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Full Length Article

Calcium Alleviates Temperature Stress by Regulating Nitrogen and Respiratory Metabolism in *Malus baccata* Roots

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Abstract

The effects of calcium on respiratory and nitrogen metabolism of apple roots (*Malus baccata* Borkh.) exposed to temperature stress (5°C ~ 20°C ~ 0°C) were investigated. Seedlings were treated with distilled water (control), calcium chloride ($CaCl_2$) or calmodulin antagonist trifluoperazine (TFP) before temperature stress. Temperature was increased from 5°C to 20°C (1°C h^{-1}) and then decreased to 0°C (1°C h^{-1}). Temperature stress decreased root vitality and increased root malondialdehyde (MDA) concentration, the effect of which was exacerbated by TFP treatment. Treatment with $CaCl_2$ improved root vitality and decreased root MDA concentration. At 20°C, exogenous $CaCl_2$ alleviated the negative effects of temperature stress on the total respiration rate by enhancing the activity of tricarboxylic acid cycle (TCA). Activities of key enzyme in nitrogen metabolism were strongly inhibited by temperature stress. Exogenous $CaCl_2$ significantly increased key enzyme activities of nitrogen metabolism compared to the control. However, the TFP treatment markedly reduced the activity of glutamate synthase (GOGAT) at 20°C and noticeably inhibited glutamate dehydrogenase (GDH) activity during the entire temperature stress period. The data showed that the Ca^{2+}-calmodulin (Ca^{2+}-CaM) signal system was involved in increase of GOGAT and GDH activity that occurred with an increase in temperature, and played a role in the increase in the total respiration rate and GDH activity which occurred with a decrease in temperature. Cultural practices that improve plant calcium (Ca) status in the early spring may mitigate damage induced by temperature stress.

Keywords: Calmodulin; Glutamate synthase; Glutamate dehydrogenase, Isocitrate dehydrogenase; Respiration rate

Introduction

Temperature limits the production and geographical distribution of fruit trees. In the cool fruit-growing region of northern China, cold air movement from Baikal Lake and Siberia can cause rapid changes in air temperature in early spring (Meng *et al.*, 2013). Large fluctuations in temperature are detrimental to apple growth and development, damage apple blossoms in northern China. Soil temperature has a significant effect on root function, which in turn affects the growth of the aerial part (Nedlo *et al.*, 2009). However, limited information is available on how rapid changes in temperature alter root function of apple trees.

Root respiration is sensitive to environmental changes. When exposed to a sudden temperature drop, plants invest more carbon (C) in root respiration (Barthel *et al.*, 2014). In addition, low temperature can reduce plant nitrogen (N) uptake capacity and the activity of enzyme in N metabolism, such as glutamate dehydrogenase (GDH) (Lu *et al.*, 2005; Lloyd *et al.*, 2011).

There is close relationship between N metabolism and respiration in plants and these two metabolic systems connect with each other via the tricarboxylic acid cycle (TCA) (Fig. 1). Nitrate (NO_3^-) is converted to amino acids by nitrate reductase (NR), glutamine synthetase (GS), and glutamate synthase (GOGAT). In this process, respiration provides energy and C skeleton (2-oxoglutarate) for N metabolism (Foyer *et al.*, 2011). The synthesis of 2-oxoglutarate (2-OG) is catalyzed by isocitrate dehydrogenase (IDH), one of the key enzymes in the TCA cycle. Very little is known on the interaction between N metabolism and respiration in roots in response to rapid changes in temperature.

Stress can trigger a sudden, transient increase in the concentration of calcium ions in the cytosol $[Ca^{2+}]_{cyt}$ initiating a series of physiological and biochemical processes by binding of Ca^{2+} to Ca^{2+}-binding proteins such as calmodulin (CaM) (White and Broadley, 2003). Previous studies have found that the adaptability of plants to temperature stress could be enhanced by exogenous calcium (Ca) treatment (Ding *et al.*, 2012). Trifluoperazine (TFP) is

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a CaM antagonist that has been used to explore Ca^{2+}-CaM dependent activation of various enzymes. It is unclear whether Ca^{2+}-CaM dependent enzyme activation occurs during respiratory and/or N metabolism responses in apple roots during the temperature changes.

Malus baccata Borkh. is native to northern China and widely used as apple rootstock in cold regions of the world because of its higher cold tolerance (Wu *et al.*, 2012). In this study, we use *M. baccata* to characterize how rapid changes in temperature affects respiration and N metabolism in roots and to determine whether Ca plays a role in the regulation of these processes.

Materials and Methods

Experimental Details and Treatments

Experimental material: Seeds of *M. baccata* were collected from Shenyang, Liaoning, China, stratified at 0-4°C. After germination, seeds were planted in 50-hole trays filled with garden soil of pH 6.3; soil organic matter, 22.9 $g \cdot kg^{-1}$; alkali-hydrolyzable nitrogen, 106 $mg \cdot kg^{-1}$; available phosphorus, 27 $mg \cdot kg^{-1}$ and exchangeable potassium of 123 $mg \cdot kg^{-1}$ and placed inside a greenhouse. After 30 d, seedlings were transplanted into separate black plastic pots (10 × 10 cm) filled with garden soil and kept in the greenhouse.

Treatments: After 60 d, a totoal of 135 seedlings (15 leaves), similar in height and vigor, were randomly divided into three groups and watered with 100 mL of distilled water (control), 100 mL of 2% (w/v) $CaCl_2$, or 100 mL of 0.1 $mg \cdot L^{-1}$ TFP. Subsquently, these seedlings were kept at 5°C in the dark for 16 h and then transferred to growth chamber (MLR-351H, SANYO Elevtric Co., Ltd, Moriguchi, Japan) at 5°C for 24 h with a photosynthetic photon flux density of 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$. After being at 5°C for 24 h, roots of 5 plants per replicate per treatment were harvested and pooled samples from 5 plants from each of three replicates (n=3; 15 plants per treatment). Samples from these plants represented the plant condition before the temperature stress event. The remaining plants in the growth chamber were then subjected to temperature stress.

Temperature Stress and Harvesting

Plants were exposed to temperature stress (5°C ~ 20°C ~ 0°C) that mimiced changing air temperature in early spring of northern China. Growth chamber temperature was increased from 5°C to 20°C (1°C h^{-1}) and maintained at 20°C for 2 h. After being exposed to 20°C for 2 h, roots of 5 plants per replicate per treatment were harvested (n=3; 15 plants per treatment). Subsequently, growth chamber temperature was decreased to 0°C (1°C h^{-1}) and maintained at 0°C for 2 h. After being exposed to 0°C for 2 h, roots of 5 plants per replicate per treatment were harvested (n=3; 15 plants per treatment). At each harvest, fine roots, which have a primary structure without lignification, were used for the analyses. All analyses were performed on pooled samples from 5 plants in each of three replicates (n=3) harvested at each targeted temperature (5°C, 20°C and 0°C).

Determination of Root Vitality and Malondialdehyde Concentration

Fresh roots (0.5 g) were homogenized with 5 mL 0.4% triphenyl tetrazolium chloride (TTC) solution and 5 mL 1/15 *M* Na_2HPO_4-KH_2PO_4 (pH 7.0). Root vitality was determined according to the methods of Zou (2000). Root vitality was calculated from: reduced TTC / (h × fresh root weight).

For determination of malondialdehyde (MDA) concentration, roots (0.5 g) were homogenized with 0.25% 2-thiobarbituric acid in 10% trichloroacetic acid and measured using a colorimetric assay (Shah *et al.*, 2001). The MDA concentration was expressed as $\mu mol \cdot g^{-1}$ FW.

Determination of Total Respiration Rate (Vt)

Total respiration rate (Vt) was measured according to the method of Bouma *et al.* (2001) using a Clark-type oxygen electrode (Hansatech Oxytherm, Hansatech Instruments Ltd, Norfolk, U.K.). Root samples (0.05 g; diameter, 1.5 mm; length, 2.0–3.0 cm) were cut into 2.0-mm pieces and added to 1.5 mL phosphate buffered saline (20 m*M* PBS, pH 6.8). The Vt was defined as the rate of O_2 uptake by roots per unit fresh root weight ($\mu mol\ min^{-1}\ g^{-1}$ FW).

Determination of Respiratory Pathway Activity (Vr)

Respiratory pathway activity (Vr) was detected according to method of Qin *et al.* (2014) using Clark-type oxygen electrode. The activities of the glycolytic pathway (EMP), TCA, and pentose phosphate pathways (PPP) were inhibited by addition of NaF (0.5 *M*), malonic acid (0.5 *M*), and Na_3PO_4 (0.5 *M*), respectively. These inhibitors were dissolved by 20 m*M* PBS (pH 6.8). Vr was calculated as (Vt – residual respiration rate). Residual respiration rate was defined as the respiration rate after adding the corresponding inhibitor to PBS.

Assay of Nitrate Reductase Activity

Root tissues (0.2 g) were homogenized with 4.0 mL cold 25 m*M* phosphate buffer (pH 7.5) containing 5 m*M* cysteine and 5 m*M* EDTA-Na_2 and then centrifuged at 4,000 *g* for 20 min at 4°C. The activitiy of nitrate reductase (NR) was measured according to the method of Zou (2000). The absorbance was measured at 540 nm. The NR activity was expressed as $\mu mol \cdot h^{-1} \cdot g^{-1}$ FW.

Assay of Glutamine Synthetase Activity

Root (0.5 g) were homogenized with 2.5 mL cold extraction buffer (0.05 *M* Imidazole-HCl, 0.5 m*M* EDTA and 1.0 m*M* dithiothreitol, pH 7.2) and centrifuged at 10,000 *g* for 20 min at 4°C. The glutamine synthetase (GS) activity was measured according to the method of Rhodes *et al.* (1975). GS activity was assayed by monitoring the formation of γ-glutamyhydroxamate at 540 nm.

Assay of Glutamate Synthase and Glutamate Dehydrogenase Activity

Root tissues (0.5 g) were homogenized in 50 m*M* KH_2PO_4 buffer (pH 7.5) containing 2 m*M* EDTA, 1.5% soluble casein, 2 m*M* dithiothreitol (DTT), and 1% insoluble polyvinylpolypyrrolidone and centrifuged at 30,000 *g* for 20 min at 4°C. The resulting extract was used to measure enzyme activity of glutamate synthase (GOGAT) and glutamate dehydrogenase (GDH) activity. GOGAT and GDH activity was measured according to the method of Groat and Vance (1981). GOGAT and GDH activity was assayed by monitoring the oxidation of NADH at 340 nm for 5 min and 3 min, respectively. One unit of GOGAT and GDH activity is defined as the oxidation of 1 μmol NADH per hour.

Assay of Isocitrate Dehydrogenase Activity

Root tissues (0.5 g) were pulverised in 3 mL extraction buffer (100 m*M* Tris-HC1, pH 7.5). The isocitrate dehydrogenase (IDH) activity was measured according to the method of Collins and Merrett (1975). IDH activity was analyzed by measuring an increase in extinction at 340 nm corresponding to the reduction of $NADP^+$.

Analysis of Calmodulin Concentration

Root tissues (0.2 g) were pulverised in 2 mL extraction buffer (10 m*M* glyoxaline, 0.25 m*M* phenylmethylsufonyl fluoride, 1 mM EGTA, 1 m*M* *β*–mercaptoethanol, 10 m*M* $MgCl_2$ and 150 m*M* NaCl, pH 7.5). The mixture was heated at 100°C for 3 min and then cooled in an ice bath. Subsequently, the mixture was centrifuged for 30 min (16,000 *g*, 4°C), and the supernatant was immediately assayed for calmodulin (CaM) concentration according to the method of Sun *et al.* (1995). The absorbance was measured at 450 nm.

Statistical Analysis

All experiments were performed in triplicate. Data were statistically analyzed by SPSS 17.0 data processing software (SPSS, Inc., Chicago, USA) using Duncan's mutiple range test at a 0.05 level. The data are expressed as the mean ± standard error (SE). Pearson's correlation analyses were used for analysis of relationships of IDH activity with key enzyme activities of N metabolism.

Results

Effects of Ca and TFP on Root Vitality and Malondialdehyde Concentration

In the control, root vitality significantly decreased with the change in temperature from 5°C to 0°C. Compared with the control, root vitality was significantly increased by the $CaCl_2$ treatment and reduced by TFP treatment throughout the entire experiment (Fig. 2A). During the temperature stress, the malondialdehyde (MDA) concentration in the control showed an increasing trend. The MDA concentration in the $CaCl_2$ treatment was relatively stable during the temperature stress and lower than of control at 20°C and 0°C. In contrast, the MDA concentration markedly increased after the addition of TFP, compared to control (Fig. 2B).

Effects of Ca and TFP on Total Respiration Rate and Respiratory Pathway Activity

Compared with the control, total respiration rate (Vt) was significanlty increased by the $CaCl_2$ treatment while it was significanlty inhibited by TFP treatment at 0°C (Fig. 3A).

Compared with the control, the $CaCl_2$ treatment significantly enhanced the respiratiory pathway acitvity (Vr) of EMP, TCA and PPP, except for EMP Vr at 0°C (Fig. 3B). Similarly, the TFP treatment significantly supressed EMP Vr at 0°C compared to the control, but inhibited the Vr of TCA throughout the entire experiment.

Effects of Ca and TFP on Nitrate Reductase, Glutamine Synthetase, Glutamate Synthase, Glutamate Dehydrogenase and Isocitrate Dehydrogenase Activity

During the temperature stress, after the exogenous application of $CaCl_2$, the nitrate reductase (NR) activity increased significantly by 34% at 20°C and 8.1% at 0°C, compared to control, while the TFP treatment had no effect on NR activity at 20°C or 0°C (Fig. 4A).

In the $CaCl_2$ treatment, the glutamine synthetase (GS) activity was significantly higher than of control at each temperature and no effect of TFP treatment during the entire experiment was observed (Fig. 4B).

The glutamate synthase (GOGAT) activity in the control and $CaCl_2$ treatment was lower at 20°C and 0°C than at 5°C (Fig. 4C), but it was significantly higher in $CaCl_2$ treatment than control at each temperature. Compared with the control, TFP treatment significantly suppressed GOGAT activity at 20°C.

Glutamate dehydrogenase (GDH) activity in the control and TFP treatment decreased from 5°C to 0°C and was significantly lower in TFP treatment than the control at each temperature (Fig. 4D). While, with $CaCl_2$ treatment GDH activity was greater than control throughout study period.

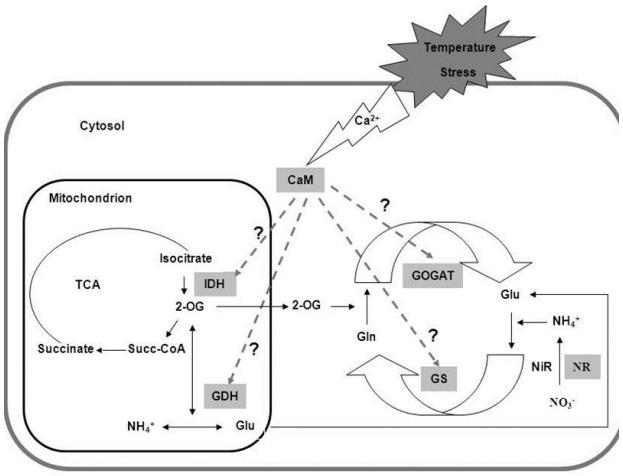

Fig. 1: A schematic model depicting the relationship between respiration and nitrogen metabolism under temperature stress. Temperature stress induces a rapid and transient increase in cytosolic calcium ion concentration. Then, calcium acts as a secondary messenger that activates a large and complex signaling cascade, including the formation of Ca^{2+}–CaM complexes. The calcium signal can effectively regulate diverse downstream targets, including the enzymes in respiratory metabolism and nitrogen metabolism, and ultimately lead to a series of physiological responses. However, whether the Ca^{2+}–CaM complex is involved in regulating these enzyme activities under rapid changes in temperature remains unclear (the dotted lines with the? marks). 2-OG, 2-oxoglutarate; CaM, calmodulin; GDH, glutamate dehydrogenase; Gln, glutamine; Glu, glutamate; GOGAT, glutamate synthase; GS, glutamine synthetase; IDH, isocitrate dehydrogenase; NiR, nitrite reductase; NR, nitrate reductase; Succ-CoA, Succinyl-Coenzyme A; TCA, tricarboxylic acid cycle

Compared with control, after the exogenous application of $CaCl_2$, isocitrate dehydrogenase (IDH) activity was increased significantly by 2.2-fold at 0°C and it was inhibited by TFP treatment at 5°C and 20°C (Fig. 4E). During the temperature stress, IDH activity of the control was positively correlated with GOGAT ($r = 0.987$; $P < 0.01$) and GDH ($r = 0.795$; $P < 0.05$) activity.

Effects of Ca and TFP on Calmodulin Concentration

Compared to the control, the $CaCl_2$ treatment significantly improved the calmodulin (CaM) concentration at 5°C and 0°C, and reduced the CaM concentration at 20°C. In addition, CaM concentration decreased significantly by 46% at 20°C and 53.3% at 0°C after the addition of TFP, compared to the control.

Discussion

Temperature stress can decrease root vitality and aggravate lipid peroxidation (Zhang and Ervin, 2008).

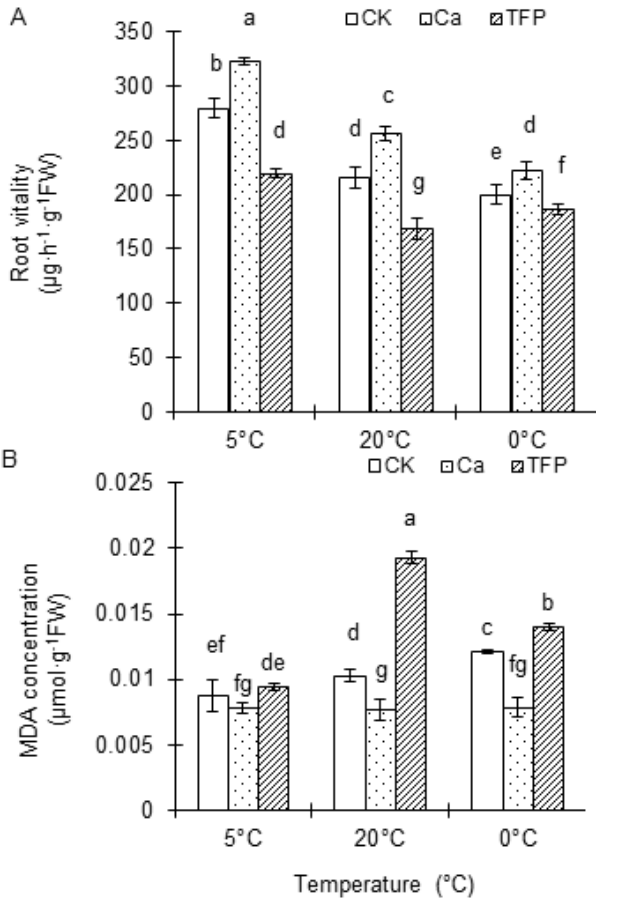

Fig. 2: Effects of Ca and TFP on root vitality (A) and malondialdehyde (MDA) concentration (B) in *Malus baccata* Borkh. roots under temperature stress. Bars represent means ± SE (n=3). Different letters on the bars indicate significant differences between the treatments ($P<0.05$)

We found that rapid changes in temperature depressed root vitality and increased MDA concentration (Fig. 2), that created a "temperature stress" condition in apple roots. Exogenous calcium treatment could alleviate the negative effect of rapid changes in temperature on root vitality and MDA concentration, however, the effect was aggravated by TFP treatment. The effects of exogenous calcium and TFP treatment on MDA concentration were similar to those described in tomato leaves [*Solanum lycopersicum* L.] under heat stress, where Ding *et al*. (2012) found that Ca^{2+}-CaM signaling pathways were involved in resistance to heat-induced oxidative stress through the induction of antioxidant enzymes and a reduction in the accumulation of oxygen-free radicals and a further reduction of MDA concentration in leaves.

Root respiration provides the energy and C skeletons necessary for biosynthesis and is easily influenced by soil temperature (Atkin *et al.*, 2000; Foyer *et al.*, 2011).

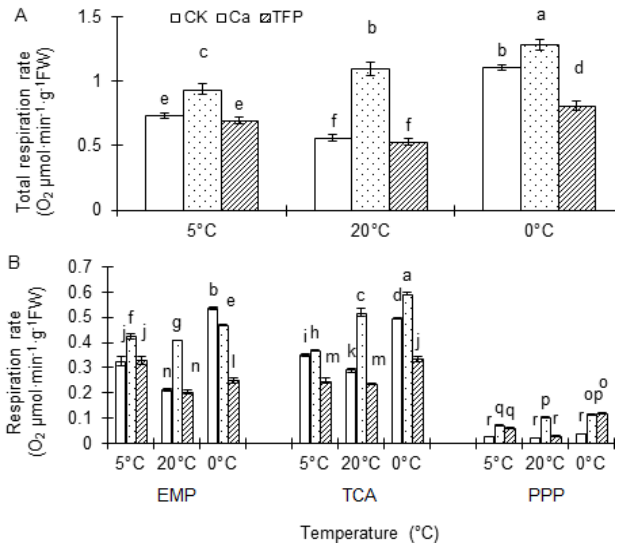

Fig. 3: Effects of Ca and TFP on the total respiration rate (A), and respiratory pathway activity from glycolysis (EMP), tricarboxylic acid cycle (TCA) and pentose phosphate pathway (PPP) (B) in *M. baccata* roots under temperature stress. Bars represent means ± SE (n=3). Different letters on the bars indicate significant differences between the treatments and the pathways (P<0.05)

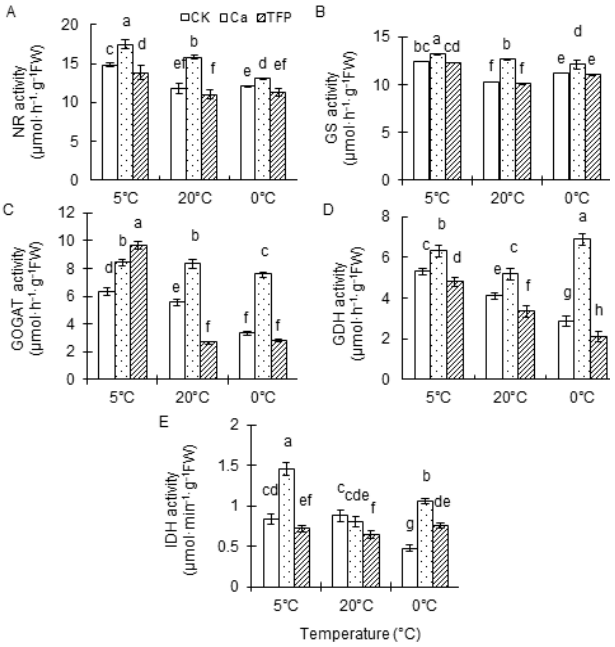

Fig. 4: Effects of Ca and TFP on key enzyme activities of nitrogen and respiratory metabolism in *M. baccata* roots under temperature stress: (A) Nitrate reductase (NR), (B) Glutamine synthetase (GS), (C) Glutamate synthase (GOGAT), (D) Glutamate dehydrogenase (GDH), and (E) Isocitrate dehydrogenase (IDH). Bars represent means ± SE (n=3). Different letters on the bars indicate significant differences between the treatments (P<0.05)

In present study, total respiration rate significantly decreased when temperature rapidly increased from 5°C to 20°C. Interestingly, the calcium treatment markedly increased the total respiration rate and the TCA activity compared with the control (Fig. 3). Previous studies have shown that TCA provide C skeleton for biosynthesis and electron donors for the mitochondrial electron transport pathway (Fernie *et al.*, 2004). Therefore, the exogenous calcium treatment strengthened root respiratory metabolism could be attributed to the increase in the activity of TCA. Moreover, the increase in PPP activity with $CaCl_2$ treatment is beneficial for alleviating low-temperature stress (Lin *et al.*, 2005).

The TCA cycle is a key metabolic hub for the interacting pathways of N assimilation and respiration metabolism (Foyer *et al.*, 2011). In this experiment, the activities of NR, GS, GOGAT and GDH were inhibited to some extent by rapid changes in temperature (Fig. 4). Similar changes have been reported for fescue (*Festuca arundinacea*) under high temperature stress (Cui *et al.*, 2006). The decrease in GS activity under temperature stress might inhibit NO_3^- reduction and NH_4^+ assimilation (Chien and Kao, 2000) and consequently, force a large number of electrons to enter into the TCA via $NADP^+$, i.e., feedback inhibited the normal process of this pathway. Such feedback inhibition on the TCA would lead to excessive accumulation of electrons and produce a series of reactive oxygen species, resulting in aggravated membrane peroxidation. Hence, the decrease in GS and GOGAT activity when

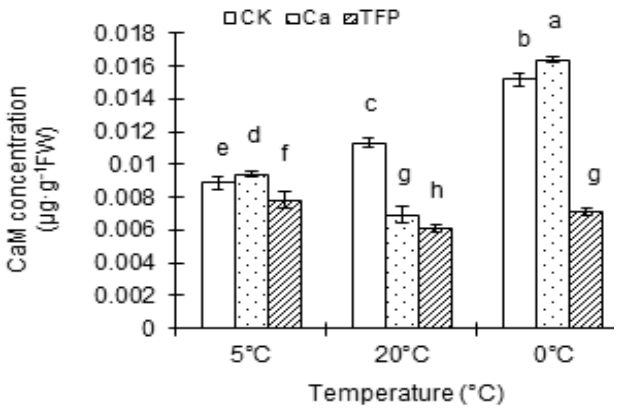

Fig. 5: Effects of Ca and TFP on calmodulin (CaM) concentrations in *M. baccata* roots under temperature stress. Bars represent means ± SE (n=3). Different letters on the bars indicate significant differences between the treatments (P<0.05)

temperatures increased from 5°C to 20°C might be one of the reasons that the MDA concentration increased and the activities of respiratory pathways decreased with increased

temperature from 5°C to 20°C. The GS/GOGAT cycle requires 2-OG as a C skeleton to produce glutamate. Thus, IDH plays an important role in the production of 2-OG for N metabolism. In present study, IDH and GOGAT activities were positively related to each other. For this reason, it is highly likely that the decrease in GOGAT activity can be attributed to the decrease in 2-OG synthesis catalyzed by IDH.

According to recent studies, NR and GS activities were activated by Ca^{2+} (Zhang *et al.*, 2011). In this experiment, the $CaCl_2$ treatment significantly increased the activities of NR, GS, GOGAT and GDH under temperature stress compared to the control (Fig. 4). The effects of applied exogenous calcium in our study are similar to those described in muskmelon (*Cucumis melo* L. var. reticulates Naud) under hypoxia-stressed conditions (Gao *et al.*, 2011). Moreover, exogenous calcium treatment significantly enhanced the IDH activity at 0°C. These results suggest that exogenous calcium may promote the consumption of $NADP^+$ and 2-OG, weaken the feedback inhibition of N metabolism on respiration induced by rapid temperature decrease, enhance the activity of TCA, increase the total respiration rate and thus, effectively alleviate the damage caused by temperature stress.

Ca^{2+}-CaM complexes play an improtant role in the process of plant response to temperature stress (White and Broadley, 2003). The increase in CaM concentration was found when temperatures rose from 5°C to 20°C in the control, but decreased with $CaCl_2$ or TFP treatments (Fig. 5). Exogenous calcium treatment markedly enhanced the activities of GOGAT and GDH compared to controls, but these activities were significantly blocked by the TFP treatment. These results indicate that the Ca^{2+}-CaM signal system was involved in the increased activities of GOGAT and GDH when temperature increased from 5°C to 20°C. When temperatures dropped from 20°C to 0°C, the CaM concentration increased in control and $CaCl_2$ treatment, and the exogenous calcium treatment had greater CaM concentration, total respiration rate and GDH activity than controls, but the result was reversed in case of TFP treatment. Ca^{2+} and CaM in the presence of "protein factor" could also directly regulate the activity of GDH (Das *et al.*, 1989). Hence, it is speculated that the Ca^{2+}-CaM signaling system is involved in improving the total respiration rate and GDH activity in *M. baccata* roots when temperatures rapidly drop.

Conclusion

Exogenous calcium counteracted the negative effects of temperature stress which was partially mediated by Ca^{2+}-CaM signaling. However, it is possible that there may have been additional Ca^{2+} sensor proteins that participated in this process which warrants further studies.

Acknowledgements

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