Exogenous Auxin-Mediated Salt Stress Alleviation in Faba Bean (Vicia faba L.)

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Abstract: Auxin not only controls the development processes, but also regulates the stress responses of plants. In this investigation, we explored the potential roles of exogenously applied indole-3-acetic acid (IAA) in conferring salt tolerance in the faba bean (Vicia faba L.). Our results showed that foliar application of IAA (200 ppm) to salt-exposed (60 mM and 150 mM NaCl) plants promoted growth, which was evidenced by enhanced root–stem traits. IAA application ensured better osmotic protection in salt-stressed plants which was supported by reduced proline and enhanced soluble sugar, soluble protein, and total free amino acid contents in the roots, stem, and seeds. IAA application also increased the number of nodules in salt-stressed plants, which may facilitate better nitrogen assimilation. Moreover, IAA mediated improvements in mineral homeostasis (K+, Ca2+, and Mg2+) and the translocation of Na+, while it also inhibited excessive accumulation of Na+ in the roots. Salt-induced oxidative damage resulted in increased accumulation of malondialdehyde, whereas IAA spraying relegated malondialdehyde by improving antioxidant enzymes, including superoxide dismutase, catalase, peroxidase, and ascorbate peroxidase. Together, these results together with a principal component analysis uncovered that foliar spraying of IAA alleviated the antagonistic effects of salt stress via enhancing osmolyte accumulation, ionic homeostasis, and antioxidant activity. Finally, exogenous IAA enhanced the yield of broad beans under high salinity conditions.

Keywords: antioxidants; broad bean; indole-3-acetic acid; nutrient homeostasis; proline; plant growth; salinity stress

1. Introduction

The existence of excessive levels of salts in agricultural fields is a serious current global issue that severely hampers crop production [1]. Recent reports show that approximately 0.80 billion hectares of terrestrial areas are disturbed by salts and this substantially limits the use of land for agricultural purposes [2,3]. From an agricultural perspective, about 20% of the crop-cultivable area and about 33% of irrigated crop-land are affected by salinity levels to varying degrees, and by 2050, this figure will exceed 50% [2,4]. Generally, exposure of plants to high concentrations of salts can inhibit growth and delay developmental processes by causing several adversities, such as an osmotic imbalance, excessive sodium (Na+) and chloride (Cl−)-induced cytotoxicity, and nutritional discrepancy [1,5]. Additionally, at a later stage, salinity-exposed plants experience enhanced oxidative stress because of the production of excessive amounts of reactive oxygen species (ROS). ROS production results in oxidative injuries to different cellular macromolecules, including lipids, proteins, and nucleic acids, which ultimately disrupts many important cellular processes in plants [1,6,7].
The responses of plants to salt stress can be split into two stages. The first phase proceeds within minutes to days, triggering ion-independent growth reductions by inducing stomatal closure and inhibiting the expansion of cells [8,9]. The buildup of salts in the rhizosphere limits the water uptake capacity of root systems, and thus, creates osmotic stress in plants [1,8]. This salinity-mediated osmotic stress causes many adversities in the physiological processes of plants, such as membrane lipid peroxidation, essential mineral aberration, impairment of ROS detoxification ability, antioxidative enzyme disparities, reduced photosynthetic capacity, and decreased stomatal aperture [9,10]. The second phase begins days or even weeks after salinity exposure and includes the accumulation of ions at higher concentrations, causing cytotoxicity. In particular, Na\(^+\) and Cl\(^-\) toxicity decelerates that action of many metabolic pathways which results in the initiation of premature senescence and cell death [1,8,11]. Sodium is a non-essential mineral for all plants, except for few C4 plants [12,13]. When the accumulation of Na\(^+\) exceeds the normal level, it becomes highly detrimental to the plants. It can alter cellular ionic homeostasis by inducing cytosolic K\(^+\) efflux from cells. Moreover, it restricts the functions of other essential minerals, including Mg\(^{2+}\) and Ca\(^{2+}\), and eventually causes a nutrient deficiency in plants. Na\(^+\) toxicity can also instigate oxidative stress in plant cells, eventually leading to growth retardation and possibly even the death of plant tissues [9,12,14].

To thrive in soils with high salt concentrations, plants have evolved different physicochemical mechanisms [8]. Examples of such mechanisms include homeostasis and the compartmentation of ions. Maintenance of nutrient homeostasis through ion absorption and translocation is not only important for normal plant growth, but is also an essential process for salt tolerance [10,14,15]. Elevated salt concentrations are not tolerated by either glycophytic and halophytic plants [10]. Excess Na\(^+\) ions are either transferred to the vacuole or sequestered into older tissues, thus, defending plants from Na\(^+\)-provoked injury [15,16]. Osmoprotectants and compatible solutes, also called osmolytes, are organic compounds with no charge that are soluble in water and do not create cell metabolism problems, even at high concentrations [17]. In plants, the commonly synthesized osmolytes are free amino acids that include proline, soluble sugars, and polyols [17]. In unfavorable environmental conditions, plant cells produce and store a higher amount of compatible solutes to adjust the water balance [15,18]. Moreover, to gain protection from oxidative stress, plants synthesize antioxidant compounds and activate several antioxidant enzymes [15]. The enzymatic antioxidant system comprises several enzymes, such as superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), and peroxidase (POX, EC 1.11.1.7), which are capable of degrading ROS to less toxic or non-toxic intermediates [15,19–21]. In addition to this, ascorbate peroxidase (APX, EC 1.11.1.1)—an enzyme of the Foyer–Halliwell–Asada pathway—performs a principal function in ROS homeostasis [19]. Overall, up-regulation of these osmoregulatory and antioxidative systems can confer stress tolerance in plants via maintenance of water balance and ROS detoxification. Moreover, the promotion of these systems by means of exogenous mechanisms helps plants to develop stress tolerance. Exogenous application of phytohormones, including auxin, represents a promising option in this regard.

One of the most important forms of auxin is indole-3-acetic acid (IAA), most of which is generated in the apical portion of plants, often in new buds and leaves [22]. IAA affects diverse growth-related and developmental aspects in plants [22,23]. It also modulates a wide range of physiological events, including the elongation of cells, and induction of root growth, flower production, and fruit production [24,25]. Treatment with IAA greatly restores the growth of maize (Zea mays L.) cultivated in saline soils [26]. It has previously been reported, that even the development of fruit in tomato plants (Solanum lycopersicum L.) is enhanced with the use of IAA [27]. Since growth promoters are involved in modifying the growth of crop plants, the undesirable consequences of salinity can be rescued by boosting growth [28]. Exogenous application of IAA improved the growth of plants by ensuring better germination, osmoregulation, nutrient homeostasis, and antioxidative systems in potato (Solanum tuberosum L.) grown in vitro in a saline medium [29], in rice (Oryza sativa
L.) grown in pots containing saline soils [30], in fenugreek (Trigonella foenum-graecum L.) grown in cadmium-contaminated soils [24], in spring wheat (Triticum aestivum L.) grown in pots containing saline soils [28], and in rice under high-temperature stress [31].

Faba bean is the third most cultivated legume crop and is grown in more than sixty countries [32]. The production of V. faba is severely restricted by saline soils, especially in semi-arid regions [33]. Therefore, in the current experiment, we attempted to determine whether exogenous IAA offers a defense mechanism to V. faba against salinity stress, as is the case for many other food crops grown under diverse environmental stress conditions. Furthermore, in the case that IAA does confer defense, we attempted to demonstrate the mechanisms that contribute to the increased ability of V. faba to withstand salinity.

2. Materials and Methods

2.1. Experimental Conditions

The seeds of V. faba, cultivar ‘Assiut 95’ were disinfected for 5 min using a mercuric chloride (0.1%) solution and then washed three times. A total amount of 5 kg of air-dried soil (sand: clay, 3:1 v/v) was loaded in each plastic pot. A total of 10 healthy-looking seeds were sown in each pot. A 15 cm long perforated tube was injected into the pot to facilitate addition of nutrient and treatment solutions. The pots were watered regularly with distilled water and a stable osmotic potential was maintained for uniform seed germination. This study was conducted in a completely randomized manner in greenhouse conditions with a relative humidity of 65–75% and an average night and day temperature ranging between 27–35 °C. One set of germinated V. faba seedlings was subjected to NaCl (60 mM and 150 mM NaCl) stress on the 14th day after sowing. Both salinity-stressed and non-stressed plants were sprayed with IAA solution (50 mL per pot for each spray) two times 10 and 20 days after salinization. The plants belong to the control treatment were sprayed with the same amount of distilled water. The concentration of IAA solution was 200 ppm. This concentration was selected based on a preliminary experiment. Thus, there were six treatments, as follows: (i) control (C), 0 mM NaCl + water spray; (ii) 60 mM NaCl + water spray (S1); (iii) 150 mM NaCl + water spray (S2); (iv) 0 mM NaCl + 200 ppm IAA (IAA); (v) 60 mM NaCl + 200 ppm IAA (IAA + S1); (vi) 150 mM NaCl + 200 ppm IAA (S2 + IAA). Treatments were repeated three times and a total of 18 pots were organized in a completely randomized design, where each pot contained 5 plants (after thinning). The experiment was continued for 114 days.

2.2. Plant Growth Traits Measurement

Plant root and stem samples were collected 100 days after salinization. At first, the root fresh weight (RFW), and stem fresh weight (SFW) were measured. Then, samples were desiccated in a ventilated oven at 80 °C for 96 h and the root dry weight (RDW) and stem dry weight (SDW) were recorded. Samples were also ground and stored for different chemical analyses. After 114 days post sowing, harvesting was performed. The number of nodules (NOD) in roots was counted and the fresh weight of the pods (fresh yield) was recorded and following drying, the dry weight of the pods (dry yield) was recorded.

2.3. Osmolytes or Primary Metabolites Content Determination

To assess the osmolytes level and primary metabolite status, different biochemical parameters, such as proline (Pro), total soluble sugars (TSS), total soluble proteins (TSP), and total free amino acid (TFAA) contents, were determined using the fresh roots, stem, and seeds of V. faba plants. An acid ninhydrin-based method was used to determine the Pro content [34]. Using anthrone-sulfuric acid, the TSS content of V. faba was determined and glucose was used as the standard [35]. The TSP content was determined using Folin phenol reagent and bovine serum albumin was used as the standard [36]. The content of TFAA was determined using ninhydrin, following a published protocol [37].
2.4. Mineral Ion Content Determination

To analyze the mineral homeostasis status of *V. faba* plants, different mineral contents—such as Na\(^+\), K\(^+\), Ca\(^{2+}\), and Mg\(^{2+}\)—of the roots, stem, and seeds were determined. After harvesting, samples were rinsed with deionized water to remove surface minerals. Dried and ground plant parts were treated with the di-acid mixture (HNO\(_3\) and HClO\(_4\) at 2:1 \(v/v\) ratio) for 2.5 h at 220 °C in a Kjeldahl digestion block, as previously described [38]. The Na\(^+\) and K\(^+\) contents were measured by an atomic absorption flame spectrophotometer (Model AA-6400 F, Shimadzu Corporation, Kyoto, Japan) [39]. The Ca\(^{2+}\) and Mg\(^{2+}\) contents were determined using the versene (disodium dihydrogen ethylene-diamine-tetraacetic acid) method of titration [40].

2.5. Lipid Peroxidation Determination

The level of lipid peroxidation in terms of the malondialdehyde (MDA) content was determined for *V. faba* leaves, following a standard procedure [41]. Fresh samples were homogenized with 5 mL of 5% trichloroacetic acid (TCA) solution and centrifuged at 15,000 \(\times\) g for 10 min at 4 °C. After this, 4 mL of 20% TCA, containing 0.5% thiobarbituric acid solution, was mixed with 2 mL of the plant extract in an air-tight glass tube. Then, the tubes were incubated at 95 °C for half an hour and then quickly transferred to iced-water. The tubes were then centrifuged at 10,000 \(\times\) g for 10 min and the supernatants were collected in a new glass tube. The tubes were again quickly placed in an ice bath and the centrifugation step was repeated for 10 min at 11,000 \(\times\) g. The absorption of the supernatant was quantified at 532 nm and 600 nm wavelength, respectively. The MDA content was calculated by using an absorption coefficient of 155 mM\(^{-1}\) cm\(^{-1}\).

2.6. Antioxidant Enzymatic Activity Measurement

First, 0.2 g fresh leaf samples of *V. faba* plants were homogenized using a pestle in a chilled mortar along with liquid nitrogen. Then, 5 mL of 100 mM potassium-phosphate (K-P) buffer (100 mM, pH 7.0) was added to disodium EDTA (0.1 mM) and polyvinylpyrrolidone (0.1 g). The homogenized samples were filtered with fine-cloth and centrifuged at 14,500 \(\times\) g for 10 min at 4 °C and the supernatants were used for antioxidant enzyme assays. The SOD activity was determined, as described earlier in the protocol [42]. A total of 3 mL of solution was prepared by adding the mixture containing K-P buffer (50 mM, pH 7.8), EDTA (0.1 mM), L-methionine (13 mM), riboflavin (2 \(\mu\)M), and nitroblue tetrazolium (75 \(\mu\)M) with 2 mL leaf extract. For initiating the reaction, the mixtures were exposed to fluorescent light (cool white) for 15 min and the blue reaction color was determined spectrophotometrically at 560 nm. Determination of CAT activity was performed based on a published protocol [43]. A total of 3 mL reaction solution, comprised of K-P buffer (50 mM, pH 7.0), H\(_2\)O\(_2\) (30%, \(w/v\)), and leaf extract, was used for the CAT activity assay. The enzymatic activity of CAT was determined by observing the changes in the absorbance of H\(_2\)O\(_2\) at 240 nm. POD activity was determined using guaiacol [44]. Leaf extract (0.5 mL) was added to a 3.0 mL reaction mixture of K-P buffer (10 mM, pH 7.0), H\(_2\)O\(_2\) (10 mM), and guaiacol (20 mM). Increased absorbance, as a consequence of the production of tetraguaiacol, was observed at 470 nm. The decreased absorbance at 290 nm as ascorbic acid was oxidized indicated APX activity [45]. APX was assayed with the reaction mixtures (3.0 mL) containing K-P buffer (50 mM, pH 7.0), ascorbic acid (0.5 mM) and H\(_2\)O\(_2\) (0.5 mM). All spectrometric readings were recorded through a Spectronic Genysis\textsuperscript{TM} 2PC spectrophotometer, Spectronic Instruments, Waltham, MA, USA.

2.7. Statistical Analysis

One-way analysis of variance (ANOVA) along with the Tukey’s test \((p < 0.05)\) was performed using the “multcompView” package in the statistical programming language R 3.6.1. The “pheatmap” package was used for constructing a heatmap and the hierarchical clustering analysis, considering the Euclidean distances, and “ggplot2”, “factoextra”, “FactoMineR” packages were used for the principal component analysis (PCA) in R 3.6.1.
3. Results

3.1. Exogenous IAA Enhanced Morphological Parameters of V. faba under Salt Stress

We investigated the effects of exogenous IAA on the salinity-induced growth inhibition of V. faba. The results show that salt-stressed S1 and S2 plants demonstrated significantly reduced levels of SFW (by 29.5 and 44.3%), SDW (by 20.3 and 35.6%), fresh yield (by 6 and 26.3%), dry yield (by 19.3 and 35.5%), and NOD (by 20.5 and 53.4%), respectively (Figure 1B,C,E–G). Whereas a significant reduction in RFW (by 25.1%) and RDW (by 53.8%) was only found in S2 plants (Figure 1A,D). Foliar application of IAA to the S1 (IAA + S1) and S2 (IAA + S2) plants significantly enhanced RFW (by 20.1 and 43.6%), SFW (by 50 and 53.8%), RDW (by 24.2 and 131.1%), SDW (by 71.5 and 50.1%), fresh yield (by 7.1 and 14.4%), dry yield (by 26.4 and 34.9%), and NOD (by 30.6 and 45.6%) compared to the S1 and S2 plants, respectively (Figure 1). Moreover, a single exposure of IAA also resulted in a significant increase of such morphological parameters over the control. These results indicate that salt stress significantly inhibit the growth of V. faba and exogenous application of IAA significantly alleviates salt-induced growth inhibition.

![Figure 1. Effects of exogenous indole-3-acetic acid (IAA) on various growth traits, such as (A) root fresh weight, (B) stem fresh weight, (C) fresh yield, (D) root dry weight, (E) stem dry weight, (F) dry weight, (G) number of nodules, of V. faba plants grown under non-stress and saline conditions. Each boxplot represents values of three independent replicates (n = 3). Different letters above the bars indicate a statistically significant difference (p < 0.05) by Tukey’s test. C, (control) 0 mM NaCl + water spray; S1, 60 mM NaCl + water spray; S2, 150 mM NaCl + water spray; IAA, 0 mM NaCl + 200 ppm IAA; IAA + S1, 60 mM NaCl + 200 ppm IAA; IAA + S2, 150 mM NaCl + 200 ppm IAA.](image-url)

3.2. Exogenous IAA Increased Osmolytes and Primary Metabolite Contents in V. faba under Salt Stress

The salt-stressed S1 and S2 plants demonstrated enhanced contents of TSS in the roots (by 12.3 and 11.9%), TSP in the stem (by 3.3 and 17.4%) and seeds (by 15.7 and 22.6%), FAA in roots (by 20.1 and 36.8%) and seeds (by 7.2 and 17.2%), and Pro in roots (by 13.2 and 40.9%), stem (by 9.9 and 45.0%), and seeds (by 12.4 and 40.2%), respectively (Figure 2). On the contrary, salt-stressed S1 and S2 plants showed reduced contents of TSS in the stem (by 6.3 and 38.3%) and seeds (by 13.8 and 26.2%), respectively (Figure 2B,C), whereas
reduced TSP content in the roots (by 6.1%) was observed in the case of S1 plants (Figure 2D). Exogenous application of IAA to the S1 (IAA + S1) and S2 (IAA + S2) plants increased the contents of TSS in the roots (by 11.8 and 8.7%), stem (by 10 and 58.7%), and seeds (by 44.5 and 109.1%); TSP in the roots (by 29.6 and 46.2%), stem (by 4.8 and 6.6%), and seeds (by 81.5 and 22.2%); TFAA in the roots (by 30.1 and 56.5%), stem (by 20.8 and 15.6%), and seeds (by 13.1 and 28.2%) and Pro in the roots (by 7.58 and 14.5%), and seeds (by 9.6 and 10.0%) compared to the S1 and S2 plants, respectively (Figure 2). However, IAA + S1 plants did not show any significant change in the contents of TSS in the stem compared to S1 plants (Figure 2K). Furthermore, a single application of IAA also increased the TSS content in the roots (by 7.1%) and seeds (by 18.3%) (Figure 2A,B), TSP content in seeds (by 86.5%) (Figure 2F), TFAA content in all organs (by 63.5, 15.7, and 11.1%) (Figure 2G–I), and Pro content in the roots (by 9.6%) and seeds (by 6.9%) (Figure 2J,L). These results indicate that foliar application of IAA enhanced the osmolyte and primary metabolite contents in *V. faba* under salt stress.

![Figure 2. Cont.](image-url)
Figure 2. Effects of exogenous IAA on various osmolytes and primary metabolites in the roots, stem and seeds, including (A–C) total soluble sugars (TSS), (D–F) total soluble proteins (TSP), (G–I) total free amino acid (TFAA), and (J–L) proline (Pro) content of *V. faba* plants grown under non-stress and saline conditions. Each boxplot represents values of three independent replicates ($n = 3$). Different letters above the bars indicate a statistically significant difference ($p < 0.05$) by Tukey’s test. C, (control) 0 mM NaCl + water spray; S1, 60 mM NaCl + water spray; S2, 150 mM NaCl + water spray; IAA, 0 mM NaCl + 200 ppm IAA; IAA + S1, 60 mM NaCl + 200 ppm IAA; IAA + S2, 150 mM NaCl + 200 ppm IAA.

3.3. Exogenous IAA Regulated Ionic Homeostasis in *V. faba* under Salt Stress

The salt-stressed S1 and S2 plants showed significantly increased Na$^+$ contents in the roots (by 231.3 and 513.8%), stem (by 201.8 and 442.8%), and seed (by 194.8 and 407.9%) compared with control plants (Figure 3A–C). A significant reduction was observed in K$^+$ content in the roots (by 25.0%), stem (by 29.5%), and seeds (by 23.5%) (Figure 3D–F). A significant reduction was also found in Ca$^{2+}$ content in the stem (by 44.2%) and seeds (by 38.4%) (Figure 3H,I), Mg$^{2+}$ content in the roots (by 14.3%) and stem (by 60.1%) (Figure 3J,K) in S2 plants compared with the control. Additionally, salt-stressed S1 plants demonstrated decreased Ca$^{2+}$ content in the seeds (by 15.8%) (Figure 3I) and Mg$^{2+}$ content in the stem (by 30.3%) (Figure 3K) compared with the control. Application of IAA to the S1 (IAA + S1) and S2 (IAA + S2) plants significantly reduced the Na$^+$ content in the roots (by 31.8 and 49.9%), stem (by 26.6 and 38.47%), and seeds (by 39.7 and 27.63%) compared to the S1 and S2 plants, respectively (Figure 3A–C), whereas IAA application to the S1 (IAA + S1) and S2 (IAA + S2) plants increased the K$^+$, Ca$^{2+}$, and Mg$^{2+}$ content in all organs compared to the S1 and S2 plants (Figure 3D–L). However, single application of IAA also increased the Ca$^{2+}$ and Mg$^{2+}$ content in all parts of the plants (Figure 3G–L) and K$^+$ content in the stem and seeds (Figure 3E,F).

3.4. Effects of Exogenous IAA Application on Minerals Translocation in *V. faba* under Salt Stress

The salt-stressed S1 and S2 plants showed significantly reduced levels of translocation factor Na$^+$ from the root to the stem (by 8.9 and 11.5%) (Figure 4A) and translocation factor Mg$^{2+}$ from the root to the stem (by 25.7 and 55.7%) (Figure 4D) compared with the control. Meanwhile, the translocation factor Ca$^{2+}$, from the root to the stem (by 30.5%), was reduced only in S2 plants compared with the control (Figure 4C). Supplementation of IAA to the IAA + S1 and IAA + S2 plants significantly increased the Na$^+$ translocation factor from the root to the stem (by 7.7 and 22.3%) compared to the S1 and S2 plants, respectively (Figure 4A). In addition, increases in translocation factor Mg$^{2+}$ from the root to the stem (by 42.1%) were observed in IAA + S2 plants compared to the S2 plants (Figure 4D). IAA + S1 plants showed significantly reduced Ca$^{2+}$ translocation factor levels from the root to the stem (by 15.1%) compared to the S1 plants (Figure 4C). Furthermore, a single application of IAA showed a significant increase in K$^+$ translocation factor levels from the root to the stem (by 6.6%) (Figure 4B), while a decrease in Na$^+$ (by 6.8%) and Ca$^{2+}$ (by 12.2%) translocation...
factors from the root to the stem was observed following the single application of IAA (Figure 4A,C).

Figure 3. Effects of exogenous IAA on various mineral contents in root, stem and seed, including (A–C) Na\(^{+}\), (D–F) K\(^{+}\), (G–I) Ca\(^{2+}\), and (J–L) Mg\(^{2+}\) contents of \(V\). \(faba\) plants grown under non-stress and saline conditions. Each boxplot represents values of three independent replicates \((n = 3)\). Different letters above the bars indicate a statistically significant difference \((p < 0.05)\) by Tukey’s test. C, (control) 0 mM NaCl + water spray; S1, 60 mM NaCl + water spray; S2, 150 mM NaCl + water spray; IAA, 0 mM NaCl + 200 ppm IAA; IAA + S1, 60 mM NaCl + 200 ppm IAA; IAA + S2, 150 mM NaCl + 200 ppm IAA.
factor Mg\(^{2+}\) from the root to the stem (by 25.7 and 55.7\%) (Figure 4D) compared with the control. Meanwhile, the translocation factor Ca\(^{2+}\), from the root to the stem (by 30.5\%), was reduced only in S2 plants compared with the control (Figure 4C). Supplementation of IAA to the IAA + S1 and IAA + S2 plants significantly increased the Na\(^{+}\) translocation factor from the root to the stem (by 7.7 and 22.3\%) compared to the S1 and S2 plants, respectively (Figure 4A). In addition, increases in translocation factor Mg\(^{2+}\) from the root to the stem (by 42.1\%) were observed in IAA + S2 plants compared to the S2 plants (Figure 4D). IAA + S1 plants showed significantly reduced Ca\(^{2+}\) translocation factor levels from the root to the stem (by 15.1\%) compared to the S1 plants (Figure 4C). Furthermore, a single application of IAA showed a significant increase in K\(^{+}\) translocation factor levels from the root to the stem (by 6.6\%) (Figure 4B), while a decrease in Na\(^{+}\) (by 6.8\%) and Ca\(^{2+}\) (by 12.2\%) translocation factors from the root to the stem was observed following the single application of IAA (Figure 4A,C).

Figure 4. Effects of exogenous IAA on translocation factor of different minerals from root to stem of *V. faba* plants grown under non-stress and saline conditions. (A) Na\(^{+}\) translocation factor: root to stem, (B) K\(^{+}\) translocation factor: root to stem, (C) Ca\(^{2+}\) translocation factor: root to stem, (D) Mg\(^{2+}\) translocation factor: root to stem. Each boxplot represents values of three independent replicates (n = 3). Different letters above the bars indicate a statistically significant difference (p < 0.05) by Tukey’s test. C, (control) 0 mM NaCl + water spray; S1, 60 mM NaCl + water spray; S2, 150 mM NaCl + water spray; IAA, 0 mM NaCl + 200 ppm IAA; IAA + S1, 60 mM NaCl + 200 ppm IAA; IAA + S2, 150 mM NaCl + 200 ppm IAA.

3.5. Exogenous IAA Energized Antioxidant Enzyme Activities and Reduces Lipid Peroxidation in *V. faba* under Salt Stress

The salt-stressed S1 and S2 plants significantly increased in MDA content (by 49.4 and 119.2\%, respectively) compared with the control (Figure 5A), whereas supplementation of IAA to the S1 (IAA + S1) and S2 (IAA + S2) plants decreased the MDA content (by 32.1 and 41.8\%, respectively) compared with the S1 and S2 plants. Similar to MDA, the salt-stressed S1 and S2 plants showed a significant increase in antioxidant enzyme activities, i.e., SOD (by 29.5 and 28.8\%), CAT (by 19.5 and 53.3\%), POD (by 15.4 and 32.9\%), and APX (by 29.4 and 66.9\%) (Figure 5B–E). Furthermore, salt-stressed plants supplemented with IAA also showed increased antioxidant enzyme activities. Additionally, the single application of IAA also enhanced the antioxidant enzyme activities (Figure 5B–E).
Figure 5. Effects of exogenous IAA on (A) malondialdehyde (MDA) content and activities of (B) superoxide dismutase (SOD), (C) catalase (CAT), (D) peroxidase (POD), (E) ascorbate peroxidase (APX) of *V. faba* plants grown under non-stress and saline conditions. Each boxplot represents values of three independent replicates (*n* = 3). Different letters above the bars indicate a statistically significant difference (*p* < 0.05) by Tukey’s test. C, (control) 0 mM NaCl + water spray; S1, 60 mM NaCl + water spray; S2, 150 mM NaCl + water spray; IAA, 0 mM NaCl + 200 ppm IAA; IAA + S1, 60 mM NaCl + 200 ppm IAA; IAA + S2, 150 mM NaCl + 200 ppm IAA.

3.6. Assessment of Treatment-Variable Interaction through Heatmap and PCA

The mean values of all morphological and biochemical parameters were employed to construct a heatmap with hierarchical clustering and PCA (Figure 6). In the variable axis, three clusters (Cluster-A, -B, and -C) were found from the hierarchical clustering (Figure 6A). Cluster-A possesses the TSS stem, K⁺ root, RFW, RDW, K⁺ stem, fresh yield, Ca²⁺ stem, NOD, Mg²⁺ stem, Ca²⁺ seed, SFW, dry yield, Ca²⁺ root, SDW, K⁺ seed, Mg²⁺ root, TSP seed, and Mg²⁺ seed of plants. The cluster-A parameters showed a decreasing trend in S1 and S2 plants and showed an increasing trend in IAA, IAA + S1, and IAA + S2 plants. Cluster-B encompassed the variables MDA, Na⁺ seed, Na⁺ root, and Na⁺ stem. The cluster-B parameters exhibited an increasing trend in S1 and S2 plants and a decreasing trend in IAA, IAA + S1, and IAA + S2 plants. The variables, Pro stem, Pro seed, TSP stem, Pro root, TFAA seed, CAT, TFAA stem, APX, TSS root, SOD, TFAA root, POD, TSS seed, and TSP root were clustered in group C. The highest values of cluster-C parameters were observed in IAA + S2 plants, followed by IAA + S1. Additionally, we performed a PCA to clarify the association of clusters-A, -B, and -C with C, S1, S2, IAA + S1, and IAA + S2 treatments (Figure 6B). The PCA components, PC1 and PC2 jointly exhibited 91.1% of the data variability (Figure 6B). The PCA illustrated that variables of cluster-A were intimately associated with IAA + S1 and IAA treatments. On the other hand, variables of cluster-B were highly correlated with the IAA + S2 treatment. Additionally, variables of cluster-C were intensely connected with S2 treatment and moderately associated with S1 treatment.
Figure 6. Hierarchical clustered heatmap and principal component analysis (PCA) were performed to illustrate the interactions between treatments and all studied parameters. (A) The scaled average values of all studied parameters of *V. faba* are shown using a heatmap with clustering approach. (B) PCA was used to analyze all data. The studied variables are root fresh weight (RFW), stem fresh weight (SFW), root dry weight (RDW), stem dry weight (SDW), number of nodules (NOD), total soluble sugars (TSS), total soluble proteins (TSP), total free amino acid (TFAA), proline (Pro), Na (Na⁺ content), K (K⁺ content), Ca (Ca²⁺ content), Mg (Mg²⁺ content), malondialdehyde content (MDA), superoxide dismutase (SOD) activity, catalase (CAT) activity, peroxidase (POD) activity, ascorbate peroxidase (APX) activity. “R” = root, “S” = stem, “Sd” = seed; C, (control) 0 mM NaCl + water spray; S1, 60 mM NaCl + water spray; S2, 150 mM NaCl + water spray; IAA, 0 mM NaCl + 200 ppm IAA; IAA + S1, 60 mM NaCl + 200 ppm IAA; IAA + S2, 150 mM NaCl + 200 ppm IAA.

4. Discussion

Among abiotic stresses, salinity is the leading stress and can reduce the growth and yield of crop-plants. Exogenous application of different phytohormones is an effective method for minimizing the effects of stressors [46–48]. In our study, salt-induced injury effects were demonstrated in terms of reduced SFW, SDW, RFW, and RDW, which resulted in fresh and dry yield losses of *V. faba* (Figure 1A–F). Similar results have been found in *V. faba* [49–51], chickpeas (*Cicer arietinum*) [52], and artichokes (*Cynara scolymus*) [53]. Foliar spraying with IAA is an effective way to overcome the injuries of cadmium stress [54], salt stress [29,55], and drought stress [56] in various crop-plants. The results presented here also show that foliar spraying with IAA repressed the salt-induced growth inhibition of *V. faba* (Figure 1A–G). Our findings exhibit that IAA + S1 and IAA + S2 plants showed a con-
siderable increase in RFW and RDW compared to salt-stressed V. faba plants (Figure 1A,D). This above-mentioned result indicates that the exogenous foliar spraying of IAA assisted V. faba plants in maintaining healthier growth of roots in saline soils. Later, the improved root traits enabled increased moisture and nutrient uptake from the soil and improved overall plant growth performance. Symbiotic nitrogen fixation via associated rhizobia bacteria is a prime source of biologically available nitrogen in legume plants [57]. Salinity stress severely hampers nodule formation and the levels of nitrogen fixation. In many legumes, the ability to fix nitrogen drops to below 50% when exposed to 50 mM of NaCl [58]. It has been shown that irrigation with 25% sea water reduced the NOD by up to 40%; with 35% sea water, no nodule formation occurred in V. faba [59]. Our results also reveal that with the increment of salinity concentrations, the NOD decreased drastically and IAA application enhanced the NOD in IAA + S2 and IAA + S1 V. faba plants (Figure 1G). Therefore, it can be assumed that IAA improved nodule formation and enabled plants to fix more biologically available nitrogen, with this subsequently facilitating growth enhancement of V. faba plants under saline conditions (Figure 1A–G). Furthermore, auxin is an essential growth hormone for plants and its biosynthesis and metabolism are down-regulated under stress conditions, including salinity [60]. Therefore, exogenous supply of IAA might improve the levels of endogenous auxin and eventually improve the growth of V. faba plants. Finally, improved growth ensured a better yield in IAA + S1 and IAA + S2 V. faba plants compared to those plants that were only treated with salt (Figure 1C,F). It is important to note here, that the PCA analysis showed that the IAA + S1 treatment is strongly associated with growth and yield traits compared to the IAA + S2 treatment (Figure 6B), suggesting that IAA + S1 plants demonstrated improved growth under stress. Moreover, cluster-A in the heatmap showed that the color intensities of C and IAA + S1 treatments are similar (Figure 6A), suggesting that application of IAA completely recovered the 60 mM NaCl-induced growth and yield reductions. Furthermore, in cluster A of the heatmap, IAA treatment showed a higher color intensity than the C treatment, and in PCA, IAA treatment had the strongest association with growth and yield traits (Figure 6), which indicates that IAA application in non-stress conditions can improve the yield of V. faba.

It has been confirmed that plants accumulate osmolytes in cells in response to diverse stress conditions and that osmolytes function in maintaining osmotic amendment in cells under unfavorable conditions [61–63]. Numerous investigations have discovered that salt stress led to higher deposition of osmoregulators such as Pro, TSP, and TFAA in rice and artichoke plants [53,64,65]. In this study, we also found that salt stress increased TSS, TFAA, and Pro contents in different organs of V. faba plants and exogenous application of IAA also boosted TSS, TSP, and TFAA contents and decreased Pro content in salt-stressed plants (Figure 2), with the PCA analysis validating the strong positive correlation of these osmolytes with the IAA + S1 and IAA + S2 treatments (Figure 6). These results indicate that IAA enhances the TSP, TSS, and TFAA contents in plants and that increased osmolytes in the stressed V. faba plants may enable plants to reduce the energy required for growth and survival via enabling better osmotic adjustment. Thus, IAA could assist V. faba plants in withstanding salt-induced osmotic stress. Moreover, higher TSP, TSS, and TFAA production in “IAA + S1” and “IAA + S2” plants (Figure 2A–I), signifies the better metabolic status of V. faba plants and improved salt stress tolerance.

The detrimental consequences of salt stress on the growth attributes of plants are interrelated with nutrient uptake and translocation discrepancy [66,67]. In the present investigation, the NaCl-induced growth retardation might be accompanied by ionic stress as a result of a significant accumulation of toxic Na+ and a significant reduction in beneficial K+, Ca2+, and Mg2+ levels in different zones of the V. faba plants (Figure 3). This result also corroborates previous findings in rice [68], Phaseolus vulgaris [69], and leafy vegetables of Brassicaceae [70] under salt stress. The PCA also displayed a very strong positive association between S2 treatment and Na+ accumulation in the roots, stem, and seeds of V. faba (Figure 6B), suggesting that salinity-induced toxicity in V. faba is mainly because of the excessive accumulation of Na+ ion. Foliar application of IAA to the saline-affected V. faba
Plants increased K\textsuperscript{+}, Ca\textsuperscript{2+}, and Mg\textsuperscript{2+} contents and reduced Na\textsuperscript{+} content in all parts of the plants (Figure 3), indicating that IAA application effectively maintained ionic homeostasis in plants. Furthermore, IAA-mediated enhancement of K\textsuperscript{+}, Ca\textsuperscript{2+}, and Mg\textsuperscript{2+} contents might play a role in improving cell membrane integrity and nutrient balance. Similar results reported by [26], stated that foliar supplementation of IAA enhances maize plant growth and biomass production by maintaining ionic homeostasis under salt stress. Our work also revealed that levels of Na\textsuperscript{+}, K\textsuperscript{+}, Ca\textsuperscript{2+}, and Mg\textsuperscript{2+} translocation from root to stem were greatly decreased (Figure 4), indicating that these nutrients were concentrated in the roots of saline-affected plants. Thus, the higher accumulation of Na\textsuperscript{+}, K\textsuperscript{+}, Ca\textsuperscript{2+}, and Mg\textsuperscript{2+} ions in the roots of V. faba led to ionic toxicity and poor root growth which further hampered essential nutrient uptake under salinity stress (Figure 1A,D and Figure 4). On the other hand, foliar application of IAA improved nutrient translocation from the roots to the stem in IAA + S2 and IAA + S1 V. faba plants (Figure 4).

Plants experience metabolic disturbances that are often accompanied by an excessive accumulation of ROS, causing lipid peroxidation, which disrupts the functioning of cellular components such as proteins, lipids, and DNA, resulting in membrane damage and cell death in plants [71–73]. Numerous studies have reported that salt stress unleashed a great burden on plant metabolism due to excessive accumulation of toxic Na\textsuperscript{+}, resulting in oxidative stress directed towards cellular constituents [74]. Our results show that salt-stressed V. faba plants experienced a significant accumulation of MDA contents (Figure 5A) and accumulation of toxic Na\textsuperscript{+} (Figure 3A–C). Additionally, PCA analysis revealed that salt-stressed Vicia faba plants had a positive correlation with MDA (Figure 6B). Furthermore, supplementation of IAA to the salt-stressed plants reduced the MDA content. These results suggest that application of IAA might ease the suffering of V. faba plants from salt stress by diminishing the oxidative damage. It has been reported that antioxidant enzyme activities are considered as an integral part of plant defense mechanisms to combat against oxidative damage under salt stress [75,76]. Many studies have reported that salt stress enhanced the activities of antioxidant enzymes and supplementation of different regulators also boosted the antioxidant enzyme activities under salt stress [62,76,77]. The present study showed that salt stress led to a significant increment in the activities of SOD, CAT, POD, and APX in V. faba plants (Figure 5B–E). Foliar spraying of IAA further enhanced SOD, CAT, POD, and APX activities (Figure 5B–E). This result is also strengthened by the heatmap and PCA analysis, which showed that salt stress enhanced the antioxidant enzyme activities and supplementation of IAA also boosted the enzyme activities (Figure 6A). These results suggest that enhanced antioxidant enzyme activities defend V. faba plants against salinity-induced oxidative damage. Taking all of the results in this study together, it is apparent that salt stress reduces V. faba plant growth (Figure 1) by accumulating toxic Na\textsuperscript{+} (Figure 3) and increasing oxidative damage (Figure 5A) and supplementation of IAA ameliorates the salt-induced growth inhibition by increasing osmolyte contents (Figure 2), maintaining ionic homeostasis (Figure 3) and enhancing antioxidant enzyme activities (Figure 5B–E).

5. Conclusions

It can be concluded that exogenous application of IAA alleviates salt-induced growth inhibition in V. faba plants by enhancing antioxidant enzyme activities and maintaining ionic homeostasis. IAA application improved the yield of V. faba plants under salt stress conditions. Moreover, exogenous IAA can be used to improve the yield of V. faba under non-stress conditions. Further field trials should be performed to validate our results in field conditions.

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