

Mitigation of salinity stress by exogenous application of cytokinin in faba bean (*Vicia faba* L.)

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Abstract

Soil salinity limits agricultural land use and crop productivity, thereby a major threat to global food safety. Plants treated with several phytohormones including cytokinins were recently proved as a powerful tool to enhance plant's adaptation against various abiotic stresses. The current study was designed to investigate the potential role of 6-benzyladenine (BA) to improve broad bean (*Vicia faba* L.) salinity tolerance. The salt-stressed broad bean plantlets were classified into two groups, one of which was sprayed with water and another was sprayed with 200 ppm of BA. Foliar applications of BA to salt-exposed plants promoted the growth performance which was evidenced by enhanced root-shoot fresh and dry biomass. Reduced proline was strongly connected to the enhanced soluble proteins and free amino acids contents, protecting plant osmotic potential following BA treatment in salt-stressed broad bean. BA balanced entire mineral homeostasis and improved mineral absorption and translocation from roots to shoots, shoots to seeds and roots to seeds in salt-stressed plants. Excessive salt accumulation increased malondialdehyde level in leaves creating oxidative stress and disrupting cell membrane whereas BA supplementation reduced lipid peroxidation and improved oxidative defence. BA spray to salinity-stressed plants also compensated oxidative damage by boosting antioxidants defence mechanisms, as increased the enzymatic activity of superoxide dismutase, catalase, peroxidase and ascorbate peroxidase. Moreover, clustering heatmap and principal component analysis revealed that mineral imbalances, osmotic impairments and increased oxidative damage were the major contributors to salts toxicity, on the contrary, BA-augmented mineral homeostasis and higher antioxidant capacity were the reliable markers for creating salinity stress tolerance in broad bean. In conclusion, the exogenous application of BA alleviated the antagonistic effect of salinity and possessed broad bean to positively regulate the osmoprotectants, ion homeostasis, antioxidant activity and finally plant growth and yield, perhaps suggesting these easily-accessible and eco-friendly organic compounds could be powerful tools for the management of broad bean growth as well as the development of plant resiliency in saline prone soils.

Keywords: antioxidants; cytokinins; faba bean; oxidative stress; salt stress; 6-benzyladenine

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Introduction

Faba bean (*Vicia faba* L.) or broad bean is one of the major legume crops belonging to the family *Fabaceae* that can grow across various meteorologic conditions (Singh *et al.*, 2013; Abdel Latef *et al.*, 2018). *Vicia faba* is a preferable and alternative source of essential proteins so that it is cultivated for collecting seeds that are consumed as human and animal feed. Along with high protein content and a balanced amino acids profile (nearly 30% of lysine), broad bean seeds are abundant in dietary fiber, minerals, vitamins, lipids, γ -aminobutyric acid and phenolic compounds which provides nourishment for humans and positively influence the antioxidant system and biological process (Giménez *et al.*, 2012; Raikos *et al.*, 2014; Mahdi *et al.*, 2021). It can be grown in a wide range of agro-ecosystem, but the performance of this legume crop is severely affected because of several environmental stresses including salinity.

Soil salinity is considered as one of the primary abiotic stress factors associated with the significant economic impact because of losing a huge amount of agricultural land and declining crop yield (Mbarki *et al.*, 2018; Fariduddin *et al.*, 2019; Abdel Latef *et al.*, 2020a). It was reported that over 800 Mha lands are salt-contaminated globally (Munns and Tester, 2008; Osman *et al.*, 2020). Therefore, nowadays, salinity is a major threat to sustainable agricultural productivity. Further to this, about 45 Mha of irrigated land are captured by salt stress-related problems worldwide, and more than 50% of arable land could be salt-affected by the next 30 years (Shrivastava and Kumar, 2015). Global soil salinization is aggravating day by day due to global warming-induced sea-level rise, saline-enriched water irrigation, and massive erosion of soils (Shrivastava and Kumar, 2015; Nachshon, 2018). The world population has been estimated to increase by about 2 billions in the next 30 years (DESA, 2015). Therefore, scientists are working to find economically feasible and efficient tactics to reduce or mitigate salt-induced stress for ensuring the nutritional security of the ever-increasing population (Geist, 2017; Noreen *et al.*, 2020; Abdel Latef *et al.*, 2021).

In crop plants, salinity stress inhibits the molecular, biochemical and physiological processes of crop plants by damaging photosynthetic activity, changing the osmotic adjustment and ionic homeostasis, producing toxic oxidants and radicals, regulating phytohormonal functions, counteracting essential metabolic pathways, and manipulating the gene expression pattern (Tang *et al.*, 2015; Yang and Guo, 2018; Morton *et al.*, 2019). Additionally, excess amounts of Na^+ and Cl^- causes ionic imbalance, osmotic stress, water deficiency, and nutrient (such as K, Ca, Mg, Fe, Zn, N and P) disorders, which consequence in the physiological malfunction in both crop and vegetable plants (Rehman *et al.*, 2019). However, excessive reactive oxygen species (ROS) generation due to the disruption of redox homeostasis leads to oxidative stress to the cellular biomolecules (Abdel Latef *et al.*, 2017a,b,c, 2018, 2019a,b, 2020b; Attia *et al.*, 2021). The plant showed a response against either osmotic or ionic stress in two mechanisms. In response to osmotic stress, the water absorption in plant root is inhibited that suppresses the cell development and growth of lateral bud, while ionic stress induces the change in the optimum ratios of K^+/Na^+ content and Na^+ and Cl^- ion levels that are detrimental for the normal cellular functions and processes (Munns and Tester, 2008; Singh *et al.*, 2017). In stressed condition, plant defense against excessive ROS is associated with the protection of cellular redox homeostasis, which is mostly conferred by some antioxidant enzymatic activity for example superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX), etc. (Tang *et al.*, 2015; Tanveer and Shabala, 2018; Abdel Latef *et al.*, 2020c; Rahman *et al.*, 2020; Dawood *et al.*, 2021). Therefore, any mechanisms that maintain optimal K^+/Na^+ content, nutritional balance, and ROS in plants may provide tolerance capability against salinity (Assaha *et al.*, 2017).

Different mitigation and adaptation approach was applied to conquer the adverse impacts of salt stress (Wang *et al.*, 2019). Application of exogenous growth regulators including cytokinins (CK) would be an effective and affordable strategy for the development of plant resiliency against the detrimental consequences of salt stress. However, 6-benzyladenine (BA) is a CK-like plant growth regulator that can significantly amplify plant CK levels. It is reported that CK helps plants to confer salt tolerance by raising the activity of antioxidant enzymes and alleviating lipid peroxidation to protect the generation and scavenging consistency of ROS

(Adam *et al.*, 1989; Bin *et al.*, 2010; Xiaotao *et al.*, 2013). At present, applications of exogenous CK including BA have been described in mitigating various abiotic stresses, e.g salinity, drought, cold and waterlogging (Abdel-Latef, 2003; Azooz *et al.*, 2004a,b; Iqbal *et al.*, 2006; Abdel Latef *et al.*, 2009; Javid *et al.*, 2011b; Wu *et al.*, 2014; Hu *et al.*, 2020). Therefore, the present investigation was operated for exploring the ameliorate role of BA for mitigating salt stress and promoting salt tolerance mechanism in broad bean plants by evaluating the following properties: (i) growth-promoting characters and yield, (ii) regulation of mineral homeostasis and translocation, (iii) level of osmoprotectants, (iv) oxidative stress and lipid peroxidation, (v) improvement of antioxidant enzymatic activity.

Materials and Methods

Plant materials and experimental design

The investigation was performed using broad bean (*Vicia faba* L. cv. 'Assiut 85/37'). Mature seeds were carefully surface-sterilized for 5 min in 0.1% mercuric chloride followed by three times washed with autoclaved distilled water. Then 10 disinfected seeds were planted in each pot where each pot was filled with 5 kg of air-dried soil (sand: clay, 3:1 v/v). The pots were daily irrigated with tap water until full germination. On the 14th day after sowing, germinated broad bean plantlets were subjected to 0 mM NaCl (control, C), 60 mM NaCl (S1), and 150 mM NaCl (S2) stress. After that, all pots were classified into 2 groups, one of which was sprayed with water and another was sprayed with 200 ppm of BA (Sigma-Aldrich, St. Louis, MI, USA) (50 mL to each pot at the 14-day interval for the total period of 100 days). Thus, there were six treatment compositions as follows: (i) control (C), 0 mM NaCl + 0 ppm BA; (ii) 60 mM NaCl + 0 ppm BA, S1; (iii) 60 mM NaCl + 0 ppm BA, S2; (iv) 0 mM NaCl + 200 ppm BA, BA; (v) 60 mM NaCl + 200 ppm BA, B+S1; and (vi) 150 mM NaCl + 200 ppm BA, B+S2. The dose of exogenous BA (200 ppm) was selected as following the report of (Iqbal *et al.*, 2006). Our investigations were conducted in a completely randomized design (CRD) with three replications where each pot containing 6 broad beans were considered as a replica.

Plant growth measurements

The root fresh weight (RFW), shoot fresh weight (SFW), fresh seed weight (FSW) and the number of nodules were estimated for determining the plant growth performance. The freshly harvested samples were packed and preserved in an aerated oven for 7 days at 80 °C. After that, the samples were completely desiccated and the root dry weight (RDW), shoot dry weight (SDW) and dry seed weight (DSW) were measured.

Determination of mineral ion contents

Root, stem and leaves were harvested from broad bean plant and rinsed these samples with deionized water for making them contamination-free. After that, fresh samples were dried in the oven for four days at 70 °C. Then finely grinded plant samples were treated with HNO₃: HClO₄ (2:1v/v) for 2 h at 220 °C by following the protocol of (Williams and Twine, 1960). Na⁺ and K⁺ contents were determined by the atomic absorption flame spectrophotometer (Model AA-6400 F, Shimadzu Corporation, Japan). The versene (disodium dihydrogen ethylene-diamine-tetraacetic acid) titration method (Schwarzenbach and Biedermann, 1948) was used for Ca²⁺ and Mg²⁺ contents were estimated using the versene (disodium dihydrogen ethylene-diamine-tetraacetic acid) method of titration. The level of translocation was determined from quantifying the value of translocation factor of nutrients in broad bean plant parts according to the procedure of (Hawrylak-Nowak, 2013):

Translocation factor (roots to shoot) = Mineral content in shoot/Mineral content in root

Translocation factor (shoot to seed) = Mineral content in seed/Mineral content in shoot

Translocation factor (roots to seed) = Mineral content in seed/Mineral content in root

Extraction and determination of antioxidant enzymatic activity

The enzyme extraction was performed according to the procedure of (Mukherjee and Choudhuri, 1983). The fresh leaf samples (0.2 g) were finely ground in a cold mortar-pestle with 5 mL of 100 mM potassium-phosphate of (K-P) buffer (100 mM, pH 7.0) having Na₂EDTA (0.1 mM) and polyvinylpyrrolidone (0.1 g). Then, the homogenate was then filtered using cheesecloth and performed centrifugation at 12000×g for 10 min at 4 °C and the resulted supernatant was used for the analysis of the following antioxidants: superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), peroxidase (POD; EC 1.11.1.7) and ascorbate peroxidase (APX; EC 1.11.1.11).

The SOD activity was estimated as followed by the protocol of (Scebba *et al.*, 1999). Leaf extracts (2 mL) were added with the mixtures containing K-P buffer (50 mM, pH 7.8), EDTA (0.1 mM), M L-methionine (13 mM), riboflavin (2 µM) and nitroblue tetrazolium (75 µM) at a final volume of 3 mL. The reaction was initiated, when the reaction mixtures were exposed to fluorescent light (cool white) for 15 min. The blue reaction colour was determined spectrophotometrically at 560 nm. The activities of CAT were assayed in 3 mL reaction solution comprised of K-P buffer (50 mM, pH 7.0), H₂O₂ (30%, w/v) and leaf extract (0.1 mL), by using the method of (Aebi, 1984). The enzymatic activity of the CAT was determined by reducing of absorbance at 240 nm resultant in H₂O₂ consumption. POD activity was determined by adding leaf extract (0.5 mL) in 3 mL reaction mixture of K-P buffer (10 mM, pH 7.0), H₂O₂ (10 mM) and guaiacol (20 mM) (Maehly and Chance, 1954). An increased absorbance as a consequence of the production of tetraguaiacol was observed at 470 nm (Klapheck *et al.*, 1990). The activity of APX was determined from the decreased absorbance at 290 nm as ascorbic acid was oxidized (Chen and Asada, 1992). APX was assayed with the reaction mixtures (3 mL) having K-P buffer (50 mM, pH 7.0), ascorbic acid (0.5 mM) and H₂O₂ (0.5 mM). All spectrometric readings were taken through a Spectronic Genysis^{1M} 2PC spectrophotometer, Spectronic Instruments, USA.

Measurement of lipid peroxidation

The amounts Lipid peroxidation determined in the term of malondialdehyde (MDA) in broad bean followed by the protocol of (Heath and Packer, 1968). Leaves materials (0.2 g) were homogenized with the solutions of 5 mL of 5% trichloroacetic acid (TCA) followed by centrifuging at 15000g for 10 min at 4 °C. Along with 20% TCA, 4 mL solutions of 0.5% were added in 2 mL aliquots of the supernatant. The supernatant absorption and the non-specific absorption value were quantified at 532 and 600 nm, respectively. The MDA contents were estimated by employing the absorption coefficient of 155 nmol⁻¹ cm⁻¹.

Measurement of proline, soluble sugars, soluble proteins and free amino acids levels

The levels of proline (Pro) in broad bean plants were determined according to the description of (Bates *et al.*, 1973). The method based on anthrone-sulfuric acid, as described by (Fales, 1951) was employed to determine the content of soluble sugars (SS). The method of (Lowry *et al.*, 1951) was used to estimate soluble proteins (SP) content as following the standard of bovine serum albumin (BSA). The content of free amino acids (FAA) was estimated as following the procedure of (Moore and Stein, 1948).

Statistical analysis

The obtained data were undertaken to a two-way analysis of variance (ANOVA) followed by performing Tukey's test ($P < 0.05$) using the 'multcompView' package of the statistical programming language R 3.6.1. The MetaboAnalyst (<https://www.metaboanalyst.ca/>) was used for constructing heatmap and hierarchical clustering considering Euclidean distances. The packages 'ggplot2', 'factoextra', 'FactoMineR' of R 3.6.1 were used to perform principal component analysis (PCA).

Results

Analysis of variance

The ANOVA of different growth and yield-contributing traits in the broad bean is shown in Supplementary Table 1. The S and BA significantly affect all growth and yield-related parameters in broad bean. However, S and BA interaction has no significant difference on SDW and No. of nodules. The analysis of variance of SS, SP, proline, and FAA content in root, shoot and seed of broad bean is shown in Supplementary Table 2. The S and BA significantly affect SS, SP and FAA content in root, shoot and seed of broad bean plants. However, there is no significant difference was exhibited for S and BA interaction with SP and FAA contents in root, SS content in shoot, SP and FAA contents in the seed of broad bean. The analysis of variance of different mineral content in root, shoot and seed of broad bean is shown in Supplementary Table 3. The S and BA significantly affect Na⁺, K⁺, Ca²⁺ and Mg²⁺ contents in root, shoot and seed of broad bean plants. However, there is no significant difference was exhibited for S and BA interaction with Mg²⁺ content in root, Na⁺ content in shoot and Ca²⁺ content in seed of broad bean. The ANOVA of different antioxidant enzymes of broad bean plants was shown in Supplementary Table 4. The S and BA significantly affect the MDA, SOD, CAT, POD and APX activity in broad bean. However, the interaction of S and BA showed significant differences for MDA, SOD, CAT and APX activity except for POD in broad bean plants.

Exogenous BA boosted broad bean growth under salinity stress

The 'S1' plants showed a considerable reduction in RFW (by 16.17%), SFW (by 7.16%), and RDW (by 29.47%) compared with that of 'C' plants (Figure 1A, B, D), but did show any effect on FSW, SDW, SDW and no. of nodules (Figure 1C, E-G). Moreover, 'S2' plants displayed a marked decrease in RFW (by 48.70%), RDW (by 70.42%), SFW (by 59.72%), SDW (by 42.14%), FSW (by 45.54%), DSW (by 53.36%) and no. of nodules (by 47.20%) compared with 'C' plants (Figure 1A-G). Foliar application of BA to 'B+S1' broad bean assuaged the adverse effects of saline toxicity by significantly improving the RFW, RDW, SFW, SDW, FSW, and no. of nodules (by 31.95%, 92.54%, 29.13%, 25.52%, 6.37%, and 31.29%, respectively), except DSW compared to 'S1' plants (Figure 1A-G). Moreover, 'B+S2' plants also exhibited significant enhancement in SFW, SDW, FSW, DSW and no. of nodules by 91.32, 36.21, 33.08, 55.45, and 84.73%, respectively, without RDW compared with the 'S2' plants (Figure 1A-G). Moreover, 'BA' plants showed amelioration in all studied growth parameters, versus 'C' plants (Figure 1A).

Exogenous BA enhanced level of osmoprotectants under salinity stress

The 'S2' plants showed decreased content of SS in the root, shoot and seed (by 55.41%, 33.43% and 36.85%, respectively), and the 'S1' plants also exhibited reduction (Figure 2A-C). However, in contrast with 'S2' plants, 'B+S2' plants exhibited an improved level of SS in the root, shoot and seed (by 43.69%, 29.15% and 55.06%, respectively) (Figure 2A-C). Moreover, 'B+S1' plants showed enhanced content SS in the root, shoot and seed compared with 'S1' plants (Figure 2A-C). Furthermore, 'BA' plants induced improvement of SS content in all studied organs, versus 'C' plants (Figure 2A-C). The 'S2' plants displayed a reduced level of SP in the root (by 28.03%), and seed (by 48.19%), whereas increased in the shoot (by 27.56%) in comparison with 'C' plants (Figure 2D-F). Moreover, 'S1' plants recorded an enhanced level of SP in the shoot (by 12.00%), whereas decreased in the shoot (by 34.14%) compared with 'C' plants (Figure 2E, F). However, 'B+S2' plants displayed an increased level of SS in the root, shoot and seed (by 48.42%, 34.15% and 62.02%, respectively) compared to 'S2' plants (Figure 2D-F). Moreover, 'B+S1' plants showed enhanced content SS in the root, shoot and seed compared with 'S1' plants (Figure 2D-F). Furthermore, 'BA' plants displayed an enhanced level of SP in all tested organs, against 'C' plants (Figure 2D-F). The 'S2' plants exhibited an increased content of FAA in the root, shoot and seed (by 44.12%, 46.15% and 27.70%, respectively), and the 'S1' plants also exhibited similar trends (Figure 2G-I).

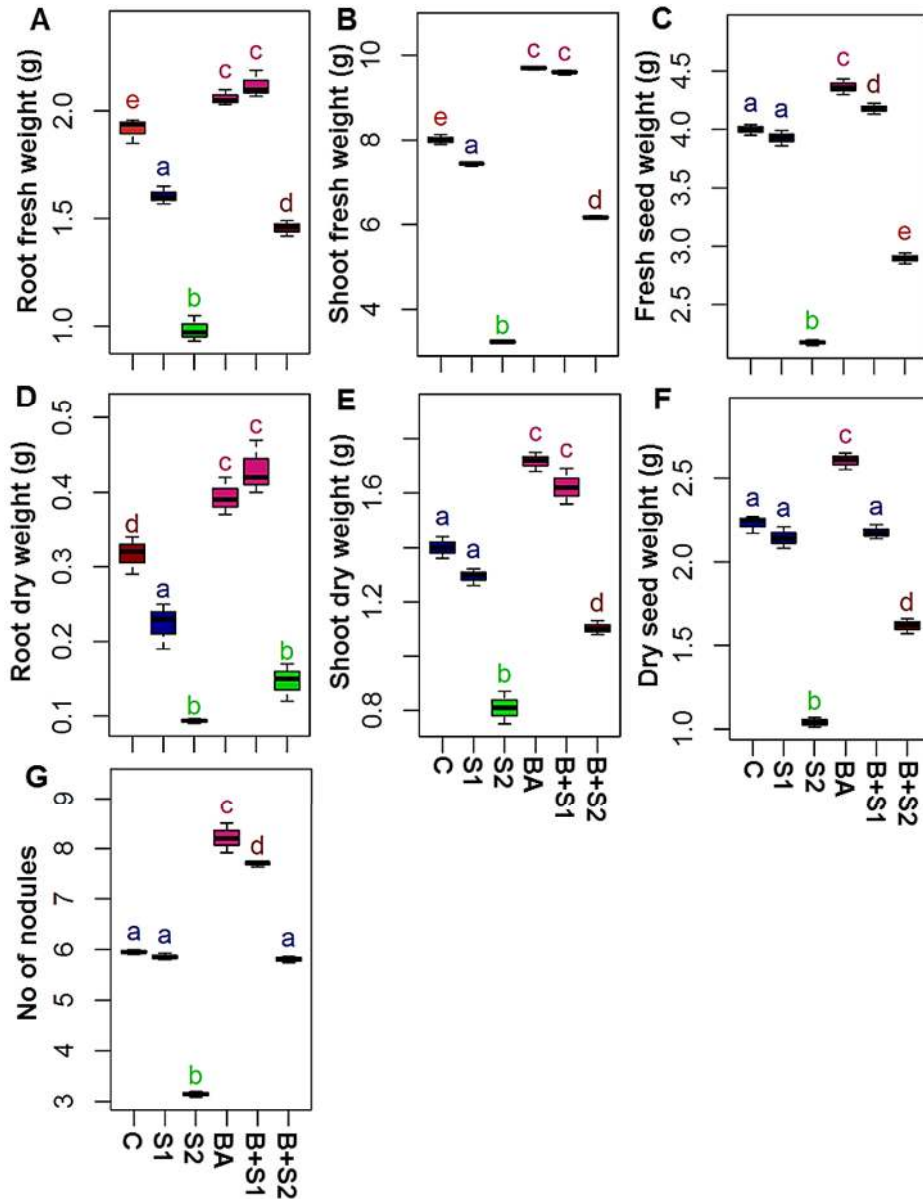


Figure 1. Different growth and yield-related parameters, (A) root fresh weight, (B) shoot fresh weight, (C) fresh seed weight, (D) root dry weight, (E) shoot dry weight, (F) dry seed weight and (G) no. of nodules of broad bean plants exposed to salinity with or without 6-benzyladenine (BA) foliar spray. For each treatment, values were obtained from three independent replicates ($n = 3$). Different letters above the bars indicate a statistically significant difference based on $P < 0.05$ by Tukey's test. 'C', 0 mM NaCl + distilled water spray (control); 'BA', 0 mM NaCl + 200 ppm BA spray; 'S1', 60 mM NaCl + distilled water spray; 'S2', 150 mM NaCl + distilled water spray; 'S1+B1', 60 mM NaCl + 200 ppm BA spray; and 'B+S2', 150 mM NaCl + 200 ppm BA spray.

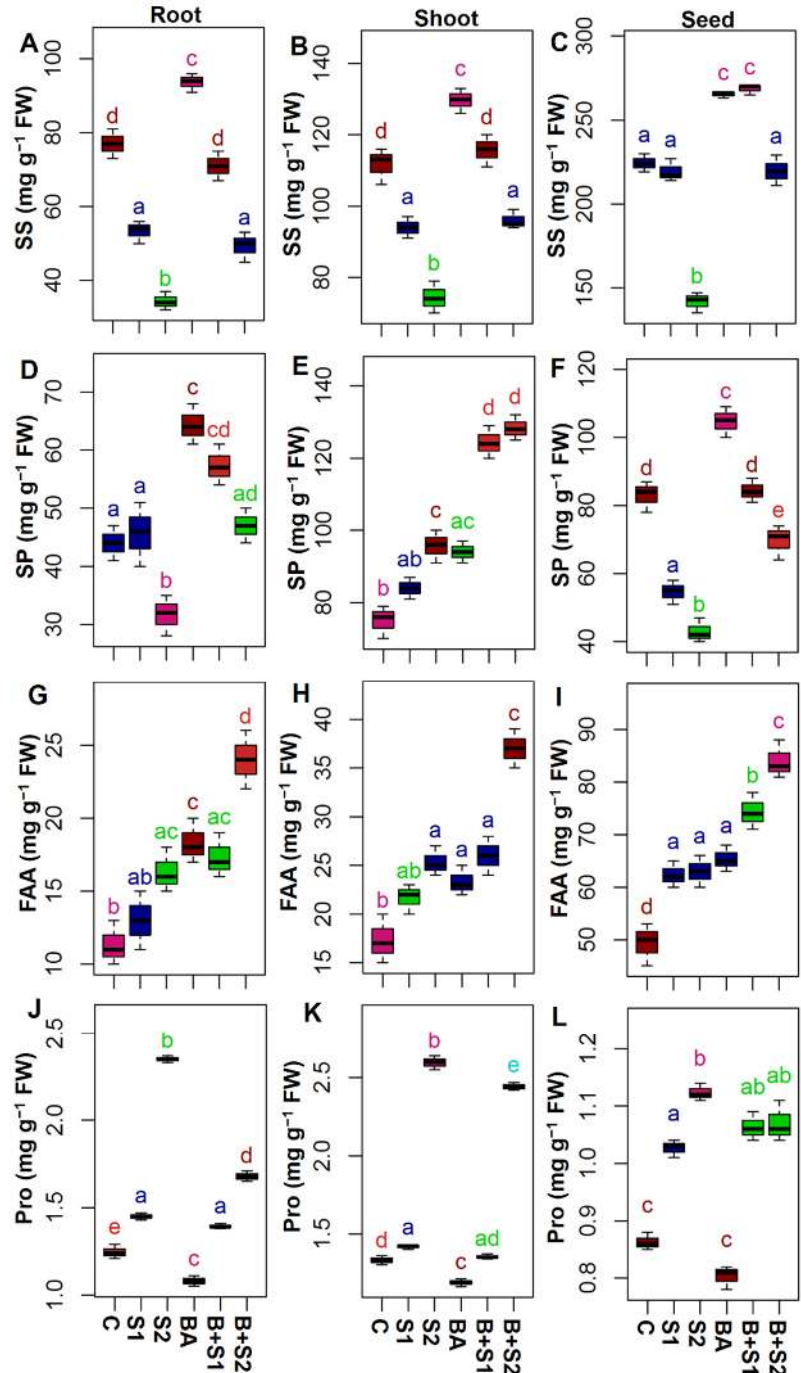


Figure 2. Effect of foliar application of 6-benzyladenine (BA) soluble sugars (SS) content in (A) root (B) shoot and (C) seed; soluble proteins (SP) content in (D) root (E) shoot (F) and seed; free amino acids (FAA) content in (G) root, (H) shoot and (I) seed; proline (Pro) content in (J) root, (K) shoot and (L) seed in broad bean

For each treatment, values were obtained from three independent replicates ($n = 3$). Different letters above the bars indicate a statistically significant difference based on $P < 0.05$ by Tukey's test. 'C', 0 mM NaCl + distilled water spray (control); 'BA', 0 mM NaCl + 200 ppm BA spray; 'S1', 60 mM NaCl + distilled water spray; 'S2', 150 mM NaCl + distilled water spray; 'S1+B1', 60 mM NaCl + 200 ppm BA spray; and 'B+S2', 150 mM NaCl + 200 ppm BA spray.

However, in comparison with 'S2' plants, 'B+S2' plants exhibited a further enhanced level of SS in the root, shoot and seed (by 46.94%, 46.05% and 33.33%, respectively) (Figure 2G-I). Moreover, 'B+S1' plants displayed similar trends (Figure 2G-I). Furthermore, 'BA' plants displayed improvement of SS content in all studied parts, compared to 'C' plants (Figure 2G-I). The 'S2' plants displayed increased content of Pro in the root (by 88.50%), and shoot (by 95.24%) and seed (by 30.12%) compared with 'C' plants (Figure 2J-L). Moreover, 'S1' plants also showed an increased level of Pro in the root, shoot and seed (by 12.00%), compared with 'C' plants (Figure 2J-L). In contrast, 'B+S2' plants exhibited diminished content of SS in the root, shoot and seed (by 34.15% and 5.91% and 4.75%, respectively), in comparison with 'S2' plants (Figure 2J-L). Moreover, 'B+S1' plants showed a reduced level of Pro in shoot and seed compared with 'S1' plants (Figure 2K, L). Furthermore, 'BA' plants displayed a decreased level of SP in root and shoot, versus 'C' plants (Figure 2J, K).

Exogenous BA regulated mineral homeostasis under salt stress

The 'S2' plants displayed increased content of Na⁺ in the root, shoot and seed (by 509.13%, 336.58% and 440.46%, respectively) in comparison with 'C' plants. Moreover, 'S1' plants showed similar results (Figure 3A-C). However, in 'B+S2' plant, the amount of Na⁺ was reduced in the root, shoot and seed (by 39.89%, 22.78% and 33.58%, respectively), relative to the content in S2' plants (Figure 3A-C). Moreover, in 'B+S1' plant, the level of Na⁺ was also reduced in the root, shoot and seed, relative to the content in S1' plants (Figure 3A-C). Furthermore, 'BA' plants displayed decreased content of SP in seed compared to 'C' plants (Figure 3C). The 'S2' plants displayed decreased content of K⁺ in shoot and seed (by 32.39% and 37.36%, respectively) whereas increased in the root (by 13%) compared with 'C' plants (Figure 3D-F). Moreover, the 'S1' plants showed decreased content of K⁺ in shoot whereas increased in root compared with 'C' plants (Figure 3D, E). However, in 'B+S2' plant, the amount of K⁺ was amplified in the root, shoot and seed (by 11.10%, 42.48% and 116.53%, respectively), relative to the content in 'S2' plants (Figure 3D-F). Moreover, in 'B+S1' plant, the level of K⁺ was also augmented in roots, shoot and seed, in comparison with 'S1' plants (Figure 3D-F). Furthermore, 'BA' plants enhanced the content of K⁺ in all tested organs compared to 'C' plants (Figure 3D-F). The 'S2' plants displayed decreased content of Ca²⁺ (by 74.44%, 59.58% and 41.67%, respectively) and Mg²⁺ (by 37.88%, 29.22% and 33.71%, respectively) in the root, shoot and seed compared with 'C' plants and 'S1' plants follow similar trends (Figure 3G-L). However, in 'B+S2' plant, the content of Ca²⁺ (by 154.70%, 46.49% and 52.38%, respectively), and Mg²⁺ were amplified in the root, shoot and seed (by 57.89%, 43.31% and 59.44%, respectively), relative to the content in 'S2' plants (Figure 3G-L). Moreover, in 'B+S1' plant, the level of Ca²⁺ and Mg²⁺ was also augmented in the root, shoot and seed, in comparison with 'S1' plants (Figure 3G-L). Furthermore, 'BA' plants enhanced the content of Ca²⁺ and Mg²⁺ in all examined organs compared to 'C' plants (Figure 3G-L).

We estimated the mineral translocation rates within different parts of broad bean, i.e. root to shoot, shoot to seed, and root to seed. 'S2' plants showed decreased translocations for Na⁺ from root to shoot and root to seed whereas increased from shoot to seed compared with 'C' plants (Figure 4A-C). Moreover, 'S1' plants showed increased translocations for Na⁺ from shoot to seed and root to seed compared with 'C' plants (Figure 4A-C). However, 'B+S2' plants showed improved translocations for Na⁺ from root to shoot and root to seed whereas declined from shoot to seed compared with 'S2' plants (Figure 4A-C).

Moreover, 'S1' plants showed decreased translocations for Na⁺ from shoot to seed and root to seed compared with 'S1' plants (Figure 4B, C). 'BA' plants showed decreased translocations for Na⁺ from shoot seed and root to seed compared with 'C' plants (Figure 4B, C). 'S2' plants showed decreased translocations for K⁺ from all different parts compared with 'C' plants (Figure 4D-F). Moreover, 'S1' plants showed reduced translocations for K⁺ from root to shoot and root to seed compared with 'C' plants (Figure 4D-F).

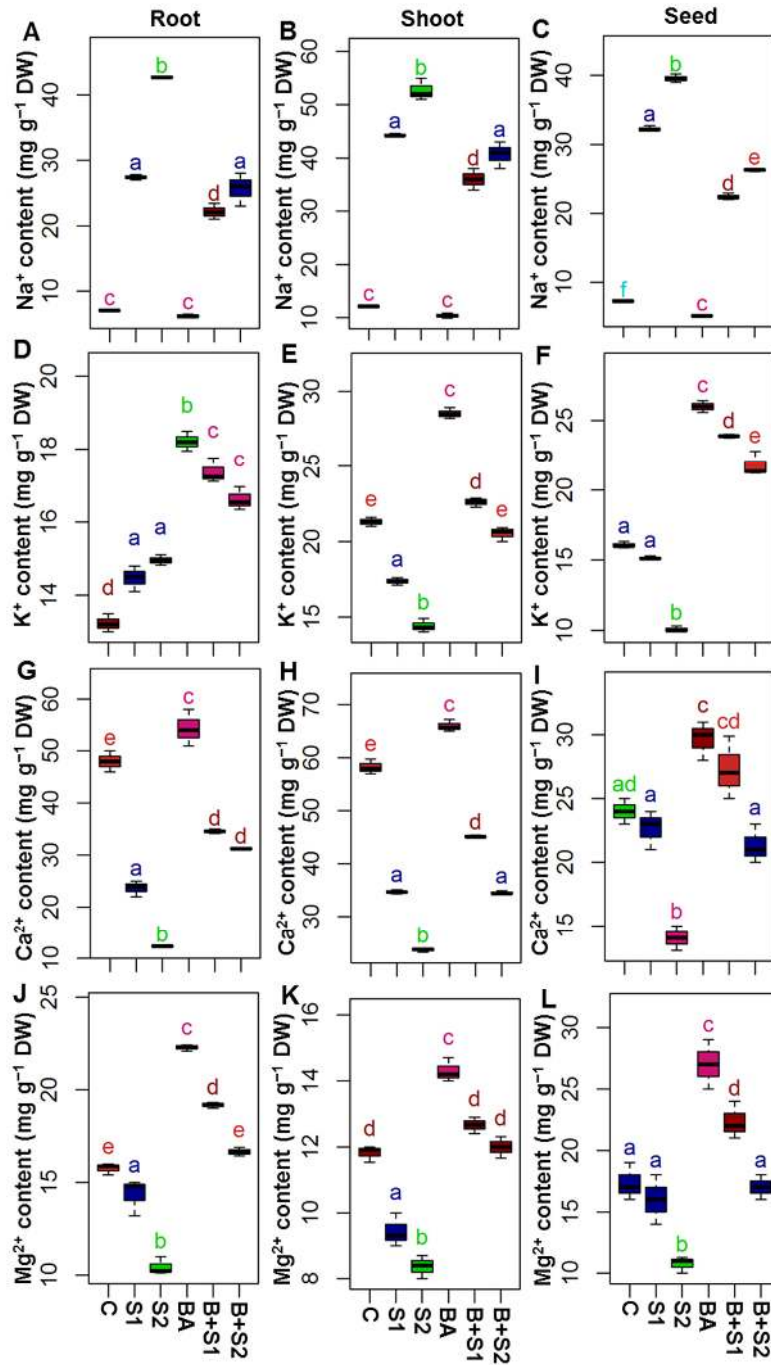


Figure 3. Nutrient contents in different parts of broad bean plants exposed to salt stress with or without 6-benzyladenine (BA) treatment. Na⁺ content in (A) root (B) shoot and (C) seed; K⁺ content in (D) root (E) shoot and (F) seed; Ca²⁺ content in (G) root, (H) shoot and (I) seed; and Mg²⁺ content in (J) root, (K) shoot and (L) seed in broad bean plants

For each treatment, values were obtained from three independent replicates ($n = 3$). Different letters above the bars indicate a statistically significant difference based on $P < 0.05$ by Tukey's test. 'C', 0 mM NaCl + distilled water spray (control); 'BA', 0 mM NaCl + 200 ppm BA spray; 'S1', 60 mM NaCl + distilled water spray; 'S2', 150 mM NaCl + distilled water spray; 'S1+B1', 60 mM NaCl + 200 ppm BA spray; and 'B+S2', 150 mM NaCl + 200 ppm BA spray.

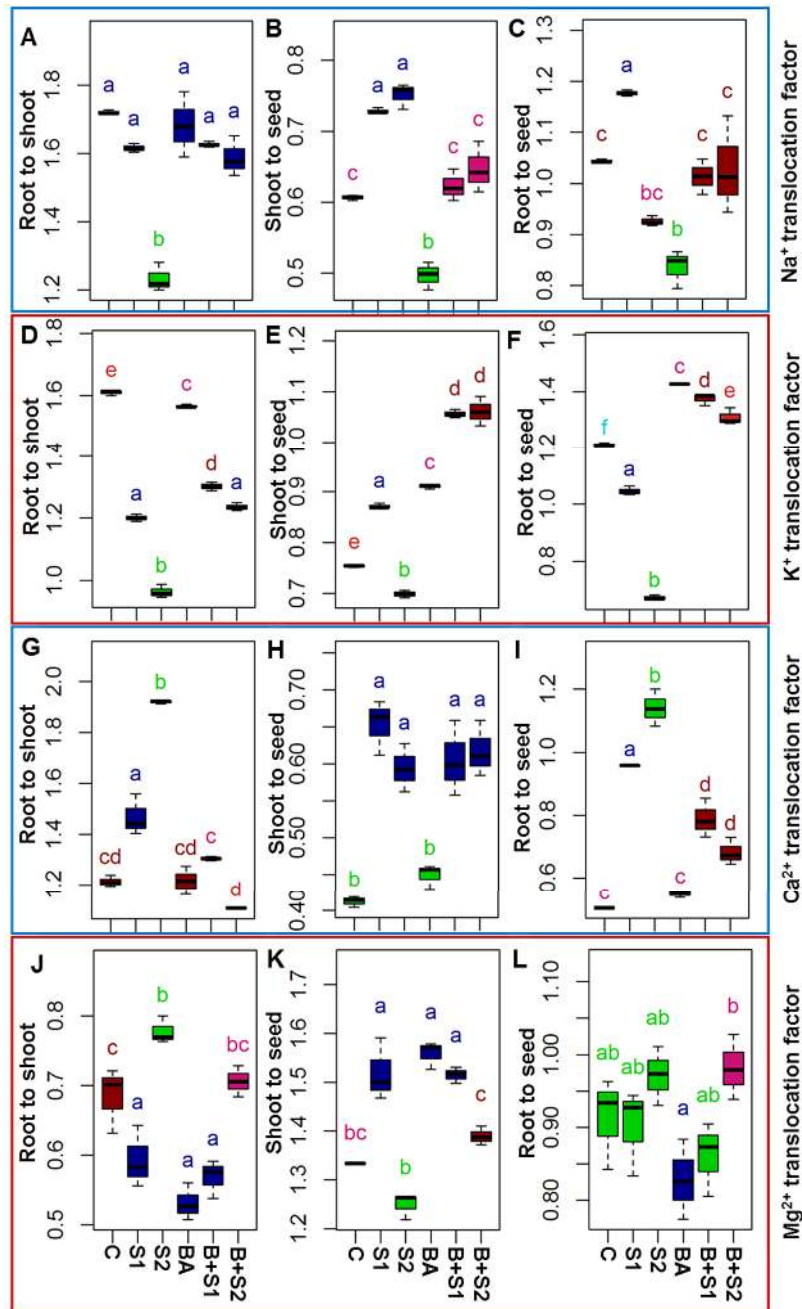


Figure 4. Translocation of nutrients in different parts of broad beans exposed to salt stress with or without 6-benzyladenine (BA) treatment. Translocation factor of Na⁺ in (A) root to shoot (B) shoot to seed (C) root to seed; K⁺ in (D) root to shoot (E) shoot to seed (F) root to seed; Ca²⁺ in (G) root to shoot (H) shoot to seed (I) root to seed; and Mg²⁺ in (J) root to shoot (K) shoot to seed (L) root to seed in broad bean plants. For each treatment, values were obtained from three independent replicates (*n* = 3). Different letters above the bars indicate a statistically significant difference based on *P* < 0.05 by Tukey's test. 'C', 0 mM NaCl + distilled water spray (control); 'BA', 0 mM NaCl + 200 ppm BA spray; 'S1', 60 mM NaCl + distilled water spray; 'S2', 150 mM NaCl + distilled water spray; 'S1+B1', 60 mM NaCl + 200 ppm BA spray; and 'B+S2', 150 mM NaCl + 200 ppm BA spray.

However, ‘B+S2’, ‘B+S1’ and ‘BA’ plants showed improved translocations for K^+ in all parts of broad bean compared with ‘S2’, ‘S1’ and ‘C’ plants, respectively (Figure 4D-F). ‘S2’ and ‘S1’ plants showed increased translocations for Ca^{2+} in all studied organs compared with ‘C’ plants (Figure 4G-I). However, ‘B+S2’ and ‘B+S1’ plants showed decreased translocations for Ca^{2+} from root to shoot and root to seed compared with ‘S2’ and ‘S1’ plants, respectively (Figure 4G, I). ‘S2’ plants showed decreased translocations for Mg^{2+} from root to shoot to seed whereas increased from shoot to seed compared with ‘C’ plants (Figure 4J, K). Moreover, ‘S1’ plants reverse this trend (Figure 4J, K). However, ‘B+S2’ plants showed declined translocations for Mg^{2+} from root to shoot whereas increased from shoot to seed compared with ‘S2’ plants (Figure 4J, K)

Exogenous BA reduced MDA content and improved antioxidant defence system under salt stress

The production of MDA was markedly increased by 7.92% and 99.32% at both S1 and S2 treatment compared with ‘C’ plants (Figure 5A). Supplementation of BA to both ‘S1’ and ‘S2’ plants showed significant abatement in MDA content by 19.90% and 46.49% in comparison with ‘S1’ and ‘S2’ salt-stressed plants, respectively (Figure 5A).

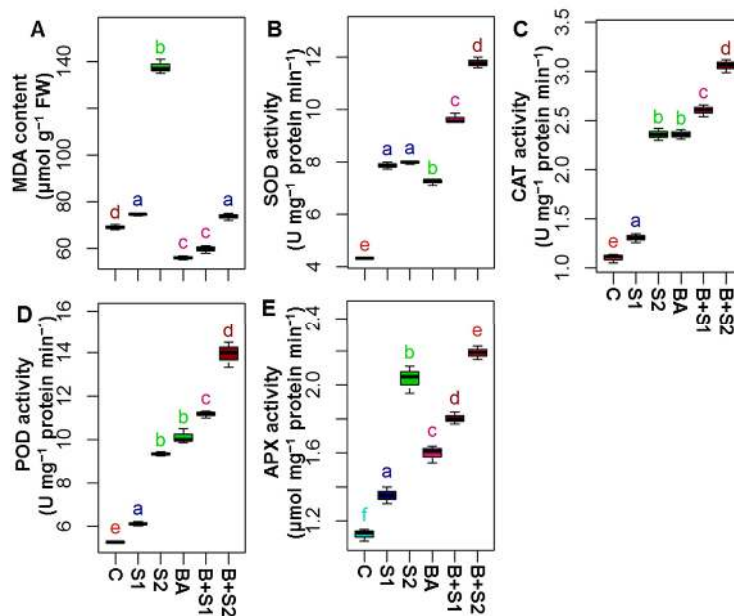


Figure 5. Levels of (A) malondialdehyde (MDA) content (B) SOD (superoxide dismutase) activity, (C) CAT (catalase) activity, (D) POD (peroxidase) activity, and (E) APX (ascorbate peroxidase) activity in broad bean plants exposed to salinity with or without 6-benzyladenine (BA) foliar spray

For each treatment, values were obtained from three independent replicates ($n = 3$). Different letters above the bars indicate a statistically significant difference based on $P < 0.05$ by Tukey’s test. ‘C’, 0 mM NaCl + distilled water spray (control); ‘BA’, 0 mM NaCl + 200 ppm BA spray; ‘S1’, 60 mM NaCl + distilled water spray; ‘S2’, 150 mM NaCl + distilled water spray; ‘S1+B1’, 60 mM NaCl + 200 ppm BA spray; and ‘B+S2’, 150 mM NaCl + 200 ppm BA spray.

Furthermore, ‘BA’ plants also showed a significant reduction in MDA content (Figure 5A). The activities of SOD, CAT, POD, and APX were significantly increased (by 85.02%, 113.90%, 77.23%, and 81.85%, respectively) in ‘S2’ plants compared with ‘C’ plants (Figure 5B-E). Moreover, ‘S1’ plants followed a similar trend in all studied antioxidant enzymes (Figure 5B-E). However, ‘B+S1’ plants showed significant improvement in the activity of SOD, CAT, POD, and APX by 22.59%, 99.23%, 82.42%, and 33.58%, respectively, in comparison to ‘S1’ plants (Figure 5B-E). Likewise, ‘B+S2’ plants markedly improved the activities of SOD, CAT, POD, and APX by 47.62%, 29.66%, 49.29% and 7.53%, respectively, in comparison

with the 'S2' plants (Figure 5B-E). Furthermore, enhanced activities of all examined antioxidant enzymes were recorded in 'BA' plants compared with 'C' plants (Figure 5B-E).

Hierarchical clustering and PCA analysis

The morpho-physiological, biochemical, and growth attributing data under both control and salinity stress conditions were subjected to hierarchical clustering, heatmap analysis, and PCA (Figure 6).

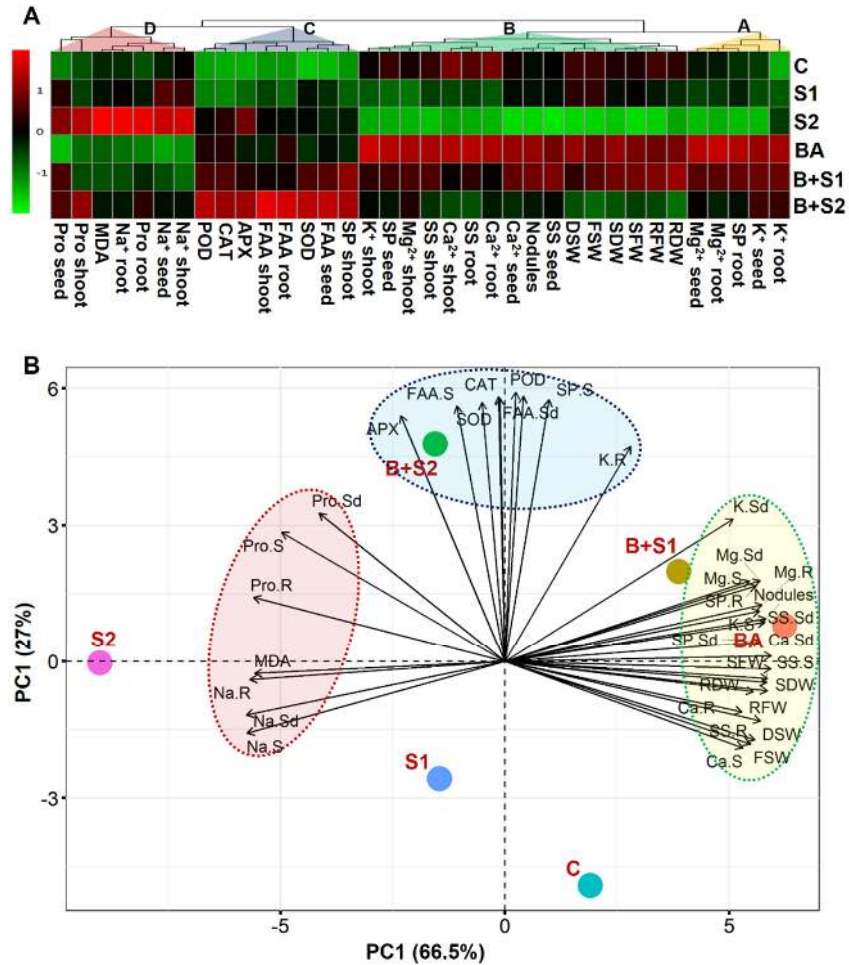


Figure 6. Hierarchical clustering and heatmap (A) to elucidate the treatment variable relationships. The mean values of the various parameters obtained in this study were normalized and clustered. The entire dataset was analysed using principal component analysis (PCA) (B)

The variables included shoot fresh weight (SFW), fresh seed weight (FSW), root dry weight (RDW), shoot dry weight (SDW), dry seed weight (DSW), no. of nodules, soluble sugars (SS), soluble proteins (SP), free amino acids (FAA), proline (Pro), malondialdehyde content (MDA), superoxide dismutase (SOD) activity, catalase (CAT) activity, peroxidase (POD) activity, ascorbate peroxidase (APX) activity. 'C', 0 mM NaCl + distilled water spray (control); 'BA', 0 mM NaCl + 200 ppm BA spray; 'S1', 60 mM NaCl + distilled water spray; 'S2', 150 mM NaCl + distilled water spray; 'S1+B1', 60 mM NaCl + 200 ppm BA spray; and 'B+S2', 150 mM NaCl + 200 ppm BA spray.

According to Hierarchical clustering results, these parameters were classified into four clusters (cluster-A–D) (Figure 6A). K⁺ root, K⁺ seed, SP root, Mg²⁺ root and Mg²⁺ seed were clustered into cluster-A. and RDW, RFW, SFW, SDW, FSW, DSW, SS seed, no. of nodules, Ca²⁺ seed, Ca²⁺ root, SS root, Ca²⁺ shoot, SS shoot, Mg²⁺, SP seed, K⁺ shoot were included in cluster-B. In comparison with 'C', all cluster-A and cluster-B

included parameter showed a negative affinity in ('S1' and 'S2') broad bean under salt stress, whereas a positive affinity was noticed in BA-treated salt-stressed ('B+S1' and 'B+S2') broad bean in comparison with salt-stressed plants (Figure 6A). It has been observed that BA treated stress-free broad bean which showed the highest level of increment of these parameters were grouped into cluster-A and cluster-B. However, cluster-C was comprised of SP shoot, FAA seed, SOD, FAA root, FAA shoot, APX, CAT and POD (Figure 6A). In comparison with 'C', every parameter of cluster-C exhibited an increasing trend in both salt-stressed ('S1' and 'S2') and BA-treated salt-stressed ('B+S1' and 'B+S2') broad bean plants (Figure 6A). Moreover, B+S2 treated stressed broad bean plants that exhibited the highest level of increment of these parameters were grouped into cluster-C. Cluster D was classified as Na⁺ shoot, Na⁺ seed, Pro root, Na⁺ root, MDA, Pro shoot, Pro seed. On the contrary to other clusters, every parameter of cluster-D showed an increasing trend in salt-affected plants ('S1' and 'S2') in comparison with control (Figure 6A). But the fall of these parameters was observed when the stressed plants are treated with BA ('B+S1' and 'B+S2') broad bean plants. 'S2' plants evidenced that the highest level of increment was observed in those parameters which grouped into cluster-D (Figure 6A).

We accomplished PCA for determining acquaintance between the treatment groups and the morpho-physiological and biochemical parameters. The PCA biplotelicits segregation clearly among the groups of six treatments ('C', 'S1', 'S2', 'B+S1', 'B+S2' and 'BA') (Figure 6B). Both the first and second components of PCA elucidate approximately 93.5% data variability (Figure 6B). Experimental results showed that heatmap cluster-A and -B variables (Figure 6A) were moderately associated with 'C' and strongly associated with 'B+S1' treatments (Figure 6B), whereas the heatmap cluster-C variables (Figure 6A) were intensely interlinked to 'B+S2' treatments (Figure 6B). The variables of heatmap cluster-B (Figure 6A) were sharply interlinked with 'S2' and moderately interlinked with 'S1' treatment (Figure 6B).

Discussion

Various small organic compounds, such as plant growth regulators and signal molecules, could be applied as a powerful tool in improving and enhancing plants' adaptability against several unfavourable environments such as soil salinity (Fahad *et al.*, 2015; Mostofa *et al.*, 2015; Ahmed *et al.*, 2019). Application of exogenous phytohormones including CK has been proven as a promising sight in the alleviation of the adverse effects of salt toxicity in several vegetables and crops, such as brinjal (*Solanum melongena* Mill.), soybean (*Glycine max* L.), mungbean (*Cucumis sativus* L.), rice (*Oryza sativa* L.) and maize (*Zea mays* L.) (Hadiarto and Tran, 2011; Javid *et al.*, 2011a; Wu *et al.*, 2014; Fahad *et al.*, 2015; Tahjib-Ul-Arif *et al.*, 2018b; Akram *et al.*, 2019; Rahman *et al.*, 2019). An important synthetic CK, BA has been recognized as the potential stress-mitigating phytohormones to the scientific community. The purpose of our current investigation is to explore the potential role of BA in enhancing salinity tolerance in broad bean plants.

In plants, salinity from soil or irrigation initially creates an imbalance in ionic homeostasis, which consequently provokes osmotic impairments and oxidative damages, both of which noticeably limit the growth and development of plants (Jiang *et al.*, 2019). The negative effects of the abiotic factor on vegetable-producing plants can be appraised by determining the physiological and morphological attributes (Akram *et al.*, 2019). The current experiment revealed that salinity elicited its obvious adverse effects on broad bean growth and biomass production, whereas the exogenous application of BA assuaged the salt-induced injurious impacts (Figure 1). To understand the underlying defense mechanisms of BA-mediated salt acclimation, we investigated various physio-biochemical characters (Figures 2-5). The RFW, SFW, FSW, RDW, SDW, DSW and no. of nodules of the salt-stressed broad bean were reduced as compared to control (Figure 1), which endured by an imbalance of mineral homeostasis and oxidative injury in plants (Figures 3,5). However, foliar application of BA recovered salt-affected damage by improving the phenotypic characteristics and plant growth features which includes RFW, SFW, FSW, RDW, SDW, DSW and no. of nodules (Figure 1), perhaps by balancing mineral homeostasis (Figure 3), and maintaining ROS production and lipid peroxidation (Figure 5).

In concord with our findings, it was mentioned that exogenous BA enhanced plant growth of brinjal (Wu *et al.*, 2014), maize (Ren *et al.*, 2017; Hu *et al.*, 2020) and wheat under salt-stress and waterlogging conditions (Iqbal *et al.*, 2006). Furthermore, our works were supported by PCA, which implied that BA treated salt injured plant of broad bean showed a stronger correlation with plant growth features as compared with 'S1' and 'S2' plants (Figure 6B), exhibiting an influential application of BA in the mitigation of the salt adverse effect on the plant growth-related features and physiology.

An important osmoprotectant, Pro was widely used as an efficient marker in plant salt tolerance mechanism because of its crucial role in the osmotic adjustment of plants (Sharma *et al.*, 2011; Uddin *et al.*, 2012). Besides, under abiotic stress conditions, osmoregulants lay an important role in the sustainable growth and development of plants (Zulfiqar *et al.*, 2020). Thus, the plant maintains its water balance and osmotic potential by accumulating essential osmolytes like Pro, SS, SP and FAA (Mansour and Ali, 2017; Hasanuzzaman *et al.*, 2019). Our result revealed that proline was rapidly accumulated by salt-exposed broad bean and subsequent increment of SP and FAA content except for SS to regulated the metabolic activity by maintaining osmotic homeostasis (Figure 2). Our findings is similar to the results obtained from the response dos salt stressed cucumber (*Cucumis sativus* L.) (Fan *et al.*, 2012), *Salvadora persica* (Parida *et al.*, 2016), maize (Fu *et al.*, 2017) and rice (Wang *et al.*, 2018), drought-stressed barley (*Hordeum vulgare* L.) (Bandurska *et al.*, 2017; Dbira *et al.*, 2018) and cold-stressed rice (Sohag *et al.*, 2020b). However, the exogenous supply of BA significantly reduced the excess Pro produced under salt stress and improved plant growth (Figures 1, 2J-L). Intriguingly, increased levels of SS, SP, FAA in 'B+S1' and 'B+S2' plants revealed that exogenous BA strongly compensated Pro by absorbing those osmoprotectants to ensure osmotic adjustments (Figure 2). A parallel result was reported in eggplant, wheat and rapeseed (*Brassica napus* L.) plants (Wu *et al.*, 2014; Nawaz *et al.*, 2015; Khan *et al.*, 2019a, 2019b).

Our study indicated that excess salt accumulation in broad bean destroyed the balance of mineral homeostasis and thus the plant growth was retired. Salt stress decreased K^+ , Ca^{2+} and Mg^{2+} contents in all parts (except K^+ content in root) (Figure 3D-L), which because of excess accumulation of Na^+ in shoot and other organs (Figure 3A-C). With the increased absorption rate of Na^+ and Cl^- ions, a significant decline in other ions (e.g., K^+ , Ca^{2+} , and Mg^{2+}) exhibited in many experiments (Hakim *et al.*, 2014; Orlovsky *et al.*, 2016; Mahmud *et al.*, 2017). Exogenous plant hormone (e.g. CK) application on salt-stressed rice and rapeseed plants was found to alleviate the salt antagonistic effects on the morpho-physiological and biochemical characteristics of these crops (Anuradha and Rao, 2001; Khan *et al.*, 2019b). However, exogenous BA application re-established ionic balance by mitigating uptake of excessive Na^+ and enhancing the K^+ , Ca^{2+} and Mg^{2+} level in broad bean which may ensure proper nourishment and normal plant growth (Figures 1, 3). Our present results are strongly correlated K^+ , Ca^{2+} and Mg^{2+} contents with 'B+S1' and 'B+S2' plants than 'S1' and 'S2' plants which was clearly and powerfully supported by PCA analysis (Figure 6B). A similar result was noticed in several experiments that were undertaken in different environmental stress conditions (Gurmani *et al.*, 2007; Ding *et al.*, 2017; Yan *et al.*, 2018; Sohag *et al.*, 2020a). Moreover, control, 'B+S1' and 'B+S2' plants accumulated higher concentrations of Ca^{2+} and Mg^{2+} (Figure 3G-L). It suggested that exogenous BA enhanced plant growth by up-regulating the uptake capacity of divalent cations in broad bean. Enhanced content of Ca^{2+} and Mg^{2+} plays a crucial role in the proper functioning of some biological processes such as protein synthesis, enzymatic stimulation, signal transduction and fortification of cell membrane structure fortification (Chen *et al.*, 2010; Guo, 2017). The abundance of nutrients especially K^+ to plants enhances the plant's ability to survive under stressed conditions (Shrivastava and Kumar, 2015).

Our study also revealed that nutrient (Na^+ , K^+ , Ca^{2+} and Mg^{2+}) translocations from root to shoot, shoot to seed and root to seed were severely attenuated as much of these ions were accumulated in roots of 'S1' and 'S2' plants (Figure 4A-L). Moreover, our findings further revealed that the preferential allocation of minerals in roots contributed to retard plant growth and injury in the aerial portion of broad bean under salt stress (Figures 1A, 4F-J). On the contrary, exogenous supplementation of BA superbly augmented mineral uptake and translocation in 'B+S1' and 'B+S2' plants (Figure 4F-J). BA-induced improvement of plant roots

branching and growth of root-shoot growth likely facilitated broad bean foraging for excess mineral and water from the neighboring and adjacent rooted layer of soil (Khan *et al.*, 2018; Nadeem *et al.*, 2019).

MDA has been recognized as an excellent marker for measuring the destruction of the membrane due to oxidative stress in plants (Miller *et al.*, 2010). ROS accumulation in response to salt stress is responsible for creating oxidative damage, showed a positive and frequent correlation with the content of MDA in plants, as noticed in soybean, broad bean, wheat and *Rosa hybrida* (Siddiqui *et al.*, 2017; Soundararajan *et al.*, 2018). The present research revealed that excess salt uptake in broad bean resulted in oxidative damage by overproducing ROS and thus MDA content was significantly increased in leaves, which ultimately promoted huge growth retardation and yield loss of broad bean (Figures 1, 5A). On the contrary, exogenous BA treatment to broad bean leaves alleviated salt-mediated oxidative injury, as strongly supported by the reduced MDA content in the of 'B+S1' and 'B+S2' plants leaves (Figure 5A), which was similar to the findings in maize under salinity and submerge conditions and faba bean under salt stress (Shah, 2011; Ren *et al.*, 2018; Tahjib-Ul-Arif *et al.*, 2018b; Ahmad *et al.*, 2019). When the plants are exposed to salt stress, it produces toxic ROS which resulted in oxidative damage and retard the growth of the plant (Tang *et al.*, 2015; Roy *et al.*, 2019). However, the increased activities of some antioxidants showed an efficient and effective remedy against ROS toxicity and oxidative damage (Hanin *et al.*, 2016; Engwa, 2018). In our current investigation, we observed that the enzymatic activity of all antioxidants (namely SOD, CAT, POD and APX) was enhanced in both salt-treated plants (Figure 5B-E). Our result was supported by experiments on soybean (Weisany *et al.*, 2012), maize (Tahjib-Ul-Arif *et al.*, 2018b) and rice (Roy *et al.*, 2019), whereas opposite findings were reported in *Pisum sativum* (Öztürk, 2012) and rice (Tahjib-Ul-Arif *et al.*, 2018a). However, foliar spraying BA to both stress-treated plant leaves, further accelerated the activities of all studied antioxidant enzymes (Figure 5B-E), suggesting an advanced antioxidant capacity of the broad bean plants in response to 'B+S1' and 'B+S2' treatment to detoxify salt-induced ROS. In current experiments, BA stimulated the activities of antioxidant enzyme SOD and POD under salt stress, according to the study in *Nigella sativa* (Shah, 2011), eggplant (Wu *et al.*, 2014) and maize (Hu *et al.*, 2020). Moreover, BA increased the activities of CAT and APX under salt stress, which results were supported by the findings in eggplant (Wu *et al.*, 2014), strawberry (Faghih *et al.*, 2017) and faba bean (Ahmad *et al.*, 2019). The enhanced activity of SOD was correlated with the salinity tolerance in plants (Ma *et al.*, 2012). Besides, POD, CAT and APX are work as H₂O₂ scavenging enzymes in cells (Mittler, 2002; Roy *et al.*, 2019).

Our results indicate that the potential activity of exogenous BA positively energizes the plant growth by providing oxidative protection which might augment plants' entire metabolic process and antioxidant capacity, thereby broad bean plant develop tolerance mechanism against salt-stress (Figures 1, 5). The PCA also suggested that 'B+S1' and 'B+S2' plants had a strong and positive correlation with SOD, POD, CAT and APX activity than 'S1' and 'S2' plants (Figure 6B).

Conclusions

It is concluded that salt stress caused severe osmotic damage, created oxidative damage, disrupted the essential mineral homeostatic balance and retarded the normal growth of plants. Our present study describes the first proof of exogenous BA-induced plant stress tolerance mechanisms against salinity in broad bean. The favourable application of exogenous BA might contribute to alleviating salt-induced excess adsorption of ROS by improving antioxidant enzymatic activity of SOD, CAT, POD and APX. Moreover, foliar application of BA might maintain osmotic potentiality and plant metabolic activity by modulating Pro, SP, and SS and FAA levels in the broad bean grown in salt-affected soils, which might assist in enhancing essential water uptake capacity and plant physiological response under salinity stress condition. Moreover, under salt stress regimes BA treatment strongly upregulated the mineral homeostasis by controlling Na⁺, K⁺, Ca²⁺ and Mg²⁺ contents. Finally, the detailed study suggests that exogenous application of BA might be considered as an effective and

powerful strategy in alleviating the antagonistic activity of salt toxicity, thereby latterly ensured sustainable growth and development of broad bean. Furthermore, the molecular investigation is needed to explore new insights into a better understanding of the extensive biological functions of BA-mediated salt stress response and tolerance mechanism in broad bean.

Authors' Contributions

Conceptualization: AAHAL; Formal analysis: AAHAL; MTUA; Funding acquisition; AAHAL; Investigation: MH, MTUA; Methodology; AAHAL; Project administration; AAHAL; Resources; AAHAL; Software: MTUA; Supervision; AAHAL; Validation: MH, MTUA; Visualization: MTUA; Writing - original draft: MH; Writing - review and editing: AAHAL, MTUA. All authors read and approved the final manuscript.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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