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Modulation of antioxidant defense system and NADPH oxidase in *Pluchea indica* leaves by water deficit stress

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ABSTRACT. We investigated changes in antioxidant content and antioxidant enzyme activity in polyethylene glycol (PEG)-induced water deficit stressed *Pluchea indica* leaves. We also used diphenylene iodonium (DPI), a plasma membrane NADPH oxidase suicide inhibitor, to examine the role of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in the induction of antioxidant defense systems. PEG decreased water content and the reduction ability of 2,3,5-triphenyltetrazolium chloride (TTC), but increased malondialdehyde (MDA), total peroxide, O_2^- and H_2O_2 contents, indicating water deficit-dependent oxidative stress. Total ascorbate (AsA), AsA contents, and AsA/oxidized AsA ratios were increased by moderate water deficit (-0.6 MPa) conditions, while total glutathione (GSH), GSH contents and GSH/GSSG ratios increased as water potential decreased. Superoxide dismutase (SOD) activity was not affected after 24 h of PEG treatment but decreased after 48 h. Catalase (CAT) activity increased as water potential decreased while peroxidase (POX) activity increased only at -1.2 MPa. Ascorbate peroxidase (APX) activity increased under moderate water deficit and glutathione reductase (GR) activity increased as water potential decreased. DPI depressed the induction of antioxidant accumulation and antioxidant enzyme activity by water deficit, indicating that reactive oxygen species (ROS) signals are involved in activating defense systems in *P. indica* in response to oxidative stress. We conclude that moderate water deficit stress induces both NADPH oxidase-mediated non-enzymatic and enzymatic oxidative defense mechanisms in ROS scavenging in *P. indica* leaves.

Keywords: Antioxidant defense system; NADPH oxidase; *Pluchea indica*; Reactive oxygen species (ROS); Water deficit.

Abbreviations: APX, Ascorbate peroxidase; **AsA,** Ascorbate; **CAT,** Catalase; **DPI,** Diphenylene iodonium; **DTT,** Dithiothreitol; **GR,** Glutathione reductase; **GSH,** reduced Glutathione; **GSSG,** oxidized Glutathione; **H_2O_2,** Hydrogen peroxide; **HO^-,** Hydroxyl radical; **NADPH,** Reduced nicotinamide adenine dinucleotide phosphate; **MDA,** Malondialdehyde; **O_2^1,** Singlet oxygen; **$O_2^{\cdot-}$,** Peroxide; **O_2^-,** Superoxide radical; **PEG,** Polyethylene glycol; **POX,** Peroxidase; **ROS,** Reactive oxygen species; **SOD,** Superoxide dimutase; **TCA,** Trichloroacetic acid; **TTC,** 2,3,5-Triphenyltetrazolium chloride.

INTRODUCTION

Water deficit stress is a major limiting factor affecting plant growth, development and production (Wilkinson and Davies, 2010). Water deficit stress can induce oxidative stress in plants resulting from the disruption of cellular homeostasis of reactive oxygen species (ROS) production from the excitation of O_2 to form singlet oxygen (O_2^1) and the transfer of 1, 2 or 3 electrons O_2 to form superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (HO^-) (Mittler, 2002; Jubany-Mari et al., 2009; 2010). ROS cause the oxidative destruction of the cell components through oxidative damage of membrane lipids, nucleic acid and protein, especially SH-containing enzymes (Imlay and Linn, 1998; Beligni and Lamatina, 1999). To counteract the toxicity of ROS, plants have developed antioxidant defense mechanisms in scavenging cellular

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ROS to cope with oxidative stress via the non-enzymatic and enzymatic systems (Bartoli et al., 1999; Mittle, 2002; Mittler et al., 2004). The antioxidants, including water-soluble ascorbate (AsA), reduced glutathione (GSH), water-insoluble α-tocopherol and carotenoids, are a non-enzymatic system for ROS scavenging in cells (Asada, 1992; Noctor and Foyer, 1998; Smirnoff and Wheeler, 2000; Lei et al., 2006). In the enzymatic ROS-scavenging pathways, superoxide dimutase (SOD; EC 1.15.1.1) converts $O_2^{\cdot-}$ to H_2O_2 and ascorbate peroxidase (APX; EC 1.11.1.11)/glutathione reductase (GR; EC 1.6.4.2) are responsible for operating the ascorbate-glutathione cycle (AGC) in the removal of H_2O_2 (Asada, 1992). Catalase (CAT; EC 1.11.1.6) (Willekens et al., 1997), and peroxidase (POX; EC 1.11.1.7) (Asada, 1992) are also involved in the reduction of H_2O_2 to H_2O.

The ability to detoxify H_2O_2 is pivotal in the antioxidant defense mechanisms against ROS in plants (Foyer and Harbinson, 1994). In addition to functioning as a toxic cellular metabolite, H_2O_2 has been recognized as a signaling molecule that mediates stimuli responses in plants (Neill et al., 2002; Miller et al., 2008). When exposed to stresses, the balance of H_2O_2 production and removal can be disrupted, and the subsequent increase in cellular H_2O_2 can initiate signaling responses (Overmyer et al., 2003; Laloi et al., 2004; Miller et al., 2008). The oxidative burst can also be produced via the increased activity of plasma membrane-bound reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, which transfer electrons from intracellular NADPH to O_2 to form $O_2^{\cdot-}$, and then dismutated to H_2O_2, and the influx of H_2O_2 as a signaling molecule (Hao et al., 2006; Pourrut et al., 2008). Plasma membrane-bound NADPH oxidase is involved in regulating the antioxidant defense mechanisms in plants in response to water deficit stress (Zhao et al., 2001; Jiang and Zhang, 2002a,b; Agrwal et al., 2003).

Pluchea indica (L.) Less (Asteraceae: Inuleae) is a traditional medicinal plant with reported anti-inflammatory, anti-ulcer, anti-amoebic and antioxidant activities (Sen et al., 2002; Biswas et al., 2007; Gomes et al., 2007; Pramanik et al., 2007). It is widespread along the coastline of southwestern Taiwan, commonly occurring in littoral areas such as mangroves, brackish swamps, and tidal flats and is also abundant in the salty, muddy, volcanic areas in Kaohsiung's Yenchao County in southern Taiwan (Peng et al., 1998). These areas are characterized by high levels of salt and water in the soil, and dry (October to May) and wet (June-September) seasonal climates.

Our preliminary examination showed that this shrub frequently suffered water deficit stress during the dry season in southern Taiwan. In this work, we investigated the induction of the non-enzymatic and enzymatic antioxidatant defense systems in detached *P. indica* leaves in response to water deficit stress by determining their water-soluble antioxidant contents (AsA and GSH) and antioxidant enzyme activities (SOD, CAT, POX, APX and GR) when exposed to varying polyethylene glycol (PEG) concentrations. We used DPI, a plasma membrane-bound NADPH oxidase inhibitor to elucidate the possible involvement of NADPH oxidase in the induction of antioxidatant defense systems (Auh and Murphy, 1995; Allan and Fluhr, 1997).

MATERIALS AND METHODS

Plant materials and treatments

Pluchea indica leaves (4.2±0.2 cm length and 1.7±0.1 cm width) were sampled from WuSanTao Mud Volcano Nature Preserve in Yenchao County of Kaohsiung in southern Taiwan. Detached leaves were transferred to the laboratory within 2 h after sampling, washed with distilled water twice, then incubated at 25°C in a plastic box containing distilled water for 12 h in the dark to allow any wounds to recover. Detached leaves were prevented from full water submersion during this process. Three healthy leaves without browning were transferred to a Petri dish (10 cm diameter) containing 10 mL distilled water with varying PEG 6000 (Merck, Darmstadt, Germany) concentrations for water deficit treatment at 0, -0.6, -1.2 and -1.8 MPa. Water potential of -0.6 MPa is moderate water deficit, which the leaves can recover fully after release of -0.6 MPa challenge. To elucidate the involvement of NADPH oxidase on water deficit- induced antioxidatant defense system, leaves were pre-treated for 12 h in distilled water containing 50 μM Diphenylene iodonium (DPI), then transferred to -0.6 MPa solution for another 12 h. In this study, a Petri dish was a replicate and there were three replicates for each treatment.

Relative water contents determination and TTC reduction ability

Relative water contents were determined according to Lee et al. (1996). Fresh weight (f. wt.) of leaves was determined immediately after treatments and then after lyophilization at -60°C to determine dry weight (d. wt.). Lyophilized leaves were stored at -70°C for antioxidant content and antioxidant enzyme activity analyses. Relative water content was calculated as the following equation: (g f. wt.-g d. wt.)/g d. wt.

To determine the cellular activities, leaves of approximately 0.05 g f. wt. were incubated in 1.5 mL of 0.8% (w/v) 2,3,5-Triphenyltetrazolium chloride (TTC) solution containing 50 mM potassium phosphate buffer (pH 7.4) and 0.01% (v/v) Tween 20 for 6 h under darkness (Chang et al., 1999). After three washes with 5 mL of distilled water, intracellular insoluble formazan was extracted twice with 5 mL of 95% ethanol at 80°C for 20 min and the absorbance of ethanol extract was determined at A_{530}. The A_{530} values of water deficit or DPI-treated leaves were calculated as the percentage of control.

Determination of lipid peroxidation, total peroxide, $O_2^{\cdot-}$, H_2O_2 and water-soluble antioxidants

0.1 g d. wt. lyophilized leaves were homogenized in 1 mL of 5% (w/v) trichloroacetic acid (TCA). After centrifu-

gation at 12,000 ×*g* for 15 min at 4°C, the supernatant was collected as TCA extract for lipid peroxidation and total peroxide determination and the H_2O_2 and water-soluble antioxidant contents. To determine $O_2^{\cdot-}$ production rate, lyophilized leaves of 0.05 g d. wt. were homogenized in 1 mL of 50 mM Tris-HCl (pH 7.5), then centrifuged at 12,000 ×*g* for 15 min at 4°C to obtain the supernatant as the $O_2^{\cdot-}$ determination extract; $O_2^{\cdot-}$ production rate was assayed within 0.5 h after extraction.

The lipid peroxidation level, that is, malondialdehyde (MDA) content, was determined from the thiobarbituric acid reacting substance (TBARS) contents resulting from the thiobarbituric acid (TBA) reaction as described by Health and Packer (1968). The MDA contents were calculated based on A_{532}-A_{600} with the extinction coefficient of 155 mM^{-1} cm^{-1}.

Total peroxide contents were determined by the absorbance of A_{480} and extinction coefficient of 13.93 μM^{-1} cm^{-1} according to Sagisaka (1976). $O_2^{\cdot-}$ production rate was determined according to the method described by Able et al. (1998). H_2O_2 contents were determined based on the decomposition of H_2O_2 by peroxidase as described by Okuda et al. (1991).

The total ascorbate (AsA) and reduced AsA content measurements were modified from the method of Hodges et al. (1996). Total AsA contents were determined in 1-mL mixture containing 200 µL of TCA extract, 50 mM potassium phosphate buffer (pH 7.4), 3 mM ethylenedinitrilotetraacetic acid (EDTA) and 1 mM Dithiothreitol (DTT). The mixture was incubated at 25°C for 10 min, 100 µL of N-ethylmaleimide was added and then 400 µL of 0.61 M TCA, 400 µL of 0.8 M orthophosphoric acid and 400 µL of α,α'-bipyridyl were added. Finally, 200 µL of $FeCl_3$ was added and the reaction mixture was incubated in a 55°C water bath for 10 min, and the absorbance was detected at A_{525}. Reduced AsA contents were determined by adding distilled water instead of DTT and N-ethylmaleimide, then followed the method as described above. Total AsA and reduced AsA contents were estimated from the standard curve of 0-40 nmole L-AsA determined by the above methods for total ascorbate and reduced AsA analysis, respectively. Oxidized AsA contents were calculated by subtracting reduced AsA from total AsA.

Total glutathione contents were determined according to Griffiths (1980). Glutathione contents were estimated from the standard curve of 0-20 nmole GSH. After removing reduced glutathione (GSH) by 2-vinylpyridine derivatization, oxidized glutathione (GSSG) contents were determined and GSH contents were calculated by subtracting GSSG contents from total glutathione contents.

Determination of antioxidant enzyme activity

Lyophilized leaves of 0.01 g d. wt. were homogenized in liquid nitrogen after which 1 mL of 50 mM sodium phosphate buffer (pH 7.0) containing 0.2% Triton X-100, 0.5 mM EDTA and 1 mM phenylmethylsulfonyl fluoride (PMSF) were added. After centrifugation at 12,000 ×g for 15 min at 4°C, the supernatant was used as for an enzyme activity assay. Enzyme activities were determined within 2 h after extraction. The soluble protein contents were determined by the Coomassie blue dye binding method (Bradford, 1976), with bovine serum albumin as standard curve.

CAT activity was measured at A_{420} for H_2O_2 decomposition rate using the extinction coefficient of 40 mM^{-1} cm^{-1} according to Kato and Shimizu (1987). Guaiacol POX activity was determined by the formation rate of tetraguaiacol detected at A_{470} with the extinction coefficient 26.6 mM^{-1} cm^{-1} according to Kato and Shimizu (1987). SOD activity was determined by the inhibition of photochemical inhibition of nitro blue tetrazolium according to Giannopolitis and Ries (1977). APX activity was determined at A_{290} for oxidized AsA according to the extinction coefficient of 2.8 mM^{-1} cm^{-1} according to Nakano and Asada (1981). GR activity was monitored A_{340} for NADPH oxidization as GSSG reduction according to the method of Schaedle and Bassham (1977).

Statistical analysis

SAS (SAS v 8.1, NC, USA) was used in this study. The results present in this study were the mean of 3 replicates with a Petri dish as a replicate. The effects of PEG and treatment time on water contents, MDA contents, total peroxide contents, $O_2^{\cdot-}$ production rate, H_2O_2 contents, water-soluble antioxidant contents and antioxidant enzyme activity were analyzed by 2-way variance analysis (ANOVA). DPI treatment effects were analyzed by 1-way ANOVA. The significant difference among means was analyzed by Duncan's new multiple range test followed by significant ANOVA at $P<0.05$.

RESULTS

Relative water contents, TTC reduction ability, $O_2^{\cdot-}$ production rate, H_2O_2 contents, MDA and total peroxide contents

To have a mechanistic causal understanding of PEG-induced water deficit injury in *P. indica* leaves, we estimated statuses of TTC reduction ability, some of the principal ROS, the $O_2^{\cdot-}$ and H_2O_2, and the level of lipid peroxidation, that is, malondialdehyde (MDA) content, which is an indicator of oxidative damage. PEG (2-way ANOVA, $P<0.05$) and treatment time ($P<0.05$) affected water potential and TTC reduction ability, and the interaction of PEG and treatment time on water potential and TTC reduction ability was significant ($P<0.05$). On exposure to water deficit, the relative water contents decreased as water potential decreased from 0 to -1.8 MPa and appeared a further decline after 48 h treatment as compared to the 24 h treatment (Figure 1A). Similarly, TTC reduction ability also decreased as water potential decreased and the decline in TTC reduction ability was more significant at 48 h than the 24 h treatment (Figure 1B). In contrast, MDA contents (Figure 1C), total peroxide contents (Figure 1D), $O_2^{\cdot-}$ production rate (Figure 1E) and H_2O_2 contents (Figure 1F) increased as water potential decreased ($P<0.05$) and treat-

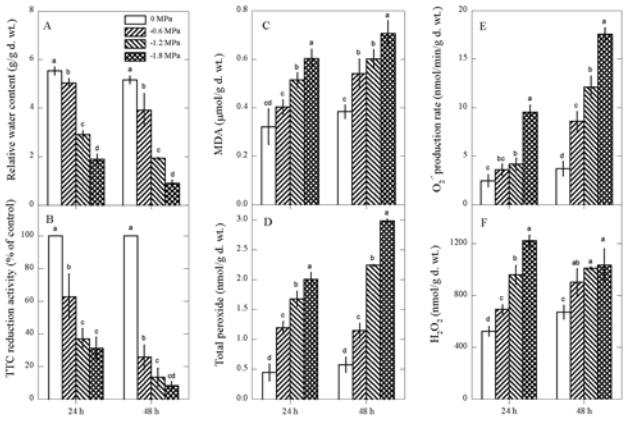

Figure 1. Changes in relative water contents (A), TTC reduction ability (B), MDA contents (C), total peroxide contents (D), $O_2^{\cdot-}$ production rate (E) and H_2O_2 contents (F) in *Pluchea indica* leaves in response to varying PEG concentrations, i.e. 0, -0.6, -1.2 and -1.8 MPa. Data are present as means±SD (n=3) and different letters indicate significant difference among treatments.

ment time advanced (P<0.05). The interaction of PEG and treatment time on $O_2^{\cdot-}$ production rate and total peroxide, MDA and H_2O_2 contents was significant (P<0.05). These indicate that PEG-induced water deficit can trigger oxidative damage in *P. indica* leaves.

Ascorbate and glutathione contents

We observed changes in antioxidant *Ascorbate and glutathione contents* during oxidative damage in *P. indica* leaves. PEG (P<0.05) but not treatment time (P>0.05) affected total AsA and reduced AsA contents while both PEG (P<0.05) and treatment time (P<0.05) affected oxidized AsA contents and reduced AsA/oxidized AsA ratios, the interaction of PEG and treatment time on oxidized AsA contents and reduced AsA/oxidized AsA ratios was significant (P>0.05). After exposure to water deficit, total AsA (Figure 2A) and reduced AsA (Figure 2B) contents and reduced AsA/oxidized AsA ratios (Figure 2D) increased in response to -0.6 MPa, but total AsA, reduced AsA and oxidized AsA contents and reduced AsA/oxidized AsA ratios decreased in response to -1.8 MPa.

Both PEG (P<0.05) and treatment time (P<0.05) affected total glutathione, GSH and GSSG contents, and the interaction of PEG and treatment time on total glutathione, GSH and GSSG contents and GSH/GSSG ratios was significant (P<0.05). After 24 h of water deficit treatment, total glutathione (Figure 2E) and GSH (Figure 2F) contents and GSH/GSSG ratios (Figure 2H) increased as water potential decreased from 0 to -1.8 MPa, but GSSG contents only showed a small increase at -1.2 MPa. After 48 h of treatment, total glutathione (Figure 2E) and GSH (Figure 2F) contents also increased, but their increments were relatively smaller than at 24 h treatment. The GSSG contents showed a marked increase after 48 h of water deficit treatment (Figure 2G), resulting in a drop of GSH/GSSG ratios (Figure 2H).

Antioxidant enzyme activities

An analysis of active oxygen-processing enzymes activities showed that SOD was affected by PEG (P<0.05) and treatment time (P<0.05). SOD activities were not affected by PEG after 24 h of treatment, but decreased after 48 h of treatment (Figure 3). CAT activities were affected by PEG (P<0.0001) but not by treatment time (P=0.2562). POX, APX and GR activities were affected by both PEG (2-way ANOVA, P<0.05) and treatment time (P<0.05). The interaction of PEG and treatment time on antioxidant enzyme activities was significant for POX, APX and GR (P<0.05) but not for CAT (P>0.05).

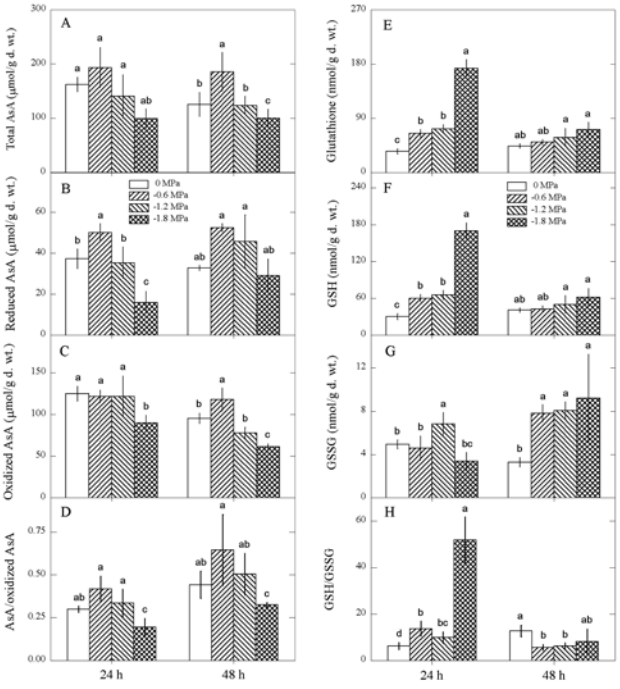

Figure 2. Changes in ascorbate: total AsA (A); reduced AsA (B); oxidized AsA (C); reduced AsA/oxidized AsA ratio (D), and glutathione contents: total glutathione (E); GSH (F); GSSG (G); GSH/GSSG ratio (H) in *Pluchea indica* leaves in response to 0, -0.6, -1.2 and -1.8 MPa. Data are present as means±SD (n=3) and different letters indicate significant difference among treatments.

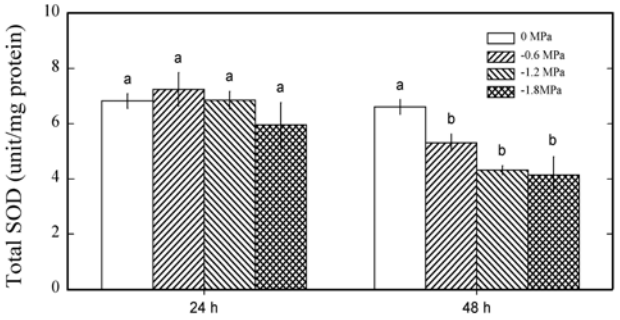

Figure 3. SOD activity in *Pluchea indica* leaves in response to 0, -0.6, -1.2 and -1.8 MPa. Data are present as means±SD (n=3) and different letters indicate significant difference among treatments.

CAT activities increased as water potential decreased from 0 to -1.8 MPa (Figure 4A) while POX activities increased only at -1.2 MPa, and this increment was more significant after 48 h treatment (Figure 4B). APX activities increased significantly after exposure to -0.6 and -1.2 MPa for 24 h and the magnitude of APX activity increment was reduced after 48 h treatment as compared to the 24 h treatment (Figure 4C). GR activities significantly increased after 24 h of exposure to water deficit, but only increased in response to -1.2 MPa (Figure 4D) after 48 h.

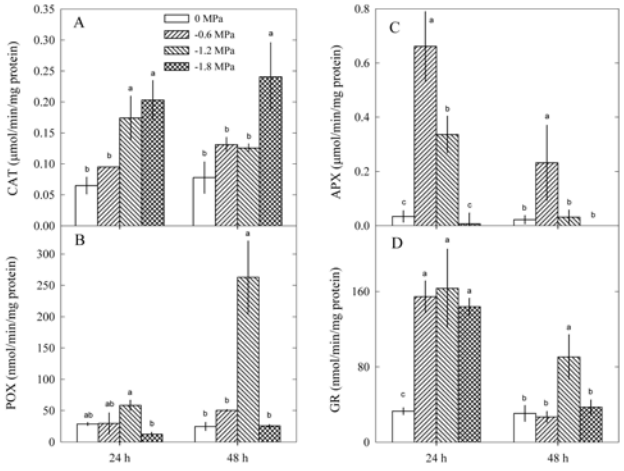

Figure 4. CAT (A), POX (B), APX (C) and GR (D) activities in *Pluchea indica* leaves in response to 0, -0.6, -1.2 and -1.8 MPa. Data are present as means±SD (n=3) and different letters indicate significant difference among treatments.

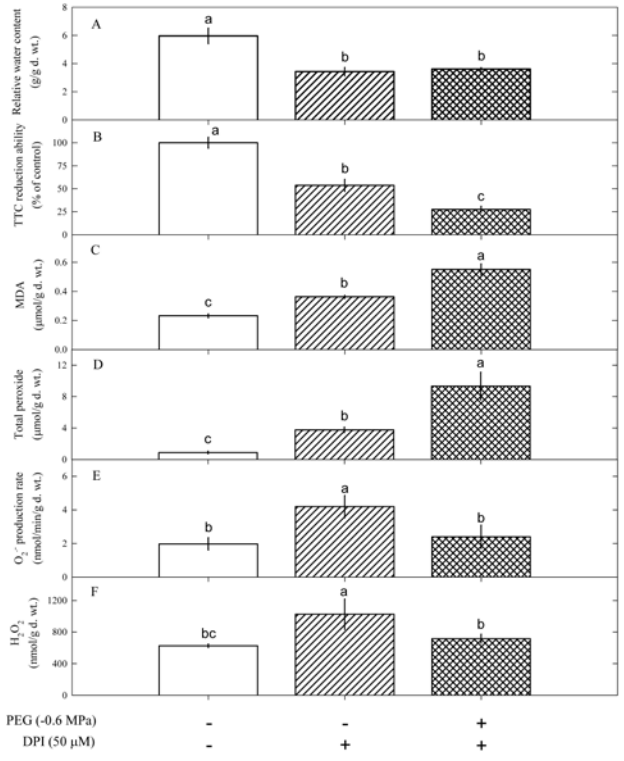

Figure 5. The effects of DPI (50 μM) pretreatment (12 h) on relative water contents (A), TTC reduction ability (B), MDA contents (C), total peroxide contents (D), $O_2^{\cdot-}$ production rate (E) and H_2O_2 contents (F) in *Pluchea indica* leaves after transferred to -0.6 MPa for 12 h. Data are present as means±SD (n=3) and different letters indicate significant difference among treatments.

Effects of DPI on water stress-induced antioxidant defense system

To elucidate the role of NADPH oxidase in inducing the antioxidatant defense system, we pretreated leaves with 50 μM DPI for 12 h, then transferred to PEG (-0.6 MPa) for another 12 h. When transferred to -0.6 MPa, the magnitude of MDA, total peroxide and H_2O_2 accumulation in DPI-pretreated leaves was higher than in untreated leaves (ANOVA, $P<0.05$) (Figure 5). In contrast, the decrease in TTC reduction ability after exposure to -0.6 MPa was more significant in DPI-pretreated leaves than in those without ($P<0.05$) (Figure 5).

After exposure to water deficit stress, total AsA and oxidized AsA contents in DPI-pretreated leaves showed a more significant decrease than in those not treated ($P<0.05$), while reduced AsA contents were not affected by DPI pretreatment ($P>0.05$) (Figure 6). DPI pretreatment induced a greater decrease in total glutathione, GSH and GSSG contents under -0.6 MPa conditions compared to those without DPI ($P<0.05$) (Figure 6). The GSH/GSSG ratio increased after exposure to -0.6 MPa ($P<0.05$), but the ratio was same in DPI-pretreated and DPI-free treatments (Figure 6).

The increase in CAT, APX and GR activities under -0.6 MPa conditions was depressed by DPI pretreatment, but SOD activity was not affected (Figure 7). DPI pretreatment increased POX activity (Figure 7).

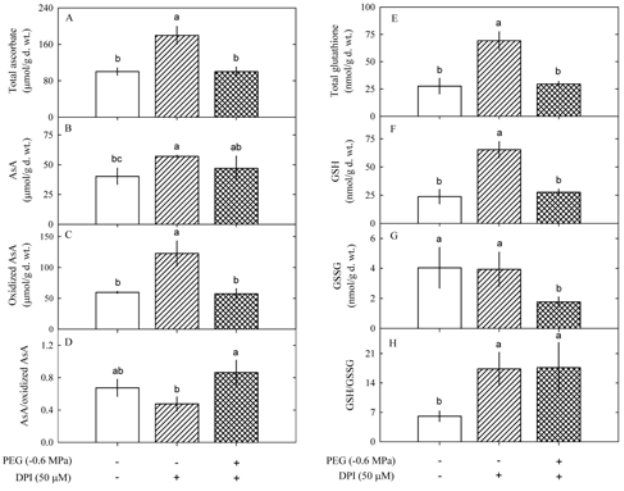

Figure 6. The effects of DPI (50 μM) pretreatment (12 h) on ascorbate: total AsA (A); reduced AsA (B); oxidized AsA (C); reduced AsA/oxidized AsA ratio (D), and glutathione contents: total glutathione (E); GSH (F); GSSG (G); GSH/GSSG ratio (H) in *Pluchea indica* leaves after transferred to -0.6 MPa for 12 h. Data are present as means±SD (n=3) and different letters indicate significant difference among treatments.

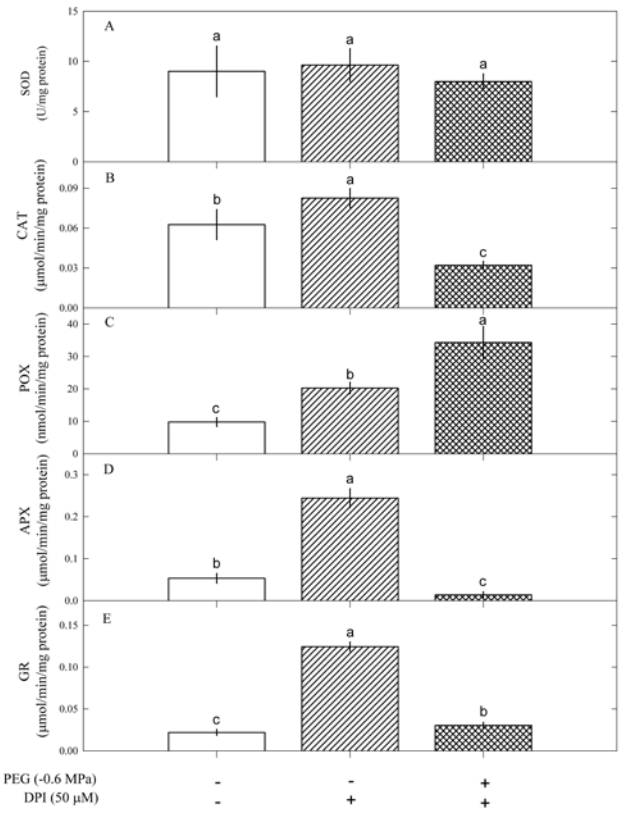

Figure 7. The effects of DPI (50 μM) pretreatment (12 h) on SOD (A), CAT (B), POX (C), APX (D) and GR (E) activities in *Pluchea indica* leaves after transferred to -0.6 MPa for 12 h. Data are present as means±SD (n=3) and different letters indicate significant difference among treatments.

DISCUSSION

Pluchea indica, an erect shrub growing in littoral areas of western Taiwan, frequently suffers water deficit stress during the dry season from October to May. The present study was aimed at better understanding the relationship between water deficit stress and oxidative stress in *P. indica* leaves. It has been known that water deficit stress can enhance ROS production resulting in oxidative stress in several plant systems (Sgherri et al., 1993; Smirnoff, 1993; Moran et al., 1994; Sgherri and Navari-Izzo, 1995; Jiang and Zhang, 2002a). We conducted this study due to a lack of information on herbaceous plants responses to water deficit, especially the antioxidant defense responses they use to adapt to the stressful conditions in harsh environments.

The results of the present study provide several lines of evidence showing that PEG-induced water deficit stress can cause oxidative stress in *P. indica* leaves by increasing lipid peroxidation (MDA), and elevating total contents of peroxide and ROS ($O_2^{\cdot-}$ and H_2O_2) (Figure 1). The magnitude of oxidative stress is not only increased by decreasing water potential but also enhanced by longer water stress treatment. This study has shown that *P. indica* leaves have developed defense mechanisms to cope with oxidative stress under moderate water deficit (-0.6 MPa) conditions.

The maintenance of antioxidant pools and enhanced scavenging capacity are essential for plant defense against oxidative stress (Noctor and Foyer, 1998). The present results from an increase in the contents of total and reduced AsA and a higher regeneration rate of reduced AsA as indicated by a rise in reduced AsA/oxidized AsA ratio in *P. indica* leaves in response to -0.6 and -1.2 MPa (Figure 2) seem to suggest that increased ascorbate pools and the ability in maintaining a high reduced per oxidized ratio of ascorbate are crucial for the proper scavenging of ROS generated under moderate water deficit conditions. It is obvious that sufficient levels of reduced AsA in combination with increased APX activities (Figure 4C) allow *P. indica* leaves to maintain the balance of their cellular ROS components under water deficit conditions, resulting in low H_2O_2 accumulation under -0.6 and -1.2 MPa. Because the ascorbate pools and the regeneration ability of oxidized AsA to reduced AsA is decreased under severe water deficit (-1.8 MPa) conditions, it is likely that significant oxidative damage occurring under these severe conditions is due in part to low capacity in maintaining normal ascorbate biosynthesis and its regeneration to reduced AsA (Figure 2A-D).

Glutathione also accumulated under water deficit conditions but showed a different pattern than did ascorbate. Total glutathione and GSH contents increased as water potential decreased, with the increase more significant after 48 h treatment. Following increasing water deficit stress, increased ability to regenerate glutathione as indicated by accumulated GSH contents and high GSH/GSSG ratio (Figure 2E-H) as well as high GR activities (Figure 4D) demonstrate there was increased capacity to reduce H_2O_2 by regenerating GSH under moderate water deficit conditions, that is, GSH could be converted to GSSG for the scavenging of ROS and the GSSG can be recycled to GSH. Because GSSG could be regenerated to GSH via GR, a decrease in GR activities after 48 h treatment indicates a reduction in GSH recovery the longer under water deficit stress. This explains the accumulation of GSSG and declined GSH/GSSG ratio after 48 h treatment. Our study results clearly suggest a tightly controlled antioxidant balance in *P. indica* leaves that helps maintain an optimal redox status under different water deficit conditions. It is obvious that increasing ascorbate and glutathione pools together with higher regeneration rates contribute to the ROS scavenging in *P. indica* leaves in response to the moderate water deficit conditions while the notable reduction of antioxidant regeneration might be responsible for the occurrence of significant oxidative stress caused by severe water deficit stress.

As crucial as is antioxidant ROS scavenging in plant defense against active oxygen species In addition to, the enzyme-mediated conversion of superoxide radical $O_2^{\cdot-}$ to H_2O_2 and the subsequent detoxification of H_2O_2 are crucial for (Foyer and Harbinson, 1994; Mittler, 2002). In this study, the maintenance of SOD activity in *P. indica* leaves

in response to 24 h of water deficit fallowed by a drop at 48 h (Figure 3) suggests that $O_2^{\cdot-}$ could be dismutated to H_2O_2 during the early water stress periods but this $O_2^{\cdot-}$ dismutation ability was depressed by longer water stress conditions. There was a significant $O_2^{\cdot-}$ accumulation after 48 h of water stress treatment and thus increased oxidative damage caused by $O_2^{\cdot-}$ attack can be expected in response to prolonged water deficit treatment.

The present results indicate that H_2O_2 generation following the $O_2^{\cdot-}$ dismutation in water stress-treated *P. indica* leaves would be alleviated by APX/GR (Figure 4C-D), and in turn, H_2O_2 did not accumulate in the conditions of both -0.6 and -1.2 MPa. It is known that APX and GR are responsible for the removal of H_2O_2 as the ascorbate-glutathione cycle. CAT may also be involved in H_2O_2 removal because its activity increased as water potentials decreased (Figure 4A). CAT is likely responsible for excess H_2O_2 removal under severe water stress conditions, while AGC operates under moderate water stress conditions. It is obvious that the H_2O_2 detoxifying enzyme expression in *P. indica* leaves is modulated differently depending on water deficit conditions; AGC is activated by moderate water deficit conditions and CAT responds to extreme water stress.

The present study suggests that antioxidant defense system induction is mediated by H_2O_2 in *P. indica* leaves. H_2O_2 has been known as the signal for plant antioxidatant defense system induction in response to biotic and abiotic stresses (Neill et al., 2002). It is now believed that $O_2^{\cdot-}$ can be generated by mammalian-like plasma membrane neutrophil NADPH oxidase and $O_2^{\cdot-}$ is automatically dismutated to H_2O_2, which influxes into cellular space as a signal to induce the antioxidant defense system in plants (Auh and Murphy, 1995; Vera-Estrella and Blumwald, 1997). The inhibition of water stress-induced ascorbate and glutathione accumulation and antioxidatant enzyme activity increase by DPI (Figures 6 and 7), the inhibitor of plasma membrane NADPH oxidase, provide evidence that the generation of ROS mediated by NADPH oxidase is involved in the induction of antioxidant defense mechanism in *P. indica* leaves. This provides an explanation for enhanced H_2O_2 production in DPI-pretreated *P. indica* leaves after their transfer to water deficit conditions (Figure 5F). The antioxidative defense systems blockage by DPI has been observed in several plant systems (Doke and Ohashi, 1988; Doke and Miura, 1995; Levine et al., 1994). The regulation of H_2O_2 production via NADPH oxidase has been suggested in cell suspension cultures of *Taxus chinensis* treated with a fungal elicitor from *Aspergillus niger* (Qin et al., 2004). NADPH oxidase is known to mediate the ROS production and signals the up-regulation of abscisic acid (ABA)-induced antioxidant defense systems in maize leaves exposed to water deficit stress (Jiang and Zhang, 2002b). It is most likely that water deficit stress activates the plasma membrane NADPH oxidase in *P. indica* leaves, resulting in the $O_2^{\cdot-}$ production that automatically dismutates to H_2O_2 that triggers the antioxidant defense system to overcome the subsequent ROS production.

We conclude that the defense mechanisms against oxidative stress occurring in *P. indica* leaves exposure to water deficit condition are antioxidant availability and antioxidatant enzymes activation, and that ROS signals mediated by plasma membrane-bound NADPH oxidase are involved in the activation of oxidative defense systems. Nitric oxide (NO) is also important to oxidative stress signaling and is likely involved in the regulation of antioxidative enzymes in defending against oxidative stress (Arasimowicz and Floryszak-Wieczorek, 2007; Shao et al., 2008a) and interacting with ROS to transmitting the signal (Mittler, 2002; del Río et al., 2006; Shao et al., 2008b). Although we did not apply chemicals such as sodium nitroprusside (SNP) to release NO (Arasimowicz and Floryszak-Wieczorek, 2007), we speculate that NO may also be involved in controlling the antioxidant defense system in *Pluchea indica* leaves. We hope to prove this in the near future.

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鯽魚膽切離葉於缺水環境下抗氧化防禦系統之調節與 NADPH 氧化酶活性之關係

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本研究探討鯽魚膽 (*Pluchea indica* (L.) Less.) 切離葉片於 PEG 誘導產生的缺水處理中，其抗氧化物質及抗氧化酶活性之變化情形，並利用原生質膜 NADPH 氧化酶抑制劑 DPI (diphenylene iodonium) 處理，了解還原態 NADPH 氧化酶 (NADPH oxidase) 所扮演之角色。經 PEG 缺水處理的鯽魚膽葉片其含水量與 TTC 活性降低，但 malondialdehyde (MDA)、total peroxide、$O_2^{\cdot-}$ 與 H_2O_2 量增加，顯示鯽魚膽葉片在缺水逆境確實可誘導氧化逆境的產生。在中度缺水的情況下，total ascorbate (AsA)，AsA contents 及 AsA/oxidized AsA ratios 皆增加，且隨著水勢增加，total glutathione (GSH) 、GSH contents 與 GSH/GSSG ratios 亦增加。而 superoxide dismutase (SOD) 活性在 PEG 處理 24 小時沒有明顯改變，但在 48 小時後則增加。catalase (CAT) 與 glutathione reductase (GR) 活性隨著水勢降低而增加，而 peroxidase (POX) 活性則只在水勢 -1.2 MPa 增加，ascorbate peroxidase (APX) 活性只在中度缺水時增加。這些藉由缺水逆境所誘導產生之抗氧化物質的累積與抗氧化酶活性之變化情形可被 DPI 抑制，顯示鯽魚膽在氧化逆境中可啟動其防禦系統中之活性氧族 (reactive oxygen species，ROS) 訊息分子。因而，鯽魚膽葉片對中度缺水逆境之反應係藉由 NADPH oxidase 所媒介之非酵素形式或酵素形式之氧化防禦機制來清除 ROS 而達成。

關鍵詞：NADPH 氧化酶；鯽魚膽；活性氧族；缺水逆境；抗氧化防禦系統。