Effects of Cold Acclimation on Several Enzyme Activities in *Euonymus radicans* 'Emorald & Gold' and Its Relation to Semi-lethal Temperature

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ABSTRACT The changes in activities of superoxide dismutase (SOD), peroxidase (POD) and ATPase in the leaves of *Euonymus radicans* were studied when seedlings were cold-acclimated (at 4 ℃) for 1 week, 2 weeks, 3 weeks and then treated for 1 d under low temperature stress (at –5 ℃). The semi-lethal temperatures of acclimated and unacclimated seedlings were also investigated. The results indicated that the activities of the three enzymes in the leaves of the seedlings treated at 4 ℃ for 1, 2 and 3 weeks were all higher than those of unacclimated seedings (treated at 22 ℃ as controls). The activities of SOD and POD increased continuously with the prolongation of the time of cold acclimation, but stepped up to summits then down to the levels of the controls. The activities of SOD culminated at the first week, and the activities of POD at the second week. When acclimated and unacclimated seedlings were both treated at –5 ℃ for 1 d, the activities of the three enzymes in the leaves of acclimated seedlings were a little lower than those before stress, but higher than those of the controls. Moreover, the decrease rate of enzyme activities was greatly lower than that of the controls. The results showed that cold acclimation could enhance the stability of the three enzymes in the leaves of seedlings under low temperature stress; the semi-lethal temperature was –19.1 ℃ when the seedlings were treated at 4 ℃ for 3 weeks, but it was –5.4 ℃ when the seedlings were treated at 22 ℃. The decline of the semi-lethal temperature caused by the adaptive changes of enzyme activities was one of the foundations of enhancing the cold tolerance.

KEY WORDS cold acclimation, *Euonymus radicans,* superoxide dismutase, peroxidase, ATPase, semi-lethal temperature, cold tolerance

1 Introduction

To meet the demand of making the city green and beautiful in winter, the authors introduced about ten varieties of evergreen and color-leaved plants including *Euonymus radicans*, from Holland in the spring of 2000. Due to the long and severe winter in Beijing, low temperature become the chief restriction factor for introducing and acclimating plants. It is found that *Euonymus radicans* has the strongest enduring ability to low temperature stress, so as to live through the severe winter. So the study of cold tolerance mechanism of *Euonymus radicans* is of great significance for greening Beijing in winter. The damage of plant cell membrane system caused by low temperature is an essential reason of plant low temperature injury (Jian 1992). The generation of some toxic materials such as free radical of oxygen and peroxidates under low temperature stress could damage cell membrane system (Chen 1991, Li *et al*. 1995). However, plants could clear up those toxic materials through raising enzymatic defense ability of membrane defense enzymes, such as SOD, POD and ATPase to protect membrane system and to enhance the plant cold tolerance (Kasamo 1988). Semi-lethal temperature is often regarded as a major index for measuring the plant cold tolerance. Integrating the changes of cell membrane defense enzymes under low temperature stress with the semi-lethal temperature could contribute to evaluating and analyzing the reliability and physiological base of semi-lethal temperature (Yan and Tan 2000). This paper deals with the effect of cold acclimation on several enzyme activities in *Euonymus radicans* and its relation to semi-lethal temperature to inquire into the adaptability mechanism of *Euonymus radicans* under the low temperature stress.

2 Materials and methods

2.1 Materials

The cuttings collected from healthy two-year-old *Euonymus radicans* in the nursery garden of Beijing Forestry University were rooted in pots in greenhouse. The seedlings that had about 20 leaves were collected for research.

2.2 Methods

2.2.1 The assay of SOD activity

Euonymus radicans seedlings were divided into two groups. The first group of seedlings were divided into three parts and respectively acclimated at 4 ℃ with a 12 h photoperiod and a light intensity of 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 1, 2 and 3 weeks, respectively. The second group of seedlings were also divided into three parts treated at 22 ℃ as control with the same

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photoperiod and light intensity as above. Then, seedlings held at 4 ℃ for 3 weeks and the controls were placed at –5 ℃ for 1 d and 2 d. 0.5 g leaves were separately collected from the middle of all above-mentioned seedlings. 10 mL of buffer (62.5 mmol·L^{-1} Na-phosphate, pH 7.8) was added to each sample group. They were grounded in an ice bath and homogenates were centrifuged at 13 000 g for 20 min (at 4 ℃), the supernatants were then used for assaying the SOD activities by the method of Giannopolitis and Ries (1977).

2.2.2 The assay of POD activity

The treatment of experimental materials and enzyme extraction was the same as above. The activities of POD were assayed according to the method of Liu and Zhang (1994).

2.2.3 The electromicroscopic-cytochemical assay of ATPase

A group of *Euonymus radicans* seedlings were held at 4 ℃ for 1 week and the other group held at 22 ℃ as control. Then they were held at –5 ℃ for 1 d. The sample blocks, about 0.5 mm × 0.5 mm in size, were respectively taken from the middle part of the first pair of extending leaves next to the top of all above-mentioned seedlings. Samples were fixed in 2.5% glutaraldehyde and 4% formaldehyde for 2 h at 4 ℃, rinsed three times with sodium cacodylate buffer, then washed two times with 0.05 mol·L^{-1} Tris-maleate buffer (pH 7.2) to remove residual glutaraldehyde and incubated for 2 h at 37 ℃ in a medium containing 50 mmol·L^{-1} Tris-maleate, 2 mmol·L^{-1} $MgSO_4$, 3 mmol·L^{-1} $Pb(NO_3)_2$, and 2 mmol·L^{-1} ATP (pH 7.2). Control specimens were incubated in the same media but without substrate. The material was washed three times at 4 ℃ in sodium cacodylate buffer, postfixed in 1% OsO_4 for 2 h at 4 ℃, rewashed three times in sodium cacodylate buffer, then dehydrated in a graded acetone series, infiltrated and embedded in Epon 812 resin. Ultrathin sections were cut with LKB-V ultramicrotome, stained with uranyl acetate, and examined with a JEOL JEM.1010 electron microscope.

2.2.4 The measurement of the semi-lethal temperature

The seedlings acclimated at 4 ℃ for 3 weeks and the controls were cooled stepwise to –3, –6, –9, –12, –15, –18, –21, –24, –27, –30 and –33 ℃, respectively, in a programmed freezer (DP-200). 6 seedlings were held for 2 h under each temperature. Then they were divided into two groups (3 seedlings each group). One group was used for measuring the electrolyte osmotic ratio immediately, and marked for 22 ℃ or 4 ℃ (before revivification), the other group was placed at 4 ℃ for 1 d and then used for the measurement of the electrolyte osmotic ratio marked for 22 ℃ or 4 ℃ (after revivification) to test the semi-lethal temperature. A series of electrolyte osmotic ratios were combined with Logistic equation $y = K/(1 + ae^{-bx})$ to obtain inflexion as plant semi-lethal temperature. Let $Y = \ln(K–y)/y$, the equation was transformed to $Y = \ln a – bx$, then $x = (\ln a)/b$, x is just the inflexion, i.e. the semi-lethal temperature.

3 Results

3.1 The changes in activities of SOD

The activities of SOD enhanced dramatically in the leaves of seedlings acclimated at 4 ℃ for 1 week, and increased by 17.8% compared with controls. After 2 weeks of cod acclimation, the activities of SOD in the leaves of seedlings gradually decreased, only increased by 5.3% compared with controls. After 3 weeks of cold acclimation, the activities of SOD were closed to level of controls, only increased by 0.7% (Fig. 1). When acclimated seedlings treated for 3 weeks and their controls (unacclimated seedlings) were both placed at –5 ℃ for 1 d, the activities of SOD in unacclimated seedlings decreased by 14.3% compared with those before stress and the activities of SOD in acclimated seedlings only decreased by 5.2%; After 2 d of low temperature stress (at –5 ℃), the activities of SOD in unacclimated seedlings decreased by 37.2%, but the activities of SOD in acclimated seedlings only decreased by 8.1% (Fig. 2).

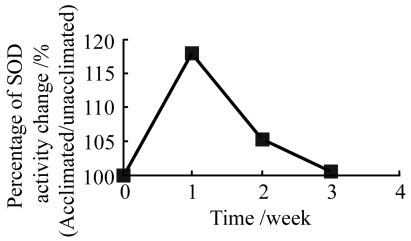

FIGURE 1 Effect of different duration of cold acclimation on SOD activity

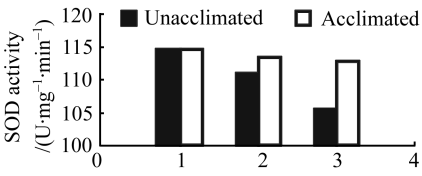

FIGURE 2 Effect of low temperature stress on SOD activity

1: before stress; 2: 1 d after stress; 3: 2 d after stress

3.2 The changes in activities of POD

After 1 week of cold acclimation, the activities of POD enhanced slightly in the leaves of seedlings, only increased by 2.1% compared with controls. After 2 weeks of cold acclimation, the activities of POD in the leaves culminated and increased by 14.2% compared with controls. After 3 weeks of cold acclimation, whereas, the activities of POD hardly went down to near the level of controls and only increased by 0.3%. Then, the 3-week acclimated seedlings and their controls were all treated at –5 ℃. After 1 d, the activities of POD in the leaves of unacclimated seedlings decreased more quickly than those in acclimated seedlings. The former decreased by 12.2%, the latter 6.2%. After 2 d, the activities of POD in the leaves of unacclimated seedlings decreased even more quickly than those in acclimated seedlings. The former decreased by 28.4%, the latter 8.9%.

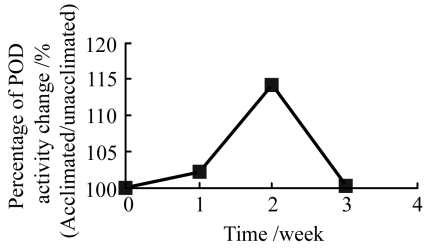

FIGURE 3 Effect of different duration of cold acclimation on POD activity

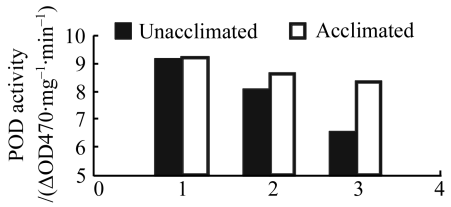

FIGURE 4 Effect of low temperature stress on POD activity

1: before stress; 2: 1 d after stress; 3: 2 d after stress

3.3 The changes in activities of ATPase

The electron-dense lead phosphate deposits, an indication of ATPase activity, mainly distributed on the plasma membrane (PM) and tonoplast in the mesophyll cells of palisade and spongy tissue, in which there is no obvious difference of ATPase distribution and activities. When seedlings were acclimated at 4 ℃ for 1 week , the ATPase activities on the PM and tonoplast were higher than those of the seedlings treated at 22 ℃ (Plate Ⅰ-1,2,3). The ATPase in mesophyll cells became inactivation and the ultrastructrue of cells was damaged seriously when the unacclimated seedlings were treated at –5 ℃ for 1d. However, when the cold-acclimated seedlings were then treated at –5 ℃ for 1 d, the ATPase activities were still observed on the PM and tonoplast, but it was lower than that before low temperature stress, and the ultrastructure of mesophyll cells remained unchanged (Plate Ⅰ-4,5). No lead phosphate deposits on the PM and tonoplast of mesophyll cells were observed when substrate (ATP) was not added into the reaction medium (Plate Ⅰ-6).

3.4 The semi-lethal temperature of acclimated and unacclimated seedlings

The electrolyte osmotic ratio and transformed electrolyte osmotic ratio deduced by the Logistic equation $y = K/(1 + ae^{-bx})$ in the leaves of *Euonymus radicans* treated at 22 ℃ for 3 weeks were shown in Tables 1 and 2 and Fig. 5. As shown in Fig. 5, lna = 0.622 6, b = 0.114 5, and the semi-lethal temperature (lna/b) was –5.4 ℃.

The leaf electrolyte osmotic ratio after revivification tested the correctness of the semi-lethal temperature. After the seedlings treated at –3 ℃ revived at 4 ℃ for 1 d, the leaf electrolyte osmotic ratio was lower than that before revivification. The result indicated that the seedlings did not suffer from lethal damage and their cell function could revive. However, after the seedlings treated at –6 ℃ revived at 4 ℃ for 1 d, the electrolyte osmotic ratio was higher than that before revivification. It was shown that the seedlings could not repair the damage caused by –6 ℃ stress and the temperature is lethal for the seedlings.

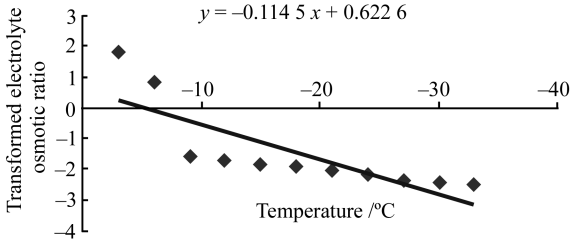

FIGURE 5 Regression of electrolyte osmotic ratio in leaves of *Euonymus radicans* at 22 ℃

The leaf electrolyte osmotic ratio and transformed leaf electrolyte osmotic ratio deduced by the Logistic equation $y = K/(1 + ae^{-bx})$ of *Euonymus radicans* treated at 4 ℃ for 3 weeks were shown in Tables 3 and 4 and Fig. 6. As shown in Fig. 6, lna = 2.815 7, b = 0.147 8, and the semi-lethal temperature (lna/b) was –19.1 ℃.

After the seedlings treated at above the semi-lethal temperature revived for 1 d, the leaf electrolyte osmotic ratio was lower than that before revivification. Whereas, the electrolyte osmotic ratio after revivification of those seedlings treated at below the semi-lethal temperature were all higher than that

before revivification.

TABLE 1 Leaf electrolyte osmotic ratio of *Euonymus radicans* at 22 ℃

Temperature /℃	–3	–6	–9	–12	–15	–18	–21	–24	–27	–30	–33
Electrolyte osmotic ratio (before revivification)	13.9	30.1	83.3	85.1	86.4	86.7	88.3	89.6	91.2	91.7	92.4
Electrolyte osmotic ratio (after revivification)	13.1	60.3	84.1	86.6	86.9	88.2	91.6	93.3	94.8	96.7	98.4

TABLE 2 Transformed leaf electrolyte osmotic ratio of *Euonymus radicans* at 22 ℃ (before revivification)

Temperature /℃	–3	–6	–9	–12	–15	–18	–21	–4	–7	–30	–33
Y	1.824	0.843	–1.607	–1.742	–1.849	–1.875	–2.021	–2.154	–2.338	–2.402	–2.498

TABLE 3 Leaf electrolyte osmotic ratio of *Euonymus radicans* at 4 ℃

Temperature /℃	–3	–6	–9	–12	–15	–18	–21	–24	–27	–30	–33
Electrolyte osmotic ratio (before revivification)	11.2	13.3	16.4	19.6	28.2	33.7	72.1	78.7	80.7	82.9	83.5
Electrolyte osmotic ratio (after revivification)	9.4	10.9	13.8	14.7	25.0	32.2	77.8	84.1	84.6	86.4	89.9

TABLE 4 Transformed leaf electrolyte osmotic ratio of *Euonymus radicans* at 4 ℃ (before revivification)

Temperature /℃	–3	–6	–9	–12	–15	–18	–21	–24	–27	–30	–33
Y	2.070	1.875	1.629	1.411	0.935	0.677	–0.949	–1.306	–1.431	–1.579	–1.621

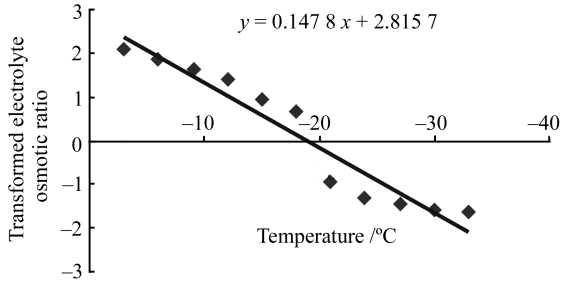

FIGURE 6 Regression of electrolyte osmotic ratio in leaves of *Euonymus radicans* at 4 ℃

4 Discussions

4.1 The relation between the cold acclimation and membrane defense SOD, POD as well as ATPase

Some studies revealed that SOD, POD, as the membrane defense enzymes, could quickly clear up the toxic materials such as free radical of oxygen and peroxidates to eliminate or reduce the damage caused by chillimg stress so as to enhance the cold tolerance of plants (Kasamo 1988, Dai *et al*. 1991, Li *et al*. 2000, Yan and Tan 2000, Wu *et al*. 2001). Our results showed that the activities of SOD and POD had a gradually enhancing process, the activities of SOD reached a summit at the first week, and the peak of activities of POD appeared at the second week. The activities of two enzymes did not always increasingly enhance with the prolongation of cold acclimation being prolonged, but gradually went up to summits then down to near the level of controls. In the following low temperature stress, the activities of SOD and POD in the leaves of acclimated seedlings were a little lower than those before stress, and the decrease rate became slower. However, the activities of two enzymes in the leaves of unacclimated seedlings decreased rapidly and the decrease rate became quicker. It was suggested that the enhancement mechanism of plant cold tolerance not only lie in the stability of enzyme activities in the low temperature stress caused by the increase of enzyme activities during the cold acclimation, more importantly, but lie in low temperature adapatability mechanism developed with the changes in activities of enzymes during the cold acclimation as well. Therefore, when the acclimated and unacclimated seedlings suffered from low temperature stress at the same time, the former had much greater ability to clear up the toxic materials than the latter to protect seedlings as a result of the development of low temperature adapatability mechanism caused by cold acclimation.

It was well-known that ATPase, as a kind of protein combined with cell membrane, carried out the transmission of K^+, Na^+, Ca^{2+}, Mg^{2+} and H^+ between the inner and outer side of the cells. The transmission of ions accompanied with the transportation of solute between the inner and outer side of the cells, the

transmission of ions caused by the enhancement of ATPase activity might be one of the procedures for developing plant cold tolerance (Mattheis and Ketchie 1990). The enhancement of ATPase activity also strengthened the hydrolysis of ATP *in vivo* of plants, so as to provide energy for the biosynthesis of RNA and protein (enzyme) related to the development of cold tolerance and the increase of the survival rates of seedlings (Lin *et al*. 2001). In our experiments, it was observed that the ATPase activities on the plasma membrane and tonoplast in the leaves of *Euonymus radicans* enhanced after cold acclimation, and in the following low temperature stresses, the acclimated seedlings had stronger ability to maintain the stability of ATPase than controls as reported for other plants (Dai *et al*. 1991, Wang *et al*. 1998, Jian *et al*. 1999). That was to say, the stability of ATPase showed a positive correlation with the enhancement of cold tolerance of seedlings. Due to the enhancement of ATPase activities caused by cold acclimation and their stability in the following low temperature stresses, it was suggested that the role of ATPase in enhancing cold tolerance of *Euonymus radicans* might involved in one or more above-mentioned mechanisms.

4.2 The relation between the semi-lethal temperature and the changes in activities of enzymes during the cold acclimation

The semi-lethal temperature, as a major index for measuring the plant cold tolerance, could directly and exactly reflect the cold tolerance of plants and the limit to endure the low temperature, which is of significance for introducing and acclimating plants. It had been reported that plants could lower the semi-lethal temperature after cold acclimation (Gao *et al*. 2003). Our experimental results showed that the semi-lethal temperature was –5.4 ℃ when *Euonymus radican*s seedlings were treated at 22 ℃ (unacclimated), whereas, the semi-lethal temperature was –19.1 ℃ after the seedlings being acclimated at 4 ℃ for 3 weeks, resulting in the enhancement of cold tolerance of seedlings. The result coincided with the survey on wintering of *Euonymus radicans* seedlings planted in the field. Those seedlings planted in the field lived through –18 ℃ in winter and still grew well, indicating their semi-lethal temperature should be below –18 ℃.

It had been reported that the decline of the semi-lethal temperature caused by the changes of membrane protection system was the foundation of raising the cold resistance (Yan and Tan 2000). The changes in activities of enzymes during the different duration of cold acclimation indicated that the seedlings could adapt themselves to low temperature after 3 weeks of cold acclimation so as to enhance the cold tolerance of plant in the following low temperature stresses. It was obvious that the decline of semi-lethal temperature caused by the adaptive changes in activities of enzymes was really one of the foundations of raising the cold tolerance.

As far as the relation between cold acclimation and plant cold tolerance was concerned, many researchers studied the changes of many materials and energy during the cold acclimation, among which the studies of membrane defense enzymes—SOD, POD and ATPase, etc., were one of the important respects. However, the adapatability to low temperature of plant cell membrane system resulted from a series of complicate processes including the response to low temperature, signal transduction, gene express and regulation, protein assembly and function acquirement on the membrane and so on. So the early revealation of mechanism of plant cold tolerance need more and further studies.

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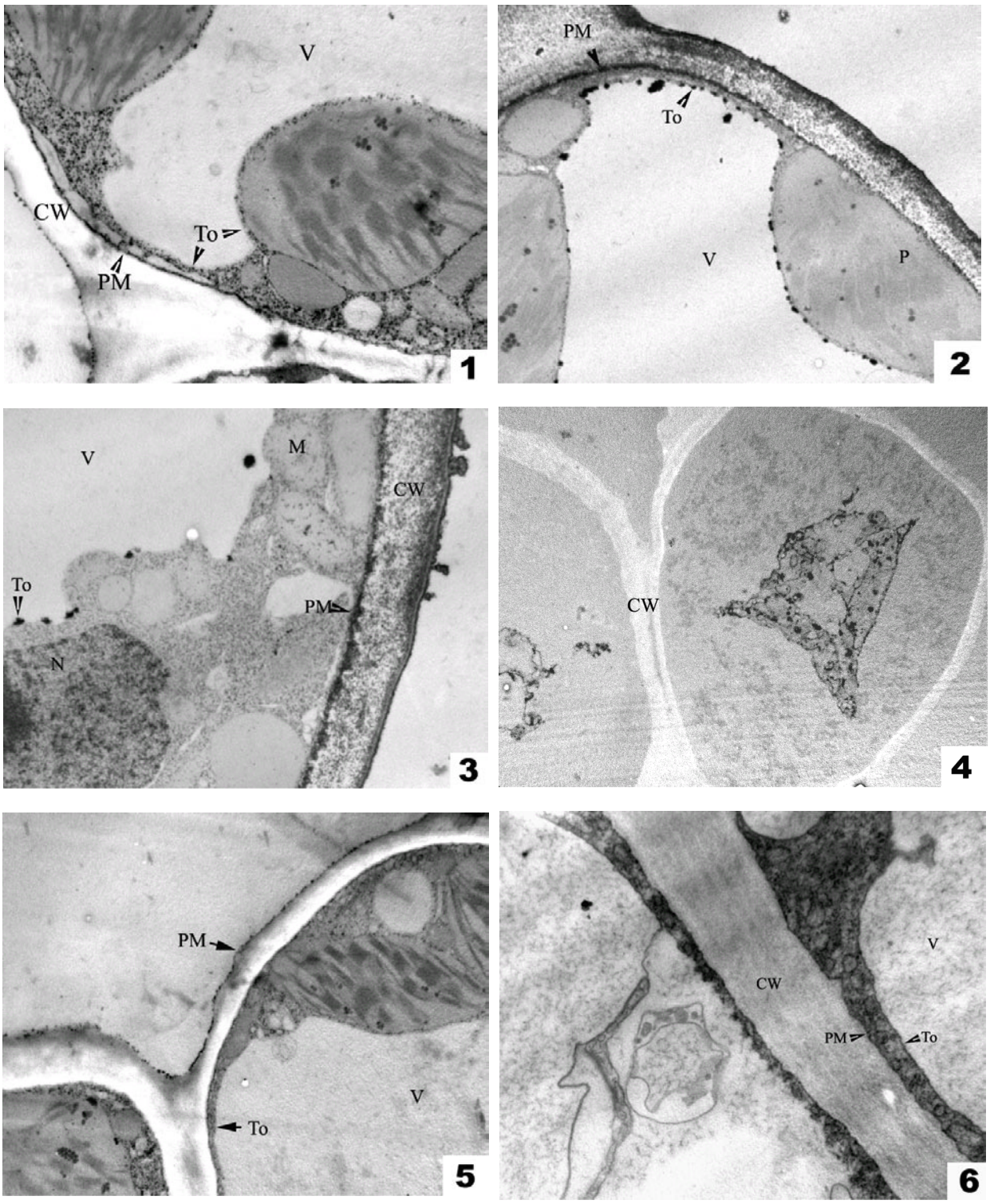
Guo Huihong *et al*.: Effects of Cold Acclimation on Several Enzyme Activities in *Euonymus radicans* 'Emorald & Gold' and Its Relation to Semi-lethal Temperature **Plate Ⅰ**



CW = cell wall; P = plastid; PM = plasma membrane; To = tonoplast; V = vacuole

1: ATPase activity on the PM and tonoplast in mesophyll cells of *Euonymus radicans* treated at 22 ℃ (15 000×);

2: ATPase on the PM and tonoplast in the mesophyll cells of *Euonymus radicans* treated at 4 ℃ (12 000×);

3: ATPase on the PM and tonoplast in the mesophyll cells of *Euonymus radicans* treated at 4 ℃ (20 000×);

4: ATPase became inactivation and ultrastructure of mesophyll cells damaged seriously for unacclimated seedlings treated at low temperature stress (6 000×);

5: ATPase on the PM and tonoplast remained active and ultrastructure of mesophyll cells was not damaged for acclimated

seedlings treated at low temperature stress (10 000×);

6: No lead phosphate deposits on the PM and tonoplast of mesophyll cells were observed when substrate was not added into the reaction medium (25 000×)

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