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# Nitric oxide alleviates freezing stress by reducing oxidative injury in Citrumelo leaves

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**Key words:** Citrumelo, Freezing stress, Oxidative stress, Sodium nitroprusside.

**Abstract**

Freezing stress causes economical losses of citrus in the world. Nitric oxide could alleviate this stress. In this study the effects of sodium nitroprusside (SNP) as NO donor (0, 0.1 and 1 mM) on temperature treatments (+9, -3, -6 and -9°C) were evaluated. Application of 0.1 mM SNP decreased the electrolyte leakage and lipid peroxidation of Citrumelo leaves significantly. The highest antioxidant activity was detected in 0.1 mM SNP at -6 °C. Maximum content of total phenolics was at -9 °C and 0.1 mM SNP application. SNP also had positive effects on antioxidant enzymes activity. Superoxide dismutase, catalase and peroxidase had highest activity at -6°C with 0.1 mM SNP, and the highest activity of APX was in 0.1 mM SNP at -9°C. The results showed nitric oxide released from SNP increased the antioxidant capacity of Citrumelo leaves against freezing induced oxidative stress.

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**Introduction**

Cold stress, including chilling and freezing, is one of the major abiotic stresses that limit the growth, productivity, and geographical distribution of many plants (Esim and Atici 2014). Freezing can cause irreversible damage to plant cells via mechanical forces that generated by the formation of extracellular ice crystals and cellular dehydration as well as chilling makes some physiological and biochemical impairments in plant cells (Sevillano *et al.* 2009).

Numerous researches have illustrated that cold stress is associated with oxidative stress (Yang *et al.* 2011) (Sala 1998). Cold stress induces the over-production of reactive oxygen species (ROS) such as superoxide radical, hydrogen peroxide and hydroxyl radical in plants. The redundant ROS react with cell components that lead to inactivation of enzymes, protein degradation, lipid per oxidation and DNA damage (Liu *et al.* 2010). However, plants have evolved specific protective mechanisms to reduce the injurious effects of ROS and protect themselves at different oxidative stresses such as low temperatures (Asada 2006). Many studies have been illustrated that the antioxidant systems play important roles in protecting plants against oxidative injury induced by different stresses such as cold stress. Antioxidant systems as the main ROS-scavenging mechanisms included enzymatic system (superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxide (APX), glutathione reductase (GR)) and non-enzymatic system (ascorbic acid (AsA) and glutathione (GSH)) (Apel and Hirt 2004). So plants can improve tolerance to cold stress by enhancing the activity of antioxidant enzymes and contents of antioxidant components that play a crucial role in ROS scavenging and cell protection (Tajvar *et al.* 2011, Yang, Wu and Cheng 2011).

Nitric oxide (NO) is an important signaling molecule involved in many physiological processes under normal and stress conditions in plant cells (Corpas *et al.* 2008, Neill *et al.* 2008). The effects of NO in various physiological functions, such as seed germination, root growth, dormancy, flowering, stomatal closure, pollen tube growth, senescence and photomorphogenesis has been proved (Böhm *et al.* 2010). Furthermore, NO has been reported as an important signal molecule that mediating plants responses to abiotic stresses (Neill, Bright, Desikan, Hancock, Harrison and Wilson 2008). It has also been observed that NO protects plant cells against oxidative stress by reducing ROS accumulation (Xu *et al.* 2010).

Literature survey showed that NO could alleviate oxidative damage and had protective effect against various stressful conditions such as salt (Tanou *et al.* 2009), drought (Hao *et al.* 2008), heavy metals (Zhang *et al.* 2011), high light (Xu, Sun, Jin and Zhou 2010) and low temperature (Zhao *et al.* 2009) (Zhu *et al.* 2008), when applied as exogenous NO donor such as sodium nitroprusside (SNP). Sodium nitroprusside (SNP) is a commonly used NO donor for its well-documented application, as well as the continuous, long-lasting NO production compared with other NO donors (Floryszak-Wieczorek *et al.* 2006).

Citrus is one of the most economically important fruit crop in tropical and subtropical regions of the world. Cold stress is one of the limiting factors for production of citrus in these regions, which causes significant damage and economic losses of them. However, commercial citrus production is done in regions that are facing the threat of cold stress, including Japan, Spain, regions of the United States and Caspian Sea coast (Yelenosky 1985). In these conditions citrus resistant rootstocks to low temperatures such as trifoliate orange (*Poncirus trifoliata*), a close relative of Citrus genus that is resistant to cold stress, is used. To obtain the resistant rootstocks to various stresses, trifoliate orange has been crossed to citrus. One of the produced rootstock is Citrumelo, which is hybrid of *P. trifoliata* and *Citrus paradasi.* It is used widely in citricultures because of beneficial characteristics such as citrus tristeza virus, phytophthora foot rot, citrus nemathode and blight tolerance. It can grow in calcareous soils with high pH. Moreover, the fruits of scions on Citrumelo have high quality specially grapefruit (Castle *et al.* 1988).

Due to the importance of NO effects in alleviation of oxidative stress damages caused by freezing stress (Zhao, Chen, Zhang and Zhang 2009), in this study the effects of different concentrations of sodium nitroprusside on antioxidants characteristics and resistance capacity of Citrumelo (as one of the important citrus rootstocks) under low temperatures were investigated.

## Materials and methods

*Plant materials and treatments*

One year old Citrumelo seedlings from Mazandaran province (north of Iran) were transferred to greenhouse in Tabriz in April 2015. They were transplanted to plastic pots, containing a mixture of perlite and peat moss (1:1, v/v), at the temperature of 20 to 33°C under natural photoperiod and 65% in average humidity. They were irrigated with modified Hogland solution. After establishment of seedlings, they were sprayed with sodium nitroprusside (SNP) at 0 (distilled water), 0.1 and 1 mM concentration containing 2% Tween 80. They were sprayed to run off on each plant. After three weeks of treatment, shoots were collected from each group randomly. After that they placed in the wet sand in the fridge at +9°C for 24 hours. Then the temperature was decreased to -3, -6 and -9°C gradually. Plants were kept at each temperature for 24 hours, and then leaves were picked as sample for analysis. Electrolyte leakage was assessed by fresh leaves, and other assessments carried out by freeze dried leaves in -80°C.

*Electrolyte leakage*

Electrolyte leakage was determined by method of (Blum and Ebercon 1981) with some modification. Six leaf discs (0.8 cm in diameter) were cut from each shoot per treatment using a stainless cork borrer and then washed with distilled water. And then the discs were placed in tubes with 20 ml double distilled water. Samples were kept at 20°C for 24 hours. The conductivity of the diffuse was measured using an electrical conductivity meter. Samples were subsequently boiled in a 100°C water bath for 20 min, and were remeasured after cooling to room temperature. Membrane permeability was expressed as the relative leakage (%), which is the ratio of the first to the second conductivity readings.

*Lipid peroxidation*

Lipid peroxidation value in leaves was measured with evaluation of malondialdehyde (MDA) content by the thiobarbituric acid reaction method (Heath and Packer 1968). Amount of 0.1 g of leaf sample was homogenized in 0.1% trichloroacetic acid (TCA) and then, centrifuged at 10000 × g for 5 min at 4°C. The supernatant was incubated at 95°C for 30 min, after that, the reaction was immediately stopped in ice and centrifuged at 3000 × g for 5 min at 4°C. MDA concentration was determined by reading the absorbance at 532 nm after subtracting the nonspecific absorption at 600 nm by using the extinction coefficient 155 $mM^{-1}.cm^{-1}$.

*DPPH radical scavenging activity*

DPPH (2, 2 diphenyl 2 picrylhydrazyl hydrate) radical scavenging activity of plant extracts was determined according to (Brand-Williams *et al*. 1995). Amount of the 0.3 g of leaf was extracted with 3 ml acidic methanol at 4°C. After centrifuge, 50 µl of the methanolic extract were rapidly mixed with 1950 µl of DPPH methanolic solution (40 mg $L^{-1}$) in a cuvette placed in the spectrophotometer (SPEKOL 1500, Analytik Jena, Germany). The absorbance at 517 nm was measured after 20 min incubation in darkness at room temperature. The radical scavenging activity of the sample was stated as the decrease in radical concentration. DPPH radical scavenge rates was calculated with following equation:

Scavenging activity (%)=$[(A_B-A_A)/A_B]\times100$, where $A_B$ stands for absorbance at 517 nm of control, and $A_A$ stands for absorbance at 517 nm of sample.

*Total phenolics content*

Total phenolics content was determined with the Folin-Ciocalteu colorimetric method (Slinkard and Singleton 1977). The extract obtained for DPPH measurement was also used for the determination of total phenol content. After extract centrifuging at 12000 × g for 10 min at 4°C, 50 µl of the supernatant was diluted with 450 µl of water.

Then, 2.5 ml of freshly prepared 50% Folin-Ciocalteu reagent was added. The solution was incubated in darkness at room temperature for 7 min. After that, 2 ml of a 7.5% sodium carbonate solution was added. Samples were incubated in darkness at room temperature for 45 min. Then, absorbance was measured at 760 nm. A gallic acid standard curve was used for the calibration, and total phenolics content was expressed as mg gallic acid equivalents per gram of fresh weight (mg GAE $g^{-1}$).

*Antioxidant enzymes activity*

Specific activity of the enzymes was expressed as mmolmin$^{-1}$ mg protein $^{-1}$ (U. mg$^{-1}$ protein). For measurement the amount of soluble protein in samples, the (Bradford 1976) method was used. Amount of the 0.5g of leaves was homogenized in 5 ml of ice cold 100 mM potassium phosphate buffer (pH 6.8) with a mortar and pestle. After centrifuge of homogenates at 12000 g for 20 min at 4°C, the supernatant fraction used as the source of protein and CAT and POD enzymes. All steps were carried out at 0-4°C.

Catalase (CAT, EC 1.11.1.6) activity was assayed by the UV method of (Aebi 1984). The reaction mixture contained 500 µL of 10 mM $H_2O_2$, 1480 µL of 100 mM potassium phosphate buffer and 20 µL of enzyme extract in a 2 ml volume. The decrease in absorbance was noted at 240 nm for 60 seconds at intervals of 5 with spectrophotometer. Specific activity of enzyme defined as mM $H_2O_2$ consumption per mg protein per minute by using the $H_2O_2$ extinction.

The activity of peroxidase (POD, EC 1.11.1.7) was estimated as guaiacol oxidation by $H_2O_2$. Mixture of 20 µl of the enzyme extraction, 1180 µl of 100 mM potassium phosphate buffer and 500 µl of 20 mM guaiacol solution was prepared, and then 300 µl of 10 mM $H_2O_2$ were added.

Increase in absorbance was recorded for 90 seconds at intervals of 5 seconds in 470 nm (Chance and Maehly 1955). By using the extinction coefficient of tetraguaiacol product (26.6 $mM^{-1}cm^{-1}$) the specificity of enzyme was expressed as mM produced tetraguaiacol per mg protein per minute.

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was determined by the (Nakano and Asada 1981) with some modification. Reaction mixture contained10 µl of enzyme extract, 1390 µl of 50 mM Hepes buffer, 200 µl of 10 mM ascorbic acid, 200 µl of 10 mM EDTA and 200 µL of 100 mM $H_2O_2$.

The reaction started with addition of $H_2O_2$. The decrease of absorbance at 290 nm was monitored in 60 second sat intervals of 5 seconds. APX specific activity was expressed as unit per mg protein per min with 2.8 $mM^{-1}cm^{-1}$ as the extinction coefficient.

The activity of superoxide dismutase (SOD, EC 1.15.1.1) was determined according to (Giannopolitis and Ries 1977) with some modification. The reaction mixture contained 300 µl of enzyme extract, 200 µl of 50 mM Hepes buffer, 300 µl of 12 mM methionine, 300 µl of 75 µM nitro blue tetrazolium (NBT) and 1 mL of 50 mM $Na_2CO_3$. At the end, 30 µl of 1 µM riboflavin was added. The reaction and control mixture were placed 20 cm in front of a 40 W fluorescent lamp for 20 min.

A complete reaction mixture without enzyme was used as control. For blank, a non-irradiated reaction mixture which did not develop a color was served.

The absorbance was recorded at 560 nm. One unit of SOD activity was taken as the quantity of enzyme that required producing 50% inhibition of NBT photochemical reduction. The specific enzyme activity was expressed as units per mg protein.

*Statistical analysis*

The experimental design was a factorial test based on a completely randomized design with four replications. Results were analyzed statistically using the Statistical Analysis Program (SPSS ver. 16.0). The mean values were calculated and compared by Duncan's multiple range tests ($P < 0.05$).

**Result and discussion**

*Electrolyte leakage percentage and lipid peroxidation*

Electrolyte leakage shows the degree of damage of plasma lemma in stresses. Fig. 1 shows the percentages of electrolyte leakage in Citrumelo leaves under the freezing stress.

Electrolyte leakage increased with increasing of cold intensity. Maximum electrolyte leakage (52.609 %) occurred at -9°C and SNP application significantly decreased it at all studied temperatures. Low temperature disrupts the balance of reactive oxygen species (ROS) metabolism. Accumulation of ROS damages plasma lemma structure and causes to electrolyte leakage and induces lipid per oxidation (Liu, Jiang, Zhao and An 2010).

Our results showed that SNP application reduced electrolyte leakage at -9 and -6°C significantly (Fig. 1). SNP displayed significant inhibition of the electrolyte leakage at freezing stress.

The low temperatures occur frequently and it's so important that we can help plants to rescue with application of SNP. (Fan and Liu 2012) also showed that SNP application reduces electrolyte leakage. MDA concentration shows the level of lipid peroxidation in plants.

The freezing stressed plants exhibited a higher rate of electrolyte leakage and lipid peroxidation and SNP reduced them. As showed in Fig. 2, although the temperature of -6 and -9°C increased MDA concentration but 0.1 mM SNP treatment reduced it significantly. In this study, SNP application of 0.1 and 1 mM decreased the electrolyte leakage and lipid peroxidation content of Citrumelo leaves signifi-cantly.SNP application reduced MDA content of leaves that exposed to freezing temperature, so it seems reasonable that SNP application enhanced the cold tolerance of leaves. This effect might be is due to NO capability to scavenge of ROS.

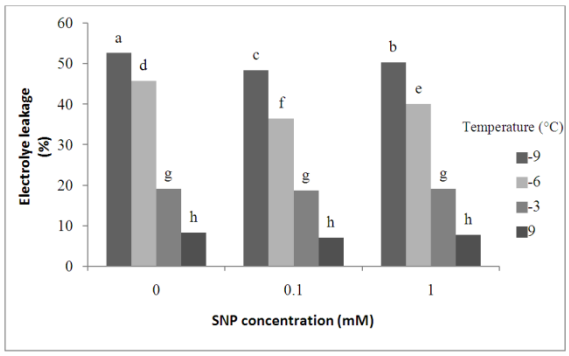


**Fig. 1.** Rate of electrolyte leakage in Citrumelo leaves in SNP (0, 0.1, 1 mM) and freezing temperatures (-9, -6, -3 and + 9°C) treatments. Bars showing the same letter are not significantly different at $p < 0.05$ as determined by Duncan's multiple range test.

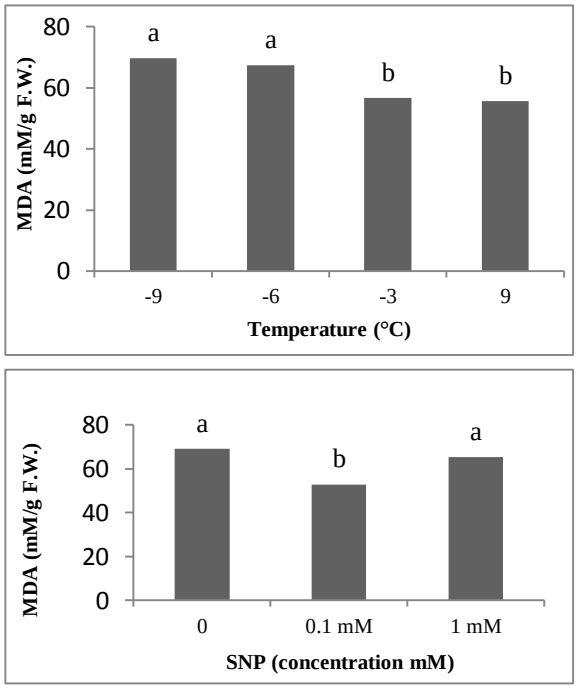


**Fig. 2.** Malondialdehyde content in Citrumelo leaves in SNP (0, 0.1, 1 mM) and freezing temperatures (-9, -6, -3 and + 9°C) treatments. Bars showing the same letter are not significantly different at $p < 0.05$ as determined by Duncan's multiple range test.

*DPPH-radical scavenging activity*

The DPPH assay often is used for the fast evaluation of antioxidant activity. DPPH-radical scavenging activity significantly increased in the leaves under freezing stress as well as in SNP treatment.

According to Fig. 3, foliar application of SNP at 0.1 mM concentration increased the average DPPH-radical scavenging activity in all temperatures and the highest antioxidant activity (70.018%) was detected in 0.1 mM SNP at -6°C.NO has been applied to reduce the frost injury in some horticultural crops such as kiwi fruit (Zhu, Sun, Liu and Zhou 2008), strawberry (Wills *et al.* 2000) and tomato (Lai *et al.* 2011). The results of present study showed the NO enforcement effects on antioxidant system of Citrumelo plants under freezing stress.

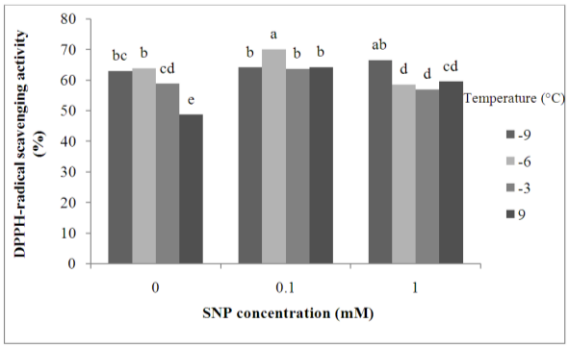


**Fig. 3.** DPPH-radical scavenging activity in Citrumelo leaves in SNP (0, 0.1, 1 mM) and freezing temperatures (-9, -6, -3 and + 9°C) treatments. Bars showing the same letter are not significantly different at $p < 0.05$ as determined by Duncan's multiple range test.

According to (Kang and Saltveit 2002) higher levels of DPPH activity was correlated with increased frost tolerance in plants. (Yang, Wu and Cheng 2011) demonstrated that exogenous NO reduced chilling injury by induction of DPPH radical scavenging activity. (Pakkish and Tabatabaienia 2016) showed that DPPH radical scavenging activity was improved by NO application under freezing stress. In agreement with these results, our study also showed that SNP application increased DPPH radical scavenging activity. Therefore, it is considered that NO increased freezing tolerance in Citrumelo leaves by increase of DPPH radical scavenging activity.

*Total phenolics content*

Increase in intensity of frost caused increase in total phenolics content, except -6°C. SNP application increased total phenolics content and most total phenolics content achieved at 0.1 mM SNP, but 1 mM SNP reduced it.

Application of SNP improved the phenolics content in Citrumelo leaves that were under low temperatures (Fig. 4). Maximum content of total phenolics was 12.228 mg GAE $g^{-1}$ FW at -9 °C and 0.1 mM SNP application. Phenolic compounds have antioxidant capacity due to their reactivity as hydrogen and electron donors, stabilize unpaired electron, chelate transiton metal ions and modification of the lipid packing order and to decrease membrane fluidity. These functions restrict diffusion of free radicals and peroxidative reactions. A positive and highly significant relationship between total phenolics and antioxidant activity in several plant products has been reported (Schroeter *et al.* 2002).

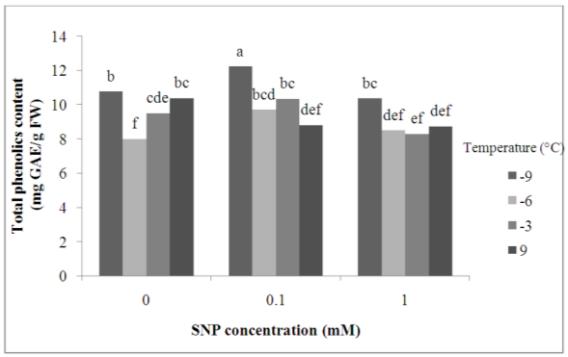


**Fig. 4.** Content of total phenolics in Citrumelo leaves in SNP (0, 0.1, 1 mM) and freezing temperatures (-9, -6, -3 and + 9°C) treatments. Bars showing the same letter are not significantly different at $p < 0.05$ as determined by Duncan's multiple range test.

*Antioxidant enzymes*

Antioxidant enzymes alleviate oxidative stress that caused by stress such as freezing stress. SOD, APX, CAT and POD are most important antioxidant enzymes that help plants to stress tolerance. Results showed that freezing stress relatively increased antioxidant enzymes activity and application of SNP also had positive effects on their activity. SOD had highest activity at -6°C with 0.1 mM SNP application (Fig. 5). APX activity increased with temperature decreases; and the highest activity of APX was in 0.1 mM SNP at -9°C (Fig. 6). 0.1 mM SNP application increased CAT activity under freezing temperatures especially at -6 °C (Fig. 7).

SNP application increased the POD activity especially in concentrations of 1 mM at -9°C and 0.1 mM at -6°C with the 0.0499 and 0.0544 unite per mg protein, respectively.

Antioxidant enzymes help plant to avoid the harmful effects of ROS. Last researches showed the improvement of frost tolerance in plants is related to enhancement in activity of antioxidant enzyme (Zheng *et al.* 2008). Antioxidant enzymes especially SOD, APX, CAT and POD have an essential protective role in the scavenging process of ROS.

The dismutation of superoxide radicals in to $H_2O_2$ by SOD is an important step in the cell protection from superoxide radicals. APX, CAT and POD are the most important enzymes that catalyze $H_2O_2$ to $H_2O$ and oxygen. (Sala 1998) showed that the cold tolerant mandarins have a higher antioxidant enzyme activity than the cold sensitive ones. We observed with reduction of temperature, the activity of SOD, APX and POD increased with some exception as well as the activity of CAT reduced.

Antioxidant enzymes are important part of antioxidant system of plants that protect them from oxidative stress caused by abiotic stresses such as freezing stress. Sudden increase of ROS caused by freezing stress might be a signal of increasing the antioxidant enzymes. In fact, plant have very efficient antioxidant enzyme systems for scavenging the newly evolved ROS, that it needs *de novo* synthesis, initiated by an over expression of antioxidant enzymes encoded gens (Baek and Skinner 2012).

Our study showed that SNP application increased SOD, APX, CAT and POD under freezing stress, and 0.1 mM concentration was more effective (Fig.s 5-7). The present study confirmed the results of previous studies on other plants under abiotic stresses, such as *Poncirus trifoliate* (Fan and Liu 2012), *Brassica napus* (Kazemi *et al.* 2010), tomato (Lai, Wang, Li, Qin and Tian 2011), soybean (Böhm, Ferrarese, Zanardo, Magalhaes and Ferrarese-Filho 2010) and tall fescue (Xu, Sun, Jin and Zhou 2010) and maize (Esim and Atici 2014).

To the best of our knowledge, our study is the first report that show NO have beneficial effects against freezing injury of Citrumelo leaves.

The protective effect of NO against ROS is due to two possibilities. First, NO has been suggested to act as an antioxidant molecule that scavenge ROS (Lamattina *et al.* 2003). Second, NO can interact with ROS such as $O_2^-$ to form nitrating agent, peroxynitrite, which may served as a signaling molecule in stress response or function in protein activity regulation (Baudouin 2011).

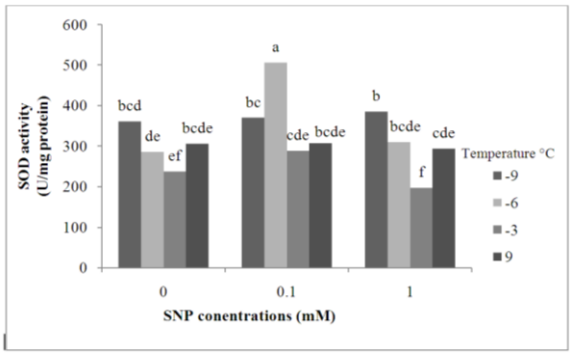


**Fig. 5.** Activity of SOD in Citrumelo leaves after SNP (0, 0.1, 1 mM) and freezing temperatures (-9, -6, -3 and + 9°C) treatments. Bars showing the same letter are not significantly different at $p < 0.05$ as determined by Duncan's multiple range test.

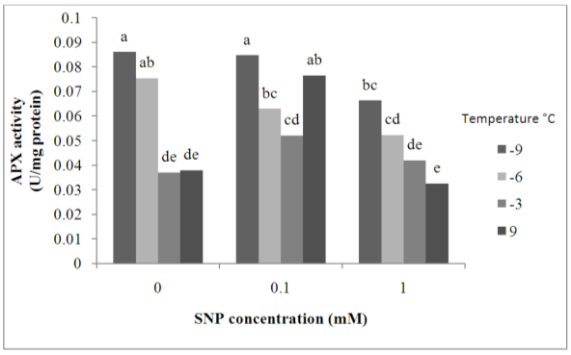


**Fig. 6.** Activity of APX in Citrumelo leaves after SNP (0, 0.1, 1 mM) and freezing temperatures (-9, -6, -3 and + 9°C) treatments. Bars showing the same letter are not significantly different at $p < 0.05$ as determined by Duncan's multiple range test.

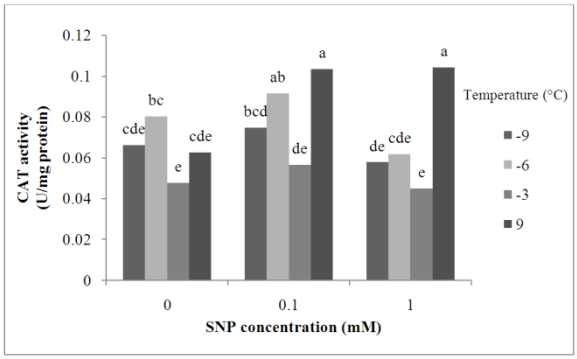


**Fig. 7.** Activity of CAT in Citrumelo leaves after SNP (0, 0.1, 1 mM) and freezing temperatures (-9, -6, -3 and + 9°C) treatments. Bars showing the same letter are not significantly different at $p < 0.05$ as determined by Duncan's multiple range test.

**Abbreviations**

NO: Nitric oxide, SNP: Sodium nitroprusside, EL: Electrolyte leakage, MDA: Malondialdehyde, DPPH: 2, 2 diphenyl 2 picrylhydrazyl hydrate, SOD: Superoxide dismutase, APX: Ascorbate peroxidase, CAT: Catalase, POD: Peroxidase, ROS: reactive oxygen species.

**Conclusion**

Freezing stress in early spring and late autumn is a very important factor that damages crops such as citrus. Citrus are subtropical plants and they are sensitive to frost. However, many areas that citrus is grown there, are in freezing risk. In this study, we demonstrated alleviative effect of SNP on freezing stress in Citrumelo; the important citrus genotype that used in citrus propagation as rootstock. NO that released from SNP improved free radical scavenging by enforcement of plant antioxidant system, so protected cells, and plants could resist to low temperature.

This study demonstrate that SNP application increased tolerance of freezing stress by increasing the activity of antioxidant enzymes, antioxidant capacity and cell viability, as well as, decrease electrolyte leakage and lipid peroxidation. Thus exogenous application of NO can be used to reduction of frost injury in plants such as Citrumelo.

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