Water Stress and Accumulation of β-*N*-Oxalyl-L-α,β-diaminopropionic Acid in Grass Pea (*Lathyrus sativus*)

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Grass pea seedlings were grown in an irrigated field. Roots of 15-day-old seedlings were treated with PEG, and leaves were studied. With the duration of PEG treatment, changes in the lipid peroxidation and activities of superoxide dismutase, catalase, peroxidase, and glutathione reductase as well as contents of hydrogen peroxide and β-*N*-oxalyl-L-α,β-diaminopropionic acid (ODAP) were assayed. The results indicate that with the duration of PEG treatment, activities of superoxide dismutase, peroxidase, and catalase decreased, whereas contents of hydrogen peroxide and ODAP, extent of lipid peroxidation, and activity of glutathione reductase increased. Both diethyldithiocarbamate and aminotriazole strongly inhibit activities of superoxide dismutase and catalase, respectively. At same time, the extent of lipid peroxidation was obviously increased. However, mannitol decreased the extent of lipid peroxidation. Diethyldithiocarbamate, aminotriazole, and mannitol do not affect the accumulation of ODAP. The observations suggest that there is no direct relationship between the accumulation of ODAP and the metabolism of free radicals. In addition, the relationship between water stress and ODAP accumulation in grass pea is discussed.

Keywords: *Water stress; PEG; lipid peroxidation; hydrogen peroxide; ODAP; Lathyrus sativus; antioxidant enzyme*

INTRODUCTION

Grass pea (*Lathyrus sativus*) is used as a nutritious food in poor semiarid areas in the world, offering high-quality protein and increasing the input of combined N_2 into the soil. However, it contains a neurotoxin, β-*N*-oxalyl-L-α,β-diaminopropionic acid (ODAP), that causes animal lathyrism after overconsumption of the seeds (Malathi et al., 1970). Therefore, the utilization of grass pea is restrained in semiarid areas. In addition, grass pea is grown in semiarid areas of Africa, Asia, and Europe. It has high drought tolerance, so it is a good sample for studying the mechanism of drought resistance and seeking genes of drought resistance. We think it is very significant work to determine whether ODAP is associated with drought tolerance.

Environmental stress is known to increase the oxygen uptake, which induces damage to cells due to the generation of oxygen radicals or due to inhibition of the antioxidant metabolism in plant (Barber and Andersson, 1992; Malan et al., 1990). Drought stress brings about damage to plants in a variety of ways (Ludlow, 1987). Plants tolerate this stress by diverse mechanisms such as osmotic adjustment (Levitt, 1980), enzymatic defense (Scandalios, 1993), or abscisic acid induced modification of plant water relations (Harris et al., 1988).

Superoxide dismutase (SOD), catalase, and peroxidase are essential for cells that utilize oxygen, as they catalytically scavenge O_2^- and H_2O_2, respectively, and are, therefore, thought to control the level of oxygen-induced damage (Quartacci and Navari-Izzo, 1992).

Implicit to the theory of free radical damage during drought stress is the notion that the cell's defensive mechanisms break down or are overloaded (Stewart, 1989). Consequently, if plants can tolerate drought stress, they must be able to maintain their defensive mechanisms or amplify them under conditions of increased stress. It has also been proposed that a capacity to limit membrane damage to a reparable level, by controlling lipid peroxidation, may be an important component of drought tolerance in plants (Dhindsa and Matowe, 1981), as during drought stress plants are susceptible to oxidative and free radical damage (Smirnoff and Colombe, 1988).

Therefore, to study the mechanism of plant membrane damage induced by drought stress and the relationship between ODAP and drought stress, 15-day-old grass pea seedling roots were treated by PEG and leaves were used as study objects. We examined (i) the activities of SOD, catalase, glutathione reductase, and peroxidase, because these enzymes are known to catalyze the destruction of O_2^- and H_2O_2 (Fridovich, 1975); (ii) lipid peroxidation of membranes that are well recognized to cause membrane deterioration (Dhindsa and Matowe, 1981); (iii) changes of the ODAP content during drought stress; and (iv) the relationship between drought stress and ODAP.

MATERIALS AND METHODS

Plant Materials. Grass pea (*L. sativus*) seeds were soaked in water and germinated in the dark at 4 °C for 6 days; the seedlings were grown in an irrigated field. Fifteen-day-old grass pea seedlings were subjected to water deficit for 24 h by immersing the root into polyethylene glycol (PEG 6000)

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solutions with the osmotic potential at −1.0 MPa [equivalent to a 20% (w/v) PEG solution], PEG solution (20%) plus DDC (inhibitor of SOD activity) at a concentration of 10^{-3} mol/L, PEG solution (20%) plus AT (inhibitor of CAT activity) at a concentration of 2×10^{-3} mol/L, and PEG solution (20%) plus mannitol (scavenger of hydroxyl radical) at a concentration of 10^{-2} mol/L, respectively. Leaves were harvested for analysis from the four treatments at the beginning of the experiment and during the treatment at 3, 12, and 24 h. At each sampling date three different samples from the control and stress treatments were taken, and each sample was analyzed twice.

Determination of Lipid Peroxidation. The level of lipid peroxidation in the leaf tissue was measured in terms of malondialdehyde (MDA) content determined by using the thiobarbituric acid (TBA) reaction (Heath and Packer, 1968). The procedure followed concurs with that described by Qiu and Liang (1995). The content of MDA was calculated using the following equation: $C = \Delta OD(532-600)/\xi b$, where C is the concentration of MAD, ξ is the extinction coefficient (= 155 L/mmol·cm), and b is the light distance (= 1 cm).

Enzyme Extraction and Assay Procedure. Leaf samples of 0.5 g were homogenized in a Waring Blendor at 4 °C. The grinding medium contained 0.05 M potassium phosphate buffer (pH 7.8), 20 mmol/L β-mercaptoethand, 1 mmol/L EDTA, and 0.1 mmol/L phenylmethanesulfonyl fluoride (PMSF) (Baker et al., 1996). Poly(vinylpyrrolidone) (PVP), 0.2 g/mL of grind, was added to the samples to scavenge leaf phenolics. Homogenates were centrifuged at 1700*g* for 15 min at 4 °C. The supernatant fractions were carried out at 0−4 °C. All activities were determined at 25 °C. We preferred to express all enzyme activities on a protein basis. Protein concentrations were measured according to the method of Bradford (1976) using BSA as a standard.

Surperoxide dismutase activity was determined by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) using the method of Beauchamp and Fridovich (1971). The 3 mL reaction mixture contained 2.4×10^{-6} mol/L riboflavin and 0.013 mol/L methionine phosphate at pH 7.8. One unit of enzyme activity was the amount of enzyme bringing about 50% inhibition of the photochemical reduction of NBT (Dhindsa et al., 1981).

Catalase activity was determined by measuring the decreasing rate in the absorbance of H_2O_2 at 240 nm (Aebi, 1984; Lu et al., 1993). One unit was defined as the amount of enzyme catalyzing the decomposition of 1 μmol/L H_2O_2 per minute, calculated from the extinction coefficient for H_2O_2 at 240 nm of 0.036 $cm^2\ \mu M^{-1}$ (Luck, 1963).

Peroxidase activity was determined using guaiacol as the substrate, with the method of Volk and Feierabend (1989). The molar extinction coefficient of tetraguaiacol (26.6 $mM^{-1}\ cm^{-1}$) was used in the calculation of the enzyme concentration.

Glutathione reductase activity was determined in 0.5 mL of reaction mixture that contained 0.1 mmol/L Hepes buffer (pH 8.0), 1.0 mmol/L EDTA, and 50−100 μL of enzyme extract. The reaction was initiated by the addition of 1.0 mmol/L oxidized glutathione (GSSG), and the rate of NADPH oxidation was monitored at 340 nm (Burke et al., 1985). Glutathione reductase activity was expressed in nanomolars of NADPH oxidized per minute per milligram of protein.

Measurement of Hydrogen Peroxide. H_2O_2 was extracted and estimated following the method of MacNevin and Uron (1953) with slight modifications (Mondal and Choudhuri, 1981). Isolation was made from 5 g of leaf tissue in ice-cold acetone by the addition of 5% (w/v) titanyl sulfate and concentrated NH_4OH solution; the absorbance was read at 415 nm against a water blank. The H_2O_2 content was calculated from a standard curve prepared in a similar way (Mukherjee and Choudhuri, 1983).

Determination of *L. sativus* Neurotoxin (ODAP). The finely powered leaf material of grass pea (20 mg) was extracted with shaking for 6 h with 2 mL of 60% ethanol, and the clear supernatant was collected. An aliquot (1 mL) of the extract was hydrolyzed with 3 mol/L KOH (2 mL) for 30 min. The mixture was centrifuged for 15 min. The supernatant hydrolyzed extract was collected for assay. Samples were assayed for ODAP using *O*-phthalaldehyde reagent (OPT). The peak absorbance was measured. The ODAP concentration of each of the original samples was calculated by reference to a calibration curve. The standard calibration curve was prepared using diaminopropionic acid, which was subjected to the procedure (Briggs et al., 1983).

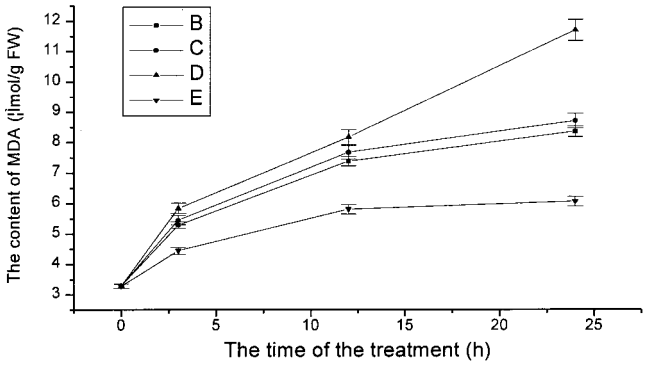

Figure 1. Differential changes in the extent of lipid peroxidation (MDA content) as the time of PEG treatment goes on: (B) PEG treatment; (C) PEG plus DDC treatment; (D) PEG plus AT treatment; (E) PEG plus mannitol treatment. Results represent mean values (n = 3) of samples extracted and assayed independently. Bars represent ±SE.

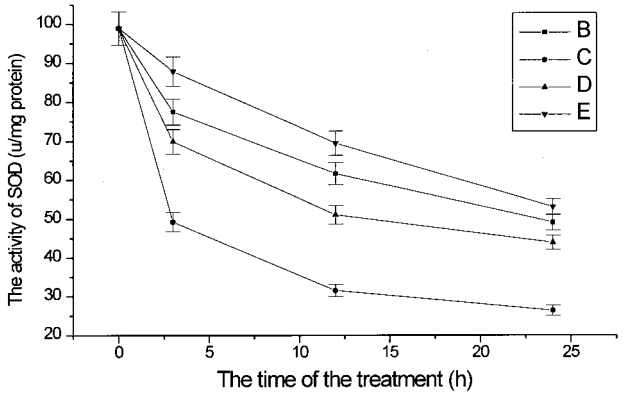

Figure 2. Differential changes in SOD activity as the time of PEG treatment goes on: (B) PEG treatment; (C) PEG plus DDC treatment; (D) PEG plus AT treatment; (E) PEG plus mannitol treatment. Results represent mean values (n = 3) of samples extracted and assayed independently. Bars represent ±SE.

RESULTS

The extent of lipid peroxidation in the plant was used as an indication of free radical damage due to stress. The extent of lipid peroxidation in PEG treatment gradually increased as the time of the treatment went on (Figure 1). Both diethyldithiocarbamate (DDC) and aminotriazole (AT) as inhibitors of SOD and catalase increase the extent of lipid peroxidation by PEG treatment. The effect of DDC is not obvious, but the effect of AT is more so (Figure 1). However, mannitol, as a scavenger of the hydroxyl radical (•OH), decreases the extent of lipid peroxidation in PEG treatment significantly (Figure 1).

After 24 h of treatment, SOD activity strongly decreases in DDC experiments as expected, and a small decrease in SOD activity is observed in AT experiments. However, a small increase in SOD activity can also be observed in mannitol treatment (Figure 2).

The peroxidase activity gradually decreases as time of PEG treatment goes on (Figure 3). Figure 3 clearly

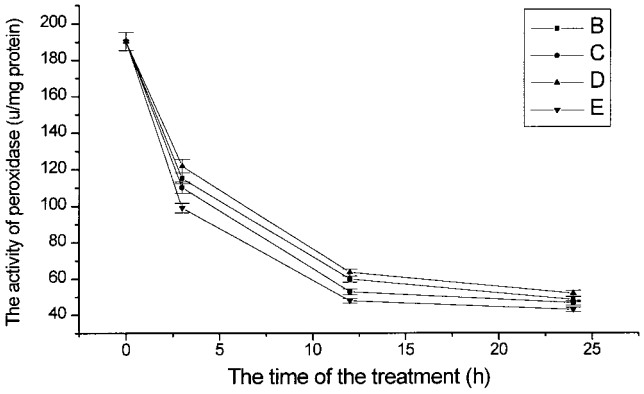

Figure 3. Differential changes in peroxidase activity as the time of PEG treatment goes on: (B) PEG treatment; (C) PEG plus DDC treatment; (D) PEG plus AT treatment; (E) PEG plus mannitol treatment. Results represent mean values (n = 3) of samples extracted and assayed independently. Bars represent ±SE.

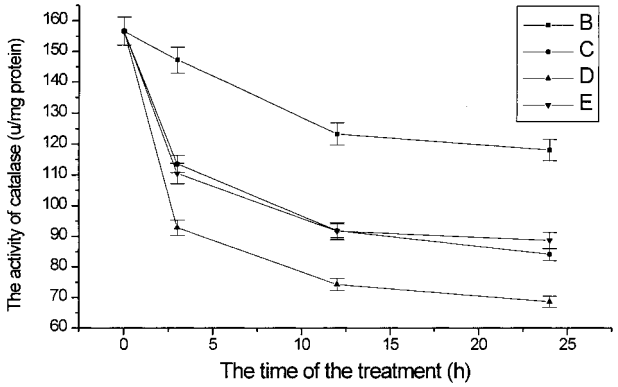

Figure 4. Differential changes in CAT activity as the time of PEG treatment goes on: (B) PEG treatment; (C) PEG + DDC treatment; (D) PEG plus AT treatment; (E) PEG plus mannitol treatment. Results represent mean values (n = 3) of samples extracted and assayed independently. Bars represent ±SE.

indicates that peroxidase activity is independent of DDC, AT, and mannitol.

The catalase activity decreased significantly with the increase of PEG treatment. AT, as an inhibitor of catalase, strongly inhibits the catalase activity. Meanwhile, catalase activity is decreased by PEG plus AT and PEG plus mannitol treatments (Figure 4). In contrast to the decrease in SOD and catalase activities, a PEG treatment-induced increase in the activity of glutathione reductase was observed as the time of PEG treatment went on (Figure 5).

Figure 6B shows that the hydrogen peroxide content gradually increases as the time of PEG treatment goes on. DDC as an inhibitor of SOD activity decreased the hydrogen peroxide content with the duration of PEG plus DDC treatment (Figure 6C). AT, as an inhibitor of catalase activity, increased the hydrogen peroxide content with the duration of PEG plus AT treatment (Figure 6D). Mannitol, as a scavenger of the hydroxyl radical (•OH), does not have an obvious effect on the hydrogen peroxide content with the duration of PEG plus mannitol treatment (Figure 6E).

The leaf toxin content in PEG treatment ranged from 0.290 to 0.497% compared to 0.274% in the control sample (Figure 7). To discover the relationship between the accumulation of ODAP and antioxidant metabolism

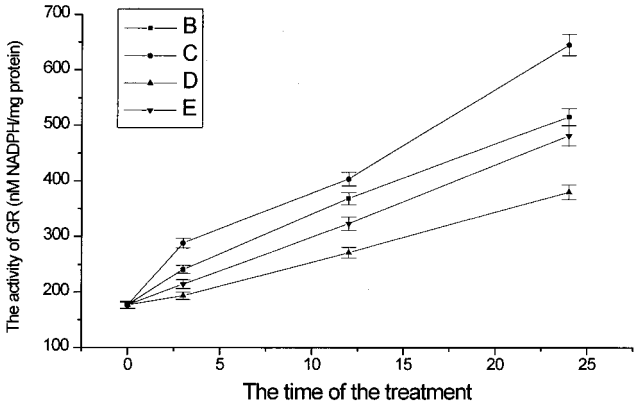

Figure 5. Differential changes in GR activity as the time of PEG treatment goes on: (B) PEG treatment; (C) PEG plus DDC treatment; (D) PEG plus AT treatment; (E) PEG plus mannitol treatment. Results represent mean values (n = 3) of samples extracted and assayed independently. Bars represent ±SE.

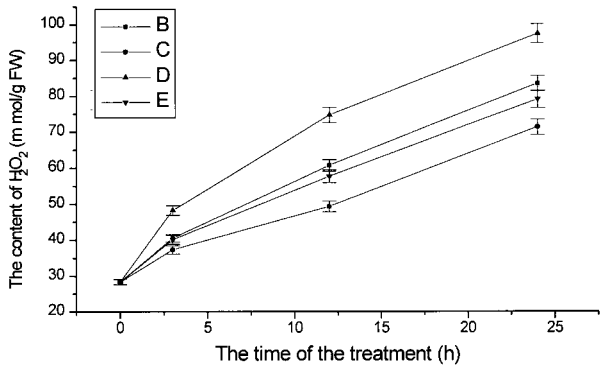

Figure 6. Differential changes in the content of H_2O_2 as the time of PEG treatment goes on: (B) PEG treatment; (C) PEG plus DDC treatment; (D) PEG plus AT treatment; (E) PEG plus mannitol treatment. Results represent mean values (n = 3) of samples extracted and assayed independently. Bars represent ±SE.

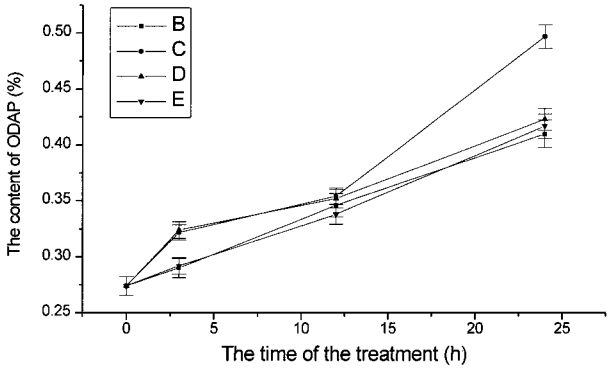

Figure 7. Differential changes in the content of ODAP as the time of PEG treatment goes on: (B) PEG treatment; (C) PEG plus DDC treatment; (D) PEG plus AT treatment; (E) PEG plus mannitol treatment. Results represent mean values (n = 3) of samples extracted and assayed independently. Bars represent ±SE.

in PEG treatment, we analyzed the ODAP content in PEG plus DDC, PEG plus AT, and PEG plus mannitol treatments. We observed that AT and mannitol do not have a clear effect on ODAP content as the time of PEG treatment goes on, and DDC increases ODAP content as the time of PEG treatment goes on, but this increase is limited.

DISCUSSION

Some authors have proposed to consider water stress as an oxidative stress (Dhindsa and Matowe, 1981; Burke et al., 1985) because lipid peroxidation causes alterations in the membrane similar to those noticed during dehydration. Our experiment results show that the extent of lipid peroxidation increases with the duration of PEG stress. Meanwhile, hydrogen peroxide content increases, too. The extent of lipid peroxidation increases in DDC and AT treatments. However, it increases more in the AT treatment than in the DDC treatment. Therefore, the effect of AT is more obvious than the effect of DDC. AT and DDC inhibit the activities of catalase and SOD, respectively, and SOD shall be used for a variety of metalloproteins catalyzing the reaction $2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$. The term catalase shall be used for a variety of metalloproteins catalyzing the reaction $2H_2O_2 + O_2 \rightarrow 2H_2O + O_2$. Therefore, it is clear that the effect of hydrogen peroxides is more obvious than the effect of superoxide radicals in the MDA generation of plasma membranes. However, the extent of lipid peroxidation is reduced in mannitol (a scavenger of the hydroxyl radical) treatment, which suggests that hydroxyl radicals play an important role in the MDA generation of plasma membranes. The relationship between the generation of MDA of plasma membranes and the metabolism of free radical is intimate. Therefore, superoxide radicals, hydrogen peroxide, and hydroxyl radicals might be involved in MDA generation of plasma membranes. The inhibition of MDA formation by SOD, catalase, and mannitol was observed (Qiu and Liang, 1995), which indicated the involvement of superoxide radicals, hydrogen peroxide, and hydroxyl radicals in the MDA generation of plasma membranes.

DDC and AT inhibit the activities of SOD and catalase, respectively. At the same time, the extent of lipid peroxidation and the content of hydrogen peroxide are increased. For this reason, we suggest that superoxide radicals, hydrogen peroxide, and hydroxyl radicals accumulated along with the decrease of SOD and catalase activities; therefore, the extent of lipid peroxidation was made greater.

In the hydrated tissues, free radical generation is normally regulated by compounds with a scavenging function such as SOD and catalase. It is possible that water loss from cells alters the equilibrium between free radical production and enzymatic defense reactions in favor of the former (Quartacci and Navari-Izzo, 1992). From the decrease in the SOD, peroxidase, and catalase activities (Figures 2−4) it can be seen that the three enzymes are no longer able to oppose the increased radical production, and the content of hydrogen peroxides is increased (Figure 6). This result was consistent with water stress (Mukherjee and Choudhuri, 1983; Roy and Choudhuri, 1985). Therefore, we think that hydrogen peroxides and hydroxyl radicals can increase the extent of lipid peroxidation and that the ability of superoxide radicals for increasing the extent of lipid peroxidation is weaker than the ability of hydrogen peroxides and hydroxyl radicals.

The activity of glutathione reductase increases as the time of PEG treatment goes on (Figure 5). Glutathione reductase, as the important component of an antioxidant system in plants, has a very important effect on stress tolerance of grass pea. Glutathione reductase has been said not only to direct electrons away from O_2 and minimize the production of superoxide radical during drought stress but may also serve to ensure the availability of $NADP^+$ to accept electrons (Foster and Hess, 1982). Therefore, we think the content of superoxide radical decreases as the glutathione reductase activities are increased by PEG treatment. This proves that the ability of superoxide radicals for increasing the extent of lipid peroxidation is weaker than that of hydrogen peroxides and hydroxyl radicals once more.

Detailed information on the rate of biosynthesis and accumulation of ODAP in different tissues and organs during plant development is not clear at present. There are a few studies about the relationship between the accumulation of ODAP and water stress. We determined ODAP accumulation of leaves during PEG treatment. The results show that the content of ODAP increases along with the duration of the PEG treatment (Figure 7). However, DDC, AT, and mannitol do not have obvious effects for ODAP accumulation of leaves in *L. sativus* (Figure 7). Therefore, we think there is no direct contact between the ODAP accumulation of leaves and the metabolism of free radicals. It seems that the increase of the ODAP content of leaves is concerned with the water deficit of the cell in PEG treatment. We infer that the content of the neurotoxin of *L. sativus* is increased as the drought tolerance of *L. sativus* increases.

The enzymatic oxalylation of α,*β*-L-diaminopropionic acid (DAPRO) with the formation of ODAP has been demonstrated. The cotyledons are presumably the site for this biosynthetic step (Lambein et al., 1990). Therefore, we think that ODAP, as a small amino acid, probably has an important effect on water stress. Perhaps ODAP, as a "signal molecule", takes effect in drought tolerance. These hypotheses remain to be studied and discussed in future work.

ABBREVIATIONS USED

AT, aminotriazole; BSA, bovine serum albumin; CAT, catalase; DDC, diethyldithiocarbamate; EDTA, ethylendiaminotetraacetate; GR, glutathione reductase; GSSG, oxidized glutathione; MDA, malondialdehyde; NBT, nitroblue tetrazolium; ODAP, *β*-*N*-oxalyl-L-α,*β*-diaminopropionic acid; PEG, polyethylene glycol; PMSF, phenylmethanesulfonyl fluoride; SOD, superoxide dismutase.

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