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Full Length Research Paper

The effects of drought stress on the activity of acid phosphatase and its protective enzymes in pigweed leaves

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A model of drought was created on pigweed and the effects of drought stress on the activity of acid phosphatase and its protective enzymes were examined. The pot-cultured pigweeds were divided into 4 groups (ten plants per group) when they reached 6 leaves. (1) In the control group, the culture media contained 70 - 85% of field moisture capacity, (2) In the second group, the mild drought stress group, the culture media contained 50 - 60% of field moisture capacity, (3) The moderate drought stress group had a culture media that contained 40 - 50% of field moisture capacity; (4) The severe drought stress group culture media contained 30 - 40% of field moisture capacity. All through the process of the present study, the pigweed plants were cultured under natural conditions on the rooftop of the laboratory building; though transferred indoor in rainy days to avoid the influence of natural precipitation. The plants were sampled and detected every five days after the administration of drought stress. The results clearly demonstrated that the drought stress significantly enhanced the activity of acid phosphatase, membrane permeability and MDA contents; though the activity of acid phosphatase declined after a certain time of drought stress, the extent of membrane permeability and MDA contents still increased with the time. The membrane permeability and MDA contents were correlated with a correlation coefficient of 0.963, 0.971 and 0.939 under mild, moderate and severe drought stress, respectively. The activity of superoxide dismutase (SOD), peroxide dismutase (POD) and hydrogen peroxidase (CAT) was also enhanced with increase in the intensity of drought stress or the prolongation of drought stress at first and then decreased some time afterwards. It was concluded that drought stress enhanced the activity of acid phosphatase, membrane permeability and MDA in the pigweed plants, which was able to resist a certain drought stress by enhancing the activity of protective enzymes. However, excessive drought stress markedly affected the metabolic systems of enzyme and decreased the activity of enzyme.

Key words: Pigweed, drought-stress, acid phosphatase, MDA, protective enzyme.

INTRODUCTION

Drought is one of the most severe constrains to crop production (Toumi et. al., 2008) and the bottleneck of agriculture development in various regions (Cao et al., 2003; McKersie and Leshem, 1994). The damage caused by reactive oxygen species (ROS) has been proved to be one of the major mechanisms of cellular membrane dysfunction in plants. Generally, the loss in the integrity and function of cellular membrane was believed to be directly correlated with the massive accumulation of ROS under drought stress. However, disputes remained regarding the primary mechanism of injury caused by ROS. Some believed that reactive oxygen species significantly increased the peroxidation of membrane lipids, which result in injury to large molecules such as proteins, nucleic

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acids, carbohydrates and lipids, (Vehiba, 1998); Bowler et al. (1992) pointed out that drought stress caused the imbalance of production and scavenging in oxygen free radicals within cells, which promote the membrane lipid peroxidation or membrane lipid de-fat and eventually, caused the formation of malondialdehyde (MDA) that harmed plants. MDA is the final product of plant cell membrane lipid peroxidation and is one important sign of membrane system injury. Wang et al. (2009) found that the increase of alfalfa membrane permeability was significantly positive correlated with the accumulation of MDA, while MDA accumulation was negatively correlated with the growth amount. ROS can attack amino acid residues of proteins to form a carbon-based derivative. H_2O_2 produced in this process, can go through Haber-Weiss reaction to produce •OH, which is more toxic and would promote further membrane lipid peroxidation (Asada and Takahash, 1987). Membrane lipid peroxidation can lead to the destruction of cellular integrity, cell dysfunction and ultimately affect the survival and reproduction of plants. While some authors argued that ROS activated phospholipase catalyzed the degrease reactions in membrane phospholipids (Wang et al., 1989), the first perspective remained dominant in the current research of mechanisms of cellular injury caused by ROS. Later studies are still rare; so far only Hou et al. (2003) has reported that drought stress markedly enhanced the activity of acid phosphatase in the cells of sunola leaves. Pigweed, also referred to as Lambsquarters, whose leaves are used not only as vegetable for human and feeding material for animals, but also in traditional Chinese medicine (Sun et al., 2005a) is regarded as being very tolerant to abiotic stresses. It can grow very well in high salinity, drought and barren soil. Previous research has found that, although pigweed is drought resistant, it still had a sensitive response to drought stress at physiological and biochemical levels (Sun et al., 1999, 2005b, 2007, 2009a, 2009b), increase and decrease of ROS, activities of SOD and POD and the process of osmosis regulation. The substances in pigweed clearly correlates to the strength of drought stress, used in the present experiments (Sun et al., 2005a). In gel electrophoresis, a new protein (drought-induced protein) band appeared in pigweed under drought stress (Sun et al., 2009b).

Arabidopsis is a model plant used in genetic analysis of plant development and other issues. Although Arabidopsis belongs to glycophyte, the research of responses to salt osmotic stress has proved Arabidopsis as a promised model and has provided valuable information about molecular mechanisms of plant stress responses (Zhu, 2000; Wang et al., 2003). In comparison with Arabidopsis model system, the disadvantage of Pigweed as model plant is the absence of genomic information and the difficulties involve in carrying out genetic experiments. But these problems can be partly solved by using orthologous sequences of Arabidopsis, which might fulfill the same functions in drought stress signaling in pigweed as in Arabidopsis (Xiong and Zhu, 2002). A plant, which is more tolerant to abiotic stress, may result from changes in the threshold of some regulatory switches or mutations in some key determinants (Xiong and Zhu, 2002). So, the comparison of the signaling components between a drought sensitive and a tolerant plant may give us a deep insight into the molecular mechanisms of how a plant survived in drought environment. Briefly, pigweed is an ideal complementary model for studying physiological processes and molecular mechanisms of plant responses to drought stress. So pigweed used as a model plant to research the drought tolerant mechanism of Plant. Sun Cunhua had reported the impact of drought stress on membrane lipid peroxidation (Sun et al., 2005b), photosynthesis (Sun et al., 2007), drought-induced protein of pigweed (Sun et al., 2009a, b) and other prominent physiological and biochemical responses of pigweed to drought stress. However, it remained controversial as to whether membrane lipid peroxidation, membrane phospholipids degrease reaction were started under drought stress. Few reports regarding these questions are presently available. In the present study, pigweed pot-cultured was used as the experimental material with manually-controlled soil moisture to investigate the possible mechanisms of impaired or strengthened structure and function of plant cells membrane through the activity of its acid phosphatase and protective enzymes. This may provide a scientific evidence for understanding the mechanism of the drought-resistance in field crops.

MATERIALS AND METHODS

Material

Pigweed (*Chenopodium album* L) was cultured in pots under natural conditions. The culture pots were 30 cm in aperture diameter and 23 cm in height. The yellow fluovo-aquic soil obtained from the Yellow River sediments was used as culture media, which was characterized by a slightly high level of calcium carbonates and soluble salts as detected by alkaline reactions. Each culture pot contained 12 kg of culture media with a soil moisture content of 12.46% and a maximal moisture content of 29.8% after saturated watering. Sufficient base fertilizer was added. Well-stacked pigweed kernels were selected and sown at the end of March. When seedling emerged, 5 pigweed plants were kept in each culture pot and fostered under natural conditions of rooftop of the Number 4 Teaching Building in Xuzhou Normal University.

Exposure to drought stress

When reaching 6 leaves (in mid-May), drought stress was imposed by irrigation while weighing method was used to control soil moisture to make the soil moisture meet the experimental requirements. 10 pots were selected randomly and the pots field moisture capacity (soil saturation water content) were measured. 10 pots averaged and the pot-cultured pigweeds were matched and divided into 4 groups (10 plants per group): (1) In the control group, the culture media contained 70-85% of field moisture capacity, (2) In the second group, the mild drought stress group, the culture media contained 50-60% of field moisture capacity, (3) The moderate drought

stress group had a culture media that contained 40-50% of field moisture capacity; (4) The severe drought stress group culture media contained 30-40% of field moisture capacity. All through the process of the present study, the pigweed plants were cultured under natural conditions on the rooftop of the laboratory building; though transferred indoor in rainy days to avoid the influence of natural precipitation. In order to qualify if the soil moisture content meet the requirements of the present study, a manual weighing method was adopted to control the soil moisture. The culture pots were weighted at 6:30 pm every day and the moisture loss the past day was replenished. A soil hygrometer was employed to detect the moisture content in different treatments of drought stress and a safe range of soil moisture content was carefully maintained. The plants were sampled and various physiological parameters were detected on the fifth day after the administration of drought stress. Functional leaves were randomly obtained from one of the pigweeds in each pot. Three repetitions were performed for each culture pot and the average of three samplings was used for analysis once every five days.

Detection methods

The detection of relative permeability of cell membrane

The method described by Zhou et al. (2006) was adopted and modified. 1 g of pigweed leaves were loaded to a 30 ml test tube, in which 10 ml double distilled water was added so that the leaves were completely submerged. The air was extracted by using syringe and the test tubes were placed in air for 30 min (with vibrations every 4 min). The electrodes of the DDS-11A Conductivity Detector were interpolated into the test tube and the conductivity of the exudates was detected, while the temperature was measured at the same time. The test tubes were then placed in boiling water bath for 10 min to deactivate tissues and cooled to the same temperature. The conductivity of the exudates was detected 3 times and averaged.

$$\text{Relative membrane permeability (\%)} = \frac{\text{L1 (the conductance value before leaf was killed)}}{\text{L2 (the conductance value after leaf was killed)}} \times 100$$

The measurement of MDA content was performed according to the methods described by Wang et al. (1986).

The measurement of the activity of protective enzymes (SOD, POD and CAT)

The crude enzyme was extracted (Tang et al.,1999): 0.05 g of fresh pigweed leaves were obtained and cut into pieces in pre-cooled mortars and 4 ml of pre-cooled phosphate buffer (0.05mol/l pH = 7.0) was added and the tissues were grinded to homogenate in ice bath. The homogenate was diluted to 5 ml and centrifuged at 4000 r/min and 4℃ for 15 min. The supernatant contained the gross extract of SOD, POD and CAT. The detection of SOD was done using the nitroblue tetrazolium method (Li, 2000). The detection of CAT was performed using modified ultraviolet method (Yang et al., 2004) in which 200 µl of enzyme was added to 3 ml of reaction reagent (pH = 7.0, 50 mmol/l phosphatase buffer mixed with 30% H_2O_2, so that the OD_{240} of the solution was in the range of 0.5 - 0.55) and the mixture was immediately detected for OD_{240} at every 30 s using ultraviolet spectrophotometer. The activity of enzyme was shown as ΔOD_{240}/g Fw·min. The detection of POD activity was carried out using the Guaiacol method (Zhang and Qu, 2003).

The detection of acid phosphatase

The method described by Sun et al. (2005) was adopted and modified. 0.05 g of pigweed leaves was obtained and grinded in 4 ml 0.05mol/l Tris-Hcl buffer in ice bath, diluted to 5 ml and centrifuged at 4000 r/min and 4℃ for 10 min. The supernatant was retrieved as gross acid phosphatase. 0.1 ml of the enzyme was diluted to 1 ml and mixed with 1 ml of 0.1 mol/l acetic acid buffers (pH 5.0) and 0.1 ml of 0.018 mol/l 4 nitrobenzne disodium hydrogen phosphate. The reaction was performed at 30℃ in a water bath for 30 min and then 1 ml of 0.5 mol/l NaOH was added, which turned the solution into yellow. Optical density was detected at 400 nm for the calculation of enzyme activity.

Data analysis

Data were analyzed with DPS data processing system.

RESULTS

The effects of drought stress on the activity of acid phosphatase

The activity of acid phosphatase was significantly increased during the early phase of drought stress (first 15 days) (Figure 1), which was markedly higher than that of the control group ($P < 0.05$). The extent of increase was in the following order: Severe stress group was a little greater than moderate stress group, moderate stress group was much greater than mild stress group and mild stress group was much greater than control group. But the activities of acid phosphatase under moderate, severe drought stress decreased rapidly and lower than that of mild drought stress group at the 20th day after exposure to drought stress. The activity of acid phosphatase in mild stress group continue to increase after 20 days and got to the maximum on the 35th day after exposure and then declined rapidly.

The effects of drought stress on the SOD activity

The prolongation of drought stress was accompanied with the increase of SOD activity at first and then fall back (Figure 2). The activity of SOD under drought stress levels was significantly higher than that of the control group ($P < 0.05$), which indicated that the pigweed was able to increase the metabolism of $O_2^{\cdot-}$ by enhancing the activity of SOD and reducing the rate of membrane lipid peroxidation to resist the effects imposed by the external environment. During the early phase of drought stress (first 25 days), the SOD activity followed the order of severe drought stress > moderate drought stress > mild drought stress > control group. Under severe drought stress, the activity of SOD peaked at the 25th day after exposure and then dramatically dropped. 35 days after exposure to drought stress, the activity of SOD was even lower than those of the moderate and mild drought stress groups. This suggested that the pigweed could use self-

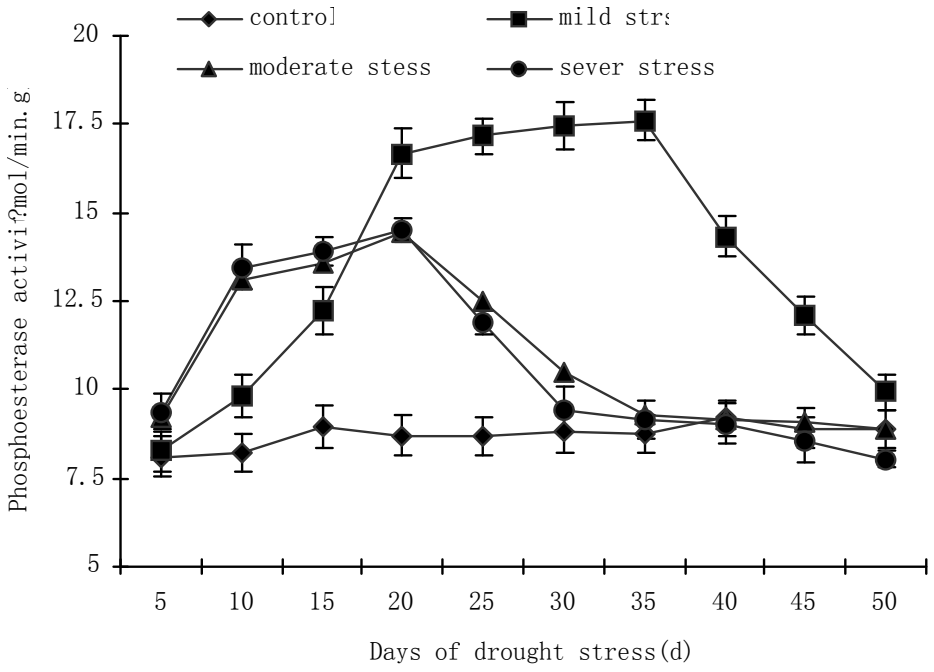

Figure 1. The effect of drought stress on the activity of acid phosphoesterase in leaves of pigweed.

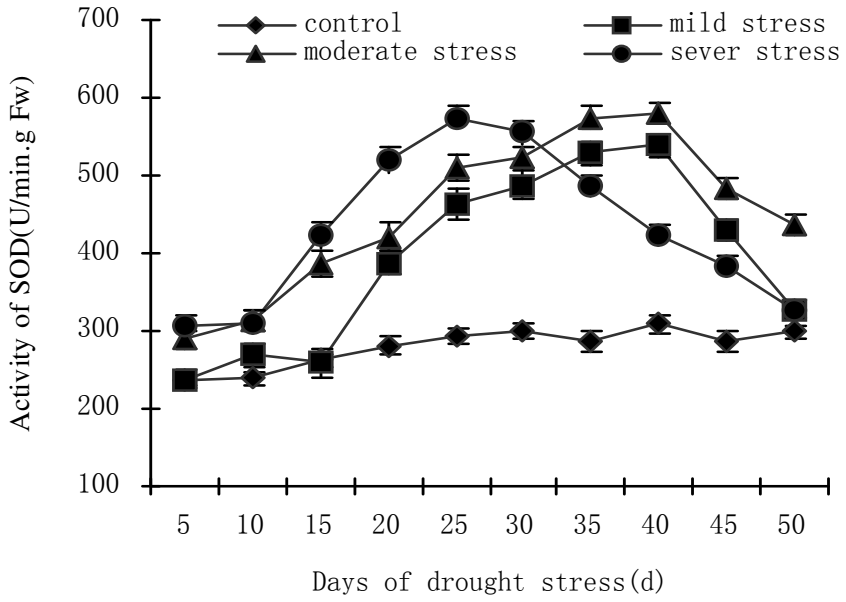

Figure 2. The effect of drought stress on the activity of SOD in leaves of pigweed.

protected response and be able to increase the activity of SOD to counteract the cellular injury caused by ROS under drought stress. However, the massive accumulation of ROS was beyond the capacity of cellular dismutase and caused significant damage to multiple cellular functions. The disruption of physiological metabolism occurred as the SOD activity dropped.

The effects of drought stress on POD activity

The effects of drought stress on Pod activity in leaves of pigweed is shown in Figure 3. The changes of POD activity was in the same tendency as that of SOD under all levels of drought stress. The POD activity under all drought stress levels was significantly higher than that of

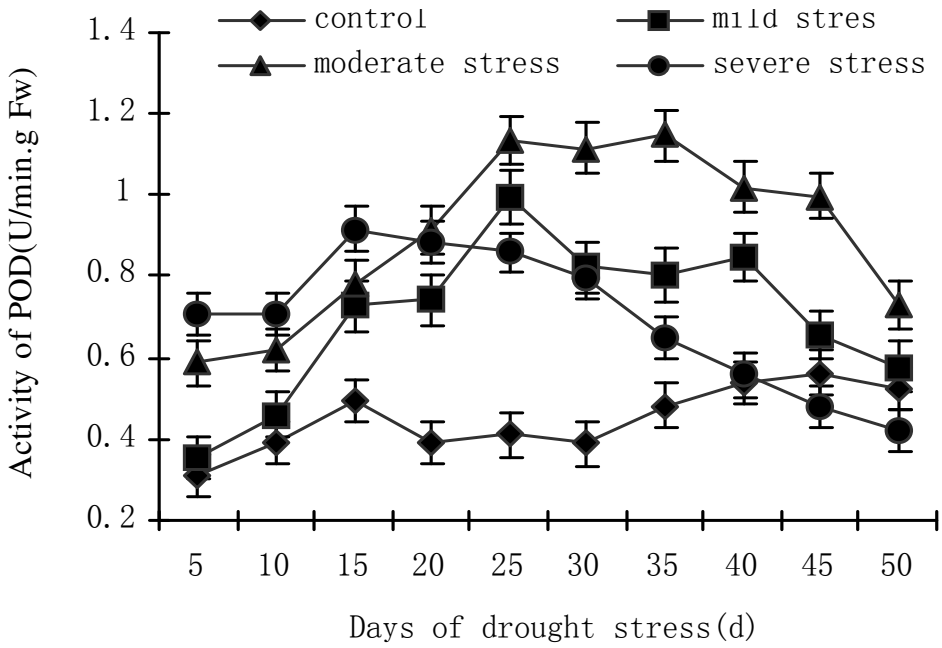

Figure 3. The effect of drought stress on the activity of POD in leaves of pigweed.

the control group ($P < 0.05$). The prolongation of drought stress was accompanied first by the increase and then the decline of POD activity. The POD activity in mild and moderate drought stress group peaked at the 25th day after exposure. The POD activity in moderate drought stress group was always higher than that of mild drought stress group. The changes of POD activity in severe drought stress group followed the same pattern, but with the peak occurring at the15th day after exposure. The POD activity in severe drought stress group dropped below that of mild and moderate group, 25 days after exposure and further below that of control group, 40 days after exposure.

The effects of drought stress on CAT activity

CAT was the scavenger of ROS in plants and its activity is usually increased under drought stress in order to diminish the accumulated ROS and lessened the damage to cellular membrane system caused by free radicals. Sun et al. (2003) suggested that the changes of CAT activity in birch (*Betula alba* L.) also followed the pattern of first increase and then decrease. Pigweeds showed the same pattern under drought stress, which, as was indicated in Figure 4, was markedly different from the control group ($P < 0.05$). Consistent with the results of Sun Guorong, the CAT activity increased in the early phase of exposure to severe and moderate drought stress, while the mild drought stress group witnessed the higher CAT activity during the late phases of exposure. The highest CAT activity appeared in the moderate group all through the study.

The effects of drought stress on permeability of cellular membrane

Figure 5 indicated that the membrane permeability gradually increased with the exacerbation of drought stress, which implicated more and more severe injury to the cellular membrane system in pigweeds.

During the early phase of drought stress (first 25 days), the increase of membrane permeability was minor in mild, moderate and severe drought stress groups, inferring little injury to the cellular membrane system. 25 days after exposure, the increase of membrane permeability was slightest in the mild exposure group, suggesting minor injury to the membranes under this stress. However, evident increase of membrane permeability was seen in the moderate and severe group as compared to the control group, the difference was significant ($P < 0.05$). The greatest augmentation of membrane permeability occurred in the severe exposure group, which amounted to 17.6% at 30th day, 23.3% at 40th day and 28.4% at 50th day. The results also clearly demonstrated that the pigweeds were irreversibly injured under long term moderate and severe drought stress.

The effects of drought stress on MDA contents

As the plants were exposed to drought stress, the dysfunction of metabolism occurred with massive accumulation of free radicals and ROS (Kato et al., 2002), which would attack cell membrane by inducing peroxidation of the membrane lipids and eventually led to the damage of cell membrane and cell death (Yang et al., 2004). MDA

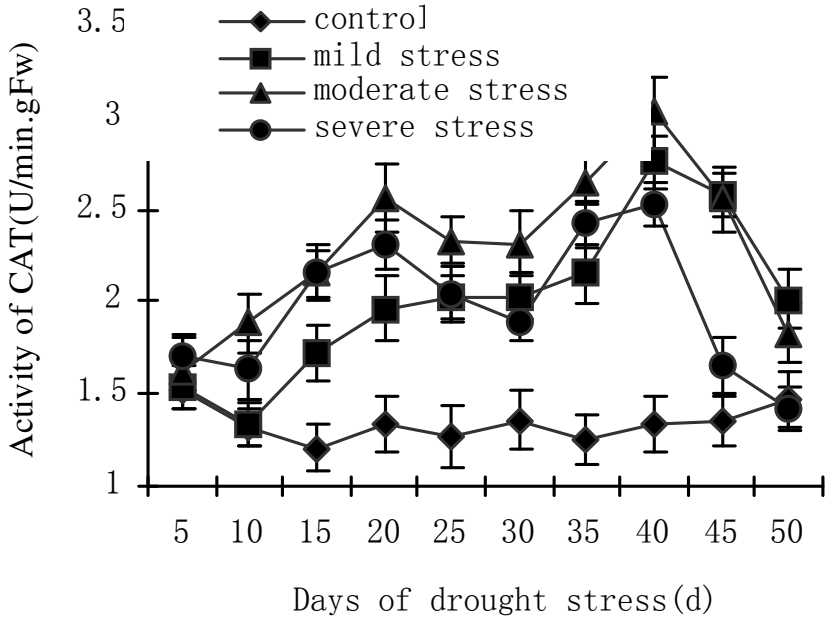

Figure 4. The effect of drought stress on the activity of CAT in leaves of pigweed.

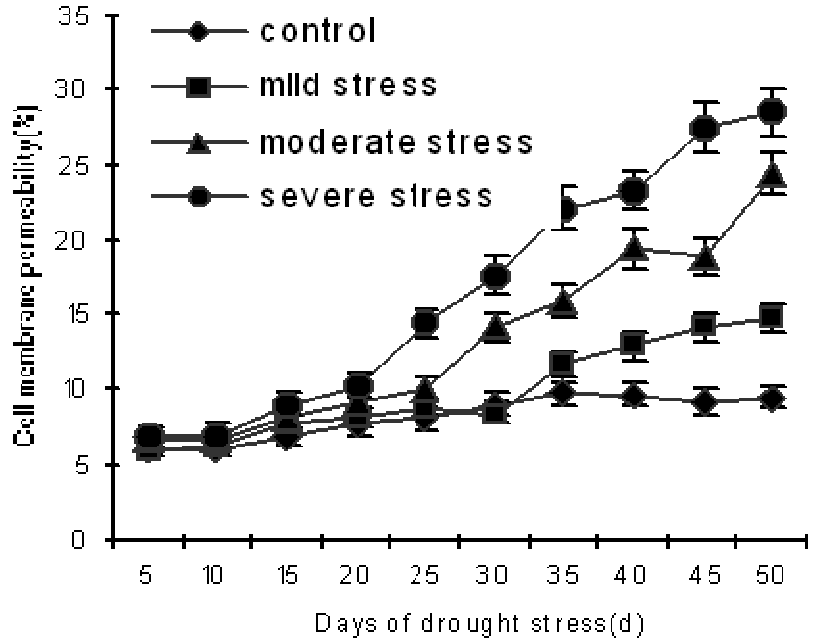

Figure 5. The effect of drought stress on cell membrane permeability in leaves of pigweed.

was the major product of membrane peroxidation and its content indicated the level of membrane lipid peroxidation and the extent of membrane injury (Zhang et al., 2004; Smirnoff, 1993). As indicated in Figure 6, the MDA content in pigweed leaves increased with the prolongation of exposure to drought stress, the extent of increase followed the order of severe drought stress > moderate drought stress > mild drought stress > control group. There was no significant difference in the MDA contents of mild exposure group and the control. Figures 5 and 6 indicated the apparent correlation of MDA content and membrane permeability with a correlation coefficient of 0.963, 0.971 and 0.939 for the mild, moderate and severe exposure, respectively.

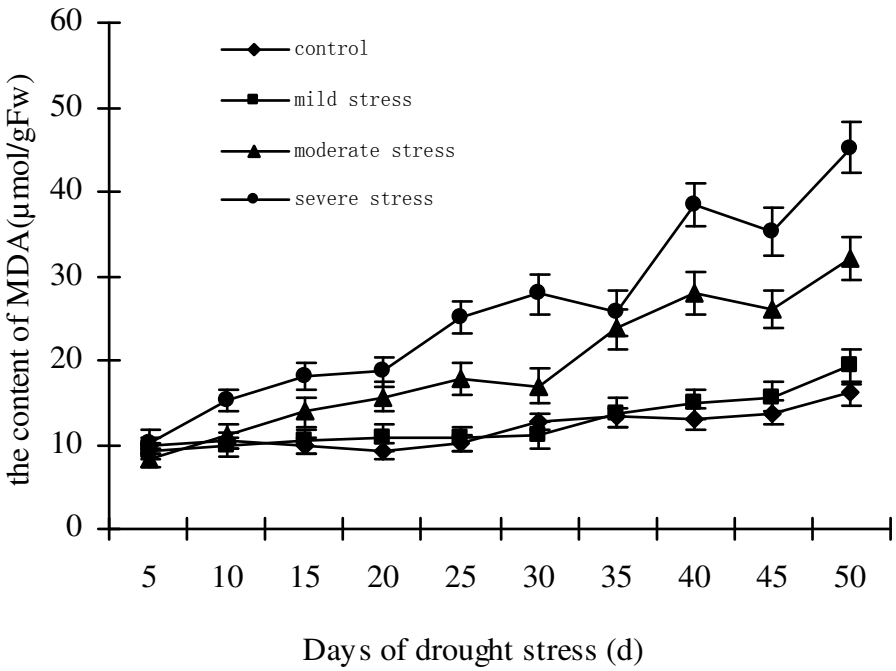

Figure 6. The effect of drought stress on MDA content in leaves of pigweed.

DISCUSSION

Acid phosphatase hydrolyzes phosphatidyl groups into free fatty acids and soluble phosphorus in plant cells. Therefore, it is usually used as an indicator of degrease reaction (Wang et al., 1988). During the early phase of drought stress, the activity of acid phosphatase increased along with the augmentation and prolongation of drought stress (Figure1), which was in accordance with the results of Lu et al. (2004) and Xu et al. (1994). Xu believed that the increased ROS under drought stress promoted the peroxidation of membrane lipids, which lowered the degree of unsaturation and fluidity of membrane. This further led to the obvious activation of lysing effects of the acid phosphatase formerly inhibited by the membranes (Xu et al., 1994). The activity of acid phosphatase in pigweed leaves rapidly decreased 20 days after exposure to moderate and severe drought stress and 35 days after exposure to mild stress, which indicated probable injury to the cellular membrane. As an enzyme constrained on cellular membranes, acid phosphatase was inactivated as the cellular membrane suffered further damage. Further investigation should be performed to understand the details of these mechanisms.

When suffered drought stress, plants produced massive amount of reactive oxygen free radicals such as $O_2^{\cdot-}$ and H_2O_2, among others (Wang et al., 2007), which would further activate peroxidation of membrane lipids. During the long process of evolution, plants developed a series of anti-oxidation protective system to avoid injuries caused by the external environment. SOD, POD and CAT were vital constituents of this system and played major roles in maintaining normal physiological functions in the plants. The present study indicated that SOD activity followed the increase-decrease pattern when exposed to mild, moderate or severe drought stress, with the activity being peaked at the 40th day and 25th day after exposure. The activity of POD in the mild, moderate exposure groups was significantly higher than that of the control group. Though the POD activity in the severe drought stress group was above that of the control at the 40th day after exposure, it drastically dropped below the level of control group afterwards. The CAT activity in all drought stress levels also followed the increase-decrease pattern with the prolongation of exposure, with higher activity in moderate and severe groups in the early phase of exposure while mild exposure group in the later phase of exposure. The moderate drought stress group had the highest CAT activity all through the experiment, which implied that the increase-decrease pattern in the changes of protective enzymes was probably induced by the expression of certain protective genes in the early stage of drought stress so that the cellular protective enzymes were strengthened to defend the attacks of ROS to membrane lipids and protect the structure and function of cellular membrane system. This might be part of the self-protection mechanisms in plants. However, as the intensity of drought stress increased and time prolonged, the disruptions of the enzyme metabolism system occurred and the expression of these protective genes and the synthesis of enzymes were inhibited. The exposure to severe drought stress further destroyed the structure of protective enzymes. These negative changes led to the decreased activity of protective enzymes, which was no

longer sufficient to counteract the damage caused by drought stress. The changes of SOD, POD and CAT were not exactly consistent, suggesting possible interactions and synergic effects among these enzymes under drought stress. The diminish activity of CAT during the later phase of drought stress might be caused on one hand by the inactivation of CAT and the disturbance of metabolism under severe drought stress and on the other hand by the occurrence of light-deficient inactivation. ^{35}S-Met label confirmed that CAT could be inactivated due to lack of light and the maintenance of catalytic activity depends on the continuous synthesis of CAT protein in the presence of light. The light repair of CAT was extremely sensitive to external factors (Qi et al., 2007) and the exposure to overwhelming drought stress probably decreased the capability of light repair. In addition, H_2O_2 and $O_2^{\cdot-}$ reacted with CAT in group or alone to form compounds, which inactivated the CAT activity (Asada, 1999).

The changes of plant membrane permeability and the damage of membrane system under drought stress were part of the essential features of drought stress-induced injuries. Peroxidation of membrane lipids was the primary mechanism of injuries on plant cell membranes (Zhang and Shan, 1997). Peroxidation of membrane lipids under severe drought stress caused membrane phase separation and changes of lipid constituents, which converted the membrane lipids from liquid crystalline state to colloidal gel state and therefore led to decreased fluidity and increased permeability of cellular membranes. On the other hand, the MDA produced in peroxidation attacked the amino groups in proteins and caused intermolecular cross link, which caused loopholes on the membrane and therefore increased permeability. Many researchers have proposed in recent years that the damage to membranes were caused by the imbalance of free radical metabolism under drought stress, in which the free radicals were not completely eliminated and accumulated only to induce the peroxidation of membrane lipids and the inactivation of protective enzymes such as SOD, POD and CAT (Jiang and Guo, 1996; Wang, 1988; Chen, 1989). The present study indicated that the membrane permeability of pigweed leaf cells demonstrated an increasing tendency following the augmentation of drought intensity and prolongation. With the first 25 days after the exposure to drought stress, the increase of membrane permeability was not significant in the mild, moderate or severe exposure groups, which suggested that the cell membranes were sill maintaining a relative stability. However, as the exposure prolonged, the membrane permeability increased drastically in the moderate and severe exposure groups, implicating the cell membranes suffered great damage. The MDA content and membrane permeability were significantly correlated, in accordance with the decrease in the activities of SOD, POD and CAT.

A lot of work on the mechanism of drought damage to plant have been done, (Li, 2007; Zeng et al., 2007; Pallavi and Rama, 2005; Liu, 2008; Jan, 2006; Toumi et. al., 2008). At present, the theory of ROS injury to the plant cell membranes attracted the most attention. It is generally believed that the integrity and function of plant cell membrane is directly related to the accumulation of ROS and a large number of the active oxygen enhanced membrane lipid peroxidation under arid conditions. But some researchers considered that it might be reactive oxygen species activating phospholipase activity, thereby contributing to the decrease of membrane phospholipids, but this still needs a systematic research to verify. Compared with previous work, this paper focus on the effects of drought stress on plant cell membrane from the angle of reactive oxygen species on phospholipase activity and the role of membrane lipid peroxidation. The result could be used as a new direction for studying the mechanism of the reactive oxygen species injury to plant cell membranes.

Conclusion

A model of drought on pigweed and the effects of drought stress on the activity of acid phosphatase and its protecttive enzymes were created. The present results clearly demonstrated that (1) the drought stress significantly elevated the activity of acid phosphatase, membrane permeability and MDA contents; though the activity of acid phosphatase fell back afterwards while the extent of membrane permeability and MDA contents continued to increase with time. The membrane permeability and MDA contents were correlated. (2) The activity of protective enzymes such as SOD, POD and CAT was also enhanced with an increase in the intensity of drought stress or the prolongation of drought stress, which decreased some time afterwards. (3) Both the peroxidation and degrease reactions of membrane phospholipids were started in pigweed leaves under drought stress. (4) Drought stress caused the elevation of the activity of acid phosphatase, membrane permeability and MDA in the pigweed plants, which was able to resist to some extent drought stress by enhancing the activity of protective enzymes. However, excessive drought stress markedly affected the activity of protein metabolism system and decreased the activity of protective enzymes. (5) Both drought and high salinity can exert osmotic stress on plants and are most limiting factors for crop productivity. A plant like Pigweed is not only drought resistant, but also salt-tolerant and can be used as a complementary model for studying plant responses to osmotic stress. The study of pigweed on drought resistant is still at the initial stage. The coincidence of our results with the results of Cao et al. (2006) and Liu et al. (2008) indicated that they may share the same signaling pathways of drought resistant mechanisms and this should be further examined.

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