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Research article

Antioxidant, physiological and biochemical responses of drought susceptible and drought tolerant mustard (*Brassica juncea* L) genotypes to rhizobacterial inoculation under water deficit stress

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ABSTRACT

Response of drought susceptible (DS) genotype Pusa Karishma LES-39 and drought tolerant (DT) mustard genotype NPJ-124, to rhizobacterial inoculation under water deficit stress, was compared in the present study to determine the influence of inoculants on biochemical and physiological attributes of these two different genotypes. Inoculation was observed to improve root and shoot dry weight in both the genotypes, although better results were observed in the DS genotype. There was variation in the response of the two genotypes to rhizobacterial inoculation, under water deficit stress. Significant improvement in most of the physiological and biochemical parameters including antioxidative enzyme activities of the DS genotype; with concomitant decrease in starch content, accumulation of H_2O_2 and lipid peroxidation upon inoculation of rhizobacteria was observed. In contrast, there was improvement in only few physiological and biochemical parameters in the DT genotype in response to inoculation with rhizobacteria. There was significant increase in catalase enzyme activity along with concomitant decrease in lipid peroxidation. Thus, drought susceptibility of the mustard genotypes, NPJ-124 and Pusa Karishma LES-39, determined their physiological, biochemical and antioxidative responses to rhizobacterial inoculation under water deficit stress. Expression of drought stress responsive genes belonging to ABA-dependent (*RD20* and *RD26*) and ABA-independent (*DREB2* and *DREB1-2*) pathways was studied in the DS genotype. Expression of *DREB2* and *DREB1-2* genes was considerably enhanced due to inoculation under water deficit stress; indicating that in *Bacillus*-mediated priming for drought stress tolerance, in this genotype, ABA-independent pathway probably played key role in enhancing tolerance to drought stress.

1. Introduction

Agricultural productivity in most of the arid and semi-arid regions of the world is severely impacted by drought. At least 70% of the world's irrigated cropped area is periodically exposed to water shortages (Brauman et al., 2016). Drought affects all the stages of plant growth including seed germination and crop yield, resulting in huge economic losses (Fahad et al., 2017). Mustard is an important oilseed crop grown in India. Low water availability during critical stages of its growth *viz.* flowering and pod filling severely impacts crop yield in mustard (Mirzaei et al., 2013). Under moisture stress, yield reductions ranging between 17 and 94% have been reported in mustard (Chauhan et al., 2011).

Rhizobacteria are soil bacteria living in close association with plant roots and many of these are directly or indirectly involved in plant growth promotion. Many workers have reported improvement in drought tolerance of plants due to inoculation with beneficial rhizobacteria (Bandeppa et al., 2015; Niu et al., 2017). Among the various plant growth promoting rhizobacteria (PGPRs) isolated, different species of *Bacillus* have also been reported to help alleviate water deficit/drought stress in various crops. Beneficial effects of different species of *Bacillus* under water deficit/drought stress were reported in wheat,

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tomato, mustard, lettuce, pepper and maize (Timmusk et al., 2014; Paul et al., 2017; Bandeppa et al., 2018). The alleviation of drought stress was attributed to the ability of *Bacillus* species to improve plant growth and alter physiological and biochemical status of the plant. Enhanced relative water content, chlorophyll content and improved accumulation of osmolytes *viz.* proline, amino acids and total soluble sugars was observed in the treated plants due to inoculation with *Bacillus* species under drought stress condition (Gagné-Bourque et al., 2016; Zhou et al., 2016). A common aspect of most of the adverse environmental conditions is the increased production of reactive oxygen species (ROS) resulting in oxidative damage to the cellular components (Mohammadi et al., 2018). Enhanced activity of different antioxidative enzymes, greater accumulation of antioxidants and reduced lipid peroxidation has been reported in response to drought stress in *Bacillus* species inoculated plants, thereby mitigating adverse effects of drought stress (Timmusk et al., 2014; Zhou et al., 2016).

Mustard, a comparatively drought tolerant crop, is usually grown under rainfed conditions, although irrigation does improve mustard yield considerably. Both drought tolerant and drought susceptible mustard genotypes, suitable for growing under rainfed and irrigated conditions, respectively have been developed (Chauhan et al., 2011; Singh et al., 2017). We have identified osmotolerant rhizobacteria, which have been able to mitigate water deficit stress in the drought susceptible mustard genotype (Bandeppa et al., 2015). Earlier also many workers have reported microbe-mediated alleviation of the deleterious effects of drought stress in various crops (Kang et al., 2014; Paul et al., 2017). However, most of the work has focussed on the effect of these rhizobacteria on crop growth and the mechanism of mitigation of drought stress by these rhizobacteria. Most of these studies were undertaken using the drought susceptible crops/crop genotypes and the effect of inoculation with these rhizobacteria on plant growth, under drought stress, was observed. The drought tolerant genotypes have their own strategies for overcoming drought stress and their responses to rhizobacterial inoculation have, generally, not been well-documented. In the present investigation, we have compared the antioxidative, physiological and biochemical responses of the drought tolerant mustard genotype NPJ-124 (Yadava et al., 2013) and the drought susceptible mustard genotype Pusa Karishma LES-39 (Chauhan et al., 2011) to rhizobacterial inoculation, under water deficit stress condition. We have also studied the expression of drought stress-responsive genes in the inoculated drought susceptible genotype, under the stressed environment. For that we have selected two genes belonging to ABA-dependent pathway (*RD20* and *RD26*) and two genes belonging to ABA-independent pathway (*DREB1-2* and *DREB2*). Several genes functioning under drought stress response in plants are regulated through the ABA-dependent and ABA-independent pathways. ABA an isoprenoid phytohormone, regulates adaptation to various abiotic stresses like drought, salt and cold stresses. In the plant, when environmental conditions are harsh, the level of ABA increases via ABA biosynthesis. The increased ABA binds to its receptor to initiate signal transduction, leading to cellular responses to stresses therefore, ABA is also called a stress hormone. ABA-dependent pathway gene *RD20* (response to dehydration20) is involved in regulating stomatal opening in response to drought stress (Aubert et al., 2010); *RD26* (response to dehydration26) is involved in up-regulation of ABA-inducible genes (Fujita et al., 2004). Though several drought stress responsive genes are expressed with the concomitant increase in the level of ABA, still many of the genes induced under drought condition are not influenced by ABA, indicating the possibility of ABA independent mechanisms of operation. The regulation of these ABA-independent genes occurs through the drought responsive element (*DRE*) and C-repeat (*CRT*) *cis*-acting elements, in combination with DRE-binding protein (*DREB*) or C-repeat-binding factor (*CBF*) transcription factors (Liu et al., 2018). These have APETALA2 (*AP2*) DNA binding domain, such as *DREB2*, playing a pivotal role in ABA-independent gene expression under drought stress.

2. Materials and methods

2.1. Bacterial cultures and plant used in the study

Rhizobacterial cultures *Bacillus cereus* strain NA D7 and *Bacillus* sp. strain MR D17, (Bandeppa et al., 2015), used in the present study, were obtained from the culture collection of Division of Microbiology, ICAR-Indian Agricultural Research Institute, New Delhi, India. These cultures had earlier been screened for their osmotic stress tolerance and reported to be osmotolerant (Bandeppa et al., 2015). The stock cultures were maintained on nutrient agar slants. For broth cultures, rhizobacterial cultures *B. cereus* strain NA D7 and *Bacillus* sp. strain MR D17 were multiplied in nutrient broth for 48 h till the OD at 600 nm reached to 0.8. Then, nutrient broth tubes containing 5 ml broth were inoculated with 100 µl inoculum from these broth cultures and incubated at 28 ± 2 °C for 48 h on an incubator shaker.

2.2. Plant genotypes

Drought tolerant (DT) mustard genotype NPJ-124 and drought susceptible (DS) mustard genotype Pusa Karishma LES-39, were used in the present study.

2.3. Pot experiment

Earthen pots (14 inch size) containing 12 kg soil were used for the experiment. The soil was taken from the farm block of the Institute. The soil belongs to family typic hyplustept, order-inceptisol (representing alluvial soil). Three seeds of Pusa Karishma LES -39 (DS genotype) and NPJ-124 (DT genotype) were sown in each pot. Prior to sowing, rhizobacterial cultures were multiplied as described earlier. Then, 10 µl of 48 h old broth cultures (approx. 1×10^7 colony forming units/ml) were, treatment wise, applied evenly on the surface of the seeds of the selected mustard genotypes and the seeds were immediately sown in pots. Thus, six pots per treatment, each pot containing three plants were maintained in a nethouse. Two moisture regimes, field capacity (normal water condition) and 50% field capacity (water deficit stress condition) were maintained during the experimental period. One week after emergence, the plants were exposed to water deficit stress. Tensiometers were installed in each pot at 15 cm depth during the entire growth period for continuous monitoring and maintaining the desired water status in soil. After 50 days of growth, sampling in triplicate, was carried out to determine the effect of water deficit stress and inoculation on root and shoot dry weight (DW).

2.4. Plant physiological and biochemical parameters

After 45 days of growth, plant sampling in triplicate, was carried out to determine the effect of water deficit stress and inoculation on plant physiological and biochemical parameters. **Membrane stability** was determined by the modified method of Ibrahim and Quick (2001). One gram sample consisting of 10 leaf segment, 7 cm long, were rinsed in distilled water and placed in test tube with 10 ml of distilled water. The tubes were held overnight at room temperature. After incubation, conductance of water was measured with an electrical conductivity meter (T1). Test tubes were then autoclaved for 10 min at 120 °C and conductance was measured again (T2). Membrane stability was expressed as MSI = (1 - T1/T2) x 100. **Relative water content** was determined by the method of Silvente et al. (2012). One gram of fully expanded leaves were collected and cut into 8 mm discs, fresh weight (FW) of these discs was determined and then these were floated over distilled water in Petri plates for 6 h. At the end of this period, surface of the discs was blotted dry and saturated weight (SW) was recorded. Thereafter, the samples were dried in an oven (70 °C) for 24 h and DW was recorded. Relative water content was then calculated as RWC = (FW – DW)/(SW – DW). **Proline content** was determined by the

method of Bates et al. (1973). For that, one g leaf samples were grinded in 5 ml of 3% 5-sulfosalicylic acid and then centrifuged at 10000 rpm for 30 min. One ml of the supernatant was mixed with 2 ml of ninhydrin reagent, boiled for 1 h, colour was extracted with 2 ml of toluene and absorbance was measured at 520 nm using a spectrophotometer. **Total phenolics content** was determined by the method of Singleton and Rossi (1965). Fresh leaf tissue (0.5 g) was grinded in 5 ml ethanol (80% v/v), centrifuged at 10000 rpm for 30 min and pellet was resuspended in 2.5 ml ethanol. The suspension was recentrifuged and an aliquot of 0.375 ml of this phenolics extract was mixed with 2.5 ml 1/10 diluted Folin-Ciocalteu reagent and 0.75 ml of 7.5% (w/v) Na_2CO_3, vortexed and then incubated at 45 °C in a shaking water bath for 15 min. Phenolics content were measured at 750 nm using a spectrophotometer. **Amino acid content** was determined by the method of Chen et al. (2007). One gm of freeze dried leaf sample was grinded in 5 ml methanol-chloroform-water (60:25:15 v/v), and then incubated at 60 °C for 2 h. After incubation, samples were centrifuged and total amino acid content was determined by heating 1 ml of the supernatant with 1 ml of 0.1 M acetate buffer (pH 4.3) and 1 ml of 5% ninhydrin (in ethanol) at 95 °C for 15 min. This was cooled to room temperature and O.D. measured at 570 nm using a spectrophotometer. **Starch content** was determined by the method of AitBarka et al. (2006). Five hundred mg of freeze-dried leaf samples were homogenized in 0.1 M phosphate buffer (pH 7.5) at 4 °C, using a mortar and pestle. The homogenates were centrifuged and the pellets were resuspended in dimethyl sulfoxide-8 M HCl (4:1 v/v). Starch was dissolved over 30 min at 60 °C with agitation and recentrifuged. After centrifugation, 0.2 ml supernatant was mixed with 0.2 ml iodine-HCl solution (0.06% KI and 0.003% I_2 in 0.05 M HCl) and 2 ml of distilled water. The absorbance was read at 600 nm after 15 min of incubation at room temperature. **Total sugar content** was determined by the method of Dubois et al. (1956). The samples were prepared as described for amino acids and 0.2 ml supernatant was taken and volume was made upto 1 ml with distilled water. To this 1 ml of 5% phenol (in water) and 5 ml of 96% sulphuric acid was added and mixed thoroughly. It was kept at room temperature for 10 min and again shaken. It was then incubated at 25–30 °C for 20 min and O.D was taken at 490 nm. **Hydrogen peroxide** was estimated by formation of titanium-hydro peroxide complex (Mukherjee and Choudari, 1983). Leaf samples (0.5 g) were homogenized in 10 ml cooled acetone in a chilled mortar and pestle, filtered through Whatman No. 1 filter paper followed by the addition of 4 ml of titanium reagent and 5 ml of ammonium hydroxide solution to precipitate the titanium–hydro peroxide complex. The reaction mixture was centrifuged at 10000 x g for 10 min. The precipitate was dissolved in 10 ml of 2 M concentrated sulphuric acid and re-centrifuged. The supernatant was read at 415 nm against blank and H_2O_2 expressed as μmol/g FW. **Lipid peroxidation** was determined by the method of Heath and Packer (1968). Leaf tissue (0.5 g) was homogenized in 10 ml of 0.1% (w/v) Trichloroacetic acid (TCA) and centrifuged for 20 min at 10000 rpm. One ml of the supernatant was mixed with 4 ml 0.5% Thiobarbituric acid (TBA) diluted in 20% TCA, incubated in water bath at 95 °C for 30 min and then cooled in an ice bath. The absorbance was measured at 532 and 600 nm. Lipid peroxidation was expressed as μmols Malondialdehyde (MDA)/g FW.

2.5. Antioxidative enzyme assay

Leaf samples (0.5 g) were homogenized in potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1% polyvinyl pyrrolidone (PVP) using a mortar and pestle, followed by centrifugation. The enzyme extracts obtained were used for determining the activity of superoxide dismutase, catalase, ascorbate peroxidase and glutathione reductase enzymes. The extraction buffer for ascorbate peroxidase enzyme additionally contained 1 mM ascorbic acid. **Superoxide dismutase** activity (SOD) was assayed by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) (Dhindsa et al., 1981). The 3 ml reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 25 mM nitroblue tetrazolium (NBT), 2 μM riboflavin, 0.1 mM EDTA, 50 mM sodium carbonate and 0.1 ml enzyme extract. Test tubes containing the reaction mixture were illuminated for 15 min at a light intensity of 3600 lux. Reaction was stopped by switching off the light. Appropriate blank and control were maintained. Absorbance at 560 nm was recorded using a spectrophotometer. One unit of SOD was defined as the amount of enzyme required for causing 50% inhibition of the reduction of NBT. The enzyme activity was expressed as U/g FW. **Catalase** activity was measured according to the method given by Aebi (1984) with some minor modifications. Three ml of reaction mixture contained 0.1 ml enzyme extract, 1.5 ml of 100 mM phosphate buffer (pH 7.0), 0.5 ml of 75 mM H_2O_2 and 950 μl of distilled water. Catalase activity was estimated by monitoring the decrease in absorbance of H_2O_2 ($\varepsilon = 39.4\ mM^{-1}\ cm^{-1}$) at 240 nm. The enzyme activity was expressed as mM of H_2O_2 reduced/ min/mg FW. **Glutathione reductase** activity was measured according to the method given by Smith (1985). The reaction mixture contained 10 mM potassium phosphate buffer, 0.33 mM EDTA, 0.5 mM 5, 5-dithiobis-2-nitrobenzoic acid (DTNB), 2.0 mM NADPH, 20 mM GSSG (Oxidized Glutathione), 0.1 ml of enzyme extract and double distilled water to make up the final volume to 3.0 ml. Reaction was initiated by adding 0.1 ml of 20 mM GSSG. Increase in absorbance at 412 nm was recorded using a spectrophotometer. The enzyme activity was expressed as glutathione oxidized/ min/mg FW. **Ascorbate peroxidase** was assayed by recording the decrease in optical density due to ascorbic acid at 290 nm (Nakano and Asada, 1981). The 3 ml reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM EDTA, 0.1 mM H_2O_2, 0.1 ml enzyme and water to make a final volume of 3.0 ml. The reaction was started with the addition of 0.2 ml of hydrogen peroxide. Decrease in absorbance for a period of 30 s was measured at 290 nm in an UV–visible spectrophotometer. The enzyme activity was expressed as ascorbic acid oxidized/ min/g FW.

2.6. Analysis of the expression of the drought stress responsive genes

Leaf samples were harvested randomly from three plants of the DS mustard genotype exposed to normal water and water deficit stress conditions, for RNA isolation at 60 DAS. Samples were immediately frozen in liquid nitrogen and stored at −80 °C for isolating RNA.

2.6.1. Total RNA extraction, first cDNA synthesis and semi-quantitative PCR

Total RNA was extracted from the harvested leaf samples using Trizol reagent (Sigma-Aldrich) according to the manufacturer's instructions. First-strand cDNA was synthesized from 2 μg of DNase-treated total RNA by reverse transcriptase (Fermentas, USA) in 20 μl reaction volume using oligo (dT) primer following the manufacturer's instructions. The reaction was terminated by heating at 70 °C for 5 min and cDNA was stored at −20 °C. Once cDNA was synthesized, semi-quantitative RT-PCR was performed for all the primers listed in Table 1, using cDNAs diluted to a working concentration of 100 ng/μl.

Semi-quantitative RT-PCR was performed using a thermocycler (Applied Biosystems) in a 25 μl final volume including 2 μl of diluted cDNA template, 2.5 μl of 10X amplification buffer (Thermo Scientific, USA), 0.5 μl of 10 mM deoxyribonucleotide triphosphates (Thermo Scientific, USA), 0.5 μl of 10 pico-molar of each primer, and 0.2 μl (1 U) of dreamtaq DNA polymerase and 18.8 μl of PCR grade water. PCR steps included an initial denaturation at 94 °C for 4 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min with a final extension at 72 °C for 10 min. PCR products were separated using 1.2% agarose gels, stained with ethidium bromide and observed on a UV transilluminator. Experiments with all the primers were carried out at least three times.

Table 1
Sequence of the primers used for semi-quantitative PCR.

Gene*	Accession no.	Oligonucleotide sequence
DREB1-2	DQ208402	Forward: 5′AAACGAGCAGGGAGGAAGAA 3′
DREB1-2	DQ208402	Reverse: 5′ ACTGCCTCAACCATAGTCGT 3′
DREB2	EU924266	Forward: 5′AGGTAAGTGGGTGTGTGAGG 3′
DREB2	EU924266	Reverse: 5′ CAAGCCATGATCCTTCGTCG 3′
RD20	AT2G33380	Forward: 5′TGCCATCACCATTATTGCCG 3′
RD20	AT2G33380	Reverse: 5′TCTCGAGGTTAACTGGGACA 3′
RD26	AT4G27410	Forward: 5′ACCCACTCGAGCTGTACCCG 3′
RD26	AT4G27410	Reverse: 5′CTCGTAGCCATGGAAGCTCC 3′
HKG-Alpha Tubulin	NM100360	Forward: 5′ GCTGGGAGCTGTACTGTCTTG 3′
HKG-Alpha Tubulin	NM100360	Reverse: 5′CAACGGAGGTAGAGACCTGTG 3′

Note: * *DREB1-2* (Drought response element binding 1–2); *DREB2* (Drought response element binding 2); *RD20* (Response to dehydration 20); *RD26* (Response to dehydration 26).

2.7. Statistical analysis

Data generated were subjected to three factor analysis of variance (ANOVA) using the statistical software, http://14.139.232.166/opstat/default.asp. The means in all these analysis were separated using the least significant difference test at $P < 0.05$. Principle component analysis (PCA) of mixed data was done in 'R' using the package FactoMineR.

3. Results

3.1. Plant growth

Significant reduction in root and shoot dry weight was observed when mustard plants were exposed to water deficit stress (Fig. 1A and B). Inoculation with the osmotolerant *Bacillus* sp. strain MR D17 and *B. cereus* strain NA D7 significantly increased all these parameters under water deficit stress condition. Response to inoculation was markedly more in the DS mustard genotype. However, there was no significant increase in the root and shoot dry weight as compared to the inoculated treatments under normal water condition.

3.2. Plant physiological status

The effect of inoculation on mustard plant under water deficit stress condition was studied in terms of physiological and biochemical status of the plant. There was significant improvement in the plant physiological status under water deficit stress condition due to inoculation with the selected osmotolerant rhizobacterial *B. cereus* strain NA D7 and *Bacillus* sp. strain MR D17 (Table 2). Membrane stability and relative water content of the inoculated plants were observed to significantly improve under water deficit stress condition. There was also significant reduction in lipid peroxidation in the inoculated plants under water deficit stress condition as compared to the uninoculated plants. Response to inoculation under stressed condition was markedly better in the DS mustard genotype as compared to the DT genotype.

Inoculation with the rhizobacterial strains also considerably improved the plant biochemical parameters (Table 2). There was nearly 4 fold, 3 fold and 1.5 fold increases in the phenolics, proline and amino acid contents of the inoculated plants, respectively in the DS genotype under water deficit stress condition, while the inoculated plants in the DT genotype showed nearly 1.7 fold increase in only the phenolics content. However, the DT genotype did not show much increase in proline and amino acid contents due to inoculation under water deficit stress condition. There was more than four-fold increase in the sugar content of the inoculated plants in the DS genotype under water deficit stress condition, while the inoculated plants in the DT genotype showed approx. only 1.7 fold increase in the sugar content. There was more than 2.5 times decrease in the starch content of the inoculated plants in the DS genotype under water deficit stress condition, while the inoculated plants in the DT genotype did not show considerable change in their starch content or even an increase in starch content in case of *Bacillus* sp. strain MR D17 treated plants.

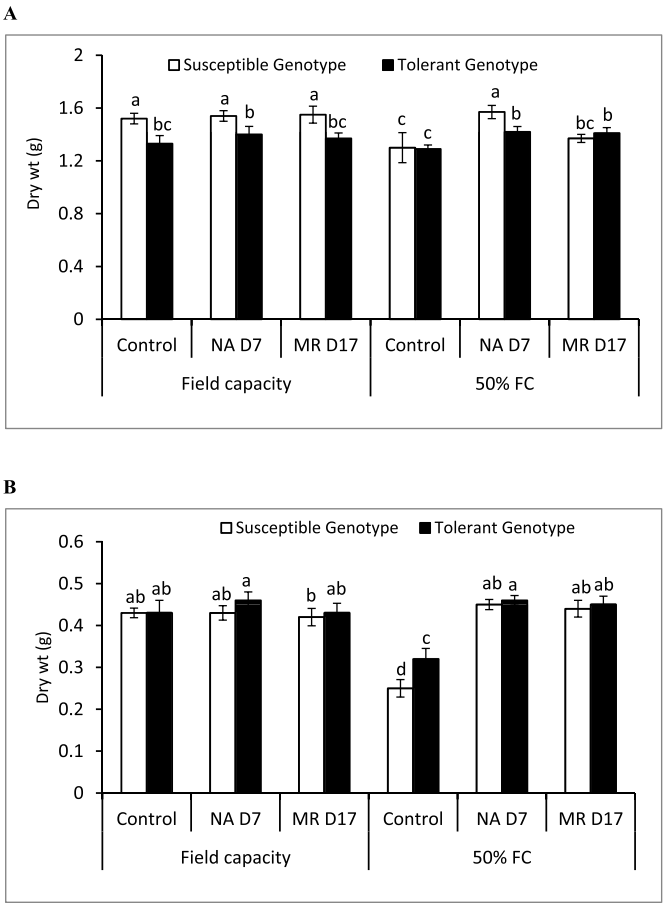

Fig. 1. Effect of inoculation with the osmotolerant *Bacillus cereus* strain NA D7 and *Bacillus* sp. strain MR D17 on plant growth under water deficit stress condition A) Shoot DW B) Root DW. Values are means of three replications. Error bars represent SE. Data followed by different letters are significantly different at $P < 0.05$.

3.3. Plant antioxidative enzymes and antioxidant status

Exposure to water deficit stress resulted in an increase in the catalase enzyme activity in the uninoculated control plants of the DS mustard genotype (Table 3). There was significant increase in the catalase, ascorbate peroxidase, glutathione reductase (GR) and superoxide dismutase (SOD) activities in the inoculated plants in the DS mustard genotype. However, in case of the DT genotype, only catalase enzyme activity increased in response to inoculation under water deficit stress condition. All the other enzyme activities *viz.* ascorbate peroxidase, glutathione reductase and superoxide dismutase in the inoculated and uninoculated treatment under water deficit stress condition were statistically at par. There was decrease in the accumulation of H_2O_2 in the inoculated plants under water deficit stress condition in the DS mustard genotype while under no stress condition it showed a variable response to inoculation. However, inoculation with these rhizobacteria did not affect the accumulation of H_2O_2 in the DT mustard genotype under water deficit stress condition. There was no effect of inoculation on the accumulation of ascorbic acid under no stress condition in the DS mustard genotype, while the DT genotype showed a variable response

Table 2
Effect of inoculation with the osmotolerant rhizobacteria on plant physiological and biochemical parameters.

Genotype		Treatments	MSI[b] (%)	RWC[c] (%)	Lipid peroxidation (nmoles of TBARS/g FW)	Phenolics (μg/g FW)	Proline (μmol/g FW)	Amino acid (mg/g FW)	Starch (mg/g FW)	Sugar (mg/g FW)
Susceptible genotype	FC[a]	Control	63.95 d	85.91 a	59.74 d	2.17 f	0.27 j	0.93 h	1.31 h	54.27 f
		NA D7	64.39 d	87.77 a	58.19 f	2.23 f	0.73 i	1.72 b	3.65 f	55.67 f
		MR D17	64.78 cd	86.61 a	59.03 e	2.65 e	0.75 i	1.42 ef	3.74 f	66.20 e
	50% FC	Control	58.34 f	74.42 c	74.32 a	1.53 g	0.85 h	1.15 g	4.27 e	38.20 g
		NA D7	68.94 a	87.08 a	52.39 i	7.10 a	2.76 b	1.91 a	1.38 h	177.07 a
		MR D17	68.94 a	88.68 a	62.42 c	6.20 b	3.29 a	1.49 c	1.65 g	155.07 b
Tolerant genotype	FC	Control	66.51 bc	78.76 bc	54.00 h	2.13 f	0.92 g	1.37 f	4.60 d	53.27 f
		NA D7	66.51 bc	79.20 bc	52.00 j	3.44 d	0.97 f	1.42 ef	4.87 c	106.00 c
		MR D17	67.54 ab	80.62 b	51.00 k	3.17 d	0.96 fg	1.43 de	4.81 cd	79.33 d
	50% FC	Control	61.06 e	75.18 c	64.00 b	2.49 ef	1.33 e	1.40 ef	5.41 b	62.13 ef
		NA D7	66.53 bc	77.78 bc	58.00 f	4.43 c	1.42 d	1.48 cd	5.62 b	110.80 c
		MR D17	66.64 b	76.44 bc	55.00 g	4.28 c	1.49 c	1.44 cde	5.86 a	102.53 c

Values represent mean of data of three replications; Different letters show significant difference ($P = 0.05$) in mean values.
[a] FC – Field capacity
[b] MSI – Membrane Stability Index.
[c] RWC – Relative Water Content.

to inoculation under similar condition. On exposure to water deficit stress condition, however, both the mustard genotypes showed a significant increase in the accumulation of ascorbic acid.

3.4. Principal component analysis

The differences between responses of the two genotypes to rhizobacterial inoculation, especially under water deficit stress conditions were also supported by the PCA analysis. As can be seen from individual factor map (Fig. 2A and B), that the response of the DS and the DT genotypes, at the two different moisture regimes, was varied with rhizobacterial inoculation. Under normal water condition, the DT genotype appeared more close to the bioinoculant *Bacillus* sp. strain MR D17, whereas under water deficit stress condition, the DS genotype was more close to *Bacillus* sp. strain MR D17 in the biplot, indicating differential response of the genotype to the bioinoculant with changing moisture level. The per cent contribution of different factors in principle component (PC) 1 and 2 differed with change in the soil moisture regime (Suppl. Fig. 1A and B; Suppl. Fig. 2A and B). Under normal water condition, the highest contribution in PC 1 was of catalase, glutathione reductase, proline and starch whereas under water deficit stress condition, total sugar (TS), phenolics and starch were the major contributors to variability in PC 1 (Suppl. Fig 1A and Suppl. Fig 3A). Similarly, the contribution of factors in PC 2 also varied under normal water condition (Suppl. Fig 1B) and under water deficit stress condition (Suppl. Fig 2B). Under normal water condition, the highest contribution was of rhizobacteria and ascorbate whereas, under water deficit stress condition, cultivar, glutathione reductase were the main contributors to the variability.

The overall covariance analysis of the qualitative variables revealed that the response to inoculation was more under water deficit stress condition as both the rhizobacterial strains (*Bacillus* sp. strain MR D17 and *B. cereus* strain NA D7) were positioned more towards water deficit stress (DR) co-ordinate. The DT genotype was spaced more towards normal water condition (FC) coordinate but the DS genotype appeared to be equally and oppositely spaced, probably due to the influence of inoculation and behaved differently under different moisture regimes (Suppl. Fig 3B). Among the quantitative variables, the contribution of root, shoot, SOD and GR was least compared to other variables (Suppl. Fig 3A). H_2O_2 production occupied relatively opposite co-ordinate with respect to other variables indicating a negative correlation of H_2O_2 production with other variables. The effect of genotype, moisture levels and rhizobacteria on different quantitative variables revealed there was distinct clustering of the DS genotype and the DT genotype, normal water condition and water deficit stress condition; while the effect of rhizobacteria was overlapping due to being correlated with each other in influencing the variables while uninoculated control formed a distinct cluster (Fig. 3). Figure in parenthesis represents the contribution of PC 1 (37.1%) and PC 2 (15.2%) to explain the variability in the data.

3.5. Expression of plant drought stress responsive genes

The effect of inoculation on the expression of drought stress responsive genes in the DS mustard genotype was determined since it was

Table 3
Effect of inoculation with the osmotolerant rhizobacteria on antioxidative enzymes activities and antioxidant status in the plant.

		Treatments	Catalase (μM H_2O_2 reduced/min/g FW)	Ascorbate peroxidase (μM ascorbic acid oxidized/min/g FW)	Glutathione reductase (μM of glutathione oxidized/min/g FW)	Superoxide dismutase (U/g FW)	H_2O_2 (μmol/g FW)	Ascorbic acid (nmoles/g FW)
Susceptible genotype	FC[a]	Control	1.904 g	0.680 d	4.96 a	1.381 def	5.46 def	696.73 d
		NA D7	2.163 f	0.760 bcd	3.37 g	1.639 bcd	4.44 g	750.83 d
		MR D17	2.224 f	0.907 ab	3.53 f	1.671 abc	5.56 cde	738.30 d
	50% FC	Control	2.249 f	0.722 cd	3.68 e	1.467 bcdef	5.98 bcd	517.60 f
		NA D7	2.980 a	0.864 abc	3.91 d	1.928 a	4.60 efg	1019.00 a
		MR D17	2.737 b	0.949 a	4.45 b	1.706 ab	5.89 bc	903.83 b
Tolerant genotype	FC	Control	2.404 e	0.720 cd	4.06 c	1.213 f	4.67 efg	599.03 e
		NA D7	2.512 de	0.728 cd	2.07 i	1.457 bcdef	4.50 fg	849.27 c
		MR D17	2.590 cd	0.740 bcd	2.22 h	1.412 cdef	6.53 abc	619.93 e
	50% FC	Control	2.500 de	0.748 bcd	3.37 g	1.346 ef	6.48 abc	573.83 ef
		NA D7	2.740 b	0.762 bcd	3.37 g	1.564 bcde	7.04 a	1041.67 a
		MR D17	2.670 bc	0.770 bcd	3.43 fg	1.499 bcde	6.62 a	940.67 b

[a] FC – Field capacity; Values represent mean of data of three replications; Different letters show significant difference ($P = 0.05$) in mean values.

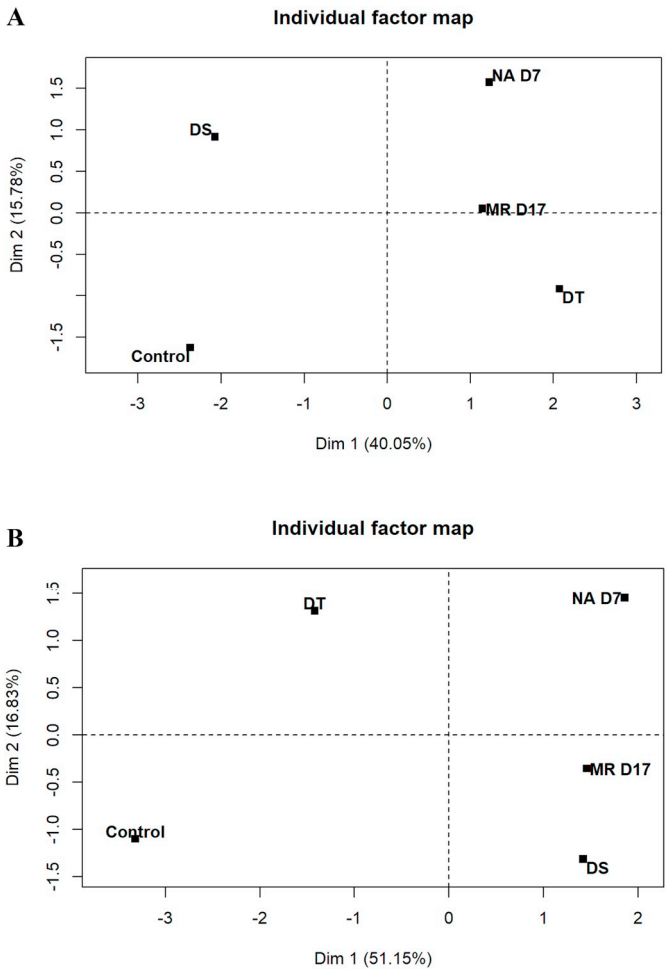

Fig. 2. Individual factor map A) at field capacity (normal water condition) B) 50% FC (water deficit stress condition).

more responsive to rhizobacterial inoculation. There was rhizobacteria mediated increased tolerance to water deficit stress. However, in an attempt to understand whether microbe-mediated increase in plant's tolerance under water deficit stress was through ABA-dependent pathway or ABA-independent pathway, expression analysis of two drought stress responsive genes *RD20* and *RD26* belonging to ABA-dependent pathway and two drought stress responsive genes *DREB2* and *DREB1-2* belonging to ABA-independent pathway was carried out, using *tubulin* as housekeeping gene (Fig. 4A and B; Suppl. Table 1). Densitometric analysis indicated that there was down-regulation of the expression of *DREB2* gene under water deficit stress condition (0.6 fold), however, inoculated plants showed enhanced expression of this gene (2.53 and 3.84 fold for *B. cereus* strain NA D7 and *Bacillus* sp. strain MR D17, respectively) under water deficit stress condition. Under normal water condition also both these rhizobacterial strains enhanced the expression of *DREB2* gene in the inoculated plants. Although, there was up-regulation of the expression of *DREB1-2* gene under water deficit stress condition (2.79 fold), inoculated plants showed considerable increase in the expression of this gene (16.2 and 18.78 fold for *B. cereus* strain NA D7 and *Bacillus* sp. strain MR D17, respectively) under water deficit stress condition. Under normal water condition also both these rhizobacterial strains considerably enhanced the expression of *DREB1-2* gene in the inoculated plants. The expression of *RD20* gene was enhanced in uninoculated plants grown under water deficit stress condition (2.59 fold). Inoculation by both the strains considerably enhanced the expression of this gene under normal water condition (2.16 and 3.09 fold for *B. cereus* strain NA D7 and *Bacillus* sp. strain MR D17, respectively). There was not much change in the expression of *RD20* gene in the inoculated plants as compared to uninoculated plants under water deficit stress condition. Up-regulation of *RD26* gene under water deficit stress condition (1.5 fold) was observed. However, there was enhanced expression of *RD26* gene in the inoculated plants under both normal and water deficit stress conditions. The enhanced expression was considerably more under normal water condition, for *B. cereus* strain NA D7 and *Bacillus* sp. strain MR D17 inoculated plants, as compared to under water deficit stress condition vis-à-vis their respective uninoculated controls. There was 1.52 and 1.87 fold increase in the expression of this gene in *B. cereus* strain NA D7 and *Bacillus* sp. strain MR D17 inoculated plants respectively, with respect to its uninoculated control under water deficit stress (Suppl. Table 1).

4. Discussion

Drought is known to greatly impact plant growth and reduce crop

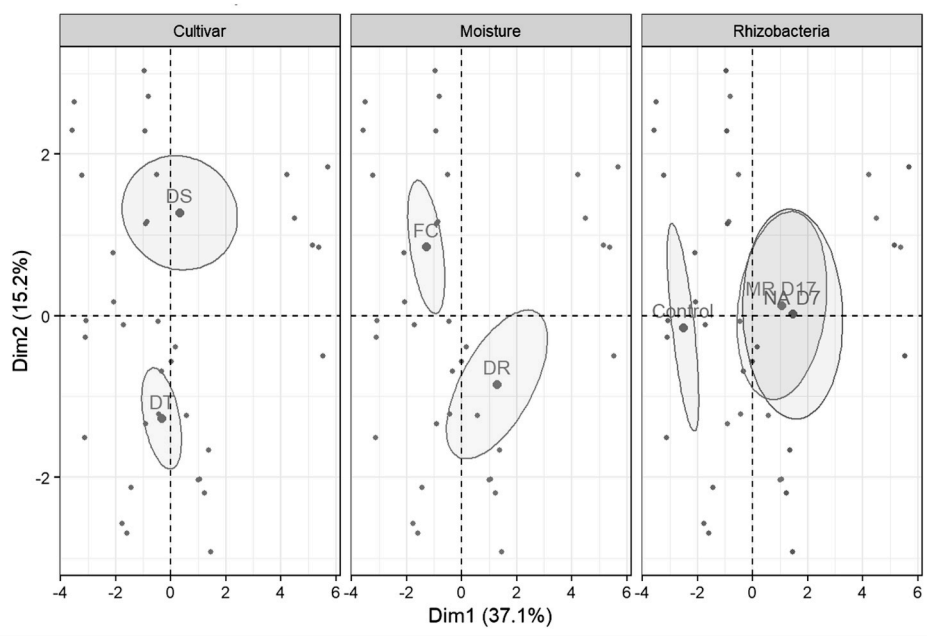

Fig. 3. Factor map of PCA analysis of different variables studied such as genotype, moisture level and rhizobacteria on biochemical and physiological parameters. The centre bigger bold dot represents the average value of all the parameters which differs in position in first two squares indicating the difference due to genotype and moisture condition on inoculation with rhizobacteria.

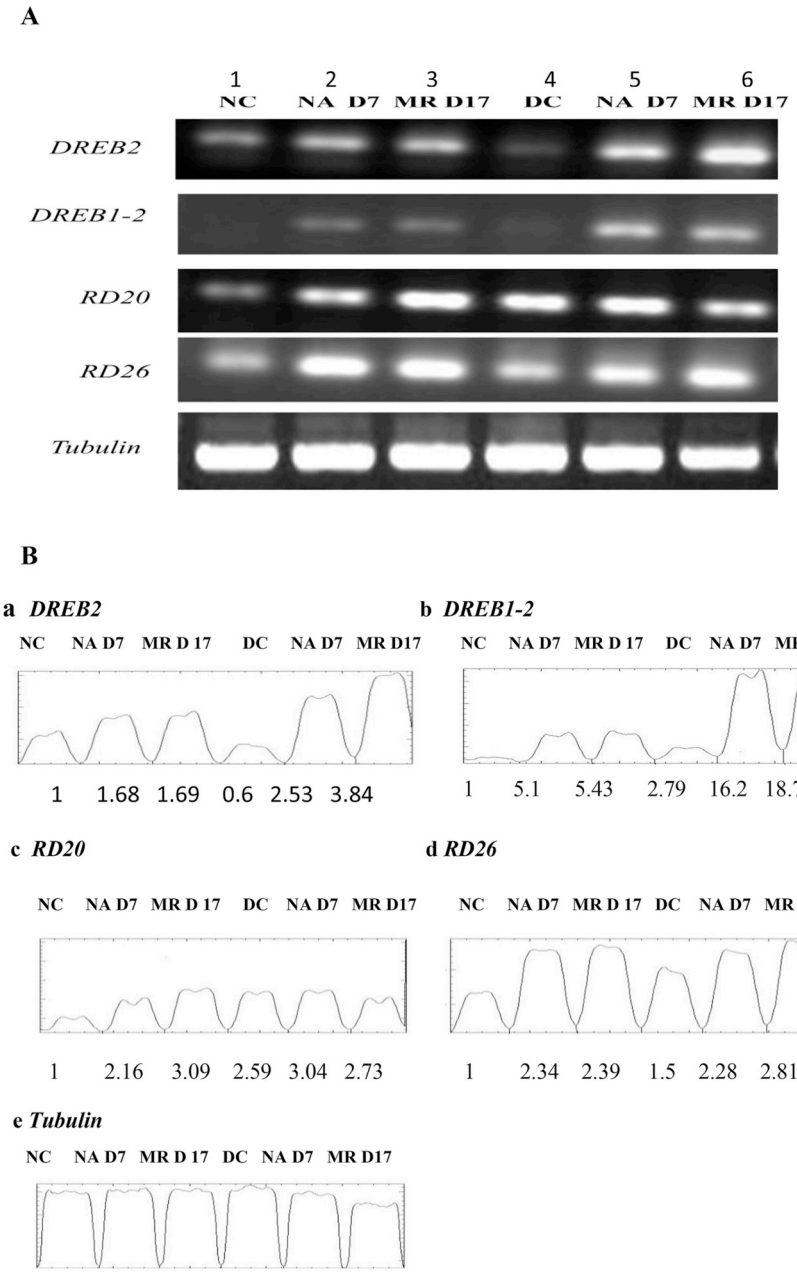

Fig. 4. A. Gel image of the semi-quantitative reverse transcriptase-polymerase chain reaction analysis (RT-PCR) for expression levels of the drought stress responsive genes in the mustard drought susceptible genotype Pusa Karishma LES-39. (1–3) Plants grown under normal water condition (1) uninoculated control under normal water condition (NC), (2) *Bacillus cereus* strain NA D7 (3) *Bacillus* sp. strain MR D17, (4–6) Plants grown under water deficit stress condition (4) uninoculated control under water deficit stress (DC), (5) *Bacillus cereus* strain NA D7 (6) *Bacillus* sp. strain MR D17. B. Relative expression levels of (a) *DREB2*, (b) *DREB1-2*, (c) *RD20*, (d) *RD26* and (e) *Tubulin* (internal control) Representative data from three independent experiments are shown. Numbers under the PCR products represent relative expression of the different genes. Relative gene expression was deduced from the band intensities of semi-quantitative RT-PCR amplicons using the ImageJ image analysis software (NIH, USA).

productivity. In the present investigation also, both root and shoot growth in mustard were considerably reduced on exposure to water deficit stress. The deleterious effect of water deficit stress on plant growth was significantly reduced due to inoculation with the selected rhizobacterial *Bacillus* sp. and *B. cereus* strains. The beneficial rhizobacteria have been reported to reduce the deleterious effects of drought to crop; improve plant growth, root biomass and yield. Abiotic stresses disturb the plant endogenous hormone homeostasis. Rhizobacteria are known to produce phytohormones such as IAA and gibberellins which help supplement the endogenous pool of plant hormones and thereby improve root and shoot growth (Egamberdieva et al., 2017). Rhizobacteria also help in reduction of ethylene levels, produced by the plants on exposure to abiotic stresses, through ACC deaminase activity, resulting in improved plant growth (Niu et al., 2017). The rhizobacteria used in the present investigation are known to produce phytohormones IAA and GA under osmotic stress conditions during the plant-endophytic association (Bandeppa et al., 2018). These rhizobacteria were also observed to reduce ethylene levels in the inoculated plants under osmotic stress conditions during the plant-endophytic association, *in vitro*. Presumably, these PGPR traits helped in improving plant root and shoot biomass on exposure to water deficit stress under pot culture conditions.

DS genotype was apparently more prone to the adverse effect of water deficit stress condition as evident from the marked reduction in the soluble sugars, phenolics and relative water content (RWC) in the uninoculated treatment in the DS genotype. Reduced water content probably had a detrimental effect on membrane integrity leading to the observed increase in lipid peroxidation. Lipid peroxidation is also associated with water deficit stress in plants (Mohammadi et al., 2018). Although, an increase in the contents of compatible solutes, proline and amino acids was noted, it was probably, for cellular osmotic adjustment (Iqbal, 2018). In contrast, the DT genotype did not show significant impact of water deficit stress and was observed to possess better physiological status than the DS genotype in the uninoculated treatment. The drought tolerant genotypes have been reported to possess an array of morphological, physiological and biochemical adaptations to endure

drought stress (Basu et al., 2016). There was no change in phenolics, amino acid, sugars and RWC in the DT genotype on exposure to water deficit stress. Silvente et al. (2012) did not observe much change in the RWC of the drought tolerant genotype of soybean on exposure to short term drought, which was in concurrence with our findings. Although, an increase in proline content was noted under water deficit stress condition, however, even under normal water condition, the DT genotype had higher proline content than the DS genotype probably by virtue of the inherent ability of the genotype and genetic modification brought during breeding of the genotype to make them thrive under stress condition irrespective of environmental cue for stresses. Omidi (2010) also reported higher proline content in the drought tolerant canola genotype in root and shoot tissues as compared to the drought susceptible genotype, under both no stress and drought stress conditions. Some impact of water deficit stress was observed in terms of increased lipid peroxidation and reduced membrane integrity. However, impact in terms of lipid peroxidation was not as severe as was observed in the DS genotype.

There was variation in response of both the DS and the DT genotypes to inoculation with the selected rhizobacterial strains under water deficit stress. The detrimental effects of water deficit stress on the DS genotype were mitigated due to inoculation with the selected rhizobacterial strains, leading to improved physiological and biochemical status of the treated plants. There was increase in membrane stability, RWC, phenolics, proline, amino acid and total sugar content. Similar results were also reported by other workers. Higher accumulation of compatible osmolytes has been reported in PGPR inoculated plants, which probably help in maintaining cell water status, membrane integrity and prevent protein denaturation under stress (Gagné-Bourque et al., 2016; Zhou et al., 2016; Mohammadi et al., 2018). Under drought stress, RWC was also considerably improved in *Bacillus megaterium* inoculated *Arabidopsis* plants (Zhou et al., 2016). The concomitant decrease in starch content presumably was due to its conversion to soluble sugars to maintain cell turgor as observed by Chaves et al. (2009). Inoculation of the DT genotype with the selected rhizobacterial strains also improved certain physiological and biochemical parameters of the treated plants under water deficit stress *viz.* membrane stability, phenolics and total sugar content; incremental increases in proline and amino acid content were also noted. However, the response to inoculation was not as high as was noted for the DS genotype. Inoculation did not improve RWC of the DT genotype. Moreover, there was change in the starch content of the DT genotype due to inoculation with only one rhizobacterial strain, under water deficit stress.

Impact of inoculation was also noted in terms of improved antioxidant status of the DS genotype, probably to mitigate the harmful effects of water deficit stress. There was enhancement of catalase (CAT), glutathione reductase (GR) and superoxide dismutase (SOD) enzyme activities and increase in antioxidant ascorbic acid content in the inoculated plants, under water deficit stress condition. An increase in the generation of ROS has been reported, under water deficit stress condition, which causes injury and thus, damages the cell membranes. Quan et al. (2004) observed a positive correlation between drought stress sensitivity and membrane damage. Plants have developed a complex antioxidant system to mitigate and repair damage caused by ROS (Fahad et al., 2017). Antioxidative enzymes like CAT, SOD, peroxidase (PRX), GR and ascorbate peroxidase (APX) are the most important components in the scavenging system of ROS. The beneficial effect of rhizobacteria on the plant's antioxidant system probably played a crucial role in improving growth of the inoculated plants of the DS genotype, on exposure to water deficit stress. Significant increases in the CAT, SOD, APX and glutathione peroxidase (GPX) activity in the leaves of water deficit stressed plants due to inoculation with PGPRs has been widely documented (Heidari and Golpayegani, 2012; Gusain et al., 2015). Mohammadi et al. (2018) reported lower H_2O_2 and MDA levels in the *Pseudomonas fluorescens* PF-135 inoculated chamomile plants under water deficit stress. In comparison, there was an increase in only CAT enzyme activity and ascorbic acid content in the inoculated plants in the DT genotype, under water deficit stress condition. No changes in the activity of other antioxidative enzymes *viz.* GR, APX and SOD were noted.

Thus, the mechanism of rhizobacteria for alleviation of drought stress in the DS genotype appeared to be through enhanced accumulation of osmolytes proline, amino acids and sugars leading to better osmotic adjustment and improved RWC in the leaf tissues. Along with this there was also simultaneous up-regulation of the activities of antioxidative enzymes CAT, GR and SOD and accumulation of antioxidants ascorbic acid and phenolics which led to detoxification of the generated ROS. This led to the observed improved membrane stability and reduced lipid peroxidation. In contrast, the DT genotype possessing an inbuilt high osmolyte accumulation trait and ability to maintain higher RWC, under both no stress and water deficit stress condition did not show similar pattern of response, as the DS genotype, to rhizobacterial inoculation. Probably due to higher tolerance of the DT genotype, there was very less negative impact of water deficit stress. Hence, inoculation led to markedly lower enhancement of the physiological and biochemical parameters; and improvement of very few antioxidant attributes involved in providing protection against water deficit stress. Thus, overall the DS genotype was observed to be more responsive to rhizobacterial inoculation than the DT genotype. In individual factor map, PCA analysis of the data also indicated differential response of the two genotypes to rhizobacterial inoculation. There was greater variability in their response under water deficit stress. This was probably due to tolerance of the DT genotype to water deficit stress; and higher response of the DS genotype to rhizobacterial inoculation under water deficit stress.

PGPRs have been reported to induce changes in the expression of drought stress responsive genes. Timmusk and Wagner (1999) were the first to report induction of drought stress responsive *ERD15* gene due to inoculation with *Paenibacillus polymyxa*. Analysis of the expression of drought stress responsive genes involved in ABA-independent (*DREB1-2* and *DREB2*) and ABA-dependant (*RD20* and *RD26*) pathways were carried out in the present study. The DS genotype was selected for these studies since this genotype was observed to be more responsive to rhizobacterial inoculation. It was observed that rhizobacterial inoculation modulated the expression of all these four genes. The expression of *DREB1-2* and *DREB2* genes in the inoculated plants was considerably enhanced under both the water conditions, although higher expression was observed under water deficit stress condition. This indicated that there was bacteria-mediated up-regulation of the expression of these genes under both the water conditions. *DREB*s (dehydration responsive element binding) are important transcriptional factors involved in the regulation of abiotic stress responsive genes in ABA-independent manner (Shinozaki and Yamaguchi-Shinozaki, 2007). *DREB*2 and *DREB1* are transcription factors for initiation of transcription of *LEA* (late embryogenesis abundant) genes. *DREB* transcription factors and *DRE* (dehydration responsive element) element serve in the signal transduction under conditions of drought, salinity and cold stress and can control the expression of several target functional genes involved in enhancing plant's tolerance to these abiotic stresses (Shinozaki and Yamaguchi-Shinozaki, 2007). It is interesting to note that the expression of the transcriptional factors was significantly enhanced under normal water condition as compared to untreated control plants, indicating priming of the plant for drought tolerance. However, rhizobacteria-mediated enhancement of expression of these genes under water deficit stress was many folds higher as compared to treated plants under normal water condition. This indicated that there was bacteria-mediated conferring of drought tolerance to the plant through marked up-regulation of the expression of transcription factors involved in ABA-independent stress tolerance pathway.

Expression of drought stress responsive *RD20* (response to dehydration20) and *RD26* (response to dehydration26) genes is dependent on ABA signalling. *RD20* is a stress inducible caleosin which plays a role

in drought tolerance by regulating stomatal opening (Aubert et al., 2010). *RD26* is a transcription factor involved in up-regulation of ABA-inducible genes (Fujita et al., 2004), especially those involved in the regulation of *GlyI* (Glyoxylase) gene. Glyoxylase catalyzes glutathione dependent detoxification of ROS. The expression of both these was enhanced under normal water condition, indicating priming of the plant for drought stress tolerance, under no stress condition. A plant which has been primed for drought tolerance is able to respond faster to drought stress as compared to a plant which has not been primed. However, inoculation led to only an incremental increase in the expression of *RD20* gene as compared to uninoculated treatment, under water deficit stress condition. This indicated that there was limited influence of inoculation on the expression of this gene, under stressed condition. In case of *RD26* gene also, inoculation led to higher induction of the gene under normal water condition. Although, under water deficit stress condition also rhizobacteria-mediated up-regulation of *RD26* gene was observed, as compared to untreated control plants. However, the expression level of these genes in the inoculated plants under normal water condition and water deficit stress condition was comparable, though other ABA-dependent biochemical parameters like proline accumulation considerably increased under water deficit stress condition, indicating simultaneous operation of both ABA-dependent and ABA-independent pathways. Proline accumulation is partially regulated by ABA, but ABA applied in the absence of stress is insufficient to induce high levels of proline (Sharma and Verslues, 2010). Also, marked up-regulation of the expression of transcription factors *DREB2A* and *DREB1-2* indicated the key role played by ABA-independent pathway in conferring water deficit stress tolerance to the inoculated mustard plants in the DS genotype. Some ABA-dependent proteins were reported to play important roles in the regulation of the drought response by interacting with the ABA-independent proteins DREB2A, DREB1A and DREB2C (Liu et al., 2018). Thus, in *Bacillus*-priming for drought stress tolerance in the DS mustard genotype, under water deficit stress, along with the ABA-dependent changes induced in the inoculated plant, ABA-independent pathway played a major role.

In conclusion, it was observed that the DT genotype showed a different pattern of physiological and biochemical response to rhizobacterial inoculation as compared to the DS genotype, under water deficit stress condition. Moreover, where the two genotypes showed similar response, the response to rhizobacterial inoculation was markedly stronger in the DS genotype as compared to the DT genotype. This was presumably because the DT genotype has its own inbuilt mechanism for evading/overcoming water deficit stress. Thus, it was not solely dependent on rhizobacteria-mediated mechanism for alleviation of water deficit stress, hence it's response to inoculation with rhizobacteria was also not very prominent; while the DS genotype was solely dependent on rhizobacteria-mediated mechanism for alleviation of water deficit stress, thus it's response to inoculation with rhizobacteria was markedly stronger. Semi-quantitative PCR analysis studies revealed that under water deficit stress condition, in the *Bacillus*-mediated priming for drought stress tolerance in the DS genotype ABA-independent pathway probably played a key role in enhancing tolerance to drought stress.

Contribution

Bandeppa S., Chandrashekar N., Deepika Kumar Umesh, A.D. Asha were responsible for designing and conducting of experiments. Bandeppa S., Chetana Aggarwal, Sangeeta Paul and Jyoti Kumar Thakur were responsible for analysis and interpretation of data. Bandeppa S., Sangeeta Paul and Jyoti Kumar Thakur were responsible for preparation of manuscript. Bandeppa S. and Sangeeta Paul were responsible for planning of experiments.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.08.018.

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