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# EFFECTS OF EXOGENOUS NO ON ASA-GSH CIRCULATION METABOLISM IN YOUNG LOQUAT FRUIT MITOCHONDRIA UNDER LOW TEMPERATURE STRESS

**JINCEHNG WU *, JINGJING WU, JIE LIANG, JIANQIN CHEN AND LIQIN CAI**

*Department of Environment and Life Science, Putian University, Xueyuan Road, Putian 351100, China*
**Corresponding author's e-mail: wjc2384@163.com; Phone: 0086-594-2631931; Fax: 0086-594-2631931*

**Abstract**

The effects of exogenous nitric oxide (NO) on antioxidant systems under low temperature stress in young loquat (*Eriobotrya japonica* Lindl. cv. Jiefangzhong) fruit mitochondria were investigated in this study. Young loquat fruits were treated with 0.2, 0.5 and 1.0 mmol $L^{-1}$ of sodium nitro-prusside (SNP) under 0°C for 6-hours. The results indicated that the concentrations of hydrogen peroxide ($H_2O_2$) was lower than the control following treatment. However, reduced glutathione (GSH) and ascorbate (AsA) concentrations resulted in increased ascorbate peroxidase (APX, EC 1.11.1.11), glutathione reductase (GR, EC 1.6.4.2), dehydroascorbate reductase (DHAR, EC 1.8.5.1), and monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) activity relative to the control ($p<0.05$). Therefore, we concluded that suitable exogenous NO concentration enhanced the mitochondrial antioxidant capacity in young loquat fruits by increasing GSH and AsA concentrations, elevating APX, GR, DHAR and MDHAR activity, and reducing $H_2O_2$ concentration. As a result, oxidative damage was reduced and the cold-resistance capacity of young loquat fruits was increased under low temperature stress conditions.

## Introduction

Loquat (*Eriobotrya japonica* Lindl.) is an evergreen tree of the Rosaceae subfamily Maloideae with origins in subtropical China. According to Ecological Types, common loquat is classified as a temperate cultivar and a tropical cultivar type. The temperate cultivar grows in the north subtropical region of China and in some regions experiences winter snow; therefore this ecological type has high cold resistance. The tropical cultivar is distributed in the south subtropical and marginal tropical zones of China. The loquat varieties cultivated in Fujian and Guangdong Province have low cold resistance and in this part of south China, young loquat fruits frequently suffer from freezing injury during January and February due to Siberian cold high-pressure air reaching the region. Consequently, low temperature stress is a limiting factor in loquat varieties growth in south China. Mitochondria are the primary organelle to produce reactive oxygen species (ROS) in the cell (Noreen *et al*., 2009). Low temperature stress induces excess accumulation of ROS, which can damage proteins and DNA, and even result in lipid peroxidation (Halliwell & Gutteridge, 1999; Neill *et al.*, 2002). ROS can also act as a signal molecule or take part in programmed cell death, resulting in growth delays (Lamb & Dixon, 1997; Bethke & Jones, 2001; Noreen & Ashraf 2008), production decline and even plant death (Chen & Xu, 1998). Nitric oxide (NO) might function as an important redox signaling and toxicity molecule. The molecule has been shown to cause defense-related gene expression and function as an anti-oxidant under stress conditions (Zhao *et al.*, 2002). Wang *et al*., (2004) found a significant protective effect of exogenous NO on wheat under osmotic stress. Ma *et al*., (2005) reported that different concentrations of SNP, a NO precursor, showed antioxidation effects on annual ryegrass. However, the direct effects of exogenous NO on young loquat fruit mitochondria under low temperature stress have not been reported. Our objective was to explore the influence of exogenous NO on the antioxidant system of young loquat fruit mitochondria using AsA-GSH circulation metabolism. We specifically investigated and clarified the cold resistance mechanism regulated by exogenous NO in young loquat fruit mitochondria under low temperature stress conditions.

## Materials and Methods

Container seedlings of three-year-old *Eriobotrya japonica* Lindl. cv. Jiefangzhong were grown at Putian University, Putian City, Fujian Province, China. We selected young loquat fruits 60 days following anthesis from trees planted in well-spaced rows growing in a regularly maintained orchard. The fruits showed no sign of pests and damage. Young fruits were sprayed with 100 ml SNP of the following concentrations: 0.2, 0.5 and 1.0 mmol $L^{-1}$. The fruits were separated into 3 groups: T1: treatments with low temperature stress and 0.2 mmol $L^{-1}$ SNP; T2: treatments with low temperature stress and 0.5 mmol $L^{-1}$ SNP; T3: treatments with low temperature stress and 1.0 mmol $L^{-1}$ SNP; CK: treatments with low temperature stress and 0 mmol/L SNP (treated with distilled water as the control). Samples were subsequently covered with plastic film and maintained for 2-hours to maintain moisture. Following treatments, all fruits were kept at 0°C for 6-hours in an Artificial Atmospheric Phenomena Simulator before equilibrating at room temperature for 10 h. Finally, the treated fruits were freezed in liquid nitrogen and stored at -70°C in an ultra-low temperature freezer.

**Mitochondria preparation:** Mitochondria were extracted from young loquat fruits applying the method developed by Xu *et al*., (2008). Mitochondria were suspended in 0.3 mol $L^{-1}$ mannitol soliquoid to determine $H_2O_2$, GSH and AsA concentrations and APX, GR, DHAR and MDHAR activity.

**Determination of $H_2O_2$, GSH and AsA content:** $H_2O_2$ content were established using the method of Zou (2000). GSH and AsA content were determined following Chen & Wang (2002).

**Determination of enzyme activity:** Determination of APX and GR activity followed the protocol in Chen & Wang (2002). $OD_{290nm}$ change by 0.01 in 1 min was defined as one unit of APX activity. $OD_{340nm}$ change by 0.01 in 1min was defined as one unit of GR activity.

Determination of DHAR and MDHAR activity followed Song *et al*., (2005). DHAR activity was expressed in μmol of AsA $min^{-1}$ $mg^{-1}$ prot, and MDHAR activity was expressed in nmol of NADH $min^{-1}$ $mg^{-1}$ prot.

**Data analysis:** Each measurement was repeated three times and an average value was obtained for data analysis. SPSS software was used to perform statistical analyses.

## Results

**Effect of NO on $H_2O_2$ content in young loquat fruit mitochondria:** $H_2O_2$ is one a primary ROS in plants. Excessive accumulation of $H_2O_2$ induces oxidation of oxygen and even results in plant mortality. Mitochondrial $H_2O_2$ content in CK was higher than those treated with SNP (Fig. 1). $H_2O_2$ content decreased significantly in both T2 and T1 groups ($p<0.01$and $p<0.05$, respectively). However, although the mitochondrial $H_2O_2$ content in fruits treated with 1.0 mmol $L^{-1}$ SNP was lower than CK, the difference was not significant ($p>0.05$). However, overall we concluded that mitochondrial $H_2O_2$ content in young fruits declined with SNP treatment.

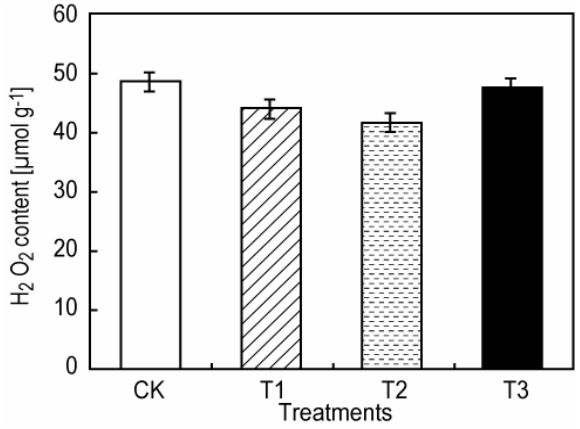


Fig. 1 Effect of NO on $H_2O_2$ content in young loquat fruit mitochondria. CK: 0 mmol $L^{-1}$ SNP Treatments with low temperature stress and 0 mmol $L^{-1}$ SNP ; T1: Treatments with low temperature stress and 0.2 mmol/$L^{-1}$ SNP; T2: Treatments with low temperature stress and 0.5 mmol $L^{-1}$ SNP; T3: Treatments with low temperature stress and 1.0 mmol $L^{-1}$ SNP.

**Effects of NO on GSH and AsA content in young loquat fruit mitochondria:** GSH is an effective peroxide scavenger in the cell, and plays a fundamental role in clearing ROS. Mitochondrial GSH content in fruits treated with 0.2, 0.5 and 1.0 mmol $L^{-1}$ SNP were higher than the control, and significantly higher at 0.2 and 0.5 mmol $L^{-1}$ SNP ($p<0.01$, $p<0.01$and $p>0.05$, respectively) (Fig. 2). These results indicated that treatments with the two lowest SNP concentrations significantly increased mitochondrial GSH content of young loquat fruits under low temperature stress.

**Effect of NO on APX and GR activity in young loquat fruit mitochondria:** APX is a key enzyme that scavenges $H_2O_2$ in plant cells. It is one of the most important

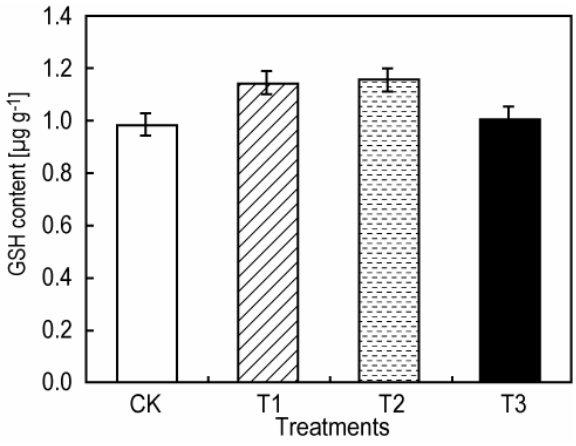


Fig. 2 Effect of NO on GSH content in young loquat fruit mitochondria. CK: 0 mmol $L^{-1}$ SNP Treatments with low temperature stress and 0 mmol $L^{-1}$ SNP ; T1: Treatments with low temperature stress and 0.2 mmol $L^{-1}$ SNP; T2: Treatments with low temperature stress and 0.5 mmol $L^{-1}$ SNP; T3: Treatments with low temperature stress and 1.0 mmol $L^{-1}$ SNP.

AsA directly eliminates $H_2O_2$ and serves an important role in ROS detoxification processes. The order of mitochondrial AsA content in fruits was as follows: T2>T1>T3>CK (Fig. 3). Differences in mitochondrial AsA content among groups T1, T2 and CK were significant ($p<0.01$). However, significant differences in mitochondrial AsA content between 1.0 mmol $L^{-1}$ SNP and the control ($p>0.05$) were not observed. These results demonstrated that SNP increased the AsA content in mitochondria and the effect of SNP on AsA and GSH content in fruit mitochondria was similar.

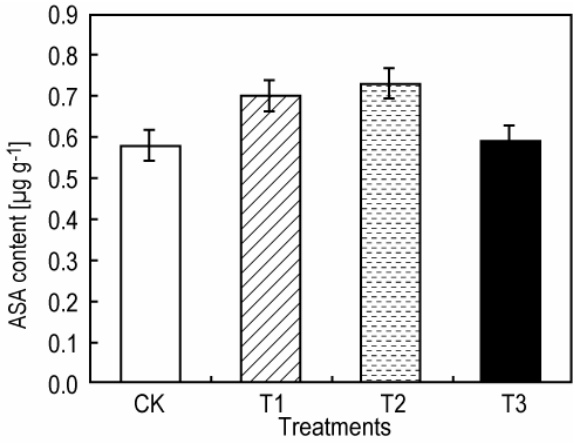


Fig. 3 Effect of NO on AsA content in young loquat fruit mitochondria. CK: 0 mmol $L^{-1}$ SNP Treatments with low temperature stress and 0 mmol $L^{-1}$ SNP ; T1: Treatments with low temperature stress and 0.2 mmol $L^{-1}$ SNP; T2: Treatments with low temperature stress and 0.5 mmol $L^{-1}$ SNP; T3: Treatments with low temperature stress and 1.0 mmol $L^{-1}$ SNP.

components of plant AsA-GSH circulation. Mitochondrial APX activity in young fruits treated with SNP was higher than the control. Significant differences were detected

between mitochondrial APX activity in young fruits treated with 0.2 and 0.5 mmol $L^{-1}$ SNP and CK ($p<0.01$). However, significant differences were not found between mitochondrial APX activity in young fruits treated with 1.0 mmol $L^{-1}$ SNP and CK ($p>0.05$) (Fig. 4). Consequently, APX activity can be increased through treatments with suitable SNP levels, enhancing ROS elimination ability and improving antioxidant capacity.

GSH is oxidized into GSSG when it clears ROS; and GSSG is reduced to GSH by GR in plant cells. GR has a central role in effective operation of AsA-GSH circulation and oxidative stress response (May *et al.*, 1998). Compared with CK, mitochondrial GR activity was highest in T2, followed by T1 and T3. In addition, relative to CK, significant increases in mitochondrial GR activity were observed in young fruits treated with 0.2 and 0.5 mmol $L^{-1}$ SNP ($p<0.01$) (Fig. 5). The difference between mitochondrial GR activity in young fruits treated with 1.0 mmol $L^{-1}$ SNP and CK was not significant ($p>0.05$). The results showed that different SNP concentrations have different effects on mitochondrial GR activity in young loquat fruits. Mitochondrial GR activity significantly increased in fruits treated with lower SNP concentrations (i.e. 0.2 and 0.5 mmol $L^{-1}$).

**Effect of NO on DHAR and MDHAR activity in young loquat fruit mitochondria:** AsA is one of the most important antioxidants in plant cells. DHAR and MDHAR play important roles in maintaining cellular AsA levels. Results showed that mitochondrial DHAR activity in loquat fruits was higher following SNP treatments (Fig. 6). Significant differences in mitochondrial DHAR activity between young fruits treated with 0.5 mmol $L^{-1}$ SNP and the control ($p<0.01$) were evident. However, differences in mitochondrial DHAR activity among other groups and CK were not significant ($p>0.05$). Fig. 7 shows that MDHAR activity in samples treated with 0.2 and 0.5 mmol $L^{-1}$ SNP were higher than CK ($p<0.05$), whereas MDHAR activity in samples treated with 1.0 mmol $L^{-1}$ SNP were slightly (but not significantly) higher than CK.

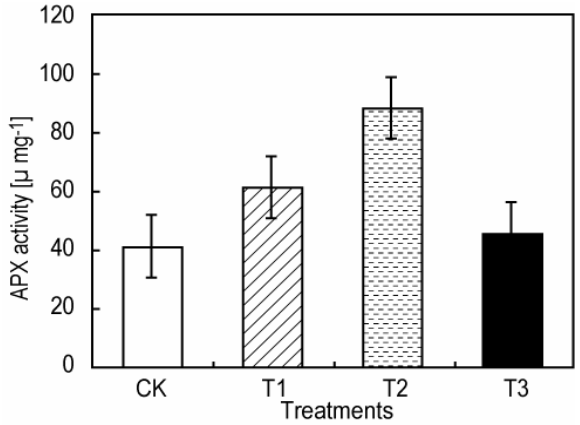


Fig. 4 Effect of NO on APX activity in young loquat fruit mitochondria. CK: 0 mmol $L^{-1}$ SNP Treatments with low temperature stress and 0 mmol $L^{-1}$ SNP ; T1: Treatments with low temperature stress and 0.2 mmol $L^{-1}$ SNP; T2: Treatments with low temperature stress and 0.5 mmol $L^{-1}$ SNP; T3: Treatments with low temperature stress and 1.0 mmol $L^{-1}$ SNP.

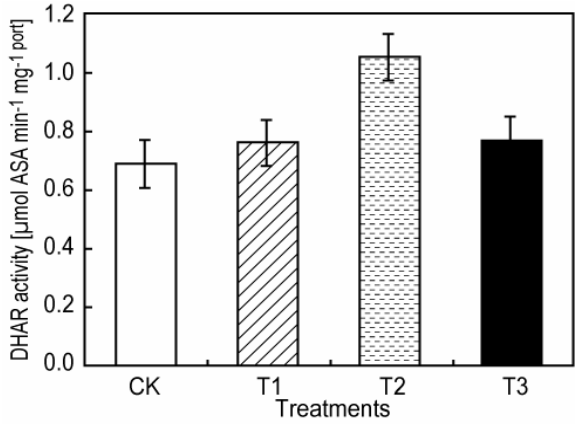


Fig. 6 Effect of NO on DHAR activity in young loquat fruit mitochondria. CK: 0 mmol $L^{-1}$ SNP Treatments with low temperature stress and 0 mmol $L^{-1}$ SNP ; T1: Treatments with low temperature stress and 0.2 mmol $L^{-1}$ SNP; T2: Treatments with low temperature stress and 0.5 mmol $L^{-1}$ SNP; T3: Treatments with low temperature stress and 1.0 mmol $L^{-1}$ SNP.

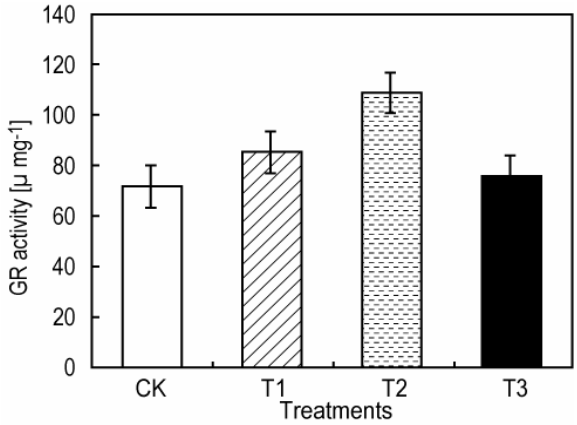


Fig. 5 Effect of NO on GR activity in young loquat fruit mitochondria. CK: 0 mmol $L^{-1}$ SNP Treatments with low temperature stress and 0 mmol $L^{-1}$ SNP ; T1: Treatments with low temperature stress and 0.2 mmol $L^{-1}$ SNP; T2: Treatments with low temperature stress and 0.5 mmol $L^{-1}$ SNP; T3: Treatments with low temperature stress and 1.0 mmol $L^{-1}$ SNP.

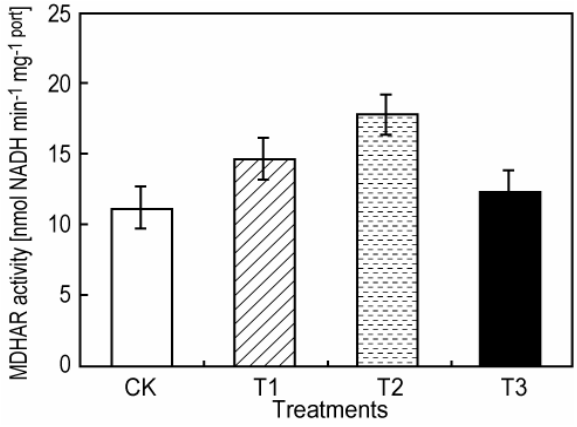


Fig. 7. Effect of NO on MDHAR activity in young loquat fruit mitochondria.

CK: 0 mmol $L^{-1}$ SNP Treatments with low temperature stress and 0 mmol $L^{-1}$ SNP ; T1: Treatments with low temperature stress and 0.2 mmol $L^{-1}$ SNP; T2: Treatments with low temperature stress and 0.5 mmol $L^{-1}$ SNP; T3: Treatments with low temperature stress and 1.0 mmol $L^{-1}$ SNP.

## Discussion

The production equilibrium and ROS clearance in plant cells is altered in response to low temperature stress. Increased ROS accumulation will initiate or aggravate membrane lipid peroxidation damage, which can even result in cell death. Low temperature stress damage can be alleviated by a rapid clearing of ROS. In plant cells, $H_2O_2$ is mainly generated in mitochondria. AsA-GSH circulation metabolism is the primary anti-oxidation mechanism to remove mitochondrial $H_2O_2$ (Luo & Song, 2002). APX can facilitate a reaction between AsA and $H_2O_2$. $H_2O_2$ is hydrogenated into $H_2O$ by accepting an electron of NADPH that is GSH-mediated to detoxify $H_2O_2$. Concurrently, AsA is oxidized into MDHA, and part of MDHA is further oxidized into DHA; and with the aid of DHAR, DHA is hydrogenated into AsA using GSH as the substrate. The GSSG produced in this reaction can be hydrogenated into GSH with GR as the enzyme; MDHA can also be hydrogenated into AsA with MDHAR as the enzym (Luo *et al.*, 2007).

Wang & Li (2002) demonstrated that in maize roots biochemical changes occurred with cold acclimation, including the accumulation of $H_2O_2$, which included cell membrane damage and cell senescence. $H_2O_2$ is inactive and can survive for long periods in cells and occupy every part of a cell as a ROS signal. In this analysis, we showed that exogenous NO reduced mitochondrial $H_2O_2$ content in young fruits. We suggest two explanations for these results. First, exogenous NO clears $H_2O_2$ directly (Wang *et al.*, 2004) or exogenous NO promotes a scavenging effect of AsA-GSH mitochondrial circulation on $H_2O_2$ by means of increasing antioxidant content and antioxidant enzyme activity.

GSH and AsA are two vital antioxidants in plant cell AsA-GSH circulation. GSH can react with free radicals directly and participate in AsA-GSH circulation; and converts to GSSG from GSH. AsA plays a prominent role in oxidative stress resistance because it directly clears $H_2O_2$. It is also involved in the regulation of enzyme activity and other metabolic processes (Ma *et al.*, 2007). Ruan *et al.*, (2005) reported that exogenous NO increased GSH content of wheat seedlings and improved salt tolerance. In the present study, following exogenous NO treatment, AsA and $H_2O_2$ content and GSH and $H_2O_2$ content were negatively correlated. The correlation coefficients (r) between GSH content and $H_2O_2$ content in mitochondria treated with 0.2, 0.5 and 1.0 mmol $L^{-1}$ SNP were -0.884, -0.500 and -0.756, respectively. The correlation coefficients (r) between AsA content and $H_2O_2$ content in mitochondria treated with 0.2, 0.5 and 1.0 mmol $L^{-1}$ SNP were a respective -0.448, -0.743 and -0.756. The above results indicate that NO treatment not only improved AsA-GSH circulation metabolism of mitochondria in young loquat fruits under low temperature stress, but also increased the mitochondrial GSH and AsA content. These factors have the potential to reduce the damage of young fruit tissue caused by low temperature stress.

APX shows marked effects at eliminating $H_2O_2$, which catalyzes the reaction of AsA and $H_2O_2$ and successfully decomposes $H_2O_2$ (Bowler *et al.*, 1992). Luo *et al.*, (2007) suggested that an increase in APX activity was beneficial to improve plant resistance. Xiang & Oliver (1998) proposed that APX was activated by $H_2O_2$, and NO induced APX gene expression. This may explain improved APX activity under low temperature conditions. Liu *et al.*, (2005) demonstrated that an increase in APX activity contributed to improvement in rice salt resistance. Our results indicated the correlation coefficients (r) between APX activity and $H_2O_2$ content in mitochondria treated with 0.2, 0.5 and 1.0 mmol $L^{-1}$ SNP were 0.961, 0.866 and 0.903, respectively.

It is clear that APX played a central role in the process of $H_2O_2$ elimination. An accumulation of $H_2O_2$ will alleviate harm to young loquat fruits when treated with 0.5 mmol $L^{-1}$ SNP through increased APX activity.
GSSG can be converted to a reduced form by GR. Its activity can directly affect the content of GSH. Higher GSH content can stabilize membrane protein structure. GSH and GR are recognized as important indicators of plant antioxidant status; and are integral in the cellular clearance of $H_2O_2$ by participating in AsA-GSH circulation (Yi *et al.*, 2007). GR activity and $H_2O_2$ content exhibited a negative correlation; correlation coefficients (r) between GR activity and $H_2O_2$ content in mitochondria treated with 0.2, 0.5 and 1.0 mmol $L^{-1}$ SNP were -0.390, -0.500 and -0.726 respectively. Therefore, data indicated the increase in GR activity was responsible for the reduction of $H_2O_2$ content. Results also indicated that increased GR activity might be related to exogenous NO as a signaling molecule involved in regulation. SNP treatment promoted conversion of GSSG into GSH that eliminated $H_2O_2$, resulting in a reduction in the accumulation of ROS in the mitochondria. As a result, the antioxidant capacity of young fruits was promoted.

AsA oxidizes into MDHA and DHA when it scavenges $H_2O_2$. MDHA and DHA regenerate AsA with DHAR and MDHAR as enzymes (Song *et al.*, 2005). Our results showed the correlation coefficients (r) between DHAR activity and AsA content in mitochondria treated with 0.2, 0.5 and 1.0 mmol $L^{-1}$ SNP were 0.403, 0.411 and 0.850, respectively; while the correlation coefficients (r) between MDHAR activity and AsA content were a respective 0.661, 0.601 and 0.955, which indicated MDHAR activity and DHAR facilitated the accumulation of AsA. The analysis further demonstrated that exogenous NO treatment induced an increase in DHAR and MDHAR activity, and enhanced the regenerative capacity of AsA maintaining the function of AsA-GSH circulation metabolism, ultimately reducing the damage to young fruits caused by low temperature stress.

High levels of GSH facilitated AsA-GSH circulation, elevate AsA content, and increase mitochondrial APX, MDHAR and GR activity (Aono *et al.*, 1995). In the present study, our results demonstrated that GSH and AsA content and APX, GR, DHAR and MDHAR activity were markedly increased in treatments with low temperature stress and 0.5 mmol $L^{-1}$ SNP. All these factors together facilitated AsA-GSH circulation and enhanced the free radical scavenging ability of mitochondria, reducing membrane lipid peroxidation. We suggest this is a primary reason that exogenous NO inhibits harm to young loquat fruits under low temperature stress. Furthermore, results derived from young loquat fruits treated with 1.0 mmol $L^{-1}$ SNP lead us

to infer that high concentrations of SNP treatment may adversely affect cold tolerance of young loquat fruits.

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