Article

Mitigation of Salinity Stress Effects on Broad Bean Productivity Using Calcium Phosphate Nanoparticles Application

Amira K. Nasrallah ¹, Ahmed A. Kheder ², Maimona A. Kord ^{1,*}, Ahmed S. Fouad ¹, Mohamed M. El-Mogy ³ and Mohamed A. M. Atia ^{4,*}

¹ Department of Botany and Microbiology, Faculty of Science, Cairo University, Giza 12613, Egypt; anasrallah@sci.cu.edu.eg (A.K.N.); ahmedsfouad@yahoo.com (A.S.F.)

² Virus and Phytoplasma Research Department, Plant Pathology Research Institute, Agricultural Research Center, Giza 12619, Egypt; ahmedkheder@hotmail.com

³ Vegetable Crops Department, Faculty of Agriculture, Cairo University, Giza 12613, Egypt; elmogy@agr.cu.edu.eg

⁴ Genome Mapping Department, Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Center (ARC), Giza 12619, Egypt

* Correspondence: kord@sci.cu.edu.eg (M.A.K.); matia@ageri.sci.eg (M.A.M.A.); Tel.: +20-01223139977 (M.A.K.); +20-1000164922 (M.A.M.A.)

Abstract: Water salinity is one of the major abiotic stresses, and the use of saline water for the agricultural sector will incur greater demand in the coming decades. Recently, nanoparticles (NPs) have been used for developing numerous plant fertilizers as a smart and powerful form of material with dual action that can alleviate the adverse effects of salinity and provide the plant with more efficient nutrient forms. This study evaluated the influence of calcium phosphate NPs (CaP-NPs) as a soil fertilizer application on the production and bioactive compounds of broad bean plants under salinity stress. Results showed that salinity had deleterious effects on plant yield with 55.9% reduction compared to control. On the other hand, CaP-NPs dramatically improved plant yield by 30% compared to conventional fertilizer under salinity stress. This improvement could be attributed to significantly higher enhancement in total soluble sugars, antioxidant enzymes, proline content, and total phenolics recorded use of nano-fertilizer compared to conventional use under salt stress. Additionally, nano-fertilizer reflected better mitigatory effects on plant growth parameters, photosynthetic pigments, and oxidative stress indicators (MDA and H₂O₂). Therefore, our results support the replacement of traditional fertilizers comprising Ca²⁺ or P with CaP-nano-fertilizers for higher plant productivity and sustainability under salt stress.

Keywords: salt stress; nano-fertilizer; calcium phosphate; antioxidant enzymes; *Vicia faba* L.; yield

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1. Introduction

Broad bean, also known as broad beans (*Vicia faba* L.), is the fifth most crucial Fabaceae crop after pea, chickpea, common bean, and lentil [1]. According to FAOSTAT (2019), it is cultivated worldwide over 2.6 million hectares (has) with an annual production of 5.4 million tons. The importance of this leguminous crop arises from its high protein content (27–40%), carbohydrate (50–60%), minerals, and vitamins [2]. Furthermore, it is a good source of L-3,4-dihydroxyphenylalanine (L-DOPA), a precursor of a drug used to treat Parkinson's disease [1]. In addition, the broad bean plant plays a role in agricultural sustainability through nitrogen-fixing capability, improving crop productivity [3].

Globally, salinity is a major abiotic stress that severely affects crop plant growth and development from seed germination to harvest [4]. As a result, it reduces 30% of global food production [5]. At a global scale, it is estimated that one billion ha is affected by soil salinization increases at 2 million ha per year [6]. More than 7% of total land and about 20% of arable lands are harmed by salinity, particularly in arid and semi-arid regions such

as Egypt [7]. Furthermore, most of world arable lands (>50%) are predicted to be salinized by 2050 [8].

Salinity affects all the physiological processes in the plant, such as photosynthesis, protein synthesis, respiration, and lipid metabolism [9]. The initial effect of salt stress in plants is to reduce leaf surface expansion rate that may be completely stopped with a subsequent significant reduction in shoot growth [10]. Adverse effects of salinity on plant growth result from osmotic stress and ionic toxicity [11]. Sodium and chloride toxicity induces not only nutritional disorders but also causes physiological drought by lowering the osmotic potential of the soil solutions [12] that stimulate the accumulation of compatible solutes including soluble sugars and proline enabling water absorption [13].

High concentrations of sodium ions (Na^+) leads to the excessive production of reactive oxygen species (ROS) as superoxide ions ($\text{O}_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\text{OH}^\bullet$), and singlet oxygen ($^1\text{O}_2$) in plant cells that are associated with membrane damage, lipid peroxidation, nutrient imbalance, altered levels of growth regulators, enzymatic, and metabolic disorder harming photosynthesis and ultimately plant death [14].

As a defense response, plants accumulate some compatible solutes, such as proline, sugars, amino acids, and proteins inside vacuoles [15]. These solutes are involved in osmotic regulation in plant cells, which maintain cellular homeostasis [16].

In addition, oxidative stress provokes and orchestrates enzymatic and non-enzymatic mechanisms to scavenge the accumulated ROS [17]. Superoxide dismutase (SOD) is the first defense line against oxidative damage [18]. It scavenges ($\text{O}_2^{\bullet-}$) by its dismutation into molecular oxygen and H_2O_2 [19]. Several enzymes detoxify H_2O_2 in plant cells, such as catalase (CAT) which is responsible for converting it into H_2O and O_2 [20], and peroxidase (POX) by the oxidation of various substrates as guaiacol and pyrogallol [21]. At the same time, metabolic processes are directed towards biosynthetic pathways that consume reduction equivalents. Consequently, the biosynthesis of reduced compounds, including phenolics and flavonoids, is enhanced [22].

Calcium and phosphorus are essential macronutrients for plant metabolic processes and vital macromolecules. They are documented to alleviate the harmful effects of soil salinization on tomato [23], rice [24], sweetsop [25], and common bean [26]. However, excessive P and Ca fertilizers use causes many problems such as micronutrient imbalance, leaching, groundwater contamination, and eutrophication [27,28]. To limit these environmental problems, raise agricultural yield, and improve plant nutrient use efficiency for sustainable global development, novel fertilizer manufacture and application approaches are required.

Nanoparticles (NPs) are defined as particles with a size of 1 to 100 nm at least in one dimension. Compared to bulk materials, they can positively change physio-chemical characteristics, as their larger surface area makes their solubility and surface reactivity usually higher [29].

Application of nanoparticles can alleviate the injurious impact of salt stress through increasing the uptake of K^+ ; decreasing the Na^+ ion toxicity; regulating ion balance; activating the antioxidative defense system; improving in membrane stability index; increasing the contents of the compatible solutes and pigments, in addition to increasing stomatal conductance as well as leaf water content [30].

Therefore, NPs are widely used to enhance salt tolerance in many field crops. Several studies have reported the salt-alleviating effect of various NPs such as nano-silicon in tomato [31], CeO_2 -NPs [32], and Zn-ONPs [33] in *Brassica napus*. Further studies have reported growth-promoting silica NPs on lupin and wheat [34], and Fe_2O_3 -NPs on soybean [35].

Recently, Rane et al. [36] reported that CaP-NPs promote growth and root maturation in maize plants. However, there are limited reports on the role of CaP-NPs in mitigating salt stress effects on plants. Therefore, we hypothesized that the use of CaP-NPs fertilizer is more effective than the corresponding conventional fertilizer and it also reduces the pollution in soil and enhances crop production under saline conditions.

Therefore, the present study aimed to evaluate the potential use of CaP-NPs to mitigate the salinity stress in *Vicia faba* at the level of agro-physiological traits.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

Seeds of broad bean (*Vicia faba* cv. Sakha 1) were surface sterilized in 1% NaClO for 2 min and then washed three times with sterilized distilled water. Sterilized seeds were sown in plastic pots (30 cm in diameter) filled with a mixture of sand, clay, and peat moss in 1:1:1 (v/v/v). Thirty days after sowing (DAS), vegetates were thinned to three uniform plants per pot, and pots were divided into six groups and arranged in randomized complete block design with three replicates per treatment under the same growth conditions.

2.2. Transmission Electron Microscope (TEM)

The CaP-NPs (containing calcium and phosphorous at 38.76% and 19.97% (m/m), respectively) used in this study were purchased from NanoTech Co., Cairo, Egypt. Based on transmission electron microscopy (JEM-1400 TEM, JOEL, Peabody, MA, USA), particles were spherical with a diameter of 6.41 to 11.3 nm (Figure 1). The experimental solution was prepared before use by suspending dry CaP-NPs powder in double-distilled water then sonication at 100 W and 30 kHz for 30 min to avoid particle aggregation.

Figure 1. The TEM micrograph of CaP-NPs.

2.3. Salinity and Fertilizer Treatments

Based on a pilot experiment, the following treatments were applied through irrigation as illustrated in (Figure 2):

- 1 Tap water (Negative control)
- 2 Tap water supplemented with CaP (16 mg L⁻¹)
- 3 Tap water supplemented with CaP-NPs (16 mg L⁻¹)
- 4 Tap water supplemented with NaCl (150 mM)
- 5 Tap water supplemented with CaP (16 mg L⁻¹) and NaCl (150 mM)
- 6 Tap water supplemented with CaP-NPs (16 mg L⁻¹) and NaCl (150 mM)

Figure 2. Scheme of the experimental setup. Horizontal lines represent different plant treatments: control, CaP, CaP-NPs, Salt, CaP-NPs + Salt, and CaP + Salt. Broad bean yield was harvested at the end of the experiment.

Each pot was irrigated with 250 mL solution every five days. For plants treated with CaP or CaP NPs, each plant received 18.13 mg of the applied fertilizer during the entire growth cycle.

At 58 DAS (the end of February 2020), the plants were harvested for each treatment. Shoots were separated from roots using a sharp knife then weighed separately to obtain their fresh weights (FW). Shoot height (cm) of each plant was measured and leaf area (cm²) was calculated using the comparison method. Dry weights of shoots and roots were recorded after oven-drying at 65 °C until constant weight.

Leaf samples were collected from each treatment at the vegetative, flowering, and pod set stage (42, 50, 58 DAS respectively) and stored at −80 °C for physiological analyses in fresh tissues. At the same time, other representative leaf samples were collected and dried at 65 °C till constant weight and devoted to analyses in dry tissues. The plants' provided leaf samples were discarded after sampling, while the remaining plants were left until the end of the experiment (98 DAS) for yield-related data. First, pods of each plant were harvested and counted, and then the seeds were collected from the pods and weighed to calculate the average weight of 1000 seeds and total yield.

2.4. Quantification of Total Chlorophyll and Carotenoids

Frozen leaves tissue (20 mg) was ground in 2 mL ice-cold 80% (v/v) acetone as described by Merwad et al. [37]. The total chlorophyll content was measured in a 96-well microplate reader (Infinite 200 PRO, Tecan Group Ltd., Männedorf, Switzerland) at 662, 644, and carotenoid at 440.5 nm using MacKinney equations Sestak et al. [38].

2.5. Determination of Osmolytes Content

Total soluble sugars content of 10 mg dry leaves was extracted in 1.5 mL of distilled water in a boiling water bath for 1 h [39] and quantified using anthrone-sulfuric acid colorimetry at 630 nm [40] with glucose as a calibration standard. The free proline in the dry leaves was extracted and estimated according to the protocol outlined by Shabnam et al. [41]. It was extracted from 10 mg of dry leaves using 1.5 mL of 3% (w/v) sulfosalicylic acid solution and estimated using 1.25% (w/v) ninhydrin reagent in a boiling water bath for 30 min. Absorbance was measured at 508 nm against a water-reagent blank in a 96-well reader and compared with the proline standard curve.

2.6. Extraction and Assay of Antioxidant Enzymes

Antioxidant enzymes were extracted according to Grace and Logan [42], where 100 mg of frozen leaf tissue was powdered in liquid nitrogen and homogenized in 1 mL potassium phosphate buffer (50 mM, pH 7.0) containing 4% (*w/v*) PVP, 2% (*v/v*) glycerol and 0.1 mM EDTA. Homogenates were centrifuged at $15,000 \times g$ for 30 min at 4 °C and the supernatants were used to analyze enzymes activities.

The peroxidase (POX, EC: 1.11.1.7) activity was assessed according to Lagrimini [43]. Briefly, 2.9 mL of the reaction mixture (50 mM phosphate buffer (pH 7.0), 0.1 mM EDTA, 30 mM H₂O₂, and 100 mM Guaiacol) was added to 100 µL of crude extract. The formation of tetraguaiacol was measured at 465 nm using the extinction coefficient $26.6 \text{ mM}^{-1} \text{ cm}^{-1}$. The POX activity was expressed as $\mu\text{mol tetraguaiacol mg}^{-1} \text{ protein min}^{-1}$.

Catalase (CAT, EC: 1.11.1.6) activity was assayed by measuring the ability of the enzyme extract to decompose hydrogen peroxide (H₂O₂) at room temperature by detecting the decrease in absorbance at 240 nm [44]. The reaction mixture was composed of 100 mM potassium phosphate buffer (PH 7) and 25 mM H₂O₂. The reaction was started by adding 100 µL crude extract in 3 mL total volume. A decrease in H₂O₂ content was calculated using the extinction coefficient $43.6 \text{ M}^{-1} \text{ cm}^{-1}$. The results were expressed as $\text{mmol H}_2\text{O}_2 \text{ mg}^{-1} \text{ protein min}^{-1}$.

Superoxide dismutase (SOD, EC: 1.15.1.1) activity was determined as described by Dhindsa et al. [45] depending on its ability to inhibit nitro blue tetrazolium (NBT) photoreduction. First, 10 µL of enzyme extract was mixed with 200 µL of reaction mixture composed of 75 µM NBT, 60 µM riboflavin, 13 mM methionine, 0.1 mM ethylenediaminetetraacetic acid (EDTA), and 50 mM phosphate buffer (pH = 7.8). Subsequently, the mixture was exposed to a 15 W fluorescent lamp for 10 min at a distance of 20 cm and then the reaction was stopped by covering the plate with a black cloth immediately. The absorbance was recorded at 560 nm. One unit of SOD represents the amount of enzyme that inhibits the nitro blue tetrazolium photoreduction by 50% and expresses as enzyme unit $\text{mg}^{-1} \text{ protein min}^{-1}$.

Total soluble protein content was determined in crude extract according to Lowry [46].

2.7. Malondialdehyde and Hydrogen Peroxide Content

Lipid peroxidation status was determined as malondialdehyde (MDA) level as described by Heath and Packer [47]. Briefly, 20 mg of fresh leaves was homogenized in 500 µL of 0.1% (*w/v*) trichloroacetic acid (TCA) at 4 °C then 50 µL of the clear extract was incubated with 150 µL of 0.5% (*v/v*) thiobarbituric acid (TBA) in 20% (*w/v*) TCA at 95 °C for 60 min to produce (TBA-MDA). The difference between specific and non-specific absorbance at 532 and 600 nm, respectively, was used to quantify MDA based on the extinction coefficient of $155 \text{ mM}^{-1} \text{ cm}^{-1}$.

Hydrogen peroxide (H₂O₂) content in fresh leaves was determined as described by Junglee et al. [48]. Leaf tissue (150 mg) was directly homogenized in an ice bath with 1 mL of extraction mixture containing 0.1% (*w/v*) TCA, 1 M KI, and 10 mM potassium phosphate buffer (pH 5.8) for 10 min. The clear extract was employed to directly estimate H₂O₂ at 390 based on the H₂O₂ standard curve.

2.8. Total Phenolic Content

Total phenolic content (TPC) was extracted from dry leaf material with 70% ethanol (*v/v*) according to the method outlined by Sauvesty et al. [49]. Therefore, 50 µL of the clear extract was used to estimate TPC according to Lowe [50], using a mixture of 100 µL of 10% (*v/v*) Folin-Ciocalteu phenol reagent (LOBA CHEMIE PVT. Ltd., Mumbai, India) and 100 µL of 20% (*w/v*) anhydrous sodium carbonate. The absorbance of the developed blue color was measured after 30 min at 650 nm and used to quantify TPC based on a gallic acid standard curve.

2.9. Total Flavonoids

Total flavonoids were extracted from 15 mg dried leaf tissues in 500 μ L 80% (*v/v*) methanol and estimated using Chang et al. [51] method. Briefly, 25 μ L of the supernatant was mixed with 150 μ L distilled water and 10 μ L of 3.75% sodium nitrite (NaNO_2), followed by 15 μ L of 10% Aluminum chloride (AlCl_3) after 6 min. The absorbance was measured at 510 nm after further 5 min incubation with 50 μ L of 1 M NaOH and compared with the rutin standard curve.

2.10. Statistical Analysis

The measured parameters in all treatments were represented as the mean of 3 replicates with calculated standard error ($\pm$ SE). The mean values were compared through the Duncan multiple comparison test at the 5% level of significance using SPSS 15 software. Person correlations between all measured traits were calculated using the same software. The correlations were represented as a matrix heatmap using MS Excel 2019.

3. Results

3.1. Plant Growth and Yield

Different growth parameters showed diverse responses following treatment with fertilizers (Figures 3–5). For CaP, root fresh weight, root dry weight, leaf area, and plant height increased reaching 117%, 130%, 126%, and 119% of control, respectively, while the remaining growth parameters remained insignificantly affected. On the other hand, CaP-NPs treatment improved growth and yield expressed by shoot fresh weight, leaf area, plant height, pods weight/plant, and weight of 1000 seeds by 25%, 46%, 26%, 25% and 20% compared to control, respectively without significant effect for other parameters. Furthermore, compared with the corresponding results recorded for CaP treatments, leaf area and pod length reflected significantly better performance for CaP-NPs while the remaining growth parameters showed no significant difference between the effects applied fertilizers.

Observing the control irrigated with tap water, salt stress significantly reduced all growth parameters and yield except shoot fresh and dry weight. Seed weight per plant was the most sensitive parameter for salt stress where it showed a 56% decrease compared with control. Both fertilizers alleviated the growth-retarding effects of salinity, which was more pronounced for nano-fertilizers observing root dry weight and seed yield per plant. Otherwise, both fertilizers showed insignificantly different mitigatory behavior.

3.2. Carotenoids and Total Chlorophyll

Compared with the control, carotenoids content decreased in response to CaP treatments in all stages (Figure 6A). However, CaP-NPs treatment was associated with significantly decreased carotenoids content during the pod set stage only. Exposure to salinity stress significantly decreased carotenoids by 22%, 19% and 20% during the vegetative, flowering, and pod set stages, respectively. Thus, the CaP-NPs nullified the inhibitory effect of salinity stress on carotenoid content at the flowering stage. Otherwise, both fertilizers failed to modify such effects significantly.

Applying CaP did not affect the green pigment content at the vegetative stage while it was associated with 21% and 23% inhibition, compared with the corresponding control at the last two stages (Figure 6B). On the other hand, CaP-NPs application was responsible for a 24% and 10% increase in chlorophyll content at the vegetative and flowering stages, respectively. However, nano-fertilizer decreased the green pigment to 80% of control at the pod set stage. Salinity stress was accompanied by a 22%, 34% and 31% decrease in chlorophyll content at vegetative, flowering, and pod set stages, respectively, compared with corresponding control (Figure 6B). Both fertilizers alleviated the harmful effects of salinity on chlorophyll content that was more obvious for CaP-NPs that eliminated such effects during vegetative and flowering stages.

Figure 3. Effect of different treatments on (A) shoot fresh weight, (B) shoot dry weight, (C) root fresh weight, (D) root dry weight, (E) leaf area, (F) pod length, (G) plant height, (H) pods weight/plant, (I) seed yield/plant, and (J) weight of 1000 seeds. The same letters indicate no significant difference ($p < 0.05$) as analyzed by the Duncan test. Each value is the average of 3 replicates $\pm$ SE.

Figure 4. Effect of treatments on pods length (cm) of broad bean under salt stress.

Figure 5. Effect of treatments on broad bean growth under salt stress.

Except for the significant decrease recorded following application of both fertilizers at the vegetative stage and application of the nano-fertilizer at the flowering stage, neither of CaP nor CaP-NPs modified carotenoids/total chlorophyll ratio was recorded for corresponding controls (Figure 6C). Salinity led to a 4% to 25% significant increase in carotenoids/total chlorophyll ratio at all stages, compared with tap water-irrigated controls. However, CaP treatment mitigated salt-induced elevations at the vegetative stage while the mitigatory effect of the other fertilizer extended to the flowering stage. Both CaP and CaP-NPs did not affect the ratio at the pod set stage.

Figure 6. Effect of different treatments on (A) carotenoids, (B) total chlorophyll content, and (C) carotenoids/chlorophyll ratio of broad bean plants in 3 growth stages. The same letters indicate no significant difference ($p < 0.05$) as analyzed by the Duncan test. Each value is the average of 3 replicates $\pm$ SE.

3.3. Proline Content and Total Soluble Sugar

At the vegetative stage, the CaP treatment showed an increase in proline content by 24% of the corresponding control (Figure 7A). Meanwhile, the CaP-NPs treatment enhanced proline content by 12%, 30%, and 24% at the vegetative, flowering, and pod set stage, respectively, compared with control (Figure 7A). Under NaCl stress, plants showed a marked accumulation in the interesting amino acid during all growth stages. Salt stress-induced proline accumulation was amplified by adding fertilizers at the vegetative stage that were more obvious when applying CaP-NPs. The amplification disappeared for CaP-NPs and reversed for CaP at the flowering stage; thereafter, it similarly reversed for both fertilizers at the pod set stage.

Figure 7. Effect of different treatments on (A) the proline content and (B) total soluble sugars of broad bean plants in 3 growth stages. The same letters indicate no significant difference ($p < 0.05$) as analyzed by the Duncan test. Each value is the average of 3 replicates $\pm$ SE.

In the absence of salinity, CaP and CaP-NPs increased total soluble sugar by 47% and 90%, respectively, at the first developmental stage (Figure 7B). Except for the 17% decrease following application of CaP at the middle stage, both fertilizers were accompanied with insignificant change, compared with the corresponding control, in total soluble sugars at the flowering and pod set stages. Under salt stress, the CaP-NPs induced increase in soluble sugar content by 71%, 23%, and 43% at the vegetative, flowering, pod set stages, respectively, compared to non-stressed plants. Except at pod set stage applying CaP, both fertilizers intensified salinity-provoked soluble sugar accumulation at all stages with better performance for nano-fertilizer.

3.4. MDA Level and H_2O_2 Content

Lipid peroxidation was expressed as an increase in MDA content in broad bean plants during the three developmental stages, as shown in (Figure 8A). CaP showed no harmful effect on membrane status at all developmental stages as indicated with a slight decrease in MDA content at the vegetative stage and no significant changes at the remaining stages. On the other hand, CaP-NPs treatment was accompanied without significant alteration in lipid peroxidation at the vegetative stage while it enhanced MDA accumulation at other stages. Salt stress stimulated MDA content reaching 144%, 196%, and 159% of the corresponding control at vegetative, flowering, and pod set stages, respectively. Both fertilizers alleviated lipid peroxidation under salinity stress that was more pronounced applying CaP-NPs.

Figure 8. Effect of different treatments on (A) MDA and (B) H₂O₂ of broad bean plants in 3 growth stages. The same letters indicate no significant difference ($p < 0.05$) as analyzed by the Duncan test. Each value is the average of 3 replicates $\pm$ SE.

The content of H₂O₂ exhibited the same response showed with MDA with two exceptions concerning CaP treatments (Figure 8B). The first, CaP enhanced H₂O₂ content at the vegetative stage, compared with the corresponding control. The second, CaP failed to ameliorate H₂O₂ accumulation associated with salinity stress.

3.5. Antioxidant Enzymes Activities

The ordinary fertilizer enhanced POX activity reaching 119% of corresponding control at the vegetative stage that was neglected at the next developmental stages (Figure 9A). On the other hand, CaP-NPs treatment was accompanied by an 18% to 29% increase in POX activity at all developmental stages. Salinity stress enhanced POX activity reaching 181%, 208%, and 187% of corresponding control at vegetative, flowering, and pod set stages, respectively. CaP significantly culminated POX activity stimulated by salt stress treatment at all stages while CaP-NPs failed to mitigate POX response to salinity except at the flowering stage.

Compared with the corresponding control, both fertilizers had a significant stimulatory effect on CAT activity that was more recognizable for CaP-NPs at the flowering stage (Figure 9B). Applying salt stress was accompanied by a 21% to 178% increase at all stages. Both fertilizers intensified the salinity effect on CAT activity that was more pronounced for CaP-NPs at the flowering stage.

Considering the corresponding control, CaP enhanced SOD activity at the vegetative stage that faded at the next developmental stages (Figure 9C). Switching to the other fertilizer correlated with 181%, 153%, and 131% of corresponding control at vegetative, flowering, and pod set stages. Salt stress enhanced SOD activity at all stages that were modified by fertilizers. CaP had an alleviatory effect on salinity-provoked SOD activity at

the first two developmental stages only. On the other side, CaP-NPs mitigated the effect of salt stress on SOD activity at the vegetative stage while enhancing such effect at the last two developmental stages.

Figure 9. Effect of NaCl stress on (A) POX, (B) CAT, and (C) SOD of broad bean plants in 3 growth stages. The same letters indicate no significant difference ($p < 0.05$) as analyzed by the Duncan test. Each value is the average of 3 replicates $\pm$ SE.

3.6. Total Phenolic Content

The CaP treatment increased the phenolics content by 21% at the vegetative stage and did not affect it at the following two stages (Figure 10A). On the other hand, the CaP-NPs elevated the content by 28%, 30%, and 31% compared to control at the three successive stages, respectively. Salinity exhibited a significant increase in total phenolic content by 83%, 72%, and 78%, respectively, at the sequential stages. CaP significantly ameliorated. The impact of salt stress on phenolics accumulation at all developmental stages was also observed for CaP-NPs but only at the pod set stage.

Figure 10. Effect of different treatments on (A) the total phenolics and (B) total flavonoid content of broad bean plants in 3 growth stages. The same letters indicate no significant difference ($p < 0.05$) as analyzed by the Duncan test. Each value is the average of 3 replicates $\pm$ SE.

3.7. Total Flavonoids Content

Both fertilizers increased flavonoids content equally at all stages except failure of CaP at pod set stage (Figure 10B). Compared with the corresponding control, plants accumulated extra total flavonoids under salt conditions by 42%, 62%, and 73% at the vegetative, flowering, and pod set stages, respectively. Both fertilizers failed to modify flavonoids response under salt stress at vegetative stage though equally amplified such response at the middle developmental stage then culminated it at pod set stage.

4. Discussion

Results of the present investigation reflected the oxidative stress exemplified by the generation of H_2O_2 as a result of experiencing salt treatment. Accumulation of H_2O_2 and other ROS explains lipid peroxidation recorded in this study as indicated by an increase in MDA in plants exposed to salinity stress. Increased polyunsaturated fatty acid peroxidation reduces membrane fluidity and increases leakiness, resulting in severe membrane damage [52] responsible for growth retardation and decreased pigment content documented in the current results following salt stress. Ahmad et al. [13] observed an increase in H_2O_2 and MDA levels in association with a decrease in the growth and pigments of pea leaves in response to salinity. Similarly, Frukht et al. [53] documented that accumulation of H_2O_2 induced more MDA in rice under salt stress.

Reduction in total chlorophyll and carotenoid content in plants under saline stress is a common occurrence that has been observed in a variety of plants [54]. Inhibition of chlorophyll production via activation of chlorophyllase enzymes and membrane degradation are two probable explanations for this decline [55]. The change in chlorophyll pigment has been attributed to oxidative stress, inactivation of the Rubisco enzyme [56] and changes of chloro-

phyll ultrastructure [57]. In addition to the function as an accessory pigment, carotenoids show antioxidant properties; the increased carotenoids/chlorophyll ratio is an approach to prevent photo-oxidation [58]. Under salt stress, increased carotenoids/chlorophyll was recorded in maize [59], *Leptochloa fusca* [58] and sugarcane [60].

As a powerful oxidant, H_2O_2 causes a variety of harmful consequences in plants when converted to hydroxyl anion. Plants have a multifaceted antioxidant system made of enzymatic and non-enzymatic processes that are engaged in detecting, detoxifying, eliminating, and neutralization of ROS overproduction to maintain cellular redox equilibrium [61].

Antioxidants are considered to be critical in the plant's defense against ROS in this situation. The ascorbate–glutathione cycle is a major hydrogen peroxide detoxification system in plant cells, in which SOD enzymes play a vital role in accelerating the conversion of H_2O_2 to H_2O using ascorbate as a particular electron donor. SOD converts superoxide radicals into H_2O_2 and oxygen, CAT decomposes H_2O_2 into water and oxygen molecules [20]. POX is responsible for the elimination of the overproduced H_2O_2 by oxidation of various phenolic substrates [21].

The present investigation reflected an increase in SOD, CAT, and POX activities in response to salinity stress. In agreement with these results, Khan et al. [62] recorded a raise in the three enzymes activities under 120 and 150 mM NaCl in pearl millet. Additionally, Ahmad et al. [23] demonstrated an elevation in SOD and CAT activities in salt-stressed tomato seedlings. Non-enzymatic antioxidant activity exhibited by flavonoids and phenolic compounds deactivate lipid-free radicals. Furthermore, as flavonoids can interact with the polar region of phospholipids, they contribute to membrane lipid homeostasis preventing envelope membrane disruption and oxidative injury by inhibiting lipoxygenase enzyme and preventing polyunsaturated fatty acids conversion to oxygen-containing derivatives [63]. Accumulation of flavonoids is a clear indicator of salt tolerance [64] due to the ability to reduce ROS generation [65].

Results presented in Figure 6 reflected an increase in compatible solutes represented by proline as a part of plant response to salt stress. Proline may operate as a non-toxic osmotic solute, maintaining the structure of macromolecules and organelles preferentially. It has been observed that a high quantity of proline protects plants from osmotic stressors by stabilizing numerous functional units such as complex II electron transport, membranes, proteins, and enzymes as RUBISCO [66]. In harmony with these results, an increase in proline accumulation was documented in wheat [8], pearl millet [62] and *Pisum sativum* [13]. Our study revealed the accumulation of soluble sugars by salt treatment at the vegetative stage. This enhancement helps the plant to adjust the osmotic balance, scavenge ROS, and provide energy to resist salinity [13,67,68]. Meanwhile, a significant decrease in soluble sugar content was detected at flowering and pod set stages that were significantly positively correlated with the reduction in chlorophyll pigment under salt stress (Table S1).

Qados et al. [69], Hassanein et al. [70], and Taie et al. [71] documented that soluble carbohydrates decreased with an increase of NaCl in soil. Similar to the results of the present investigation, Qados et al. [69] proved that the application of Si NPs alleviated salt stress by decreasing soluble sugar synthesis. NaCl stress limits photosynthesis, causing plants to shift carbohydrate synthesis to secondary metabolites. As shown in Table S1, soluble sugars are significantly negatively correlated with total phenolics and flavonoids.

Results recorded for growth parameters and pigments content reflected the ameliorating effect of the applied fertilizers against salt stress that was undoubted for CaP-NPs. It has been reported that Ca^{2+} and P supplements stimulated several defense mechanisms to reduce NaCl toxicity. Both Ca^{2+} and P fertilizers play a key role in K^+ homeostasis in plant cells. P stimulates the accumulation of Na^+ in the vacuole and, therefore, the concentration of K^+ increases in the cytosol [72]. The following is an explanation of how the administration of P stimulates the buildup of Na^+ in the vacuole: Both H^+ adenosine triphosphatase and pyrophosphatase pumps acidify the inside of the vacuole with H^+ , prompting the H^+/Na^+ antiport protein to substitute the cytosol's Na^+ with the vacuole's H^+ [73]. Similar to our results, Roy et al. [24], Ahmad et al. [23], de Andrade et al. [25], and

Mohamed et al. [26] reported that P or Ca^{2+} supplements improved the crops' resistance to soil salinity.

Furthermore, a sufficient supply of Ca^{2+} controls the ion homeostasis pathways in plants by limiting the entry of Na^+ . In addition, calcium plays a role in the regulation of phospholipid signaling in response to osmotic stress [74]. Ca^{2+} -phospholipid signaling system communication regulates phospholipids, inositol, fatty acids, and ROS. It also maintains cellular osmotic equilibrium [75]. On the other hand, P is a critical component of biological substances such as nucleic acids, nucleotides, phospholipids, and phosphoproteins making it the most important plant growth macronutrient [76,77]. In addition, it is involved in various metabolic activities, including energy transfer, signal transduction, macromolecular biosynthesis, photosynthesis, and respiration [78]. Phosphate molecules serve as the "carbon currency" within plants [79].

Comparing the behavior of the recorded parameters in response to different treatments suggests amplification of catalase activity and the resultant decrease in H_2O_2 content accompanied with a decrease in lipid peroxidation as a potential mechanism underlying the observed role of fertilizers against salinity that was more pronounced for nano-type. Supporting this point of view, Upadhyaya et al. [80] reported that CaP-NPs decreased MDA and ROS at 10 and 20 mg L^{-1} by elevating antioxidant enzymes such as CAT. Additionally, Xu et al. [66] observed a significant increase in SOD, CAT, and POX and a simultaneous decrease in ROS production in *Calligonum mongolicum* plants supplemented with Ca^{2+} under oxidative stress. The ability of Ca^{2+} to enhance SOD and CAT was also confirmed in tomato [23] and rice [81].

Results of the current study also reflected the priority of nano form over ionic one in the stimulation of SOD. Similarly, SOD activity is enhanced by the application of other NPs including Mn NPs on pepper [82], nano-silicon on alfalfa [69], Ag NPs on pearl millet [62] under salt stress.

Results recorded for compatible solutes suggest a potential role for fertilizers to create an osmoregulatory mechanism independent of proline under salinity stress conditions. Such a mechanism allowed allocation of more assimilated carbon to its normal sinks reflected in more or less normal growth and yield upon application of CaP-NPs to salt-stressed plants. As indicated in the present study, the application of CaP and CaP-NPs alleviated the inhibitory effect of salt stress by stimulation of new sugars synthesis. In this context, Qados et al. [69] reported that nano-silicon increased sugar in broad bean and El-Sharkawy et al. [83] also reported these osmo-protectants in alfalfa by application nano K under salt stress. Mohamed et al. [17] also demonstrated the Ag NPs-mediated increase in soluble sugars content of wheat leaves under salt stress. One of the salt-mitigatory mechanisms of some fertilizers is an increasing in soluble sugars content which is reported in P application on potato [25], potassium fertilizer on soybean [84] and Ca^{2+} supplementation on *Calligonum mongolicum* [66].

It has been reported that the application of some fertilizers ameliorated abiotic stresses by increasing soluble sugars synthesis to maintain osmotic balance independent on proline, as using silicon to limit salt toxicity on *Glycyrrhiza uralensis* [85], and tobacco [86]. Additionally, it occurred by applying combined fertilizer (NPK, PGPR, and vermicompost) on *Brassica napus* under drought stress [87].

However, the reduction in proline has been observed in several studies as in Wang et al. [88] and Rossi et al. [89], who documented that proline did not participate in salt tolerance using CeO_2 NPs and plant improve other defense mechanisms as photosynthesis and antioxidant system. Avestan et al. [90], recorded salt tolerance mechanisms in strawberry using SiO_2 NPs through improving growth, chlorophyll content, relative water content, and limiting proline accumulation. In the case of salt-stressed grape plants, using Fe NPs and potassium silicate together mitigated salt injury by lowering MDA, proline, and increasing total proteins, SOD, APX, and POX activities [91].

The present investigation has shown that sugars play a more important role in osmotic adjustment than proline in CaP-NPs treatment under salt stress. Similarly, Yang et al. [92]

reported that using calcium silicate slag lowered proline and accumulated soluble sugars in rice leaves under drought stress. The authors explained the opposite correlation between proline and sugar to the competition for carbon skeletons. In addition, P fertilizers reduced proline under salinity stress in *Cicer arietinum* [93], and drought stress in dwarf bamboo [94].

The negative correlation recorded between proline and chlorophyll (Table S1) provides another possible explanation for proline reduction where glutamate is a common precursor in proline and chlorophyll synthesis [95]. The applied fertilizer reduced proline to improve the synthesis of green pigments in leaves as reported by Mamnabi et al. [87]. The current results showed the better performance of CaP-NPs against salt stress compared with CaP as CaP-NPs accumulated higher total soluble sugars, especially in the flowering and pod set stages to mitigate the osmotic stress. Additionally, it induced CAT and POX activities as antioxidant ROS scavengers resulted in less MDA and less H₂O₂ content.

It could be added that CaP-NPs provide Ca²⁺ and P to plants on a nanoscale, which may reduce ion toxicity caused by salt stress. P nutrients in a nano-formulation are more effective than traditional fertilizer [96]. In case of changes in pH or presence of some cations in soil, the P NPs may remain relatively stable at the suspension and less damaged by solution pH, coexisting ions, or solids, while soluble phosphate will be removed more quickly from a solution phase through precipitation. In phosphorus NPs, there was substantially more P left in the growth media for plant roots to absorb.

5. Conclusions

Salinity adversely affects the growth and yield in broad bean plants due to the induction of osmotic stress, ionic toxicity, and excessive generation of toxic ROS that negatively influence cell functional integrity, and cause oxidation of cell molecules such as proteins, lipids, and chlorophyll. Our results demonstrated that the applied fertilizers (CaP and CaP-NPs) generally mitigated these injuries by enhancing the defense mechanisms, especially antioxidant enzymes and accumulation of soluble sugars. Furthermore, nano-fertilizer was the first runner being more resistant to alterations in soil conditions. Therefore, this study put a solid base for further open-field studies aiming to evaluate the future of replacing the conventional fertilizers containing Ca²⁺ or P with their nano form to alleviate the harmful effect of salinity stress and to improve the productivity of broad beans. In addition, our study would be beneficial for other studies involved in the application of nano-fertilizer in agriculture.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/horticulturae8010075/s1>, Table S1: The correlation matrix heatmap shows the values of the Pearson correlation coefficient for all studied parameters.

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