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Greenhouse Evaluation of Nitric Oxide-Mediated Responses of Yaghooti Grapevine Under Low-Temperature Stress

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ABSTRACT

Low-temperature is one of the environmental stresses that affect the growth, development and performance of the grapevine. Nitric oxide (NO) is a relatively stable gas radical that prevents the production of reactive oxygen radicals at low concentrations. The present study aimed to investigate the physiological changes created in response to the foliar application of NO (0, 100 and 200 µM) in Yaghooti variety grape plants under two temperature treatments (24 ºC and 4ºC) in greenhouse conditions as a factorial experiment based on a completely randomized design. Based on the results, the application of NO increased the membrane stability in the leaf cells of the treated vines, which documented by lower ion leakage and malondialdehyde content in the vines sprayed with this growth regulator under low-temperature stress. On the other hand, in vines under low-temperature stress, the use of NO resulted in maintaining the concentration of chlorophyll, increasing the content of proline, total soluble sugar, total phenol, total flavonoid, soluble proteins and also caused a significant increase in the activity of antioxidant enzymes under low-temperature stress. By affecting compatible osmolytes, antioxidant enzymes, nutritional elements such as magnesium and iron, NO increased the tolerance of grape plants under low-temperature stress conditions. According to the results, foliar spray of NO especially at 200 µM is recommended to increase tolerance to low-temperature stress in grapes.

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1. Introduction

Low-temperature is one of the important environmental stresses that, through damage to the structure and function of biological membranes and disruption of cell metabolism, ultimately leads to reduced or stopped growth, poor yield, and gradual death of the plant (Karimi, 2020; Guo *et al*., 2025). One of the harmful consequences of low-temperature stress is the disruption of the function and structure of biological membranes, which affects the activity of enzymes and important metabolic processes such as photosynthesis in the cell (Guo *et al*., 2025; Zaeri-Behrooz *et al*., 2026).

Under low temperature conditions oxidative stress usually occurs, which is accompanied by increased electrolyte leakage, the formation of reactive oxygen species (ROS) such as superoxide (O^{-2}), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-), and singlet oxygen (O^-) (Karimi *et al*., 2016; Guo *et al*., 2025). Low-temperature stress causes oxidative stress in the plant by increasing ROS in plant cells. Also, under low-temperature stress conditions, potassium absorption decreases and osmotic regulation of cells is weakened, which disrupts transpiration, photosynthesis, and nutrient absorption by the plant (Negrao *et al*., 2017). In order to overcome oxidative stress caused by low-temperature and reduce the damage of ROS, plants are equipped with a series of enzymatic protective systems such as superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), and ascorbate peroxidase (APX), and non-enzymatic metabolites such as ascorbic acid, phenolic compounds, and glutathione (Karimi *et al*., 2016; Guo *et al*., 2025).

The application of compounds that cause homeostasis of cell metabolism and stability of biological membranes under stress conditions is one of the management methods of plants under environmental stresses (Karimi *et al*., 2016; Cui *et al*., 2025; Jafari et al., 2026). Nitric oxide is a relatively stable radical that can act as a molecule in the phenomenon of signal transduction in plants and interfere in various physiological processes such as seed germination, stomatal closure, response to pathogens and root development (Duan *et al*., 2007). On the other hand, NO can participate as an intermediary in the action of plant growth regulators and the metabolism of ROS. It is also involved in signal transduction and response to biotic and abiotic stresses (Cui *et al*., 2025). In some plants, exogenous application of NO has reduced damage caused by some stresses such as heavy metals, herbicides, low-temperature, ultraviolet radiation and salt stress (Babuta *et al*., 2025; Guo *et al*., 2025). In grape vines under salt stress, application of NO has led to an increase in chlorophyll, proline, total soluble sugar, total phenols, total flavonoids, soluble proteins content and also caused a significant increase in the activity of antioxidant enzymes (Ebrahimi *et al*., 2019). In broad bean (*Vicia faba* L.), the application of three levels of 0, 50, and 100 µM NO led to improved photosynthetic and physiological traits of this plant under low temperature stress (Nabati *et al*., 2025).

Grapes (*Vitis vinifera* L.) are one of the most important horticultural crops in the world and Iran, which is cultivated in more than 90 countries due to its numerous by-products. This extensive cultivation area has exposed grapevines to various environmental stresses, including low-temperature stress (Karimi, 2020; Guo *et al*., 2025). The 'Yaghooti' grape is one of the earliest-ripening grape cultivars in the Iran; due to its status as an early-season crop and its rapid market entry, it generates significant economic value for grape-growers. However, owing to its tropical origins, this cultivar is sensitive to low-temperature; consequently, in the context of climate change and the arrival of sudden low-temperature air masses, it often loses a substantial portion of its yield (Ershadi *et al*., 2016; Eshghi et al., 2024; Jabbari et al., 2025). Therefore, increasing the low-temperature tolerance of this grapevine variety in order to have sustainable production, at least through the use of exogenous protective compounds, seems more necessary than ever. No research has been conducted on the effect of NO treatments on grapevine plants under low-temperature stress. The aim of this study is to study the physiological and biochemical mechanisms of the effect of NO in reducing the effects of low-temperature stress, which has been evaluated on 'Yaghooti' grapevine under greenhouse conditions.

2. Materials and Methods

2.1. Plant materials and experimental design

In this experiment, the interaction effects of low-temperature stress and NO on physiological indices of grapes were evaluated in a completely randomized. Nitric oxide was applied at three levels (0, 100, and 200 µM) and temperature treatment was applied at two levels (24°C as greenhouse conditions and 4°C as low-temperature stress). Each experimental unit consisted of three cuttings, and each treatment was represented by three independent experimental units, giving a total of nine cuttings per treatment. After preparing one-year-old grape cuttings in mid-winter (5 Feb. 2024) from the research vineyard (#1) of the Grape and Raisin Research Institute of Malayer University, the cuttings were transferred to an educational-research greenhouse. After disinfecting the cuttings with 0.2% Benomyl, the cuttings were treated with the hormone indole-3-butyric acid (IBA) (1000 mg/L for 5 seconds) for fast and uniform rooting. In the third week of March, the rooted cuttings (a total of 10 per each treatment) were transferred to 10-liter plastic pots with a mixture of cocopeat and perlite (1:1; v/v) and placed in glasshouse with 1150 µmol m^{-2} s^{-1} PPFD; 24±1 ºC /18 ºC day/night temperatures and 67 % RH). It should be

noted that the cuttings randomly allocated to each experimental units and positions within the greenhouse also randomized. During this period, the pots were irrigated twice a week with a 0.5% solution of NPK20-20-20 fertilizer (Ziegler, Germany) and were subjected to other growth care for 60 days (approximately 15-20 leaf stage. Once the plants reached this growth stage, a NO treatment (0 and 100 µM sodium nitroprusside as a NO donor compound), was applied via foliar spraying in two stages, with a three-day interval between applications.

2.2. Temperature treatments

The temperature treatments considered for this experiment included 24°C (optimal growth temperature in the greenhouse) and 4°C (chill temperature). For this purpose, 48 hours after foliar spraying, potted vines treated with different concentrations of NO (0, 100, and 200 µM) were divided into two groups. The first group remained to a greenhouse at a temperature of 24±1°C and samples were taken after 12 hours. In the second group, the pots were first insulated with glass wool and then transferred to a cold chamber (Radelectronic, Tehran, Iran). The vines were gradually exposed to the desired low-temperature treatments (4°C) over a 12-hour period during the night. The starting temperature of the cold chamber was based on the ambient temperature at the time of pot transfer and the rate of temperature decrease was 2°C/h. After 12 hours, the temperature of the cold chamber was gradually increased and reached 15°C within 5 hours. After that, the low-temperature -treated vines were removed from the cold chamber and placed in a greenhouse at 24°C for three days to allow the vines to recover. Their fully developed upper leaves were harvested to measure the amount of soluble proteins and antioxidant enzymes (CAT, GPX, and APX) and immediately frozen in liquid nitrogen and stored in a -80°C freezer. Leaf samples were also harvested and transferred to the Horticultural Research Laboratory of Malayer University for measurement of chlorophyll and membrane lipid peroxidation.

2.3. Photosynthetic pigments

To measure the concentration of photosynthetic pigments such as chlorophylls a, b and carotenoids, 0.125 g of fresh leaf tissue was ground with 10 ml of 80% acetone in a porcelain mortar until it became a uniform mass. This operation was performed in low light and cool environment. Then the resulting extract was centrifuged at 6000 rpm for 10 minutes (Model R 320 Universal, Hettich, Germany) and the supernatant was removed and its light absorption was read at wavelengths of 663 nm (maximum light absorption of chlorophyll a), 645 nm (maximum light absorption of chlorophyll b) and 470 nm (maximum light absorption of carotenoids) by a spectrophotometer (Model Spekol 2000, Analytic Jena, Germany) using 80% acetone as a control. The concentration of each pigment in the extract was calculated in mg/L.

2.4. Proline

To extract proline, first half a gram of frozen leaf tissue was ground in a mortar using 5 ml of 95% ethanol and the upper part of the solution was separated. The extraction was repeated once more by adding 5 ml of 70% ethanol to the previous sediments. The extract was centrifuged for 15 minutes at 6000 rpm. To measure proline in each sample, one ml of extract was diluted with 9 ml of distilled water. To the above solution, 5 ml of ninhydrin reagent (consisting of: 0.125 g of ninhydrin + 2 ml of 6 M phosphoric acid + 3 ml of glacial acetic acid) was added along with 5 ml of glacial acetic acid and after slight shaking, it was placed in a steam bath (model WNB14, Memmert, Germany) at a temperature of 100 ºC for 45 minutes. After cooling the samples in ice water, 4 ml of toluene was added to each sample and shaken thoroughly so that proline entered the toluene phase. After 30 min, the absorbance of the toluene phase of each sample was determined with a spectrophotometer (model Spekol 2000, Analytic Jena, Germany) at a wavelength of 515 nm (Bates *et al*., 1973). Proline concentration was determined based on a pure proline standard curve and expressed in µg /g of leaf fresh weight (FW).

2.5. Soluble sugars and proteins

The extraction of soluble sugars was similar to proline. To measure soluble carbohydrates, 0.1 ml of the obtained alcoholic extract was mixed with 3 ml of freshly prepared anthrone (150 mg anthrone + 100 ml of 72% sulfuric acid). To start the color reaction, the tubes were placed in a 90°C hot water bath for ten minutes. After cooling, the absorbance of the samples was read with a spectrophotometer (Spekol 2000 model, Analytic Jena, Germany) at a wavelength of 625 nm (Irigoyen *et al*., 1992). The concentration of soluble sugars was determined based on the glucose standard curve and expressed as mg/g FW.

To measure the content of soluble proteins, 0.5 gr of fresh leaf tissue was ground with 5 ml of extraction buffer (Tris with a concentration of 1 mM and pH 7) in a Chinese mortar. It was thoroughly crushed and the mixture was centrifuged for 20 minutes at 6000 rpm. Then, 100 µl of the upper clear solution was mixed with 5 ml of Bio-Rad's reagent [(10% glacial acetic acid + 25% ethanol + 65% distilled water + 0.1% (v/w) Coomassie Brilliant Blue 250 solution)] and its absorbance was read with a spectrophotometer at a wavelength of 595 nm (Bradford, 1979). Using a standard curve prepared from different concentrations of bovine albumin, the concentration of soluble proteins was calculated as mg/g FW.

2.6. Total phenol and flavonoids

To measure the total phenol content in the leaves, first 0.5 gr of fresh leaf sample was thoroughly ground in 4 ml of ethanol to prepare a homogeneous solution and after 20 minutes of centrifugation at 9500 rpm, the clear supernatant solution was separated. 300 µL of the extract was mixed with 1200 microliters of 7% sodium carbonate and half a ml of 10% Folin-Ciocalteu and placed in a dark place for 20 minutes. After the required time, the absorbance was measured by a spectrophotometer at a wavelength of 725 nm and then, using the gallic acid standard diagram, the phenol content was obtained in terms of mg of gallic acid per FW (Karimi, *et al*., 2019).

To measure the amount of total flavonoids, the aluminum chloride colorimetric method was used (Chang *et al*., 2002). In this method, first 0.1 ml of 10% aluminum chloride was poured into the test tube, then 0.1 ml of 1 M potassium acetate was added to the tubes and mixed with it, and then 2.8 ml of distilled water was added to the tubes. In the final step, 0.5 ml of methanolic leaf extract solution was added to the mixture. The samples were placed in a dark environment for 30 minutes and finally the absorption of the samples at a wavelength of 415 nm was determined by a spectrophotometer. The amount of total flavonoids for each of the extracts was calculated as mg of quercetin per gram of FW.

2.7. Relative water content (RWC) and electrolyte leakage (EL)

To measure the RWC of the leaves, first leaf pieces with a radius of one cm were prepared and their FW was determined. After the leaf pieces were placed in distilled water (in a refrigerator for 24 hours), the turgor weight (TW) of the leaves was determined. To measure dry weight (DW), leaf pieces were dried in an oven (35-liter Hoshmand model, Shimaz Company, Iran) at 80 °C for 24 hours and then weighed. The RWC (%) was obtained from the following equation (Kirnak *et al*., 2001):

Equation 1: RWC (%) = (FW- DW) / (TW- DW) ×100

To measure EL, leaf samples (1 cm in diameter) were immersed in 70 mL plastic tubes containing 30 ml of distilled water and shaken on a shaker (model KS260 digital, IKA, Germany) (speed 120 rpm) for 20 hours. After this period, the electrical conductivity (EC) of the solution containing the samples was read using a conductivity meter (model Cond 720, WTW, Germany) (EC1). The tubes containing leaf pieces were autoclaved at 121 °C for 15 minutes (model 75 L, Reyhan Teb, Iran). After gradual cooling, their EC was read again (EC2) and finally the percentage of EL was calculated using the following equation (Campos *et al*., 2003).

Equation 2: EL (%) = (EC1/EC2) × 100

2.8. Malondialdehyde (MDA) and antioxidant enzymes

Membrane lipid peroxidation was measured in 200 mg of each leaf sample, which was homogenised in 5 ml of 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged at 6,000 × g for 5 min. The supernatant was collected and lipid peroxidation was measured in terms of malondialdehyde content, according to Heath and Packer (1968).

To measure the activity of antioxidant enzymes, an enzyme extract was first prepared. For this purpose, the frozen leaf tissue was first ground in the presence of liquid nitrogen in a Chinese mortar and 0.1 g of it was added to a plastic tube containing one mL of extraction buffer and mixed. After passing through the filter, the prepared extract was centrifuged for 15 minutes at a speed of 10,000 rpm and a temperature of 4 ºC, and the upper clear solution was gently separated. This solution was used to measure the activity of each of the antioxidant enzymes as follows:

To determine the activity of CAT, first 50 µL of plant extract was mixed with 3 mL of extraction buffer containing 50 mM sodium phosphate (pH 7) and 2 mM ethylene di-amine tetra acetic acid (EDTA). The CAT enzyme reaction was initiated by adding 5 µL of 30% H_2O_2 to the above mixture. Changes in light absorption of the samples were recorded at a wavelength of 240 nm for one minute. Each unit of CAT enzyme activity was considered as the amount of enzyme that reduces one µM of H_2O_2 per minute. The enzyme activity was expressed in units per mg of leaf protein (Bergmeyer, 1970).

To measure the activity of GPX enzyme, first 50 µL of plant extract was mixed with 3 ml of extraction buffer containing 50 mM sodium phosphate (pH 7) and 2 mM EDTA. The reaction of GPX enzyme was started by adding 5 µL of 30% H_2O_2 and 5 µL of guaiacol to the above mixture. The changes in light absorption of the samples at a wavelength of 465 nm, which indicates the degree of destruction and reduction in H_2O_2concentration, were recorded for one minute. Each unit of GPX enzyme activity was considered as the amount of enzyme that causes a reduction of one µmol of H_2O_2 per ml per minute. The GPX activity was expressed in units per mg of leaf protein (Herzog and Fahimi, 1973).

To measure the activity of APX enzyme, first 50 µL of plant extract were mixed with 3 ml of extraction buffer containing 50 mM sodium phosphate (pH 7), 2 mM EDTA, 1% polyvinylpyrrolidine-40 (w/v), 0.1% Triton-100 (v/v), and 1 mM ascorbate. The reaction of APX enzyme was initiated by adding 4.5 µL of 30% H_2O_2 to the above mixture. The changes in optical absorption of the samples at a wavelength of 290 nm, which indicates the degree of oxidation and reduction of ascorbate concentration, were recorded for one minute. Each unit of APX enzyme activity was considered as the amount of enzyme that causes the oxidation of one µmol of ascorbate per minute. The APX activity was expressed in units per mg of leaf protein (Nakano and Asada, 1981).

2.9. Leaf nutrients

For leaf nutrients analysis, the sampled leaves were digested in nitric acid after oven drying and their macronutrients (N, P, and K) and some microelements (Fe and Zn) were measured. An ANALYST70 model atomic absorption spectrophotometer (Perkin Elmer, USA) was used for Fe, and Zn analysis. Phosphorous was measured by Spekol 2000 spectrophotometer (Analytic Jena, Germany). For N and K measurement the samples were subjected to Kjeldahl and 405G Flame-photometry (Crouse, Germany) devices in respectively (Karimi *et al*., 2019).

2.10. Data analysis

Data were subjected to a two-way analysis of variance (ANOVA) using the GLM procedure in the SAS software package (SAS Institute Inc., Cary, NC, USA). Means separation was done using Duncan's multiple range test at a probability level of $P \leq 0.05$.

3. Results

3.1. Total chlorophyll

The effect of NO, temperature and their interaction on total leaf chlorophyll content was significant at the 1% level (Table 1). At 24 ºC, the leaf chlorophyll concentration of vines treated with NO increased so that in vines treated with 200 µM concentration this value reached 2.66 mg/g FW (Table 1).

Table 1. The combined effect of low-temperature and NO treatments on the amount of chlorophyll a, chlorophyll b, total chlorophyll and carotenoid in Yaghooti grape leaves.

Temperature regimes (ºC)	Nitric oxide (µM)	ChL a (mg/g FW)	ChL b (mg/g FW)	Total ChL (mg/g FW)	Carotenoids (mg/g FW)
24	0	1.62b	0.637b	2.26c	0.97b
24	100	1.86a	0.653ab	2.52b	0.85d
24	200	1.98a	0.677a	2.66a	0.85d
4	0	1.33c	0.436e	1.79e	1.11a
4	100	1.48b	0.551d	2.03d	0.92c
4	200	1.57b	0.585c	2.16d	0.90c

The same letters indicate no significant difference at the probability level of P<0.05.

The chlorophyll content in vines treated with low-temperature without NO treatment showed a significant decrease compared to the remaining plants at 24 °C. The trend of changes in chlorophyll a and b was similar to total chlorophyll and under low-temperature stress the content of these two types of chlorophyll was higher in vines treated with NO at 200 µM concentrations (Table 1). Carotenoid content in vines under low-temperature stress was higher than in vines treated with NO, indicating the role of this growth regulator in maintaining chlorophyll structure under low-temperature stress and was higher compared to carotenoid content. In low-temperature-stressed vines, NO treatment-maintained chlorophyll stability and even slightly increased it after foliar spraying with 100 and 200 µM concentrations of this growth regulator (Table 1).

3.2. Total soluble sugar

The effects of NO, low-temperature stress and their interaction on leaf soluble sugars content were significant at the 1% probability level (Table 2). By applying gradual temperature stress from 24 to 4°C, the leaf content of soluble sugars increased in all stressed vines. However, the increase of these osmoregulant in NO- treated vines was greater than control plants (0µM NO) under low temperature stress condition (Table 2). In the temperature treatment of 4 °C, the highest soluble sugars content was related to vines treated with 200 µM NO concentrations (Table 2). Vines under low-temperature stress without NO treatment had higher concentrations of soluble sugars compared to control vines under 24 °C (Table 2).

3.3. Proline

The effect of NO, low-temperature stress and their interaction on leaf proline concentration was significant at the 1% probability level. The trend of changes in proline concentration under the influence of temperature

treatments and different concentrations of NO was largely similar to the pattern of changes in soluble carbohydrates (Table 2). With a decrease in temperature from 24 to 4 °C, leaf proline content increased, and this increase was much greater in plants treated with NO compared to the control. The highest proline content was observed in temperature treatments of 4 °C after the application of 200 µM NO concentrations, which, of course, did not show a statistically significant difference with the amount of this amino acid in vines treated with a concentration of 100 µM (Table 2).

Table 2. The combined effect of low-temperature and NO treatments on the amount of chlorophyll a, chlorophyll b, total chlorophyll and carotenoid in Yaghooti grape leaves.

Temperature regimes (ºC)	Nitric oxide (µM)	Soluble sugar (mg/g FW)	Proline (mg/g FW)	Soluble protein (mg/g FW)	Phenol (mg/g FW)	Flavonoids (mg/g FW)
24	0	14.44e	2.61c	1.85e	12.2e	2.89e
24	100	15.78de	2.56c	2.65d	15.3cd	4.60d
24	200	16.00d	2.63c	3.17c	17.6c	6.20b
4	0	23.42c	5.36b	3.60c	23.2b	5.32cd
4	100	30.00b	8.77a	5.89b	26.9a	7.99a
4	200	35.04a	9.43a	6.65a	28.7a	8.36a

The same letters indicate no significant difference at the probability level of $P<0.05$.

3.4. Total soluble protein

The effects of NO, temperature and their interaction on leaf soluble protein content were significant at the 1% probability level (Table 2). The soluble protein content increased in vines treated with low temperature (4ºC) compared to vines kept at 24ºC. Nitric oxide application slightly increased the soluble protein content in vines kept at 24ºC. However, a significant difference was observed between the leaf soluble protein content of control vines (0µM NO) and vines treated with different concentrations of NO. In vines that had experienced low-temperature stress (4ºC), NO increased the soluble protein content to a greater extent (i.e., compared to control vines; Table 2). Under low temperature stress, the content of soluble proteins in vines treated with 200 µM NO was 46% higher than that in control vines (0µM NO; Table 2).

3.5. Total phenol

The effect of NO, low-temperature stress and their interaction on leaf phenol content was significant at the 1% probability level (Table 2). In general, a decrease in temperature caused a relative increase in leaf total phenol content in all treatments. The minimum total phenol was observed in control plants in all temperature treatments, and vines treated with 200 and 100 µM concentrations (without significant differences) had higher total phenol in their leaves than plants treated with 0 µM NO concentration in low-temperature treatments of 4°C. The highest leaf phenol content was observed in plants treated with 200 µM NO concentration under 4°C temperature, which was not statistically different from the 100 µM concentration (Table 2).

3.6. Total flavonoids

The effect of NO, low-temperature stress, and their interaction on total leaf flavonoid content was significant at the 1% probability level. In general, reducing temperature to 4 ºC caused a relative increase in total leaf phenolic concentration in all treatments. Also, vines treated with 200 and 100 µM NO concentrations had higher total flavonoids content in their leaves than plants treated with 0 µM NO in low-temperature treatments of 4 ºC. The highest leaf flavonoid content was observed in plants treated with 200 µM NO under temperature treatment of 4 ºC, which was not statistically significantly different from the 100 µM concentration (Table 2).

3.7. Relative water content

The effects of NO, low-temperature stress and their interaction on leaf RWC were significant at the 1% probability level. At 24ºC, foliar application of NO did not significantly affect the RWC of vines treated with this growth regulator compared to the control (Table 3). Regardless of NO treatment, transferring vines from 24ºC to low temperature significantly reduced RWC. However, this reduction was greater in vines treated with different concentrations of NO than in the control (Table 3). Under temperature treatments of 4 ºC, the highest leaf RWC was observed in control vines and the lowest values without significant differences were related to vines treated with 100 and 200 µM NO concentrations (Table 3).

3.8. Electrolyte leakage

The effect of NO, low-temperature stress and their interaction on the percentage of leaf EL was significant at the 1% probability level. Under a temperature of 24ºC, no statistically significant difference was observed between the control and NO-treated vines, in other words, in this temperature treatment, NO spraying had no effect on the percentage of leaf EL (Table 3). Transferring the vines to a low temperature (4ºC) led to an increase in leaf EL in all treatments (0, 100 and 200 µM NO). However, under low-temperature stress, the percentage of leaf EL in vines treated with NO at 200 µM was lower than in other treatments, so that it was 22% lower compared to the measured EL in control vines in this temperature treatment (4ºC; Table 3).

Table 3. Combined effect of low-temperature and NO treatments on leaf relative water content (RWC), electrolyte leakage (EL) and malondialdehyde (MDA) of Yaghooti grape.

Temperature regimes (ºC)	Nitric oxide (µM)	RWC (%)	EL (%)	MDA (nmol/g FW)
24	0	85.3a	12.9d	26.4d
24	100	86.7a	11.7d	24.7d
24	200	85.5a	12.6d	22.3d
4	0	77.0b	48.2a	67.9a
4	100	76.9b	41.7b	46.4b
4	200	74.4c	37.5c	39.3c

The same letters indicate no significant difference at the probability level of P<0.05.

3.9. Membrane lipid peroxidation

Membrane lipid peroxidation was measured based on the malondialdehyde (MDA) index produced during membrane damage. The effects of NO, temperature, and their interaction on leaf MDA production were significant at the 1% level (Table 4). Low temperature stress increased MDA production (Table 4). Under both temperature treatments, a significant difference was observed between the MDA production in control vines and vines treated with NO. In this regard, the application of 100 and 200 µM NO concentrations had an effective role in reducing MDA production in vines, indicating the protective role of this growth regulator in membrane stability (Table 4).

3.10. Antioxidant enzymes

3.10.1. CAT

The effect of NO, temperature and their interaction on leaf CAT enzyme activity was significant at the 1% level (Table 4). Exogenous application of NO in vines maintained at 24ºC resulted in an increase in CAT enzyme activity compared to control vines in this temperature treatment. The highest activity of this enzyme was observed at 24 ºC in vines treated with 200 µM NO (Table 4). Regardless of NO treatment, low-temperature stress itself increased CAT enzyme activity. Exogenous application of NO significantly increased CAT enzyme activity in vines treated at 4ºC. Catalase enzyme activity at 4ºC was dependent on the concentration of NO used, and the highest enzyme activity recorded (8.46 U/mg protein) in this temperature treatment was observed in vines sprayed with 200 µM NO (Table 4).

Table 4. Combined effect of low-temperature and NO treatments on the levels of some antioxidant enzymes in Yaghooti grape leaves.

Temperature regimes (ºC)	Nitric oxide (µM)	CAT (Unit /mg protein)	GPX (Unit /mg protein)	APX (Unit /mg protein)
24	0	1.36e	2.11d	2.00d
24	100	2.87d	3.55d	3.20c
24	200	3.4c	3.89d	3.10c
4	0	2.94cd	7.24c	4.66b
4	100	6.77b	9.12b	7.80a
4	200	8.46a	13.88a	9.31a

The same letters indicate no significant difference at the probability level of P<0.05.

3.10.2. GPX

The effect of NO and temperature on leaf GPX enzyme activity was significant at the 1% probability level. However, their interaction on the activity of this enzyme was not significant (Table 4). The activity of the GPX enzyme was higher in vines that received a temperature treatment of 4ºC compared to vines maintained at a temperature of 24 ºC (Table 4). NO treatment increased the activity of this enzyme in both groups of vines compared to the control (no NO treatment). The highest activity of the GPX enzyme (13.88 U/mg protein) was

observed in vines that had been sprayed with a concentration of 200 µM NO before experiencing low temperatures (Table 4).

3.10.3. APX

The effect of NO, temperature and their interaction on the activity of leaf APX was significant at the 5% level (Table 4). Low temperature treatment significantly increased the activity of APX. Regardless of the temperature regime applied, NO treatment had a positive effect on the activity of APX, and a significant difference was observed between the activity of this enzyme in control vines (0µM NO) and vines treated with 100 and 200 µM NO concentrations. Under low-temperature stress, the highest activity of this enzyme was observed in vines treated with 200 µM NO concentrations, which, of course, did not differ significantly with NO at 100 µM (Table 4).

3.11. Leaf nutrient content

The effect of NO, temperature and their interaction effects on the content of phosphorus, potassium, nitrogen and zinc in grape leaves was significant at the 5% level, but the effects of these treatments on the content of magnesium and iron were significant at the 1% level (Table 5). There was no significant difference between the leaves phosphorus content of the control vines and those treated with two concentrations of NO under a temperature of 24 ° C. However, in this temperature treatment, a significant difference was observed between the content of nitrogen, potassium, magnesium, iron and zinc in the leaves of the control vines and the vines treated with NO, especially at a concentration of 200 µM (Table 5).

Table 5. The combined effect of low-temperature and NO treatments on the concentration of nutrients in Yaghooti grape leaves.

Temperature regimes (ºC)	Nitric oxide (µM)	N (% DM)	P (% DM)	K (% DM)	Mg (% DM)	Fe (ppm)	Zn (ppm)
24	0	1.56cd	0.25a	1.75b	1.23e	65.4c	57.4b
24	100	1.83b	0.26a	1.83a	1.44b	76.4b	65.8a
24	200	2.3a	0.26a	1.89a	1.67a	80.0a	66.1a
4	0	1.40d	0.20c	1.50d	0.95f	57.1d	45.2c
4	100	1.75c	0.23b	1.64c	1.28d	66.6c	53.9b
4	200	1.78bc	0.23b	1.66c	1.40c	67.0c	54.5b

The same letters indicate no significant difference at the probability level of $P<0.05$.

4. Discussion

In the present study, nitric oxide-mediated responses in 'Yaghooti' grapes under low-temperature stress were evaluated under greenhouse conditions. Chlorophyll content was significantly reduced in vines exposed to low-temperature without NO treatment. The reduction in chlorophyll content under low-temperature stress may be related to oxidative damage to chloroplast membranes due to low-temperature or increased chlorophyllase enzyme activity (Zaeri-Behrooz *et al*., 2026). Oxidative damage induces peroxidation of thylakoid membrane lipids and then chlorophyll degradation (Guo *et al*., 2025). In the present study, chlorophyll stability under low-temperature stress was higher in vines treated with NO compared to the control. No change or even an increase in chlorophyll content has been reported in plants treated with NO in grape (Ebrahimi *et al*., 2019) and rice (Huang *et al*., 2020) under salinity stress and in broad bean under low-temperature stress (Nabati *et al*., 2025). The ability of NO to reduce the peroxidation of membrane lipids in chloroplasts, especially thylakoid membrane structures, through increasing the activity of free radical-neutralizing enzymes is one of the possible mechanisms for reducing chlorophyll degradation under low-temperature stress (Cui *et al*., 2025). This finding is one of the reasons that can explain the improvement in gas exchange indices in plants treated with NO under chilled condition.

Nitric oxide treatment increased the content of soluble sugars, proline and soluble protein in the leaves of treated vines subjected to low-temperature stress. Biosynthesis and accumulation of osmolyte solutes, including sugars, is one of the important regulatory reactions of plants in response to abiotic stress (Karimi, 2017; Ghasemi *et al*., 2026). In this way, the plant accumulates osmolyte solutes, including soluble sugars, in the vacuole and cytoplasm so that, by protecting and adjusting osmotic pressure, it can continue to absorb water, store carbon and nitrogen, and scavenge ROS. However, with increasing stresses and their destructive effects, this stops and the plant suffers irreparable damage (Guo *et al*., 2025; Ghasemi *et al*., 2026). In the present study, NO treatment increased the accumulation of sugar in the intercellular and intracellular space, thus increasing the low-temperature tolerance of the treated vines by creating greater osmotic regulation and maintaining the structure of the cell membrane. An increase in cellular sugar levels in response to NO application has been reported in some plants under various environmental stresses (Ebrahimi *et al*., 2019; Nabati *et al*., 2025), which confirms the results of the present study.

In grape low-temperature stress also increased proline content in vine leaves (Karimi *et al*., 2016; Karimi, 2020), this confirms the results of the present study. The increase in proline content may be due to the presence of a common precursor with chlorophyll (glutamine), which is less chlorophyll produced under stress conditions due to the production of proline to modulate the cells osmotic condition (Fozouni *et al*., 2012; Guo *et al*., 2025). Under salinity stress in rice seedlings, foliar application of NO significantly increased proline concentration in seedling leaves, which was associated with increased transcription of the key gene for proline biosynthesis, delta-pyrroline-5-carboxylate synthase (Feng *et al*., 2021). Intracellular proline, accumulated during stress, not only provides stress tolerance but is also stored as organic nitrogen during the recovery period from stress (Fozouni *et al*., 2012). Moreover, the increase in soluble proteins is one of the most common biochemical changes that can protect the plant from lethal dehydration injuries (Karimi *et al*., 2016). Soluble protein, like proline, is also a compatible solution that is considered a factor in improving plant resistance to low-temperature stress (Feng *et al*., 2025). Consistent with the results of the present study, leaf protein content in grapevines plant under salinity stress has been shown to increase with NO treatment at 100 mM (Ebrahimi *et al*., 2019). Also, in a study on rice seedlings, the application of NO caused increased gene expression of stress-related proteins such as heat shock proteins (Feng *et al*., 2021).

In the present study, NO application resulted in higher total phenolic and flavonoids accumulation than untreated grapes under low-temperature stress. The increase in phenolic compounds in these grapes may act as an adaptive mechanism to overcome low-temperature-induced oxidative stress. Foliar application of NO in grapes has increased stress tolerance through an increase in phenolic compounds (Ebrahimi *et al*., 2019), indicating the involvement of this substance in the pathways of phenolic compound synthesis, especially in stressed plants. In a study, phenolic compounds have also increased the ability of grapevine plants to scavenge free radicals under stress (Karimi *et al*., 2016). The importance of flavonoids is due to the role they play in non-enzymatic defense systems (Ebrahimi *et al*, 2019). Environmental factors have a significant effect on the flavonoids content, and when the plant senses the presence of stress, the plant's defense system, including flavonoids, is activated and increased to cope with the stress (Li *et al*., 2025).

Under low-temperature stress, RWC in vines treated with NO decreased more severely compared to control vines. The reduction in RWC in low-temperature-treated leaves causes water stress, which may be an adaptive factor for increased frost tolerance in vines. Also, under low temperatures, relatively high concentrations of abscisic acid may enable mildly stressed plants to fix carbon dioxide, which is associated with a small amount of relative water loss (Karimi *et al*., 2016). The present results are consistent with research conducted on grapes (Karimi and Ershadi, 2015; Ebrahimi *et al*., 2019). Moreover, RWC was declined at a lower level in vines treated with NO. The reduction in RWC in vines exposed to low temperatures and NO treatment can be due to the binding of free cellular water with macromolecules such as sugars and antifreeze proteins, which occurs in order to increase frost tolerance (Karimi, 2020). On the other hands, in some cases (stomatal regulation), NO is thought to play a crucial role in ABA-mediated signaling pathways (Cui *et al*., 2025) which can decrease RWC and enhance cold resistance in plants by regulating the biosynthesis of NO (Babuta *et al*., 2025)

Under low-temperature stress, the percentage of leaf EL in vines treated with NO at 200 µM was lower than in other treatments. The degree of temperature reduction and the duration of exposure to low-temperature play an important role in increasing the rate of EL (Orooji *et al*., 2024). In fact, with a decrease in temperature, the rate of EL increased. This will lead to changes in the selective permeability of biological membranes, leakage of substances from the membrane and changes in the activity of membrane-bound enzymes. Therefore, measuring EL is an indicator of measuring the amount of oxidative damage to the membrane and cell (Campos *et al*., 2003; Karimi, 2017). Also, the percentage of EL is inversely related to the health and stability of the membrane, and the higher EL reflecting the unstable membrane and the more sensitive of it to stress condition (Guo *et al*., 2025). The reason for the reduction of EL with NO consumption may is the increase in membrane stability and the prevention of oxidative stress caused by low-temperature on the membrane.

In plants, the cell membrane is one of the first sites to be damaged when exposed to low temperatures. Increased levels of MDA are one of the indices that indicate the extent of membrane damage, in other words, it reflects oxidative damage to membrane lipids (Feng *et al*., 2025). In the present study, MDA increased in leaves of chilled vines. In strawberries (Gulen *et al*., 2008) and grapes (Karimi *et al*., 2016), MDA content also increased sharply in chilled plants. The significant increase in MDA production occurred due to an increase in lipid peroxidation of biological membranes. Therefore, low-temperature stress probably disrupts electron transport in mitochondria and chloroplasts and leads to oxidative damage to biological membranes by producing ROS (Gulen *et al*., 2008; Feng *et al*., 2025). Exogenous application of NO significantly reduced MDA production in low-temperature-stressed vines. In the study by Ebrahimi *et al*. (2019), application of NO reduced MDA production in leaves of grapevines under salt stress. NO probably reduces the content of superoxide and H_2O_2 radicals by affecting the activity of antioxidant enzymes, preventing the activity of the lipoxygenase enzyme and the breakdown of membrane fatty acids, and ultimately reducing oxidative damage to membrane lipids during low temperatures (Karimi, 2020; Cui *et al*., 2025).

Exogenous application of NO remarkably increased antioxidant enzymes activities in vines under 4ºC.This change in enzyme activity indicates an adaptive response and protective regulation of the plant against low-temperature stress conditions. Low activity of antioxidant enzymes is considered to be an indicator of normal environmental conditions for plant growth. Therefore, their increase was due to the fact that, as an antioxidant free radical enzyme, it neutralizes the damage caused by ROS caused by low-temperature stress while protecting macromolecules and cell membranes (Karimi *et al*., 2016). Increased activity of antioxidant enzymes is a protective mechanism of the photosynthetic apparatus in the face of oxidative stress (Karimi *et al*., 2016). Our results showed that NO can significantly increase the activity of CAT and GPX enzymes in grapes under low-temperature stress. Application of NO to rice seedlings under salt stress resulted in increased antioxidant enzyme activity, which was accompanied by a decrease in the production of membrane damage markers such as MDA production, oxygen peroxide, and ultimately less EL (Feng *et al* 2021), which is consistent with the results of the present study. Metabolic changes caused by NO lead to changes in the levels of oxygen free radicals, which underlie the induction of the antioxidant defense system (Cui *et al*., 2025). It seems that NO and ROS, especially H_2O_2, are involved in the cellular signaling system and act as second messengers to induce antioxidant defense under stress conditions (Feng *et al*., 2021). Ascorbate peroxidase is one of the main and important plant antioxidants, and under stress conditions, plant cells produce this compound to protect against oxidative damage, which acts as a free radical scavenger system (Ebadi *et al*., 2016). Exogenous application of NO in cucumber (Feng *et al*., 2021) reduced the production of oxygen peroxide, which is consistent with the results of the present study. One of the adverse effects of low-temperature stress is the accumulation of ROS in membranes and the formation of membrane lipid peroxidation, which, as shown in the results of this study, increased the amount of H_2O_2 in grapes under low-temperature stress. One of the important roles of NO is the activation of antioxidant enzymes such as CAT, GPX and APX (Feng *et al*., 2021; Cui *et al*., 2025), which was documented in this study. The cellular antioxidant system plays an important role in plants under low-temperature stress. The most important antioxidant enzymes that neutralize ROS, act as frontline defense enzymes against the damaging effects of low-temperature stress (Karimi *et al*., 2016). In the present study, the activity of all three antioxidant enzymes was higher in chilled vines compared to vines remaining at normal temperature. Increased antioxidant activity is one of the important mechanisms of plants that play an important role in improving the efficiency of ROS neutralization activity in plants under low-temperature stress (Karimi *et al*., 2016).

Low temperature reduced the content of leaf elements compared to the content of vine elements at greenhouse temperature. However, vines treated with NO, especially at a concentration of 200 µM, showed higher content of nitrogen, magnesium, phosphorus, potassium, iron and zinc compared to control vines under low-temperature stress, and the content of these elements in these vines was higher compared to the amount of these elements in control vines under stress. Nitrogen plays a role in the structure of chlorophyll and amino acids, including proline (Karimi *et al*., 2019). Therefore, increasing the content of nitrogen in vines treated with NO under low-temperature stress can lead to increased low-temperature tolerance in vines while maintaining the structure of chlorophyll and also increasing the accumulation of proline and soluble proteins (Karimi *et al*., 2019). Based on the results, it was determined that the use of NO affects the metabolism of elements including potassium, phosphorus, zinc and iron, and these changes are aimed at regulating the osmotic pressure induced by potassium, providing the necessary nitrogen to maintain the structure of chlorophyll, proline and soluble protein and phosphorus to provide energy or membrane phospholipids, and inducing the oxidation and reduction properties of iron and zinc in the electron transport chain (Karimi, 2017; Zaeri-Behrooz *et al*., 2026). The content of potassium ions in plant tissues indicates the presence of an important cation, which is one of the important components in the osmotic regulation of cells and maintaining cell swelling under cold stress (Karimi, 2017; Mozafari et al., 2026). Potassium is one of the most important elements that plays a role in the balance of anions and cations within the cell. This element also has a significant effect on the absorption of other elements by the root and helps to eliminate the adverse effects of imbalance of some nutrients in the soil (Karimi, 2017; Kazemi et al., 2025). Improvement of macronutrient absorption status due to the application of NO has also been observed in a study on lettuce (Babuta *et al*., 2025), which is consistent with the results of the present study.

Conclusion

Based on the results obtained, NO increased the tolerance of grape plants under low-temperature stress conditions by affecting compatible osmolytes, antioxidant enzymes, potassium and magnesium. As a result, 200 µM was the most effective concentration among those evaluated and is recommended for 'Yaghooti' grape variety to increase tolerance to low-temperature stress (4 °C). Although field-based evaluation of nitric oxide application—particularly at a concentration of 200 µM—could increase our confidence in the results of this experiment regarding enhanced low-temperature tolerance of grapevine.

Conflict of interest

The authors declare that there are no conflicts of interest.

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