NanoImpact 28 (2022) 100423

Contents lists available at ScienceDirect

NanoImpact

journal homepage: www.elsevier.com/locate/nanoimpact

Calcium nanoparticles (Ca-NPs) improve drought stress tolerance in *Brassica napus* by modulating the photosystem II, nutrient acquisition and antioxidant performance

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ARTICLE INFO

Editor: Bernd Nowack

Keywords:
Chlorophyll fluorescence
Calcium nanoparticles
Antioxidants
Hormones
Secondary metabolites
Brassica napus

ABSTRACT

Foliar-application of nano-particles enhanced the foliar nutrient status and crop growth and yield. It is hypothesized that being second messenger molecule, supplementation of Ca^{2+} via calcium nanoparticles (Ca-NPs) can trigger various signaling pathways of physiological processes which can lead to alleviate the adverse effects of drought stress on the growth of canola (*Brassica napus* L.). Nano-enabled foliar-application could be an ideal strategy for advancing agricultural productivity. The present study explored the role of calcium nanoparticles (Ca-NPs) in alleviating drought stress in hydroponic *Brassica napus* (*B. napus*) plants. The foliar applied Ca-NPs were spherically shaped with an average size of 86 nm. Foliar application of 100 mg L^{-1} Ca-NPs enhanced biomass of canola plants and considered as optimal dose. Ca-NPs at 100 mg L^{-1} has a greater favorable impact on mesophyll ultrastructure, PSI and PSII efficacy, gas exchange parameters, chlorophyll content, and mineral absorption. The Ca-NPs treatment increased NPQ and Y(NPQ) under drought condition, indicating a higher PSII protective response to stressed conditions with better heat dissipation as a photoprotective component of NPQ. Ca-NPs application also reduced oxidative stress damage as measured by a reduction in reactive oxygen species (ROS) generation in terms of hydrogen peroxide and malondialdehyde (H_2O_2 and MDA). Furthermore, Ca-NPs induced drought tolerance response corresponded to an increased in key antioxidative defense enzymes (SOD, POD, CAT, APX), as well as non-enzymatic components (protease, lipoxygenase, proline, total soluble protein contents, endogenous hormonal biosynthesis), and secondary metabolite expression in *B. napus* plants. Taken together, the results of this study offer new insights into the physiological and molecular mechanisms by which *B. napus* responds to Ca-NPs exposure.

1. Introduction

Nanotechnology, because of the unique properties of nanomaterials (NMs), offers a promising alternative in plant protection and has numerous advantages over conventional products, which are associated with enhanced efficacy, reduced input, and lower eco-toxicity (Muraisi et al., 2022). The development of a suitable growth stimulating compounds such as nano-fertilizers, can significantly boost agricultural productivity (Verma et al., 2022). Increased crop productivity and global food security mostly depend upon the application of fertilizers made up of calcium, phosphorus, potassium or nitrogen sources. Le Bot et al. (2021) summarized the role of essential beneficial elements in plants and Ca among those that promote plant vigor. Calcium (Ca) is an essential macronutrient for plant development as its play important structure and signaling role.Ca is required for cell wall membrane maintenance, hormonal biosynthesis, enzyme activity enhancement,

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https://doi.org/10.1016/j.impact.2022.100423
Received 31 May 2022; Received in revised form 29 August 2022; Accepted 30 August 2022
Available online 7 September 2022

and intracellular physiological events that control plant responses to stressful conditions (Azeez et al., 2020; Meier et al., 2020; Azeez et al., 2021). It stimulates metabolic processes by inhibiting the intake of other minerals and increasing cell elongation, which strengthens hormonal processes (Ramírez-Rodríguez et al., 2020; Gandhi et al., 2021).

A growing body of literature suggests that metal and metal oxide nanoparticles (NPs) may mitigate water stress and other abiotic and biotic pressures in crops (González-Fontes et al., 2017). For maize, growth and yield increase under drought when SiO_2 NPs and potassium/silica NPs are applied. Si and SiO_2 NPs also aid barley under drought stress. Similarly Nazir et al. (2022) also reported alleviating effects of CaO nanoparticles against arsenic stress. Therefore, it has been suggested that engineered nanomaterial-based nanofertilizer can be judiciously used for efficient utilization of soil nutrients including Cato boost the crop productivity. This may reduce the fertilizer applications as well as prevent ecosystem pollution (Liu et al., 2016). Calcium nanoparticles (Ca-NPs) function as nanofertilizers by modulating pH, organic matter, and cation exchange capacity in order to provide nutrients like as calcium and make certain bound minerals accessible (Ditta and Arshad, 2016; Badihi et al., 2021; Liu et al., 2021).

Plants grown in fields are affected by one or more abiotic stresses (Zafar et al., 2017). Drought is one of the most significant environmental constraints to agricultural productivity in the world (Javed et al., 2021). Nutrient mobility and uptake by plants can be impeded in low moisture with potentially cascading effects on metabolism (e.g., hormones and chlorophyll), phenological development and acquisition of nutrients (Tombuloglu et al., 2019). Not surprisingly, plant macronutrients use efficiencies that are already low, <50%, under normal soil moisture conditions (Dimkpa et al., 2017) are exacerbated by drought, further reducing fertilizer efficacy.

Overproduction of ROS impairs membrane integrity and cellular function by interfering with photosynthesis, mineral absorption, and assimilation (Ghani et al., 2022). The ROS generation induced due to drought manifests as superoxide around PSI through the Mehler process or singlet oxygen species at PSII (El Sabagh et al., 2019). Drought primarily disrupts photosynthetic pigment production, electron transport, and the Calvin-Benson cycle via increased membrane lipid peroxidation and thylakoid disintegration, which eventually impairs photosynthesis's light and dark reactions (Mathur et al., 2014; Roach and Krieger-Liszkay, 2019; Bano et al., 2021b). To mitigate elevated ROS production and metabolic protection, plants up-regulated the antioxidant system and enhanced osmolytes accumulation (Ahanger et al., 2021). Antioxidants are both enzymatic and non-enzymatic, which stabilize the cells by removing excess ROS (York-Duran et al., 2019). Different kinds of osmolytes, including amine, compounds (glycine betaine), proline, and soluble sugars are key regulators of water content, enzyme functions, and stress signaling (Ahanger et al., 2014; Ejaz et al., 2020).

Brassica napus (*B. napus*) is one of the most important oilseed crops in the world, generating a significant quantity of edible oil and proteins for human and animal use. Rapeseed has been grown on about 36.4 million hectares of land globally, with a gross volume of around 72.5 million tonnes, and is consumed raw or as a component in other foods (Ivanova et al., 2016; Chmielewska et al., 2021). However, deliberate selection for significant quality features, such as low glucosinolate level and minimum erucic acid concentration, has manifested in genetic bottlenecks, restricting genetic variety in today's new cultivated oilseed rape gene pools (Wanasundara et al., 2016). Drought, a worldwide issue, has a substantial influence on rapeseed growth and productivity. This paper will address whether growth matrix supplementation to *B. napus* plants with Ca in the presence of sufficient phytoavailable nutrients from dissolved mineral salts, provides "nano-enabled" mitigation of water stress.

Various studies had revealed a numbers of good attempts to understand the impact of nanoparticles on plants growth (Upadhyaya et al., 2017a, 2017b; Tombuloglu et al., 2019), however, the effect of Ca-NPs under drought conditions is not well explored. Therefore, in the present investigation, it was hypothesized that foliar application of Ca-NPs benefit to *B. napus* seedlings by strengthening the tolerance mechanisms and protects from detrimental effects of drought stress by up-regulating the antioxidant system, osmoregulation, mineral nutrient acquisition, and secondary metabolites accumulation. Here, the impacts of the Ca-NPs and drought treatments on the *B. napus* leaf photosynthetic potential were also measured in situ with chlorophyll fluorescence measurements for the operating quantum yield for photosystem II (ΦPsII).

2. Materials and methods

2.1. Experimental design and growth conditions

Rapeseed oil seed obtained from College of Agriculture and Biotechnology, Zhejiang University, China, were sterilized by 0.1% Sodium hypochlorite (NaClO) solution for about 10 min. After sterilization seeds were rinsed by distilled water and sown on a Whatman filter paper in a petri plate. After germination, one-week-old healthy plants were transferred into one-liter plastic pot having half strength Hoagland nutrient solution. The seedlings were monitored on daily basis and nutrient solution was changed after every third day. After three weeks of acclimatization the plants were divided into two groups control and drought with treatments as follows; control (Ck), Ca-NPs 25 mg L^{-1}, 50 mg L^{-1}, 100 mg L^{-1} and 150 mg L^{-1} sprayed thrice in a day on both the upper and lower sides of plant leaves until it runoff before one week of drought. According to literature (Ayyaz et al., 2021), the plants were exposed to drought by using 15% PEG-6000 (Polyethylene Glycol) in full strength Hoagland nutrient solution for about seven days. (1) Seedlings under control treatments were exposed to Ca-NPs treatment; (2) for drought treatment, Hoagland solution was supplemented with 15% PEG-6000 and given to plants. The Ca-NPs in the form of (CaO) brought out commercially from Shanghai Aladdin Biochemical Technology Co., Ltd. (CAT# 1305–78-B). Each treatment comprises of four biological replicates. The experiment was designed according to completely randomized block design (CRBD).

2.2. Morphological parameters and quantum yield (QY=Fv/Fm)

After harvesting, the plants were separated into shoot and root and fresh and dry biomass were observed by using digital electric balance, and after that moved to an electric oven at 85 °C for 72 h (Ulhassan et al., 2019). For chlorophyll fluorescence measurements fully matured randomly selected light adopted leaves at 11:00 a.m. to 13:00 p.m. Data regarding PSII quantum yield (QY) was recorded by using FluorPen FP-110 MX-LM (Photon System Instruments, Czech Republic).

2.3. Relative water content (RWC)

Fresh weight (FW) of fully mature randomly selected leaves of *B. napus* was taken and imbibed for 24 h in distilled water, then turgid weight (TW) was measured. Samples were oven dried for 24 h at 80 °C, and then the dry weight of seedlings was calculated (Shahzadi et al., 2021).

2.4. Chlorophyll fluorescence

Fluorometer FP 110 (Photon System Instruments, Drasow, Czech Republic) was used to record the chlorophyll fluorescence (Bilger and Björkman, 1991). For the measurement of light response curve, leaves were dark adapted for 20 min by wrapping aluminum foil. Then rapidly fix the leaf in Fluorometer leaf holder after removing foil and initiate rapid light curve program under fluorescence and absorbance mode. Minimum (F_o) and maximum fluorescence (Fm) was measured by providing actinic light. Max. QY of photosystem II was estimated by (Fm – Fo)/ Fm. Red actinic light (Saturating pulse of 3000 μmol m^{2-} s^{1-} intensity was provided after every 30 μs interval for 2 min stepwise to

increase the photosynthetically active radiation (PAR). The values of different parameters for PSII were plotted graphically such as Fv/fm, ETR and NPQ (Bano et al., 2021a).

2.5. Photosynthetic pigments and leaf gas exchange parameters

For chlorophyll content estimation, 0.2 g of leaf tissue ground in 80% acetone with final volume of 10 ml (Arnon, 1949). The OD was observed using a fluorescence spectrophotometer (Hitachi F-4600, Japan).

For gas exchange parameters analysis, photosynthesis system (LI-COR 6400XT) was used of *B. napus* leaves between 9:00 and 11:00 h. Photosynthetic rate (Pn), stomatal conductance (Gs), intercellular CO_2 concentration (Ci), and transpiration rate (Tr) were determined on the top most fully expanded leaf. The fully expanded leaves were kept in a chamber at 1000 μmol m^{-2} s^{-1} photon flux density, irradiance (PAR) 100 w m^{-2} (400–700 nm) with a gas flow rate at 200 μmol s^{-1} with leaf temperature 28 °C. The ambient CO_2 concentration was kept at 380 μmol CO_2 mol^{-1} air by keeping the vapor pressure deficit at 2.0 kPa.

2.6. Total soluble protein (TSP) and proline content

Fresh leaf sample (0.2 *g*) was ground and centrifuged at 6000*g* for 15 min in 4 ml of 50 mM phosphate buffer (pH 7). For protein, quantification was observed at 595 nm (Bradford, 1976). While, proline contents were evaluated according to Bates et al. (1973).

2.7. Lipid peroxidation and reactive oxygen species (ROS)

Membrane lipid peroxidation was observed in terms of MDA content, Reaction mixture comprises of TBA (thiobarbituric acid) reaction. Fresh leaf samples weighing 0.25 g crushed in 5 ml of 0.1% TCA (trichloroacetic acid). Then, the samples centrifuged at 12000*g* for 15 min. The supernatant was collected and kept at 4 °C. A test tube containing 0.5% TBA with 1.5 ml of sample extract and 5% TCA heated in a water bath. After cooling down, the absorbance of the samples measured at 600 and 532 nm against a blank using a double beam spectrophotometer at (Hitachi U2000, Japan) according to (Ali et al., 2018). For H_2O_2 analysis, the leaf sample (0.25 g) ground in 0.1% TCA and centrifuged at 12,000 rpm for 15 min, with the supernatant kept at 4 °C. In test tubes, 10 mM potassium phosphate buffer (0.5 ml), supernatant (0.5 ml), and KI (1 ml) combined to estimate hydrogen peroxide. Double beam spectrophotometer (Hitachi U2000, Japan), was used to measure the absorbance at 390 nm (Farooq et al., 2018).

2.8. Biochemical assay of antioxidant enzymes

Leaf sample (0.5 g) homogenized in 10 ml of 50 mM phosphate buffer for the evaluation of antioxidant enzyme activities. The mixture was centrifuged at 13000 rpm for 10 min and stored at 4 °C. The capacity of superoxide dismutase (SOD) to prevent NBT photochemical degradation was measured according to (Gill et al., 2017). A reaction mixture of total volume 3 ml, including 50 mM PBS pH (7.8), 2 mM riboflavin, 13 mM methionine, 75 mM NBT, and 0.1 mM EDTA, as well as 100 l enzyme extract. One unit of SOD activity corresponds to the amount of enzyme necessary to prevent 50% of NBT degradation detected using a spectrophotometer at 560 nm. The activity of guaicol peroxidase as determined by Farooq et al. (2016). Catalase activity was observed according to Aebi (1984). Reaction mixture comprise of 50 mM phosphate buffer (2.8 ml), sample extract (0.1 ml), and 300 mM H_2O_2 (0.1 ml) in a test tubes. The data was recorded at 240 nm by a spectrophotometer. The H_2O_2 dependent oxidation rate of ascorbic acid (AsA) was used to determine APX activity, as described by (Islam et al., 2016).

2.9. Protease and lipoxygenase (LOX) activity

To determine the level of plant protease the ELISA kit assay (Cat#-CA01083P Shijiazhuang Simer Technology Co., Ltd) of the leaf sample, in covered microtiter plate wells. The reaction mixture comprises of 50 μl standard to micro Elisa strip plate, 40 μl sample dilution and 10 μl testing sample, incubated at 37 °C for 30 min in dark. The mixture was diluted 30 fold with distilled water. Then, uncover the closure plate washout the plate five times, discard the liquid, dry by swing and washed again using washing buffer four times. Add 50 μl HRP-conjugate reagent to each well and incubate at 37 °C for 30 min in dark and washout five times. Add 50 μl chromogen A and chromogen B solution respectively and incubate at 37 °C for 10 min in dark. Add 50 μl stop solution to each well, terminate the reaction (the blue color change to yellow). Read absorbance at 450 nm using Multifunctional Microplate Reader (BioTek Synergy H1).

The ELISA kit (Cat#G0906F Suzhou grid Rui Si Biological Technology Co., Ltd) test of the leaf sample in fully covered microtiter plate wells was used to quantify the level of plant LOX. The reaction solution containing 50 μl standard solution, 40 μl sample dilution and 10 μl testing sample to micro Elisa strip plate and incubated at 37 °C for 30 min in dark. The mixture was diluted 30 times with distilled water. Then, uncover the plate and wash it five times with washing buffer, remove the liquid, dried by swing, and wash out four times with washing buffer. In each well, add 50 μl HRP-conjugated reagent, incubate for 30 min in the dark at 37 °C, and wash out again five times. After the addition of 50 μl chromogen A and chromogen B solutions incubate reaction solution at 37 °C for 10 min in the dark. The concentration of plant LOX in the samples was determined by comparing the samples OD to the standard curve using Multifunctional Microplate Reader (BioTek Synergy H1).

2.10. Endogenous hormones determination

2.10.1. Abscisic acid (ABA)

Endogenous ABA assay was performed by using ELISA kit (Catalog # H251–1-2; Nanjing Jiancheng Institute of Biological Engineering Co., Ltd). The reaction solution comprising the ABA antibodies and plant tissues were incubated at 37 °C for 30 min. The enzyme substrate reaction was stopped through the addition of sulfuric acid. Data was collected at 450 nm using a Multifunctional Microplate Reader (BioTek Synergy H1).

2.10.2. Jasmonic acid (JA)

To estimate JA, an ELISA kit (Catalog # F4976-B; MLBIO Tech.) was used as directed by the manufacturer. The reaction solution comprises of plant samples and reagents provided. The HRP conjugated reagent and plant samples were incubated at 37 °C for 30 min. The JA pre-coated antibody inhibits the reaction concerning JA and HRP-conjugated JA reaction solution. Data was collected at 450 nm using a Multifunctional Microplate Reader (BioTek Synergy H1).

2.10.3. Salicylic acid (SA)

Endogenous SA content, an ELISA kit (Jiangsu Enzyme Industry Co., Ltd., Catalog # MM-3372202) was used as per manufacture instructions. Leaf tissue was homogenized in 5 mL of PBS at a pH of 7.4 and centrifuged at 1000 *g* for 20 min. The reaction mixture, comprising of 10 μl supernatants diluted with 50 μl PBS and 10 μl of enzyme-labeled reagent added to the wells, was incubated at 37 °C for 60 min. The reaction was halted by the addition of 50 μl of stop solution. A standard curve of SA supplied with the kit was used for quantitative analysis. Data was collected at 450 nm using a Multifunctional Microplate Reader (BioTek Synergy H1).

2.11. Plant mineral status

Leaf samples were ash at 550 °C for 20 h in a muffle furnace to determine the micro and major mineral concentrations. The ash was then incubated at 70 °C for about 2 h after adding 31% HNO_3 (m/v) and 17.5% H_2O_2 (*v*/v). Data was recorded using inductively coupled plasma mass spectrometry.

2.12. Secondary metabolites

For secondary metabolites, expression 100 mg leaf sample mixed with 1 ml of 70% methanol solution placed in a 2 ml LEP tubes. Leaf tissues were ground with metallic steel ballast 50HZ for 5 min, and refrigerated at 4 °C for about 8 h. Later on, samples centrifuged at 13000 r/min, at 4 °C for 15 min, after centrifugation the supernatant filtered over the 0.22 μm membrane and data collected by using LC-MS analysis.

2.13. Leaf ultra-structural observation by transmission electron microscopy

Leaf fragments without veins (about 1 mm^2) were fixed in glutaraldehyde overnight and then washed three times with PBS. The samples were post fixed in OsO4 for one h and washed again three times with PBS. After that, the samples were dehydrated in a graded series of ethanol for 15–20 min each and then in absolute acetone for 20 min. After dehydration, the samples were embedded in Spur's resin overnight. The specimens were heating at 70°C for 9 h, the ultra-thin sections (80 nm) were cut and mounted on copper grids for the transmission electron microscope (TEM 1230EX, JEOL, Japan) at 60.0 kV.

2.14. Statistical analysis

For statistical analysis, completely randomized analysis of variance (two-way ANOVA) was used on rapeseed obtained from various factors. The significance of factors is denoted by the symbol '*' while non-significant factors are denoted by the symbol 'ns' in figures. The means of treatments are compared using LSD (least significant difference). MS-Excel (for descriptive statistics) and COSTAT v.6.4 (Cohort, California, USA) used for the entire statistical study. The data for each parameter is presented in terms of mean with standard errors, bar and line graphs were made using Graph Pad Prism (8.0.1).

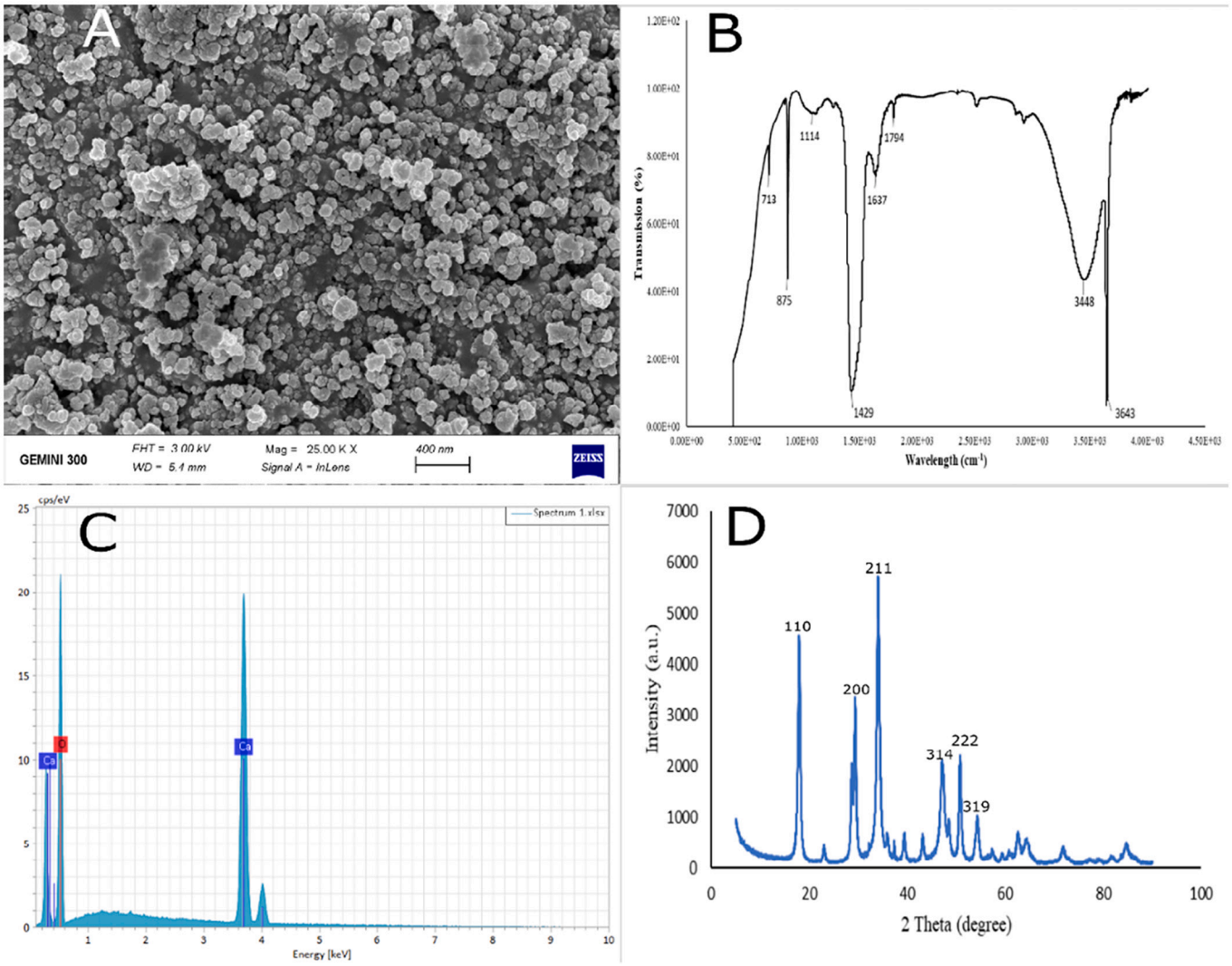

Fig. 1. Morphological, compositional and spectroscopic characterization of Ca-NPs (A) SEM [scale bar = 200 nm] (B) FTIR spectrum (C) EDS analysis (D) XRD pattern.

3. Results

3.1. Characterization of Ca-NPs

The morphological, compositional and spectroscopic characterization of Ca-NPs illustrated in Fig. 1. The Ca-NPs comprises of 98% purity with particle size range from 60 to 90 nm confirmed by using scanning electron microscope (ZEISS Gemini SEM 300, Germany). SEM graph showed a spherical, regular surface with less micro pores on its surface (Fig. 1A). These results are in evidence with the SEM analysis (Nazir et al., 2022). The Fourier Transform Infrared (FTIR) spectroscopy (model: Nico LET iS50 FTIR spectrometer) used for the detection of the presence of functional groups on the surface of Ca-NPs. The FTIR spectra showed a wide range from 400 to 4000 cm^{-1} (Fig. 1B). The wider peaks at 3643, 3448, 1794, 1637, 1429 and 1114 cm^{-1} depicts the stretching of O—H group of alcohol, C═C, C═O stretching and presence of CH_2 polysaccharide functional group and (P-O-C) bonding in FTIR spectrum respectively. In addition, the peaks at 875 and 713 cm^{-1} suggested the presence of C—C or O—C bonding and OH functional group of Ca-NPs respectively (Fig. 1B). These results suggest that the presence of functional groups are very important and helps out the nanoparticles in the structural and functional modulation which results in alteration of surface reaction, change in surface behavior, hydrophilicity and increased electrical or catalytic activities of Ca-NPs (Vijai Anand et al., 2021). The energy dispersive X-ray spectroscopy (EDX) analysis was performed by (FEI QUANTA FEG 650) and EDS by (EDAX Inc. Genesis XM) to analyze the elemental composition of calcium and oxygen which were 55% and 44% in Ca-NPs (Fig. 1C). Our results suggested the phenomenological lattice constant of Ca describes the stable crystals with maximum yield strength. The XRD spectra of Ca-NPs revealed the crystalline planes and crystalline nature of Ca-NPs (Fig. 1D). The diffraction peaks of Ca-NPs observed at 18.84°, 29.66°, 34.80°, 47.17°, 51.86°, 54.68°. Various diffraction angles of Ca-NPs at 2θ degrees coincided at 110, 200, 211, 314, 222, 319 respectively, of the cubic phase of Ca-NPs with a space group of Fm-3 m and are in agreement with the standard JCPDS File No. 37–1497 (Gandhi et al., 2021).

The hydrodynamic size and zeta potential of CaO-NPs were observed at scattering angle of 90° at 25 °C by using NanoPlus "Particle Size & Zeta Potential Analyzer" (Particulate Systems a division of Micromeritics, 4356 Communications Drive, Norcross GA, 30093, USA) (Hussain et al., 2020). The zeta potential degree describes the electrostatic repulsion degree among the adjacent and similar charged particles during the dispersion state. The Ca-NPs exhibited the hydrodynamic size of 244 nm along with the zeta potential of +28.0 mV. Zeta potential is considered as the effective technique in describing the stability of nanoparticles. The nanoparticles having high zeta potential values of +30 mV are considered as electrically stable and low zeta potentials than −30 mV are known unstable (Jiang et al., 2009; Morga et al., 2013).

3.2. Plant dry biomass and RWC content increased by Ca-NPs application

The effects of different concentrations (0, 25 mg L^{-1}, 50 mg L^{-1}, 100 mg L^{-1}, and 150 mg L^{-1}) of Ca-NPs on the shoot and root biomass accumulation of rapeseed seedlings under drought-stressed and control conditions were significant. Drought reduced plants shoot and root dry biomass by 63% and 42%, respectively as compared to control. Application of Ca-NPs alleviated the drought stress and showed positive effect on *B. napus* plants growth attributes and significantly increased the shoot dry biomass accumulation by (35%–82%) and (23%–41%),and root dry biomass by (28%–67%) and (21%–29%)under control and drought stress conditions, respectively (Fig. 2A and B). The 100-mg L^{-1} Ca-NPs treatment under drought treatment improved plants growth and showed maximum increase in shoot and root dry biomass accumulation by 82% and 67%, respectively (Fig. 2A and B). Plants exposed to drought treatment showed restricted growth, while Ca-NPs amendment to the drought-treated seedling helped them to retain their vigor and growth. Drought also considerably reduced the plants RWC content by 59% as compared to control plants. While, application of Ca-NPs different concentrations (0, 25 mg L^{-1}, 50 mg L^{-1}, 100 mg L^{-1}, and 150 mg L^{-1}) to rapeseed oil seedlings significantly increased the RWC content ranged from(39%–71%) and (23%-48)under control and drought stress, respectively (Fig. 2C). The 100-mg L^{-1}Ca-NPs treatment under drought treatment showed maximum increase in RWC content by 51%, respectively relative to control plants (Fig. 2C).

3.3. Ca-NPs up-regulate the photosynthetic pigments, gas exchange parameters and NPQ in Brassica seedlings under drought stress

The effect of Ca-NPs and drought stress treatment on the contents of chlorophyll *a*, b, total chlorophyll, carotenoids and xanthophyll in *B. napus* plants is illustrated in (Fig. S1A—F). Drought cause considerable reduction of photosynthetic pigments such as chlorophyll *a* by 69%, chlorophyll *b* 28%, chlorophyll *a*/b 11%total chlorophyll 77%, carotenoids 54% and xanthophyll 32%, respectively relative to non-treated plants (Fig. S1A—F). However, the Ca-NPs supplementation to plants showed a substantial increase in chlorophyll *a* ranging from (24%–41%), chlorophyll *b* (18%–38%), chlorophyll *a*/b (10%–26%), total chlorophyll (34%–76%), carotenoids (20%–54%) and xanthophyll (10%–18%) as compared to control, respectively(Fig. S1A—F). Similarly, Ca-NPs application to rapeseed seedlings under drought stress resulted in a significant increase in chlorophyll *a* (28%–66%), chlorophyll *b* (23%–58%), chlorophyll *a*/b (18%–44%), total chlorophyll (34%–88%), carotenoids (24%–64%) and xanthophyll (14%–36%) compared to control plants (Fig. S1A—F).The 100-mg L^{-1} Ca-NPs treatment exhibited the maximum increase in chlorophyll *a* by 66%, chlorophyll *b* 58%, chlorophyll *a*/b 44%, total chlorophyll 88%, carotenoids 64% and xanthophyll 36%, respectively relative to control plants.

Drought stress reduced plants net photosynthetic rate (Pn) by 53%,

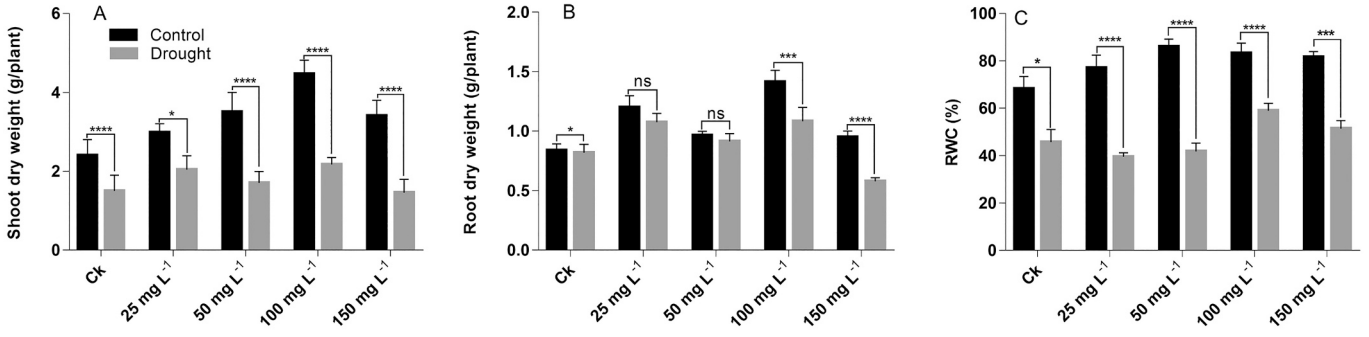

Fig. 2. Effect of different concentrations of Ca-NPs (0, 25, 50, 100 and 150 mg L^{-1}) onset to drought (15% PEG-6000) treatments on *Brassica* plants growth and morphology (A) shoot dry weight, (B) root dry weight and (C) relative water content respectively. Different letters represents significance level as * = 0.05, ** = 0.01, *** = 0.001, **** = 0.0001, ns = non-significance.

stomatal conductance (Gs) 68%, carbon dioxide intake (Ci) 48%, and transpiration rate (Tr) 56%, respectively as compared to control plants (Fig. 3A-D). However, the application of Ca-NPs to plants considerably increased the Pn, Gs, Ci and Tr ranging from 18%–44%, 27%–56%, 21%–37%, and 32%–48%, respectively that of control plants. Likewise, under drought stress plants onset to different Ca-NPs treatments demonstrated a substantial increase in Pn, Gs, Ci, and Tr ranging from 23%–73%, 27%–83%, 20%–63% and 17%–75%, respectively as compared to control (Fig. 3A-D). The concentration of 150-mg L^{-1} Ca-NPs treatment under drought stress showed significant increase in Pn, Gs, Ci, and Tr by 73%, 83%, 63%, and 75% respectively relative to control plants, which shows maximum recovery under NPs treatments.

Drought stress negatively affected the photosynthetic machinery and decreased the photosystem II (PSII) activity by 41%, QY 30%, ETR 38% and NPQ 34% respectively as compared to control plants (Fig. 4A-D). Application of different Ca-NPs treatments to plants under control conditions showed substantial increase in PSII efficiency ranging from 14%–32%, QY 17%–32%, ETR 21%–36% and NPQ 18%–38%, respectively that of control plants. While, the Ca-NPs treatments significantly ameliorated the drought and enhanced the PSII efficiency ranging from 21%–48%, QY 24%–52%, ETR 27%–56%, and NPQ 25%–62% respectively as compared to control (Fig. 4A-D).The 100-mg L^{-1} Ca-NPs application under stress variably influenced the chlorophyll fluorescence parameters by improving PSII efficiency by 48%, QY 52%, ETR 56% and NPQ 62%, which suggest the maximum recovery of plants.

3.4. Ca-NPs suppressed the oxidative stress enhanced proline and total soluble proteins (TSP) contents

Drought stress increased ROS generation in the form of H_2O_2, which favors higher membrane lipid peroxidation in terms of MDA content as compared to control. Under drought, the rapeseed oil plants showed increasedH_2O_2 content by 109% and MDA 129%, respectively relative to control. Although, Ca-NPs treatments under drought stress significantly ameliorated the ROS production by lowering H_2O_2 and MDA content ranging from 42%–68% and 57%–78%, over control, respectively (Fig. 5A and B).Despite this, the 100-mg L^{-1}Ca-NPs treatment reduced H_2O_2 and MDA contents by 68% and 78%, respectively relative to control plants.

Drought stressed *Brassica* seedlings exhibited higher levels of proline and total soluble protein with the rise being more pronounced in the Ca-NPs treatment. Drought increased proline by 78% while decreasing TSP content by 65% (Fig. 5C and D). Application of Ca-NPs under control condition enhanced the proline and TSP contents ranging from 44%–68% and 26%–65% respectively that of control plants. However, Ca-NPs treatment under drought stress considerably increased the proline and TSP contents with the maximum increase of 64%–123% and 36%–82%, respectively as compared to control plants (Fig. 5C and D). Data show that100-mg L^{-1} Ca-NPs treatment under drought stress significantly increased the proline content by 123%, and 150-mg L^{-1} Ca-NPs treatment under stress condition enhanced the TSP by 82%, respectively as compared to control plants (Fig. 5C and D).

3.5. Ca-NPs upregulated the antioxidative defense potential, protease, and lipoxygenase enzymes activities of plants

Drought significantly increased antioxidant enzyme activity such as SOD, POD, CAT and APX as shown in (Fig. 6A-F). Drought treated plants showed tremendous increase in SOD activity by 95%, POD 69%, CAT 88%, APX 74%, protease 97% and lipoxygenase 108% respectively than that of control plants. The application of Ca-NPs treatments under control conditions considerably enhanced the antioxidant enzyme activities including SOD activity ranging from 48%–88%, POD 28%–66%, CAT 43%–72%, and APX 45%–70%,and protease activity by 21%–56%

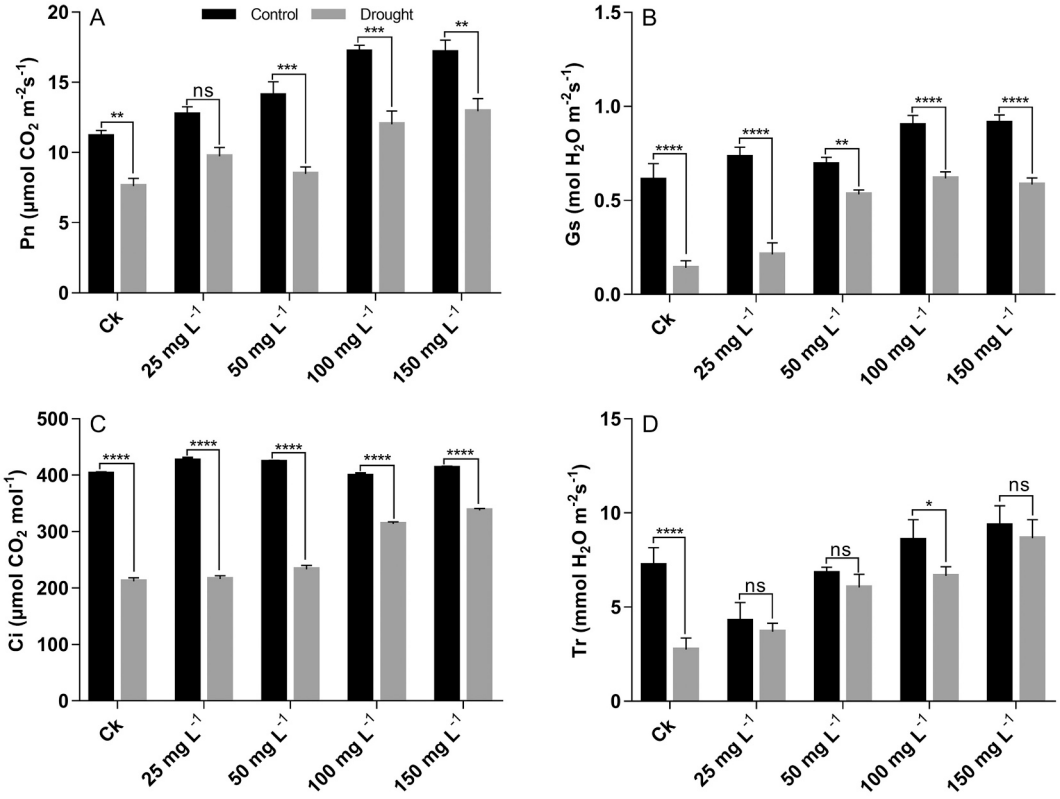

Fig. 3. Effect of Ca-NPs (0, 25, 50, 100 and 150 mg L^{-1}) and drought (15% PEG-6000) treatment on *B. napus* seedlings, (A) net photosynthesis (Pn), (B) stomatal conductance (Gs), (C) carbon dioxide intake (Ci), and (D) transpiration rate (Tr), respectively. Different letters represents significance level as * = 0.05, ** = 0.01, *** = 0.001, **** = 0.0001, ns = non-significance.

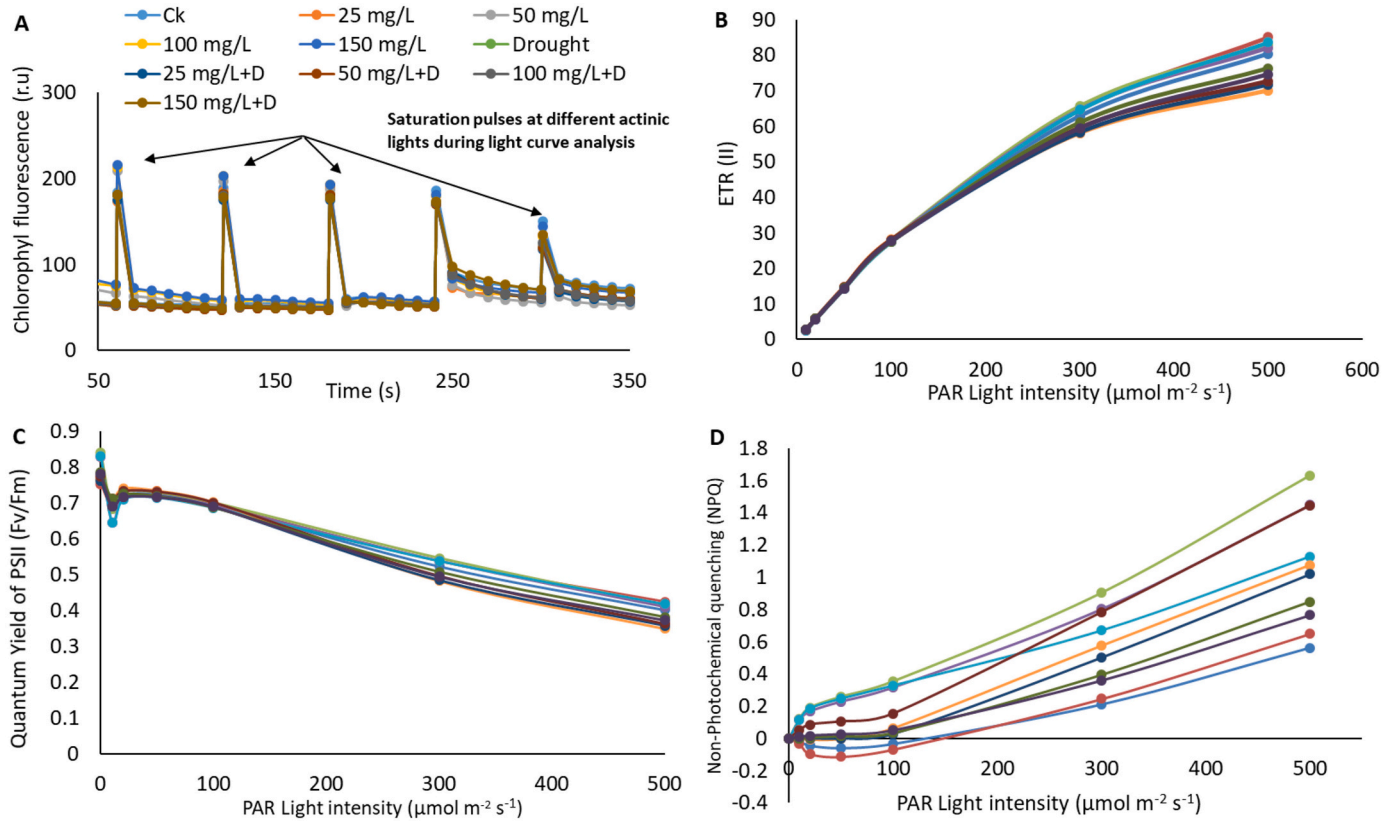

Fig. 4. Effect of Ca-NPs (0, 25, 50, 100 and 150 mg L^{-1}) and drought (15% PEG-6000) treatment on *B. napus* seedlings, (A) relative values of chlorophyll fluorescence, (B) electron transport rate (ETR), (C) quantum yield of PSII (QY), and (D) non-photochemical quenching (NPQ) of PSII respectively. Different letters represents significance level as * = 0.05, ** = 0.01, *** = 0.001, **** = 0.0001, ns = non-significance.

and lipoxygenase activity by 33%–69%, respectively as compared to control plants (Fig. 6A-F). However, Ca-NPs application under drought considerably enhanced the antioxidant enzymes activities in terms of enhanced SOD activity ranging from 120%–164%, POD 62%–97%, CAT 65%–178% and APX 78%–104%, respectively as compared to control plants (Fig. 6A-D). In addition, the protease and lipoxygenase activities under different Ca-NPs and drought stress treatments declined significantly ranging from 21%–56% and 33%–78%, respectively that of control plants (Fig. 6E and F). Under drought stress, 100-mg L^{-1} Ca-NPs treatment showed the maximum increase in SOD, CAT and APX activities with increase of 164%, 178% and 104%, respectively as compared to control plants (Fig. 7A-D). Furthermore, 150-mg L^{-1} Ca-NPs concentration under drought significantly enhanced the POD activity by 97% compared to control (Fig. 6C). While, 100-mg L^{-1} Ca-NPs treatment under drought stress the protease and lipoxygenase enzymes activities decreased by 56% and 78% respectively that of control plants (Fig. 6E and F).

3.6. Ca-NPs upregulated the endogenous hormonal expression and mineral status of Brassica seedlings

Drought stress treated plants exhibited a significant reduction in endogenous hormonal levels including abscisic acid (ABA) 41%, jasmonic acid (JA) 56% and salicylic acid (SA) 66% respectively as compared to control plants. While, Ca-NPs application under control conditions enhanced the endogenous hormone in terms of ABA, JA and SA by 34%–56%, 24%–73% and 36%–79% respectively as compared to control plants (7A-C). However, the different Ca-NPs concentrations treated under drought stress enhanced the expression of endogenous hormones ABA, JA, and SA by 38%–92%, 29%–84% and 49%–118%, respectively as compared to control plants (7 A-C). A maximum increase in ABA 92% was observed in 150-mg L^{-1}Ca-NPs treatment under drought stress. While, maximum increase in JA 84% and SA 118% levels were observed in 100-mg L^{-1} Ca-NPs treatment under drought stress.

Mineral status in the leaves of *Brassica* plants are illustrated in (Fig. S2A—I). The uptake of major and trace minerals by rapeseed plants were considerably reduced by drought stress (Fig. S2A—I). For example, drought decreased the Ca content by38%, potassium (K) 44%, phosphorous (P) 43%, magnesium (Mg) 34%, manganese (Mn) 52%, boron (B) 36%, iron (Fe) 74%, copper (Cu) 39%, and zinc (Zn) 26%, respectively when compared to control plants(Fig. S2A—I). While, Ca-NPs treatments under control conditions enhanced the plants mineral status with maximum increase of Ca ranging from (34%–65%), K (20%–52%), P (25%–47%), Mg (17%–56%), Mn (28%–68%), B (19%–48%), Fe (40%–60%), Cu (24%–47%) and Zn (14%–39%), respectively as compared to control plants (Fig. S2A—I). However, the Ca-NPs treatments significantly improved plants mineral status under drought, with maximum increases in Ca ranging from (44%–78%), K (38%–69%), P (32%–76%), Mg (29%–82%), Mn (36%–86%), B (28%–66%), Fe (48%–92%), Cu (34%–69%), and Zn (26%–64%), respectively as compared to control plants (Fig. S2A—I). The maximum increase in P content was 76%, Mg 82%, Mn 86%, B 66%, Fe 92%, Cu 69%, and Zn 64% at 50-mg L^{-1} Ca-NPs under drought stress. While, 100-mg L^{-1} Ca-NPs treatment onset to drought stress resulted in the maximum increase in Ca by 78%. In addition, the 150-mg L^{-1} Ca-NPs treatment under drought enhanced K content by 69% as compared to control plants (Fig. S2A—I).

3.7. Comparison of secondary metabolic compounds under drought and Ca-NPs treatments

Application of Ca-NPs treatments to plants effectively modulates the biosynthesis of secondary metabolites under control and stressful

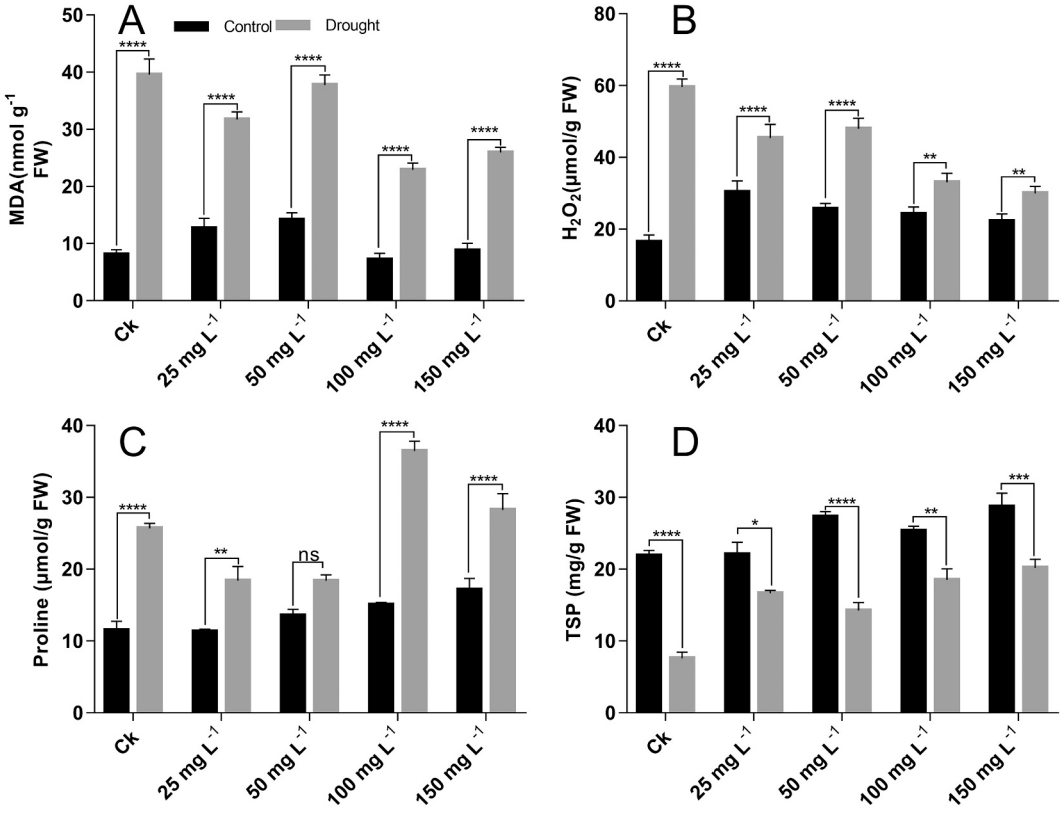

Fig. 5. Effect of Ca-NPs (0, 25, 50, 100 and 150 mg L^{-1}) and drought (15% PEG-6000) treatment on *B. napus* seedlings, (A) malonaldehyde (MDA), (B) hydrogen peroxide (H_2O_2), (C) proline content, and (D) total soluble proteins (TSP), respectively. Different letters represents significance level as * = 0.05, ** = 0.01, *** = 0.001, **** = 0.0001, ns = non-significance.

conditions. Secondary metabolites profiling majorly comprises of phytochemical compounds including flavonoids, terpenoids, coumarins and derivatives, alkaloids and derivatives, quinone, phenylproponoids and lignin. Metabolites profile under control conditions, majorly include the fisetinidin chloride, adenine, epigallocatechin, sudan I, taxifolin. Relative to control plants drought stress showed significant increase in 5, 7-dihydroxychromone, bisdemethoxycurcumin, dimethoxy L-glutamine, pyridoxal 5-phosphate, norisoboldine, sertraline, prim-o-glucosylcimifugin, bilobalide, feruloyltryptamine were prominent. The Ca-NPs pretreatment plants under drought showed substantial increase in 7-methoxy-4-methyl coumarin, cis-4- hydroxyl-D-proline, germacrone, L-tryptophan, N-propiomyl glycine, purpurin over control respectively (Fig. S3).

3.8. Cell ultrastructure changes in leaf

The ultrastructure study further confirmed the impact of Ca-NPs on *Brassica* plants with and without drought stress relative to control (Fig. 8A and B). Under control condition the plants had well-developed cell wall, normal nucleus with well-developed nucleolus and nuclear membrane, integrated chloroplast having compactly stacked grana along with several plastoglobuli enclosed in smooth and clear membrane. Furthermore, several mature and spherical mitochondria having apparent shaped cristae were found in micrograph of control plants (Fig. 8A). Drought, on the other hand, induced significant changes in mesophyll cell structure and function as well as vacuolation, resulting in severe organelle damage. Drought treated plants had distorted cell wall, disheveled nucleus with broken nucleolus and disordered chloroplasts having fewer plastoglobuli and large sized starch grain along with swollen mitochondria and peroxisomes. Moreover, the plastoglobuli was huge and occupying the most of the chloroplast surface, which ultimately squeezes the thylakoid membrane (Fig. 8B). Plants treated with Ca-NPs supplementation under drought stress had intact and smooth cell wall, improved chloroplasts structure with less grana, distended loosen thylakoid membrane and osmiophilic plastoglobuli as compared to control (Fig. 8B). The increased number of plastoglobuli and recovered structure of chloroplasts along with mitochondria depicts the improved cellular stability and tolerance to stressful conditions.

4. Discussion

4.1. Ca-NPs potentially improve plant growth under drought stress

The field of nanotechnology has great potential within plant sciences and plant production systems. Herewith, we reported the potential applications of Ca-NPs ameliorated the drought effects and increased the biomass of the *B. napus* seedlings in comparison with the plants exposed to drought only. According to our results, drought-stressed rapeseed seedlings showed a considerable decrease in plant biomass and chlorophyll content. The dramatic effects of drought on plant growth has been extensively studied in *Brassicaceae* species (Raza et al., 2020), *B. napus* (Batool et al., 2022), *B. carinata* (Samanta et al., 2020), and *B. oleracea* (He et al., 2020). Khan et al. (2019) investigated that rapeseed plants exposed to drought showed reduction in plant growth, plant turgor pressure, affects the cell division, cell wall biosynthesis and metabolism. Recent studies has shown that using nanoparticles can effectively reduce the drastic effects of drought on plants (Cunningham et al., 2018; Juárez-Maldonado et al., 2019; Sanzari et al., 2019; Alabdallah et al., 2021). Ca-NPs significantly improved plant growth in drought stressed rapeseed (Fig. 2A and B). These findings are consistent with those of calcium phosphate nanoparticles, which significantly increased rice growth characteristics (Upadhyaya et al., 2017a, 2017b). The improved plant growth by Ca-NPs under drought stress might be attributed to reduce cellular damage, increased chlorophyll pigments and

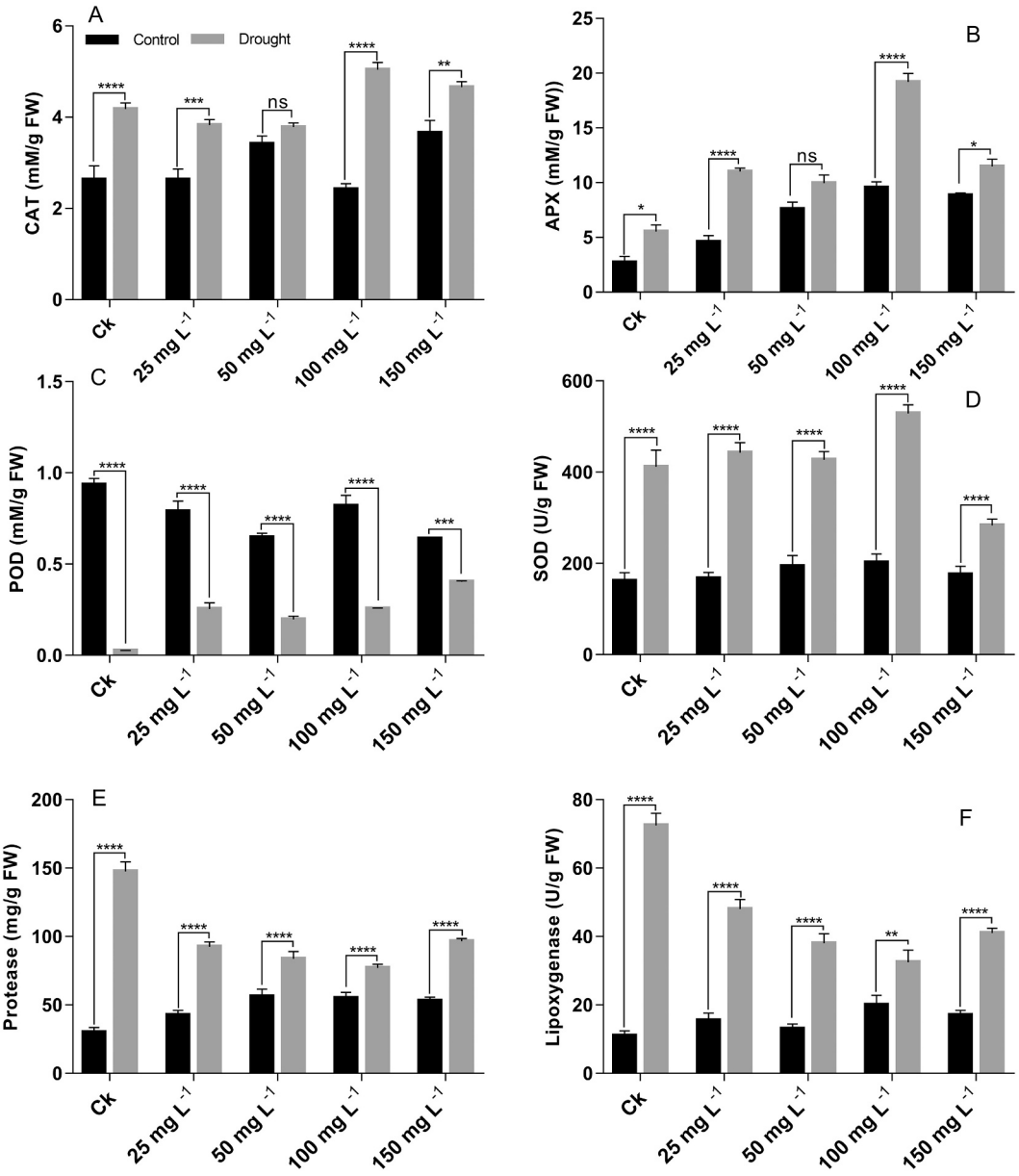

Fig. 6. Effect of Ca-NPs (0, 25, 50, 100 and 150 mg L^{-1}) and drought (15% PEG-6000) treatment on *B. napus* seedlings, (A) catalase (CAT), (B) ascorbate peroxidase (APX), (C) peroxidase (POD), (D) superoxide dismutase (SOD), (E) protease, and (F) lipoxygenase activity respectively. Different letters represents significance level as * = 0.05, ** = 0.01, *** = 0.001, **** = 0.0001, ns = non-significance.

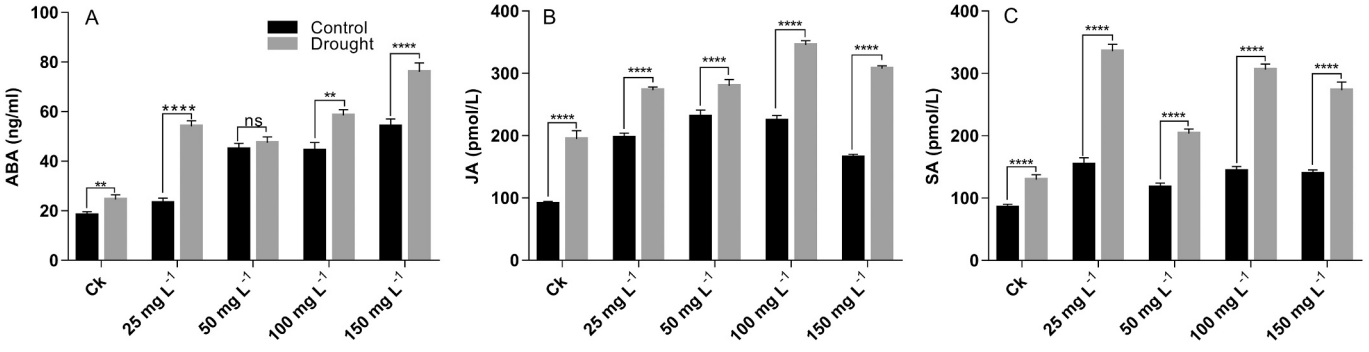

Fig. 7. Effect of Ca-NPs (0, 25, 50, 100 and 150 mg L-1) and drought (15% PEG-6000) treatment on endogenous hormones of *B. napus* seedlings including (A) abscisic acid (ABA), (B) jasmonic acid (JA), and (C) salicylic acid (SA) content, respectively. Different letters represents significance level as * = 0.05, ** = 0.01, *** = 0.001, **** = 0.0001, ns = non-significance.

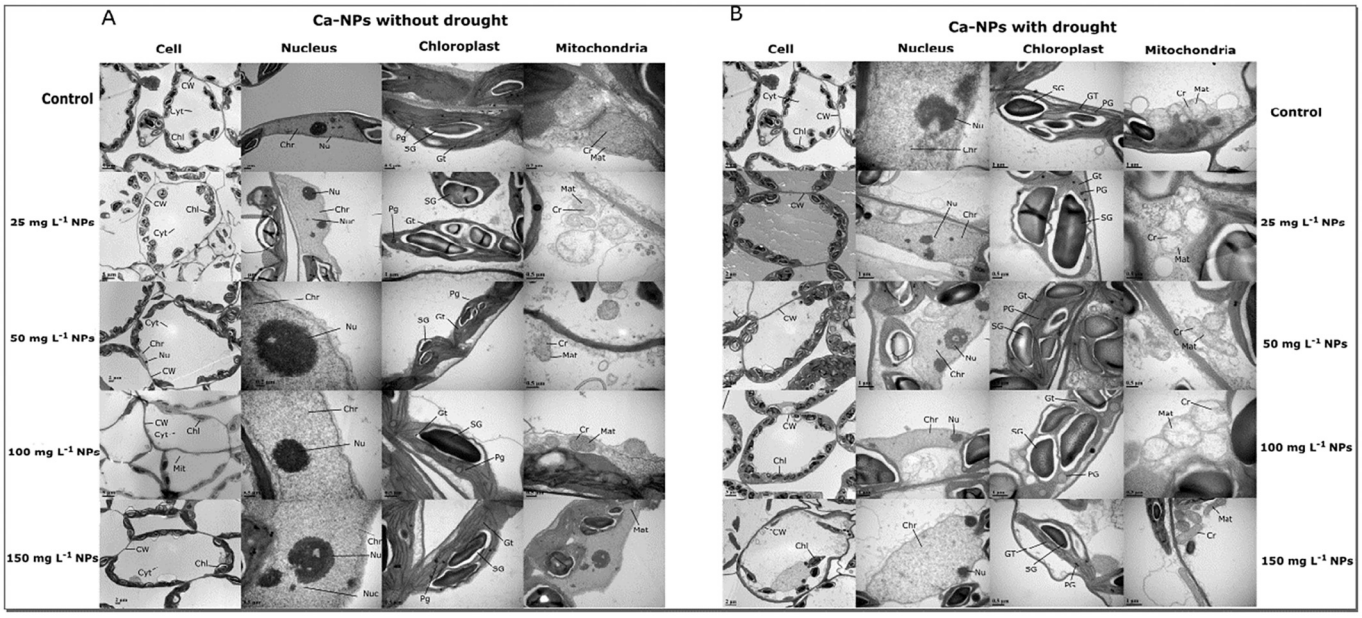

Fig. 8. Effect of Ca-NPs (0, 25, 50, 100 and 150 mg L^{-1}) and drought (15% PEG-6000) treatment on *B. napus* leaves mesophyll cells ultrastructure. (A) Dispersion of nucleolus and chromatin in nucleus matrix, mitochondria, and well-organized chloroplasts with many plastoglobuli and larger starch grains under control conditions (B) condensation of chromatin in nuclear matrix and nucleolus, and swelling and vacuolized chloroplast and thylakoidal membrane distortion and mitochondrial swelling and vague cristae of plants after 7 d of drought stress. Chr, chromatin; CW, cell wall; Gt, grana thylakoid; Nu, nucleus; Nuc, nucleolus Pg, plastoglobule; SG, starch grain; Mat, matrix; Cr, cristae.

photosynthetic efficiency in terms of PSII performance.

4.2. Ca-NPs regulates the chlorophyll pigments, photosynthesis and PSII performance under drought stress

Photosynthesis is considered as key indicator of plant adaptation to environmental stimuli. Under drought stress, the *Brassica* plants showed a substantial decrease in chlorophyll pigment (Fig.S1). This might occur due to increased chlorophyllase enzyme activity, oxidative pigment destruction, thylakoid membrane deformation, ornutritional deficiency (Rehman et al., 2016; Gholamin and Khayatnezhad, 2020). According to our data (Fig. 2C and 3A-D) drought significantly reduces plant growth, causing leaf wilting as well as a decrease in leaf gas exchange parameters including RWC and (Pn, Tr, Gs, and Ci), indicating the dehydration effects of drought on plants, respectively. These findings are in correspondence with previous research conducted under water deficit conditions (Ghobadi et al., 2013; Anjum et al., 2017; Bhuiyan et al., 2019; Demirel et al., 2020). The decreased gas exchange parameters might be attributed to photosynthetic pigments degradation and the resulting distortion of water transport from root to shoot, which resulted in lower Gs and Tr, as well as a decrease in Pn (Grieco et al., 2020). However, the exogenous application of Ca-NPs regulate the drought induced negative impact on photosynthetic pigments and gas exchange parameters in terms of increased PSII efficiency by improving the Rubisco activity under abiotic stress, which was associated with the higher photosynthetic rate in leaves (Figs. 3 and 4) (Ditta and Arshad, 2016). According to our data Fig. (3B) the exogenous application of Ca-NPs showed significant increase in stomatal conductance (Gs) of *Brassica* plants, suggesting that calcium nanoparticles can regulate the opening and closing of plant leaf stomata. The regulatory function of Ca^{2+} includes some key processes such as Calvin–Benson–Bassham cycle and providing $NADP^{+}$ as the terminal electron acceptor for photosynthesis (Hochmal et al., 2015). Consistent to our results Betterle et al. (2017) reported that Ca-NPs mediated enhanced photosynthesis process is accompanied with increased proportion of open reaction centers and electron transfer rates in PSII, and relieved photo inhibition under drought stress. It is documented that calcium can regulate the photosynthetic pigment functioning and prevents its disintegration to maintain the photosynthetic electron transport (Hu et al., 2007). Moreover, application of calcium to plants maintains the membrane function and enhances cell membrane structure stability by forming calcium salts with phospholipid molecules in the thylakoid membrane (Betterle et al., 2015).

Drought also has a considerable impact on plant photosynthetic efficiency and Fv/Fm, the primary yield of PSII photochemistry and a potential indication of photosynthetic capacity (Bresson et al., 2015). Variations in Fv/Fm are often confirmed by changes in the concentrations of specific photosynthetic pigments and cell structure modulations, which can be influence by a variety of environmental factors that affect PSII activity, including light, nutrient status, and temperature (Brestic et al., 2016). Likewise, Rane et al. (2015) and Gandhi et al. (2021) suggested that Ca-NPs might enhance the plants photosynthetic efficiency by lowering down the damage of chloroplast thylakoid membrane and maintaining cellular homeostasis under stressful conditions. In addition, our findings also indicate that Ca-NPs and drought treated plants has less effect on the QY that of control (Fig. 4C), which suggests the protective effects of Ca-NPs on plants.

Similarly, the decrease in Fv/Fm of PSII under drought stress notably, related to PSII down regulation through sustained components of protective NPQ mechanism (Nath et al., 2017). Our findings suggested that the decrease in PSII efficiency due to significant increase of light intensity (0–700 μmol m^{-2} s^{-1}) is positively correlated with the enhanced electron transport rate at PSII and increased NPQ under drought treatment in Brassica seedlings. Drought resulted in reduction of ETR(II) and increased NPQ in plants exposed to drought stress, suggesting the maximum electrons accumulation at PSII acceptor site that mediates the higher non photochemical quenching (Hu et al., 2018; Vijai Anand et al., 2021). While, our results suggested that the photoprotective components NPQ substantially increased in *Brassica* seedlings under Ca-NP_S and drought treatment (Fig. 4D). These results suggest that the exogenous application of Ca-NPs under drought stress provides maximum protection from photo-damage at PSII site and reduced photochemistry might be correlated by rapid recovery of NPQ through xanthophyll cycle (Meier et al., 2020; Badihi et al., 2021).

Interestingly, the exogenous application of Ca-NPs under stressful conditions impairs the chloroplast structure and thylakoid membrane and photosystems activities by binding with extrinsic luminal proteins PsbO and sustains the oxygen-evolving complex (OEC) (Betterle et al., 2015; (Terashima et al., 2012). Moreover, Ca^{2+} ions effectively impart in cellular photo-protective mechanism by improving the expression of LHC stress related proteins (Hochmal et al., 2015). Our data suggests that the application of Ca-NPs to plants relived the damage to the PSI acceptor-side under drought stress by inducing a rapid increase in the cyclic electron flow, thereby protecting the PSI reaction center (Gandhi et al., 2021).

4.3. Ca-NPs application reduces ROS accumulation and upregulates the proline, TSP and antioxidative defense system of Brassica plants

Environmental stressful conditions including drought causes significant increase in cellular ROS production, which leads to the membrane lipid peroxidation and degradation of antioxidant enzymes, ultimately causing genetic changes. The ROS generation occurs frequently under stressful conditions, regardless of plant species. According to our findings, the drought treated *B. napus* plants produced significantly more ROS (Fig. 5). ROS generation has been considered as the fundamental symptom of phytotoxicity, and this process has been extensively studied in plants under abiotic stress (Rejeb, 2015; Banerjee and Roychoudhury, 2018; Sharma et al., 2019). The application of Ca-NPs under drought stress resulted in a significant increase in cellular antioxidative enzymes potential and osmo-protectants in terms of SOD, POD, CAT, and APX activities and (TSP and proline content), as well as a decrease in ROS levels in plants, implying that oxidative stress is alleviated (Fig. 6). Increased ROS generation and membrane lipid peroxidation are accompanied by increased lypoxygenase and protease activity, which alters lipid and protein content and may lead to structural and functional changes in key macromolecules such as proteins and fatty acids (Khanna-Chopra, 2012; Ahmad and Tahir, 2016; Ahanger et al., 2016). Several studies have found that application of Ca-NPs under stressful conditions improves antioxidative defense and lower the ROS accumulation in plants (Ditta and Arshad, 2016). Similarly, López-Moreno et al. (2018) found that exogenous calcium nanoparticle treatment significantly boosted antioxidative defense in plants exposed to abiotic stress. Furthermore, some previous studies have also shown that calcium nanoparticles treatment resulted in an increase in antioxidant enzyme activities as well as low $OH^{\cdot}$ radicals, superoxide ($O^{\bullet-}$), H_2O_2, and MDA concentrations in plants (Anantharaman et al., 2016; Shukla et al., 2019; Azeez et al., 2020; Mittal et al., 2020; Thirugnanasambandan, 2021). Antioxidant enzymes are important for scavenging superoxide ions and the prevention of lipid peroxidation.Ca-NPs significantly boosted SOD activity under drought stress when compared to the control group (Fig. 6). This shows that Ca-NPs treated plants performed better in terms of reducing superoxide ion-induced oxidative stress, which leads to lipid peroxidation and membrane breakdown. These findings support with previous research that found increased SOD activity in lettuce and zucchini (Anantharaman et al., 2016; Meier et al., 2020). Ca-NPs treatment of drought-stressed plants increased POD activity significantly more than control plants (Fig. 6). Increased POD activity in plants, according to these results, allows them to tolerate drought stress. These findings are comparable to those of prior investigations on drought-stressed wheat plants (Kirova et al., 2021). During drought stress Ca-NPs-treated plants had higher constitutive levels of CAT and APX than control plants (Fig. 6). Increased CAT and APX activity, as well as TSP and proline levels, indicating that the plants are more drought tolerant (Ramli et al., 2019). Previous research has shown that CAT and APX activities in agricultural plants increased the onset to calcium oxide nanoparticles under stressful conditions (Rane et al., 2015; Anantharaman et al., 2016). Overall, the considerable increase in antioxidant enzyme efficacy during drought stress may be attributed to tolerance mechanisms based on fine-tuned control of its redox state.

4.4. Ca-NPs mediated upregulation of endogenous phytohormones under drought stress

Present study investigated the expression of endogenous hormones (ABA, JA and SA) and their putative role in the onset of physiological responses to Ca-NPs and drought stress (Fig. 7). Previous studies provide ample evidence that ABA play a role in plant growth and adaptation to drought stress (Kumar et al., 2019; Li et al., 2020; Jogawat et al., 2021). In this study, the ABA level in *Brassica* plants treated with Ca-NPs increased significantly more than in control plants (Fig. 7A). Our findings correspond to previous studies (Chen, 2018; Almutairi, 2019; Linh et al., 2020; Mustafa et al., 2021). However, in current study the exogenous application of Ca-NPs mediates the upregulation of endogenous phytohormones levels with and without drought stress (Fig. 7A). As in case of zinc and copper nanoparticles drought treatment to wheat plants appears to mediate endogenous ABA production and translocation into the neighboring and far neighboring tissues (Taran et al., 2017). Our results indicated that, in addition to ABA the JA and SA might also play a role in reducing oxidative stress damage in *Brassica* seedlings exposed to drought (Fig. 7B). JA has been shown to either play a role in the regulation of stress responses in plants, directly or indirectly (Abeed et al., 2021). Under drought stress, Ca-NPs treatment appears to increase JA biosynthesis (Fig. 7B). A number of studies showed the key role of JA in plants response to nanoparticles titanium oxide treatment under drought (Karamian et al., 2020). Our results are in line with the previous studies, indicating that wheat plants develop drought tolerance to copper and silver nanoparticles under drought stress (Ahmed et al., 2021). Similarly, Naseer et al. (2021)found that metal nanoparticles and drought treatments significantly enhanced the expression of cellular JA biosynthesis related genes and the jasmonate ZIM-domain (JAZ). In the absence of JA the JAZ proteins supposed to bind to and regulate the activity of various transcription factors including MYC2. However, JA shows robust increase under stressful conditions which is supposed to upregulate the active transcription factors and stress responsive genes expression (Ali et al., 2017). Our results also showed that Ca-NPs application to plants resulted in a significant increase in endogenous SA levels under drought stress (Fig. 7C). Under drought stress, sunflower seedlings treated with titanium dioxide nanoparticles showed a similar additive response (Khattak et al., 2021). Similarly, Naseer et al. (2021) demonstrated the potential role of metal nanoparticles counterpart in SA production as well as the physiological impact under drought stress. According to Kumaraswamy et al. (2019), the chitosan nanoparticles application to plants causes a significant increase in SA biosynthesis and enters cellular translocation. Entering the cellular translocation of neutral SA becomes more favorable in the acidic cell wall and more negative charge in cytoplasm, leading an “ion trap” uptake mechanism of translocation in plant cell (Ilyas et al., 2021). SA mediated improved plant growth in the presence of Ca-NPs and drought treatment is supposed to involve non-expresser of NPR1 gene expression and signaling (Shaukat et al., 2022). Higher SA concentrations reduce the environment causing the conversion and translocation of NPR1 multimeric unit to the nucleus (Gorni et al., 2020). NPR1 binds SA inside the nucleus, causing a conformational shift that frees its C-terminal activation domain from inhibition, allowing transcription to occur. Endogenous SA level appear to increase in plants exposed to iron nanoparticles and drought treatments, with cellular SA level in strawberries roots increasing fivefold and twice respectively (Havas and Ghaderi, 2018). Similarly, Ilyas et al. (2021) discovered a significant association between Ca^{2+} supplementation and SA content generation in plants under drought stress.

4.5. Ca-NPs potentially increased the plants mineral status including Ca^{2+} content in plant tissue

Proper nutrition and water availability are considered key proper and suitable crop management strategies. Improved nutrient absorption

and utilization potential can help to mitigate the drastic effects of drought. Because of nutrient limitation, the consequences of plant metabolism and development are altered. Even if enough nutrients are available, drought makes it very difficult for plants to acquire minerals from root to shoot (Pandey, 2018). Drought significantly reduce the ionic status of plants as compared to control indicates the restriction in nutrients uptake under stressful conditions due to robust decline in transpiration rate, decreased transpiration rate and low membrane permeability (Hu and Schmidhalter, 2005; Ahanger et al., 2014; Hasanuzzaman et al., 2018; Nawaz et al., 2020). In current study drought, lead a considerable reduction in macro and micro minerals uptake in rapeseed plants including Ca, K, Mg, Mn, B, Fe, Cu and Zn compared to normal conditions, respectively (Fig. S2). Shahriari (2021) observed that drought stress caused significant reduction in plant growth as well as macro-minerals including the K, Mg, P, N, and Ca and micro-minerals Mn, Cu, and Fe. However, the application of Ca-NPs to rapeseed improved plants growth and helped for better utilization of mineral elements under drought stress (Fig. S2). Our finding are in accordance with previous studies on the onset of zinc oxide nanoparticles and drought stress in cucumber (Ghani et al., 2022), and sorghum (Dimkpa et al., 2019). Several studies reported that Ca^{2+} ions modulates the cellular signaling and response and play a vital role in increasing plant tolerance under stressful conditions (Azeez et al., 2020). Experiments reveal that application of CaO nanoparticles play a key role in signaling processes that reduces the effects by increasing the mineral uptake and ion homoeostasis in plants (Carmona et al., 2020; Ramírez-Rodríguez et al., 2020).

4.6. Ca-NPs mediates the secondary metabolites expression in Brassica seedlings

Drought stress resulted in significant biosynthesis of secondary metabolites in terms of flavonoids, terpenoids, coumarins and derivatives, alkaloids and derivatives, quinone, phenylproponoids and lignin in *Brassica* seedlings (Fig. S3). Our findings are in line with previous studies, suggesting that the increased biosynthesis of secondary metabolites imparts in plant growth tolerance under drought (Marchiosi et al., 2020; Ackah et al., 2021). However, a few studies have focused on the influence of exogenously applied Ca-NPs on secondary metabolites expression onset to drought. Recent study highlighted the effect of Ca-NPs application on plants oxidative damage, membrane lipid peroxidation, and membrane stability in drought stressed *Brassica* seedlings. Nanomaterial application promotes secondary metabolites biosynthesis and mediates the decline in induced oxidative stress as a result of cumulative impact on flavonoids, terpenoids in terms of festetidin, sertraline and L-tryptophan production, as well as antioxidant enzymes activities (Verma and Shukla, 2015; Ashraf et al., 2018; Jan et al., 2021; Yadav et al., 2021). According to our data (Fig. 6E and F) the protease and lipoxygenase activities were increased under drought stress, but significantly reduced after Ca-NPs treatment indicating that Ca-NPs plays a positive role in proteins and lipids protection in *Brassica* seedlings exposed to such stressful conditions. It was hypothesized enhanced secondary metabolites accumulation in Ca-NPs and drought was associated to counter ROS triggered damage (Fig. S3). Our results are similar with previous studies on *Stevia rebaudiana* (Javed et al., 2018) and *Zatariamultiflora* (Mosavat et al., 2019), which found that ZnO and CuO nanoparticles increased secondary metabolites expression in terms of bisdemethoxycurcumin, methoxy 4- methyl coumarins, cis-4-hydroxy-D-proline, germacrone and N-proionyle glycine (Lala, 2021). According to (Khalil et al., 2019) increased ROS scavenging potential in plants with Ca-NPs application concomitant with enhanced secondary metabolites expression. Although more research is needed to investigate the exact mechanism, overexpression of secondary metabolites caused by calcium nanomaterial improves drought-interceded decrease in plant growth and photosynthesis.

4.7. Ca-NPs regulates the ultra-structure modulation in Brassica seedlings exposed to drought stress

The examination of ultrastructure changes in plant leaf cells under drought is a highly valuable tool for exploring and understanding the process involved in conferring drought resistance. Our findings suggested that at ultrastructural level, under control conditions the cellular organelles such as chloroplasts usually had normal oblong shape along with distinctive stroma, and thylakoid membrane structures (Fig. 8A). However, under drought stress the plant leaves showed a few number of grana, along with swollen and vesiculated thylakoid membranes. Because chloroplasts are a primary source of reactive oxygen species (ROS), they are vulnerable to oxidative stress (Fig. 8B). Brassica seedlings supplemented with Ca-NPs responded better and maintained cellular integrity under drought stress. The enhanced antioxidative potential of Ca-NPs in *Brassica* seedlings under stressful conditions may be a response to reduce the ROS overproduction in the chloroplasts. Exogenous application of zinc oxide nanoparticles mediates drought stress regulation in eggplants by modulating mesophyll ultrastructure and antioxidant defense system (Semida et al., 2021). Iron nanoparticles effectively improve wheat plants growth by enhanced photosynthesis and protecting the ultrastructure of mesophyll cells from drought stress (Adrees et al., 2020). Sun et al. (2020) suggested that the zinc oxide nanoparticles and melatonin supplementation promotes maize seedlings growth by mesophyll ultrastructure modification, increasing cellular metabolism and antioxidant potential under drought stress. However, a few studies have explored the involved mechanism and plant response to stressful conditions. Although, our results indicated that ultrastructural changes in the mesophyll cells of *Brassica* seedlings cause minimum cellular damage when exposed to Ca-NPs and drought stress.

5. Conclusion

Present study investigated the potential role of Ca-NPs in the mitigation of drought stress induced oxidative damage in the *B. napus* seedlings. Drought significantly reduced the plants growth by affecting the water status and photosynthesis activity through enhanced ROS generation. According to our findings, Ca-NPs treatment considerably improved the plant growth and physiological or biochemical processes in terms of maintaining RWC content, proline accumulation, photosynthetic performance, photosystem II efficacy, chlorophyll content, antioxidative enzymes activities along with protease and lipoxygenase activities under drought stress. In addition, manage over excitation of PSII by heat dissipation safely through photo-protective component of NPQ, regulate the electron transport through PSII and PSI, as well as enhanced activity of osmolytes, mineral status, secondary metabolites expression and mesophyll ultrastructure onset to Ca-NPs supplementation unveil its positive role in declining drought mediated ROS accumulation in *Brassica* seedlings.

Credit author statement

Ayyaz A, Farooq MA, and Zhou W conceived the idea and designed the experiment, Ayyaz A performed the experimental work, Fang R, Ma J, Hannan F, and Huang Q collected the data and did the data analysis, Ayyaz A, Athar H.R, and Ali S wrote, review & edited the first draft of the manuscript. Javed M, and Sun Y revised the first draft, and Zhou W further corrected and improved the manuscript.

Declaration of Competing Interest

Authors declared there is no conflict of interest.

Data availability

Data will be made available on request.

Acknowledgments

This work was supported by the Science and Technology Department of Zhejiang Province (2021C02064-2), the National Natural Science Foundation of China (31950410554), Collaborative Innovation Center for Modern Crop Production co-sponsored by Province and Ministry (CIC-MCP), and the Agriculture and Rural Affairs Department of Zhejiang Province (2021XTTGLY0202).We thank Rui Sun and Weizhen Hu from Agricultural Experiment Station of Zhejiang University for their assistance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.impact.2022.100423.

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