus, the PGPRs help in the depletion of ethylene levels, and enhance abiotic stress tolerance in plants. In the present study, two rhizobacterial strains, i.e., *Bacillus cereus* and *B. haynesii,* isolated from *Vigna mungo* and *Phaseolus vulgaris,* were used. These strains were taxonomically identified by 16S rDNA sequencing as *B. cereus* and *B. haynesii,* with NCBI accession numbers LC514122 and LC 514123, respectively. The phylogeny of these strains has also been worked out based on homology, with data available on NCBI GenBank. The strains were screened for their plant growth-promoting traits, and quantified in the same way. The enzymatic activity and molecular weight of the ACC deaminase obtained from both bacterial strains have also been determined. An in vitro drought tolerance study was done by using PEG 6000. These bacterial strains exhibited higher ACC deaminase activity (~5 to 6 µmol/mL), exopolysaccharide yield (15 to 18 mg/10 mL protein), and indole acetic acid (27–32 µg/mL). These characteristics indicate that the bacterial strains under present study may be helpful in enhancing the drought tolerance of the crops with enhanced yield. *Bacillus cereus* has been found to be a tolerant strain to As, Ba, and Ni, based on the plate assay method, and so it has the potential to be used as biofertilizer in fields affected by these metals.

Keywords: PGPR characterization; ACC deaminase; exopolysaccharides; molecular diversity

Citation: Andy, A.K.; Rajput, V.D.; Burachevskaya, M.; Gour, V.S. Exploring the Identity and Properties of Two *Bacilli* Strains and their Potential to Alleviate Drought and Heavy Metal Stress. *Horticulturae* **2023**, *9*, 46. https://doi.org/10.3390/horticulturae9010046

Academic Editors: Adalberto Benavides-Mendoza, Yolanda González-García, Fabián Pérez Labrada and Susana González-Morales

Received: 30 November 2022
Revised: 22 December 2022
Accepted: 23 December 2022
Published: 2 January 2023

1. Introduction

The human population keeps on increasing year by year, and the total population is expected to reach nine billion by 2050. This increase is accompanied by rapid urbanization and industrialization, which inevitably leads to environmental stress and damage. The consequences are higher evaporation and transpiration, which eventually cause global warming [1]. This leads to reduced agriculture land and soil fertility. To attain food security, agricultural production has been increased using synthetic fertilizers and chemicals at a very high rate, which has damaged soil health and, consequently, resulted into abiotic stress and ultimately decreased the quantity and quality of crops, considerably [2].

Meanwhile, the genetic potential of crop yield is hindered by natural abiotic stresses [3], among which drought is one of the worst constraints for agricultural production. The mechanism of drought tolerance has been explored in several crops, and a cluster of gene network has been identified to regulate the plant–water relationship [4]. Drought affects the physiology, morphology, and biochemistry of plants, and it also reduces soil fertility. These factors ultimately lead to a reduction in crop productivity, effecting global economics as well.

Salt tolerance in crops, which has been attained by genetic modifications to improve crop production, helps plants grow in saline conditions [5]. Studies indicate that micro-

organisms such as plant growth-promoting rhizobacteria (PGPR) have the potential to restore the degraded soil, and can enhance crop productivity even under diverse stress environmental conditions [6,7].

Micro-organisms are microscopic living creatures also found in diverse extreme conditions, and mainly include bacteria, cyanobacteria, algae, fungi, yeast, actinomycetes, and myxomycetes. Almost all existing natural materials are decomposed by these microbes. Such micro-organisms can transform naturally available organic matter into plant nutrients and make them freely available to the plants. Bacteria and mycorrhizal fungi present in agricultural fields help plants uptake water and minerals [2]. Many of these microbes protect the crops from various diseases and nutrient deficiency, and enrich the soil by acquiring enhanced available P and N. They can also increase the water retention capacity of soil, and thereby enhance soil fertility. PGPRs are known to be helpful for plants to grow even in abiotic stress conditions, as they have the potential to fix nitrogen, solubilize phosphorus, and sequester iron by siderophore production, and produce different phytohormones (auxins, gibberellins, cytokinins) [8]. Further, PGPRs are also able to produce ACC deaminase, which degrades 1-aminocyclopropane- 1-carboxylic acid (ACC) into α-ketobutyrate and ammonia, which inhibits ethylene production and ultimately help root growth, and thereby support plants to grow well even under stress conditions [9,10]. The association of PGPRs with soil helps in the formation of aggregates of soil particles, thereby increasing soil aeration.

Microbiological approaches have also attracted researchers, due to their potential to remove/sequester heavy metals and relieve plants from the pollution of these metals. Various biological approaches (bioleaching, bio-stimulation, bioventing, composting, bioaugmenting, land forming, and bioremediation) have been explored by soil scientists using microbes (single or in combination) to enhance soil health and increase yield. Such eco-friendly strategies received public acceptance at a large scale [11,12].

In several developing nations of the world, pulses and vegetables belonging to the leguminous family stand as the major sources of dietary proteins for millions of people, and play an important role in mitigating protein malnutrition [13], especially for the poor residing in parts of South Asia and sub-Saharan Africa; here, chickpea remains a most important source [14].

In the past decades, increased drought frequency and severity have been noticed in different parts of the world [15]. It is pertinent that we develop a strategy to make the crops drought-tolerant, or facilitate crop growth using biofertilizers to enhance yield, and thereby ensure food security. Chickpea is one of the major crops globally grown with other crops such as finger millet, and groundnut is branded as a forgotten crop, though it provides a good income to poor farmers. In chickpea production, South Asia contributes about 72%, while only 28% is contributed by the rest of the world [16].

The literature reveals that chickpea produces ethylene under drought and salt stress, which leads to a negative impact on plant growth. The impact of drought on chickpea production had reduced the yield by 33% globally. PGPRs could help chickpeas to tolerate the stress by secreting ACC deaminase [17], as noted earlier by Khan et al. (2019), where they identified that some PGPRs, such as *Bacillus subtilis*, *Bacillus thuringiensis*, and *Bacillus megaterium*, helped chickpeas to cope with drought stress [18].

The potential of our isolated rhizobacterial strains possessing PGPR capabilities were assessed. Out of 25 isolates, seven isolates were characterized for Gram's staining, biochemical characteristics [Methyl red (MR) test, Voges–Proskauer (VP) test, Catalase test], and PGPR potential (Phosphate solubilization, Siderophore production, Exopolysaccharide yield, ACC deaminase production, Nitrogen fixation) [19]. In the present manuscript, the investigation and results are described for two bacterial isolates, AV-7 from our previous study [19], and AV-12, a new strain which is reported for the first time.

Based on our present study, a new isolate AV-12 and a previously reported isolate AV-7 are further characterized based on biochemical assays such as IMViC tests (Indole test, Methyl red (MR) test, Voges–Proskauer (VP) test, catalase test). Our study also

includes other important biochemical characteristics such as the citrate utilization test, phosphate solubilisation, amylase hydrolysis, and carbohydrate fermentation test, where D-glucose, sucrose, and maltose are utilized. Then, these strains were studied for their PGPR (qualitative and quantitative) potentials, which mainly included IAA production, ammonia production, HCN production, siderophore production, and ACC deaminase. These bacterial strains have also been studied for exopolysaccharide production under normal and induced water-stress conditions. The bacterial strains have been taxonomically identified using their 16S rRNA sequence.

2. Materials and Methods

Soil samples were collected from rhizospheric zones of *Vigna mungo* and *Phaseolus vulgari*, grown in agriculture farm (cultivated with many varieties of crops including different legumes) of Sam Higginbottom University of Agriculture, Technology and Sciences (SHUATS), Allahabad with GPS location of 25°24′36″ N 81°51′11″ E. The soils were analyzed for their physical and chemical properties including the presence of heavy metals. Heavy metals were analyzed by Atomic Absorption Spectrophotometer [19].

The bacterial strains were studied for Gram staining (Figure 1), and preliminary qualitative biochemical tests were performed as described previously [19]. The experimental methodologies did not add in details here, as full details can be obtained from Andy et al. [19].

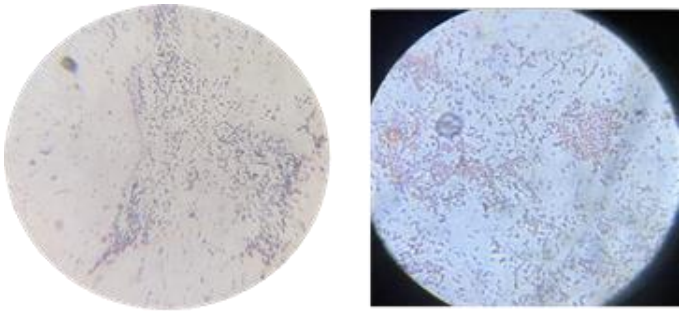

Figure 1. Slides observed under light microscope (100× oil immersion resolution) of two strains after Gram staining.

Biochemical characteristics

The bacterial isolates were characterized biochemically by various assays, namely indole test, methyl red test, Voges–Proskauer test, catalase test, Simmons citrate test, carbohydrate hydrolysis test, carbohydrate fermentation test [20], and amylase hydrolysis test [21].

Plant Growth Promoting Rhizobacterial traits evaluation

Phosphate solubilization efficiency of the bacterial isolates was studied by using Pikovskaya medium, which contained inorganic phosphate as main ingredient. The isolates were inoculated into the media and incubated at 30 °C for 24 h [22] (Figure S1). Then, the final OD was read at 600 nm, and the obtained values of cultured isolates were compared with the non-inoculated control values. The amount of phosphate released by the bacterial isolates was studied using the standard curve of potassium di-hydrogen phosphate as a source of P in the range of 10–100 mg/mL.

HCN production by the bacterial isolates was determined by adapting the method proposed by Lorck [23]. In this method, nutrient agar media was amended with glycine (4.4 g/L), and then the isolates were streaked in it. Then, the Petri plates were placed with Whatman filter paper, previously soaked in 2% sodium carbonate in picric acid (0.5%) solution. After sealing the plates with parafilm, these were incubated for 4 days at 28 °C. The filter paper changed from greenish-yellow to reddish-brown in the testing procedure and was

concluded to be HCN-positive (Figure S1). The concentration of HCN was determined by spectrophotometric measurements at 424 nm, with standard curve of KCN.

Indole-3-acetic Acid (IAA) production by bacterial isolate has been estimated in nutrient broth containing 0.5 mg/mL L-tryphtophan. The cultures were incubated for 24 h at 30 °C. After incubation, when 1 mL of bacterial culture was mixed with 2 mL of Salkowski's reagent (7.5 mL 0.5 M$FeCl_3 \cdot 6H_2O$, 150 mL concentrated H_2SO_4, 250 mL distilled water), it resulted in appearance of reddish-orange colour. It indicates production of IAA by the cultured strain (Figure S2) [24]. For quantitative estimation of IAA, optical density was recorded at 530 nm by spectrophotometer and the concentration of IAA was calculated using standard curve of IAA (Hi-media) constructed in the range of 10–100 µg/mL.

For quantitative estimation of siderophore, CAS-shuttle assay (CAS: Chrome Azurol S agar) was performed [25]. Siderophore removes the iron from the dye complex, causing reduction in the intensity of blue colour, which was recorded at 630 nm. In this test, minimal medium was used as blank and percentage siderophore units were calculated.

Bacterial isolates were tested for their potential to produce ammonia in peptone water using Nessler's reagent [26] (Figure S2). Bacterial isolates were grown in peptone water broth for 48 h at 28 °C temperature; development of brown to yellow colour indicated production of ammonia, and its optical density was measured at 450 nm using spectrophotometer for its quantification. The concentration of ammonia was estimated from the standard curve of ammonium sulphate in the range of 1–10 µmol/mL.

2.1. Identification of Bacterial Strains Based on 16s rRNA Sequence

The total genomic DNA of the bacterial isolates was extracted and quantified at 260/280 nm by using spectrophotometer and analyzed on agarose gel, and the same was used in PCR amplification as a template for the 16S rDNA. The universal primers, 27F (5′- AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (5′-ACG GCT ACC TTG TTA CGA CTT-3′), were used for amplification of 16S rDNA region as described by Weisburg et al. [27]. The PCR reactions were performed in 20 µL volumes containing 2 µL of the genomic DNA. The PCR master mix was having 0.16 mM dNTP Mix, 20 pmol of forward and reverse primers, and 0.75U Taq DNA polymerase (MBI, Fermentas, Lithuania). Two drops of mineral oil were overlaid on mixes contained in PCR tubes. Amplification was carried out in a thermal cycler (Eppendorf Master cycler nexus 230 V/50–60 Hz) [28]. The PCR conditions used in this study were as follows: an initial denaturation at 95 °C for 6 min, 40 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 1 min, and extension at 72 °C for 1 min. Final extension was performed at 72 °C for 10 min. Gel electrophoresis with agarose (2%) and ethidium bromide was used to analyze the obtained PCR products. The DNA bands were observed by Gel Documentation System (Zenith, Gel Documentation System; Model No. Gel.LUMINAX-312) (Figure 2).

The PCR product was sequenced based on Sanger's Method [29]. BioEdit (an online) tool was used for alignment of obtained forward and reverse sequence, then the query sequences were identified, considering *E* value a $<1 \times 10^{-5}$ and maximum hits (99 or 100%) with a species in the reference NCBI database. Nucleotide BLAST and MEGA X were used for construction of phylogenetic tree by employing Maximum Parsimony (MP) program using the fast minimum evolution method [30].

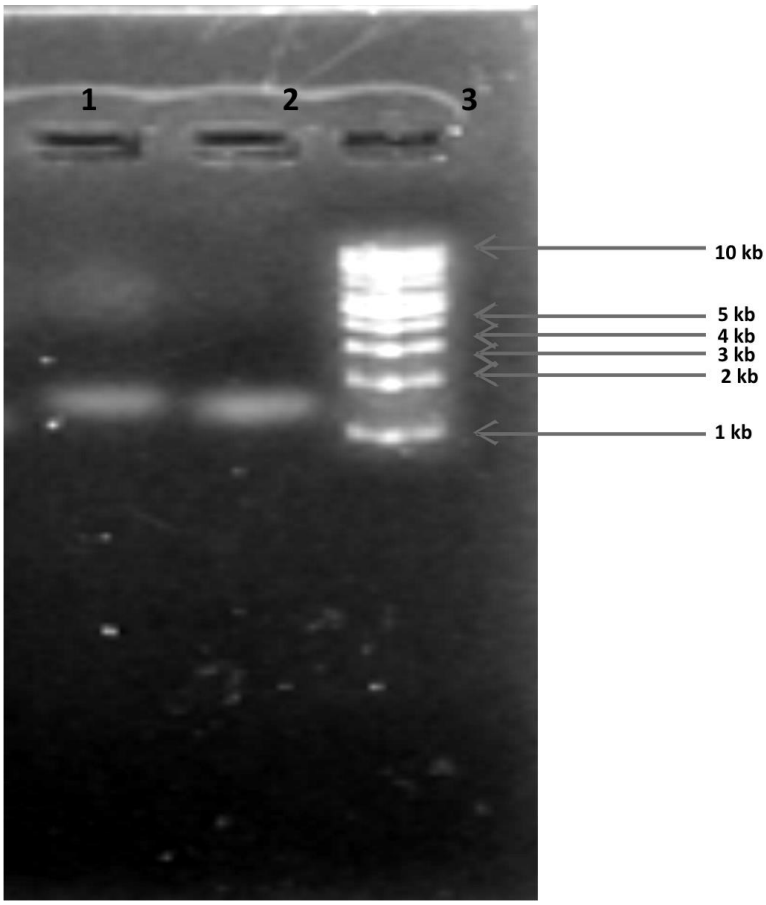

Figure 2. 16s rRNA gene amplification for bacterial isolates, where Lane 1 well was loaded with the *Bacillus cereus* sample, Lane 2 well was loaded with *Bacillus haynesii* sample, and as a check, a 10 Kb marker was loaded in Lane 3 well.

2.2. Screening ACC Deaminase Activity from Isolated Rhizobacteria

2.2.1. ACC Deaminase Activity (Qualitative)

For qualitative estimation of ACC deaminase, the bacterial isolates were serial diluted in LB (Luria–Bertani) medium, which contains 10 g of peptone, 5 g of yeast extract, and 10 g of NaCl per liter. This medium was supplemented with agar (1.5%). The appropriate dilutions (0.1 mL) of the sample bacterial isolates were cultured on LB agar medium and incubated at 28 °C for 24 h.

After incubation, distinct colonies (morphologically) were identified, and the same were observed for ACC deaminase production and its activity simultaneously on sterile Dworkin and Foster (DF) minimal salts media [31] (Figure S1). DF salts minimal media was prepared as per standard protocol and $(NH_4)_2SO_4$ was replaced by 3 mM ACC. Under this condition, bacteria will secrete ACC deaminase and use ACC as a sole source of N. Therefore, the bacteria which survive on this medium will definitely have ACC deaminase activity.

2.2.2. ACC Deaminase Quantified from Selected Bacterial Isolates

ACC deaminase activity was quantified using the method described by Penrose and Glick [32], where α-ketobutyrate produced by activity of ACC deaminase has been calculated by spectroscopic observations [32].

2.2.3. Measurement of ACC Deaminase (Enzyme) Activity

ACC deaminase converts ACC into α-ketobutyrate, and so, indirectly, ACC deaminase was estimated by estimating α-ketobutyrate production at 540 nm by spectroscopic method [32].

2.2.4. Standard Curve of α-Ketobutyrate

The standard solutions of α-ketobutyrate were prepared with 1–10 µmol concentrations. 300 µL of 2, 4-dinitrophenylhydrazine was added to each tube and gently vortexed. Then, the tubes were incubated at 30 °C for 30 min, then 2 mL of 2N sodium hydroxide was added to each tube, and then the absorbance was read at 540 nm, and standard curves were constructed accordingly.

2.3. Portrayal of Partially Purified ACC Deaminase Enzyme

Purification of ACC deaminase enzyme was performed by ammonium sulfate precipitation method followed by dialysis [33], where silica-based column chromatography was used. 10 g of silica gel was weighed and mixed into sodium phosphate buffer until it is completely dissolved. Then, the gel is poured into Pasteur pipette by using beaker (10 mL). The column was left until the silica gel settles down completely. Finally, when the buffer and gel become separated, the buffer was allowed to flow down the column drop by drop. The solvent level was monitored for its flows through the silica gel and left until its level reaches 3 mm layer, which indicated the sample was completely poured out. At the end of the process, pure sample in different concentrations were collected.

2.4. Quantification of Partially Purified Protein by Bradford Method

Bradford reagent was prepared by dissolving 100 mg Coomassie Brilliant Blue G-250 in 50 mL 95% ethanol, and then 100 mL 85% (*w*/*v*) phosphoric acid was added. Solution was diluted to 1 L. When the dye was completely dissolved, it was filtered through Whatman No. 1 paper. Standard solutions were prepared containing a range of 5 to 100 micrograms protein (albumin) in 100 µL volume. Then, 5 mL dye reagent was added in each solution and incubated for 5 min. Absorbance at 540 nm was measured using spectrophotometer and standard curve was plotted. Amount of protein present in the sample was calculated by comparing absorbance by sample with the standard curve.

2.5. Determination of Molecular Weight of ACC Deaminase

The molecular weight of ACC deaminase was determined by using SDS PAGE, where the bands of ACC deaminase were compared with ladder of 1 Kb [34].

2.6. Estimation of Exopolysaccharide (EPS)

The bacterial isolates were grown in nutrient broth containing 5% of sucrose as carbohydrate source. 10 mL of culture suspension was collected after 6 days and centrifuged at 30,000 rpm for 45 min. Then, thrice the volume of chilled acetone was added to the supernatant. EPS was separated from the mixture in the form of a slimy precipitate. Precipitate was collected on a filter paper. The precipitates were allowed to dry overnight at 50 °C temperature. Then, by gravimetric method, weight of EPS was recorded [35].

2.7. Protein Content in EPS: PGPRs Potentials at Various Levels of Drought

For estimating protein in EPS media with different water potentials (−0.05, −0.15, −0.30, and −0.49 MPa) in trypticase, soya broth (TSB) was prepared by adding appropriate quantity of polyethylene glycol (PEG 6000) [9], then 1% overnight-raised bacterial cultures were inoculated in the screw-capped tubes. These tubes were incubated at 120 rpm for 24 h at 28 °C. The cultures grown at minimum (−0.05 MPa) and maximum (−0.49 MPa) stress level were analyzed for their ability to produce EPS, for which the culture grown was centrifuged for 25 min at 20,000 rpm and the supernatant was collected. Intracellular polysaccharides extraction was possible, so it was ruled out by adding 2, 4 Diphenylalanine (DPA) reagent for the presence of DNA in the collected supernatant. Nitrocellulose membrane (0.45 µm) was used to filter supernatant, and then it was extensively dialyzed with chilled water (4 °C). To remove insoluble material (if present) from obtained dialysate, it was centrifuged at 20,000 rpm for 25 min by mixing with ice-cold (3 volumes) absolute ethanol and incubated at 4 °C overnight. The mixture was centrifuged at 10,000 rpm for

15 min to obtained EPS. Further, the precipitate was purified by repeated dialysis and precipitation process again, after suspended in water. The growth in cultures was estimated by measuring the OD at 590 nm. The total protein content in the precipitated EPS was determined by comparing with the standard curve of Bovine serum albumin (BSA) by Bradford's assay.

2.8. Heavy Metal Tolerance Test by Plate Method

To study the tolerance of As, Ba, and Ni in the bacterial strains, the bacterial strains were cultured on the nutrient agar media amended with 14.74 mg/L and 29.48 mg/L of As, 6.28 mg/L and 12.56 mg/L of Ba, and 2.963 mg/L and 5.926 mg/L of Ni separately. Arsenic (III) oxide (As_2O_3), barium chloride ($BaCl_2 \cdot 2H_2O$), and nickel (II) chloride ($NiCl_2 \cdot 6H_2O$) were used for this study [19]. The bacterial strain which could survive on amended media with heavy metal has been considered as tolerant strain.

Each experiment has been repeated thrice and the average value is presented in this manuscript.

3. Results

3.1. Soil Properties

The soil samples were analyzed for both physical and chemical properties. The physical properties of the soil included pH (7.6), and electric conductivity (0.20). The soil texture was sandy loam and had 53% sand, 27% silt, and 20% clay. Soil bulk density was found to be 1.26 Mg/m^3 and soil particle density was determined to be 2.86 Mg/m^3. The soil has been found to be non-saline. The chemical analysis of soil indicate presence of 0.68% organic carbon, 54 kg/ha nitrogen, 28.8 kg/ha phosphorous, and 230 kg/ha potassium. Thus, the soil had high organic carbon, low N, high P, and moderate K content. The presence of these nutrients allowed proliferating bacteria belonging to families *Enterococcaceae*, *Bacillaceae*, *Coccidae, Pseudomonadaceae*, and *Pseudomonadaceae aerobe*. The soil was also studied for heavy metals like As, Ba, and Ni, out of which As and Ba were found to be below detectable levels and Nickel was found to be 0.66 ppm.

3.2. Bacterial Properties

The morphological features, biochemical characteristics, PGPR characteristics, and molecular characteristics of the two bacterial isolates are summarized in Table 1. The stain AV-7 has been found to be Gram-negative cocci with round and smooth colonies. The bacterium has been found to be positive for various biochemical tests except indole test and citrate utilization (Table 1). It was not capable of fermenting D-glucose and sucrose in carbohydrate fermentation test (Table 1). The bacterial strain AV-12 has been found similar to AV-7 with white convex elevated circular colonies. However, this bacterial strain has potential to utilize D-glucose and sucrose in carbohydrate fermentation test (Table 1).

Based on morphological and Gram's staining results, the bacterial strains were identified to be *Bacillus species*.

3.3. Identification of PGPRs by 16s rRNA Sequence

The quantitative analysis of isolated genomic DNA of both the strains was recorded as 7.57 µg/mL for AV-12 and 7.145 µg/mL for AV-7. The 16S rRNA sequences were found to be 1377 bp for AV-12 and 1340 bp for AV-7. AV-12 and AV-7 were the isolates from rhizospheric soils of *Vigna mungo* and *Phaseolus vulgaris*, respectively [19]. The 16S rRNA sequence for both bacterial strains are submitted to DNA Database Bank of Japan (DDBJ), which is a NCBI collaborator. The isolates were assigned with accession no. LC514122 and LC514123 for *Bacillus cereus* (AV-12) and *Bacillus haynesii* (AV-7), respectively. The obtained partial 16S rRNA sequences from both bacterial isolates were compared with existing sequences database from NCBI GenBank and found to be related with other reported strains (Figure 3) [30].

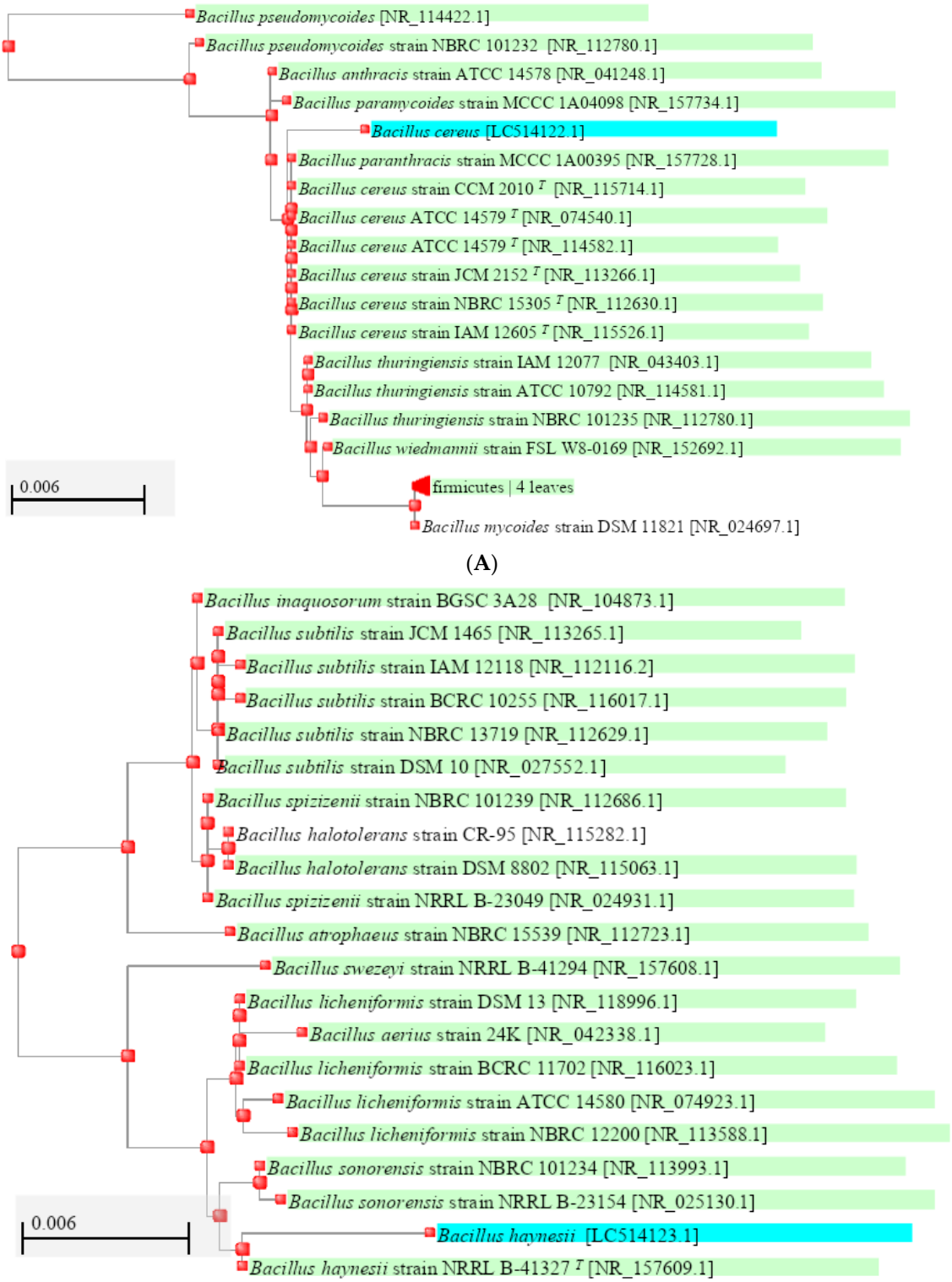

Figure 3. (**A**) Phylogenetic tree (NCBI BLAST): Tree Method used in constructing this picture was Fast minimum evolution with maximum sequence distance as 0.75. Sea-green-colour-indicated query (*Bacillus cereus*) was searched for 16S rRNA partial sequences from Bacteria and Archaea, bottle-green-colored strains are from type material, and red colour is an indication for Firmicutes. The Type strains are superscripted with T. (**B**) Phylogenetic tree (NCBI BLAST): Tree Method used in constructing this picture was Fast minimum evolution with maximum sequence distance as 0.75. Sea-green-colour-indicated query (*Bacillus haynesii*) was searched for 16S rRNA partial sequences

from Bacteria and Archaea, bottle-green-coloured strains are from type material, and red colour is an indication for Firmicutes. The Type strains are superscripted with T.

Table 1. Source of rhizosphere soil and morphological, biochemical, and molecular characteristics of bacterial isolates.

Characteristics	AV-12	AV-7
Soil sample source	*Vigna mungo*	*Phaseolus vulgaris*
Morphology		
Cell morphology	Round and Smooth colonies	Round and Smooth colonies#
Gram reaction	Gram-Negative	Gram-Negative #
Shape of organism	Bacilli (rod)	Cocci #
Spore formation	Observed	Observed
Arrangement of cells	Cells form clusters	Cells form clusters
Culture		
Colony colour	White	White
Elevation	Convex	Convex
Biochemical tests		
Indole test	Negative	Negative
Methyl red test	Positive	Positive #
VP test	Positive	Positive #
Catalase test	Positive	Positive #
Citrate utilization test	Positive	Positive
Phosphate solubilization	Positive	Positive #
Amylase hydrolysis	Positive	Positive
Carbohydrate production test	Positive	Positive
Carbohydrate fermentation		
D-Glucose	Negative	Positive
Sucrose	Negative	–
Maltose	Negative	Positive
PGP Traits		
Phosphate solubilization (mg/mL)	0.063	1.881
Ammonia production (µmol/mL)	0.518	0.413
HCN production (µmol/mL)	21.30	30.58
Siderophore production (%)	48.71	42.30
Exopolysaccharide yield (mg/10mL)	18	15
ACC deaminase production (µmol/mL)	5.484	6.008
Molecular		
BLAST Comparison (16S rDNA)	*Bacillus cereus*	*Bacillus haynesii*
Accession Number	*LC514122*	*LC514123*

These results have already reported in our previous study [19].

3.4. Screening ACC Deaminase Activity from Rhizobacteria Isolates

3.4.1. ACC Deaminase Qualitative Test

Both the AV-7 and AV-12 rhizobacterial strains were found to produce ACC deaminase in Petri plate method.

3.4.2. Quantification of ACC Deaminase

ACC deaminase activity for *B. cereus* and *B. haynesii* were estimated and presented in Table 2. *B. cereus* and *B. haynesii* produced 5.484 µM/mL and 6.008 µM/mL concentration of α- ketobutyrate, respectively.

Table 2. Performance of bacterial isolates under in vitro drought and heavy metal stress.

Properties	*B. cereus*	*B. haynesii*
Cell density (Number/mL)	108×10^6	108×10^6
EPS (mg/mg protein) No Stress	2.26	1.29
EPS (mg/mg protein) Stress	5.88	4.84
ACC deaminase activity (µM αKB/mg/min)	12.6	11.0
Total protein in crude extract (mg)	2.87	1.98
After purification		
ACC deaminase activity (µM/mg/min)	3.33	2.85
Mol weight of ACC deaminase (35-42 kDa)	35	40
Heavy metal stress at two concentrations of each heavy metal		
Arsenic (As) [14.74 mg/L]	Resistant	Sensitive
Arsenic (As) [29.48 mg/L]	Resistant	Sensitive
Barium (Ba) [6.28 mg/L]	Resistant	Sensitive
Barium (Ba) [12.56 mg/L]	Resistant	Sensitive
Nickel (Ni) [2.963 mg/L]	Resistant	Sensitive
Nickel (Ni) [5.926 mg/L]	Resistant	Sensitive

3.4.3. Measurement of ACC Deaminase Activity

The catalytic activity of ACC deaminase was found to be 12.6 µM αKB/mg/min for *B. cereus* and 11.0 µM αKB/mg/min for *B. haynesii*.

3.4.4. Characterization of Partially Purified ACC Deaminase Enzyme

The total protein was precipitated using ammonium sulphate to obtain partially purified segment of the ACC deaminase enzyme using chromatography. Initially, the total protein in crude extract was estimated to be 2.87 mg/mL for *B. cereus* and 1.98 mg/mL for *B. haynesii*. The ACC deaminase activity of crude protein extract was found to be 12.6 and 11.8 µM α keto butyrate/mg/min for *B. cereus* and *B. haynesii*, respectively (Table 2). The crude protein was further purified using silica gel column chromatography and obtained partially purified enzyme, whose specific activity was noted as 3.33 µM/mg/min and 2.85 µM/mg/min for *B. Cereus* and *B. haynesii*, respectively (Table 2).

3.4.5. Quantification of Partially Purified Protein Extraction by Bradford Method

Bacillus cereus and *B. haynesii* were tested for protein content in the partially purified enzyme. The average protein was quantified by using α-ketobutyrate standard curve and the concentrations were found to be 0.372 µg/mL for *B. cereus* and 0.272 µg/mL for *B. haynesii*.

3.4.6. Determination of Molecular Weight of ACC Deaminase

ACC deaminase is a multimeric sulfhydryl enzyme, and each subunit has molecular mass ranging 35–42 kDa approximately and is always tightly bound with pyridoxal phosphate (PLP) as co-factor [19]. Molecular weight of purified ACC deaminase enzyme of one subunit from *B. Cereus* and *B. haynesii* were found to be 35 kDa and 40 kDa, respectively (Figure 4).

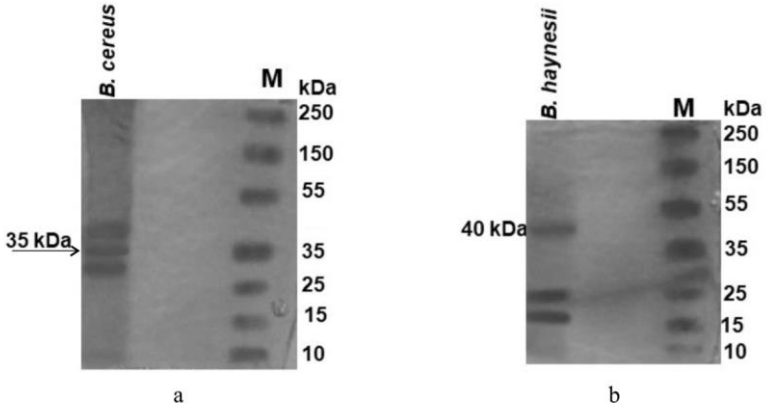

Figure 4. Purified 1-aminocyclopropane-1-carboxylate (ACC) deaminase derived from (**a**) *Bacillus cereus strain* (35 KDa) and (**b**) *Bacillus haynesii* (40 KDa).

3.5. Quantification of EPS

EPS yield was recorded after 6 days of inoculation of the bacterial strains and the results indicated that *B. cereus* and *B. haynesii* produced 18 mg/10 mL and 15 mg/10 mL EPS, respectively (Table 2).

Protein content in EPS

EPS contains protein, carbohydrates, uronic acid, and little amount of DNA. The protein and other content in the EPS help the PGPRs to stick on the surface of the roots of the crop [6]. To study the efficacy of the PGPRs under varied stress conditions, 5% PEG 6000 (−0.05 MPa); 10% PEG 6000 (−0.15 MPa); 15% PEG 6000 (−0.30 MPa), and 20% PEG 6000 (−0.49 MPa), to produce protein content in EPS; the protein content in the EPS from both the bacterial strains has been compared. The results revealed that *B. cereus* produced 2.27 µg/mg, 3.73 µg/mg, 4.61 µg/mg, and 5.87 µg/mg protein in its EPS at −0.05 MPa, −0.15 MPa, −0.30 MPa, and −0.49 MPa water potential, respectively. Similarly, *B. haynesii* also produced increased amount of protein content in EPS with a decrease in osmotic potential. It produced 1.31 µg/mg, 2.07 µg/mg, 3.47 µg/mg, and 4.82 µg/mg of protein in EPS at −0.05 MPa, −0.15 MPa, −0.30 MPa, and −0.49 MPa water potential, respectively.

3.6. Heavy Metal Tolerance

Out of these two rhizobacterial isolates, only *B. cereus* (AV-12) was capable of growing on nutrient agar supplemented with As, Ba, and Ni (Table 2). Therefore, AV-12 can tolerate these heavy metals *in vitro*, but the rest of the isolates including *B. haynesii* (AV-7) could not grow on the provided media and remained sensitive towards these heavy metals.

4. Discussion

In the present study, the comparative analysis of 16S rRNA gene sequence indicated that the bacterial isolate AV-12 is *B. cereus.* This bacterium belongs to: Species: *B. cereus*, Genus: *Bacillus*, Family: *Bacillaceae*, Order: *Caryophanales*, Class: *Bacilli,* and Phylum: *Firmicutes*. The scientific name of this bacterium was assigned by Frankland and Frankland (1887). The type strains are ATCC 14579, CCM 2010, JCM 2152, BCRC 10603, BCRC 11026, IAM 12605, NRRL B-3711, DSM 31, NBRC 15305, CGMCC 1.3760, LMG 6923, CECT

148, NCIB 9373, NCTC 2599, CECT 5050, CCUG 7414, CIP 66.24, IFO 15305, HAMBI 1887, IAM 14174, KCTC 3624, NCCB 75008, NCAIM B.02078, NCIMB 9373, VKM B-504, VTT E-93143, and NCFB 1771. Hence, during 16S rRNA nucleotide sequence BLAST performance, ATCC 14579 was selected as a reference sequence, whose similarity with AV-12 was found to be 99.64%, and other strains showed similarity ranging from 99.64% to 95.36% in the phylogenetic tree. *B. cereus* group comprises of other closely related species, namely: *B. cereus sensu stricto* (referred to herein as *B. cereus*), *B. anthracis*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides*, and *B. cytotoxicus*.

In a similar way, the other bacterial isolate AV-7 was identified as *B. haynesii*. This bacterium belongs to: Species: *B. haynesii*, Genus: *Bacillus*, Order: Caryophanales, Family: Bacillaceae class: Bacilli, and phylum: Firmicutes. The scientific name of this bacterial strain was assigned by Dunlap et al. (2017) [36]. The type strains are NRRL B-41327, and CCUG 70178. Hence, during 16S rRNA nucleotide sequence BLAST performance, NRRL B-41327 (*B. haynesii* Dunlap et al., 2017, Accession No. 3EC4C1) was selected as a reference sequence, whose similarity was found to be 99.18%, and 20 other *B. cereus* group type strains showed similarity ranging from 98.964 to 97.61% in the phylogenetic tree.

Diverse organisms with a wide range of ACC deaminase activity act as PGPR. A low level of ACC deaminase activity, namely approximately $\geq$20 nmol α-ketobutyrate/mg/h, is sufficient to permit a bacterium to grow on ACC and to act as a PGPR, and can support root elongation in crops [37]. Another similar study was carried out with 841 rhizobacterial isolates, isolated from 74 chickpea plants, out of which 743 isolates were determined to be *Bacillus* and *Pseudomonas* by using taxonomically selective and enrichment isolation protocols. Out of these 743 isolates, 19 isolates were closely related to *Bacillus* spp. and could produce ACC deaminase activity ranging from 0.35 to 7.32 µmol α KB/h, and it was sufficient to alleviate drought stress [37]. In another study, Duan et al. (2021) isolated seven bacterial isolates from grapevine rhizosphere. Out of these seven isolates, two (DR3 and DR6) isolates were found to produce high amount of protein in EPS, which ranged from 41.18 to 60.11 µmol α-KB/(mg/Protein/h) and showed drought tolerance potentials [38]. A wide range of bacteria belonging to the genera *Bacillus*, *Burkholderia*, *Pseudomonas* and *Variovorax* showed ACC deaminase production ability which reduced ethylene production under various abiotic stress conditions.

A study was conducted in chickpeas by Kumar et al. (2016), who studied the potential of two PGPR (*Bacillus amyloliquefaciens* and *Pseudomonas putida*) to alleviate drought stress in chickpeas, as these strains had multiple PGP traits including ACC deaminase activity when performed in vitro. In this study, PGPRs colonization in chickpea rhizosphere was visualized with gfp labelling. The in vivo experiments disclosed that a combined application (in consortia) of these microbes ameliorated negative effects of drought stress in chickpeas, as it was evident by increased biomass. The increased colonization, enhanced ACC deaminase activity, increased chlorophyll content, and proline accumulation were observed in chickpeas, which indicates that the bacterial consortium was able to alleviate drought stress [39].

In a similar study, Sharma et al. (2013) isolated 47 bacterial isolates from rhizospheric soils of chickpeas in Punjab (India), and they found that 25 and 22 strains were of *Bacillus* and *Pseudomonas*, respectively. Further, they found that out of these only ten strains could use ACC as sole source of N. They reported that *Bacillus* isolate 23-B and *Pseudomonas* 6-P in combination with *Mesorhizobium ciceris* can help the plant to mitigate the water stress for *Cicer arietinum* (Kabuli L-552 and Desi GPF-2) and enhanced all growth parameters even under water stress conditions [17].

It was noted that the molecular weight of ACC deaminase from *Pseudomonas* strains are in a range of 35 kDa to 41 kDa [40]. The molecular mass of the whole ACC deaminase enzyme isolated from *Pseudomonas* sp. UW4 was found to be 168 kDa [41]. The molecular mass of ACC deaminase has been reported in various studies such as *Methylobacterium nodulans* ORS2060 (Homotetramer 144 kDa) [42], *Methylobacterium radiotolerans* JCM2831 (Homotetramer 144 kDa) [43], and *Amycolatopsis methanolica* 239 (Homotetramer 144 kDa) [43].

Water retention and cementing properties are the unique qualities of exopolysaccharide which have vital roles in stabilizing soil aggregates, forming biofilm, and enhancing nutrient flow up to plant roots. *Bacillus cereus* and *B. haynesii*, isolated in this study, also produced fairly good amounts of exopolysaccharides, due to which they can result as promising bioinoculants in various stress conditions, especially drought. EPS production in significant amounts have been reported in several studies. According to Sandhya et al. [44], out of 81 bacterial strains isolated from alfisols, vertisols, inceptisols, oxisols, and aridisols, 26 isolates could tolerate the maximum level of drought stress (−0.73 MPa) and produced EPS. Further, they observed that *P. putida* GAP-P45 had higher drought tolerance capacity [44].

Heavy metal tolerance was studied in *Bacillus* sp., which was collected and isolated from heavy-metal-polluted soil. This bacterial isolate was helpful in the detoxification of trivalent and tetra valent chromium [45]. Similarly, *Bacillus subtilis* was found to be instrumental in alleviating stress caused by high concentration of Cd in soil for carrots [46]. The inoculation of this bacterial strain could immobilize Cd through bioaugmentation, and enhance the plant shoot and root growth 16% and 55%, respectively [46]. *Acidithiobacillus caldus* and *Sulfobacillus thermotolerans* were reported for their heavy metal bioleaching properties for Cu, Cd, Pb, Zn, Mn, Hg, and As [47]. Some other endophytic bacterial isolated from *Tridax procumbens* were reported for heavy metal detoxification including Zn, Pb, and As [48].

In the present study, efforts have been made to isolate the rhizobacterial strains from the rhizospheric soil collected from two legume crops. Two bacterial strains were characterized based on morphology and biochemical properties. Both, strains were Gram-negative. These bacterial isolates were evaluated for their potential as PGPR. *Bacillus cereus* and *B. hyanesii* had potential to produce acid during fermentation and are capable of producing catalase; these tests reveal their capacity to scavenge reactive oxygen species. These strains have potential to solubilize P and produce siderophore. *Bacillus cereus* can withstand heavy metals (As, Ba, and Ni), while *B. haynesii* was sensitive to said heavy metals. These strains can be used to promote growth of the crops cultivated in the soil lacking available P. These two strains also were able to tolerate drought under in vitro conditions. These strains also produce exopolysaccharides and these were also capable of producing the stress-releasing enzyme ACC deaminase. Looking at the various characteristics of these rhizobacterial isolates, they can be considered a potential PGPR bacteria to support the growth and yield of the legumes. In comparison to the use of synthetic hazardous chemicals in agriculture, the use of PGPRs is considered as the best alternative, as an eco-friendly and sustainable approach for agriculture. Applications of PGPRs as biofertilizers have the chance of increase agriculture productivity. Further, field study of these strains with crops will reveal their potential as a future bio-fertilizer [49].

5. Conclusions

The present study reveals that both bacterial strains (*B. cereus* and *B. haynesii*) produce EPS and ACC deaminase in considerable amounts, sufficient for mending abiotic stress, and showed their potential towards plant-growth-promoting properties. The molecular weight for ACC deaminase monomers was found to be 35 kDa and 40 kDa for *B. cereus* and *B. haynesii*, respectively. The heat stress tolerance potential of the bacterial strains has been further evaluated by looking into EPS-based protein production at various drought stress levels. The results indicate that the *B. cereus* bacterial strain has better protein yield than *B. haynesii* strain in both normal and induced water-stress conditions. Hence, from the present study, it can be concluded that both bacterial strains have PGPR potential. However, the *B. cereus* strain has shown better performance in vitro for PGPR properties with reference to ACC deaminase activity and also for protein quantity in EPS, both in normal and drought conditions, proving its potentiality as a biocontrol agent, biofertilizer, or as part of a bio-inoculum, especially as it can be one of the best solutions for abiotic stresses. The *Bacillus cereus* strain also showed heavy metal resistance to arsenic, barium, and nickel heavy metals, whereas *Bacillus haynesii* was sensitive towards these heavy

metals. Further studies are required to look into the performance of these bacterial strains in association with chickpeas, initially in a pot experiment and later in field trials.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae9010046/s1, Figure S1: PGPR traits (qualitative): A: Control; B: Bacillus cereus; C: Bacillus haynesii. First row: Phosphate solubilization; Second row: HCN production; Third row: ACC deaminase production. Figure S2: PGPR traits (qualitative): A: Production of ammonia- both the isolates showed positive response B: Production of IAA- both the bacterial isolates were positive for the test.

Author Contributions: V.S.G.: Concept, experimental design, and help in writing manuscript. A.K.A.: Conducted the experiments and collected the data, compiled the data, and wrote the manuscript. V.D.R. and M.B.: Provided critical suggestions, and edited and improved the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The authors would like to thank the dean of JIBB, SHUATS for conducting the experiment in their laboratory. V.D.R. acknowledges the support by the laboratory «Soil Health» of the Southern Federal University with the financial support of the Ministry of Science and Higher Education of the Russian Federation, agreement No. 075-15-2022-1122. M.B. would like to recognize the Grant of the Ministry of Education and Science of the Russian Federation for the development of youth laboratories, within the framework of the implementation of the Tula State Lev Tolstoy Pedagogical University program "Priority 2030" under Agreement No. 073-03-2022-117/7.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Barnawal, D.; Singh, R.; Singh, R.P. Role of Plant Growth Promoting Rhizobacteria in Drought Tolerance: Regulating Growth Hormones and Osmolytes. In *PGPR Amelioration in Sustainable Agriculture*; Singh, A.K., Kumar, A., Singh, P.K., Eds.; Woodhead Publishing: Sawston, UK, 2019; pp. 107–128. [CrossRef]
2. Sahu, P.K.; Jayalakshmi, K.; Tilgam, J.; Gupta, A.; Nagaraju, Y.; Kumar, A.; Hamid, S.; Singh, H.V.; Minkina, T.; Rajput, V.D.; et al. ROS generated from biotic stress: Effects on plants and alleviation by endophytic microbes. *Front. Plant Sci.* **2022**, *13*, 1042936. [CrossRef] [PubMed]
3. Tuteja, N.; Sopory, S.K. Chemical signaling under abiotic stress environment in plants. *Plant Signal. Behav.* **2008**, *3*, 525–536. [CrossRef] [PubMed]
4. Kour, D.; Khan, S.S.; Kaur, T.; Kour, H.; Singh, G.; Yadav, A.; Yadav, A.N. Drought adaptive microbes as bioinoculants for the horticultural crops. *Heliyon* **2022**, *8*, e09493. [CrossRef]
5. Ashraf, M.; Akram, N.A. Improving salinity tolerance of plants through conventional breeding and genetic engineering: An analytical comparison. *Biotechnol. Adv.* **2009**, *27*, 744–752. [CrossRef] [PubMed]
6. Ahmed, B.; Shahid, M.; Syed, A.; Rajput, V.D.; Elgorban, A.M.; Minkina, T.; Bahkali, A.H.; Lee, J. Drought tolerant enterobacter sp./Leclercia adecarboxylata secretes indole-3-acetic acid and other biomolecules and enhances the biological attributes of *Vigna radiata* (L.) R. Wilczek in water deficit conditions. *Biology* **2021**, *10*, 1149. [CrossRef]
7. Upadhyay, S.K.; Rajput, V.D.; Kumari, A.; Espinosa-Saiz, D.; Menendez, E.; Minkina, T.; Dwivedi, P.; Mandzhieva, S. Plant growth-promoting rhizobacteria: A potential bio-asset for restoration of degraded soil and crop productivity with sustainable emerging techniques. *Environ. Geochem. Health* **2022**. [CrossRef]
8. Kour, D.; Rana, K.L.; Sheikh, I.; Kumar, V.; Yadav, A.N.; Dhaliwal, H.S.; Saxena, A.K. Alleviation of drought stress and plant growth promotion by *Pseudomonas libanensis* EU-LWNA-33, adrought-adaptive phosphorus-solubilizing bacterium. *Proc. Natl. Acad. Sci. India Sect. B Biol. Sci.* **2020**, *90*, 785–795. [CrossRef]
9. Ali, S.Z.; Sandhya, V.; Rao, L.V. Isolation and characterization of drought-tolerant ACC deaminase and exopolysaccharide-producing fluorescent *Pseudomonas* sp. *Ann. Microbiol.* **2014**, *64*, 493–502. [CrossRef]
10. Vacheron, J.; Desbrosses, G.; Bouffaud, M.L.; Touraine, B.; Moenne-Loccoz, Y.; Muller, D.; Legendre, L.; Wisniewski-Dye, F.; Prigent-Combaret, C. Plant growth-promoting rhizobacteria and root system functioning. *Front. Plant Sci.* **2013**, *4*, 356. [CrossRef]
11. Fulekar, M.H.; Sharma, J.; Tendulkar, A. Bioremediation of heavy metals using biostimulation in laboratory bioreactor. *Environ. Monit. Assess.* **2012**, *184*, 7299–7307. [CrossRef]

12. Pande, V.; Pandey, S.C.; Sati, D.; Bhatt, P.; Samant, M. Microbial interventions in bioremediation of heavy metal contaminants in agroecosystem. *Front. Microbiol.* **2022**, *13*, 824084. [CrossRef] [PubMed]
13. Choudhary, A.K.; Kumar, S.; Patil, B.S.; Bhat, J.S.; Sharma, M.; Kemal, S.; Ontagodi, T.P.; Datta, S.; Patil, P.; Chaturvedi, S.K.; et al. Narrowing yield gaps through genetic improvement for Fusarium wilt resistance in three pulse crops of the semi-arid tropics. *SABRAO J. Breed. Genet.* **2013**, *45*, 341–370.
14. Araujo, S.S.; Beebe, S.; Crespi, M.; Delbreil, B.; Gonzalez, E.M.; Gruber, V.; Lejeune Henaut, I.; Link, W.; Monteros, M.J.; Prats, E.; et al. Abiotic stress responses in strategies used to cope with environmental challenges. *Crit. Rev. Plant Sci.* **2015**, *34*, 237–280. [CrossRef]
15. Fahad, S.; Bajwa, A.A.; Nazir, U.; Anjum, S.A.; Farooq, A.; Zohaib, A.; Sadia, S.; Nasim, W.; Adkins, S.; Saud, S.; et al. Crop Production under Drought and Heat Stress: Plant Responses and Management Options. *Front. Plant Sci.* **2017**, *8*, 1147. [CrossRef] [PubMed]
16. Available online: http://www.fao.org/faostat/en/#data/QC (accessed on 7 September 2022).
17. Sharma, P.; Khanna, V.; Kumar, P.I. Efficacy of aminocyclopropane-1-carboxylic acid (ACC)-deaminase-producing rhizobacteria in ameliorating water stress in chickpea under axenic conditions. *Afr. J. Microbiol. Res.* **2013**, *7*, 5749–5757. [CrossRef]
18. Khan, N.; Bano, A.; Rahman, M.A.; Guo, J.; Kang, Z.; Babar, M. Comparative physiological and metabolic analysis reveals a complex mechanism involved in drought tolerance in chickpea (*Cicer arietinum* L.) induced by PGPR and PGRs. *Sci. Rep.* **2019**, *9*, 2097. [CrossRef]
19. Andy, A.K.; Masih, S.A.; Gour, V.S. Isolation, screening and characterization of plant growth promoting rhizobacteria from rhizospheric soils of selected pulses. *Biocatal. Agric. Biotechnol.* **2020**, *27*, 101685. [CrossRef]
20. Sharma, K. *Manual of Microbiology: Tools & Techniques*; Ane Books: Delhi, India; pp. 141–171.
21. Bhaskara, R.K.V.; Ashwini, K.; Gaurav, K.; Karthik, L. Optimization, production and partial purification of extracellular á-amylase from *Bacillus* sp. marini. *Arch. Appl. Sci. Res.* **2011**, *3*, 33–42.
22. Pikovskaya, R.I. Mobilization of phosphorus in soil in connection with vital activity of some microbial species. *Mikrobiologiya* **1948**, *17*, 363–370.
23. Schwyn, B.; Neilands, J. Universal chemical assay for the detection and determination of siderophores. *Anal. Biochem.* **1987**, *160*, 47. [CrossRef]
24. Gordon, S.A.; Weber, R.P. Colorimetric estimation of indole acetic acid. *Plant Physiol.* **1951**, *26*, 192–195. [CrossRef] [PubMed]
25. Cappuccino, J.C.; Sherman, N. *Microbiology: A Laboratory Manual*, 3rd ed.; Benjamin/Cummings Pub. Co.: New York, NY, USA, 1992; pp. 125–179.
26. Lorck, H. Production of hydrocyanic acid by bacteria. *Physiol. Plant* **1948**, *1*, 142–146. [CrossRef]
27. Weisburg, W.G.; Barns, S.M.; Pelletier, D.A.; Lane, D.J. 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* **1991**, *173*, 697–703. [CrossRef] [PubMed]
28. Jain, D.; Kachhwaha, S.; Jain, R.; Kothari, S.L. PCR based detection of cry genes in indigenous strains of Bacillus thuringiensis isolated from the soils of Rajasthan. *Indian J. Biotechnol.* **2012**, *11*, 491–494.
29. Deharvengt, S.J.; Petersen, L.M.; Jung, H.S.; Tsongalis, G.J. Chapter 13—Nucleic acid analysis in the clinical laboratory. In *Contemporary Practice in Clinical Chemistry*, 4th ed.; Academic Press: Cambridge, MA, USA, 2020; pp. 215–234.
30. Tamura, K.; Stecher, G.; Peterson, D.; Filipski, A.; Kumar, S. MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* **2013**, *30*, 2725–2729. [CrossRef]
31. Dworkin, M.; Foster, J.W. Experiments with some microorganisms which utilize ethane and hydrogen. *J. Bacteriol.* **1958**, *75*, 592. [CrossRef]
32. Penrose, D.M.; Glick, B.R. Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria. *Physiol. Plant* **2003**, *118*, 10–15. [CrossRef]
33. Maxton, A.; Singh, P.; Masih, S.A. ACC deaminase-producing bacteria mediated drought and salt tolerance in *Capsicum annuum*. *J. Plant Nutr.* **2018**, *41*, 574–583. [CrossRef]
34. Jia, Y.J.; Kakuta, Y.; Sugawara, M.; Igarashi, T.; Oki, N.; KisAKi, M.; Shoji, T.; Kanetuna, Y.; Horita, T.; Matsui, H.; et al. Synthesis and degradation of 1-aminocyclopropane-1-carboxylic acid by *Penicillium citrinum*. *Biosci. Biotechnol. Biochem.* **1999**, *63*, 542–549. [CrossRef]
35. Chug, R.; Mathur, S.; Kothari, S.; Gour, V.S. Maximizing EPS production from *Pseudomonas aeruginosa* and its application in Cr and Ni sequestration. *Biochem. Biophys. Rep.* **2021**, *26*, 100972. [CrossRef]
36. Dunlap, C.A.; Schisler, D.A.; Perry, E.B.; Connor, N.; Cohan, F.M.; Rooney, A.P. *Bacillus swezeyi* sp. nov. and *Bacillus haynesii* sp. nov., isolated from desert soil. *Int. J. Syst. Evol. Microbiol.* **2017**, *67*, 2720–2725. [CrossRef] [PubMed]
37. Alemneh, A.A.; Zhou, Y.; Ryder, M.H.; Denton, M.D. Is phosphate solubilizing ability in plant growth-promoting rhizobacteria isolated from chickpea linked to their ability to produce ACC deaminase? *J. Appl. Microbiol.* **2021**, *131*, 2416–2432. [CrossRef] [PubMed]
38. Duan, B.; Li, L.; Chen, G.; Su-Zhou, C.; Li, Y.; Merkeryan, H.; Liu, W.; Liu, X. 1-Aminocyclopropane1-carboxylate deaminase-producing plant growth-promoting rhizobacteria improve drought stress tolerance in grapevine (*Vitis vinifera* L.). *Front. Plant Sci.* **2021**, *12*, 706990. [CrossRef]

39. Kumar, M.; Mishra, S.; Dixit, V.; Kumar, M.; Agarwal, L.; Chauhan, P.S.; Nautiyal, C.S. Synergistic effect of Pseudomonas putida and *Bacillus amyloliquefaciens* ameliorates drought stress in chickpea (*Cicer arietinum* L.). *Plant Signal. Behav.* **2016**, *11*, e1071004. [CrossRef] [PubMed]
40. Glick, B.R.; Nascimento, F.X. Pseudomonas 1-Aminocyclopropane-1-carboxylate (ACC) Deaminase and Its Role in Beneficial Plant-Microbe Interactions. *Microorganisms* **2021**, *9*, 2467. [CrossRef]
41. Hontzeas, N.; Zoidakis, J.; Glick, B.R.; Abu-Omar, M.M. Expression and characterization of 1-aminocyclopropane-1-carboxylate deaminase from the rhizobacterium *Pseudomonas putida* UW4: A key enzyme in bacterial plant growth promotion. *Biochim. Biophys. Acta* **2004**, *1703*, 11–19. [CrossRef]
42. Fedorov, D.N.; Ekimova, G.A.; Doronina, N.V.; Trotsenko, Y.A. 1-Aminocyclopropane-1-carboxylate (ACC) deaminases from *Methylobacterium radiotolerans* and *Methylobacterium nodulans* with higher specificity for ACC. *FEMS Microbiol. Lett.* **2013**, *343*, 70–76. [CrossRef]
43. Ekimova, G.A.; Fedorov, D.N.; Doronina, N.V.; Trotsenko, Y.A. 1-aminocyclopropane-1-carboxylate deaminase of the aerobic facultative methylotrophic actinomycete *Amycolatopsis methanolica* 239. *Microbiology* **2015**, *84*, 584–586. [CrossRef]
44. Sandhya, V.; Ali, S.Z.; Grover, M.; Reddy, G.; Venkatesvarlu, B. Alleviation of drought stress effects in sunflower seedlings by exopolysaccharides producing *Pseudomonas putida* strain GAP-P_{45}. *Biol. Fertil. Soils* **2009**, *46*, 17–26. [CrossRef]
45. Syed, S.; Chinthala, P. Heavy metal detoxification by different *Bacillus* species isolated from solar salterns. *Scientifica* **2015**, *2015*, 319760. [CrossRef]
46. Wang, T.; Sun, H.; Jiang, C.; Mao, H.; Zhang, Y. Immobilization of Cd in soil and changes of soil microbial community by bioaugmentation of UV-mutated *Bacillus subtilis* 38 assisted by biostimulation. *Eur. J. Soil Biol.* **2014**, *65*, 62–69. [CrossRef]
47. Panyushkina, A.E.; Babenko, V.V.; Nikitina, A.S.; Selezneva, O.V.; Tsaplina, I.A.; Letarova, M.A.; Kostryukova, E.S.; Letarov, A.V. *Sulfobacillus thermotolerans*: New insights into resistance and metabolic capacities of acidophilic chemolithotrophs. *Sci. Rep.* **2019**, *9*, 15069. [CrossRef] [PubMed]
48. Govarthanan, M.; Mythili, R.; Selvankumar, T. Bioremediation of heavy metals using an endophytic bacterium *Paenibacillus* sp. RM isolated from the roots of *Tridax procumbens*. *3 Biotech* **2016**, *6*, 242. [CrossRef]
49. Basu, A.; Prasad, P.; Das, S.N.; Kalam, S.; Sayyed, R.Z.; Reddy, M.S.; El Enshasy, H. Plant Growth Promoting Rhizobacteria (PGPR) as Green Bioinoculants: Recent Developments, Constraints, and Prospects. *Sustainability* **2021**, *13*, 1140. [CrossRef]