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Full Length Research Paper

Effects of water stress and seed mass on germination and antioxidative enzymes of *Xanthoceras sorbifolia*

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The effects of water stress and seed mass on germination, as well as antioxidative enzymes, in *Xanthoceras sorbifolia* seed were studied. The germination percentage decreased gradually in all seeds with decreasing water potential. The reduction was more significant under -0.6 MPa treatment than under the -0.2 MPa treatment in all seeds. The germination percentage of the big seeds was the highest in all treatments and had the earliest initiated germination compared with those of the medium and small seeds. Lipid peroxidation (malondialdehyde), osmotic substances (free proline), and antioxidative enzymes (peroxidase, superoxide dismutase, and catalase) increased in all seeds with increasing water stress. The values of all parameters indicated that water was a critical factor in *X. sorbifolia* seed germination, and the big seeds displayed increased tolerance to water stress as measured by germination percentage, osmoregulatory substance, and antioxidant enzymatic activities.

Key words: Germination percentage, PEG 6000, malondialdehyde, free proline, antioxidant enzymes, *Xanthoceras sorbifolia.*

INTRODUCTION

Germination is a complex process that includes three phases, namely, imbibition, plato phase and radicle protrusion (Giba et al., 2004). The period of germination is one of the most sensitive life stages of a plant because this stage affects seriously seedling and crop establishment (Koochaki, 1991). The germination process is associated with various innate and external factors. Seed mass is positively related to germination, seedling survival, and seedling vigour (Shieh and Mcdonald, 1982; Zhang and Mann, 1990; Raveendranth and Singh, 1991; Cordazzo, 2002; Upadhaya et al., 2007; Aziz and Shaukat, 2010). Big seeds have an advantage over small seeds within a population during germination and seedling establishment, especially under limited resources (Jurado and Westoby, 1992). Bigger seeds require less time to germinate and achieve greater germination percentage compared with the small seeds in *Castanopsis chinensis* under limited light (Du and Huang, 2008). Apart from the innate mechanisms, various environmental factors, such as soil water, temperature, and light, disturb metabolic reactions and affect germination negatively (Basra and Basra.1997; Jevgenija and Gederts, 2007), which were reported in some species, such as *Artemisia spicigera* and *Artemisia fragrans* (Azarnivand et al., 2007), *Ziziphus lotus* (Maraghni et al., 2010), and wetland *Carex* species (kettenring et al., 2006). Water stress is one of the important restriction factors to seed germination because of the delay in its initiation or decrease in the final germinability (Hardegree and Ermmerich, 1990; Gorai et al., 2009; Mantovani and Iglesias, 2010). This phenomenon may be attributed to the increases in osmotic pressure and decreases in matric potential caused by water stress which leads to difficult germination (Naseri, 2003). Superoxide dismutase

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Abbreviations: PEG, Polyethylene glycol; **EDTA,** ethylenediaminetetraacetic acid; **MDA,** malondialdehyde; **CAT,** catalase; **POD,** peroxidase; **SOD,** superoxide dismutase; **PVP,** polyvinylpyrrolidone; **TCA,** trichloroacetic acid; **NBT,** nitroblue tetrazolium; **TBA,** thiobarbituric acid.

(SOD), peroxidase (POD), and catalase (CAT) are the main protective enzymes because they are engaged in the removal of free radicals and activated oxygen species (Blokhina et al., 2003). Reactive oxygen species (ROS) are accumulated during the imbibition phase of seed germination (Bailly, 2004). Seed germination essentially requires signaling (low levels) of ROS. Considering the role of antioxidant enzymes in controlling the ROS levels during germination, these enzymes are particularly important for the completion of germination (Roberts, 1972).

Although many studies have reported the effect of water stress or seed mass on germination and antioxidant enzymes in several seeds, such as *C. chinensis* (Du and Huang, 2008), *Ipomoea Sindica*, *Cleome viscose* and *Digera muricata* (Aziz and Shaukat, 2010), *Picea asperata* (Yang et al., 2010) and *Jatropha curcas* (Cai et al., 2011), little is known about *Xanthoceras sorbifolia*. In addition, few researchers investigated on the effect of interaction between water levels and seed mass to germination and antioxidant enzymes. *X. sorbifolia* seeds were examined to investigate the individual, as well as combined, influence of water levels and seed mass on germination and antioxidant enzymes of *X. sorbifolia*.

This plant, an oil shrub or small tree endemic to China, is distributed naturally in many areas in China (28°34' to 47°20' N , 73°20' to 120°25' E). The northern Loess Plateau of China, a shrub state in the given region, is the main distribution area of *X. sorbifolia*. The species has a well-developed root and is a perfect ecology plant for the conversion of cropland to forest as well as for barren hill management in the northern Loess Plateau of China. Moreover, this species is an economic plant because its seeds have significant oil content, which is a suitable raw material for the production of biodiesel (Wang et al., 2011), edible oils, and medicine (Hou et al., 2011). However, few seedlings could be currently found among the undergrowths in the northern Loess Plateau of China, which might have been caused by low germination percentage and high seedling mortality of the *X. sorbifolia* forest. The northern Loess Plateau of China is dry and has little rain, resulting in water as a limiting factor to plant growth. *X. sorbifolia* seed varies greatly in mass. Thus, whether water and seed mass influence the germination of *X. sorbifolia* in this region must be determined. The primary objectives of this current study were as follows: (1) to analyze individually the effect of water stress and seed mass as well as their interaction on the germination of *X. sorbifolia*; (2) to analyze the seed germination physiology that can help in the evaluation of safe-site availability because seed germination trait determines the number and timing of seedling emergence, and (3) to offer a reference opinion for plantation management of *X. sorbifolia* in the northern Loess Plateau of China. Significant economic benefits are presented by the results because seed germination is a major factor that affects the cost such that low germination rate requires more seed, resulting in a more expensive production. In additional, these results have important ecological significance to vegetative recovery in the northern Loess Plateau of China.

MATERIALS AND METHODS

Plant materials

X. sorbifolia seeds were harvested in July 2009 from the northern Loess plateau (35°45′ N, 109°10′ E), Fu county, Shanxi province, China. The seeds were divided into the following three categories based on mass variation: small (<0.6 g), medium (0.6 g to 1.0 g) and big (<1.0 g). The three seed categories required a dormancy-breaking treatment by wet sand storage at 4°C prior to experiment.

Stress treatment

The seeds of sand storage were surface sterilized with 2% potassium permanganate solution and then rinsed thrice with distilled water. *X. sorbifolia* seeds were sowed in 200 mm diameter glass Petri dishes. Filter paper sheets that were previously autoclaved and moistened with 20 ml of test solution with 0.2% nystatin were used as a substrate (Silva et al., 2001). Germination tests were conducted at 25°C with 12 h of light exposure (100 µmol m^{-2} $s^{-1)}$. Osmotic potentials of -0.2 and -0.6 MPa for polyethylene glycol (PEG 6000) were considered as slight and moderate water stress (Kaya et al., 2006; Zhu et al., 2005). Each Petri dish with 50 seeds was tested for germination in five replicates. Germination was observed after every 24 h. The authors sampled and determined the biochemical parameters after the seeds were treated for 15 days. The germination criterion was the emergence of the radicle from the seed coat. Germination percentage = SN_1 / SN_0 × 100, where SN_1 is the number of germinated seed, and SN_0 is the total number of seeds (50 seeds).

Lipid peroxidation

Lipid peroxidation was determined by estimating the malondialdehyde (MDA) content according to the method of Zhang et al. (2010). A sample containing 0.5 g of plant material was mixed with 5 ml of 5% trichloroacetic acid and centrifuged at 12,000 ×*g* for 25 min. Two milliliters of the supernate was mixed with 2 ml of 0.67% thiobarbituric acid solution and heated for 30 min at 100°C. After cooling, the precipitate was removed by centrifugation. The absorbance of the sample was measured at 450, 532, and 600 nm using a blank containing all the reagents. The MDA content of the sample was calculated using the formula, C/μmol/L= 6.45(A_{532} - A_{600}) -0.56 A_{450}.

Enzymatic assays

Samples (0.3 g) in 3 ml 0.05 M Na phosphate buffer (pH 7.8) including 1 mM EDTA and 2% (w/v) polyvinylpyrrolidone (PVP) were homogenized in a prechilled mortar and pestle in an extraction medium at 4°C. The homogenate was centrifuged at 12,000 rpm for 20 min at 4°C and the supernate was used for the enzymatic activity assays. All assays were performed at 4°C.

According to Beauchamp and Fridovich (1971), SOD activity was determined based on the method which measured inhibition in the photochemical reduction of nitroblue tetrazolium (NBT)

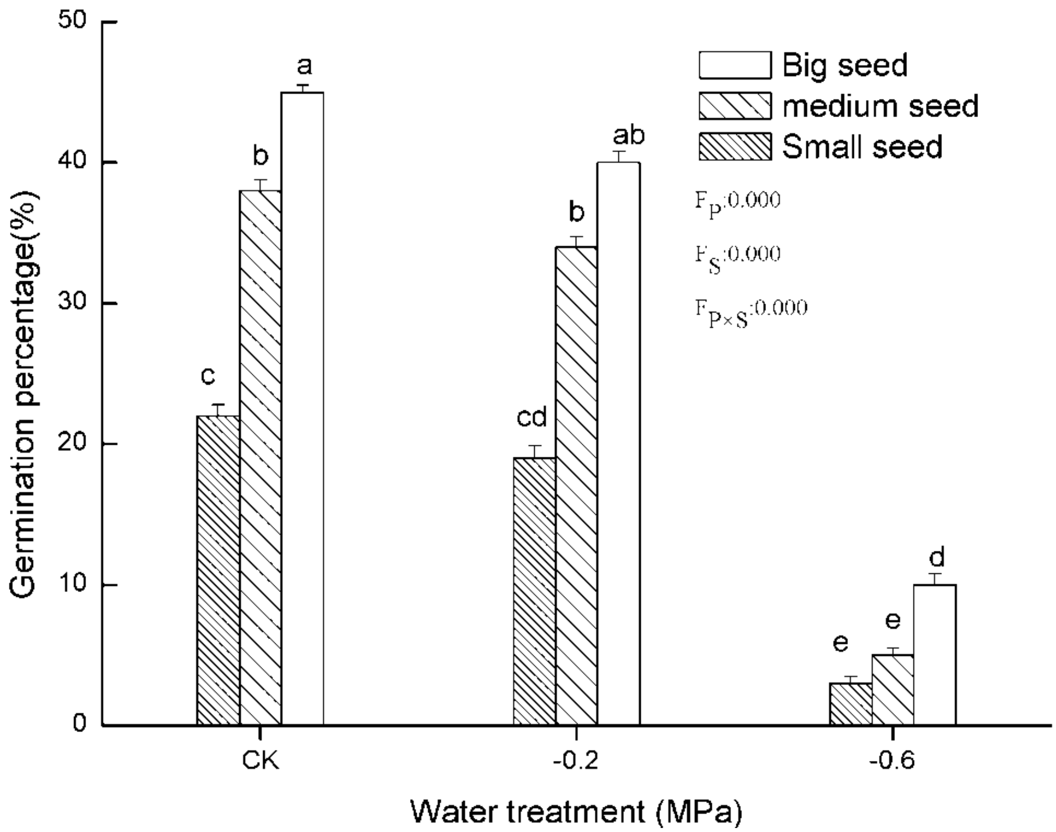

Figure 1. Effects of water stress and seed mass on germination of *Xanthoceras sorbifolia*. Data were shown as means ± SE. Different letters indicated significant differences between treatments ($p < 0.01$). F_P, PEG effect; F_S, seed mass effect; $F_{P\times S}$, PEG × seed mass interactive effect.

spectrophotometrically at 560 nm. The reaction mixture contained 50 mM Na phosphate buffer (pH 7.8), 33 µM NBT, 10 mM L-methionine, 0.66 mM EDTA, and 0.0033 mM riboflavin. Reactions were conducted at 25°C under light intensity of approximately 300 $\mu mol^{-1} m^{-1} s^{-1}$ for 10 min. Peroxidase activity (POD) was analyzed according to Prochazkova et al. (2001). The 3 ml reaction mixture contained 28 µl guaiacol, 2 mM H_2O_2, 0.1 M phosphate buffer (pH 6.0), and 0.1 ml enzyme. Absorbance was recorded at 470 nm.

CAT was performed according to Prochazkova et al. (2001), which measured the induction of H_2O_2 by absorbance at 240 nm. The 3 ml reaction mixture contained 0.1 mM phosphate buffer (pH 7.0), 10 mM H_2O_2, and 0.2 ml enzyme extracts.

Free proline content

Free proline content was determined according to the method of Li (2000). Samples (0.3 g) including 5 ml of 3% sulfosalicylic acid were homogenized and centrifuged at 3,000 rpm for 20 min. The supernate was added to 2 ml of glacial acetic acid with 2 ml acidic ninhydrin. The mixture was heated at 100°C for 25 min. After the liquid was cooled, the mixture was added to 4 ml toluene. The absorbance of the extracts was read at 520 nm.

Statistical analysis

All data analyses were conducted with SPSS18.0 statistical software package for Windows. Analysis of variance (ANOVA) was applied to test the different seed mass and water stress treatments. Least significance difference (LSD) multiple comparison tests were used to separate significant differences among all treatments at the 0.01 level. Standard error (SE) was calculated, and results were shown in figures and tables.

RESULTS

Germination percentage

Germination percentages were higher in the control treatment than those in PEG treatments regardless of the seed mass (Figure 1). The germination percentage was enhanced with decreasing PEG levels (-0.6 MPa to -0.2 MPa) or decreasing water stress. Significant decrease in the germination percentage (77.6, 87.7, and 87.9% in the big, medium, and small seeds, respectively) was observed under the -0.6 MPa conditions compared with those under the -0.2 MPa conditions, in which the germination percentage reductions in the big, medium, and small seeds were 10, 11, and 15%, respectively (Figure 1, $p < 0.01$). Big seeds exhibited the highest germination percentage compared with the other seeds. Significant interaction between PEG treatments and seed mass was found in the germination percentage (Figure 1, $p < 0.01$). The first count of germination showed that big

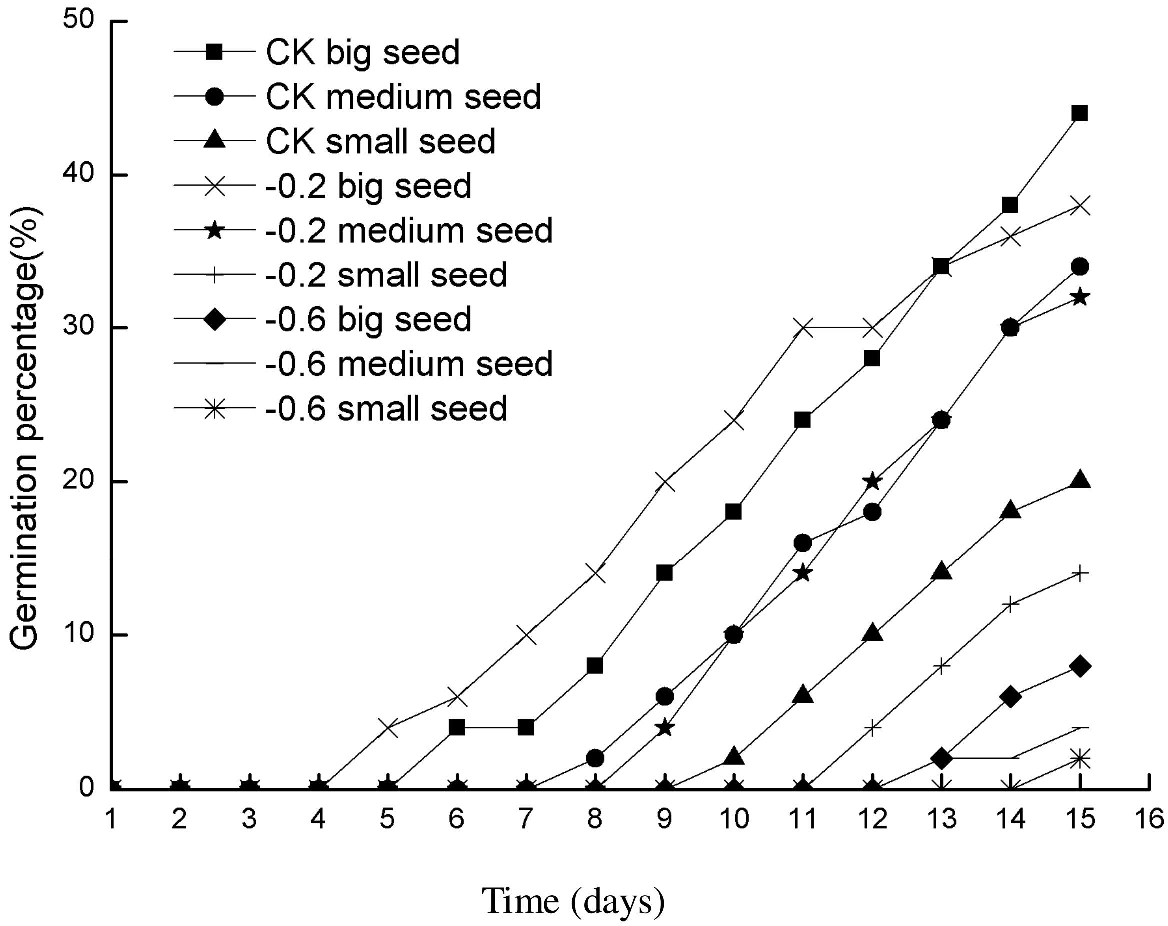

Figure 2. Germination of *Xanthoceras sorbifolia* over time as influenced by seed mass under water stress.

seeds under the -0.2 MPa treatment started germination earlier compared with all the seeds under the other treatments (Figure 2). Although the initiation time of the big seed germination was earlier under the -0.2 MPa conditions than the control conditions, the germination percentage of the big seeds was bigger under the control conditions than that under the -0.2 MPa condition after 15 days. The slowest initiation time of germination of all the seeds was under the -0.6 MPa condition.

MDA content

MDA content was used extensively to measure the degree of lipid peroxidation (Michel and Kaufmann, 1973). The level of MDA increased with decreasing PEG treatment in all the seeds (Figure 3). MDA content in the big seeds, medium, and small seeds increased by 7.8, 13.3, and 13.9%, respectively, under the -0.2 MPa conditions. Similarly, MDA content increased by 33, 43, and 58%, respectively, under the -0.6 MPa condition. The increased levels of MDA were significant in the medium and small seeds under the -0.6 MPa condition (Figure 3, $p < 0.01$). MDA content was highest in the small seeds compared with those in the other seeds. No significant interaction between PEG treatment and seed mass was found in MDA content (Figure 3, $p > 0.05$).

Free proline content

Free proline is the important organic osmoregulatory substance. Accumulation of this substance in plants reduces tissue penetration under adverse conditions (Chen et al., 2003). Significant accumulation of free proline content was observed in all seeds under water stress (Figure 4). The magnitude of the increase in free proline content under the -0.2 MPa condition was more significant in the big seeds (174%) than those in the other seeds (145 and 101%, respectively). The increase in the free proline content under the -0.6 MPa condition was more significant in all seeds than under the -0.2 MPa treatment. Free proline content was highest in the big seeds compared with those in the other seeds in all treatments. Significant interaction between PEG treatment and seed mass was observed in free proline

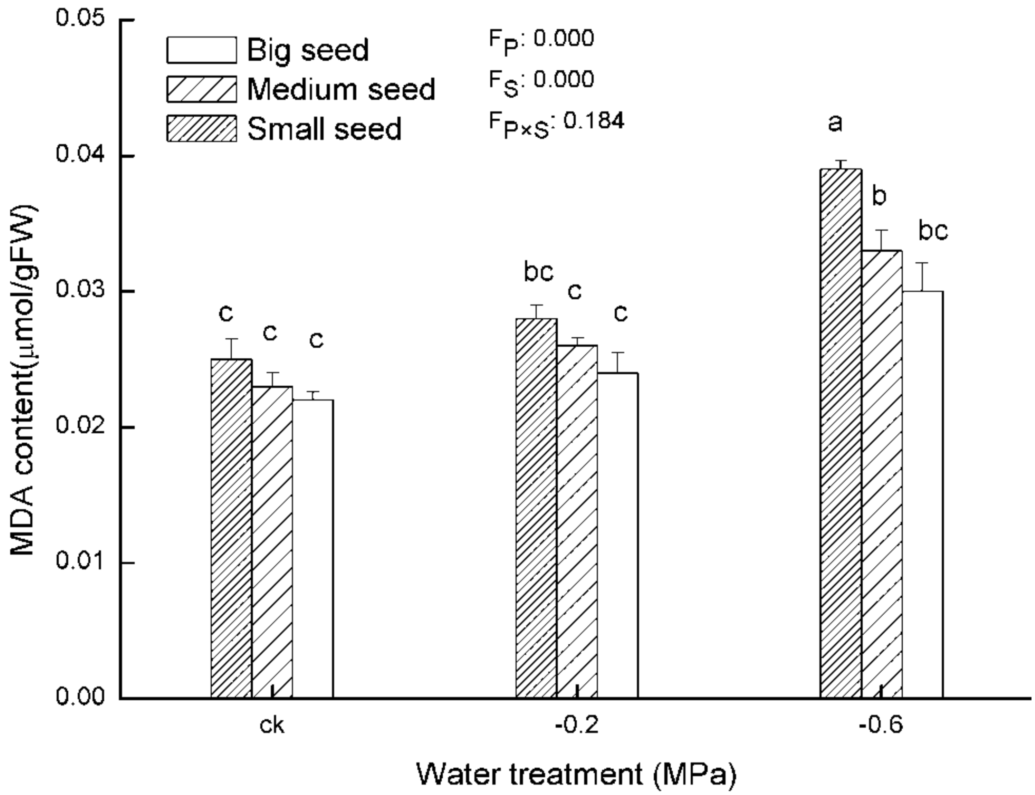

Figure 3. Effect of water stress and seed mass on MDA content during germination of *Xanthoceras sorbifolia*. Data were shown as means ± SE. Different letters in the table indicated significantly differences between treatments ($P < 0.01$). F_P, PEG effect; F_S, seed mass effect; $F_{P\times S}$, PEG × seed mass interactive effect.

content ($p < 0.01$, Figure 4).

Protective enzyme

POD, CAT and SOD activities are shown in Table 1. The enzymatic activities gradually increased in all seeds with decreasing water potential. Seed mass had significant effect on the POD, CAT, and SOD activities.

POD activities were significantly increased in all seeds under the -0.2 MPa condition ($p < 0.05$). However, the increased in SOD and CAT activities was insignificant in the small seeds. The increase of CAT activity was insignificant in the medium seeds under the -0.2 MPa conditions ($p > 0.05$). SOD and POD activities were insignificant in the small seeds under the -0.6 MPa conditions ($p > 0.05$). The highest POD, CAT, and SOD activities were observed in big seeds in all treatments. The combined effect of PEG treatment and seed mass was insignificant on the POD, SOD, and CAT activities.

DISCUSSION

Water stress may result in delayed, reduced, or complete prevention of germination (Hegarty, 1977). This current study also reveals the same pattern of germination with PEG treatment (Figures 1 and 2). Similar results have been reported in *Ougeinia dalbergioides* (Uniyal and Nautiyal, 1998), *A. spicigera*, *A. fragrans* (Azarnivand et al., 2007), *restinga* (Mantovani and Iglesias, 2010), and *P. asperata* (Yang et al., 2010). Germination percentage decreased in all seeds with increasing water stress. Seed size variance is associated with a variety of fitness-related traits, such as the probability and timing of germination (Schaal, 1980; Roach, 1987; Winn, 1988; Biere, 1991; Platenkamp and Shaw, 1993). This current research results also indicate that seed mass significantly affect germination percentage and mean daily germination. Big seeds had the highest germination percentage and earliest initiated germination compared with the other seeds in all treatments, which might contribute to the drought stress tolerance of big seeds. These results are in agreement with the results from others species, such as *Xanthium strumarium* (Zimmerman and Weis, 1983), *Agropyron psammophilum* (Zhang and Maun, 1990), *Prunus jenkinsii* (Upadhaya et al., 2007), *I. sindica, C. viscose* and *D. muricata* (Aziz and Shaukat, 2010). In addition, germination was initiated early in the big seeds under the -0.2 MPa condition

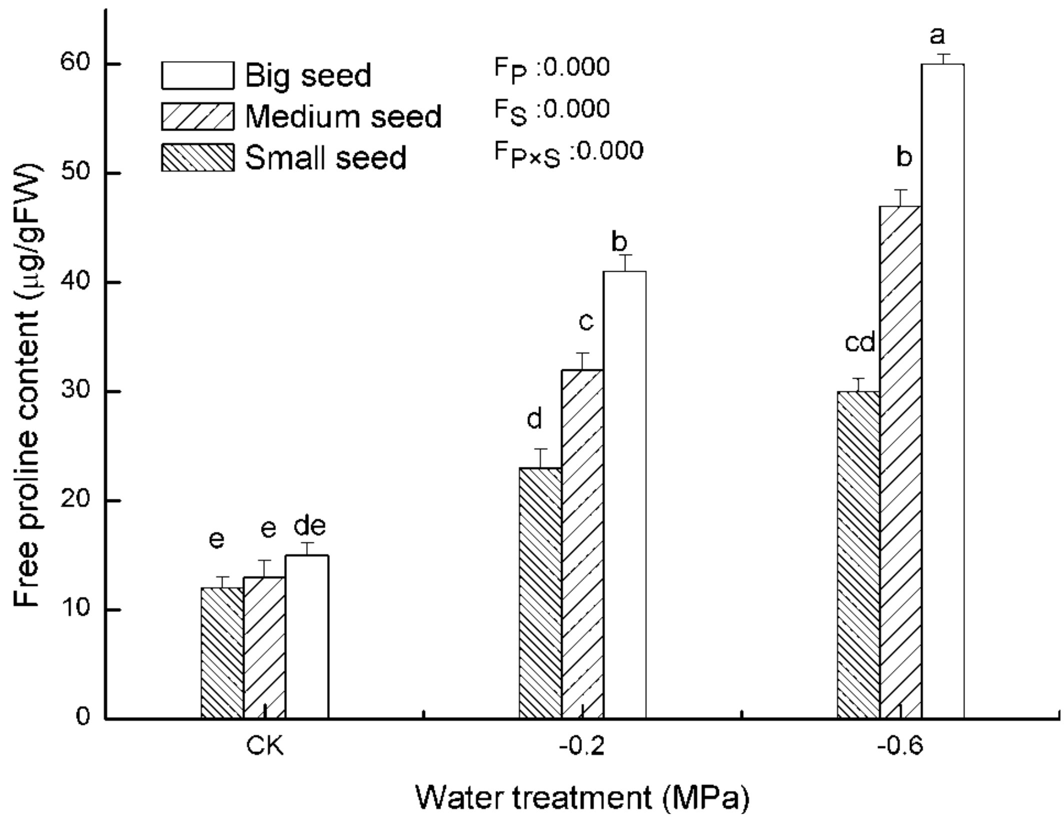

Figure 4. Effect of water stress and seed mass on free proline content during germination of *Xanthoceras sorbifolia*. Data were shown as means ± SE. Different letters in the table indicated significantly differences between treatments ($P < 0.01$). F_P, PEG effect; F_S, seed mass effect; $F_{P\times S}$, PEG × seed mass interactive effect.

because seed germination essentially requires ROS signaling (Bailly, 2004). The increased levels of MDA are an indicator of membrane damage that is closely associated with the uncontrolled accumulation of ROS caused by water stress. Water stress affected seed germination. The germination percentage of all seeds under the -0.6 MPa condition decreased evidently compared with those under the control and -0.2 MPa treatments. Seed germination is considered the most critical phases for the establishment of any species. Thus, tolerance of seeds to various stresses during germination should be determined (Uniyal and nautiyal, 1998) to understand the performance of the seed in field conditions. The results indicate that the big seeds of *X. sorbifolia* were more drought-tolerant than the medium and small seeds. Therefore, *X. sorbifolia* seeds >1.0 g should be selected in the afforestation of the northern Loess Plateau of China.

MDA is one of the main products of lipid peroxidation and is strongly toxic. MDA levels that accumulate in the cell can reflect the dynamics of the free radical and degree of impairment of a plant. MDA content increased with decreasing water potential in all the *X. sorbifolia* seeds. Similar results were obtained in *Cercidiphyllum japonicum* (Mai et al., 2009) and *P. asperata* (Yang et al., 2010). The increasing amounts of MDA content was accompanied by decreasing germination percentage in all the *X. sorbifolia* seeds. The increased level of MDA is an indicator of membrane damage that is closely associated with the uncontrolled accumulation of ROS caused by water stress (Menconi et al., 1995; Cai et al., 2011). The levels of MDA content were connected with seed mass under water stress. Significant increase in the levels of MDA was found in the medium and small seeds under the -0.6 MPa conditions. The medium and small seeds had weaker resistances to moderate stress (-0.6 MPa) compared with the big seeds. The change in the levels of MDA according to the different seed masses might have resulted from the ROS-mediated lipid peroxidation estimated using MDA and differences in the roles of protective enzymes in controlling the ROS level present in seeds.

The increase in free proline content is one of the self-defense reactions during water stress in plants, protecting the enzyme and epicyte system (Liu et al., 2008). Free proline content increased significantly in all the seeds with increasing water stress. Similar results have been reported in *P. asperata* (Yang et al., 2010)

Table 1. Effect of water stress and seed mass on POD, CAT and SOD activities during germination of *Xanthoceras sorbifolia* seed.

Treatment	SOD (μ/gFW)	POD (μ/gFW.min)	CAT (μ/gFW.min)
Big seed			
CK	336.3 ± 12.7^{bc}	80.0 ± 1.6^{d}	17.0 ± 0.39^{bc}
-0.2MPa	461.8 ± 11.1^{ab}	113.8 ± 7.6^{c}	19.8 ± 0.38^{ab}
-0.6MPa	508.4 ± 9.6^{a}	199.7 ± 3.9^{a}	22.3 ± 0.23^{c}
Medium seed			
CK	303.9 ± 12.9^{c}	62.4 ± 5.0^{d}	13.7 ± 0.14^{c}
-0.2MPa	398.2 ± 15.8^{b}	100.1 ± 2.9^{c}	15.8 ± 0.35^{bc}
-0.6MPa	431.8 ± 21.8^{ab}	189.8 ± 1.8^{ab}	17.8 ± 0.29^{b}
Small seed			
CK	298.9 ± 24.6^{c}	40.8 ± 5.6^{e}	11.3 ± 0.35^{c}
-0.2MPa	325.5 ± 13.9^{c}	80.4 ± 5.7^{d}	12.2 ± 0.22^{c}
-0.6MPa	333.3 ± 14.3^{bc}	180.0 ± 2.9^{b}	13.5 ± 0.30^{bc}
F_P	0.000	0.000	0.000
F_S	0.000	0.000	0.000
$F_{P\times S}$	0.111	0.307	0.459

CAT, Catalase; POD, peroxidase; SOD, superoxide dismutase.

and *Cajanas cajan* (Bhamburdekar and chavan, 2011). Free proline content varied with seed mass in all treatments. The big seeds had the highest free proline content in all treatments compared with the other seeds. The magnitude of increase of free poline content under PEG treatment was more significant in the big seeds than those in the other seeds. The big seeds are more tolerant than other seeds under adverse conditions. Antioxidant enzymatic activities constitute the major part of the plant antioxidant defense system (Celikkol Akcay et al., 2010). SOD converts the toxic O_2^- to H_2O_2, which must be scavenged to O_2 and H_2O by the antioxidant enzymes, such as CAT and POD (Pan et al., 2006). POD is among the major enzymes that scavenge H_2O_2 in chloroplasts, which are produced through dismutation of O_2^- catalyzed by SOD (Asada and Takahashi, 1987). CAT eliminates H_2O_2 by breaking it down directly to water and oxygen (Tsang et al., 1991). POD, SOD, and CAT activities increased in all seeds with decreasing water potential corresponding to a decrease in germination (Figures 1 and 3). Similar increases in the POD, CAT, and SOD activities were reported in the stressed seeds (Kopyra and Gwo´z´dz´, 2003; Zhang et al., 2010; Yang et al., 2010). The increase in the levels of the antioxidant enzymes was the physiological response of *X. sorbifolia* seeds to resist drought. The magnitudes of the antioxidant enzymatic activities are related to the capacity of the plant to resist adversity (Liu, 1997). POD, CAT, and SOD activities were significantly increased in the big seeds with decreasing PEG treatment. Moreover, big seeds exhibited the highest antioxidant enzymatic activities in all the treatments compared with the other seeds. The big seeds of *X. sorbifolia* were the most tolerant of drought compared with the other seeds.

These current results demonstrate that water affects *X. sorbifolia* seed germination, and is the foremost critical factor in seed germination of *X. sorbifolia* in the northern Loess Plateau of China. In addition, the results indicate that the seed mass of *X. sorbifolia* affects the germination percentage and a seed mass >1.0 g could evidently increase germination percentage. Soil water conditions and seed mass caused the low number of seedlings in the undergrowths in the northern Loess Plateau of China. The results from this present study can provide a guide to the afforestation practice of *X. sorbifolia* in the northern Loess Plateau of China. This plant has received considerable attention because of the increasing demand for biodiesel. The plantation area of *X. sorbifolia* will expand in the northern Loess Plateau of China. The authors suggest that forest planting method be adopted for the afforestation of *X. sorbifolia* in the northern Loess Plateau. A seed mass >1.0 g should be selected in the northern Loess Plateau for direct seeding. This measure presents significant ecological, as well as economic, benefits and can improve the survival rate and reduce afforestation cost. Temperature, light, and seed treatment method affect seed germination of *X. sorbifolia* (Xu, 2006). Whether these conditions are the restrictive factors for *X. sorbifolia* seed germination must be investigated. Aside from germination, whether the survival of *X. sorbifolia* seedlings affects the number of seedlings in the undergrowths needs further research.

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