**Bacillus megaterium** strain A12 ameliorates salinity stress in tomato plants through multiple mechanisms

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1. Introduction

According to an estimate, nearly 7% of the land is affected by salinity (Ruizlozano et al. 2012; Abdel-Ghani et al. 2015). Abiotic stresses inhibit plant growth and cause considerable yield reduction (Baniasadi et al. 2015; Khan et al. 2018). Salinity stress may cause >30% losses in crop plants in saline-affected fields (Ashraf and PjC 2004). The area of salinity affected agricultural land is increasing because of natural processes and conventional agricultural practices (Berger et al. 2012). High levels of salinity adversely affect plant growth and development (Hashem et al. 2016). Salinity causes osmotic and ionic stresses to limit plant growth (Flowers and Colmer 2008). Additionally, high salinity manifests an oxidative stress inside plant body, resulting in malfunctioning of photosynthesis and ion homeostasis to retard plant growth (Porcel and Ruiz-Lozano 2012; Alqarawi, Hashem, et al. 2014; Rahneshan et al. 2018).

Abiotic stresses affect photosynthesis in plants (Ozakca 2013). The changes in photosynthesis process are associated with distresses in carbon and nitrogen assimilation pathways (Tejera et al. 2004). These stress cause reduction of the electron transport chain leading to photo-oxidation (Grbić and Bleecker 1995; Balota et al. 2004). Furthermore, abiotic stresses can damage the PSII protein system of the photosynthetic machinery, that is an unavoidable process (Gururani, Mohanta, et al. 2015; Gururani, Venkatesh, et al. 2015). Plants have developed the process to recover the damaged PSII through a specific repairing system (Melis 1999; Murata et al. 2007). Reactive oxygen species (ROS) are key deleterious products hindering plant metabolism under stress conditions. It has been proved that ROS hinder the PSII repairing mechanism by obstructing the formation of the D1 protein of PSII system encoded by PsbA gene (Nishiyama et al. 2011; Gururani, Venkatesh and Tran 2015; Yu et al. 2015). Secondly, the increased accumulation of ROS causes oxidative damage and affects the integrity of the cell (Ahmad et al. 2010). ROS accumulation in leaves causes the oxidation of certain molecules and ultimately programmed cell death (Pang and Wang 2008).

Plants cope with a suite of biotic and abiotic stress factors in natural habitats (Nguyen et al. 2016). Plants employ self-defense mechanisms to prevent oxidative damages under salinity stress. The hyper activation of antioxidant systems and increased production of compatible osmolytes are included among several defensive strategies used by plants against abiotic stress conditions (Vardharajula et al. 2011; Alqarawi, Allah, et al. 2014). The enzymatic and non-enzymatic components work together to neutralize toxic ROS in antioxidant systems of plants. Osmolytes like free proline, sugars and amino acids help to sustain the water level to regulate cellular metabolism and functionality (Hossain et al. 2015). Recently, the plants respond to abiotic stress through changes in some major metabolic pathways such as photosynthesis, TCA cycle and (Lewis et al. 2001; Rai 2002; Lotfi et al. 2010). Different authors have contributed equally.

**ABSTRACT**

Plants must cope with the stress conditions to survive. Plant growth promoting rhizobacteria can improve plant growth either directly or indirectly under stress conditions. However, the possible mechanisms remain unclear. Here we report that *Bacillus megaterium* strain A12 (BMA12) maintains hormonal and redox homeostasis and restores the photosynthetic efficacy of tomato plants through multiple mechanisms to survive under salinity stress conditions. Tomato plants were co-cultivated with BMA12 under saline conditions. The application of BMA12 significantly increased plant growth and photosynthetic capacity. BMA12 decreased production of ROS and ethylene but increased expression levels of selected genes responsible for repairing of damaged photosynthetic apparatus and maintenance of redox homeostasis. Furthermore, BMA12 significantly altered metabolic profile to restore perturbations of tomato plant physiology impaired with salinity stress. This study proves that BMA12 can be used in the conventional agriculture system in the salinity-affected fields.
metabolites may participate in plant stress tolerance. However, the knowledge regarding salinity tolerance-related metabolomics is limited. Metabolomic analyses can help to determine the specific responses of plant physiological systems to cope environmental stresses (Oliver et al. 2011). A variety of small signaling molecules modulate molecular responses of plants against abiotic stresses through complicated networks (Pieterse et al. 2012; Vleesschauwer et al. 2014). Phytohormones play a key role in controlling certain molecular mechanisms inside plant body and hence optimize plant responses against abiotic stresses (Nguyen et al. 2016). Abscisic acid (ABA) is involved in many developmental processes like growth inhibition, stomatal conductance and primary root growth (Liang et al. 2014). This hormone is an important controlling factor of plant responses to different environmental stresses as salinity (Sharp and Lenoble 2002; Agata and Iwona 2013).

Previous studies have suggested that beneficial soil microbes including plant growth promoting rhizobacteria (PGPR) rescue plant growth and yield under stress conditions (Berg 2009; Cho et al. 2015; Abd_Allah et al. 2018). Some important roles of PGPR include bio-fertilization, restriction of pathogen growth and induction of abiotic stresses tolerance in plants (Adesemoye et al. 2008; Bhattacharyya and Jha 2012; Jing et al. 2018). PGPR can promote plant growth by both directly and indirectly mechanisms. The direct mechanisms included root growth promotion, rhizoremediation and stress mitigation (Vaishnav et al. 2016; Jha and Subramanian 2018). In addition to that, PGPR modulate physiological processes through perturbation of metabolism of plants (Illangumaran and Smith 2017). It is important to know how PGPR repair osmotic homeostasis and photosynthesis process after onset of salinity stress in plants and what inducible mechanisms make plants to survive salinity stress in the presence of these beneficial microbes. In our previous research, B. megaterium strain A12 (BMA12) stimulated the growth of tomato plants under salinized conditions (Aslam et al. 2018). The objectives of this study were to understand the responses, adaptation and tolerance of salinity stress in tomato plants at physiological, molecular and biochemical levels under influence of BMA12. In this research work, the possible adaptations are also described made by tomato plants to alter osmotic homeostasis and improve photosynthesis under stress conditions mediated by this beneficial bacterium.

2. Materials and methods
2.1. Strains and culture condition

The pure B. megaterium strain A12 (BMA12) culture was procured from the conservatory of the Plant Biotechnology laboratory, Institute of Agricultural Sciences, University of the Punjab, Lahore, Pakistan, and grown in a nutrient agar medium overnight on a rotating shaker (200 rpm) at 30°C.

2.2. Plant materials and bacterial inoculation

Tomato seeds (L. esculantum cv. RioGrande) were purchased from commercial seed market. The seeds were surface sterilized using standard sodium hypochlorite method. Following the sterilization, the seeds were germinated in plastic pots of 6-inch diameter filled with sterilized commercial potting mix. After germination, one healthy seedling was left in each pot. As in our previous study, the plant’s growth was the most retarded at 200 mM NaCl (Aslam et al. 2018). Therefore this concentration was used for experiments. Plants were treated after 10 days of emergence. Treatment details are as follow: Control = 100 mL of distilled sterilized water to serve as non-treated control. T1 = 100 mL of 200 mM aqueous NaCl solution to act as salinity control. T2 = 100 mL of aqueous BMA12 formulation (1 × 10⁷ colony forming units mL⁻¹) to serve as bacterial control, T3 = 100 mL of 200 mM NaCl and 100 mL of aqueous formulation of BMA12 (1 × 10⁷ colony forming units mL⁻¹). The pots were irrigated with distilled sterilized water when needed. Each experiment was repeated twice with five replicate plants of each treatment. After ten days of treatment applications, the plants were analyzed for growth attributes and rest of the analyses.

2.3. Leaf pigment analysis

Young leaves of tomato plants were excised from plants after ten days of treatment applications. The leaf material was powdered in liquid nitrogen and extracted in 30 mL of solution (1:1 (v:v) acetone and ethanol) overnight at 30°C. Chlorophyll a, b and carotenoid contents in the leaves were quantified by the spectrophotometric method as described by (Kaźmierzak 1998).

2.4. Photosynthetic rate measurements

The fully expanded leaves of tomato plants from each treatment were selected for photosynthetic parameters measurements using LI-6400 system (Li-Cor Inc., Lincoln, NE, USA). The samples were illuminated with the saturated photosynthetic photon flux density (PPFD) with the help of a light-emitting diode (LED) light source for half hour prior to measurements for full induction of the photosynthesis process. Afterwards, the net photosynthetic rate (Pn), transpiration rate (Tr), and stomatal conductance (Gs) were measured simultaneously. All parameters for measurement were adopted as described by (Chen et al. 2010). Intrinsic water use efficiency (iWUE) was calculated from the ratio of Pn and Tr.

2.5. Analysis of changes in soluble sugars, free amino acids, soluble protein contents and some related metabolites

Tomato leaf material was grinded as fine powdered in liquid nitrogen and (0.1 g) was extracted with 80% (w/v) ethanol at 80°C. This extract was used to determine total soluble sugars, sucrose, and free amino acid. Total soluble sugar contents were measured adopting the anthrone reagent method (Turakainen and Hartikainen Hseppanen 2004). Five milliliters of anthrone sulfuric acid solution (75% v:v) was added to 0.1 mL of supernatant previously prepared. This mixture was warmed up to 90°C for 20 min and cooled in water bath in cold water. OD was taken at 620 nm. Free amino acid contents were determined using ninhydrin reagent (Moore and Stein 1954). One milliliter of acetate buffer (pH = 5.4), 1 ml chromogenic agent and 1 mL of extraction material were mixed thoroughly and heated in boiling water bath for twenty
minutes. The solution was cooled, 3 mL ethanol (60%, v/v) was further added and OD was taken at 510 nm. Lastly, soluble protein contents were quantified using method of (Kriger 1988).

2.6. Quantifications of enzymatic and non-enzymatic antioxidants

For quantification of enzymatic antioxidants, plants protein was extracted in protein extraction buffer (50 mM Tris–HCl buffer (pH7.0) containing 3 mM MgCl2, 1 mM EDTA) as described by (Kang et al. 2014). Here ascorbate (APX) activity was determined by measuring the oxidation of ascorbic acid substrate at 290 nm as suggested by Nakano and Asada (1981) and expressed as mol ASA min\(^{-1}\) substrate at 290 nm as suggested by Nakano and Asada (1981) and expressed as mol ASA min\(^{-1}\). Superoxide dismutase (SOD) activity was measured by monitoring the photoreduction of nitroblue tetrazolium (NBT) at 560 nm, as advised by Beyer and Fridovich (1987). One unit of SOD was defined as the amount of enzyme that caused a 50% decrease of the SOD-inhibited NBT reduction. Catalase (CAT) activity was determined as advised by Azevedo et al. (1998). For that purpose, the initial rate of decrease in ascorbate concentration caused by the consumption of H\(_2\)O\(_2\) was measured at 240 nm and expressed as mm H\(_2\)O\(_2\) min\(^{-1}\). Peroxidase (POD) activity was based on the determination of guaiacol oxidation at 470 nm caused by H\(_2\)O\(_2\) as suggested by Putter (1974) and expressed as µmol oxidized guaiacol. Polyphenoloxidase (PPO) activity was quantified by measuring the rate of increase in absorbance at 410 nm in the presence of 0.1 M catechol substrate as suggested by Halpin and Lee (1987).

To measure changes in quantities of non-enzymatic antioxidants, plant leaf samples were homogenized in 5% trichloro acetic acid and clear supernatant was collected by centrifugation at 12000 rpm for 15 min at 4°C. Ascorbate (ASC) and dehydro-ascorbate (DHA) were quantified using methodology of (Arrigoni et al. 1992). Reduced glutathione (GSH) and oxidized glutathione (GSSH) contents were estimated by method of (Chevone and Hess 1992).

2.7. H\(_2\)O\(_2\) quantification

Hydrogen peroxide was extracted by snap freezing 100 mg of plant material in liquid nitrogen (Veljovic-Jovanovic et al. 2002) Plant material was taken from the top, middle and bottom part of tomato plants and homogenized in 1.5 mL of 1 M HClO\(_4\). Phenolic compounds were removed by using insoluble polyvinylpyrrolidone. This mixture was centrifuged at 12000 x g for 15 min at 4°C. H\(_2\)O\(_2\) contents were quantified as described by Cheeseman (2006). Briefly, 60 µL of this material was mixed with 600 µL of eFOX reagents (250 Mm ferrous ammonium sulfate, 100 µM sorbitol, 100 µM xylenol orange, and 1% ethanol in 25 mM H\(_2\)SO\(_4\)). OD was taken at 550 and 800 nm and the difference was recorded. H\(_2\)O\(_2\) quantity was estimated using a standard H\(_2\)O\(_2\) curve.

2.8. Analysis of metabolomic perturbations and phytohormones quantification by UPLC-ESI MS/MS

This analysis was performed to observe perturbations in important physiological processes and assessment of changes in quantities of some important liquid phytohormones. For that purpose, UPLC-ESI MS/MS analysis was performed to simultaneously quantify phytohormones and some other plant metabolites as described by (Yu et al. 2015).

To observe metabolic perturbations, leaf material was grinded to fine powder in liquid nitrogen. This powdered material was mixed in pure methanol following sonication for 5 min. The solution was passed through cellulose filters (0.2 µm pore size). Whereas, optimized sample preparation strategy was used for phytohormones quantification as described by (Yu et al. 2015). Afterwards 0.2 µL of prepared samples were injected into ‘UPLC/ESI-Qtof/ MS’ instrument separately. The chromatographic separation was performed on a Waters ACQUITY UPLC I-class system (Waters Corporation, Dublin, Ireland) fitted with Waters ACQUITY UPLC BEH C18 column. The composition of mobile phases and details of flow rate parameters can be seen in (Molina-Calle et al. 2017). The chromatography system was coupled with electrospray ionization (ESI) to a Waters Xevo Qtof-MS, operating in full scan mode. The parameters of ESI source and mass spectrometer were exactly followed as described by (Vieira et al. 2017). Cinnamic acid was used as an internal standard for phytohormones quantifications, as suggested by (Yu et al. 2015).

MzMine version 2.30 (mzmine.github.io) was used for both qualitative and quantitative analysis of UPLC-ESI MS/MS data regarding phytohormones and other metabolites. The alignment was carried out as a function of retention time, using a tolerance window of 0.2 min and 10 ppm mass accuracy (Molina-Calle et al. 2017). Metabolites were identified by matching mass spectra with mass spectral libraries (NIST and Wiley) and using online database MassBank (http://www.massbank.jp/). Metabolites were considered identified with a spectral match factor higher than 800.

2.9. Analysis of changes in ethylene production

Ethylene quantification was performed by gas chromatography as described by (Yasin et al. 2018). Briefly, freshly removed leaf pieces were placed in 1 mL of water in falcon tube. The tube was immediately covered with a gas-proof septum and placed in dark at 30°C for 4 h. Afterwards, 1 mL gas was withdrawn using Hamilton gastight syringe and injected inside a gas chromatograph for ethylene quantification.

2.10. RNA extraction and qRT-PCR analysis

qRT-PCR analysis was performed to analyze changes in transcriptome levels of some selected genes involved in photosynthesis system, maintenance of redox homeostasis and stress related processes. Total RNA was isolated from leaves using the TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Quantitative RT–PCR (qRT–PCR) was performed using the SYBR green based qRT–PCR Kit (TaKaRa, Dalian, China). Details of primers are given in the supplementary (Table 1). The details of the qRT-PCR process can be seen in (Hu et al. 2016). The reactions were carried out in triplicate of each treatment. Actin gene was used as internal standard.
2.11. Statistical analysis

All the experiments were repeated twice with five technical replicates. One-way ANOVA was performed using the DSAASTAT software (Onofri, Italy). The significant differences between different treatments were determined through Duncan’s new multiple range test (DNMRT) test.

3. Results

3.1. Resistance to salt stress

Inoculating tomato plants with halotolerant BMA12 allowed for an evaluation of the ameliorative effect on plants against the salinity stress. The growth indices attained by the plants in either conditions are shown in Table 1. Plants cultivated with B. megaterium A12 grew to a significantly greater extent in comparison to the plants that were raised without bacterium (Table 1). Fresh and dry weights accumulation was significantly greater in bacterially treated plants under either condition (Table 1). Plant growth was inhibited in the absence of bacteria in salinized growth media (Table 1). It was seen that the plants cultivated with BMA12 and salinity stress accumulated fresh and dry weights to nearly similar as non-inoculated control plants raised in the absence of salinity stress (Table 1).

3.2. Photosynthetic pigment analysis

The chlorophyll (Chl a, Chl b) and carotenoid contents in leaves of tomato plants were increased with BMA12 treatment (Figure 1). The leaf Chl a and Chl b contents were higher by 38.1 and 21.5% respectively in tomato plants inoculated with BMA12 under salinized conditions (Figure 1). Changes in the carotenoid contents in salinized tomato plants showed a same trend and increased by 32.1% with BMA12 symbiosis compared to the control plants (Figure 1). This beneficial bacterium also increased chlorophyll and carotenoids contents of tomato plants under non-salinized conditions compared to the control plants (Figure 1).

3.3. Leaf gas exchange and photochemistry

The photosynthetic rate, transpiration rate, stomatal conductance and water use efficacy in tomato plants were greatly affected by BMA12, under both salinized and normal growth media (Table 2). Compared to the plants under salinity stress alone, these parameters were increased from 19% to 43% in tomato plants inoculated with BMA12 (Table 2). Salinity stress significantly affected the photosynthetic rate in tomato plants. Bacterial inoculated tomato plants showed significantly higher photosynthetic rate even after being salinized (Table 2). This bacterium demonstrated high recovery potential for photosynthetic rate as compared to transpiration rate and stomatal conductance and led to higher water use efficiency (photosynthetic rate/stomatal conductance) compared to the salinized control plants (Table 2).

When the effects on transpiration rate were evaluated, the salinity stress reduced the transpiration rate in tomato plants.

| Parameters                  | Control (With salinity) | BMA12 (With salinity) | Control (Without salinity) | BMA12 (Without salinity) |
|-----------------------------|-------------------------|-----------------------|----------------------------|--------------------------|
| Plant height (cm)           | 21.36 ± 1.32D           | 29.34 ± 1.70D         | 33.85 ± 1.25D              | 41.58 ± 2.79D            |
| Root length (cm)            | 09.58 ± 05.36D          | 13.87 ± 0.83D         | 19.27 ± 0.73D              | 26.47 ± 1.07D            |
| Fresh biomass (g)           | 23.87 ± 1.07D           | 38.57 ± 2.50D         | 57.68 ± 3.29D              | 74.05 ± 5.32D            |
| Dry biomass (g)             | 01.98 ± 0.28D           | 02.82 ± 0.19D         | 04.33 ± 0.26D              | 6.07 ± 0.44D             |
| Leaf area (cm²)             | 11.28 ± 0.93D           | 13.56 ± 1.61D         | 17.23 ± 0.99D              | 21.58 ± 1.91D            |

Note: Data presented here are mean values of replicates of same treatment. Values with ± represents standard error. Capital letters represents level of significance as governed by ANOVA and DNMRT at = p ≥ 0.05.
The treatment of BMA12 modulated the redox status under saline conditions (Table 3). Salinity stress alone also changed the concentrations of these enzymes but the effects were more evident in the presence of BMA12 (Table 3).

Similarly, the levels of non-enzymatic antioxidants were significantly changed in BMA12 treated tomato plants compared to their respective control plants grown without BMA12 (Table 2). The symbiosis of BMA12 increased water use efficiency by 23.7% in tomato plants grown under salinity condition as compared to the plants raised under salinity stress alone (Table 2).

### 3.4. Changes in redox homeostasis

The treatment of BMA12 modulated the redox status under salinity stress. BMA12 treated tomato plants were differed in the pool of the antioxidant enzyme system during salinity stress (Table 2). These differences were more evident in SOD, APX, and PPO activities. The activities were increased up to 2.5, 1.3 and 2.8 folds in leaves of tomato plants co-cultivated with BMA12 than with control plants under salinized conditions (Table 3). Salinity stress alone also changed the concentrations of these enzymes but the effects were more evident in the presence of BMA12 (Table 3).

Similarly, the levels of non-enzymatic antioxidants were significantly changed in BMA12 treated tomato plants under stress conditions in a similar manner to that antioxidant enzymes (Table 4). The comparison of quantities of these non-enzymatic antioxidants showed varying trends. The total quantities of DHA (15.8%), GSH (49.5%) and GSSG (37.9%) were higher in the BMA12 treated plants under salinity stress conditions compared to the plants grown without BMA12 under salinity stress conditions (Table 4). However, the reduced ascorbate contents were higher in salinized tomato plants in the absence of BMA12 (Table 4). The composition of these non-enzymatic antioxidants was slightly varied with salinity alone, whereas major increases were induced by BMA12 (Table 4).

### 3.5. Phytohormones profile

The concentrations of ABA, GA, SA and ethylene were significantly changed in tomato plants after salt and BMA12 treatment compared with the control condition (Figure 2). The well-known stress response hormones ethylene and ABA increased significantly (2.1 and 1.7 folds) in tomato plants under salinity stress. The ethylene quantity was decreased (1.8 folds) after BMA12 treatment in comparison to the salinity control plants (Figure 2). In contrast, the quantities of IAA (1.2 folds), ABA (1.6 folds), salicylic acid (1.2 folds) and GA4 (2.3 folds) were increased in tomato plants receiving BMA12 as compared to the plants grown under salinity stress alone (Figure 2).

### 3.6. Changes in expression levels of photosynthesis and stress related genes

The transcriptome levels of selected genes of the photosynthesis process (PsbA, PBGD, Chlase), redox regulation (TrxI, Trx2, Trx m1/2) and stress related (SOS1, APX1, LERBOH1) genes in tomato plants were analyzed...
after 07 days of treatments application. Regarding photosynthesis related genes, the salinity stress significantly decreased expression levels of PsbA and PBGD genes as compared to the control plants (Figure 3). When tomato plants treated with NaCl along with BMA12, the expression of both genes was up-regulated to a level significantly higher than the salinity control plants (Figure 3). In the same way, NaCl increased expression level of Chlase gene (Figure 3). However, BMA12 was unable to have significant effects on expression levels of this gene (Figure 3).

In the same way, symbiosis of BMA12 showed altered expression of redox regulation and stress related genes in tomato plants (Figure 3). Interestingly, the exposure of salinity stress also increased Trxf, Trxm2 and Trm x1/2 genes expression levels but at varying extent. The presence of BMA12 in combination to salinity stress showed more pronounced expression of these genes (Figure 3). When salinized tomato plants received BMA12, the expression of Trxf gene was scientifically increased as compared to the non-treated control plants (Figure 3). Even in the absence of salinity, B. megaterium positively increased expression levels of Trxf.
gene (Figure 3). Regarding Trxm2 gene, salinity exposure significantly increased its expression levels (Figure 3). However, BMA12 increased expression levels of Trxm2 in combination to salinity stress but with non-significant differences as compared to the salinized control plants (Figure 3).

BMA12 symbiosis positively influenced stress related genes (SOS1, APX1) of tomato plants both under salinized and non-salinized conditions. Here salinity stress alone was also effective enough to up-regulate expression levels of these genes at significant places (Figure 3). Contrastingly, BMA12 decreased expression levels of LERBOH1 gene. It showed maximum expression levels under salinity stress alone (Figure 3). Taken together, BMA12 mostly increased expression levels of selected genes governing photosynthesis process, stress management and redox regulation in tomato plants under stress conditions.

3.7. Change in soluble sugars, soluble proteins, free amino acid contents and related metabolites in tomato plants

Results showed pronounced effects of salinity and BMA12 applications on soluble sugars contents (SSC), free amino acids contents (FAAC), soluble protein contents (SPC) and some related metabolites (Table 5). Seven days after treatment, concentrations of SSC (39.0%), FAAC (18.7%), and SPC (37.8%) significantly increased in tomato plants exposed to BMA12 under salinity stress conditions as compared to salinized control plants (Table 5). The same increase was seen for proline that is considered an important osmolyte in plants. Importantly, under the exposure of salinity stress, SSC, FAAC and SPC were 24.6%, 44.8%, and 26.6% lower in comparison to the non-treated control plants respectively (Table 5). In the same way, onset of salinity stress decreased ß-carotene contents (39.3%) in tomato plants as compared to the non-treated control plants (Table 5). Whereas, BMA12 increased ß-carotene contents up to 21.6 and 07.4% in tomato plants under salinized and normal conditions as compared to the non-treated control plants respectively (Table 5).

3.8. Changes in photosynthesis related metabolism

UPLC-ESI MS/MS analysis detected changes in concentrations of several metabolites. We compared the metabolite’s peak area with internal standard to make comparative analysis among different treatments. We observed that the concentrations of several metabolites belonging to glycolysis process were different in the tomato plants with varying treatments (Figure 4). We focused on perturbations in photosynthesis related metabolism in tomato plants under salinity and BMA12 (Figure 4). Sugars such as mannose, xylose, fructose and glucose were more abundant in tomato plants under the influence of BMA12 under all conditions (Figure 4). Salinity stress decreased the quantities of most of the sugars inside tomato plants. However, symbiosis of BMA12 significantly increased sugar production under all conditions (Figure 4).

Similarly, the concentrations of most of the tricarboxylic acid (TCA) compounds were increased in tomato plants co-cultivated with BMA12 in comparison to the respective control plants (Figure 4). However, some deviations were seen from this trend. For example, changes in citrate and oxaloacetate were opposite from the above-mentioned scenario. Apart of sugars and organic acids, some amino acids (glycine, threonine) were also increased by BMA12 under salinity and normal conditions (Figure 4).

4. Discussions

Abiotic stress not only effect the normal growth of plant and crop productivity but also the extent of recovery after the damage has taken place. High salinity is a major factor that significantly limits crop productivity. Plants exposure to salt stress conditions cause various morphological, physiological and biochemical changes (Yin et al. 2017). The establishment of plant-BMA12 interaction, showed multiple positive effects in tomato plants under salinized conditions. BMA12 was previously shown to induce salinity tolerance in tomato plants, as well as increasing plant growth under both stress and normal conditions (Aslam et al. 2018). In this study we elucidated the possible mechanisms behind stress tolerance of tomato plants mediated by BMA12 using molecular, physiological and biochemical techniques. The relevant role of BMA12 is proved in the acclimation of tomato plants grown under salinized conditions by different mechanisms including the maintenance of redox homeostasis and the restoration of photosynthetic capabilities (Figure 5). The details of these ameliorative mechanisms are discussed below.

4.1. Symbiosis of BMA12 can rescue damaged photosynthetic machinery of tomato plants under salinity stress by multiple mechanisms

Abiotic stress severely affect the photosynthesis processes leading to photoinhibition (Gururani et al. 2015). It is sensible to measure photosynthetic parameters to study the response of plants during stress conditions (Gururani et al. 2015). Abiotic stress can affect photosynthesis of plants by stomatal and non-stomatal limitation factors (Zhou et al. 2016). Salinity stress inhibited net photosynthetic rate. In the same way, transpiration rate, stomatal conductance and water use efficacy were adversely affected in tomato plants under salinized conditions. However, in the symbiosis of

| Parameters | With salinity | Without salinity |
|------------|--------------|------------------|
|            | Control      | BMA12            | Control     | BMA12          |
| SSC (mg g⁻¹ FW) | 77.28 ± 8.37EC | 128.26 ± 7.63E    | 102.40 ± 9.34E  | 136.39 ± 12.70E |
| SPC (mg g⁻¹ FW) | 06.98 ± 4.36ED | 11.15 ± 0.62E     | 12.07 ± 1.51E  | 15.28 ± 0.88E  |
| FAAC (mg g⁻¹ FW) | 13.25 ± 1.25ED | 16.39 ± 1.50E     | 21.84 ± 1.60E  | 29.35 ± 2.48E  |
| SC (µg g⁻¹ FW) | 08.37 ± 0.64ED | 13.25 ± 0.86E     | 11.67 ± 1.33E  | 17.98 ± 0.95E  |
| PC (µmol g⁻¹ FW) | 05.67 ± 0.68E  | 07.27 ± 0.40E     | 03.35 ± 0.27E  | 4.91 ± 0.58E   |
| ß-carotene (µg g⁻¹ FW) | 0.29 ± 0.032D  | 0.37 ± 0.019F    | 0.38 ± 0.067H  | 0.62 ± 0.081I  |

Note: Data presented here are mean values of replicates of same treatment. Vales with ± represents standard error. Capital letters represents level of significance as governed by ANOVA and DNMRT at p = 0.05.
BMA12, the photosynthetic parameters were increased significantly. This may have been caused by the regulation of stomatal and non-stomatal limitation factors by BMA12.

Some physiological indices like chlorophyll contents are closely linked with the plant photosynthetic process (Foyer and Shigeru 2011) and considered to assess the plant’s tolerance to stress conditions (Orellana et al. 2010). The total chlorophyll contents decreased up to 40% in the leaves of tomato plants under salt stress as compared to the non-treated control plants. Whereas, this parameter decreased only up to 07% in the leaves of tomato plants co-cultivated with BMA12 in the same regards, showing that the total chlorophyll degradation rate in BMA12 treated plants was slower than the plants cultivated under salt stress alone. As chlorophyll contents imitate the extent of damage of photosynthetic machinery, these results showed that symbiosis of BMA12 can minimize the adverse effects of salt stress on photosynthetic machinery of tomato plants.

**Figure 4.** Changes in metabolomics of tomato plants induced by Effect of *Bacillus megaterium* strain A12 (BMA12) and salinity stress. Metabolites were quantified by UPLC/ESI MS/MS after one week after treatments applications. Metabolites were extracted from leaf samples of tomato plants. Results provided here are mean values of two independent experiments.

**Figure 5.** Possible mechanisms of induced salinity tolerance in tomato plants mediated by *Bacillus megaterium* strain A12 (BMA12).
It has been proved that excessive salt accumulation lowers the transcription and translation of PsbA gene responsible for the biosynthesis of the D1 protein of PSII (Allakhverdiev et al. 2002). This protein plays a key role in repairing of damaged photosystem after stress induced photoinhibition (Krishna et al. 2013). Secondly, the stress mediated increased accumulation of ROS in plants impair the biosynthesis of D1 protein after induced photoinhibition (Nishiyama et al. 2011; Yoshi-taka and Norio 2014). RT–PCR analysis showed the higher expression levels of PsbA gene that encoded D1 protein, in tomato plants under influence of BMA12. Here possible mechanism behind increased transcriptome levels of D1 protein encoding gene can be the reduced accumulation of ROS in tomato plants co-cultivated with BMA12.

Chlorophyll biosynthesis involves porphobilinogen deaminase enzyme encoded by the PBGD gene (Roberts et al. 2012). This enzyme plays key role in the formation of tetrapyrrole molecules. Whereas, chlorophyllase (Chlase) encoded by Chlase, catalyzes the breakdown of chlorophyll thorough de-esterification process (Harpazsaad et al. 2007). We observed decreased expression of PBGD gene but increased expression of Chlase gene after onset of salinity in tomato plants. These results are consistent with chlorophyll quantifications as its quantity decreased significantly under saline conditions. Hence, the decrease of chlorophyll contents under salinity stress could be due to decline of PBGD activity or increased Chlase activity. Here symbiosis of BMA12 reversed the scenario by increasing expression levels of PBGD that may have led to the increased biosynthesis of chlorophyll in tomato plants.

4.2. BMA12 mediated decrease in ROS can help in restoration of photosynthetic activity

ROS can cause oxidation of different molecules and disturbance of normal cellular processes leading to cell death (Andrzej et al. 2010). Redox signals are the key regulators of plant photosynthesis, metabolism, growth and development (Foyer and Allen 2003). Secondly, redox regulation is of crucial importance for the biosynthesis of the photosynthetic apparatus and its efficacy (Kieselbach 2013). Therefore, the aerobic organisms have developed an enzymatic and non-enzymatic antioxidant systems against ROS. Salinity-induced alterations in the ion homeostasis can contribute to the malfunctioning of photosynthetic apparatus of tomato plants. Inactivation of enzymatic antioxidants like SOD and APX are considered as limitations of photosynthetic efficiency under different stress conditions in plants (Ishikawa and Shigeoka 2008) and thus potential targets for improvement (Foyer and Shigeru 2011).

It was seen that co-cultivation of BMA12 mostly increased the quantities of non-enzymatic antioxidants and activities of antioxidant system related enzymes in tomato plants. The increased activities of antioxidant system related enzymes may contribute to the recovery of the redox state under stress conditions. In the same way, increases in non-enzymatic antioxidants (DHA, GSH and GSSG) pools of tomato plants induced by BMA12 can help plants to better adopt stress conditions (Orellana et al. 2010). The redox state of cell is also involved in the regulation of photosynthetic electron flow (Andrzej et al. 2010) and activity of some of the photosynthesis related enzymes (Rochaix 2011). As evident from results, the treatment of BMA12 can assist tomato plants to retain the redox balance and it might be a contributing factor to restore the functionality of photosynthetic system.

The benefits of BMA12 can be further seen regarding its effect of expression of some redox regulation (Trxf, Trxm2) genes. Compared with the non-treated control plants, BMA12 significantly increased expression levels of both genes. Trxf gene displayed reduced expression under salinity. Presence of BMA12 in the rhizosphere of tomato plants significantly increased its expression levels. Regarding Trxm2 gene, salinity stress alone positively influenced its expression levels. The possible reason can be the oxidative signaling during salinity stress conditions that can act as an elicitor for this gene (Fernández-Trijueque et al. 2012). Here BMA12 resulted in further increase of expression levels of Trxm2 gene but with non-significant differences as compared to the salinized control plants.

Likewise, different regulation of SOS1, APX1 and LERBOH1 was observed in tomato plants in response to salinity stress and BMA12 symbiosis. The SOS pathway is of critical importance in regulating Na⁺/K⁺ homeostasis and salinity stress tolerance (Munns 2002). APX1 gene is involved in scavenging of ROS. BMA12 induced increased expression levels of both of these genes in tomato plants. This suggests that this beneficial bacterium enhanced the sensitivity of tomato plants towards salinity stress. The application of salinity alone also led to a significant upregulation of LERBOH1 gene involved in ROS biosynthesis which is consistent with the increased H₂O₂ quantities observed under salinity stress. This fact could also serve to explain the lowering of H₂O₂ contents in tomato plants receiving BMA12 that significantly decreased expression levels of this gene.

4.3. BMA12 induced changes in plant hormones can modulate photosynthesis and salinity stress response

The symbiosis of BMA12 was responsive to the phytohormones production in tomato plants grown under salinity conditions suggesting that BMA12 played a key role in hormonal signal transduction. We found that BMA12 showed maximum increase in ABA production in tomato plants under salinity stress that may have acted in ABA dependent signaling pathways in response to the abiotic stress conditions. In addition, salinity stress alone also increased ABA production in tomato plants but at lower extent. Ethylene affects plant stress tolerance and regulates senescence (Bleecker and Kende 2000). Increased ethylene level can induce senescence in plants (Grbić and Bleecker 1995, Morgan and Drew 1997). Ethylene increase in plants under abiotic stresses including salinity (Balota et al. 2004; Hays et al. 2007). Decrease in the levels of ethylene production has been correlated with stress tolerance in plants (Hays et al. 2007). As demonstrated by the results, ethylene levels were decreased significantly in tomato plants co-cultivated with BMA12. These findings also indicate that this balance between ethylene and abscisic acid might have regulated the response of tomato plants to mitigate salinity stress.

Similarly, cytokinins have been shown to slower the degradation of photosynthetic protein and increased expression of photosystem related genes under stress conditions (Hare et al. 1997; Rivero et al. 2010). The exogenous applications of cytokinins have been shown to increase abiotic stress tolerance in bent grass (Zhao et al. 2008; Merewitz et al. 2010). We observed increased levels of cytokinins in tomato plants
induced by BMA12. These results indicate that BMA12 mediated changes in phytohormones production may play an important role in adaptation to salinity stress conditions. As phytohormones effect the expression of photosynthesis related genes and play the role in PSII damage repair mechanism (Bartoli et al. 2013; Anne et al. 2014), hence, it is quite imperative to consider these changes in the levels of hormones interlinked with improved photosynthesis efficacy of tomato plants under influence of BMA12.

4.4. BMA12 positively influences osmolites production and restores metabolomic perturbations in tomato plants

Findings of these studies have demonstrated that besides improving photosynthesis efficacy, BMA12 also played a positive role by restoring the perturbations in metabolomics of tomato plants induced by salinity stress. Under salinity stress plants accumulate compatible solutes that are known for their osmo-protection activity (Chellichaabouni et al. 2010). This is one of the common responses of plants to change in the external osmotic potential (Hasegawa et al. 2000). Proline act as a biochemical marker of salt stress level in plants (Shamshiri and Fattahi 2014). This acts as a free radical scavenger, stabilize cytosolic pH for subcellular structures and balance cell redox process (Verbruggen and Hermans 2008). In this study, salinity induced an increase in the proline content in the leaves of tomato plants. The increase was significantly remarkable in bacterized tomato plants under salinized conditions. Likewise, symbiosis of BMA12 increased sugar production in tomato plants. Sugars help in storage and transportation of carbon inside plants body fixed through photosynthesis (Xu et al. 2013). Sugars also act as signaling molecules to regulate other physiological process in plants (Koch 2004). Some disaccharides accumulate under stress conditions and help to maintain the membrane integrity and cell hydration levels (Dracup et al. 1986; Koch 2004). Here increased production of some sugars was found in tomato plants under influence of BMA12. This further highlights the active photosynthetic supply of carbohydrates and increased carbon reserves that can help to rescue growth of tomato plants under salinity stress. Apart of sugars, some amino acids were clearly increased in tomato plants co-cultivated with BMA12. Opposite to symbiosis of BMA12, a predominate decrease in the amino acid concentrations was seen in tomato plants under salinity stress.

Globally, changes in the production of sugars and amino acids discriminated differential effects of salinity and BMA12. Their accumulation was reduced under salinity stress alone, whereas symbiosis of BMA12 increased biosynthesis of these photosynthesis intermediates. These metabolites are recognized as important players in the growth and development of plants and abiotic stress tolerance.

5. Conclusion

Our results showed that BMA12 symbiosis in tomato plants restored redox homeostatis and restored photosynthesis system, consequently improving the growth of tomato plants against salinity stress. This study suggests that BMA12 could act as a source to ameliorate salinity stress and, possibly, offers a source that can be used in conventional agriculture system to make plants survive under salinity stress.

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WA, GL, HA, AA carried out the experiments. WA, GL, AA, TA, NAY, TW and WL performed data analysis. WA, JG, TW drafted the manuscript. WA, TA designed the experiments. SA, TA, supervised the whole work.

Disclosure statement

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