



## A multifaceted rhizobacterium *Paenibacillus lentimorbus* alleviates nutrient deficiency-induced stress in *Cicer arietinum* L.



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### ABSTRACT

Nutrient deficiency in soil is one of the limiting factors responsible for stunted growth and poor flowering/fruitletting of crops which result in decline in overall agricultural productivity. However, one important strategy to overcome the problem of nutrient deficiency and to avoid use of chemical fertilizers is the use of plant growth promoting rhizobacteria (PGPR). *Paenibacillus lentimorbus* NRRL B-30488 (hereafter B-30488), an efficient PGPR has been reported to have various plant growth promoting traits that help crops to mitigate various environmental stresses. Therefore, the present work was designed to examine the application of B-30488 on chickpea growth under nutrient stress condition. Plants inoculated with B-30488 showed positive modulation in physio-biochemical behaviour and mineral nutrient uptake for better growth and development. Alteration in gene expression and metabolic profile under nutrient stress condition in chickpea also supported the stress amelioration capability of B-30488. Principal component analysis statistically proved that improved growth performance of chickpea plants under nutrient stress was mainly due to B-30488 induced modulation of metabolic pathways. To the best of our knowledge, this is the first study for analysis of growth promotion and stress alleviation in chickpea plants subjected to nutrient stress in presence of PGPR B-30488.

### 1. Introduction

Chickpea (*Cicer arietinum* L.), a cost-effective protein-rich legume plant, is widely cultivated in many parts of the world (Li et al., 2017). Globally, its production accounts for nearly 13.1 million tonnes, and India is one of the leading producers of chickpea with production of 67% (Thudi et al., 2016). Chickpea is an essential source of proteins, minerals, carbohydrates, fibers and other beneficial secondary metabolites having several health-promoting effects (White and Brown, 2010; Bar-El Dadon et al., 2017; Badhan et al., 2018). Beside, chickpea is a rustic plant which is mainly cultivated on infertile and water stressed soils which affect its productivity and yield (Valenciano et al., 2011; Bar-El Dadon et al., 2017). The deep tap root system makes it competent to grow on a wide range of soils and enhances its capability to withstand several stresses (Considine et al., 2017). Therefore, it is being considered as an important food security crop targeted to fulfill the nutritional requirement of increasing world population (Bar-El Dadon et al., 2017). However, deficiency of nutrients is a major constraint for chickpea production, especially in developing countries like India where its production is mostly done on marginal and sub-marginal lands, which are poor in several nutrients (Srinivasrao et al., 2002; Bhadouria et al., 2017). Decline in chickpea productivity due to

mineral nutrient deficiency has been reported by several studies. For example, nitrogen (N) and phosphorus (P) deficiencies have caused yield losses of 709,000 and 653,000 t/year, all around the world (Rasool et al., 2015; Bhadouria et al., 2017). Iron and zinc deficiency induced impairment is also reported in chickpea (Rasool et al., 2015). Moreover, losses in chickpea production due to micronutrient deficiencies have been calculated approximately to 360,000 t/year (Rasool et al., 2015). Nutrient deficiency also makes chickpea crop more prone to several pathogens like fungi, bacteria, viruses, nematodes and mycoplasmas leading to yield losses globally (Nene et al., 2012; Ghosh et al., 2017a, 2017b; Jendoubi et al., 2017).

Nonetheless, to overcome the problem of nutrient deficiency, excessive and inappropriate use of chemical fertilizers is being promoted, which has many environmental and economical drawbacks (Savci, 2012). The agronomists are developing genomics-assisted breeding and transgenic approaches to alleviate adverse effects of abiotic stresses; but both these technologies are time-consuming and labor-intensive, therefore, are inappropriate to be implemented right away to resolve the problem. The association of chickpea with soil rhizobia helps in improving productivity and soil fertility but at the same time literature available suggests that rhizobia are sensitive to soil environment (Kulkarni and Nautiyal, 2000; Surange et al., 1997; Wolde-meskel et al.,

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2018). It is also documented in previous reports that mineral nutrient deficiencies limit nitrogen fixation by the legume-*Rhizobium*-symbiosis in agricultural soils and nodulation is impeded and hence, cannot be used under such conditions (O'Hara et al., 1988; Karmakar et al., 2015). Therefore, use of plant growth promoting rhizobacteria (PGPR) as an alternate and efficient technology in nutrient limited conditions will be helpful for sustainable chickpea production. The PGPR are a group of rhizosphere colonizing bacteria that improve the plant growth and health by various mechanisms (Kumar et al., 2018). Multiple evidences indicate that PGPR help to improve tolerance to several biotic and abiotic stresses in crop plants (Yaoyao et al., 2017; Shameer and Prasad, 2018). One such rhizosphere inhabiting PGPR is *Paenibacillus lentimorbus* NRRL B-30488 (henceforth B-30488). B-30488 isolated from the milk of Sahiwal cow (DasGupta et al., 2006), is a widely reported PGPR having multifarious plant growth promotion traits like natural antioxidants alteration (Nautiyal et al., 2006, 2008), bio-control against *Fusarium oxysporum* f. sp. ciceri, *Alternaria solani* and *Sclerotium rolfsii* (DasGupta et al., 2006; Khan et al., 2012b; Dixit et al., 2016), bioremediation of Chromium-contaminated soil (Khan et al., 2012a), elicitation of ISR to reduce the incidence of CMV and growth promotion in Tobacco (Kumar et al., 2016). Therefore, it was imperative for us to explore the potential of PGPR B-30488 on chickpea plants grown under nutrient-deficient conditions. Though, some review suggests that PGPR might help in amelioration of degraded soil (Meena et al., 2016, 2017; Khan et al., 2017a,b) but till now no one has studied the role of PGPR in crop plants under suboptimal nutrient level. Therefore, the objectives of present study were to evaluate nutrient deficiency induced stress amelioration ability of B-30488 on chickpea plants and the possible mechanism involved in it.

## 2. Materials and methods

### 2.1. Hydroponics screening

To determine the optimum nutrient concentration for chickpea growth, the screening experiment was performed in a plant growth chamber at CSIR-National Botanical Research Institute, Lucknow, India for 15 days with temperature  $25 \pm 2^\circ\text{C}$  during the day and  $20 \pm 2^\circ\text{C}$  at night to assess the effect of different nutrient concentrations on chickpea growth. A popular chickpea cultivar K850 was used for this study. Chickpea seeds were surface sterilized with 1% sodium hypochlorite solution for 5 min followed by 4–5 times washing with sterile MilliQ water and then soaked in sterile water for overnight. Next day the seeds were transferred on moist autoclaved Whatman No. 1 filter paper and kept for germination in petriplates. Uniformly germinated seeds were then grown hydroponically in different nutrient concentrations of Hoagland's solution (Hoagland and Arnon, 1950) with six replicates of each treatment.

### 2.2. Plant test

On the basis of the screening test an extended plant test was performed under a green house at CSIR- National Botanical Research Institute, Lucknow, India with temperature fluctuating between  $25 \pm 2^\circ\text{C}$  (day) and  $20 \pm 2^\circ\text{C}$  (night) under natural light; with two different set of parameters in each treatment namely control and B-30488 inoculated ( $10^8$  CFU  $\text{mL}^{-1}$ ). Vermiculite, an inert growth substrate, was used to maintain the exact nutrient composition throughout the experiments. Chickpea seeds were sown in pots filled with autoclaved vermiculite supplemented with half strength and one-fourth strength of Hoagland solution, respectively (Appendix 1). Four different treatments in chickpea plants were named as  $\text{N}^+$  for nutrient sufficient,  $\text{N}^+ + \text{B}$  for nutrient sufficient inoculated with B-30488,  $\text{N}^-$  for nutrient deficiency, and  $\text{N}^- + \text{B}$  for nutrient deficiency inoculated with B-30488. Three replicates of each treatment, with three plants per pot, were maintained during the experiment for one month and the

potential of B-30488 to alleviate low nutrient stress in chickpea was evaluated. Stressed and control plant tissues were harvested at the same time to avoid any diurnal variation. All morpho-physiological data and biochemical analyses were recorded on the same day of harvesting. Leaf samples for qRT-PCR analyses were harvested, kept in liquid nitrogen and stored at  $-80^\circ\text{C}$  until further use.

### 2.3. Determination of photosynthetic pigments, relative water content and membrane integrity

The quantification of photosynthetic pigments was done following the method of Zhang et al., (2009) with some modifications. 100 mg of fresh leaf samples were crushed in 80% acetone and centrifuged. The supernatant was collected, and the absorbance was measured at 480 nm, 510 nm, 645 nm and 663 nm on a UV-vis spectrophotometer.

The Relative water content (RWC) was determined according to Barrs and Weatherley (1962) with some modifications. Fresh tissue samples were collected from plants and taken immediately to record fresh weight (FW). Then these tissue samples were soaked in 30 ml Milli Q water for 4 h at room temperature after which turgid weight (TW) was measured. Finally, the samples were dried at  $60^\circ\text{C}$  in a hot air oven for 48 h, and dry weight (DW) was recorded. RWC was calculated according to the formula:  $\text{RWC}\% = (\text{FW}-\text{DW})/(\text{TW}-\text{DW}) \times 100$ .

To analyse the integrity of membrane, determination of electrolytic leakage (EL) and lipid peroxidation (LP) was performed. To determine EL, a method described by Lata et al. (2011) was used. About 100 mg fresh tissue samples were soaked in 15 ml deionised water, and kept at rotary shaker at 100 rpm, for 3 h, and initial conductivity (E1) was measured. In order to release all electrolytes in the solution these culture tubes were boiled for 30 min and cooled to room temperature, and then the final conductivity (E2) was measured. EL (%) was expressed according to the formula:  $\text{E1}/\text{E2} \times 100$ . LP was determined by a modified protocol of Heath and Packer (1968). Fresh tissue (0.1 g) was ground in 1% TCA and centrifuged at 10,000 rpm for 5 min. 500  $\mu\text{l}$  of supernatant was collected and mixed with 1.5 ml of 0.5% TBA and kept at  $95^\circ\text{C}$  in the water bath for 30 min. The mixture was cooled and then centrifuged at 5000 rpm for 5 min. Finally, the absorbance was recorded at 532 nm, and 600 nm and the concentration of MDA was calculated using the extinction coefficient of  $155 \text{ mM}^{-1} \text{ cm}^{-1}$ .

### 2.4. Estimation of total proline content and total soluble sugar

The total proline content in the chickpea samples was measured according to Carillo and Gibbon (2011). 100 mg of fresh tissue was homogenized in 1 ml of 70% ethyl alcohol and centrifuged. 50  $\mu\text{l}$  of ethanolic extract was collected from the supernatant and allowed to react with ninhydrin (1% w/v) prepared in acetic acid (60% v/v) and ethanol (20% v/v). Then the reaction mixture was incubated at  $95^\circ\text{C}$  for 20 min, and finally, the absorbance was measured at 520 nm. Total soluble sugar (TSS) in chickpea samples was determined according to DuBois et al., (1956) with some modifications. About 100 mg of fresh tissue was homogenized in 2.5 ml methanol (80% v/v) and was incubated in a water bath at  $70^\circ\text{C}$  for 1 h. After incubation, 500  $\mu\text{l}$  of supernatant was taken and mixed with 500  $\mu\text{l}$  of phenol (5% w/v) and 5 ml of  $\text{H}_2\text{SO}_4$  (95% v/v) and incubated for 15 min in the dark. After that, the absorbance was measured at 490 nm.

### 2.5. Antioxidant enzymes assays and assessment of oxidative stress index (OSI)

For different antioxidant enzymes fresh tissue (0.5 g) was ground under chilled conditions with extraction buffer containing phosphate buffer (50 mM, pH 7.8), EDTA, (0.1 mM), and 1% (w/v) PVP. Then, the resulting homogenate was centrifuged at 12,000 g for 10 min at  $4^\circ\text{C}$  and supernatant was collected. The activity of superoxide dismutase (SOD) (EC1.15.1.1), Catalase (CAT) (EC1.11.1.6), and ascorbate peroxidase

(APX) (EC1.11.1.11) were measured by Beyer and Fridovich (1987); Aebi (1984) and Nakano and Asada (1981), respectively. OSI was used to estimate overall oxidative stress according to (Pérez-Palacios et al., 2017).

## 2.6. Quantification of nutrient elements

0.2 g of dry samples were digested with concentrated sulfuric acid to determine the N concentration according to the Kjeldhal method (Nelson and Sommers, 1973) using Automatic Nitrogen Distillation System (Kjelplus). For the estimation of other elements, 0.1 g of dried samples were digested with nitric acid and perchloric acid. The digest was filtered, and deionized water was added to make up the final volume to 50 ml. The element concentrations i. e., Mg, Mn, Cu, Zn, and Fe were measured using Atomic Absorption Spectrophotometer while Ca and K concentration was determined by flame photometer. The P concentration of the digest was measured according to Jackson (1973) using UV–vis spectrophotometer at a wavelength of 690 nm.

## 2.7. Evaluation of anatomy in cross-sections of chickpea roots

Chickpea roots samples of all the treatments were fixed in formalin–acetic acid–alcohol (FAA) according to Paolillo and Zobel (2002). The preserved roots were cut into 5-mm-long segments behind the root tip from a distance of 10 mm to a distance of 10 mm below the basal region, i.e., the root-shoot junction. Then these segments were manually cut into thin cross-sections with the help of double-edge razor blades. Subsequently, the cross-sections were stained with Johansen's safranin and fast green (Johansen, 1940) and rinsed with a clearing solution containing 1 vol. each of ethanol and xylene; and 2 vol. of methyl salicylate to capture fine images and fast microscopic observation. Photographs were taken on EVOS FL Cell Imaging System (Thermo Fisher Scientific).

## 2.8. Total RNA Extraction, cDNA synthesis and qRT-PCR analysis

Total RNA was extracted manually from ~100 mg tissue of chickpea plant samples using TRIzol reagent (Invitrogen Co., CA, USA). DNA contamination from all the RNA samples was removed using RNase-free DNase (Genei Laboratories Pvt Ltd, India) and the RNA was quantified using NanoDrop spectrophotometer. 1 µg of DNase free total RNA was reverse transcribed into cDNA using Maxima H Minus M-MuLV reverse transcriptase (Thermo Scientific, United States) following manufacturer's instructions. Relative gene expression levels were measured using 2X Brilliant III SYBR Green QPCR (Agilent Technologies, United States) on Stratagene Mx3000 P (Agilent Technologies, United States) in triplicates. GAPDH gene was used as an internal reference gene for normalization of semi-quantitative-RT-PCR (Garg et al., 2010). Primers for stress-responsive genes used in this study were obtained from Tiwari et al., 2016 (Appendix 2). The transcript accumulated for each target gene was normalized to the internal control and examined using  $2^{-\Delta\Delta Ct}$  method (Livak and Schmittgen, 2001).

## 2.9. Determination of metabolites in plant tissues

One gram of dry samples was extracted three times with 50% methanol on the orbital shaker for 2 h. The pooled plant extract was filtered through a Whatman filter paper no. 1, and fractionated by ethyl acetate. All the fractions of ethyl acetate were pooled and concentrated using a rotary evaporator. All residues obtained were then dissolved in 1 ml methanol and filtered through a membrane filter before injecting in HPLC system (Shimadzu, Kyoto, Japan) equipped with dual Shimadzu LC-10 ATVP reciprocating pumps, SPD-M20 A PDA detector, and SIL 20 AC HT autosampler. Running conditions were similar to those described by Niranjana et al., (2011). Gallic, chlorogenic, ferulic, caffeic, coumaric, anisic, rutin, syringic, p-hydroxy benzoic acid,

quercetin and kaempferol were used as standards. Concentrations of the metabolic compounds were calculated in  $\mu\text{g g}^{-1}$  by comparing peak areas of the samples with those of standards run under the same elution conditions.

## 2.10. Statistical analysis

Significance between mean values of different treatments applied was checked by one-way analysis of variance (ANOVA), and comparison was carried out using Duncan multiple range test at  $P < 0.05$  with SPSS software package version 16.0 (SPSS Inc./IBM Corp. Chicago, USA). The principal component analysis (PCA) was applied to understand and reveal the correlation among variables to assess the relationship between treatments and variables using R 3.5.1 package.

## 3. Results

### 3.1. Hydroponics screening

For the determination of optimum nutrient concentration on chickpea growth hydroponics screening was performed. After 15 days of growth, the effect of different nutrient concentration on chickpea growth was clearly visible (Appendix 3). When only distilled water (DW) was used, the rate of plant growth was markedly inhibited. At one-fourth concentration of nutrients, plant growth was less inhibited as compared to plants grown in DW only. When the concentration of nutrients was reduced to half the plant growth inhibition was not observed; plants were healthy, attained the proper height and didn't show any deficiency or osmotic stress symptoms. However, when the higher concentration of the nutrient solution was used plant growth was inhibited, and symptoms related to osmotic stress were more prominent (Appendix 3).

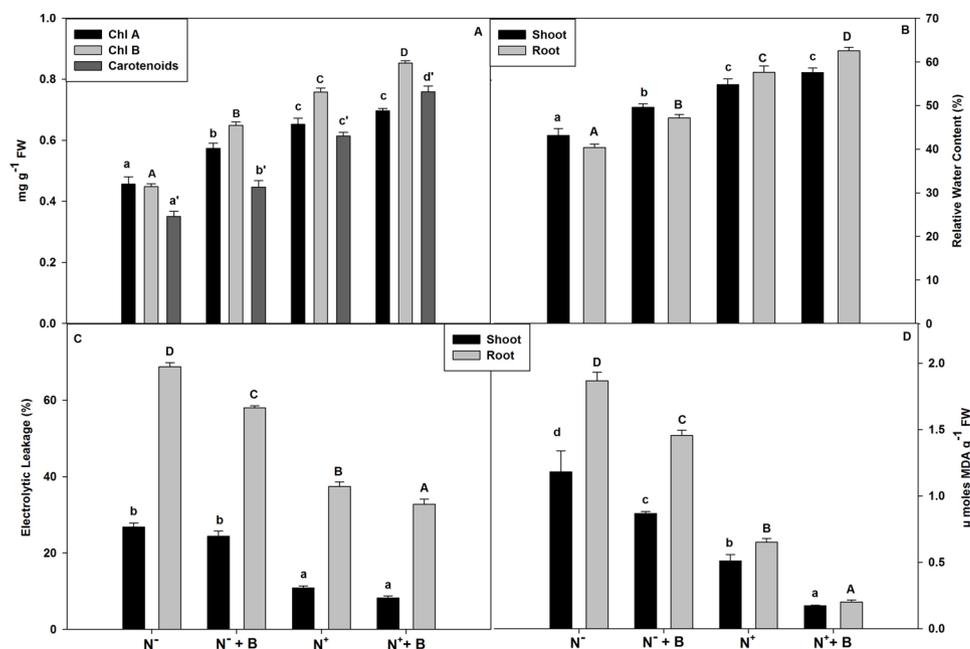
### 3.2. Effect of B-30488 inoculation on growth parameters of Chickpea

On the basis of hydroponic screening, two concentration of nutrients were used for further study namely, half-strength Hoagland solution as nutrient sufficient and one-fourth strength Hoagland solution as the nutrient deficient condition under both uninoculated ( $N^+$  and  $N^-$ ) and inoculated treatments ( $N^+ + B$  and  $N^- + B$ ), respectively.

Plants grown under sufficient nutrient supply showed higher biomass than those grown under nutrient deficient conditions (Appendix 4). In comparison to uninoculated plants, inoculation of B-30488 under both nutrient deficient and sufficient condition lead to an increase in biomass by ~8% and ~28% respectively. Comparatively, root length was found to be higher in nutrient sufficient than nutrient deficient plants. Under both nutrient deficient and sufficient condition root length was more in B-30488 inoculated plants by ~34% and ~4% than their respective uninoculated plants (Appendix 4). Similarly, B-30488 inoculation increased the shoot length by ~11% significantly under nutrient sufficient condition in comparison to uninoculated plants (Appendix 4). However, the shoot length of plants grown at nutrient deficient condition had no significant difference in shoot length regardless of the bacterial treatment. The significant increment in these parameters indicates that *P. lentimorbus* improved stress endurance capacity of chickpea under stress.

### 3.3. Effect of B-30488 inoculation on photosynthetic pigments, relative water content and membrane integrity of chickpea

The photosynthetic pigments, RWC, EL and malondialdehyde (MDA) content were estimated in this study and found to be altered in B-30488 inoculated chickpea plants. Deficiency of mineral nutrients reduced the photosynthetic pigments concentration by almost 30–45% in chickpea plants when compared to the uninoculated chickpea plants grown in the presence of sufficient nutrients (Fig. 1A). However,



**Fig. 1.** Changes in photosynthetic pigments (A), relative water content (RWC) (B), electrolytic leakage (C) and malondialdehyde (MDA) (D) of the chickpea plants after 30 d of treatments (N<sup>-</sup>, N<sup>-</sup> + B, N<sup>+</sup> and N<sup>+</sup> + B). All the values are means of 3 replicates (n = 3) ± S.E. Different letters indicate different significant values among the treatments (DMRT, p ≤ 0.05).

inoculation with B-30488 increased the level of these pigments significantly by ~27–45% as compared to uninoculated plants grown in deficient conditions. RWC was significantly decreased by 21% in shoots and 29% in roots under nutrient deficient conditions compared to plants grown with sufficient nutrients (Fig. 1B). In comparison to uninoculated plants, B-30488 treatment significantly increased this parameter by ~15% and ~17% in shoot and root, respectively; under nutrient deficient condition; indicating that B-30488 inoculation helps in maintenance of water balance in plants even when sub-optimal level of nutrients are available to plants. B-30488 treated plants were also able to improve membrane integrity as comparatively less ion leakage of ~8% and ~16% in the shoot and root respectively was observed when compared to non-treated plants grown with fewer amounts of nutrients (Fig. 1C). The EL was significantly lower in case of plants grown in unstressed conditions while B-30488 treatment further reduced the EL by ~24% and ~13% in shoot and root respectively in these plants. MDA content which is considered as a marker of membrane injury due to lipid peroxidation upon exposure to stress was significantly increased by ~130% in the shoot and ~186% in root in uninoculated plants under nutrient stress condition compared to the plants under nutrient sufficient condition (Fig. 1D). In comparison to uninoculated chickpea plants under nutrient stress, B-30488 inoculation partially attenuated the deleterious effect of nutrient stress on membranes by ~27% in the shoot and ~22% in roots in this crop.

### 3.4. Effect of B-30488 inoculation on proline and total soluble sugar content

Proline and total soluble sugar are the osmoprotectants which are considered to protect plants against stress. Under deficient conditions, proline and TSS content were significantly increased by ~118% and ~160% in proline (Fig. 2A) and ~103% and ~130% in TSS (Fig. 2B) of uninoculated chickpea plants in shoot and root, respectively; however, B-30488-treatment led to significant reduction in proline content by ~30% and ~24% in TSS, in both shoot and root, respectively; as compared to the uninoculated plants under nutrient deficiency.

### 3.5. Antioxidant enzymes and evaluation with OSI

Under limited nutrient conditions, the significant increase in CAT, SOD and APX activities were observed in untreated chickpea plants,

though B-30488 inoculation decreased the level of these antioxidant enzymes significantly (Fig. 3A–C). Also, it was observed that the level of these antioxidant enzymes were lower in nutrient sufficient condition both in uninoculated and inoculated chickpea plants when compared to the plants facing nutrient deficiency. Our results also showed that OSI values (Appendix 5) varied among different treatments; the highest OSI values were recorded in non-inoculated plants grown under nutrient deficient conditions while inoculation with B-30488 led to reductions in OSI. Plants were grown with sufficient nutrients also had low values for this parameter whether inoculated or uninoculated.

### 3.6. Accumulation of nutrient elements

In the present study, when plants were exposed to nutrient deficiency the level of N, P, K, Mn, Fe, Mg, Cu, Zn and Ca were significantly reduced when compared to plants grown with sufficient nutrients (Table 1). However, supplementation of B-30488 altered the level of these nutrient elements in roots and shoots of chickpea plants under both the conditions, i.e., sufficient and deficient, respectively. An increase in the accumulation of nutrients viz., N, P, K, Fe, Mn, Cu and Zn in roots (26%, 28%, 48%, 123%, 309%, 6% and 8%, respectively) except Mg and Ca was observed on B-30488 inoculation to plants facing deficiency of nutrients as compared to uninoculated ones. While, in shoots, the increase was more prominent for N, P, K, Ca and Mg which were found to be increased by 54%, 87%, 48%, 40%, and 11%, respectively than the uninoculated plants.

### 3.7. Evaluation of chickpea roots anatomy on B-30488 inoculation under low nutrient stress conditions

As shown in Fig. 4, significant variation in lignin deposition was observed in root sections of different treatments. Lignin deposition was maximum in untreated chickpea plants as compared to those treated with B-30488 under stress. Chickpea roots grown under nutrient sufficient condition with or without B-30488 showed the least lignin deposition. Also, under the nutrient deficient condition the central portion of the stellar region was highly damaged, and cambium cells were not developed; nevertheless, no such damage was observed, and cambium cells were developed in case of other three treatments.

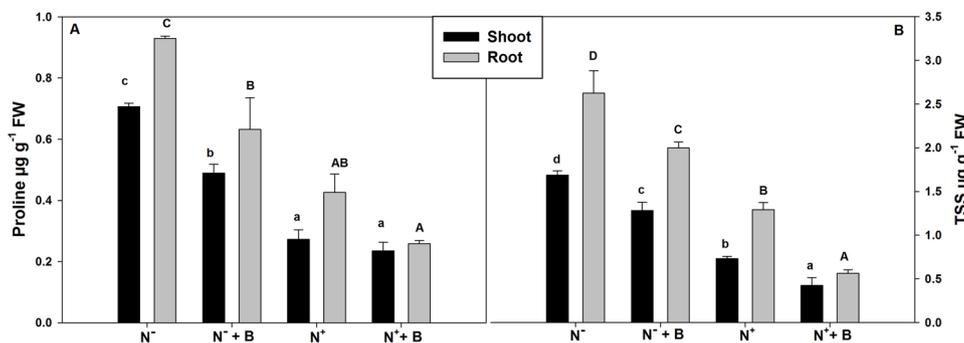


Fig. 2. Changes in proline (A) and total soluble sugar (TSS) (B) in roots and shoots of the chickpea plants after 30 d of treatments (N<sup>-</sup>, N<sup>-</sup>+B, N<sup>+</sup> and N<sup>+</sup>+B). All the values are means of 3 replicates (n = 3) ± S.E. Different letters indicate different significant values among the treatments (DMRT, p ≤ 0.05).

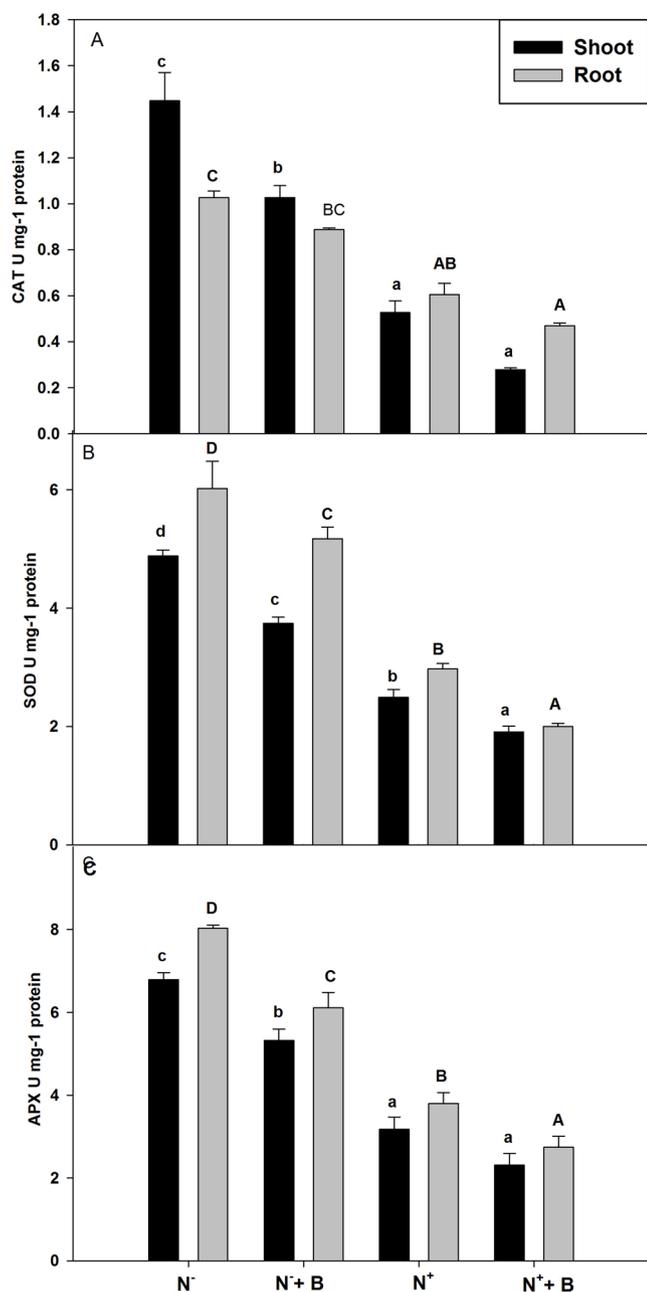


Fig. 3. Changes in antioxidant enzymes in roots and shoots of chickpea plants after 30 d of treatments (N<sup>-</sup>, N<sup>-</sup>+B, N<sup>+</sup> and N<sup>+</sup>+B). All the values are means of 3 replicate (n = 3) ± S.E. Different letters indicate different significant values among the treatments (DMRT, p ≤ 0.05).

### 3.8. Effect of B-30,488 inoculations on gene expression in chickpea

In order to verify our results for biochemical, and physiological analyses expression levels of several stress-related marker genes namely, catalase (CAT), ascorbate peroxidase (APX), glutathione S-transferase (GST), NAC transcription factors (NAC1) and ACC oxidase (ACO) were investigated using qRT-PCR (Fig. 6). The expression level of gene encoding antioxidative enzymes namely CAT, GST and APX were found to be up-regulated by ~3 to ~5.5-fold in plants subjected to nutrient deficiencies. Relative expression of ACO gene was also found to be upregulated by ~2-fold under nutrient stress condition. Similarly, NAC 1 transcription factor (TF) known to play a role in plant development also showed upregulation by ~3.6 fold under nutrient deficient condition. However, the relative expression abundance of these genes was found to be lower in B-30488 inoculated plants than uninoculated ones, indicating that B-30488 helps plants to cope with the stressful condition. Interestingly, under nutrient sufficient condition, the expression of these genes was found to be basal. Further, the gene expression data were also found to be in correlation with our other results under stress condition.

### 3.9. Alteration of plant metabolites on B-30488 inoculation

To expand our knowledge on the role of PGPR-plant interaction on plant metabolism under low nutrient stress, HPLC analysis of chickpea shoot and root samples under different treatments was done. Fig. 5(A and B) shows the significant variations in the amount of different compounds quantified in the shoot and root of inoculated and uninoculated chickpea plants grown in the presence or absence of nutrients. However, the B-30488 induced changes were more prominent under stress condition as compared to the unstressed condition. The highest induction under stress condition was detected in ferulic acid and rutin in both shoot and root amongst all the compounds when inoculated with B-30488. The results clearly indicate that B-30488 treatment alters the metabolic pathways involved in the biosynthesis of these compounds under both nutrients sufficient and deficient conditions.

### 3.10. PCA analysis

The results of the multivariate PCA analysis are shown in the score plots and its corresponding scattered plots between PC1 and PC2 (Appendix 6). PCA analysis revealed that different metabolites are associated with different treatments in chickpea viz., nutrient deficiency, nutrient deficiency supplemented with B-30488, sufficient nutrients and sufficient nutrients supplemented with B-30488, suggesting a clear distinction in the metabolite accumulation under the four treatments.

## 4. Discussion

A balanced supply of plant nutrients is essential for commercial

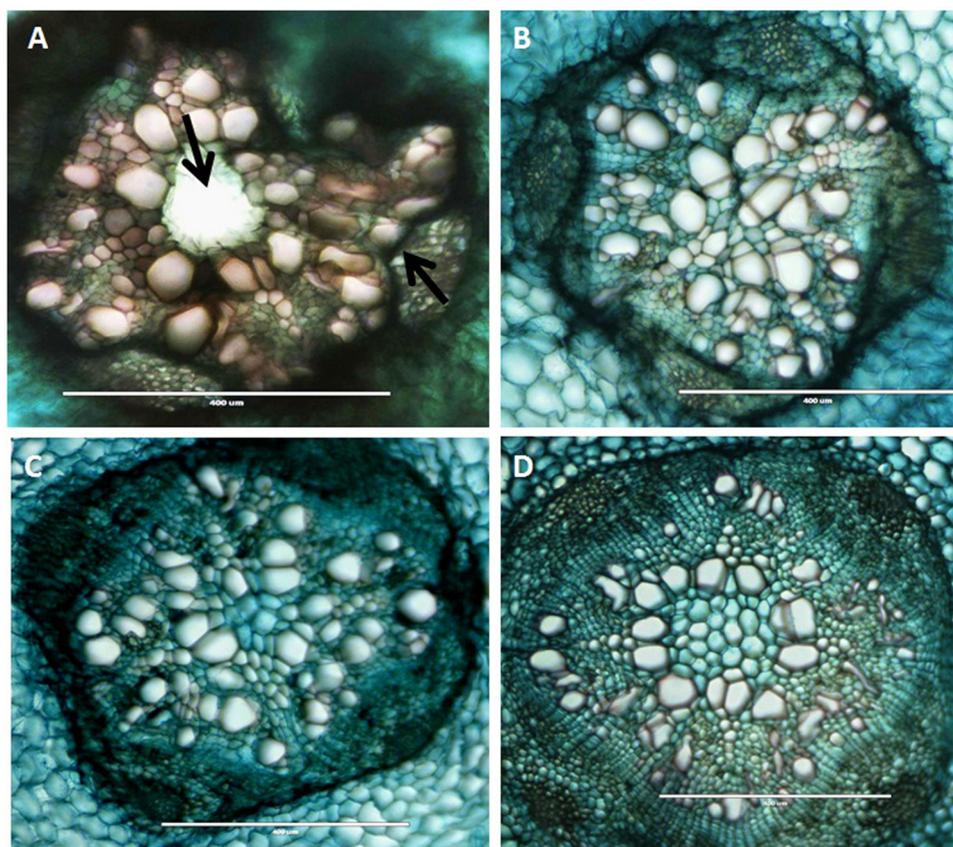
**Table 1**  
Effects of B-3-0488 treatment on accumulation of macro and microelements in shoot and root of chickpeaplants.

Element	Tissue	Treatments			
		N <sup>-</sup>	N <sup>-</sup> + B	N <sup>+</sup>	N <sup>+</sup> + B
N (mg g <sup>-1</sup> )	Shoot	15.58 ± 0.56a	24.02 ± 0.21b	23.97 ± 0.30b	26.22 ± 0.28c
	Root	18.52 ± 0.59a	23.42 ± 0.29b	25.44 ± 0.29b	29.31 ± 0.80c
P (mg g <sup>-1</sup> )	Shoot	4.25 ± 0.053a	7.96 ± 0.027b	8.62 ± 0.21b	12.04 ± 1.49c
	Root	5.29 ± 0.28a	6.80 ± 0.07b	8.87 ± 0.20c	11.38 ± 0.59d
K (mg g <sup>-1</sup> )	Shoot	11.49 ± 0.24a	17.11 ± 0.05b	21.16 ± 0.29c	48.06 ± 0.089d
	Root	18.49 ± 0.59a	27.46 ± 0.59c	48.29 ± 0.59d	23.21 ± 0.08b
Ca (mg g <sup>-1</sup> )	Shoot	7.94 ± 0.29a	11.12 ± 0.21b	12.48 ± 0.084c	17.57 ± 0.56d
	Root	7.87 ± 0.14a	7.92 ± 0.23b	10.80 ± 0.29c	12.09 ± 0.21d
Mg (mg g <sup>-1</sup> )	Shoot	108.16 ± 1.19a	120.7 ± 0.26b	142.16 ± 0.23d	132.83 ± 1.19c
	Root	134.33 ± 1.49a	131.76 ± 1.10a	133.04 ± 0.85a	161.83 ± 0.29b
Fe (µg g <sup>-1</sup> )	Shoot	83.28 ± 0.56a	62.1 ± 26.83a	72.93 ± 0.59a	145.91 ± 1.19b
	Root	67.88 ± 0.59a	151.75 ± 0.89b	366.21 ± 2.98c	626.74 ± 0.22d
Mn (µg g <sup>-1</sup> )	Shoot	2.33 ± 0.10b	1.51 ± 0.14a	3.31 ± 0.021c	12.16 ± 0.24d
	Root	3.13 ± 0.08a	12.83 ± 0.24b	25.68 ± 0.58c	12.43 ± 0.29b
Cu (µg g <sup>-1</sup> )	Shoot	4.71 ± 0.20b	4.61 ± 0.20b	5.33 ± 0.029c	3.86 ± 0.11a
	Root	4.16 ± 0.14a	4.41 ± 0.02a	4.92 ± 0.06b	7.48 ± 0.02c
Zn (µg g <sup>-1</sup> )	Shoot	12.53 ± 0.59b	10.66 ± 0.29a	17.33 ± 0.20d	14.73 ± 0.29c
	Root	12.98 ± 0.29a	14.11 ± 0.23a	18.13 ± 0.50c	16.81 ± 0.026b

All the values are means of three replicates ± S.E. Different letters indicate different significant values among the treatments (DMRT,  $p \leq 0.05$ ).

production of crop plants. Either deficiency or excess of nutrients is reported to cause severe damage, consequently leading to yield losses and even plant death (Riedelsberger and Blatt, 2017). When plants face nutrition deficiencies major physiological, biochemical, anatomical and molecular changes are induced, making plants to adapt the adverse conditions. But these adaptation and acclimation responses are not sufficient for crop plants to grow better and provide adequate yields (Ahanger et al., 2017). However, the application of PGPR is reported to

play an important role in augmenting soil nutrient and moisture content by various mechanisms (Vurukonda et al., 2017). In the present study, deficiency of nutrients led to a decline in many plant growth parameters of chickpea plants, though, application of B-30488 significantly alleviated those inhibitory effects. In plants experiencing nutrient deficiency root length was reduced and root system architecture was altered which is in accordance to Liang et al., 2017. However, inoculation with B-30488 increased the root length and altered root system



**Fig. 4.** Effect of different treatments on chickpea root anatomy. (A) limited nutrients (N<sup>-</sup>), (B) limited nutrients in presence of B-30488 (N<sup>-</sup> + B), sufficient nutrients (N<sup>+</sup>) and sufficient nutrients along with B-30488 inoculation (N<sup>+</sup> + B). Scale bar: 400 µm.

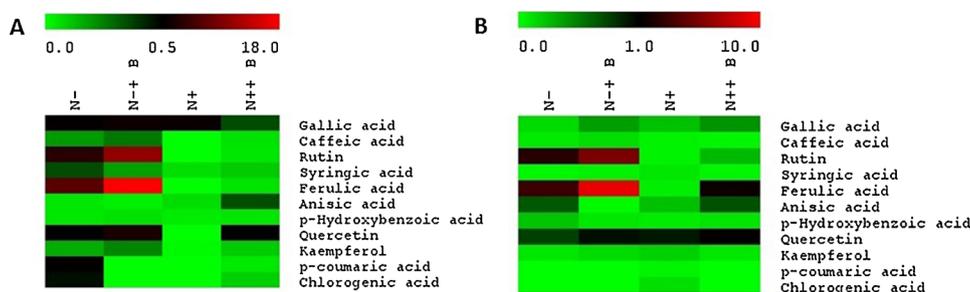


Fig. 5. Differential accumulation of metabolites in shoot (A) and root (B) of chickpea plants after 30 d of treatments ( $N^-$ ,  $N^- + B$ ,  $N^+$  and  $N^+ + B$ ). The heat map has been generated based on the concentration values of different metabolites in different treatments. The colour scale is shown at the top.

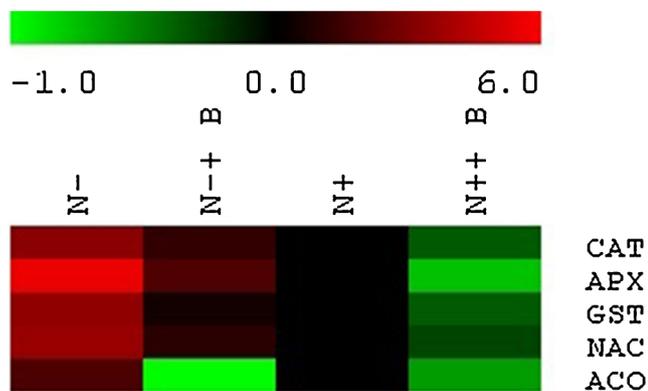


Fig. 6. Differential expression of genes in chickpea exposed to different treatments ( $N^-$ ,  $N^- + B$ ,  $N^+$  and  $N^+ + B$ ). The heat map has been generated based on the fold-change values among different treatments used. The colour scale for fold-change values is shown at the top.

architecture as compared to uninoculated plants under stress conditions (Appendix 4). So, it might be possible that these alterations in the root system by B-30488 made chickpea plants to adapt the prevailing deficient conditions better. In our experiments, we also co-inoculated chickpea compatible *Rhizobium* along with B-30488 but no nodule formation was observed and plants showed symptoms of nutrient deficiency. This was in accordance to previous reports which suggested that nodule formation and thus nitrogen fixation is affected under mineral nutrient deficiencies (O'Hara et al., 1988; Kulkarni and Nautiyal, 2000; Karmakar et al., 2015 (data not shown)). Therefore, we excluded *Rhizobium* from our further study.

The concentrations of photosynthetic pigments were significantly lower in stressed plants, but their levels were found higher in plants to which B-30488 had been applied. Kalaji et al., (2014) have reported that stress factors inhibit plant growth by inhibiting photosynthesis and that may be due to the lower concentration of photosynthetic pigments. The increase in photosynthetic pigments level in our study might be responsible for higher biomass accumulation in plants inoculated with B-30488 as compared to uninoculated plants. Therefore, even though photosynthetic pigments level under limited nutrient regime was reduced, exogenous application of B-30488 significantly eased the adverse effect by making photosynthetic pigments stable and thereby increased the plant biomass.

Several studies have reported that deficiency of minerals induce oxidative stress in plants (Tewari et al., 2006; Ahmad et al., 2012; Juszczuk et al., 2001; Hippler et al., 2018) and hampers plants physiological functions (Sirohi et al., 2016). Therefore, it was necessary for us to find the biochemical and physiological status of the chickpea plants facing nutrient limitation and inoculated with PGPR. For this several biochemical and physiological assays were performed and the results indicated that RWC, which is an important marker for plant's water balance, was decreased and EL, which is inversely associated to

membrane integrity of the cell, was found to be significantly increased under deficient conditions. However, B-30488 inoculation led to better maintenance of both plant water status and membrane integrity which is in confirmation with other earlier studies (Kang et al., 2014; Tiwari et al., 2016). MDA is used to explicate the extent of damage due to peroxidation and is induced during several abiotic stresses (Savicka and Škute, 2010). Similarly, in our study MDA was found to be higher in plants grown under nutrient deficient conditions; however, B-30488 inoculation helped in overcoming membrane damage by lowering MDA content as compared to uninoculated plants. Similar findings were also observed by Yasin et al., (2018) where inoculation with halotolerant PGPR decreased the MDA content in *Capsicum* during salt stress.

The significant increase in osmolytes concentration like proline and total soluble sugar was found in uninoculated plants under stress condition. High concentration of these osmolytes under nutrient stress in chickpea plants might also be responsible for inhibition of growth while B-30488 inoculation reduced proline and TSS accumulation and thereby alleviates growth inhibition in these plants (Sarkar et al., 2017). Similar observations were also recorded in chickpea under drought stress where PGPR-priming reduced the level of stress-induced osmolytes in plants (Tiwari et al., 2016). The presence of stress induces high oxidative stress in crop plants leading to the production of various antioxidant enzymes (CAT, SOD, APX, GPX, etc.) to balance the production of ROS (Almeselmani et al., 2006). Interestingly, in our study nutrient deficient condition induced the level of ROS quenching enzymes in chickpea plants whereas the reduction in the level of these enzymes by B-30488 inoculation was observed. The reduced level of antioxidant enzymes in B-30488 inoculated plants may be ascribed to the fact that the PGPR reduce the adverse effect of stress via regulating antioxidant enzymes activity. Khan et al., (2018) have also reported that the exogenous application of PGPR leads to a decline in the level of antioxidant enzyme activities in the leaves of chickpea plants exposed to stress. Earlier, some other studies have also shown the reduction in antioxidant enzymatic activity by PGPR application (Mahsa Hosseini et al., 2015; Khan et al., 2017a,b). The levels of oxidative stress index also corroborate with our results where OSI level was lower for plants inoculated with B-30488 than those of uninoculated plants grown under deficient conditions, indicating that oxidative stress induced by nutrient deficiency can be alleviated by PGPR application. Similar observations were also reported by Paredes-Páliz et al., (2018) in *Spartina densiflora* upon PGPR inoculation. Several earlier studies have summarized the mechanism of increased nutrient uptake on PGPR inoculation (Dobbelaere et al. 2003; Mantelin and Touraine, 2004; Saffronova et al., 2006). Similarly, in our experiments, significant variations in the concentrations of N, P, K, Fe, Zn, Ca and Mg in uninoculated and inoculated chickpea plants under deficit conditions were observed. It is likely that the increased accumulation of nutrients in inoculated chickpea plants was due to root system modulation by B-30488 which maximized the surface area for nutrient absorption. Alternatively, it can also be supported by the fact that B-30488 has several traits which contribute to the enhanced availability of nutrients to the plants (Chaudhry et al., 2013). This nutritional effect of B-30488 can

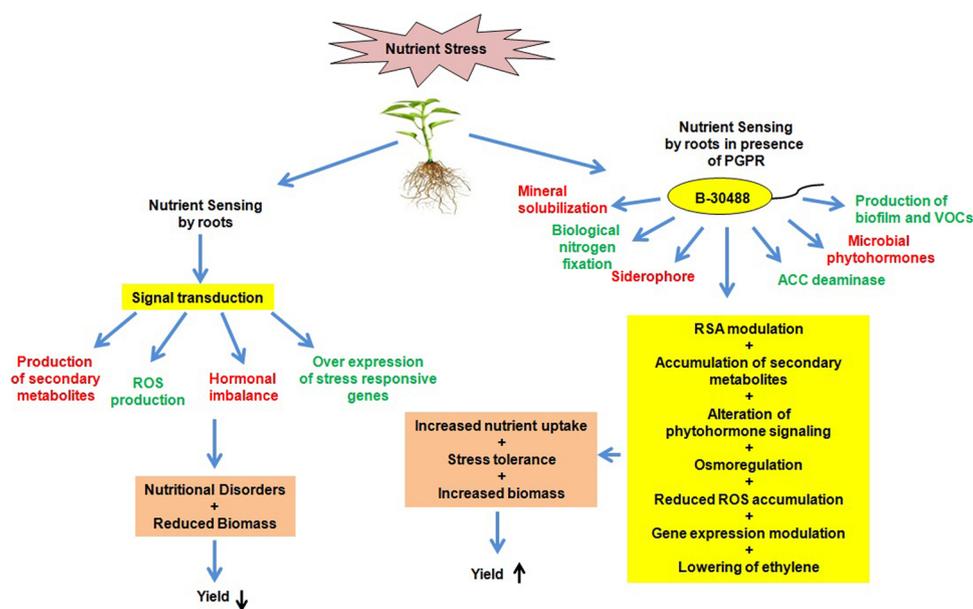


Fig. 7. A hypothetical model created based on the differential response of the enzyme assays, physiological, and molecular analysis under nutrient deficient condition in chickpea in presence and absence of PGPR and other well-known concepts.

also be the one possible reason for the ability of chickpea plants to withstand stress and support plant under stressful conditions. Our results are also in agreement to Liang et al., (2017) where they reported that application of dopamine significantly increase the nutrients and provide tolerance to apple plants facing nutrient deficiency.

It is previously reported that abiotic stresses enhance production of lignin that in due course inhibits plant growth (Neves et al., 2010). Less lignification in inoculated plants as compared to the uninoculated ones can probably due to developmental effects exerted by B-30488 which helped chickpea plants to counteract the low nutrient stress-induced lignin formation. It is worth mentioning that under nutrient-deficient conditions the central portion of the stellar region was highly damaged while B-30488 inoculation reduced this damage to a greater extent, simultaneously the cambium cells were also not found under deficient condition. The possible reason why all these changes were not observed in B-30488 inoculated plants under stress might be due to the fact B-30488 inoculation did not let chickpea plant to sense the stress owing to their tolerance to such extreme conditions.

Previously, many studies have reported PGPR-mediated modulation of numerous genes during various abiotic stresses in many crop plants (Ghosh et al., 2017a; Tiwari et al., 2017; Ambreetha et al., 2018) but the effect of this interaction in nutrient stress tolerance in chickpea by altering gene expression remain largely unknown. NAC TFs has been earlier reported to be involved in abiotic stress tolerance as well as in growth and developmental pathways (Shen et al., 2017). *NAC1* gene expression increased on exposure of chickpea plants to nutrient deficiency and its decreased transcript level in B-30488 inoculated plants illustrates the negative regulation of *NAC1* gene by B-30488 under stress. Elevated expression of genes encoding antioxidant enzymes namely *CAT*, *APX*, and *SOD* were observed in uninoculated chickpea plants exposed to nutrient stress. However, lower level of expression in B-30488 inoculated chickpea plants exposed to nutrient stress indicates that B-30488 is able to alleviate stress and reinstates normal growth conditions in inoculated plants as compared to the uninoculated ones. Several environmental stresses including both biotic and abiotic are known to increase the rate of ethylene production (Lynch and Brown, 1997) and further the role of PGPR to reduce the level of ethylene in plants are also known (Glick et al., 1998). Increase in ethylene production means the higher activity of ethylene biosynthesis enzymes like ACO. Accordingly, the higher expression of ACO under low nutrient

stress in chickpea and its comparatively low level of expression in B-30488 inoculated plants, suggests ethylene production is reduced on B-30488 inoculation due to ACC deaminase activity of B-30488. The higher expression of *GST* is considered as a marker for plants facing stress (Kumar and Chattopadhyay, 2018). Increased expression of *GST* under deficiency condition while decline expression on PGPR inoculation was observed, indicating the role of B-30488 in alleviating stress under deficiency conditions.

It is previously reported that plants synthesize these specific compounds to acclimatize themselves to severe environmental biotic and abiotic stresses including the nutrient stress (Ramakrishna and Ravishankar, 2011; Nakabayashi and Saito, 2015; Khan et al., 2018). In the present study, accordingly, metabolites increased significantly under nutrient deficient condition. However, alteration in these metabolites was observed when chickpea plants were inoculated with B-30488. This variation in inoculated and uninoculated plants clearly indicate that B-30488 inoculation altered the metabolic pathways in chickpea which in part played an important role in conferring tolerance to plants against nutrient stress. Significantly high accumulation of rutin and ferulic acid in PGPR-inoculated chickpea plants under deficit condition, suggests their antioxidant activity in improving plant tolerance to stress (Ismail et al., 2015; Yildiztugay et al., 2018). Similar to our observations del Rosario Cappellari et al., 2017 have demonstrated the role of PGPR induced plant metabolites in improving the nutrient status of plants and improving plant growth.

In conclusion, chickpea though a rustic plant, respond to nutrient stress by decline in its growth parameters which ultimately decrease its productivity. However, when chickpea plants were supplemented with PGPR, *P. lentimorbus* B-30488 they displayed greater flexibility and tolerance to nutrient deficiency due to PGPR induced alteration in gene expression and metabolic pathways. Our study is an initial step for understanding the mechanism of PGPR-mediated nutrient stress tolerance in crop plants. Further, studies should focus more on this area of interest to explain more clearly the mechanism behind this interaction. Based on amelioration of nutrient stress by B-30488 in chickpea with the several other published studies and well-known concepts a hypothesis has also been elaborated in Fig. 7.

## Conflict of interest

The authors declare that there is no conflict of interest.

## Author contribution statement

PSC and NB conceived the idea and designed the experiments. NB and ST performed the experiments. PCS contributed to the microscopic analysis. AN performed the HPLC. NB, ST, and PSC wrote and edited the MS.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.micres.2019.04.007>.

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