

Calcium nanoparticles mitigate Cd-induced cellular damage by regulating redox state, antioxidant defense, and photosynthetic performance in *Vicia faba* L.

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ABSTRACT

Cadmium (Cd) is a highly toxic metal that impairs plant growth and yield. Thus, Cd-mitigating cost-effective and eco-friendly strategy is highly desirable. The aim of the study was to investigate the effects of calcium nanoparticles (N-Ca) at 0.1 mM (N-Ca-1) and 0.2 mM (N-Ca-2) in mitigating Cd toxicity in faba beans. In this study, foliar treatment with N-Ca-1 or N-Ca-2 significantly improved faba bean growth and physio-biochemical parameters by regulating plant growth regulators, antioxidant enzymes, and expression of key antioxidant genes in response to Cd stress. Faba bean plants remarkably induced Cd accumulation, leading to excessive reactive oxygen species (ROS), which caused Cd-induced oxidative damage via elevated lipid peroxidation and ion leakages. However, both N-Ca treatments (N-Ca-1 and N-Ca-2) effectively reduced ROS ($O_2^{\cdot-}$ and H_2O_2) levels and oxidative damage in both Cd-stressed and normal plants compared to N-Ca-0. The application of Cd-2 combined with N-Ca-2 maximized plant antioxidant capacity, indicating the ability to scavenge ROS and enhance photosynthetic performance. Interestingly, both N-Ca treatments enhanced leaf integrity by regulating relative water content and membrane stability index (MSI), as well as enhancing antioxidant enzyme activities, osmoregulation, and phytohormones. Supplementation of Ca-nanoparticles improved Ca content while reducing Cd absorption, thereby lowering Cd levels in both faba bean roots and shoots, which minimized ROS levels and oxidative damage. Expressions of key antioxidant genes (SOD, CAT, APX, and GR) suggest that they were fully active in response to Cd stress, which led to alleviating Cd toxicity. Notably, N-Ca-2 induces better yield compared to N-Ca-1. Therefore, these findings reveal N-Ca-mediated Cd toxicity mitigation is a cost-effective, eco-friendly, and efficient alternative for sustainable faba bean production in Cd-toxic soils.

INTRODUCTION

As a major legume species, faba bean (*Vicia faba* L.) is broadly cultivated for its importance in supporting human and animal diets. According to FAO data from 2003 to 2023, China (34.6%) and Ethiopia (16.9%) accounted for over 50% of global faba bean production. Furthermore, the data reveal that seven countries together account for 75% of total global production: China, Ethiopia, the United Kingdom (7.6%), Australia (6.7%), France (5.4%), Egypt (3.9%), and Germany (2.3%) [1]. In Egypt, faba beans are essential, and their production does not meet the demands of both the population and livestock. This issue can be addressed by increasing faba bean cultivation, exploiting arable soils, including those contaminated with heavy metals (HMs) such as cadmium (Cd). Bean seeds contain many essential nutrients, proteins, amino acids, fiber, and carbohydrates [2]. Additionally, plants can fix atmospheric N through symbiotic relationships, fulfilling over 80% of their N needs. This process improves soil quality and productivity, enhances N-use efficiency, and decreases the necessity for chemical N fertilizers [2, 3]. Contamination of arable soil with heavy metals (HMs), including Cd, is one of the major



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global challenges. Cd is a highly mobile and toxic HM that can cause serious damage to plants. Using sewage sludge, wastewater, phosphate fertilizers, and industrial processes in agriculture are major human activities that can release high amounts of Cd into the environment. Consequently, it poses serious damage to plants, humans, and animals [4]. The allowable concentrations of Cd range from 3 to 8 ppm, depending on soil texture, from light to heavy. Exceeding these levels can cause serious damage to plants. Due to its high solubility and fluidity, Cd is easily taken up through symplastic and apoplastic pathways in plant roots. Studies suggest that Cd is transported to the plant's shoots and sequestered in cell vacuoles and walls for detoxification [5]. In addition, Cd can cause lethal effects on plants. It alters plant phenotypic characteristics and disrupts phytohormone balance and cellular redox status, thereby inhibiting growth and root development [2, 6]. It negatively affects the biosynthesis of photosynthetic pigments, photosynthesis, gas exchange, and water movement, hindering plant metabolism and thus restricting plant growth and development [6, 7]. Cd toxicity disrupts various physio-biochemical and structural attributes in plants. It also disrupts antioxidant defense systems by excessive production of ROS (e.g., $O_2^{\bullet-}$, 1O_2 , H_2O_2 , $OH^{\bullet}$, etc.) in plants, thereby inducing oxidative stress. Consequently, cell death may occur depending on the Cd dose and exposure time. The plant can tolerate oxidative damage from Cd stress by reinforcing antioxidant levels, whether induced or constitutive [2, 8]. Thus, eco-friendly and cost-effective strategies are highly desirable for mitigating Cd-toxicity in plants. Calcium (Ca) nanoparticles could be an effective solution for mitigating Cd-toxicity in plants.

Ca is an essential nutrient for healthy plant growth and regulating cellular functions. It preserves cell wall membranes and promotes the activity of many enzymes, the biosynthesis of hormones, and intracellular physiological functions that control stressed plant responses [9, 10]. Foliar treatment with Ca can enhance physiological functions and plant production. Compared to soil Ca supplements, it has greater benefits in addressing Ca deficiency. As a result, it optimizes photosynthesis, growth, biomass, production, and the quality of plant yield [11]. This results from the rapid absorption and distribution of Ca throughout the plants, enhancing water relations and osmotic enhancers (OEs) such as proline and soluble sugars, while also limiting ROS generation, including superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), under stress [10]. To maximize Ca benefits, it has recently been used in nanoparticle form as a foliar spray on plants [9, 10, 11].

Contamination of soils with Cd poses serious risks to agricultural sustainability and food safety. Therefore, foliar supplementation of calcium nanoparticles (N-Ca) is crucial for efficient and eco-friendly crop improvement and ensuring food security. This approach would be effective in minimizing Cd-toxicity in plants and sustainable agricultural production. *Brassica alboglabra* plants are subjected to Cd stress, and foliar application of N-Ca reduces Cd accumulation while also boosting antioxidant defense systems and promoting plant growth. Thus, this strategy is both scientifically important and practically beneficial [12]. In agriculture, nanotechnology is an emerging technology that can serve as an economic strategy when applied through appropriate and well-studied methods. Nano-fertilizers can help repair innovative cultivation systems and significantly enhance food security. They effectively optimize plant production and protect against environmental stresses [2, 11]. N-Ca can help reduce the amount of conventional Ca applied to crops and contribute to precision agriculture by minimizing fertilizer loss and environmental pollution from unacceptable agronomic practices, thus maximizing agricultural outputs [11, 13]. Foliar-applied N-Ca can effectively enhance plant growth, productivity, physiological and biochemical traits, photosynthetic capacity, phytohormone and nutrient balance, antioxidant capacity, enzymatic activity, and key antioxidant gene expression, while minimizing oxidative stress markers and their

damage under stressful conditions. These positive findings may be attributed to N-Ca's various physicochemical properties, including its small molecular size and high surface-to-volume ratio, which enhance plant cell functions, including metabolism. In addition, N-Ca can easily penetrate plant cell walls, enhancing the transport of biomolecules and micronutrients, stabilizing genomic templates, and overall improving plant viability [10, 13]. It is essential to use nanoparticles at concentrations appropriate to the plant's age, species, and nutrient type to prevent unexpected toxic effects. High concentrations of nutrient nanoparticles can damage plants and decrease biomass production [14]. N-Ca can be strategically used in HMs bioremediation in legumes by enhancing plant tolerance, immobilizing toxic ions, and working in synergy with microbial partners. Therefore, future research should focus on the role of N-Ca in reducing Cd and other HMs, while also improving legume plant performance and enhancing symbiotic N fixation [13, 14].

Although N-Ca has been used to mitigate Cd stress effects on some crop plants, it has not yet been applied as a foliar treatment for faba beans under Cd stress. This research hypothesized that foliar treatment with N-Ca would minimize the toxic effects of Cd stress on faba bean plants and enhance plant tolerance to such stress. The objective of the current study was to determine the effects of foliar N-Ca treatment on Cd-stressed faba bean plant performance, along with several parameters. These include growth traits, photosynthetic efficiency, osmoregulation, leaf integrity, various enzymatic and non-enzymatic antioxidants, and key antioxidant gene expression. Indicators of Cd-toxicity induced oxidative stress and damage, as well as Cd status in plants, were also examined.

MATERIALS AND METHODS

Synthesis and characterization of N-Ca

To control particle size, analytical grade bulk CaCO_3 (a precursor), ultra-pure H_2O (resistivity $18.2 \text{ M}\Omega\cdot\text{cm}$), PVP K-30 (a stabilizing agent), and Tween-80 were used. A 750 W high-intensity ultrasonic processor operating at 20 kHz was used, along with a 13 mm diameter titanium alloy probe, a high-speed [$15,000 \text{ revolutions min}^{-1} \text{ (rpm)}$] centrifuge, a vacuum freeze-dryer, and a temperature-controlled reactor vessel. A dynamic light scattering analyzer (DLS), a high-resolution transmission electron microscope (HR-TEM), and an X-ray diffraction (XRD) system were also utilized.

A 45% saturated CaCO_3 solution in ultra-pure H_2O at $25 \pm 1^\circ\text{C}$ was prepared. PVP K-30 (1.5%, w/v) and Tween-80 (0.5%) were added under magnetic stirring. The solution pH was adjusted to 3.5 ± 0.2 by adding HCl or NaOH. Using a $0.45 \mu\text{m}$ pore-size membrane, the solution was pre-filtered to obtain a contaminant-free precursor for N-Ca synthesis. The sonochemical synthesis was performed in a temperature-controlled vessel maintained at $15 \pm 2^\circ\text{C}$ using a circulating cooling bath. An ultrasonic processor operated in pulse mode (30s on/10s off) at 70% amplitude for one hour was employed. The N-Ca configuration was organized by operating the system by adding PVP K-30 as a stabilizer, whereas Tween-80 lowered surface tension and inhibited particle aggregation.

Purification of the nanoparticle suspension was involved multiple steps: initial separation was achieved by centrifugation for 20 minutes at 12,000 rpm and 4°C , followed by three sequential washes with 70% ethanol. After each wash, the particles were re-dispersed through brief sonication (30 seconds at 30% amplitude). Finally, the purified nanoparticles were converted into N-Ca powder via freeze-drying at -50°C and 0.001 mbar for 24 hours. The morphology and particle size distribution of the n-Ca powder were analyzed by HR-TEM (JEM-2100, JEOL, Japan) operated at 200 kV (Suppl. Figure S1).

Plant material, experimental setup, and N-Ca treatment

Faba bean seeds (*Vicia faba* cv. Giza-716) were obtained from the Agricultural Research Center in Giza, Egypt, to implement three trials simultaneously from November 5 to December 15, 2024. A 5% NaOCl solution was used to sterilize the seed surface. Then, the disinfection solution was removed from the seed surface by washing with distilled H₂O. In each of the three trials, one hundred thirty-five (135) plastic containers, each with a diameter and depth of 38 and 35 cm, respectively, were used to sow seeds and grow plants. Three seeds were planted on November 5, 2024, in each container filled with synthetic composite medium (SCM) [15] with minor modification. Acid-treated (ion-free) sand (25.0%), vermiculite (25.0%), and peat moss (50%) were the main components of the SCM by weight. The SCM received 12.5 mg of the fungicide Moncut SC and 25.0 mg of humic acid kg⁻¹ (Glass Co., Ltd., Tokyo, Japan). Each kg of the SCM was enriched with 0.20, 0.15, 0.15, 0.15, 0.10, 0.10, and 0.10 mg of ammonium nitrate, calcium superphosphate, potassium sulfate, magnesium sulfate, iron, zinc, and manganese, respectively. To regulate the SCM pH, 500 mg CaCO₃ was added per kg. The pots were filled with a mixture of SCM (98.0%) and compost (2.0%). Under natural weather conditions, containers were randomly ordered in an open greenhouse. The mean temperature was 23 ± 3°C, the relative humidity was 62 ± 5%, and the photoperiod was 11/13 hours of day/night with natural sunlight intensity. After 15 days of planting (DAP), the containers were arranged in three strips, each containing 45 containers (15 replicates for each treatment). The three strips represented three cadmium chloride (Cd) treatments: 0 mM (Cd-0), 1 mM (Cd-1), and 2 mM (Cd-2). These treatments were applied with irrigation water. Each set of Cd treatments was divided into three subsets for nano-calcium carbonate (N-Ca) treatments: 0 mM (N-Ca-0), 0.1 mM (N-Ca-1), and 0.2 mM (N-Ca-2). These treatments were applied as foliar sprays. The Cd and N-Ca treatments were then initiated directly on 15 DAP. The Cd-0 treatment was irrigation with pure water free of Cd, and the N-Ca-0 treatment was foliar spraying with distilled H₂O. Therefore, 9 treatments were as follows: (1) Cd-0 + foliar spray with N-Ca-0, (2) Cd-0 + foliar spraying with N-Ca-1, (3) Cd-0 + foliar spraying with N-Ca-2, (4) Cd-1 + foliar spray with N-Ca-0, (5) Cd-1 + foliar spraying with N-Ca-1, (6) Cd-1 + foliar spraying with N-Ca-2, (7) Cd-2 + foliar spray with N-Ca-0, (8) Cd-2 + foliar spraying with N-Ca-1, and (9) Cd-2 + foliar spraying with N-Ca-2. The N-Ca concentrations (0.1 and 0.2 mM) were chosen from a preliminary trial (Table S1) for this study.

Foliar applications of N-Ca solutions (N-Ca-0, N-Ca-1, and N-Ca-2) were carried out in the morning after dew evaporation. Spraying was performed using a 2-L portable pressure sprayer equipped with an adjustable nozzle (model 0417.02.00; Guarany Ind. & Com. Ltd). Depending on the treatment, plants received three applications at 15, 25, and 35 DAP. To enhance penetration into leaf tissues, 0.1% (v/v) Tween-20 was added as a surfactant.

The trials were ordered in a randomized complete block design and ended 40 DAP. For stressful treatments, evaluations were performed periodically using an ICP-AES (IRIS-Advan type, Thermo, USA) to possibly stabilize the SCM's Cd level (1.0 and 2.0 mM). The containers were watered using the weighing method once every two days. Each container was watered to the SCM's full capacity. This involved calculating the difference between the SCM-filled container weight and the SCM-free container weight. During the experimental duration, the containers were rotated to mitigate the inconsistent effects of local conditions alongside irrigation. Conventional crop protection practices were applied to manage weed growth and suppress plant pathogens in the area.

Faba bean plant samples were collected at 40 DAP at 6:00 am (10 ± 2 °C) to minimize environmental fluctuations. Refrigerated boxes with insulated walls were used to store

the samples until they were transported to the relevant laboratories for analysis. From each treatment, a sample of 9 plants was randomly selected and used to examine all growth traits. Another sample of 9 plants was randomly selected and used to evaluate all physio-biochemical determinations. A third sample containing 9 plants was randomly selected and used to assay the antioxidant system (enzymatic and non-enzymatic). A fourth sample containing 9 plants was randomly selected and used to assay key antioxidant gene expression and phytohormones.

Plant growth traits and photosynthetic efficiency

Plant shoot samples were used to determine morphological traits; leaf numbers were recorded, and leaf area per plant (dm²) was measured using a Digital-Planix-7 planimeter. After weighing to obtain fresh weight (FW, g) using an electric balance, shoots were stored at 70 °C until a stable dry weight (DW, g) was obtained.

Fresh leaf content of chlorophyll and carotenoids (mg g⁻¹ FW) was evaluated in a 80% acetone-extract [16]. At 470, 647, and 662 nm, the OD (optical density) was documented using a Shimadzu spectrophotometer (UV-160A, Japan). Chlorophyll “a” fluorescence (Fv/Fm) was also evaluated using a fluorometer (PAM-2000, Heinz-Walz) [17].

Determination of osmotic stress indicator and leaf integrity

To evaluate the soluble sugar content (mg g⁻¹ DW), ethanol (96%) was used to extract it from dried leaves [18]. Each 0.1 mL extract required 3 mL of anthron reagent. The mixture was boiled for 10 minutes and then cooled. The OD was documented at 625 nm. To evaluate the proline content, sulphosalicylic acid (3.0%) was utilized to extract it from dried leaves [19]. After centrifuging (10,000 ×g, 10 min) the extract, the produced supernatant required an acid-ninhydrin (freshly prepared) solution. The supernatant-ninhydrin mixture was stored at 90°C for 30 min. To terminate the reaction, an ice bath was used, and another extraction was performed to obtain the toluene phase, and the OD at 520 nm was recorded. These estimates were evaluated spectrophotometrically (UV-160A, Shimadzu, Japan).

To evaluate the glycine betaine (GB) content, a mixture of 3 parts water, 5 parts chloroform, and 12 parts methanol (v/v/v) was utilized to homogenize a liquid nitrogen-frozen sample [20]. Afterwards, the homogenates were incubated at 4 °C overnight. After purifying, the extract was centrifuged (5000×g for 10 min) to remove the ion exchange resin. The supernatant was filtered and loaded into the Shimadzu-V HPLC system. The GB content was detected by a UV detector at 230 nm and then quantified using a pure GB standard. To evaluate the K⁺ content (mg g⁻¹ DW), HNO₃ and H₂O₂ were utilized to suspend and digest the samples [21] and then quantified by utilizing Optima 7900DV ICP-MS (Perkin-Elmer, Waltham, MA, USA).

Relative water content (RWC) [22] and membrane stability index (MSI) [23] were evaluated in a leaf sample, and the values were obtained from the formula:

$$RWC (\%) = \left[\frac{(fresh\ sample - dry\ sample)}{(turgid\ sample - dry\ sample)} \right] \times 100$$

The fresh, turgid, and dry samples of 2 cm-diameter leaf discs indicate the weight at preparation, after water-saturating in the dark for 24 h, and after drying at 70°C until a stable weight was obtained, respectively.

$$MSI (\%) = \left[1 - \left(\frac{EC1}{EC2} \right) \right] \times 100$$

The EC1 indicates the electrical conductivity (EC) of 0.2 g leaf sample solution after heating to 40°C for 30 minutes, and EC2 indicates the EC of a similar sample boiled for 10 min, respectively.

Root activity, Ca and Cd contents, and Cd translocation factor

Root activity was evaluated using the tetrazolium (2,3,5-triphenyl-2H-tetrazolium chloride) reduction method [24]. A 0.5 g fresh root tissue was stored in the dark with 5 mL of 0.4% tetrazolium chloride solution and 5 mL of phosphate buffer at 37 °C for 2 h. Two mL of 1.0 M H₂SO₄ was added to terminate the reaction. The samples were then extracted with ethyl acetate, and the resulting red extract was diluted to 10 mL (total volume) with ethyl acetate. The OD was recorded at 485 nm, and the obtained value was considered an indicator of root activity.

A Perkin-Elmer Atomic-Absorption model 3300 was utilized to determine the root and shoot contents of Cd and Ca [25]. The dried samples were digested for 12 h in a mixture of 1 vol. perchloric acid (80%) per 5 vol. H₂SO₄ (Conc.). Then, sterile distilled H₂O was added to dilute the samples (to 100 ml), and Cd and Ca contents were evaluated. The Cd TF was calculated as follows:

$$TF = \left[\frac{\text{Shoot Cd content}}{\text{Root Cd content}} \right] [26]$$

Determination of oxidative stress and damage indicators

Levels of hydrogen peroxide (H₂O₂) [27] and superoxide (O₂^{•-}) radical [28], as oxidative stress indices, were assessed. The damage induced by the oxidants was estimated as lipid peroxidation (expressed as malondialdehyde; MDA) and leaf electrolyte leakage (EL) [23, 29].

$$EL (\%) = \left[\frac{(EC2 - EC1)}{EC3} \right] \times 100$$

The electrical conductivities EC1, EC2, and EC3 were measured for three solutions. The first solution contained untreated midrib-free leaf discs, the second solution contained similar discs heated at 45°C–55°C for 30 min, and the third solution contained similar discs boiled for 10 min.

Non-enzymatic antioxidants, redox states, and total antioxidant activity

For the AsA level (μmol g⁻¹ FW), a mixture of 0.03 M K-P buffer (pH 7.4) + TCA (2.5%) + H₃PO₄ (8.4%) + bipyridyl (0.8%) + FeCl₃ (0.3%) + AsA extract for reaction at 40°C for ½ h [30]. The OD was recorded at 525 nm. The DHA (oxidized AsA) + AsA levels were estimated after the reaction of the extract and 500 μmol DTT. The OD was documented at 525 nm.

$$AsA \text{ redox state } (\%) = \left[\frac{AsA}{(AsA + DHA)} \right] \times 100$$

For the GSH level (μmol g⁻¹ FW), a mixture of GSH extract received 0.13 M and 7.0 mM buffers of Na-P (pH 7.4 and 6.8, respectively), and 6.0 mM DTNB to react at 30 °C for 10 min [31]. The OD was documented at 412 nm. The total GSH level obtained from the reduction of GSSG to GSH was estimated, and the content of GSSG+GSH and GSH was evaluated (pure GSH was the standard).

$$GSH \text{ redox state (\%)} = \left[\frac{GSH}{(GSH + GSSG)} \right] \times 100$$

The method (the HPLC system) was used to determine α -tocopherol content [32]. Total contents of phenolic compounds [33] and flavonoids [34] were estimated. Overall antioxidant activity (OAA) was evaluated using the DPPH radical-scavenging assay [35]. The extract (0.1 ml) was reacted with 1 ml of a 20 mg L⁻¹ DPPH solution (with methanol as the blank) and incubated in the dark for 20 min. The OD of the DPPH solution was documented at 517 nm. These estimates were performed spectrophotometrically (UV-160A, Shimadzu, Japan). The OAA, expressed as an inhibition (%) for DPPH-free radicals, was calculated:

$$\text{Inhibition (\%)} = \left[\frac{(\text{Blank OD} - \text{Sample OD})}{\text{Blank OD}} \right] \times 100$$

Enzyme activities, soluble protein content, and key antioxidant gene expression

The enzyme extract (EEx) was produced by extracting 0.5g of leaf, and the homogenate was centrifuged for 10min at 15,000 × g [36]. The following were assayed for their activities in the supernatant (EEx): catalase (CAT) [52], ascorbate peroxidase (APX) [37], superoxide dismutase (SOD) [38], and glutathione reductase (GR) [39].

APX activity was assayed in EEx supplemented with a K-P buffer (pH 7.0), H₂O₂, and AsA. CAT activity was assayed in EEx supplemented with a P-buffer (pH 7.8) and H₂O₂. Declined OD at 240nm indicates the activity for both APX and CAT. GR activity was assayed by the increase in OD at 412 nm due to the reduction of 5,5'dithiobis (2-nitrobenzoic acid) by GSH in EEx. SOD activity was assayed by assessing the inhibition of photochemically reduced nitroblue tetrazolium (NBT). Elevated OD at 560nm indicates the activity. The enzyme quantity required to inhibit 50.0% of the NBT reduction rate was identified to be 1.0 SOD unit. Enzyme activities were expressed in units g⁻¹ protein. The procedure in [40] was practiced evaluating soluble protein content.

Leaf tissues were processed for total RNA extraction according to the manufacturer's instructions. The transcripts of key antioxidant genes were quantified using qRT-PCR using SYBR Green Master Mix (TakaRa, Japan) on a LightCycler® 96 real-time PCR platform (Roche, Germany). Details of the primer sequences are presented in Table S2, with actin serving as the internal reference gene. The nucleotide sequences of the targeted key antioxidant defense genes were retrieved from the NCBI database (<https://www.ncbi.nlm.nih.gov>). Relative gene expression levels were determined using the 2- $\Delta\Delta$ Ct calculation method [41].

Phytohormone quantification and identification

Phytohormones were determined using GC-MS following established protocols used earlier [42]. Briefly, 100mg of Faba bean leaf tissue was homogenized in 2 mL of cold extraction buffer containing 80 units methanol, 19 units water, and 0.1 parts HCl (6N). The homogenate was then centrifuged at 25,000 × g for 5 min at 4°C, and the supernatant was reduced to 50 μ L under a N stream before storage at -80°C.

In order to isolate auxins, 50 μ L of extract was reacted with 40 μ L of methyl chloroformate (MCF), concentrated to 20 μ L under N, and treated with 0.5mg sodium sulfate to separate residual moisture. To isolate cytokinins (CKs) and gibberellic acid (GA₃), 50 μ L of extract was evaporated and derivatized with 100 μ L of N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) by heating at 85°C for 45 min.

For GC-MS analysis, 1 μL of each derivatized sample was injected into a Clarus 680 GC coupled to an SQ8-T mass spectrometer (Perkin Elmer, Waltham, MA, USA) equipped with an Elite-5MS capillary column (30 m $\times$ 0.25 mm $\times$ 0.025 μm film thickness). Helium served as the carrier gas at a flow rate of 1 mL min⁻¹. For auxins: the column was maintained at 50°C for 3 minutes, ramped to 200°C at 4°C minute⁻¹, and held for 5 minutes. For CKs and GA₃: the column was held at 60°C for 2 minutes, increased to 160°C at 20°C minute⁻¹, then raised to 290°C at 5°C min⁻¹.

Injector and detector temperatures were set at 250°C and 260°C, respectively. Chromatographic data were processed using TurboMass software (version 6.1, Perkin Elmer). Identification of auxins, CKs, and GA₃ was achieved by comparing retention times, linear retention indices (LRIs), and diagnostic ions with authentic standards [43].

Statistical analysis

A two-way ANOVA based on a randomized complete block design was performed to evaluate the data, after verifying the homogeneity of error variance [44] using the InfoStat statistical software [45]. Differences among treatment means were further assessed using Duncan's Multiple Range Test at a 5% probability level.

RESULTS

Faba bean plants were grown under three Cd concentrations: 0 mM (Cd-0), 1.0 mM (Cd-1), and 2.0 mM (Cd-2). Additionally, the non-stressed (Cd-0) or stressed (Cd-1 and Cd-2) plants were foliar-sprayed with three levels of nano-calcium carbonate (N-Ca): 0 mM (N-Ca-0), 0.1 mM (N-Ca-1), and 0.2 mM (N-Ca-2). Overall, responses of plant growth, physiological and biochemical attributes, antioxidant capacity, and phytohormone levels to N-Ca were more noticeable under Cd stress than under stress-free conditions.

Impacts of N-Ca on growth and photosynthetic efficiency of Cd-stressed plants

Cd stress markedly decreased plant growth and photosynthetic efficiency. The Cd-1 and Cd-2 treatments caused reductions of 30.7% and 78.3% in the number of plant leaves, 34.0% and 77.3% in leaf area, 34.3% and 80.1% in shoot fresh weight, and 24.2% and 72.0% in shoot dry weight, respectively. Additionally, both Cd treatments led to decreases of 30.8% and 63.2% in total chlorophyll content, 26.8% and 50.0% in total carotenoid content, 22.2% and 61.1% in Fv/Fm, and 22.6% and 47.4% in photochemical activity, respectively, compared to the control (Cd-0) (Suppl. Figure S2, and Figure 1).

Under normal (Cd-0) or Cd stress conditions, applying the N-Ca-1 or N-Ca-2 treatment significantly improved plant growth and photosynthetic efficiency. The N-Ca-2 treatment outperformed N-Ca-1, resulting in increases of 19.0, 49.1, and 234.3% in the number of plant leaves, 28.2, 62.0, and 196.6% in leaf area, 47.0, 68.5, and 210.9% in shoot fresh weight, and 43.5, 39.4, and 190.9% in shoot dry weight under Cd-0, Cd-1, and Cd-2 conditions, respectively. Additionally, the N-Ca-2 treatment led to increases of 21.8, 56.5, and 82.7% in total chlorophyll content, 30.8, 48.8, and 61.5% in total carotenoid content, 16.7, 35.7, and 92.9% in Fv/Fm, and 26.0, 46.4, and 38.6% in photochemical activity under Cd-0, Cd-1, and Cd-2, respectively, compared to the corresponding controls (Figure 1).

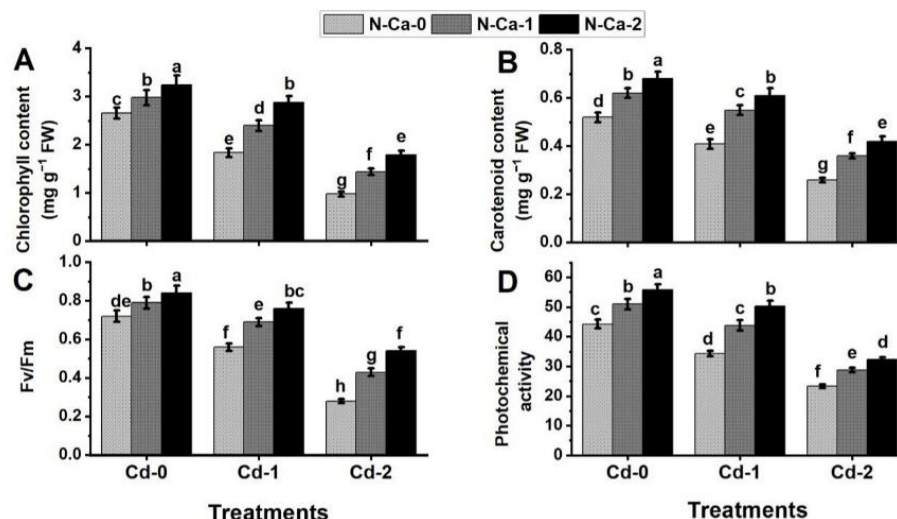


Figure 1. Response of photosynthetic efficiency, including A) chlorophyll content, B) carotenoid content, (C) Fv/Fm, and D) photochemical activity of faba bean plants to foliar spraying with calcium nanoparticles under cadmium stress conditions. The represented data are means $\pm$ SE labeled by the same letter are not significantly different in each plot according to Duncan's Multiple Range Test ($p \leq 0.05$). Cd-0; without cadmium, Cd-1 and Cd-2; cadmium added to the soil at 1.0 mM and 2.0 mM, respectively, N-Ca-0; foliar spraying with distilled water, N-Ca-1 and N-Ca-2; foliar spraying with calcium nanoparticles at 0.1 mM and 0.2 mM, respectively, Chls; total chlorophylls, Car; total carotenoids, Fv/Fm; efficiency of PSII in photosynthesis, Photo-Ac; photochemical activity that is the chemical reaction initiated by the absorption of energy in the form of light, and FW; fresh weight.

Role of non-enzymatic antioxidant and total DPPH-RSA content

Under control or Cd stress conditions, supplementation of N-Ca-1 or N-Ca-2 significantly improved non-enzymatic antioxidants and antioxidant activity. The N-Ca-2 treatment outperformed N-Ca-1 in this regard. In this context, the N-Ca-2, α -tocopherol (α -ToC) increased by 19.1% and 40.8%, total phenols increased by 22.3% and 36.8%, total flavonoids increased by 31.4% and 53.9%, and antioxidant activity by 25.3% and 55.2% under Cd-0 and Cd-1, respectively (Supp. Figure S3).

Effects of N-Ca on osmolytes and leaf integrity of Cd-stressed plants

Cd stress declined leaf integrity while increasing the contents of osmotic stress indicators. Specifically, the Cd-1 and Cd-2 treatments resulted in decreases of 15.9% and 37.9% in leaf relative water content (RWC), and 22.9% and 48.3% in leaf membrane stability index (MSI), respectively. Conversely, both Cd (1.0 and 2.0 mM) treatments stimulated increases of 21.8% and 55.6% in total soluble sugar content, 54.2% and 125.0% in free proline (Pro) content, 26.3% and 68.6% in glycine betaine (GB) content, and 13.2% and 36.8% in potassium ion (K^+) content, compared to the control (Cd-0) (Supp. Figure S4).

Under stress-free (Cd-0) or Cd stress conditions, applying N-Ca-1 or N-Ca-2 treatment significantly enhanced leaf RWC and MSI, while further increasing OEs contents. Under Cd-0, Cd-1, and Cd-2 conditions, the N-Ca-2 treatment outperformed N-Ca-1, resulting in increases of 10.7, 20.5, and 29.2% in leaf RWC and 11.9, 27.6, and 46.7% in leaf MSI, respectively. Further, the N-Ca-2 treatment increased soluble sugar content by 22.5% and 26.6%, free Pro content by 31.3% and 48.6%, GB content by 23.7% and 28.9%, and K^+ content by 13.8% and 19.8% under Cd-0 and Cd-1 conditions, respectively. However, these increases were not observed with N-Ca treatments under Cd-2 conditions compared to the corresponding controls (Suppl. Figure S4).

Impacts of N-Ca on Cd and Ca contents of Cd-stressed plants

Cd stress damaged root activity, reduced root and shoot Ca contents, while increasing root and shoot Cd contents and Cd translocation factor (Cd-TF). The Cd-1 and Cd-2 treatments resulted in reductions of 21.0% and 46.8% in root activity, 24.5% and 49.5% in root Ca content, and 28.0% and 46.8% in shoot Ca content, respectively. Conversely, Cd treatments (1.0 and 2.0 mM) resulted in increases of 1143.8% and 1650.5% in root Cd content, 1314.3% and 2074.5% in shoot Cd content, and 12.9% and 24.2% in Cd-TF, respectively, compared to the control (Figure 2).

Under stress-free (Cd-0) or Cd stress conditions, applying N-Ca-1 or N-Ca-2 treatment significantly improved root activity and Cd content in roots and shoots, while reducing root and shoot Cd content and Cd-TF. The N-Ca-2 treatment outperformed N-Ca-1, resulting in increases of 19.4, 28.6, and 51.5% in root activity, 26.7, 31.8, and 53.8% in root Ca content, and 75.3, 124.6, and 90.9% in shoot Ca content under Cd-0, Cd-1, and Cd-2 conditions, respectively. Conversely, the N-Ca-2 treatment decreased root Cd content by 60.8, 71.6, and 36.0%, shoot Cd content by 67.6, 74.9, and 53.6%, and Cd-TF by 17.7, 12.9, and 28.6% under Cd-0, Cd-1, and Cd-2 conditions, respectively, compared to the corresponding controls (Figure 5).

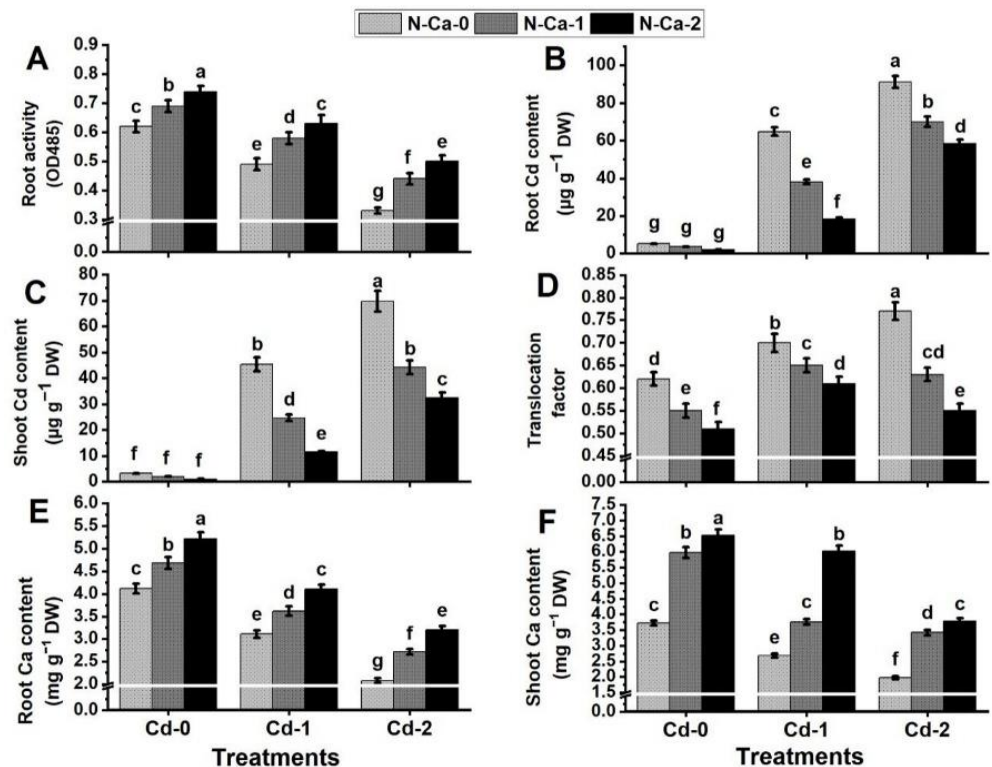


Figure 2. Response of A) root activity, B) root Cd content, C) shoot Cd content, D) translocation factor of Cd, E) root Ca content, and F) shoot Ca content of faba bean plants to foliar spraying with calcium nanoparticles under cadmium stress conditions. The represented data are means $\pm$ SE labeled by the same letter are not significantly different in each plot according to Duncan's Multiple Range Test ($p \leq 0.05$). Cd-0; without cadmium, Cd-1 and Cd-2; cadmium added to the soil at 1.0 mM and 2.0 mM, respectively, N-Ca-0; foliar spraying with distilled water, N-Ca-1 and N-Ca-2; foliar spraying with calcium nanoparticles at 0.1 mM and 0.2 mM, respectively, OD; optical density, and DW; dry weight.

Role of N-Ca on oxidative damage in Cd-stressed plants

The levels of oxidative stress indicators [superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2)] increased, along with malondialdehyde (MDA) and electrolyte leakage (EL), indicating oxidative damage under Cd stress conditions. Specifically, the levels of $O_2^{\bullet-}$ rose by 63.6% and 122.7%, and H_2O_2 increased by 71.3% and 135.6%, respectively. Furthermore, MDA levels increased by 70.0% and 126.7%, and EL increased by 150.7% and 277.4% under the Cd-1 and Cd-2 conditions, respectively, compared to the control (Cd-0, Figure 6).

The application of N-Ca-1 or N-Ca-2 treatment significantly decreased the levels of oxidative stress markers and stress damage under stress-free (Cd-0) or Cd stress conditions. The N-Ca-2 treatment outperformed the N-Ca-1 treatment, reducing the levels of $O_2^{\bullet-}$ by 15.9, 41.7, and 42.9%, H_2O_2 by 29.9, 43.6, and 34.1%, MDA by 36.7, 43.1, and 29.4%, and EL by (not significant), 60.3, and 59.1% under Cd-0, Cd-1, and Cd-2 conditions, respectively, compared to the corresponding controls (Figure 3).

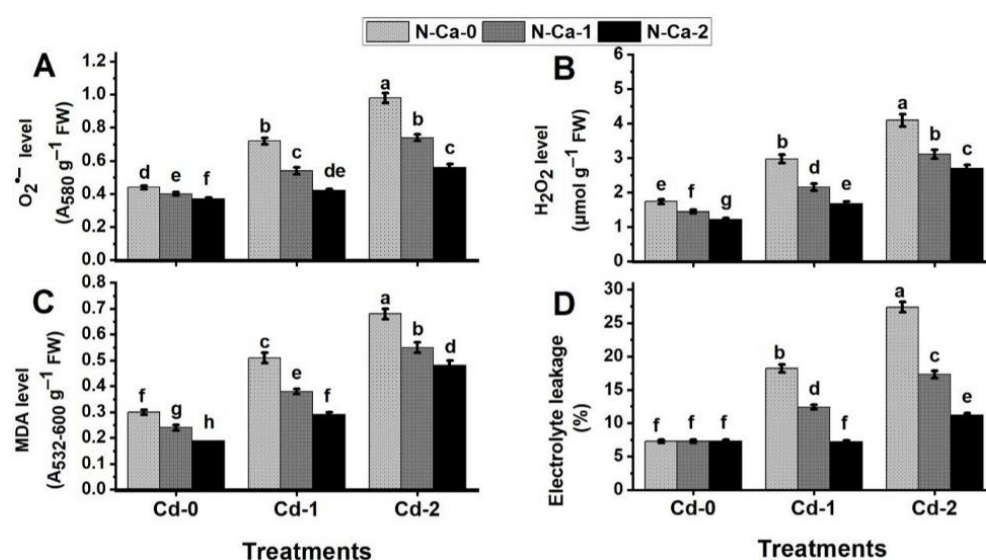


Figure 3. Response to the levels of oxidative stress markers, such as A) superoxide ($O_2^{\bullet-}$) content and B) hydrogen peroxide (H_2O_2) content, and oxidative damage indicators such as C) malondialdehyde (MDA) content, and D) electrolyte leakage (EL) in faba bean plants to foliar spraying with calcium nanoparticles under cadmium stress conditions. The represented data are means $\pm$ SE labelled by the same letter are not significantly different in each plot according to Duncan's Multiple Range Test ($p \leq 0.05$). Cd-0; without cadmium, Cd-1 and Cd-2; cadmium added to the soil at 1.0 mM and 2.0 mM, respectively, N-Ca-0; foliar spraying with distilled water, N-Ca-1 and N-Ca-2; foliar spraying with calcium nanoparticles at 0.1 mM and 0.2 mM, respectively, A; absorption, and FW; fresh weight.

N-Ca responsive regulation of antioxidant capacity of Cd-stressed plants

The non-enzymatic antioxidants studied demonstrated significantly elevated levels and antioxidant activity under Cd stress conditions. The levels of ascorbate (AsA) increased by 31.1% and 62.2%, glutathione (GSH) increased by 35.0% and 67.5%. Additionally, the AsA redox state improved by 20.8% and 48.8%, the GSH redox state by 29.8% and 51.8%, and antioxidant activity by 25.3% and 55.2% under the Cd-1 and Cd-2 conditions, respectively, compared to the control (Figure 4).

Under stress-free (Cd-0) or Cd stress conditions, the application of N-Ca-1 or N-Ca-2 treatment further elevated non-enzymatic antioxidant levels and antioxidant activity. The N-Ca-2 treatment outperformed the N-Ca-1 treatment, increasing AsA level by

17.6% and 22.7%, GSH by 22.5% and 22.2%, α -ToC by 19.6% and 17.3%, total phenols by 13.8% and 12.1%, and total flavonoids by 29.4% and 31.3%, respectively. Additionally, the AsA redox state increased by 20.5% and 22.7%, the GSH redox state increased by 20.9% and 16.5%, and antioxidant activity increased by 23.9% and 24.8% under Cd-0 and Cd-1, respectively.

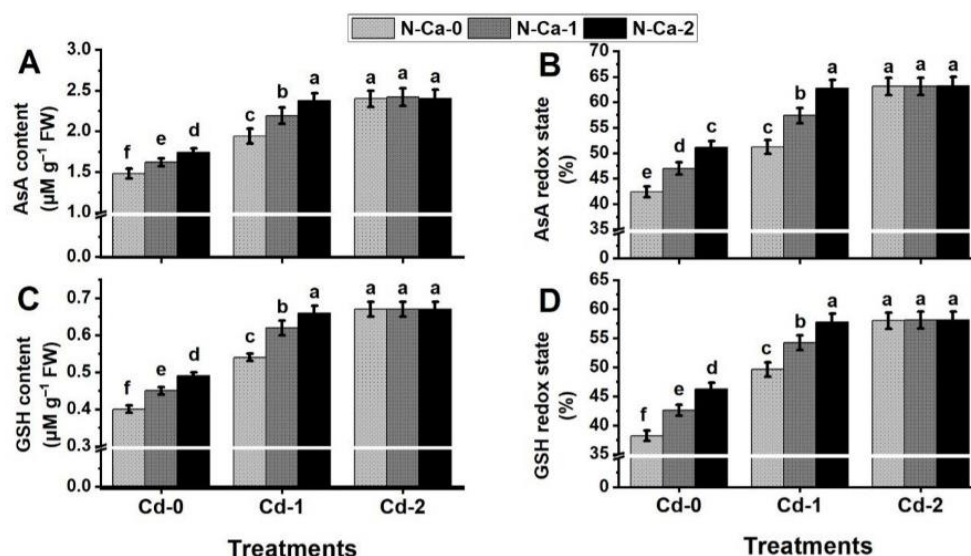


Figure 4. Response of A) ascorbate (AsA), B) AsA redox state, C) glutathione (GSH), and D) GSH redox state in faba bean plants to foliar spraying with calcium nanoparticles under cadmium stress conditions. The represented data are means $\pm$ SE labeled by the same letter are not significantly different in each plot according to Duncan's Multiple Range Test ($p \leq 0.05$). Cd-0; without cadmium, Cd-1 and Cd-2; cadmium added to the soil at 1.0 mM and 2.0 mM, respectively, N-Ca-0; foliar spraying with distilled water, N-Ca-1 and N-Ca-2; foliar spraying with calcium nanoparticles at 0.1 mM and 0.2 mM, respectively.

Impacts of N-Ca on enzyme activities of Cd-stressed plants

The enzymes studied showed high activity and key antioxidant gene expression, while protein content decreased in protein content in faba bean plants subjected to Cd stress. Specifically, protein content decreased by 34.0% and 53.1%. In contrast, key enzyme activities increased significantly: superoxide dismutase (SOD) by 20.3% and 42.5%, catalase (CAT) by 64.6% and 119.5%, ascorbate peroxidase (APX) by 33.8% and 86.8%, and glutathione reductase (GR) by 43.4% and 64.6%, respectively. Furthermore, the relative expressions of these enzymes also increased, with *SOD* rising by 55.0% and 125.0%, *CAT* by 50.0% and 70.0%, *APX* by 40.0% and 95.0%, and *GR* by 55.0% and 80.0% under the Cd-1 and Cd-2 conditions, respectively, when compared to the control (Figure 5).

Under stress-free (Cd-0) or Cd stress conditions, applying N-Ca-1 or N-Ca-2 treatment further increased enzyme activity, key antioxidant gene expression, and protein content. The N-Ca-2 treatment outperformed the N-Ca-1 treatment, further increasing the activities of studied enzymes: *SOD* by 17.9% and 18.8%, *CAT* by 52.2% and 49.5%, *APX* by 31.1% and 38.6%, and *GR* by 33.0% and 27.6%, respectively. Furthermore, the relative expressions of these enzymes also increased, with *SOD* rising by 50.0% and 41.9%, *CAT* by 35.0% and 26.7%, *APX* by 40.0% and 35.7%, and *GR* by 35.0% and 25.8% under Cd-0 and Cd-1, respectively (Figure 6).

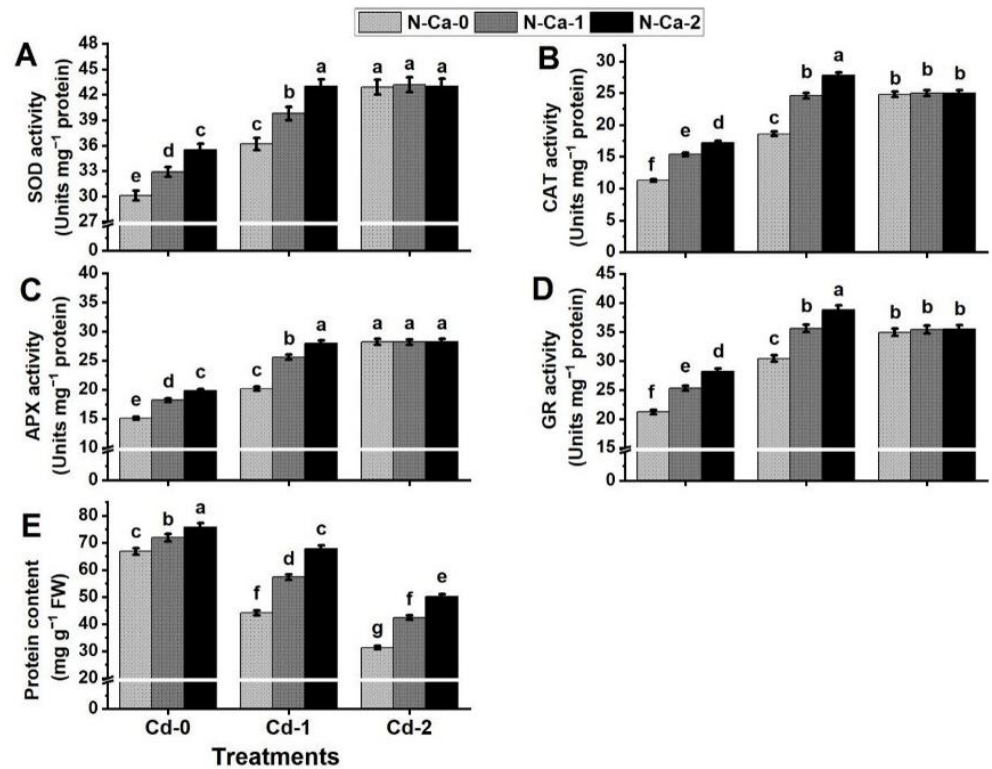


Figure 5. Response of the leaf enzymatic [A] superoxide dismutase (SOD), B) catalase (CAT), C) ascorbate peroxidase (APX), and D) glutathione reductase (GR)] activities, and (E) total soluble protein content of faba bean plants to foliar spraying with calcium nanoparticles under cadmium stress conditions. The represented data are means $\pm$ SE labeled by the same letter are not significantly different in each plot according to Duncan's Multiple Range Test ($p \leq 0.05$). Cd-0; without cadmium, Cd-1 and Cd-2; cadmium added to the soil at 1.0 mM and 2.0 mM, respectively, N-Ca-0; foliar spraying with distilled water, N-Ca-1 and N-Ca-2; foliar spraying with calcium nanoparticles at 0.1 mM and 0.2 mM, respectively, and FW; fresh weight.

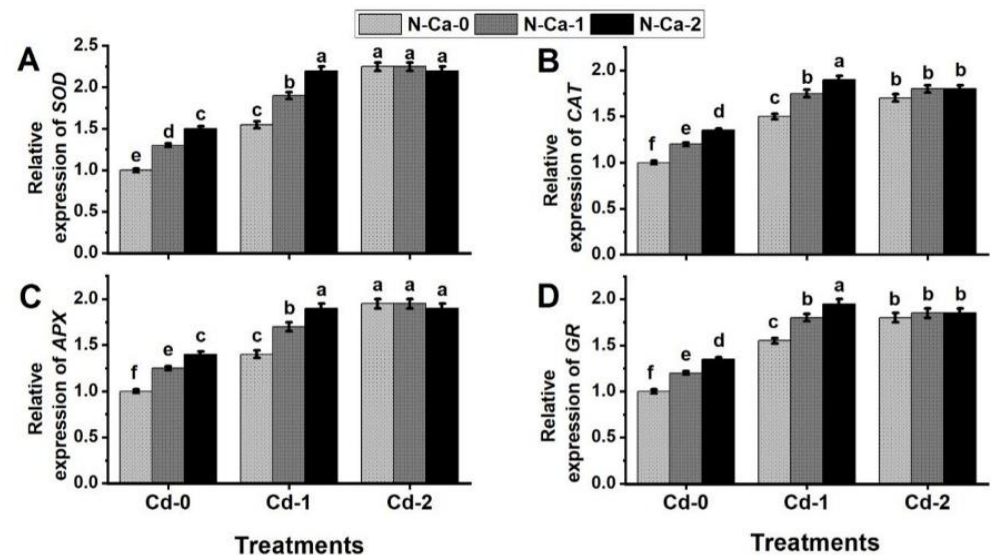


Figure 6. Response of the enzyme gene [A] SOD, B) CAT, C) APX, and D) GR] expressions of faba bean plants to foliar spraying with calcium nanoparticles under cadmium stress conditions. The represented data are means $\pm$ SE labeled by the same letter are not significantly different in each plot according to Duncan's Multiple Range Test ($p \leq 0.05$). Cd-0; without cadmium, Cd-1 and Cd-2; cadmium added to the soil at 1.0 mM and 2.0 mM, respectively, N-Ca-0; foliar spraying with distilled water, N-Ca-1 and N-Ca-2; foliar spraying with calcium nanoparticles at 0.1 mM and 0.2 mM, respectively, SOD; superoxide dismutase, CAT; catalase, POD; peroxidase, APX; ascorbate peroxidase, and GR; glutathione reductase.

Effects of N-Ca on phytohormone status of Cd-stressed plants

Cd stress led to an imbalance in the phytohormone contents of faba bean plants. Specifically, it significantly decreased indole-3-acetic acid (IAA), GA₃, and CKs levels, while increasing abscisic acid (ABA) content. The Cd-1 and Cd-2 treatments caused reductions of 27.6% and 48.5% in IAA content, 30.9% and 55.3% in GA₃ content, and 37.8% and 60.3% in CKs content, respectively. Conversely, both Cd (1.0 and 2.0 mM) treatments resulted in increases of 80.6% and 140.3% in ABA content, respectively, compared to the control (Cd-0, Figure 11).

Under stress-free (Cd-0) or Cd stress conditions, the application of N-Ca-1 or N-Ca-2 treatment resulted in a balance in the phytohormone content of faba bean plants. This significantly augmented the levels of IAA, GA₃, and CKs, while reducing ABA levels. The N-Ca-2 treatment outperformed N-Ca-1, resulting in increases of 27.0, 46.5, and 22.8% in IAA content, 28.4, 66.1, and 57.5% in GA₃ content, and 16.0, 61.3, and 15.3% in CKs content under Cd-0, Cd-1, and Cd-2 conditions, respectively. Conversely, the N-Ca-2 treatment decreased ABA content by 18.5, 50.4, and 58.7% under Cd-0, Cd-1, and Cd-2 conditions, respectively, compared to the corresponding controls (Figure 7).

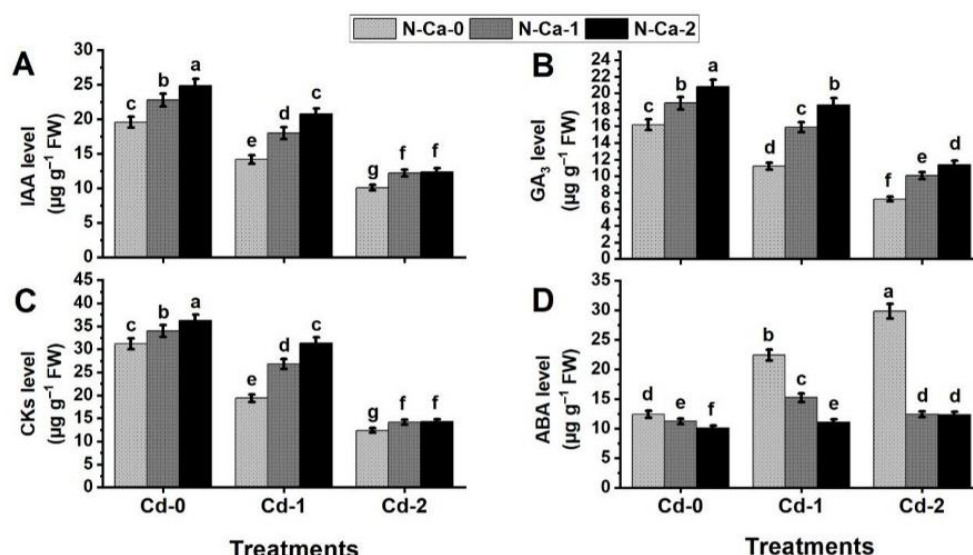


Figure 7. Response of the levels of phytohormones [A) indole-3-acetic acid (IAA), B) gibberellic acid (GA₃), C) cytokinins (CKs), and D) abscisic acid (ABA)] of faba bean plants to foliar spraying with calcium nanoparticles under cadmium stress conditions. The represented data are means ± SE labeled by the same letter are not significantly different in each plot according to Duncan's Multiple Range Test ($p \leq 0.05$). Cd-0; without cadmium, Cd-1 and Cd-2; cadmium added to the soil at 1.0 mM and 2.0 mM, respectively, N-Ca-0; foliar spraying with distilled water, N-Ca-1 and N-Ca-2; foliar spraying with calcium nanoparticles at 0.1 mM and 0.2 mM, respectively, μg; microgram, and FW; fresh weight.

DISCUSSION

Calcium nanoparticles (N-Ca) have been previously applied to various stressed crops [10, 11, 13, 46]. However, N-Ca has not yet been applied as a foliar treatment for faba beans under Cd stress. N-Ca concentrations used were 0.1 mM (N-Ca-1) and 0.2 mM (N-Ca-2) under Cd concentrations of 1.0 mM (Cd-1) and 2.0 mM (Cd-2). N-Ca application offers an eco-friendly, cost-effective, and efficient alternative for supplying Ca to plants. Positive results were obtained due to the large surface area and high solubility of N-Ca, which cause rapid absorption by plant parts [46]. N-Ca can penetrate through multiple layers of the leaf. The negatively charged pores (less than 5.0 nm in size) in the hydrophobic waxy cuticle facilitate the passage of positively charged nutrients such as

Ca²⁺. Another pathway, leaf stomata with larger pores, can easily pass N-Ca when they are opened. N-Ca can be transported either through apoplastic pathways or symplastic pathways. Once Ca enters the plant through the stomata, epidermis, and cuticle, it is redistributed to plant parts via the phloem. Due to their smaller molecular size and larger surface area, they are rapidly absorbed compared to traditional calcium (T-Ca), enabling them to perform their functions more quickly [13]. Consequently, plant growth, physiological and biochemical attributes, and nutrient (K⁺ and Ca²⁺) uptake were upregulated by N-Ca, positively impacting the performance of faba bean plants under Cd stress.

Faba bean plant growth was inhibited under Cd stress. The Cd toxicity-induced plant growth impairment was higher under Cd-2 than Cd-1. This finding was consistent across various crops [6, 47]. However, Cd declined chlorophyll synthesis, which led to impaired photosynthesis and metabolic pathways. The consequence of Cd toxicity affects the mitotic division in epidermal cells, reducing leaf surface area and plant biomass [47]. In contrast, the improvement of plant growth in response to N-Ca-1 or N-Ca-2 treatment, with N-Ca-2, indicates significant benefits. This finding demonstrates that N-Ca promotes plant growth and enhances tolerance to Cd toxicity, most likely through increased Ca content, which supports greater cell elongation and expansion [11–14]. Antioxidant activities, gene expression, osmotic enhancers (OEs), and hormone levels were enhanced, while reducing Cd content, transport factor (Cd-TF), and stress indicators through N-Ca foliar treatment. These findings led to improved plant growth. N-Ca can maintain membrane structure, protein synthesis, membrane function, and expansion in plant cells to withstand cadmium stress. This finding is achieved by increasing antioxidant activity, which helps recover oxidative damage [9, 10]. Study suggests that Ca²⁺ acts as a second messenger to maintain metabolic processes, helping to enhance plant growth and biomass. N-Ca utilization improves membrane stability (MS) and leads to enhanced Ca²⁺ and K⁺ accumulation, which contribute to maintain growth of stressed plants [48].

Impairment of photosynthetic efficiency, chlorophyll and carotenoid contents, Fv/Fm, and photochemical activity due to Cd stress, suggesting that dose Cd-2 is critical for faba bean compared to Cd-1. Study suggests that Cd-induced degradation or reduced synthesis of chlorophyll and carotenoids. Cd also disrupts the pigment-protein complex in thylakoid membranes and both PSI and PSII in photosynthesis, impairing photosynthetic efficiency [49]. However, N-Ca treatment significantly improved photosynthetic efficiency, especially in response to N-Ca. N-Ca inhibits chlorophyll degradation under Cd stress by maintaining the thylakoid membrane structure and activating CKs [13]. Ca helped to minimize thylakoid breakdown and increase expression of genes related to chlorophyll synthesis. Carotenoid content and photochemical activity, and therefore cellular homeostasis, are maintained under stress [13, 50]. N-Ca can activate PSI and PSII (Hill reaction; photophosphorylation) and the Calvin cycle, thus enhancing photosynthetic efficiency [50]. Furthermore, N-Ca can activate Rubisco and enhance expression of the *psbA* gene, which encodes the D1 protein—a crucial structural and functional component of the PSII reaction center [13, 50–52].

The high content of OEs [soluble sugars, proline, glycine betaine (GB), and K⁺], and the decline in leaf integrity (RWC and MSI) in faba bean plants were more pronounced under Cd-2 compared to Cd-1. Cd stress induces chlorophyll degradation and affects Hill's reaction and Rubisco activity [13, 49]. However, the soluble sugar content increases concurrently with the rise in proline, GB, and K⁺. These increased OEs helped maintain leaf integrity (RWC and MSI) under Cd stress. These findings align with those in [47, 53],

which indicate increased soluble sugar content in *Pistia stratiotes* and *Glycine max* under Cd stress. In addition, proline, GB, and K⁺ are utilized in plants for signal transduction and stress responses. However, N-Ca application further elevated the soluble sugars, proline, GB, and K⁺ contents in normal and stressed faba bean plants. These increases helped the plant leaves recover by increasing RWC and MSI. The N-Ca-mediated enhancement of photosynthesis further increased the OEs contents in stressed plants. These findings confirm those recently obtained [13, 48], likely due to the N-Ca-mediated increases in CO₂ fixation and Rubisco activity [10–13]. Applying N-Ca enhances N metabolism, sugar transport, and the function of plasmalemma-bound enzymes. It also improves nucleic acid and antioxidant metabolism and transport, upregulates cell wall and membrane integrity, facilitating beneficial K⁺ and Ca²⁺ exchange [13]. Proline and GB biosynthesis increase under stress and act as efficient OEs and antioxidants [54]. High K⁺ and Ca²⁺ contents, with increased OEs contents, helped maintain RWC and MSI under Cd stress after N-Ca application.

Root activity and root and shoot Ca contents were reduced, while increasing Cd content and Cd-TF, with more damage under Cd-2 compared to Cd-1. The elevated levels of H₂O₂ and O₂^{•-} (oxidative stress indicators) and stress damage (MDA and EL) in plants under Cd stress led to these negative findings. These findings confirm those in [10–12, 54–58], demonstrating that Cd notably damages root activity and elevates both the Cd content and Cd-TF, inducing oxidative stress and damage in several crops. However, N-Ca treatment alleviated Cd toxicity and notably reduced the levels of O₂^{•-}, H₂O₂, EL, and MDA in faba bean plants. These reductions are attributed to decreased Cd-TF and Cd content in plant organs. Cd-stressed plants can induce Cd reduction mechanisms, including cytoplasmic chelation, cell wall detoxification, and vacuole sequestration [5]. The greater the sequestration of root vacuoles, the less Cd translocation to the leaves. The transport proteins *HMA3*, *CAX4*, and *CAX2* play crucial roles in chelating Cd in the vacuole. Cd also binds to pectin and polygalacturonic acid in the cell wall after entering the roots, minimizing the Cd content in the cytosol. Endodermic Casparian strips in the roots also contribute to reducing Cd in the cell. Thus, vacuole sequestration and chelation of Cd are essential detoxification mechanisms [13, 59]. N-Ca boosted the efficiency of Cd-limiting mechanisms by increasing the Ca²⁺ and K⁺ contents. Furthermore, N-Ca notably enhanced the plant's defense systems, including both non-enzymatic and enzymatic antioxidants, and key antioxidant gene expression. These, in addition to membrane stabilization (MSI and RWC), contribute to decreased ROS (O₂^{•-} and H₂O₂) and damage (EL and MDA) in Cd-stressed crop plants following N-Ca application [13, 48]. The reduction of ROS and damage following N-Ca application indicates that Ca contributes to alleviating the impacts of Cd stress. It has been demonstrated that Ca application reduces ROS production and lipid peroxidation in Cd-stressed rice seedlings [13, 51, 52].

The increased non-enzymatic and enzymatic antioxidant levels and activities, and key antioxidant gene expression under Cd stress were greater under Cd-2 than Cd-1. These findings confirm those in [13, 42, 47, 54]. The supplantation of N-Ca minimized the harmful influences of Cd due to antioxidant defense. More specifically, the N-Ca-2 enabled faba bean plants to effectively tolerate Cd stress and control ROS (O₂^{•-} and H₂O₂) levels. This tolerance was achieved and regulated by N-Ca-mediated optimization of the levels of AsA and GSH, along with their respective redox states. Additionally, the rise in other non-enzymatic antioxidants (α -tocopherol, total phenolic compounds, and flavonoids), and overall antioxidant activity (OAA), played a significant role in the faba bean's tolerance to Cd stress. The enhancement of antioxidant enzymes, including SOD, CAT, APX, and GR, and key antioxidant gene expression, also boosted Cd stress tolerance in faba bean plants. These enhanced findings indicate that N-Ca application effectively minimized the overproduced levels of O₂^{•-}, H₂O₂, EL, and MDA. N-Ca serves

as an anti-stress element that enhances antioxidant metabolism and transport, thereby improving plant tolerance to stress [9–13]. Our findings indicated that faba bean plants resist the harmful influences of ROS through two key antioxidant mechanisms. As reported in [13, 47], non-enzymatic pathways are the primary mechanism by which several metabolites, including GSH and proline, directly scavenge ROS. This is in addition to phenolic compounds that contribute to minimizing ROS levels and the “AsA-GSH pathway”, where H_2O_2 is eliminated. The second major antioxidant mechanism is the minimization of ROS levels through enzyme activities [13, 47, 49, 60]. The high pattern of enzymatic activities and key antioxidant gene expression (closely correlated with enzyme activities) and the low pattern of oxidation products indicate that N-Ca-treated faba bean plants have less stress impact, thus less damage to cell membranes and more efficient plant growth than Cd-stressed plants without N-Ca. The reduction in Cd-stimulated oxidative damage in N-Ca-treated plants is likely due to the plants' elevated antioxidant response.

The loss of phytohormone balance (decreased levels of IAA, GA_3 , and CKs, with increased levels of ABA) under Cd stress was more pronounced under Cd-2 compared to Cd-1. These findings confirm what was reported in [47, 60]. However, applying N-Ca mitigated the Cd stress damage and re-balanced phytohormones (increased levels of IAA, GA_3 , and CKs, with decreased levels of ABA). In this study, faba bean plant growth and defense pathways were up-regulated by N-Ca-mediated re-balanced phytohormones due to their irreplaceable mechanisms of tolerance to Cd stress. The enhanced growth of Cd-stressed plants was attributed to elevated metabolite levels with plant development, re-balancing of phytohormones, and key antioxidant gene expression. N-Ca-mediated re-balanced phytohormones up-regulate stability and permeability of cell membranes, enzyme activities, secondary metabolism, and plant growth and development [13, 60]. As an important mechanism, N-Ca-mediated optimized GA_3 stimulates hormonal balance and Cd stress tolerance in plants by optimizing gene expression [47]. As a crucial mechanism, N-Ca-mediated optimized CKs stimulate stress tolerance and improve protein biosynthesis, secondary metabolism, and plant growth by eliminating ROS and antagonizing ABA [47, 60].

Furthermore, N-Ca-mediated optimized IAA activates the AsA-GSH cycle and antioxidant activities, overcoming Cd toxicity [13, 60]. Phytohormones rebalanced by N-Ca can contribute to the increase in antioxidant capacity/OAA to restrict the levels of $\text{O}_2^{\bullet-}$ and H_2O_2 [47] and lipid peroxidation while optimizing membrane stability to maintain healthy growth of faba bean plants. Optimal phytohormone balance by applying N-Ca, particularly N-Ca-2, was associated with increased photosynthesis, antioxidant capacity, defense systems, and key antioxidant gene expression. It was also linked to minimal $\text{O}_2^{\bullet-}$, H_2O_2 , EL, and MDA levels due to minimizing Cd-TF and Cd contents in plant shoots and roots. These findings align with a report [60] indicating that nanoparticles interact with heavy metals by regulating hormone levels and candidate gene functions.

CONCLUSIONS

This study explores the mechanistic insights of cadmium-NPs-induced regulation of metabolism, growth, and mitigation of Cd-stress in faba bean plants (Figure 8). Foliar treatment with 0.2 mM calcium nanoparticles enables plants to withstand cadmium toxicity stress. It rebalances phytohormones and nutrients, and improves photosynthetic parameters, enzymatic and non-enzymatic antioxidant defense, and antioxidant activity and key antioxidant gene expression. These positive results reflect minimized levels of reactive oxygen species-induced oxidative stress (via reduced hydrogen peroxide and

superoxide) and damage (via reduced malondialdehyde and ion leakage), as well as cadmium translocation factor under cadmium stress. However, more research is needed to understand the complex physio-biochemical and molecular mechanisms that influence the response of faba bean plants treated with calcium nanoparticles to cadmium toxicity. Specifically, the molecular basis of calcium nanoparticles' impact on endogenous calcium levels, which regulate signaling pathways or stimulate calcium-regulating genes, may provide precise insight into the cadmium stress tolerance in faba bean plants. Therefore, the present study recommends foliar application of calcium-based nanoparticles as a successful, eco-friendly, cost-effective, and efficient alternative for supplying calcium to plants. This strategy may enhance the sustainable faba bean cultivation by improving its productivity in cadmium-contaminated soils.

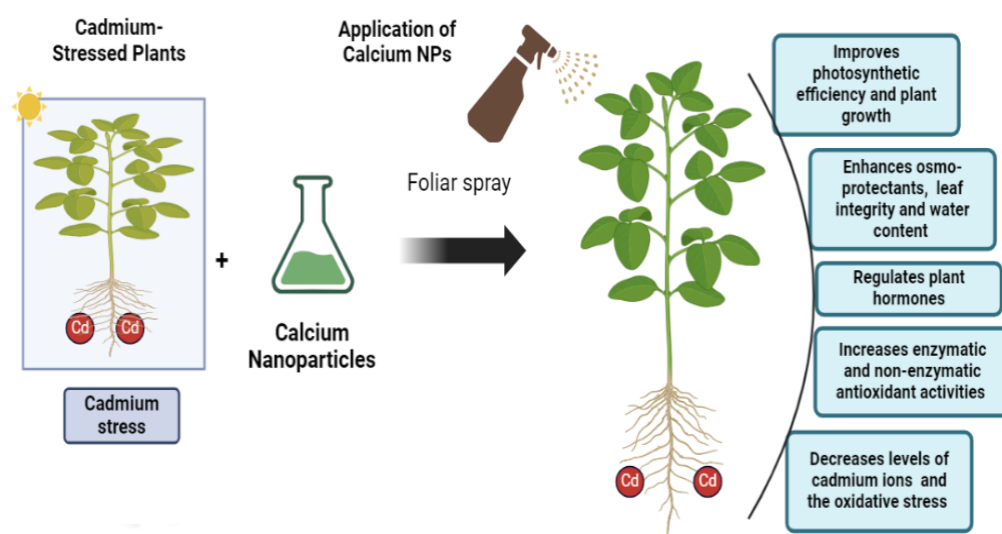


Figure 8. The summary demonstrates the minimization of cadmium stress-induced harmful effects in *Vicia faba* plants by foliar application of nano calcium carbonate.

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AUTHOR CONTRIBUTIONS

MMR: Data curation, Investigation, Validation, Visualization, Writing – original draft, Writing – review & editing. HMA: Data curation, Investigation, Validation, Visualization, Writing – original draft, Writing – review & editing. DEAS: Data curation, Validation, Visualization, Writing – original draft. RKK: Data curation, Investigation, Validation, Visualization, Writing – original draft, Writing – review & editing.

CONFLICTS OF INTEREST

There is no conflict of interest among the authors.

SUPPLEMENTARY MATERIALS

Supplementary Figure S1-S4, HR-TEM images of calcium nanoparticles, and SAED pattern, growth parameters, non-enzymatic and total free radical scavenging activity, and response of the levels of cell osmotic stress indicators. Table S1, A preliminary trial

to identify the best performance of different physiological parameters following foliar spraying with calcium carbonate and nano-calcium carbonate levels, and Table S2, primer sequences for RT-qPCR of stress-related genes in *Vicia faba* ([Supplementary Materials](#)).

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