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Mitigating Cd stress in alfalfa: the role of melatonin and nano-calcium oxide in enhancing photosynthesis and antioxidant defense

Maqsood Ul Hussan[1], Umer Mahmood[2], Saeedeh Zarbakhsh[3*], Sadam Hussain[4], Muhammad Bilal Hafeez[5], Noreen Zahra[6], Munib Ahmed[7], Quanzhen Wang[1*] and Mingke Yan[1*]

Abstract

Background Heavy metal contamination, particularly cadmium (Cd), is a significant threat to agricultural productivity and food security. Melatonin, as a key biostimulant, plant growth regulator and stress resistance hormone, along with nano-calcium oxide (nCaO), can enhance plant resilience to stress. However, few studies have investigated the combined effects of melatonin and nCaO in mitigating Cd stress in plants. This study examined the effects of the combined application of melatonin (100 µM) and nCaO (100 mg kg^{-1}) on alfalfa (*Medicago sativa* L.) seedlings.

Results Under Cd stress, alfalfa seedlings exhibited a 43% reduction in shoot length, 50% in root length, and 60% in chlorophyll content, while increased MDA, hydrogen peroxide (H_2O_2) and $O_2^{\bullet-}$ accumulation. However, the combined application of melatonin and nCaO significantly alleviated Cd toxicity, enhancing shoot and root growth by 46% and 49%, respectively. Photosynthetic efficiency improved by 70%, while chlorophyll content and Fv/Fm ratio increased by 68% and 81%, respectively. This treatment also reduced Cd accumulation in roots and shoots by 47% and 75%, while increasing calcium uptake by 84% in roots and 63% in shoots. Antioxidant enzyme activities (SOD, POD, CAT, and APX) were upregulated by 59%, 42%, 62%, and 49%, respectively, mitigating oxidative damage. The effect of combined treatment on ultrastructural analysis revealed restored chloroplast integrity and stomatal functionality under Cd stress.

Conclusion The combined application of melatonin + nano calcium oxide shows great promise for ecologically acceptable alleviation of Cd stress in alfalfa by lowering its absorption.

Keywords Calcium bioaccumulation, Gene expression, Reactive oxygen species, Stomatal conductance, Transcriptomic analysis, Ultrastructural damage

*Correspondence:
Saeedeh Zarbakhsh
saeedeh.zarbakhsh@yahoo.com
Quanzhen Wang
wangquanzhen191@163.com
Mingke Yan
yanmk@nwafu.edu.cn

Full list of author information is available at the end of the article

Introduction

The global community faces significant challenges, including ensuring food security for a growing population and addressing soil pollution caused by toxic trace elements [1–3]. Heavy metals (HMs), particularly cadmium (Cd), create severe threats to ecosystems and human health due to their environmental persistence and ability to accumulate in the food chain [4, 5]. Cd, a non-essential and highly toxic element, does not degrade naturally in soil which lead to long-term environmental contamination [6]. Its accumulation in agricultural soils is often exacerbated by the use of contaminated irrigation water, further intensifying the risk of Cd uptake by crops [7]. Even at low concentrations, Cd toxicity adversely affects plant growth and physiological processes by disrupting photosynthesis, impairing biochemical activities, and altering cellular ultrastructure [8, 9]. Cd is absorbed by plant roots, transported via the xylem, and distributed throughout plant tissues, where it disrupts cellular redox homeostasis and generates reactive oxygen species (ROS). This oxidative stress damages lipid membranes and organelles, leading to cellular dysfunction [10, 11]. Moreover, Cd bioaccumulation in crops poses serious health risks to humans, including lung, skin, and kidney cancers, as well as cardiovascular and neurological disorders [12]. Plants have evolved various strategies to combat metal stress through either "avoidance" (limiting metal absorption) or "tolerance" (managing elevated internal levels) [13]. One such approach is the antioxidant defense system, which comprises both non-enzymatic and enzymatic antioxidants that help maintain redox balance by scavenging ROS. Superoxide dismutase (SOD) plays an essential role in converting produced singlet oxygen (O_2^-) into H_2O_2, while other enzymes such as peroxidase (POD) and catalase (CAT) further reduce H_2O_2 to water and oxygen molecules. This defense mechanism helps to reduce the accumulation of ROS and improve tolerance to oxidative damage [14].

Physiochemical and agronomic strategies for remediating HM-contaminated soils often encounter limitations, including high costs, ecological risks, and adverse impacts on soil microbial communities [15, 16]. Consequently, innovative and sustainable approaches are needed to mitigate Cd toxicity and ensure food safety [17]. Recent studies have highlighted the potential of essential nutrients, such as calcium (Ca), zinc (Zn), and iron (Fe), to alleviate Cd stress due to their chemical similarity and competitive interactions with Cd in soil-plant systems [18, 19]. Calcium, a vital structural component of cell walls and membranes, functions in maintaining cellular integrity and signaling under stress conditions [20]. It functions as a secondary messenger in various metabolic pathways and developmental processes in response to abiotic stressors [21]. Calcium deficiency has been linked with reduced antioxidant capacity, impaired photosynthetic efficiency, and compromised plant growth in several species, including poplar and peach [22, 23].

The advent of nanotechnology has opened new avenues for addressing agricultural challenges, including HM stress. Nanoparticles (NPs) of essential nutrients, such as calcium oxide (CaO-NPs), have shown promise in enhancing plant growth and antioxidant defense systems under various biotic and abiotic stresses, including salinity [24], drought and heat [25, 26], arsenic contamination [27], lead toxicity [28], and fungal pathogens such as *Botrytis cinerea* [29]. For instance, CaO-NPs application in barley has been shown to improve growth, photosynthetic activity, and antioxidant defense under Cd stress [30]. Similarly, CaO-NPs combined with organic amendments have effectively mitigated Cd toxicity in mung bean (*Vigna radiata* L.) by enhancing physiological and biochemical traits [31]. Furthermore, synergistic interactions between Ca-NPs and phytohormones, such as abscisic acid, have been reported to improve drought tolerance by promoting nutrient uptake, photosynthetic efficiency, and stress-responsive protein expression [32].

Melatonin (MT), a key biostimulant, plays a crucial role in enhancing plant resilience to environmental stresses, including extreme temperatures, salinity, UV radiation, drought, and HM toxicity [33]. It protects plants from oxidative damage by promoting growth, enhancing photosynthetic efficiency and chlorophyll content, and reducing ROS generation [34]. The exogenous application of melatonin has been linked to enhanced antioxidant capacity, activation of stress-related enzymes, regulation of polyamine metabolism, and improved ROS scavenging in plants exposed to Cd stress [35, 36]. Studies on alfalfa have demonstrated that melatonin application under salt stress [37] and drought stress [38] has been shown to improve photosynthetic pigments, stomatal conductance, nutrient uptake, and antioxidant enzyme activity, thereby mitigating ROS accumulation and lipid peroxidation.

Alfalfa (*Medicago sativa* L.), a widely cultivated forage crop, is particularly vulnerable to Cd stress. As a high-protein fodder crop, it plays a critical role in livestock nutrition and soil conservation, especially in northern China. Its ability to fix atmospheric nitrogen through symbiotic association with *Rhizobia* spp. further enhances its agricultural value [39, 40]. However, Cd contamination reduces alfalfa's growth and nutritional quality while raising toxic metal accumulation in the food chain, posing risks to both animal and human health [41]. Given its agricultural importance and sensitivity to Cd, alfalfa serves as an ideal candidate for investigating the protective role of soil-applied nCaO and exogenous MT in response to Cd stress.

The agricultural sector has increasingly recognized the advantages of using combined amendments over single

treatments for remediating soil pollution. For instance, the simultaneous application of zinc oxide nanoparticles (ZnO-NPs) and melatonin in wheat under Cd stress significantly improved growth, chlorophyll content, Zn concentration, and yield while reducing Cd accumulation in grains [42]. Similarly, the combined use of melatonin and silicon nanoparticles (Si-NPs) in *Brassica oleracea* significantly enhanced growth, photosynthetic pigments, antioxidant activity and osmotic regulation under salinity stress [43]. Nevertheless, the combined application of nCaO and MT to mitigate Cd toxicity in alfalfa seedlings has received limited attention. This study aims to explore the interactive effects of soil applied nCaO and exogenous melatonin on Cd detoxification in alfalfa. It focuses on evaluating morphological, physiological, and ultrastructural changes, assessing the expression of antioxidant enzyme-related genes, and analyzing the impact of nCaO on Cd availability in alfalfa seedlings.

Materials and methods

Preparation of soil amendments and analyse

The soil utilized for the experimental study was procured from three different spots in research fields at depth of (0–20 cm) from Northwest Agriculture and Forestry University Yangling (34°20′ N, 108°24′ E, and 466.7 m elevation), China. The collected soil was initially air dried in shade and all objectionable debris material like root and plant biomass were removed to make soil homogenous. After that homogenized soil was sieved with a 2 mm nylon sieve and transport to lab for pot filling and further analysis. The soil was analyzed for key soil properties at the beginning, such as soil texture [44], pH and electrical conductivity (EC) [45], as well as organic matter contents and total metal concentration was evaluated by already detail procedure of [46], and [47], respectively. The soil physiochemical characteristics are listed in Table S1. Additional details on nCaO characteristics are available in [48].

Experimental design and growth condition of plants

A pot experiment was planned and conducted in small growth chamber of plant protection department. The uncontaminated soil was spiked with cadmium sulphate ($CdSO_4$) at 30 mg kg^{-1} and placed for 4 weeks prior to sowing at 70% field capacity for treatment stabilization. The Cd-spiked soil was mixed with prepared soil amendment nCaO (100 mg kg^{-1}) in plastic pots having (top dimension 24 cm, bottom dimension 20 cm and height 30 cm) containing capacity of 4 kg soil. The dosage for nCaO (100 mg kg^{-1}) and MT (100 µM) were determined based on previous reports [37, 48]. Each treatment was replicated three times in the experimental setup.

The surface sterilized seeds of alfalfa variety (Sanditi) were used for sowing of 24 pots, provided by Beijing Rytway Ecotechnology Co., LTD. The sterile seeds were evenly dispersed on moist filter paper, subsequently placed in petri dish and cultured at 25 ± 1 °C for 16 h light and 8 h dark period for 5 days. Once the seedlings achieved cotyledon stage with taproots of 4–6 cm long, ten healthier seedlings were shifted to each pot. The plants were grown for a period of 60 days in a small growth chamber with a humidity level of 60%, a photoperiod of 14/10 hours (day/night) with photon flux density (700–800 µmol m^{-1} s^{-1}), and a temperature of 25 °C. Seedlings were fertilized twice to thrice a week with ½ strength Hoagland solution without (Ca $(NO_3)_2.4H_2O$) and watered to field capacity.

Quantification of plant and soil Cd contents with morphological characteristics

Plants were separated into roots and shoots after harvesting and subsequently cleansed with tap water to eliminate the adherent dust particles. The plant specimens were firstly allowed to air dried and later on oven dried at 70 °C until constant dry weight. After that, take 1 g precise samples of both shoots and roots in open flask digestion system and were digested using a mixture of nitric acid (HNO_3) and perchloric acid ($HClO_4$) in a 2:1 volume ratio as described by [49]. The cadmium concentrations in the digested samples of shoots and roots were accurately measured using Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Furthermore, plant growth attributes including shoot length (SL), and root length (RL) in centimeters, shoot and root fresh weight (SFW and RFW) in grams as well as shoot and root dry weight (SDW and RDW) in grams were measured.

Chlorophyll contents and photosynthetic attributes with ultrastructural observation

This study employed comprehensive methodologies for the evaluation of photosynthetic attributes, including net photosynthetic rate (Pn), stomatal conductance (gs), intercellular CO_2 concentration (Ci), and transpiration rate (Tr). These traits were recorded using a portable gas exchange photosynthesis system (Li-Cor 6800XT, Lincoln, NE, USA). Measurements were conducted on the second fully expanded leaf from each plant during the day, from 9:00 to 11:00, in full sunlight adhering to the methodology advocated by [50]. Subsequently, the relative chlorophyll content of the same leaf was determined with a spectrophotometer. Fresh 0.1 g leaf samples were soaked in acetone (85%) for 72 h and centrifugation at 5000 g for 12 min and after taking resulting solution the chlorophyll contents were measured at 452.5 and 644 nm using a spectrophotometer (UV-2600) and computed according to the previous studies [51]. Furthermore, the procedure was followed by [52] to process and determine the cortical cells (2–3 mm) from leaf mesophyll cells

under the high-resolution scanning electron microscopy (SEM) and transmission electron microscopy (TEM) in order to analyze stomatal aperture, guard cells, and intracellular structure in plant tissues. Detailed protocols for the assessment of chlorophyll fluorescence (Fv/Fm), are delineated in the supplementary material.

Assay of enzymatic antioxidant in alfalfa tissues

For the enzyme activity assays, fresh shoot samples weighing 0.3 g were homogenized using pre-cooled mortar and pestles in phosphate buffer (pH 7.8). The homogenate's total volume was adjusted to 8 mL with the same buffer, followed by centrifugation at 10,000 rpm for 15 min at 4 °C. The supernatant obtained, which contains the enzyme extract, was used to determine the activities of the aforementioned antioxidant enzymes utilizing a UV-Spectrophotometer (Model UV-2600). The (SOD) extraction required 50 mM phosphate buffer at pH = 7.8 and 1.33 mM diethyl-diaminopenta-acetic acid. The SOD activity was measured by measuring the O_2-induced reduction of nitroblue tetrazolium using xanthine-xanthine oxidase system. The spectrophotometric measurement was observed 560 nm.The POD were quantified following the methods detailed by [53], employing wavelengths of 470 nm, with the analysis conducted over a period of 60 s. For catalase determination, a mixture of reactants was produced with 50 mM K_3PO_4 buffer at pH 7.0 and 100 µL enzyme extract. After that, 10 mM hydrogen peroxide was added to initiate the reaction, followed by spectrophotometer absorbance measurement at 240 nm within a 30-second timeframe, whereas for the ascorbate peroxidase experiment, a reaction mixture was formulated including 50 mM K_3PO_4 buffer at pH 7.0, 100 µL of enzyme extract, 0.1 mM H_2O_2, and 0.1 mM ascorbate, with absorbance measured at 290 nm using a spectrophotometer [52].

Quantitative analysis of malondialdehyde (MDA), hydrogen peroxide (H_2O_2), electrolyte leakage (EL) and superoxide anion ($O_2^{\bullet-}$), and histochemical staining by NBT and DAB

The supplementary material provides comprehensive methodologies for quantifying oxidative stress indicators such as malondialdehyde (MDA), hydrogen peroxide (H_2O_2), electrolyte leakage (EL) and superoxide anion ($O_2^{\bullet-}$). Furthermore, the quantities of H_2O_2 and $O_2^{\bullet-}$ in the leaves of alfalfa seedlings were differentiated, as per [54], by employing 3,3-diaminobenzidine (DAB) and nitro blue tetrazolium (NBT) for staining, respectively. In detail the photochemical reduction approach with p-nitro blue tetrazolium chloride was employed to stain superoxide radicals ($O_2^{\cdot-}$). The NBT solution was made by dissolving 0.1 g of nitro blue tetrazolium (NBT) in 50 mM sodium phosphate buffer to achieve a final volume of 50 mL, thereafter mixed using a magnetic stirrer. Simultaneously, the DAB solution was prepared by dissolving 50 mg of DAB in 45 mL of distilled water, followed by pH adjustment to 3.8. The leaf sample was subsequently immersed overnight in DAB and NBT staining solution to identify H_2O_2 and $O_2^{\cdot-}$, while preventing light penetration into the sample. After that, solution was subsequently drained, and the sample was boiled with absolute ethanol to eliminate chlorophyll for optimal stain visualization. Subsequently, the leaf samples were transferred to a paper towel containing 60% glycerol for imaging purposes. H_2O_2 was observed as brownish spots by the DAB reaction, whereas $O_2^{\cdot-}$ was identified as dark blue spots, reflecting the outcomes of the NBT reaction.

Statistical analysis methodology

This experiment was carried out in a completely randomized design (CRD) with factorial arrangement. The data was analyzed by Variance Analysis utilizing SPSS 20 software. Results are presented as the mean ± standard error (SE) based on a minimum of three replicated experiments. Statistical significance was evaluated through ANOVA, followed by least significant difference (LSD) test. A probability (p-value) of ≤ 0.05 was considered to indicate statistical significance. For visual representation, figures and graphs were crafted using OriginPro version 10.0.5 (Origin Lab Corporation, USA, 1991–2023), effectively illustrating the data patterns and results.

Results

Effects of melatonin and nCaO on growth indices of alfalfa

The current study sought to examine the combined effect of MT and nCaO on the growth of alfalfa seedlings subjected to cadmium stress. A variety of growth attributes were monitored including (SL and RL), (SFW and RFW) and (SDW and RDW) (Fig. 1). In comparison to control (Con) plants, alfalfa seedlings exposed to Cd stress exhibited a substantial decrease in SL (43%), RL (50%), SWF (40%), RFW (65%), SDW (47%), and RDW (112%). However, the combination of MT + nCaO under Cd stress effectively ameliorates the adverse effect of Cd and considerably enhances the shoot length, and fresh and dry biomass. Notably, the maximum increase was observed with the application of MT (100 µM) in conjunction with nCaO (100 mg kg^{-1}) under Cd stress, resulting in marked increase of SL, RL, SFW, RFW, SDW, and RDW by 46%, 49%, 42%, 55%, 43%, and 104%, respectively (Fig. 1) in contrast with respective Cd treatment. Combined application of MT + nCaO revealed ($p \leq 0.05$) the highest increase in morphological attributes compared to all other treatments without stress and markedly enhanced the SL (18%), RL (20%), SFW (25%), RFW (15%), SDW (40%) and RDW (35%) over con seedlings.

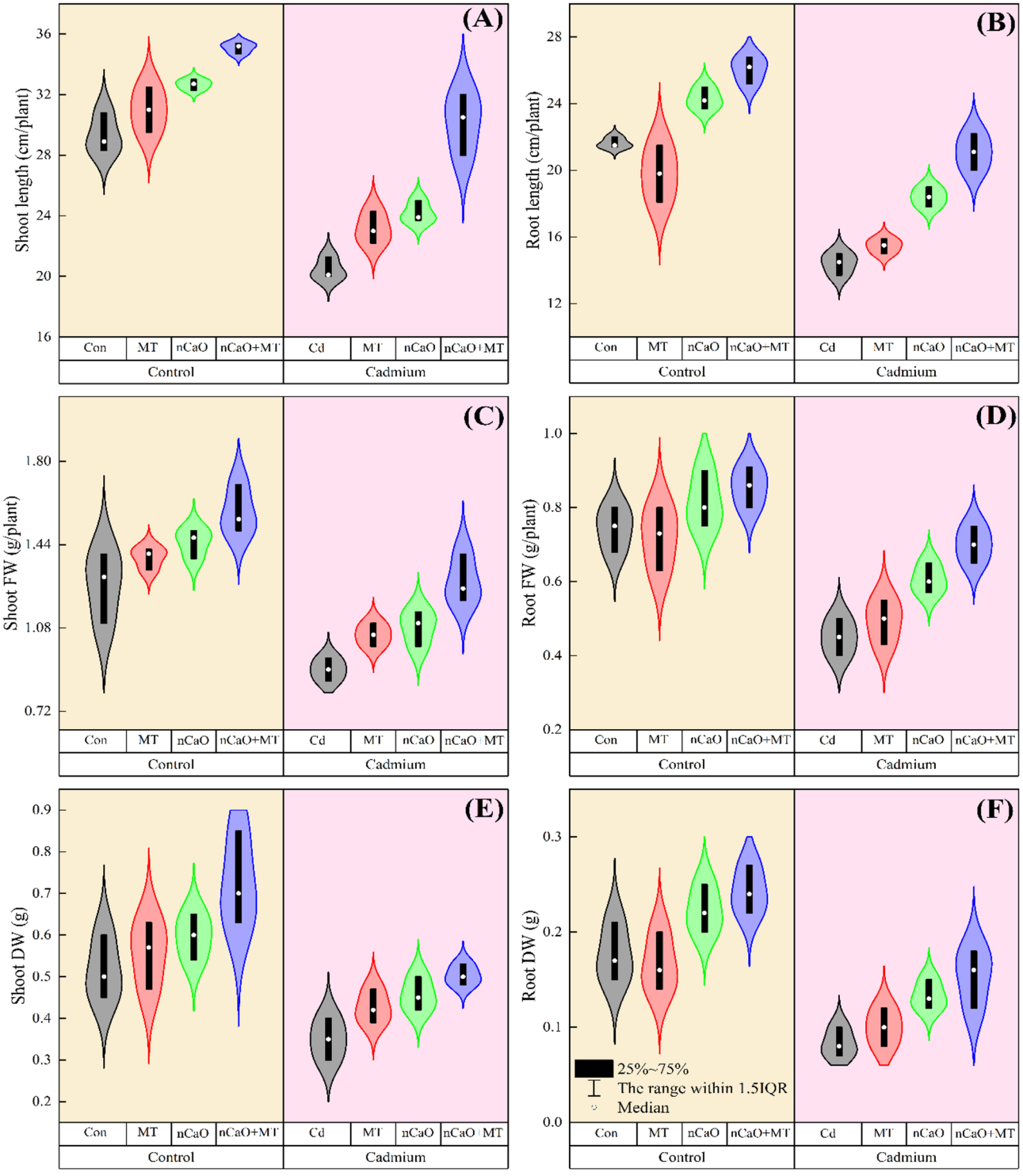

Fig. 1 Alfalfa physio-morphological traits in control (Con) and cadmium stress conditions as a result of the combined effects of soil nano calcium oxide and exogenous melatonin. **A** shoot length; **B** root length; **C** shoot fresh weight; **D** root fresh weight; **E** shoot dry weight, and **F** and root dry weight. The data are presented as the mean ± standard error of three separate biological replicates

Effects of MT and nCaO on gaseous exchange and chlorophyll contents

The results of various treatments on IRGA traits and chlorophyll contents in alfalfa leaves has been documented in (Fig. 2). In comparison to con seedlings, single application of MT and nCaO has beneficial impact on photosystem II (PSII), gaseous exchange attributes and chlorophyll concentration in leaves of alfalfa plant.

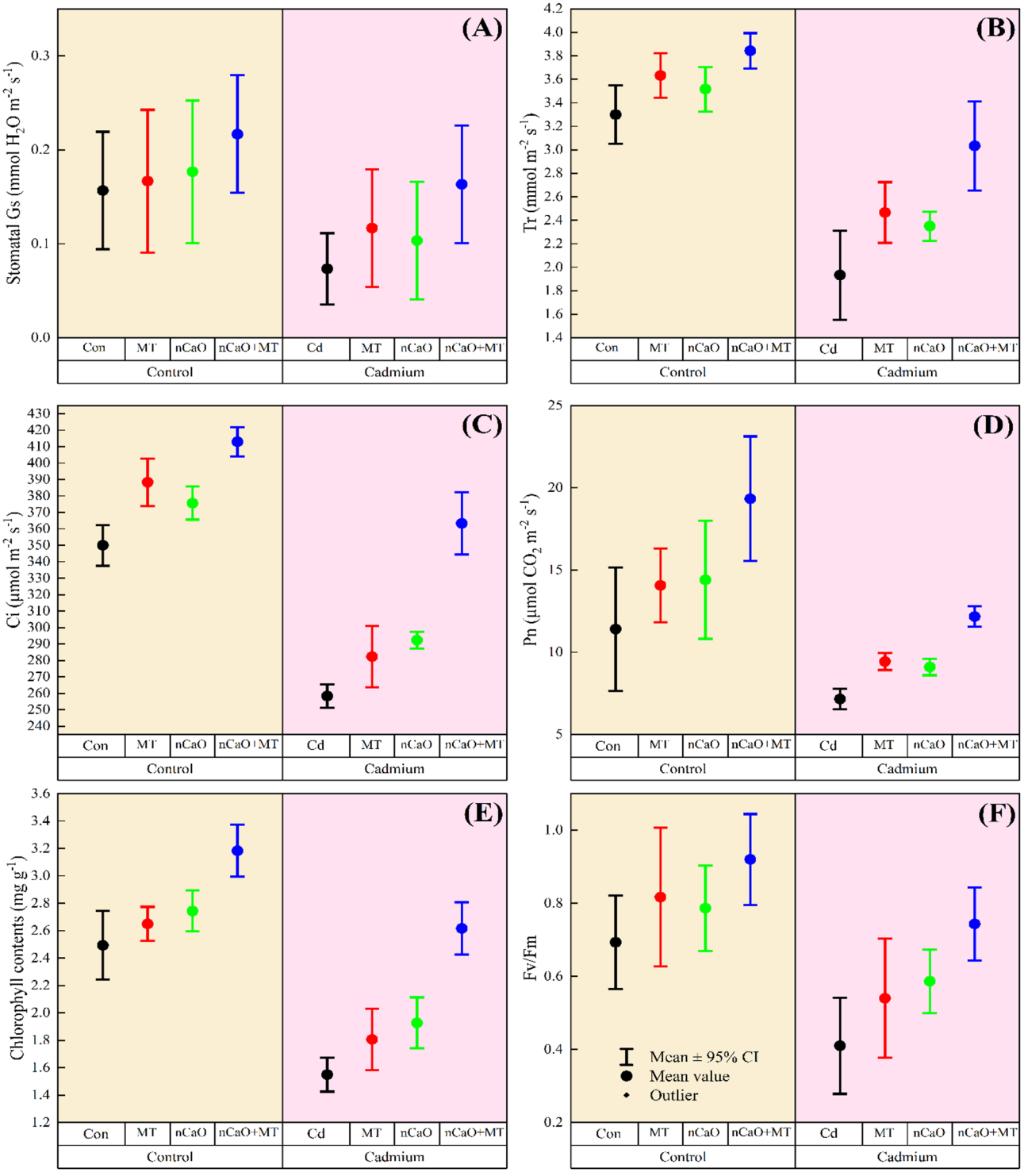

Fig. 2 Attributes of gas exchange in alfalfa under control (Con) and cadmium stress conditions due to the synergistic effects of soil nano calcium oxide and exogenous melatonin. **A** stomatal conductance (Gs); **B** transpiration rate (Tr); **C** intracellular carbon dioxide (Ci); **D** photosynthesis (Pn); **E** chlorophyll contents, and **F** Fv/Fm. The data are presented as the mean ± standard error of three separate biological replicates

The Cd poisoning in this study revealed harmful impact on Pn, Tr, gs, and Ci and decreasing their values by 59%, 70%, 113% and 35%, respectively, when compared to con seedlings grown under non contaminated soil. In alfalfa, incorporation of 100 mg kg^{-1} nCaO alone enhanced the Pn (27%), Tr (21%), gs (40%), and Ci (13%), when compared to their Cd treated control seedlings. This effect was further intensified by foliar application of MT (100

μM) with the same amount of nCaO and increased these traits by 70%, 56%, 122%, and 40% in comparison to their respective control. Furthermore, in terms of chlorophyll contents and PSII activity, Cd toxicity caused a notable decrease by 60% and 69%, respectively, in alfalfa leaves over con treatment (non-contaminated soil). However, introduction of nCaO + MT exhibited the maximum amelioration of Cd poisoning and caused significant increase of chlorophyll contents (68%) and Fv/Fm (81%), over Cd sole treated seedlings. Exogenous treatment of MT at 100 μM alone under Cd stress enhanced the chlorophyll contents and PSII activity (Fv/Fm) by 16% and 31%, respectively, when compared to their respective control. The pseudo-color pictures of alfalfa leaves displayed a shift from dark purplish-blue to light blue/green, indicating a reduction in the Fv/Fm ratio during sole-Cd treatment. The addition of combine nCaO in soil and exogenous application of MT significantly restores leaf coloration (Fig. 3A), indicating that this treatment successfully mitigates the adverse effects of Cd on plants.

Antioxidant defense system and oxidative stress in alfalfa plants with staining

We observed in boosting of defense activity (SOD, POD, CAT and APX) of alfalfa seedlings with combine treatment of nCaO + MT under non contaminated soil. Application of 100 mg kg^{-1} of soil nCaO alone and with MT (100 μM) resulted in substantial changes in the antioxidant enzymatic response (SOD, POD, CAT, and APX), under Cd stress compared to the con. Plants exposed to Cd stress resulted in higher antioxidant enzymatic activity for SOD, POD, CAT and APX by 8%, 24%, 19% and 37%, respectively, compared to con seedlings. Exogenous application of MT (100 μM) alone showed most pronounced effect on antioxidant activity and led to enhancement of SOD (34%), POD (23%), CAT (29%), and APX (56%), compared to Cd treated control seedlings. Soil amendment of nCaO + exogenous MT under Cd stress manifested most remarkable upregulation of antioxidant activity (including, SOD, POD, CAT and APX) by 59%, 42%, 62%, and 49%, respectively, in contrast with respective control (Fig. 4).

In plants, MDA and ROS such as $O_2^{\bullet-}$ and H_2O_2, are function as secondary messengers and exert an impact on physiological processes. Alfalfa seedlings under Cd stress produced a remarkable amount of ROS along with MDA and EL compare to con seedlings. Notably, seedlings subjected to sole Cd stress generate H_2O_2, $O_2^{\bullet-}$, MDA, and EL level that were increased by 41%, 45%, 53% and 59%, respectively, compared to con plants. Exogenous application of MT at 100 μM considerably reduces the content of H_2O_2 (22%), MDA (15%), $O_2^{\bullet-}$ (20%), and El (35%), compared to Cd treated control seedlings. Moreover, combine soil application of nCaO and exogenous MT under Cd stress demonstrated significant effectiveness to alleviate the ROS production (H_2O_2, $O_2^{\bullet-}$) by 37% and 41%, respectively, whereas same treatment under Cd stress also decreases level of MDA (41%) and EL (56%) when compared with control plants (Fig. 5). In addition, the existence of H_2O_2 and $O_2^{\bullet-}$ buildup after sole Cd treatment was further established by histochemical staining of leaves using DAB and NBT (Fig. 6A-B). Alfalfa leaves showed increased amounts of dark blue and brown marks when subjected to sole Cd treatment (Fig. 6A-B). Conversely, the introduction of combine application of nCaO and MT notably reduce the presence of dark brown and dark blue stains in the tissues of alfalfa seedlings exposed to Cd stress. A comparison study revealed a substantial decrease in dark blue and brown spots after nCaO + MT + Cd treatment, underscoring its efficacy in mitigating ROS accumulation.

Content of Ca and Cd in leaves

The treatment group exposed to sole Cd had the greatest levels of Cd in alfalfa seedlings compare to other treatments (Fig. 7B, D). Notably, the soil inclusion of

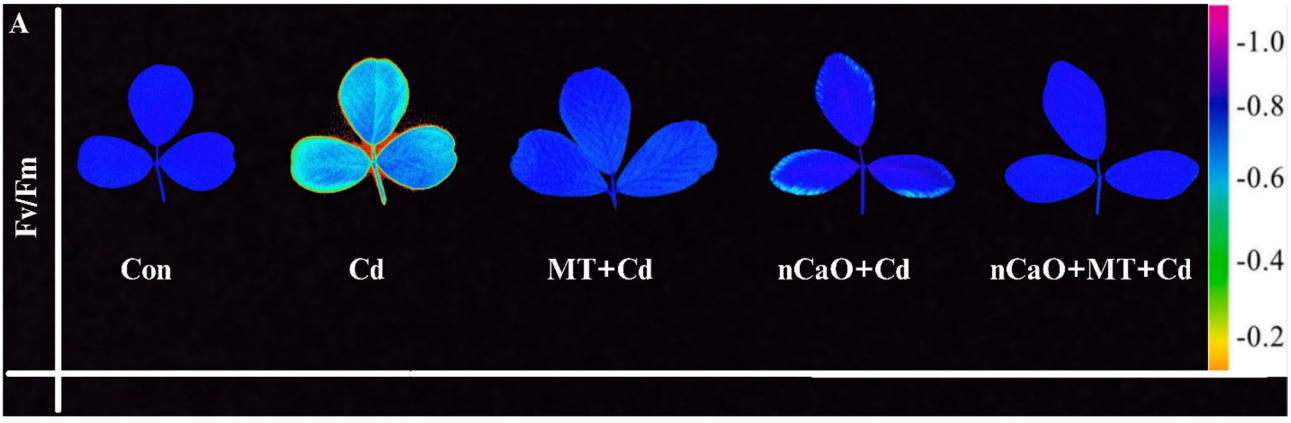

Fig. 3 Pseudo color images of alfalfa represent the fluorescence efficiency (Fv/Fm) under cadmium and control (Con) conditions

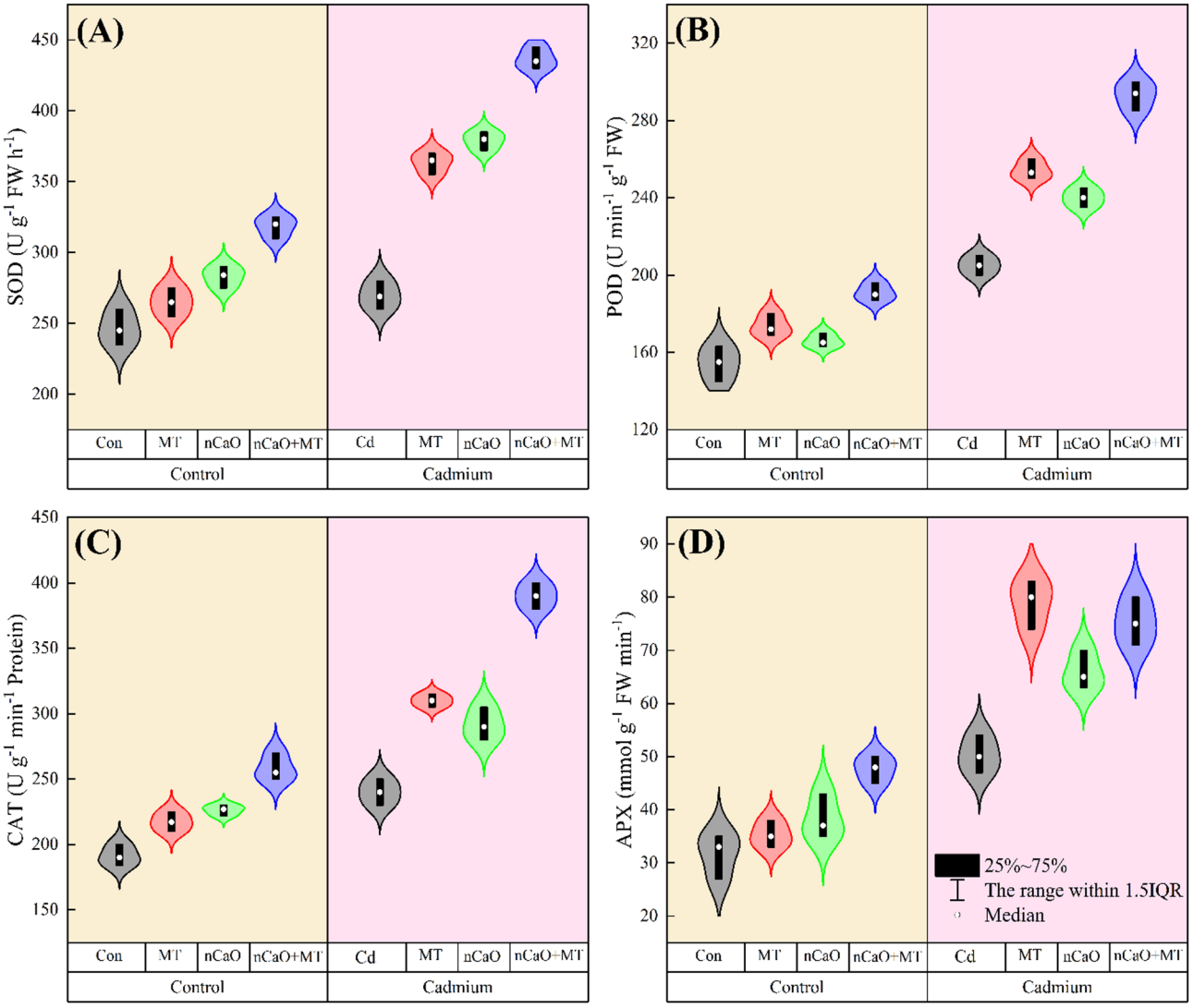

Fig. 4 Effects of the combined application of exogenous melatonin and soil nano calcium oxide on the enzymatic antioxidant activities of alfalfa under both control (Con) and cadmium stress conditions. **A** superoxide dismutase (SOD); **B** peroxidase (POD); **C** catalase (CAT); and **D** ascorbate peroxidase (APX). The data are expressed as the mean ± standard error from three distinct biological replicates

(nCaO) + exogenous (MT) resulted in the most substantial reduction, showing a 47% decline in root Cd concentration and a 75% decrease in shoot Cd concentration compared to the Cd-control treatment (Fig. 7). Single application of MT and nCaO treatment in alfalfa seedlings under Cd stress yielded a reduction of (07% and 19%) as well as (16% and 27%) in root and shoot, respectively, compare to relative control (Fig. 7A, C). In case of Ca content in alfalfa tissues, maximum reduction was observed in Cd treated control seedlings among all other treatments. Cd induced toxicity caused remarkable reduction of Ca concentration in roots and shoots by 65% and 47%, respectively, over control seedlings. Single application of nCaO treatment showed significant escalation in Ca contents in both tissues under contaminated and non-contaminated soil. Highest content of Ca was noted in roots and shoots were observed where combine application of nCaO + MT was done in both contaminated and non-contaminated soil. Notably, the pronounced enhancement of Ca content was observed in roots (84%) and shoots (63%) under combine application of both amendments as compared to Cd treated control group.

Influence of soil applied nCaO and exogenous melatonin on Cd damage on stomata and guard cells

The micrographs of SEM showed that Cd induced toxicity produce detrimental effect on plant stomata, guard cells along with impaired stomatal aperture as observed by notable distortion in stomatal morphology (Fig. 8). Furthermore, the presence of Cd

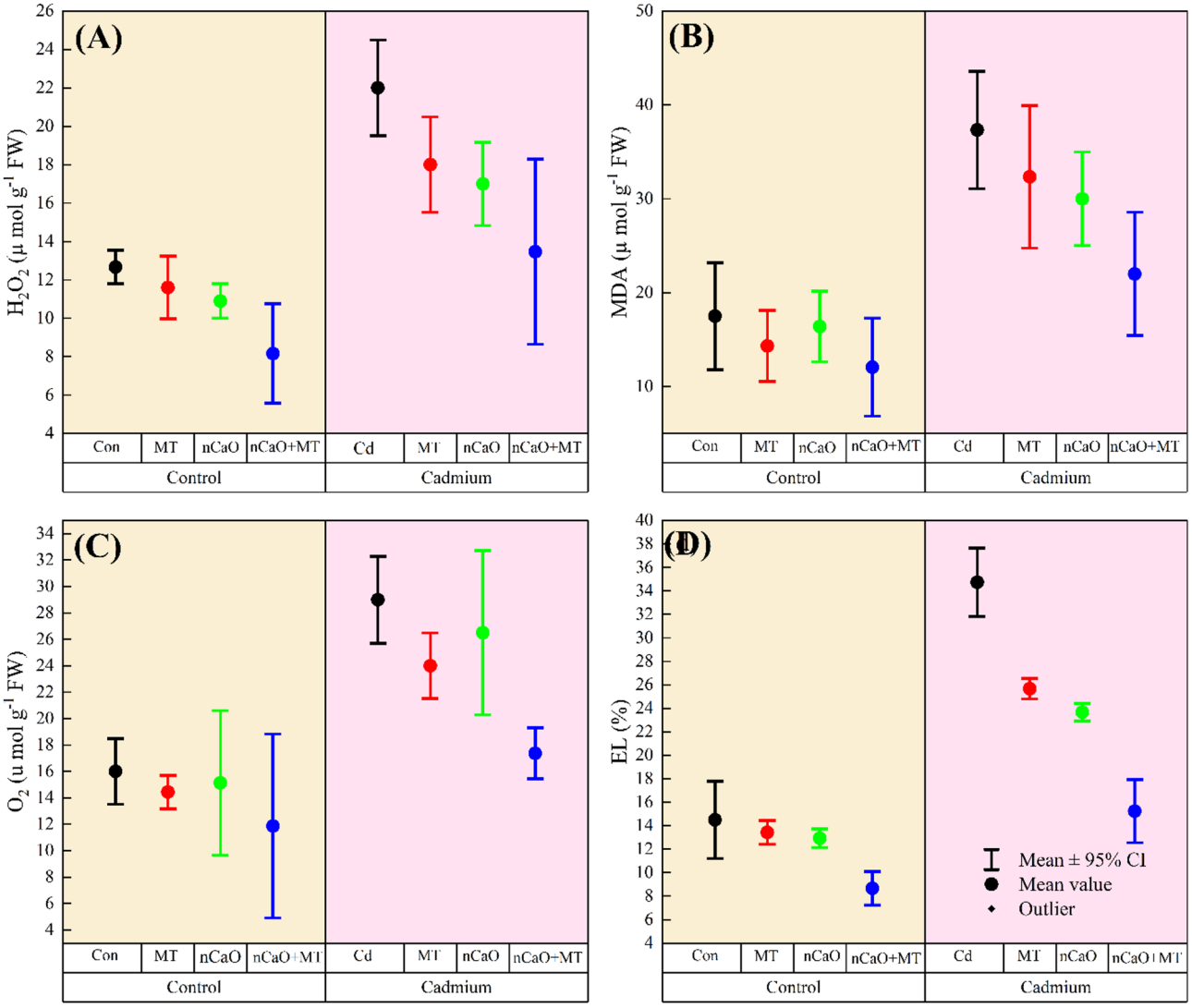

Fig. 5 Alfalfa reactive oxygen species characteristics in control (Con) and cadmium stress conditions as a result of the combined effects of soil nano calcium oxide and exogenous melatonin. **A** Hydrogen peroxide (H_2O_2); **B** malondialdehyde (MDA); **C** singlet oxygen (O^-_2) and **D** electrolyte leakage (EL). The data are presented as the mean ± standard error of three separate biological replicates

stress resulted in plasmolysis, characterized by the sunken appearance of the guard cells. This finally led to a decrease in the overall stomatal count and the incomplete or total closure of stomata (Fig. 8B, E). In contrast, the amendment of nCaO (100 mg kg^{-1}) + exogenous MT (100 μM) treatment exhibited improved development of refined stomatal structures with well-formed guard cells and an ideal stomatal aperture. This treatment effectively protected plants against Cd stress and facilitated stomatal opening. These findings collectively suggested that addition of soil nCaO + MT + Cd application can mitigate the hazardous effect of Cd and minimizing harm to guard cells. Administration of same treatment enhanced stomatal mobility and promoted the gaseous exchange and associated physiological processes. Our SEM results indicated that Cd stress exerts serious damage on guard cells and stomatal functioning. Nevertheless, combine application of nCaO + MT greatly improved stomatal ultrastructure which in turn alleviate Cd stress and improved physiological processes in alfalfa seedlings.

Role of nCaO + MT for mitigation of ultrastructural damages in plants

We employed transmission electron microscopy (TEM) to assess the harmful impact of Cd stress and the possible mitigating effect of nCaO + MT on alfalfa ultra-cellular structure. The control group exhibited leaf cells with intact cell walls (CW), normal nuclei (Nuc), and well-shaped chloroplasts (Chl) with organized thylakoids (Thy), suggested healthy

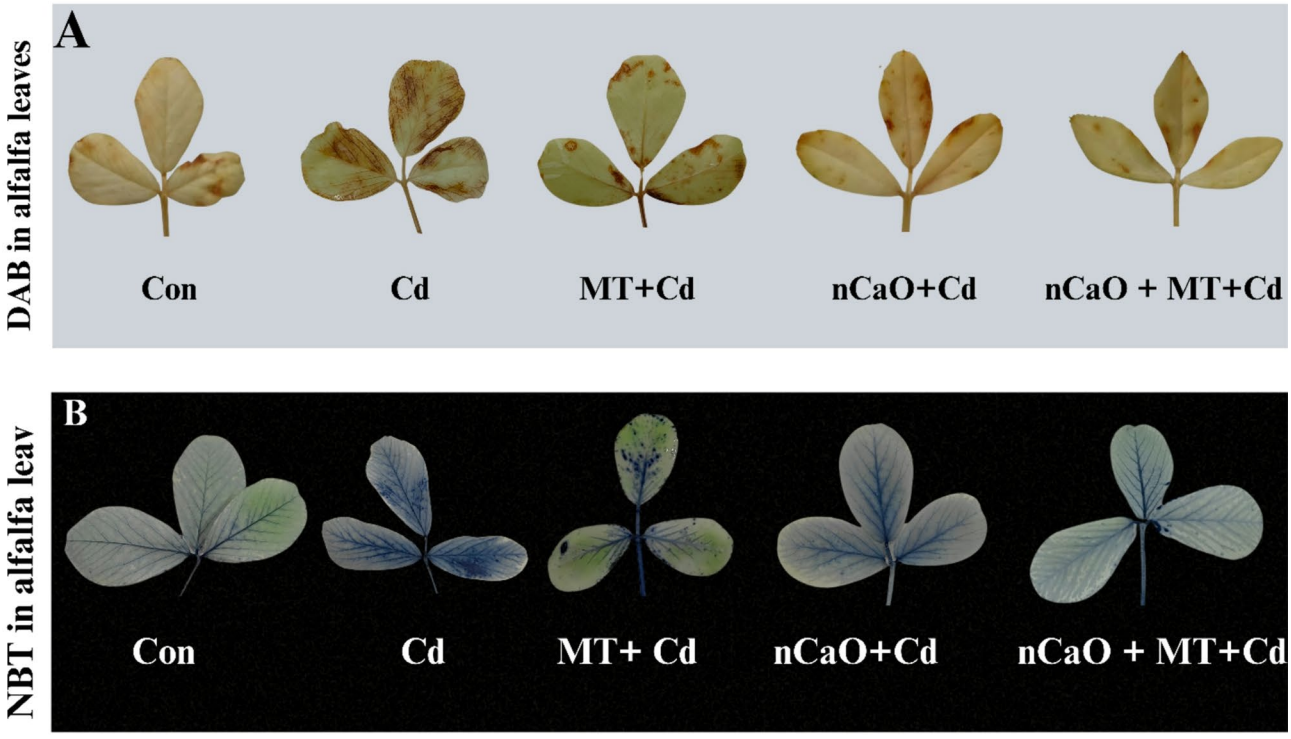

Fig. 6 DAB and NBT staining of alfalfa leaves under cadmium and control (Con) condition as a result of combined application of soil nano calcium oxide and exogenous melatonin

ultrastructure of plant cells (Fig. 9A, D). The investigation demonstrated that Cd-stress inflicted significant damage on the ultra-cellular architecture of plant shoots, particularly affecting cell organelles. Seedlings exposed to Cd stress showed considerable structural modifications, including dysfunctional cell wall (CW), aberrant structure of nucleus (Nuc), indistinct nuclear membrane (NM) and disturbed grana thylakoid (GT). Additionally, we observed an increase in the amount of plastoglobuli (PG) and chloroplasts (Chl) with abnormal shapes, as well as merging thylakoids (Thy), in the mesophyll cells of the leaves (Fig. 9B, E). In contrast, the application of the nCaO + MT + Cd resulted in notable restoration of the ultra-cellular structure in leaves mesophyll cells. The recovery was marked by enlarged nucleoli, distinct nuclear membranes (NM), and well-defined cell walls (CW) in the shoot cells. Furthermore, in shoot cells, chloroplasts exhibited better development and well-structured thylakoids (Thy) as well as prominent membranes. These findings emphasized the potential of nCaO + MT for mitigating the Cd stress in mesophyll cells and this treatment protects chloroplasts and associated cell organelles, thereby improving the intracellular structure of plants. Similarly, these structural enhancements can directly enhance the efficiency of plant photosynthetic processes and the overall growth traits of alfalfa seedlings.

Discussion

Alfalfa is a fodder crop, included in Cd sensitive indicator plants [55]. Utilizing alfalfa as an ecotoxicity receptor is an efficient method to investigate the mechanisms by which NPs with bio-stimulant regulate host plant Cd resistance. Cadmium contamination constitutes a critical environmental disaster that presents substantial health risks to all living species. Exposure to Cd significantly impedes plant growth, metabolism, development, and agricultural productivity [56]. Cadmium stress impairs seedling growth via osmotic damage and ionic toxicity, markedly diminishing the root system's ability to absorb water and nutrients [57]. Diminishing Cd absorption, bio-accumulation, and translocation from the upper soil layer can be a crucial strategy to improve crop resilience to Cd toxicity [13]. Therefore, current study was executed to investigate the impact of soil-applied nCaO and exogenous MT on the morpho-physiological, biochemical, and molecular traits of alfalfa seedlings under Cd stress.

The findings of this investigation revealed that Cd stress at concentration of 30 mg kg^{-1} decreased shoot and root length, as well as their fresh and dry weights, compared to the control that aligns with the findings of [58]. Numerous studies have documented the adverse effects of Cd on plant growth, including wheat, soybean, maize, lucerne, and rice, highlighting the deleterious influence of Cd poisoning on plant development [13, 59–62]. The

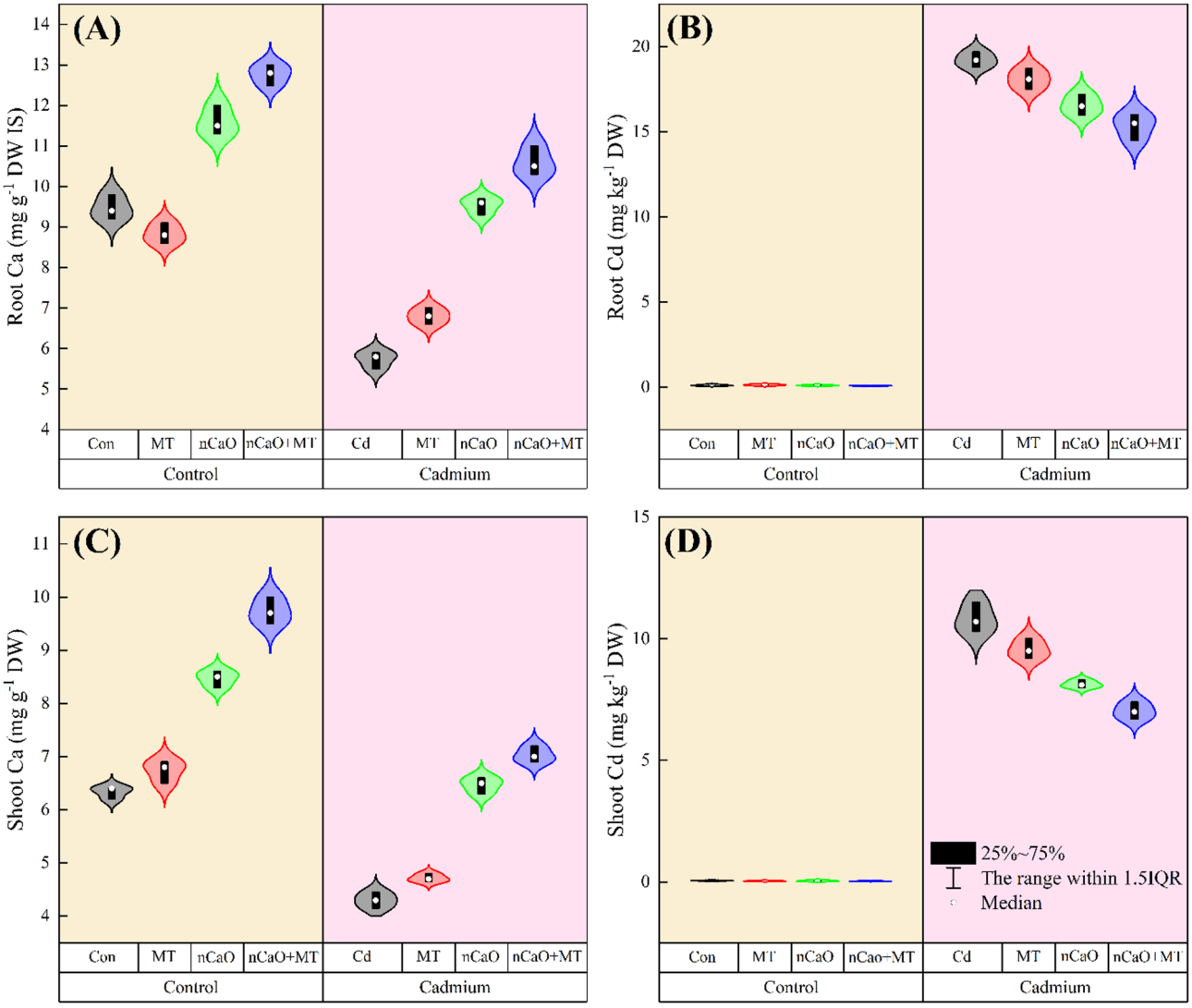

Fig. 7 Effects of the combined application of exogenous melatonin and soil nano calcium oxide on content of calcium and cadmium of alfalfa under both control (Con) and cadmium stress conditions. **A** root calcium (Root Ca); **B** root cadmium (Root Cd); **C** shoot calcium (shoot Ca); **D** and shoot cadmium (shoot Cd). The data are expressed as the mean ± standard error from three distinct biological replicates

rationale for this stunted growth of plants induced by Cd may be ascribed to compromised cellular metabolism, reduced cell turgor due to heightened membrane permeability, tissue damage, and a general reduction in cell expansion [63, 64]. Nonetheless, soil-applied nCaO and foliar-applied MT significantly improved growth traits under both con and Cd stress conditions. Under stress conditions, better seedling growth was mainly associated with improved photosynthetic traits, as shown in (Fig. 1). There is growing evidence that MT functions as a growth hormone and a stress-regulating molecule, which is advantageous for promoting seedling growth and development [65, 66]. For instance, Zhang et al. [67] demonstrated that melatonin application significantly improved seedling growth in alfalfa under waterlogged conditions. Moreover, application of melatonin enhances seedling growth, which can be ascribed to elevated stomatal conductance and leaf water status, improved photosynthesis, pigment formation, CO_2 exchange, upregulation of PSII efficiency, and augmented starch and sugar production in stressful conditions [68–70].

Nano-calcium oxide, as macronutrient-based fertilizer, can promote seedling growth under HM stressors including Cd, as reported in barley [30], and alfalfa crops [58]. Improved seedling growth due to the application of nCaO can be explained in different ways. Nano calcium oxide serves a good source of Ca^{2+}, which can enhance the uptake of other nutrients and improve crop nutrient use efficiency [30, 71]. In plants, Cd^{2+} and Ca^{2+} utilize similar apoplastic and symplastic pathways during their transit to the xylem and phloem, competing for occupancy within the plant [72]. Due to their lower size and

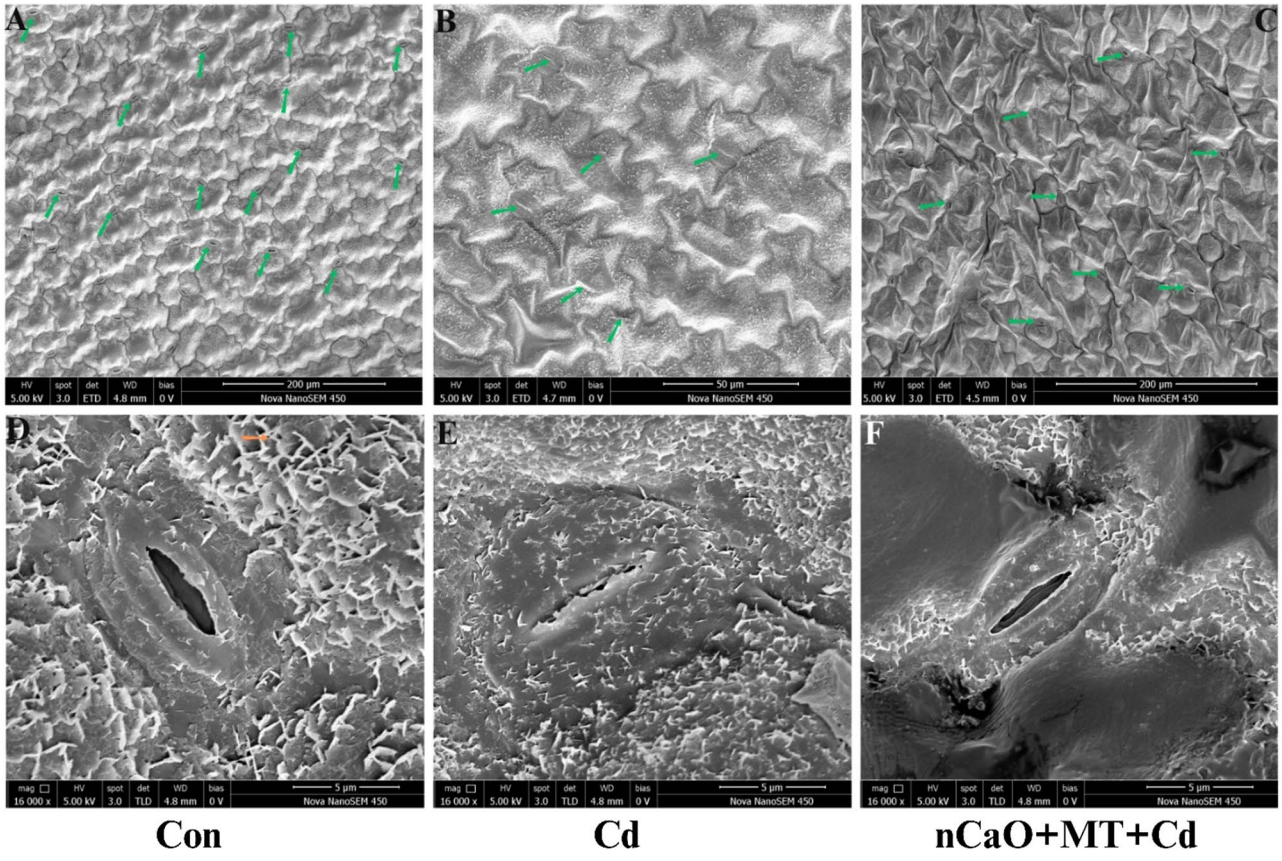

Fig. 8 Effect of nano-calcium oxide (nCaO) and melatonin (MT) on stomatal conductance and guard cells on alfalfa leaves under cadmium (Cd) stress and control (Con) conditions. (**A**, **D**); control, (**B**, **E**); Cd stress, and (**C**, **F**); nCaO + MT + Cd

increased availability, nCaO can quickly fill plant tissues, minimizing metal uptake and translocation. Moreover, nCaO enhances Ca supply to plant roots, improving its accumulation in the upper parts of the plants, thereby promoting better seedling growth [73]. Previous studies have also shown that the combined application of melatonin and NPs significantly accelerated seedling growth under stress conditions [43]. Interestingly, there is limited evidence on the combined application of MT and nCaO under Cd stress. Recently [43], demonstrated that the combined application of melatonin and Si-NPs potentially improved seedling growth under salt stress conditions compared to only one application of these agents and our results are consistent with these findings. We interpret that enhanced seedling growth was strongly linked to high Ca uptake and its translocation to the aerial part of the plants under the combined application of these elements. Moreover, we found that the improved growth of stressed plants under combined MT and nCaO supplements was due to better chlorophyll pigment formation, higher photosynthetic activity, enhanced chlorophyll fluorescence, and reduced Cd accumulation in plant organs. However, the exact mechanisms behind the improved growth resulting from the simultaneous application of MT and nCaO need further investigation.

Chlorophyll fluorescence traits are highly vulnerable to HM stresses and can serve as marker of a seedling's response to metal stresses, including Cd [74, 75]. In our study, Cd stress significantly reduced physiological traits, including pigment content, photosynthetic and transpiration rates (Tr, Ci), and the maximum quantum efficiency of PSII (Fv/Fm), compared to the control [48, 57] (Fig. 2). The reduction in Fv/Fm values under Cd stress are largely associated with decreased light absorption and energy utilization in PSII [76], while Cd-induced damage on chloroplast structure has been well-reported previously [77]. However, the combined application of soil-applied nCaO and foliar MT significantly improved chlorophyll fluorescence and other physiological traits under Cd stress. Melatonin treatment improved photosynthesis by increasing quantum yield (Fv/Fm), electron transport efficacy, and protecting PS proteins [68]. Furthermore, MT has been shown improved action of the Rubisco enzyme and other constituents of the Calvin cycle in seedlings [78]. In addition to direct effects on photosynthesis, MT interacts with various growth hormones to enhance plant resistance in stressful conditions

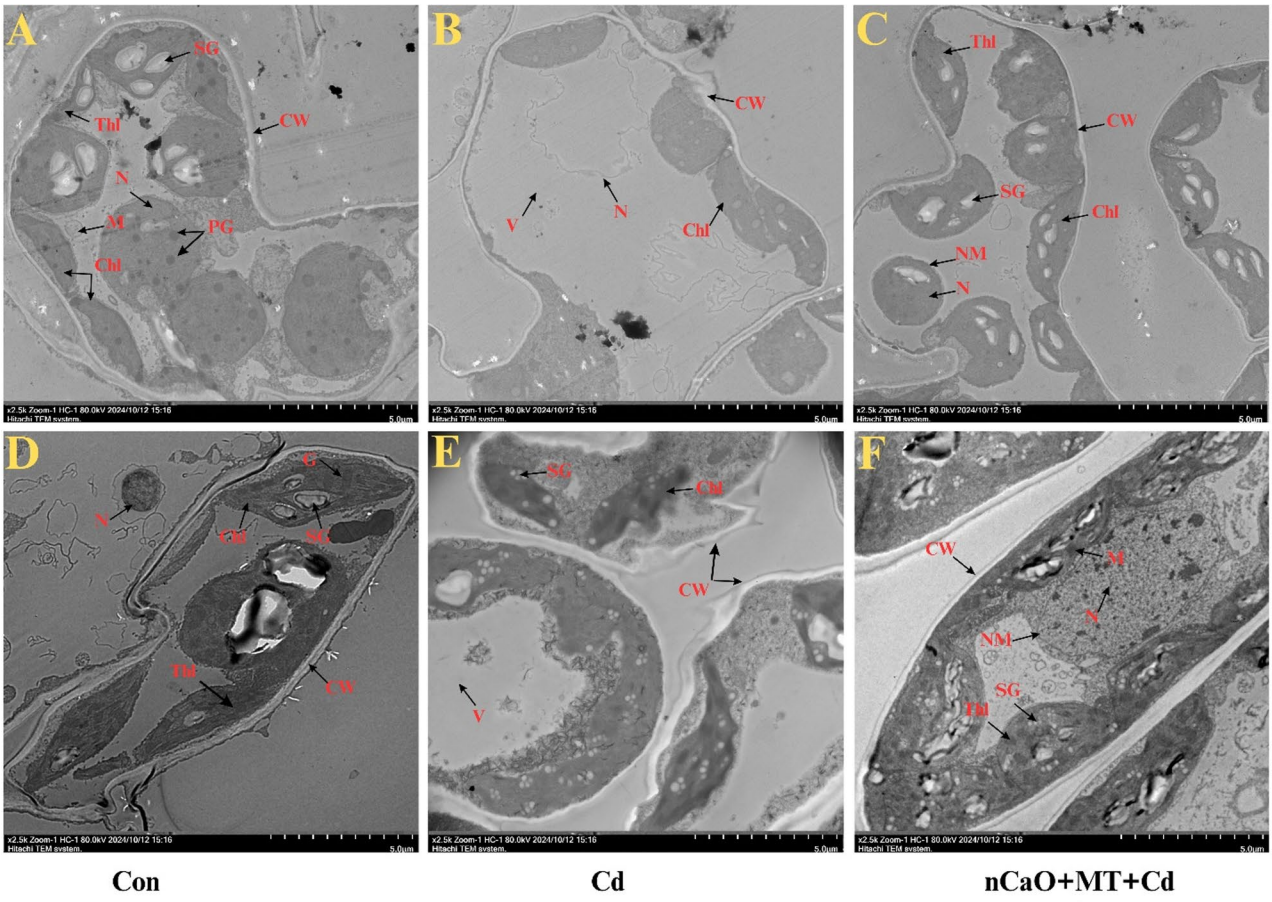

Fig. 9 TEM micrograph representation of alfalfa mesophyll cell by soil amendment of nano calcium oxide (nCaO) and exogenous melatonin (MT) under control (Con) and cadmium (Cd) stress condition. Label points showed cell wall (CW), plastoglobuli (PG), thylakoid (Thl), nucleus (N), nuclear membrane (NM), starch granules (SG), vacuole (V), chloroplast (Chl) and grana (G). (**A**, **D**); control, (**B**, **E**); Cd stress, and (**C**, **F**); nCaO + MT + Cd

[43]. Melatonin application increases ABA accumulation in plants, thereby improving stress tolerance [79]. The improved tolerance confirmed by MT application may also be associated with increased stomatal closure and reduced water loss under stressful conditions [43, 79]. In another study, Ahmad and Anjum [80] established that melatonin application to maize seedlings has resulted in elevated levels of various hormones, including abscisic acid (ABA) and jasmonic acid (JA), and the expression of related genes, which enhanced stress tolerance. In addition to MT, nCaO has also shown to improve the physiological traits of alfalfa seedlings, particularly under Cd toxicity. The enhanced physiological performance of Cd-exposed seedlings treated with CaO-NPs may be attributed to the increased uptake and translocation of Ca, which is a key component of the NAD^+ kinase enzyme that plays a crucial role in chloroplast function [58]. In another study, Nazir et al. [30] reported similar findings, stating that the improved performance of photosynthetic traits under nCaO application is strongly linked to higher Ca uptake by the aerial part of the plant and reduced Cd accumulation. This study also recorded that nCaO facilitated Ca uptake under Cd stress. Previously, Mubeen et al. [81] reported similar results for brassica seedlings. Furthermore, Ayyaz et al. [25] established that CaO-NPs are involved in the improved functioning of PSII, as the high availability of Ca^{2+} plays a fundamental role in the formation of plastid structures. The signaling and sensing functions of Ca^{2+} under abiotic stresses have been well documented in previous studies [82]. It is commonly believed that healthy seedlings can utilize available resources, including light, more effectively, which helps plants survive under stress conditions. Therefore, the findings suggest that soil-applied nCaO and exogenous MT enhance alfalfa's Cd tolerance by improving physiological traits when compared to the Cd control. However, the mechanisms underlying the improved photosynthetic traits, particularly under the combined application of MT and nCaO, remain elusive.

Plants's ability to withstand HM stresses is strongly interpreted to their antioxidative defense mechanisms [28, 83]. Under Cd-induced oxidative stress, plants activate antioxidative defense enzymes to scavenge ROS. This antioxidative defense system includes the activities

of SOD, POD, and CAT. In this study, we observed that Cd stress increased the activities of antioxidant enzymes (Fig. 4). Interestingly, soil-supplemented nCaO and foliar-applied MT further boosted the activities of these antioxidative enzymes. nCaO may engage with the plasma membrane of cells, effectively scavenging ROS and safeguarding the cell membrane from oxidative damage. Our investigation shown that the application of nCaO and MT significantly decreased the levels of MDA, H_2O_2, and O_2 in plants subjected to Cd poisoning. These results are aligned with recent findings byMukherjee et al. [84] and [48], who reported that both melatonin and nCaO treatments increased antioxidative activities while reduced ROS production. In another study, Ni et al. (2018) demonstrated that melatonin enhances wheat tolerance to Cd stress by scavenging ROS and enhancing the activities of antioxidative enzymes. Similar mechanisms have been drawn for CaO-NPs in previous studies [30], which confirm our current research. For instance, Hussan et al. [48] documented that CaO-NPs enhanced Cd tolerance in alfalfa seedlings by increasing antioxidative activities and decreasing ROS production. The protective roles of melatonin and nCaO are well documented in improving oxidative stress tolerance by scavenging ROS and boosting antioxidative activities. These increased antioxidant activities enabled the plants to counteract the additional ROS generations in response to stressful signals. Consequently, through the current study for the first time, we described the effectiveness of combined MT and nCaO application in enhancing Cd stress tolerance in alfalfa seedlings. However, the precise mechanisms behind the improved oxidative stress tolerance under the combine application of MT and nCaO require further investigation.

Several studies have evaluated alfalfa seedling chloroplast and nucleoli ultrastructure exposed to Cd or NPs treatment. These findings revealed that chloroplasts of alfalfa plants subjected to Cd had ultrastructural alterations, including disorganization of thylakoid stacking, a decrease in both the number and size of grana, and modifications to the chloroplast membrane [48, 85]. Nucleoli displayed modifications, including variations in size, shape, and the quantity of nucleoli per cell. Our results indicated that alfalfa seedlings treated with both nCaO and MT under Cd toxicity significantly reduced damage to their chloroplasts and nucleoli compared to those seedlings exposed alone to Cd stress (Fig. 9C, F). The chloroplasts of the nCaO and MT treated plants exhibited highly ordered grana thylakoids (Thl), an abundance of starch granules (SG), and a well-defined nucleus, showing that nCaO and MT maintained the structural integrity of the chloroplasts exposed to Cd stressed. The augmentation of antioxidant enzyme activity and the scavenging of ROS can safeguard the plant against the detrimental impacts of HMs poisoning. These results align with the findings of Hassan et al. [85] and Kareem et al. [56], who observed that alfalfa seedlings subjected to Cd poisoning had deformed nuclei with aberrant nuclear membranes and punctured chloroplasts with compromised thylakoids.

Stomata play a vital role in gas and water exchange between plants and their environment. Understanding how environmental stress affects crop development involves examining plant stomatal activity [37]. This study found that Cd stress significantly damaged the stomatal aperture compared to other treatments (Fig. 8). The distortion in stomatal opening were directly associated with the phytotoxic effects of Cd on the alfalfa seedlings. The alteration of guard cell morphology under Cd Stress can be generated by suppressing metabolic processes that maintain turgor pressure in guard cells [61]. Cd-induced modifications in stomatal aperture may be linked to the overproduction of ROS in alfalfa leaves. Interestingly, supplementing with nCaO in combination with exogenous MT reduced oxidative stress and preserved the morphology of stomata. These findings line up with earlier studies of [56]. They asserted that higher ROS production led to oxidative damage in guard cells under the Cd stress and cobalt, resulting in the closure of stomata in alfalfa and maize, respectively.

Conclusion

In conclusion, this study demonstrates the tremendous potential of combining melatonin and nano-calcium oxide to combat Cd-induced stress in alfalfa. The combined application increased growth and photosynthetic efficiency and significantly reduced Cd accumulation and oxidative damage. By enhancing calcium uptake and activating antioxidant defense mechanisms, this approach offers a sustainable and innovative solution to heavy metal contamination, such as Cd stress in agriculture. The restoration of cellular ultrastructure and modulation of stress-responsive genes further demonstrate the efficacy of this combined treatment. These findings contribute to a better understanding of melatonin and nanotechnology's role in future research into the molecular mechanisms underlying Cd detoxification and stress tolerance, providing insights for developing resilient crops in heavy metal-polluted environments. Ultimately, this study highlights of biostimulants and nanotechnology interplay potential to safeguard food security and environmental health encounters of escalating HMs pollution at the farm level.

Abbreviations

Cd	Cadmium
nCaO	Nano-Calcium Oxide
MT	Melatonin
ROS	Reactive Oxygen Species

MDA	Malondialdehyde
H_2O_2	Hydrogen Peroxide
$O_2^{\bullet}$	Superoxide Anion
SOD	Superoxide Dismutase
POD	Peroxidase
CAT	Catalase
APX	Ascorbate Peroxidase
EL	Electrolyte Leakage
DAB 3,3'	Diaminobenzidine
NBT	Nitroblue Tetrazolium
DEGs	Differentially Expressed Genes
PSII	Photosystem II
Fv/Fm	Maximum Quantum Efficiency of PSII
Pn	Net Photosynthetic Rate
gs	Stomatal Conductance
Ci	Intercellular CO_2 Concentration
Tr	Transpiration Rate
CW	Cell Wall
PG	Plastoglobuli
Thl	Thylakoid
Nuc	Nucleus
NM	Nuclear Membrane
SG	Starch Granules
V	Vacuole
Chl	Chloroplast
G	Grana
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy
ICP	MS-Inductively Coupled Plasma Mass Spectrometry
RNA	seq-RNA Sequencing
CRD	Completely Randomized Design
ANOVA	Analysis of Variance
LSD	Least Significant Difference
SE	Standard Error

Supplementary Information

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Supplementary Material 1.

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Authors' contributions
M.U.H.: Experimentation, Conceptualization Data analysis, Writing- Original draft preparation. U.M.: Conceptualization, Formal analysis, Data curation. S.Z.: Formal analysis, Investigation and Reviewing and Editing. S.H.: Writing- Original draft preparation, Methodology. M.B.H.: Methodology, review & editing. N.Z.: Writing- Reviewing and Editing. M.A.: Formal analysis, Investigation. Q.W.: Conceptualization, Project administration, Writing- Reviewing and Editing. M.Y.: Conceptualization, Visualization, Reviewing and Editing

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Data availability
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Author details
[1]College of Grassland Agriculture, Northwest A&F University, Yangling 712100, China
[2]Department of Plant Breeding, Swedish University of Agricultural Sciences, Vaxxtskyddsvagen 11, Alnarp 234 56, Sweden
[3]Department of Horticultural Science, Shiraz University, Shiraz, Iran
[4]College of Horticulture, Northwest A&F University, Yangling 712100, China
[5]Department of Agronomy, University of Agriculture Faisalabad, Faisalabad 38000, Pakistan
[6]Department of Botany, Government College Women University Faisalabad, Faisalabad 38000, Pakistan
[7]College of Life Sciences, Northwest A&F University, Yangling 712100, China

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