



Application of *Trichoderma viride* and *Bacillus subtilis* Leads to Changes in Antioxidant Enzymes, Proline, and Lipid Peroxidation under Salinity Stress in Chickpea (*Cicer arietinum* L.)

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Authors' contributions

This work was carried out in collaboration between both authors. both authors read and approved the final manuscript.

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ABSTRACT

The study was carried out to evaluate the influence of application of *Trichoderma viride* and *Bacillus subtilis* on antioxidant enzymes, proline and lipid peroxidation to decrease the impact of salinity stress on chickpea (*Cicer arietinum* L.), a salinity sensitive crop. A pot experiment was conducted with contrasting set of genotypes (tolerant vs. sensitive) under salinity stress compared to control soil conditions in completely randomized design with three replications. Microbial inoculation was done through seed priming and application to soil at 20 days after sowing (DAS). Content of antioxidant enzymes, proline, and lipid peroxidation were assessed in leaves at flowering stage. Results showed that antioxidant enzymes viz., catalase, peroxidase, and superoxide dismutase were significantly increased under salinity stress compared to control condition and they were further increased with application of microbes either as seed priming alone or in combination with soil application at 20 DAS in both the genotypes under saline as well as control conditions. The content of lipid peroxidation increased significantly under salinity stress, and it was stronger pronounced in sensitive genotype while the lipid peroxidation content was decreased by application of microbes. Proline content increased under salinity stress, and it was

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further enhanced by the microbial inoculation. The study thus conclusively proved that *Bacillus subtilis* and *Trichoderma viride* positively increased content of antioxidant enzymes, proline, and lipid peroxidation in leaves of chickpea grown under salinity stress conditions. The best microbe species was *Trichoderma viride* as seed priming plus soil application. This can be an important additional approach to decrease the impact of salinity stress on chickpea crop.

Keywords: Chickpea; salinity stress; antioxidant; *Bacillus subtilis*; *Trichoderma viride*.

1. INTRODUCTION

Chickpea (*Cicer arietinum* L.) is popular crop grown under a wide range of climatic conditions but it is highly sensitive to salinity stress which causes yield loss of 8 to 10% globally [1]. Soil salinity is one of the major inevitable problems in arid and semi-arid regions where evapotranspiration exceeds precipitation, affecting about 80 million ha of arable land [1]. Salinity adversely affects several morphological features and physiological processes leading to reduction in growth, decrease in water status, chlorophyll, ion balance, photosynthesis, nodulation, nitrogen fixation etc., and increase in reactive oxygen species (ROS), lipid peroxidation thus leading to membrane dysfunction [1,2]. Research reports suggest that salinity affects growth and development of plants by developing oxidative, osmotic stresses and ionic imbalances. Naturally, reactive oxygen species are expeditiously scavenged by antioxidative systems, but this elimination is diminished under salt stress [3]. Proline and carbohydrates are accumulated in plant tissue under saline stress, and these substances contribute to osmotic adjustment enhancing salt tolerance. As a protective system to control the levels of ROS, plants have evolved a complex series of enzymes including superoxide dismutase (SOD), peroxidase (PER) and catalase (CAT) along with non-enzymatic antioxidants like ascorbates and glutathione that detoxify lipid peroxidation products. Proline has been reported to counteract growth inhibition induced by salinity, for example in rapeseed [4] and rice [5]. Salinity tolerance is therefore considered to be an extremely complex phenomenon in majority of plant species, since there are several mechanisms at cellular, tissue, organ, or whole plant levels.

It is of utmost significance to develop strategies to enhance plants' salt tolerance in agricultural production. Beneficial soil microbes are known to improve soil-water-plant relations through intricate pathways and subtle signaling cues which are yet to be scrutinized [6]. *Trichoderma* spp. are known to be an endophytic plant

symbiont widely used as biofertilizer for stimulation of plant growth and also as a biocontrol agent against various plant diseases [7]. *Trichoderma* strains are capable of amplifying tolerance of plant to biotic and abiotic stresses [8], through promotion of root growth, nutritional uptake and by increasing some of enzymatic antioxidant defence system (SOD, CAT and PER) thus inducing protection against oxidative damage. Salinity negatively impacts the bacterial diversity associated with crops. However, *Bacillus* spp. due to their great metabolic/genomic background and spore formation, have shown high resilience to abiotic stress enhancing the tolerance of plants to saline soils. *Bacillus* strains have been reported to influence plant growth, nutrient uptake and yield of crops under saline conditions [9,10]. These bacteria can also enhance the activity of ROS-scavenging enzymatic antioxidants such as SOD, PER, CAT, glutathione reductase and non-enzymatic antioxidants like ascorbic acid and glutathione [11]. Hence, as an different and additional approach to decrease the impact of salinity stress on chickpea plants, this study was carried out to know influence of application of *Trichoderma viride* and *Bacillus subtilis* on content of antioxidant enzymes, proline, and lipid peroxidation in contrasting genotypes grown under salinity stress compared to control conditions.

2. MATERIALS AND METHODS

2.1 Plant Material and Experimental Conditions

Twenty-two chickpea genotypes of desi group, obtained from ICAR- Indian Institute of Pulse Research, Kanpur, India were initially screened under in vitro conditions in Petri dishes at 0.0, 4.0, 6.0 and 8.0 dSm⁻¹ salinity levels and based on morpho-physiological traits viz., germination percent, root length, shoot length, shoot dry weight, root dry weight and vigour index, as recorded in 8-day old seedlings (data not shown), contrasting set of genotypes were identified. One salinity tolerant (BG 212) and one

salinity sensitive (BPM) chickpea genotype was selected to study physio-biochemical responses of microbial application under saline (4.8 dSm⁻¹) and non-saline/ control (1.2 dSm⁻¹) conditions.

Sterilization of seeds was done with 0.1 percent mercuric chloride solution for two minutes followed by thorough washing with distilled water prior to sowing. The salt solution was prepared by taking NaCl: CaCl₂: Na₂SO₄ in the ratio of 7:3:1 (w/v) and electrical conductivity of different salinity levels (4.0, 6.0 and 8.0 dSm⁻¹) were measured by Conductivity Meter (Systronics Model-303). These solutions were used to impose different salinity stress at seed germination.

The identified tolerant and sensitive genotypes were sown in pots in two-factor completely randomized design with three replications (one replication consisted of one pot having five seedlings). There were five treatments viz., seed priming with *Bacillus subtilis* [B(SP)], seed priming with *Trichoderma viride* [TR(SP)], seed priming with *Bacillus subtilis* + its soil application at 20 DAS [B(SP+SA)], seed priming with *Trichoderma viride* + its soil application at 20 DAS [TR(SP+SA)] and the control, each under saline and non-saline (control) conditions. The seeds were sterilized by dipping in 1% sodium hypochlorite solution for 10 minutes, then washed with distilled water and air-dried. After that the seeds were primed with respective treatments. Seeds were sown in plantation pots (diameter×height=250×250 mm) filled with field soil having salinity 1.2 dSm⁻¹ EC (control), and 4.8 dSm⁻¹ EC (saline soil). Soils of higher salinity was obtained from naturally occurring salt affected lands (*usar* lands) with EC varying from 8 to 9 dSm⁻¹, and then leaching it to desired level of salinity level for pot experiment. Soil salinity was measured as electrical conductivity (EC) of a 1:5 soil: distilled water suspension following 1 h of mixing by Conductivity Meter (Systronics Model-303). The salinity level (4.8 dSm⁻¹) was maintained by the method of Hardie and Doyle [12] at 15 days interval. After germination five plants per pot were maintained. Pots were irrigated as per the requirement, to retain field capacity and salinity levels.

2.2 Application of Microbial Inoculants

Talc formulation of *Trichoderma viride* (Strain NRCL-T-1) was obtained from ICAR- National Research Centre on Litchi, Muzaffarpur, Bihar and liquid formulation of *Bacillus subtilis* was

obtained from the Department of Microbiology, College of Basic Sciences & Humanities, RPCAU, Pusa, Bihar. Seed priming with *Trichoderma viride* was done at the rate of 10 g kg⁻¹ seed while soil application was done at the rate of 10 g per pot (1.0 kg *Trichoderma* formulation was mixed in 100 kg compost and distributed in 100 pots @ 1 kg mixture per pot filling only the top 8-10 cm portion of pot). Similarly, seed priming with *Bacillus subtilis* was done at the rate of 4.0 mL kg⁻¹ seed (4.0 mL formulation mixed with 1.0 L water) and soil application @ 10 mL per pot (1.0 kg *Bacillus* formulation was mixed in 100 kg compost and distributed in 100 pots @ 1 kg mixture per pot filling only the top 8-10 cm portion of pot).

2.3 Assay for Antioxidant Enzymes Activity and Proline Content

The observations for activities of enzymes catalase, peroxidase, superoxide dismutase, lipid peroxidation and proline content were recorded at 50 DAS during flowering in leaf samples. For the extraction of enzyme, 0.5 g of fresh leaf sample (taken from fully expanded young leaves from top of plant) was triturated in 3 mL of pre-chilled extraction buffer (0.1 M phosphate buffer, pH 6.7) followed by centrifugation at 10000× g for ten minutes. The supernatant was collected and used for the assays of enzymatic activities. All steps in the preparation of the enzyme extract were carried out at 4 °C [13].

Catalase activity was determined by consumption of H₂O₂ using the method of Dhindsa et al. [14]. Peroxidase activity was determined spectrophotometrically (UV-Vis Spectrophotometer Tometl model NV 230) using the method of Amako et al. [15] and Salama et al. [16]. SOD activity was determined by measuring its ability to initiate photochemical reduction of Nitro blue tetrazolium (NBT) according to the method of Giannopolitis and Ries [17]. The amount of lipid peroxidation was determined in terms of malondialdehyde (MDA) content, a product of lipid peroxidation measured by thiobarbituric acid reaction [18].

Proline content was determined in dried leaf material, according to Bates et al. [19]. Extraction of proline was done by homogenizing 0.1 g of leaf sample in 10 mL of 3% sulpho salicylic acid. The reaction mixture was centrifuged for 10 minutes at 10000 × g. Supernatant was collected for the estimation of proline. The quantity of proline was calculated using standard curve which was prepared by taking 10-50 µg proline

from the stock solution (10 mg/ 100 mL) of L-Proline dissolved in water.

2.4 Statistical Analysis

Data of three separate replications were reported as the mean ± SD. The data were subjected to analysis of variance (ANOVA) using statistical computing software SAS version 9.2 software (SAS Institute, Cary, NC, USA). The F value, least significant differences (LSD) between means at 5% level of significance (P = 0.05) and the standard error (SE) of means were calculated. Microsoft Excel program was used to present the figures.

3. RESULTS AND DISCUSSION

3.1 Catalase Activity

Catalase activity in leaves of chickpea at flowering was positively influenced by the application of microbes both under non-saline and saline soils conditions (Fig. 1). Application of *Trichoderma* (SP+SA) led to greatest increase in catalase activity compared to other treatments

and the control, increase being more in tolerant genotype (BG 212) under control (non-saline) soil (21.44%) than saline soil (18.95%) conditions. Catalase activity notably increased in the order of treatment, *Bacillus* (SP), *Trichoderma* (SP) and *Bacillus* (SP+SA) in soil without NaCl in the genotypes BG 212. Similar trend was observed in the genotype BPM, highest increase being with treatment *Trichoderma* (SP+SA) (9.68%) followed by *Bacillus* (SP+SA), *Trichoderma* (SP) and *Bacillus* (SP) under control condition while the increase was 9.15, 8.60, 3.07, and 2.39 % respectively under saline soil conditions. The data of the present study revealed that, catalase activity was enhanced in the chickpea genotypes when exposed to salinity stress which corroborates with the results of Latef and Chaoxing [20] in tomato and Khomari and Davari [21] in soybean. Catalase is responsible for scavenging of H₂O₂ [22]. Mittal et al. [23] reported that increased catalase activity of mustard contributed to high salinity tolerance. Higher increase was also confirmed in the *Trichoderma* treated plants under salt stress condition by Khomari and Davari [21].

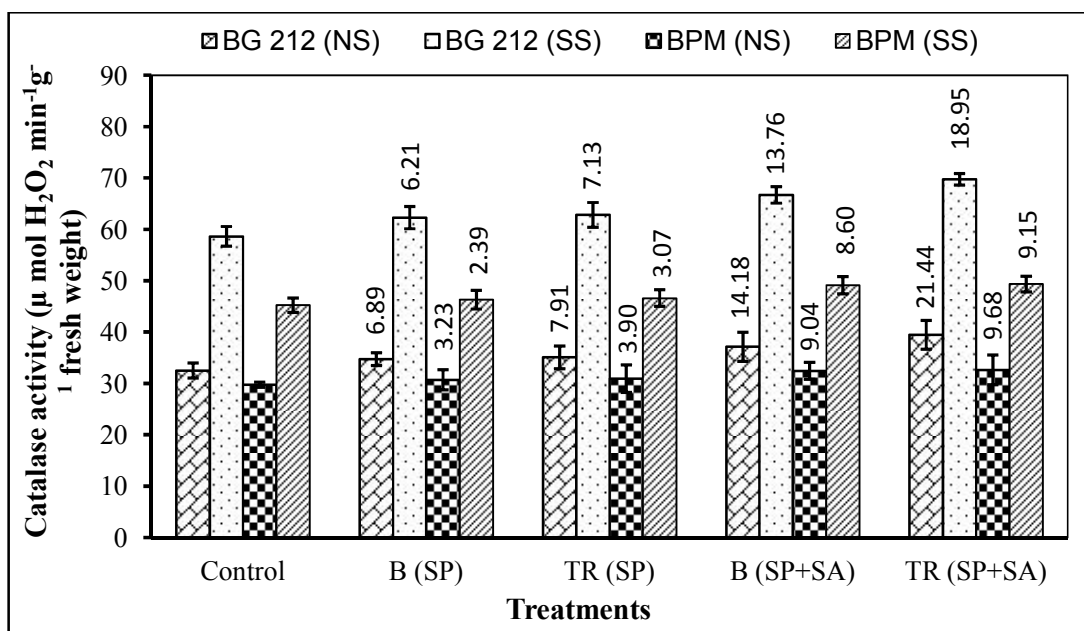


Fig. 1. Effect of application of microbes on catalase activity in leaves of two chickpea genotypes (BG 212, BPM) at flowering stage under control (NS) and saline soil (SS) conditions; B= Bacillus, SP= Seed priming, TR=Trichoderma, SA= Soil application. The vertical bar indicates standard error (SE) of the mean whereas the values above the bars indicate percent increase over control. The least significant difference (LSD) at P=0.05 for 'soil conditions', 'treatments' and interaction 'soil conditions×treatments' are 1.03, 1.45, and 1.59, respectively for the genotype BG 212, and 1.45, 2.29, and non-significant, respectively for the genotype BPM

3.2 Peroxidase (PER) Activity

The results showed that exposure of plants to salt stress triggered increase of peroxidase activity in both tolerant and sensitive genotypes, and application of microbes resulted in further increase in peroxidase in leaves at flowering stage, increase being more in saline soil than control soil conditions (Fig. 2). The maximum value of peroxidase activity (5.38 units mg⁻¹ fresh weight) was recorded in the treatment with *Trichoderma* (SP+SA) followed by *Bacillus* (SP+SA), *Trichoderma* (SP) and *Bacillus* (SP) in the salinity-tolerant genotypes BG 212 grown in saline soil. Similar trend of result was observed in the salinity sensitive genotype BPM. Data revealed that the interaction effect of soil conditions and microbial treatments was significant at flowering stage in both genotypes.

Similar findings were reported by Ahmad et al. [24] in mustard and Ferreira et al. [25] in maize who observed an increase in peroxidase production when plants were exposed to salt stress. The greater activity of peroxidase in tolerant than in sensitive genotype indicates relatively higher salt tolerance with high capacity to decompose H₂O₂ generated by superoxide dismutase, thereby providing protection against oxidative stress. Acceleration of peroxidase activity in chickpea plants by *B. subtilis* could be attributable to enhanced lignin biosynthesis and other associated protective compounds in order to reduce oxidative stress [11]. Application of *Trichoderma harzianum* to salinity treated mustard plants led to increase in peroxidase showing the defensive nature of *Trichoderma* on mustard seedlings under NaCl stress [24].

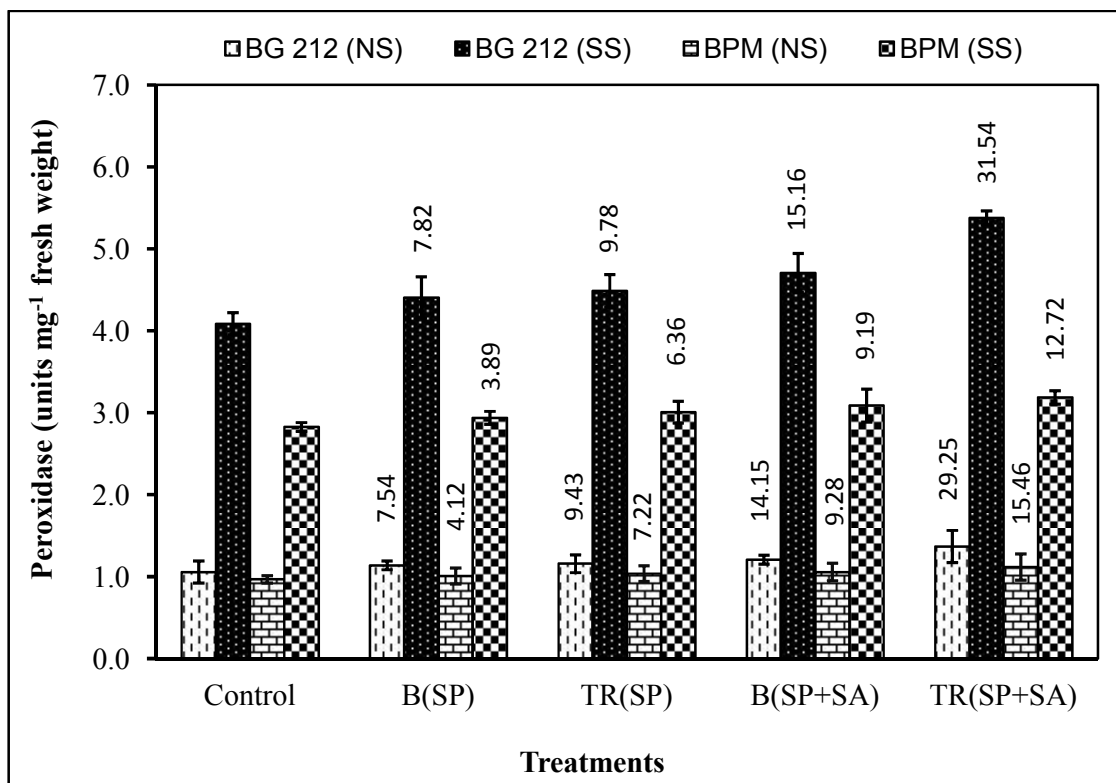


Fig. 2. Effect of application of microbes on peroxidase activity in leaves of two chickpea genotypes (BG 212, BPM) at flowering stage under control (NS) and saline soil (SS) conditions; B= Bacillus, SP= Seed priming, TR=Trichoderma, SA= Soil application. The vertical bar indicates standard error (SE) of the mean whereas the values above the bars indicate percent increase over control. The least significant difference (LSD) at P=0.05 for 'soil conditions', 'treatments' and interaction 'soil conditions×treatments' are 0.15, 0.24, and non-significant, respectively for the genotype BG 212, and 0.09, 0.14, and non-significant, respectively for the genotype BPM

3.3 Superoxide Dismutase (SOD) Activity

An enhanced specific activity of superoxide dismutase (SOD) was observed when plants were exposed to salinity stress. It increased from 24.68 to 56.37 and 24.18 to 36.41 mg⁻¹ protein in leaves of the chickpea genotype BG 212 and BPM, respectively in control which further increased with all the microbial application (Fig. 3). The maximum value reached up to 70.12 mg⁻¹ protein in the treatment Trichoderma (SP+SA) in the salinity tolerant genotype BG 212 and 42.83 mg⁻¹ protein in the genotype BPM. This was followed by Bacillus (SP+SA), Trichoderma (SP) and Bacillus (SP) treatments. It was evident that inoculation of microbes further improved the activity of SOD in both the genotypes with the increase being the maximum in case of tolerant genotype BG 212. The percent increase was in the decreasing order of treatments viz., T(SP+SA) 28.28% and 19.60%; B(SP+SA) 24.88% and 14.81%; T(SP) 15.19% and 9.64%, and B(SP) 14.30% and 6.66% under control soil in the genotypes BG 212 and BPM, respectively. Similar trend was observed under saline soil in

both the genotypes. SOD is the primary defence enzyme against ROS. In chloroplast, mitochondria, cytoplasm, apoplast and peroxisome, O₂ is transformed to H₂O₂ by SOD [22]. The results of the current study indicated that activity of SOD increased with increasing salinity (Fig. 3), which corroborates the findings of Latef and Chaoxing [20] in tomato, Ahmad et al. [24] in mustard, and Khomari and Davari [21] in soybean. Inoculation of *B. subtilis* in chickpea might have ameliorated the salt stress by reducing the amount of ROS along with regulating genes involved in the production of antioxidants and absorption of nutrients as well [26]. While NaCl therapy has stimulated the development of antioxidant enzymes, inoculation of *B. subtilis* further activated the antioxidant mechanism, resulting in accelerated removal of toxic ROS [11]. Application of *Trichoderma harzianum* further increased the SOD activity in saline condition over the control [24]. Khomari and Davari [21] reported that salinity levels as well as *Trichoderma* isolate promoted the activity of SOD in soybean seedlings.

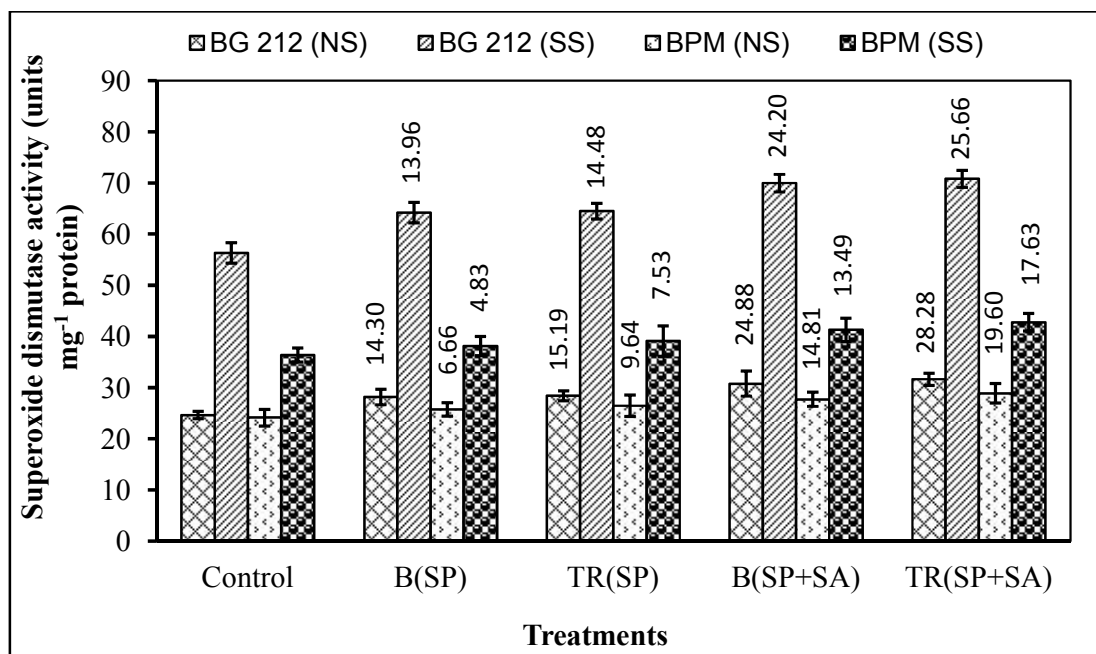


Fig. 3. Effect of application of microbes on superoxide dismutase activity in leaves of two chickpea genotypes (BG 212, BPM) at flowering stage under control (NS) and saline soil (SS) conditions; B= Bacillus, SP= Seed priming, TR=Trichoderma, SA= Soil application. The vertical bar indicates standard error (SE) of the mean whereas the values above the bars indicate percent increase over control. The least significant difference (LSD) at P=0.05 for 'soil conditions', 'treatments' and interaction 'soil conditions×treatments' are 1.87, 1.95, and non-significant, respectively for the genotype BG 212, and 1.52, 1.41, and non-significant, respectively for the genotype BPM

3.4 Lipid Peroxidation

Exposure of chickpea plants to saline environment led to enhanced lipid peroxidation in both genotypes, however the rise being more prominent in sensitive genotype BPM (Fig. 4). Lipid peroxidation value under saline soil in the control of both tolerant and sensitive genotypes were 19.14 and 27.64 nmol MDA g⁻¹ fresh weight, respectively while under control soil, the values were 13.48 and 14.93 nmol MDA g⁻¹ fresh weight, respectively. Application of microbes reduced lipid peroxidation in the leaves of chickpea genotypes under both control as well as saline conditions. The minimum amount of MDA content 7.93 and 10.57 nmol MDA g⁻¹ fresh weight under control condition in the genotype BG 212 and BPM, respectively with application of Trichoderma (SP+SA). This was followed by Bacillus (SP+SA), Trichoderma (SP) and Bacillus (SP) treatments. Assessment of level of lipid peroxide serve as a dependable indicator of oxidative damage caused due to abiotic stress [26]. Salinity affects the polyunsaturated fatty acid composition, leading to membrane

dysfunction [27]. Abd-Allah et al. [11] reported that salinity stress resulted in enhanced production of hydrogen peroxide, which led to lipid peroxidation and ultimately affected membrane structural integrity. Abd-Allah et al. [11] suggested that *B. subtilis* is expected to regulate the membrane activities by maintaining the optimal ratio of polyunsaturated to saturated fatty acids in addition to limiting the production of harmful free radicals, like hydroxyl and H₂O₂. Excess generation of reactive oxygen species triggers the peroxidation of unsaturated lipid components, causing the loss of membrane integrity along with leakage and desiccation. In the present study, biopriming with *Trichoderma* as well as bio-priming+soil application decreased lipid peroxidation by reducing the content of MDA in both chickpea genotypes which is in close conformity with findings in many other crops like tomato [26], soybean [21], and maize [28]. *Trichoderma* also induces expression of stress related proteins like glutathione S-transferase (GST), glutathione dependent formaldehyde dehydrogenase and peroxidase, which could reduce the MDA content [24].

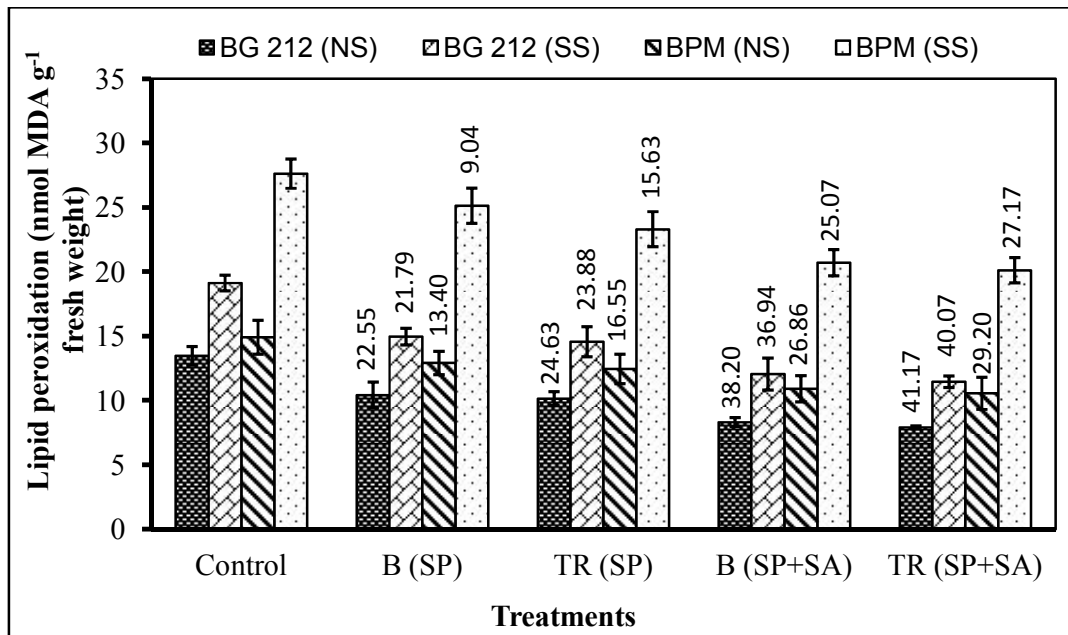


Fig. 4. Effect of application of microbes on lipid peroxidation in leaves of two chickpea genotypes (BG 212, BPM) at flowering stage under control (NS) and saline soil (SS) conditions; B= Bacillus, SP= Seed priming, TR=Trichoderma, SA= Soil application. The vertical bar indicates standard error (SE) of the mean whereas the values above the bars indicate percent decrease over control. The least significant difference (LSD) at P=0.05 for ‘soil condition’, ‘treatments’ and interaction ‘soil conditions× treatments’ are 0.69, 1.10, and non-significant, respectively for the genotype BG 212, and 1.48, 1.70, and non-significant, respectively for the genotype BPM

3.5 Proline Content

Proline content in leaves increased significantly in response to salinity stress in both tolerant and sensitive genotypes. The chickpea genotype BG 212 showed the maximum accumulation of proline which increased from 0.76 mg g⁻¹ dry weight under control condition to 1.27 mg g⁻¹ dry weight in saline condition. In the genotype BPM it enhanced from 0.69 mg g⁻¹ dry weight to 1.08 mg g⁻¹ dry weight respectively in control and saline conditions (Fig. 5). The results pertaining to the effect of different microbial applications on proline accumulation in leaves of chickpea genotypes at flowering showed increased values in all the microbial treatments, the maximum being in the treatment Trichoderma (SP+SA). This was followed by Bacillus (SP+SA), Trichoderma (SP) and Bacillus (SP) treatments. Trend was similar in both control and saline conditions. Further, the data revealed that the interaction effect of different microbial treatments and soil conditions was statistically significant at flowering in salinity-tolerant genotype whereas it was non-significant in salinity sensitive genotype. When exposed to salinity, several plants naturally tend to accumulate high levels of

osmolytes which include proline that enables plants to maintain an osmotic balance even under low water potential [29]. In the present study, the data revealed that proline content increased when plants were exposed to salinity stress which corroborates the findings of Rawat et al. [30] in wheat and Qurashi and Sabri [31] in chickpea. Inoculation with microbes improved levels of proline accumulation as compared to un-inoculated control in control as well as salinity stressed environments [11]. Under salt stress environments proline has a key role in the maintenance of water balance in cells and can help scavenge ROS protecting proteins and other important bio-molecular structures thus ameliorating the toxic effects of salinity [11]. Accumulation of proline induced by *B. subtilis* could be a result of altered expression of proline-metabolizing enzymes, in addition to up-regulation of biosynthetic along with down-regulation of degradative enzymes [32]. Proline has antioxidant properties and hence scavenges ROS in addition to its prominent role in energy storage under saline soil conditions. Rawat et al. [30] had also reported more accumulation of proline in wheat plants inoculated with *Trichoderma harzianum* under salt stress.

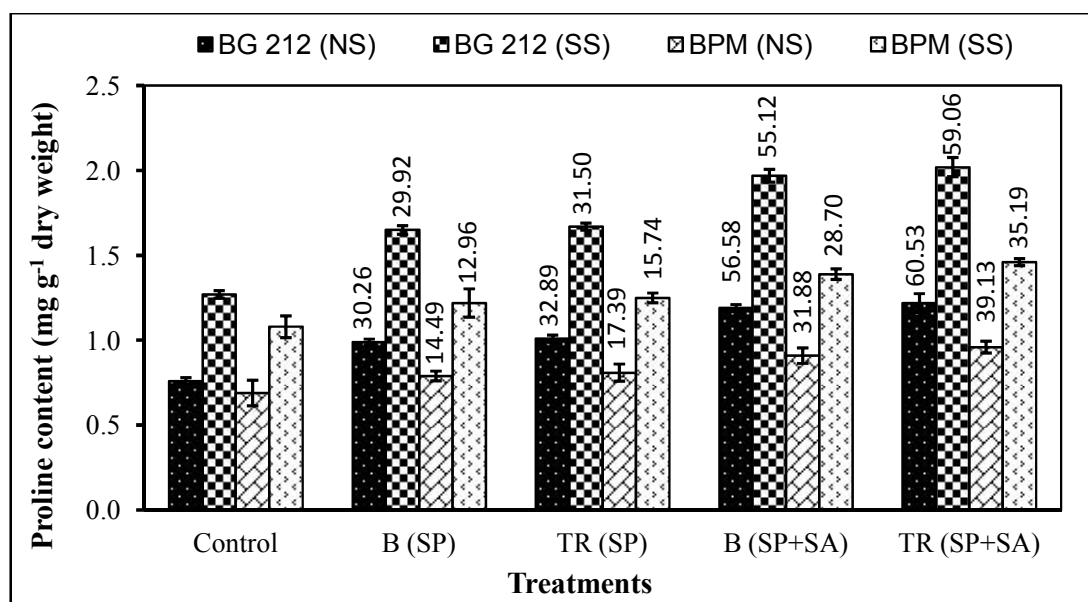


Fig. 5. Effect of application of microbes on proline content in leaves of two chickpea genotypes (BG 212, BPM) at flowering stage under control (NS) and saline soil (SS) conditions; B= Bacillus, SP= Seed priming, TR=Trichoderma, SA= Soil application. The vertical bar indicates standard error (SE) of the mean whereas the values above the bars indicate percent increase over control. The least significant difference (LSD) at P=0.05 for ‘soil condition’, ‘treatments’ and interaction ‘soil conditions×treatments’ are 0.02, 0.04, and 0.06, respectively for the genotype BG 212, and 0.03, 0.05, and non-significant, respectively for the genotype BPM



Fig. 6. Effect of application of microbes on growth of chickpea grown in saline soil. Left to right: A. *Trichoderma viride* (Seed priming + Soil application 20 DAS), B. *Bacillus subtilis* (Seed priming + Soil application 20 DAS), C. Seed priming with *Trichoderma viride*, D. Seed priming with *Bacillus subtilis*, and E. Control

4. CONCLUSION

The biochemical parameters *viz.*, antioxidant enzymes, lipid peroxidation and proline were adversely affected under salinity stress but were changed under saline as well as control conditions with the application of microbes (*Bacillus subtilis* and *Trichoderma viride*) as seed priming alone or seed priming in combination with soil application at 20 DAS, the best being *Trichoderma viride* as seed priming plus soil application (Fig. 6). The study conclusively proved efficacy of microbes as additional approach to decrease the impact of salinity stress on chickpea crop.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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