

Original Research Article

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Isolation and Characterization of Drought Tolerant Bacteria from Rice Phyllosphere

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ABSTRACT

Tamil Nadu is one of the top rice production states in India. Southwest monsoon failure of recent years in Tamil Nadu hits with severe drought, especially in the regions of Ramanathapuram districts. The main objective of present study is to isolate drought tolerant bacteria from phyllosphere of drought tolerant rice varieties cultivated in paramakudi region. Rice phyllosphere constitutes the diversified microbial community. The phyllosphere bacteria were isolated in different media Nutrient agar, Tryptic soy agar, R2A agar, and King's B media from four drought tolerant rice varieties Anna R(4), PMK3, Nootripattu and Mattaikar. A total of 50 different bacterial isolates were purified and screened for abiotic stress tolerance such as drought stress at PEG6000 (-0.15MPa, -0.49MPa, -0.73MPa and -1.2MPa), salt stress at 1.2M NaCl and temperature stress at 50°C. The isolates were named with series PB. The best 8 isolates PB3, P17, PB18, PB23, PB24, PB37, PB46 and PB50 was first biochemically characterized and screened for production of plant growth hormones IAA and GA₃ at different PEG6000 stress. The result shows PB50 and PB46 has highest production of plant growth hormones and sustain drought stress. Future works are characterization of isolates for Plant growth promotion activities and application of isolates on rice for drought mitigation.

Keywords

Nootripattu and
Mattaikar,
Phyllosphere

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Introduction

Water is essential for all living organisms. A key challenge for plant growth is global water shortage, limiting crop yields already today in more than 70% of arable lands, and the drought limitations further gain in importance in the near future as agricultural activities

expand to less fertile areas to satisfy growing demands for food. Moisture stress during the crop ontogeny has been one of the main constraints for sustainable rice productivity, especially in uplands (Hossain, 2014). Integrating microbes as component of agricultural system for enhanced drought tolerance in plants needs to be endorsed to

boost sustainable crop production (Trivedi *et al.*, 2017). The aerial habitat colonized by microbes is termed as Phyllosphere and the microorganisms are called as epiphytes. Most of the Phyllosphere microorganism occupies the surface area of leaves, flowers and fruits, and work has been focused mainly on Phyllosphere microbiology on leaves, a dominant aerial structure of plants. Bacteria are the dominant microbiome on leaf with the numbers averaging 10^6 to 10^7 cells/cm² of leaf (H. Andrews and F. Harris, 2000). Phyllosphere is an extreme and unstable habitat where it is exposed to different environmental factors, mainly high light, UV radiation, temperature and desiccation. It has the characteristics of oligotrophy limitation in carbon and nitrogen sources. It is reported that microorganism in Phyllosphere utilize the plant metabolites available on leaf surface such as soluble sugars, polyols, amino acids, amines and VOC (Bringel and Couée, 2015). Proline is the most abundant osmolytes produced in both eukaryotes and prokaryotes. Glutamate acts as precursor of proline, during stress condition bacteria utilize three enzyme mechanism glutamate kinase, glutamate-γ-semialdehyde dehydrogenase and pyrroline-5-carboxylate reductase for converting glutamate to proline (Sugiura and Kisumi, 1985). Proline slakes the drought stress by two strategies, one by acting as chemical chaperone which directly scavenges the reactive oxygen species generated during stress and other strategy by indirect mechanism, which activates signalling pathway and balances the cellular energy (Liang *et al.*, 2013). Bacteria uses several strategies to protect from osmotic stress, they produce quaternary ammonium compounds like Glycine Betaine (GB), Choline and Proline betaine, which serves as osmoprotectant (Slama *et al.*, 2015). GB and choline are a dominant osmoprotectant in bacteria followed by proline, they act as protein chaperons and stabilize the protein

from denaturation during stress condition, it is proven that both the osmolytes stabilize the enzymatic activity of citrate synthase during thermal stress in *E. coli* strains (Caldas *et al.*, 1999). Though Choline and GB acts as osmoprotectant, Choline is the precursor of glycine betaine. The homeostasis between choline and GB has been studied in *Pseudomonas aeruginosa*, during osmotic stress certain level of choline is oxidized to GB using enzymes choline oxidase and betaine aldehyde dehydrogenase (Fitzsimmons *et al.*, 2012). In This present study we have isolated drought tolerant bacterial isolates from four drought tolerant rice varieties. The ability of drought tolerant bacterial isolates to produce phytohormone was assessed.

Materials and Methods

Sample collection site

We studied four rice drought varieties maintained in the fields of the Agricultural research station, Paramkudi (9°33'01.8" N 78°34'53.7" E) with soil pH 9.3 ± 0.2 and EC 0.177 ± 0.008 dsm⁻¹, which is considered to be an alkaline soil. Temperature was recorded to be 40°C during collection. The varieties are Mattaikar, Nootripattu, Anna R (4) and PMK3, which are at the vegetative phase. The 4th and 5th leaves of main tillers were collected randomly from the field in the morning using sterile plastic bags and was transported to the laboratory in an icebox and analysed immediately.

Isolation of phyllosphere bacteria

The leaf samples were washed using sterile water in order to isolate putative bacteria colonized on the leaf surface. To isolate bacterial population on the adaxial and abaxial leaf surface, Both the sides of leaf were imprinted on different agar media,

Nutrient agar, Tryptic soy agar, R2A agar, and King's B media, Individual leaf of size 5 cm each was placed on agar media and the imprint was done by pressing the leaf sample using a sterile glass rod (Yadav *et al.*, 2010). The plates were incubated for 24hr at 30°C and the morphologically different colonies were picked and purified in the respective agar plates and final purification was done tryptic soy agar. Finally, 50 isolates were obtained and screened for abiotic tolerance. The isolates were named from PB1 to PB50. Isolate PB1 to PB21 belongs the Anna R(4); isolates PB22 to PB 35 belongs to PMK3; isolates PB35 to PB42 belongs Nootripattu and isolates PB43 to PB50 belongs Mattaikar. Then bacterial isolates were preserved in 50% glycerol stock at -20°C.

Screening of bacterial isolates for abiotic stress

All the isolates were subjected to check their ability to tolerate different drought stresses such as high temperature (50°C), salinity (1.2 M), and drought (-0.15, - 0.49, -0.73 and -1.2 MPa) using tryptone soy broth (TSB). The Initial inoculum for screening the isolates were grown in TSB broth for 6 h to 7 h and the final bacterial population was adjusted to same optical density (0.1). The growth of all isolates in stress conditions were recorded using spectrophotometer at 600 nm with sterile medium as blank and this was done in triplicate. Bacterial isolates with OD 0.5 and more are considered as stress tolerant bacteria (Praveen Kumar *et al.*, 2014)

High temperature tolerance

Ten mL of TSB was dispensed into 30 mL test tubes and autoclaved. Then the sterile broth was inoculated with 0.1ml uniform cell density (0.1) of exponentially grown fresh bacterial cultures and tubes were incubated at 50°C on a shaker (120 rpm) for 24 h and OD were recorded at 600nm.

Drought tolerance

For screening the drought tolerant isolates, a known quantity of TSB medium amended with four different concentration of polyethylene glycol-6000 was used. - 0.15 MPa [110 gm PEG per 1L media (w/v)], - 0.49 MPa [210 gm PEG per 1L media (w/v)], - 0.73 [260 gm PEG per 1L media (w/v)] MPa and - 1.2 MPa [326 gm PEG per 1 L media (w/v)] was dissolved by heating in a microwave oven, and then the final volume was made up to 1L with PEG amended medium(Michel and Kaufmann, 1973). The liquid medium was dispensed in 30 mL capacity test tubes and autoclaved. Fresh cultures of test strains grown for 6 h to 7 h on a shaker incubator, population were adjusted to OD 0.1 and used as initial inoculum. The inoculated tubes were incubated at 28°C for 24 h on a shaker (120 rpm) and OD was recorded

Salinity tolerance

To the 100ml conical flask 30mL of TSB amended with 1.2M NaCl [70 gm of NaCl in 1L of TSB broth (w/v)] was dispensed and autoclaved. Fresh cultures of test isolates grown for 6 h to 7 h on a shaker incubator were adjusted to the OD of 0.1 and used as initial inoculum. The inoculated tubes were incubated at 28°C for 24 h on shaker (120 rpm) and OD was recorded.

Biochemical characterization

Biochemical characterization test such as indole production, Methyl red test, Vogus-Proskauer test, Citrate utilization test, Amylase test, Catalase test, Urease test, Pectinase test, Cellulase test, Gram staining, 3% KOH test, cell shape, Hydrogen sulphide production test and Carbon assimilation test was performed according to (Thayer and Murray, 1977).

For all the biochemical tests 24h grown bacterial isolates were used and results were denoted in positive and negative.

Bacterial diversity analysis

The positive and negative data from biochemical characterization was represented in binary data with positive as one and negative as zero. With the binary data obtained, dendrogram was developed using NTSYS software (Rohlf, 1988) and bootstrap value was generated using R programming.

Estimation of IAA production at different PEG 6000 concentration

The bacterial isolates were grown in TSB broth for 24h. Then isolates were transferred to 10ml of TSB broth without and with PEG at three different concentration (-0.15 MPa, -0.49 MPa and -0.73 MPa) amended with 0.1% of Tryptophan in triplicate. Then incubated for 48h in a shaker at 28°C (120 rpm). The IAA production was determined using the Salkowski method (Rahman *et al.*, 2010). After 48h of growth, the isolates were centrifuged and cell-free supernatants were used for IAA determination. To the 10ml of supernatant, 2ml of Salkowski reagent (2ml of 0.2 M Ferric chloride, 49 ml of Perchloric acid and 49 ml of Distill water) was added and incubated for 10 minutes. The blank was prepared using sterile broth with Salkowski reagent. Then the samples were checked for absorbance at 530nm. IAA standard graph curves were calibrated using Indole acetic acid (Himedia) in TSB broth at different concentration (5, 10, 20, 50 and 100 µg/ml) and sample IAA concentration was calculated.

Estimation of GA₃ production at different PEG 6000 concentration

The 24 h old bacterial culture was taken and inoculated in 15ml test tubes containing

sterile TSB broth without and with different PEG concentration (-0.15 MPa, -0.49 MPa and -0.73 MPa) in triplicate. Gibberellic acid was estimated using potassium ferrocyanide precipitation method (Holbrook *et al.*, 1961). The cultures were incubated at 30°C in a shaker (180 rpm) for 7 days. After incubation, the cultures were centrifuged at 10,000 rpm for 10 mins. To the 15 ml of samples, 2ml of zinc acetate was added and after two minutes 2 ml of potassium ferrocyanide was added and allowed to precipitate. The samples were centrifuged at 8,000 rpm for 10 mins, 5ml of each supernatant was taken and added to 5ml of 1N HCl. Then the samples were incubated at 25°C for 75 mins. The sterile broth with 1N HCl was taken as blank. Absorbance was taken at 254 nm in UV-Vis spectrophotometer. GA₃ concentration in samples was calculated the standard curve graph prepared using gibberellic acid (Himedia) in sterile TSB broth (0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml).

Statistical analysis

All the experiments were carried out with replication (n=3). The mean data obtained were analyzed by one-way analysis of variance (ANOVA) and ranked using Duncan's multiple range test, correlation coefficient squared was calculated using R programming. Least significant difference was considered at $p < 0.05$ for comparing difference means of treatments.

Results and Discussion

Bacterial population in rice phylloplane

Leaf imprint technique was implemented to isolate rice phylloplane bacteria. Among four varieties Anna R(4) constitutes the highest bacterial population followed by Nootripattu, PMK3 and Mattaikar (Table 1). The highest population obtained when compared to medium used was TSB medium. Hence all the

bacterial isolates from different media were purified in TSB medium.

Screening of abiotic stress tolerant bacteria

The isolates subjected to drought stresses were checked for OD at 600nm. Among the different osmotic stresses, all the bacteria attain maximum growth at -0.49MPa stress.

Hence the OD of isolates above 0.5 under all abiotic stress except at -1.2MPa was noted since the growth was below 0.5 OD comparatively. A total of 8 isolates were screened down, they are PB3, PB17, PB18, P23, PB24, PB37, PB46 and PB50. Isolate PB18 was noted with prominent growth under drought stress at -1.2MPa, whereas PB3 was said to be best temperature stress tolerance

and PB18 and PB23 has the highest growth under salt stress (Table 2). Screened isolates were further characterized using biochemical test.

Biochemical characterization

The results from the biochemical characterization were represented in (Table 3) and the diversity analysis using biochemical characters was represented in (Figure 1). Diversity analysis shows that isolates PB46, PB50, PB3 and PB37 classified under separate clade, among them PB50 and PB46 shows close relation. Another separate clade with isolates PB17, PB18 and PB23 shows they common ancestor. The isolate PB24 remains as out group.

Table.1 Bacterial population on rice Phylloplane

Rice varieties	Population (cfu/sq.cm)			
	NA media	TSB media	King's B media	R2A media
Anna R (4)	11.13 (0.98) ^a	11.2 (0.6) ^a	10.13 (0.90) ^a	10 (0.72) ^a
PMK3	10.66 (1.47) ^b	10.26 (1.52) ^d	9.733 (0.76) ^b	9.86 (0.64) ^a
Nootripattu	11.33 (0.80) ^a	10.8 (1.21) ^b	9.533 (0.46) ^b	9.13 (0.61) ^b
Mattaikar	9.4 (1.05) ^c	10.53 (0.92) ^c	9.066 (0.83) ^c	8.8 (0.4) ^c

Note: The numerical values are mean and SD of different observations; Same values in superscript indicates no significant difference between observations.

Table.2 Growth of isolates under different abiotic stress

Isolates	PEG6000 (-1.2MPa)	NaCl (1.2M)	Temperature (50°C)
PB3	0.167(0.003) ^c	1.011(0.037) ^a	0.673 (0.011) ^b
PB17	0.128(0.005) ^f	0.853(0.034) ^f	0.459 (0.007) ^a
PB18	0.327(0.017) ^a	0.846(0.007) ^b	0.772 (0.009) ^a
PB23	0.293(0.004) ^b	0.683 (0.0043) ^b	0.788 (0.005) ^a
PB24	0.122(0.006) ^f	0.638(0.0036) ^e	0.541 (0.007) ^d
PB37	0.157(0.006) ^e	0.529 (0.014) ^e	0.567 (0.010) ^c
PB46	0.185(0.004) ^d	0.604 (0.004) ^c	0.580 (0.009) ^c
PB50	0.236(0.002) ^c	0.585(0.006) ^d	0.547 (0.023) ^d

Note: The numerical values are mean and SD of different observations; Same values in superscript indicates no significant difference between observation.

Table.3 Biochemical characterization of drought tolerant bacteria

	PB3	PB17	PB18	PB23	PB24	PB37	PB46	PB50
Indole test	-	-	-	-	-	-	-	-
MR	+	+	+	+	-	+	+	+
VP	-	+	+	+	-	-	-	+
Citrate utilization	+	-	-	-	-	+	+	+
Amylase test	-	+	+	-	-	+	+	-
Pectinase test	-	-	-	-	-	+	+	-
Cellulase test	-	-	-	-	+	+	+	+
Urease test	+	-	-	-	-	+	+	+
Catalase test	+	+	+	+	+	+	+	+
H2S test	-	-	-	-	-	-	-	-
Gram Stain	Gram Positive							
KOH test	Gram Positive							
Shape	Rod	Rod	Rod	Rod	Coccus	Rod	Rod	Rod
Carbon assimilation:								
Glucose	+	+	+	+	+	+	+	+
fructose	+	+	+	+	+	+	+	+
Sucrose	+	-	+	+	-	-	+	+
Mannitol	+	+	+	+	-	-	+	+
Lactose	-	-	+	-	-	-	-	-
Trehalose	-	-	+	+	+	+	+	+
Succinic acid	+	-	-	-	-	+	+	+
Malic acid	+	-	-	+	+	+	+	+
Oxalic acid	-	-	-	-	-	-	-	-
Methanol	-	-	+	-	-	-	+	+

Note: + and – sign in above table indicates positive and negative for the biochemical test.

Fig.1 Dendrogram using the biochemical characterization of drought tolerant bacterial isolates

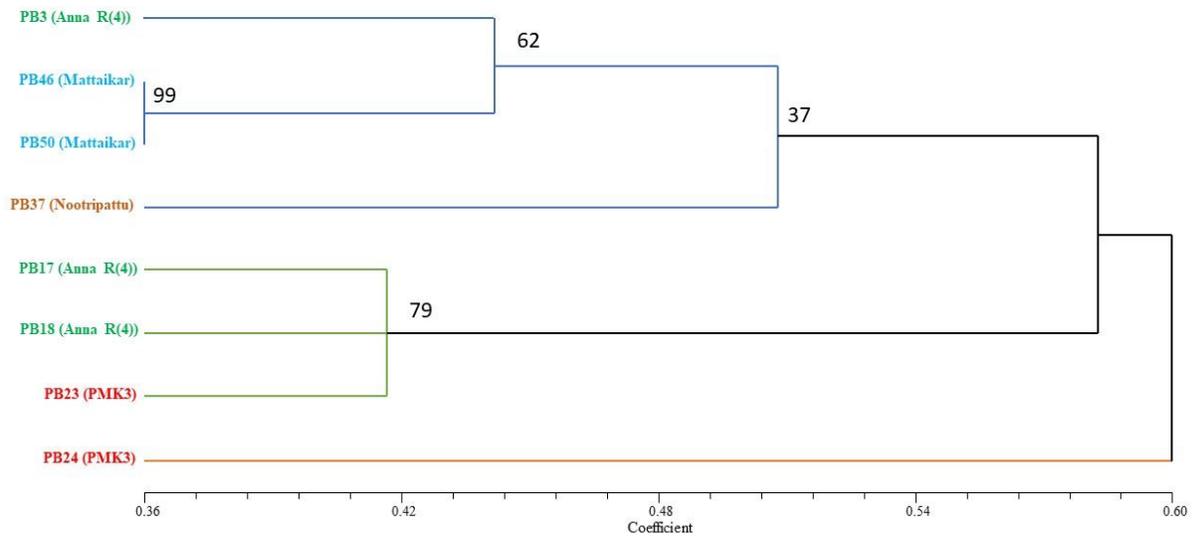
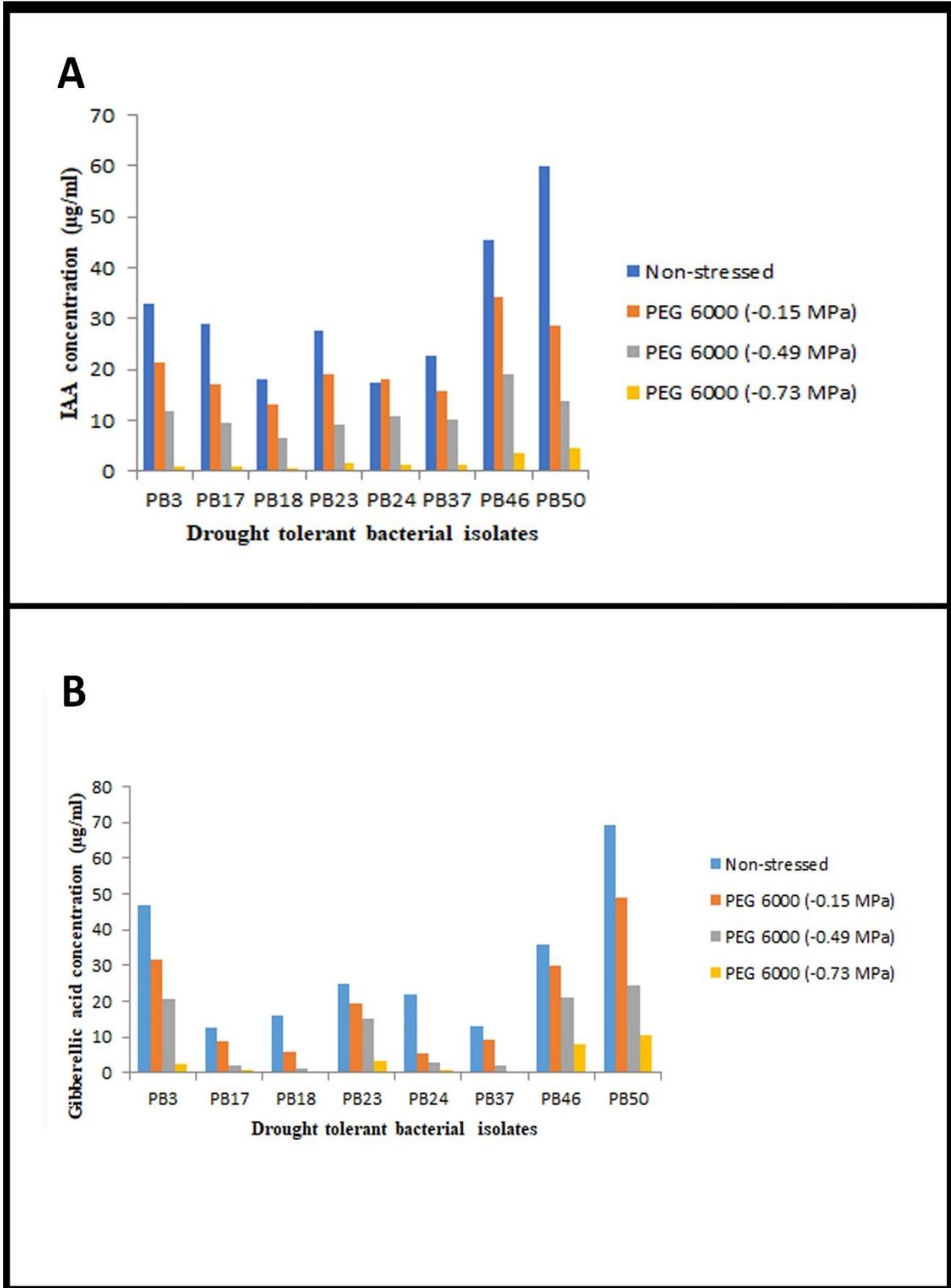


Fig.2 (A) IAA production (B) Gibberellic acid production by drought tolerant bacterial isolates



Phytohormone production

Drought tolerant bacterial isolates were assessed for the production of phytohormones IAA and gibberellic acid. The isolate PB50 was recorded with the highest production of IAA, 60 µg/ml under non-stress condition and 4.72 µg/ml under -0.73 osmotic stress conditions. Followed PB50, PB46 has the highest IAA production and production by other isolates has no significant difference (Figure 2A). In case of gibberellic acid production, isolate PB50 produces 69µg/ml under non-stress condition and 10.3µg/ml under -0.73 MPa osmotic stress condition. Followed by PB50, the isolates PB3 and PB 46 have the highest production of gibberellic acid (Figure 2B). In both the cases, all the isolates were recorded with gradual decrease in phytohormone production with increase in osmotic stress.

Bacteria on phylloplane constantly exposed to different stress factors and could sustain in that environment. A drought tolerant bacterium with PGP traits could help the plants from drought mitigation. In present study a total of 50 bacterial isolates were obtained, among them 8 isolates are identified to grow well under abiotic stress condition. The isolates may undergo cellular mechanism of osmotic adaptation through compatible solute and osmolyte productions (Madkour *et al.*, 1990). From the biochemical characterization and diversity analysis, it is shown that all the isolates except PB24, belongs to *Bacillus* species. The ability of *Bacillus* to produce endospore under extreme condition made them to thrive on rice phylloplane under drought condition (Nicholson *et al.*, 2000). The bacterial isolates from individual rice varieties are closely related and it shows diversified drought tolerant bacterial isolates were present on each rice varieties. Phylloplane is said to be highly variable environment, the bacterial

population size and species varies within the plants of same species and within the same area (Hirano and Upper, 2000). All the drought tolerant bacteria isolated from rhizosphere of soybean (*Glycine max*) was reported to *Bacillus* sp. and has noted with IAA production and Exopolysaccharide production capability (Susilowati *et al.*, 2018). Similarly, drought tolerant *Bacillus* sp. from maize shows plant growth promotion and reported to accumulate proline and sugars under drought condition, thus supports the growth of maize under drought stress (Vardharajula *et al.*, 2011). The isolates PB46 and PB50 was recorded to produce high IAA and gibberellic acid production and ability to withstand under abiotic stress condition.

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