Journal of Plant Physiology and Breeding
Print ISSN: 2008-5168 Online ISSN: 2821-0174
University of Tabriz

2025, 15(2): 37-63

Physiological protection of basil plant (*Ocimum basilicum* L.) against cold stress using L-arginine-coated calcium hydroxide nanoparticles

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Article Info

Article type:
Research article

Article history:
Received: February 18, 2025
Revised: October 27, 2025
Accepted: November 13, 2025
Published online: November 30, 2025

Keywords:
Calcium hydoxide nanoparticles,
Cold stress,
Nanoparticles,
Ocimum basilicum L.

Abstract

Objective: Low temperatures can limit crop productivity, which is a major concern for agricultural and horticultural crops worldwide. Cold stress can cause damage to plants during sensitive growth stages, leading to reduced crop performance. One such crop that is particularly susceptible to cold stress is *Ocimum basilicum* L., commonly known as basil. In this article, we present a study on the use of Ca (OH)2 nanoparticles (NPs) coated with L-arginine to decrease cold stress in this plant.

Methods: Basil plants were divided into two groups after two weeks of growth. One group was sprayed with distilled water, while the other group was sprayed with Arg-$Ca(OH)_2$ nanoparticle solutions every other day for a week. To induce cold stress, the plants were placed at 3 °C for 5 hours and then transferred to a greenhouse. After 24 hours, samples were taken and frozen in liquid nitrogen. The photosynthetic pigments, lipid peroxidation, protein oxidation, proline content, total soluble sugars, total protein, and enzymes' activity, including superoxide dismutase, ascorbate peroxidase, catalase, guaiacol peroxidase, and lipoxygenase, were measured.

Results: The findings of this study revealed that cold stress decreased chlorophyll levels in basil plants while increasing activity of antioxidant enzymes, including malondialdehyde, proline, soluble sugars, protein oxidation, and lipoxygenase activity. However, treating the plants with the NPs significantly reduced malondialdehyde, proline, and protein oxidation. It also prevented chlorophyll degradation, boosted soluble sugar content, and increased antioxidant enzyme activity in the cold-stressed plants compared to untreated ones.

Conclusion: Overall, this research highlighted a beneficial role of the Ca(OH)2 NPs in mitigating the adverse effects of cold stress on basil plants.

Cite this article: Physiological protection of basil plant (*Ocimum basilicum* L.) against cold stress using L-arginine-coated calcium hydroxide nanoparticles. J Plant Physiol Breed. 15(2): 37-63. https://doi.org/10.22034/jppb.2025.65959.1361

Introduction

Basil (*Ocimum basilicum* L.) is native to tropical and subtropical regions in Asia and has been introduced to South and North America, Africa, and Europe. It belongs to the genus *Ocimum* within the Lamiaceae family and includes around 160 distinct species, many of which are fragrant and yield essential oils. The most well-known species of sweet basil is widely used commercially as a dry herb or fresh plant (Novak and Blüthner 2020). Research indicates that various secondary metabolites, like anthraquinones, polyphenols, phenolic acids, and terpenoids, are found in basil leaves. Studies suggest that these secondary metabolites from *O. basilicum* possess biological activities including antibacterial, antifungal, antioxidant, and allelopathic effects (Shahrajabian *et al.* 2020; Brandão *et al*. 2022). Furthermore, basil is known for its medicinal uses, including anti-cancer, radioprotective, immunomodulatory, anti-inflammatory, anti-tension, anti-diabetic, anti-pyretic, anti-arthritic, and antioxidant properties, as well as prophylactic and cardiovascular diseases' benefits (Shahrajabian *et al*. 2020).

Most basil production is limited to tropical climates and subtropical climates due to its tropical origin. Exposure to low temperatures induces chilling stress and negatively impacts basil commercial yield (Jakovljević *et al*. 2021). Chilling stress significantly affects physiological, biochemical, and morphological characteristics, impacting plant growth and development, metabolism, and productivity. (Mehrotra *et al*. 2020; Saleem *et al*. 2021).

Plants detect the cold signal through cell membrane receptors, with factors like light and temperature influencing the severity of this stress. This signal triggers a cascade of responses involving cold-responsive genes, calcium as a second messenger, and transcription factors, leading to visible symptoms such as reduced germination, stunted growth, leaf chlorosis, wilting, impaired reproductive development, and cell death. Low-temperature stress also results in membrane damage, decreased scavenging enzyme activity, and protein destabilization (Manasa *et al*. 2022). This stress has a significant impact on chloroplast structure, energy production, photosynthesis, and crop yield (Zhao *et al.* 2020). Furthermore, it hampers photosynthetic electron transport efficiency in plants, leading to the generation of reactive oxygen species (ROS), which cause oxidative damage and increase membrane rigidity (Zhao *et al.* 2020). Cold stress also disrupts the carbon reduction cycle and the regulation of stomatal conduction (Gusain *et al*. 2023). As plants face various abiotic stresses, including cold stress, they need to develop specific strategies to thrive in such conditions (Saleem *et al*. 2021).

Plant scientists are investigating strategies to reduce damage from low temperatures. Nanotechnology has emerged as a significant approach to alleviating multiple stresses. It is a rapidly advancing field that has had remarkable progress in the fabrication and application of nanomaterials (Pardha-Saradhi *et al*. 2014; Mehrotra *et al.* 2020).

Nanotechnology is widely applied in various fields due to the unique properties of nanoparticles (NPs), like size-dependent optical, physicochemical, and biological characteristics. There is a growing belief in soil-plant nutrition that NPs can contribute to sustainable agricultural development with minimal environmental impact (Tavallali *et al*. 2019; Tavallali *et al*. 2020). The application of nanoparticles at low doses enhances nutrient uptake and improves nutrient use efficiency. Additionally, nanotechnology-based techniques significantly enhance the performance of agricultural inputs by enabling targeted delivery, controlled release, increased solubility, and extended shelf life. These advancements not only boost productivity but also minimize the risk of environmental pollution (Gholinezhad *et al*. 2024). Nanoparticles have been shown to enhance crop production by improving physiological processes such as seed germination rate, photosynthetic activity, seedling growth, synthesis of compounds such as proteins and sugars, and nitrogen metabolism (Tavallali *et al*. 2019; Tavallali *et al*. 2020).

Calcium is an essential macronutrient for all plants, as it acts as a structural component of cell membranes and as an intracellular second messenger. Proper regulation of calcium uptake, distribution, and storage is a high priority for plant structure and signaling. This element strengthens cell walls by forming bonds during biosynthesis, and its deficiency can lead to increased membrane permeability and cell death in meristematic regions. Additionally, calcium regulates membrane stability by influencing lipid composition and the functionality of ion transporters (Thor 2019; de Bang *et al*. 2021; Naeem *et al*. 2023). As a secondary messenger, this element modulates the level of free calcium in the cytosol in consideration of external and internal signals, initiating downstream processes that are crucial (Tang and Luan 2017; Demidchik *et al*. 2018). Calcium plays an important role in numerous physiological and developmental processes in plants. It contributes to all plants' defense strategies, growth, development, plant immunity signaling, regulation of plant mineral uptake and transport, and responses to environmental stressors (Shabbir *et al*. 2022; Wang *et al*. 2023).

Arginine, with its four nitrogen atoms, plays an important role in nitrogen circulation within plants and is essential for maintaining nitrogen balance throughout the plant life cycle. In agriculture, arginine is commonly used as a bio-stimulant to enhance crop quality and yield (Nasibi *et al*. 2011; Kawade *et al*. 2023). It is a precursor for essential compounds like nitric oxide, proline, and polyamines, which are involved in signaling responses to abiotic stresses (Hasanuzzaman *et al.* 2018;

Freitas *et al*. 2023). It has been shown that arginine delays senescence, promotes growth and development, enhances photosynthesis, influences amino acid metabolism, and boosts antioxidant levels in plants, ultimately improving overall plant health and performance (Ramadan *et al*. 2019; Hussein *et al*. 2022; Kawade *et al*. 2023). In this study, arginine-coated calcium hydroxide $Ca(OH)_2$ nanoparticles were prepared by the precipitation method using L-arginine as the amine ligand to improve basil plant resistance under low temperature conditions.

Materials and Methods

Preparation of arginine-coated calcium hydroxide Ca $(OH)_2$ nanoparticles

$CaCl_2$, NaOH pellets, ethanol, and L-arginine were procured from Merck. All chemicals used were of analytical reagent grade. A low-cost, mild chemical route was used to synthesize the $Ca(OH)_2$ NPs (Mirghiasi *et al*. 2014).

Preparation and characterization of NPs

Firstly, stock solutions of 0.5M $CaCl_2$, 2M NaOH, and 1 molar L-arginine in double distilled water were prepared in separate beakers. For the synthesis of $Ca(OH)_2$ NPs using L-arginine as a capping agent, 3 mL of 2 molar sodium hydroxide was mixed with 50 mL double-distilled water under vigorous stirring for 15 minutes at room temperature. Then, 1 mL L-arginine was added dropwise into this solution and stirred for another 15 min. At the end, 6 mL $CaCl_2$ was added dropwise into the solution, which instantly resulted in white precipitate. The precipitate was then centrifuged at 3000 rpm for 20 minutes and washed 2-3 times with ethanol. White precipitate was then dried in an electric oven at 55 °C for 12 h. Solid precipitate was finely ground in an agate mortar to turn into smaller $Ca(OH)_2$ NPs. The Nps structure was analyzed using various techniques. An X-ray diffractometer (XRD; EXPLORER, GNR, Italy, 40kV, 30mA) was used to identify molecular structures. Molecular structure analysis was carried out using Fourier-transform infrared spectroscopy (FTIR) with the Spectrum Two model from PerkinElmer. Microstructure images of the materials were acquired using field emission scanning electron microscopy (FE-SEM) technology with the ZEISS company's FESEM device, specifically the Sigma VP model. In addition, electronic equipment for energy dispersive spectroscopy (EDS) detectors from Oxford Instruments UK was used in the analysis (Mirghiasi *et al*. 2014).

Plant material and treatments

The seeds of sweet basil used in this research were provided by the Pakan Bazr company, Isfahan,

Iran. The seeds were surface-sterilized by immersing them for 1 minute in 0.1% sodium hypochlorite and then washed with distilled water once. After soaking in distilled water for 1 hour, the seeds of basil were sown in plastic pots filled with washed suitable soil. The pots were placed in a greenhouse under conditions of a long-day photoperiod (16 hours light/8 hours dark) with regulated temperatures ranging from 18 to 28 °C and 60-70% relative humidity. The basil plants were grown under these conditions for two weeks and irrigated twice a week with Hoagland's solution. After two weeks of growth, the basil plants were divided into two groups. The pre-treatment group was sprayed with arginine-modified $Ca(OH)_2$ nanoparticles solution (1000ppm) every other day for a week, while the control group was sprayed with distilled water. In a preliminary experiment, the optimal concentration of arginine-modified $Ca(OH)_2$ was determined by treating plants with four different concentrations: 500, 1000, 1500, and 2000 ppm. It was found that the concentration of 1000 ppm of the nanocomplex yielded the most favorable results. This concentration was selected as the optimal concentration for subsequent steps. To apply cold stress, the basil plants were placed in a refrigerator at 3 °C for 5 hours. After this, the plants were immediately transferred to the greenhouse and kept for 24 hours. Both the control and stressed plants were frozen in liquid nitrogen and transferred to a freezer for further analysis. The experiments were conducted using a completely randomized design with three replications.

Photosynthetic pigments

The photosynthetic pigments, such as chlorophyll a and b, and total chlorophyll, were measured following the method outlined by Lichtenthaler (1987). To do this, 0.2 g of the plant sample was milled thoroughly with 15 mL of 80% acetone. After centrifugation, the resulting solution's absorbance was measured using a spectrophotometer at wavelengths of 646.8 and 663.2 nm. The pigments were then quantified in milligrams per gram of fresh weight using the following equations (Lichtenthaler 1987):

$$\text{Chl a } (\mu g/mL) = 12.25\ A663.2 - 2.79\ A646.8$$

$$\text{Chl b } (\mu g/mL) = 21.21\ A646.8 - 5.1\ A663.2$$

$$\text{Chl t (Chl a + Chl b)} = 7.15\ A663.2 + 18.71\ A646.8$$

Lipid peroxidation assay

The malondialdehyde (MDA) content in *Ocimum basilicum* L. leaves was determined following specific protocols. In summary, 0.2 grams of plant tissue was ground in 5 mL of 0.1% trichloroacetic acid (TCA) and then centrifuged. One milliliter of the supernatant obtained from centrifugation was

homogenized in 4 mL of 20% TCA containing 0.5% thiobarbituric acid. This mixture was incubated in water at 95 °C for 30 minutes, and then the reaction was stopped in an ice bath. Subsequently, centrifugation at 10,000 rpm for 20 minutes was performed, and the absorbance of the supernatant was measured at 450, 532, and 600 nm. The MDA content was expressed as mmol MDA per g fresh weight and calculated with an extinction coefficient of 155 mM^{-1} cm^{-1}. For other aldehydes calculation, non-specific absorption at 600 nm was subtracted from the 455 nm reading, and the extinction coefficient of 0.458 × 105 mM^{-1} cm^{-1} was used. The other aldehyde content was expressed as mmol per g fresh weight (Heath and Packer 1968).

Protein oxidation measurement

To estimate oxidative damage to proteins, the oxidation of amino acids available in proteins can be measured by assessing groups of carbonyls. The reaction of groups of carbonyls with 2,4-dinitrophenylhydrazine (DNPH) produces the dinitrophenylhydrazone product, which is used to estimate protein oxidation (Levine *et al*. 1994). In this method, 0.2 grams of fresh plant tissue was ground in 2 mL of 50 mM potassium phosphate buffer with pH=7.4 containing 1 mM EDTA. The resulting extract was then centrifuged at 15,000 x g for 20 minutes, and the supernatant was used to measure the amount of carbonyl groups. Next, 800 microliters of 10 mM DNPH solution (prepared in 2.5 M hydrochloric acid) was added to 200 microliters of the extract. The mixture was well vortexed and kept for 1 hour at room temperature in a dark place. The tubes were vortexed once every 15 minutes. Following this, 1 mL of 20% TCA was added to the tube, and the mixture was placed on ice for 10 minutes to precipitate the protein. The tubes were then centrifuged at 3500 g for 20 minutes, and the supernatant was carefully separated and discarded. This process was repeated twice with 10% TCA, and the sediment was thoroughly washed three times with 800 microliters of ethyl acetate: ethanol solution (1:1) to remove DNPH and remaining oils. The remaining protein precipitate was dissolved in 400 microliters of 6 M guanidine hydrochloride (in 20 mM potassium phosphate buffer with pH 2.3) and incubated for 10 minutes at 37 °C. The mixture was then centrifuged at 4000 g for 5 minutes to remove undissolved substances. Finally, the absorbance of the resulting sample was read at 370 nm, and the extinction coefficient equal to 22000 $M^{-1}cm^{-1}$ (for aliphatic hydrazone) was used to calculate the concentration of carbonyl groups (Levine *et al*. 1994).

Proline Content

A 0.02 g of frozen plant tissue was ground in 10 mLof 3% sulfosalicylic acid solution. The resulting extract was then centrifuged at 4000 g for 5 minutes. Subsequently, 2 mL of the supernatant was

combined with 2 mL of the ninhydrin powder reagent and 2 mL of pure acetic acid liquid. The mixture was then heated in a hot water bath at 100 °C for one hour. After thorough mixing, 4 mL of toluene was added, and the tubes were shaken well. Two distinct layers were formed after allowing the tubes to stand still for 15 to 20 seconds. The absorbance of the upper colored layer containing toluene and proline was measured at 520 nm. The proline standard curve was used to calculate the amount of proline in terms of micromoles per gram of plant fresh weight (Bates *et al*. 1973).

Total soluble sugars

The total soluble sugars were determined using anthrone reagent with glucose as a standard (Fales 1951). Initially, 0.1 gram of the plant sample was ground with 2.5 mL of 80% ethanol and placed in a hot water bath at 95 °C. The carbohydrates were then extracted, and the resulting extracts were filtered by the filter paper, followed by alcohol evaporation. Subsequently, the remaining solution was dissolved in 2.5 mL of distilled water. 5 mL of anthrone reagent (150 mg of anthrone dissolved in 72% H_2SO_4) was added to each of the 500 µl samples and then heated in a water bath at 95 °C for 10 minutes. After cooling, the absorbance was measured at 625 nm using a spectrophotometer. Various concentrations of glucose were used to create the standard curve. The amount of soluble carbohydrates was quantified in milligrams per gram of fresh weight (Fales 1951).

Protein extraction and enzyme activity assay

After homogenizing 300 milligrams of fresh weight with 3 mL of 50 mM potassium phosphate buffer at 4 °C, the plant extract was centrifuged at 10000 g for 20 minutes. The resulting supernatant was then separated and used to assay the activities of various enzymes and to determine protein content.

Superoxide dismutase enzyme activity (SOD, E.C.1.15.1.1)

The superoxide dismutase enzyme activity was assessed by measuring the inhibition of the nitro blue tetrazolium chloride (NBT) photoreduction reaction at 560 nm. The reaction mixture contained phosphate buffer, NBT, riboflavin, and enzyme extract. A control sample without the enzyme extract was used to establish 100% photoreduction of NBT. The difference in absorbance between the treated samples and the control indicated the inhibition of NBT photoreduction by the SOD enzyme. The enzyme activity was quantified in terms of enzyme units per 100 microliters of extract using the Bradford method to measure the total protein content (Giannopolitis and Ries 1977).

Ascorbate peroxidase enzyme activity (APX) (EC 1.11.1.11)

The enzyme activity of APX was determined by measuring the decrease in absorbance at 290 nm. This was done in a reaction mixture containing potassium phosphate buffer, ascorbate, hydrogen peroxide (H_2O_2), and the enzyme extract. The absorbance change at 290 nm was used to calculate the amount of the remaining ascorbate, using the extinction coefficient of $\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$. One unit of enzyme activity was defined as the amount that oxidizes one micromole of ascorbate in one minute. The enzyme activity was reported in units per mg protein in the extract (Nakano and Asada 1981).

Catalase enzyme activity (CAT) (EC 1.11.1.6)

The activity of the CAT enzyme was measured by monitoring the decrease in H_2O_2 absorption at 240 nm. A reaction mixture containing potassium phosphate buffer and hydrogen peroxide was used, with enzyme extract added to initiate the decomposition of H_2O_2. Absorbance change per minute was calculated, and the amount of H_2O_2 remaining after one minute was determined using the quenching coefficient and Beer-Lambert law. Enzyme activity was reported in enzyme units per total protein present in the extract, with one unit defined as the amount that decomposes one micromole of H2O2 in one minute (Dhindsa *et al.* 1981).

Guaiacol peroxidase activity (GPX) (EC1.11.1.7)

The GPX (EC 1.11.1.7) activity was measured based on the method of Plewa *et al*. (1991). A reaction mixture consisting of 50 mM potassium phosphate (pH 7.0), 0.3% (v/v) H_2O_2, 1% (v/v) guaiacol, and 100 microliters of the enzyme extract. The enzyme activity was recorded as units per milligram protein, where one unit of enzyme activity was considered as the amount of enzyme that produced one micromole of tetraguaiacol per minute (Plewa *et al*. 1991).

Lipoxygenase enzyme activity (LOX)(EC 1.13.11.12)

The reaction mixture contained linoleic acid as a substrate, phosphate buffer, and the enzyme extract, with absorbance readings taken over 90 seconds at 234 nm. The enzyme activity was reported in units per milligram of protein present in the extract, with one unit defined as the conversion of one micromolar linoleic acid into the reaction product in one minute (Minguez-Mosquera *et al*. 1993).

Total protein content

The total protein content in the leaves of *Ocimum basilicum* L. was measured using Bradford's (1976) method. To perform this analysis, 0.1 mL of the protein extract was mixed with 5 mL of the biuret

reagent in test tubes and vigorously vortexed. The solutions were then left to incubate for a period ranging from two minutes to one hour. After incubation, the absorbance was measured at a wavelength of 595 nm using a spectrophotometer. The protein concentration was determined by comparing it to an albumin standard curve and expressed in milligrams per gram of fresh weight (Bradford 1976).

Results

As depicted in Figure 1, after 24 hours of exposure to cold stress, the difference in the appearance of plants was quite apparent when they were in the greenhouse. Despite some damage to the control plants, the pre-treated basil plants with arginine-coated calcium hydroxide $Ca(OH)_2$ nanoparticles (Ca-Arg NP) demonstrated much less damage**.**

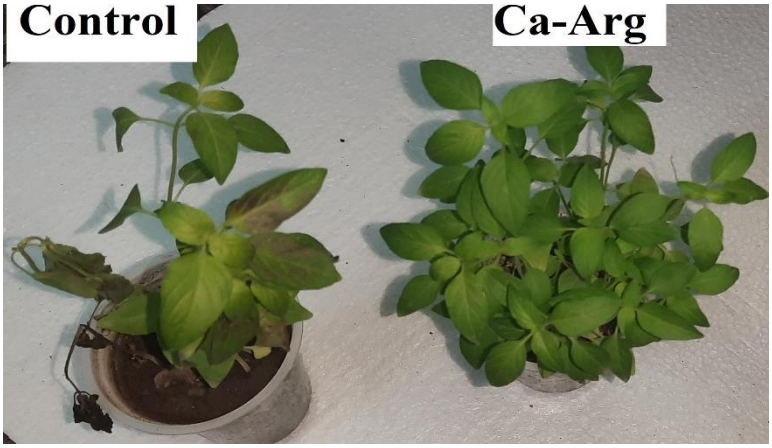

Figure **1.** Effect of Ca-Arg NP: $Ca(OH)_2$ nanoparticles modified with L-arginine on pre-treated *Ocimum basilicum L.*, 24 hours after cold stress treatment.

XRD

X-ray diffraction patterns of calcium hydroxide ($Ca(OH)_2$) samples are presented in Figure 2. All the diffraction peaks in this figure were accordant with the standard structure of $Ca(OH)_2$ (Portlandite, JCPDS card No. 00- 004-0733) with hexagonal crystal system and lattice parameters (a = 3.5956 A˚, b = 3.5956 A˚, c = 4.9280 A˚).

The mean particle size of $Ca(OH)_2$ NPs was estimated to be roughly 73 nm according to the Debye-Scherrer equation $D= (K\lambda)/(\beta \cos\theta)$, where λ is the wavelength of the X-ray used for the diffraction, K is a constant called shape factor (K = 0.89), θ is peak position (2θ/2) in radians, and β is the full width at half-maximum severity in radians (Patterson 1939).

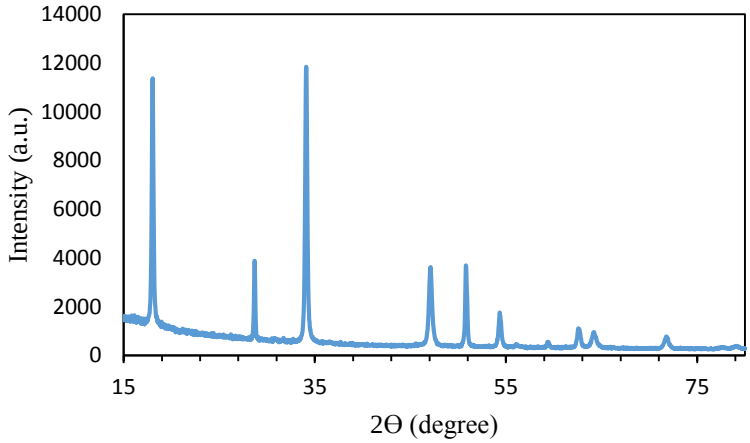

Figure 2. X-ray patterns of $Ca(OH)_2$ nanoparticles modified by L-arginine.

SEM image and EDS spectrum

Figure 3 indicates the SEM images of the treated modified $Ca(OH)_2$ nanoparticles, which show that most of the NPs have a smoother surface with the morphology of a single plate and a hexagonal shape with an average surface diameter of 155 nm and a thickness of 55 nm. The EDS spectrum as the synthesized $Ca(OH)_2$ NPs capped using L-arginine confirms the purity of the prepared samples. Spectrum clearly shows peaks corresponding to Ca and O. Energy peaks corresponding to the elements C and N are due to the presence of L-arginine. NPs were coated with gold before SEM to increase conductivity.

FTIR spectral analysis of Ca-Arg NC

The FTIR spectrum of Ca-Arg NP revealed absorption peaks at 406.83, 542.72, 626.29, 803,97, 870.19, 1030.56, 1097,04, 1260.92, 1454.91, 1616.41, 1744.89, 2308.45, 2875.37, 2925.47, 3421.19, 3642.58, 3778.32, and 3822.12 cm^{-1} (Figure 4). The peaks at 406.83, 542.72,626.29, 1030.56, and 1097,04 cm^{-1} could be linked to oxygen (-O) vibrations, while the peak at 1616.41 cm^{-1} might be associated with C=O vibrations. The peak of 870.19 cm^{-1} is due to the rocking vibration of $–NH_2$ and the absorbance peaks at 1260.92 and 1744.89 cm^{-1} could be ascribed to the stretching vibration of chains -C-O-C- and C=O, respectively. The peaks at 2925.5, 2857.4, 3421.2, and 3778.3 cm^{-1} are designated for N-H stretching (Senthilkumar *et al*. 2017; Petit and Puskar 2018; Senthilkumar *et al*. 2019).

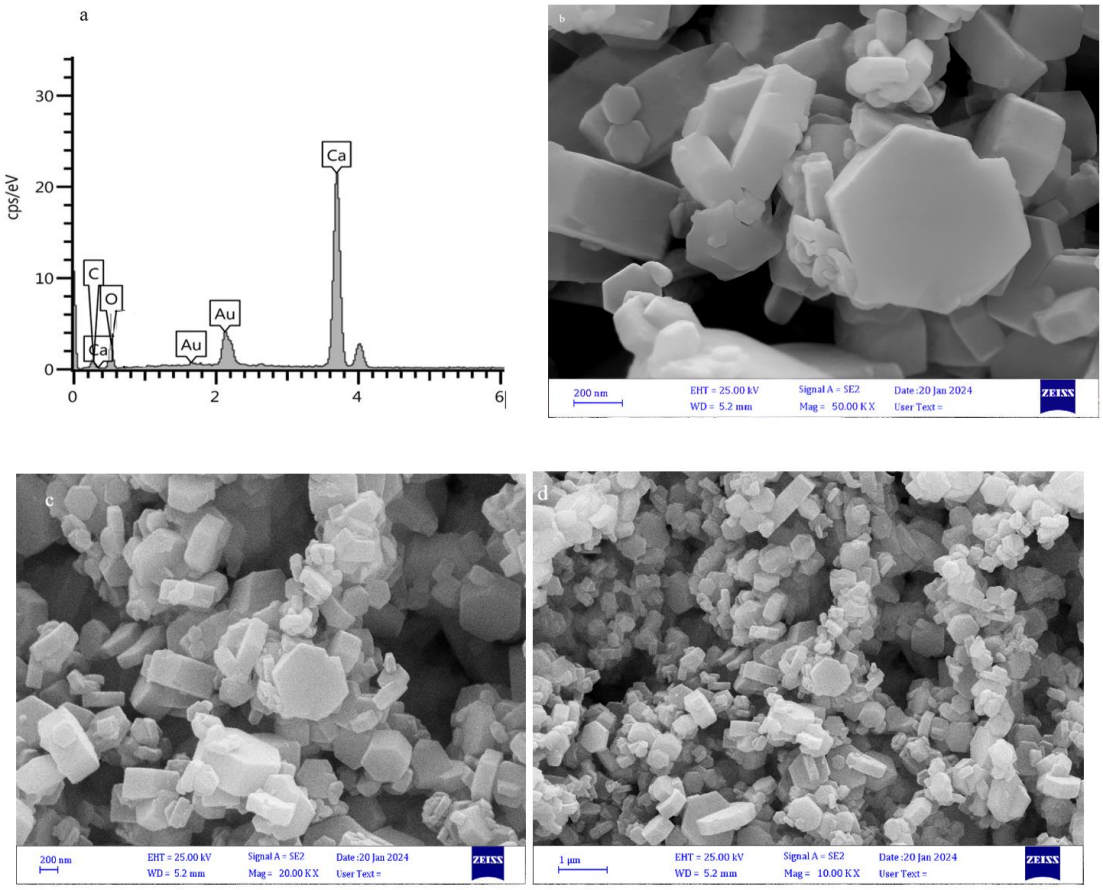

Figure 3. The SEM images and EDS spectrum of $Ca(OH)_2$ nanoparticles modified by L-arginine; SEM: Scanning electron microscopy, EDS: Energy dispersive spectroscopy.

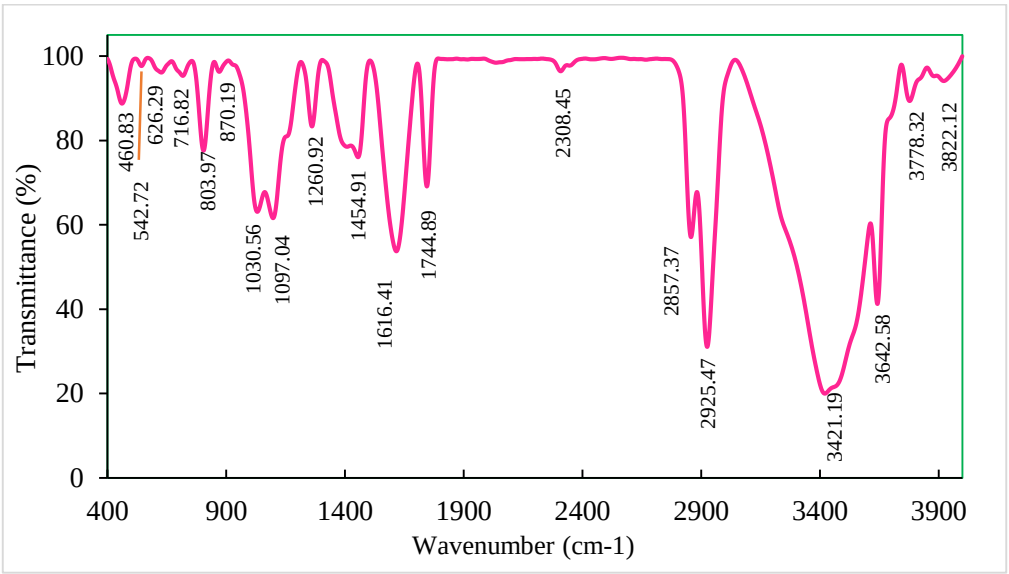

Figure 4. FTIR spectra of arginine-coated calcium hydroxide $Ca(OH)_2$ nanoparticles; FTIR: Fourier transform infrared.

Quantification of the photosynthetic pigment

Based on Figure 5, the analysis of photosynthetic pigments demonstrated that exposure to cold stress led to a significant decrease in the levels of chlorophyll a, b, and total chlorophyll in basil plants. However, when the plants were pretreated with the modified Ca-Arg NPs before the stress, the concentration of chlorophyll exhibited a noticeable increase in comparison to the non-pretreated plants.

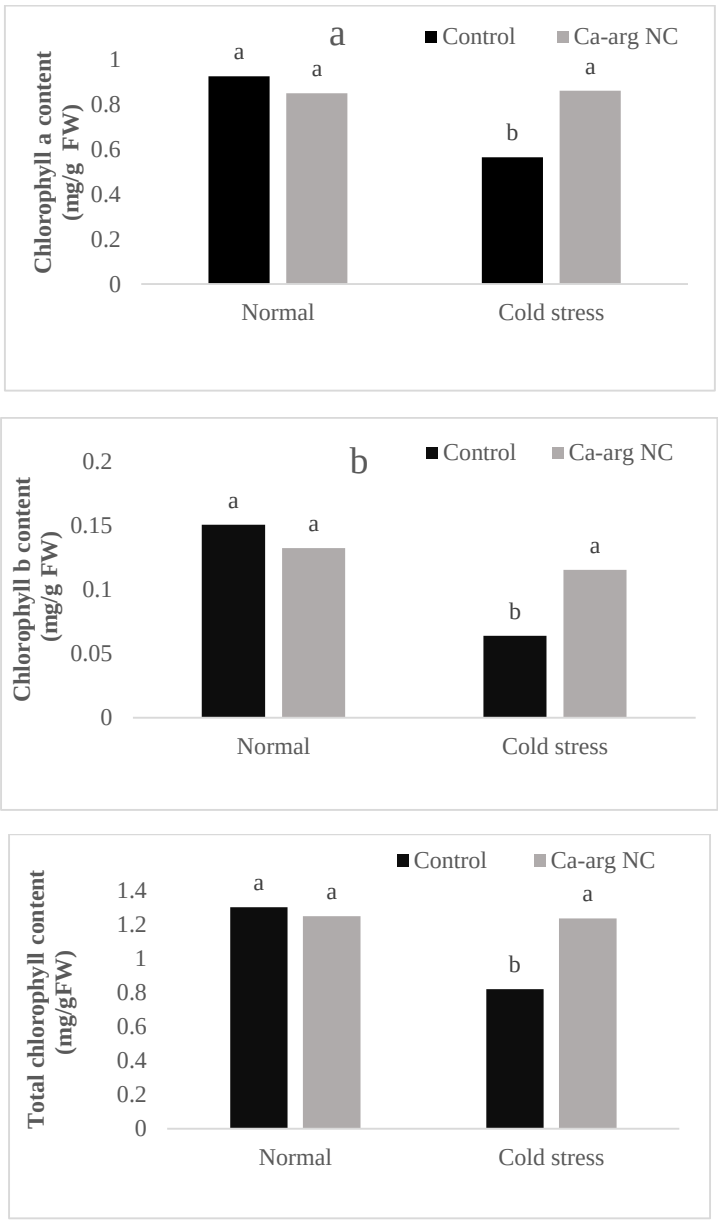

Figure 5. Impact of Ca-Arg nanoparticles pretreatment on photosynthetic pigments content in leaf tissue of *Ocimum basilicum L*. under control and cold stress conditions; Means denoted by different letters are significantly different, based on Duncan's multiple range test at $p \leq 0.05$.

Lipid peroxidation and lipoxygenase enzyme activity

The measurements of lipid peroxidation in leaf tissues showed that plants exposed to cold stress had a significant enhancement in MDA and other aldehydes as compared to the normal conditions (Figure 6). However, when the plants were pretreated with the modified Ca-Arg nanoparticles, lipid peroxidation declined. Cold stress also enhanced the activity of the LOX enzyme by more than two folds. Nevertheless, pre-treating the plant with the modified Ca-Arg nanoparticles reduced the activity of this enzyme by about 60% under low-temperature stress conditions.

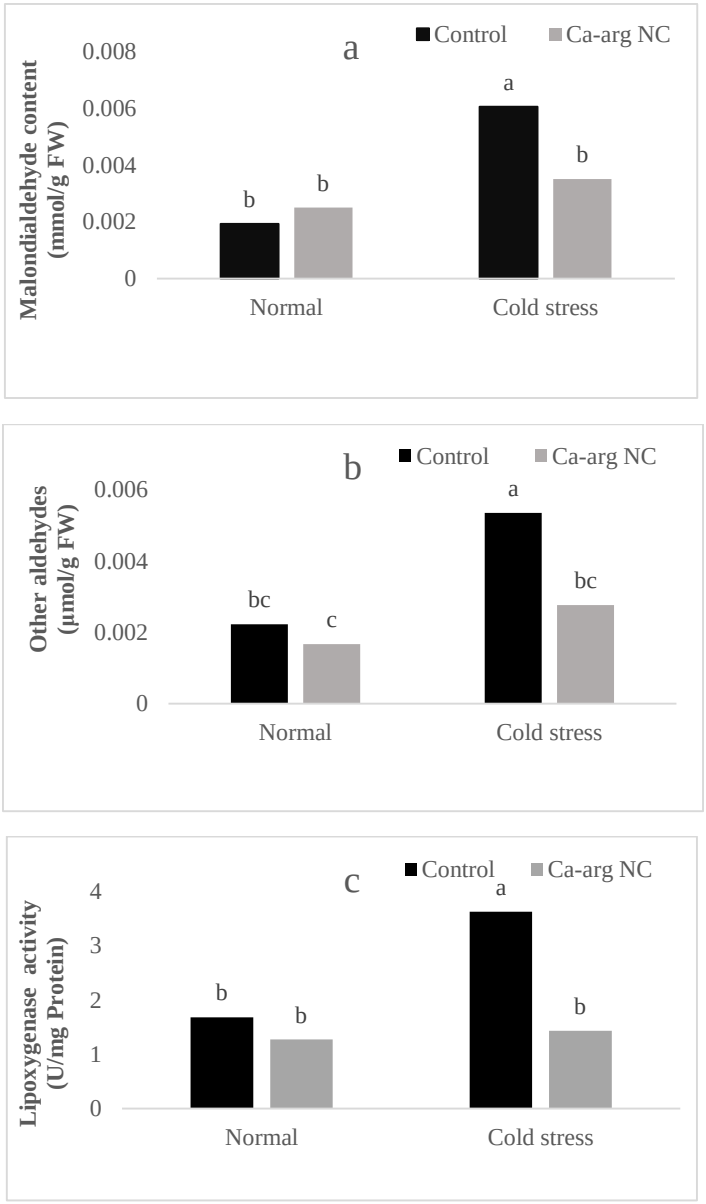

Figure 6. Impact of Ca-Arg nanoparticles pretreatment on MDA (a), other aldehydes' content (b), and lipoxygenase enzyme activity (c) in leaf tissue of *Ocimum basilicum L*. under control and cold stress conditions; MDA: Malondialdehyde; Means denoted by different letters are significantly different, based on Duncan's multiple range test at $p \leq 0.05$.

Oxidation of proteins (carbonyl groups)

The results indicated that cold stress led to an enhance in protein oxidation in the texture of basil leaves. However, pretreating the plants with the modified Ca-Arg nanoparticles notably reduced the oxidation of proteins in the cold stress conditions (Figure 7).

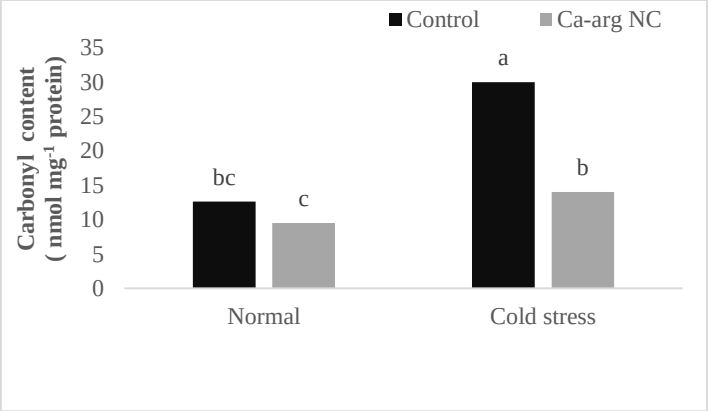

Figure 7. Impact of Ca-Arg nanoparticles pretreatment on protein oxidation in the leaf tissue of *Ocimum basilicum L.* under control and cold stress conditions; Means denoted by different letters are significantly different, based on Duncan's multiple range test at $p \leq 0.05$.

Total soluble sugars

The analysis of the total soluble sugars showed a significant enhancement in cold stress. Pretreatment with the modified Ca-Arg nanoparticles increased the total soluble sugar content under control and cold stress conditions (Figure 8).

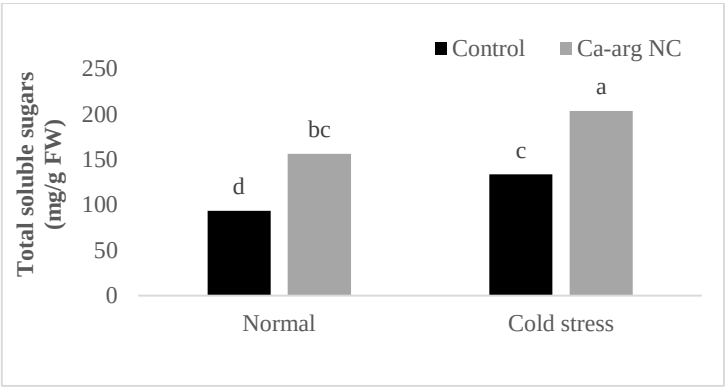

Figure 8: Impact of Ca-Arg nanoparticles pretreatment on total soluble sugars in the leaf tissue of *Ocimum basilicum L.* under control and cold stress conditions; Means denoted by different letters are significantly different, based on Duncan's multiple range test at $p \leq 0.05$.

Proline content

As illustrated in Figure 9, under cold stress, basil plants showed a significant enhance in the proline content, but this was reduced when basil plants were pre-treated with the modified Ca-Arg nanoparticles.

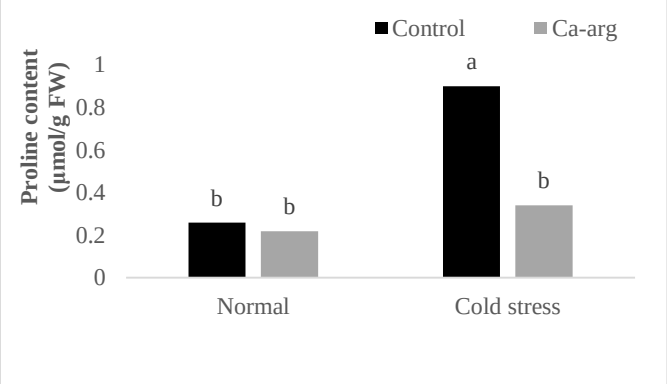

Figure 9. Impact of Ca-Arg nanoparticles pretreatment on proline content in the leaf tissue of *Ocimum basilicum L*. under control and cold stress conditions; Means denoted by different letters are significantly different, based on Duncan's multiple range test at $p \leq 0.05$.

Activity of antioxidant enzymes (SOD, APX, CAT, and GPX)

The results of this research showed that low temperature stress had a significant effect on the antioxidant enzyme activities in the *Ocimum basilicum* L. plants, except for GPX. The activities of SOD, CAT, and APX increased under cold stress. However, when plants were treated with the modified Ca-Arg nanoparticles, the activities of these enzymes were further enhanced under cold stress conditions. Conversely, neither calcium pretreatment nor the low-temperature stress had a significant impact on the GPX enzyme activity (Figure 10).

Discussion

When plants are exposed to cold and frost, their metabolism gets affected, which results in reduced crop quality and yield. Cold stress affects crucial physiological processes like water regulation, photosynthesis, mineral nutrition, metabolism, respiration, and causes permanent damage such as damage of cell proteins and disruption of plasma membrane integrity (Kumar *et al.* 2018). Nanotechnology is a promising tool that can enhance plant resilience against environmental stressors. In this study, low temperatures were found to reduce the amount of photosynthetic pigments; however, this reduction was alleviated by pre-treating the plants with Ca-Arg NPs. Chlorophyll is a vital biomolecule that plays a crucial role in photosynthesis process by absorbing light and transform

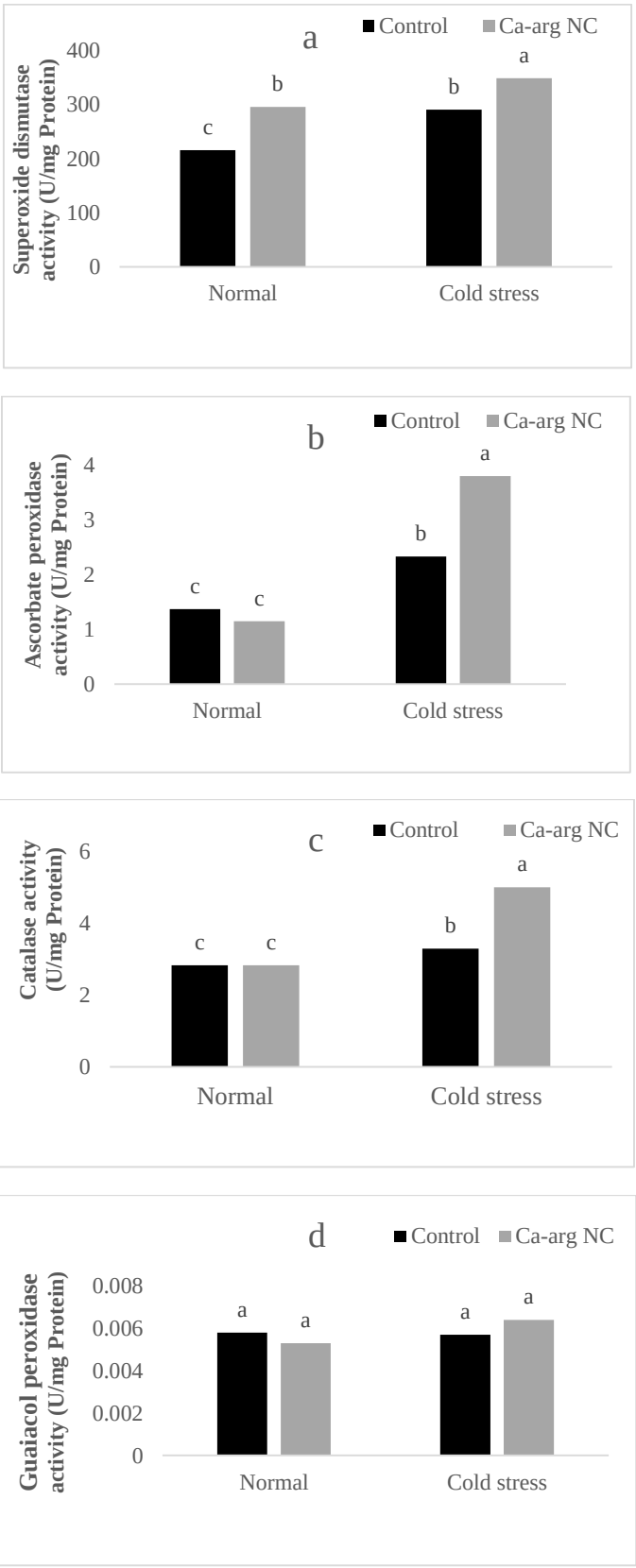

Figure 10. Impact of Ca-Arg nanoparticles pretreatment on SOD (a), APX (b), CAT (c) and GPX (d) enzymes in leaf tissue of *Ocimum basilicum L*. under control and cold stress conditions; SOD: Superoxide dismutase, APX: Ascorbate peroxidase, CAT: Catalase, GPX: Guaiacol peroxidase; Means denoted by different letters are significantly different, based on Duncan's multiple range test at $p \leq 0.05$.

it into energy (Dey *et al.* 2021). Exposure to low-temperature stress can cause an agglomeration of ROS, which can damage photosynthetic membranes and reduce the amount of chlorophyll content (Ritonga *et al.* 2021). This reduction may be due to the enhanced activity of the chlorophyllase, chlorophyll-degrading enzyme, or the inhibition of chlorophyll biosynthesis, which is influenced by the chloroplast biogenesis (Dey *et al.* 2021). Hamperring the chlorophyll biogenesis leads to a decrease in thylakoid membrane proteins and granum lamellae (Ritonga *et al.* 2021). Cold stress can also hinder thylakoid protein synthesis, leading to a significant reduction in the total chlorophyll and b, as observed in plants like *Stevia rebaudiana* (Hajihashemi *et al.* 2018). The research indicates that the utilization of Ca-Arg nano-complex leads to an increase in the chlorophyll content. This effect can be attributed to the presence of both calcium and arginine in this composition. Calcium element plays a significant role in the chlorophyll synthesis and preservation of photosynthetic membranes, such as chloroplast and thylakoid membranes under cold stress conditions, resulting in an increase of chlorophyll content (Zhao *et al*. 2023). There are circumscription reports on the direct use of arginine in reducing stress. However, multiple studies have shown that substances such as polyamines and nitric oxide, that are derived from arginine, possess antioxidant properties. This can help scavenge ROS, reduce oxidative damage in the photosynthetic apparatus, and enhance chlorophyll content under cold stress (Nasibi *et al.* 2011; Ramadan *et al.* 2019; Freitas *et al.* 2022).

The data of this research indicated that cold stress increases lipoxygenase enzyme activity and raises MDA levels in basil plant cells. When the plants are exposed to cold temperatures, their cell membrane is the first to sense the stress. However, it is also the first component to be damaged. Cold stress triggers the peroxidation of lipid molecules in the cell membrane, which causes compromised membrane permeability and stability. This, in turn, peroxidation of unsaturated fatty acids by ROS in plant cells, ultimately results in the agglomeration of MDA (Valizadeh Kamran *et al.* 2011; Liu *et al.* 2023). In basil plant cells, cold stress causes an enhance in the activity of lipoxygenase (LOX) enzyme and the MDA content. This indicates that the cell membrane was damaged by the stress. LOX activity and the presence of high levels of MDA lead to ROS, which affect the cell membrane permeability under environmental stresses. This can cause a decrease in unsaturated fatty acids and a loss of membrane integrity, which can eventually cause cell death in plants (Javidi *et al.* 2022). Calcium is important in maintaining the structural integrity of plant cells under stress conditions by stabilizing membrane structure and regulating membrane permeability (Guo *et al.* 2021). Calcium nanoparticle has been shown to appreciably affect the activity of LOX enzymes and reduce the MDA level, thereby decreasing lipid peroxidation (Sahin *et al.* 2023). A decrease in MDA with calcium treatment under drought stress was observed in *Trachyspermum ammi* L. (Mazhar 2023). Arginine is also a potent

antioxidant, preventing membrane dysfunction by binding to polyanionic compounds on the membrane surface. Previous research has shown that nitric oxide is effective in reducing the amount of ROS, LOX activity, reaction with peroxyl and lipid alkoxyl radicals, and lipid peroxidation (Hasanuzzaman *et al.* 2018). In this research, part of the efficacious effect of the modified Ca-Arg NP can be related to the role of arginine or its products.

This research on basil plant has shown that cold stress leads to an increase in carbonyl groups, while, Ca-Arg, decreases the carbonyl groups. Proteins are one of the primary targets of ROS produced during cold stress. Cold stress can cause harmful effects by accumulating ROS, which can damage proteins (Hmmam *et al*. 2023). If the amount of ROS exceeds a certain limit, it can lead to damage to some macromolecules such as proteins, and eventually protein oxidation and other changes occur. (Tambussi *et al.* 2014; Ramazan *et al.* 2021; Javidi *et al.* 2022). Our study showed that the pretreatment of the *Ocimum basilicum* plants with Ca-Arg NPs reduced protein oxidation. Previous research has shown that treatment with calcium can decrease protein oxidation in the *Fusarium graminearum*-infected wheat seedlings (Sobhy *et al.* 2023). Research has shown that the activities of SOD, CAT, and APX enzymes in plants are significantly affected by the calcium ions. By regulating the level of enzymes, can help reduce oxidative stress and eliminate ROS (Rezayian *et al.* 2020; Sahin *et al.* 2023). However, arginine has a protective effect in reducing protein oxidation, which is related to its role in the synthesis of polyamines and nitric oxide. (Nejadalimoradi *et al.* 2014; Eslami *et al.* 2019; Ramadan *et al.* 2019; Freitas *et al*. 2022). These substances act as antioxidants in plants.

When plants are subjected to cold stress, they react by accumulating certain substances known as compatible osmolytes. Proline and carbohydrates are two such osmolytes that have an important effect on protecting the plant's biological membranes, detoxifying harmful compounds, and preserving cellular structures. These osmolytes are crucial for the plant's stress-protective mechanisms (Nahar *et al.* 2016; El-Mahdy *et al.* 2018;). Under challenging conditions, such as low temperatures, plants rapidly accumulate proline as an early stress indicator. Proline maintains plant growth and adaptation by regulating cellular osmotic balance, nitrogen and energy metabolism, protein stabilization, inhibition of lipid peroxidation, and regulation of the antioxidant system. Additionally, proline helps to mediate ROS (Liu *et al.* 2013; Yang *et al.* 2016; El-Mahdy *et al.* 2018). Variations in this amino acid metabolism and aggregation in response to low temperature stress have been reported in the melon plants (Li *et al.* 2023). A reduction in the concentration of proline in plant leaves after treatment may indicate that the plants have experienced a lower level of stress (Bhattacharya 2022). This is in agreement with the results of our research. In our experiment, we realized that the amount of proline in *Ocimum basilicum* plants increased almost fourfold in cold

conditions. However, before the stress, when the plants were treated with the modified Ca-Arg NP, the amount of proline was significantly reduced. Canola plants that were treated with calcium during drought stress showed a reduction in proline levels (Rezayian *et al.* 2020). In the present research, the reduction in proline and other stress indicators, such as protein oxidation and lipid peroxidation, in the pre-treated plants confirms that the treatment of basil plants with the modified Ca-Arg NPs has caused less damage to the plant in the cold stress conditions.

Plants synthesize carbohydrates through photosynthesis, which serve as important osmolytes. Exposure to low temperatures can cause changes in these water-soluble carbohydrates, which function as osmoprotectants, stabilizers of cell membranes, scavengers of ROS, and signaling molecules for enhancing chilling and freezing stress tolerance (Bhattacharya 2022; Eom *et al.* 2022). Previous reports have shown that low temperature conditions in corn also increases the carbohydrate content (Ramazan *et al.* 2021). In our research, pretreatment of plants with the modified Ca-Arg NPs reduced the sugar content compared to the untreated plants in the cold stress conditions. It appears that Ca-Arg NPs acts as an antioxidant and help maintain cell structures, especially cell membranes. This leads to reduced cold stress and less need for osmoprotectant synthesis in the treated basil plants.

The antioxidant enzymes such as APX, CAT, GPX, and SOD play a key role in protecting the cells by controlling free radicals and preventing their harmful effects. This helps to maintain stable conditions between free radicals and antioxidants, which is essential for optimal cell function. When plants are under cold stress, they may experience an excess of ROS that can lead to oxidative damage. However, plants have an antioxidant defense system that works to reduce the impact of ROS and protect cell structures and membranes. The mentioned defense system has different strategies, among them, enzymatic or non-enzymatic antioxidant molecules can be mentioned (Yu *et al.* 2020; Guo *et al.* 2022). This study has shown that plants that were treated with amine-modified nanoparticles had an increase in the activity of antioxidant enzymes. Consequently, the oxidative stress indicators, including MDA content, were reduced. This indicates that the modified Ca-Arg NPs played an important antioxidant role in the cold stress conditions. Previous research has demonstrated that $Ca2^{+}$ pretreatment can reduce ROS production in plants by activating antioxidant enzymes, which is mainly attributed to the role of $Ca2^{+}$ (Lamnai *et al.* 2022). Additionally, $Ca2^{+}$ functions as a secondary messenger in response to stress signals (Gao *et al.* 2023). Research by Zhao *et al.* (2023) has shown that exogenous application of Ca2+ can increase the accumulation of antioxidant enzymes in *Rosa hybrida* L. under drought conditions. Furthermore, it has been stated in research that arginine can effectively modulate the antioxidant response by regulating the activity of CAT, SOD, and APX enzymes (Ramadan *et al.* 2019; Freitas *et al.* 2022).

Conclusion

The results showed that cold stress increased oxidative damage by increasing LOX activity and lipid peroxidation, as well as protein oxidation, while reducing the photosynthetic pigments. However, pre-treatment with the modified Ca-Arg NPs considerably improved the plant's cold resistance by increasing the activity of antioxidant enzymes and reducing oxidative damage. It seems that this amine-modified nanoparticle has a potential antioxidant role in cold conditions due to the presence of two essential substances, calcium and arginine. It is important to note that further research is required to understand this nanocomplex's underlying mechanism of action comprehensively. Additionally, a detailed analysis of its performance is necessary to determine its potential applications.

Conflict of Interest

The authors declare that they have no conflict of interest.

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