Influence of Drought on Oxidative Stress and Flavonoid Production in Cell Suspension Culture of *Glycyrrhiza inflata Batal*

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The effect of water deficit on flavonoid production and physiological parameters characteristic for oxidative stress were studied in a cell suspension culture of *Glycyrrhiza inflata Batal* to investigate its drought tolerance. The result indicated that appropriate water deficit enhanced biomass accumulation of 27.1 g L^{-1} and flavonoid production of 151.5 mg L^{-1}, which was about 2-fold and 1.5-fold of the control, respectively. But it decreased the water content. Drought stress led to hydrogen peroxide accumulation more than in the control. Moreover, under drought conditions, malondialdehyde content, the activities of catalase and peroxidase increased to a greater extent than the control, and each reached a maximum value of 91.3 μmol g^{-1} dry weight, 85.6 U and 1951 U g^{-1} dry weight per min, which was 1.5-, 1.7- and 3.7-fold of the control, respectively. All above showed that appropriate water deficit could activate the antioxidative defense enzymes system to maintain stability in plants subjected to drought stress. On the contrary, the activity of phenylalanine ammonia lyase of the control increased in company with the biosynthesis of flavonoids, which indicated that phenylalanine ammonia lyase might play an important role in the path of the biosynthesis of flavonoids.

Key words: Drought, Flavonoid Production, *Glycyrrhiza inflata Batal*

Introduction

Reactions of plant antioxidant systems upon drought have been intensively investigated for several crop plants, such as maize (Li and Staden, 1998) and wheat (Selote *et al.*, 2004). In different drought-tolerant species or genotypes, decreased oxidative damages have been associated with an increased expression of the antioxidant system (Smirnoff, 1993). In fact, manipulation of the antioxidant system might be considered as a promising target for the production of stress-tolerant transgenic plants, since it is possible that an increase in one or a few enzymes could increase the tolerance of plants to the conditions causing oxidative damages (Smirnoff, 1995; Allen *et al.*, 1997).

Glycyrrhiza inflata Batal is used extensively in traditional Chinese medicine which not only has anti-inflammatory, antibacterial and antiviral activities, but also has immunomodulating, antioxidant and free radical scavenging activities (Shetty *et al.*, 2002). It grows mainly in drought-, cold- and hot-stressed environments of the northwestern part of China (Wang *et al.*, 2001). In this regard, the species may be able to withstand drought. Therefore, it is a promising material for studying the drought tolerance of the cells and its mechanisms of water deficit.

To model water deficit, polyethylene glycol-6000 (PEG-6000) was added to cell suspension cultures. Polyethylene glycol 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. Moreover, PEG has a characteristic impermeability to cell walls, that is to say, PEG itself does not contribute to the osmotic adjustment and enter the cells (Kaur *et al.*, 1998).

In the present study, cells of *G. inflata Batal* were cultured in liquid medium containing different contents of PEG from 5 to 30% to investigate the effects of PEG on physiological parameters characteristic for oxidative stress and flavonoid production and to examine its drought tolerance.

Materials and Methods

Suspension culture establishment and cell growth determination

The cells were derived from the seeds of *G. inflata Batal*, which were obtained from wild plants

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growing in the desert of Xinjiang in China, donated by an incorporated company of Xinjiang KunLunshennong of Northwestern China, and identified by the planting centre of *Glycyrrhiza* of Xingjiang in China. Seeds were set in a beaker in which 98% H_2SO_4 was added to facilitate germination. Then the seeds were cleaned under running tap water and surface-sterilized by placing them in 75% ethanol for several minutes, followed by two treatments of 10 min rinse in 0.1% mercuric chloride solution and washed with sterile distilled water for five times. After surface-sterilization, the seeds were placed separately in 30 mL Murashige and Skoog (MS) basal medium in 100 mL flasks, and kept in the dark before germination. The cotyledons and hypocotyls were excised when they grew to a length of 3–4 cm, and placed on the surface of MS medium containing 3% sucrose and 0.8% agar supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D, 1.0 mg L^{-1}), naphthalene acetic acid (NAA, 1.0 mg L^{-1}) and 6-benzyladenine (6-BA, 1.0 mg L^{-1}). The media used for all the experiments were autoclaved at 121 °C for 20 min, and the pH value of the media was adjusted to 5.8 prior to autoclaving.

The suspension cells were derived from the callus tissue. 5 g callus per 250 mL flask with 80 mL of liquid MS culture medium containing 2,4-D (0.5 mg L^{-1}), NAA (0.5 mg L^{-1}) and 6-BA (0.5 mg L^{-1}) were subcultured with 5% inoculum every week until the cells showed continuous and stable accumulation of biomass. In our study, cells were cultured in liquid medium containing different contents of PEG from 5 to 30% at (25 ± 1) °C on a rotatory shaker with a speed of 120 rpm under 16 h photoperiod illumination. Cells grown in the culture medium without PEG addition were considered as the controls. The cells were harvested from the suspension cultures by filtration via a Buchner funnel with a filter paper, washed with distilled water to remove residual medium, and then filtered again under vacuum. After filtration the fresh cells were dried at 50 °C to constant dry weight (DW). Cell growth was measured based on dry weight.

Assay of flavonoids

The dried pulverized cell samples were weighed (0.10 g exactly) and put into a 10 mL measuring flask. The flavonoids were extracted with 30 volumes of ethanol/water (70:30, v/v) by sonication using a ultrasonic cleaning instrument (KQ-100DB, Kunshan Ultrasonic Instrument Co., Kunshan, China) for 1 h at room temperature. After centrifugation at 5500 × *g* for 6 min, the supernatant was extracted three times with EtOAc, and then with 95% ethanol. The flavonoid content was determined by colorimetry according to Zhang *et al.* (2001). Flavonoid content in the samples was the combination of flavonoids in cells and media. Rutin was used as the standard sample.

In addition, samples were harvested at various times after treatment to measure the H_2O_2 content, malondialdehyde (MDA) content, activities of CAT, POD and phenylalanine ammonia lyase (PAL).

Contents of H_2O_2 were measured by monitoring the absorbance at 415 nm of the titanium-peroxide complex according to Brennan and Frenkel (1977).

MDA assay

Lipid peroxidation was determined by measuring the MDA content following the method described by Heath and Packer (1968) with slight modifications. Fresh cells (0.5 g) were homogenized with 5 mL of 5% (w/v) trichloroacetic acid (TCA) and quartz sand. After centrifugation at 2000 × *g* for 15 min, 2 mL of supernatant were mixed with 2 mL of 0.67% (w/v) thiobarbituric acid (TBA). The mixture was incubated at 100 °C for 30 min, and quickly cooled in an ice-bath. Then the samples were centrifuged at 2000 × *g* for 5 min, and the absorbance of supernatant was determined at 532, 600 and 450 nm. The content of MDA-TBA complex (red pigment) was obtained using the formula: c (μmol L^{-1}) = 6.45 ($A_{532}-A_{600}$) − 0.56 A_{450}, where c is the content of MDA, A_{532} is the maximal absorbance of the MDA-TBA complex, A_{600} is the minimal absorbance of the MDA-TBA complex and A_{450} is the error led by sucrose.

CAT activity assay

The activity of CAT was determined by measuring the rate of decomposition of H_2O_2 that followed by the decline in absorbance at 240 nm (Havir and McHale, 1987). Each 3 mL reaction solution contained 50 mM K-phosphate buffer (pH 7.0), 12.5 mM H_2O_2 and 0.1 mL enzyme extracted from fresh cells. One unit of CAT (U) was defined as the change of 0.1 OD per min under the assay

conditions. The enzyme activity was expressed as U g^{-1} DW min^{-1}.

POD activity assay

0.5 g fresh cells was homogenized with 0.05 M phosphate buffer (pH 5.5), and centrifuged at 1400 $\times g$ in a refrigerated centrifuge. The supernatant was taken as the enzyme extract. The POD activity was measured according to the method of Lee and Liu (1996) with modifications. The assay mixture contained 1 mL phosphate buffer (pH 5.5), 1 mL 50 mM guaiacol, 1 mL 0.1 M H_2O_2 and 0.1 mL enzyme extract. The increase in absorbance at 470 nm was recorded every minute within 5 min after adding H_2O_2. One POD unit (U) was defined as the change of 0.01 OD per min at 37 °C under the assay conditions. The enzyme activity was expressed as U g^{-1} DW min^{-1}.

PAL activity assay

Phenylalanine ammonia lyase was extracted and its activity determined by the modified methods of Li *et al.* (2003). Fresh cells (0.5 g) were homogenized with quartz sand, 10 mL extraction buffer (0.1 M sodium borate, pH 8.0, 5 mM mercaptoethanol) and 0.5 g polyvinylpyrrolidone (PVP). The homogenate was then centrifuged at 5500 $\times g$ for 15 min. The supernatant was assayed as follows: 1 mL enzyme extract and 1 mL 0.02 M L-phenylalanine as substrates were incubated at 30 °C for 30 min, and the amount of cinnamic acid was then determined by measuring the absorbance at 290 nm. Tubes containing the enzyme preparation without substrate were used as a control. The change of 0.01 OD per min under the assay conditions was defined as one enzyme unit (U). The enzyme activity was expressed as U g^{-1} DW min^{-1}.

Statistical analysis

All data in the figures are represented as the means ± standard errors (SD). Every experiment was repeated at least 3 times. The significant difference between the treatment and the control was statistically evaluated by analysis of variance (ANOVA).

Results and Discussion

The biomass dry weight, flavonoid content, flavonoid production and water content in suspension cultures of *G. inflata Batal* added by different contents of PEG from 5 to 30% are shown in Fig. 1. The cell biomass and flavonoid production of the control were 13.9 g L^{-1} and 106.1 mg L^{-1}, respectively. All treatments changed the biomass and flavonoid production. When the PEG content was below 10%, the cell biomass based on DW and flavonoid production increased with elevated PEG contents, and reached 27.1 g L^{-1} and

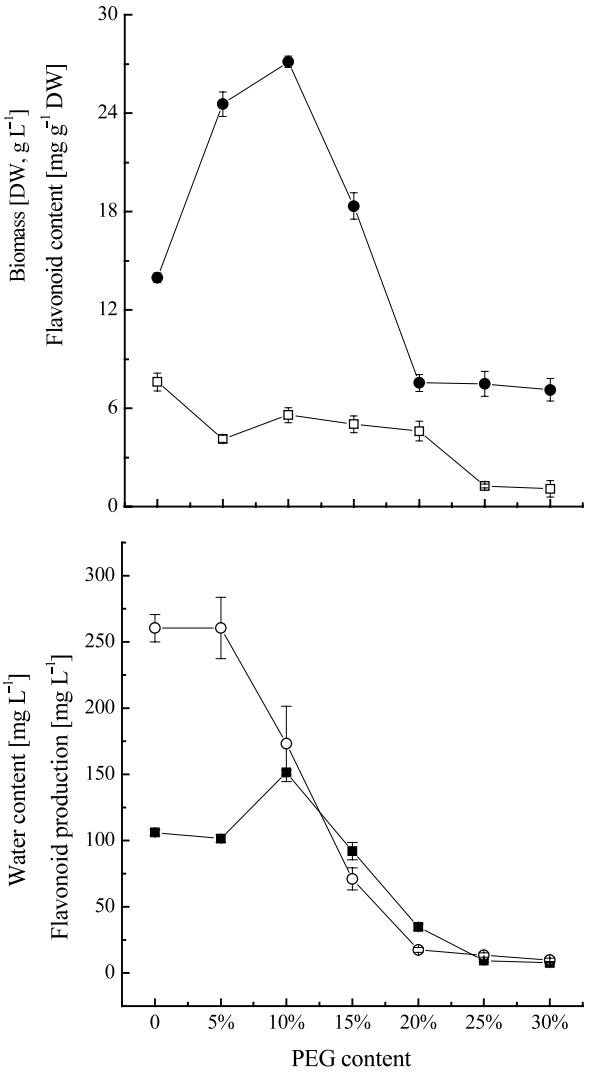

Fig. 1. Influence of PEG content on the cell biomass (dry weight) (●), flavonoid content (□), water content (○) and flavonoid production (■) of *G. inflata Batal*. Varied contents of PEG were added to a 10-d-old culture. Each value is the mean ± SD from three independent experiments.

151.5 mg L^{-1}, which was about 2-fold and 1.5-fold of the control, respectively. But if the PEG content continued to increase, the biomass and flavonoid production decreased. This result was consistent with the observation of Gupta *et al.* (1993) who found that water stress induced by 15% PEG decreased the length and biomass of epicotyl and hypocotyls. In the medium containing PEG from 20% to 30%, it was observed that some cells became brown and died, and survived cells grew very slowly. Consequently, the biomass became very little in comparison to the control. The reduction of biomass under stress conditions might be due to membrane lipid peroxidation or other reasons such as the inhibition of cell division, cell elongation induced by the treatments (Kaur *et al.*, 1998). There was no significant difference of the cell biomass between the culture containing 20% and 30% PEG ($P > 0.05$). However, the significant difference of flavonoid production was observed when the medium contained from 10% to 30% ($P < 0.05$). These results indicated that appropriate water deficit enhanced biomass accumulation and flavonoid production. Moreover, the addition of any content of PEG decreased significantly the flavonoid content as observed from Fig. 1. The higher content of PEG led to relative lower flavonoids content. The maximum flavonoid content obtained in the 10% PEG culture was only 73% of the control, which illuminated that the increase of flavonoid production was owing to the biomass accumulation. Water content, determined by the difference of fresh weight and dry weight, decreased significantly with increasing PEG contents. Significant difference in water content was observed between the culture treated by 10% PEG and the control ($P < 0.05$). The result was similar to what Egert and Tevini (2002) had found that drought stress could decrease the water content in leaves of chives. As a result, we selected 10% PEG addition to study the mechanism of water deficit because of reasonable biomass and flavonoid production obtained in this medium.

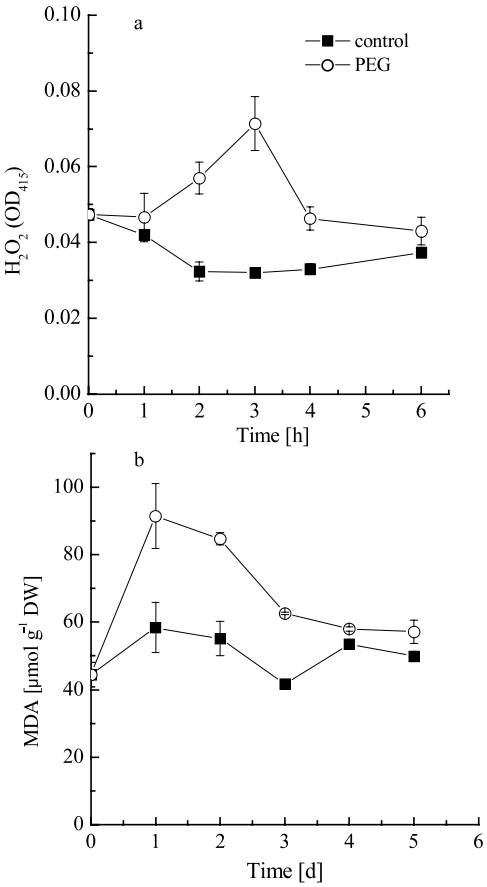

Fig. 2. Time course of H_2O_2 content (a) and MDA content (b) in cell suspension culture of *G. inflata Batal.* 10% PEG was added to a 10-d-old culture. (■) and (○) represent the control and PEG-treated culture, respectively. The rate of H_2O_2 accumulation and the MDA content were both determined at various times in PEG-treated cultures and the untreated control. Each value is the mean ± SD from three independent experiments.

Effect of water stress on H_2O_2 production

The effect of water stress on H_2O_2 production in cell suspension cultures of *G. inflata Batal* is shown in Fig. 2a. The H_2O_2 production in the treated cell culture started to increase within 2 h after treatment, and reached the maximum at about the third hour quickly; later it decreased slowly. Treatment of cell cultures with PEG stimulated the H_2O_2 accumulation, what agreed with the observations in several plants such as wheat (Selote *et al.*, 2004), in which water deficit led to 3-fold and 2-fold increase of H_2O_2 in the leaves and roots compared to the control, respectively. Cell cultures without treatment did not accumulated H_2O_2, on the contrary, the H_2O_2 content decreased a little at the first hour. Significant differences in H_2O_2 production between treated culture and the control appeared from the second to the forth hour ($P < 0.05$).

Effect of water stress on MDA content

Water stress induce the accumulation of free radicals, which damages cells by initiating or accelerating the membrane lipid peroxidation. The occurrence of MDA, one of the terminal products of the peroxidation of polyunsaturated fatty acids, was considered as the important landmark of cell membrane lipid peroxidation. That is to say, the content of MDA indicated the level of lipid peroxidation resulting from oxidative stress. PEG treatment caused the MDA content to improve rapidly (Fig. 2b), reaching to a peak of 91.3 μmol g^{-1} DW at the first day (24th h) after treatment, which was about 1.5-fold of the control. Significant difference was observed in MDA content between the PEG-treated culture and the control at the first day ($P < 0.05$). Thereafter, the content of MDA in PEG-treated cultures began to decrease, and levelled off at the fifth day, which was still a little higher than the control value. On the other hand, the MDA content of the control remained unchanged, about 50 μmol g^{-1} DW, during the whole course of cultivation. The increases in lipid peroxidation observed herein were in agreement with the results of other studies (Moran *et al.*, 1994; Zhang and Kirkham, 1994). Lipid peroxidation of biological membranes might lead to structural alterations in drought-stressed plants. As mentioned above, our results of the increase of MDA content under drought stress strongly suggested that water deficit might lead to the appearance of cell membrane lipid peroxidation, even to cell death.

Effect of water stress on enzyme activity

Water deficit affected the relationship between antioxidative enzymes and the generation of ROS that was demonstrated in many species (Li and Staden, 1998; Egert and Tevini, 2002). CAT and POD played an essential role in scavenging from the H_2O_2 toxicity. The combined action of CAT and POD converted the toxic superoxide radical and H_2O_2 to water and molecular oxygen (O_2). Therefore, the cellular damage under unfavorable conditions like water stress could be averted to some extent.

The effects of water stress on CAT, POD and PAL activity in cell suspension cultures of *G. inflata Batal* were are shown in Fig. 3. Trends in CAT activity were similar in the PEG-treated culture and the control. CAT activity started to increase within 2 h after treatment, and reached the maxi-

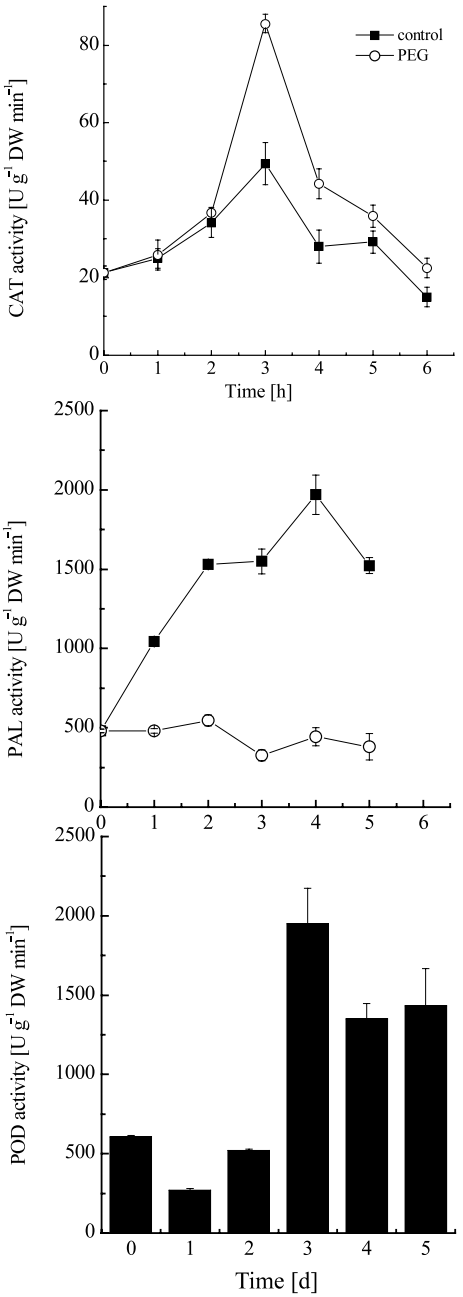

Fig. 3. Time courses of CAT, PAL and POD activity in cell suspension cultures of *G. inflata Batal*. 10% PEG was added to a 10-d-old culture. The activities of enzymes were determined at various times in PEG-treated cultures and the untreated control. In the time course of CAT and PAL activity, (■) and (○) represent the control and PEG-treated culture, respectively. In addition, the value at 0 min was the POD activity of the control. Each value is the mean ± SD from three independent experiments.

mum values of 85.6 U and 49.3 U g^{-1} DW min^{-1} in the PEG-treated culture and the control, respectively, at about the third hour then both began to decrease slowly. Significant difference in CAT activity between the treated culture and the control was observed at the peak ($P < 0.05$). With regards to POD activity, it decreased quickly after PEG treatment, and then increased slowly until it attained a peak of 1951 U g^{-1} DW min^{-1} at the third day, which was about 3.7-fold of the control. Thereafter, the POD activity began to decrease slowly, to 1400 U g^{-1} DW min^{-1} at the fifth day, which was still higher than the control. Under drought conditions, the enzymes activities were higher in PEG-treated cultures than in the control. These enzymes, which resulted in the production of O_2 and H_2O_2, were quantitatively the major responsible for superoxide removal (Asada, 1999). The higher activity in the treated culture suggested a more effective H_2O_2 removal than in the control. Water deficiency caused increases in CAT and POD activity to a great extent, which was consistent with the observations by Li and Staden (1998) who found that the CAT and POD activity significantly increased when calli of maize cultivars were subjected to water stress. By contrast, several studies had reported loss in CAT activity as the water deficit progressed (Dhindsa and Matowe, 1981; Chowdhury and Choudhuri, 1985; Zhang and Kirkham, 1994). This might be explained by the continuous enzyme photoinactivation, especially under severe photooxidation conditions (Smirnoff, 1995). To sum up, significant differences both in CAT and POD activity were observed between the treated and control cultures, which indicated that appropriate water deficit could activate the antioxidative defense enzymes system to maintain stability in plants subjected to adverse environmental conditions. As a whole, adaptation to drought might depend on different mechanisms, including the capacity to maintain high levels of antioxidants and/or through the induction of antioxidant enzymes.

PAL played an important role in the path of the biosynthesis of flavonoids which catalyzed the first committed step in the synthesis of defense-related phenylpropanoids, the conversion of phenylalanine into cinnamic acid (Zon *et al.*, 2002). On the whole, the PAL activity remained unchanged during the whole course of cultivation, which was always about 500 U g^{-1} DW min^{-1} in the PEG-treated culture in comparison to the control. On the contrary, the PAL activity of the control began to increase slowly during the whole culture time, and attained to 2000 U g^{-1} DW min^{-1} at the forth day which was about 4-fold of the value of the treated culture. Significant difference was observed between the cultures of water stress and the control ($P < 0.05$). In the biosynthesis approach of the flavonoids, PAL acted as a key limiting enzyme speed (Bin *et al.*, 2000) which was directly correlated with the flavonoid content. Above results showed that the culture treated by PEG increased the biomass and total flavonoid production, but decreased the flavonoid content. The reduction of flavonoid content was consistent with the decrease of PAL activity in the treated culture. Consequently, a conclusion could be drawn that appreciate water stress improved the biomass production, but led to the appearance of cell membrane lipid peroxidation.

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