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Determination of Cold Tolerance in Local Alfalfa (M. sativa L.) Cultivars Grown in Türkiye under In Vitro Conditions through MsPYL3-3 and MsPYL5-1 Genes

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Abstract: Alfalfa is an important legume cultivated worldwide. Cold stress adversely affects alfalfa's growth and development. Recently, the use of phytohormone salicylic acid (SA) and nanoparticles are attracting attention as effective approaches for gaining tolerance in both plant development and stress defense mechanisms. This study investigated how the SA and magnesium oxide nanoparticles (MgO NPs) influence the expression of MsPYL3-3 and MsPYL5-1 genes in two alfalfa varieties, Denizli and Van, under cold stresses of 10°C and 4°C. Initially, plants were grown under controlled conditions (25°C, 16-hours light/8-hours dark photoperiod, 60% humidity) for one month before being exposed to cold treatments. The treatments included foliar applications of 1 mM and 2 mM SA, as well as 5 ppm and 20 ppm MgO nanoparticles. Gene expression was measured using RT-qPCR analysis. Results showed that lowering temperatures induced upregulation of MsPYL3-3 and MsPYL5-1 in both varieties. Moreover, treatment with SA and MgO nanoparticles further increased the expression levels of these genes. Notably, the gene expression responses varied between the Denizli and Van varieties, indicating genotype-specific differences.

Keywords: Medicago sativa L., Salicylic acid, MgO nanoparticle, MsPYL3-3, MsPYL5-1

INTRODUCTION

Alfalfa (*Medicago sativa* L.) is a legume highly valued for its exceptional nutritional content and significant economic importance. Often referred to as the "queen of forages," it is abundant in essential minerals, fiber, proteins, and vitamins. Beyond its role as a nutritious feed, alfalfa also contributes positively to soil health by enhancing soil structure and fertility. Alfalfa reduces the need for synthetic fertilizers and offers significant environmental advantages thanks to the symbiotic relationships it establishes with soil bacteria (Samac et al., 2006; Yang et al., 2010).

Cold stress is a major factor limiting plant growth, yield, and survival. Therefore, breeding or developing alfalfa varieties that can tolerate low temperatures is critically important from both scientific and practical viewpoints (Li et al., 2009). Cold stress affects plants when temperatures range between 0 and 15°C, and its impact becomes even more severe below freezing (<0°C). Low temperatures can drastically reduce seed germination rates and negatively influence both vegetative growth and reproductive processes during the growing season. In addition, exposure to cold stress triggers an increase in reactive oxygen species, causing extensive damage at the cellular level (Wu et al., 2008). Freezing temperatures, in particular, result in significant injury and yield loss in plants, making cold stress a key challenge in forage crop production. Given these productivity constraints, sustainable, cost-

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effective and environmentally friendly approaches are needed to cope with cold stress in crops (Szczerba et al., 2021; Adhikari et al., 2022). Employing such strategies to mitigate cold stress is essential to improve alfalfa productivity.

Nanoparticles have emerged as promising tools to combat various abiotic stresses affecting plants. Developments in nanotechnology offer new opportunities to boost crop productivity by supporting plant growth and mitigating stress-related damage (Shang et al., 2009). These nanoparticles can be produced using physical, chemical, or biological synthesis methods (Kumari & Nath, 2018). While MgO, ZnO, TiO_2, and CuO nanoparticles have raised concerns due to their potential toxicity, green synthesis techniques using biological materials have gained attention for producing nanoparticles with reduced harmful effects. Such biologically derived nanoparticles often exhibit antioxidant, antimicrobial, and anticancer activities, along with lower toxicity levels (Iravani, 2011; Nejati et al., 2022). Consequently, green-synthesized nanoparticles sourced from plants and microorganisms are favored because they are eco-friendly, stable, affordable, and safe (Ahmed et al., 2017; Bhardwaj et al., 2020). Among these, magnesium oxide (MgO) nanoparticles are particularly notable for their low cost, non-toxic nature, and multifunctional properties, including antibacterial, anticancer, and antioxidant effects. MgO nanoparticles have been reported to enhance plant stress mechanisms, while also enhancing chlorophyll concentration and photosynthetic efficiency (Pugazhendhi et al., 2019; Shen et al., 2020).

The usage of plant hormones is of invaluable importance in increasing stress tolerance and increasing vegetative and reproductive success of the plant. SA compounds, found in limited amounts in plants, function as a signaling molecule that increases resistance to various stresses. (Karlidag et al., 2009). As a key plant growth promoter, SA is essential for numerous physiological processes, including seed germination, fruit maturation, and photosynthesis (Khan et al., 2015; Hernández et al., 2017; Klessig et al., 2018). In addition, SA contributes to the development of cytological, morphological, physiological and biochemical properties by regulating the expression of genes associated with cold stress responses in plants, thus supporting the increase of cold tolerance. (Miura and Tada, 2014; Arif et al., 2020; Wang et al., 2020; Gondor et al., 2022).

MsPYL3-3 and *MsPYL5-1* genes make important contributions to regulating plant response mechanisms to cold stress by participating in hormonal signaling networks. These genes belong to the *PYR*/*PYL* receptor family, which mediates abscisic acid (ABA) signaling and regulates the expression of downstream stress-responsive genes that contribute to enhanced cold tolerance. In Arabidopsis, overexpression of genes encoding *C-repeat binding factors* (*CBFs*), which are functionally related, has been shown to significantly improve cold acclimation and increase stress resilience (Zhao et al., 2016). The interaction between cold stress and hormonal signaling especially involving genes like *MsPYL3-3* and *MsPYL5-1* offers an essential regulatory basis for enhancing cold tolerance in various plant species. Additionally, elevated expression of both genes has been correlated with the upregulation of protective proteins that help alleviate cold-induced damage, as documented in other studies examining plant stress responses (Kidokoro et al., 2015; Deng et al., 2018). Therefore, elucidating the functional roles of *MsPYL3-3* and *MsPYL5-1* may provide a valuable framework for novel acquisitions to enhance tolerance to cold stress in plants.

Although some studies in the literature have emphasized on the contribution of SA and nanoparticles to cold tolerance in plants, studies on the improvement of cold tolerance with SA and magnesium oxide nanoparticles in different alfalfa genotypes are quite limited. In this context, this study aimed to reveal the changes in the expression levels of *MsPYL3-3* and *MsPYL5-1* putative cold stress genes in alfalfa varieties, using qRT-PCR.

MATERIALS AND METHODS

Plant Materials and Stress Treatments

This study was conducted in the Molecular Genetics Laboratory of the Life Sciences and Technology Application and Research Center at Kafkas University. Salicylic acid (Merc) was purchased commercially from companies. MgO nanoparticles were obtained by Green Biosynthesis in the Molecular Biology and Genetics and Chemistry departments of Atatürk University (Türkiye). Afterwards, these NPs were characterized by SEM (Scanning Electron Microscope) and FTIR (Fourier transform infrared) analyses (Akçay et al., 2025). The plant material used in the study consisted of *Denizli* and *Van* alfalfa varieties. The seeds were obtained from local farmers. Initially, surface sterilization of the seeds was achieved using a 0.5% sodium hypochlorite (NaClO) solution. For sowing, trays (vials) with a depth of approximately 7 cm were used. An equal number of seeds were sown in each tray, followed by irrigation. After planting, the trays were placed in a climate chamber with 25°C, 60% relative humidity and 16/8 h light/dark photoperiod settings to initiate the growth process. During the four-week growth period, plants were watered only with drinking water. At the end of the fourth week, pre-planned cold stress, hormone and nanoparticle treatments were applied. Cold stress levels and applied hormone SA and nanoparticle (MgO) concentrations were determined based on preliminary experiments confirmed by literature (Akçay et al., 2025). The treatment groups used in the study are presented in Table 1. Solutions of SA and MgO nanoparticles were prepared using distilled water. These solutions were applied exogenously to plant leaves and kept at 25°C for one day to ensure absorption. Then, the first stage of cold stress treatment was initiated with the application of 10°C temperature with the above photoperiod conditions of the climate chamber. Plants were exposed to this condition for 2 weeks. At the end of the 2-weeks period, samples were taken from some of the plants for RNA isolation and stored at −80°C. The remaining plants were treated with SA and MgO nanoparticles again. These plants were kept at 10°C for one day to facilitate further absorption. Following this, the second stage of cold stress treatment was initiated by setting the climate chamber to 4°C under the same humidity and photoperiod conditions. The plants were exposed to this low temperature for 10 days, after which samples were collected for RNA isolation and stored at −80°C.

Table 1. *SA and MgO nanoparticle concentration applications*

Treatments	Concentrations
Control (25ºC)	0 mM SA, 0 ppm MgO
Cold Control (10ºC, 4ºC)	0 mM SA, 0 ppm MgO
SA (Salycyclic Acid)	1 mM SA
	2 mM SA
MgO (Nanoparticle)	5 ppm MgO
	20 ppm MgO

Determination of *MsPLY3-3* and *MsPLY5-1* Gene Expression Levels

In order to determine the changes in *MsPLY3-3* and *MsPLY5-1* gene expression levels in *Denizli* and *Van* alfalfa varieties exposed to SA and MgO nanoparticle applications under cold stress (10 and 4ºC), first RNA isolation, then cDNA synthesis and finally qRT-PCR procedures were carried out.

Total RNA Isolation

Total RNA was isolated in accordance with the TRIzol reagent (Thermo Fisher Scientific) protocol, following a series of carefully controlled steps. To begin, the centrifuge was set to cool at 4°C in preparation for the procedure. Approximately 0.2 grams of plant tissue were precisely measured using an analytical balance and then pulverized in liquid nitrogen with a mortar and pestle. Immediately after homogenization, 1 mL of TRIzol reagent was added to the powdered sample to facilitate thorough cell lysis. The mixture was left at room temperature for 5 minutes to allow the reagent to act. Next, 200 µL of chloroform was introduced to the tube, followed by vigorous shaking, and the sample was allowed to rest for 3 minutes. Centrifugation was then carried out at 12,000 rpm for 15 minutes to separate the phases. The upper aqueous layer, containing RNA, was carefully transferred to new microcentrifuge tubes. Then, 250 µL of isopropanol was added to each sample, and the tubes were incubated at room temperature for 10 minutes. A second round of centrifugation was conducted at 12,000 rpm for 10 minutes. The supernatant was removed, and the resulting RNA pellet was rinsed with 1 mL of 70% ethanol that had been chilled to −20°C, followed by centrifugation at 7,500 rpm for 5 minutes. After discarding the ethanol, the pellet was left to air-dry until all residual ethanol had evaporated. The final step involved dissolving the RNA pellet in 40 µL of nuclease-free water. The amount of RNA was measured using a nanodrop device (Thermo Scientific Multiscan SkyHigh /A51119700). Finally, purity was determined by calculating the Abs260/280 nm and Abs260/230 nm ratios.

cDNA Synthesis

cDNA synthesis from RNA obtained by RNA isolation was performed according to the Thermoscientific RevertAid First Strand cDNA Synthesis Kit protocol using 6 µL of RNA and stem-loop primers, and the final volume was 20 µL. The reaction mixture contained 5X buffer, RNase inhibitor, dNTPs, RevertAid reverse transcriptase (RT), RNase-free water, random primers, and RNA samples in appropriate volumes, which were transferred into PCR tubes. The thermal cycling conditions for the reaction were as follows: an initial incubation at 65°C for 5 minutes, followed by 25°C for 5 minutes, and then 42°C for 60 minutes. Upon completion of the reaction, 50 µL of ultrapure (dH_2O) was added to each cDNA sample to finalize the synthesis. The resulting cDNA samples were subsequently used for Real-Time PCR analysis.

Real Time PCR (RT-qPCR)

Quantitative Real-time PCR was performed on Applied Biosystems, StepOne PlusTM S/N: 720012158 using the protocol of the Solis Biodyne Hot Firepol Evagreen Real-Time qPCR Supermix, 5X kit. Following the manufacturer's protocol, each 10 µL reaction mixture included 2 µL of Solis BioDyne EvaGreen Supermix, 0.2 µL of each primer (forward and reverse), 2 µL of cDNA template, and 5.6 µL of nuclease-free water. The reaction mixtures were transferred into 96-well PCR plates, sealed with adhesive film, and placed in the RT-qPCR system. The thermal cycling conditions began with an initial denaturation step at 95 °C for 12 minutes (one cycle), followed by 40 cycles consisting of denaturation at 95 °C for 15 seconds, annealing at 58 °C for 20–30 seconds, and extension at 72 °C for 20–30 seconds. The genes examined in this experiment *MsPYL3-3*, *MsPYL5-1*, and *Mtactin* along with their corresponding primer sequences, are detailed in Table 2. The *Mtactin* gene was included as the reference housekeeping gene. Gene expression was quantified using the $2^{-\Delta\Delta Ct}$ method based on the obtained RT-qPCR data.

Table 2. *Genes and primer sequences*

Gene	Forward	Reverse
MsPLY3-3	TTCAACCCTACAACCGATGCAACC	GCCGTGGGTGGTGGTGTTTG
MsPLY5-1	CAAGAGACAACCGCCTCCATCAC	CACATCACCGTCGCCACCAAC
Mtactin	TGACGGAGAATTAGGGTTCG	CCTCCAATGGATCCTCGTTA

RESULTS AND DISCUSSION

Fold Change in *MsPLY3-3* and *MsPLY5-1* Gene Levels in Leaf Samples of *Denizli* and *Van* Varieties

In the *MsPYL3-3* gene, while it was down-regulated by 5 ppm MgO (0.9) in the 10 °C *Denizli* variety with 25 °C and cold controls (1, 1.99), it was up-regulated by 1 mM SA (2.74), 2 mM SA (4.64), 20 ppm MgO (2.52). In the 4 °C *Denizli* variety with 25 °C and cold controls (1, 2.05), 1 mM SA (2.81), 2 mM SA (5.32), 5 ppm MgO (2.23) and 20 ppm MgO (3.23) it was up-regulated (Figure 1). In the 10 °C *Van* variety, 2 mM SA (1.12) was up-regulated, while 1 mM SA (0.41), 5 ppm MgO (0.54) and 20 ppm MgO (0.73) were down-regulated compared to 25 °C and cold controls (1, 0.89). In the 4 °C *Van* variety, 5 ppm MgO (0.64) and 20 ppm MgO (0.89) were down-regulated compared to 25 °C and cold controls (1, 1.69), while 1 mM SA (3.33) and 2 mM SA (1.75) were up-regulated (Figure 2).

In the *MsPYL5-1* gene, 1mM SA (3.79), 2mM SA (3.55), 5 ppm MgO (3.9) and 20 ppm MgO (3.62) were up-regulated in the 10 °C *Denizli* variety compared to cold controls (3.44). In the 4 °C *Denizli* variety, 1mM SA (5.09), 2 mM SA (4.61) were up-regulated, while 5 ppm MgO (2.72) and 20 ppm MgO (3.87) were down-regulated compared to cold controls (4.56) (Figure 3). In the 10 °C *Van* variety, 1mM SA (0.51), 2 mM SA (0.22), 5 ppm MgO (0.79) and 20 ppm MgO (0.64) were down-regulated with 25 °C and cold controls (1, 1.26). In the 4 °C *Van* variety, 1mM SA (1.95), 2 mM SA (1.59), 5 ppm MgO (1.41) and 20 ppm MgO (1.47) were up-regulated with 25 °C and cold controls (1, 1.33) (Figure 4).

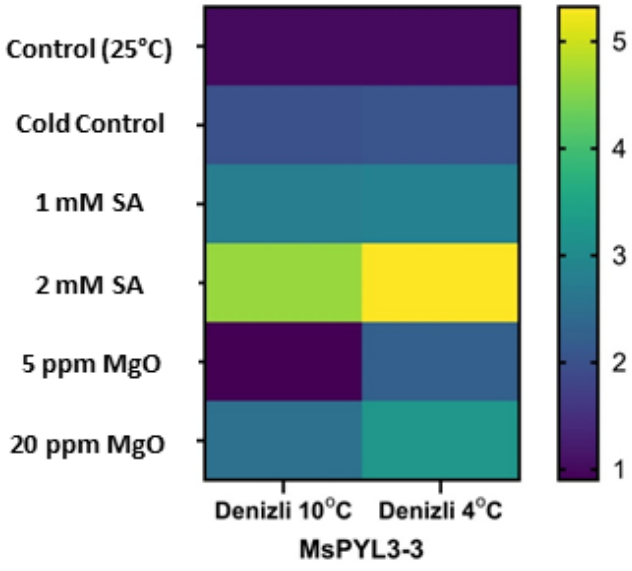

Figure 1. *Heat map of MsPYL3-3 gene level fold change obtained by* $2^{-\Delta\Delta CT}$ *method for the Denizli variety. The color scale indicates fold changes in gene expression.*

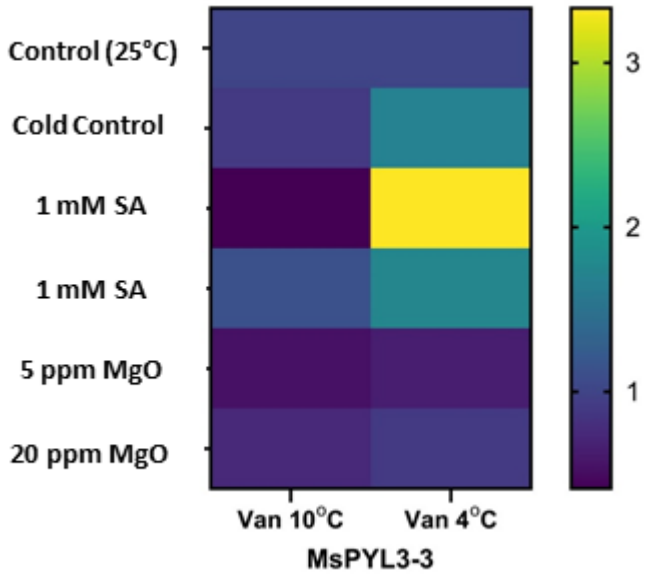

Figure 2. *Heat map of MsPYL3-3 gene-level fold change obtained by the $2^{-\Delta\Delta CT}$ method for the Van variety. The color scale indicates fold changes in gene expression.*

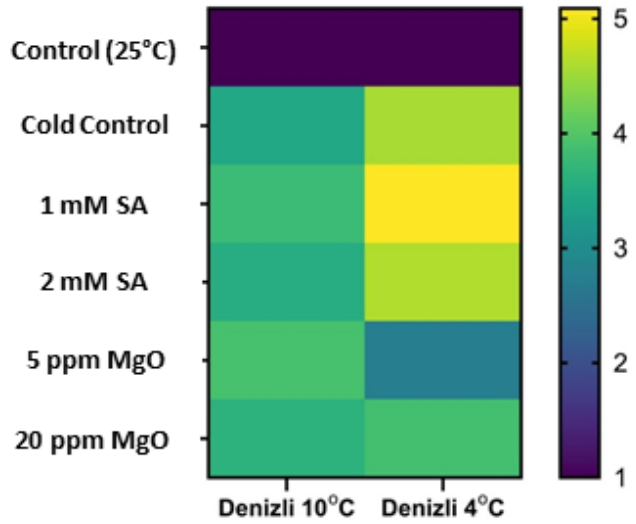

Figure 3. *Heat map of MsPYL5-1 gene level fold change obtained by $2^{-\Delta\Delta CT}$ method for Denizli variety. Color scale shows fold changes in gene expression.*

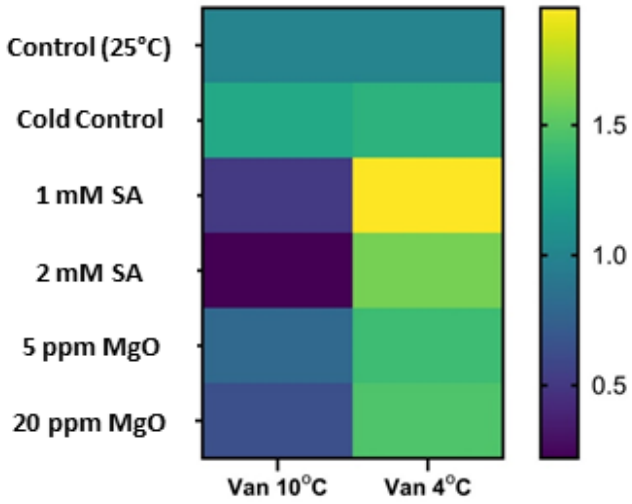

Figure 4. *Heat map of MsPYL5-1 gene level fold change obtained by $2^{-\Delta\Delta CT}$ method for Van variety. Color scale shows fold changes in gene expression.*

The results demonstrate that the expression patterns of *MsPLY3-3* and *MsPLY5-1* vary notably under cold stress, depending on the alfalfa variety and the treatment applied. This variation suggests that different alfalfa genotypes may possess unique mechanisms to eliminate low temperature stress. Both genes were generally upregulated in response to cold in the two studied varieties; however, the *Denizli* variety exhibited a stronger transcriptional response at both 10°C and 4°C compared to the *Van* variety (Fig. 1, 2, 3, 4). This enhanced gene expression indicates that *Denizli* may have a more efficient regulatory mechanism contributing to greater cold tolerance. In support of this idea, Ren et al (2022) reported that the *VaPYL4* gene from *Vitis amurensis* also increased resistance to cold stress when overexpressed in *Arabidopsis thaliana*. This highlights the broader role of *PYL* family genes in stress resilience across plant species. Similarly, Nian et al. (2021) identified several PYL genes in *Medicago sativa* L., most of which were upregulated in response to 4°C cold treatment. Their study found that *MsPLY3-3* was particularly induced under long-term cold exposure, while *MsPYL5-1* showed increased expression only during short-term stress and was suppressed with extended exposure. These observations emphasize that gene activation is affected not only by temperature severity but also by the duration of the stress period.

Evaluation of exogenous treatments revealed that SA applications at both 1 mM and 2 mM concentrations influenced gene expression levels in a variety- and temperature-dependent manner. In the *Denizli* cultivar, treatment with 2 mM SA induced the highest expression of the *MsPLY3-3* gene, whereas 1 mM SA triggered the most significant upregulation of *MsPYL5-1* (Fig. 1, 3). These results highlight the potential role of SA in developing defense mechanisms against environmental stress factors in plants. Supporting this, Zhao et al. (2020) demonstrated that during seed germination in *Arabidopsis thaliana*, the expression of several *PYL* family genes, particularly *PYL11* and *PYL12*, was elevated in response to external ABA treatment, influencing both germination and seedling growth. Based on this, it is plausible that SA may function similarly to plant hormones, enhancing gene expression and contributing to cold stress resistance during early growth phases. As for MgO nanoparticle applications (5 and 20 ppm), *MsPLY3-3* gene expression tended to decrease under both temperature conditions in the *Van* variety (Fig. 2). In contrast, *MsPYL5-1* was upregulated under 10°C cold stress (Fig. 4) but showed reduced expression at 4°C in the *Denizli* variety (Fig. 3). Overall, both *MsPLY3-3* and *MsPYL5-1* showed increased expression at 10°C (Fig. 1, 2, 3, 4). These findings emphasize not only the genotype-specific

nature of stress responses but also the importance of temperature and concentration in determining the regulatory impact of nanoparticle treatments on gene expression.

The *MsPYL3-3* and *MsPYL5-1* genes are integral to plant cold stress adaptation, functioning within regulatory networks that respond to environmental stimuli. These genes are part of the *PYR/PYL/RCAR* family, which encodes receptors for abscisic acid (ABA) a central phytohormone known to coordinate plant responses to abiotic stress (Zhang et al., 2012; Sun et al., 2022). Data from this study indicate that the expression of these genes triggers a cascade of adaptive processes, including regulation of genes associated with cold stress and cellular mechanisms that facilitate acclimation to low temperatures. When plants encounter cold environments, they initiate a series of molecular and physiological changes. One of the most critical responses involves the increased expression of *Cold-Responsive (COR) genes* and *C-repeat Binding Factors (CBFs)* (Miura et al., 2011; Vijayakumar et al., 2016). These gene families contribute to enhanced freezing tolerance by regulating transcription factors within the *CBF* pathway and activating downstream *COR* genes, helping to stabilize internal cellular conditions during stress (Zhao et al., 2016; Hong & Ryu, 2023). Additionally, research has confirmed that upregulation of *PYL* genes is linked to improved cold resistance. This is believed to occur through their role in modulating stress response pathways, including those that influence stomatal closure and transcriptional regulation during adverse conditions (Seki et al., 2002; Zhang et al., 2012).

The significance of *MsPYL3-3* and *MsPYL5-1* is highlighted by the crucial need to understand how plants cope with the cold stress, especially in crops exposed to low temperatures during vital developmental phases. Studies involving comparative transcriptome analysis have revealed that genes related to cold tolerance show varied expression patterns across different plant varieties or genotypes. This variation implies that certain *PYL* genes, including those from the *MsPYL* family, might play key roles in the mechanisms plants use to endure cold conditions (Hall et al., 2008; Cofer et al., 2018). Furthermore, research has underscored the importance of tightly controlled gene regulation to counteract cold stress, particularly through the interactions between reactive oxygen species (ROS) signaling pathways and cold-responsive genes (Yu et al., 2014; Liu et al., 2022). These previous findings serve as a valuable framework supporting the aims of this study.

CONCLUSION

This research explored how the phytohormone Salycylic acid and magnesium oxide nanoparticles influence the expression of *MsPYL3-3* and *MsPYL5-1* genes in two alfalfa varieties (*Denizli* and *Van*) subjected to cold stress at 10°C and 4°C. The findings proved that cold stress generally led to an upregulation of these genes in both varieties, with Denizli showing a notably stronger response. Additionally, treatment with SA and MgO nanoparticles under cold conditions further amplified gene expression, again with a greater effect observed in the *Denizli* variety. Overall, the study demonstrates that both SA and MgO nanoparticles modulate *PYL* gene expression during cold stress, with variations dependent on the specific cultivar. These results point toward the potential use of SA and nanoparticle applications as effective methods to boost cold tolerance in plants. Future research focusing on the regulatory mechanisms of these genes and cultivar-specific reactions could offer important insights for enhancing crop resilience to environmental stress.

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AUTHOR CONTRIBUTIONS

The authors contributed equally to this study.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

REFERENCES

Adhikari, L., Baral, R., Paudel, D., Min, D., Makaju, S. O., Poudel, H. P., Acharya, J. P., & Missaoui, A. M. (2022). Cold stress in plants: Strategies to improve cold tolerance in forage species. *Plant Stress*, *4*, 100081.

Ahmed, S., Chaudhry, S. A., & Ikram, S. (2017). A review on biogenic synthesis of ZnO nanoparticles using plant extracts and microbes: a prospect towards green chemistry. *Journal of Photochemistry and Photobiology B: Biology*, *166*, 272-284.

Akçay, M., Yazıcılar, B., Kassa, S. B., Ilhan, D., Shadıdızajı, A., & Bezırganoglu, I. (2025). Synergistic effects of salicylic acid and magnesium oxide nanoparticles on cold stress and miRNA expression in alfalfa (*Medicago sativa* L.) genotypes. *Plant Cell, Tissue and Organ Culture (PCTOC)*, *161*(3), 1-18.

Arif, Y., Sami, F., Siddiqui, H., Bajguz, A., & Hayat, S. (2020). Salicylic acid in relation to other phytohormones in plant: A study towards physiology and signal transduction under challenging environment. *Environmental and Experimental Botany*, *175*, 104040.

Bhardwaj, B., Singh, P., Kumar, A., Kumar, S., & Budhwar, V. (2020). Eco-friendly greener synthesis of nanoparticles. *Advanced pharmaceutical bulletin*, *10*(4), 566.

Cofer, T., Engelberth, M., & Engelberth, J. (2018). Green leaf volatiles protect maize (zea mays) seedlings against damage from cold stress. Plant Cell & Environment, 41(7), 1673-1682. https://doi.org/10.1111/pce.13204

Deng, X., Wang, J., Li, Y., Wu, S., Yang, S., Chao, J., Chen, Y., Zhang, S., Shi, M., & Tian, W. (2018). Comparative transcriptome analysis reveals phytohormone signalings, heat shock module and ros scavenger mediate the cold-tolerance of rubber tree. Scientific Reports, 8(1). https://doi.org/10.1038/s41598-018-23094-y

Gondor, O. K., Pál, M., Janda, T., & Szalai, G. (2022). The role of methyl salicylate in plant growth under stress conditions. *Journal of Plant Physiology*, *277*, 153809.

Hall, J., Cobb, J., Iqbal, M., Abidali, M., Liu, Z., & Mount, D. (2008). Uvh6, a plant homolog of the human/yeast tfiih transcription factor subunit xpd/rad3, regulates cold-stress genes in arabidopsis thaliana. Plant Molecular Biology Reporter, 27(2), 217-228. https://doi.org/10.1007/s11105-008-0076-x

Hong, J. and Ryu, H. (2023). Identification and functional analysis of cold-signaling-related genes in panax ginseng. Journal of Plant Biotechnology, 50. https://doi.org/10.5010/jpb.2023.50.028.225

Hernández, J. A., Diaz-Vivancos, P., Barba-Espín, G., & Clemente-Moreno, M. J. (2017). On the role of salicylic acid in plant responses to environmental stresses. *Salicylic acid: a multifaceted hormone*, 17-34.

Iravani, S. (2011). Green synthesis of metal nanoparticles using plants. *Green chemistry*, *13*(10), 2638-2650.

Karlidag, H., Yildirim, E., & Turan, M. (2009). Exogenous applications of salicylic acid affect quality and yield of strawberry grown under antifrost heated greenhouse conditions. *Journal of Plant Nutrition and Soil Science*, *172*(2), 270-276.

Khan, M. I. R., Fatma, M., Per, T. S., Anjum, N. A., & Khan, N. A. (2015). Salicylic acid-induced abiotic stress tolerance and underlying mechanisms in plants. *Frontiers in plant science*, *6*, 462.

Kidokoro, S., Watanabe, K., Ohori, T., Moriwaki, T., Maruyama, K., Mizoi, J., Htwe, N. M. P. S., Fujita, Y., Sekita, S., Shinozaki, K., Shinozaki, K. Y., & Yamaguchi-Shinozaki, K. (2015). Soybean dreb1/cbf-type transcription factors function in heat and drought as well as cold stress-responsive gene expression. The Plant Journal, 81(3), 505-518. https://doi.org/10.1111/tpj.12746

Klessig, D. F., Choi, H. W., & Dempsey, D. M. A. (2018). Systemic acquired resistance and salicylic acid: past, present, and future. *Molecular plant-microbe interactions*, *31*(9), 871-888.

Kumari, R., & Nath, M. (2018). Synthesis and characterization of novel trimethyltin (IV) and tributylltin (IV) complexes of anticoagulant, WARFARIN: Potential DNA binding and plasmid cleaving agents. *Inorganic Chemistry Communications*, *95*, 40-46.

Li, Y., Liu, G. B., Gao, H. W., Wang, Z., Zhao, H. M., Xie, N., & Wang, Y. H. (2009). Study on comprehensive evaluation of drought resistance of *Medicago sativa* L. germplasm at seedling stage. *Acta Agrestia Sinica*, *17*(6), 807.

Liu, Y., Cai, Y., Li, Y., Zhang, X., Shi, N., Zhao, J., & Yang, H. (2022). Dynamic changes in the transcriptome landscape of arabidopsis thaliana in response to cold stress. Frontiers in Plant Science, 13. https://doi.org/10.3389/fpls.2022.983460

Ma, Z., Huang, B., Xu, S., Chen, Y., Li, S. and Lin, S. 2015. Isolation of high-quality total RNA from chinese fir (*Cunninghamia lanceolata* (Lamb.) Hook). PLoS One, 10(6), e0130234.

Miura, K., Ohta, M., Nakazawa, M., Ono, M., & Hasegawa, P. (2011). Ice1 ser403 is necessary for protein stabilization and regulation of cold signaling and tolerance. The Plant Journal, 67(2), 269-279. https://doi.org/10.1111/j.1365-313x.2011.04589.x

Miura, K., & Tada, Y. (2014). Regulation of water, salinity, and cold stress responses by salicylic acid. *Frontiers in plant science*, *5*, 4.

Nejati, M., Rostami, M., Mirzaei, H., Rahimi-Nasrabadi, M., Vosoughifar, M., Nasab, A. S., & Ganjali, M. R. (2022). Green methods for the preparation of MgO nanomaterials and their drug delivery, anti-cancer and anti-bacterial potentials: A review. *Inorganic Chemistry Communications*, *136*, 109107.

Nian, L., Zhang, X., Yi, X., Liu, X., Ain, N. U., Yang, Y., Li, X., Haider, F. U., & Zhu, X. (2021). Genome-wide identification of ABA receptor PYL/RCAR gene family and their response to cold stress in *Medicago sativa* L. *Physiology and Molecular Biology of Plants*, *27*, 1979-1995.

Pugazhendhi, A., Prabhu, R., Muruganantham, K., Shanmuganathan, R., & Natarajan, S. (2019). Anticancer, antimicrobial and photocatalytic activities of green synthesized magnesium oxide nanoparticles (MgONPs) using aqueous extract of Sargassum wightii. *Journal of Photochemistry and Photobiology B: Biology*, *190*, 86-97.

Ren, C., Kuang, Y., Lin, Y., Guo, Y., Li, H., Fan, P., Li, S., & Liang, Z. (2022). Overexpression of grape ABA receptor gene VaPYL4 enhances tolerance to multiple abiotic stresses in *Arabidopsis*. *BMC Plant Biology*, *22*(1), 271.

Samac, D. A., & Jung, H. J. G, Lamb JFS (2006): Development of alfalfa (*Medicago sativa* L.) as a feedstock for production of ethanol and other bio products.

Seki, M., Narusaka, M., Ishida, J., Nanjo, T., Fujita, M., Oono, Y., Kamiya, A., Nakajima, M., Enju, A., Sakurai, T., Satou, M., Akiyama, K., Taji, T., Shinozaki, K. Y., Carninci, P., Kawai, J., Hayashizaki, Y., & Shinozaki, K. (2002). Monitoring the expression profiles of 7000 arabidopsis genes under drought, cold and high-salinity stresses using a full length cdna microarray. The Plant Journal, 31(3), 279-292. https://doi.org/10.1046/j.1365-313x.2002.01359.x

Shang, Y., Hasan, M. K., Ahammed, G. J., Li, M., Yin, H., & Zhou, J. (2019). Applications of nanotechnology in plant growth and crop protection: a review. *Molecules*, *24*(14), 2558.

Shen, Y., He, L., Yang, Z., & Xiong, Y. (2020). Corrosion behavior of different coatings prepared on the surface of AZ80 magnesium alloy in simulated body fluid. *Journal of Materials Engineering and Performance*, *29*, 1609-1621.

Sun, C., Zhu, L., Cao, L., Qi, H., Liu, H., Zhao, F., & Han, X. (2022). Pks5 confers cold tolerance by controlling stomatal movement and regulating cold-responsive genes in arabidopsis. Life, 12(10), 1633. https://doi.org/10.3390/life12101633

Szczerba, A., Płażek, A., Pastuszak, J., Kopeć, P., Hornyák, M., & Dubert, F. (2021). Effect of low temperature on germination, growth, and seed yield of four soybean (*Glycine max* L.) cultivars. *Agronomy*, *11*(4), 800.

Vijayakumar, H., Thamilarasan, S., Shanmugam, A., Natarajan, S., Jung, H., Park, J., Kim, H., Chung, M. Y., & Nou, I. (2016). Glutathione transferases superfamily: cold-inducible expression of distinct gst genes in brassica oleracea. International Journal of Molecular Sciences, 17(8), 1211. https://doi.org/10.3390/ijms17081211

Wang, W., Wang, X., Zhang, J., Huang, M., Cai, J., Zhou, Q., Dai, T., & Jiang, D. (2020). Salicylic acid and cold priming induce late-spring freezing tolerance by maintaining cellular redox homeostasis and protecting photosynthetic apparatus in wheat. *Plant Growth Regulation*, *90*, 109-121.

Wu, L., Zhang, Z., Zhang, H., Wang, X. C., & Huang, R. (2008). Transcriptional modulation of ethylene response factor protein JERF3 in the oxidative stress response enhances tolerance of tobacco seedlings to salt, drought, and freezing. *Plant physiology*, *148*(4), 1953-1963.

Yang, S. S., Xu, W. W., Tesfaye, M., Lamb, J. F., Jung, H. J. G., VandenBosch, K. A., Vance, C. P., & Gronwald, J. W. (2010). Transcript profiling of two alfalfa genotypes with contrasting cell wall composition in stems using a cross-species platform: optimizing analysis by masking biased probes. *BMC genomics*, *11*, 1-18.

Yu, G., Jiang, L., Ma, X., Xu, Z., Liu, M., Shan, S., & Cheng, X. (2014). A soybean c2h2-type zinc finger gene gmzf1 enhanced cold tolerance in transgenic arabidopsis. Plos One, 9(10), e109399. https://doi.org/10.1371/journal.pone.0109399

Zhang, F., Huang, L., Wang, W., Zhao, X., Zhu, L., Fu, B., & Li, Z. (2012). Genome-wide gene expression profiling of introgressed indica rice alleles associated with seedling cold tolerance improvement in a japonica rice background. BMC Genomics, 13(1). https://doi.org/10.1186/1471-2164-13-461

Zhao, C., Zhang, Z., Xie, S., Si, T., Li, Y., & Zhu, J. (2016). Mutational evidence for the critical role of cbf transcription factors in cold acclimation in arabidopsis. Plant Physiology, 171(4), 2744-2759. https://doi.org/10.1104/pp.16.00533

Zhao, H., Nie, K., Zhou, H., Yan, X., Zhan, Q., Zheng, Y., & Song, C. P. (2020). ABI5 modulates seed germination via feedback regulation of the expression of the PYR/PYL/RCAR ABA receptor genes. *New Phytologist*, *228*(2), 596-608.