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Research Report

Effects of Drought Stress on the Antioxidant Systems in Three Species of *Diospyros* L.

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Abstract. Drought stress is a limiting factor for plant cultivation in many areas of China and persimmon has good capacity to withstand water shortage. Three species of *Diospyros* (three accession of *Diospyros lotus*, referred to as accession No. 824, 846, and 847; one accession of *Diospyros kaki* var. *sylvestris* referred to as 869; and one accession of *Diospyros virginiana* referred to as 844) were chosen for drought stress analysis. We withheld water from healthy two-year-old potted seedlings for 20 days, and compared the effects of water stress on malondialdehyde (MDA), superoxide free radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), antioxidative enzyme, glutathione (GSH), and ascorbic acid (AsA) levels in the leaves of the three species. In the treatment group, water stress increased the membrane lipid peroxidation in the three species, but a more significant increase was observed in the *D. kaki* var. *sylvestris* 869 than in the *D. virginiana* 844. Moreover, accumulation of $O_2^{\cdot-}$ and H_2O_2 was faster in weakly drought-resistant hybrids than in the highly drought- resistant hybrids. Though the activities of antioxidant enzymes and antioxidant contents were increased in the three species under drought stress, the activities of superoxide dismutase, catalase, peroxidase and ascorbate peroxidase were stimulated to a greater extent in *D. virginiana* 844 than in *D. lotus* and *D. kaki* var. *sylvestris* 869. In addition, the GSH, AsA, and relative water contents were increased significantly in *D. virginiana* 844, but not in *D. kaki* var. *sylvestris* 869. In the control group, no significant changes in lipid peroxidation and relevant antioxidant parameters were detected among the three species. These results indicated that changes of MDA, $O_2^{\cdot-}$, and H_2O_2 content, antioxidative enzyme activities, and GSH and AsA concentrations were correlated to drought resistance in the different species. *D. virginiana* 844 had a higher antioxidation capacity in response to drought than the other two species.

***Additional key words*:** antioxidant content, antioxidant enzyme, persimmon rootstock, reactive oxygen species, water stress

Introduction

Abiotic stress is a major limiting factor for plant cultivation in many areas all over the world. Its effects will become more severe as desertification claims the vast majority of the world's arable land. Among the various environmental stresses, drought has the greatest effect on agriculture worldwide (Hayano-Kanashiro et al., 2009). Many studies have focused on the responses of physiological and biochemical parameters to drought stress in fruit crops, including apple (Wang et al., 2012; Zhang et al., 2010), olive (Boussadia et al., 2008), coconut (Gomes et al., 2010), and watermelon (Yoshimura et al., 2008). For drought stress, plants have their own resistance mechanisms such as osmotic adjustment, photosynthesis, and enhanced antioxidative capacity. Analyses of these protective strategies provide us with a basic understanding of the tolerance and resistance of plants. Great efforts have been made to improve plant resistance to water stress, but little is known about how this trait is conferred due to the complexity of droughts, different species, genotypes, developmental phases, and the adaptation to and duration of the droughts (Li et al., 2009; Xiong et al., 2006).

When being subjected to drought stress, plants increase their antioxidant capacity to adapt to the environment (Li et al., 2009; Yao et al., 1993). It is well known that scarcity of water causes an overproduction in plants of reactive oxygen species (ROS), which are highly reactive and toxic and can lead to damage to proteins, lipids, cellular structures, and carbohydrates while reacting with all components of the DNA (Halliwell, 2009; Miller et al., 2010). The ROS includes

superoxide radicals ($O_2^{\cdot-}$), alkoxy radicals (RO·), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH·). An over accumulation of ROS among the various environmental stresses is a major factor for the loss of crop productivity around the world (Khan and Sarvajeet, 2008; Sarvajeet et al., 2011; Tuteja, 2010).

Antioxidant defense machinery protects plants against oxidative stress damages. ROS accumulation induced by various stresses is counteracted by antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), peroxidase (POD), glutathione reductase (GR), glutathione peroxidase (GPX), guaiacol peroxidase (GOPX), and glutathione-*S*-transferase (GST), and nonenzymatic low molecular-weight metabolites including ascorbic acid (AsA), glutathione (GSH), alkaloids, phenolic compounds, nonprotein amino acids, and α-tocopherol, and β-carotene (Mittler et al., 2004; Sarvajeet et al., 2011). These enzymes and metabolites not only protect plants from cellular damage, but also fine tune the concentrations of ROS in order to optimize their functions in metabolism (Becana et al., 2010). Since high concentrations of ROS are harmful for plant cells, many recent studies have focused on the ROS in a steady-state level as the substrates and signals in plant cells (Foyer and Shigeoka, 2011; Sarvajeet and Tuteja, 2010) that engage in the control of plant growth and development and as beneficial transduction signaling molecules during normal metabolism and in response to different stresses (Nauseef, 2008).

The antioxidant capacity of the persimmon is higher than that of apple, grape, tomato, blueberry, and strawberry (Chen et al., 2008; George and Redpath, 2008). There are many valuable persimmon germplasm resources in China. *Diospyros lotus* was used frequently as the rootstock for persimmon in the North of China due to its hardiness and capacity for drought tolerance, whereas *Diospyros kaki* var. *sylvestris* is generally used as a kind of rootstock for persimmon in the South of China. *Diospyros virginiana* originated from America and now is used in Israel as a rootstock for Japanese persimmon and is particularly well adapted to damp soil and very cold-hardy (Tao and Sugiura, 1992). In this study, the three species, which are cultivated in different regions, were selected to determine how the different accessions respond to the lack of water.

Materials and Methods

Plant Material and Growing Conditions

Five accessions of three species, *D. lotus*, *D. kaki* var. *sylvestris,* and *D. virginiana,* were employed in this study (Table 1). All the trees were grown in the National Field Genebank for Persimmon of Northwest A&F University, located in Yangling (34′20″ N, 108′24″ E), Shaanxi Province, China. Standard horticultural practices and pest management were applied to all trees. Sixty uniform one-year-old seedlings per species, which came from seeds sown the previous spring, were transplanted to plastic pots (30 cm in diameter and 50 cm in height) filled with a garden soil/sand mixture (3:1) in the early spring of 2012. All the pots were maintained under greenhouse conditions with a relative humidity of 60-75%, at temperatures from 15 to 37°C and were irrigated daily to maintain the soil water content close to field capacity.

Experimental Design

At the beginning of our water-stress treatments (September 2012), 30 plants of the same size (about 0.9 m tall) per species were selected for the experiment, with 10 being used as the controls and 20 being used for the drought treatment. The control trees were irrigated daily to maintain saturated soil water content. In a preliminary trial, we had determined that it was 20 days after treatment (DAT) at which leaves showed extreme wilting. The fifth to seventh fully expanded leaves were collected from five plants with same soil water content at 10:00 am at 4-day intervals. The collected leaves were immediately frozen in liquid nitrogen and stored at -70°C for subsequent analyses of enzyme activity and antioxidant concentrations. The recovery experiment was performed on the seedlings of three persimmon species at 20 DAT, and we investigated the survival rate of the three species.

Table 1. Materials used for drought treatment

Persimmon species	Accession No.	Chromosome number	Location of the species of original collection	Hundred-grain weight of experimental seeds (g)	Color and luster of experimental seeds	Average fruit weight (g)	Fruit shape
D. Lotus	824	2n=2X=30	Mei county, Shanxi, China	13.7	dark and glossy	5.34	circular
D. Lotus	846	2n=2X=30	Mei county, Shanxi, China	16.4	dark and glossy	5.74	circular
D. Lotus	847	2n=2X=30	Mei county, Shanxi, China	14.7	brown and dull	6.40	circular
D. kaki var. *sylvestris*	869	2n=6X=90	Zhejiang, China	38.8	dark and glossy	15.32	oblate
D. virginiana	844	2n=6X=90	Israel	42.1	dark and glossy	18.34	oblate

Measurement of Leaf Relative Water Content and Soil Water Content (v/v)

The method for determining leaf relative water content (RWC) was as described by Barrs and Weatherley (1962). The RWC of the seventh leaf from each species subjected to drought and normal watering was measured at the same time at 4-day intervals. The RWC was calculated as follows:

$$RWC(\%) = \frac{FW - DW}{TW - DW} \times 100,$$

where FW = leaf fresh weight, DW = leaf dry weight, and TW = leaf turgid weight. After the fresh tissue was weighed, the same leaf was floated on deionized water for 12 h under low irradiance, and then, the turgid tissue was quickly blotted dry prior to determine its turgid weight. Dry weight was determined after oven drying the leaf at 80°C for 72 h until it reached a constant weight. Soil moisture contents (v/v) were obtained daily by an HH2 Moisture Meter (Delta-T Devices, Cambridge, UK).

Measurement of Lipid Peroxidation, H_2O_2, and $O2^{\cdot-}$ Generation

The level of lipid peroxidation in the leaf tissue was measured in terms of malondialdehyde (MDA) content as described by Gao (2000). A total of 0.5 g leaf sample was used. The absorbance value at 532 nm and the value for the nonspecific absorption at 600 nm were detected to calculate the MDA content using its extinction coefficient of 155 $mM^{-1} \cdot cm^{-1}$.

The H_2O_2 concentration and $O_2^{\cdot-}$ generation rate were detected using the methods described by Alexieva et al. (2001) and Gao (2000), respectively. For H_2O_2 concentration measurements, a 0.5 g leaf sample was homogenized in 5 mL ice-cold 3% trichloroacetic acid followed by centrifuging at 12,000 × *g* for 20 min at 4°C. A total of 1 mL supernatant was transferred to a new tube and mixed with 0.1 mL 10 mM PBS (pH 7.0) and 2 mL 1 M potassium iodide. The absorbance of the solution was read at 410 nm.

For $O_2^{\cdot-}$ generation ratio detection, another 0.5 g leaf sample was ground with a pestle in an ice-cold mortar with 4 mL 65 mM pre-chilled PBS buffer. The homogenate was centrifuged at 12,000 × *g* for 20 min at 4°C. Meanwhile, a mixture containing 0.1 mL 10 mM hydroxylamine hydrochloride and 0.5 mL 65 mM PBS (pH 7.8) was prepared and incubated for 10 min at 25°C. After centrifugation, 0.5 mL supernatant was added into the above mixture and incubated for 20 min at 25°C. Then 1 mL of 17 mM sulfanilamide and 1 mL 7 mM α-naphthylamine were added and incubated for another 20 min at the same temperature. The absorbance value of the final mixture was read at 530 nm. Both the H_2O_2 concentration and $O_2^{\cdot-}$ generation rate were calculated according to the standard curve method based on the respective absorbance value.

Enzyme Activity Determination of SOD, CAT, POD, and APX

Samples (0.5 g) were ground in a chilled mortar with a pestle and then homogenized in 8 mL of 50 mM pre-cooled potassium phosphate buffer (pH 7.8), which contained 0.1 M ethylenediaminetetraacetic acid (EDTA) and 1% (w/v) polyvinylpyrrolidone (PVP). The homogenates were centrifuged at 13,000 × *g* for 20 min at 4°C, and the supernatants were used for the enzyme assays are described below.

The SOD activity was assayed by the nitroblue tetrazolium (NBT) method. The 3 mL reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 75 μM NBT, 20 μM riboflavin, 0.1 mM EDTA-Na_2, and 25 μL enzyme extract. The reaction mixture without an enzyme was used as the control. All the reaction was initiated by adding riboflavin and mixing well in the dark. Then, the reaction mixtures in the glass test tubes were irradiated under fluorescent lamps (4000 lx) for 36 min. After the color of the reaction mixture became blue black from yellow, the absorbance was read at 560 nm. The reaction mixture without enzyme and illumination was used to zero the absorbance at 560 nm. One unit of SOD activity was defined as the amount of enzyme that caused 50% inhibition in the reduction of the NBT as monitored at 560 nm.

The CAT activity was analyzed following the method described by Gao (2000) with a little modification. The reaction solution (3 mL) contained 50 mM PBS (pH 7.0) with 15 mM H_2O_2 and 50 μL enzyme extract. The reaction was started by the addition of the H_2O_2. Absorbance changes at 240 nm were followed for 3 min. One unit of CAT activity was defined as the changes in absorbance of 0.01 $U \cdot min^{-1}$.

The activity of the POD was determined by the method of Gao (2000) with some modifications. The 3 mL reaction mixture contained 100 mM PBS (pH 6.0), 25 mM guaiacol, 15 mM H_2O_2, and 50 μL enzyme extract. The reaction was initiated by the adding H_2O_2. The increase in the absorbance at 470 nm was followed for 6 min. Decomposition of 1 mM H_2O_2 min^{-1} was defined as one unit of the POD.

The APX activity was estimated according to Nakano and Asada (1981). Monitoring was done to observe the decrease in absorbance at 290 nm as the ascorbate was oxidized. The reaction solution contained 50 mM PBS (pH 7.0), 9 mM reduced ascorbate (ASA), 15 mM H_2O_2, and 50 μL enzyme extract in a final volume of 3 mL. The reaction was started by adding H_2O_2. Measurements that showed 1 mmol ascorbate oxidized per min was defined as one unit of APX activity.

Determination of Ascorbic Acid and GSH

Leaf tissue (0.5 g) was ground in a cooled mortar and homogenized in 3 mL of pre-chilled metaphosphoric acid (5%, w/v) and then centrifuged at 14,000 ×*g* for 10 min at 4°C; the supernatant was collected for analysis of the AsA and GSH content. The AsA and GSH content were measured by the methods described by Tanaka et al. (1985) and Lu et al. (1999), respectively. The 3 mL reaction mixture of AsA contained 75 mM sodium dihydrogen phosphate (pH 7.4), metaphosphoric acid (10%, w/v), phosphoric acid (44%, w/v), 2,2-bipyridine (4%, w/v), ferric trichloride (3%, w/v), and 200 μL enzyme extract, which was mixed well. The mixture was incubated for 60 min at 37°C, and measurements were calibrated. The 3 mL reaction mixture of GSH contained 150 mM sodium dihydrogen phosphate (pH 7.7); 5,5-dithiobis-nitrobenzoic acid (DTNB); and 200 μL enzyme extract, which were mixed well. The mixture was incubated for 5 min at 30°C, and measurements were made on a spectrophotometer at 412 nm. A standard curve of the GSH was used for calibration.

Results

Effects of Drought on Leaf RWC and Soil Water Content

In this study, results showed that the leaf RWC and soil water content decreased under drought stress. For the control plants of each species, the soil water contents were very similar: 37.0% at a depth of 10 cm during the entire period (Fig. 1A). Under stress conditions, the soil water content of all species decreased compared with the controls. The soil water content of the three species decreased gradually with the increase of the extent of the drought until 20 DAT, when soil water content reduced from 37.0% to 15.0-20.0%. The three species differed in soil water content but not significantly.

In the control group, the plants from the three species had similar and stable leaf RWC, which was about 90.0% during the entire experimental period. After receiving the drought treatment, *D. kaki* var. *sylvestris* 869 and *D. virginiana* 844 exhibited decreased leaf RWC by 53.35 and 12.81%, respectively, at 20 DAT (Fig. 1B). Among the three accessions of *D. lotus*, the strongest and the earliest decrease in the RWC was observed in 846, which decreased by 40.74%, followed by 847 and 824, which decreased by 23.07 and 17.07%, respectively. *D. virginiana* 844 had more ability to maintain leaf RWC than the other two species among the three species.

Lipid Peroxidation

The MDA concentrations in the leaves of the three species

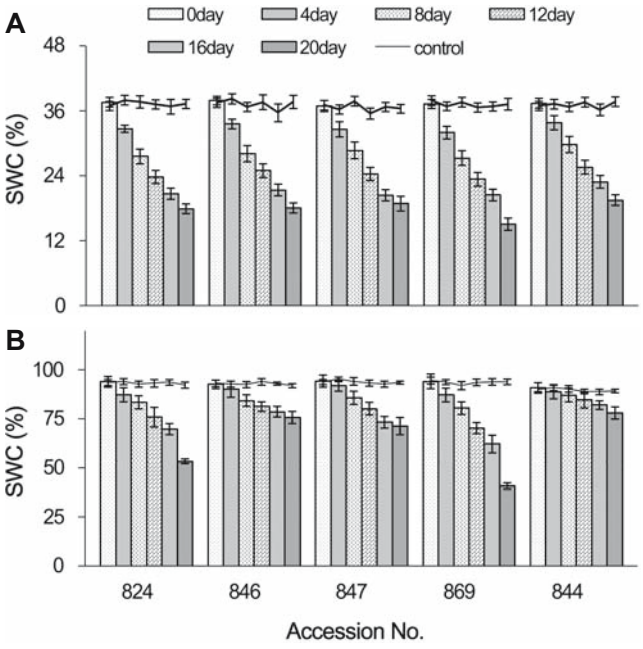

Fig. 1. Effects of drought stress on the soil water content (SWC) (A) and relative water content (RWC) (B) in the leaves of three species of persimmon rootstocks.

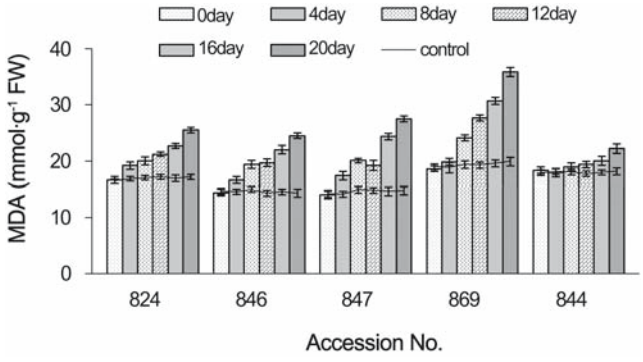

Fig. 2. Effects of drought stress on malondialdehyde (MDA) content in the leaves of three species of persimmon rootstocks.

all increased with water stress (Fig. 2). The MDA level in both *D. virginiana* 844 and *D. kaki* var. *sylvestris* 869 was high at 20 DAT and had increased by 21.15 and 92.39%, respectively. In *D. lotus*, the MDA content of 824, 846, and 847 increased by 52.92, 69.35, and 95.79%, respectively. The MDA concentration in *D. virginiana* 844 was higher than that in *D. lotus* under controlled conditions, but the extent of growth of the MDA content in *D. virginiana* 844 was much lower than in *D. lotus* under stress. This indicated that leaf cells of *D. kaki* var. *sylvestris* 869 were more easily damaged compared with other species.

Generation of H_2O_2 and $O2^{-}$

In a normally managed environment, changes in the H_2O_2 content in the leaves of the three species were not obvious

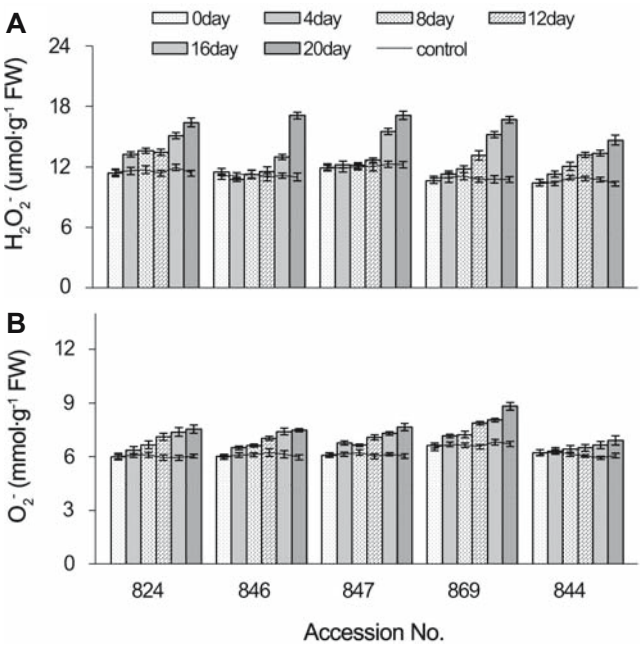

Fig. 3. Effects of drought stress on peroxide (H_2O_2) concentration (A) and superoxide free radical ($O2^-$) formation (B) in the leaves of three species of persimmon rootstocks.

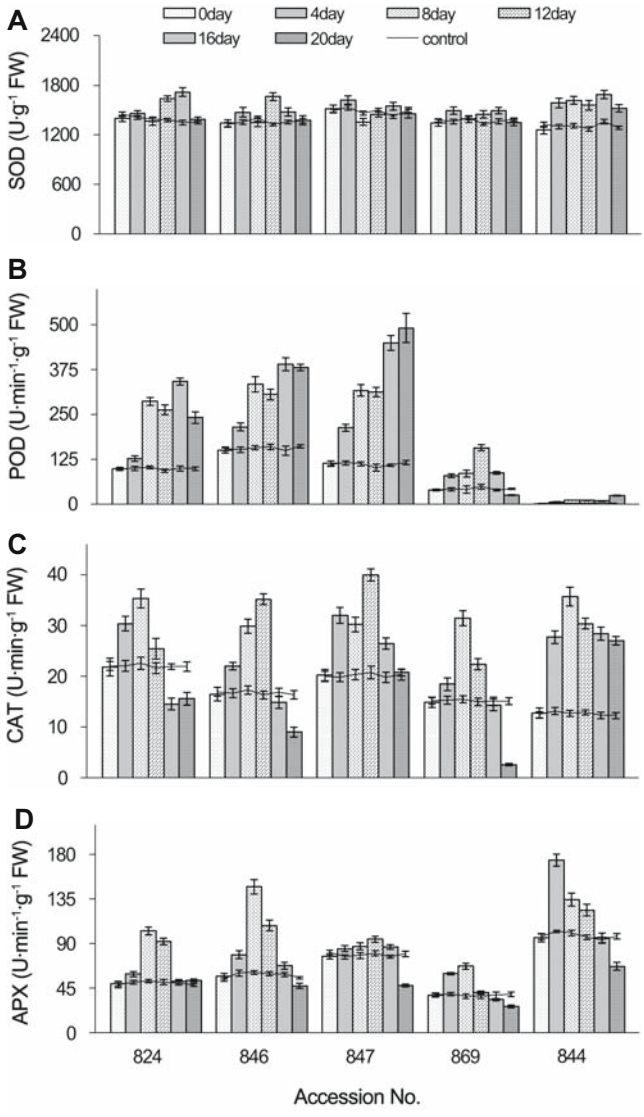

Fig. 4. Effects of drought stress on activities of superoxide dismutase (SOD) (A), peroxidase (POD) content (B), catalase (CAT) (C), and ascorbate peroxidase (APX) (D) in the leaves of three species of persimmon rootstocks.

(Fig. 3A). When subjected to drought stress, the H_2O_2 concentration in the leaves of all the species increased gradually (Fig. 3A). Furthermore, at 20 DAT, the H_2O_2 content in *D. virginiana* 844 and *D. kaki* var. *sylvestris* 869 had increased by 40.66 and 57.01% compared with that of the control plants, respectively. In *D. lotus*, the H_2O_2 concentration had increased by 44.27, 48.94, and 43.83% in 824, 846, and 847, respectively, at 20 DAT. These results indicate that the tolerance of the three species to reactive oxygen had moderate interspecific differences, and *D. kaki var. sylvestris* 869 accumulated more H_2O_2 than that by the other two species.

Production of $O_2^{\cdot-}$ remained nearly unchanged in the leaves of the three species over time under control conditions (Fig. 3B). The $O_2^{\cdot-}$ concentration in the leaves of the three species showed similar patterns under conditions of drought (Fig. 3B). The $O_2^{\cdot-}$ content in both *D. virginiana* 844 and *D. kaki* var. *sylvestris* peaked at 20 DAT, and was higher than that of the control plants by 11.07 and 33.01%, respectively. Among the three accessions in *D. lotus*, levels of $O_2^{\cdot-}$ in 824, 846, and 847 increased by approximately 25.48, 23.96, and 25.92%, respectively. This indicated that the stress induced all species to produce and accumulate $O_2^{\cdot-}$, and that *D. virginiana* 844 had a greater ability to scavenge reactive oxygen compared with the other two species.

Antioxidant Enzymes

Activities of antioxidant enzymes showed significant changes in the three species after water treatment. The stress-induced activity of the SOD in the leaves was obviously different for the three species (Fig. 4A). The curve of activities of antioxidant enzymes of *D. virginiana* 844 showed two peaks, at 8 DAT and 16 DAT, which was marked by approximately 28.55 and 33.91%, respectively. The curve of *D. kaki* var. *sylvestris* 869 had a peak 16 DAT, which is higher than that of the control by 10.94%. In *D. lotus*, the SOD activity of the leaves for the three accessions, 824, 846 and 847, increased until 16, 12, and 4 DAT, by 22.75, 24.04, and 6.98%, respectively and then, they decreased. The SOD levels were similar for the controls for all accessions.

The POD activity increased sharply in the leaves for the three species within 16 days of beginning the drought treatment, except for *D. kaki* var. *sylvestris* 869 (Fig. 4B). In *D. virginiana*

844, the POD activity increased to a maximum level at 20 DAT and was approximately nine-fold higher than that of the control plants, but its activity was much lower than that of the other two species. The POD activity in *D. kaki* var. *sylvestris* 869 was a little higher than that in *D. virginiana* 844, but was much lower than that in *D. lotus*. In *D. lotus*, both 824 and 846 reached their highest POD activity 16 DAT, which was higher than the corresponding controls by 244.77 and 159.05%, respectively. The POD activity in 847 increased consistently over the course of the entire experiment, and finally, POD activity was fourfold that of the control.

When plants were subjected to water stress, the CAT activity in the leaves increased to the maximum and then declined. The changing pattern of the APX activity was similar to that of CAT (Fig. 4C). CAT activity in both *D. virginiana* 844 and *D. kaki* var. *sylvestris* 869 peaked 8 DAT, with levels exceeding those of their corresponding control plants by 179.17 and 110.71%, respectively. In *D. lotus*, the CAT activity of 824 was the highest among all three accessions of *D. lotus* and, reached the maximum at 8 DAT, which was higher than that of the control by 61.71%; both *D. lotus* 846 and *D. lotus* 847 reached the maximum at 12 DAT and exceeded CAT activity levels of their controls by 112.90 and 97.37%, respectively.

APX activity increased by 81.16% in *D. virginiana* 844 at 4 DAT and by 77.46% in *D. kaki* var. *sylvestris* 869 at 8 DAT, respectively, after drought treatment (Fig. 4D). Meanwhile, the APX activity in both *D. lotus* 824 and 846 peaked at 8 DAT, showing increases of 107.53 and 158.22%, respectively, and in *D. lotus* 847, the APX activity increased by 22.07%, with the curve peaking 12 at DAT. Although, the speed of the APX activity increase in *D. virginiana* 844 was slightly less than that in *D. lotus*, the APX activity of *D. virginiana* 844 was much higher than in the other two species.

AsA and GSH Concentrations

Under water stress, ascorbate concentrations in the leaves of the three species reached the maximum value and then decreased. Furthermore, the content of the AsA in the leaves of *D. virginiana* 844 was higher than in the other two species for the entire experiment. The AsA content in *D. virginiana* 844 and *D. kaki* var. *sylvestris* 869 peaked at 12 and 8 DAT, which showed an increase of 93.04 and 38.48%, respectively, before decreasing. The AsA content in *D. lotus* 824, 846, and 847 increased by 34.76, 113.46, and 86.47%, respectively, and all *D. lotus* accessions peaked at 12 DAT (Fig. 5A).

The changing pattern of the GSH concentration was similar to that of the ASA concentration in the leaves for all species. The GSH content increased by 38.97% in *D. virginiana* 844 at 8 DAT and by 43.16% in *D. kaki* var. *sylvestris* 869 at 12 DAT, and then it decreased in both. The GSH content in *D. lotus* 824, 846, and 847 all reached peaks at 12 DAT, which increased by 41.14, 50.91, and 34.18%, respectively (Fig. 5B).

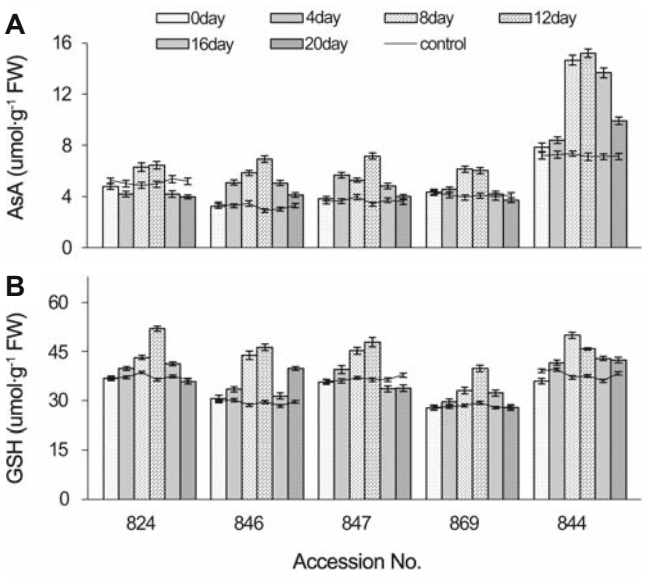

Fig. 5. Effects of drought stress on ascorbic acid (AsA) content (A) and glutathione (GSH) content (B) in the leaves of three species of persimmon rootstocks.

Recovery experiment

All *D. virginiana* 844 plants survived in the recovery experiment, which was conducted at 20 DAT; the survival rates of *D. lotus* 824, 846, and 847 were 60.0, 70.0, and 70.0%, respectively. Among the three studied species, *D. kaki* var. *sylvestris* 869 had the lowest survival rate, at only 45.0%. From the recovery experiment result, *D. virginiana* 844 was found to be the species with the strongest drought resistance.

Discussion

Relative water content was used to measure drought stress in field-grown barley across Mediterranean environments (Teulat et al., 2003) and was shown to be an appropriate parameter for representing the stress level of plants. After drought, the high leaf water content of a plant is usually due to its strong water holding ability; likewise, a lower degree of damage in the cell membrane is due to its strong drought resistance. Rampino et al. (2006) found that RWC was the first indication that wheat was undergoing drought. The results in our experiment also showed that with the increase of stress in drought treatment, the RWC of *D. kaki* var. *sylvestris* 869 decreased quickly and showed the lowest RWC value, whereas *D. virginiana* 844 had the highest RWC among the three different *Diospyros* species. The RWC level can be reflected in the morphological appearance of the potted

seedlings, and *D. virginiana* 844 has the strongest drought resistance.

When a plant experiences drought, membrane lipid peroxidation is initiated by the excessive activated oxygen generated in many tissues in the plants (Sairam et al., 2005), which would produce the MDA (Munné-Bosch et al., 2001), and the MDA content reflects the degree of membrane lipid peroxidation. As the MDA accumulates, the defense ability of the plant declines. In previous studies, the maintenance of low levels of MDA was related to high drought resistance in plants (DaCosta and Huang, 2007; Moran et al., 1994; Sánchez-Rodríguez et al., 2010, 2012). The MDA content of the plants could be increased in apple (Wang et al., 2012) and wheat (Esfandiari et al., 2007) after water stress. In our study, MDA content was increased to a different degree in the three species of *Diospyros* under stress; our results showed that the five accessions of these three different species responded differently to drought. The extent of the increase in leaf MDA contents in *D. virginiana* 844 was the lowest among the three species. The MDA content in *D. kaki* var. *sylvestris* 869 increased gradually with the increase of water stress, and was always higher than for the other two species for the entire experiment, indicating that *D. virginiana* 844 had higher drought-resistance than the other two species.

Many studies have shown that $O_2^{\cdot-}$ accumulates excessively in plant cells under stress, and the superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) were three major protective enzymes needed to scavenge reactive oxygen. When plants are subjected to drought, the SOD dismutatd the $O_2^{\cdot-}$ into H_2O_2 (Bowler et al., 1992), and the SOD activity shows different changes according to the plant species, intensity, and duration of drought. The SOD activity increased in two clones of *Coffea canephora* under drought stress, and the activity of the SOD in the drought-tolerant clone120 was higher than that of drought-sensitive clone109A (Lima et al., 2002). DaCosta and Huang (2007) discovered that SOD activity of three bentgrasses all decreased, and the smallest decrease in SOD activity was found in drought-tolerant *Agrostis canina velert* under water stress. The SOD activity in *Sorghum* was almost unchanged under moderate water stress (Zhang and Kirkham, 1996). Our experimental results showed that SOD activity of the three species increased gradually with increasing stress and time. The biggest increase of SOD activity was found in *D. virginiana* 844 (by 33.91%), followed by the three accessions of *D. lotus* (824 increased by 22.75%, 846 increased by 24.04%, and 847 increased by 6.98%), with *D. kaki* var. *sylvestris* 869 having the least increase (10.94%). Our results were consistent with other studies on persimmon, wheat, apple, cool-season grass, Phaseolus acutifolius, and Phaseolus vulgaris (Yao et al., 1993；Esfandiari et al., 2007; Wang et al., 2012; Huang and Fu , 2001; Turkan et al., 2005).

The CAT and POD converted H_2O_2 into H_2O (Apel and Hirt, 2004; Bowler et al., 1992). Under drought, the CAT activity was increased in some plants (Fazeli et al., 2007; Zlatev et al., 2006), decreased in others (DaCosta and Huang, 2007; Yao et al., 1993; Zlatev et al., 2006), and remained stable in some others (Esfandiari et al., 2007; Turkan et al., 2005). In our study, the largest increase in the CAT activity among the three species was in *D. virginiana* 844 and increased by 179.17% 8 DAT, while CAT activity in other species was just beginning to decline at 12 DAT. For POD activity, *D. virginiana* 844 and 847 of *D. lotus* increased consistently during the entire period of stress, and the other two accessions of *D. lotus* and *D. kaki* var. *sylvestris* 869 peaked at 16 and 12 DAT, respectively. For *D. virginiana* 844, the POD activity was much lower than in the other two species, which indicated that in *D. virginiana* 844, POD played a less important role in scavenging ROS, and this conclusion was consistent with findings in a study on spinach (Tanaka et al., 1990) and *S. pallescens Freyn* (Castillo, 1996). The experimental results show that the increased activities of protective enzymes were the kinds of adaption to mild and moderate water stress, which can be used effectively to scavenge ROS and improved the drought tolerance of plants. When the levels of stress became severe, a decrease in the activities of the protective enzymes and the excessive accumulation of $O_2^{\cdot-}$ and H_2O_2 disturbed the balance between the production of ROS and an effective antioxidant system (Breusegem et al., 1998).

Antioxidants (glutathione GSH and ascorbic acid AsA) were still required for plants to scavenge ROS. AsA played an important role in reducing the lipid peroxidation (Candan and Tarhan, 2003), which was not only used as a substrate for ascorbate peroxidase (APX) in the Hallwell-Asada cycle, but also acted directly as antioxidants in scavenging ROS (Gupta et al., 1993). The result showed that APX activity increased gradually under water stress in three species with the extension of time and increasing stress. $O_2^{\cdot-}$ and H_2O_2 accumulated rapidly, which led to the consumption of APX, GSH, and AsA, largely to for use in scavenging ROS, and this also resulted in a decrease in APX and antioxidants. *D. virginiana* 844 had higher contents of APX, AsA, and GSH, and had a much stronger ability to scavenge ROS as well.

In this study, the $O_2^{\cdot-}$ and H_2O_2 contents of the three species was increased with the increase in stress. The drought-sensitive seedlings *D. kaki* var. *sylvestris* 869 accumulated most of the $O_2^{\cdot-}$ and H_2O_2 contents, while accumulation of $O_2^{\cdot-}$ and H_2O_2 concentrations in the drought-tolerant *D. virginiana 844* seedlings were the lowest. The same trend was also found in the tomato (Sánchez-Rodríguez et al., 2012) and apple (Ma et al., 2008; Wang et al., 2012). The rate of generation of $O_2^{\cdot-}$ in the leaves of the seedlings started to increase at 4 DAT, while the SOD activity increased,

and the H_2O_2 concentration was transformed by the $O_2^{\cdot-}$, which was certain to increase, as the POD activity, the AsA, and the GSH increased and enhanced scavenging ability for H_2O_2, which prevented the H_2O_2 content from increasing rapidly. After moderate stress at 12 DAT, the generation rate of $O_2^{\cdot-}$ continued to increase, the SOD activity also increased further, while the CAT activity and GSH decreased significantly. Although the POD activity increased significantly, the production of the ROS was beyond the capacity of the plant to clear, which led to the accumulation of too much ROS. Once the stress had continued until 20 DAT, the metabolism in the plant tended to become erratic, so that the expression of the SOD and POD genes were inhibited, and the SOD and POD activity declined.

In this study, we compared the responses of three species of *Diospyros* L. persimmons to drought stress by withholding water from two-year-old potted healthy plants for 20 days. Then, we identified and compared the effects of water stress on the MDA, $O_2^{\cdot-}$, H_2O_2, antioxidative enzymes (GSH and AsA) in the leaves of the three species. Some differences were noted between the rootstock damage in the drought-sensitive and drought-tolerant species, and the main reason for the damage from water stress was disturbance to the oxygen metabolism. Higher protective enzyme activities along with more AsA and GSH contents in the drought-tolerant rootstock, allowed scavenging of the free oxygen, and therefore, reduced the damage to the membrane system. Under water stress, the $O_2^{\cdot-}$ generation rate, H_2O_2 content, MDA content, GSH content, AsA content, and activities of the protective enzymes in the leaves of the plants from different rootstocks were closely related to drought resistance.

The drought resistance of the persimmon rootstock is a complicated quantitative trait (Teulat et al., 2003), and the adaption mechanism of these plants to an arid environment is not very clear. We propose the discussion of drought resistance from a horticultural point of view. The leaf of *D. virginiana* 844 is smaller and thicker than those of the other two species. The young trees of *D. virginiana* 844 have taproots with good water retention capacity, but develops lesser amounts of fibrous roots than those of *D. lotus* and *D. kaki*, when the seedlings were subjected to water stress. Further, the water loss by transpiration from *D. virginiana* 844 leaves was lower than that from the other two species. From these observations, we confirmed that *D. virginiana* 844 has higher drought resistance than the other two species. Future studies should address the response of the persimmon rootstocks to water stress at different growth stages, the ROS concentrations that induce damage, and the mechanisms of the ROS-inducing antioxidant system.

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