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MODULATION OF BARLEY (*Hordeum vulgare*) DEFENSE AND HORMONAL PATHWAYS BY *PSEUDOMONAS* SPECIES ACCOUNTED FOR SALINITY TOLERANCE

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 # Equal Contribution
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In the present study, we investigated two *Pseudomonas* species strains (*P. fluorescens* SBW25 and *P. putida* KT2440) for their use in plant tolerance under salt stressed condition. Barley (*Hordeum vulgare* L.) plants inoculated with PGPR strains were subjected to 200mM salinity stress and were analyzed in comparison to non-treated stressed plants based on height, leaf area and fresh and dry weights of shoots. Our data revealed significant enhancement of root fresh and dry weights, chlorophyll content and relative water content in PGPR applied plants. Further investigation of various phytohormones (ABA, JA, ethylene, SA and IAA), stress responsive transporters and antioxidant enzymes genes at molecular level revealed that among the selected genes of ABA biosynthesis and regulated genes including *NCED, DHN5, DRF1* and *WRKY18* were significantly down-regulated, while *P5CS1* and *DHN1* genes were significantly up-regulated by PGPR applications. Except *LOX2* all the selected genes (*FAD3, LOX1, AOS, AOC, OPR3, PLDα1*and *PI (SD10)*) of JA biosynthesis pathway were significantly up-regulated by *P. putida* KT2440 strain. KT2440 strain also significantly induced the expression of ethylene biosynthesis *ACCO* and *ACCS* and SA biosynthesis *ICS* and protein kinase *MAPKK* genes. For auxin pathway, *P. fluorescens* SBW25 strain helped in significant induction of *TDC* expression, while *P. putida* KT2440 strain significantly boosted the expression of *T5M* gene. Both PGPR strains, displayed significant down-regulation of *NHX1* antiporter, while showed an up-regulation of *GR* antioxidant gene. A significant enhanced expression of nitrate transporter *NRT2.2,* and antioxidant *CAT2* genes was observed in *P. fluorescens* SBW25 inoculated barley roots compared to control roots. The findings of our study revealed effectiveness of selected PGPR strains for enhancing salinity tolerance by modulating the expression of major defense mediated pathways, stress responsive transporters and of antioxidant enzymes genes. Both PGPR strains showed improved effects, but *P. putida* KT2440 showed more pronounced significant effects comparatively. Conclusively, these PGPR species are an effective source of plant stress tolerance and elevated growth of barley plants.
Keywords: Salt stress, *Hordeum vulgare* L., PGPR, ABA, Jasmonic acid, Ethylene.

INTRODUCTION

The most important challenge for the agriculture world today is to produce 70% more food crops for an additional 2.3 billion people, which are to be expected by 2050 worldwide (FAO, 2009). The severity of abiotic stresses, particularly salt stress, disturbs the perspectives of crop production (Shahbaz and Ashraf, 2013; Donohue, 2005). Salinity is an abiotic stress which is common in arid and semiarid regions and is substantially declining the crop yield by more than 50% (Yang *et al.*, 2009). Soil is becoming saline in these regions because of poor irrigation management. According to an estimation, worldwide more than 20% of the cultivated and 33% of the irrigated agriculture land is salinized due to continuous increase of soil salinity and is increasing day by day and if it continues with this pace, then by the year 2050, 50% of the cultivable land would be salt affected (Jamil *et al.*, 2011). In recent times, high soil salinity have become a very serious factor that is limiting the agricultural production worldwide (Bybordi and Tabatabaei, 2010) and hence a major hindrance to fulfill the increasing demand of food crops.

During salt stress soluble salts deteriorate the soil fertility and affect the plant growth and development adversely (Munns and Tester, 2008). Salt stress imparts osmotic and ionic stress as well as secondary stresses like oxidative stress and nutritional imbalances (Hussain *et al.*, 2008). Initially it is known that soil salinity through osmotic stress restricts the plant growth which is then followed by ionic stress (James *et al.*, 2011). Osmotic stress builds up due to increase in salt concentrations around the roots, which reduces water uptake, therefore salt stress is also known as hyperosmotic stress (Munns, 2005). Salt stress is also known as hyperionic stress. The ionic stress builds due to increase of Na^+ concentration above the threshold level in plants mainly in leaves (Munns and Tester, 2008). Increase uptake of sodium and chloride ions causes severe ionic imbalances. High concentration of

Na^+ inhibits K^+ uptake which reduces productivity of plant (James *et al.*, 2011). Salinity stress also increases the production of reactive oxygen species (ROS) (Apel and Hirt, 2004; Ahmad and Umar, 2011), causing oxidative damages in lipids, proteins and DNA. Many metabolic and physiological changes occur in plants in response to salt stress (Saharan and Nehra, 2011). Both osmotic and ionic stresses cause leaf chlorosis, affect the photosynthesis, transpiration, translocation of nutrients, hormonal status and other metabolic activities (Munns, 2002).

Against such stressful conditions defensive strategy of plant encircles primary responses like changes in ionic, osmotic levels and stomatal closure etc. and secondary responses like phytohormones and secondary metabolites etc. Plant hormones play a fundamental role in the plant's ability to adapt to environmental stresses. Abscisic acid (ABA) is known as the major regulator of plant responses and tolerance to abiotic stresses (Peleg and Blumwald, 2011). Jasmonic acids (JA) also play relevant functions by regulating tolerance against salt and water stress along with other abiotic stresses (Khan and Khan, 2013; Riemann *et al.*, 2015). Ethylene regulate plant growth and is also known as stress hormone (Saleem *et al.*, 2007). Both biotic and abiotic stresses accelerate endogenous ethylene production, initially effecting the root growth followed by the whole plant. Salicylic acid (SA) is also known to participate in plant responses to abiotic stresses (Fragnière *et al.*, 2011). Indole-3-acetic acid (IAA) is the best studied phytohormone playing important role in cell division, differentiation, elongation, phototropism and gravitropism (Korasick *et al.*,2013), but recent studies also show its involvement in plant responses to different stress conditions. Similarly, intracellular antiporter proteins in plants are also known to improve tolerance to salt stress (Rodríguez-Rosales *et al.*, 2008).

Naturally occurring PGPR are usually associated with plant rhizosphere, a part of the soil having direct interactions with the plant roots (García-Fraile *et al.*, 2015). These PGPR are important for rescuing plant growth under stress (Kang *et al.*, 2014). As salt stress generates osmotic and ionic imbalance, microbial activities can help plants to increase their resistance against such stressful condition through various mechanisms like osmoregulation, ionic homeostasis (which maintains balance of Ca^{2+}: Na^+ and K^+: Na^+ ratio), by boosting antioxidant defense system (to compensate production of harmful ROS), maintenance of photosynthetic activity and regulation of root water uptake. Improved water uptake capacity of PGPR inoculated plants increase the photosynthetic rates under water deficit condition (Gururani *et al.*, 2013). Moreover, PGPR are also known to regulate the endogenous phytohormones under stress conditions (Kang *et al.*, 2014). PGPR containing 1-aminocyclopropane-1-carboxylate (ACC) deaminase reduce production of stress ethylene by breaking down ACC (a precursor of ethylene) into α-ketobutyrate and ammonia (Farajzadeh *et al.*, 2012). Various PGPR genera such as *Psudomonas* and *Bacillus* are reported to increase osmolytes synthesis in plants under abiotic stress conditions (Choudhary, 2012).

There exist genetic variations for salt tolerance in plants. The degree of salt tolerance varies with plant species and even with varieties within a species. Among the major food cops, barley (*Hordeum vulgare* L.) has the higher degree of salt tolerance than wheat and rice (Pang *et al.*, 2010; Ábrahám *et al.*, 2011). Barley is mainly grown as a grain and forage crop in the arid and several semi-arid regions (Al-Karaki, 2001). In many parts of the world it is used as an important source of food (Gupta *et al.*, 2010). Despite being a tolerant crop its growth and development are affected by salt stressed conditions due to osmotic and ionic imbalances (Mahmood, 2011).

With considerable increase in the knowledge of PGPR mediated salinity stress tolerance in host plants, now efforts are being made to understand the mechanism of tolerance at the gene expression level. PGPR induce diverse changes in plants and promote their growth through complex and unique combinations of different PGPR induced mechanisms (Bashan *et al.*, 2004). In barley, expression of various genes has been associated with stress (Bassil *et al.*, 2012). In the current work, we report the transcriptional responses of PGPR inoculated and non-inoculated barley plants to gradually imposed salt stress.

MATERIALS AND METHODS

PGPR inoculation, plant growth and salt stress treatment: *P. fluorescens* SBW25 and *P. putida* KT2440 strains were assessed for their potential ameliorating role for salt stress. PGPR were grown in Laurea broth (LB) medium for 24h on continuous shaking in an incubator (200 rpm) at 28 ± 2°C followed by centrifugation to collect pellets which were washed thrice and resuspended in sterile distilled water to set an OD (Optical Density) of 1 at 600nm = 8×10^8cfu/ml. Seeds (var. Snober-96) of barley obtained from National Agricultural Research Centre (NARC), Islamabad, Pakistan were surface sterilized using chlorox 10% solution (3 min) and subsequently washed with 95% ethanol and water. Surface sterilized seeds were used for inoculation of PGPR in the form of suspensions and in distilled water (control) for 3h and grown in petri plates with moistened cotton in the dark. After three to five days emergence of the radical and plumule, seedlings were placed in pots of sterilized soil and sand (3:1) for growth under controlled environmental conditions. Stress treatment (30 plants per treatment) was applied ~30 days after planting. Salt stress was given according to Habib *et al.* (2016) gradually to prevent salinity shock in plants with an increment of 50mM per day until the 200mM final salt concentration was attained in four days (Shahid *et al.*, 2011).

Measurement of plant growth parameters: Plant height of control and inoculated stress treated plants was measured

Table 1. List of barley genes with primer sequences used for qRT-PCR.

Genes	Forward Primers	Reverse Primers
HvFAD3	GAGACATAATCTACTACCAAACTG	TCCACCTGCTTGAATTGC
HvLOX 1	GCCATCGACCAAGGTAATCA	GATCCAGACACATCCATCCATC
HvLOX 2	GTGGATGAGTGGAACAAC	CGCCTAGTTGAGTTACAC
HvAOS	GGCACCAAGGTTGAGTTC	CGGTGTAAGGATCGTTGC
HvAOC	CACCGAGCCACACGCATG	GCAACACACGGAGATTCATTCAAC
HvOPR3	TACACCGACTACCCGTTCC	CCCAAACCCATCTACCATCAC
HvPLDα1	CCATCCTCACCACATAGATTGC	CACAAGTTCTGAATCACCAAAGG
HvPI(SD10)	CATTGTGCCGGTAGGAAGTAT	CTTAGCCAATCTTGGGAACCT
HvACCS	GCTGGTGCATACATGGATG	CCGTAAACAAGCAAAACAAAG
HvACCO	CGAGACACAAATTAAGAAGTTC	TGAGTAGCTAGAGCAAGTG
HvDhn1	GCAACAGATCAGCACACTTCCA	GCTGACCCTGGTACTCCATTGT
HvDhn5	GGCGTCATGGAGAACATCAA	GCCAGTCATTTCGGTGTCTT
HvDRF1	ACTTGTGGAGCAGAGGAAAG	AGGTACCCATCTCAGTCATAGT
HvWRKY18	GGAGGAGGAGATACTGGATGA	CTCCGTTGAACACCGATAGTAG
HvTDC	GTCAACCGCCTTCTAATGG	GCTGGTAGTCTTCTTGATGAG
HvT5M	CACGAAGATGATAAACTGATGAAC	GACAAGAGACTGGATTAATTGAAC
HvAmidase	CTGGCTATCTCACCTGTTAG	GCTCGCATTATCTTCTCAAG
HvNHX1	AAGCACCTTCCTTGGAGTATTT	GGTAGGCCATGAGCATCATAAG
HvNRT 2.2	CTATCATCCGCGACAACCTAAA	GAGGAAGGCGCATCCATATC
HvCAT2	CCACACCTTCTTCTTCCTCTTC	AACTTGACGTAGTGGGACTTG
HvGR	GTTGAAGTTACCCAGCCAGAT	AACTCTGGTCAACATTCCTGG
HvActin	ACTGGTGTTATGGTTGGTATGG	CTCCATGTCATCCCAGTTGTT

from the base of the plant to the tip of longest leaf. Leaf area of the plants was calculated using the following formula (McKee, 2010):

Leaf Area = Length of leaf (cm) ×Width of leaf (cm) × 0.74

Root and shoot fresh and dry weights were measured for harvested salt stressed barley plants with and without PGPR inoculations. For the measurement of dry weights, plants were kept in an oven for 72 h at 70°C.

Quantitative RT-PCR: Total RNA from the roots of control (non-inoculated) and PGPR (*P. fluorescens* SBW25 and *P. putida* KT2440) inoculated salt stressed barley plants was extracted using TRIzol reagent (MRC, TR#118). RNA was quantified using Nanodrop spectrophotometer (Titertek Berthold, Germany) and for the removal of genomic DNA contamination treated with RNase-free DNase I (Thermo Scientific Cat. # EN052). We used 1-µg of total RNA for cDNA synthesis by using RevertAid First Strand cDNA Synthesis kit (Thermo Scientific Cat. # K1622) as recommended by the manufacturer. Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo scientific Cat. #K0221) was used for qRT-PCR using MyGo Pro Real-Time PCR system (IT-IS international Ltd, UK). Thermal cyclic conditions were: 95ºC for 10 min followed by 40 cycles of denaturation at 95ºC for 15 sec, annealing at 60ºC for 30 sec and extension at 72ºC for 30 sec. Sequences of previously reported primers (Marimuthu and Smith, 2012) and designed using IDT PrimerQuest Tool used in this study are listed in Table 1. *HvActin* was used as internal control. The mRNA transcript levels were expressed as $2^{-\Delta\Delta CT}$. Fold changes in transcript abundance for inoculated salt stressed plants were analyzed and compared with control stressed plant.

Statistical Analysis: For statistical analysis statistical program SPSS version 20.0 was used. Means and standard errors (SE) of means of three replicates each were determined for control and inoculated stress treated plants. Data were analyzed using One-way analysis of variance (ANOVA) followed by Tukey's HSD post hoc test for multiple comparisons at $p \leq 0.05$ significance level.

RESULTS AND DISCUSSION

***Effect of PGPR on growth parameters of barley under 200mM salt stress condition*:** Plant growth is an important character for the determination of plant's salt tolerance ability. It is well known that growth of the plants is affected by salinity stress (Parida *et al.*, 2002) through influencing the fresh and dry weights of shoots and roots (Ashraf *et al.*, 2003). Long term root growth is also affected due to having in direct contact with toxic salts (Tyerman and Skerrett, 1998), which ultimately affects the biomass production. Our current work showed that salt stress influenced the growth attributes like plant height, leaf area, shoot and root fresh and dry weights in non-inoculated control plants, while PGPR strains (*P. putida* KT2440 and *P. fluorescens* SBW25) were found to have ameliorative effects on barley growth. Application of these strains improved the growth of barley plant by increasing the plant height, leaf area and fresh and dry weights of shoots with respect to non-treated plants under saline condition

(Fig. 1A&B and Fig. 2A&B). Similar results were also reported by Baniaghil *et al.* (2013), where the *Pseudomonas* and *Azospirilum sp.* under salinity stress increased the growth of canola plants. Our results also corroborate with previous findings of Singh *et al.* (2013) and Rojas-Tapias *et al.* (2012), where the *Pseudomonas putida* UW4 treated tomato plant showed increased shoot growth under salt stress condition. For roots, PGPR inoculation found to be more effective for enhancing fresh weight significantly with more pronounced effect observed for *P. fluorescens* SBW25. Similarly, *P. fluorescens* SBW25 also displayed significant effect for root dry weight (Fig. 2C&D).

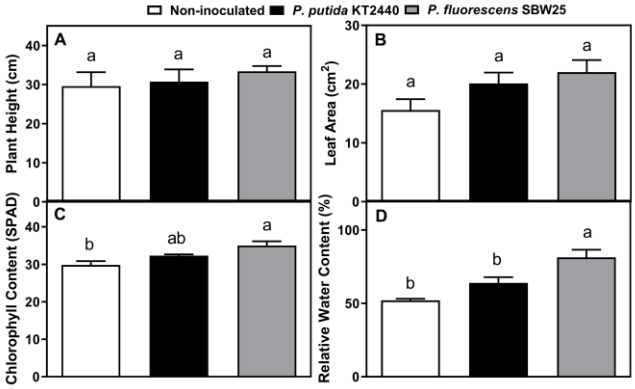

Figure 1. Effect of PGPR (*P. fluorescens* SBW25 and *P. putida* KT2440) on plant height, leaf area, chlorophyll content and relative water content of barley plants under 200mM salt stress condition.(A) Plant height; (B) Leaf area; (C) Chlorophyll content; and (D) Relative water content. Bars are mean ± SE, while n=3. Bars represented by different letters are significantly different at $p \leq 0.05$.

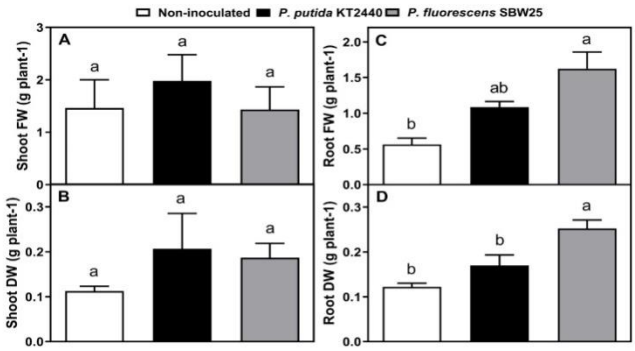

Figure 2. Effect of PGPR (*P. fluorescens* SBW25 and *P. putida* KT2440) on shoot and root fresh and dry weightsof barley under 200mM salt stress condition. (A) Shoot FW (Fresh weight); (B) Shoot DW (Dry weight); (C) Root FW; and (D) Root DW. Bars are mean ± SE, while n=3. Bars represented by different letters are significantly different at $p \leq 0.05$.

The low water potential (which lowers the cell turgor and reduces cell division and elongation) in the rooting medium and the toxic effects of Na^+ and Cl^- ions might be the reasons of depressed growth of plants under salinity stress (Silveira *et al.*, 2009). Likewise under saline conditions, assimilation of CO_2 also reduces, which ultimately reduces the growth of root (Kasukabe *et al.*, 2004).

Photosynthesis is the vital process affected by salt stress (Hayat *et al.*, 2010). In addition to stomatal closure leading to reduced assimilation of CO_2, non-stomatal factors such as decrease in green pigments and reduced leaf area also contribute to decrease photosynthesis during salt stress. Salts usually affect ionic content, photosynthetic enzymes and chlorophyll content (Misra *et al.*, 1997). Leaf chlorophyll content is the best indicator of salt tolerance (Percival *et al.*, 2003). In our current study, chlorophyll content was found to be significantly higher in barley plants receiving bacterial suspension, hence showing that beneficial association can undermine such stresses. *P. fluorescens* SBW25 showed more pronounced significant effect than that of *P. putida* KT2440 when compared to control plant (Fig. 1C). Similarly, high chlorophyll content was also observed in PGPR inoculated salt stressed cucumber (Kang *et al.*, 2014) and rice plants (Deivanai *et al.*, 2011). Likewise, Rojas-Tapias *et al.* (2012) also reported that PGPR inoculation increased chlorophyll content in leaves and enhanced stress tolerance. Decreased chlorophyll content in salt stressed control plant might be due to excessive amount of Na^+ and Cl^- ions and ROS formation that degenerate cell organelles (Hassine and Lutts, 2010). While the higher chlorophyll content and dark green leaves in inoculated plants might be owing to the maintenance of photosynthetic efficiency by PGPR.

Photosynthetic parameters like leaf relative water content is also affected by salinity (Nadeem *et al.*, 2006; Lee *et al.*, 2005). Water status of leaf is also important for the growth of plant. Salinity reduces soil water potential (Lloyd *et al.*, 1989). The shortage of available water causes cellular dehydration. It has been reported that PGPR help in maintaining the hydraulic conductance of root, which under saline environment help in maintaining plant water status (Marulanda *et al.*, 2007). RWC is an indicator of water stress, which decreases during salinity stress. In our current investigation, the RWC of PGPR inoculated plants was observed to be higher than that of control during salinity stress. The effect of *P. fluorescens* SBW25 inoculation was found to be significant for enhancing RWC (Fig. 1D). Our findings are in agreement with previous study (Marulanda *et al.*, 2010), where PGPR inoculated maize plants helped in reducing salinity stress by fetching more water compared to control when exposed to salt stress. Bacterial exopolysaccharides play a role in protecting plants from water stress (Vardharajula *et al.*, 2011).

***Regulation of phytohormones biosynthesis by PGPR under 200mM salt stress condition*:** It has been found that plant's

response to abiotic stress is controlled by complex network of hormonal signals (Bartsch *et al.*, 2010; Lumba *et al.*, 2010). Phytohormones such as ABA, JA and ethylene are well documented to be involved in plant responses to abiotic stress (Großkinsky *et al.*, 2016). In addition, SA and auxin also play a role for improving plant tolerance to abiotic stresses (Kazan, 2013; Silva *et al.*, 2017). During salinity stress regulation of gene expression includes a broad range of mechanisms used by the plants to up or down regulate the production of specific gene products. PGPR are known to mitigate stress response by regulating hormonal balance in plants and inducing systemic resistance to stress. PGPR have ability of synthesizing phytohormones (Etesami *et al.*, 2015), which affect the metabolism of endogenous phytohormones (Sorty *et al.*, 2016). In our current investigation, we assessed the regulation of endogenous phytohormones in barley plant by PGPR under 200mM salt stress application.

***Expression anlaysis of ABA biosynthesis and ABA-regulated genes*:** The role of ABA in tolerance to salinity and other types of stress appears to be the regulation of water balance in the plant. Isopentenyl diphosphate is the precursor of ABA biosynthesis which leads to the formation of zeaxanthin, antheraxanthin, and trans-violaxanthin which is then converted into 9-cis-neoxanthin. 9-cis-neoxanthin is then cleaved to form 15C xanthoxine compound. This step is the rate limiting step catalyzed by the enzyme 9-cis-epoxy carotenoid dioxygenase (*NCED*). Xanthoxin is oxidized to ABA-aldehyde and then finally converted to ABA (Wasilewska *et al.*, 2008). The effect of PGPR on ABA status of plant is controversial; some studies found that PGPR enhance salinity induced ABA accumulation, while other reports explored a decrease in ABA accumulation. As elaborated by earlier reports this effect may fluctuate depending on different microorganisms and plant species (Yang and Crowley, 2000; Evelin *et al.*, 2009). According to our current findings expression of ABA biosynthesis gene *NCED* was significantly down-regulated both in *P. putida* KT2440 inoculated (~0.09-fold) and in *P. fluorescens* SBW25 inoculated (0.03-fold) barley roots with respect to non-PGPR interaction (Fig. 3A). Chen *et al.*, (2016) also reported the same down-regulation of *NCED* expression in PGPR inoculated seedlings under salinity stress. Our findings are also supported by various other studies reporting low level of ABA under PGPR applied and stress condition (Iqbal and Ashraf, 2013; Kang *et al.*, 2014). Higher ABA level under salt stress inhibits leaf expansion and shoot development, however PGPR application counteract the adverse effect of stress by decreasing the ABA level and increasing leaf area.

Rhizobacteria also have the ability of improving plant water relations by increasing the accumulation of osmolytes in plants. Expression of the genes important for stress tolerance like enzymes of osmoprotectant synthesis was also found to be regulated by ABA (Fujita *et al.*, 2011). Proline is an osmolyte and proline carboxylate synthase (*P5CS*) gene is responsible for its synthesis from glutamate. Various researchers determined the relation between proline biosynthesis *P5CS* gene expression and proline accumulation in PGPR treated plants and found that up-regulation of *P5CS* gene expression in plant roots leads to free proline accumulation (Kumari *et al.*, 2015). Our study also revealed the significant (4.2-fold) induction of proline biosynthesis *P5CS1* gene expression in *P. putida* KT2440 applied plants, whereas *P. fluorescens*SBW25 inoculation also showed higher expression level with respect to control stressed plants (Fig. 3B). Our results are confirmatory to previous study (Kim *et al.*, 2014), where the PGPR treatment increased the expression level of proline biosynthesis (*P5CS1* and *P5CS2*) genes in Arabidopsis plant. *P5CS* was also expressed in Arabidopsisand rice plants when exposed to ABA and salt stress (Kishor and Sreenivasulu, 2014).

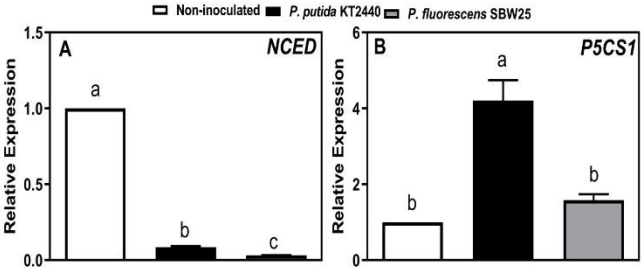

Figure 3. Effect of PGPR (*P. fluorescens* SBW25 and *P. putida* KT2440) on ABA biosynthesis and regulated genes expression of barley plant under 200mM salt stress condition. (A) *NCED*; (B) *P5CS1*. Bars are mean ± SE, while n=3. Bars represented by different letters are significantly different at $p \leq 0.05$.

Dehydrins (DHNs) belong to LEA II group of LEA proteins and are also referred to as Responsive to ABA (RAB) proteins. Dehydrins (DHNs) are known to be induced by different stresses (salinity, drought, cold, heat etc.) causing cell dehydration. Over expression of these dehydrins in several crop plants has been reported to provide tolerance against various abiotic stresses (Kumar *et al.*, 2014). In this study, dehydrin gene *DHN1* also exhibited significantly higher expression in both *P. putida* KT2440 (5.3-fold) and *P. fluorescens* SBW25 inoculated (5-fold) barley roots when compared to control roots (Fig. 4A). Under salt, drought and cold stresses increased expression of *DHN* has also been reported previously in barley, wheat (Kosová *et al.*, 2014) and in rice (Kumar *et al.*, 2014). Expression level of *DHN5* was found to be reduced significantly in both PGPR treated plants with statistically more significant reduction recorded in *P. putida* KT2440 inoculated plants with respect to control plants (Fig. 4B).

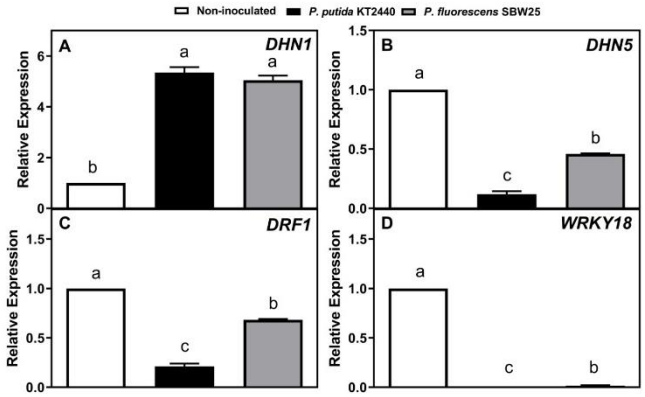

Figure 4. Effect of PGPR (*P. fluorescens* SBW25 and *P. putida* KT2440) on ABA-regulated genes expression of barley plant under 200mM salt stress condition. (A) *DHN1*; (B) *DHN5*; (C) *DRF1*; (D) *WRKY18*. Bars are mean ± SE, while n=3. Bars represented by different letters are significantly different at $p \leq 0.05$.

The dehydration responsive factor 1 (*DRF1*) gene belongs to DREB family and is known to play a role in abiotic stress response. Several evidences indicate that *HvDRF1* in barley plays a role in ABA-mediated gene regulation (Xue and Loveridge, 2004). Similarly, transcription factors (TFs) responsible for transcript regulation constitute a major group of genes which are also induced by abiotic stresses (Hsieh *et al.*, 2010). Among them WRKY TFs are one of the major groups in plants known to participate in ABA mediated signaling (Dong *et al.*, 2003). Our study revealed a significant reduction of *DRF1* and *WRKY18* genes in both PGPR treated plants compared to control plants (Fig. 4 C & D). Lower expression of these genes along with *DHN5* might be associated with the lower expression of *NCED* gene, as these genes are known to be regulated by ABA. PGPR inoculations facilitate the plant growth under the stress by repressing *DHN5, HvDRF1* and*WRKY18* genes expression and thus play positive role in amelioration of salt stress.

***Expression analysis of JA biosynthesis genes*:** JA is synthesized by the octadecanoid pathway, its synthesis begins by the peroxidation of trienoic fatty acids at C-13 catalyzed by lipoxygenase (*LOX*), as a result 13-hydroperoxide is generated, which is further modified to allene oxide fatty acid and then cyclized to 12-oxo-phytodienoic acid (*OPDA*). JA is synthesized from OPDA by the reduction of double bond and the three consecutive rounds of β-oxidation. Jasmonic acid can further be converted enzymatically into various derivatives (Vick and Zimmerman, 1983). Expression analysis of various JA biosynthesis related genes was done under salt stress condition. Our data showed significant (~2-folds) induction of *FAD3* transcripts in *P. putida* KT2440 inoculated salt stressed barley roots with respect to control plants (Fig. 5A). Salt stress significantly boosted the *LOX1* gene expression in both PGPR (6-folds and 10.5-folds) treated barley roots. Among the two strains tested *P. fluorescens* SBW25 showed more pronounced significant (10.5-fold) induction (Fig. 5B). On the other hand, significant reduction of *LOX2* gene expression was noticed in PGPR applied plants with more significant reduction (0.09-fold) observed in *P.*

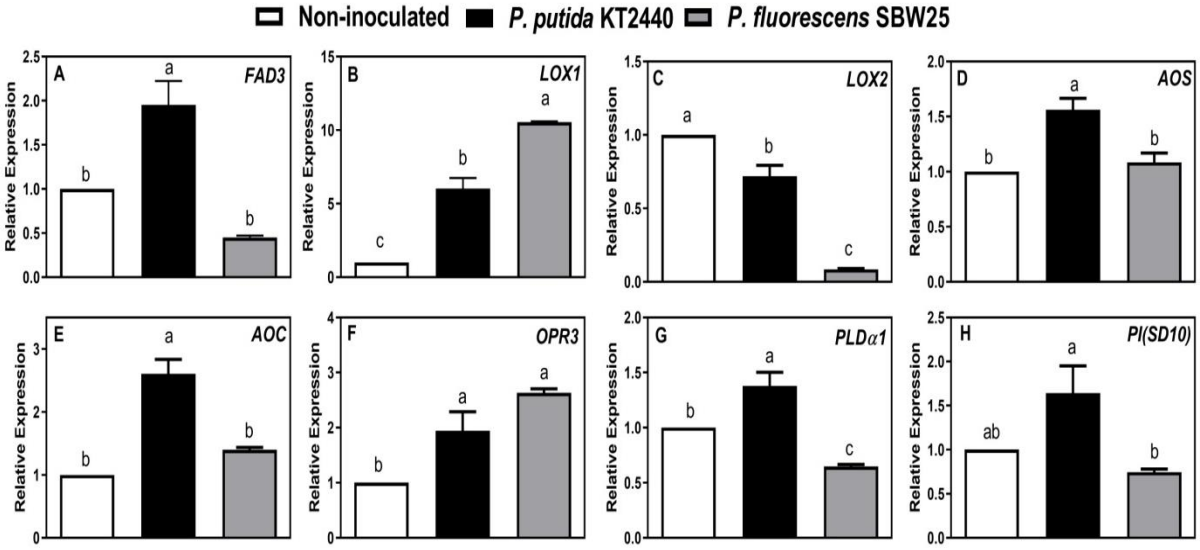

Figure 5. Effect of PGPR (*P. fluorescens* SBW25 and *P. putida* KT2440) on JA biosynthesis genes expression of barley plant under 200mM salt stress condition. (A) *FAD3*; (B) *LOX1*; (C) *LOX2*; (D) *AOS*; (E) *AOC*; (F) *OPR3*;(G) *PLDα1;* (H) *PI (SD10)*. Bars are mean ± SE, while n=3. Bars represented by different letters are significantly different at $p \leq 0.05$.

fluorescens SBW25 inoculated plants when compared to control stressed plants (Fig. 5C). *AOS* and *AOC* genes displayed significant (1.6-fold and 2.6-fold) induction in *P. putida* KT2440 treated barley roots, while *P. fluorescens* SBW25 application also showed higher transcript levels compared to control plants (Fig. 5D & E). Application of both PGPR also found to be useful for up-regulating (~2-fold and 2.6-fold) the *OPR3* gene expression significantly with respect to control plants (Fig. 5F). Our data also showed significant induction of *PLDα1*gene expression in *P. putida* KT2440 (~1.4-fold) inoculated barley roots, while a significant (0.6-fold) reduction was noticed in *P. fluorescens* SBW25 inoculated barley plants (Fig. 5G). Protease inhibitor gene *PI (SD10)* exhibited significantly (1.6-fold) higher expression in *P. putida* KT2440 treated plants, while exhibited a significantly (0.7-fold) lower expression in *P. fluorescens* SBW25 inoculated plants (Fig. 5H). Conclusively, *P. putida* was efficient enough to enhance most of the JA biosynthetic genes transcript levels contributing towards increased resistance to salt stress**.** Another study of Pedranzani *et al.* (2003) also showed higher accumulation of mRNA of *LOX*, *AOS* and protease inhibitor under saline treatment.

***Expression analysis of ethylene biosynthesis genes*:** Ethylene in plant is synthesized by Yang cycle, in which S-Adenosylmethionine is converted to ACC by ACC synthase (*ACS*), which is then converted to ethylene by ACC oxidase (*ACO*) (Yoon and Kieber, 2013). Synthesis of ethylene increases under salt stress (Kamei *et al.*, 2005), however in some cases a decrease in production was also observed e.g. under prolonged water storage conditions (Morgan and Drew, 1997). Some authors also suggested that ethylene play no major role in plant response to abiotic stress (Cao *et al.*, 2007; El-Khallal *et al.*, 2009). Our current investigation displayed significant induction of both *ACCS* (~4-fold) and *ACCO* (8-fold) genes in *P. putida* KT2440 inoculated salt stressed barley plants (Fig. 6A & B). Significant up-regulation of *ACS* and *ACO* genes may trigger ACC accumulation in plants during the initial phase of stress. As reported previously, application of ACC or ethylene improves plant tolerance to high salt stress most probably through increasing the expression of ROS scavengers (Peng *et al.*, 2014). On the other hand *P. fluorescens* SBW25 inoculated plants showed statistically no significant difference in expression of *ACS* and *ACO* genes with respect to control plants (Fig. 6A & B).

***Expression analysis of SA biosynthesis and kinase genes*:** Salicylic acid (SA) production initiates induced systemic resistance (ISR) and improves performance of plants under both biotic and abiotic stress conditions (Pozo and Azcón-Aguilar, 2007). SA biosynthesis starts from chorismate and proceeds through isochorismate. Isochorismate synthase (*ICS*) is the rate-limiting enzyme for this pathway catalyzing the transformation of chorismate to isochorismate. SA level increases with the increase of SA biosynthetic enzyme activity under saline condition (Sawada *et al.*, 2006). Our study also revealed extraordinarily higher expression level (908-fold) of *ICS* gene in *P. putida* KT2440 treated plants, while *P. fluorescens* SBW25 treated plants also exhibited increase in expression level (45-fold) with respect to control plants (Fig. 7A). Our results are in accordance with previous findings (Nazar *et al.*, 2011; Jayakannan *et al.*, 2013), where the higher level of SA was found to improve salinity tolerance in plants.

Mitogen activated protein kinase (MAPK) in eukaryotes play a role for transducing extracellular stimuli into intracellular responses. Stress tolerance in plants is also mediated by protein phosphorylation, which is the central theme of cell signaling (Zhu, 2002). It is reported that osmotic stress induces the expression of several protein kinases. In the current study, transcript levels of *MAPKK* gene was induced significantly (~1.4-fold) in *P. putida* KT2440 applied plants, while *P. fluorescens* SBW25 inoculated roots also exhibited higher expression level compared to control barley roots (Fig. 7B).

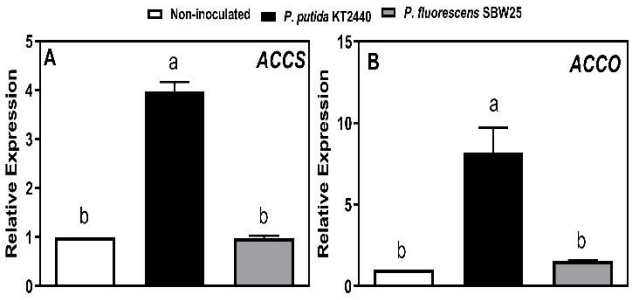

Figure 6. Effect of PGPR (*P. fluorescens* SBW25 and *P. putida* KT2440) on ethylene biosynthesis genes expression of barley plant under 200mM salt stress condition. (A) *ACCS*; (B) *ACCO*. Bars are mean ± SE, while n=3. Bars represented by different letters are significantly different at $p \leq 0.05$.

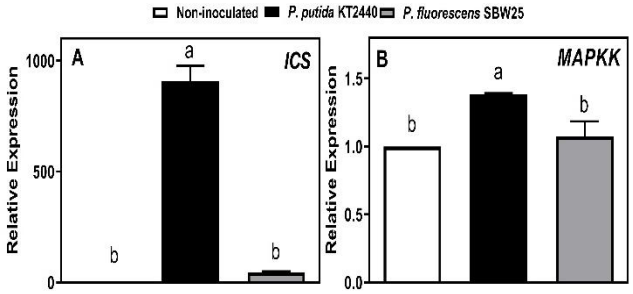

Figure 7. Effect of PGPR (*P. fluorescens* SBW25 and *P. putida* KT2440) on SA biosynthesis and kinase genes expression of barley plant under 200mM salt stress condition. (A) *ICS*; (B) *MAPKK*. Bars are mean ± SE, while n=3. Bars represented by different letters are significantly different at $p \leq 0.05$.

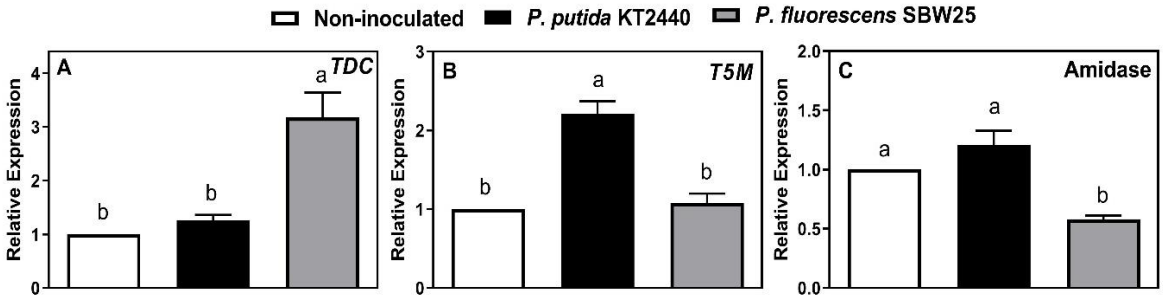

Figure 8. Effect of PGPR (*P. fluorescens* SBW25 and *P. putida* KT2440) on IAA biosynthesis genes expression of barley plant under 200mM salt stress condition.(A) *TDC*; (B) *T5M*; (C) Amidase. Bars are mean ± SE, while n=3. Bars represented by different letters are significantly different at $p \leq 0.05$.

***Expression analysis of IAA biosynthesis genes*:** Saline condition was found to affect the plant growth by accumulating IAA in the root which affects the cell elongation. Moreover, it also inhibits synthesis of cytokinins (Dodd *et al.*, 2005). Two major pathways are known for auxin biosynthesis in plants, Trptophan (Trp)-dependent and independent pathways (Chandler, 2009). Typtophandecrboxylase (*TDC*) is a key enzyme that converts tryptophan to tryptamine (Rizvi *et al.*, 2016). Amidsases (AMIDASE 1(*AMI1*)) can hydrolyze Indole-3-acetamide (IAM) into IAA (Pollmann *et al.*, 2003). In our present investigation, expression of IAA biosynthesis gene *TDC* was up-regulated significantly (3.2-fold) in *P. fluorescens* SBW25 inoculated plants, while *P. putida* KT2440 inoculated barley plants also displayed increased expression with respect to control plants (Fig. 8A). Tryptophan-5-monooxygenase (*T5M)* gene was induced significantly (2.2-fold) in *P. putida* KT2440 inoculated barley plants, whereas induction in *P. fluorescens* SBW25 inoculated plants was not found to be statistically significant in comparison to control plants (Fig. 8B). Expression of amidase gene was reduced significantly (~0.6-fold) in *P. fluorescens* SBW25 inoculated barley plants, while *P. putida* KT2440 treatment exhibited higher expression level compared to control plants (Fig. 8C). In addition to phytohormones, transcript analysis of other stress responsive ion and nitrate transporters genes and antioxidant enzymes genes was also done using control and PGPR inoculated barley root samples grown under salt stress conditions.

***Expression analysis of ions and nitrate transporter genes*:** *NHX1* catalyses Na^+ accumulation in vacuole and is known to be involved in plant adaptation to salt stress (Gaxiola *et al.*, 1999). Even though over-expression of antiporters is known to improves salt tolerance in plants (Rodríguez-Rosales *et al.*, 2008), but our data showed significant reduction of *NHX1* antiporter both in *P. putida* KT2440 (~0.3-fold) and *P. fluorescens* SBW25 (~0.2-fold) inoculated plants with respect to control barley plants (Fig. 9A), that might be their ameliorative role for stress tolerance. Our this finding is not in agreement with previous finding (Pinedo *et al.*, 2015), where PGPR colonized *Arabidopsis* plants exhibited higher NHX transcript level when exposed to long term salt stress. However, another study of Ouziad *et al.* (2006) revealed no changes in expression of *NHX1* and *NHX2* antiporter genes under saline condition.

Nitrate transporters (NRT) in plants are responsible for the uptake of nitrate. Abiotic stress, such as salinity, severely affects nitrogen uptake and its assimilation in plants. The qRT-PCR analysis revealed more significant (~219-fold) up-regulation of nitrate transporter gene *NRT2.2* in *P. fluorescens* SBW25 inoculated plants, while statistically no significant difference in expression of *NRT2.2* was noticed between *P. putida* KT2440 inoculated and control plants (Fig. 9B).

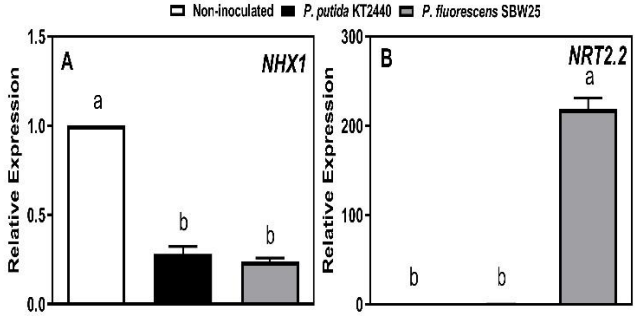

Figure 9. Effect of PGPR (*P. fluorescens* SBW25 and *P. putida* KT2440) on ions and nitrate transporter genes expression of barley plant under 200mM salt stress condition. (A) *NHX1*; (B) *NRT2.2*. Bars are mean ± SE, while n=3. Bars represented by different letters are significantly different at $p \leq 0.05$.

***Expression analysis of antioxidant enzyme genes*:** The antioxidant enzymes in plants have the ability of removing free radicals generated during abiotic stress conditions (Mittler, 2002; Abogadallah, 2011). Our results revealed that mRNA levels of the genes encoding antioxidant enzymes *CAT* and *GR* were increased in the PGPR treated salinized

plants compared to control plants. Expression level of *CAT2* was up-regulated significantly (13-fold) in *P. fluorescens* SBW25 inoculated plants, while it was slightly decreased in *P. putida* KT2440 inoculated salinized plants with respect to control plants (Fig. 10A). Expression of *GR* was up-regulated significantly both in *P. putida* KT2440 inoculated (~12-fold) and *P. fluorescens* SBW25 inoculated (5.4-fold) plants in comparison to control stressed plants (Fig. 10B). Hence, we provided evidences that plant acquired protection against salinity stress are due to PGPR applications. Our results are in agreement with the findings of Gururani *et al.* (2013), where an increase in the expression levels of *CAT* and *GR* in PGPR treated potato plants under 200mM salt stress was observed. Habib *et al.* (2016) also reported similar findings of increase of transcript levels of antioxidant enzymes in PGPR inoculated okra plants under salt stress condition.

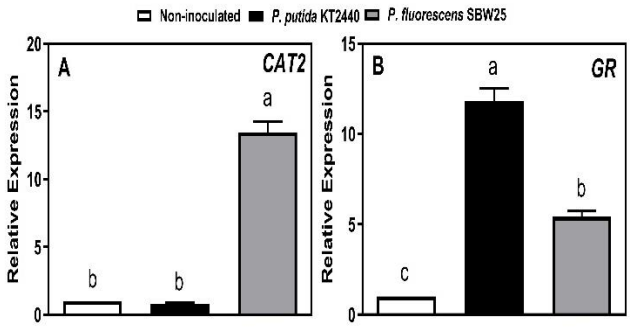

Figure 10. Effect of PGPR (*P. fluorescens* SBW25 and *P. putida* KT2440) on antioxidant enzyme genes expression of barley plant under 200mM salt stress condition.(A) *CAT2*; (B) *GR*. Bars are mean ± SE, while n=3. Bars represented by different letters are significantly different at $p \leq 0.05$.

***Conclusion*:** In conclusion, the results of this study suggest that *P. putda*KT2440 and *P. fluorescens* SBW25 bacterial strains have ability to improve the productivity of barley plants by reducing the adverse effects of salinity stress by modulating defense and hormonal pathways. Inoculation of barley plants with these strains conferred tolerance against 200mM salt stress at physiological level and also at molecular level by modulating the expression of various genes related to defense mediated phytohormones pathways, stress responsive transporters and antioxidant enzymes under salt stress condition. Among the two strains tested *P. putda* KT2440 was shown to be effective in stress tolerance and plant growth promotion of barley. Conclusively, these *Pseudomonas* strains can be used to benefit barley plants or related crops for their growth promotion and tolerance under salt stress and the candidate genes used in this study can be used as potential markers of tolerance against salt stress.

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