



# The Role of Phyllosphere Bacteria in Improving Cotton Growth and Yield Under Drought Conditions

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Cotton is a valuable fiber and cash crop in Telangana, India. This study examines how crop growth and fiber yield are affected by the uneven distribution of rainfall. Cotton phyllosphere bacterial isolates were gathered from the Rangareddy and Warangal Districts of Telangana, and in total, 31 phyllosphere bacterial isolates were obtained. These isolates were screened for drought tolerance and it was found that fifteen suitable isolates grew at  $-1.25$  Mpa stress level with copious amounts of exopolysaccharides production. These isolates were further screened for ACC deaminase production and we observed  $0.13$ – $0.40$  mM of  $\alpha$ -ketobutyrate per milligram of cellular protein per hour. Five efficient bacterial isolates, namely *Pseudomonas stutzeri*, *Acinetobacter* sp., *Bacillus mojavensis*, *Pseudomonas chlororaphis*, and *Enterobacter asburiae* were found to produce ACC deaminase and were able to grow at  $-1.25$  Mpa stress level. The cotton variety ADB-542 (drought susceptible) was treated with drought-tolerant five isolates. *Acinetobacter* sp. treated seeds had the highest seed germination and seedling vigor of 76.67 and 45.81%, respectively. The nutrient status of inoculated plots was considerably improved. The root length, fresh weight, proline content, and the number of bolls were increased by 28.52, 41.9, 28.78, and 12.99%, respectively, with the inoculation of *Acinetobacter* sp., to plants at  $-0.75$  Mpa water potential. Overall the performance of cotton was improved significantly with the inoculation of phyllosphere bacteria to seeds; hence they can be recommended for the application of field crops as bio-inoculants.

**Keywords:** drought, water potential, ACC deaminase, phyllosphere, exopolysaccharides (EPS)

## INTRODUCTION

Cotton is mainly cultivated as a rainfed crop in the Telangana districts of Warangal and Rangareddy due to a lack of irrigation facility. In recent years, cotton production was declined due to severe climate change biotic stress, increased temperature, inconsistent distribution of rainfall, decreased water availability, and crop yield (Gosal et al., 2009). As per Cotton Advisory Board (CAB) estimates, cotton production in India during 2017–18 was 377 lakh bales (170 kg) from 122 lakh hectares with a productivity of 524 kg lint/ha (CAB, AICRP for Cotton 2017–18). The quantity and quality of the fiber produced in cotton plant crops are directly related to water availability during the different phenological phases of development (Zakaria and Sabrina, 2017).

In many cases, the cotton economic yield was severely affected by drought stress in arid and semi-arid areas. Drought impedes germination, seedling growth, flower and bud drop, interferes with photosynthesis, and increases CO<sub>2</sub> loss through transpiration, thus increasing the competition between vegetative and reproductive aspects for nutrients and carbohydrates (Qian et al., 2020). Moisture conservation practices have improved the crop stand by 10–15%; however, low yields were incurred due to peak moisture stress at the square formation stage. A lack of efficient moisture stress management technology makes the crops more vulnerable to drought conditions.

Developing climate-resilient varieties and hybrids takes several years and demands extensive labor and money. The potential for phyllosphere microorganisms to promote plant growth under various abiotic stress conditions, especially drought, has been of particular interest to researchers in recent years. Illustrating that the phyllosphere microflora is extremely useful for elucidating underlying plant stress mitigation pathways and identifying possible future candidates. However, there is a lack of detailed evidence on the phyllosphere bacterial population of cotton and their functions in abiotic stress mitigation. Beneficial phyllosphere microorganisms help plants manage abiotic stress more efficiently by interacting with roots and leaves (Glick et al., 2007; Chauhan et al., 2015; Ripa et al., 2019). Drought stress induces plants to produce more abscisic acids (ABA), which predisposes plant tissues to produce stress-related proteins and causes stomatal closure (Sah et al., 2016). The concentration of ABA in plants is influenced both favorably and adversely by bacterial interactions with plants. Inoculation of *Bacillus pumilis*, and *Azospirillum lipoferum* increased the ABA concentration several-fold in planta (Cohen et al., 2015; Yasmin et al., 2017). Several researchers have suggested using phyllosphere bacteria in drought stress mitigation in several crops (Timmusk and Wagner, 1999; Marulanda et al., 2008). Phyllosphere microorganisms tend to produce and share compatible solutes, amines, and sugars with plants and incite Induced Systemic Tolerance (IST) against drought stress (Yang et al., 2009).

It has also been reported that phyllosphere microorganisms produce exopolysaccharides (EPS), thus indirectly improving plants' water availability (Creus et al., 2004). In addition, they were capable of producing phytohormones like cytokinin and auxins with concomitant production of stress response enzyme-ACC deaminase under abiotic stress conditions (Madhaiyan et al., 2005; Chinnadurai et al., 2009). The phyllo-rhizosphere isolates, *Pantoea agglomerans*, and *Azospirillum brasiliense* produce nitric oxide on leaves, a signaling molecule for IAA-producing pathways, and help with adventitious root formation which effectively checks the drought stress in plants (Molina-Favero et al., 2008). The phyllosphere bacteria are more competitive in stressed ecosystems because they are continually exposed to harsh conditions, as shown by the high microbial numbers. The common phyllosphere bacteria encountered are *Pseudomonas* sp., *Xanthomonas* sp., *Bacillus* sp., *Erwinia* sp. (*Pantoea* sp.), and *Methylobacterium* sp. (Holland et al., 2002; Kumar et al., 2019). In recent years, there has been raised

concern about the possible role of phyllosphere microorganisms in foodborne illnesses and possible metabolic interaction with applied foliar microbes (Legein et al., 2020). Rhizosphere application of microbes potentially avoids foodborne illness through physical separation. Furthermore, because drought is primarily defined by a lack of moisture in the root zone, phyllosphere bacteria in the root zone have a more significant impact than phyllosphere application.

There is still a lack of research on the application of phyllosphere microorganisms to soil or the rhizosphere and their potential roles in drought mitigation (Saghafi et al., 2020). In response, this study aimed to find the prospective phylobacteria for drought mitigation in the rhizosphere. The cotton-growing regions of Telangana (Warangal and Rangareddy districts) have witnessed intermittent rainfall or drought for the past few decades, and it is essential to find a suitable solution to the problem. In this view, the present study is focused on the isolation and characterization of drought-tolerant cotton phyllosphere-associated bacterial strains, which were screened for stress alleviating characters such as IAA, ACC deaminase, and exopolysaccharides production. They were then further evaluated for inducing drought tolerance in cotton under pot culture conditions.

## MATERIALS AND METHODS

### Collection of Leaf Samples

Leaf samples were collected from cotton crop growing areas in the Rangareddy and Warangal Districts of Telangana State. All samples were separately bagged, brought to the laboratory in sterile polythene bags, and stored at 4°C to isolate phyllosphere bacteria later.

### Isolation of Cotton Phyllosphere Microorganisms

Different phyllosphere bacterial strains were isolated by leaf imprinting technique, using suitable media and serial dilution method. Ammonium Mineral Salts (AMS) medium (Whittenbury et al., 1970) amended with filter-sterilized solutions of cycloheximide (50 mg/ml), vitamins (Colby and Zatman, 1973), and 0.5% v/v methanol was used as a selective medium for isolating phyllosphere bacteria and methylotrophs, and the final pH of the medium was adjusted to pH 7.0. Fresh leaves of cotton were imprinted on the AMS medium so that each plate should have one side of the leaf (Corpe et al., 1985). Finally, the plates were incubated at 28 ± 2°C for 5 days in the incubator.

### *In vitro* Responses of Isolates Under Stress Drought Tolerance Screening Test

Trypticase Soy Broth (TSB) with different water potentials (0 Mpa, -0.25 Mpa, -0.50 Mpa, -0.75 Mpa, and -1.25 Mpa) was prepared by adding 2.4, 4.7, 6.5, and 9.4 g of polyethylene glycol (PEG-6000) in 100 ml of distilled water (Michel and Kaufmann, 1973; Sandhya et al., 2015). Pure bacterial isolates were inoculated into TSB and incubated at 30°C for 24 h at 120

rpm, and shaken in a rotary shaker. Growth measurements were recorded at 600 nm in a spectrophotometer for the treatments, including control (TSB without bacterial culture). Isolates with an increased optical density of 48 h incubation were considered drought-tolerant bacteria.

### Exo Polysaccharide Production (EPS)

The production of exopolysaccharides by isolates at stress-induced conditions was estimated as per the procedure given by Ali and Kim (2018). In brief, isolates were inoculated in trypticase soy broth with  $-0.75$  Mpa stress level and incubated at  $30^{\circ}\text{C}$  for 3 days in the shaker at 200 rpm. Bacterial cells were harvested with  $500\ \mu\text{l}$  of 1 mM EDTA and centrifuged at 9,000 rpm for 10 min. Then the supernatant was separated and added with cold acetone solution. Biomass deposited in exopolysaccharide was washed twice with distilled water and dried at  $60^{\circ}\text{C}$  for 24 h. Dry weights of biomass produced by the bacterial isolates were expressed as mg/ml.

### Morphological, Physiological, and Molecular Characterization of Isolates

Morphological characters such as shape, size, and gram responses were studied using standard protocols (Cappuccino and Sherman, 1992). The physiological/biochemical characters such as indole production (Isenberg and Sundheim, 1958), methyl red test (Macfaddin, 2000), Voges-Prausker test (Macfaddin, 2000), Citrate utilization test (Macfaddin, 2000), starch hydrolysis test (Macfaddin, 2000), catalase test (Rangaswami and Bagyaraj, 1993), and oxidase test (Collins and Lyne, 1970) were performed for all isolates. The production of HCN by bacterial isolates was tested on AMS medium amended with glycine at 4.4 g/l (Castric and Castric, 1983). Production of brown or reddish-brown was considered positive and scored as moderate (++) or strong (+ + +) based on color intensity.

The genomic DNA of bacteria was isolated by following the standard method given by Ausubel et al. (1999). The extracted DNA was initially quantified against the lambda DNA standard (1 Kb). The 16S rRNA gene of the target bacterial isolates was amplified by using universal primers as reported by Heddi et al. (1998). The reaction mixture was incubated in a thermal cycler (Eppendorf, Germany), and the conditions for PCR amplification were  $94^{\circ}\text{C}$  for 5 min for initial denaturation, followed by 35 cycles of  $95^{\circ}\text{C}$  for 1 min (denaturation),  $60^{\circ}\text{C}$  for 45 s (annealing),  $72^{\circ}\text{C}$  for 1 min 30 s (extension) and a final extension at  $72^{\circ}\text{C}$  for 7 min. Electrophoresis used 1% agarose gel to resolve the amplified PCR products in 1 X Tris-acetate EDTA buffer, a 1 kb DNA ladder (Bangalore Genei) marker. PCR products of the 16S rRNA gene of five efficient bacterial isolates were freeze-dried in a lyophilizer. The purified PCR products were sequenced using the forward primer 5'-AGA GTT TGA TCC TGG CTC AG-3' and reverse primer 5'-CGG TTA CCT TGT TAC GAC TT-3' in Sanger dideoxy method. The partial 16S rRNA sequences of bacterial isolates were compared with the available sequences in GenBank-BLAST (Basic Local Alignment Search Tool) Database (<http://www.ncbi.nih.gov/blast>).

### In vitro Solubilization of Inorganic Zinc, Phosphorous, and Potassium Minerals

Isolates were tested for their ability to solubilize tri-calcium phosphate [ $\text{Ca}_3(\text{PO}_4)_2$ ], zinc phosphate, and potassium release on Pikovaskaya's agar medium (Pikovaskaya, 1948), Tris minimal medium (Saravanan et al., 2003), and Aleksandrov's medium supplemented with mica (Prajapati and Modi, 2012), respectively. The plates were incubated at  $37^{\circ}\text{C}$  for 3–5 days. The isolates showed a clear halo zone of solubilization around the colony were taken as solubilizers, and solubilization was expressed in mm.

### Indole-Acetic Acid (IAA) Production

Indole-Acetic Acid production by isolates was measured by the method given by Gordon and Weber (1951). In short, bacterial cultures were inoculated to Luria-Bertani broth containing 5 mM tryptophan and incubated for 3–4 days. They were followed by centrifugation at 10,000 rpm for 20 min. Two ml of the supernatant were mixed with two drops of orthophosphoric acid and 4 ml of Salkowski reagent and incubated at room temperature for 25 min. The pink colors developed after 30 min, which was considered positive for IAA production. The intensity of the pink color developed was measured at 530 nm in a spectrophotometer. A standard graph determined the quantity of IAA in the culture filtrate and expressed as  $\mu\text{g/ml}$  of the medium.

### Siderophores Production

Isolates were tested for siderophore production on Chrome azurol sulfonate (CAS) agar medium (Schwyn and Neilands, 1987). CAS agar medium was prepared by dissolving 60.5 mg CAS in 50 ml of double-distilled water, mixed with 10 ml of iron (III) solution (1 mM  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  in 10 mM HCl) was added to 72.9 mg of hexadecyl trimethyl ammonium bromide (HDTMA) in 40 ml of distilled water. This reagent was added to the PIPES agar medium (30.24 g of PIPES buffer dissolved in 750 ml of distilled water + 15 g of agar, and its pH was adjusted to 6.8 using 1.06 g of NaOH pellets). The medium was then allowed to solidify in the plates, and isolates were spot inoculated and incubated at  $30^{\circ}\text{C}$  for 3–4 days. Siderophore-producing isolates produced an orange color around the colony.

### Measurement of ACC Deaminase Activity

The ACC deaminase production by isolates was assayed using the protocol laid down by Honma and Shimomura (1978). In this method, the amount of  $\alpha$ -ketobutyrate produced after the cleavage of ACC by ACC deaminase was measured in mM/mg/h of  $\alpha$ -ketobutyrate, which was compared to a standard curve of  $\alpha$ -ketobutyrate based on the absorbance at 540 nm in a spectrophotometer.

### Preparation of Bacterial Inoculum

The selected bacterial inoculum was prepared in the Tryptic Soya Broth (TSB) and incubated at  $28 \pm 2^{\circ}\text{C}$  in an orbital shaking incubator at 160 rpm for 3–5 days. The cells were harvested at  $15,000 \times \text{g}$  for 10 min and then suspended in 0.03 M  $\text{MgSO}_4$  to obtain the requisite cell concentration ( $10^9$  CFU/ml) at  $\text{OD}_{600}$ . The compatibility of the five bacterial isolates was

examined on TSA plates using the dual culture technique for microbial consortium preparation (Kunova et al., 2016). The bacterial consortium was prepared by inoculating TSB medium with equivalent quantities of 3-day old bacterial suspensions of CPB23, CPB24, CPB25, CPB27, and CPB31.

### Germination Percentage

Cotton (*G. hirsutum* L.) variety ADB-542 seeds were surface sterilized in 70% ethanol for 30 s, then 0.1 per cent mercuric chloride for 3 min, followed by sterile distilled water rinses. The cotton seeds were incubated for 1 h in the appropriate suspension of bacterial cultures, then air-dried aseptically in the laminar airflow for bacterial inoculation. Cotton seeds that had been surface-sterilized but not primed were used in the control group and immersed in a 0.03 M MgSO<sub>4</sub> solution for 5 min. Surface-sterilized seeds were aseptically placed on 0.8 percent seedling agar and incubated for 48–96 h to test germination (Islam et al., 2009).

### Microcosm Experiment

A microcosm experiment was carried out at the Department of Agricultural Microbiology and Bioenergy, the College of Agriculture, PJTSAU, Rajendranagar, Hyderabad during *Rabi*-2018 (September 20, to December, 20). The mean temperature during the crop growth period was  $\sim 22 \pm 2^\circ\text{C}$ . The soil was collected from College Farm, PJTSAU, Hyderabad, which had physiochemical properties, namely, red color with pH-7.25, Electrical Conductivity (EC)-0.498 (dS m<sup>-1</sup>), organic carbon (OC) 0.65%, available Nitrogen (N)-148.18 Kg ha<sup>-1</sup>, available phosphorous (P<sub>2</sub>O<sub>5</sub>)-68.22 Kg ha<sup>-1</sup>, available potassium (K<sub>2</sub>O)-270.12 Kg ha<sup>-1</sup>, and bulk density 1.7 mg m<sup>-3</sup>. This soil was autoclaved (121°C for 15 min at 15 psi) three times and cooled before filling into pots, and each pot was filled with 15 kg of soil pot<sup>-1</sup>. Seeds of ADB-542 variety were obtained from RRC, Rajendranagar, Hyderabad. The inoculum was prepared by growing selected isolates in LB broth for 24 h. Treatments were imposed by inoculating the drought susceptible cotton variety (ADB-542) seeds with 2 ml (10<sup>9</sup> CFU/ml) of efficient strains (Whittenbury et al., 1970); seeds without inoculation served as control. Three seeds per pot were sown at a depth of 5 cm, and the experiment was laid out in Complete Randomized Block Design (CRD) with eight treatments (five isolates, consortium, and two controls) and three replications. After 15 days of sowing, drought was imposed by adding PEG-6000 at 10, 20, and 30% in pots with 50% field capacity. After 20 days of sowing, cultures were sprayed on leaves with a hand sprayer at the rate of 25 ml/plant (Holland and Polacco, 1994). The recommended fertilizer (RDF) dose for cotton was 120-60-60 kg of NPK ha<sup>-1</sup>. Irrigations were given based on the soil field capacity status.

### Biometric and Yield Observations

Biometric observations *viz.*, germination percentage (20 DAS), seedling vigor index [Seedling vigor index = (Root length + shoot length) x germination %] as given by Abdulbaki and Andersons (1973) and shoot length (cm), root length (cm), fresh weight (g), dry weight (gm, after oven drying at 60–65°C for 1 h for 3 consequent days), number of leaves, and number of

branches were recorded at 30, 60, and 90 days after sowing in five competitive plants selected at random from each treatment and the mean per plant was worked out. Yield attributes including the number of bolls, boll weight (g), and seed cotton yield (Kg ha<sup>-1</sup>).

### Enumeration of Rhizosphere and Phyllosphere Bacterial Population

The bacterial population count on Tryptic soya Agar (TSA) was recorded at 30, 60, and 90 DAS in soil and leaf samples. In brief, leaf samples and rhizosphere soil samples were collected from eight treatments. Leaf samples were processed as per the procedure given by Yadav et al. (2010), and soil samples were processed as per the procedure laid down by Parmar et al. (2016).

### Proline Estimation

The amount of proline in cotton leaf samples was determined based on proline's reaction with ninhydrin (Bates et al., 1973) at the flowering stage in pot culture (60 Days After Planting). Fresh cotton leaves (0.5 g) were hand-homogenized in 5 ml of 3% (w/v) sulfosalicylic acid. The homogenate was filtered through Whatman no. 2 filter paper. In another test tube, 2 ml of acid ninhydrin and 2 ml of glacial acetic acid were added to the 2 ml of filtrated extract. The resulting mixture was incubated at 100°C in a hot water bath. The reaction was then stopped using an ice bath, and the contents were extracted with 4 ml of toluene and mixed vigorously using a stirrer for 15–20 s. The chromophore containing toluene was aspirated from the aqueous phase and thawed to room temperature, and the absorbance of the solution was measured at 520 nm using a spectrophotometer. The proline concentration was determined from a standard curve prepared with L-proline and expressed as µg/g FW (Fresh weight).

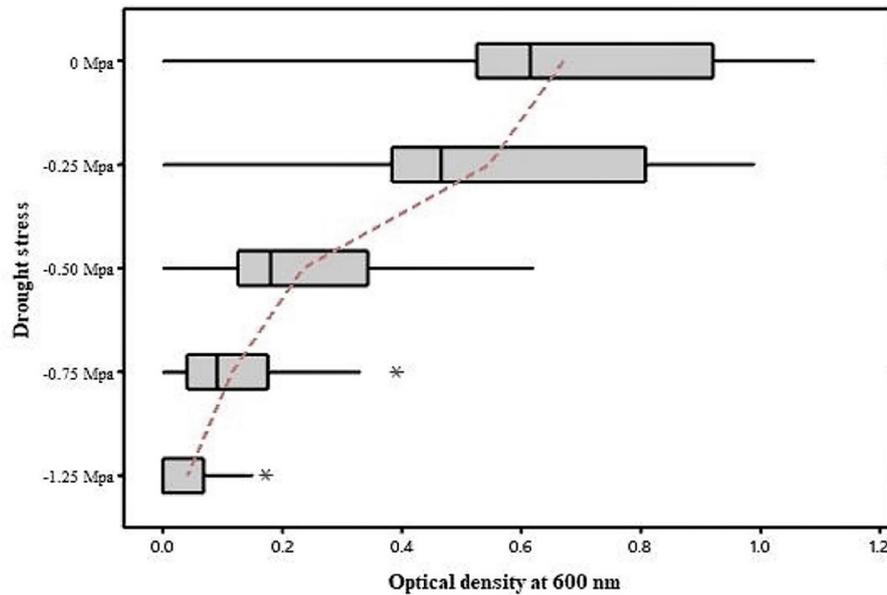
### Statistical Analysis

The data obtained in different treatments were statistically analyzed using a Completely Randomized Design (CRD) described by Yates (1937). Data on other characters *viz.*, growth parameters, NPK analysis, bacterial population, proline estimation, yield attributes, and yield were subjected to analysis of variance (ANOVA) procedures outlined for CRD (wherever necessary). Statistical significance was tested by *p*-value at 0.01 level of probability, and the critical difference was worked out where the effects were significant.

## RESULTS

### Isolation and Screening for Drought Tolerance in Phyllosphere Bacteria

A total of 31 isolates from the Telangana districts of Rangareddy (17 isolates) and Warangal (14 isolates) were isolated and purified based on variations in colony morphology on the AMS medium. In the Gram staining test, seventeen isolates retained crystal violet (Gram-positive) and fourteen isolates retained safranin (Gram-negative). Microscopic studies (Olympus) using an oil immersion lens (Numerical aperture-0.95) revealed that eighteen isolates were rods and thirteen were oval. At 0 Mpa (Megapascal) stress level, all the isolates grew well in TSB, but their growth was slowed as PEG-6000 concentration increased (**Figure 1**).



**FIGURE 1** | Growth of bacterial Isolates under drought stress. As the concentration of PEG-6000 increases, many isolates' growth was inhibited. Few isolates tolerated the higher concentrations of PEG-6000, which were labeled as Drought tolerant bacteria. \*indicates that the isolate that doesn't fall in the mean values.

Growth at  $-0.25$ ,  $-0.50$ ,  $-0.75$ ,  $-1.25$  Mpa stress levels was recorded in spectrophotometer at  $OD_{600}$ , values were in the range of  $0.2$ – $1.09$  (31 isolates),  $0.14$ – $0.99$  (31 isolates),  $0.09$ – $0.62$  (31 isolates),  $0.02$ – $0.39$  (28 isolates), and  $0.02$ – $0.17$  (15 isolates), respectively. Drought tolerant isolates had  $OD_{600}$  values  $>0.10$  at a stress level of  $-1.25$  Mpa. We also noticed that isolates grew well at stress levels ranging from  $-0.25$  to  $-0.75$  Mpa.

Furthermore, these isolates were tested for their ability to develop EPS at a stress level of  $-0.75$  Mpa. Nine isolates were able to produce exopolysaccharides with concentrations ranging from  $0.20$  to  $0.99$   $\text{mg ml}^{-1}$ . Drought stress caused a significant amount of EPS demand. CPB 24 had the most EPS ( $0.99$   $\text{mg ml}^{-1}$ ) under similar conditions (Table 1). The isolates with the maximum *in vitro* drought tolerance and EPS output were selected for *in vivo* cotton growth promotion under drought stress.

## Biochemical and Molecular Characterization of Selected Isolates

The physiological or biochemical characteristics of isolates are illustrated in Table 1. Selective isolates such as CPB 23, CPB 24, CPB 25, CPB 27, and CPB 31 were characterized using 16S rRNA sequencing. BLAST analysis of 16S rRNA isolates showed homology *Pseudomonas stutzeri*, *Acinetobacter* sp., *Bacillus mojavensis*, *Pseudomonas chlororaphis*, and *Enterobacter asburiae*, respectively. These isolates were submitted to NCBI-GenBank and obtained accession numbers i.e., MZ614657, MZ614623, MZ614631, MZ614656, and MZ614653, respectively.

## Inorganic Chemical/Minerals Solubilization

Phosphate solubilizers were characterized by forming a clear halo zone around the colony on Pikovoskay's media after 5 days of

incubation. Fifteen isolates tested positive in this test, with CPB-24 (*Acinetobacter* sp.) having the maximum solubilization of 21 mm (Table 1). Solubilization zones ranged from 2 to 21 mm in diameter. Potassium release on Aleksandrov's medium was recorded in 13 isolates with solubilization zones ranging from 3 to 18 mm, isolate *Acinetobacter* sp., showed a maximum of 18 mm solubilization zone. Zinc phosphate solubilization assay was performed on Tris Minimal media Solubilization, and observation was recorded after 5 days of inoculation. Thirteen isolates were positive for this test. The diameter of solubilization zones was in the range of 3–22 mm. Highest zinc phosphate solubilized by *Enterobacter asburiae* (22 mm).

## Plant Growth-Promoting Attributes

The production of IAA by 31 phyllosphere bacteria was quantified at 530 nm in a spectrophotometer. The isolates released different amounts of indole acetic acid, with 9 preferring to grow in L-tryptophan's presence. They produced significant amounts of IAA in the range of  $3.81$ – $17.93$   $\mu\text{g ml}^{-1}$ . When combined with 0.1% of L-tryptophan, *Acinetobacter* sp. produced the maximum IAA of  $17.93$   $\mu\text{g/ml}$ , followed by *Enterobacter asburiae* with  $14.44$   $\mu\text{g/ml}$ . ACC deaminase activity test was performed on Dworkin and Foster (DF) salts minimal broth and found seven positive isolates viz., CPB 11, *Pseudomonas stutzeri*, *Acinetobacter* sp., *Bacillus mojavensis*, *Pseudomonas chlororaphis*, and *Enterobacter asburiae*. Quantification of ACC deaminase activity was performed based on the production of  $\alpha$ -ketobutyrate through deamination reaction in DF minimal media. The observation was recorded at 540 nm in the spectrophotometer, and ACC deaminase activity was recorded in the range of  $0.13$ – $0.40$  mM of  $\alpha$ -ketobutyrate per milligram of

**TABLE 1** | Biochemical and plant growth attributes: physiological/biochemical responses of efficient isolates and production of EPS, ACC deaminase, IAA, HCN under *in vitro* conditions.

S.No.	Characteristics	Isolate name				
		CPB23	CPB24	CPB25	CPB27	CPB31
1	IMVIC test	+	+	+	+	+
2	Catalase test	+	+	+	+	+
3	Oxidase test	+	+	+	+	+
4	Starch hydrolysis	+	+	+	+	+
5	P-solubilization (mm)	13.00	21.00	2.00	16.00	10.00
6	K-solubilization (mm)	3.00	18.00	3.00	8.00	13.00
7	Zn-solubilization (mm)	7.00	5.00	-	14.00	22.00
8	IAA-production ( $\mu\text{g/ml}$ )	10.59	17.93	10.32	7.19	14.44
9	HCN production	+++	+++	+++	++	+++
10	Siderophore	++	+++	++	+++	+++
11	ACC deaminase ( $\alpha$ -ketobutyrate mM/mg/h)	0.32	0.40	0.28	0.18	0.26
12	EPS production (mg/ml)	0.68	0.99	0.70	0.77	0.82
13	OD <sub>600</sub> @-1.25 Mpa	0.13	0.17	0.11	0.14	0.15
14	16S rRNA identification	<i>Pseudomonas stutzeri</i> <i>Acinetobacter</i> sp. <i>Bacillus mojavensis</i> <i>Pseudomonas chlororaphis</i> , <i>Enterobacter asburiae</i>				

cellular protein per hour. The highest ACC deaminase activity was reported in the bacterial isolate *Acinetobacter* sp. (0.40 mM  $\alpha$ -ketobutyrate mg protein<sup>-1</sup> h<sup>-1</sup>) followed by *Pseudomonas stutzeri* (0.32 mM  $\alpha$ -ketobutyrate mg protein<sup>-1</sup> h<sup>-1</sup>) (Table 1). The highest IAA production was reported in *Acinetobacter* sp. (17.93  $\mu\text{g ml}^{-1}$ ), followed by *Enterobacter asburiae* (14.44  $\mu\text{g ml}^{-1}$ ).

### HCN and Siderophores Production

HCN production was observed in 21 isolates. Eight were classified as vigorous HCN producers and 13 isolates as moderate HCN producers based on the brown color intensity developed in picric acid filter paper. Siderophore production was observed among the 13 isolates; of these, 3 isolates were classified as the highest siderophore producers and 10 isolates as moderate producers based on the size of the yellow halo zone around the colony.

### Effect of Inoculation of ACC Deaminase Producing Isolates on Cotton Growth Under Drought Stress

The influence of the five highest ACC deaminase, IAA producing and phosphate, zinc, and potassium solubilizing PGPR strains, namely, *Pseudomonas stutzeri*, *Acinetobacter* sp., *Bacillus mojavensis*, *Pseudomonas chlororaphis*, and *Enterobacter asburiae*, on growth promotion of cotton under no stress and drought stress conditions, were evaluated through pot trials. Soil nutrient status was improved over control plants. The soil nitrogen (224 kg/ha), phosphorous (99.8 kg/ha), and potassium (547.3 kg/ha) content were highest in *Acinetobacter* sp. treated soils (Table 2). The compatibility test between the isolates was satisfactory and did not find any inhibitory activity between the five efficient isolates.

**TABLE 2** | Details of the nutrient status of soil.

Treatments	N (kg/ha)	P <sub>2</sub> O <sub>5</sub> (kg/ha)	K <sub>2</sub> O (kg/ha)
T <sub>1</sub> : Control	116.3 ± 3.44	41.6 ± 1.10	274.8 ± 15.32
T <sub>2</sub> : Control + 30% PEG-6000	112.8 ± 4.17	27.9 ± 1.03	401.8 ± 12.08
T <sub>3</sub> : <i>Pseudomonas stutzeri</i> + 30% PEG-6000	157.3 ± 6.01	49.8 ± 1.96	389.4 ± 9.50
T <sub>4</sub> : <i>Acinetobacter</i> sp. + 30% PEG-6000	224.0 ± 5.50	99.8 ± 2.02	547.3 ± 5.08
T <sub>5</sub> : <i>Bacillus mojavensis</i> + 30% PEG-6000	126.3 ± 2.69	47.6 ± 0.41	364.2 ± 1.18
T <sub>6</sub> : <i>Pseudomonas chlororaphis</i> + 30% PEG-6000	174.3 ± 7.54	54.3 ± 1.13	390.7 ± 1.22
T <sub>7</sub> : <i>Enterobacter asburiae</i> + 30% PEG-6000	214.5 ± 5.04	91.7 ± 0.31	455.2 ± 10.66
T <sub>8</sub> : Consortium of all isolates + 30% PEG-6000	189.4 ± 3.73	78.9 ± 0.33	401.8 ± 12.08
SE ( $\pm$ m)	4.991	1.219	9.74
CD (0.01)	15.091	3.685	29.45

The adverse effects of drought stress resulted in a reduction in growth and yield parameters such as germination percentage, seedling vigor index, number of bolls per plant, boll weight, seed cotton yield per plant, plant height, root length, number of leaves, number of branches, fresh and dry weight of root and shoot of cotton plants in uninoculated plants compared to non stressed cotton plants. However, the plant growth and yield parameters in root and shoot length, fresh and dry weight, boll weight, and cotton yield were significantly ( $p < 0.01$ ) increased in bacterial inoculated plants under drought-stressed conditions. The isolate *Acinetobacter* sp. application enhanced cotton plant growth exposed to drought-stressed conditions (30% PEG-6000).

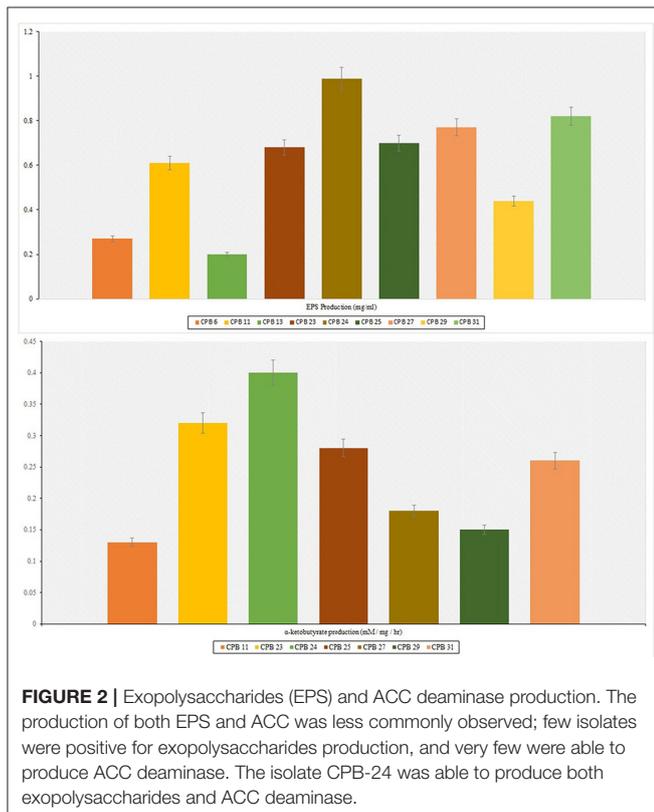
**TABLE 3 |** Influence of phyllosphere bacteria on the cotton crop: significant increase in fresh and dry weights of cotton in bacteria primed treatments.

Treatments	Fresh weights (g/plant)			Dry weight (g/plant)			Total bacterial population (soil)			Total bacterial Population (Leaf)			Boll weight (g)	Seed cotton yield plant <sup>-1</sup> (g)	Seed cotton yield (Kg ha <sup>-1</sup> )
	30 DAS	60 DAS	90 DAS	30 DAS	60 DAS	90 DAS	30 DAS	60 DAS	90 DAS	30 DAS	60 DAS	90 DAS			
T <sub>1</sub> : Control	1.98 ± 0.06	3.62 ± 0.05	7.12 ± 0.06	0.3 ± 0.01	1.22 ± 0.01	2.41 ± 0.01	2.13 ± 0.04	3.1 ± 0.03	2.79 ± 0.02	1.57 ± 0.04	2.13 ± 0.01	1.79 ± 0.03	4.36 ± 0.03	36.26 ± 0.8	727.01 ± 14.7
T <sub>2</sub> : Control + 30% PEG-6000	1.43 ± 0.05	3.12 ± 0.06	6.6 ± 0.11	0.22 ± 0.01	1.08 ± 0.02	2.29 ± 0.01	1.96 ± 0.02	2.16 ± 0.02	2.03 ± 0.04	1.1 ± 0.01	1.94 ± 0.03	1.61 ± 0.02	3.7 ± 0.37	28.66 ± 0.5	530.74 ± 11.5
T <sub>3</sub> : <i>Pseudomonas stutzeri</i> + 30% PEG-6000	2.33 ± 0.01	4.2 ± 0.02	8.23 ± 0.02	0.42 ± 0.01	1.34 ± 0.01	2.72 ± 0.02	2.28 ± 0.03	3.7 ± 0.04	3.2 ± 0.02	2.03 ± 0.03	3.75 ± 0.02	3.2 ± 0.10	4.83 ± 0.19	41.86 ± 0.8	775.18 ± 6.5
T <sub>4</sub> : <i>Acinetobacter</i> sp. + 30% PEG-6000	4.6 ± 0.17	7.56 ± 0.29	11.36 ± 0.05	0.96 ± 0.02	1.9 ± 0.02	3.93 ± 0.02	4.65 ± 0.07	5.7 ± 0.04	5.2 ± 0.03	4.75 ± 0.02	6.25 ± 0.01	5.6 ± 0.03	5.36 ± 0.03	55.5 ± 0.56	1027.77 ± 8.3
T <sub>5</sub> : <i>Bacillus mojavensis</i> + 30% PEG-6000	2.2 ± 0.13	4.62 ± 0.04	7.76 ± 0.04	0.39 ± 0.02	1.43 ± 0.01	2.63 ± 0.2	2.17 ± 0.03	3.4 ± 0.01	3.05 ± 0.02	1.84 ± 0.02	3.5 ± 0.01	2.47 ± 0.01	4.53 ± 0.01	41.4 ± 0.28	766.66 ± 12.4
T <sub>6</sub> : <i>Pseudomonas chlororaphis</i> + 30% PEG-6000	2.87 ± 0.05	5.56 ± 0.03	9.2 ± 0.05	0.68 ± 0.01	1.64 ± 0.01	3.07 ± 0.02	3.75 ± 0.02	4.4 ± 0.01	4 ± 0.07	3.15 ± 0.02	4.35 ± 0.03	3.95 ± 0.03	4.93 ± 0.02	44.9 ± 0.63	831.48 ± 2.55
T <sub>7</sub> : <i>Enterobacter asburiae</i> + 30% PEG-6000	3.37 ± 0.09	5.96 ± 0.03	9.9 ± 0.04	0.82 ± 0.01	1.78 ± 0.006	3.26 ± 0.01	4.05 ± 0.03	5.3 ± 0.02	4.65 ± 0.03	4.35 ± 0.03	5.7 ± 0.01	4.95 ± 0.04	5.1 ± 0.03	47.8 ± 0.51	885.18 ± 4.7
T <sub>8</sub> : Consortium of all isolates + 30% PEG-6000	2.54 ± 0.05	5.2 ± 0.02	8.76 ± 0.01	0.53 ± 0.01	1.52 ± 0.009	2.88 ± 0.06	3.4 ± 0.01	4.25 ± 0.02	3.8 ± 0.01	3.7 ± 0.02	4.8 ± 0.03	3.75 ± 0.03	5.03 ± 0.03	45.83 ± 0.54	848.7 ± 3.1
SE (±m)	0.039	0.052	0.168	0.009	0.033	0.081	0.082	0.088	0.085	0.074	0.083	0.078	0.12	0.68	19.86
CD (0.01)	0.122	0.153	0.524	0.021	0.117	0.255	0.261	0.292	0.276	0.225	0.268	0.242	0.44	2.02	74.1

*Efficient phyllosphere bacteria positively influenced the bacterial population in soil and leaves.*

The seed germination was significantly higher in T<sub>4</sub> (*Acinetobacter* sp., + 30% PEG-6000) 76.67%, which was 17% higher than the control treatment. The seedling vigor index increased by 45.81% in T<sub>4</sub> compared to the control under drought stress conditions. The plant height had improved ( $P < 0.01$ ) by 10% in T<sub>4</sub> treatment than corresponding uninoculated control plants. However, no significant difference was found in the case of shoot length between individual strain applications.

Similarly, the isolate *Acinetobacter* sp. treatment had a tremendous influence on root length, number of leaves, and the number of secondary branches of cotton plants on exposure to drought-stressed conditions. The root length was increased by ~28.52% in T<sub>4</sub> treatment than controls with an unstressed and stressed plants group. Furthermore, increased fresh and dry weights under drought stress were recorded in *Acinetobacter* sp. and *Enterobacter asburiae* treated cotton plants (Table 3). Fresh weight was significantly ( $p < 0.01$ ) increased by 41.9 and 33.3%, while dry weight improved by 41.7 and 29.7% at T<sub>4</sub> and T<sub>7</sub> treatments when compared to the stressed control (T<sub>2</sub>). A significant increase in the proline content was reported in inoculated cotton plants over the control plants. The proline content in cotton plants under drought stress was increased by 28.78% in T<sub>4</sub> treatment, followed by 25.39 at Treatment T<sub>7</sub>. The number of bolls and boll weight were significantly higher in *Acinetobacter* sp., treated cotton plants (Table 3). The number of bolls was markedly higher in T<sub>4</sub> (12.66 per plant), followed by T<sub>7</sub> (11.66 per plant). The boll weight in cotton was highest in T<sub>4</sub> (5.36 g), followed by T<sub>7</sub> (5.10 g). Similarly, the seed cotton yield was highest in T<sub>4</sub> (1027.77 kg ha<sup>-1</sup>).



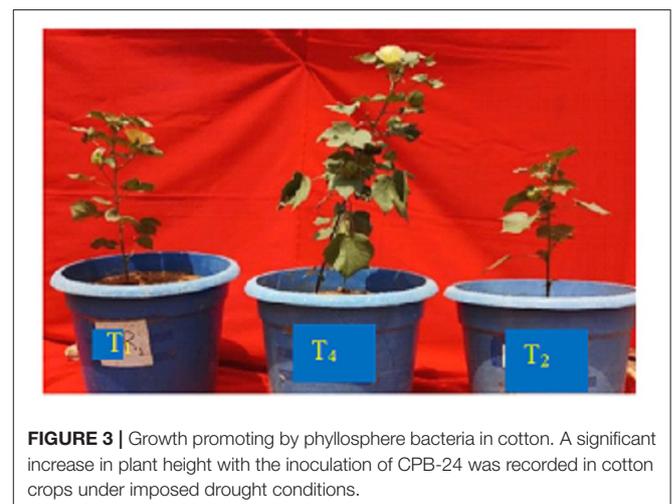
## Bacterial Population Dynamics in Rhizosphere and Phyllosphere

Cultivable bacterial populations in rhizosphere and phyllosphere samples were analyzed on Nutrient Agar plates. The bacterial population in the rhizosphere and phyllosphere samples were increased up to 60 days of sowing in all treatments; the maximum population was reported in *Acinetobacter* sp., treated cotton plants (Table 3). The cultivable bacterial population in the rhizosphere and phyllosphere were recorded at 60 DAS as  $5.70 \times 10^7$  and  $6.25 \times 10^5$  CFU gm<sup>-1</sup>.

## DISCUSSION

Drought conditions are better controlled by the soil application of microorganisms as drought mainly refers to soil conditions. In addition, soil application will enhance their chances of survival in the rhizosphere, as the rhizosphere is a nutrient-rich environment and better coordinates with plants' defense mechanisms to control soil-borne pathogens. The phyllosphere microorganisms with ACC deaminase activity play a significant role in promoting plants grown under different stress conditions (Chandra et al., 2018). When the plant is under stress, an intermediate compound of ethylene called 1-aminocyclopropane-carboxylic acid (ACC) makes its way into the rhizosphere as a root exudate. Rhizosphere microbes produce a special kind of enzyme called ACC deaminase, which breaks ACC and produces ammonia and α-ketobutyrate (Zhang et al., 2010). Ultimately, the ethylene production in plants was interrupted by rhizosphere microorganisms and usually helped the plant function (Barnawal et al., 2014).

In the present work, 31 phyllosphere bacterial isolates were tested for drought tolerance under *in vitro* conditions. Five isolates grew at high moisture stress (−1.25 Mpa) and were identified as *Pseudomonas stutzeri*, *Acinetobacter* sp., *Bacillus mojavensis*, *Pseudomonas chlororaphis*, and *Enterobacter asburiae* based on BLAST analysis. The cotton plants inoculated with the highest EPS (0.99 mg ml<sup>-1</sup>) and ACC deaminase-producing (Figure 2) isolate *Acinetobacter* sp., (CPB 24) showed maximum

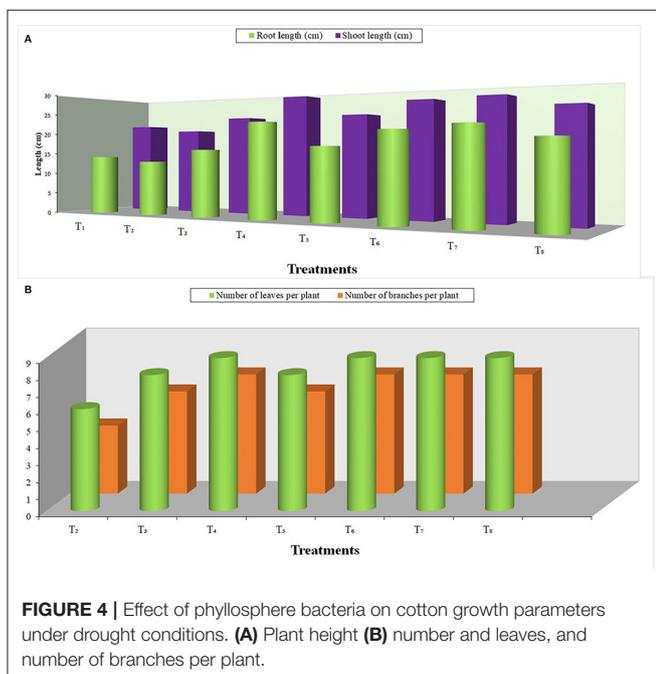


nutrient uptake and soil nutrient status. Bashan et al. (2004) reported, inoculation with EPS producing *Azospirillum* sp. increased soil nutrient status, structure, and aggregation. Several strains of *Pseudomonas* sp., *Bacillus* sp., and *Acinetobacter* sp. were reported to produce EPS, IAA, ACC deaminase, and solubilize insoluble minerals under different strains (Verma et al., 2018; Nagaraju et al., 2020). The precursor (L-tryptophan) concentration directly affects IAA produced (Nagaraju et al., 2020). According to current research, isolates preferred to grow at a five mM concentration of L-tryptophan and generated variable amounts of IAA, which was confirmed spectrophotometrically at 600 nm optical density.

In many cultivated plants, nutrient uptake was impeded by drought stress; however, in the current study, inoculated plants showed increased plant nutrient status and fresh and dry weights. The highest levels of soil nitrogen, phosphorous, and potassium were recorded in *Acinetobacter* sp. treated plots. Concomitant observations made under *in vitro* conditions showed that phosphorous and potassium solubilization were highest in the same treatment. Earlier reports suggested that phosphate solubilization and chelation by beneficial soil microorganisms are the primary reason for the higher nutrient status in soil (Kour et al., 2020). Several findings support early reports on zinc, phosphate, and potassium solubilization and confirm their universal soil occupancy (Kour et al., 2019). Solubilization of the minerals/chemicals are accomplished by lowering the pH of the surroundings, production of several organic acids by *Pseudomonas* sp., *Enterobacter* sp., and *Bacillus* sp., was reported by Nagaraju et al. (2016, 2020). In addition, several microbes produce siderophores when the available forms of iron and zinc are limited in soil (Priyanka et al., 2015). Several reports have highlighted the importance of siderophore production in

promoting plant growth. However, little work has been carried out under drought-stress conditions. In the present investigation, we highlighted the production of siderophores by drought-tolerant phyllosphere bacteria.

Intergenic compatibility i.e., seed and microbes, were tested by inoculating drought-tolerant PGPR bacterial isolates to seeds under drought stress and found a significant improvement in germination percentage and seedling vigor, compared with the untreated control. The compatibility between the bacterial isolates was confirmed in the dual culture test and recorded their cumulative positive effects in cotton crop growth when applied as a consortium under a pot culture experiment (Figure 3). In nature, microbes work synergistically with some microbes (Morris et al., 2013). Thus, applying a combination of two or more beneficial microbes to higher populations may positively impact the growth and yield of crops under different stresses (Triveni et al., 2013; Barra et al., 2016; Nagaraju et al., 2020). However, several researchers have reported that individual cultures might perform on par or higher than consortium because of their variable inhibitory activity under *in vivo* conditions when compared to *in vitro* conditions (Kour et al., 2019). In the current investigation, mono-inoculation with *Acinetobacter* sp. individual culture inoculated plants outperformed the control. In the present research, control plants showed the lowest root and shoot length and recorded the lowest proline content. Plants produce stress hormones under drought stress, which result in decreased yields. Several researchers suggest that applying plant growth-promoting bacterial isolates manifests ethylene production in plants and thus confers drought tolerance and crop growth (Safari et al., 2018). In the pot culture experiment, seed bacterization of PGP bacterial isolates enhanced the root, shoot length, fresh and dry weights, boll weight, number of bolls per plant, number of leaves per plant, and secondary branches per plant because of their multifarious PGP traits (Figure 4). Our study emphasizes that using a single strain or a consortium enhances crop growth under stressed and non-stressed conditions. Therefore, the application of stress-bursting microbes induces tolerance and increases crop growth under harsh climatic conditions. Furthermore, these can be multiplied and commercialized as biofertilizers or bio inoculants after careful evaluation in pot and field trials.



## CONCLUSIONS

The current study reports on the isolation of drought-tolerant ACC deaminase-producing bacteria from the phyllosphere of cotton. Five potential strains, *Pseudomonas stutzeri*, *Acinetobacter* sp., *Bacillus mojavensis*, *Pseudomonas chlororaphis*, and *Enterobacter asburiae*, were found to have plant growth-promoting potentials such as siderophore production, IAA production, phosphate, zinc, and potassium solubilization. Screening for drought tolerance in pot culture conditions revealed that inoculating drought-tolerant isolates improved cotton crop growth and yield. It is also unclear if inoculating other plants with these drought-tolerant isolates has a comparable effect on plants other than cotton, allowing them

to produce more grain or fruit under various stresses. As a result, more emphasis should be given to multi-location trials with multiple crops in future research.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in NCBI-GenBank online repositories. The names of the NCBI-GenBank

and accession number(s)- MZ614657, MZ614623, MZ614631, MZ614656, and MZ614653 can be found in the article.

## AUTHOR CONTRIBUTIONS

SS conducted all the experiments under the guidance of ST. ST, PL, and BV were involved in designing the experiment. YN analyzed the data and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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