



Synergistic effects of salicylic acid and magnesium oxide nanoparticles on cold stress and miRNA expression in alfalfa (*Medicago sativa* L.) genotypes

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Received: 10 December 2024 / Accepted: 4 May 2025 / Published online: 20 May 2025
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Abstract

This study aimed to investigate the responses of alfalfa (*Medicago sativa* L.) genotypes (Van/Denizli) to cold stress and to evaluate the effects of exogenous salicylic acid (SA), magnesium oxide nanoparticles (MgO NPs) and their combination (SA+MgO NPs) on these responses. Alfalfa genotypes were subjected to treatments with SA (1 and 2 mM), MgO NPs (5 and 20 ppm), and SA + MgO NPs, followed by exposure to cold stress at 10 °C and 4 °C for 14 days each. Changes in biochemical parameters, scanning electron microscope (SEM) image analysis, and alterations in miRNA (miRNA156 and miRNA173) gene expression levels were examined to assess the mitigating effects of the treatments on cold-induced damage. The results indicate that under cold conditions, SA and MgO NPs singularly reduced lipid peroxidation (LPO) and hydrogen peroxide (H₂O₂) contents while increasing proline content. The combination of 2 mM SA+20 ppm MgO NPs exhibited the most significant alleviating effects against cold stress. Analysis of miRNA gene expression levels revealed differential regulation patterns, with miR156 and miR173 showing distinct responses to temperature and treatment combinations. Our study underscores the potential of MgO NPs and SA as promising agents to enhance cold tolerance and mitigate crop losses in agriculture. Future research endeavors exploring the efficacy of these substances across diverse plant genotypes under cold stress conditions could offer valuable insights into developing strategies for enhancing cold stress resilience and improving agricultural productivity.

Key Message

Salicylic acid and MgO NPs improved the resistance of *M. sativa* genotypes conditions cold stress enhancing antioxidant enzyme activity, biochemical parameteres, miRNA gene expression and electronic scanning analysis.

Keywords *M. sativa* L. · MgO NPs. · SA · Cold stress · mtr-miR156a · mtr-miR173-5p

Communicated by Mohammad Faisal.

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Introduction

Medicago sativa L., commonly referred to as lucerne or alfalfa, is recognized as the preeminent forage crop within scientific discourse. This botanical entity, classified within the genus *Medicago* and situated in the Faboideae subfamily of the Fabaceae family, garners the esteemed epithet of the "queen of forage crops" (Hossain et al. 2020). With a worldwide cultivation expanse encompassing an estimated 32 million hectares, representing 2.5% of arable land, alfalfa emerges prominently for its nutritional abundance, minimal fiber composition, elevated protein content, and superior digestibility attributes. These characteristics collectively render it an invaluable dietary choice for livestock (Radović et al. 2009).

Within the intricate tapestry of ecological relationships, environmental variables assume a pivotal role in molding plant development. Optimal environmental conditions are indispensable for the robust proliferation of plants. Nonetheless, exposure to adverse conditions can instigate stress, thereby testing a plant's adaptive prowess and metabolic pathways (Shao et al. 2008). Cold stress, delineated by temperatures falling below the threshold conducive to optimal growth, emerges as a noteworthy abiotic stressor, imposing deleterious impacts on the morphology, physiology, and metabolic dynamics of plants (McKersie et al. 1994; Mahajan and Tuteja 2005). During cold stress, plants encounter disturbances in their electron transport systems within chloroplasts and mitochondria, culminating in the buildup of reactive oxygen species (ROS) and subsequent oxidative stress (Suzuki and Mittler 2006). ROS, such as hydrogen peroxide (H_2O_2), impose deleterious effects on biological molecules, leading to DNA damage, enzyme inactivation, and lipid peroxidation (Navrot et al. 2007). To mitigate the adverse impacts of cold stress, plants deploy adaptive mechanisms, which encompass the synthesis and accumulation of osmolytes, such as proline, glycine, and soluble sugars (Eruçar 2006). Osmolytes play a critical role in upholding osmotic equilibrium, safeguarding structural integrity, and scavenging free radicals, consequently augmenting plant resilience against cold stress (Tester and Davenport 2003).

Salicylic acid (SA) emerges as a crucial phytohormone synthesized by plants, widely distributed throughout plant tissues, albeit its precise transport mechanism remains elusive. Research indicates that SA is translocated via the phloem from externally applied sites to diverse plant organs. Functioning as a signaling molecule of plant origin, SA and its derivatives trigger the activation of defense-related genes, enhancing plant resistance to various stressors and bolstering their resilience. Its multifaceted effects range from regulating cell growth and stomatal closure to modulating the

expression of genes associated with senescence and fruit yield (Raskin 1992).

The influence of salicylic acid (SA) on plants demonstrates variability, contingent upon environmental factors, application techniques, plant genetic makeup, and dosage. While lower concentrations of SA promote growth, higher levels may impede it. In suboptimal environmental conditions, endogenous SA levels might increase, whereas external applications of SA can offer adaptive benefits by mitigating stress (Bagautdinova et al. 2022).

The potential of nanotechnology in the 21st century is undeniable, as it revolves around particles sized from 1 to 100 nanometers, encompassing various forms like nanocrystals, nanowires, nanorods, and nanoparticles (Hulkoti and Taranath 2014). Nanotechnology, a multidisciplinary science, commands substantial attention across an array of fields. Its applications span rapid disease detection, molecular therapies, pathogen control, biosensor development, improved nutrient utilization in agriculture and plants, as well as contributions to engineering, textiles, automotive manufacturing, construction, industrial processes, space exploration, and environmental sustainability (Mbonyiru-ivuze et al. 2015).

In the realm of agriculture, the incorporation of nanotechnology offers compelling prospects to slash production expenses, promote robust plant growth, and combat environmental contamination by minimizing reliance on chemical inputs (Daghan 2017). Magnesium (Mg) stands out as a paramount element, holding the rank of the 8th most abundant constituent in the Earth's crust and securing its position as the second most prevalent cation in plants (Senbayram et al. 2015). Crucial for optimal plant growth, magnesium plays indispensable roles in activating enzymes, facilitating photosynthesis, and synthesizing proteins and nucleic acids (Cowan 2002). Plants fortified with sufficient magnesium demonstrate heightened resilience to diverse abiotic stresses (Ahmed et al. 2023).

Magnesium oxide nanoparticles typically range in size from 5 to 120 nm, providing a specific surface area of 25–50 m²/g. Distinguished by their diminutive dimensions, pronounced ionic characteristics, and distinctive crystal structure, magnesium oxide nanoparticles hold significant promise for a wide array of applications (Bindhu et al. 2016). These nanoparticles are easily synthesized, non-toxic, cost-effective, and exhibit antimicrobial properties, in line with the tenets of green and sustainable agriculture. In agricultural contexts, magnesium oxide nanoparticles show low genotoxicity, biotoxicity, and phytotoxicity, while concurrently stimulating seedling development, amplifying plant growth, and conferring resilience against diverse stressors. The application of magnesium oxide nanoparticles via foliar spraying as nanofertilizers and enhancers of

light absorption has been pivotal in bolstering the growth and yield of numerous crops (Cai et al. 2018).

Stress induces intricate regulatory mechanisms in plants, including the modulation of gene expression by microRNAs (miRNAs), which may exhibit over or under-expression or generate new miRNAs to counteract stressors. Recent advancements in high-throughput sequencing technology have facilitated the discovery of numerous miRNAs across various plant species, shedding light on their roles in mitigating biotic and abiotic stresses such as drought, salinity, cold, mechanical stress, and heavy metal toxicity. MiRNAs, small single-stranded non-protein-coding RNA molecules spanning 21–24 nucleotides, exert regulatory functions by binding to target mRNAs, thereby modulating gene expression through mRNA degradation or repression. Emerging studies emphasize the crucial role of miRNAs in plant stress responses, enhancing the stability and reliability of current miRNA gene editing techniques (Zhou et al. 2008; Zhong et al. 2020).

An array of abiotic stress factors, comprising low and high temperatures, drought, salinity, nutritional deficiencies, and metal elements, have been implicated in modifying the expression patterns of miRNAs linked to the progression of plant growth across various plant species (Shriram et al. 2016). Among the extensively studied miRNAs, miR156 stands out as a prominent member, demonstrating high conservation across plant species. Initially identified in *Arabidopsis*, miR156, consisting of 20 nucleotides, targets the SPL (SQUAMOSA promoter binding protein) and SBP (SQUAMOSA promoter binding protein) gene families, thereby regulating pivotal aspects of plant growth, reproduction, and development (Reinhart et al. 2002). In addition to miRNAs, small regulatory RNAs include heterochromatic siRNAs (hc-siRNAs), repeat-associated siRNAs (ra-siRNAs), natural sense-antisense transcript siRNAs, trans-acting siRNAs (ta-siRNAs), and the recently discovered Piwi-interacting RNAs.

The discovery of trans-acting siRNAs (ta-siRNAs) in *Arabidopsis* has bridged the miRNA and siRNA pathways, offering insights into their intricate regulatory networks. Ta-siRNAs, initially elucidated through the study of RDR6 and gene silencing suppressor (SGS3) genes, are triggered by the cleavage of primary TAS transcripts targeted by miRNAs. Subsequently, aided by RDR6 and SGS3, the cleavage products undergo conversion into double-stranded RNA (dsRNA), ultimately processed by DCL4 to yield ta-siRNAs. Notably, *Arabidopsis* harbors four TAS gene families, with miR173 targeting TAS1a, TAS1b, and TAS1c, while TAS2 is solely targeted by miR173. The resultant ta-siRNAs regulate a wide array of genes, including the pentatricopeptide repeat (PPR) genes, which constitute a significant protein family in land plants. PPR proteins serve various molecular

functions and physiological roles in plant growth, development, photosynthesis, and responses to environmental stimuli (Peragine et al. 2004; Allen et al. 2005; Yoshikawa et al. 2005).

In this study, we explore the effects of exogenously applied salicylic acid and magnesium oxide nanoparticles on biochemical responses and the expression levels of miRNA156 and miRNA173 genes in Van and Denizli alfalfa genotypes under cold stress conditions. Our investigation seeks to elucidate nutritional requirements and improve product quality in response to the challenges posed by population growth and escalating production costs.

Materials and methods

Preparation of blackcurrant (*Ribes nigrum*) fruit extracts

Blackcurrants (*Ribes nigrum*) were harvested from the Atatürk University Campus in Turkey. The fruits were meticulously washed in tap water, followed by additional rinses in distilled water (3–4 times). Subsequently, they underwent freeze-drying at -40°C . A total of 10 g of dried blackcurrant fruit were pulverized into a fine powder. This powder was then combined with 100 ml of distilled water and heated to 80°C for one hour with continuous stirring. Following the heating process, the mixture was allowed to cool to room temperature. To isolate the extract, the cooled mixture underwent centrifugation at 8500 rpm for 10 minutes. The resulting supernatant was then filtered using Whatman filter paper to obtain a pure liquid extract of the blackcurrant fruit.

Green synthesis of MgO NPs

For this, 2 g of magnesium nitrate ($\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$) was combined with 50 ml of blackcurrant fruit extract and stirred for 30 minutes at room temperature. Subsequently, the mixture was heated at 90°C for 2 hours. Upon reaching this temperature, the pH was adjusted to 11 using a 2 M NaOH solution, resulting in a color change of the solution to dark brown. Following cooling, the solution underwent centrifugation (4°C) twice for 20 minutes at 8500 rpm, followed by multiple washes with deionized water. The resulting solid MgO NPs were then dried in an oven at 60°C overnight. Finally, the dried MgO NPs were subjected to calcination in a heating furnace (Thermolyne/Dubuque III) at 500°C for 3 hours. The resulting green synthesized MgO NPs were utilized for further characterization and various applications (Nijalingappa et al. 2019).

Characterization of MgO NPs

The crystal structure and orientation of MgO NPs, synthesized through the green method, were investigated employing a Rigaku Miniflex diffractometer utilizing Cu K α radiation (1.5406 Å). Morphological characterizations of the nanoparticles were conducted using an FEI Quanta scanning electron microscope (SEM). For detailed chemical composition analysis, an energy-dispersive X-ray spectroscopy (EDX) system was integrated with the SEM (FEI INSPECT550). Furthermore, Fourier transform infrared (FTIR) data spanning the range of 400–4000 cm⁻¹, providing insights into the functional groups present in the synthesized metal oxide nanoparticles, were recorded using a Spectrum One FT-IR Spectrometer with the KBr pellet method.

Plant material and cold stress, SA, and MgO NPs applications

The seeds of Van and Denizli genotypes of alfalfa (*Medicago sativa* L.) used in the study were obtained from local farmers. Before sowing, the seeds were sterilized with 70% ethyl alcohol for 10 min and 0.05% Tween® 20 and 20% commercial bleach (with 3% hypochlorite) for 7 minutes, respectively, and then washed thoroughly with deionized water for several minutes. The germinated seeds were sown in pots (54 cm length $\times$ 28 cm width) carefully covered with soil (soil: perlite: peat (1:1:1)). The seedlings were placed in a climate-controlled growth chamber (Panasonic MLR-352H-PE) with a temperature of 25 °C, 60% relative humidity and a lighting period of 16/8 (day/night) with 15,000 lux illumination. Following the germination, the seedlings were grown under the control conditions for 4 weeks (28 days). Following this stage, seedling leaves in each pot were treated with one of the following: (1) salicylic acid (SA) alone (1 or 2 mM in 0.1% Tween-20), (2) MgO nanoparticles alone (5 or 20 ppm), or (3) various combinations of SA and MgO nanoparticles (1 mM SA+5 ppm MgO NPs; 1 mM SA+20 ppm MgO NPs; 2 mM SA+5 ppm MgO NPs; 2 mM SA+20 ppm MgO NPs). The concentrations were selected based on preliminary trials supported by existing literature. Each solution was applied using a 100 mL atomizer until complete leaf coverage (runoff) was achieved. The control plants were treated with the same amount of distilled water containing 0.1% Tween-20. To ensure thorough absorption of the applied compounds by the leaves, all the plants were incubated in the same climate chamber for 24 hours under conditions of 16/8 hour light/dark cycle, 60% humidity, and 25 °C. Then, the ambient temperature was lowered to 10 °C (16/8 hour light/dark cycle, 15,000 lux, and 40%

humidity), and the seedlings were grown under these conditions for an additional 14 days. At the end of this stage, half of the pots from each treatment group were harvested (with at least 3 pots per group), and the remaining pots were treated again with the same applications of SA and MgO NPs, along with their respective combinations, following the procedure described earlier. Twenty-four hours after the application, all pots were exposed to 4 °C conditions (16/8 hour light/dark cycle, 15,000 lux, and 40% humidity), and the seedlings were grown under these conditions for another 14 days. Then, the plants were harvested. Plant samples harvested at both stages (10 °C and 4 °C) were transferred to a deep freezer at -80 °C until used in the experiments.

Determination of total soluble sugar content

Total soluble sugar content was determined by DNSA (3,5-dinitrosalicylic acid) method. Plant samples (0.1 g) were homogenized in a mortar using liquid nitrogen. Then 5 mL of 2.5 N HCl was added and homogenization was completed. The samples were centrifuged at 15,000 rpm for 10 minutes and 2 mL of the supernatant was taken into a glass tube. After adding 2 mL of DNSA to the tube, it was incubated in a 90 °C water bath for 20 min and cooled in an ice bath to stop the reaction. Aliquots of 100 μ L were taken from each sample including the blank sample, and the absorbance was measured at 550 nm on a multiscan spectrophotometer (Thermo Scientific, Multiskan GO Microplate Spectrophotometer).

Determination of H₂O₂ content

The concentration of H₂O₂ was determined according to the previously described protocol (Sousa et al. 2013). For this, fresh tissue (0.2 g) was homogenized using a mortar and pestle, first with liquid nitrogen and then with 2 mL of potassium phosphate buffer (50 mM, pH 6.5). The homogenization was completed with 2 mL of 0.1% TCA (trichloroacetic acid). After centrifugation at 15,000 rpm for 15 minutes at 4 °C, 1 mL of the supernatant was combined with 0.15 mL of 5% Ti(SO₄)₂ and 0.3 mL of 19% NH₄OH. The resulting extract was centrifuged at 15,000 g for 15 minutes at 4 °C, and the pellet was dissolved in 3 mL of 2 M H₂SO₄. The absorbance of the mixture was measured at 415 nm on the multiscan spectrophotometer. The absorbance values were then correlated to micrograms of H₂O₂ using a standard graph prepared with pure H₂O₂. The results were expressed as μ mol/g⁻¹.

Determination of lipid peroxidation level

The level of lipid peroxidation (LPO) was determined by assessing the malondialdehyde (MDA) content (Dhindsa and Matowe 1981). Plant samples (0.2 g) were ground in mortar using liquid nitrogen. Homogenization was achieved by adding 2 mL of 0.1% TCA and then centrifuging at 15,000 rpm (4 °C) for 15 minutes. The supernatants (0.5 mL) was mixed with 1 mL of 1% TBA (thiobarbituric acid). This mixture exposed to incubation in a water bath at 95 °C for 30 minutes and was then promptly cooled to room temperature on an ice bath. The samples were subjected to centrifugation once again at 3000 rpm for 5 minutes, and the absorbance of the resulting supernatant was measured at 440, 532, and 600 nm using a microplate reader. The calculations were performed based on the formula specified by (Hodges et al. 1999), and the results were expressed as $\mu\text{mol/g}^{-1}$.

$$\text{MDA (nmol/mL)} = \left[\frac{[(A_{532} - A_{600}) - [(A_{440} - A_{600}) \times (0.0571)]]}{15700} \right] \times 10^6 \quad (1)$$

Determination of proline content

The proline content was determined according to (Ahmad et al. 2012) with slight modifications. Firstly, 0.4 g of plant leaves were finely ground using liquid nitrogen in a porcelain mortar. Subsequently, 7.5 mL of 3% sulfosalicylic acid was added to the samples and homogenized again. After centrifugation at 12,000 rpm for 10 minutes, 2 mL of the resulting supernatant was carefully transferred to clean glass tubes. Then, 1 mL of acetic acid and 1 mL of acid ninhydrin were sequentially added to the tubes. The samples were then incubated in a 100 °C water bath for 1 hour. After incubation, the tubes were rapidly cooled in an ice bath. Next, 4 mL of toluene was added to each cooled tube, followed by thoroughly mixing. The absorbance of the toluene

phase in the tubes was measured using a spectrophotometer at a wavelength of 520 nm.

SEM analysis (Image acquisition with scanning electron microscope)

Plant samples were kept in 0.025 M buffer (pH 6.8) at room temperature for 1 hour. Then they were kept in fixative buffer (pH 6.8) at room temperature (25 °C) for 2 hours and then 0.025 M buffer (pH 6.8) was kept on ice for 1 hour. After washing 3 times with dH_2O , they were kept in distilled water for 30 minutes. Starting with 10% ethyl alcohol ($\text{C}_2\text{H}_5\text{OH}$), they were kept in 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100% ethyl alcohol concentrations for 15 min each at room temperature, respectively (Fowke 1995). Imaging was performed with a Quante FEG 250 scanning electron microscope.

RNA isolation and Real time PCR

In order to determine the changes in mtr-miR156a and mtr-miR173-5p gene expression levels in Van and Denizli alfalfa cultivars exposed to cold, SA and MgO NPs treatments, first total RNA isolation, then cDNA synthesis and finally RT-qPCR were performed. RNA isolation was performed from frozen leaves (0.2 g) using a commercial kit (PureLink RNA/Invitrogen by Thermo Fisher Scientific) according to a protocol, and the RNA quantity was measured using a nanodrop device (Thermo Scientific Multiscan SkyHigh / A51119700). Finally, the purity was determined by calculating $\text{Abs}_{260}/\text{Abs}_{280}$ nm and $\text{Abs}_{260}/\text{Abs}_{230}$ nm ratios. cDNA synthesis from the RNA obtained through RNA isolation was carried out according to the protocol of the Thermo Scientific RevertAid First Strand cDNA Synthesis Kit using 6 μL of RNA and stem loop primers, with a final volume of 20 μL . Real time PCR was performed on Applied Biosystems, StepOne Plus™ S/N: 720012158 using the protocol of the Solis Biodyne Hot Firepol Evagreen Real-Time qPCR Supermix, 5X kit. The RT-qPCR conditions consisted of an initial denaturation at 95 °C for 12 minutes, followed by denaturation at 95 °C for 15 seconds (40 cycles), annealing at 58–72 °C for 30 seconds (40 cycles), and finally, extension and elongation. The reference U6, mtr-miR156a and mtr-miR173-5p forward and reverse primer sets used in the reaction protocol were designed using the NCBI database and the primers used are given in Table 1. below. Each sample was run in 3 replicates and the Ct values and fold values obtained as a result of the Real-Time PCR reaction were calculated by the ($2^{-\Delta\Delta\text{Ct}}$) method.

Table 1 mtr-miR156a, mtr-miR173-5p, and U6 primer sequences

mtr-miR156a	
Stem Loop	GTCGTATCCAGTGCAGGGTCCGAGG TATTCGCACTGGATACGACTGTGCT
Forward (5'-3')	ACCGGGGTGACAGAAGAGAGAG
Reverse (3'-5')	CCAGTGCAGGGTCCGAGGTA
mtr-miR173-5p	
Stem Loop	GTCGTATCCAGTGCAGGGTCCGAGG TATTCGCACTGGATACGACGTGATT
Forward (5'-3')	ACCGGTTTCGCTTGCAGAGAGA
Reverse (3'-5')	CCAGTGCAGGGTCCGAGGTA
Reference U6	
Forward (5'-3')	ATTGTAACATACAGGA
Reverse (3'-5')	CGATAAAATTGGACCATTTCTG

Statistical analysis

The data obtained with the post-reaction were analyzed using the SPSS 26.0 package program. The Kolmogorov-Smirnov test was used to determine whether the data were normally distributed. Analysis of variance was performed with the two-way ANOVA test, and $P < 0.05$ confidence level was determined using Duncan's multiple comparison test.

Results

SEM, FT-IR, and XRD analyses of MgO NPs

The structural phase of MgO NPs was determined by X-ray diffraction spectra. (Fig. 1) shows the XRD spectrum of MgO NPs synthesized by the green method. As shown in the figure, the diffraction peaks observed at $2\theta = 36.631$, 42.451 , 61.841 , 74.111 and 78.041 are associated with the [111], [200], [220], [311] and [222] planes, respectively, in good agreement with the literature. The XRD spectrum (Fig. 1a) provides insights into the crystalline structure of the CaO NPs. The peaks at specific angles (17.98° , 31.71° , 34.13° , and 50.75°) indicate the presence of $\text{Ca}(\text{OH})_2$ units, offering valuable information about the crystallographic composition of the synthesized nanoparticles. This analysis contributes to the overall understanding of the material's structure. The d-range values of the peaks (from JCPDS data) were found to be almost equal to the measured d-range values (from the Bragg equation). The lattice parameters of the crystal structure are $a=b=c=0.4211$ nm. Moreover, no peaks of other impurities were observed, which is a strong indication of the high purity of the synthesized nanoparticles. At the same time, the strong and sharp diffraction peaks are evidence of good crystallization of MgO NPs.

The Fourier transform infrared spectrum of MgO NPs synthesized using the environmentally friendly green method is shown in (Fig. 1A). The peaks at low frequencies of 652 and 836 cm^{-1} are associated with the vibrational mode of the metal-oxygen bond of MgO (Ansari et al. 2018). The corresponding band is observed at 1409 cm^{-1} and, as is known, chemical absorption of atmospheric carbon dioxide causes the formation of this group. The bands detected at 2902 and 2977 cm^{-1} are due to C–H stretching modes. The band observed at 3676 cm^{-1} is due to hydroxyl groups or defects in low coordination sites. The observation of bands related to H_2O and CO_2 groups in Fourier Transform Infrared Spectrometry is attributed to the chemical absorption of these molecules by magnesium oxide when exposed to the atmosphere.

SEM image with a magnification of $5\text{ }\mu\text{m}$ to determine the morphology of the synthesized MgO NPs is given in Fig. 2. It is noticeable from this FESEM image that the average particle size varies between $100\text{--}120\text{ nm}$. The EDX graph in the FESEM image in Fig. 2 shows that the stoichiometric ratio of Mg to O is approximately one.

Total soluble sugar content findings

The results from leaves of plants exposed to 10°C and 4°C for 14 days were analyzed through two comparisons: (1) cold stress controls (10°C and 4°C) versus the 25°C control group, and (2) treatments applied under cold stress versus their respective temperature controls (10°C or 4°C). In the Van genotype, the 10°C cold control showed a 0.89 mg/g increase in soluble sugar content relative to the 25°C control, while the 4°C cold control demonstrated a more pronounced increase of 1.14 mg/g . Among the cold-stress treatments, the combination of $2\text{ mM SA}+20\text{ ppm MgO NPs}$ yielded the highest soluble sugar accumulation: 2.386 mg/g at 10°C and 1.781 mg/g at 4°C compared to

Fig. 1 A XRD spectrum of MgO NPs synthesized by green method. B FTIR spectrum of synthesized MgO nanoparticles

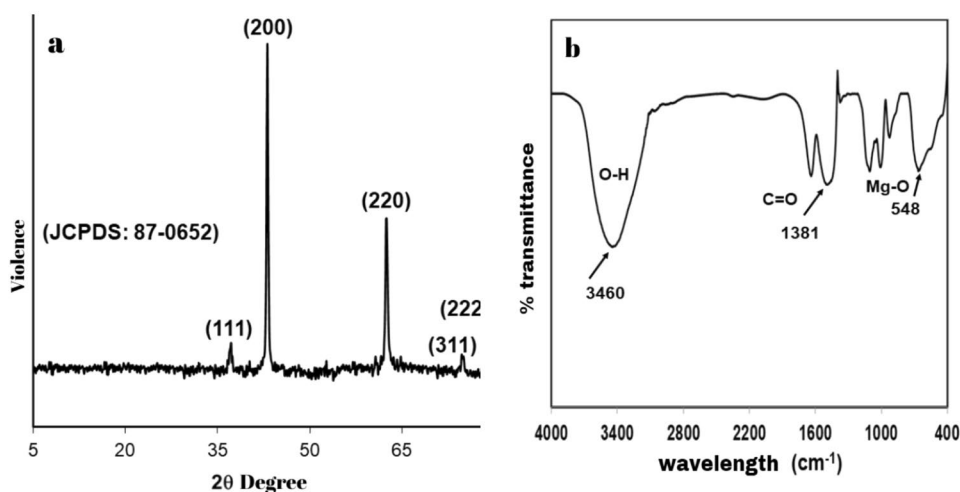
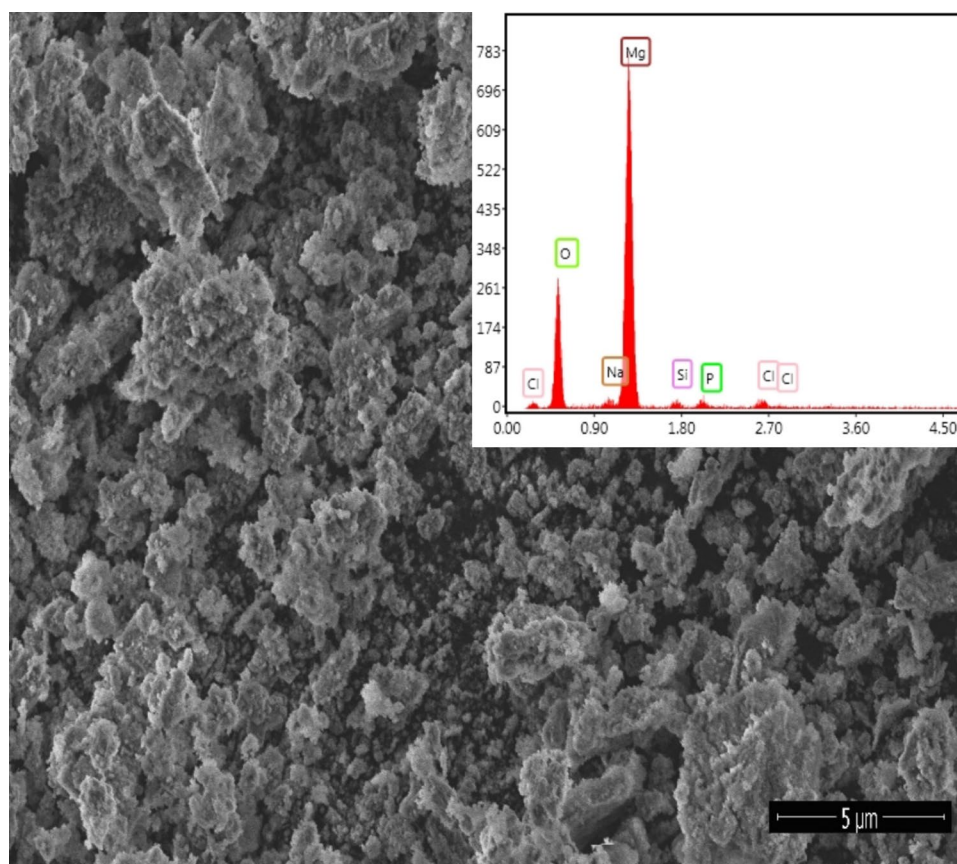


Fig. 2 SEM image and EDX spectrum of synthesized MgO nanoparticles



their respective cold controls. The Denizli genotype demonstrated similar trends, with its 10 °C cold control showing a 0.995 mg/g increase in soluble sugar content relative to the 25 °C control, while the 4 °C cold control displayed a greater accumulation of 1.212 mg/g. Among cold stress treatments, the 2 mM SA+20 ppm MgO NPs combination produced the maximum increase at 10 °C (2.680 mg/g), whereas at 4 °C, the 1 mM SA treatment showed the highest elevation (1.901 mg/g) compared to respective temperature controls. Comparative analysis revealed that the Denizli genotype consistently accumulated higher soluble sugar content than the Van genotype across both cold stress conditions (Fig. 3).

H₂O₂ content findings

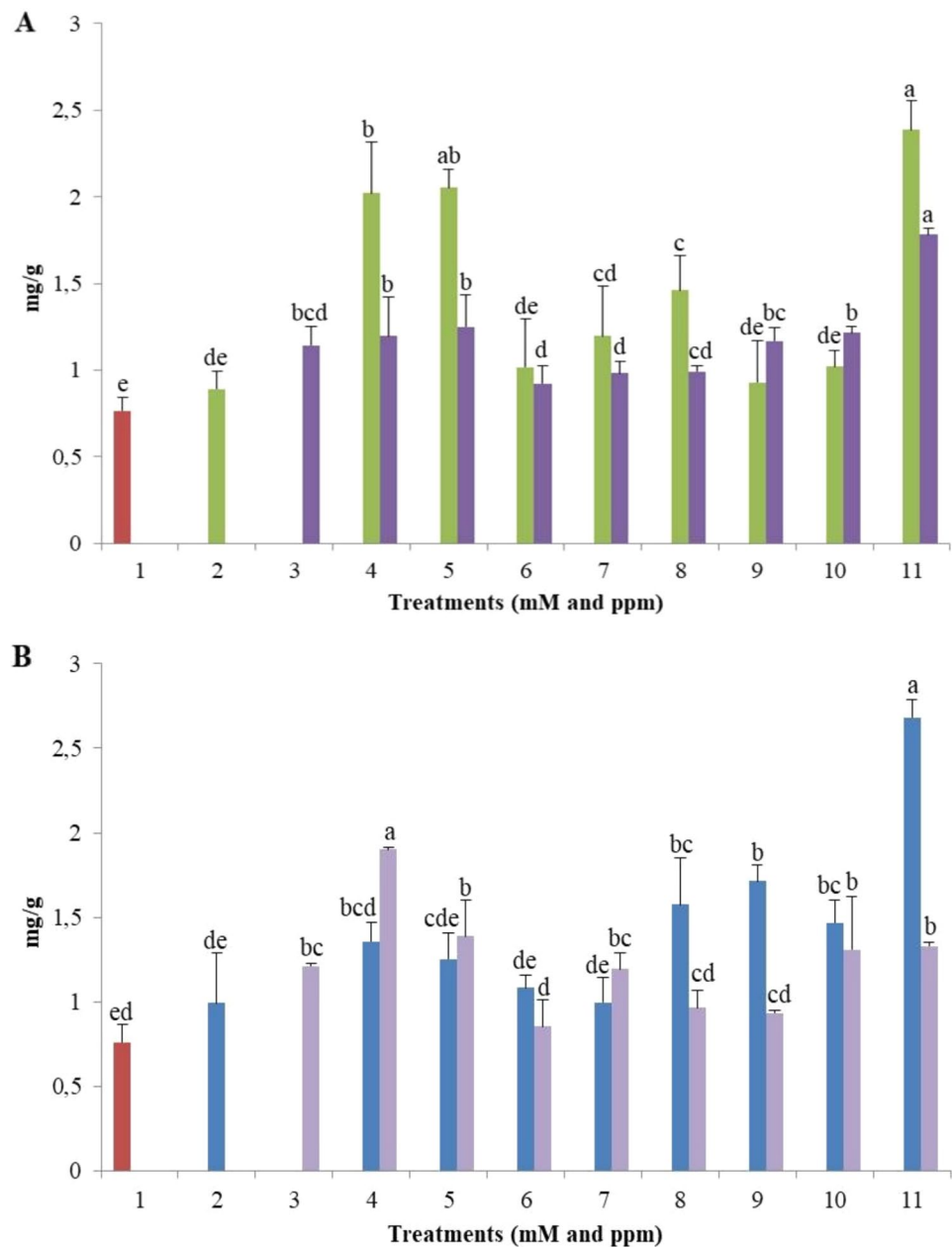
The H₂O₂ content in the Van genotype showed distinct temperature-dependent patterns, ranging from 0.319 to 1.291 μmol/g FW at 10 °C and 0.482 to 1.054 μmol/g FW at 4 °C. Compared to the 25 °C control, the 10 °C cold control displayed a 1.291 μmol/g FW, whereas the 4 °C treatment showed 0.831 μmol/g FW. Among cold stress treatments, the 2 mM SA+ 20 ppm MgO NPs combination most effectively mitigated H₂O₂ accumulation, reducing levels to 1.291 μmol/g FW at 10 °C and 0.831 μmol/g FW at 4 °C

relative to their respective cold controls. The Denizli genotype exhibited more pronounced H₂O₂ accumulation, with concentrations ranging from 0.469 to 1.191 μmol/g FW at 10 °C and 0.486 to 0.955 μmol/g FW at 4 °C. Notably, the 10 °C cold control showed a 1.191 μmol/g FW compared to the optimal temperature control, while the 4 °C treatment increased to 0.783 μmol/g FW. Treatment efficacy differed between temperatures: 20 ppm MgO NPs alone produced the maximal reduction at 10 °C, whereas the 2 mM SA+5 ppm MgO NPs combination was most effective at 4 °C. Collectively, both genotypes demonstrated significant attenuation of cold-induced H₂O₂ accumulation following SA and MgO NPs application (Fig. 4). The Van genotype showed greater responsiveness to combined treatments, while Denizli exhibited stronger responses to single-component applications, revealing genotype-specific mitigation patterns.

Lipid peroxidation content findings

The Van genotype exhibited differential malondialdehyde (MDA) accumulation under cold stress, with the 10 °C treatment reaching 0.87 μmol/g FW relative to the 25 °C control, while the 4 °C treatment demonstrated 0.77 μmol/g FW. Under 10 °C stress, the most effective MDA mitigation

Fig. 3 Total soluble sugar content in leaves of two alfalfa (*Medicago sativa* L.) genotypes: (A) Van and (B) Denizli. Treatment codes: 1: 25 °C Control, 2: 10 °C Cold Control, 3: 4 °C Cold Control, 4: 1 mM SA, 5: 2 mM SA, 6: 5 ppm MgO NPs, 7: 20 ppm MgO NPs, 8: 1 mM SA+5 ppm MgO NPs, 9: 1 mM SA+20 ppm MgO NPs, 10: 2 mM SA+5 ppm MgO NPs, 11: 2 mM SA+20 ppm MgO NPs. ^{a,c} Values with different superscript letters in the same column indicate significant differences



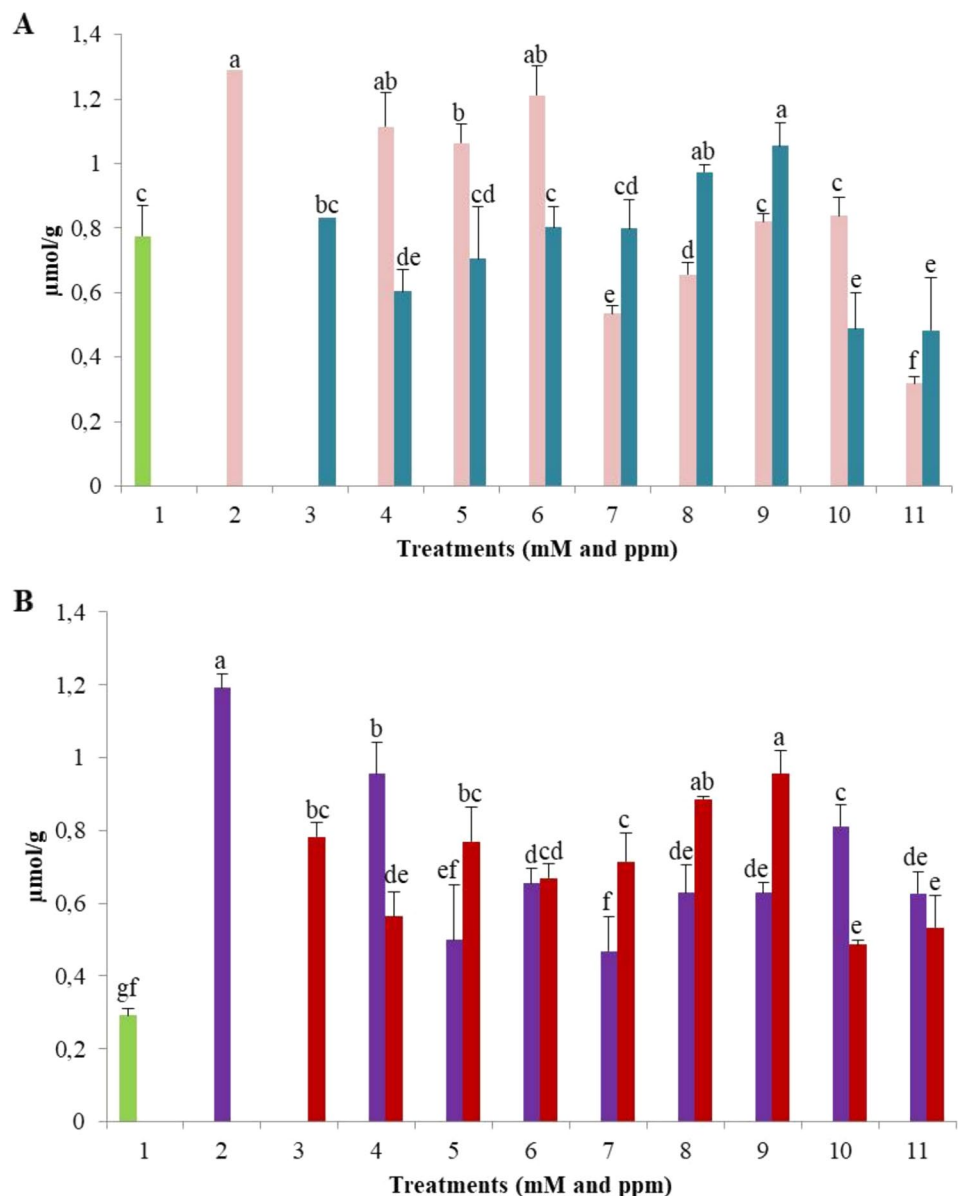
was achieved by combined SA and MgO NP treatments, with 2 mM SA+5 ppm MgO NPs and 2 mM SA+20 ppm MgO NPs reducing concentrations to 0.408 $\mu\text{mol/g}$ FW and 0.436 $\mu\text{mol/g}$ FW, respectively, compared to the cold control. In contrast, the Denizli genotype displayed greater cold-induced oxidative damage, as evidenced by MDA levels of 0.902 $\mu\text{mol/g}$ FW at 10 °C and 1.063 $\mu\text{mol/g}$ FW at 4 °C relative to controls. The 2 mM SA+ 20 ppm MgO NP treatment proved most effective at 10 °C, yielding 0.106 $\mu\text{mol/g}$ FW, while at 4 °C, 2 mM SA+5 ppm MgO NPs produced 0.483 $\mu\text{mol/g}$ FW in MDA content (Fig. 5). Notably, the Denizli genotype showed approximately 1.2–2.7-fold higher cold-induced MDA accumulation compared to Van,

suggesting greater inherent sensitivity to low-temperature oxidative stress. These genotype-specific responses highlight the importance of tailored stress mitigation strategies in *Medicago sativa* cultivars.

Proline content findings

Both genotypes exhibited substantial cold-induced proline accumulation, with treatment-enhanced responses. In the Van genotype, proline concentrations reached 3.146 $\mu\text{g/g}$ FW at 10 °C and 4.471 $\mu\text{g/g}$ FW at 4 °C relative to 25 °C controls. The 2 mM SA+20 ppm MgO nanoparticle treatment

Fig. 4 H_2O_2 content in leaves of two alfalfa (*Medicago sativa* L.) genotypes: (A) Van and (B) Denizli. Treatment codes: 1: 25 °C Control, 2: 10 °C Cold Control, 3: 4 °C Cold Control, 4: 1 mM SA, 5: 2 mM SA, 6: 5 ppm MgO NPs, 7: 20 ppm MgO NPs, 8: 1 mM SA+5 ppm MgO NPs, 9: 1 mM SA+20 ppm MgO NPs, 10: 2 mM SA+5 ppm MgO NPs, 11: 2 mM SA+20 ppm MgO NPs. ^{a,g} Values with different superscript letters in the same column indicate significant differences

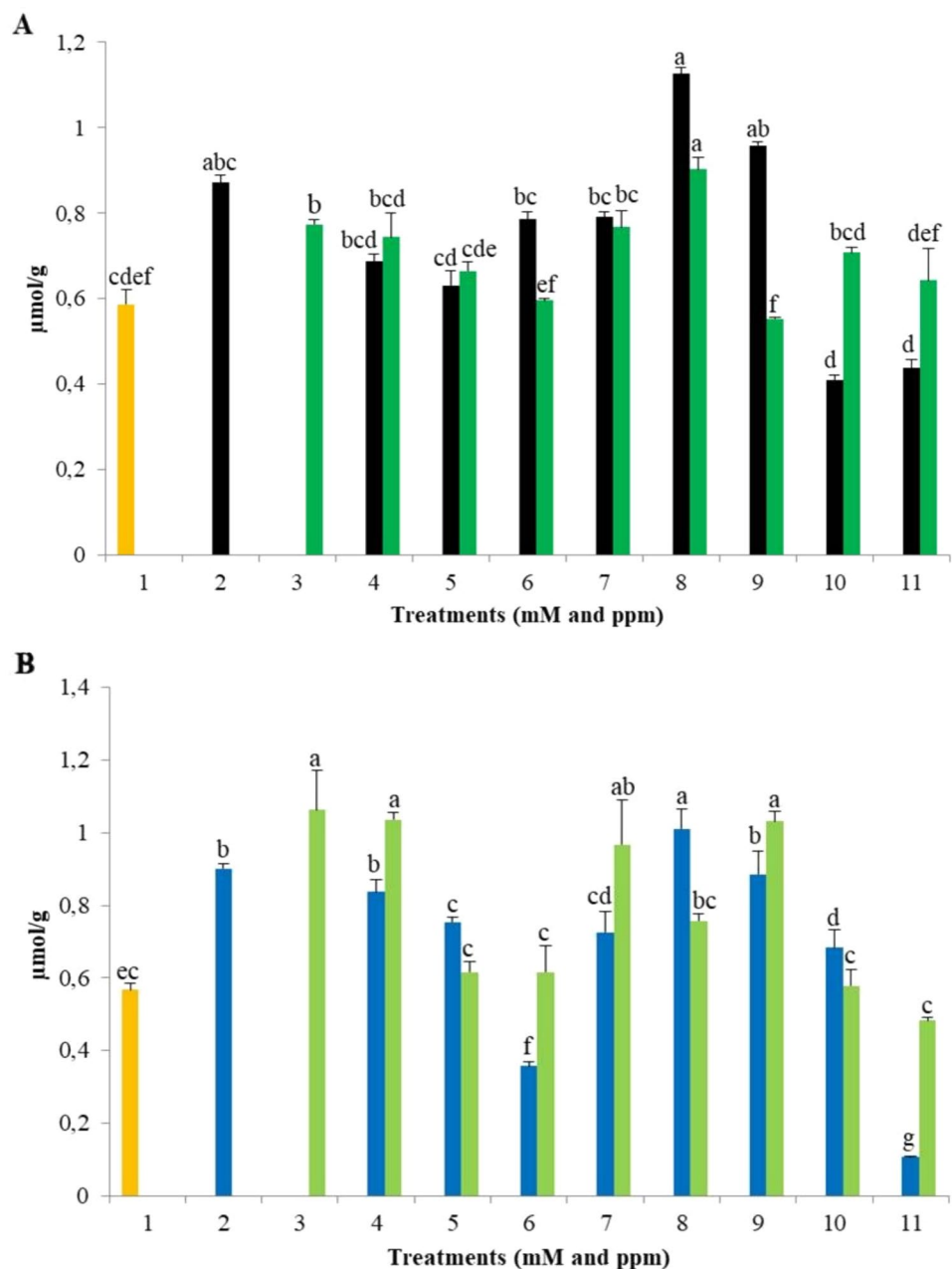


elicited maximal proline accumulation, yielding 7.300 $\mu\text{g/g}$ FW and 6.500 $\mu\text{g/g}$ FW at 10 °C and 4 °C, respectively, compared to temperature-matched controls. The Denizli genotype showed more pronounced cold-stress responses, with proline levels reaching 3.040 $\mu\text{g/g}$ FW at 10 °C and 3.751 $\mu\text{g/g}$ FW at 4 °C. The same SA+MgO NP combination demonstrated consistent efficacy, enhancing proline concentrations to 5.065 $\mu\text{g/g}$ FW and 5.260 $\mu\text{g/g}$ FW under 10 °C and 4 °C stress, respectively (Fig. 6). Notably, while absolute proline accumulation was higher in Van, Denizli exhibited greater relative increases from baseline levels.

SEM analysis findings

SEM analysis was conducted to examine the intracellular structures within the leaves of Van and Denizli genotypes treated with SA and MgO NPs under cold stress conditions. The results are presented in (Fig. 7). Observations of SEM images of the Van genotype revealed globular structures in 10 °C control 4 °C control, 10 °C 2 mM SA treatments. However, upon treatment with 25 °C control and 2 mM SA+5 ppm MgO NPs, spherical structures became more prominent, accompanied by noticeable stomatal apertures (Fig. 7A, F). The application of 4 °C 20 ppm MgO NPs resulted in the observation of membrane-like structures, while a combination of 2 mM SA and 5 ppm MgO NPs exhibited very fine fibrous structures. In the Denizli variety, fibrous

Fig. 5 MDA content in leaves of two alfalfa (*Medicago sativa* L.) genotypes: **(A)** Van and **(B)** Denizli. Treatment codes: 1: 25 °C Control, 2: 10 °C Cold Control, 3: 4 °C Cold Control, 4: 1 mM SA, 5: 2 mM SA, 6: 5 ppm MgO NPs, 7: 20 ppm MgO NPs, 8: 1 mM SA+5 ppm MgO NPs, 9: 1 mM SA+20 ppm MgO NPs, 10: 2 mM SA+5 ppm MgO NPs, 11: 2 mM SA+20 ppm MgO NPs. ^{a,g} Values with different superscript letters in the same column indicate significant differences

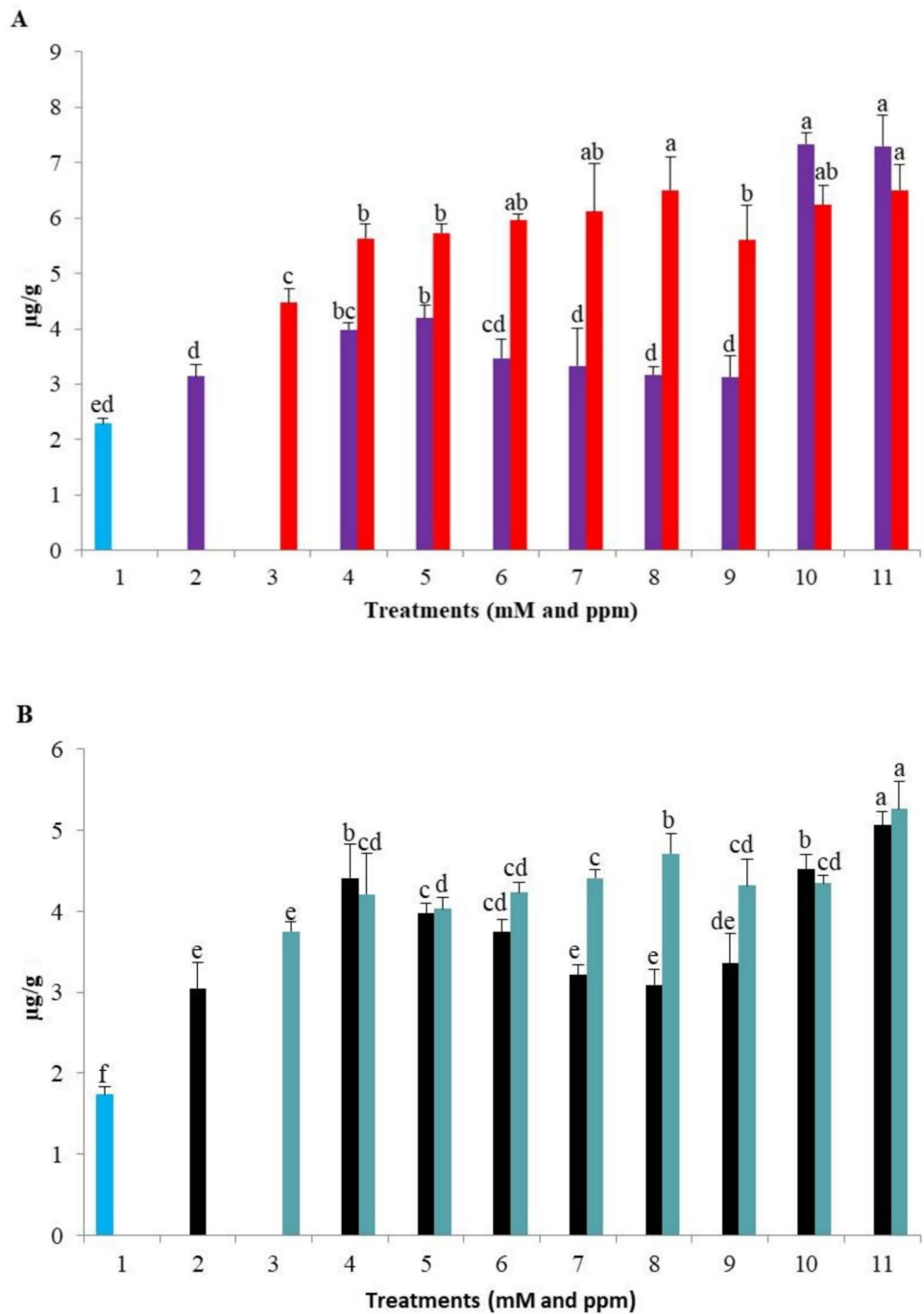


structures were evident in both the 25 °C and 10 °C control treatments, with pronounced stomatal openings observed on 25 °C Control and 10 °C 2 mM SA application (Fig. 7a, d). Dense spherical structures were observed with the combination of 2 mM SA+20 ppm MgO NPs. SEM images of the Van genotype at 25 °C, 10 °C and 4 °C control depicted globular structures, transitioning to spherical structures with 4 °C 20 ppm MgO NPs application. Combination treatment of 10 °C 2 mM SA+20 ppm MgO NPs. exhibited compact fibrous structures, and 2 mM SA with 20 ppm MgO NPs demonstrated small stomatal openings in Denizli genotype (Fig. 7f). Stomatal openings of both Van and Denizli genotypes were closed at 10 °C and 4 °C controls (Fig. 7B, b).

Evaluation of mtr-miR156a and mtr-miR173-5p gene expression altered by RT-qpcr

The gene expression patterns of mtr-miR156a and mtr-miR173-5p exhibited variations under cold stress conditions and treatments in both Van and Denizli genotypes. Comparisons between the 10 °C and 4 °C cold controls and the 25 °C control within each genotype group revealed distinct trends. In the Van genotype, there was a decrease in mtr-miR156a gene expression level under 10 °C cold control (0,424) and an increase under 4 °C cold control (1,775). Conversely, in the Denizli genotype, there was a significant

Fig. 6 Proline content in leaves of two alfalfa (*Medicago sativa* L.) genotypes: (A) Van and (B) Denizli. Treatment codes: 1: 25 °C Control, 2: 10 °C Cold Control, 3: 4 °C Cold Control, 4: 1 mM SA, 5: 2 mM SA, 6: 5 ppm MgO NPs, 7: 20 ppm MgO NPs, 8: 1 mM SA+5 ppm MgO NPs, 9: 1 mM SA+20 ppm MgO NPs, 10: 2 mM SA+5 ppm MgO NPs, 11: 2 mM SA+20 ppm MgO NPs. ^{a,f} Values with different superscript letters in the same column indicate significant differences



decrease in mtr-miR156a gene expression level under both 10 °C and 4 °C cold controls (0,245-0,076).

Upon analyzing treatments in the Van genotype under 10 °C cold stress, it was observed that mtr-miR156a gene expression generally increased, with the highest fold increase (1.723) observed in the 2 mM SA + 20 ppm MgO NPs combination treatment. Similarly, in the Van genotype under 4 °C cold stress, treatments with 5 ppm MgO NPs and 1 mM SA + 5 ppm MgO NPs combination led to decreased gene expression levels (1,526-0,753), while other treatments

generally increased mtr-miR156a gene expression. Notably, the 2 mM SA + 20 ppm MgO NPs combination treatment exhibited the strongest increase (5.375-fold change). In the Denizli genotype, both 10 °C and 4 °C cold treatments showed an overall increase in mtr-miR156a gene expression compared to cold controls alone. The 2 mM SA + 20 ppm MgO NPs combination exhibited the highest increases in mtr-miR156a gene expression, with a 6.330-fold change at 10 °C and a 3.521-fold change at 4 °C (Fig. 8).

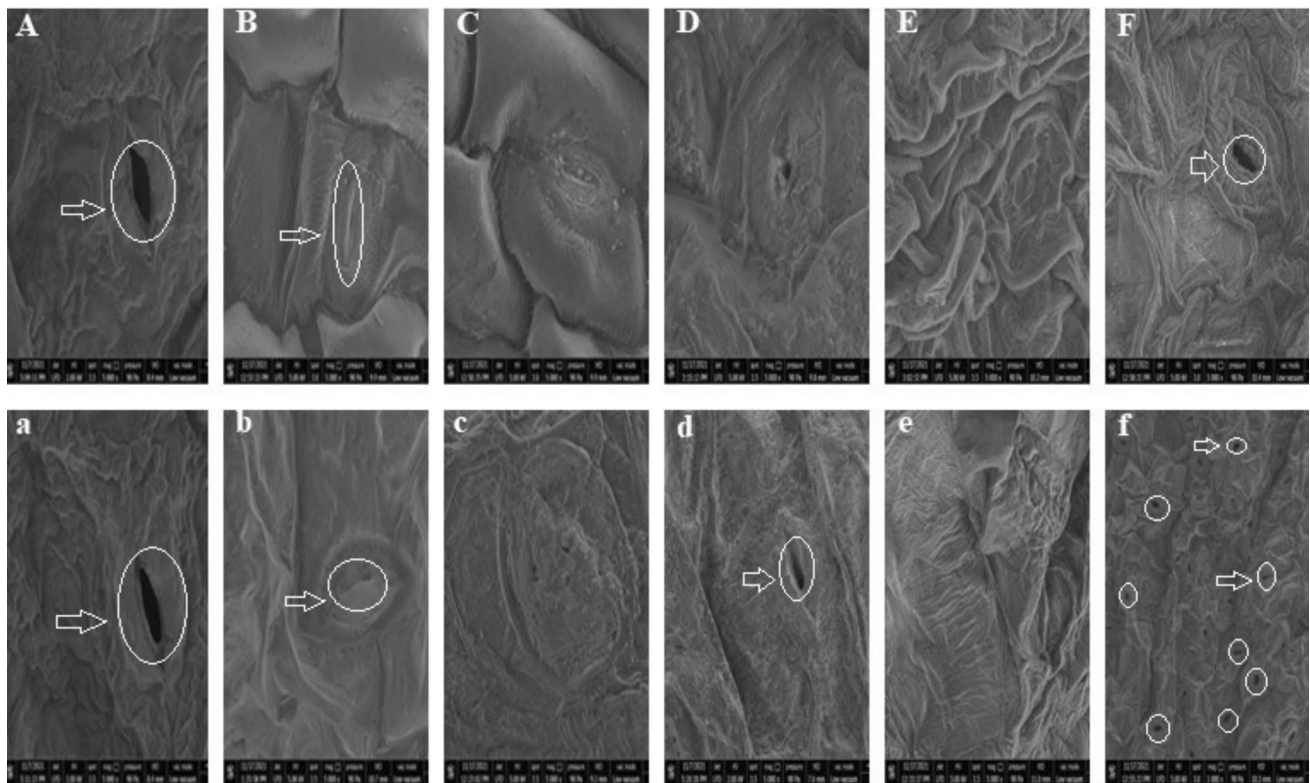


Fig. 7 1) Van genotype 10 °C and 4 °C SEM images **A**: 25 °C Control **B**: 10 °C Control **C**: 4 °C Control **D**: 10 °C 2 mM SA **E**: 4 °C 20 ppm MgO NPs **F**: 10 °C 2 mM SA+5 ppm MgO NPs 2) Denizli genotype

10 °C and 4 °C SEM images **a**: 25 °C Control **b**: 10 °C Control **c**: 4 °C Control **d**: 10 °C 2 mM SA **e**: 4 °C 20 ppm MgO NPs **f**: 10 °C 2 mM SA+20 ppm MgO NPs

When comparing the Denizli genotype with the 25 °C control within their respective groups, the *mtr-miR173-5p* gene expression in the Van genotype showed a 1.506-fold change under the 10 °C cold control and a 0.821-fold change under the 4 °C cold control. In contrast, the Denizli genotype exhibited an increase in *mtr-miR173-5p* gene expression at both 10 °C and 4 °C cold controls, with 3.508-fold change and 4.130-fold change, respectively. Upon comparing the treatments in the Van genotype at 10 °C and 4 °C cold stress with the cold controls alone, a general decrease in gene expression levels was observed. At 10 °C, the lowest decrease in expression level was observed in the 2 mM SA + 20 ppm MgO NPs combination treatment, with a fold change of 0.273, while at 4 °C, the lowest decrease in expression level was observed in the 1 mM SA + 5 ppm MgO NPs combination treatment, with a fold change of 0.616. Similarly, when comparing the treatments in the Denizli genotype at 10 °C and 4 °C cold stress with the cold controls alone, a general decline in gene expression levels was observed. At 10 °C, the lowest decrease in expression level was observed in the 2 mM SA + 20 ppm MgO NPs combination treatment, with a fold change of 0.316. At 4 °C, the lowest decrease in gene expression level was noted in the 1 mM SA treatment, with a fold change of 0.601 (Fig. 9).

Discussion

During prolonged exposure to cold stress, plants elevate the levels of water-soluble carbohydrates to bolster their tolerance. Intracellular carbohydrate accumulation contributes to cold tolerance by depressing the freezing point within cells (Smirnov 1995). In this study, both cultivars of alfalfa (Van and Denizli) exhibited elevated total soluble sugar levels under the cold stress treatments at 10 °C and 4 °C alone, relative to the control. Furthermore, considering the cold stress treatments conducted at 10 °C and 4 °C on both Van and Denizli genotypes, it was observed that the total soluble sugar content increased more markedly, particularly in response to the combined application of high concentrations of SA and MgO nanoparticles (Fig. 3). These results are consistent with previous studies reporting similar trends across various plant species. For instance, wheat plants (*Triticum aestivum* L.) exhibited enhanced carbohydrate accumulation at cold stress. Additionally, the application of MgO nanoparticles to black chickpea plants resulted in increased carbohydrate content in both roots and shoots. Similarly, nine species of medicinal plants, including *Stevia rebaudiana* Bertoni, exhibited elevated carbohydrate levels under low temperature conditions (Javadian et al. 2010;

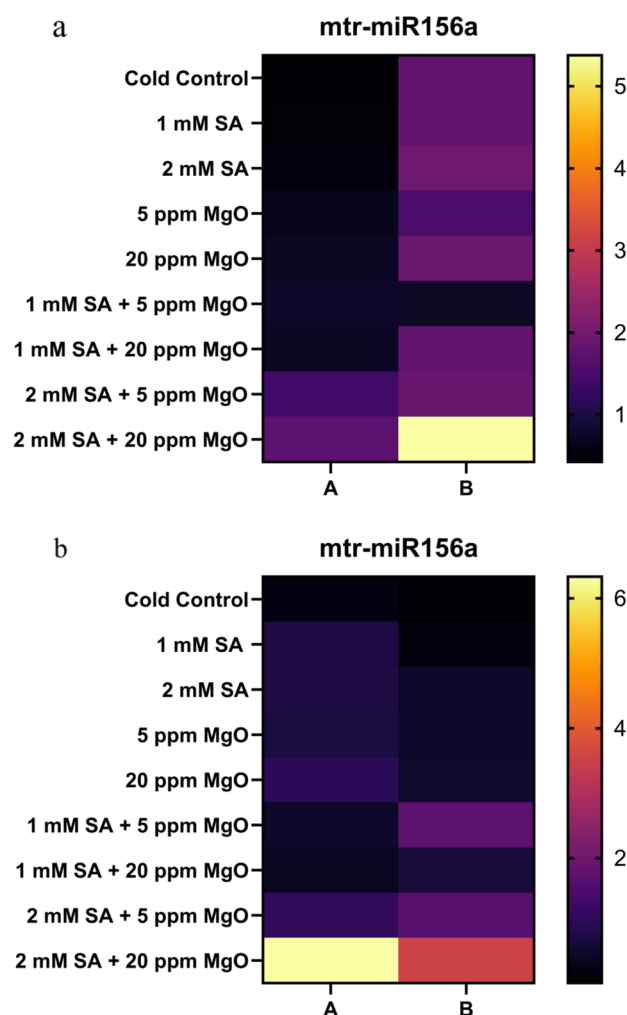


Fig. 8 Fold change heat map at mtr-miR156a gene level obtained by $2^{-\Delta\Delta CT}$ method. Colour scale indicates fold changes in gene expression. **a:** Van genotype **b:** Denizli genotype A: 10 °C B: 4 °C

Hajhashemi et al. 2018; Sharma et al. 2022). Additionally, under cold stress conditions (4 °C), the exogenous application of salicylic acid to *Vitis riparia* × *V. labrusca* seedlings led to an increase in soluble sugar content (Li and Wang 2021). Our study revealed that both cultivars of alfalfa responded to cold stress (10 °C and 4 °C) with a significant increase in total soluble sugar content. This increase was more pronounced with the combined application of SA and MgO NPs, suggesting a synergistic effect.

Abiotic stress factors including cold stress in plants induce the production of ROS, such as H_2O_2 , ultimately leading to oxidative stress (Mittler et al. 2004). For instance, it was observed that the level of H_2O_2 increased in the leaves of wheat (*Triticum aestivum*) under cold stress conditions (5/2 °C), while its levels decreased upon application of exogenous sodium nitroprusside (SNP). Similarly, although arsenic stress alone led to an increase in H_2O_2 levels, the application of MgO nanoparticles reduced this

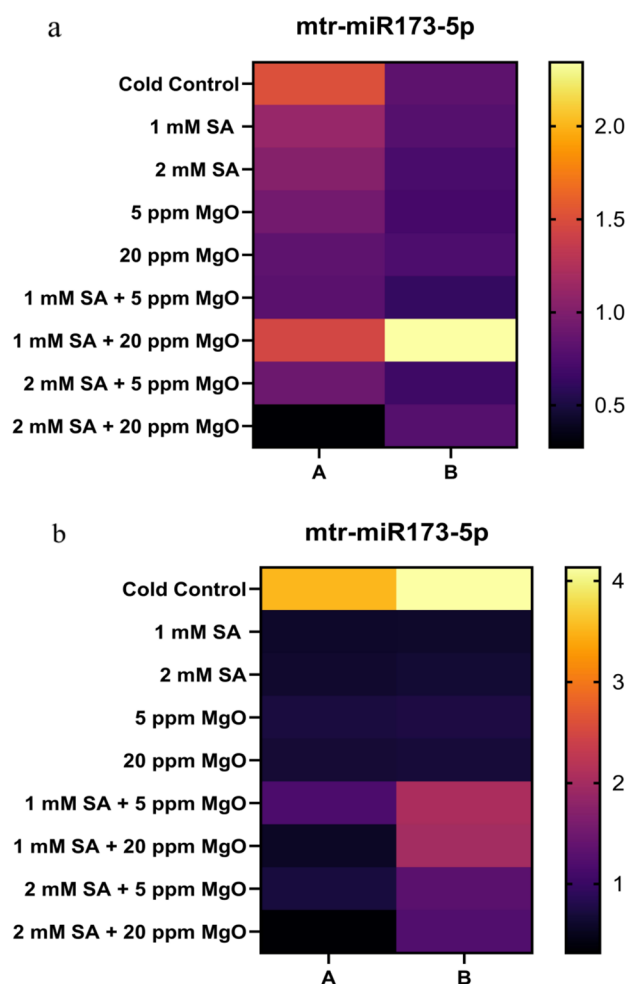


Fig. 9 Fold change heat map mtr-miR173-5p gene level obtained by $2^{-\Delta\Delta CT}$ method. Colour scale indicates fold changes in gene expression. **a:** Van genotype **b:** Denizli genotype A: 10 °C B: 4 °C

accumulation; a comparable reduction was also observed in *Hibiscus sabdariffa* under stress conditions following MgO nanoparticle treatment. Likewise, in winter wheat exposed to cold stress, H_2O_2 levels increased, but this rise was mitigated by the application of SA (Esim et al. 2012; Kiapour et al. 2020; Ahmed et al. 2021). Our investigation revealed an increase in H_2O_2 levels in both cultivars at 10 °C and 4 °C compared to the control. Interestingly, in both Van and Denizli cultivars, all treatments combined with cold stress led to a decrease in H_2O_2 levels at 10 °C. However, at 4 °C, the combination of a low dose of SA with both low and high doses of MgO NPs resulted in an increase in H_2O_2 levels. Conversely, applications of SA, MgO NPs, and high concentrations of SA and MgO NPs combinations actively contributed to enhancing cold tolerance by significantly reducing H_2O_2 levels, thereby mitigating the increase in reactive oxygen species (Fig. 4).

MDA is the final product of lipid peroxidation, and assessing MDA levels under stress conditions serves as a

marker for detecting irreversible membrane damage. Accumulation of MDA indicates compromised integrity of cell membranes (Balestrasse et al. 2010). The reduced concentration of MDA observed under stress conditions indicates a correspondingly lower degree of damage to the plant. Loss of cell membrane integrity is recognized as one of the primary physiological responses to cold stress. However, cold stress often leads to fluctuations in MDA levels, typically resulting in increased MDA accumulation in wheat plants as an indicator of lipid peroxidation and oxidative damage (Esim et al. 2012). An increase in MDA content has been reported under cold stress in both susceptible and resistant barley cultivars (Mutlu et al. 2016), as well as under salt stress in two *Pistacia vera* L. species (Gharibiyani et al. 2023). Conversely, the application of SA was found to mitigate this effect by reducing MDA accumulation. Similarly, the increased MDA content in chickpea (*Cicer arietinum* L.) genotypes under cold stress (4 °C) has shown a decrease with NPs application (Mohammadi et al. 2013). Our observations revealed an increase in MDA content in both cultivars at temperatures of 10 °C and 4 °C compared to the control representing cold stress conditions alone. However, application of SA and MgO NPs in combination with the cold stress generally mitigated the increase in MDA content across both Van and Denizli cultivars at both temperatures. However, the application of low doses of SA at 10 °C in both cultivars might have been insufficient to counteract the effects of cold stress when combined with MgO NPs, leading to an increase in MDA content. At 4 °C, MDA content increased when low concentrations of MgO NPs were combined with low concentrations of SA in the Van variety, and when high concentrations of MgO NPs were combined with low concentrations of SA in the Denizli variety. Notably, combinations of high concentrations of SA and MgO NPs significantly decreased MDA content and inhibited lipid peroxidation. This effect may be attributed to the stimulation of antioxidant enzyme systems by SA and MgO NPs, which facilitate the removal of free radicals responsible for the increase in MDA content (Fig. 5).

Proline serves as a critical component synthesized by organisms as a defense mechanism during stressful situations (Kaur et al. 2011). Several studies have reported an accumulation of proline in plants subjected to cold stress, highlighting its role in stress adaptation mechanisms. For instance, both cabbage and wheat leaves exhibit elevated levels of proline at lower temperatures (Atici et al. 2003). Similarly, it was reported that cold-tolerant cultivars of different walnut (*Juglans regia* L.) cultivars exhibited the highest proline content (Aslamaraz et al. 2011). Additionally, exogenous application of SA under cold stress conditions has been shown to enhance proline accumulation in various plant species, contributing to improved cold tolerance. For

instance, SA treatment at 4 °C increased proline levels in bean plants (Soliman et al. 2018), while 0.1 mM SA applied at temperatures ranging from 2 °C to −4 °C promoted proline accumulation and enhanced frost tolerance in wheat (Wang et al. 2022). We observed an elevation in proline concentration across all treatments and cultivars at temperatures of 10 °C and 4 °C compared to both the control subjected to cold stress alone and the control (Fig. 6). Furthermore, in both Van and Denizli varieties, the combination treatments of SA and MgO NPs demonstrated a more pronounced and statistically significant increase in proline content. This enhancement in proline content can be attributed to the antioxidant properties of SA and MgO NPs, which facilitate the removal of reactive oxygen species and protect membranes (Fig. 6).

miRNAs play pivotal roles in regulating gene expression (Bartel 2009). Currently, research has identified numerous studies investigating plant responses to diverse stresses, particularly elucidating the sensitivity of miRNAs to these stressors (Wei et al. 2009). The miR156 family targets the Squamosa Promoter Binding Protein-Like (SPL) genes, which encode various plant-specific transcription factors. The expression of miR156 and its target genes is closely linked to critical developmental processes and stress tolerance (Zhou and Tang 2019). Exogenous application of ZnO NPs to wheat leaves exhibited distinct transcriptional responses. At a toxic dose of 10 mg/L ZnO NPs, miR156 was down-regulated, whereas at a toxic dose of 50 mg/L, miR156 was induced. The overexpression of miR156 at phytotoxic levels of ZnO NPs resulted in the suppression of spike/grain development, achieved through the down-regulation of remobilization-related genes such as NAM and SUT (Niazi et al. 2023). Elevated expression of miR156 in alfalfa (*Medicago sativa* L.) enhances forage yield, delays flowering, and promotes increases in both root length and trichome density (Aung et al. 2015). miR156a exhibits up-regulation in response to 16 °C cold stress in *Arabidopsis* (Lee et al., 2010). A study investigating the identification of microRNA genes responsive to iron (Fe) deficiency in *Arabidopsis*, and their role in such conditions, revealed dynamic expression patterns of miR173. Initially, miR173 expression in shoots was up-regulated followed by down-regulation, indicating its responsiveness to Fe deficiency (Kong and Yang 2010). In *Arabidopsis* plants, the expression levels of miR173 were found to be down-regulated under conditions of sulfur (S) deficiency and excess cadmium (Cd) (Barciszewska-Pacak et al. 2015).

In this study, the expression of the mtr-miR156a and mtr-miR173-5p microRNA genes was examined in the Van and Denizli genotypes under cold stress at 10 °C and 4 °C (Fig. 8, 9). The results obtained in this study demonstrate that the response of these microRNAs varies across different

genotypes and cold stress conditions, suggesting their significant role in regulating cold tolerance in plants.

In the Van genotype, the expression of mtr-miR156a decreased under 10 °C cold stress compared to the control (Fig. 8), which is consistent with previous studies that suggest miR156 expression is downregulated under low temperatures (Xie et al. 2006). However, under 4 °C cold stress, the expression of mtr-miR156a was upregulated, suggesting a positive regulation of this microRNA at mild cold stress levels. This could indicate that miR156 helps plants adapt to moderate cold stress. Treatment with 2 mM SA + 20 ppm MgO NPs combination at both 10 °C and 4 °C induced the highest expression levels of mtr-miR156a. At 10 °C, the fold increase was 1.723, and at 4 °C, the fold increase was 5.375. This suggests that SA and MgO NPs have a synergistic effect in enhancing cold tolerance by modulating the expression of miR156. This finding is in line with previous studies, where SA was shown to modulate cold stress responses (Zhang et al. 2014) and NPs were found to improve stress tolerance in plants (Zhao et al. 2017).

In the Denizli genotype, the expression of mtr-miR156a was generally downregulated under both 10 °C and 4 °C cold stress conditions compared to the control (Fig. 8). This result is consistent with previous reports suggesting that miR156 expression is negatively regulated under cold stress conditions (Xie et al. 2006). However, the treatment with 2 mM SA + 20 ppm MgO NPs combination led to significant upregulation of mtr-miR156a, with fold increases of 6,33 (10 °C) and 3,521 (4 °C). This demonstrates that the combined application of SA and MgO NPs can effectively modulate miR156 expression, suggesting a role in enhancing cold stress tolerance. This is supported by the findings of Zhang et al. (2014) and Stief et al. (2014), who highlighted the importance of miR156 in regulating plant responses to environmental stresses, including cold.

These findings highlight the complex regulation of microRNA expression in response to cold stress across different genotypes. In the case of miR156, the expression patterns were genotype-dependent, with the Van genotype showing upregulation under 4 °C and downregulation under 10 °C, while the Denizli genotype exhibited a more consistent downregulation under both cold stress conditions. The treatment with SA and MgO NPs showed a positive effect on miR156 expression in both genotypes, supporting the idea that these treatments may enhance cold tolerance through modulation of this microRNA.

The expression of mtr-miR173-5p was also analyzed under cold stress conditions. In the Van genotype, expression levels increased 1.506-fold under 10 °C cold stress but decreased 0.821-fold under 4 °C. In contrast, the Denizli genotype exhibited a consistent upregulation of mtr-miR173-5p, with 3.508-fold and 4.130-fold increases

observed at 10 °C and 4 °C, respectively, compared to the control (Fig. 9). This suggests that miR173 might play a role in promoting cold tolerance through its increased expression in response to cold stress, as previously reported by (Mao et al. 2018), who identified miR173 as a key player in cold stress tolerance. When comparing treatments to cold controls, in the Van genotype, the lowest decrease in mtr-miR173-5p expression was observed in the 2 mM SA + 20 ppm MgO NPs combination at 10 °C, with a fold change of 0.273. At 4 °C, the lowest expression decrease was recorded in 1 mM SA + 5 ppm MgO NPs combination with 0.616 fold change. In Denizli genotype, 2 mM SA + 20 ppm MgO NPs combination at 10 °C caused a decrease with 0.316 fold change, while 1 mM SA application at 4 °C caused a decrease with 0.601 fold change. For miR173, both genotypes showed increased expression under cold stress, with the Denizli genotype exhibiting a more pronounced response. The results indicate that miR173 may be involved in promoting cold tolerance, as observed in previous studies. The treatment with SA and MgO NPs also influenced the expression of miR173, although the effects were more variable depending on the genotype and treatment combination. These findings suggest that miR173 may be associated with cold tolerance, and that genotype differences significantly influence the response to cold stress. Additionally, the combination of SA and MgO nanoparticles has been observed to modulate miR173 expression, potentially playing a role in regulating cold stress responses.

Conclusion

This study highlights the synergistic effects of SA and MgO NPs on the cold stress tolerance in the two alfalfa genotypes, Van and Denizli. Our findings indicate that both SA and MgO NPs play a crucial role in enhancing cold tolerance by modulating several physiological and molecular responses, particularly under prolonged cold stress conditions. Both genotypes demonstrated increased total soluble sugar content under the cold stress, with a more pronounced accumulation in response to the combined treatment of SA and MgO NPs. This suggests that these treatments may help lower the freezing point within plant cells, contributing to cold tolerance. Moreover, the reduction in oxidative damage, as indicated by lower H₂O₂ and MDA levels, supports the notion that SA and MgO NPs effectively mitigate oxidative stress by stimulating antioxidant defenses. In addition to the physiological changes, the expression of stress-related miRNAs, such as miR156 and miR173, was significantly affected by the combined treatments. The genotype-specific regulation of miRNAs observed in this study further underscores the complexity of the molecular responses to cold stress and the

potential for using SA and MgO NPs to enhance stress resilience through targeted gene regulation. These findings may have broader implications for improving the cold tolerance of other crops, especially in regions where low temperatures adversely affect agricultural productivity. Further research is needed to explore the underlying molecular mechanisms and the wider applicability of these treatments across various plant species.

Author contributions MA: Resource, materials, data collection and processing, analysis and interpretation, writing. BY: Materials, data collection and processing, analysis and interpretation. SBK: Materials. DI: Data collection and processing. AS: Writing, critical reviews. IB: Supervision, Concept, resource, writing, critical reviews.

Funding Open access funding provided by the Scientific and Technological Research Council of Türkiye (TÜBİTAK). We are greatly thankful to Erzurum Technical University Scientific Research Projects Coordination for funding the research work project no. 2022/001.

Data availability Data will be made available on request.

Declaration

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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