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Pseudomonas putida attunes morphophysiological, biochemical and molecular responses in *Cicer arietinum* L. during drought stress and recovery

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Abstract

Drought is one of the most important abiotic stresses that adversely affect plant growth and yield potential. However, some drought resistant rhizosphere competent bacteria are known to improve plant health and promote growth during abiotic stresses. Present study showed the role of *Pseudomonas putida* MTCC5279 (RA) in ameliorating drought stress on cv. BG-362 (*desi*) and cv. BG-1003 (*kabuli*) chickpea cultivars under in vitro and greenhouse conditions. Polyethylene glycol-induced drought stress severely affected seed germination in both cultivars which was considerably improved on RA-inoculation. Drought stress significantly affected various growth parameters, water status, membrane integrity, osmolyte accumulation, ROS scavenging ability and stress-responsive gene expressions, which were positively modulated upon application of RA in both chickpea cultivars. Quantitative real-time (qRT)-PCR analysis showed differential expression of genes involved in transcription activation (*DREB1A* and *NAC1*), stress response (*LEA* and *DHN*), ROS scavenging (*CAT*, *APX*, *GST*), ethylene biosynthesis (*ACO* and *ACS*), salicylic acid (*PR1*) and jasmonate (*MYC2*) signalling in both chickpea cultivars exposed to drought stress and recovery in the presence or absence of RA. The observations imply that RA confers drought tolerance in chickpea by altering various physical, physiological and biochemical parameters, as well as by modulating differential expression of at least 11 stress-responsive genes. To the best of our knowledge, this is the first report on detailed analysis of plant growth promotion and stress alleviation in one month old *desi* and *kabuli* chickpea subjected to drought stress for 0, 1, 3 and 7 days and recovery in the presence of a PGPR.

***Key words*:** Amelioration, Germination, Osmolytes, PGPR, Rhizosphere, Rhizobacteria

Abbreviations

ACO	1-aminocyclopropane-1-carboxylate oxidase
ACS	1-aminocyclopropane-1-carboxylate synthase
APX	Ascorbate peroxidase
CFU	Colony forming unit
DHN	Dehydrin
DREB1A	Dehydration responsive element binding 1A
GST	Glutathione S-transferase
LEA	Late embryogenesis abundant
MYC2	Myelocytomatosis 2
NAC1	NAM, ATAF and CUC 1
PGPR	Plant growth promoting rhizobacteria
PR1	Pathogenesis related protein 1
ROS	Reactive oxygen species
TBA	2-thiobarbituric acid
TCA	Tri-chloro acetic acid

1. Introduction

Chickpea (*Cicer arietinum* L.) is the second most important food legume cultivated by resource deprived farmers dwelling in arid and semi-arid regions across the globe, and is also considered a suitable source of dietary protein for human consumption owing to its excellent amino acid composition (Thudi et al. 2014). It is cultivated on an area of 13.54 million ha worldwide with a total production of 13.1 million tonnes and a productivity of 0.97 tonnes/ha (FAOSTAT 2013). India ranks first among chickpea producing countries with a total production of 8.83 million tonnes from an area of 9.6 million ha (FAOSTAT 2013). The domesticated chickpea has been broadly grouped into two distinct types namely, microsperma or small-seeded '*desi*' with brown-colored seed coat, and macrosperma or large-seeded '*kabuli*' with beige-colored seed coat (Thudi et al. 2014). Despite its economic importance, chickpea production has not witnessed any increase in yield or area under cultivation in past few decades owing to various biotic and abiotic constraints that challenges its production and productivity (Thudi et al. 2014). Drought is one of the most important abiotic stresses adversely affecting chickpea production leading to 40-50% decline in its yield potential regardless of the fact that it usually grows in relatively dry and less irrigated lands and some of its cultivars also adapt well to water-deficit conditions (Ahmad et al. 2005).

Drought stress response is a complex trait affected by several factors including environment, genotype, developmental stage, and severity and duration of stress (Lata et al. 2015). The morphophysiological and biochemical traits related to drought stress include leaf wilting, reduction in leaf area and chlorophyll content, root elongation, decline in RWC, and generation of reactive oxygen species (ROS) (Lata et al. 2011). ROS impairs the normal functions of cells and cause oxidative damage by reacting with proteins, lipids and deoxyribonucleic acid. Membrane components of plants are also damaged due to generation of ROS when exposed to drought stress (Lata et al. 2011). Apart from various physiological and cellular changes, several genes and gene products also get affected by drought stress at transcriptional, post-transcriptional and translational levels (Lata et al. 2015). Taken together all these factors contribute towards impaired growth and development ultimately leading to yield loss in crop plants. Therefore it is important to develop superior varieties or resort to alternate technologies for sustainable agricultural production. In the recent years there has been enormous accumulation of genetic and genomic information in chickpea due to genome sequencing of both *desi* and *kabuli* types (Jain et al. 2013; Varshney et al. 2013). This has encouraged several agronomists and researchers to utilize genomics assisted breeding and transgenic approach to alleviate the effects of abiotic stresses particularly drought in chickpea (Thudi et al. 2014). However improvements regarding drought

stress tolerance remain largely elusive, as it is a quantitative trait and drought stress response and adaptation is a part of the multigenic response observed under water-deficit conditions (Nautiyal et al. 2013). Further since plant breeding and genetic engineering is a labour intensive and time consuming process, there is a need to develop newer strategies or techniques that would be helpful for sustained chickpea production and productivity. One such alternate technology is the use of plant growth promoting rhizobacteria (PGPR) for abiotic stress amelioration which also holds quite significance nowadays in the context of changing climate and excessive fertilizer use in agricultural soils (Nautiyal et al. 2013).

Numerous Gram positive and negative PGPR are known to colonize plant rhizosphere and bestow favourable effects through several direct and indirect mechanisms such as biofilm formation; chemotaxis; siderophore, exopolysaccharide and indole acetic acid (IAA) production; and 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity (Srivastava et al. 2012; Nautiyal et al. 2013). Recently there have been several studies where PGPR are also reported as potential elicitors for abiotic stress tolerance including drought and salinity (Yang et al. 2009; Nautiyal et al. 2013). However the molecular basis of plant-PGPR interaction in rhizosphere is yet not fully understood as it is not a case of characteristic "gene-to-gene" interaction (Nautiyal et al. 2013). *Pseudomonas* sp. is one of the largest groups of PGPR which naturally occur in agricultural soils and known to possess several phytobeneficial traits (Srivastava et al. 2012). A *Pseudomonas putida* strain MTCC5279 (RA) has been isolated from the desert regions of Rajasthan and its physiological characterization for various plant growth promotional attributes and abiotic stress tolerance such as IAA production, phosphate solubilisation and growth at different concentrations of polyethylene glycol (PEG-6000) and salt (NaCl) stress were carried out in an earlier study from our laboratory (Srivastava et al. 2012). The ACC deaminase activity of this strain was also determined in a separate experiment in our lab (data not shown). Considering its excellent phytobeneficial and abiotic stress tolerance properties, it has been proposed as a very good PGPR for agricultural crops. Therefore, the aim of the present study was to investigate the effect of RA-inoculation on various morphophysiological and biochemical parameters as well as on expression profiles of a few stress responsive genes in two chickpea types, '*desi*' and '*kabuli*' during different durations of drought stress and subsequent recovery conditions.

2. Materials and Methods

2.1 Germination assay

This study was conducted in a growth chamber of the Division of Plant-Microbe Interactions, CSIR-National Botanical Research Institute (CSIR-NBRI), Lucknow, India. The experiment was laid out in a completely randomized block design with three replications and with ten seeds per

replication (n=30). Two distinct types of chickpea namely, cv. BG-362 (*desi*) and cv. BG-1003 (*kabuli*) were used for this study to see the effects of RA-inoculation on germination under PEG-induced osmotic stress. The PEG-6000 solutions were prepared according to weight by volume at two different concentrations viz. 15% and 30%. Seeds germinated in MilliQ water were considered as control. Chickpea seeds were first surface sterilized with 1% NaOCl for 5 min followed by several washings with sterile MilliQ water and soaked overnight. Ten seeds each of both chickpea types were then placed on Whatman No. 1 filter paper in 90 mm Petri dishes and kept in growth chamber at 25±2ºC and 70% relative humidity. The same three experimental sets of control, 15% and 30% PEG were used for RA-inoculated chickpea seeds. For RA-inoculation, seeds were bacterized for 6 h in RA suspension (~10^7 $CFUmL^{-1}$) grown in Nutrient broth (NB) medium at 28ºC with shaking at 250 rpm. The data on germination percentage were recorded after 3 days of treatment. Seeds were considered germinated only when radical measured at least 5 mm.

2.2 Green house experiment, inoculation and drought stress

The green house experiment was conducted at CSIR-NBRI, Lucknow, India (11º 24´ N/79 º 044´ E) during October-February 2014-2015 with temperature oscillating between 25±2°C (day) and 20±2°C (night) under natural sunlight. The experiment was designed with two parameters on *desi* and *kabuli* chickpea types namely, control and RA-inoculated plants exposed to different durations of drought stress viz. 0, 1, 3, and 7 days and recovery. The surface sterilized non-bacterized and bacterized seeds of cv. BG-362 and cv. BG-1003 were germinated, sown in separate 9´´pots filled with 5 kg autoclaved NBRI garden soil maintaining three replicates of each treatment with six plants in each pot. After one week of germination, RA-inoculated seedlings were again supplemented with 1% bacterial suspension (~10^7 $CFUmL^{-1}$). Non-inoculated control plants received the same amount of growth medium without bacteria. Plants were well-watered till one month, and then were subjected to drought stress by withholding water for aforementioned stress durations. Plants were then rewatered for 3 days for recovery. Stressed and control tissues (leaves and roots) were harvested at the same time to avoid any diurnal variation. All morphophysiological data including root length, number of lateral roots, number of nodules, shoot length, number of nodes, fresh and dry weight; and biochemical analyses were recorded on each day of harvesting. Leaf samples for qRT-PCR analyses were harvested, snap frozen in liquid nitrogen and stored at -80ºC until further use. All experimental data are means of at least three independent biological replicates and ~100 mg tissue samples were collected for each experiment.

2.3 Relative water content

The RWC was determined in control as well as stressed leaf samples of both chickpea cultivars as described elsewhere (Lata et al. 2011). The uppermost fully expanded fresh leaf samples from

plants were taken to immediately record fresh weight (FW). Then the leaves were soaked in 30 ml MilliQ water for 4 h at room temperature after which turgid weight (TW) was measured. Finally dry weight (DW) was recorded after drying the leaf samples at 60°C in a hot air oven for 48 h. RWC was calculated according to the formula : RWC % = (FW-DW) / (TW-DW)*100 (Barrs and Weatherly 1962).

2.4 Electrolyte leakage

Electrolytic leakage (EL) was assessed according to the method described by Lata et al. (2011) with some modifications. About 100 mg fresh root samples were taken and put in 15 ml deionised water for 1 h in sterile culture tubes at 100 rpm using a rotory shaker at room temperature, and then the initial conductivity (E1) was measured using a conductivity meter (Orion 5 star, Thermo scientific, US). The tubes were then placed in boiling water for 30 min in order to release all electrolytes in the solution, cooled to room temperature, and then the final conductivity (E2) was recorded. Results were expressed as the ratio of conductivity before boiling to that of after boiling according to the formula: E1/E2×100.

2.5 Lipid peroxidation

The lipid peroxidation (LP) level in control and stressed leaf samples were estimated by measuring malondialdehyde (MDA) content via 2-thiobarbituric acid (TBA) reaction using modified protocol of Heath and Packer (1968). Leaf tissues (100 mg) were homogenized in 500 µl of 0.1% (w/v) TCA and centrifuged for 10 min at 13,000 g at 4°C. The supernatant (500 µl) was then mixed with 1.5 ml 0.5% TBA and incubated at 95°C for 25 min. Reaction was ended by incubating on ice for 5 min. Absorbance was measured at 532 nm and 600 nm in a microplate reader (Spectrum max plus; Molecular devices, California, US). The level of LP was derived from the difference in absorbance at 532 nm and 600 nm using an extinction coefficient of 156 mM^{-1} cm^{-1} and expressed as micromoles of MDA formed.

2.5 Proline

Proline content was analyzed according to the protocol described by Carillo and Gibbon (2011). The ethanolic extract was prepared by homogenizing ~100 mg leaves in 1 ml of 70% ethanol. The 100 µl reaction mixture constituted 1% w/v ninhydrin in 60% v/v acetic acid and 20% v/v ethanol, mixed with 50 µl of ethanolic extract. The reaction mixture was then incubated at 95°C for 20 min, cooled to room temperature, and absorbance was recorded at 520 nm in a microplate reader.

2.6 Total soluble sugar

Total soluble sugar (TSS) in control and stressed chickpea leaf samples were determined according to Dubois et al. (1956) with some modifications. About 200 mg of fresh leaf tissue were homogenized in 5 ml of 80% methanol and was incubated in water bath at 70°C for 30 min. After

incubation, 1 ml of extract was mixed with 1 ml of 5% phenol and 5 ml of 95% H_2SO_4 and further incubated in dark for 15 min. Absorbance was then measured at 490 nm in a microplate reader.

2.7 Antioxidative enzymes assay

Leaf samples (100 mg) were homogenized under chilled condition in 1 ml of extraction buffer containing 100 mM sodium phosphate buffer (pH 7.8), 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1% w/v polyvinyl pyrrolidone (PVP) and 0.5% v/v triton X-100. The homogenate was then centrifuged at 12,000×g for 10 min at 4°C to obtain the supernatant and protein estimation for enzyme assay was done using BSA as standard (Lata et al. 2011).

Catalase (CAT) (EC 1.11.1.6) activity was determined according to the method described by Lata et al. (2011) with some modifications. The reaction mixture contained 50 mM phosphate buffer (pH 7.0), 20 mM H_2O_2 and 0.1 ml enzyme extract. Decrease in absorbance of H_2O_2 was measured for 3 min at 240 nm on a microplate reader. One unit of CAT activity is the amount of enzyme required to oxidize 1 µ mol of H_2O_2 per minute.

Superoxide dismutase (SOD) (EC 1.15.1.1) activity was measured by its ability to inhibit photochemical reduction of nitrobluetetrazolium (NBT) by the method of Beauchamp and Fridovich (1971). Enzyme extract (100 µl) was mixed with reaction mixture (2.5 ml) containing 100 mM phosphate buffer, 100 mM L-methionine, and 57 µM NBT. Then 400 µl of 4.4% riboflavin was added and immediately initial absorbance was recorded at 560 nm. Final absorbance was taken at same wavelength after an incubation of 7 min in light. One unit of SOD is defined as 50% reduction of NBT.

2.8 Quantitative real time (qRT) PCR analysis of drought stress responsive genes from chickpea

Total RNA was isolated from leaf samples of 30 day old both chickpea cultivars subjected to different durations of drought stress and recovery with or without RA-inoculation, using Spectrum™ Plant Total RNA Kit (Sigma, USA). DNase treatment was done to remove DNA contamination from total RNA samples using TURBO DNase (2 Units/µl, Ambion, USA). RNA concentrations were determined at 260 nm using a spectrophotometer (Nanodrop 1000, Thermo Scientific, USA). The OD_{260}/OD_{280} nm absorption ratio (1.98-2.01) and OD_{260}/OD_{230} (≥2.0), was used to determine the quality and purity of RNA preparations. The integrity of the samples was established by 1.2% formaldehyde-agarose gel electrophoresis. The first strand of cDNA was synthesized using 1 µg of DNase free total RNA primed with oligodT primers in a 20 µl reaction mix using Maxima H Minus M-MuLV reverse transcriptase (Thermo Scientific, USA) following manufacturer's instructions. The cDNA products were then diluted 5-fold with deionized water before using as a template in qRT-PCR. Real time PCR was performed using 2X Brilliant III SYBR® Green QPCR (Agilent Technologies, USA) on Stratagene Mx3000P (Agilent, USA) in

triplicates. A constitutive gene glyceraldehydes 3-phosphate dehydrogenase (*GAPDH;* GenBank accession # AJ010224; Garg et al. 2010) from chickpea was used as an internal control. The amount of transcript accumulated for each target gene normalized to the internal control was examined using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The primers used for qRT- PCR analysis were either designed from sequences of the respective genes downloaded from National Centre for Biotechnology Information (NCBI) using the IDT Primer Quest software or from previous studies (Appendix 1). The qRT-PCR cycling conditions were: initial denaturation at 95°C for 10 min, 95°C for 30 s, and 60°C for 1 min for 40 cycles followed by melt curve analysis at 95°C for 1 min, 60°C for 30 s, and 95°C for 30 s. The heat map for gene expression profiles were generated using TIGR MultiExperiment viewer (MeV 4) software package (Saeed et al 2003).

2.9 Statistical analysis

All experimental data obtained are the means of three independent biological replicates and the results are expressed as mean with standard deviation (mean±SD) or standard error (mean±SE). One way analysis of variance (ANOVA) was used to test significance between mean values of control and stressed plants or RA-inoculated unstressed and stressed plants, and comparison among means was carried out using Duncan multiple range test at $P<0.05$ with the help of SPSS software version 16.0 (SPSS Inc./IBM Corp. Chicago, USA). The results were graphically presented using Graph Pad Prism software (version 5.03, San Diego, California, USA).

3. Results

3.1 Effect of RA-inoculation on germination of chickpea under drought stress

To see the effects of RA on germination of *desi* and *kabuli* chickpea types, a germination assay was performed using PEG-induced osmotic stress. Germination of both cv. BG-362 and cv. BG-1003 seeds were tested at 15% and 30% PEG-6000 concentrations wherein increasing PEG concentrations led to reduced and delayed emergence of radical and plumule from both types of chickpea varieties (Table 1). A reduction in germination (30% and 43%) was observed at 15% and 30% of PEG stress respectively, for cv. BG-362 seeds while germination percentage dropped by 63.3% and 80% in cv. BG-1003, as compared to control where 100% germination was recorded for both chickpea cultivars. The RA-treatment led to increased germination percentage (30% and 71%) at both concentrations of PEG stress as compared to uninoculated cv. BG-1003 seeds indicating its ability to ameliorate drought stress. However RA-treatment gave no significant advantage to cv. BG-362 seeds during germination at both concentrations of PEG stress (Table 1).

3.2 Effect of RA-inoculation on various growth parameters of chickpea

To determine the response of RA-inoculation on root and shoot parameters as well as biomass of *desi* and *kabuli* chickpea types subjected to drought stress, plants were regularly monitored at 0, 1, 3 and 7 days of water stress or recovery after 3 days of rewatering in both RA-treated and non-treated plants (Appendix 2). Our results demonstrated that both primary root length and number of lateral root increased with increasing stress duration in both uninoculated and RA-inoculated chickpea cultivars (Table 2). RA-inoculated cv. BG-362 and cv. BG-1003 showed an increment of 11% and 25% in primary root length and 68% and 17.5% in number of lateral roots, respectively as compared to individual uninoculated cultivars at 7^{th} day of stress. While drought recovered RA-treated plants showed an increment of 13.3% and 9.3% in root length and 34% and 20% in number of lateral roots in cv. BG-362 and cv. BG-1003, respectively as compared to non-treated plants. Interestingly no significant difference was observed in the number of root nodules in both non-treated and RA-treated cv. BG-362 on exposure to drought stress or recovery (Table 2). While uninoculated and RA-inoculated cv. BG-1003 plants showed 105% and 155% increase in number of root nodules at 7^{th} day of drought stress. After recovery phase ~93% more nodules were observed in RA-treated cv. BG-1003 as compared to non-treated plants. Drought stress imposed no significant difference in the shoot length of both cultivars whether inoculated or uninoculated, however recovered plants showed ~16% more growth in RA-treated cv. BG-362 (Table 2). In another finding the number of nodes on branches significantly increased in both the cultivars viz. 40%, 80% and 65% in cv. BG-362 and 36%, 54% and 63% in cv. BG-1003 after 1, 3 and 7 days of drought stress, respectively as compared to day-0 uninoculated plants (Table 2). While RA-inoculated plants showed 24% and 31% more number of nodes in cv. BG-362 and cv. BG-1003 respectively, after 7 days of drought stress as compared to uninoculated plants at same stress duration. RA-inoculated drought stress recovered cv. BG-362 and cv. BG-1003 showed 17% and 12.4% more number of nodes respectively, as compared to uninoculated plants after recovery phase. Significant decline in fresh weight and dry weight of both chickpea cultivars was visible only on 7^{th} day of drought stress and it was also evident that RA-inoculation improved stress endurance capacity of chickpea since less reduction in biomass was observed at same duration (Table 2).

3.3 RA-inoculation alters physiological and biochemical parameters in chickpea

To assess the water balance of plants, leaf RWC was determined in both cv. BG-362 and cv. BG-1003 at different durations of drought stress and recovery with or without RA-inoculation. Stress treatments led to significant decline in RWC of both cultivars at all durations of stress (Fig. 1A). However after 7 days of drought stress RA-treated cv. BG-362 and cv. BG-1003 showed 16% and

11.2% fewer declines in RWC respectively, as compared to their respective non-treated plants indicating that RA-inoculation helps in better maintenance of plant water balance in both *desi* and *kabuli* chickpea. Both treated and non-treated cv. BG-362 plants were also able to better maintain membrane integrity on exposure to drought stress as no significant difference was observed in ion leakage at all durations of stress and recovery as compared to cv. BG-1003 plants which showed significant ion leakage at all-time points (Fig. 1B). However, RA-inoculated cv. BG-1003 showed 34.5% and 24% less EL post 7 days of drought stress and recovery as compared to non-treated plants at same durations. Similarly MDA content significantly increased with drought stress progression in both untreated and RA-treated chickpea; however RA-inoculation reduced the level of lipid peroxidation in this crop, as for example RA-inoculated cv. BG-362 and cv. BG-1003 showed 38.6% and 123%, respectively decline in MDA content at 7^{th} day of drought stress as compared to their individual inoculated plants (Fig. 1C). Further accumulation of compatible osmolytes namely, proline and TSS in leaves of both cv. BG-362 and cv. BG-1003 increased significantly with progression of drought stress and restored to unstressed levels in cv. BG-362 after recovery (Fig. 2A, B). However compared to non-treated plants, RA-treatment led to significant decline in proline and TSS content at all stress durations with a reduction of 114% and 214% in proline, and 50% and 198% reduction in TSS in cv. BG-362 and cv. BG-1003, respectively at 7^{th} day of drought stress. This study also indicated a significant increase in SOD and CAT activities with the progression of drought and restoration to normal levels in both chickpea cultivars after recovery. It is also interesting to observe that cv. BG-362 is able to maintain a higher SOD or CAT activity at all-time points under study suggesting it has better ROS scavenging activity than cv. BG-1003 under drought stress (Fig. 3A, B). Compared to untreated seedlings, RA-treated cv. BG-362 and cv. BG-1003 showed 79% and 70% respectively, less activity for SOD, and >100% less activity for CAT at 7^{th} day of drought stress suggesting that RA-inoculation is helpful in reducing the effects of drought stress by minimizing the ROS production in plants. Taken together all these data emphasizes that RA-inoculation exerts positive effects on chickpea seedlings by protecting them from membrane damage and oxidative stress under drought stress.

3.4 RA modulates gene expression profiles of drought stress responsive genes in chickpea

To elucidate the molecular basis of mutualistic interaction between PGPR and their host plants, the expression analysis of 11 stress responsive marker genes including two ethylene biosynthesis genes and one gene each for salicylic acid (SA) and jasmonate (JA) signalling was done using qRT-PCR in both chickpea cultivars at all treatments (Fig. 4A, B). Variability in expression pattern was observed for all 11 genes under drought stress in uninoculated and inoculated plants

of both chickpea types. *DREB1A* was up-regulated at all stress durations with up to 4-fold expression in cv. BG-362 and 10-fold expression in cv. BG-1003 at 7th day of drought stress as compared to respective control. However compared to the uninoculated plants, RA-inoculation repressed *DREB1A* expression by 1.28-fold in cv. BG-362 and 2.5-fold in cv. BG-1003 at same duration (7th day) of drought stress. The expression of *NAC1* gene was higher up to 5-fold in cv. BG-362 and up to 2.6-fold in cv. BG-1003 at 7th day of drought stress. RA-inoculation reduced the accumulation of transcript by 2-fold in cv. BG-362 whereas in cv. BG-1003 its expression was almost at the basal level. *LEA* and *DHN* showed up-regulation at all stress durations in both RA-inoculated and uninoculated cv. BG-362 while they were only activated on 3rd day and 7th day of drought stress in cv. BG-1003. A repression in their expression was observed on RA-inoculation in both chickpea types (up to 5-fold in cv. BG-362 and 4-fold in cv. BG-1003) upon drought stress while near equivalent expression was observed in drought recovered plants. The expression of genes encoding all three antioxidative enzymes under study showed a constant up-regulation (up to 6-fold for CAT; and >2-fold for APX and GST) with progression of drought stress in cv. BG-362. CAT and APX showed maximum expression of 2.9- and 2.6-fold respectively, at 7th day of drought stress in cv. BG-1003 while GST was constantly up-regulated (up to 6.8-fold) at all stress durations in this cultivar. There was almost 2.5-fold reduction in the transcript accumulation of all the three antioxidative genes upon RA-inoculation in both chickpea types under drought stress. The relative expression of ethylene biosynthesis genes namely ACO and ACS was found to be maximum (up to 3-fold in cv. BG-362 and 6-fold in cv. BG-1003) under drought stress as compared to control in both chickpea cultivars. A relative decline in their expression was observed upon RA-inoculation at all stress durations. *PR1*, a key SA signalling gene showed basal level expression at all stress durations in both uninoculated and inoculated cv. BG-362 with a slight down regulation in non-stressed RA-treated plants. While approximately 2.5-fold induction was observed at all stress durations in cv. BG-1003 with or without RA-inoculation as compared to control. *PR1* was found to be up-regulated during recovery in both chickpea cultivars. On the other hand *MYC2* an important gene in JA-dependent signalling pathway was constantly up-regulated (up to 4.5-fold) under drought stress and recovery as compared to control in cv. BG-362, however a comparative decline in its transcript abundance was observed upon RA-inoculation at all conditions. The expression of this gene was at basal level in uninoculated and inoculated cv. BG-1003 at all stress durations and recovery. The relative gene expression profiles of the genes under study were in conformity with the biochemical and physiological analysis conducted in both cultivars.

4. Discussion

Drought stress is one of the most common adverse environmental conditions that reduce crop production and productivity globally. Improving drought stress tolerance of crop plants either through breeding or genetic engineering could be one of the most reasonable approaches for enhanced agricultural productivity. However since both approaches are long drawn, labor and cost intensive, and also due to the stigma of environmental and ethical issues associated with genetically engineered crops, use of plant growth promoting microbes for improving stress tolerance of crop plants is gaining momentum of late (Nautiyal et al. 2013). These plant root associated rhizobacteria are known to elicit physical and chemical changes in plants that result in induced systemic tolerance (IST) against abiotic stresses (Yang et al. 2009). The present study demonstrates the positive regulatory role of an abiotic stress tolerant PGPR, *P. putida* RA in promoting growth as well as drought stress alleviation in chickpea. Germination is one of the most critical stages in a crop development cycle which is known to be significantly affected by drought stress (Sleimi et al. 2013). A better germination percentage reflects better seedling growth and development which is essential for a subsequent good yield. An overall increase in germination percentage at both concentrations of PEG stress in cv. BG-1003 on RA-inoculation is in accordance with an earlier study on PGPR-mediated osmotic stress amelioration (Nautiyal et al. 2013).

Drought stress is primarily perceived and responded by plant roots, particularly under field conditions. Therefore length and distribution of roots plays an important role in influencing the ability of plants to absorb water and nutrients from soil. It has been postulated that deeper root systems with greater root densities is a great stress management tool as it not only facilitates better extraction of soil water but also helps the plant to sustain optimal growth and development under drought stress conditions (Lopes et al. 2011). It has been reported that the number of lateral and fine roots increase under drought stress in several crop species which not only increases root surface area for water absorption but also increases root hydraulic conductivity (Miyahara et al. 2011). Accordingly greater increase in primary root length as well as number of lateral roots than the control plants on progression of drought stress was observed in this study. Interestingly significant increase in these root parameters upon RA-inoculation in both chickpea cultivars as compared to control during drought stress progression and subsequent recovery was also observed which can be supported from the fact that RA is an auxin-producing rhizobacteria (Srivastava et al. 2012). Auxin in turn is associated with better root growth and/or enhanced lateral roots and root hair formation (Overvoorde et al. 2010). Legumes usually fix atmospheric N_2 owing to their ability to form nodules which host symbiotic bacteria. Generally it has been suggested that legumes and their symbiotic root nodule bacteria are sensitive to abiotic stresses. However there

404 are reports that co-inoculation of PGPR with N_2 fixing bacteria augment nodule number of
405 legumes grown in green house or field situations under normal or drought stress conditions
406 (Figueiredo et al. 2008). Our results also suggested an overall increase in nodule number in both
407 chickpea types upon RA-inoculation under drought stress. This is the first report of increase in the
408 number of root nodules upon single PGPR inoculation rather than co-incoulation with a N_2 fixing
409 bacteria in a legume. The increase in nodulation may be explained, at least partially, by its auxin-
410 producing properties since IAA production has been positively correlated with nodule formation
411 (Ghosh et al. 2013). Alternately it may elicit secretion of *nod* gene-inducing signals as some
412 *Pseudomonas* strains are also known to be putative non-invasive non-rhizobial endophytes (Aeron
413 et al. 2015). Though RA-inoculation increased shoot length in both cultivars as compared to the
414 uninoculated control plants under both normal and stress conditions, the difference was not
415 statistically significant. Similar observation was also reported in loblolly and slash pine seedlings
416 by Enebak et al. (1996) where inoculation with PGPR strain(s) do not improve shoot growth.
417 Increase in fresh and dry weight of RA-inoculated chickpea plants as compared to uninoculated
418 ones under control, drought stress progression and recovery phases can be correlated with the
419 increase in the number of shoot lateral branches as well as increase in the primary root length and
420 number of lateral roots at these time points. Similar observation has also been accounted in
421 several previous studies (Yang et al. 2009; Grover et al. 2014).
422 RWC is considered as an important marker to assess the water balance of plants (Lata et al. 2011).
423 On the other hand EL is inversely related to cell membrane integrity and the ability to avoid or
424 repair membrane damage has generally been correlated with abiotic stress tolerance (Lata et al.
425 2011). RWC and EL of both cultivars declined significantly under drought stress; however RA-
426 inoculation led to better maintenance of plant water status and membrane integrity which is in
427 confirmation with other earlier studies (Figueiredo et al. 2008; Kang et al. 2014). MDA
428 accumulation is an indication of stress-induced LP of cellular membrane lipids and is often
429 considered a marker for increased oxidative damage (Lata et al. 2011). Our findings are in
430 conformity with an earlier study where LP has already been established as a function of
431 membrane integrity in 7 days old dehydration stressed chickpea plants, and together with EL was
432 ascertained as a direct indicator of dehydration stress tolerance (Bhushan et al. 2007). RA-
433 inoculation helped in overcoming membrane damage by lowering MDA accumulation as
434 compared to uninoculated plants. Accumulation of compatible osmolytes such as proline and
435 soluble sugars help plants to overcome drought stress by maintaining osmotic turgor (Grover et al.
436 2014). Their accumulation has been reported to increase manifold during stress conditions (Lata et
437 al. 2015). Accordingly this study also reports an increase in proline and TSS content in both

chickpea cultivars under drought stress and subsequent restoration to normal levels after recovery. However the proline and TSS content of inoculated chickpea plants showed significant decline during drought stress progression as compared to non-inoculated plants. This may be due to RA-induced IST response of chickpea plants since its inoculation may have stimulated root exudation, biofilm formation and conservation of soil moisture as evident from increased soil moisture content (Appendix 3) which may have resulted in enhanced root growth and nutrient uptake thereby improving plant health under stress condition. Further degradation of ethylene precursor ACC by bacterial deaminase may also be one of the reasons for relieving plant stress and rescuing normal plant growth under drought stress (Yang et al. 2009). Similar observations have also been reported in sorghum by Grover et al. (2014). Drought stress cause oxidative damage via production of toxic ROS which need to be scavenged by low molecular weight antioxidants and antioxidative enzymes (Lata et al. 2011). SOD is a defence enzyme that catalyzes the conversion of superoxide (O_2^-) radical into less damaging species such as molecular O_2 or H_2O_2. The H_2O_2 so generated is finally broken down into water and oxygen without any requirement of reducing power by the action of CAT (Lata et al. 2011). Comparatively less activity of SOD and CAT in RA-inoculated plants than the uninoculated ones at all stress durations suggests that low level of oxidative stress is convened by RA-inoculated chickpea plants. This observation is in accordance to Kang et al. (2014) who inferred that PGPR reduces adverse effects of osmotic stress by regulating phytohormones and antioxidants in cucumber.

PGPR-mediated stress resistance with the activation of numerous genes in response to abiotic stresses have recently been identified in many crop plants (Nautiyal et al. 2013; Kim et al. 2014). However molecular basis of PGPR-plant interactions with respect to drought tolerance in chickpea remained largely unknown until now. Therefore in order to delineate the expression of a few drought responsive genes, qRT-PCR analysis was performed in both cultivars subjected to drought stress and recovery with or without RA-inoculation. Dehydration responsive element binding (*DREB*) genes are important class of TFs expressed primarily under abiotic stresses such as drought, salt and cold and are known to regulate the expression of several downstream stress responsive genes such as *rd29, lea* etc. (Lata and Prasad 2011). The up-regulation of *DREB1* gene under drought stress in both cultivars is in conformity to earlier reported studies (Chu et al. 2014). Its down-regulation in presence of RA at all durations emphasizes its role in protecting chickpea plants against drought stress. NAC TFs have been reported to play important role in developmental pathways as well as in abiotic stress tolerance (Nakashima et al. 2009). Increased expression of *NAC1* gene on exposure to drought stress in both cultivars is in accordance to previous studies (Nguyen et al. 2015), and its comparatively decreased transcript level in RA-

inoculated plants shows negative regulation of *NAC1* by RA under stress. LEA and dehydrins are mainly involved in protection of macromolecules under abiotic stress and hence act as key players in plant stress tolerance (Gao et al. 2008). Their overexpression is also known to provide tolerance to abiotic stresses (Lata and Prasad 2011). In this study, the expression of these genes was also found to be activated with drought stress progression with maximum expression at 7 days of drought stress, while RA-inoculation comparatively repressed their expression at all stress durations suggesting its important role in drought stress alleviation. Significantly lower level of expression of genes encoding antioxidant enzymes namely CAT, APX, and GST in RA-inoculated chickpea plants of both varieties exposed to drought stress indicates that RA is capable of relieving stress and restoring normal growth conditions for inoculated plants as compared to the uninoculated ones. Since drought stress is multidimensional in nature, its affects are manifested at various levels including changes in endogenous phytohormones which help in generating signal transduction network(s) leading to events responsible for physiological adaptation of the plants to stress (Lata et al. 2015). Increase in the rate of ethylene production is known to be associated with various environmental stresses including drought stress (Yang et al. 2009). PGPR help to lower the concentration of ethylene in plants (Yang et al. 2009). Higher rates of ethylene production shows higher activity of enzymes involved in ethylene metabolism, such as ACS and ACO. Accordingly the relative expression of both ACO and ACS was higher under drought stress in both chickpea types while their expression showed relatively low level of expression in RA-inoculated plants, suggesting less production of ethylene in chickpea due to ACD activity of this PGPR. Though SA is classically associated with biotic stress response, its role under abiotic stresses including drought stress is well accepted and extensively reviewed (Fujita et al. 2006). It has been reported that drought-sensitive genotypes usually contained slightly higher amount of SA as compared to the tolerant ones in rice without any significant correlation with the degree of drought tolerance (Pal et al. 2014). An elevated SA content may be responsible for SA-responsive gene expression e.g. *PR1*. Accordingly higher expression of *PR1* was observed in the *kabuli* genotype as compared to *desi* at all conditions in this study. Strong induction of this gene in both drought recovered chickpea cultivars suggested the role of SA in stress recovery in accordance to an earlier reported study in bean and tomato (Senaratna et al. 2000). There has also been a progressively rising body of evidence for the involvement of jasmonates in drought stress (Fujita et al. 2006). A significant up-regulation of jasmonate signalling pathway gene *MYC2* in the *desi* chickpea cultivar under drought stress and recovery is in confirmation with an earlier reported study which established the role of jasmonates in the early drought stress signalling and/or its association with the tolerance mechanism of the drought-tolerant chickpea variety (De Domenico

et al. 2012). Taken together our results indicate that the drought stress amelioration capacity of chickpea plants have been significantly improved with RA-inoculation. Similar result has also been reported for *Bacillus amyloliquefaciens* strain SN13 mediated salt stress amelioration in rice (Nautiyal et al. 2013). Further since *kabuli* type chickpea cultivars are generally sensitive to drought stress as compared to *desi* (Wang et al. 2006), and the subsequent improvement in its drought tolerance capacity upon RA-inoculation as evident from its enhanced root and shoot growth parameters, improved physiological and biochemical responses; and the mutually interactive effects in RA-facilitated stress responsive gene expression, also strengthens the positive regulatory role of this PGPR in overcoming the effects of drought stress in sensitive cultivar(s) of chickpea.

5. Conclusion and future perspectives

In the present work, a tripartite "plant-soil-microbe" interaction was demonstrated by exploiting *Pseudomonas putida* RA in ameliorating drought stress in *desi* and *kabuli* chickpea. Drought stress progression significantly affected the growth and development of both chickpea cultivars by affecting root length, number of lateral roots and nodules, shoot length and branching, reduced RWC, increased EL and MDA content, enhanced osmolytes and ROS production, and up-regulation of various stress responsive genes. Conversely various drought-induced symptoms in chickpea such as plant growth, water status, membrane integrity, accumulation of osmoprotectants, antioxidative enzyme activities and gene expression were significantly improved or restored in presence of RA. RA-inoculation also had positive effects on better recovery of drought stressed chickpea plants. Based on differential responses of contrasting chickpea cultivars subjected to drought stress and recovery along with the published literature and well known concepts on cellular stress tolerance in other crop species, a working hypothesis for the mechanism of PGPR-mediated drought tolerance in chickpea has also been elaborated (Fig. 5). Interestingly RA is not only improving the growth of *desi* chickpea but also satisfactorily improving the drought stress ameliorating capabilities of relatively drought sensitive *kabuli* type cultivar, indicating its greater potential in enhancing agricultural yield of this economically important legume. Our results thus set up an initial step towards understanding the physiological and molecular basis of PGPR-mediated drought stress response and adaptation in chickpea. Thus applicability of RA in drought stress amelioration at field level should be worked out and the possibility to develop it as bioinoculant for drought affected areas may also be taken up.

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Author Contribution Statement: CL, PSC and CSN conceived and designed research. ST and CL conducted experiments. ST, CL and PSC analyzed data. CL and ST wrote the manuscript. PSC critically reviewed the manuscript. All authors read and approved the manuscript.

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693

694 **Legends to figures**

695 **Fig. 1:** Determination of RWC (A), EL (B) and LPX (C) in cv. BG-362 and cv. BG-1003 exposed
696 to drought stress at 0, 1, 3 and 7 days and recovery in the presence or absence of RA. Data
697 represent the means ± SD of three independent experiments. Different letters on the graph indicate
698 significant differences according to Duncan's test ($P \leq 0.05$).

699 **Fig. 2:** Determination of Proline (A) and TSS (B) in cv. BG-362 and cv. BG-1003 exposed to
700 drought stress at 0, 1, 3 and 7 days and recovery in the presence or absence of RA. Data represent
701 the means ± SD of three independent experiments. Different letters on the graph indicate
702 significant differences according to Duncan's test ($P \leq 0.05$).

703 **Fig. 3:** Determination of SOD (A) and Catalase (B) in cv. BG-362 and cv. BG-1003 exposed to
704 drought stress at 0, 1, 3 and 7 days and recovery in the presence or absence of RA. Data represent
705 the means ± SD of three independent experiments. Different letters on the graph indicate
706 significant differences according to Duncan's test ($P \leq 0.05$).

707 **Fig. 4:** Differential expression of genes in chickpea cultivars cv. BG-362 (A) and cv. BG-1003
708 (B) exposed to drought stress at 0, 1, 3 and 7 days and recovery in the presence or absence of RA.
709 The heat map has been generated based on the fold-change values in the treated sample when
710 compared with its unstressed control sample. The colour scale for fold-change values is shown at
711 the top.

712 **Fig. 5:** A model of the physiological, biochemical, and molecular basis of drought stress tolerance
713 operating in chickpea is created based on the differential response of both contrasting *desi* and
714 *kabuli* cultivars. The enzyme assays and physiological parameters estimated in this study are
715 indicated in normal font and well-known concepts reported in model species are shown in italics.

716

717 **Legends to Tables**

718 **Table 1:** Effects of PEG stress on seed germination of cv. BG-362 and cv. BG-1003 in the
719 presence or absence of RA. Data represent the mean ± SE from three biological replicates.
720 Different letters in the same column indicate significant differences according to Duncan's test (P
721 ≤ 0.05).

722 **Table 2:** Effects of drought stress on various parameters of cv. BG-362 and cv. BG-1003 in the
723 presence or absence of RA. Data represent the mean ± SE from three biological replicates.
724 Different letters in the same column indicate significant differences according to Duncan's test (P
725 ≤ 0.05).

726

727 **Legends to Supplementary Materials**

Appendix 1: List of primers used in qRT-PCR analysis.

Appendix 2: Morphological changes in the one month old chickpea cv. BG-362 and cv. BG-1003 exposed to drought stress at 0, 1, 3 and 7 days and recovery in the presence and absence of RA.

Appendix 3: Determination of soil moisture content in cv. BG-362 and cv. BG-1003 exposed to drought stress at 0, 1, 3 and 7 days and recovery in the presence or absence of RA. Data represent the mean ± SE from three biological replicates. Different letters in the same column indicate significant differences according to Duncan's test ($P \leq 0.05$).

Table 1: Effects of PEG stress on seed germination of cv. BG-362 and cv. BG-1003 in the presence or absence of RA.

Seed Germination (%)		
Treatment	**15% PEG**	**30% PEG**
BG-362 Control	70	57
BG-362 RA	73	53
BG-1003 Control	37	21
BG-1003 RA	71	43

Table 2: Effects of drought stress on various parameters of cv. BG-362 and cv. BG-1003 in the presence or absence of RA.

	Treatments	Root Length (cm.)	No. of Lateral Roots	No. of Root Nodules	Shoot Length (cm.)	No. of nodes	Fresh weight (gm.)	Dry weight (gm.)
Day-0	BG-362 Control	13.2±1.06 a	30.33±2.3 ab	18±3.5 a	19.87±0.27 a	23.33±1.2 a	1.62±0.11 ab	0.25±0.01 a
	BG-362 RA	13.07±0.78 a	29.67±1.2 a	21±1.5 ab	21.27±0.35 abc	29.67±0.67 abc	1.89±0.14 abc	0.27±0.02 ab
	BG-1003 Control	13.17±1.1 a	45.67±10.3 abc	14±1.5 a	21.9±1.3 abcd	35±2 bcde	2.28±0.09 abcd	0.32±0.01 ab
	BG-1003 RA	15.43±0.98 abcdef	44.33±8.1 abc	16.33±1.4 a	23.87±0.45 cde	37±1.1 cdef	2.42±0.22 abcd	0.31±0.02 ab
Day-1	BG-362 Control	14.13±1.5 abc	35.67±4.1 ab	13.33±0.67 a	19.83±0.32 a	26.67±2.9 ab	1.53±0.23 ab	0.23±0.04 a
	BG-362 RA	14.43±1.6 abc	53.33±12.1 abcd	15.67±2.7 a	21.93±0.29 abcd	32.67±3.3 abcd	1.9±0.14 abc	0.28±0.01 ab
	BG-1003 Control	19.03±1.2 cdefg	35.33±6.8 ab	14.33±0.8 a	22±2.15 abcd	42.33±0.4 defgh	2.32±0.36 abcd	0.34±0.05 ab
	BG-1003 RA	19.93±2.9 defg	66.67±6.8 cdef	17±1.1 a	22.4±0.15 abcd	47.67±1.8 ghij	2.58±0.06 abcd	0.38±0.02 ab
Day-3	BG-362 Control	14.8±1.1 abcde	48±5.3 abc	12.33±2.3 a	22.33±0.17 abcd	36.67±3.4 bcdef	1.85±0.13 abc	0.26±0.02 ab
	BG-362 RA	15.03±1.5 abcde	71±6.6 cdef	15.33±3 a	24.53±0.85 de	42±3.5 defgh	2.18±0.03 abcd	0.32±0.01 ab
	BG-1003 Control	17.17±0.64 abcdef	58±6.7 ef	16±2.1 a	20.1±0.21 a	41±4.6 defgh	2.26±0.27 abcd	0.34±0.05 ab
	BG-1003 RA	14.2±0.86 abc	81±12.4 bcde	21.67±0.6 ab	24.17±0.63 cde	54±3 ij	3.48±0.30 d	0.5±0.06 ab
Day-7	BG-362 Control	17.3±0.57 abcdef	50.33±12.3 abcd	15±2.3 a	21.9±0.87 abcd	33±2.1 abcd	1.29±0.43 a	0.2±0.07 a
	BG-362 RA	18.83±0.58 bcdefg	71±9.4 cdef	19±2 ab	23.83±0.77 cde	38.67±3.2 cdefg	1.68±0.13 abc	0.28±0.02 ab
	BG-1003 Control	20.7±2.7 fg	77.33±5.9 def	28.67±1.8 bc	20.73±1.3 ab	46.33±6.1 fghi	1.74±0.34 abc	0.54±0.20 abc
	BG-1003 RA	24±3.7 g	85.33±5.9 f	41.67±10.9 d	24.83±0.97 de	57.33±2.6 j	2.97±0.85 cd	0.53±0.15 abc
Recovery	BG-362 Control	18.47±1.8 abcdef	44±3.6 abc	19±1.1 ab	23.23±1.35 bcd	44.33±4.5 efghi	2.23±0.39 abcd	0.3±0.10 ab
	BG-362 RA	20.23±1 efg	54.33±12.7 abcde	22±2.6 ab	26.43±0.99 e	48.33±3 ghij	3.43±0.83 d	0.61±0.17 abc
	BG-1003 Control	13.5±1.6 ab	57.33±3.4 abcde	20±1.5 ab	22.6±0.42 abcd	46.33±5 fghi	2.7±0.18 bcd	0.44±0.04 ab
	BG-1003 RA	14.73±1 abcd	66.67±12.1 cdef	33±2.5 cd	22.1±0.76 abcd	50.67±1.4 hij	3.36±0.25 d	0.84±0.23 c

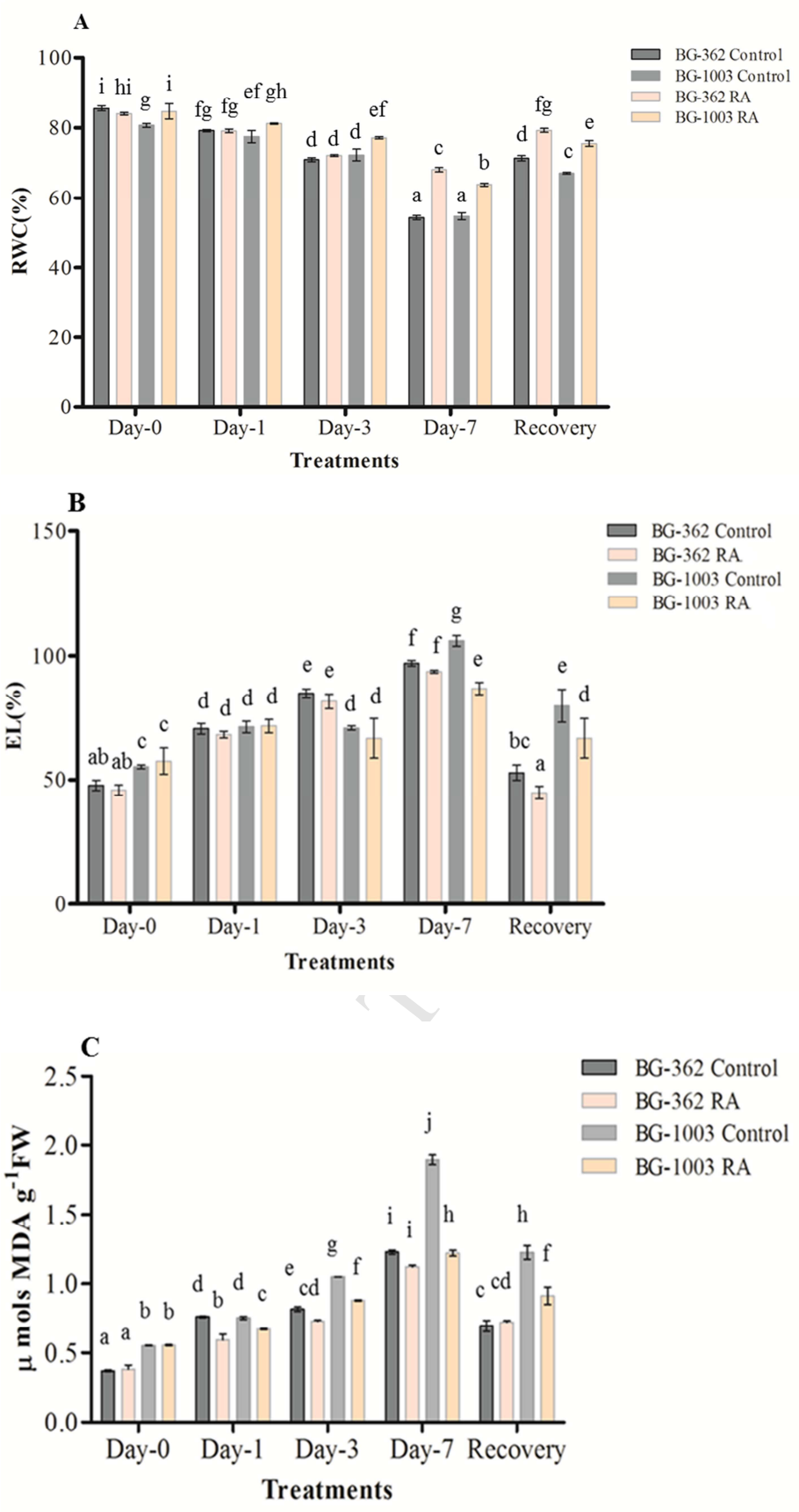
A
RWC(%)
Treatments
Day-0
Day-1
Day-3
Day-7
Recovery
BG-362 Control
BG-1003 Control
BG-362 RA
BG-1003 RA
B
EL(%)
Treatments
C
μ mols MDA g-1FW
Treatments

Fig. 1

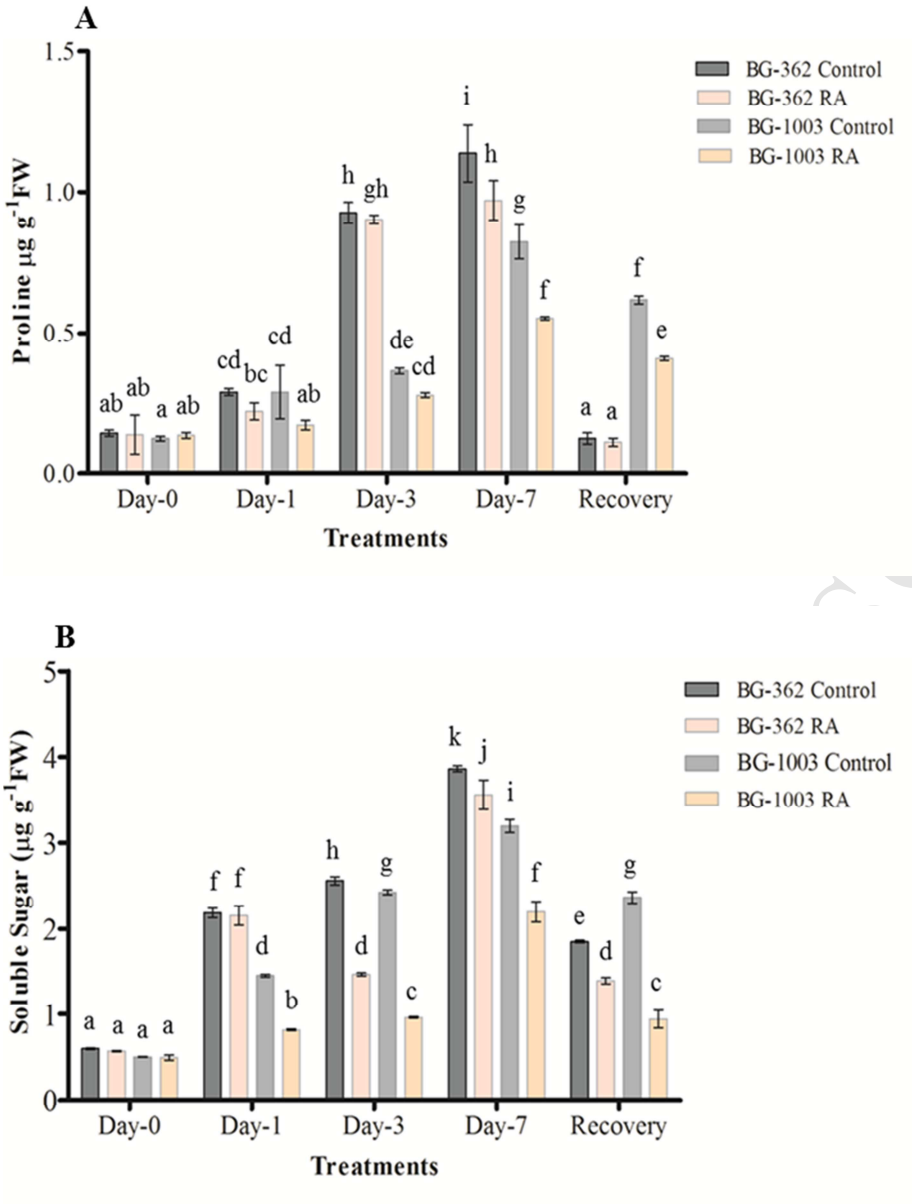
A
Proline µg g-1FW
1.5
1.0
0.5
0.0
BG-362 Control
BG-362 RA
BG-1003 Control
BG-1003 RA
ab ab a ab
cd bc cd ab
h gh de cd
i h g f
a a f e
Day-0
Day-1
Day-3
Day-7
Recovery
Treatments
B
Soluble Sugar (µg g-1FW)
5
4
3
2
1
0
BG-362 Control
BG-362 RA
BG-1003 Control
BG-1003 RA
a a a a
f f d b
h d g c
k j i f
e d g c
Day-0
Day-1
Day-3
Day-7
Recovery
Treatments

Fig. 2

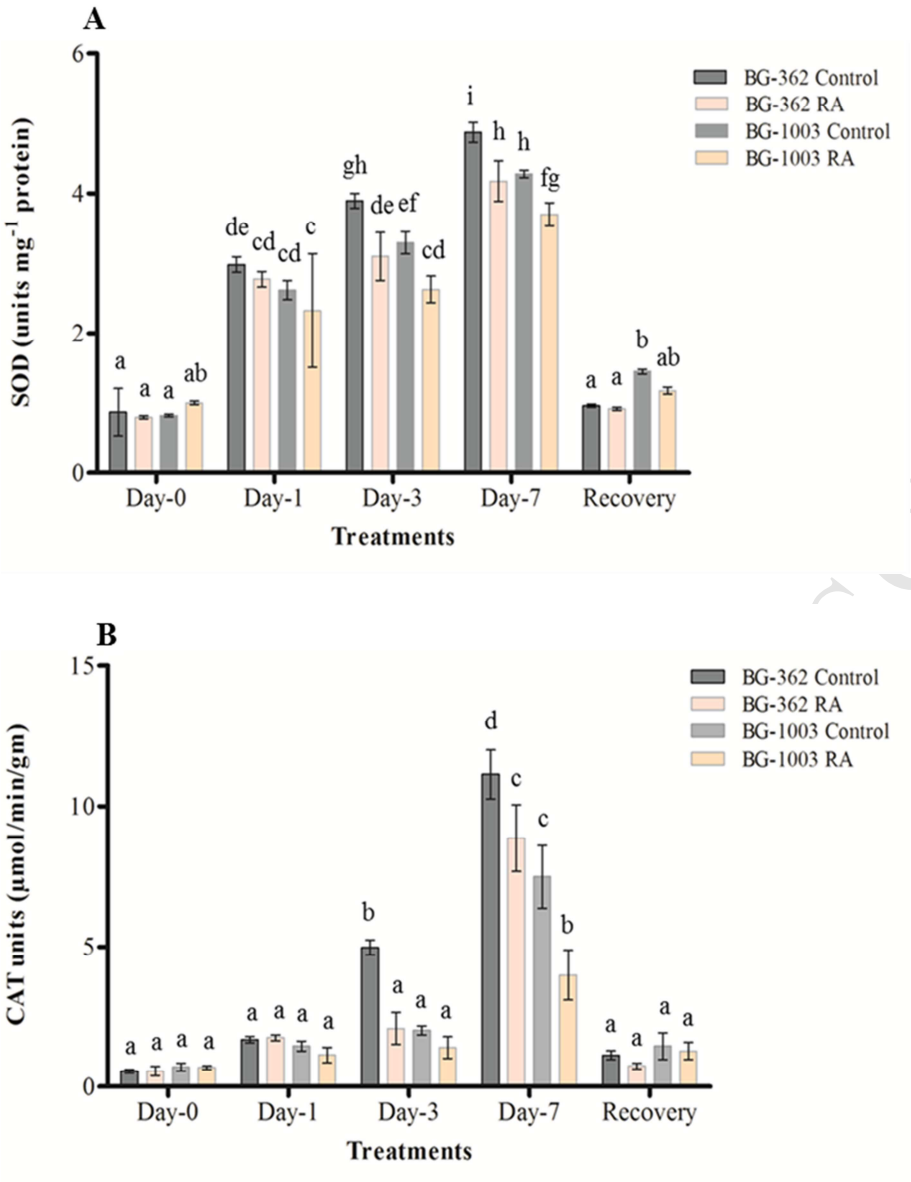
A
SOD (units mg-1 protein)
BG-362 Control
BG-362 RA
BG-1003 Control
BG-1003 RA
Day-0
Day-1
Day-3
Day-7
Recovery
Treatments
B
CAT units (µmol/min/gm)
Treatments

Fig. 3

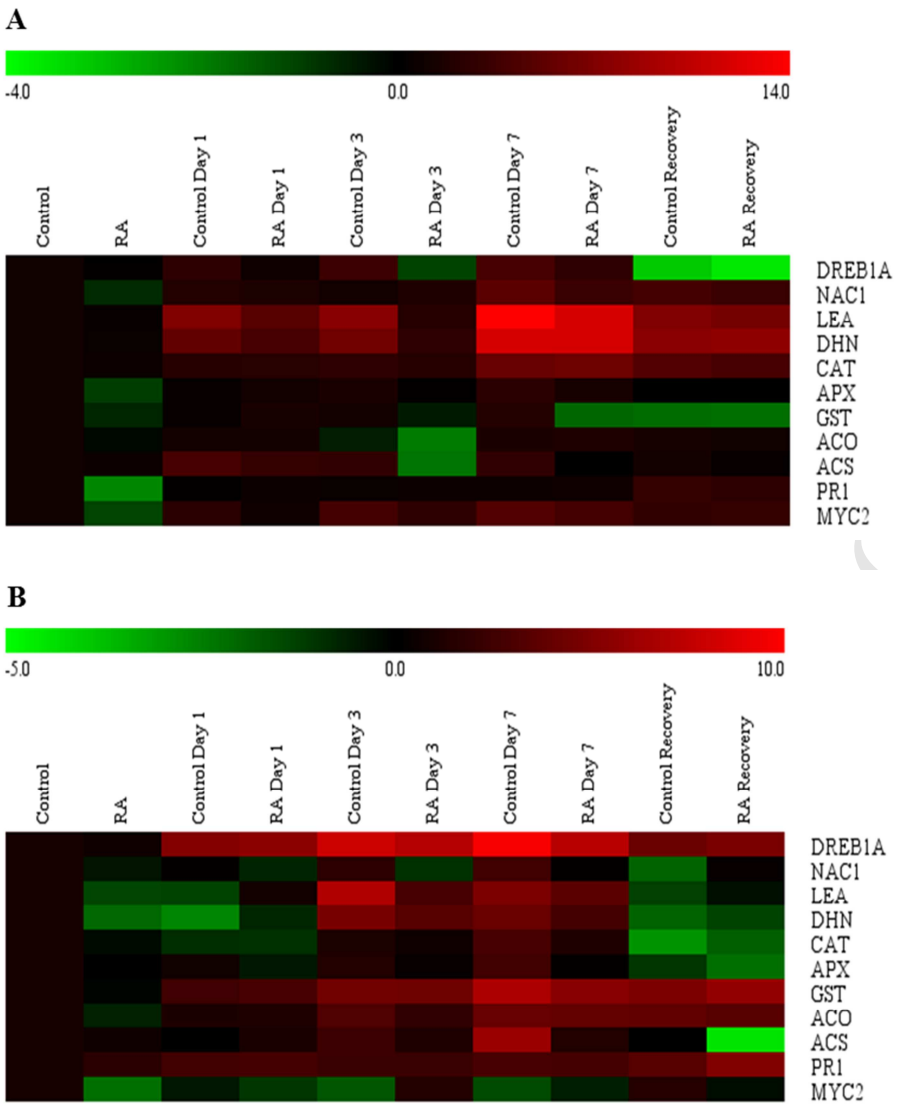

Fig. 4

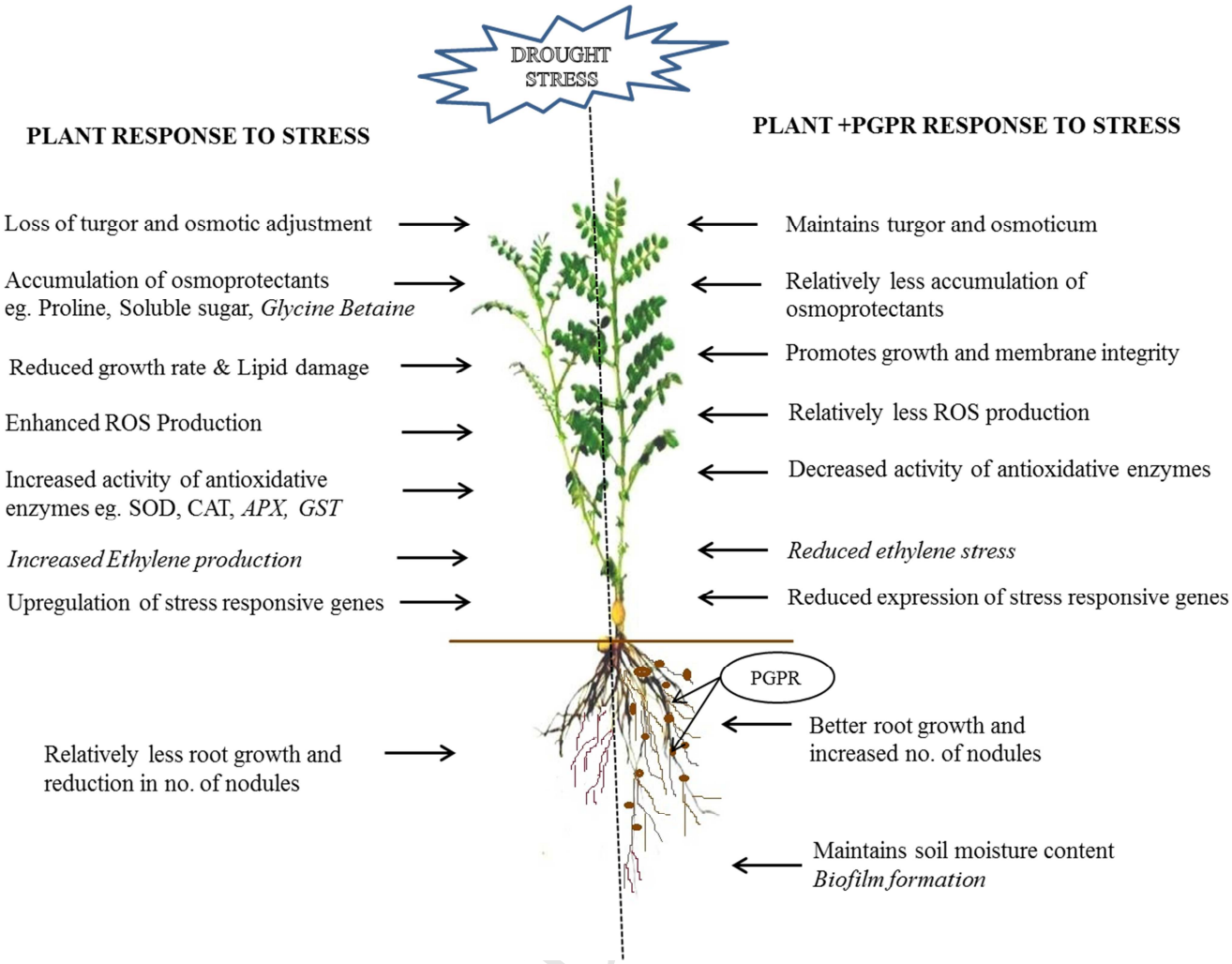

Fig. 5

Highlights

- *Pseudomonas putida* NBRIRA inoculation improves drought stress tolerance as well as assists in better recovery of both *desi* and *kabuli* chickpea.
- Promotes comparatively better seed germination during stress condition than uninoculated seeds.
- *P. putida* inoculation confers drought tolerance by altering physical, physiological and biochemical parameters.
- Inoculation reduces expression of stress responsive gene in chickpea cultivars.

Author Contribution Statement: CL, PSC and CSN conceived and designed research. ST and CL conducted experiments. ST, CL and PSC analyzed data. CL and ST wrote the manuscript. PSC critically reviewed the manuscript. All authors read and approved the manuscript.