

## EVALUATION OF INDOLE-3-ACETIC ACID DEFICIENT MUTANTS OF *PSEUDOMONAS MORAVIENSIS* AND ITS ROLE IN MITIGATION OF SALT STRESS IN *CICER ARIETINUM* L.

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### Abstract

Present investigation aims to characterize *Pseudomonas moraviensis* (wild type) and its IAA-deficient mutants and further to evaluate their role on physiology of chickpea plants (*Cicer arietinum* L.) under induced salt stress. Extracellular and whole cell protein contents as well as proline production in the M9 media and M9 media containing 4% NaCl of wild type of *P. moraviensis* and its mutants were determined. The functional group of PGPR both wild type and mutants were determined by Fourier transform infrared spectroscopy analyses (FTIR) of the culture. A significant increase (62-293%) was found in most of the mutants in the extracellular protein and whole cell protein (45-304%) than that of wild type. *P. moraviensis* wild type and its mutants had increase in the salt tolerance potential with time up to 75 mM NaCl treatments. Mutants exhibited higher tolerance to NaCl applied. Mutants convert tryptophan to IAA, 61-92% lower than that of the wild type. The plants inoculated with wild type showed increased chlorophyll, protein contents, proline contents and enhanced the peroxidase and superoxide dismutase. Some mutants produce proline much higher as compared with wild type under unstressed and salt stress. Mutants lower in proline production induce higher SOD activity in host plants but decrease the catalase activity as than that of the wild type. Thus it is concluded that the difference in the response of mutants may be attributed to the difference in their FTIR spectra and their ability of extracellular protein production.

**Key words:** Chickpea; *Cicer arietinum* L., *Pseudomonas moraviensis*; IAA deficient mutants; Bio-inoculant; Salinity.

### Introduction

Salt stress adversely affects the crop growth and yield due to osmotic stress (Ondrasek *et al.*, 2011; Oliveira *et al.*, 2013; Ahmad *et al.*, 2021). Beneficial micro-organisms have the potential to increase tolerance against stresses and enhance productivity as well as lower the dependency on chemical fertilizers (Mishra & Rhee, 2014). Implication of plant growth promoting rhizobacteria (PGPR) are environment friendly, energy efficient as well as economically viable approach to retrieve salinity affected land (Berg *et al.*, 2013; Issar *et al.*, 2012).

Numerous PGPR strains are tolerant up to 3% NaCl and can survive up to 40°C (Egamberdieva *et al.*, 2011). Salt tolerant PGPR had beneficial impact on plants growing in stressed environments, because of their higher salt tolerance potential even they can live in plant rhizosphere in saline arid soil (Upadhyay *et al.*, 2011). PGPRs significantly enhanced the activities of superoxide dismutase (SOD) and peroxidase enzymes (Stefan *et al.*, 2013). PGPR can tolerate salinity stress and protect the plant to its adverse effects by inducing hydraulic and ionic balance, maintaining greater osmotic conductance and sequestering toxic Na<sup>+</sup> concentration (Dodd & Perez-Alfocea, 2012).

*Pseudomonas moraviensis* significantly increase the growth, physiology and yield of several important crops in the saline soil (Hassan & Bano, 2016). *P. moraviensis* is gram-negative, non-spore-forming bacteria and can tolerate high levels of selenate and selenite (Staicu *et al.*, 2015). *Pseudomonas moraviensis* as IAA producer as

well as bio-inoculants on banana has also been documented (Ngamau *et al.*, 2012).

PGPR produce different types of phytohormones and secondary metabolites that improve the plant microbial interaction (Egamberdieva, 2009; Bhattacharyya & Jha, 2012). Staicu *et al.*, (2015) and Malik & Sindhu (2008) reported significant production of IAA by *Pseudomonas* sp. in LB media supplemented with L-tryptophan. IAA is amongst best characterized auxin and has great role in the plant microbial interaction and enhance the plant growth and physiology (Bhattacharyya & Jha, 2012; Reetha *et al.*, 2014).

The use of transposon elements are very important in the molecular biology techniques used for internal removal and re-organization of DNA (Gilbert *et al.*, 2002). Transposon insertion mutant library is important source for the selection of mutants (Langridge *et al.*, 2009).

IAA deficient mutant of *P. putida* (GR12-2) was prepared by the insertion of mutagens of Indole pyruvate decarboxylase (encoded by *ipdc* gene), that catalyzes the indole pyruvic acid for IAA synthesis. There were marked differences between application of *ipdc* mutants and wild type on root morphology (Patten & Glick, 2002; Malhotra & Srivastava, 2008). Malik and Sindhu (2008) derived low IAA producer and IAA overproducer mutants of *Pseudomonas* strains in culture media supplemented with tryptophan (Patten & Glick, 2002).

*Cicer arietinum* is third most important pulse crops and has important role in world food production (Dias *et al.*, 2015). Due to its importance as pulse crop, it is cultivated in barani areas of Pakistan during winter (Hassan *et al.*, 2012).

Thus present investigation was aimed to analyze the response of *P. moraviensis* wild type and its IAA deficient mutants under induced salt stress and to ascertain their role as bio-inoculant grown under axenic condition in pots and exposed to salinity stress.

## Materials and Methods

The present study was performed in stress Physiology lab at Quaid-i-Azam University, Islamabad. *P. moraviensis* was isolated from the rhizospheric soil *Cenchrus ciliaris* of the Khewra Salt Range (Hassan & Bano, 2015). IAA deficient mutants of *P. moraviensis* were prepared in the Molecular Microbiology Lab at University of Calgary Canada.

**Transposon mutagenesis:** For construction of mutants diparental mating method purposed by Thoma & Schobert (2009) was adapted. The *P. moraviensis* and donor strains of *E. coli* ST were allowed to grow on the LB media consisting of 100µg/ml of ampicillin and 50µg/ml of kanamycin (Data not presented). A TA cloning kit was used for cloning of amplified DNA into topo cloning vector (pCR 2.1), and then transformed it into the *Escherichia coli* XL1. After that, constructs were sequenced by using Quintarbio University of California.

The culture S17-1 pir D hem (ST18) having Km, Amp, Cm were grown on LB media. EZ-10 mini prep kit EZ-10 spin column plasmid DNA of pJQ15-6, pJQ15-7, pJQ15-8, pJQ118, pMH1701, pMH1801 and POT 182. 10µl of isolated plasmid DNA was mixed with *E. coli* competent cell (NEB, Canada) then allowed to cool for 30 min on ice after that placed at 42°C for heat shock for 1 minute. Then LB broth media (1µl) was added into the mixture and put at 37°C for 1 hour. This solution was plated having in culture media having δ-aminolevulinic acid and antibiotic. After incubation period of 3-7 d, colonies were streaked in LB broth and allowed to flourish overnight and diluted with 5ml of LB broth. Then recipient strain of *P. moraviensis* was cultured in the same way as described by Thoma & Schobert (2009). After that recipient and donor strains (2:200 ratio) were mixed and centrifuged at 16000 g for 1 min; then supernatant was spread on agar medium and grow for 6 h at 37°C. After 6 h, cells were scratched and mixed in 1 ml of LB broth. Serial dilutions were prepared for checking the mating efficiency. Then *P. moraviensis* was allowed to grow on AB minimal medium containing tetracycline and L-tryptophan. Mutants were determined by growing on LB agar medium supplemented with Chloramphenicol and Gentamicin (*E. coli* ST18) used for selecting against donor in broth i.e. with or with tetracycline supplemented LB agar media.

**Protein contents of PGPR:** Total protein contents were analyzed by following the procedure purposed by the method described by Lowry *et al.*, (1951). Bacterial culture (24h old) containing  $10^9$  cells were centrifuged at 3000 g for 15 min. After centrifugation, supernatant and pellet was separated for determination of extracellular and bacterial cell metabolites, respectively. Lowery reagent (7

ml) was put into the test tube and heated for 20 min under dark. 0.1ml of Folin Phenol Reagent was mixed into the mixture and mixed well by vortexed and incubate for 30 min. Then absorbance was recorded at 750nm. Calculations for total protein were made via slope intercept formula.

**Proline contents of PGPR:** Proline contents of PGPR were measured by following methods of Bates *et al.*, (1973). For that purpose, strains were grown on the M9 media and M9 media supplemented with 4% NaCl for 24 h at 30°C. All the PGPR isolates were harvested by centrifugation from 24 hours old culture. After centrifugation 100 mg of pellet was added in 1.2 ml of  $C_7H_6O_6S$  and vortexed for 1 min and allowed to centrifuge at 13000g for 10 mints. 500 µl of supernatant was picked and diluted with  $dH_2O$  and mixed with 1ml of  $C_2H_4O_2$  and 1ml of ninhydrin. After that, solution was incubated on  $H_2O$  bath for 1 h at 90°C. At last test tubes were kept in ice bath and for 3 min and toluene (2 ml) was mixed and vortexed for 2 min. Upper layer was isolated and absorbance was determined at 520nm by spectrophotometer (HITACHI, U1500).

**PGPR's salt tolerance potential:** PGPR's salt tolerance potential (mutant and wild type) was analyzed by using the method of Tank & Saraf (2009). Aqueous solutions of different concentrations (25, 50 and 75 mM) of NaCl were added to LB medium and fresh cultures were inoculated and put in the incubator shaker for 3 d at 120 g shaking speed. The optical density of the culture was recorded after every 24 hours using UV 1800 spectrophotometer (Shimadzu Japan) at 660 nm and taken as measure of growth of the bacteria that described the effect of NaCl on IAA deficient mutants and wild type.

**Colorimetric quantification of IAA indolic compound:** Colorimetric quantification of IAA indolic compound was determined by the method of Dworken & Foster (1958). For this purpose, wild type and IAA deficient mutants was grown on Dworkin Foster salts (DF) minimal medium. 20 µl of broth were added into 5ml of Dworkin Foster salts (added with 100 µg/ml of tryptophan) and centrifuged for 10 min at 5500 g. After centrifugation, supernatant (1ml) was mixed with 4ml Salkowski's reagent and allowed to stand at 37°C for 20 min. After 20 min, absorbance was recorded at 535 nm on the spectrophotometer (HITACHI, U1500) against IAA standard.

**Extraction, purification and quantification of bacterial IAA by HPLC:** Bacterial cultures (24 h old) were grown on LB broth supplemented with tryptophan (100 µ g/ml) or tryptophan free LB and incubated at 30 °C on the incubator shaker (ECELLA E24, USA) for 3-4 days at 100 g of continuous shaking to get  $10^7$  CFU/ml. After that, strains were centrifuged at 1,000g for 15 min at 4°C. After centrifugation, pH of the supernatant was maintained up to 2.8 using 1 N HCL. Method described by Tien *et al.*, (1979) was followed for determination of phytohormones. Ethyl acetate in equal volume was thoroughly mixed 3x with

supernatant in separating funnel and mixed well as a result two layers were formed. The ethyl acetate layer was separated and dried at 35°C by evaporation under vacuum generated by using rotay thin film evaporator. The residues were finally dissolved in 1000µl of HPLC grade methanol (Sigma, USA), 100µl of sample was filtered by 0.45 millipore filter. After filtration, sample was injected into the HPLC column for the detection of Phytohormones. Samples were run on the HPLC (Agilent 1100) equipped with C18 Column (39×300 mm) (BondaPack Porasil C-18, 37/50 µm, Waters, Eschbom, BRD and variable UV detector). At HPLC methanol and water (30:70 v/v) was used for mobile phase at the rate of 1,500 µl min<sup>-1</sup> and 20 min run time for each sample. IAA was determined at 280 nm on spectrophotometer (HITACHI, U1500) (Sarwar *et al.*, 1992) and culture medium without inoculation was taken as a blank.

**Fourier transformed infrared spectroscopy (FTIR):** FTIR of samples were measured by following the method of Lu *et al.*, (2004). Samples were extracted with ethyl acetate. Data of extract was recorded at 500 cm<sup>-1</sup> to 400 cm<sup>-1</sup> and OMNIC software was used for analysis. Spectrum of the sample was detected on Nicolet FTIR spectrometer along with OMNI sampler attenuated total reflectance accessory.

**Preparation of inoculum and inoculation of seeds:** Fresh culture was inoculated in 100 ml of LB broth and incubated on the shaker incubator (Excella E24 incubator shaker series) for 48 h. After 48 h, broth was centrifuged for 10 min at 1,000 g. After centrifugation, supernatant was removed and pellet was dissolved in dH<sub>2</sub>O and set optical density up to 1 at 660 nm.

Chickpea seeds variety Bhakkar 2011 was get from National Agricultural Research Council Islamabad. Ethanol (95%) and chlorox (10%) solution was used for surface sterilization of seed and consecutively washed with sterilized water. After seed sterilization, seeds were put in the inoculum for 3-4 h and then seeds were sown in the pots having sterilized soil and sand in 3 and 1 ratio. Then pots were kept in the growth chamber (IMCBL 2016, Percival Scientific) that was adjusted at 25°C and 12 h photoperiod. Salt treatments were induced in the rhizosphere soil by adding 150mM of aqueous solution of NaCl after 10 days of germination.

**Chlorophyll contents of chickpea leaves:** Chlorophyll contents were recorded by chlorophyll meter SPAD (SPAD-502, Minolta Camera Co., Ltd., Japan) before harvesting.

**Soluble protein contents of leaves:** Protein contents of fresh leaves were measured according to the procedure of Lowry *et al.*, (1951). 0.1 g of sample was grinded in 1 ml of phosphate buffer (pH 7.5) under 4°C and make homogenate mixture. After grinding mixture was centrifuged for 10 min for 3000 g. 100 µl of the supernatant was diluted by dH<sub>2</sub>O and made final volume up to 1 ml. 1 ml of reagent C (0.4 g NaOH (0.1N), 0.5 g

of CuSO<sub>4</sub>.5H<sub>2</sub>O, 1 g of Na-K tartrate and 2 g of Na<sub>2</sub>CO<sub>3</sub> was dissolved in 100 ml dH<sub>2</sub>O and horizontally shaken for 10 min. 0.1 ml of reagent D was mixed with extracted solution and absorbance was measured at 650 nm against Phosphate buffer as blank by using Bovine Serum Albumin as standard.

**Proline contents:** Proline contents of fresh leaves were determined according to Bates *et al.*, (1973). 0.1 g of sample was homogenate with 3% sulphosalicylic acid (4 ml) and kept at 5°C overnight. Then the mixture was centrifuged at 3,000g for 5min. 2 ml of supernatant was mixed with acidic ninhydrin and solution was incubated in water bath for 1 h at 100 °C and cools the mixture at 4°C. 4 ml of toluene was added in mixture and absorbance was measured by using toluene blank.

**Superoxide dismutase (SOD) activity:** SOD was measured according to the method of Beauchamp & Fridovich (1971). For this purpose, 0.5 g of fresh leaves of chickpea was ground with extracting buffer (1 g of PVP and 0.0028 g of Na<sub>2</sub>EDTA in 100ml of phosphate buffer). Then homogenate was centrifuged for 10 min at 3,000g and supernatant was used as enzyme extract. 3 ml of solution mixture (13mM methionine, 0.1 mM EDTA, 0.02 mM riboflavin and 0.075 mM NBT in 50 mM phosphate buffer) was homogenized with 0.1 ml of enzyme extract and absorbance was recorded at 560 nm and expressed as units/100 g of fresh weight.

**Peroxidase (POD) activity:** POD was measured by using process described by Reddy *et al.*, (1995). 1 g of samples were ground with 10 ml of phosphate buffer (0.1 M, pH 6.5) and centrifuged for 10 min at 3,000 g. Absorbance was noted at zero and 430 nm. 0.5 ml of H<sub>2</sub>O<sub>2</sub> was added in the cuvette and mixed well for testing the cuvette. The change in absorbance was recorded at 430 nm as one unit of peroxidase.

**Catalase activity:** Catalase activity was measured by following the method of Luck (1965). For this purpose, 0.5 g of fresh leaves were homogenate with 8ml of phosphate buffer (pH 7) and homogenate mixture was centrifuged at 3,000 g for 15 min and get supernatant that used as enzyme extract. 50 µl of enzyme extract was mixed with phosphate buffer (3 ml) and absorbance was noted at 240 nm on spectrophotometer (HITACHI, U1500) was measured. After that H<sub>2</sub>O<sub>2</sub> (3 ml) was added to experimental cuvette, and again absorbance was calculated. One enzyme unit was determined as the amount of enzyme required to decrease by 0.05 units of absorbance at 240nm.

### Statistical Analysis

Analysis of variance (ANOVA) was applied on the obtained data by using software Statistix version 8.1 by following Complete Randomized Design (CRD). Least significant difference was determined at p=0.05 (Steel & Torrie, 1980). Pearson correlation coefficients were calculated between different growth and physiology parameters by Statistix 8.1.

## Results

**Protein contents:** A significant increase (45-304%) was observed in all mutants except PM-3 and PM-5 as compared with wild type (Fig. 1) for whole cell protein. Highly significant increases (173%-304%) were observed in mutants PM-1, PM-2 and PM-7 than that of wild type. Extracellular protein was significantly high in almost all mutants except P.M-3. Maximum increases (293%) were observed in mutants PM-4=PM-5=PM-6=PM-7 over wild type. A positive correlation was observed between whole cell protein and bacterial growth in the absence of tryptophan ( $r = 0.48$ ) and in the presence of tryptophan ( $r = 0.66$ ).

**Proline production:** Bacterial cultures containing  $10^9$  cells were grown for 72h in M9 medium and M9 medium containing 4% NaCl. Results revealed that in M9 medium, mutants PM-1, PM-2= PM-8 had significant increases (48-100%) compared to wild type (Fig. 2). Mutants PM-2= PM-8 > PM-5 exhibited significant increases (20-37%) in the production of proline in M9 medium supplemented with 4% NaCl while mutants PM-4, PM-6 and PM-7 significantly reduced (27-39%) over wild type.

A significant positive correlation ( $r = 0.64$ ,  $p < 0.05$ ) was noted between proline produced under salt stressed condition and bacterial auxin production with and without the addition of tryptophan in culture medium ( $r = 0.55$ ,  $P = 0.05$ ).

**Salt tolerance Potential of *P. moraviensis* and its mutants against salt tolerance:** At 25 mM NaCl, no significant difference in the salt tolerance potential of wild type and mutants were recorded at 24 h and 48 h, except PM-4 and PM-8 (Fig. 3). At 72 h, the PM-1 and PM-3 exhibited greater salt tolerance potential over wild type.

At 50 mM NaCl treatment, PM -8 PM -2= PM-3 exhibited significant increases in salt tolerance potential (72-268%) over wild at 24 h (Fig. 4). A significant increase (51%), was observed at 48h, PM -8 > PM-5 in all mutants while PM-6 showed significant decreases compared to wild type. At 72 h, of salt treatment, mutant PM-6 showed significant increase (12%), whereas mutants PM-1, PM-4, PM-7 and P.M-8 exhibited decrease (11-65%) compared with wild type.

A significant increase was observed at 24 h for salt potential in all mutants at 75 mM NaCl treatment as compared to wild type except PM-1 and PM-4 (Fig. 5). At 48 h, there were significant increases (16-85%) in the salt tolerance potential of the mutants over wild type except PM-4. At 72 h of salt treatment, the mutants PM-1= PM-2= PM-3 showed significant increases (27-36%) in the salt tolerance potential over wild type, whereas PM-4 showed significant decrease. There was strong significant correlation ( $r = 0.52$ ,  $P = 0.05$ ) among the salt tolerance potential of mutants at 72 h and proline production ( $r = 0.35$ ,  $P = 0.05$ ) at 48 h.

**IAA contents:** Results revealed that the mutants have negligible IAA production as compared to wild type as revealed by the colorimetric method of determination using Salkowski's reagent (Fig. 6). Though, in presence of tryptophan some mutants produced IAA significantly lower than that of wild type. Similar trend was observed by HPLC analysis (Table 2). Wild type strain produced higher IAA in the absence ( $46.5 \mu\text{g ml}^{-1}$ ) and in the presence ( $64.4 \mu\text{g ml}^{-1}$ ) of tryptophan. Among mutants P.M-6 produced higher ( $0.62 \mu\text{g/ml}$ ) IAA in the absence of L-tryptophan while in the presence of tryptophan PM-7 produced  $2.88 \mu\text{g ml}^{-1}$  IAA.

Correlation between IAA production and salt tolerance potential measured after 24 h was medium (significant) ( $r = 0.4$ ,  $p = 0.05$ ) in the absence of tryptophan and ( $r = 0.45$ ) in the presence of tryptophan. The correlation between IAA production ( $r = 0.55$ ,  $r = 0.05$ ,  $P = 0.05$ ) and salt tolerance potential at 48 h were significant. Positive strong correlations were observed between IAA contents and salt tolerance potential of mutants at 72 h without tryptophan ( $r = 0.9$ ) and between IAA and salt tolerance potential of mutants with tryptophan ( $r = 0.98$ ).

## Fourier transformed infrared spectroscopy (FT-IR):

In wild type *P. moraviensis* results indicated the presence of O-H stretch and H bonds representing alcohol and phenols, C-H stretch representing alkanes, H-C=O:C-H stretch representing aldehyde group, -C=C- stretch representing the alkenes, C-C stretch in ring form vibrations in cyclic aromatic structures and C-N stretch representing aliphatic amines (Table 1) (Fig. 1).

Mutant-1 differed from wild type having functional group of  $1^\circ$ ,  $2^\circ$  amines and amides (containing N-H stretch) but lacked the alcohols, phenols and aldehydes group (Table 1) (Fig. 2). Mutant-2 differed from that of wild type in having functional group of  $1^\circ$ ,  $2^\circ$  amines, amides and carboxylic acids (having O-H stretch) but lacked the alcohols, phenols and aldehydes group (Table 1) (Fig. 3). Mutant-3 differed from that of wild type in having functional group of  $1^\circ$ ,  $2^\circ$  amines and amides but lack the alcohols, phenols, aldehydes and aromatic group (Fig. S<sub>4</sub>). Mutant-4 differed from that of wild type in having functional group of  $1^\circ$ ,  $2^\circ$  amines, amides, carboxylic acid and alkyl halides (contained C-H wag (-CH<sub>2</sub>X)) but lacked the alcohols, phenols group and aldehyde group (Fig. S<sub>5</sub>). Mutant-5 differed from that of wild type in having functional group of  $1^\circ$ ,  $2^\circ$  amines, amides, carboxylic acid and alkynes (contained -C≡C-H: C-H bend) but lacked the alcohols, phenols group and aldehyde group (Table 1) (Fig. 6). Mutants-6 and mutant-7 were similar to mutant 2 in having similar functional groups (Table 1). Mutant-8 differed from that of wild type in having functional group of  $1^\circ$ ,  $2^\circ$  amines, amides, carboxylic acid, nitriles (contained C≡N stretch) and alkyl halides but lacked the alcohols, phenols group and aldehyde group (Table 1).

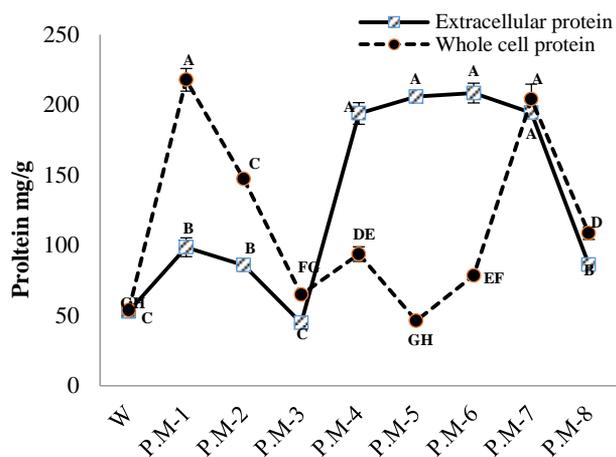


Fig. 1. Extracellular and whole cell protein produced by *P. moraviensis* and its mutants. W= *P. moraviensis* (wild type), P.M-1= Mutant 1, P.M-2= Mutant 2, P.M-3= Mutant 3, P.M-4= Mutant 4, P.M-5= Mutant 5, P.M-6= Mutant 6, P.M-7= Mutant 7, P.M-8= Mutant 8. Results are means of triplicates ± Standard Deviation.

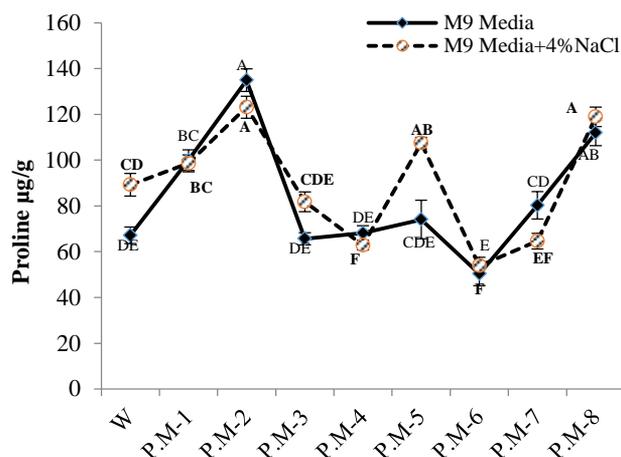


Fig. 2. Proline accumulation by *P. moraviensis* and its mutants growing in M9 medium and M9 medium supplemented with 4% NaCl. W= *P. moraviensis* (wild type), P.M-1= Mutant 1, P.M-2= Mutant 2, P.M-3= Mutant 3, P.M-4= Mutant 4, P.M-5= Mutant 5, P.M-6= Mutant 6, P.M-7= Mutant 7, P.M-8= Mutant 8. Results are means of triplicates ± Standard Deviation.

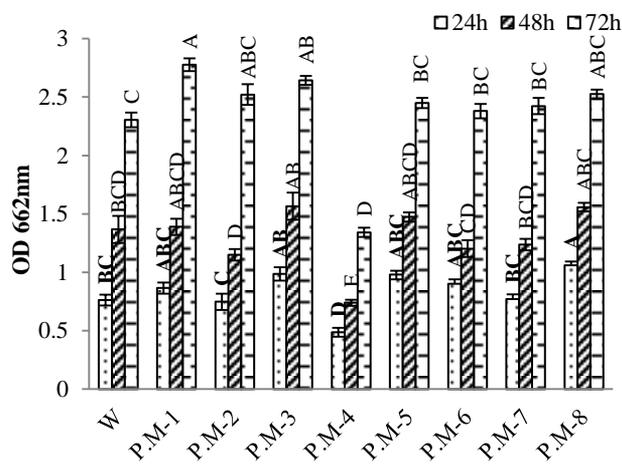


Fig. 3. Salt tolerance potential of *P. moraviensis* and its mutants in L.B broth media supplemented with 25 mM NaCl. W= *P. moraviensis* (wild type), P.M-1= Mutant 1, P.M-2= Mutant 2, P.M-3= Mutant 3, P.M-4= Mutant 4, P.M-5= Mutant 5, P.M-6= Mutant 6, P.M-7= Mutant 7, P.M-8= Mutant 8. Results are means of triplicates ± Standard Deviation.

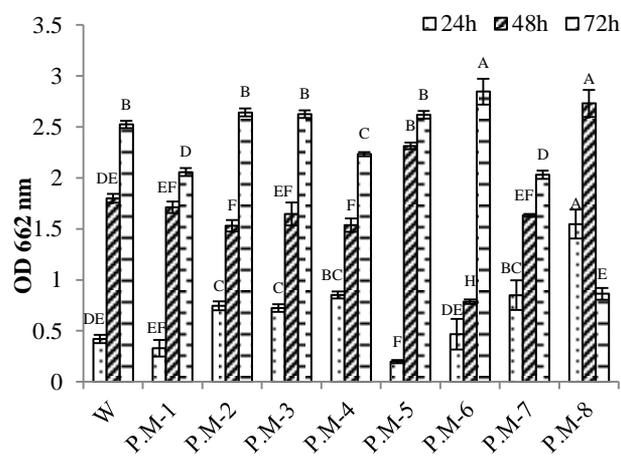


Fig. 4. Salt tolerance potential of *P. moraviensis* and its mutants in L.B broth media supplemented with 50 mM NaCl. W= *P. moraviensis* (wild type), P.M-1= Mutant 1, P.M-2= Mutant 2, P.M-3= Mutant 3, P.M-4= Mutant 4, P.M-5= Mutant 5, P.M-6= Mutant 6, P.M-7= Mutant 7, P.M-8= Mutant 8. Results are means of triplicates ± Standard Deviation.

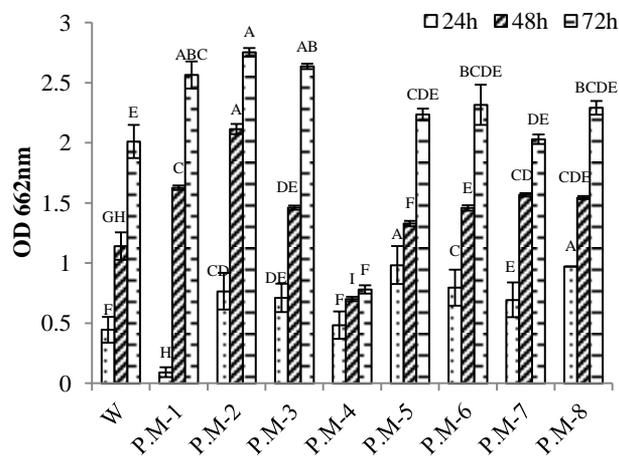


Fig. 5. Salt tolerance potential of *P. moraviensis* and its mutants in L.B broth media supplemented with 75 mM NaCl. W= *P. moraviensis* (wild type), P.M-1= Mutant 1, P.M-2= Mutant 2, P.M-3= Mutant 3, P.M-4= Mutant 4, P.M-5= Mutant 5, P.M-6= Mutant 6, P.M-7= Mutant 7, P.M-8= Mutant 8. Results are means of triplicates ± Standard Deviation.

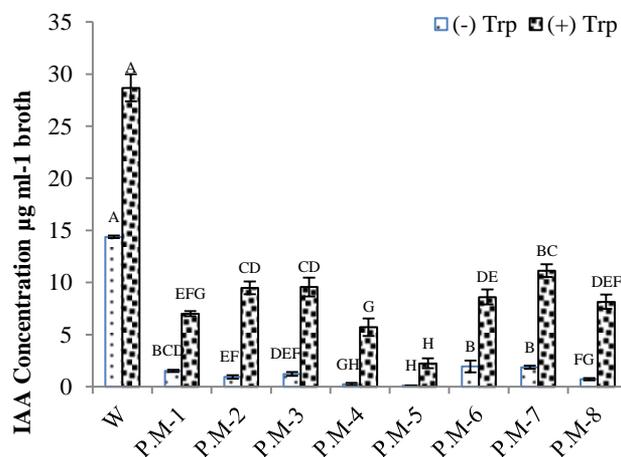


Fig. 6. IAA production by *P. moraviensis* and its mutants growing in LB broth and LB broth supplemented with 100µg/ml of tryptophan. W= *P. moraviensis* (wild type), P.M-1= Mutant 1, P.M-2= Mutant 2, P.M-3= Mutant 3, P.M-4= Mutant 4, P.M-5= Mutant 5, P.M-6= Mutant 6, P.M-7= Mutant 7, P.M-8= Mutant 8. Results are means of triplicates ± Standard Deviation.

**Table 1. Chlorophyll contents, Shoot fresh weight, Root fresh weight, Protein contents, Proline, SOD, POD and Catalase activity of chickpea leaves inoculated with *P. moraviensis* and its mutants after 17 days of germination and under salt stress treatment (Plants harvested after 7 days of treatment).**

Strains	Chlorophyll contents (SPAD value)	Shoot fresh weight (g)	Root fresh weight (g)	Protein (mg/g)	Proline ( $\mu\text{g/g}$ )	SOD ( $\mu\text{mol g}^{-1}$ Fw of shoot)	POD ( $\mu\text{mol g}^{-1}$ Fw $\text{min}^{-1}$ )	Catalase ( $\mu\text{mol g}^{-1}$ F. wt. of shoot $\text{min}^{-1}$ )
<b>Un-stressed</b>								
Control	39.6E ( $\pm 0.72$ )	0.94B ( $\pm 0.02$ )	0.06DE ( $\pm 0.00$ )	143.86I ( $\pm 19.6$ )	93.09C ( $\pm 1.39$ )	0.35EF ( $\pm 0.05$ )	0.80D ( $\pm 0.00$ )	0.08FG ( $\pm 0.00$ )
W	52.5A ( $\pm 0.49$ )	1.1A ( $\pm 0.03$ )	0.09CD ( $\pm 0.01$ )	420.14ABC ( $\pm 5.10$ )	174A ( $\pm 3.39$ )	1.08C ( $\pm 0.06$ )	1.39A ( $\pm 0.08$ )	1.95A ( $\pm 0.05$ )
P.M- 1	47.9ABC (0.86)	1.12A (0.03)	0.06DE (0.00)	336.86EF (17.18)	151.72ABC (3.86)	1.65B (0.07)	1.29AB (0.12)	0.40E (0.02)
P.M- 2	49DE ( $\pm 1.22$ )	0.55F ( $\pm 0.03$ )	0.08CD ( $\pm 0.00$ )	394.22BCD ( $\pm 5.33$ )	170.76A ( $\pm 2.24$ )	2.19A ( $\pm 0.08$ )	1.12BC ( $\pm 0.07$ )	0.71D ( $\pm 0.10$ )
P.M- 3	42.5 ( $\pm 0.78$ )	0.89BC ( $\pm 0.01$ )	0.07CDE ( $\pm 0.00$ )	241.47H ( $\pm 4.15$ )	98.46C ( $\pm 2.64$ )	1.47B ( $\pm 0.03$ )	0.98CD ( $\pm 0.01$ )	0.40E ( $\pm 0.05$ )
P.M- 4	48.2DE ( $\pm 1.04$ )	0.63EF ( $\pm 0.03$ )	0.04E ( $\pm 0.00$ )	379.06CDE ( $\pm 13.95$ )	97.995C ( $\pm 2.76$ )	0.44EF ( $\pm 0.05$ )	0.97CD ( $\pm 0.09$ )	1.1C ( $\pm 0.01$ )
P.M- 5	50.8ABC ( $\pm 0.78$ )	0.75DE ( $\pm 0.03$ )	0.12B ( $\pm 0.01$ )	404.94ABCD ( $\pm 24.12$ )	99.40C ( $\pm 3.00$ )	0.89CD ( $\pm 0.08$ )	1.30AB ( $\pm 0.02$ )	0.70D ( $\pm 0.02$ )
P.M- 6	44.6CDE ( $\pm 1.50$ )	0.81CD ( $\pm 0.02$ )	0.05E ( $\pm 0.01$ )	424.93ABC ( $\pm 1.57$ )	95.78C ( $\pm 3.53$ )	2.04A ( $\pm 0.04$ )	1.12BC ( $\pm 0.00$ )	0.92CD ( $\pm 0.06$ )
P.M- 7	51.1DE ( $\pm 1.25$ )	0.73DE ( $\pm 0.03$ )	0.18A ( $\pm 0.01$ )	447.66AB ( $\pm 54.29$ )	107.11BC ( $\pm 1.64$ )	0.19F ( $\pm 0.04$ )	1.06BCD ( $\pm 0.00$ )	0.03G ( $\pm 0.00$ )
P.M- 8	43DE ( $\pm 1.40$ )	0.80CD ( $\pm 0.02$ )	0.19A ( $\pm 0.03$ )	458.25A ( $\pm 92.0$ )	103.1C ( $\pm 0.95$ )	1.17C ( $\pm 0.01$ )	1.13BC ( $\pm 0.00$ )	0.28EF ( $\pm 0.01$ )
<b>75 mM NaCl</b>								
Control	40.53DE ( $\pm 0.54$ )	0.83B ( $\pm 0.02$ )	0.05E ( $\pm 0.00$ )	399.06D ( $\pm 2.49$ )	103.6EFG ( $\pm 1.44$ )	0.99CDEF ( $\pm 0.10$ )	0.99E ( $\pm 0.01$ )	1.06B ( $\pm 0.01$ )
W	45.47ABCD ( $\pm 1.13$ )	0.98A ( $\pm 0.04$ )	0.07CDE ( $\pm 0.01$ )	424B ( $\pm 0.91$ )	114.5DE ( $\pm 2.28$ )	1.74B ( $\pm 0.22$ )	1.44A ( $\pm 0.04$ )	1.95.26A ( $\pm 0.07$ )
P.M- 1	47.8AB ( $\pm 1.34$ )	1.10A ( $\pm 0.04$ )	0.04E ( $\pm 0.00$ )	301.06H ( $\pm 1.04$ )	155.34A ( $\pm 1.25$ )	0.64EF ( $\pm 0.05$ )	1.01DE ( $\pm 0.01$ )	0.37FG ( $\pm 0.02$ )
P.M- 2	42.25BCDE (0.95)	0.65CD (0.02)	0.13B (0.00)	338.03G ( $\pm 6.67$ )	97.41G ( $\pm 2.76$ )	1.18BCDE ( $\pm 0.06$ )	1.05DE ( $\pm 0.01$ )	0.45EF ( $\pm 0.01$ )
P.M- 3	38.87E ( $\pm 1.15$ )	0.72BC ( $\pm 0.02$ )	0.03F ( $\pm 0.00$ )	310.59H ( $\pm 0.80$ )	127.9BC ( $\pm 1.46$ )	1.48BCD ( $\pm 0.02$ )	1.37A ( $\pm 0.02$ )	0.68D ( $\pm 0.01$ )
P.M- 4	47.37AB ( $\pm 1.17$ )	0.55DE ( $\pm 0.02$ )	0.03F ( $\pm 0.01$ )	382.59DE ( $\pm 0.78$ )	95.43G ( $\pm 1.78$ )	3.47A ( $\pm 0.04$ )	1.34A ( $\pm 0.01$ )	0.99BC ( $\pm 0.00$ )
P.M- 5	49.4A ( $\pm 1$ )	0.72BC ( $\pm 0.01$ )	0.18A ( $\pm 0.01$ )	287.73H ( $\pm 3.92$ )	118.79CD ( $\pm 4.64$ )	3.05A ( $\pm 0.06$ )	1.07DE ( $\pm 0.01$ )	0.3GH ( $\pm 0.01$ )
P.M- 6	47.17ABC ( $\pm 1.33$ )	0.75BC ( $\pm 0.03$ )	0.09CDE ( $\pm 0.02$ )	368.09FF ( $\pm 3.92$ )	100.33F ( $\pm 1.54$ )	1.11BCDEF ( $\pm 0.09$ )	1.03DE ( $\pm 0.04$ )	0.55DE ( $\pm 0.04$ )
P.M- 7	42.13BCDE ( $\pm 1.01$ )	0.68C ( $\pm 0.03$ )	0.06DE ( $\pm 0.01$ )	466.48A ( $\pm 13.07$ )	110.38DE ( $\pm 1.44$ )	0.83DEF ( $\pm 0.07$ )	1.22BC ( $\pm 0.01$ )	0.88C ( $\pm 0.01$ )
P.M- 8	41.53CDE ( $\pm 1.35$ )	0.74BC ( $\pm 0.02$ )	0.06DE ( $\pm 0.03$ )	345.37FG ( $\pm 2.98$ )	126.96BC ( $\pm 3.91$ )	0.40F ( $\pm 0.07$ )	1.18C ( $\pm 0.03$ )	0.27GH ( $\pm 0.03$ )
LSD	7.36	4.9	2.05	11.2	8.93	4.1	2.3	1.6

W= *P. moraviensis* (wild type), P.M-1= Mutant 1, P.M-2= Mutant 2, P.M-3= Mutant 3, P.M-4= Mutant 4, P.M-5= Mutant 5, P.M-6= Mutant 6, P.M-7= Mutant 7, P.M-8= Mutant 8

**Table 2. IAA production by *P. moraviensis* and its mutants growing in LB broth and LB broth supplemented with 100µg/ml of tryptophan. Values given are mean of three replicates ± SE. Values followed by different letters are significantly different ( $P= 0.05$ ).**

strains	Inoculated broth	Tryp (+)
<i>P. moraviensis</i>	46.5A (± 3.35)	64.4a (± 3.8)
P.M-1	0.44C (± 0.007)	1.78e (± 0.03)
P.M-2	0.26E (± 0.001)	2.44c (± 0.09)
P.M-3	0.37D (± 0.005)	2.4c (± 0.07)
P.M-4	0.09G (± 0.00)	1.37d (± 0.04)
P.M-5	0.04H (± 0.00)	0.52f (± 0.007)
P.M-6	0.62B (± 0.008)	2.23d (± 0.05)
P.M-7	0.55BC (± 0.009)	2.88b (± 0.07)
P.M-8	0.2F (± 0.001)	2.13c (± 0.09)

W= *P. moraviensis* (wild type), P.M-1= Mutant 1, P.M-2= Mutant 2, P.M-3= Mutant 3, P.M-4= Mutant 4, P.M-5= Mutant 5, P.M-6= Mutant 6, P.M-7= Mutant 7, P.M-8= Mutant 8

**Effects of *P. moraviensis* and IAA deficient mutants on growth and physiology of chickpea:** Under salt-stressed and un-stressed conditions, all mutants showed significant decreases (23-50%) in shoot fresh weight except plant inoculated with mutant1 which was at par to plant inoculated with *P. moraviensis* under unstressed and salt stressed condition.

All the mutants under un-stressed and salt-stressed conditions showed non-significant decreases in root fresh weight except mutant 4 and mutant 6, whereas, mutant 5, mutant 7 and mutant 8 showed significant increases ranging from 16-87% over wild type (*P. moraviensis*) under unstressed condition. Under salt-stress condition, significant increases (9-16%) in root fresh weight were observed due to mutant 2 and mutant 5 than that of wild type. In the presence of tryptophan, correlations between root fresh weight and IAA production were significant under salt stress ( $r = 0.56$ ,  $P = 0.05$ ) and highly significant under unstressed conditions ( $r = 0.97$ ,  $P = 0.05$ ). Correlation among root fresh weight and IAA production without tryptophan was non-significant under salt stress ( $r = 0.32$ ,  $p = 0.05$ ) but was significant under unstressed condition ( $r = 0.97$ ,  $p = 0.05$ ).

Results revealed that under unstressed condition, *P. moraviensis* (wild type) significantly increased (32%) the chlorophyll contents over control (un-inoculated). All mutants showed a significant reduction (14-20%) compared to wild type in chlorophyll content except PM-1 and PM-5 which did not differ significantly with wild type (Table 1). Positive significant correlations were observed between chlorophyll content and IAA under stress condition ( $r = 0.9$ ,  $P = 0.05$ ).

The inoculation with wild type (*P. moraviensis*) showed significant increases in protein contents under unstressed and salt stressed condition control (un-inoculated). PM-1 and PM-3 the mutants did not differ significantly with wild type. Under salt-stressed condition all the mutants showed significant decreases (9-32%) over *P. moraviensis* (wild type) (Table 1).

There was a significant increase (87%) in proline treatment with *P. moraviensis* content of leaves following inoculation with wild type under unstressed condition (Table 1). Under unstressed condition, all mutants except mutant 1 and mutant 2 showed significant decreases as compared to wild type. Under salt stress, mutants PM-1,

PM-3, PM-5, and PM-8 significantly increased (4-36%) proline content over that of wild type.

Results showed that SOD activity was significantly higher (76-205%) in *P. moraviensis* wild type treatment over control (un-inoculated) both in unstressed and salt-stressed conditions. Under unstressed condition, mutants significantly increased (36-102%) SOD except mutant 4, 5 and 7, 8 (Table 1). Under salt-stressed condition, significant decreases were observed in all mutants except mutant 4 and mutant 5 (which exhibited significant increase) as compared to *P. moraviensis* (wild type).

Under salt-stress and unstressed condition, POD activity was significantly higher (40-78%) in *P. moraviensis* (wild type) over untreated control (Table 1). Significant decreases (21-32%) were observed in mutants except mutant 1 and 5 under unstressed condition over that of wild type. Under salt-stressed condition, most of the mutants decreased 11-27% POD activities over wild type, except mutant 3 and mutant 4.

*P. moraviensis* (wild type) showed maximum increase (25-71%) in catalase activity over control. All mutants showed decrease as compared to wild type (Table 1). Under salt stress, chlorophyll content was significantly from increased over control in all the treatments including wild type and mutants did not differ significantly wild type except PM-3. Similar was the case under salt stressed condition.

## Discussion

Under unstressed condition mutants 4, 5, 6 and 7 produced more extracellular protein whereas, the whole cell protein was higher in mutants 1, 2, 4 and 7 as compared to wild types. These mutants when used as bio-inoculants did not make any significant difference with the wild type on the leaf protein content of the chickpea plant (*Cicer arietinum*) but under salt stress except PM-7 other mutants were unable to induce new protein in the leaves of the plant. Protein content of the bacteria did not correlate directly with the growth of the bacteria in culture as evident from (Fig. 1), possibly their mutants synthesized more stress induced proteins than that of wild type.

The observed higher protein production by the mutants might be attributed to their greater salt tolerance potential as moderate significant positive correlation ( $r=0.66$ ,  $P=0.05$ ) was determined between protein and salt tolerance potential (measured as O.D of the culture) of mutants with 75 mM NaCl. The extracellular protein might assist to endure the abiotic stress (Limoli *et al.*, 2015).

The protein profiling and gene expression of plants needs modification in adaptation of salinity stress. Protein profiling is not inert but somewhat interactive with internal and external factors (Zhu *et al.*, 1995). Zorb *et al.*, (2004) investigated that under salinity, the process of analyzing the gene expression involving the identification of salt tolerance mechanism and verification of regulatory proteins. The main cause of loss of many protein bands in *Brassica parviflora* was salinity. The proportion of this protein band loss in *Brassica parviflora* was decreasing with increasing of NaCl in external environment (Parida *et al.*, 2004).

To produce resistance in plants against many stress factors such as salinity, PGPR are main that improve the antioxidants production (Younesi & Moradi, 2014). Many reports are there to describe the production role of PGPR in producing many antioxidants that improves the growth of plants under salinity (Jha & Subramanian, 2013; Kang *et al.*, 2014; Younesi & Moradi, 2014). Szabados & Savoure (2010) demonstrated that proline enhanced activities of various enzymes by maintaining the structure of enzymes under abiotic stresses (Ali *et al.*, 2013). PM-4 showed decrease (29%) in proline contents in the M9 media supplemented with 4% NaCl, with corresponding decrease in the salt tolerance potential at 75mM NaCl but P.M-1, P.M-2 and P.M-3 had significantly higher salt tolerance potential. Corresponding proline production was also higher in PM-2. Proline production was strongly (significant) correlated with salt tolerance potential of mutants ( $r=0.52$ ,  $P=0.05$ ) and IAA production ( $r=0.64$  and  $P=0.05$ ). All the mutants except PM-1, PM-3 and PM-8 were unable to increase proline production in chick pea (*Cicer arietinum*) plants under salt stress.

The IAA content of mutants and wild type were moderately (significant) correlated with the salt tolerance potential ( $r = 0.4$ ,  $P = 0.05$ ) at 24 h at 48 h ( $r = 0.55$ ,  $P = 0.05$ ) but strongly (significant) correlated at 72 h ( $r = 0.9$ ,  $P = 0.05$ ) as measured by OD representing the growth rate of the culture. The PM 1, 2 and 3,5,6,7 and 8 exhibited higher salt tolerance potential at 75mM NaCl. PM-2, 3, 6, 7 and 8 were also more efficient to convert tryptophan to IAA.

All mutants had functional group of 1°, 2° amines, amides that were absent in wild type. Rhizosphere associated bacteria are capable of producing carboxylic acids, aldehydes and other bioactive metabolites (Qiu *et al.*, 2006). Mutants 4 and 8 also have alkyl halide and mutant 8 showed nitriles in addition but lacked alcohol aldehyde and phenols. Pandey *et al.*, (2010) confirmed the presence of absorption peaks ranging from  $667.37\text{ cm}^{-1}$  to  $3302.13\text{ cm}^{-1}$  via FT-IR analysis of *Aeromonas hydrophila*. Another study conducted by Xiao *et al.*, (2007) reported same results in *Bacillus cereus*.

As bio-inoculants mutants behaved differently which might be attributed to the observed differences in their functional group as revealed by their FTIR spectra (see FTIR of mutants 4, 7 and 8). The differences in the response of the mutants were also recorded under salt stresses and unstressed condition. Under unstressed condition, all mutants produced chlorophyll content significantly lower than that of wild type under salt stress condition; chlorophyll content did not differ significantly as compared to wild type. Chlorophyll contents were strongly (significant) correlated with IAA produced in presence of tryptophan ( $r = 0.9$ ,  $P = 0.05$ ).

Phytohormones production like gibberellic acid and IAA that are produced by certain microbes is linked with the plant tolerance to the stress conditions (Berg *et al.*, 2013). Inoculation of plants with PGPR induced the production of IAA that promotes the growth of plants (Beneduzi *et al.*, 2012). IAA produced by PGPR induced salt tolerance and provide resistance against many diseases (Egamberdieva *et al.*, 2015). IAA treatments prevented the adverse effects of salt stress in wheat and these could be adopted as a potential growth regulator or antioxidant to improve wheat growth under moderate salt stress (Barakat *et al.*, 2013).

IAA not only improves the plant for nutrition absorption and accumulation but also play major role in root proliferation (Kazen *et al.*, 2013). As compared to wild type root growth of chickpea was not significantly affected following inoculation with mutant in general, the root growth exhibited significant increases over wild type in mutants PM-5, PM-7 and PM-8.

Noteworthy, the PM-2 and PM-5 exhibited significant increases in root fresh weight under salt stress over that of *P. moraviensis* (wild type). These mutants also showed significant increase in the salt tolerance potential over wild type as measured by their growth in presence of 75 mM NaCl. Shokri & Emtiazi (2010) indicated that yeast extract inoculation of *P. putida* in the seeds of *Brassica campestris*, produced less IAA that resulted in increase in the root length of seedlings.

The decrease in the fresh shoot weight in the results be due to decreased uptake of water and nutrients as a consequence of reduced fresh weight of root combined with the modulation of promoter and inhibitors i.e. IAA and ABA level (Cheng *et al.*, 2016). Kumari *et al.*, (2016) described that mutant *P. simian* strain AU-M4 provided significant resistance to mung bean under drought stress that increase production of IAA, ACC deminase activity and solubilization of inorganic phosphate than that of wild type and other mutants under drought stress (Kumari *et al.*, 2016).

PGPR produced several major hormones that are directly linked with stress resistant and enhanced growth (Gray & Smith, 2005). PGPR produced auxins that regulate plant physiological processes and enhanced growth, biomass and yield (Khan & Doty, 2009; Molina-Favero *et al.*, 2008).

The observed higher proline content in wild type under unstressed condition but decrease in the proline production under salt stress demonstrate the potential of wild type *P. moraviensis* strain to escape from salt induced osmotic stress.. This was in contrast to IAA deficient mutants (mutant 1, mutant 3 and mutant 8), which exhibited proline production greater than that of wild type in response to salt stress, possibly for osmoregulation and bioenergetics of the cell. Lee *et al.* (2008) also described that proline contents increased significantly in *Paspalum vaginatum* under salinity stress that maintained the homeostasis by maintaining the osmotic homeostasis.

The mutants (Mutant 1, 2, 3 and 6) showed significantly higher increase in SOD over wild type under unstressed condition indicating possibly the requirement for ROS scavenging system, mutants 4 and mutant 5 respond to salt by rising SOD and POD performance greater than that of wild type. The wild type mitigates oxidative stress by increasing the activities of antioxidant enzymes. Under salt stressed condition the mutant 4 and mutant 5 had higher SOD activity whereas, mutant 3 and mutant 4 had greater POD activities over wild type. Possibly the mutants deficient in IAA have different strategy to encounter stress. Antioxidants plays vital role in tolerance against numerous stresses in plants by producing of antioxidants in higher concentration (Gill & Tuteja, 2010; Tunc-Ozdemir *et al.*, 2009). Mittler (2002), investigated that alterations in the level of antioxidants contents might activate the ROS transduction as well as scavenging processes.

Plants inoculated with *P. moraviensis* mitigated the salt induced oxidative stress by stimulating their antioxidant enzymes system. Previous study described that SOD activity increased under salt stress (Lee *et al.*, 2001). Even under unstressed condition the wild type have very efficient ROS scavenging system by enhanced SOD and POD. The observed increase in the antioxidant enzymes SOD, POD etc. activities in wild type inoculated plants both under unstressed and more so under salt stressed may be helpful to recognize the important impact of PGPRs for detoxification of ROS (Faize *et al.*, 2011).

## Conclusions

The IAA deficient mutants have different strategy to combat salt stress. Inoculation with IAA deficient mutants reduced growth, decrease in water and nutrient uptake and in the protein production, ability in response to salinity and encounter osmotic as well as oxidative stress differently than the wild type. Nevertheless there are some mutants which supersede wild type in proline production and antioxidant enzymes suggesting their capability to encounter osmotic and oxidative stresses in much better way than wild type. Protein content of the bacteria does not correlate directly with the growth of the bacteria in culture. Possibly their mutants synthesize more stress induced proteins than that of wild type. Some mutants exhibit better tolerance to applied NaCl as compared with wild type and produce greater protein (both total cell protein and extracellular protein) and proline contents indicating that IAA alone is not the only criteria for salt tolerance.

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