

# N-acyl Homoserine Lactone Mediated Quorum Sensing Exhibiting Plant Growth-promoting and Abiotic Stress Tolerant Bacteria Demonstrates Drought Stress Amelioration

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## Abstract

Multiple plant growth-promoting attributes with N-acyl homoserine lactone (AHL)-mediated quorum sensing exhibiting bacterial strains can help plants to withstand varying abiotic and biotic stress conditions for improving the plant health and productivity. In total, 306 bacterial isolates were isolated from diverse locations and sites. In our exploration, bacterial isolates were screened based on AHL production, plant growth-promoting attributes, abiotic stress tolerance, and antagonistic activity against phytopathogenic fungi. Among the screened 306 isolates, 4 (11VPKHP4, 7VP51.8, P51.10, NBRI N7) were selected based on their efficiency in AHL production, biofilm formation, enduring different abiotic stress conditions, exhibiting plant growth-promoting attributes, and antagonistic activity. Based on 16S rRNA gene sequencing analyses of the selected 4 isolates belong to *Pseudomonas* genera. Selected isolates 11VPKHP4, 7VP51.8, P51.10, and NBRI N7 were also proficient in biosurfactant production, emulsification, suggesting that all isolates fabricate emulsifiers. The plant growth promotion potential of selected 4 bacterial isolates showed significant growth enhancement in all the vegetative parameters of *Zea mays* under control as well as drought stress condition. Biochemical parameters and defense enzymes under drought stress conditions were also modulated in the PGPR treated plants as compared to their uninoculated respective controls. With quorum sensing, multiple PGPR attributes, stress tolerance, biofilm formation, and EPS production the selected isolates have the potential to facilitate enhanced plant growth, rhizosphere colonization, maintenance of soil moisture content under normal and diverse stresses.

**Keywords:** Quorum sensing, AHL, PGPR, Biofilm, Drought

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## INTRODUCTION

All living beings on this planet are affected by varying climatic conditions such as prolonged drought, intense rains, flooding, high and low temperatures that also exert a detrimental impact on plants as well as on soil microbes too. In the coming years, globally it is expected to have double food demand<sup>1</sup> and crop productivity loss by 30%, due to abiotic stresses.<sup>2</sup> Among all abiotic stresses, drought is a major constrain,<sup>1,3</sup> and drought-affected arable land has increased more than twice from the 1970s to early 2000s, and the unaffected land areas are expected to reduce to half by 2050, presenting a great threat to worldwide crop productivity.<sup>4</sup> Drought stress affects *Zea mays* growth during vegetative and reproductive growth<sup>5</sup> by altering morphological responses and physiological functions.<sup>6</sup> Reactive oxygen species (ROS) produced by plants cause injury to macromolecules and DNA<sup>7</sup> facing drought stress. Many approaches are being used to cope up with this critical condition and among them, researchers are focusing on agro-eco-friendly sustainable technology by exploitation of plant growth-promoting rhizobacteria (PGPR) based formulations for improving stress amelioration, soil fertility, and crop productivity.

PGPR possesses multiple plant growth-promoting attributes including phosphate solubilization, phytohormone and siderophore production, nitrogen fixation, nutrients immobilization, exopolysaccharide production, biofilm formation.<sup>8-12</sup> PGPR with multiple PGP attributes, enzymatic activities, and abiotic stress tolerance assists host plants in maintaining their health, growth, and development under normal as well as under stressed conditions. Among several reported PGPR, rhizobacteria belonging to *Pseudomonas* genera are very well worked out and considered as plant probiotics.<sup>13</sup> Earlier studies have explored the importance of intercommunication between bacteria in respect to plant growth promotion. Signaling occurs between microbe-microbe: intra- and interspecies via diverse, small molecules like autoinducers, acyl-homoserine lactones (AHL). Quorum sensing via AHL molecules, typically produced by *Pseudomonas* genera in a cell-density-dependent manner, regulates numerous physiological processes including biofilm

formation, virulence, competence, stationary phase, biosurfactant production, antibiotic production, hydrolytic enzymes, catalase activity, motility.<sup>14,15</sup> Among genus *Pseudomonas*, quorum sensing is very well depicted in *P. aeruginosa*, *P. putida*, *P. aureofaciens*, *P. fluorescens*, *P. syringae*, *P. corrugata* and *P. fuscovaginae* and reported to produce AHLs. Apart from genus *Pseudomonas*, this sort of signaling has also been recognized in *Agrobacterium* and *Rhizobium*, *Ochrobactrum*, *Serratia*, and *Ensifer*.<sup>14,16,17</sup> Root colonizing *Pseudomonads* together with multifarious plant growth-promoting attributes (abiotic stress endurance, biofilm formation, EPS and alginate, phosphate solubilization, IAA production, siderophore production, enzymatic activities, biosurfactant production, and inorganic pollutant degradation) will assist plant growth and development in diverse stress curse conditions. The PGPR endow plant defense by inhibiting the antioxidant biochemical system and enhancing the photosynthetic physiological system under stress conditions. Among all PGPR, the *Pseudomonas* genus has been well characterized to enhance total plant vegetative parameters in *Zea mays*, finger millet, sunflower, peas, and ornamental crops facing drought and resource limiting conditions.<sup>18-22</sup> Strains from *Pseudomonas* genera were also explored for stress amelioration in maize, pea, wheat, mung bean, tomato.<sup>23,24</sup> Based on earlier researches these bacteria have been isolated from different sources and characterized for the presence of PGP attributes but different geographical locations possessing QS exhibiting *Pseudomonas* spp. are not yet reported. Moreover, very negligible information on the role of stress-tolerant, quorum sensing exhibiting *Pseudomonas* spp. in enhancing *Zea mays* growth under drought stress was reported. Therefore, our main objective of the present study is to exploit the QS exhibiting *Pseudomonas* spp. from different geographical locations of India to characterize them for enhancing the drought stress tolerance in *Zea mays*.

## MATERIALS AND METHODS

### Soil samples collection and isolation of bacteria

The soil samples for the isolation of bacteria were collected from various geographic locations from India as depicted in Table S1. These

samples were then subjected to sieving with 2 mm mesh to separate plant debris and fauna. After sieving, each was used for the isolation of bacteria. Soil samples were collected in properly labeled sterile polythene sampling bags. For isolation of bacteria from collected soil samples, 1g of each soil sample was serially diluted in sterile saline water (0.85% NaCl) followed by shaking at 150 rpm for 15 min. 100 µL of each dilution was spread on Nutrient Agar (NA; Hi-Media, India) and Petri plates were incubated at 28°C for 24 h. Pure cultures of the bacterial isolates were maintained on NA and simultaneously in 30% glycerol for preservation and stored at -80°C.

#### **Estimation of plant growth-promoting attributes, abiotic stress tolerance, and antagonistic activity of bacterial isolates**

Bacterial isolates were subjected to qualitative evaluation for a wide array of PGP traits, abiotic stress tolerance, and antagonistic activity. Among PGR attributes, bacterial isolates were mainly evaluated for phosphate solubilisation<sup>25</sup> and indole acetic acid (IAA) production.<sup>26</sup> Further, all the isolates were examined for their tolerance against abiotic stresses such as drought/water stress (30%, 45%, and 60% polyethylene glycol 6000; w/v), salt (8% and 12% NaCl; w/v), temperature (37°C and 45°C), and pH (pH3, pH5, pH9, and pH11). All the bacterial isolates were grown in a nutrient broth for 24 h with the continuous shaking of 180 rpm at 28°C (except those tested for temperature stress). Viable cells (CFU/ml) of selected bacterial isolates were counted at different time intervals of 24 h up to 10 days by serial dilution plating on nutrient agar plates. The antagonistic activity was evaluated by comparing the fungal mycelial diameter on control and test plates where all the isolates were streaked on the periphery of Potato Dextrose Agar medium (PDA; HiMedia, India) while the pathogenic fungal

mycelium of *Rhizoctonia solani* and *Sclerotium rolfsii* at the center. The plates were incubated at 28°C for 7 days. Control plates were prepared with the fungal agar plug without the bacterial streaks. Screening and selection of bacterial isolates demonstrate quorum sensing

Two biosensors, *Agrobacterium tumefaciens* (A136; for short-chain AHLs) and *Chromobacterium violaceum* (CV026; for long-chain AHLs) were used to screen AHL production of all bacterial isolates. Screening of all the bacterial isolates for production AHLs was carried out by agar plate diffusion assay with some modifications.<sup>27</sup> The test isolates for induction of AHL of CV026 were streaked on Luria agar (LA; Hi media, India) plates in parallel to the monitor biosensor. Similarly, discovering for AHL production against A136 was done in an analogous mode by supplementing the LA with 45 µg/mL of X-gal (Sigma Aldrich). *Agrobacterium tumefaciens* KYC6 used as a positive control (AHL producer) and *P. putida* as a negative control. The Petri plates were incubated for 24 to 48 h at 28°C and the production of purple pigment violacein and blue coloration by β-galactosidase activity was observed as a positive test for AHLs production.<sup>27</sup> Quantitative evaluation of PGP attributes of bacterial isolates

Selected bacterial isolates were quantitatively evaluated for biofilm formation,<sup>28</sup> exopolysaccharide,<sup>29</sup> and alginate<sup>30</sup> production. Production of auxin/ indole acetic acid (IAA),<sup>26</sup> P-solubilization,<sup>25</sup> and siderophore synthesis<sup>31</sup> were also determined to understand their potential to be utilized for protecting the host plant under stressed conditions.

#### **Evaluation of other characteristic features of selected bacterial isolates**

After estimating bacterial isolates for their

**Table 1.** Sampling sites along with their geographical coordinates, selected 4 strains with their corresponding 16S rRNA gene Gen Bank nucleotide accession numbers and closest taxonomic relationship

Sample site	Location	Strains	Accession No.	Identification
Kasol, Himachal Pradesh	32.00N, 77.18E	11VPKHP4	MZ268103	<i>P. Sesamii</i>
Nagpur	18.31N, 73.50E	7VP51.8	MZ268104	<i>P. hunansii</i>
Pune agriculture field	73.50E	P51.10	MZ268105	<i>P. asiatica</i>
Kutch, Gujrat	23.73N, 69.85E	NBRI N7	MZ268106	<i>P. aeruginosa</i>

PGP traits, they were subjected to drop collapse assay for biosurfactant production described by Jain et al.<sup>32</sup> and modified by Bodour and Miller-Maier.<sup>33</sup> In addition, the emulsification index (E24) was determined by the method described by Cooper and Goldenberg.<sup>34</sup> All the selected bacterial isolates were subjected to evaluation for swimming, swarming, and twitching motility based on zone diameter formation.<sup>34</sup> The activity of 1-aminocyclopropane-1-carboxylate (ACC) deaminase among all bacteria was qualitatively estimated by following the protocol of Penrose and Glick.<sup>36</sup> Furthermore, selected bacterial isolates were qualitatively evaluated for protease, and cellulose production respectively.<sup>37,38</sup> Catalase production was investigated by adding and assorting a drop of 3% hydrogen peroxide to 48h grown bacterial colony on an uncontaminated glass slide. The presence of effervescence is the indicator of catalase activity. Gelatinase activity was determined by preparing and inoculating bacterial isolates in gelatine tubes. Inoculated tubes were incubated at 28°C for 48h. After the incubation period, all tubes were kept on ice. Tubes that get solidified were considered gelatinase positive. Nitrate reduction was scrutinized by using the mannitol motility nitrate medium (MMNM; M1320, HiMedia, India).

#### **16S rRNA gene PCR amplification and phylogenetic analysis of selected isolates**

All the selected bacterial isolates were evaluated for 16s rRNA gene sequence amplification. For this bacterial genomic DNA was extracted from 24 h grown culture using a DNA isolation kit (Qiagen) and amplification was carried out for 16s rRNA gene using primers: forward (8F) primer (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse (1392R) primer (5'-ACGGGCGGTGTGTAC-3').<sup>39</sup> Further, PCR products were purified by QIAquick® PCR purification kit (Qiagen, Germany) followed by sequencing (3730XL DNA analyzer, Applied Biosystems, USA). Further, the sequences were compared with reference type isolate sequences available 16S rRNA database (<https://www.eztaxon.org>).

#### **Evaluation of bacterial isolates for plant growth promotion under greenhouse condition**

Following the screening of bacterial isolates based on their PGP attributes, quorum sensing activity via acyl-homoserine production,

and other attributes *in vitro*, the selected ones were tested for their capability of PGP under plant test using *Zea mays* ("Maharaja") as a model plant under control and water stress conditions. Experiments were accomplished in a wholly randomized block design under greenhouse conditions with 4 replicates using non-sterile field soil (2.5 kg soil per pot) from the experimental farm of CSIR-National Botanical Research Institute, Lucknow. Surface sterilized *Z. mays* seeds were bacterized with four AHL producing isolates i.e., 11VPKHP4; 7VP51.8; P51.10; NBRI N7 as described earlier.<sup>40</sup> Simultaneously, two sets with 4 replicates for each treatment and control were grown in a greenhouse for 45 days. After 45 days of seed sowing, drought stress for fifteen days was given by refraining the irrigation in the water stress set. After 60 days of the plantation, plants were harvested from both sets i.e. non-stress as well as drought stress conditions. Vegetative parameters such as shoot and root length, fresh and dry shoot, and root weight were instantly taken and compared with the treated and control plants of both non-stress (NS) and drought stress (DS). For evaluation of dry weights, roots and shoots were kept at 50°C in a hot air oven.

#### **Plant biochemical and antioxidant enzymatic assays**

Total chlorophyll and carotenoid content in the leaves were determined accordingly by the protocol of Arnon.<sup>41</sup> The proline and soluble sugar assay for leaf tissue were determined accordingly protocols of Bates et al.<sup>42</sup> and Dubois et al.<sup>43</sup> For determining different antioxidant enzyme activity spectrophotometrically, 500 mg of fresh tissue was homogenized with 100 mM of potassium phosphate buffer (pH 7) containing 0.1 mM EDTA and 1% polyvinylpyrrolidone (w/v) under chilled conditions followed by centrifugation at 15,000 g for 10 min. at 4°C. The collected supernatant was stored at -20°C for further analysis of Superoxide dismutase (EC1.15.1.1), Ascorbate peroxidase (EC1.11.1.11), Guaiacol peroxidase (EC 1.11.1.9) and Catalase (EC1.11.1.6) according to protocols of Beauchamp and Fridovich,<sup>44</sup> Nakano and Asada,<sup>45</sup> Beyer, and Fridovich,<sup>46</sup> Aebi,<sup>47</sup> respectively.

#### **Statistical analyses**

All data sets in this study were analyzed using SPSS ver 20.0. Initially, means were tested for homogeneity of variance to evaluate the

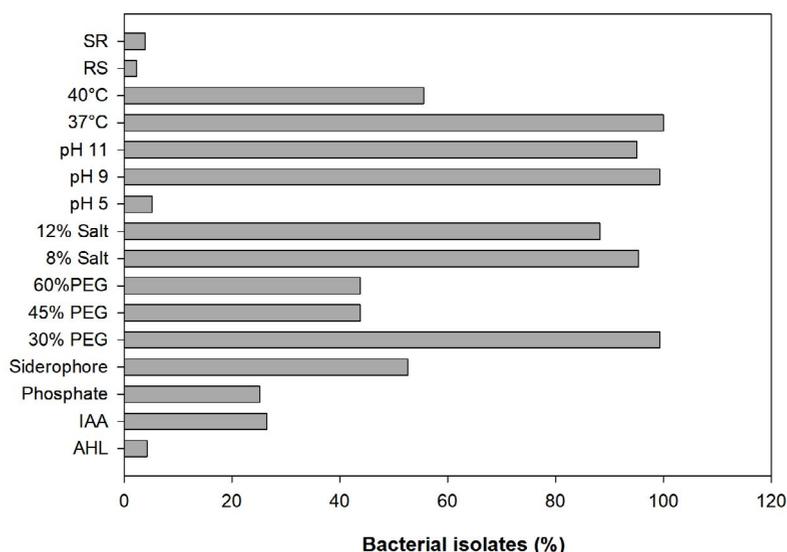
variation among obtained values. Further, these means were compared by analysis of variance (ANOVA), followed by Duncan's test to determine significance ( $p \leq 0.05$ ).

## RESULTS

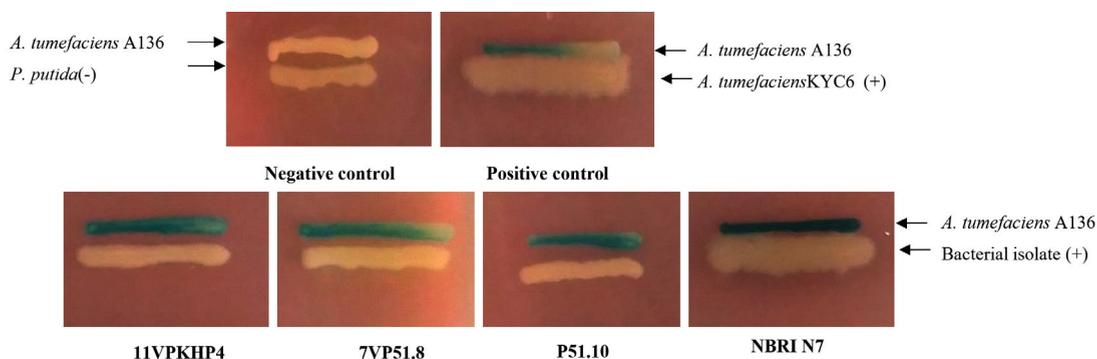
### Isolation and *in vitro* screening of bacterial isolates for AHL production, PGP attributes, abiotic stress tolerance, and antagonistic activity

In the present study, a total of 306 bacterial isolates were (Supplementary Table S1) screened for AHL production, plant growth-promoting attributes, abiotic stress tolerance, and antagonistic activity. Results revealed that only 4 out of 306 bacterial isolates were identified as AHL

producers with A136; coupling of the *traR* gene (*luxR* homolog) with *lacZ* provides a basis for the detection of acyl-homoserine lactones based on  $\beta$ -galactosidase activity (Fig. 1 and 2) whereas, none of the isolates were found to be positive with CV026. Screening of PGP attributes i.e. IAA production, P solubilization, and siderophore production for all the isolates was carried out. Among 306 isolates, 26.47% exhibited IAA production while 25.16% were able to solubilize phosphate (Fig. 1). Isolates were screened for siderophore production by chrome azurol S (CAS) assay, reported 52.61% out of total 306 isolates positive (Fig. 1). Drought stress tolerance was exhibited by 99.34% isolates at 30% PEG 6000



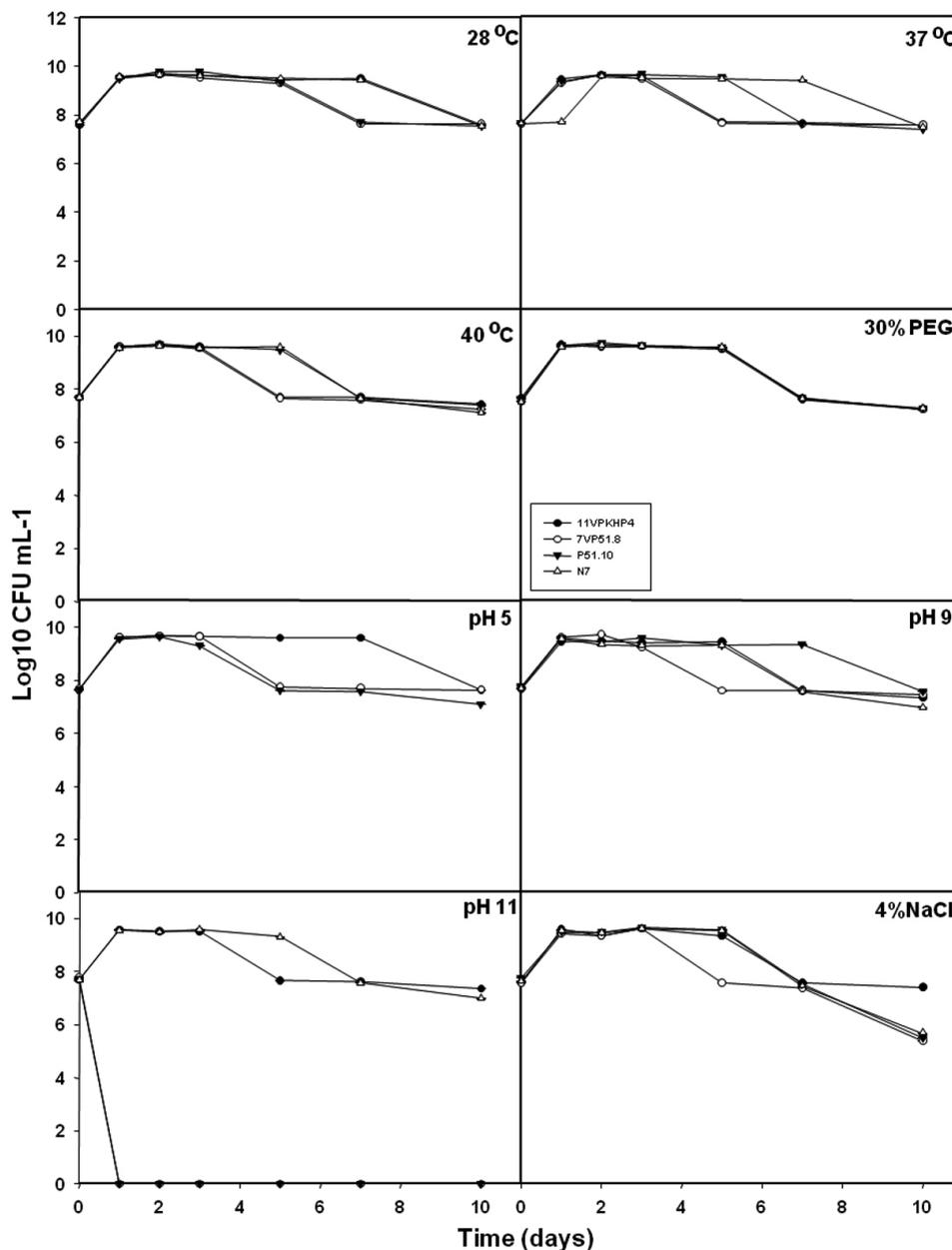
**Fig. 1.** Qualitative evaluation for PGP attributes and stress tolerance of all bacterial isolates.



**Fig.2.** Qualitative evaluation for screening of acyl-homoserine lactone production in all bacterial isolates using *Agrobacterium tumefaciens* (A136). (-) non-AHL producer, (+) AHL producer.

(w/v) while 43.79% isolates at 45% and 60% PEG 6000 (w/v) (Fig. 1). Salt stress tolerance showed by 95.42% and 88.23% isolates at 8% and 12% NaCl (w/v) and 5.22%, 99.34% and 95.09% isolates tolerant at pH 5, 9 and 11, respectively (Fig. 1). All isolates were able to grow at 37°C and 40°C while 55.55% were able to survive at 45°C. In the

case of biotic stress, 2.28% and 3.92% isolates exhibited antagonistic activity against *Rhizoctonia solanii* and *Sclerotium rofsii*, respectively (Fig. 1). Therefore, results of qualitative screening of 306 bacterial isolates lead to the selection of 4 isolates demonstrating AHL production together with PGP attributes and abiotic stress tolerance.



**Fig. 3.** Effect of abiotic stress on the growth pattern of selected bacterial isolates.

### Abiotic stress tolerance of AHL producing selected bacterial isolates

Further, selected 4 isolates were subjected for their abiotic stress tolerance i.e., temperature (37 and 40°C), drought (30%), pH (5, 9, and 11), and salt (NaCl 4%). Under control conditions, all isolates survived with 7 Log<sub>10</sub> CFU ml<sup>-1</sup> for over 10 days of the experiment (Fig. 3). At temperature 37°C and 40°C, all isolates demonstrated their survival between 7.09 to 7.42 Log<sub>10</sub> CFU ml<sup>-1</sup> for over the 10<sup>th</sup> day (Fig. 3). Under drought stress i.e., 30% PEG, all isolates were competent to survive at the end of the 10<sup>th</sup> day with 7.22 to 7.27 Log<sub>10</sub> CFU ml<sup>-1</sup>. At pH 5 and pH9, all isolates were able to survive over the 10<sup>th</sup> day with 7 Log<sub>10</sub> CFU ml<sup>-1</sup> (Fig. 3) but at pH11, only 11VPKHP4 and NBRI N7

have shown their survival at the end of the 10<sup>th</sup> day with 7.34 Log<sub>10</sub> CFU ml<sup>-1</sup> and 6.98 Log<sub>10</sub> CFU ml<sup>-1</sup> respectively (Fig. 3). Moreover, at 4% salt stress, 11VPKHP4 was competent to survive with 7.41 Log<sub>10</sub> CFU ml<sup>-1</sup> whereas 7VP51.8, P51.10, and NBRI N7 with 5.37, 5.51, and 5.68 Log<sub>10</sub> CFU ml<sup>-1</sup> at the end of the 10<sup>th</sup> day (Fig. 3). Further, four best performing bacterial isolates representing different geographic locations were selected based on their AHL producing ability, PGP, and abiotic stress tolerance ability.

### Identification of bacterial isolates

All four selected potential AHL producers were subjected for identification based on partial 16S rDNA sequences, revealed that all the bacterial isolates belong to *Pseudomonas* spp. 11VPKHP4,

**Table 2.** PGP attributes of selected AHL producing PGPR strains

PGP attributes	11VPKHP4	7VP51.8	P51.10	NBRI N7
Biofilm formation (O.D. at 590 nm)	2.09±0.04b	1.33±0.008a	2.09±0.03b	1.33±0.03a
Exopolysaccharide production (µg/ml)	256.62±0.46d	127.27±0.07b	120.12±0.32a	146.16±0.61c
Alginate (µg/ml)	110.78±0.09b	183.71±0.32c	223.77±0.05d	39.93±0.11a
IAA production (µg/ml)	73.64±1.68c	65.75±0.34b	66.15±1.61b	47.78±0.22a
Phosphate solubilisation (µg/ml)	34.12±0.49c	31.48±0.31b	52.97±0.74d	26.78±0.26a
Siderophore production	+	+	+	+

Values are mean±SE (n=3), compared by analysis of variance (ANOVA), and followed by Duncan's test. Statistically, differences were determined at p ≤ 0.05 by using SPSS ver 20. "+" siderophore producing.

**Table 3.** Biosurfactant production, emulsification index and enzymatic activities of selected AHL producing PGPR strains

Characteristics	11VPKHP4	7VP51.8	P51.10	NBRI N7
Biosurfactant production	-	-	+	+
Emulsification index (E24%)	18.53±1.14a	19.44±1.38a	25.75±1.51a	54.66±2.66b
Twitching motility	-	-	+++	++
Swarming motility	-	+	++	-
Swimming motility	+++	++++	++++	++
ACC deaminase	+	+	+	+
Proteolytic	-	-	-	+
Amylotic	-	-	-	-
Cellulolytic	-	-	-	-
Nitrate reduction	-	-	+	-
Catalase	+	+	+	+
Gelatinase	-	+	+	-

+ positive, - negative; ++++ more than 2.5 cm, +++ 2 to 2.5 cm of bacterial colony diameter; ++ 1.5 to 2.0 cm of bacterial colony diameter; + 1.0 to 1.5 cm of bacterial colony diameter; - 0.5 cm of bacterial colony diameter.

7VP51.8, P51.10 and NBRI N7 showed the closest homology with *P. Sesamii* (99.36%), *P. hunansis* (99.86%), *P. asiatica* (100%), and *P. aeruginosa* (99.28%) respectively (Table 1).

#### Plant growth-promoting attributes of quorum sensing exhibiting *Pseudomonas* spp.

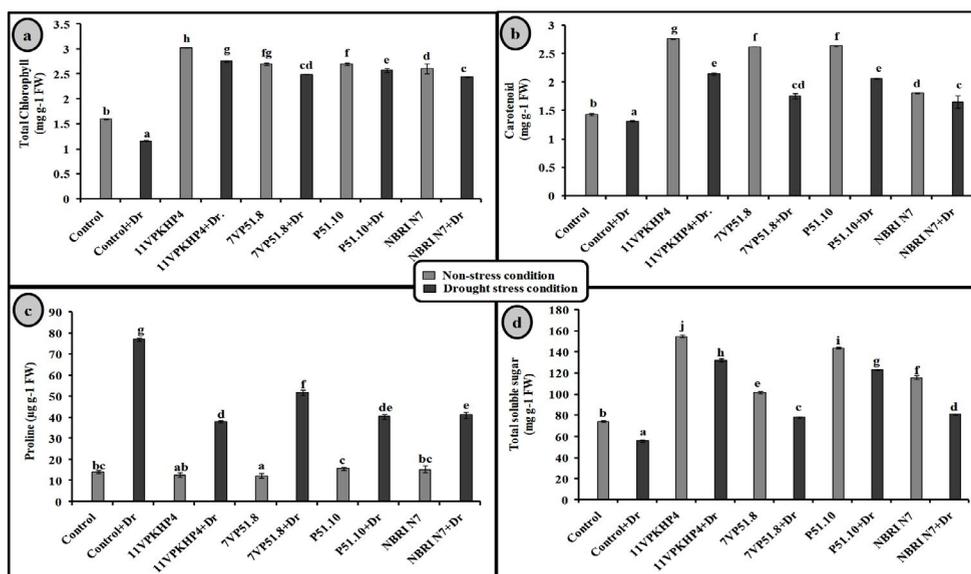
In the present study, four selected AHL producing isolates with abiotic stress tolerance

ability were further evaluated for multiple PGP attributes. All the selected isolates illustrated biofilm formation under *in vitro* conditions. Significant differences among the isolates were monitored, according to absorbance ranging from 1.0 to 2.2 at 590 nm, depicted in Table 2. Furthermore, out of four bacterial isolates, P51.10 and 11VPKHP4 demonstrated maximum biofilm

**Table 4.** Plant growth promotion by AHL producing selected PGPR strains using *Zea mays* under control and drought stress

Bacterial isolates	Root Length (cm)	Shoot Length (cm)	Fresh Weight (g)		Dry Weight (g)		Ratio RDW/SDW
			Root	Shoot	Root	Shoot	
Control	36.0±0.40a	56.7±0.61b	3.88±0.37b	7.44±0.32b	1.11±0.02b,c	1.83±0.36b	0.60±0.01d
Control+D	37.05±0.40a	48.72±1.76a	2.07±0.23a	4.51±0.18a	0.51±0.00a	1.15±0.06a	0.44±0.01b
11vPKHP4	42.2±2.36b	99.85±0.41h	5.09±0.63c	17.16±0.65f	1.8±0.06d	4.27±0.01g,h	0.42±0.01b
11vPKHP4+D	47.4±0.42c	95.8±0.39g	4.49±0.11b,c	14.88±0.24de	1.2±0.16b,c	3.87±0.22e,f	0.31±0.03a
7VP51.8	56.65±0.63d	75.97±0.76d	4.69±0.29b,c	12.70±0.90d	1.35±0.08c	3.21±0.02d	0.42±0.02b
7VP51.8+D	57.95±0.55d	66.37±1.30c	3.61±0.10b	9.52±0.21c	0.97±0.08b	2.46±0.12c	0.30±0.02a
P51.10	55.25±0.75d	85.95±1.44f	7.4±0.54d	17.42±0.17f	2.29±0.17c	4.35±0.02h	0.52±0.03c
P51.10+D	61.62±0.40e	85.87±0.69f	7.25±0.27d	14.91±0.11e	1.66±0.15d	3.76±0.16e,f	0.44±0.03b
N7	43.3±0.31b	80.3±0.50e	4.62±0.27b,c	17.03±0.41f	1.34±0.07c	3.97±0.04f,g	0.33±0.01a
N7+D	44.52±0.65b	77.15±0.45d	3.72±0.27b	14.98±0.35e	0.90±0.03b	3.55±0.17e	0.25±0.01a

Values are mean±SE (n=3), compared by analysis of variance (ANOVA), and followed by Duncan's test. Statistically, differences were determined at  $p \leq 0.05$  by using SPSS ver 20.



**Fig. 4.** Effect of PGPR on (a) Total chlorophyll (b) carotenoid content (c) proline and (d) Total soluble sugar in *Zea mays* plants grown under normal and drought stress conditions.

Values are mean±SE (n=3), compared by analysis of variance (ANOVA), and followed by Duncan's test. Statistically, differences were determined at  $p \leq 0.05$  by using SPSS ver 20.

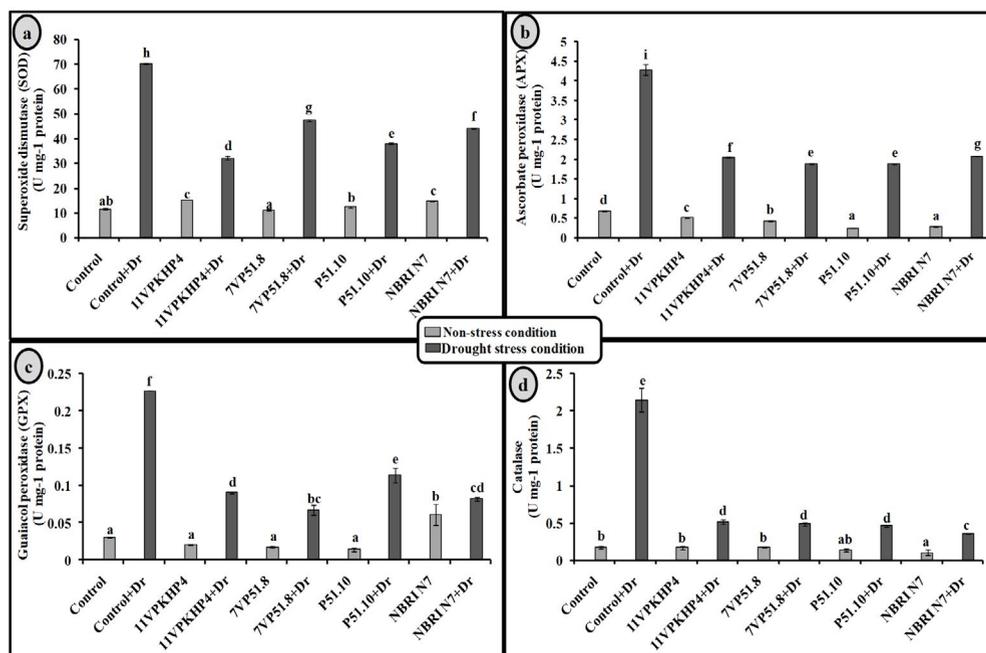
production i.e., 2.10 and 2.02 absorbance at 590 nm.

In the case of exopolysaccharide production after 72 h of growth, significant differences were revealed among all isolates, as varying levels of its production ranges between 120 to 256  $\mu\text{g mL}^{-1}$  (Table 2). However, among 4 isolates, 11VPKHP4 exhibited maximum EPS (256.62  $\mu\text{g mL}^{-1}$ ) production. In the case of alginate production, 72 h of growth revealed varying levels of its production among four isolates, ranging between 39 to 223  $\mu\text{g mL}^{-1}$  (Table 2). Among all four, isolate P51.10 produced maximum alginate 223.77  $\mu\text{g mL}^{-1}$  followed by 7VP51.8 i.e. 183.71  $\mu\text{g mL}^{-1}$ . Notably, varying levels of IAA production were observed among all isolates, ranging between 47 to 73  $\mu\text{g mL}^{-1}$  as depicted in Table 2. Furthermore, maximum IAA production is reported for 11VPKHP4 at 73.64  $\mu\text{g mL}^{-1}$  followed by 66.15  $\mu\text{g mL}^{-1}$  and 65.75  $\mu\text{g mL}^{-1}$  in P51.10 and 7VP51.8, respectively. In the matter of quantitative estimation of P-solubilisation, all four isolates illustrate the varying levels of phosphate solubilization ranging from 26 to 52  $\mu\text{g mL}^{-1}$ . Maximum phosphate solubilization exhibited by

P51.10 (52.97  $\mu\text{g mL}^{-1}$ ) whereas isolates 11VPKHP4 and 7VP51.8 illustrated phosphate solubilization 34.12 and 31.48  $\mu\text{g mL}^{-1}$ , respectively (Table 2).

#### Biosurfactant production, emulsification index, motility, and enzymatic activities of selected AHL producing *Pseudomonas* spp.

In an evaluation for drop collapse assay for biosurfactant production, out of four AHL producers, only P51.10 and NBRI N7 were proficient in biosurfactant production. In addition, all the isolates were tested positive for emulsification and produces in the range of 18 to 54%, suggesting that all isolates fabricate emulsifiers (Table 3). It is conceivable that twitching motility, swarming as well as swimming motility was observed based on zone diameter formation. Twitching motility of isolates P51.10 and NBRI N7 assessed in between 2.0 to 2.5 cm and 1.5 to 2.0 cm diameter. Swarm diameter of P51.10 and 7VP51.8 assessed in between 1.5 to 2.0 cm and 1.0 to 1.5 cm respectively (Table 3). Swimming motility of isolates P51.10 and 11VPKHP4 was assessed more than 2.5 cm whereas that of 7VP51.8 and NBRI N21 in between 2.0 to 2.5 cm of diameter respectively (Table 3). Furthermore,



**Fig. 5.** Effect of PGPR on (a) superoxide dismutase (SOD), (b) ascorbate peroxidase (APX) (c) guaiacol peroxidase (GPX) and (d) catalase (CAT) in *Zea mays* plants are grown under normal and drought stress conditions. Values are mean  $\pm$  SE (n=3), compared by analysis of variance (ANOVA), and followed by Duncan's test. Statistically, differences were determined at  $p \leq 0.05$  by using SPSS ver 20.

all four isolates were proficient in ACC deaminase and catalase activity. However, among all the four isolates, only NBRI N7 demonstrated protease activity. In addition, 7VP51.8 and P51.10 were tested positive for gelatinase activity whereas only P51.10 tested positive for nitrate reduction. However, amylolytic and cellulolytic activity was not exhibited by any of these isolates under study (Table 3).

#### **Evaluation of plant growth promotion under control and drought condition in *Zea mays***

In the present study, greenhouse results demonstrated the enhanced plant growth parameters in PGPR treatments as compared to uninoculated controls under non-stress as well as drought stress conditions (Table 4). Significant differences in plant root and shoot length along with the fresh and dry mass of root have been recorded, in which 11VPKHP4 illustrated maximum enhanced shoot length followed by P51.10 (Table 4). However, P51.10 has been verified for maximum enhanced root length followed by 7VP51.8 and 11VPKHP4 under drought stress conditions (Table 4). Moreover, under drought stress conditions, 11VPKHP4 and P51.10 enhanced shoot and root length by 96.61%; 27.93%, and 76.24%; 66.32% respectively. In addition, P51.10 enhanced fresh root weight and dry root weight by 249.21% and 225.52% while fresh shoot weight and dry shoot weight enhanced by 230.65% and 227.17%. However, 11VPKHP4 enhanced fresh root weight and dry root weight by 116.24% and 134.26% while fresh shoot weight and dry shoot weight enhanced by 230.09% and 237.17% respectively (Table 4).

#### **Effect of *Pseudomonas* spp. on biochemical and antioxidant enzymes of *Zea mays***

Inoculation with all four *Pseudomonas* spp. has significantly increased the chlorophyll (total), carotenoid, proline, and soluble sugar as compared to uninoculated plants under drought stress conditions (Fig. 4). However, 11VPKHP4 revealed a maximum increase in total chlorophyll content by 137.18%, while 56.64%, 49.72%, and 48.31% by P51.10, NBRI N7, 7VP51.8, respectively under drought stress condition whereas carotenoid content was enhanced by 62.29%, 56.50%, 32.98% and 25.17% in 11VPKHP4, P51.10, NBRI N7, 7VP51.8, respectively (Fig. 4). Plants inoculated with 11VPKHP4 and P51.10

revealed maximum enhanced total soluble sugar by 136.23% and 85.87% while 20.4% and 16.95% by NBRI N7 and 7VP51.8 under drought stress conditions (Fig. 4). Plants under drought stress revealed increased levels of proline but modulated by 50.76% and 47.47%, 46.47%, and 32.85% inoculation of 11VPKHP4, P51.10, NBRI N7, and 7VP51.8, respectively (Fig. 4). Our results revealed that plants growing under drought stress conditions have demonstrated a significant increase in all antioxidant enzyme activities. Bacterial isolates, 11VPKHP4, P51.10, NBRI N7 and 7VP51.8 modulated SOD activity by 54.12%, 45.72%, 37.04%, and 32.40% respectively (Fig. 5). In the case of APX, inoculation of P51.10, 7VP51.18, 11VPKHP4, and NBRI N7 modulated plants by 56.30%, 55.89%, 52.34%, and 51.50%, respectively. GPX activity was modulated by 16.02%, 14.44%, 13.61%, and 11.30% by inoculation of 7VP51.18, NBRI N7, 11VPKHP4, and P51.10, respectively. Similar to other soil enzymes, catalase activity was also modulated 83.62%, 78.61%, 77.45%, and 76.211% by inoculation of NBRI N7, P51.10, 7VP51.18, and 11VPKHP4, respectively (Fig. 5).

#### **DISCUSSION**

Abiotic stresses affect our agro-ecosystem every year by generating a large area of uncultivable land and therefore impact food security, significantly. To overcome this problem, soil-inhabiting microbes with multifarious PGP and abiotic stress mitigating attributes have emerged as a solution.<sup>48,49</sup> However, intensive research has been done towards characterizing the bacterial isolates from different locations for the presence of PGP attributes and their efficiency for enhancing plant growth under abiotic stresses.<sup>48,49</sup> Our findings demonstrated that the *Pseudomonas* spp. with quorum sensing potential isolated from different geographic locations demonstrated multiple PGP attributes and stress ameliorating abilities. The information available on the characterization of QS exhibiting stress-tolerant *Pseudomonas* spp. along with other attributes for drought stress amelioration in *Zea mays* is very limited. However, few studies have exposed the presence of QS in bacteria.<sup>50,51</sup> In our study, out of 306 bacterial isolates, 4 potent bacterial isolates were screened and selected based on exhibiting quorum sensing activity via

AHL induction. These isolates belong to different geographical locations having different climatic conditions and soil types. In the present study, all 4 quorum sensing exhibiting bacterial isolates i.e., 11VPKHP4, 7VP51.8, P51.10, and NBRI N7, with multiple PGP attributes, and abiotic stress tolerance are reported from different geographical locations (Table 1). Notwithstanding, the novel attribute of quorum sensing activity via AHL production has been earlier reported in other members of *Pseudomonas* but for the first time reported in *P. sesamii*, *P. hunansis*, and *P. asiatica*.<sup>51</sup> Moreover, quorum sensing signaling interaction between rhizospheric isolates was reported to produce AHLs.<sup>50,51</sup> AHLs also act as inter-kingdom interacting signal molecules affecting plant growth, gene expression, and induction of systemic resistance in plants.<sup>53</sup> On recognition of AHL molecules by plants, these AHL molecules modify root, shoot gene expression, amending defense, and cell growth responses, and also lead to lateral root development.<sup>54</sup>

In the present study, all isolates possess multifarious plant growth-promoting attributes like biofilm formation, exopolysaccharide production, alginate production, auxin production, phosphate solubilization, siderophore production, ACC deaminase activity, and abiotic stress endurance. Although, previous studies have also well documented that PGPR belonging to *Pseudomonas* genus with the PGP attributes demonstrated plant growth promotion under diverse abiotic stresses.<sup>55,56</sup> In the context of AHL production, all four isolates were found to be the best biofilm formers (Table 2) as well as able to produce exopolysaccharides (EPS). Biofilm-forming PGPR confers resistance against various abiotic stresses, produces antibacterial compounds, enhances protection from desiccation.<sup>15</sup> As compared to planktonic cells, biofilms formed from *Pseudomonas*, *Bacillus*, *Trichoderma*, *Bradyrhizobium*, *Brevibacterium*, *Pantoea*, and *Acinetobacteria* showed superior production of auxin, phosphate solubilization, siderophore production, nitrogenase activity.<sup>49,57</sup> These were also found potent in plant growth promotion under diverse environmental stress conditions.<sup>57</sup> Also, in the context of biofilm formation, all the isolates were proficient against drought (30% PEG), salt (4% salt), pH (5, 9 and 11), and temperature

(37°C and 40°C) abiotic stresses as compared to control conditions till 10<sup>th</sup> day. Our findings are in corroboration with earlier studies,<sup>58,49</sup> therefore, it is expected that all promising isolates will be able to withstand abiotic stress and abiotic stress amelioration. EPS secretion maintains soil structure and fertility by sustaining the water holding capacity and moisture content of the soil. Among all reported PGPR, the *Pseudomonas* genus has been well characterized to enhance plant vegetative parameters in maize, finger millet, sunflower, peas, and ornamental crops facing drought and resource limiting conditions.<sup>55,22,20</sup> Among all the selected four isolates, 11VPKHP4 exhibited maximum EPS production (Table 2). However, Konnova et al.<sup>59</sup> reported the role of EPS in the defense of *A. brasilense* Sp245 cells against desiccation. A considerable correlation was observed between the EPS amount produced by cowpea Bradyrhizobium strains and desiccation tolerance. *P. putida* KT2440 strain was able to synthesize four types of EPS, performing various functions in plant growth promotion.<sup>60</sup> Alginate, an extracellular polysaccharide produced by a variety of Gram-negative bacteria including *Azotobacter vinelandii*, *P. fluorescens*, and *P. aeruginosa*. Earlier, Nivens et al.<sup>61</sup> demonstrated that alginate production is not crucial for biofilm development but it plays role in biofilm 3-dimensional structure formation. In our findings, among all four isolates, P51.10 produced maximum alginate followed by 7VP51.8 (Table 2). Furthermore, in the present study, maximum IAA production was observed in 11VPKHP4 followed by P51.10 and 7VP51.8 (Table 2). Since, IAA secreted by bacteria may promote plant growth directly, by stimulating tissue differentiation, cell elongation, or cell division and tactic responses,<sup>62</sup> the observed positive effect of our isolates on the biomass of *Zea mays* plants could be due to the mentioned mechanism. A higher level of IAA production by *Pseudomonas* was reported by other workers also.<sup>63</sup> Numerous forms of phosphate including inorganic and organic phosphate persist in soil. PGPR, potent in the mineralization of most organic phosphorous compounds carried out employing phosphatase enzymes. Many solubilization reactions including acidification, chelation, and secretion of organic acids as gluconic acid for conversion of an insoluble form of P to a soluble form, carried out

by diverse groups of microorganisms belonging to the genera *Pseudomonas*, *Serratia*, *Acinetobacter*, *Bacillus*, *Burkholderia*, *Paenibacillus*, *Pantoea*, *Acinetobacter*, *Enterobacter*.<sup>64</sup> In the present study, maximum phosphate solubilization was exhibited by P51.10 followed by 11VPKHP4 and 7VP51.8 (Table 2). Recently, it was explored that phosphate solubilizing strain P34-L of *Pseudomonas* was able to elevate wheat growth by developing root system and phosphorous content.<sup>64</sup> Phosphate solubilizing *Pseudomonas libanensis* EU-LWNA-33 was evaluated for wheat growth promotion under drought stress.<sup>24</sup> Few PGPR enhance plant growth by siderophore production. Siderophore-producing bacteria chelate iron from a nearby medium, making it accessible to plants. In the present study, we found that all four *Pseudomonas* sp. were efficient in siderophore production. Earlier, Shah et al.<sup>66</sup> explored that the siderophore producing RSP5 strain of *Pseudomonas aeruginosa* was able to enhance iron content of all vegetative parameters in *Zea mays*, thereby enhancing overall plant growth and development. Therefore, it is expected that in our exploration, all four bacterial isolates promising for siderophore production shall also execute iron content in crop plants. Moreover, all four isolates were tested positive for ACC deaminase and catalase enzymatic activity whereas only NBRI N7 was positive for proteolytic activity. Based on recent past year studies, *Pseudomonas* spp. have also been characterized for enzymatic activities including catalase, nitrate reduction, and gelatinase.<sup>67</sup> Isolates 7VP51.8 and P51.10 illustrated gelatinase activity while only P51.10 illustrate nitrate reduction. Numerous studies have reported that with their ACC deaminase assistance, PGPR is capable of suppressing the ethylene levels in plants by cleaving ACC (the precursor of ethylene) and converting it into  $\alpha$ -ketobutyrate and ammonia which re-establishes the plant's normal growth and function.<sup>68</sup> Earlier, researchers have proved that consortium or all alone ACC deaminase producing PGPR (*Pseudomonas*, *Bacillus*, *Ochrobactrum*) with other PGP attributes elevate plant health under water deficit conditions which can be further employed for sustainable agriculture.<sup>58,48,49</sup> Synergism of *P. aeruginosa* (LSE-2) and *Bradyrhizobium* (LSBR-3) reveals Soybean growth promotion via improved nutrient acquisition and soil health.<sup>69</sup>

One of the most interesting characteristic features of the four isolates is motility and motility helps in colonization. Microbial factors such as the release of quorum sensing signaling molecule, ability to form biofilm on the root surface, and motility are required to establish a relationship with the plants. Another characteristic feature of bacteria is biosurfactant production and emulsification index, isolate P51.10 and NBRI N7 illustrate biosurfactant production while all isolates illustrated emulsification (Table 3). All isolates are thus considered emulsifiers. Biosurfactants are low molecular weight surface-active compounds widely produced by bacteria, yeast, and fungi. Biosurfactant produced by PGPR, effectively employed for oil recovery, improving soil quality by removing hydrocarbons as well as heavy metals,<sup>70,71</sup> plant-microbe interaction, pharmaceutical, cosmetics, petroleum, and food industries.<sup>72</sup>

In the present study, *Zea mays* growth promotion was evaluated by considering the vegetative plant growth parameters as root and shoot length, fresh, dry root and shoot weight, and dry root and shoot weight ratio. Results indicated that all the four PGPR isolates showed significantly enhanced growth in all parameters under drought stress as compared to uninoculated control. Mishra et al.<sup>48</sup> have reported the role of biofilm-producing abiotic stress-tolerant PGPR for ameliorating drought stress and enhancing maize productivity. Several other studies are indicating that inoculation with biofilm-forming PGPR has better plant growth promotion as compared to non-biofilm former inoculants.<sup>73</sup> Earlier, extensive studies have revealed the profound effects of EPS producing PGPR in ameliorating the drought stress and enhancing plant growth. It is speculated that the EPS producing PGPR assist plants to cope up with drought stress. Increased *Zea mays* vegetative parameters including root and shoot length, leaf area, leaf protein, and sugar contents under drought stress conditions were observed on bacterization of *Zea mays* seeds with EPS producing PGPR. Similar results were also observed by Khan et al.<sup>74</sup> for wheat plants under the sandy soil. Our results could be corroborated with these findings as our PGPR also produces different levels of EPS.

In our present study, we elucidated the treatment of stress-tolerant *Pseudomonas* spp. exhibiting quorum sensing activity has significantly increased plant enzymatic and non-enzymatic biochemical systems (total chlorophyll, carotenoid, proline, soluble sugar, and antioxidant enzymes) as well as vegetative parameters which found to be in covenant with earlier studies findings.<sup>49,48,21</sup> Accretion of osmolytes as proline and total soluble sugar induced by PGPR lead plants to cope up with drought stress by preventing electrolyte leakage through stabilizing membrane integrity thereby bringing ROS levels back to normal range via sustaining osmotic turgor.<sup>75,49,48</sup> As a result, this eradicates oxidative damage in plants. Our results of *Pseudomonas* inducing a substantial increase in soluble sugar levels in plants under drought stress were covenant with previous findings.<sup>49,48</sup> Under drought stress, our results also revealed a substantial increase in proline accretion levels in plants but our PGPR treatment eliminates those induced substantial increase levels of proline accretion as compared to untreated ones. Thus, our findings of PGPR treatment in eliminating the substantial increase of proline levels in plants to cope up with drought stress are in corroboration with earlier findings.<sup>29,48,21</sup>

It is speculated that a substantial increase in hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) levels in plants growing under stress conditions leads to cellular metabolites damage and worsening stress resistance power.<sup>76</sup> In our present study, we observed an increase in H<sub>2</sub>O<sub>2</sub> levels in untreated plants facing drought stress but reduced in all four *Pseudomonas* PGPR treatments with slight variation in reduction levels. Earlier researchers have speculated for the reduced levels of H<sub>2</sub>O<sub>2</sub> in plants on PGPR treatment but the exact mechanisms for this are still not wholly known. Among all four *Pseudomonas* isolates, maximum SOD levels were reduced by 11VPKHP4 and P51.10 followed by NBRI N7 and 7VP51.8 while in APX by 7VP51.8 and 11VPKHP4 followed by NBRI N7 and P51.10. Plants facing drought stress, GPX activity was reduced maximum by 7VP51.8, NBRI N7, 11VPKHP4 followed by P51.10. Catalase activity in plants was modulated by all four isolates under stressed conditions, thus, these findings are in agreement with the earlier findings.<sup>49,48</sup>

Bacterial strains efficient in quorum

sensing and biofilm formation together with multifarious plant growth-promoting attributes contributed to enhanced maize biomass under drought stress. It can be hypothesized that strains with their quorum sensing activity (AHL production) facilitate the plant-microbe interaction and nutrient acquisition. Their proficiency in biofilm formation and EPS production will further help in rhizosphere colonization, maintenance of soil moisture content for a longer duration under normal and diverse stresses. Detailed research work is required to emphasize the effects of quorum sensing and its genes engage in multifarious activity for enhancing crop productivity through signaling under diverse stresses.

#### SUPPLEMENTARY INFORMATION

Supplementary information accompanies this article at <https://doi.org/10.22207/JPAM.16.1.69>

**Additional file:** Additional Table S1.

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#### CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

#### AUTHORS' CONTRIBUTION

PSC and AS conceived the idea and designed the experiments. AS performed the experiments. AS and PSC inscribed the manuscript. AS and PSC edited the manuscript. Both the authors read and approved the final manuscript for publication.

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#### DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

#### ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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