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Full Length Article

Calcium and Nitric Oxide are involved in Signal Transduction for Low-Temperature Stress Tolerance of *Eriobotrya japonica* Seedlings

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Abstract

Two-year-old container seedlings of *Eriobotrya japonica* Lindl. cv. Zaozhong No. 6 were used as materials to investigate the effects of treatment with various agents, i.e., sodium nitroprusside (SNP, an exogenous nitric oxide [NO] donor), $CaCl_2$, 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO, a NO scavenger), and N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W7, a calmodulin [CaM] antagonist), on the response of loquat seedlings to low-temperature stress by enhancing or impeding the transduction of Ca^{2+} and NO signals in an attempt to understand the regulatory mechanisms through which Ca^{2+} and NO increase the hardiness of loquat seedlings. The results showed that $CaCl_2$ and SNP treatment increased the CaM content and the activities of superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX) in the leaves of seedlings under low-temperature stress and reduced the cellular accumulation of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA). Co-treatment with $CaCl_2$ + SNP showed a synergistic effect. In two treatment combinations (cPTIO + $CaCl_2$ and W7 + SNP), both cPTIO and W7 inhibited the physiological effects that were otherwise induced by SNP and $CaCl_2$ treatment while blocking the synergistic effect of $CaCl_2$ + SNP co-treatment and exacerbating oxidative damage to seedlings exposed to low temperature. The results suggest that interactions in Ca^{2+} and NO signal transduction occur in loquat seedlings in response to low-temperature stress. © 2020 Friends Science Publishers

Keywords: *Eriobotrya japonica*; Low-temperature stress; Ca^{2+}; NO; Signal transduction

Introduction

Loquat (*Eriobotrya japonica* Lindl.), an evergreen fruit tree native to subtropical regions of China, is sensitive to low-temperature stress; this is an important factor affecting the geographical distribution and production of loquats (Wu *et al*. 2016). Tropical loquat varieties cultivated in the southern subtropical and tropical marginal areas (*e.g*., Fujian and Guangdong provinces in China) display poor low-temperature tolerance in the southern areas of China. In particular, the major loquat cultivar Zaozhong No. 6 suffers from severe frost damage, even leading to a total loss of yield (Wu *et al*. 2010). Northern China has cold weather, thus making it unsuitable for loquat cultivation. In recent years, small-scale production of loquat has been developed in some northern cities, such as Beijing and Yingkou, using cultivation facilities according to the local conditions. Therefore, it is necessary to investigate how to improve the frost resistance in loquat.

Nitric oxide (NO) and Ca^{2+} are ubiquitous second messengers in plants, which play important roles in regulating various physiological and metabolic processes in response to stress (Lamattina *et al*. 2003; Reddy *et al*. 2011; Zhang *et al.* 2019). It was found that sodium nitroprusside (SNP) treatment significantly enhanced the activities of antioxidant enzymes, reduced the level of reactive oxygen species (ROS), and decreased the level of membrane lipid peroxidation in ginger leaves, which alleviated the heat damage of ginger leaves and increased the heat resistance of ginger plants (Li *et al.* 2014). SNP treatment obviously increased antioxidant enzyme activities in pumpkin seedlings under cold stress, decreased the accumulation of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA), showed protective effects against oxidative damage of the seedlings, and enhanced the adaptability of the plants to cold stress (Wu *et al*. 2016). In previous studies, we found that appropriate SNP treatment of young fruit induced protective enzyme activities, decreased the level of membrane lipid peroxidation, and improved the fruit's cold resistance (Wu *et al*. 2010).

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Numerous studies have shown that exogenous Ca^{2+} improves plant resistance to various stresses, including low temperature, high temperature and drought, which are associated with ROS metabolism (Ramanjulu and Sudhakar 2001; Sulochana and Rao 2002). Calcium treatment significantly increased the activities of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) in tomato under high-temperature stress and prevented the damage of photosynthetic organs caused by heat-induced ROS, thereby improving the heat resistance of the plants (Qi *et al.* 2015). Calcium treatment also enhanced the activities of SOD, CAT and POD in muskmelon and alleviated the peroxidation damage of membrane lipids due to the accumulation of ROS under cold stress, thus enhancing its cold resistance (Li *et al.* 2011). We found that calcium treatment activated SOD and CAT activities in loquat seedlings exposed to low-temperature stress and decreased the level of membrane lipid peroxidation, thus increasing the frost resistance of loquat seedlings (Wu *et al.* 2016). Although it has been confirmed that NO and Ca^{2+} play regulatory roles in the frost resistance in many plant species, it is still not clear whether the NO and Ca^{2+} pathways are crosslinked in response to low-temperature stress in loquat. In this study, we investigated the association between Ca^{2+} and NO signalling pathways in loquat seedlings in response to low-temperature stress. The results could provide a theoretical basis for the prevention of frost damage to loquat.

Materials and Methods

Plant materials and treatments

Plant materials: Two-year-old container seedlings of loquat (*Eriobotrya japonica* Lindl. cv. Zaozhong No. 6) with normal and uniform growth were provided by the Putian Institute of Pomology and used as test materials.

Treatments: Sodium nitroprusside (SNP, an exogenous nitric oxide [NO] donor), $CaCl_2$, 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO, a NO scavenger), and N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W7, a calmodulin [CaM] antagonist) were used to treat loquat seedlings, respectively. The seedlings were randomly divided into eight groups that were subjected to different treatments: Control (CK), H_2O; Treatment 1 (T1), $CaCl_2$; T2, 2-4-carboxyphenyl-4, 4, 5, 5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) + $CaCl_2$; T3, SNP; T4, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W7) + SNP; T5, $CaCl_2$ + SNP; T6, cPTIO + $CaCl_2$ + SNP; and T7, W7 + $CaCl_2$ + SNP. The treatments were performed according to the methods of Yang *et al.* (2015), Wu *et al.* (2016) and Zhang *et al.* (2018). The final concentration of SNP, $CaCl_2$, cPTIO and W7 in different treatments were 0.5, 5, 0.5 and 0.5 mmol·L^{-1} (each treatment solution was supplemented with 1% Tween-20), respectively. These treatment solutions were sprayed on the leaves of plants at room temperature (25°C) at 17:00 until it dripped off the leaves. Spraying was carried out every two days and repeated three times. Twenty-four h after spraying, the container seedlings were exposed to low-temperature stress at -3°C as described by Wu *et al.* (2010). Fifteen container seedlings from each treatment group were placed in an artificial climate chamber (relative humidity: 70%; light intensity: 20 W/m^2), and the temperature was lowered to -3°C for 6 h; the temperature was then increased to 25°C and maintained at that level for 12 h before the samples were taken. The sampling was performed according to Wu *et al.* (2016). The 3rd to the 5th leaves from the top were collected, frozen in liquid nitrogen and stored in a -70°C freezer. Three biological replicates were collected, and each replicate was a mixed sample of five container seedlings. SNP (a NO donor), W7 (a calmodulin [CaM] antagonist), and cPTIO (a NO scavenger) were purchased from Sigma (Merck KGaA, Darmstadt, Germany). Other chemicals used in this study were analytical grade reagents from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Determination of H_2O_2, MDA and CaM

The H_2O_2 content was determined based on the method of Patterson *et al.* (1984). For this purpose, 2 g of leaf tissue was ground in liquid nitrogen and homogenised with chilled 100% acetone and then centrifuged at 10,000 × g for 20 min at 4°C. The supernatant was used for analysis of H_2O_2, which was expressed as μmol g^{-1} FW (fresh weight). The content of MDA was analysed following the method of Hodges *et al.* (1999). Two grams of leaf tissue was ground in liquid nitrogen and homogenised with 80% ethanol, followed by centrifugation at 10, 000 × g for 20 min. An appropriate volume of supernatant was added to the reaction mixture. The supernatant was appropriately added to a reaction mixture containing 20% (w/v) trichloroacetic acid and 0.01% butylated hydroxytoluene. The reactions were mixed and heated at 95°C for 25 min, followed by centrifuging at 3000 × g for 10 min after cooling. The absorbances were read at 440 nm, 532 nm and 600 nm. The MDA content was calculated and expressed as mmol g^{-1} FW. The CaM content was measured by enzyme-linked immunosorbent assay (ELISA) using the Plant CaM ELISA Kit (Kmaels, Shanghai, China) according to the user manual. Each assay was repeated three times. The CaM content was expressed as μg g^{-1} FW.

Enzyme activity determination

Enzyme extraction and SOD activity analysis were performed according to Stewart and Bewley (1980) method. Two grams of leaf tissue were ground in liquid nitrogen and homogenised in cold 50 m*M* phosphate buffer (pH 7.0). The homogenate was then centrifuged at 15,000 × g for 30 min.

The supernatant was retained as the enzyme extract. Fifty micrograms of enzyme extract was added to 1 mL of solution for the measurement of enzyme activity. The measurement of SOD activity was assayed in a reaction mixture containing 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 μm nitroblue tetrazolium (NBT), 2 μm riboflavin, 100 nM EDTA and the enzyme extract. The amount of enzyme required to inhibit the reduction of NBT by 50% was defined as one activity unit (U) of SOD. The activities of CAT and POD were measured as described by Cakmak and Marschner (1992), and the activity of ascorbate peroxidase (APX) was based on the method of Nakano and Asada (1981). CAT activity was assayed in a reaction mixture containing 25 mM phosphate buffer (pH 7.0), 10 mM H_2O_2 and the enzyme extract. The POD activity was assayed in a reaction mixture containing 25 mM phosphate buffer (pH 7.0), 0.05% guaiacol, 10 mM H_2O_2 and the enzyme extract. The APX activity was assayed in a reaction mixture containing 50 mM phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.1 mM H_2O_2, 100 μM EDTA and the enzyme extract. One activity unit (U) of CAT, POD and APX was defined as the amount of enzyme that produced a change of 0.01 in optical density (OD) at 240 nm, 470 nm and 290 nm, respectively, in 1 min. Each assay was repeated three times.

Data analysis

Each treatment group included 15 container seedlings and three replicates were set randomly for the determinations of H_2O_2, MDA, CaM and enzyme activity. Significance tests of differences between groups and correlation analyses were performed using Excel 2003 and SPSS 19.0 statistical software; the means of three replicates were used in the analyses.

Results

Effects of treatments on H_2O_2 content

Fig. 1a shows that in the seedlings that received the single treatments $CaCl_2$ and SNP, and in those that received co-treatment $CaCl_2$ + SNP, the leaf H_2O_2 contents were significantly lower than that of control plants ($p < 0.05$); the H_2O_2 contents of the leaves were in the order CK (H_2O) > $CaCl_2$ > SNP > $CaCl_2$ + SNP, indicated that exogenous SNP or $CaCl_2$ treatment effectively reduced the intracellular accumulation of H_2O_2. Co-treatment with SNP + $CaCl_2$ had a notable synergistic effect, while the H_2O_2 scavenging capacity of $CaCl_2$ was lower than that of SNP. The leaf H_2O_2 content of seedlings treated with cPTIO + $CaCl_2$ + SNP was slightly higher than that of the seedlings in the $CaCl_2$ group, indicated that cPTIO, a NO scavenger, not only abolished the ability of SNP to scavenge cellular H_2O_2 but also weakens the ability of $CaCl_2$ to scavenge cellular H_2O_2. The leaf H_2O_2 content of plants that received the W7 + SNP treatment or W7 + $CaCl_2$ + SNP treatment was significantly higher than that of CK (H_2O), indicating that W7, a CaM antagonist, not only inhibits the effects induced by exogenous $CaCl_2$ and SNP but also weakens the ability of the cells to resist low-temperature oxidative stress; as a result, under low-temperature stress conditions, cellular H_2O_2 may be not removed in a timely manner and thus remains at a high level.

Effect of treatments on MDA content

Fig. 1b shows that when seedlings were exposed to low-temperature stress at -3°C, the leaf MDA content of the seedlings that received the single treatments $CaCl_2$) or SNP or the co-treatment $CaCl_2$ + SNP was significantly lower than that of the seedlings in the CK group ($p < 0.05$); the leaf MDA content showed the order SNP > $CaCl_2$ > $CaCl_2$ + SNP, indicating that the treatments had inhibitory effects on membrane lipid peroxidation in the leaf cells and that the inhibitory abilities of the three treatments differed. The inhibitory effect of $CaCl_2$ + SNP was higher than those of $CaCl_2$ and SNP. The MDA contents of leaves treated with cPTIO + $CaCl_2$ and cPTIO + $CaCl_2$ + SNP, both co-treatments, were significantly higher than those of plants treated with $CaCl_2$ and $CaCl_2$ + SNP to varying degrees ($p < 0.01$). The MDA content of the leaves of seedlings treated with W7 + SNP and W7 + $CaCl_2$ + SNP was significantly higher than the plants treated with SNP and $CaCl_2$ + SNP, and the differences were significant ($p < 0.01$). These results indicated that cPTIO and W7 promoted cell membrane lipid peroxidation and weakened the inhibitory effects of $CaCl_2$ and SNP on cell membrane lipid peroxidation; the inhibitory effect of the NO scavenger cPTIO was weaker than that of the CaM antagonist W7. The leaf MDA content of seedlings treated with cPTIO + $CaCl_2$ was higher than that of seedlings treated with $CaCl_2$, whereas the leaf MDA contents of seedlings treated with W7 + SNP and W7 + $CaCl_2$ + SNP were significantly higher than those of seedlings treated with $CaCl_2$, SNP, $CaCl_2$ + SNP and CK ($p < 0.01$). The results indicated that the inhibitory effect of $CaCl_2$ on membrane lipid peroxidation was partially mitigated by cPTIO, whereas W7 not only abolishes the inhibitory effects of SNP and $CaCl_2$ on cell membrane lipid peroxidation but also exacerbates oxidative damage to the membrane.

Effects of treatments on CaM content

Fig. 2 showed that under low-temperature stress conditions, the leaf CaM content of seedlings treated with $CaCl_2$), SNP) or $CaCl_2$ + SNP was significantly higher than that of the CK seedlings ($p < 0.05$), indicating that $CaCl_2$ and SNP treatment improves the content of CaM. $CaCl_2$ treatment had the most profound upregulating effect, whereas co-treatment with $CaCl_2$ + SNP produced a synergistic effect. The difference in the leaf CaM content of seedlings treated with the co-treatments cPTIO + $CaCl_2$ and cPTIO + $CaCl_2$ + SNP was

slight, and the leaf CaM contents of seedlings treated with cPTIO + $CaCl_2$ and cPTIO + $CaCl_2$ + SNP were lower than those of seedlings treated with $CaCl_2$, SNP and $CaCl_2$ + SNP, indicating that cPTIO treatment inhibits the expression of *CaM*. The leaf CaM content of seedlings treated with W7 + SNP was lower than that of seedlings treated with CK, indicating that W7 not only eliminates the increased *CaM* expression induced by SNP but also further inhibits *CaM* expression. The leaf CaM content of seedlings treated with W7 + $CaCl_2$ + SNP was significantly higher than that of seedlings treated with W7 + SNP or CK ($p < 0.01$), indicating that W7 inhibits the expression of *CaM*. However, the $CaCl_2$ used in the T7 (W7 + $CaCl_2$ + SNP) co-treatment promoted the cellular production of CaM, leading to higher leaf CaM content in seedlings treated with W7 + $CaCl_2$ + SNP compared to seedlings treated with W7 + SNP or CK. $CaCl_2$ and SNP promote the synthesis of CaM in leaf cells, while cPTIO and W7 inhibit its synthesis.

Treatments effects on the activities of antioxidant enzymes

Under low-temperature conditions, the leaf CAT activities of seedlings treated with $CaCl_2$, SNP or $CaCl_2$ + SNP were higher than that of the CK seedlings; CAT activity showed the order $CaCl_2$ + SNP > SNP > $CaCl_2$ > CK, indicating that $CaCl_2$ and SNP have the physiological effect of improving cellular CAT activity and that SNP enhances CAT activity more effectively than $CaCl_2$ (Fig. 3a). Co-treatment with $CaCl_2$ + SNP produced a synergistic effect in regulating and activating CAT activity. The leaf CAT activities of seedlings treated with cPTIO + $CaCl_2$, W7 + SNP, cPTIO + $CaCl_2$ + SNP and W7 + $CaCl_2$ + SNP were significantly lower than seedlings treated with $CaCl_2$, SNP and $CaCl_2$ + SNP, and the leaf CAT activity of seedlings treated with W7 + $CaCl_2$ + SNP was lower than seedlings treated with cPTIO + $CaCl_2$ + SNP, indicated that cPTIO and W7 inhibited leaf CAT activity and that the latter had a more profound inhibitory effect.

Fig. 3b showed that, under cold stress conditions, the leaf POD activity of seedlings treated with cPTIO + $CaCl_2$, W7 + SNP, cPTIO + $CaCl_2$ + SNP and W7 + $CaCl_2$ + SNP was lower than that of the seedlings treated with $CaCl_2$, SNP and $CaCl_2$ + SNP. Moreover, leaf POD activity of seedlings treated with W7 + SNP and W7 + $CaCl_2$ + SNP was lower than that of seedlings treated with cPTIO + $CaCl_2$ and cPTIO + $CaCl_2$ + SNP. The results indicated that both cPTIO and W7 inhibited leaf POD activity, but the inhibitory abilities of the two compounds were different; compared with cPTIO, while W7 showed a stronger inhibitory effect on leaf POD activity. The leaf POD activities showed the order $CaCl_2$ + SNP > SNP > $CaCl_2$ > CK, but the differences were non-significant ($p > 0.05$), indicated that the effect of SNP and $CaCl_2$ on leaf POD activity was non-significant, and co-treatment with SNP + $CaCl_2$ indicated no synergistic effect on leaf POD activity.

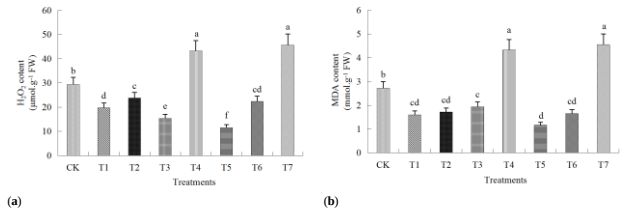

Fig. 1: Effects of different treatments on the H_2O_2 and MDA contents of loquat leaves under low-temperature stress. (**a**) H_2O_2; (**b**) MDA. CK: H_2O; T1: $CaCl_2$; T2: cPTIO + $CaCl_2$; T3: SNP; T4: W7 + SNP; T5: $CaCl_2$ + SNP; T6: cPTIO + $CaCl_2$ + SNP; T7: W7 + $CaCl_2$ + SNP; FW: fresh weight. Different letters indicate significant differences at the 0.05 level via a Tukey-Kramer test for multiple comparisons

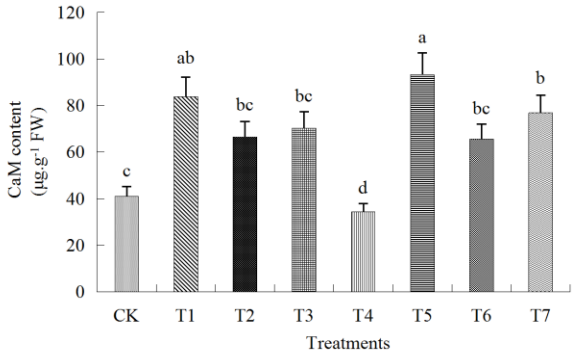

Fig. 2: Effects of different treatments on the CaM content of loquat leaves under low-temperature stress. CK: H_2O; T1: $CaCl_2$; T2: cPTIO + $CaCl_2$; T3: SNP; T4: W7 + SNP; T5: $CaCl_2$ + SNP; T6: cPTIO + $CaCl_2$ + SNP; T7: W7 + $CaCl_2$ + SNP. Different letters indicate significant differences at the 0.05 level via a Tukey-Kramer test for multiple comparisons

Fig. 3c showed that the leaf SOD activity exhibited the order $CaCl_2$ + SNP > $CaCl_2$ > SNP > CK; the leaf SOD activity of seedlings treated with $CaCl_2$, SNP or $CaCl_2$ + SNP were significantly higher than that of CK, indicated that SNP and $CaCl_2$ had an activating effect on leaf SOD activity and that the activating effect of $CaCl_2$ on SOD activity was higher than that of SNP, while co-treatment with SNP + $CaCl_2$ produces a noticeable synergistic effect. The leaf SOD activity of seedlings treated with W7 + $CaCl_2$ + SNP or W7 + SNP were significantly lower than those of seedlings treated with $CaCl_2$, SNP, $CaCl_2$ + SNP or CK, while the leaf SOD activities of seedlings treated with cPTIO + $CaCl_2$ or cPTIO + $CaCl_2$ + SNP were significantly lower than those treated with SNP or $CaCl_2$ but significantly higher than that of CK, indicating that cPTIO and W7 inhibited leaf SOD activity and the inhibitory effect of cPTIO was lower than that of W7. The leaf SOD activities of seedlings treated with cPTIO + $CaCl_2$ and cPTIO + $CaCl_2$ + SNP were similar, indicating that, during co-treatment of cPTIO + $CaCl_2$ + SNP, the activating effect of SNP on cellular SOD activity was mitigated by cPTIO and that the enhancing effect of $CaCl_2$ on cellular SOD activity was also

weakened. The leaf SOD activities of seedlings treated with W7 + SNP and W7 + $CaCl_2$ + SNP were significantly lower than those of seedlings treated with $CaCl_2$, SNP and $CaCl_2$ + SNP, indicated that the stimulatory effects of SNP and $CaCl_2$ on SOD activity were strongly suppressed by W7.

Data revealed that under low-temperature stress conditions, the leaf APX activities of seedlings treated with $CaCl_2$, SNP or $CaCl_2$ + SNP were significantly higher than that of CK ($p < 0.01$); the leaf APX activities showed the order $CaCl_2$ + SNP > SNP > $CaCl_2$ > CK, indicated that the SNP and $CaCl_2$ treatments significantly promoted leaf APX activity in plants undergoing low-temperature stress (Fig. 3d). The activating effect of SNP on APX activity is higher than that of $CaCl_2$, and the two treatments produce a synergistic effect. The leaf APX activity of seedlings treated with cPTIO + $CaCl_2$ was significantly higher than that of seedlings treated with W7 + $CaCl_2$ + SNP ($p < 0.01$), and the synergistic effect of SNP + $CaCl_2$ treatment on APX activity was inhibited by NO scavenger cPTIO and the CaM antagonist W7. The greater inhibitory effect of W7 may be because the upstream and downstream signal regulatory sites affected by W7 differ from those affected by cPTIO; this idea is supported by the observation that the leaf APX activity of seedlings treated with cPTIO + $CaCl_2$ was higher than that of seedlings treated with W7+SNP.

Correlation analysis of physiological indexes

Correlations among different physiological indexes are high and significant at the 0.01 level ($p < 0.01$). The CaM content and CAT, POD, SOD and APX activities were significantly positively correlated, and the correlation coefficients were all higher than 0.55 (Talbe 1). In contrast, the CaM contents and the activities of the four protective enzymes were negatively correlated with the H_2O_2 and MDA contents. The results indicate that the CaM content could be regulated collaboratively by the Ca^{2+} and NO signal pathways, thereby regulating the antioxidant abilities of loquat seedlings under low-temperature stress (Table 1).

Table 1: Correlation analysis of different physiological indexes

Variables	CaM	H_2O_2	MDA	CAT	POD	SOD
H_2O_2	-0.556**					
MDA	-0.525**	0.963**				
CAT	0.652**	-0.886**	-0.766**			
POD	0.558**	-0.987**	-0.913**	0.941**		
SOD	0.754**	-0.946**	-0.893**	0.909**	0.937**	
APX	0.603**	-0.962**	-0.893**	0.940**	0.977**	0.932**

The Pearson's indexes were calculated by analyze-correlate-bivariate of SPSS 19.0
** The correlation was significant at the 0.01 level（$p < 0.01$）

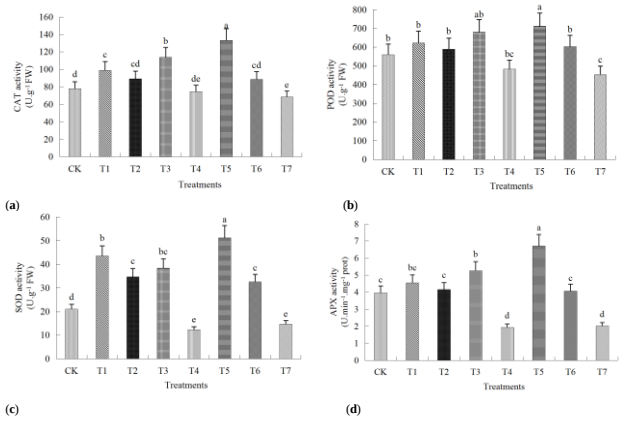

Fig. 3: Effects of different treatments on the protective enzyme activities of loquat leaves under low-temperature stress. (**a**) CAT; (**b**) POD; (**c**) SOD; (**d**) APX. CK: H_2O; T1: $CaCl_2$; T2: cPTIO + $CaCl_2$; T3: SNP; T4: W7 + SNP; T5: $CaCl_2$ + SNP; T6: cPTIO + $CaCl_2$ + SNP; T7: W7 + $CaCl_2$ + SNP. Different letters indicate significant differences at the 0.05 level via a Tukey-Kramer test for multiple comparisons

Discussion

When exposed to low-temperature stress, plant cells generated large amounts of H_2O_2 (Okuda *et al*. 1991; Guo *et al*. 2006). The excessive H_2O_2 accumulation triggered an imbalance in ROS metabolism in the cells, which could aggravate peroxidative damage to membrane lipids and cause the degradation of biomacromolecules, thereby leading to the frost damage of plants (Guo *et al*. 2006; Mishra *et al*. 2013). The activity of SOD, POD, CAT and APX in loquat leaves and young fruits were reduced after suffering low-temperature stress, while the MDA content was increased (Zheng *et al*. 2009; Wu *et al*. 2010). Various methods, including physical methods (e.g., cold acclimation), chemical methods (e.g., application of exogenous Ca^{2+}, NO, SA, etc.), and biological methods (e.g., genetic improvement), have been employed to increase the activities of cellular antioxidant enzymes, to reduce the accumulation of active oxygen in the cells and to alleviate the peroxidative damage to the membrane lipids in plants under low-temperature stress, thus improving the cold resistance of plants. Application of appropriate amounts of exogenous NO to ryegrass (*Lolium perenne*) seedlings increased the activities of protective enzymes such as SOD, CAT and POD, thus enhancing the cold tolerance of ryegrass under low-temperature stress (Ma *et al*. 2005). In previous studies, we found that appropriate exogenous $CaCl_2$ and NO treatment increased the activity of the protective enzymes (SOD, CAT and POD) and reduced the peroxidative damage of membrane lipids in different loquat organs under low-temperature stress, thereby improving the cold resistance in loquat (Wu *et al*. 2010; 2016). In this study, loquat seedlings under low-temperature stress were treated with exogenous $CaCl_2$, SNP, and $CaCl_2$ + SNP, and it was found that all three treatments increased the activities of the protective enzymes (CAT, POD, SOD and APX) and facilitated the removal of endogenous active oxygen to varying degrees, thereby decreasing the intracellular H_2O_2 and MDA contents and maintaining them at low levels. The results indicated that treatment with both exogenous $CaCl_2$ and SNP effectively enhances the antioxidant capacity and thus the cold tolerance of loquat seedlings.

Treatment of seedlings with different exogenous agents induced similar biological effects, especially the synergistic effect of $CaCl_2$ and SNP co-treatment. The correlation between the $CaCl_2$-induced and SNP-induced enhancement of frost resistance in loquat seedlings is currently unclear. It was reported that the activity of NO synthase in plants required Ca^{2+} (or CaM) as a cofactor, and NO could also induce an increase in intracellular Ca^{2+} concentration (Zottini *et al*. 2007; Jeandroz *et al*. 2016). Studies have shown that both NO and Ca^{2+} signals were both involved in a series of physiological and biochemical processes induced by heavy metal stress in *Ulva compressa* and *Pisum sativum* (Rodríguez-Serrano *et al*. 2009; González *et al*. 2012). Therefore, in this study, the NO scavenger cPTIO and the CaM antagonist W7 were used in combination with $CaCl_2$ and SNP to treat loquat seedlings before the exposure to low-temperature stress. The results showed that the activities of CAT, POD, SOD and APX and the CaM content of the leaves of seedlings co-treated with cPTIO + $CaCl_2$ were lower than those of seedlings treated with $CaCl_2$ alone, leading to higher leaf MDA and H_2O_2 contents. These results indicated that when cPTIO removed intracellular NO, it might also inhibit the activity of NO synthase by blocking the synthesis of CaM and thus synergistically reducing the intracellular NO level, which directly or indirectly led to decreased activity of protective enzymes in the cells. Therefore, during low-temperature stress, the H_2O_2 was not scavenged in a timely manner, thereby resulting in aggravated membrane lipid peroxidation (i.e., increase in the MDA content). These results illustrated that the NO scavenger cPTIO inhibited the $CaCl_2$-induced positive regulation of cold resistance of loquat seedlings, which implied NO might be involved in these processes.

By comparing the results of co-treatment with W7 + SNP with those obtained after treatment with SNP alone, we found that the CAT, POD, SOD and APX activities and the CaM content of the leaves of seedlings treated with W7 + SNP were lower than those of seedlings treated with SNP, and the H_2O_2 and MDA contents were higher. In addition, the inhibitory effect of the CaM antagonist W7 on the antioxidant capacity of loquat seedlings under low-temperature stress was higher than that of the NO scavenger cPTIO. These observations suggested that W7 inhibited the SNP-induced positive regulation of the freezing resistance of loquat seedlings by causing a malfunction of the Ca^{2+}-CaM messenger system. Ca^{2+} might be one of the indispensable components of the NO signalling pathway in loquat plants under low-temperature stress. Therefore, we predicted that NO and Ca^{2+} signals were both involved in a series of responses of loquat seedlings to low-temperature stress. This was confirmed by co-treatment with SNP + $CaCl_2$, which showed a positive regulatory synergistic effect on the cold resistance of loquat seedlings.

The results obtained with two different combinations of co-treatments, i.e., cPTIO + $CaCl_2$ + SNP and W7 + $CaCl_2$ + SNP, revealed that although both cPTIO and W7 had inhibitory effects on the increased antioxidant capacity of loquat seedlings induced by co-treatment with $CaCl_2$ + SNP, the degree of inhibition differed in that the inhibitory effect of the CaM antagonist W7 was greater than that of the NO scavenger cPTIO. It implied the presence of signalling interactions between NO and Ca^{2+} in response to low-temperature stress. Different signalling pathways ultimately acted on target enzymes, such as CAT, POD, SOD and APX, through the Ca^{2+}-CaM messenger system, which in turn regulated the antioxidant activity in loquat cells and affected the frost resistance of loquat seedlings.

Conclusion

$CaCl_2$ and SNP treatment increased the CaM content and the activities of SOD, CAT, POD and APX in the leaves of seedlings under low-temperature stress and reduced the cellular accumulation of H_2O_2 and MDA. Co-treatment with $CaCl_2$ + SNP showed a synergistic effect. In two treatment combinations cPTIO + $CaCl_2$ and W7 + SNP, both cPTIO and W7 inhibited the physiological effects that were otherwise induced by SNP and $CaCl_2$ treatment while blocking the synergistic effect of $CaCl_2$ + SNP co-treatment and exacerbating oxidative damage to seedlings exposed to low temperature. The results suggested that interactions in Ca^{2+} and NO signal transduction occur in loquat seedlings in response to freezing stress.

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