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Full Length Research Paper

Activities of some enzymes associated with oxygen metablolism, lipid peroxidation and cell permeability in dehydrated *Malus micromalus* seedlings

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Measurements were made on the relative water content, cell permeability, superoxide dismutase (SOD) activity, catalase (CAT) activity and malondialdehyde (MDA) content in *Malus micromalus* seedling during dehydration *in vitro* in whole seedling and during dehydration in culture of different PEG 6000 concentration. The results indicated that SOD and CAT activities increased during dehydration from 0 to 3 h and decreased after dehydration for 3 h; MDA content decreased before 3 h dehydration and the MDA content and cell permeability increased significantly after 3 h in various treatments of dehydration (*in vitro* and in whole seedling). SOD and CAT activities in 20 days seedlings were higher than those in 10 days seedlings whereas the MDA content and cell permeability in 20 days seedlings were lower than those in 10 days seedlings. In 20 days seedlings during dehydration *in vitro,* SOD and CAT activities in leaves were higher than those in root and the latter was higher than those in stem. However, the MDA content and cell permeability in leaves were lower than those in root which in turn were lower than those in stem. Significant correlations and regressions exist among the changes observed in the SOD activity, CAT activity, MDA content, cell permeability and the relative water content.

Key words: Drought stress, plant metabolism, reactive oxygen, lipid peroxidation, apple plant.

INTRODUCTION

Apple (*Malus domestica* Borkh.) is one of the most widely cultivated tree, and is one of the biggest economic in fruit trees in China. While apple trees are relatively sensitive to drought stress, apple production is influenced easily by the stress (Kaynas et al., 1996). Therefore, researches on mechanisms of plants to drought tolerance and screening out of drought tolerance plants are of much importance in apple trees for higher fruit production. *Malus micromalus* Mak belongs to the family Rosaceae, sub-family pomoideae. It is one of the high nutritional value small fruits and is usually used as the rootstock of apple in the north of China for its higher resistance (Atkinson et al., 1997).

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Abbreviations: SOD, Superoxide dismutase; **CAT,** catalase; **MDA,** malondialdehyde; **RWC,** relative water content; **POD,** peroxidase; **ROS,** reactive oxygen species; **PERM,** permeability.

Drought stress can induce membrane damage, increase membrane permeability and the accumulation of free radicals in plants. As a response, antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) can be produced to remove the active oxygen radicals or reduce the concentrations of free radicals (or reactive oxygen species) in the living tissues (Wang et al., 2002; Sun et al., 2003). Being highly destructive to certain functional group present in bimolecular, these products are potent agents of oxygen toxicity, particularly the superoxide radicals. A number of studies have been reported on the activity of SOD and extent of lipid peroxidation in higher plants. According to Dhindsa and Reid, (1982), free radicals initiate lipid peroxidation and this results in deterioration of cell membranes, which is accompanied by a decrease in SOD activity during leaf senescence (Jiao, 2003). Studies by Rabinowitch and Sklan, 1981, Rabinowitch et al., 1982, revealed that the activity of SOD passes through a minimum at mature-green and breaker stages of repining in tomatoes as well as in cucumber and peppers; and that artificial induction of

tolerance to photodynamic damage by controlled beat treatment was accompanied by an increasing SOD activity. George and Brooklebank, (1985) showed that iron-catalyzed with *Epilobium hirsutum* L. roots (iron-sensitive plant) lead to superoxide radical formation and the induction of superoxide dismutase and the immediate product, H_2O_2 appears to accumulate in the absence of catalase and low activity of root peroxidase, resulting in hydroxyl radical formation, increased lipid peroxidation and gross cellular damage during water-logging. Most of the studies on SOD activity during dehydration or low temperature stress proved free radical cell injury in higher plants (Lin et al., 1984; Wu and Glen, 1985; Guo et al., 2001). It has been reported that membranes are subject to rapid damage with increasing water stress. Further damage to long chain fatty acids could result in production of small chain hydrocarbon fragments including malondialdehyde (MDA) (Alscher et al., 2002).

The present study focuses on the relative significance of antioxidant enzymes, MDA content and cell permeability in *M. micromalus* seedlings to dehydration. The aim was to observe if there would be any difference among different organs and among seedlings at different earlier developmental stages and to demonstrate the mechanism of free radical injury in the cell involved in the *M. Micromalus* plant and provide a theoretical basis for breeding drought-resistant rootstock varieties and apple drought-resistant cultivation.

MATERIALS AND METHODS

Germination and dehydration treatments

M. micromalus seeds were germinated between 12 cm filter discs in Petri plates which had been moistured with 5 ml distilled H_2O. After 3 days, the unique seedlings were chosen and transplanted into plastic pots, and were thereafter cultured for 10 or 20 days at 25℃. They were then exposed to an atmosphere of 50-60% relative humidity, under a light intensity of 2500 lux, at 25-27℃. Treatments were designed as 0, 3, 6, 9 and 12 h *in vitro* and in whole seedlings respectively. 10 days seedlings after being cultured in potted vermiculite of fine sand were transplanted to hermetic boxes containing 0, 5, 15, 20, 25, 30, 35 and 40% PEG 6000 solution, respectively. Measurements were made after 24 h under the condition previously described.

Preparation of extracts

Leaves, stems or roots from each dehydrated treatments were collected, washed and mixed. Random samples of 0.2 g were thoroughly ground with a cald matar and pestle in an ice bath, until no fibrous residues could be seen. The grinding medium (4-6 ml/g fresh weight) consisted of 0.1 M potassium phosphate and 0.1 mM EDTA (PH 7.8) plus homogenizing quartz. The homogenate was centrifuged at 13000 g for 15 min in a refrigerated centrifuge at 0 - 4. The suspension hereafter was crude SOD extract and was used for determination of the SOD activity in the tissue.

Protein content

The water soluble protein content of all crude SOD, CAT and MDA extracts were determined by the method of Department of Biology in Beijing University. After precipitation with 10% trichloroacetic acid, bovine serum albumin was used as a standard.

SOD assay

All extracts were assayed for SOD activity photometrically using the assay system consisting of methionine, ribiflavin and NBT. The reaction mixture was composed of 1.3 M riboflavin, 3 mM methionine, 63 M NBT, 0.05 M sodium carbonate (PH 10.2) and appropriate volume of extract. Distrilled H_2O was added to bring to the final volume of 3 ml. One unit of SOD activity is defined as the amount that inhibits the NBT photoreduction by 50% (According to Giannopilitis and Stanley, 1977).

Catalase assay

0.02 g of samples was homogenized in 0.05 M Tris-Hcl buffer (pH 7.0) containing 0.001 M EDTA and 0.003 M $MgCl_2$ and the extract prepared as earlier described (Sanklaetal 1985). The reaction mixture of catalase contained 2.5 ml of 20 mM phosphate buffer (pH 7.4) and 0.1 ml of 1% hydrogen peroxide which is added last. The catalase activity was assayed by measuring the rate of disappearance of hydrogen peroxide (Maehly and Chance, 1959).

Lipid peroxidation

Malondialdehyde (MDA) was measured by a colorimetric method. 0.2 g of samples was homogenized in 5 ml of distilled H_2O and then 2 ml of extract in a 15 ml test tube, 0.5 ml of 40% trichloroacetic acid, 0.25 ml of 5 N HCl and 0.5 ml of 2% 2-thiobarbituric acid were added. After mixing, the test tube was placed in boiling water for 10 min, cooled and centrifuged at 2500 – 3000 rpm for 15 min. Absorbance of the supernatant was read at 532 nm, with correction for nonspecific turbidity by subtracting the absorbance at 600 nm. The amount of MDA present was calculated from the extinction coefficient of 156 nm (Heath and Packer, 1968).

RESULTS

Changes in SOD activity

During dehydration in whole seedlings, SOD activity of both the 10 and 20 days leaves increased when subjected to dehydration from 0 to 3 h, and decreased after dehydration at 3 h (Table 1), as did changes in SOD activity of both leaves and stems of the seedlings during dehydration *in vitro*. The SOD activity in roots declined during dehydration *in vitro* and in whole seedlings (Figure 1A). Changes of SOD activities when dehydrated by cultured in different solutions of PEG 6000 concentration appeared to be increased from 0 to 15% and to be decreased from 15 to 40% PEG 6000 in 20 days seedlings (Figure 2)

Changes in CAT activity

CAT activity in both 10 days and 20 days seedling leaves decreased significantly during dehydration in whole

Table 1. Changes of several indices of the leaves of both 10 days and 20 days seedlings with time of dehydration in whole *M. micromalus* seedling.

Time of Dehydration (h)	RWC (%)	PERM (%)	SOD activity (units/mg protein)	CAT activity (units/mg protein)	MDA content (units/mg protein)
10 days seedling					
0	89.60 A*	48.88 Aa**	3.83 Cc	2.88 A	3.84 C
3	66.08 B	49.98 Aa	5.84 Aa	2.84 A	3.04 D
6	58.56 C	58.82 Bb	4.50 Bb	1.32 B	3.77 C
9	51.67 D	73.06 Cc	3.49 Cc	1.24 B	4.01 B
12	48.15 E	86.61 Dd	1.78 Dd	0.86 C	4.74 A
20 days seedling					
0	80.11 A	20.82 A	6.30 ABab	9.67 A	2.94 C
3	71.38 B	28.06 B	6.88 Aa	8.77 B	1.58 E
6	56.44 C	38.81 C	6.05 Bb	7.80 C	2.41 D
9	50.61 D	51.89 D	4.21 C	6.74 C	3.18 B
12	47.10 E	77.44 E	2.50 D	4.32 E	4.03 A

*Duncan's multiple range test ($P < 0.01$); **Duncan's multiple range test ($P < 0.05$).
PERM, Cell permeability.

seedling (Table 1). Under dehydration *in vitro*, CAT activity in leaves, stems and roots of the 20 days seedlings decreased slowly from 0 to 3 h, while decreased markedly after 3 h (Figure 1B). CAT activity increased slowly from 0 to 5% PEG 6000 solution, while it decreased when the PEG 6000 concentration increased from 5 to 40% in 0 days seedlings (Figure 2).

Relative water content (RWC) and cell permeability

RWC of the leaves, stems and roots of seedlings at the 10 and 20 days all significantly decreased with the lengthening of time dehydration (*in vitro* and in whole seedlings) (Table 1 and Figure 1D) and with the increasing concentration of PEG 6000. However, the cell permeability increased during various treatments especially after 3 h *in vitro* and in whole plant dehydration (Figure 1E) as well as in the treatments of 10% PEG 6000 concentration. Therefore, we concluded that cell membrane injury occurred at 3 h dehydration and in the treatment with 10% PEG 6000 (Figure 2).

Comparison of enzyme activities and lipid peroxidation in organs and in stages of development in seedlings

The SOD and CAT activities in roots of 20 days seedlings were lower than those in leaves but higher than those in stems. MDA content of the leaves was lowest, but that in stems was highest. This indicated that cell permeability of stems was highest, followed by roots and that of leaves was lowest during dehydration *in vitro*. It can be suggested that the ability of dehydration resistant of leaf appeared to be high compared with stem and root; that of stem was higher than that of root (Figure 1).

Compared with 10 days seedlings, the SOD and CAT activities in 20 days seedlings were higher, but the MDA content and cell permeability in 20 days seedlings were lower, which demonstrated that there were differences in the dehydration-resistance in stages of seedling development during dehydration in whole *M. micromalus* plant (Table 1). Thus, it can be inferred that the stress tolerance mechanism exists at different stages of development of *M. micromals* seedlings.

Correlation and regression of the enzyme activities and lipid peroxidation

In the experiment of dehydration by different PEG6000 concentrations, the correlation and regression of SOD activity, CAT activity, MDA content, and cell permeability - RWC; MDA content-SOD activity, and MDA content - CAT activity; cell permeability-MDA content; and, SOD and CAT activities were analyzed in Table 2. Except for SOD - RWC and cell permeability-SOD, all others appeared to be significant correlations and regressions, which may show metabolism relation in plants during water stress (Table 2).

DISCUSSION

Generally, plants responded through alteration in physiological and biochemical processes when subjected to drought stress. The effects of dehydration on plants are manifold and reflect the multiple functions of water as a

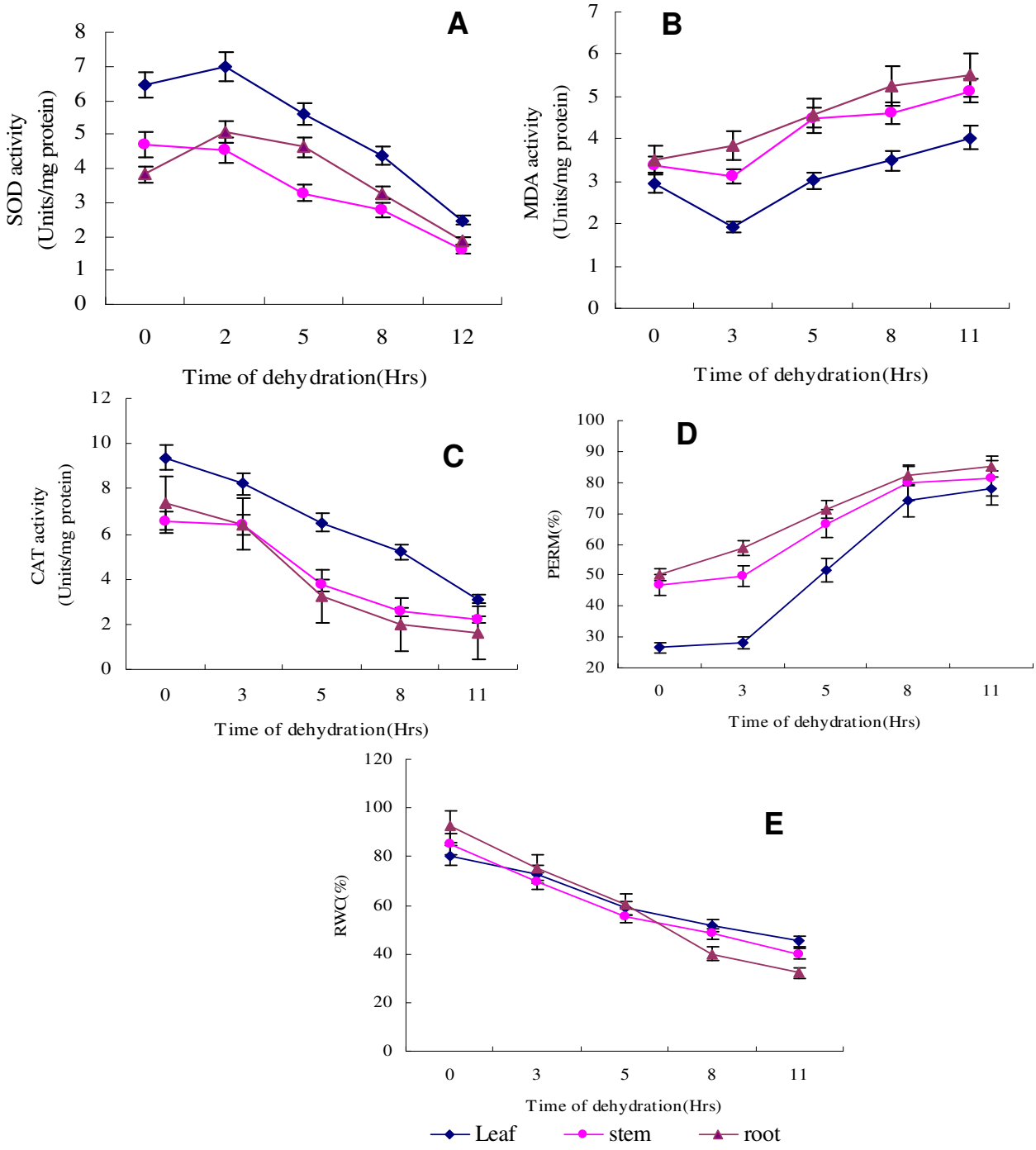

Figure 1. Effect of dehydration *in vitro* on several physiological indices in 20 days of *M. micrcmalus* seedling.

medium, a substrate, and a major constituent of active tissues. In plants, superoxide anion (O_2^-) formation could easily damage the cell either by direct reaction with its components or by the generation of hydroxyl radical (·OH) and single oxygen molecule, and the peroxidation of poly-unsaturated fatty acid. But, enzymatic antioxidant systems including SOD, peroxidase (POD) and CAT played an important role in scavenging harmful oxygen species.

A number of experiments have observed that O_2^- generating systems stimulate the peroxidation of fatty acids of membranes (Barber and Sharpe, 1971; John et al., 2006). Enzymes such as SOD and CAT catalyze the breakdown of O_2^- to ·OH and hydrogen peroxide (H_2O_2) found in a wide range of aerobic organisms (Fridovich, 1973). The O_2^- produced in contrast to that formed by the

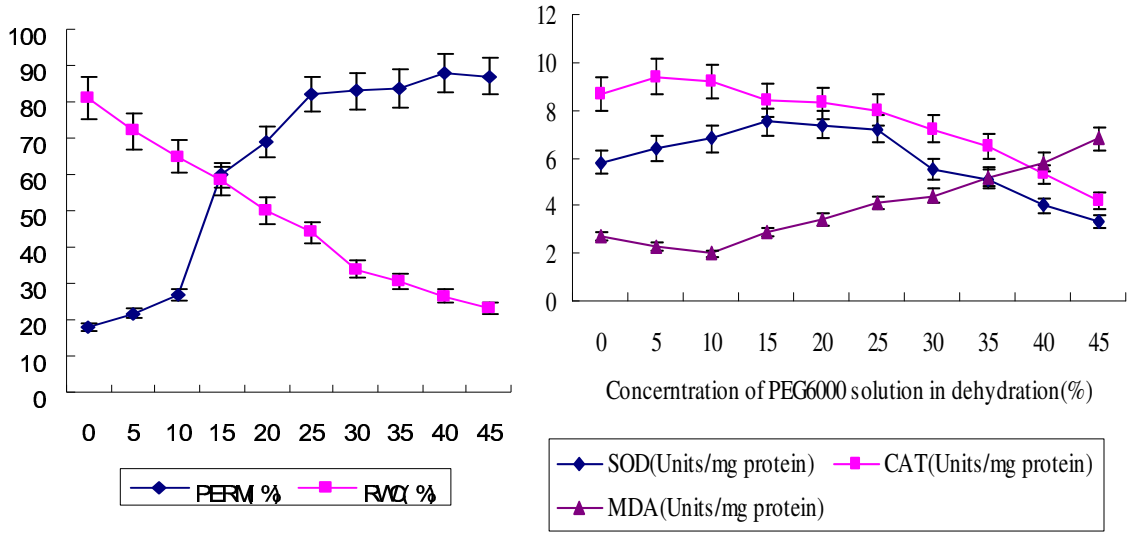

Figure 2. Changes of the several physiological indices of leaf of 20 days seedling of *M. micromalus* with PEG 6000 solution concentration.

Table 2. The regression and correlation of several indicas during dehydration in different concentration PEG 6000 solutions in *M. micromalus* seeding.

Indicas	Regression	Correlation
SOD-RWC	Y = 0.026 X +4.83	0.41
CAT-RWC	Y = 0.065 X +4.11	0.79*
MDA-RWC	Y = -0.071 X +7.62	-0.92**
PERM-RWC	Y = -1.44 X +130.40]	-0.98**
MDA-SOD	Y = -0. 80 X +9.07	-0.67*
MDA-CAT	Y = -0.91 X +11.05	-0.97**
PERM-MDA	Y = 17.15 X -10.93	0.90**
PERM-SOD	Y = -6.95 X +103.18	-0.31
PERM-CAT	Y = -13.515 X +161.79	-0.82**

*t test (P < 0.05); **t test (P < 0.01); PERM, cell permeability.

non-enzymatic dismutation of O_2^-, is in the ground state. The presence of superoxide dismutase active in aerobes suggests a role in production against oxygen free-radicals in experiments *in vitro*. SOD has been shown to protect sub-cellular organelles and bacteria against oxidative damage (Pederson, 1973). Other experiments showed that the activities of antioxidant enzymes SOD, POD and CAT increased with a suitable nitrogen level under water deficit (Sun et al., 2003). The rise in MDA content under stress conditions suggested that water stress could induce membrane lipid peroxidation by means of reactive oxygen species (ROS) (Sairam and Srivastava, 2002). The lower membrane stability index reflects the extent of lipid peroxidation, which in turn is a consequence of higher oxidative stress due to water stress conditions.

In this study, dehydration was accompanied by the decreased activities of SOD and CAT, and the increased level of MDA content which represented level of lipid peroxidation. The ability of ·OH and O_2^- to initiate lipid peroxidation has been demonstrated as described above. Thus the decline of SOD and CAT activities could result in a greater availability of free radicals, increased lipid peroxidation and then intensified membrane deterioration. In addition, the behaviors of SOD and CAT activities, MDA content, permeability (PERM) and RWC during dehydration vary from species (cultivars) to species (cultivars) and from 10 days seedlings to 20 days seedlings which could be proved that there were differences in water stress-resistance among species and between seedling stages. In this study, the decrease of CAT activity and increase of the PERM and MDA content was correlated with decrease levels of RWC, respectively, during dehydration. The increase of MDA content was also correlated with PERM and CAT. It could be suggested that the behavior of SOD, CAT activities, MDA content and PERM indicate procedures of metabolism in higher plants during water stress or drought.

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Malondialdehyde (MDA) was measured by a colorimetric method. 0.2 g of samples was homogenized in 5 ml of distilled H_2O and then 2 ml of extract in a 15 ml test tube, 0.5 ml of 40% trichloroacetic acid, 0.25 ml of 5 N HCl and 0.5 ml of 2% 2-thiobarbituric acid were added. After mixing, the test tube was placed in boiling water for 10 min, cooled and centrifuged at 2500 – 3000 rpm for 15 min. Absorbance of the supernatant was read at 532 nm, with correction for nonspecific turbidity by subtracting the absorbance at 600 nm. The amount of MDA present was calculated from the extinction coefficient of 156 nm (Heath and Packer, 1968).

RESULTS

Changes in SOD activity

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Changes in CAT activity

CAT activity in both 10 days and 20 days seedling leaves decreased significantly during dehydration in whole

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12	47.10 E	77.44 E	2.50 D	4.32 E	4.03 A

*Duncan's multiple range test ($P < 0.01$); **Duncan's multiple range test ($P < 0.05$).
PERM, Cell permeability.

seedling (Table 1). Under dehydration *in vitro*, CAT activity in leaves, stems and roots of the 20 days seedlings decreased slowly from 0 to 3 h, while decreased markedly after 3 h (Figure 1B). CAT activity increased slowly from 0 to 5% PEG 6000 solution, while it decreased when the PEG 6000 concentration increased from 5 to 40% in 0 days seedlings (Figure 2).

Relative water content (RWC) and cell permeability

RWC of the leaves, stems and roots of seedlings at the 10 and 20 days all significantly decreased with the lengthening of time dehydration (*in vitro* and in whole seedlings) (Table 1 and Figure 1D) and with the increasing concentration of PEG 6000. However, the cell permeability increased during various treatments especially after 3 h *in vitro* and in whole plant dehydration (Figure 1E) as well as in the treatments of 10% PEG 6000 concentration. Therefore, we concluded that cell membrane injury occurred at 3 h dehydration and in the treatment with 10% PEG 6000 (Figure 2).

Comparison of enzyme activities and lipid peroxidation in organs and in stages of development in seedlings

The SOD and CAT activities in roots of 20 days seedlings were lower than those in leaves but higher than those in stems. MDA content of the leaves was lowest, but that in stems was highest. This indicated that cell permeability of stems was highest, followed by roots and that of leaves was lowest during dehydration *in vitro*. It can be suggested that the ability of dehydration resistant of leaf appeared to be high compared with stem and root; that of stem was higher than that of root (Figure 1).

Compared with 10 days seedlings, the SOD and CAT activities in 20 days seedlings were higher, but the MDA content and cell permeability in 20 days seedlings were lower, which demonstrated that there were differences in the dehydration-resistance in stages of seedling development during dehydration in whole *M. micromalus* plant (Table 1). Thus, it can be inferred that the stress tolerance mechanism exists at different stages of development of *M. micromals* seedlings.

Correlation and regression of the enzyme activities and lipid peroxidation

In the experiment of dehydration by different PEG6000 concentrations, the correlation and regression of SOD activity, CAT activity, MDA content, and cell permeability - RWC; MDA content-SOD activity, and MDA content - CAT activity; cell permeability-MDA content; and, SOD and CAT activities were analyzed in Table 2. Except for SOD - RWC and cell permeability-SOD, all others appeared to be significant correlations and regressions, which may show metabolism relation in plants during water stress (Table 2).

DISCUSSION

Generally, plants responded through alteration in physiological and biochemical processes when subjected to drought stress. The effects of dehydration on plants are manifold and reflect the multiple functions of water as a

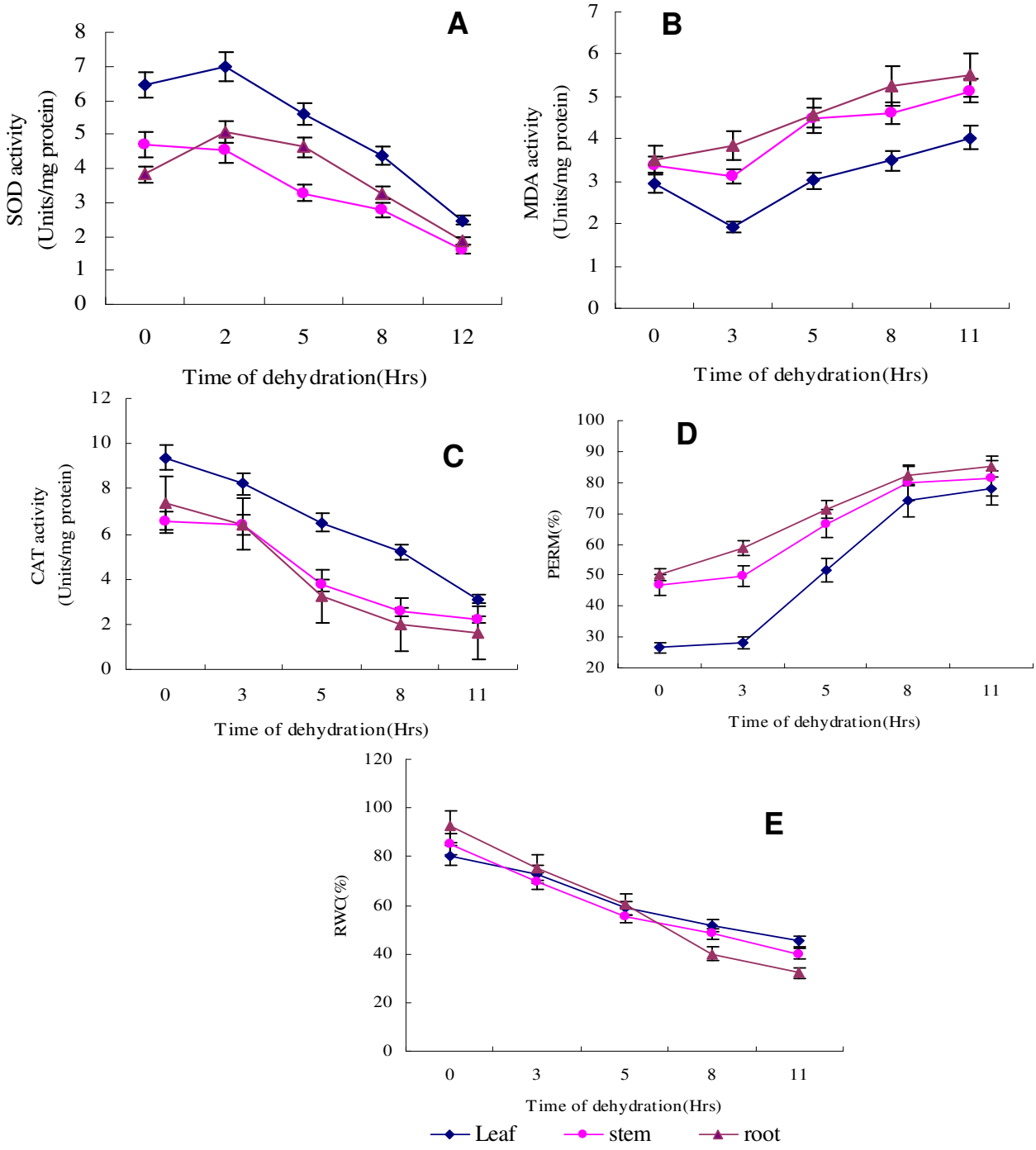

Figure 1. Effect of dehydration *in vitro* on several physiological indices in 20 days of *M. micrcmalus* seedling.

medium, a substrate, and a major constituent of active tissues. In plants, superoxide anion (O_2^-) formation could easily damage the cell either by direct reaction with its components or by the generation of hydroxyl radical (·OH) and single oxygen molecule, and the peroxidation of poly-unsaturated fatty acid. But, enzymatic antioxidant systems including SOD, peroxidase (POD) and CAT played an important role in scavenging harmful oxygen species.

A number of experiments have observed that O_2^- generating systems stimulate the peroxidation of fatty acids of membranes (Barber and Sharpe, 1971; John et al., 2006). Enzymes such as SOD and CAT catalyze the breakdown of O_2^- to ·OH and hydrogen peroxide (H_2O_2) found in a wide range of aerobic organisms (Fridovich, 1973). The O_2^- produced in contrast to that formed by the

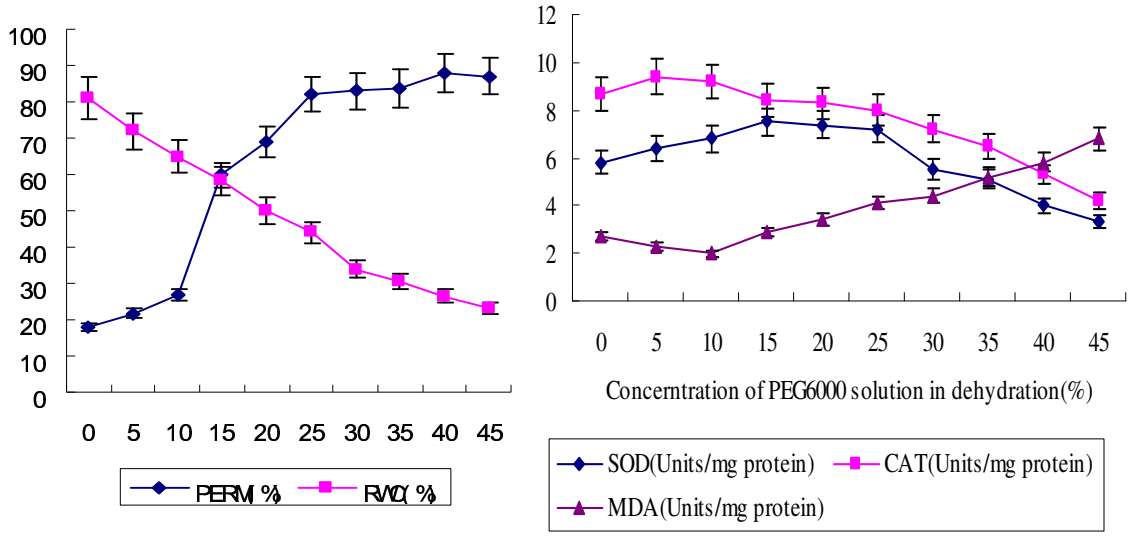

Figure 2. Changes of the several physiological indices of leaf of 20 days seedling of *M. micromalus* with PEG 6000 solution concentration.

Table 2. The regression and correlation of several indicas during dehydration in different concentration PEG 6000 solutions in *M. micromalus* seeding.

Indicas	Regression	Correlation
SOD-RWC	Y = 0.026 X +4.83	0.41
CAT-RWC	Y = 0.065 X +4.11	0.79*
MDA-RWC	Y = -0.071 X +7.62	-0.92**
PERM-RWC	Y = -1.44 X +130.40]	-0.98**
MDA-SOD	Y = -0. 80 X +9.07	-0.67*
MDA-CAT	Y = -0.91 X +11.05	-0.97**
PERM-MDA	Y = 17.15 X -10.93	0.90**
PERM-SOD	Y = -6.95 X +103.18	-0.31
PERM-CAT	Y = -13.515 X +161.79	-0.82**

*t test (P < 0.05); **t test (P < 0.01); PERM, cell permeability.

non-enzymatic dismutation of O_2^-, is in the ground state. The presence of superoxide dismutase active in aerobes suggests a role in production against oxygen free-radicals in experiments *in vitro*. SOD has been shown to protect sub-cellular organelles and bacteria against oxidative damage (Pederson, 1973). Other experiments showed that the activities of antioxidant enzymes SOD, POD and CAT increased with a suitable nitrogen level under water deficit (Sun et al., 2003). The rise in MDA content under stress conditions suggested that water stress could induce membrane lipid peroxidation by means of reactive oxygen species (ROS) (Sairam and Srivastava, 2002). The lower membrane stability index reflects the extent of lipid peroxidation, which in turn is a consequence of higher oxidative stress due to water stress conditions.

In this study, dehydration was accompanied by the decreased activities of SOD and CAT, and the increased level of MDA content which represented level of lipid peroxidation. The ability of ·OH and O_2^- to initiate lipid peroxidation has been demonstrated as described above. Thus the decline of SOD and CAT activities could result in a greater availability of free radicals, increased lipid peroxidation and then intensified membrane deterioration. In addition, the behaviors of SOD and CAT activities, MDA content, permeability (PERM) and RWC during dehydration vary from species (cultivars) to species (cultivars) and from 10 days seedlings to 20 days seedlings which could be proved that there were differences in water stress-resistance among species and between seedling stages. In this study, the decrease of CAT activity and increase of the PERM and MDA content was correlated with decrease levels of RWC, respectively, during dehydration. The increase of MDA content was also correlated with PERM and CAT. It could be suggested that the behavior of SOD, CAT activities, MDA content and PERM indicate procedures of metabolism in higher plants during water stress or drought.

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