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Full Length Research Paper

The physiological response of suspension cell of *Capparis spinosa* L to drought stress

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This study was conducted to investigate the drought tolerance and initial mechanism of *Capparis spinosa* L suspension cell to water stress. The suspension callus inoculated in liquid Murashige and Skoog medium contained 0 to 15% (m/v) polyethylene glycol (PEG 8000) and physiological index were assessed. The result indicated that drought stress led to hydrogen peroxide accumulation more than in the control which led the activities of peroxidase (POD) increased at low-concentration PEG treatment and then greatly declined. The soluble sugar followed the same trend, the content of fatty acid and total unsaturated fatty acid (TUFA) increased consequently. The longer and more severe the drought stress is, the higher the content of malondialdehyde (MDA), proline and soluble protein are. The study also demonstrated that when cells suffered more severe injury (over 8%), the soluble sugar, POD and unsaturated fatty acid exhibited decrease trend as cell membranes broke and endogenous protective enzyme activities decreased and MDA accumulated.

Key words: *Capparis spinosa* L, suspension culture, drought stress.

INTRODUCTION

Capparis spinosa L is better known as a medicinal plant for its antibiosis, anti-inflammatory, anti-oxygen and rheumatism treatment because more effective components were existed which includes; volatile oil, alkaloids, flavonoids, terpene, mustard oil glycoside, and so forth (Mattaus and Ozcan, 2002; Ali et al., 2004; Eddouks et al., 2005). *C. spinosa* L is believed as one of typical eremophytes, which can effectively used the groundwater resources for its more developed root and the xylem system, it extensively distributes in drought-, cold- and hot-stressed environments, such as in Mediterranean countries, Xinjiang, Gansu and Tibet of China (Khanafar et al., 2003; Levizou et al., 2004; Sakcali et al., 2008). *C. spinosa* L dynamically recovers even in the warmest hours of the day and under drought conditions. The long roots and wide ecological amplitude allow it to withstand harsh environments. The species thus appears to be a suitable candidate for the protection of degraded areas. Most research has focused on the modulation mechanism of *C. Spinosa* L under drought condition, but few researches have been reported on the adaptive mechanisms of *C. spinosa* L suspension cell to being cultivated under water stress (Levizou et al., 2004; Sakcali et al., 2008). Water stress inhibits cell enlargement more than cell division, it reduces plant growth by affecting various physiological and biochemical processes, such as photosynthesis, respiration, translocation, ion uptake, carbohydrates, nutrient metabolism and growth promoters (Duan et al., 2007; Farooq et al., 2009; Praba et al., 2009). *C. spinosa* L is famous of drought stress adaptation plant, but the resistance mechanism of suspension cell was not expatiated integrated.

Numerous studies have demonstrated that the membrane damage and the free radicals accumulation were frequently occurred in adverse conditions in plants

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(Kreps et al., 2002; Mahajan and Tuteja, 2006). Water availability is one of the principal limitations of plant growth, particularly in the arid and semiarid region, otherwise, the *C. spinosa* L is largely free of competition for water with other species in those areas (Rhizopoulou and Psaras, 2003). Antioxidative enzymes and some small molecule substances, such as proline, soluble sugar and soluble protein, can be produced to remove the active oxygen radicals and increase the osmotic potential (Kavi et al., 2005; Yamada et al., 2005; Matos et al., 2010). Other research revealed that in response to drought, total lipid contents increased progressively, and the most striking changes were an increase in unsaturated fatty acid (Júnior et al., 2008; Toumi et al., 2008). The responses of soybean suspension cells to PEG induced water stress were also studied, substantial osmotic adjustment was observed in adapted lines, due mainly to increased glucose, fructose and sucrose, proline concentration increased up to 40-fold in adapted and 12-fold in unadapted cells (Elsayed and Kirkwood, 1992). Water stress tolerant callus line of *Helianthus annus* L. Cv. Myak was isolated by plating the cell suspension on agar-solidified medium containing the same solute potential of PEG, this selected line grew better than the non-selected one on various levels of water deficit induced by PEG (Hassan et al., 2004). Plant cell suspension cultures have been used not only for plant propagation but also to investigate the physiological, biochemical, and molecular aspects of various cellular functions (Dong et al., 2010). Suspension cell responded directly to the physiology and biochemistry factor, maybe it is a good model for study the impact of cell under drought stress.

Polyethylene glycol (PEG) has been used to induce water stress as it decreases the water potential in the medium and has been suggested as being superior to other solutes inducing water stress because of its chemical inertness, non-toxic nature and not being readily absorbed by intact plants (Moon et al., 1995; Yang et al., 2007). So it was frequently used to simulate water deficit. In this paper, the physiological response of *C. spinosa* L to PEG-induced drought stress was studied. The main objective of this study is to understand the possible physiological and biochemical response of suspension cell of *C. spinosa* L in drought condition, and constitute the cell culture system under drought stress, and then served for adaptive cell line selecting and variety breeding in the future.

MATERIALS AND METHODS

The suspension cell were derived from the leaves of *C. spinosa* L, which were obtain from wild plants growing in the experimental field of Huazhong University of Science and Technology (Li et al., 2007). The suspension cells were cultivated in liquid MS culture medium which contained 2, 4-D (1.0 mg L^{-1}) and 6-BA (1.5 mg L^{-1}). The pH value of the media that used for suspension cell culture, was 5.8, the suspension cell was cultured at 25°C, and the intensity of illumination were 2000 and <50 Lux for light culture (14 h) and dark (10 h) culture.

PEG treatment

The suspension cells were cultured in liquid MS medium that containing 4, 8, 12, and 15% PEG respectively. Suspension cells that cultured in the MS medium without PEG were used as control. The cells were harvested every 4 days during a cell growth period after beginning treatment.

The method for cell harvesting was as follows, the cells were filtrated from liquid MS medium and then washed with distilled water to remove residual medium. Each treatment was replicated in three times.

Free proline content assay

The acid ninhydrin method was used for extracting and quantifying the free proline content as described by Tang et al. (2008). 0.5 g of fresh cells and 3% 5 ml of extraction buffer (sulfosalicylic acid) were mixed and boiled for 10 min. The mixture was filtrated after it cooling down, the filtrate was taken as the proline extract. The proline assay mixture was including 1 ml proline extract, 2 ml glacial acetic acid and 2 ml acor ninhydrin as substrates, the mixture was boiling for 30 min. Added 4 ml toluene to the this mixture and then oscillated, the absorbance of the supernatant was measured at 520 nm while toluene was taken as the control. The content of proline was calculated according to standard curve. All samples were tested in three independent experiments with three replicates each.

Soluble protein content assay

The soluble protein content assaying was according to the method of Riccardi et al. (1998). 0.2 g fresh cell were homogenized with 5 ml distilled water, and then refrigerated centrifuged at 8000 g/min for 10 min. The 1 ml supernatant was mixed with 5 ml coomassie brilliant blue G-250, layed up 2 min and determined at 595 nm. The content of soluble protein was calculated according to standard curve.

Soluble reducing sugar content assay

The soluble reducing sugar content assaying was according to the method of Ranney et al. (1991). 0.1 g of fresh cells was homogenized with 5 ml distilled water. The homogenate was then centrifuged at 8000 g/min for 10 min. The soluble reducing sugar content was assayed as follows: 1.0 ml supernatant and 0.5 ml 3.5-Dinitrosalicylic acid (DNS) were mixed and then treated in boiling water for 5 min, and the absorbance was measured at 520 nm. The content of reducing sugar was calculated according to standard curve.

Malondialdehyde assay

The analysis for malondialdehyde (MDA) content was according to the methods of Gülen et al. (2008). 0.5 g of fresh cells were homogenized with 5 ml 5% 2,4,6-trichloroanisole (TCA), after centrifuged at 3000 revolution/min for 10 min. The supernate was mixed with 2 ml 0.67% 2,4,6-tribromoanisole (TBA) and then boiling for 30 min, the mixture was centrifuged again after it cooled down. The absorbance of supernatant was measured at 532, 600

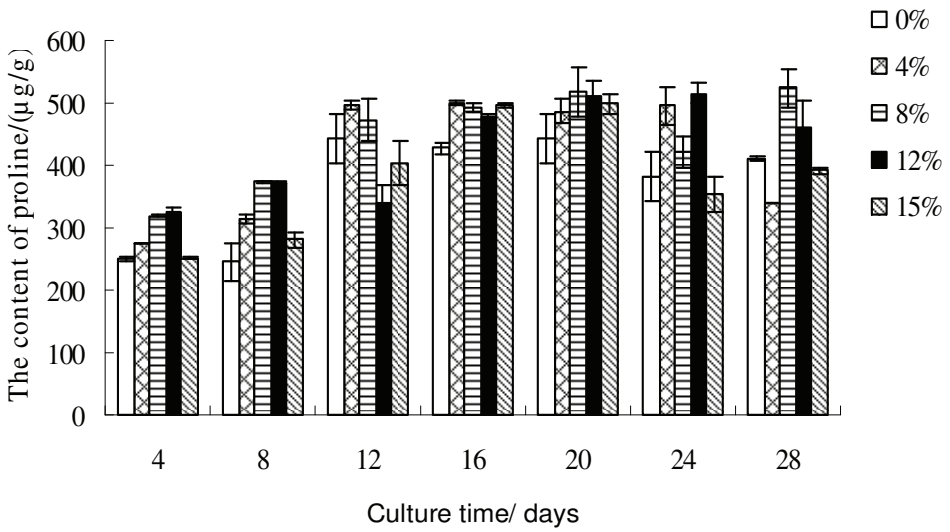

Figure 1. The changing tendency of free proline content under different PEG concentrations.

and 450 nm, the MDA content was figured out according to formula:

$C/umol/L = 6.45 (A_{532} - A_{600}) - 0.56A_{450}$.

POD activity assay

The POD activity analysis was according to the method of Lamikanrao and Watson (2001). 0.5 g of fresh cells were homogenized with phosphate buffer and then centrifuged, the supernatant was used for further experiment. The reaction system for POD activity assay was including 2.9 ml 0.05 mol/L phosphate buffer, 1.0 ml 2 % H_2O_2 and 1.0 ml 0.05 mol/L guaiacol. The supernatant boiled for 5 min was considered as the control. After adding supernatant (enzyme fluid) to the reacting system, the mixture was kept at 37°C for 3 min and the increase of absorbance at 470 nm was recorded every minute within 5 min. One POD unit (U) was defined as the change of 0.01 OD per min.

The GC-MS analysis of fatty acid composition under PEG treatment

The suspension cells treated by different PEG concentrations were harvested at logarithmic growth phase, and then dried at 50°C to constant dry weight. 2.0 g dry cells were extracted with ligarine (30 to 60°C boiling range) twice for 20 min and then centrifuged, the supernatant was then evaporated under room temperature. The concentrated lipids were mixed with 1 ml of 0.5 M KOH-methanol solution, and treated at 60°C for 15 min, add 2 ml 14% Boron trifluoride diethyl ether-methanol solution into the above mixture, and then heated at 60°C with continuous shaking for 2 min. The FAMEs were extracted with 1 ml N-hexane and 1 ml saturate NaCl solution, and then stored at –20°C. GC-MS analysis was performed on a HP INNOWax (30 m × 250 mm × 0.25 μm film) using helium as the carrier gas. The oven temperature was programmed as follows: the first 180°C, which was increased to 200C at 5°C/min, and then increased to 220°C at 2°C/min, where it was maintained for 5 min. The analyses were processed with the Star GC Workstation software.

RESULTS

Effect of water stress on free proline accumulation

It was obvious that the free proline level remained substantially increased initially and reduced during the decline phase (Figure 1). Osmotic adjustment through the accumulation of proline was positively related to PEG concentration. The free proline of the normal suspension cells varied between 250 to 410 μg/g, obviously increase in free proline was observed in the suspension cells subjected to water stress. The accumulation of free proline increased as the stress prolonged and attained maximal levels at 8 h after the polyethylene glycol treatment. The levels remained constant thereafter up to 12 h of the treatment. There was a marginal decrease in the accumulated proline 24 h after the treatment. However, the increase in free proline was as much as 65 fold, even on continuing the treatment for 24 h.

Effect of water stress on soluble protein and soluble sugar accumulation

Increasing PEG concentration (0 to 12%) was associated with a progressive production of soluble protein and the tendency was increase first and then drop down (Figure 2). When the PEG concentration achieved 15%, the callus was exposed to the severe damage, resulting in the decrease of soluble protein which was still higher than that of the control. It was obviously that the content of soluble protein remained substantially increased after PEG treatment and reached the maximum value at the 8th day (Figure 2), afterward,

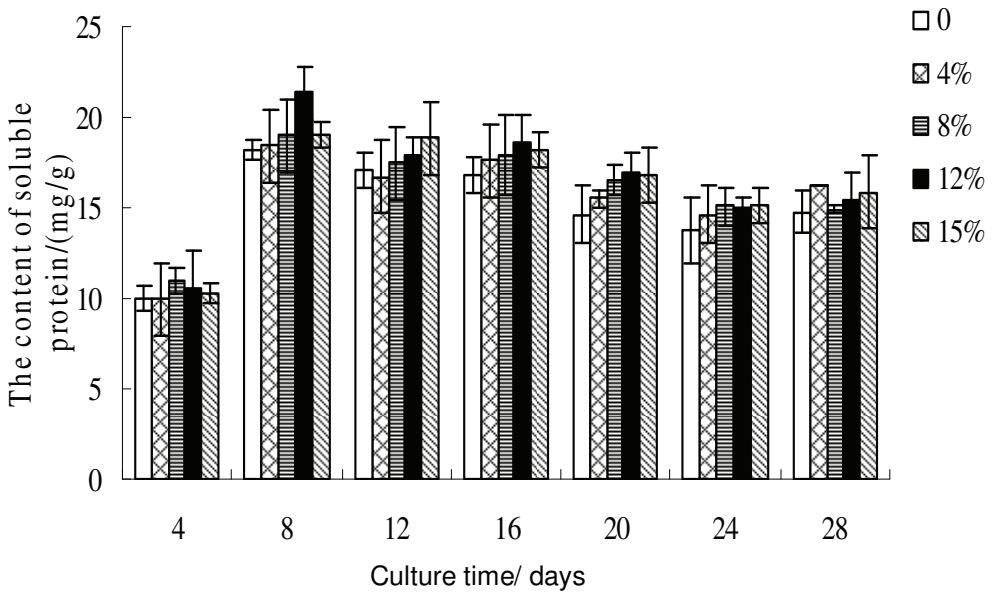

Figure 2. The changing tendency of soluble protein content under different PEG concentrations.

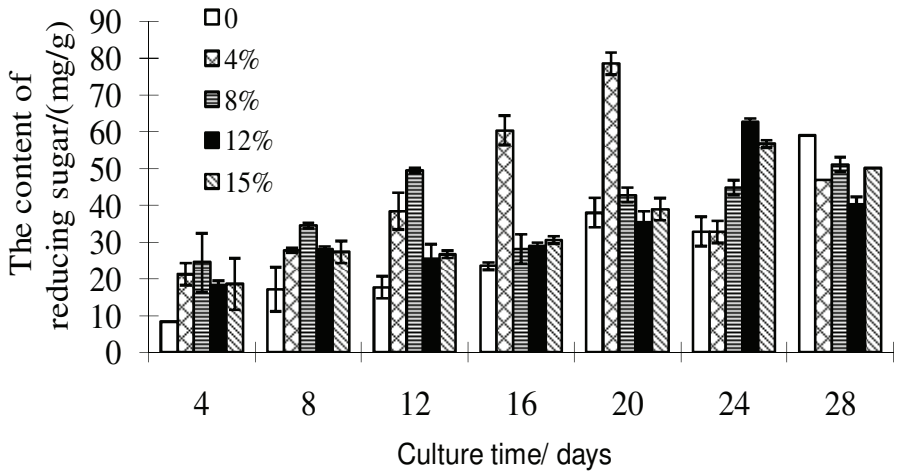

Figure 3. Influence of PEG concentration on reducing sugar content.

it fell down continually. During the whole course of lag phase, the bulk of protein was used in the cell division, simultaneously it was also contributive to enhancing the cell osmotic potential under drought condition.

When plants were subjected to drought conditions, they will usually accumulate some soluble sugar to increase the osmotic potential, and thus, will help to adapt the changes of external environments. It is manifested that the addition of certain content of PEG increased the soluble sugar content as observed from Figure 3, with 4% PEG treatment the most significant. But if the PEG content continued to increase, the soluble sugar content decreased and presented a little higher in comparison to the control.

Effect of water stress on MDA content

Under drought stress, the content of MDA increased constantly independence of drought intensity and suspension cell development stage (Figure 4). Along with the increased of PEG concentration, the cell membrane lipid peroxidized and the content of its primary product MDA increased correspondingly

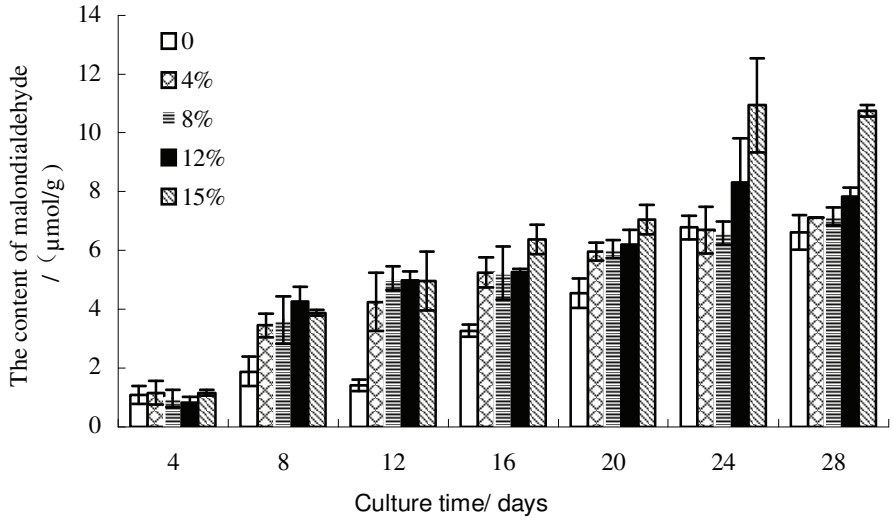

Figure 4. The changing tendency of MDA content under different PEG concentrations.

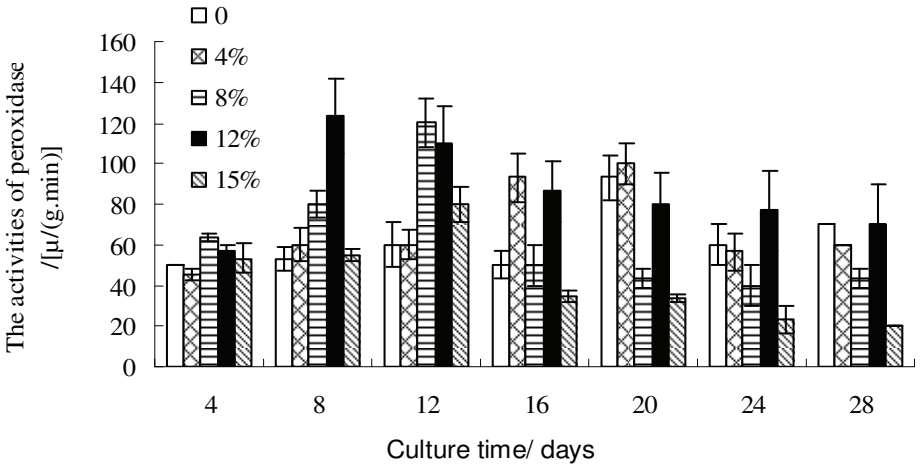

Figure 5. The changing tendency of POD activity under different PEG concentrations

(Figure 4). The MDA content increased drastically at 15% PEG treatment, reaching to a peak of 10.94 μmol/g at the 24th day, which was about 1.6-fold than that of the control. On the other hand, the MDA content still accumulated in the callus over the whole course of cultivation. During the previous 24 days of the experiment, the MDA content has raised constantly. Thereafter, the content of MDA in PEG-treated cultures began to decrease at decline phase, which was still higher than the control value.

Effect of water stress on POD activity

The activity of protective enzymes POD under water stress was shown in Figure 5. The trend of POD activity were similar between the 4% PEG-treated and the control whose peak was 100 μ/(g.min) and 93.3 μ/(g.min) at the 20th day. With regards to other PEG concentrations, POD activity increased drastically and reached the maximum values at the 8th day (12% PEG) and the 12th day (8%), which was about 1.32- and 1.29-fold higher than those of control.

Effect of water stress on fatty acid composition

The Table 1 shown that the fatty acid composition of *C. spinosa* suspension cell, which mainly included: Pentadecanoic acid (15:0), hexadecanoic acid (16:0), hexadecenoic acid (16:1), octadecanoic acid (18:0), octadecenoic acid (18:1), octadecadienoic acid (18:2),

Table 1. The change of fatty acid composition of *C. spinosa* L suspension culture on PEG treatment.

Fatty acid (%)	Control	4% PEG	8% PEG	12% PEG	15% PEG
Pentadecanoic acid (15:0)	1.096	1.178	2.14	2.411	1.687
Hexadecanoic acid (16:0)	34.067	29.98	27.889	26.87	26.524
Hexadecenoic acid (16:1)	1.918	1.582	2.971	3.112	1.816
Octadecanoic acid (18:0)	18.099	16.782	16.839	11.203	12.74
Octadecenoic acid (18:1)	13.003	12.561	9.966	22.118	25.185
Octadecadienoic acid (18:2)	3.105	9.402	11.663	3.987	1.382
Octadecatrienoic acid (18:3)	0.625	0.824	1.513	1.851	2.011
Eicosanoic acid (20:0)	4.964	3.921	2.031	2.003	2.285
Heneicosanoic acid (21:0)	0.643	0.511	0.453	0.382	0.357
Docosanoic acid (22:0)	7.028	5.811	3.337	2.140	2.584
TUFA	18.651	24.369	26.133	31.068	30.394

TUFA: Total unsaturated fatty acid.

octadecatrienoic acid (18:3), eicosanoic acid (20:0), heneicosanoic acid (21:0), docosanoic acid (22:0). Among which, the contents of hexadecanoic acid (16:0), octadecanoic acid (18:0) and octadecenoic acid (18:1) were the most abundant three components which reached approximately 30, 15 and 10 to 20%, respectively (Table 1).

When the suspension cells were subjected to PEG treatment (below 12%), the content of total unsaturated fatty acid of *C. spinosa* increased progressively from moderate to severe drought stress, and on the contrary, total saturated fatty acid decreased with PEG content increase (Table 1). The total unsaturated fatty acid (TUFA) was about 1.5 and 2-fold than that of control. As the PEG concentration increased above 12%, there was a slight decrease in TUFA content which indicated that the excess damage towards suspension cell membrane. Further analysis shown that the content of hexadecenoic acid (16:1), octadecenoic acid (18:1), octadecadienoic acid (18:2), octadecatrienoic acid (18:3) behaved a different change trend at certain PEG concentration, similar phenomenon was found in the content change of every saturated fatty acid.

DISCUSSION

Water stress is one of the most important environmental constraints limiting plant growth and agricultural productivity because it can cause osmotic stress and nutrient deficiency. In the long process of evolution, plant developed a series of mechanisms to adapt to water stress in biochemistry and molecular level, the synthesis of protective substances permeability is also a good measure, plants accumulate up to the amino acid and its derivatives are mainly proline and betaine (Kavi et at., 2005; Yamada et at., 2005; Matos et at., 2010). Plants produced various osmotic regulation materials to stabilize cellular structures such as proline, sugars, and proteins. Proline can act as a signaling molecule to modulate mitochondrial functions, influence cell proliferation and trigger specific gene expression, which can be essential for plant recovery from stress (Al-Khayri, 2002; Szabados and Savoure, 2009). The proline content increase as the drought stress progressed and reached a peak as recorded after 10 days stress, and then decreased under severe water stress as observed after 15 days of stress in water stressed maize plants (Anjum et al., 2011). Under drought stress, the soluble sugar also acted as the osmotic regulation substance and the dehydrator, enhancing the ability of cell to retain water (Watanabe et al., 2000).

The soluble protein content is an important indication of protein damage in the plant metabolism process, owing to its hydrophilic colloid nature, the soluble protein can help the cells retain more water and increase the duration under drought condition (Mohammadkhani and Heidari, 2008; Chattopadhyay et al., 2011). The suspension cell of *C. spinosa* L exhibited some modulation to PEG-induced water stress, the longer and more severe the drought stress is, the higher the content of proline and soluble protein are, but when cells suffered more severe injury (over 8%), the soluble sugar, free proline and soluble protein exhibited decrease trend.

Drought induces oxidative stress in plants by generation of reactive oxygen species (ROS), which is one of the earliest biochemical responses of eukaryotic cells to biotic and abiotic stresses triggered subsequent defense reaction in plants (Farooq et al., 2009). Plants have an internal protective enzyme-catalyzed clean up system, which is fine and elaborate enough to avoid injuries of active oxygen, thus guaranteeing normal cellular function (Apel and Hirt, 2004; Horváth et al., 2007; Helena and Carvalho, 2008; Anjum et at., 2011). The balance between ROS production and activities of antioxidative enzyme determines whether oxidative signaling and damage will occur, therefore, the cellular

damage under water stress could be averted to some extent (Moller et al., 2007; Zhu et al., 2009). In this study, the results clearly suggested that when suspension cell of *C. spinosa* L were subjected to water stress conditions, the plant antioxidant systems would take action. Increasing PEG concentration was associated with a progressive rising in POD, but the POD acitivity began to drop down compared to the control when water deficiency caused severe damages (over 8%) beyond the duration of the callus.

The content of MDA has been considered an indicator of oxidative damage, indicating the level of lipid peroxidation resulting from oxidative stress and the tolerance of plants towards adverse conditions (Moller et al., 2007; Farooq et al., 2009). A decrease in membrane stability reflects the extent of lipid peroxidation caused by ROS, levels of lipid peroxidation in leaves of pea (*Pisum sativum*) increased two to four fold with an increase in drought stress, and this was highly correlated with protein peroxidation (Moran et al., 1994; Yang and Miao, 2010). Our study also demonstrated that MDA content in stressed increased correlated with capacity to withstand water stress. The reason was possibly that when cells were transferred to the arid environment initially, it generated intense reaction to adapt the sudden changes, which led to the sharp raise of MDA content. During the whole course of cultivation, the increases in lipid peroxidation observed herein were in agreement with the results of other studies (Tang et al., 2004). Lipids are important membrane components, and changes in their composition may help to maintain membrane integrity and preserve cell compartmentation. The most striking changes in lipid composition were an increase in unsaturation fatty acid. In response to drought, total lipid contents increased progressively. A similar relationship has already been established in the case of low temperature (Moon et al., 1995; Routaboul et al., 2000) and salinity stresses (Allakhverdiev et al., 2001). Our results show that *C. apparis* displays a strong capacity to tolerate water deficits at the cell level, as shown by their capacity to maintain their polar lipid contents and the stability of their lipid composition under severe water loss conditions. Despite the increase in total lipid content, lipid class distribution remained relatively stable until the stress became very severe (above 12%). Moreover, it displays several characteristics indicative of a so far unknown adaptation capacity to drought-stress at the cellular level, such as an increase in unsaturated fatty acid.

In a word, based on the completed protection mechanism, cells of *C. spinosa* L exhibited higher tolerance to PEG-induced water stress. Understanding plant responses to drought is of great importance and also a fundamental part for making the crops stress tolerant (Ramachandra et al., 2004; Zhao et al., 2008). Although these investigations shed light on the cellular mechanisms of adaptation of *C. spinosa* L cells to water deficits induced with PEG, it should be cautioned that these mechanisms may be implemented, it remains to be seen whether regenerated plants from water stress-adapted cells exhibit increased tolerance to osmotic stress at the whole plant level. Understanding the mechanisms by which plants perceive environmental signals and transmit the signals to cellular machinery to activate adaptive responses is of fundamental importance to biology. It has become imperative to elucidate the responses and adaptation of crops to water deficit, and take actions to improve the drought resistance ability of crop plants and to ensure higher crop yields against unfavorable environmental stresses.

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