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RESEARCH ARTICLE

# Nanoparticle innovations: impact of biogenic CaP nanoparticles in mitigating the adverse effects of excessive nitrate application

**Mai A. El-Esawy · Eman A. Elkhateeb · Amira M. Hassan · Doaa E. Elsherif**

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**Abstract**

*Aims* The overuse nitrogen fertilizer has resulted in significant environmental pollution and may also affect the ability of plants to withstand stress and productivity. The goal of this study was to create a nanofertilizer that may improve the resistance of lupine (*Lupinus termis L*) nitrate stress while simultaneously releasing a micronutrient that supports plant growth.

*Methods* Calcium phosphate nanoparticles (CaP-NPs) were created via *Jania rubens* extract and sprayed on lupine plants as a foliar spray at various dosages (0, 25, 50, and 100 mg/L) to mitigate the harmful effects of high nitrate stress (200 mM nitrate) on the seedling stage of lupine plants.

*Results* The biogensis CaP-NPs were characterised to have a spherical form with a size of $29 \pm 3.2$ nm. The findings demonstrated that, in contrast to the control, nitrate stress negatively affected the growth metrics of lupines; however, foliar spraying with CaP-NPs increased these parameters during nitrate stress. Moreover, treatment with CaP-NPs greatly reduced the increased levels of indicators of oxidative stress (MDA, $H_2O_2$, proline, nitrate-nitrogen ($NO_3^{--}N$), and ammonium-nitrogen ($NH_4^+$-N)). Additionally, the administration of CaP-NPs under conditions of elevated nitrate stress induced the activity of antioxidant enzymes (peroxidase (POD), ascorbate peroxidase (APX), polyphenol oxidase (PPO), and phenylalanine (PAL)) as well as non-enzymatic markers such as total antioxidant capacity (TAC), total flavonoid content, total phenolic content, ascorbic acid, and glutathione. The administration of CaP-NPs resulted in an increase in the expression of antioxidant genes, including *ferritin (FER1), phenylalanine (PAL), catalase2 (CAT2), superoxide dismutase1 (SOD1), chalcone synthase (CHS), and flavonol synthase (FLS)*. The highest level of upregulation was observed at 100 mg/L CaP-NPs.

*Conclusion* Foliar CaP-NPs nanofertilizer application in agriculture may increase yield while reducing the harmful effects of nitrate stress on plants. Thus, our work provides a solid basis for further investigations aimed at assessing the substitution of CaP nanofertilizers for traditional $Ca^{2+}$ or P fertilizers to mitigate the deleterious effects of nitrate stress and improve lupine output.

**Keywords** Nitrate stress · Nanoparticles · Nanofertilizer · Calcium phosphate · Antioxidant enzymes · Gene expression · *Lupinus termis* L

**Abbreviations**

| | |
|---|---|
| CaP-NPs | Calcium phosphate nanoparticles |
| TEM | Transmission electron microscopy |

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M. A. El-Esawy (✉) · E. A. Elkhateeb · A. M. Hassan · D. E. Elsherif
Botany Department, Faculty of Science, Tanta University, Tanta 31527, Egypt
e-mail: mai.elesawy@science.tanta.edu.eg

| | |
|---|---|
| FTIR | Fourier transform infrared spectroscopy |
| APX | Ascorbate peroxidase |
| POD | Peroxidase |
| PPO | Polyphenol oxidase |
| *PAL* | Phenylalanine ammonia lyase |
| *CHS* | Chalcone synthase |
| *FLS* | Flavonol synthase |
| *SOD1* | Superoxide dismutase1 |
| *CAT2* | Catalase2 |
| *FER1* | Ferritin1 |
| TAC | Total antioxidant capacity |
| ASA | Ascorbic acid content |
| $H_2O_2$ | Hydrogen peroxide |
| MDA | Malondialdehyde |
| $NO_3^-$N | Nitrate-nitrogen |
| $NH_4^+$-N | Ammonium-nitrogen |
| PCA | Principle component analysis |
| ANOVA | Analysis of variance |

## Introduction

The anthropogenic activities intended to increase crop production may encourage the buildup of unwanted chemicals in plants and negatively impact the nutritional value of soil and water (Martínez-Ballesta et al. 2010). Since nitrogen fertilizer is a reasonable insurance against crop losses and economic ramifications, increasing amounts of nitrogen fertilizer are being applied to fields (Lemaire and Gastal 2018). However, plants can no longer absorb nitrogen when the supply exceeds need; as a result, nitrogen builds up in the soil, usually as nitrates (Nosengo 2003). In a three-decade in situ tracer experiment, scientists examined the fate of isotopically labelled nitrogen fertilizers and reported that plants consumed 61–65% of the applied fertilizer N, with the remaining 35–39% ending in soil (Sebilo et al. 2013). Thus, nitrogen continues to accumulate in the soil environment and has an impact on plant growth if nitrogen fertilizer is sprayed annually. The plant nitrate content is impacted by this nitrogen imbalance in the soil and elevated nitrate levels in groundwater supplies (Dapoigny et al. 2000; NAAS 2005; Vieira et al. 1998).This may have an effect on the plant's ability to withstand stress and produce more, in addition to causing significant contamination of the environment (Schlesinger 2009). According to research, applying an excessive amount of nitrogen fertilizer can lower agricultural sustainability and negatively influence tomato fruit and legumes such as alfalfa output and quality (Ronga et al. 2020; Chen et al. 2021).

Research has shown that the accumulation of high nitrate in plants results in the production of nitrite, which is then transformed into nitric oxide (NO). Nitrate reductase can then quickly catalyze the conversion of $NO^+$ and $O_2^-$into peroxynitrite ($ONOO^-$), which is extremely toxic to plants (Durner and Klessig 1999; Lamattina et al. 2003). Consequently, plant development is deleteriously impacted by high nitrate accumulation in plants (Chen et al. 2004; Ikemoto et al. 2002; Ishiwata et al. 2002). Additionally, increases in soil $NO_3^-$ions promote the exchange of $Ca^{+2}$ and $K^+$ ions from the solid soil phases to form a variety of nitrates. These nitrates can adversely affect nitrogen metabolism, reduce crop yield, and cause oxidative damage and metabolic disorders in plants (Du et al. 2017). Excessive nitrate has been shown to increase osmotic stress, ionic toxicity, and reactive oxygen species (ROS), which all negatively impact crop quality and yields. It also decreases photosynthesis and enzyme activity (YANG et al. 2010; Zhang et al. 2017a). Thus, novel strategies to improve plant resistance to elevated nitrate stress and maintain agricultural yield under nitrate stress conditions are desperately needed.

Nanofertilizers, and nanopesticides are common applications of nanotechnology in agriculture. These nanoforms have the ability to track nutrient levels, promote productivity and growth, and strengthen plant resistance to stress (Bhatt et al. 2020; Daniel et al. 2024). Owing to their special physicochemical characteristics, which include their high surface area and high reactivity, nanofertilizers allow for the targeted delivery of nutrients and bioactive substances to plants (Nongbet et al. 2022). Additionally, it is possible for nanofertilizers to improve biochemical activity, such as raising proline and chlorophyll levels, and lessen the effects of oxidative stress (Devi et al. 2023). Additionally, they aid in maintaining the relative water content and lessen the accumulation of dangerous compounds including hydrogen peroxide and malondialdehyde (Dos Santos et al. 2022). Furthermore, under stress condition, plants depend on nanofertilizers to maintain their ionic equilibrium (Devi et al. 2023). Consequently, there has been much interest in the application of nanofertilizers to improve the ability of plants to tolerate stress.

One interesting area of biotechnological research is the green manufacture of nanoparticles (NPs) smaller than 100 nm (Sharma et al. 2019). Comparison with other traditional physical and chemical approaches, this biofabrication method is efficient, less hazardous, eco-friendly, and economical (Kulkarni et al. 2023). Owing to thier ease of use, affordability, dependability, and environmental friendliness, the biofabrication of nanofertilizers, such as CaP-NPs, is currently gaining traction (Pokale et al. 2014). The stability of nanoparticles is largely dependent on the active ingredients included in their biological extracts (Rónavári et al. 2017). For plant metabolic processes and critical macromolecules, calcium and phosphorus are macronutrients that are absolutely necessary. Calcium has numerous vital functions in plants, including supporting healthy cell elongation, strengthening cell walls, taking part in the metabolic process of absorbing other nutrients, and the hormonal process (White and Broadley 2003). A key component of ATP, the molecule that gives plants "energy" for various functions including nutrient translocation, protein synthesis, photosynthesis, respiration, and the inheritance of genetic traits, is phosphorus (Khan et al. 2023). According to Ahmed et al. (Ahmed et al. 2018), phosphorus has been shown to promote root growth and have affect early maturity, crop quality, and straw strength.

An ancient leguminous plant known as *lupinus termis* L, or white lupine, has been farmed and utilized as livestock and human food for more than 2,000 years (Petterson and Fairbrother 1996). In Africa and the Middle East, lupine seeds are applied topically to cure acne and are used as anti-diabetic agents in traditional medicine (Santiago Quiles et al. 2010). Owing to their high alkaloid content, nutritional value, protein richness, and medicinal qualities, lupine seeds have recently gained popularity (Omer et al. 2016). Excessive nitrogen can prevent lupine plants from developing nodulation and cluster roots, which are essential for phosphorus uptake in the event of a phosphorus shortage. As a result, plant growth, and photosynthesis may be restricted (Pueyo et al. 2021; Wang et al. 2019). To maximize lupin sustainability, and productivity, a balanced strategy for nitrogen management is essential (Pueyo et al. 2021). However, little research has been conducted on how nanoparticles control how plants respond to high nitrate stress. This study aimed to assess the impact of an eco-friendly biosynthetic CaP-NP fertilizer on the molecular and physiological mechanisms of lupine under nitrate stress. Consequently, a thorough grasp of how exogenous CaP-NPs enhance lupine nitrate stress tolerance was given.

## Materials and methods

### Preparation of CaP-NPs

After being harvested from Ras Gharib, Gulf of Suez, Red Sea Governorate, Egypt, the macroalgae *Jania rubens* were cleaned with distilled water, allowed to air dry for three to four days, and then milled into a fine powder. After homogenizing roughly 5 g of the dried powder with 100 ml of distilled water, the mixture was centrifuged. The method outlined by Pokale et al. (2014) for CaP-NP biosynthesis involves collecting the supernatant and using it for the process. Specifically, 1.5 ml of *Jania rubens* aqueous extract was combined with 7.5 ml of 12.5 mM dibasic sodium phosphate solution and 7.5 ml of 12.5 mM calcium chloride, all while stirring continuously for 10—15 min. For thirty minutes, the mixture was sonicated. After twice washing the resulting CaP-NPs precipitate with ethanol and distilled water to get rid of any contaminants, they were centrifuged for ten minutes at 6000 rpm. The precipitate was dried for 12 h at 60 °C in a vacuum oven.

### Characterization of CaP-NPs

Using KBr disks, CaP-NPs were subjected to Fourier transform infrared (FT-IR) spectral analysis via an FTIR-4100 spectrometer from JASCO, covering a frequency range of 4000 to 400 cm-1. CaP-NP samples were examined optically and the band gap was calculated via UV–visible spectroscopy (Shimadzu 240). The average particle size and morphology of the nanoparticles were further examined via transmission electron microscopy (TEM).

### Experimental design

The Agricultural Research Center in Dokki, Cairo, Egypt, provided lupine (*Lupinus termis* L. cv. Giza-2) seeds. Before sprouting in pots, these seeds were sterilized for five minutes with 1% NaClO and then

repeatedly cleaned with distilled water. Next, five sterile seeds were planted in pots, each of which held five kilograms of air-dried soil (sand: clay, 2:1 v/v). The physicochemical properties of the soil used revealed the following concentrations: $N = 0.7$, $P = 2.4$, K = 34.3, Ca = 5.6, Mg = 23.2, Na = 6.1, and Cl = 6.69 mg/kg. The soil had a slightly alkaline pH of 7.1 and an electrical conductivity (EC) of 3.5 mS/cm. Tap water was used to irrigate the pots every day until complete germination. The lupine seedlings were exposed to 200 mM nitrate and 0 mM nitrate on the fourth day following germination. On average, potassium nitrate ($KNO_3$) and sodium nitrate ($NaNO_3$) make up the nitrate. After that 200 mM nitrate-irrigated pots were subjected to foliar spraying of CaP-NPs twice weekly in 4 groups as follows: 200 mM nitrate, 200 mM nitrate + 25 mg/L CaP-NPs, 200 mM nitrate + 50 mg/L CaP-NPs, and 200 mM nitrate + 100 mg/L CaP-NPs (The nitrate stressed plants were irrigated with tap water or 200 mM nitrate alternately every week until harvest). Following a period of 15 days, the plants were harvested estimate various factors related to plant growth performance, including growth metrics, several markers of oxidative stress, and antioxidant levels.

Growth parameters

To estimate the plant growth performance, measurements of the shoot length, root length, fresh weight, and dry weight were made.

Measurement of several oxidative stress markers

*Determination of malondialdehyde content (MDA)*

According to Heath and Packer (1968), the thiobarbituric acid (TBA) reaction was used to measure the amount of malondialdehyde (MDA), which serves as an indicator of lipid peroxidation. A sample (0.5 g) of fresh tissue (leaf) was homogenized in 10 ml 5% (w/v) of trichloroacetic acid (TCA). Then the homogenate was then centrifuged at 4000 rpm for 15 min. Two ml of this supernatant was mixed with 2 ml of 0.677% (w/v) of thiobarbituric acid (TBA). This mixture was heated in a boiling water bath for 15 min and then cooled immediately in cold water for 10 min. Measurements of the absorbance were made at 600 and 532 nm. The MDA concentration was calculated as n.mol/g f.wt. and expressed using an extinction coefficient of 1.55 µM-1 cm-1.

*Determination of hydrogen peroxide ($H_2O_2$)*

With the use of an extinction coefficient of 0.28 µM–1 cm–1 and an absorbance measurement at 390 nm, the hydrogen peroxide concentration was measured using the methodology outlined by Velikova et al. (2000). Leaf tissue (200 mg) was grinded 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 and then the result was reported as µmol/g f.wt.

*Determination of proline content*

The proline content was calculated using the Bates et al. (1973) method in which leaf tissue was grinded in sulfosalicylic acid aqueous solution (3%). Leaf extract was boiled with acid ninhydrin reagent (prepared by warming ninhydrin in glacial acetic acid and phosphoric acid) for 1 h. After 520 nm absorbance was measured, proline content was calculated and represented as mg/g d.wt. using a proline-prepared reference graph.

*Determination of nitrate-nitrogen ($NO_3^-$-N) and ammonium-nitrogen ($NH4^+$-N) concentrations*

Zhang et al. (2017b) state that fresh leaf samples were used to assess the amounts of $NO_3^-$-N and $NH_4^+$-N. Fresh samples (0.5 g) were ground in 10 mL of water and then held in a boiling water bath for 30 min. The supernatant was diluted to 25 mL. Then, 0.1 mL solution was mixed with 0.4 mL of 5% salicylic-$H_2SO_4$ to react for 20 min. And 9.5 mL of 8% NaOH was added. After cooling to room temperature, absorbance was measured at 410 nm for $NO_3^-$-N. Meanwhile, $NH_4^+$-N concentrations, the fresh samples were ground in 10 mL of 10% acetic acid and the volume was made up to 100 mL with distilled water. Then, 2 mL of the solution, 3 mL of ninhydrin hydrate and 0.1 mL of 1% ascorbic acid were mixed and heated in boiling water for 15 min. After being cooled to room temperature, absorbance was measured at 580 nm.

Measurement of some non-enzymatic and enzymatic antioxidants

*Determination of total antioxidant capacity*

Phosphomolybdenum technique for measuring total antioxidant capacity (TAC). In accordance with Prieto et al. (1999), sulfuric acid (0.6 M), ammonium molybdate tetrahydrate (4 mM), and sodium phosphate dibasic solution (28 mM) were added to create the TAC reagent. TAC reagent was combined with ethanolic extract and cooked for ninety minutes. Sample absorbance was measured at 765 nm after cooling.

*Determination of ascorbic acid content (ASA)*

Oser (1965) provided an estimate of the ascorbic acid content (ASA) in which leaf tissue was grinded in 5% sulfosalicylic acid. The reaction mixture consist of 2% Na-molybedate, 0.15 N H2SO4, 1.5 mM Na2HPO4, and leaf extract. The reaction mixture incubated at 60 °C in a water bath for 45 min, cooled and centrifuged. At 660 nm, the absorbance was measured. Using a predefined calibration curve, the ASA content was determined in mg/g d.wt.

*Determination of reduced glutathione content (GSH)*

According to Anderson (1985), the reduced glutathione (GSH) content was estimated. A known weight (0.1 g) of the fresh leaf tissue was homogenized in 5 ml of 3% (w/v) sulfosalicylic acid then centrifuged at 10,000 rpm for 10 min. The reaction mixture contains 0.5 ml of tissue extract, 0.5 ml of 0.5 mM potassium phosphate buffer (PH 7.0) and 50 µl of 3 mM 5,5-dithio-bis 2-nitrobenzoic acid (DTNB). Appropriate amounts were added. Using the standard GSH curve, the absorbance was measured at 412 nm, and the GSH content was computed.

*Determination of total phenolic content*

We evaluated the total phenolic content using the Jindal and Singh (1975) approach. A known weight (0.1 g) of the dried tissues was extracted by 10 ml of 95% ethanol; the clear supernatants were combined and completed to a known volume (10 ml). Then 1 ml of this extract was mixed with 0.1 ml Foline reagent and 1 ml $Na_2CO_3$ then completed up to a known volume (5 ml) with distilled water. The absorbance was measured at 650 nm following a half-hour incubation period. Using various gallic acid concentrations as the previous technique, a standard curve was created and used to calculate the total phenolic content (mg/g d.wt.).

*Determination of total flavonoids content*

The ethanolic extract's total flavonoids were measured using the protocol outlined by Chang et al. (2002) by adding 0.1 ml of 10% AlCl3, 0.1 ml of 1 M potassium acetate and 1.5 ml of ethanol (95%) to 0.5 ml ethanolic extract. After 30 min, the absorbance of the mixture was measured at 417 nm using quercetin as a reference flavonoid. The total flavonoids were expressed as mg/g d.wt.

*Determination of peroxidase, ascorbate peroxidase, polyphenol oxidase and phenylalanine ammonia lyase activity*

The antioxidant enzyme extraction procedure followed Grace and Logan (1996) instructions. The method used to evaluate the peroxidase activity (POD) was Kato and Shimizu (1987). Using the extinction coefficient of 26.6 mM-1 cm-1 at 470 nm, the activity of POD was computed. The ascorbate peroxidase activity (APX) was measured using the Nakano and Asada (1981)approved method. The absorbance at 290 nm decreased after ascorbate underwent hydrogen peroxide-dependent oxidation. The method of measuring polyphenol oxidase activity (PPO) was outlined by Kumar and PA (1982). The enzyme activity was expressed as µM/g f.wt. min-1, and the absorbance was measured at 420 nm using an extinction value of 26.40 M-1 cm-1. El-Shora (2002) was modified to measure phenylalanine ammonia lyase activity (PAL). The enzyme activity was measured at 290 nm and represented as µM/g f.wt. min-1.

Molecularestimation

*Total RNA extraction, cDNA synthesis and RT-PCR*

Total RNA was isolated from *Lupinus termis* L. leaves via the Qiagen RNase Mini Kit. Through reverse RNA transcription, 20 µl of complementary DNA (cDNA) was obtained using a thermocycler

(MJ Research, Inc., PTC-100TM Programmable thermal controller, USA). A first enzyme activation cycle of 42 °C for one hour and a second enzyme inactivation cycle of 95 °C for five minutes were part of the reaction conditions. SYBR Green PCR Master Mix (Fermentas, USA) was used to create the qRT-PCR in triplicate. For each reaction, a 25 µl mixture including the primer pairs (*PAL, CHS*, *FLS*, *SOD1*, *CAT2*, and *FER1*) listed in Table 1 was utilized. Using a Rotor-Gene 6000 (QIAGEN, ABI System, USA), the reaction was carried out. The reference gene for the gene was ACTIN. The relative expression of the genes under study was measured and computed using Livak and Schmittgen (2001).

### Statistical analysis

The means of the replicates ± standard errors (SEs) were used to show all the data. Using XLSTAT software (version 2014.5.03), the difference between treatments for the various measured variables was assessed via one-way variance (ANOVA), and Duncan's test. A significant difference ($P < 0.05$) was found.

## Results

### Characterization of CaP-NPs

#### *UV–visible spectrum of CaP-NPs*

The reaction mixture's color changed from yellowish to white, indicating the biofabrication of CaP-NPs (Fig. 1). The absorption spectra of the CaP-NPs biosynthesized with *Jania rubens* aqueous extract were validated via the UV–visible technique. As shown in Fig. 2, the absorbance of the biosynthesized CaP-NPs was visible in the UV–Vis spectrum at approximately 240 nm.




**Fig. 1** Changes in the color of *Jania rubens* extracts from (**A**) yellowish to (**B**) white

**Table 1** Specific primers used the current study

| Gene name | Abbreviation | Forward (F) and reverse (R) primer 5′–3′ |
|---|---|---|
| Reference gene | *ACTIN* | F: 5′-GGTAACATTGTGCTCAGTGGTGG-3′<br>R: 5′-AACGACCTTAATCTTCATGCTGC-3′ |
| Phenylalanine ammonia lyase | *PAL* | F:5′-GCAAGGAAAGCCCGAGTTTAC-3′<br>R: 5′-GGACCTTTTTGGCTACTTGGC-3′ |
| Chalcone synthase | *CHS* | F:5′-AAGCTCTCACTCTCCGGT-3′<br>F:5′-TCGTGTGAGTCCCTTGCT-3′ |
| Flavonol synthase | *FLS* | F: 5′-TTAAAGGAAGGTCTCGGTGGCGAA-3′<br>R: 5′- TCATTGGTGACGATGAGTGCGAGT −3′ |
| Superoxide dismutase1 | *SOD1* | F: 5′-TTCGAAGGGCAGAGTCGAAG-3′<br>R: 5′-CAAACGAGGCATGCCCAAAA-3′ |
| Catalase2 | *CAT2* | F:5′-TGACTGCGGATTCGAGCAA-3′<br>R: 5′-AACGCAAGAAGAGGGGTTGA-3′ |
| Ferritin1 | *FER1* | F:5′-CCCCAACACAAATCTTAGCCG-3<br>R: 5′-CCAACACTGGGTTTGGGAGA-3′ |

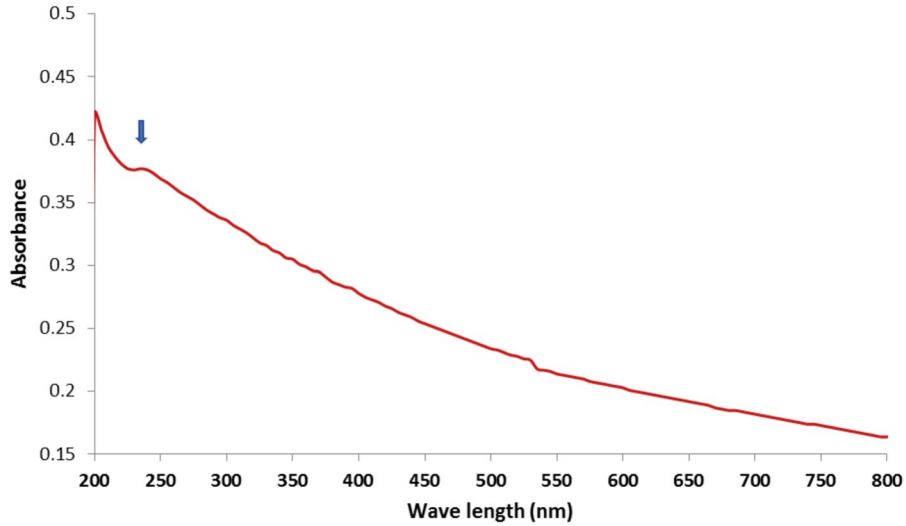



**Fig. 2** UV–Vis spectrum of biosynthesized CaP-NPs

*Transmission electron microscopy of CaP-NPs*

The average particle size and shape of the biosynthesized CaP-NPs were examined via transmission electron microscopy (TEM) to provide additional characterisation. As shown in Fig. 3, the TEM investigation revealed the presence of spherical CaP-NPs with an average size of $29 \pm 3.2$ nm.

*Fourier transform infrared spectroscopy of CaP-NPs*

As illustrated in Fig. 4, the biosynthesized CaP-NPs absorption peaks from the *Jania rubens* aqueous extract were measured via Fourier transform infrared spectroscopy (FT-IR) at 3853.42, 3743.39, 3445.70, 2361.81, 1647.48, 1541.65, 1516.80, and 1098.1 cm-1. Furthermore, 3743.53, 3442.79, 2361.26, 1646.41, 1544.51, 1516.08, 896.59, and 572.29 cm-1 were the primary absorption peaks of the *Jania rubens* aqueous extract.

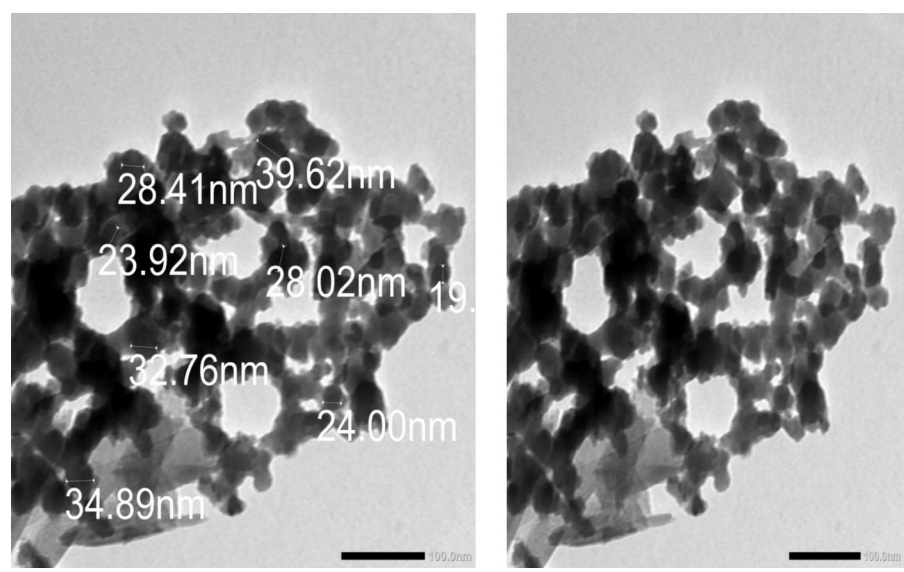



**Fig. 3** TEM image of biosynthesized CaP-NPs

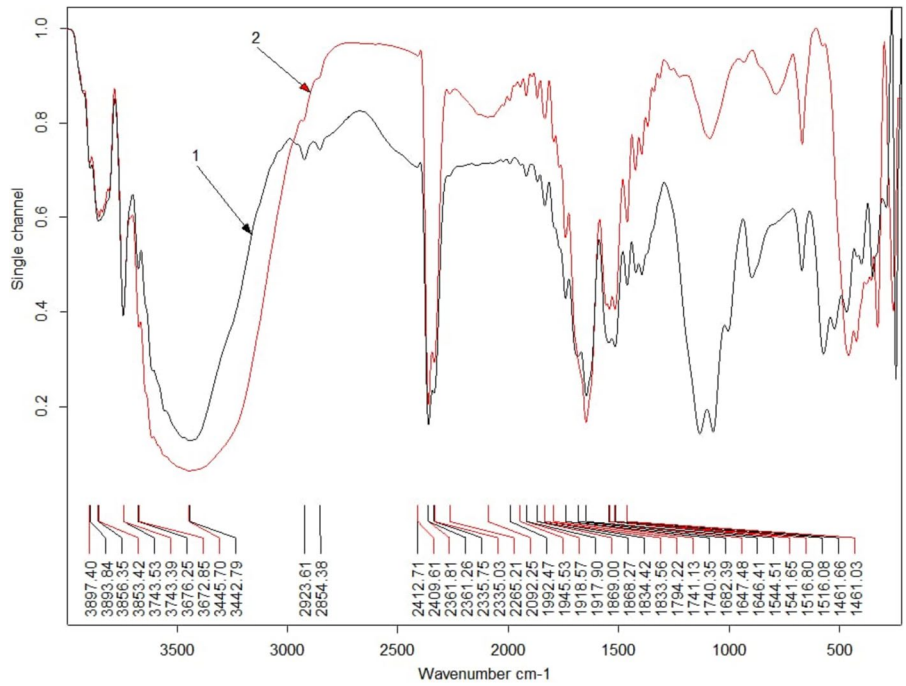



**Fig. 4** FT–IR spectra of (1) *Jania rubens* aqueous extract and (2) biosynthesized CaP-NPs

Effects of CaP-NPs on several growth parameters and

According to the data in Figs. 5 and 6, treating lupine seeds with 200 mM nitrate dramatically reduced the fresh weight, dry weight, shoot length, and root length of 15-day-old lupine seedlings by 30%, 53%, 31%, and 23%, respectively, in comparison to the control samples. Conversely, nitrate-stressed lupine seedlings significantly improved in all evaluated growth parameters under foliar treatment with 25, 50, and 100 mg/L CaP-NPs. Compared with those of lupine seedlings under nitrate stress, the ratios of enhancement were 16%, 20%, and 35% for shoot length, 52%, 75%, and 81% for root length, 21%, 24%, and 39% for fresh weight, and 16%, 19%, and 32% for dry weight.

Effects of CaP-NPs on oxidative stress

Figure 7(A) illustrates how treating lupine seeds with 200 mM nitrate considerably raised the MDA content




**Fig. 5** Effects of different doses of CaP-NPs on lupine seedling growth under nitrate stress (200 mM nitrate)

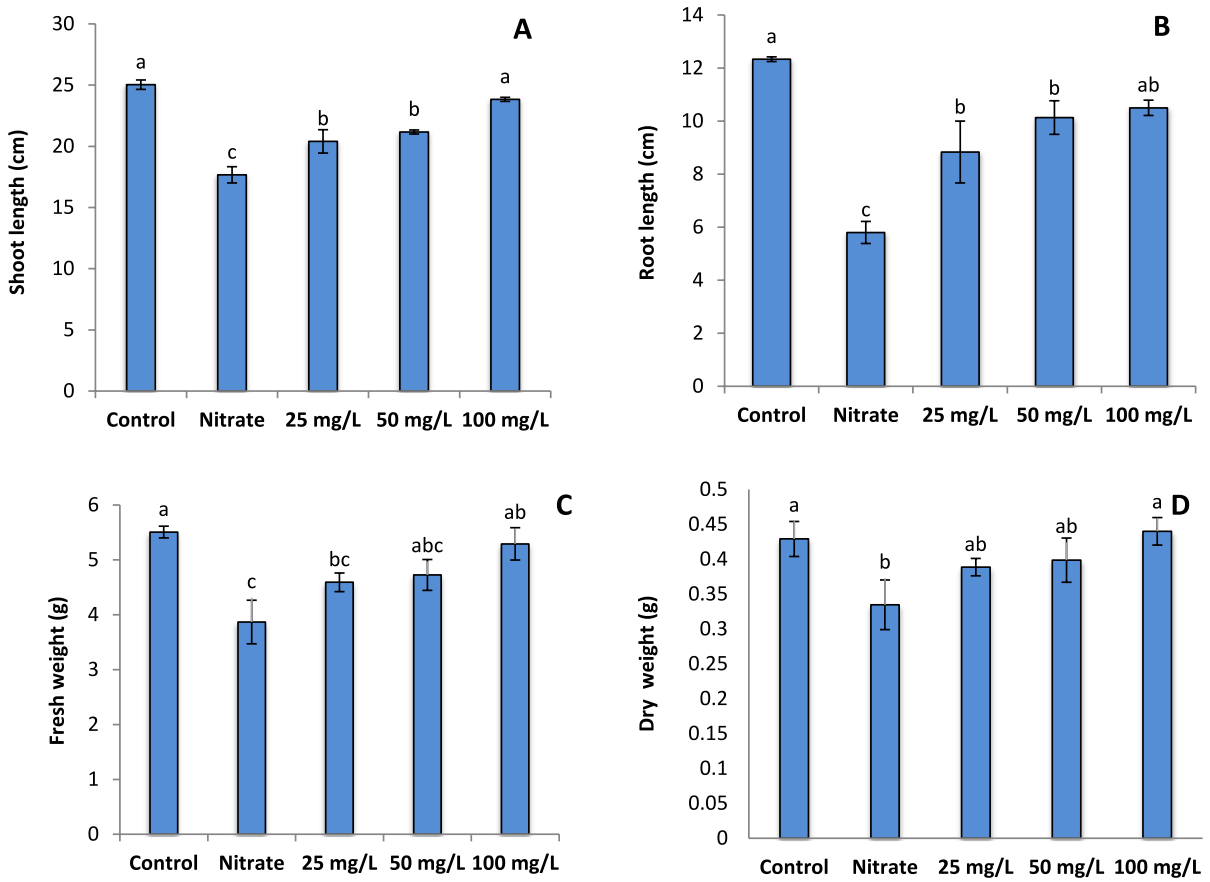



**Fig. 6** Effect of foliar spray with CaP-NPs (25, 50 and 100 mg/L) on (**A**); shoot length, **B**; root length, **C**; fresh weight, and (**D**); dry weight of nitrate-stressed lupine seedlings

of 15-d old lupine seedlings by 74% compared with that of the control group. Conversely, foliar treatment with 25, 50, and 100 mg/L CaP-NPs significantly reduced the MDA of lupine seedlings under nitrate stress by 27%, 37%, and 48%, respectively. As demonstrated in Fig. 7 (B), nitrate stress also caused a notable 150% rise in $H_2O_2$ levels in 15-day-old lupine seedlings when compared with those in the control group. In contrast, foliar treatment with 25, 50, and 100 mg/L CaP-NPs reduced the $H_2O_2$ concentration of lupine seedlings under nitrate stress by 72%, 73%, and 63%, respectively. Furthermore, in comparison with the control, nitrate stress caused a significant 91% increase in the proline concentration in 15-day-old lupine seedlings (Fig. 7C). Nevertheless, the proline content of nitrate-stressed lupine seedlings was reduced by 18%, 28%, and 36%, respectively, by foliar application of 25, 50, and 100 mg/L CaP-NPs. Furthermore, in comparison with the results of the the control group, nitrate stress led to significant increases in the contents of $NO_3^-$-N and $NH_4^+$-N of approximately 17% and 62%, respectively (Fig. 7D, E). Conversely, foliar application of 25, 50 and 100 mg/L CaP-NPs decreased the contents of $NO_3^-$-N and $NH_4^+$-N in 15-d old nitrate-stressed lupine seedlings by approximately 14%, 14% and 13%, respectively for $NO_3^-$-N and 35%, 36% and 34%, respectively, for $NH_4^+$-N.

## Effects of CaP-NPs on non-enzymatic and enzymatic antioxidants

### *Non-enzymatic antioxidants*

As shown in Fig. 8, nitrate stress dramatically reduced the total antioxidant capacity (TAC), total

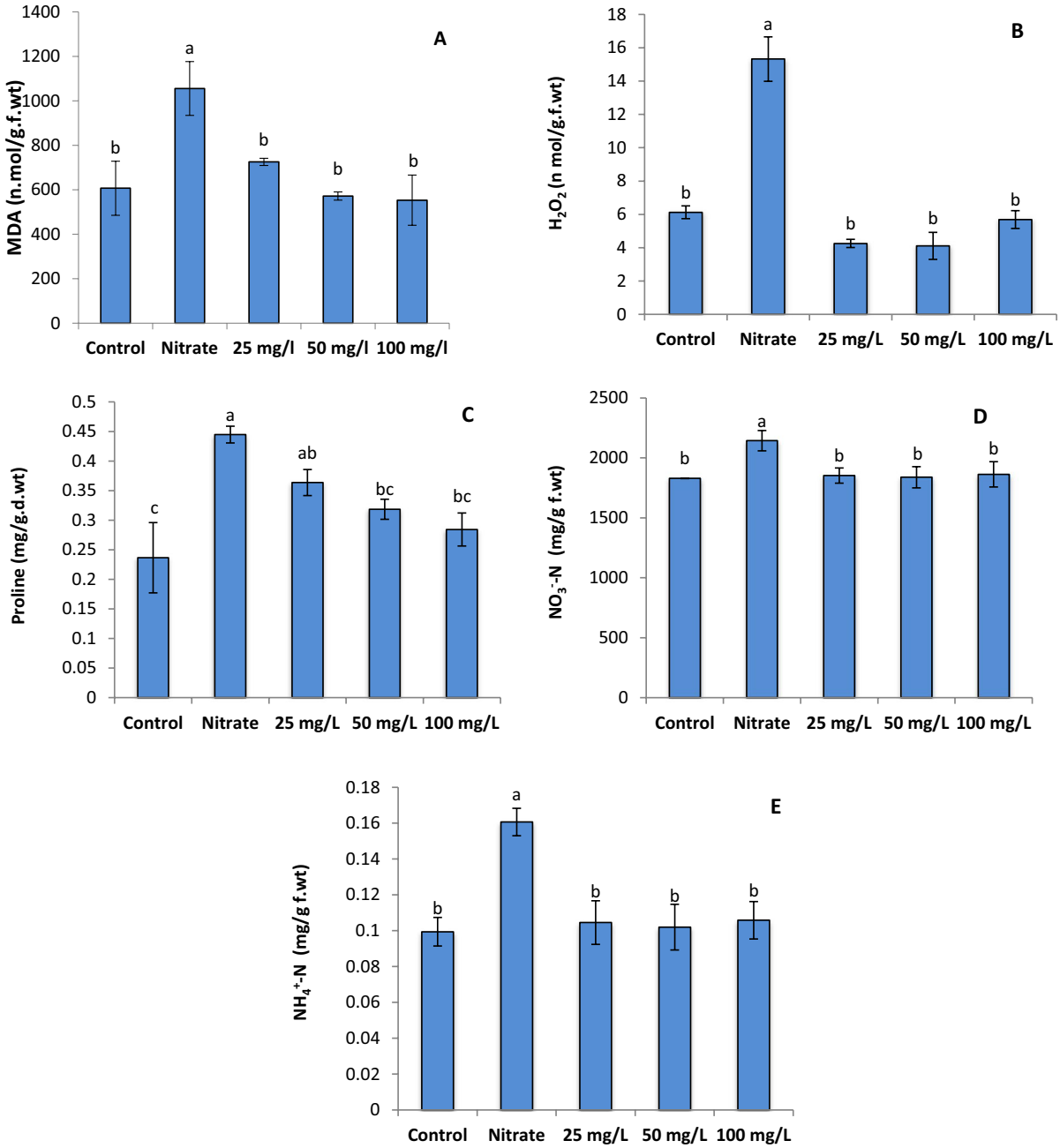



**Fig. 7** Effects of foliar spraying with CaP-NPs (25, 50 or 100 mg/L) on (**A**); MDA, **B**; $H_2O_2$, **C**; proline, **D**; nitrate-nitrogen and (**E**); ammonium-nitrogen contents of nitrate-stressed lupine seedlings

flavonoid content, total phenolic content, ascorbic acid content, and glutathione content of 15-day-old lupine seedling's by 29%, 54%, 52%, 15%, and 28%, respectively, compared with those of the control group. Nevertheless, all tested non-enzymatic antioxidants substantially increased in response to foliar treatment with 25, 50, and 100 mg/L CaP-NPs. Comparing the total antioxidant capacity enhancement to the nitrate-stressed lupine seedlings, the ratios were 14%, 25%, and 57% for the

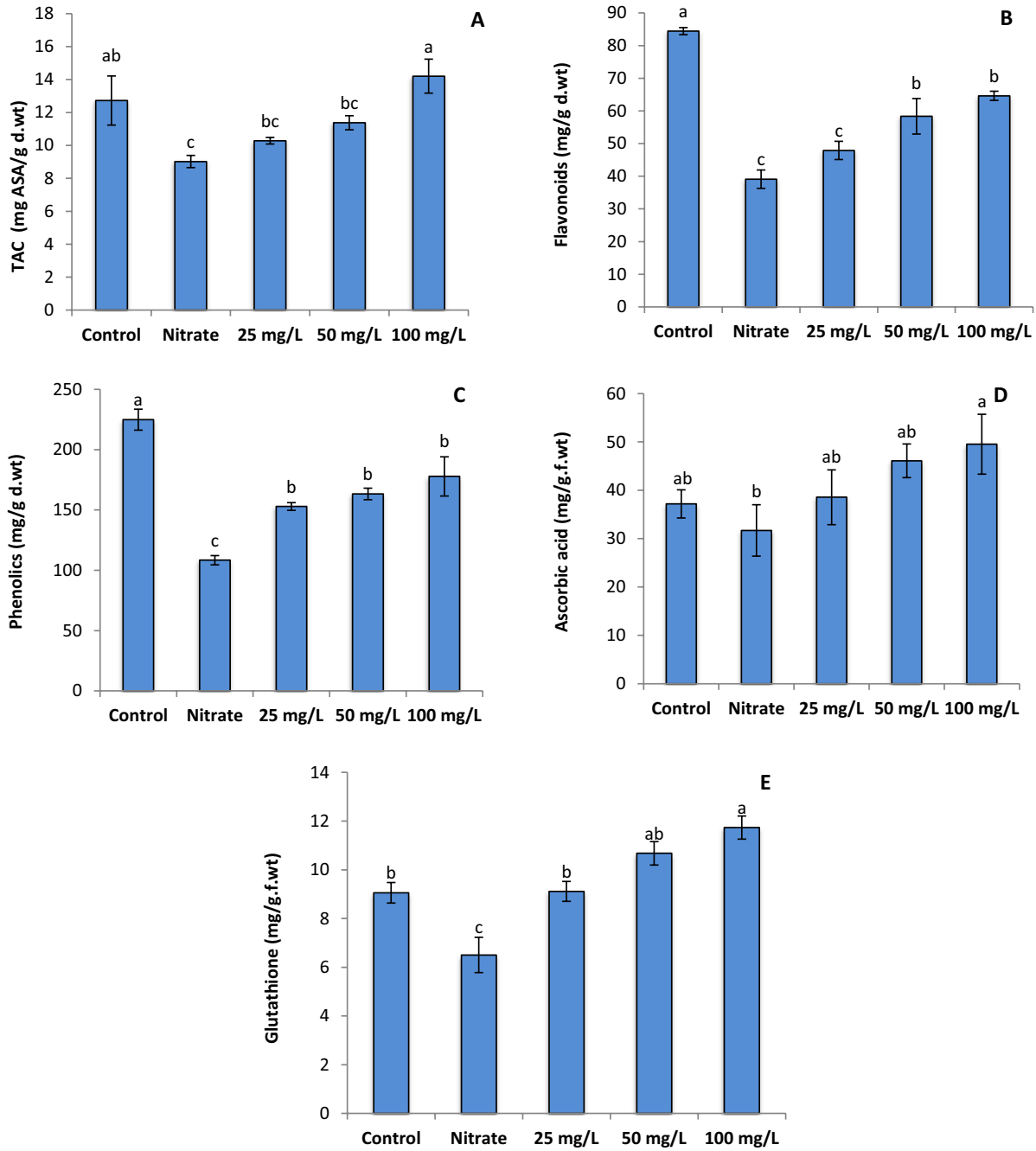



**Fig. 8** Effects of foliar spraying with CaP-NPs (25, 50 or 100 mg/L) on (**A**); TAC, **B**; flavonoids, **C**; phenolics, **D**; ascorbic acid and (**E**); reduced glutathione of nitrate-stressed lupine seedlings

total flavonoid content; 23%, 49%, and 65% for the total phenolic content; 41%, 51%, and 64% for the ascorbic acid content; and 40%, 63%, and 80% for the glutathione content.

*Enzymatic antioxidants*

As shown in Fig. 9, spraying lupine seeds with 200 mM nitrate significantly increased the APX and

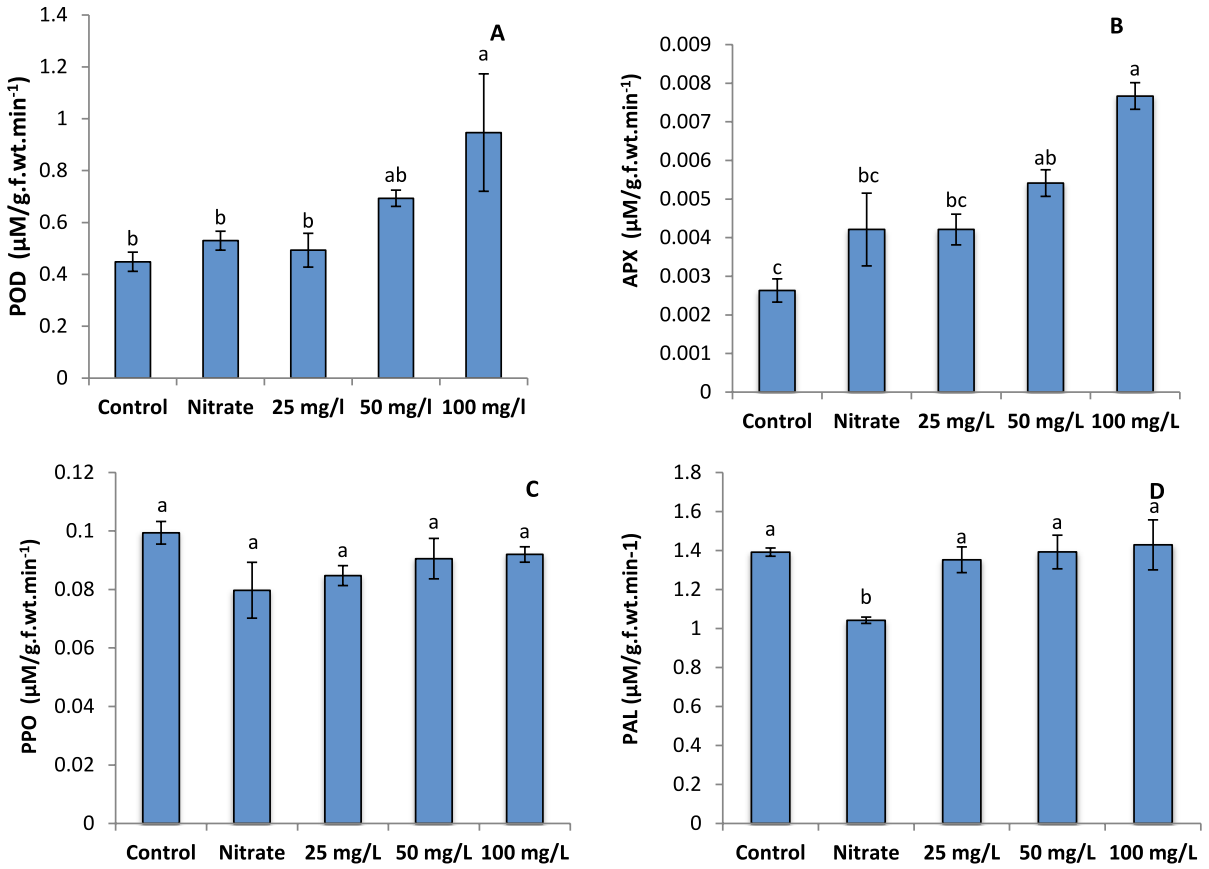



**Fig. 9** Effects of foliar spraying with CaP-NPs (25, 50 or 100 mg/L) on (**A**); POD, **B**; APX, **C**; PPO, **D**; and PAL activity in nitrate-stressed lupine seedlings. Different letters denote significant variations measured by Duncan's multiple range test at $p < 0.05$

POD activities of 15-d old lupine seedlings by 57% and 62%, respectively. In contrast, nitrate stress decreased the activities of phenylalanine ammonia lyase (PAL) and polyphenol oxidase (PPO) by 20% and 25%, respectively, in comparison with those in the control samples. Moreover, the activities of POD, APX, PPO, and PAL of 15-day-old nitrate-stressed lupine seedlings were enhanced by 6%, 14%, and 15%, respectively, for PPO, and by 30%, 34%, and 37%, respectively, for PAL, when 25, 50, and 100 mg/L CaP-NPs were applied topically. Applying 50 and 100 mg/L CaP-NPs topically also increased the activity of APX by 29% and 81%, respectively. Moreover, applying foliar spray containing 100 mg/L CaP-NPs increased the POD activity of nitrate-stressed, 15-day-old lupine seedlings by 36%.

### Effects of CaP-NPs on the relative gene expression of *PAL, CHS, FLS, SOD1, CAT2,* and *FER1*

As seen in Fig. 10, the influence of CaP-NPs on the expression patterns of *PAL, CHS, FLS, SOD1, CAT,* and *FER1* in 15-day-old nitrate-stressed lupine seedlings was assessed. The findings demonstrated that, in comparison with those of control samples, irrigation with 200 mM nitrate considerably reduced the expression levels of *CHS, PAL, FLS, SOD1, CAT2,* and *FER1*. Conversely, foliar treatment with 25, 50, and 100 mg/L CaP-NPs enhanced the expression levels of *FER1, SOD1, CAT2, FLS, PAL,* and *CHS,* especially at the 100 mg/L concentration compared with those in lupine seedlings under nitrate stress.

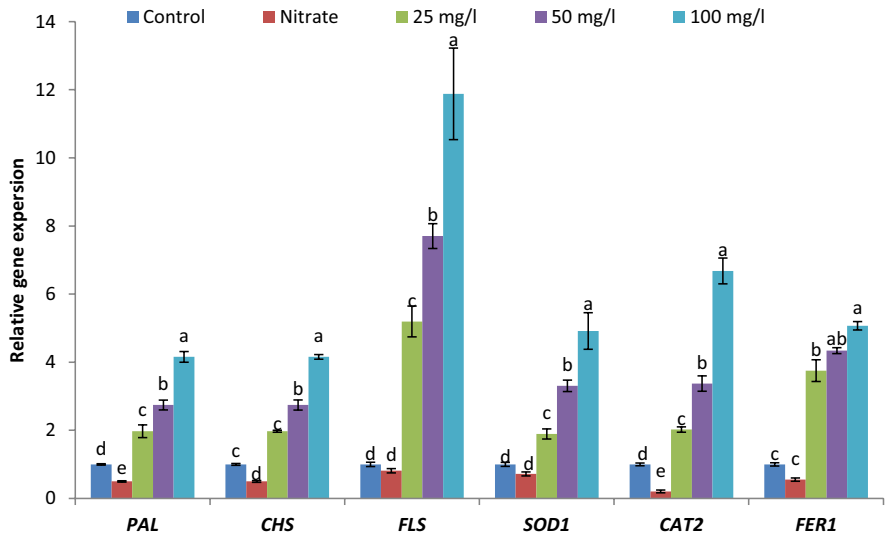



**Fig. 10** Effects of foliar spraying with CaP-NPs (25, 50 or 100 mg/L) on the relative gene expression of *PAL, CHS, FLS, SOD1, CAT2,* and *FER1* in nitrate-stressed lupine seedlings. Different letters denote the significant variations measured by Duncan's multiple range test at $p < 0.05$

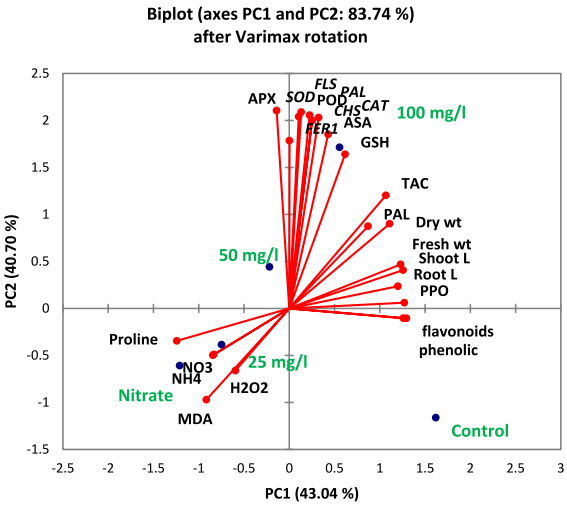



**Fig. 11** Principal component analysis of the relative expression of growth, genes and physiological characteristics assessed at different CaP-NP doses in lupine seedlings under nitrate stress

## Principal component analysis (PCA)

The PCA results connected the physiological variables and gene expression data relative to those of the CaP-NP treatments (Fig. 11). The results of the research showed that the first four PCs with eigenvalues > 1 explained 83.47% of the variation. PC1 accounted for 43.04% of the total variation, making it the most significant. In descending order, flavonoids, PPO, phenolic, shoot length, fresh weight, root length, dry weight, TAC, and PAL were the parameters that demonstrated strong correlation with the PC1. PC2, which accounted for 40.70% of the total change, was most affected, in decreasing order, by APX, POD, *SOD1, FLS, CAT2*, *PAL, CHS,* ASA, *FER1,* and GSH. PC3, which accounted for 15.64% of the variation, showed significant correlations with MDA, $H_2O_2$, $NO_3^-$-N, and $NH_4^+$-N, as well as negative effect variables. Furthermore, PCA revealed that 100 mg/L CaP-NPs had a favour effect on gene expression, growth, and physiological traits. However, there was a substantial link between nitrate stress and MDA, $H_2O_2$, $NO_3^-$-N, and $NH_4^+$-N.

## Discussion

High nitrate stress is a global problem that negatively impacts human health as well as plant growth (Niu et al. 2022). Numerous researches have shown that plant stress can be alleviated by applying calcium and phosphorus externally as a mineral addition (Hosseini et al. 2019; Khan et al. 2023). The significance of nanotechnology has increased in relation to crop plants' ability to withstand stress. Biofabrication is the process of synthesizing nanoparticles from biological sources, with an emphasis on the development of more practical methods for producing benign, nontoxic, and environmentally friendly nanoparticles (Kulkarni et al. 2023). Thus, the present study evaluated the effectiveness of *Jania rubens* extract in the

production of CaP-NP nanoparticles, which were subsequently used to improve the resistance of lupine plants to high concentrations of nitrates. The reaction mixture's color changed from yellowish to white, allowing the formation of biogenic CaP nanoparticles (Fig. 1). The resulting UV–visible spectrum indicated that CaP-NPs presented a peak at 240 nm and highly symmetrical single-band absorption. According to earlier research, CaP-NPs have absorption spectra between 230 and 260 nm (Hou et al. 2013). Furthermore, the TEM reasults revealed the size of 29 ± 3.2 nm spherically-shaped CaP-NPs.

Stretching H-bonded alcohols and phenol hydroxyl (-OH) groups are responsible for the prominent absorption peaks at 3743.53 and 3442.79 cm-1 in the infrared spectra of *Jania rubens* extract. This band was displaced in the produced CaP-NPs, most likely as a result of Ca or P ions attaching to H atoms (Beigoli et al. 2021). The carbonyl group (C = O) found in ketones, aldehydes, and carboxylic acids is thought to be responsible for the band at position 2361.26 (Mubayi et al. 2012). Both the extract and the artificial CaP-NPs exhibited a band at 1646.41 cm-1, which is indicative of the N–H functional group that is frequently present in proteins. Among the synthesized CaP-NPs, 1541.65 and 1098.1 cm-1 is characteristic bands of carbonate ($CO_3^{2-}$) and phosphate ($PO_4^{3-}$), respectively; hence these peaks represent the CaP nanoparticles (Berzina-Cimdina and Borodajenko 2012; Destainville et al. 2003; Raynaud et al. 2002). The green produced CaP-NPs and *Jania rubens* extract may include monosubstituted aromatic aromatics that bend the skeleton at the C–C and aromatic C-H positions, which could explain the absorption bands at 896.69 and 572 to 467 cm-1, respectively. Overall, the FT-IR spectra of the *Jania rubens* extract and the biosynthesized CaP-NPs showed distinct peaks that matched functional groups like -OH, C = O, and-N–H. Therefore, it can be proposed that, in addition to proteins, water-soluble aromatic compounds such as flavonoids and phenolics carry out a reduction process that results in the production of CaP-NPs and their stability. Khalifa et al. (2024) reported comparable results for green synthesized SNPs.

Inhibited physiological and biochemical growth indicators are significant characteristics linked to environmental stress (Du et al. 2017; Yuan et al. 2015). In this study, the fresh weight, dry weight, shoot length, and root length of the lupine seedlings were significantly decreased by nitrate stress. In line with other research on cucumber, spinach, and other vegetables (Guo et al. 2018; Xu et al. 2016), adenosine triphosphate (ATP) also decreased under nitrate stress (Chen et al. 2021). Total phosphorous concentrations decreases as a result of nitrate stress, which generates excess $NO_3^-$ (Chen et al. 2021; Yuan et al. 2015). Additionally, the findings concerning growth parameters demonstrated the ability of fertilizer to mitigate nitrate stress in CaP-NPs, particularly at concentrations of 100 mg/L. When exposed to abiotic stressors, chemically synthesized CaP-NPs can enhance growth indices as leaf area and number, shoot height, fresh weight, and dry weight (Nasrallah et al. 2022). Phospholipid signalling is regulated by calcium in response to osmotic stress (Tahjib-Ul-Arif et al. 2018). Fatty acids, ROS, inositol, and phospholipids are all regulated by $Ca^{2+}$-phospholipid signalling system communication. It also maintains the stability of the osmotic equilibrium of cells (Han et al. 2019). Since P is necessary for biological components like, phospholipids, phosphoproteins, nucleic acids, and nucleotides, it is the most vital macronutrient for plant development (Vance et al. 2003; White and Hammond 2008). Phosphorus is a vital component of ATP, the molecule that provides plants with "energy" for a variety of processes, including respiration, nutritional intake, protein synthesis, photosynthesis, and the inheritance of genetic features (Khan et al. 2023).

The findings of current study demonstrated how nitrate stress causes the production of $H_2O_2$, MDA, proline $NH_4^+$-N, and $NO_3^-$-N, which are all indicators of oxidative stress. Furthermore, the correlations of nitrate stress with $H_2O_2$, MDA, proline $NH_4^+$-N, and $NO_3^-$N were determined via principal component analysis (PCA) (Fig. 11). The lipid peroxidation observed in this study, which is shown by an increase in MDA in plants exposed to nitrate stress, can be explained by the accumulation of $H_2O_2$ and other ROS. Similarly, the cucumber roots of both cultivars presented relatively high $H_2O_2$ and MDA contents due to nitrate stress (Xu et al. 2012). Under nitrate stress, however, the CaP-NPs treatments reduced $H_2O_2$ and MDA. This decline could be the result of CaP-NPs facilitating cell membrane repair, which boosts plant health and lowers the possibility of damage from nitrate stress. Upadhyaya et al. (2017) reported, in accordance with our findings, that

the application of chemical synthesis of CaP-NPs to rice resulted in a decline in $H_2O_2$ and MDA levels, a reduction in free radicals, and increased plant growth. Plants cannot grow and develop when they absorb too much nitrogen. This is because they store too much $NH_4^+$-N and $NO_3^-$-N in their bodies, which causes secondary salinization of the soil and plant salt toxicity (Britto and Kronzucker 2002; Roosta and Schjoerring 2007; Zhang et al. 2017a). In the current data, biosynthesis of CaP-NPs improved the ability of plant to reduce nitrate and assimilate ammonia, which decreased the degree of harm caused by excessive nitrate stress. Researches have shown that proline content of plant increases noticeably in response to drought, salt, high temperature, and heavy metals because proline acts as both an osmotic regulator and a scavenger of harmful free radicals, allowing them to protect plant cells from the damaging impacts of these stresses (Krishnan et al. 2008; Liu et al. 2020). Hence, the proline content increased under the nitrate stress and dropped when CaP-NPs alleviated high-nitrate stress.

Numerous studies have demonstrated that stress causes plants to generate ROS. As a defense mechanism, this causes antioxidant enzyme and nonenzymatic antioxidant levels to rise, which indicates the production of ROS and the plant's capacity to adapt to them (Sharma et al. 2012).The findings of present study show that growing lupine in nitrate environments increases TAC, flavonoid, phenolic, ascorbic acid glutathione, and antioxidant enzymes (PPO, POD, and APX). However, the foliar administration of 25, 50, or 100 mg/L CaP-NPs significantly increased the levels of each non-enzymatic antioxidant that was evaluated. Owing to its ability to donate an electron and provide two-form stability, ascorbate is essential for the ascorbate/glutathione (AsA-GSH) cycle's ability to regulate ROS (Hasanuzzaman et al. 2019). The AsA-GSH cycle is also an important cofactor for violaxanthin deepoxidase and other enzymes necessary for secondary metabolite synthesis, which help regulate cellular growth. Similarly, ascorbic acid has the potential to improve the growth of alfalfa under high-nitrate stress (Smirnoff 2011). Similarly, alfalfa development under high-nitrate stress may benefit from the use of ascorbic acid (Chen et al. 2021). $H_2O_2$ is detoxified by stimulating APX and POD activities, which stops any potential harm from occurring (Gupta and Gupta 2005). Furthermore, heightened activity of PPO under stress suggests that it has the ability to oxidize and break down harmful chemicals, which are known to accumulate under stress (Farhangi-Abriz and Torabian 2017). Strong correlations were detected for APX, POD, *SOD1*, *FLS, CAT2*, *PAL*, *CHS*, ASA, and GSH, according to the PCA (Fig. 11). In line with these findings, antioxidant enzymes like, POX, SOD, CAT, GPX, APX, GR, and GST work in concert with non-enzyme antioxidants including, GSH, AsA, and flavonoids, to prevent the overproduction of ROS (Laxa et al. 2019). Biogenic nanoparticles can strengthen the antioxidant defense system and counteract the overproduction of ROS like, copper in barley (Shaikhaldein et al. 2024), and ZnO nanoparticles in melon (Rehman et al. 2023). The increase in both enzymatic and nonenzymatic activities contributed to the lupine plant's resilience to nitrate stress, maintained its healthy development, and may have contributed to the improvements observed in a number of growth indicators.

The phenylpropanoid pathway, which is the primary source of phenolic and flavonoid production, regulates polyphenols, which make up the bulk of secondary metabolite groups (Zagoskina et al. 2023). PAL initiates the phenylpropanoid synthesis pathway (Ferreira and Antunes 2021). The synthesis of flavonoids involves the sequential actions of multiple enzymes, beginning with chalcone synthase (CHS) and chalcone isomerase (CHI). These enzymes are essential for catalyzing crucial steps that lead to the production of a wide range of flavonoids (Biała and Jasiński 2018). Moreover, a major and critical role in the production of flavonols is played by flavonol synthase (FLS) (Lei et al. 2023). According to reports, flavonoids regulate a variety of biological interactions in plants, enhancing their ability to withstand stress. Several studies have shown that PAL activity and phenolic accumulation are positively correlated (Bauer et al. 2011). According to this study, the overexpression of the *PAL* gene is primarily responsible for the notable increase in PAL activity (Figs. 9 and 10). Moreover, the lupine plants treated with 100 mg/L CaP-NPs presented greater PAL activity and *PAL* gene expression than did those under nitrate stress, which may explain the greater accumulation of phenolics and flavonoids in these plants (Fig. 8). Plant stress resistance can be increased by the addition of NPs by increasing the accumulation of these phenolic

compounds during stressful situations. According to Rasheed et al. (2022), NPs can promote the manufacture of phenolic chemicals. For instance, according to recent research, plants under salinity and drought stress can produce more phenolic compounds when AgNPs and ZnONPs are applied (Kim et al. 2024; Kumari et al. 2022).

The primary function of SOD and CAT is to stop the buildup of $H_2O_2$, which is a consequence of plant metabolism (Apel and Hirt 2004; Mhamdi et al. 2010). One possible explanation for the detected effect of nano-type fertilizers on stress conditions is the amplification of SOD and CAT activity and the subsequent decrease in $H_2O_2$ concentration, which coincided with a decline in lipid peroxidation (Nasrallah et al. 2022). Our data revealed that, compared with those under nitrate stress, the *SOD1* and *CAT2* genes were upregulated in seedlings exposed to CaP-NPs. According to previous reports, plants become more resilient to oxidative stress when *SOD* genes are expressed (Wang et al. 2005). Like other antioxidant genes, the upregulation of *SOD* during stressful situations can function as a strong defensive mechanism to impede $H_2O_2$ (Mehrabad Pour-Benab et al. 2019). Upadhyaya et al. (2017) reported that CaP-NPs reduce the levels of ROS, and MDA by increasing the levels of antioxidant enzymes such as CAT, which lends credence to this theory. Additionally, under oxidative stress, Xu et al. (2017) found that, in *Calligonum mongolicum* plants fed $Ca^{2+}$, there was a considerable rise in CAT, SOD and POX and a concurrent reduction in ROS generation. It has also been demonstrated that $Ca^{2+}$ can increase SOD and CAT in rice (Rahman et al. 2016)and tomatoes (Ahmad et al. 2018).

A family of proteins known as ferritins is specifically responsible for storing iron in all living organisms (Briat et al. 2006). Owing to its capacity to buffer iron (Fe) fluxes and maintain this element temporarily in a safe and accessible state, the iron-storage protein ferritin, which is found in plant plastics, functions during development and under stressful situations (Briat 1996). Ferritins have been identified as genes that are crucial for the protection of rice, maize, bananas, and chickpeas, and against oxidative stress (Müller et al. 2015; Parveen et al. 2016; Yadav et al. 2017). According to our study, the *FER1* gene was upregulated when the seedlings were exposed to CaP-NPs as compared with nitrate stress. In addition to its role in protecting against oxidative stress upon photoinhibition, ferritin has been linked to the regulation of ROS as demonstrated by earlier results from transgenic plants overexpressing ferritins (Murgia et al. 2001). According to Parveen et al. (2016), *CaFer1*-overexpressing chickpea seedlings revealed improved growth and development as well as increased in iron buffering and oxidative stress. Furthermore, multifunctional CaFer1 appears to be a primary candidate gene for enhanced growth and development, stress tolerance, and Fe-equilibrium, as demonstrated by Parveen et al. (2016). The regulation of genes like *FER1, PAL, CAT2, SOD1, CHS,* and *FLS* is integral to enhancing plant resilience under stress conditions. Their over-expression can lead to improved antioxidant capacity, enhanced secondary metabolite production, and better overall health of the plants (Jiang et al. 2023; Yang et al. 2023). Consequently, this translates into long-term benefits for plant growth and yield potential in challenging environments.

## Conclusion

In conclusion, this study demonstrated that the application of biofabrication of CaP-NPs as a foliar spray can be an effective strategy to mitigate the harmful effects of high nitrate stress on lupine seedlings. The results showed that CaP-NP application improved growth parameters of lupine seedlings, reduced the levels of oxidative stress indicators, induced the activity of antioxidant enzymes) and non-enzymatic antioxidants and upregulated the expression of key antioxidant genes under nitrate stress. Overall, the results of this study indicate that the foliar application of biofabrication CaP-NPs as a nanofertilizer has the potential to increase crop yields while mitigating the deleterious effects of nitrate stress on lupine plants. These findings provide a strong basis for further investigations into the use of CaP-nanofertilizers as a sustainable alternative to traditional calcium and phosphorus fertilizers. Future research should investigate the influence of CaP-NPs on mitigating the adverse effects of a wider range of nitrate concentrations to optimize yield outcomes.

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**Data Availability** All data supporting the findings of this study are already included in this published manuscript.

**Declarations**

**Ethics approval and consent to participate** Not applicable.

**Consent for publication** Not applicable.

**Competing interests** The authors declare that they have no competing interests.

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