

Full Length Research Paper

# Physiological and antioxidant responses of three leguminous species to saline environment during seed germination stage

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The study investigated the physiological behaviors and antioxidant responses of *Medicago sativa*, *Melilotus officinalis* and *Astragalus adsurgens* to saline environment during seed germination stage. At 300 mM NaCl treatments, the final germination percentage of *M. officinalis* was much higher than that of *M. sativa* and of *A. adsurgens* and the initiation of germination process of *A. adsurgens* was 5 days later than the others. Moreover, after being treated in 300 mM NaCl, much weaker catalase (CAT) activity in seed of *M. officinalis*, peroxidase (POD) activity in seed of *A. adsurgens* and glutathione reductase (GR) activity in seed of *M. sativa* were found in the controls and salt treatments. Thus, oxidative stress could be an influential component of salt stresses on plant seeds during seed germination stage.

**Key words:** Germination, *Medicago sativa*, *Melilotus officinalis*, *Astragalus adsurgens*, osmotic stress, malondialdehyde, antioxidant enzymes.

## INTRODUCTION

High salinity is the most widespread abiotic stress and constitutes the most stringent factor that limits plant growth and development (Vernon and Bohnert, 1992). Germination is a key stage in the life cycle of plants in saline environments as it determines whether or not the plants can establish successfully in certain areas (Ungar, 1991). Studies on salt stress in seed germination have shown that during this stage the seeds are particularly sensitive to saline environments (Bewley and Black, 1982). Therefore, it is very important to understand the adaptive mechanisms of plants to saline environment during seed germination stage.

Plants vary greatly in their tolerance to salt. Some halophytes can complete their life cycle under hyper-saline conditions (McKell, 1994), while glycophytes, though generally more sensitive to saline stress, range widely between species and even among varieties in their tolerances (Tobe et al., 2001). In general, non-halophytes and halophytes respond to salinity in a similar way during the germination stage; in some species, the initial

germination process is often delayed under salt stress (Keiffer and Ungar, 1997; Khan and Ungar, 1997). The effect of salinity on seed germination can be attributed to an osmotic effect and/or specific ion toxicity, depending on the plant species (Petruzzelli et al., 1992; Poljakoff-Mayber et al., 1994).

Salt stress can stimulate formation of active oxygen species (AOS), such as superoxide, hydrogen peroxide and hydroxyl radicals. These activated oxygens injure the cellular components of proteins, membrane lipids and nucleic acids (Foyer et al., 1994). Malondialdehyde (MDA) is the decomposition product of polyunsaturated fatty acids of membranes and shows greater accumulation under salt stress (Gossett et al., 1994; Dionisio-Sese and Tobita 1998; Sudhakar et al., 2001). In order to avoid these oxidative injuries, plants have developed enzymatic systems for scavenging these highly active forms of AOS, superoxide is converted by SOD enzyme into H<sub>2</sub>O<sub>2</sub>, which is further scavenged by CAT and various peroxidases. Ascorbate peroxidase (APX) and GR also play a key role by reducing H<sub>2</sub>O<sub>2</sub> to water through the Halliwell-Asada pathway (Noctor and Foyer, 1998). However, under salt stress, the mechanisms developed by seeds to scavenge AOS are still poorly understood.

*Medicago sativa*, *Melilotus officinalis* and *Astragalus*

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*adsurgens* are all important leguminous forage species in China. During seed germination and seedling stage, *M. sativa* is more sensitive to salinity, while the mature plant bears with better tolerance to salt stress (Allen et al., 1986); *M. officinalis* is a salt tolerant plant (Chen and Jia, 2001) and *A. adsurgens* is drought-resistant and exhibits high growth rates in the summer months of North China. We hypothesize that the adaptabilities of these three species seeds to saline environment are related to their ion toxicity and activities of antioxidative enzymes. Therefore, our work is to evaluate the germination percentage under salt stress of the three species and the effects of 300 mM NaCl on the activity of antioxidative enzymes and the lipid membrane peroxidation, in order to better understand their adaptive mechanisms in saline environment during seed germination stage.

## MATERIALS AND METHODS

### Germination experiment

Seeds of *M. sativa*, *M. officinalis* and *A. adsurgens* were obtained from Forage Seed Laboratory (ISTA member Laboratory), China Agriculture University. Treatment solutions of NaCl were adjusted to 50, 100, 200 and 300 mM and a control (distilled water). Seeds of three species were germinated in 150 mm covered Petri dishes on two layers of filter paper moistened with 30 ml of treatment solution. Four replicate dishes, each with 100 seeds, were used for each treatment and were placed in an incubator in constant darkness at 20°C. Every 2 days the solution in each Petri dish was renewed. Each day for 12 days, the germinant seeds were recorded and removed from the Petri dishes. Seeds were considered to have germinated when the emerging radicles were at least 2 mm in length. Final germination percentage was calculated as the total cumulative germination of a treatment group over the experimental period. Daily germination percentage was calculated as the one day germination percentage in a treatment group over the experiment period.

### Determination of enzyme activity and MDA

Seeds of three species were moistened in 150 mm Petri dishes with a 300 mM NaCl solution and were placed in an incubator in constant darkness at 20°C. After one-, two-, or four-day treatment with 300 mM NaCl solution, the seeds of each treatment and of the control (moistened in distilled water for 1 day) were surface-dried with filter paper and 0.5 g of seed samples were homogenized with 50 mM sodium phosphate buffer (pH 7.0) containing 1 mM ethylenediaminetetraacetic acid (EDTA) 1 mM ascorbate and 2% (w/v) polyvinylpyrrolidone (PVP) at 4°C. Following centrifugation at 10,000 g for 15 min at 4°C, the supernatants were collected and used for assaying enzyme activity. Four replicate dishes were used for each treatment. Protein concentrations in the enzyme extract were determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Catalase (CAT, EC 1.11.1.6) activity was assayed through according to the method developed by Bergmeyer (1970). The final reaction mixture contained 50 mM sodium phosphate buffer (pH 7.0) and 2% H<sub>2</sub>O<sub>2</sub>. The activity was expressed as units (μmol H<sub>2</sub>O<sub>2</sub> consumed per minute) per mg of protein.

Peroxidase (POD, EC 1.11.1.17) activity was determined according to Tatiana et al. (1999) with small modification. The reaction mixture contained 0.05 M sodium phosphate buffer (pH

5.5), 2% H<sub>2</sub>O<sub>2</sub>, 0.05 M guaiacol and 0.1 ml enzyme extract. The formed tetraguaiacol was measured with a spectrometer at 470 nm. One unit of enzyme was defined as the amount of enzyme to decompose 1 μmol of H<sub>2</sub>O<sub>2</sub> per min at 25°C.

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was determined according to the method of Nakano and Asada (1981). The reaction mixture contained 50 mM potassium phosphate (pH 7.0), 0.2 mM EDTA, 0.5 mM ascorbic acid, 2% H<sub>2</sub>O<sub>2</sub> and 0.1 ml enzyme extract. The decrease in absorbance at 290 nm in 1 min was recorded and the amount of ascorbate oxidized was calculated using extinction coefficient ( $\epsilon = 2.8 \text{ mM}^{-1} \text{ cm}^{-1}$ ). One unit of APX was defined as 1 mmol ml<sup>-1</sup> ascorbate oxidized per minute at 25°C.

Glutathione reductase (GR; EC 1.6.4.2) activity was measured according to Foyer and Halliwell (1976). The assay medium contained 0.025 M Na-phosphate buffer (pH 7.8), 0.5 mM oxidized glutathione (GSSG), 0.12 mM NADPH.Na4 and 0.1 ml enzyme of extract in a final assay volume of 1 ml. NADPH oxidation was determined at 340 nm. Activity was calculated using the extinction coefficient ( $\epsilon = 6.2 \text{ mM}^{-1} \text{ cm}^{-1}$ ) for GSSG. One unit of GR was defined as 1 mmol ml<sup>-1</sup> GSSG reduced per minute.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was estimated as the reduction of nitro-blue tetrazolium (NBT) at 560 nm (Beauchamp and Fridovich, 1971). The reaction mixture (3 ml) consisted of 50 mM Na-phosphate buffer (pH 7.8) 13 mM L-methionine, 75 μM nitroblue tetrazolium (NBT), 10 μM EDTA-Na<sub>2</sub>, 2.0 μM riboflavin and 0.3 ml enzyme extract. The reaction mixture was kept at 35°C for 10 min under 4,000 lx. One unit SOD activity was defined as the amount of enzyme required to result in a 50% inhibition of the rate of NBT reduction measured at 560 nm.

Malondialdehyde content was measured by a thiobarbituric acid reaction according to Madhava Rao and Sresty (2000).

### Statistical analysis

Data were determined by analysis of variance using SPSS software (10.0). Differences between treatment means were separated by the least significant difference (L.S.D.) at a 0.05 probability level.

## RESULTS

### Effects of NaCl on final germination and daily germination percentage

The final germination percentages of seeds of the three legume species were over 89% in distilled water, but the percentages decreased with the increasing NaCl concentration (Figure 1). In the 50 mM NaCl concentration treatment, final germination percentage for *M. sativa* seeds was significantly decreased by 8.7%, while for *M. officinalis* and *A. adsurgens*, the decrease was 7.3 and 0.5%, respectively. At 300 mM NaCl concentration, final germination percentage of *M. officinalis* was much higher than that of *M. sativa* and *A. adsurgens* and the final germination percentage values of *M. sativa*, *M. officinalis* and *A. adsurgens* were 41, 49 and 36%, respectively.

Seeds of the three species germinated rapidly in distilled water during the initial 2 days and the maximal values of daily germination percentage appeared on the first day for *M. sativa* and *M. officinalis* and the second day for *A. adsurgens* (Figure 2). With the increasing of NaCl concentration, the appearing of maximal values of daily germination percentage was postponed in all species.

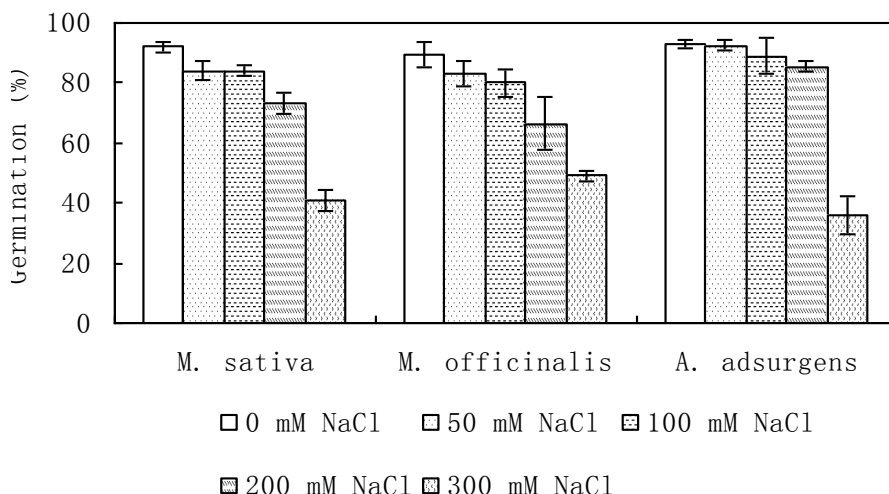


**Figure 1.** Final germination percentage for seeds germinated in contact with different NaCl in continuous darkness at 20 C for 12 d. Seeds were considered to have germinated when the emerging radicle was at least 2 mm.

At 300 mM NaCl concentration, the delaying period of appearing of maximal values of daily germination percentage were 1 day for *M. sativa*, 2 days for *M. officinalis* and 5 days for *A. adsurgens* compared with the control.

#### Effects of NaCl on SOD, POD, CAT, APX, GR activities and MDA

NaCl treatment led to an increase of SOD activity in the seeds of *M. sativa*, but a decrease in the seeds of *M. officinalis* and *A. adsurgens*, compared with the control (Table 1). However, the SOD activity of the three species treated with a 300 mM NaCl solution decreased with time increasing. The average values of SOD activities were 2.8, 2.7 and 3.4 units / mg protein in *M. sativa*, *M. officinalis* and *A. adsurgens*, respectively, for 1, 2 or 4 day salt treatments.

*A. adsurgens* seeds displayed the highest CAT activity among the three species at control treatment, whereas the CAT activities of all three species seeds were inhibited by salt solution (Table 1). The CAT activities of *M. sativa* seed were found to significantly decrease, not significantly change for *M. officinalis* seed and increase for *A. adsurgens* seed as the treating time lengthened. At 2 or 4 day treatment in 300 mM NaCl solution, the CAT activities of *A. adsurgens* seed were 0.23 or 0.22 units / mg protein, which was 2.0 or 2.2 times that in the seeds of *M. sativa* seed and 5.4 or 6.6 times that of the *M. officinalis* seed.

One- or two-day NaCl treatments led to an increase in the activity of POD in the seeds of *M. sativa* and *M. officinalis*. However salt did not change the POD activity in the seeds of *A. adsurgens* (Table 1). The average POD

activities of *M. sativa* seed under all treatments were 3.6 times higher than those of *M. officinalis* seed and 30.2 times higher than those of *A. adsurgens* seed.

Salinity stress resulted in an increase in the APX activity of seeds of the three species (Table 1). However, with the lengthened stress time, the APX activity of the *M. sativa* and *M. officinalis* seeds decreased, while the APX activity in the *A. adsurgens* seed remained the same, though the treating time increased. After NaCl stress, the average seed APX activities of *M. sativa* were 2.5 and 3.2 times that in *M. officinalis* and *A. adsurgens*, respectively.

GR activity decreased after NaCl treatment with in *M. sativa* seed, while the GR activities in *M. officinalis* seed and *A. adsurgens* seed increased. The average seed GR activity of *M. sativa* under NaCl stress was only 29% of the GR activity of *M. officinalis* seed and 24% of the GR activity of *A. adsurgens* seed (Table 1).

NaCl stress led to a significant increase in the levels of MDA content in all species. At the 4th day treatment of 300 mM NaCl concentration, the seed MDA content increased by 36%, 35% and 16% of that of the control seed of *M. sativa*, *M. officinalis* and *A. adsurgens*, respectively (Table 1).

#### DISCUSSION

The effect of salinity on seed germination is due to an osmotic effect and/or ion toxicity. However, variation of adaptive mechanisms exists in different species (Rehman et al., 1996). Salt stress can induce both a reduction of seed germination and a delay of initiation of germination process in glycophytes and to a lesser extent in halo-phytes (El-Keblawy, 2004). In the present study, 50 mM NaCl treatment resulted in a significant decrease in the

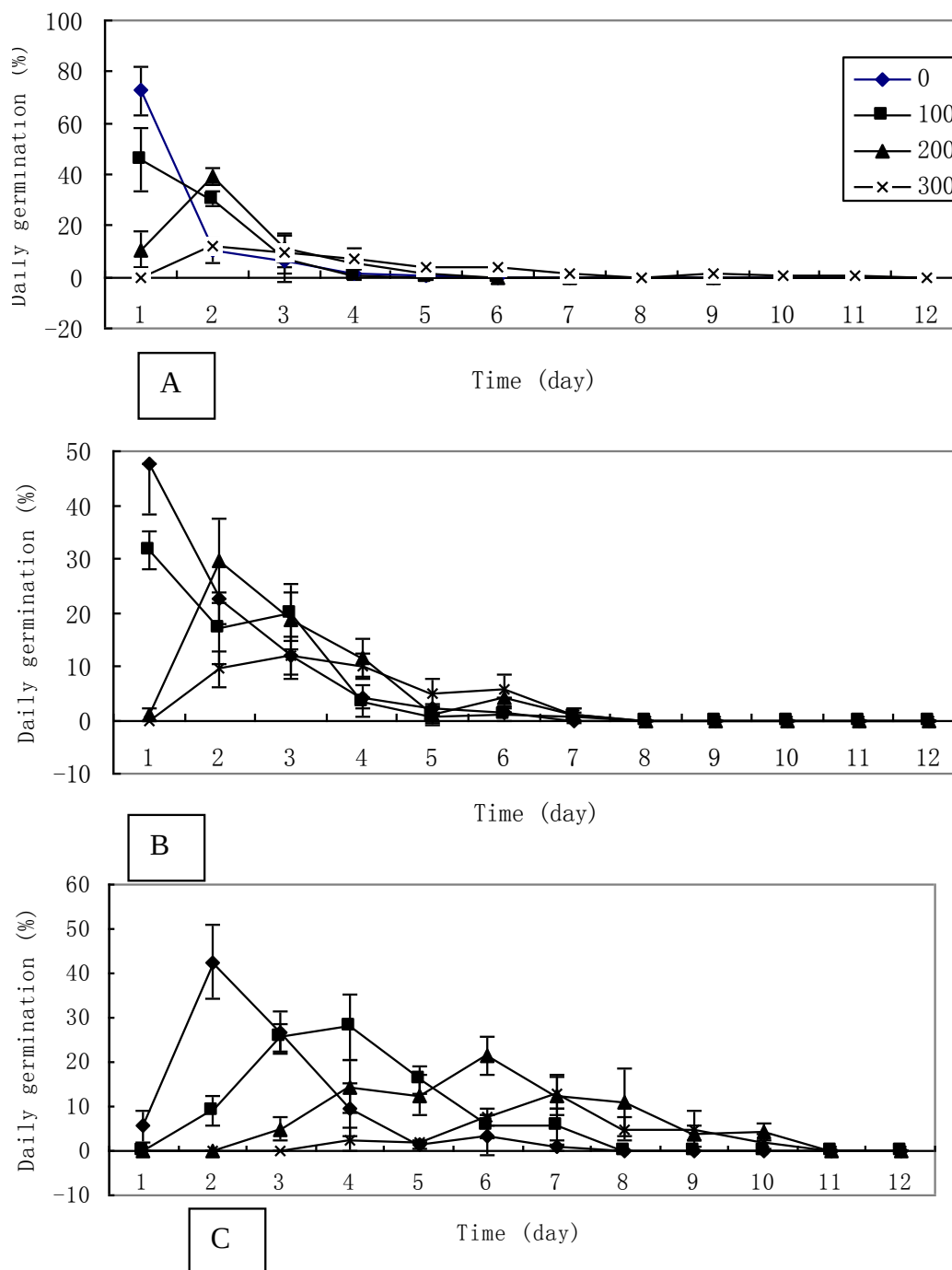


**Figure 2.** Daily germination (%) for the 12 d when seeds were placed in contact with distilled water (controls) or solutions of NaCl in continuous darkness at 20 C in (A) *M. sativa*, (B) *M. officinalis* and (C) *A. adsurgens*. Seeds were considered to have germinated when the emerging radicle was at least 2 mm.

final germination percentage for *M. sativa* seed. In a 300 mM NaCl concentration, final germination percentage of *M. officinalis* seed was much higher than that of *M. sativa* seed and *A. adsurgens* seed and a significant delay of initiation of germination process of *A. adsurgens* seed was found (Figure 1). These results indicated that in the

seed germination stage, *M. sativa* was most sensitive to even low salt stress; *M. officinalis* could adapt to stronger salt stress; while the *A. adsurgens* seed possessed the important mechanism of delaying initiation of germination process in order to adapt to the saline environment.

There are increasing evidences that membrane injury

**Table 1.** Activities of enzymes and MDA contents in seeds moistened with 300 mmol/L NaCl solution in constant darkness at 20°C for 1, 2 and 4 days and seeds moistened with distilled water in constant darkness at 20°C for 1 day were control.

| Activities of enzymes and MDA contents | Control        | Time of 300 mM NaCl treated (day) |                 |                |
|----------------------------------------|----------------|-----------------------------------|-----------------|----------------|
|                                        |                | 1                                 | 2               | 4              |
| SOD (units/mg protein)                 |                |                                   |                 |                |
| <i>M. sativa</i>                       | 2.34ac ± 0.43  | 3.27ab ± 0.60                     | 3.00b ± 0.22    | 2.19c ± 0.17   |
| <i>M. officinalis</i>                  | 3.61a ± 0.59   | 2.77a ± 0.30                      | 2.90a ± 0.73    | 2.52b ± 0.42   |
| <i>A. adsurgens</i>                    | 5.03a ± 0.30   | 3.98b ± 0.54                      | 2.98b ± 0.67    | 3.32b ± 0.39   |
| CAT (units/mg protein)                 |                |                                   |                 |                |
| <i>M. sativa</i>                       | 0.18a ± 0.02   | 0.16a ± 0.02                      | 0.11b ± 0.02    | 0.10b ± 0.01   |
| <i>M. officinalis</i>                  | 0.07a ± 0.01   | 0.04b ± 0.01                      | 0.04b ± 0.01    | 0.03b ± 0.01   |
| <i>A. adsurgens</i>                    | 0.26a ± 0.04   | 0.16b ± 0.01                      | 0.23a ± 0.04    | 0.22a ± 0.04   |
| POD (units/mg protein)                 |                |                                   |                 |                |
| <i>M. sativa</i>                       | 1.35a ± 0.25   | 1.64a ± 0.12                      | 1.64a ± 0.33    | 0.94b ± 0.12   |
| <i>M. officinalis</i>                  | 0.25b ± 0.04   | 0.37ab ± 0.15                     | 0.46a ± 0.08    | 0.46a ± 0.12   |
| <i>A. adsurgens</i>                    | 0.04a ± 0.01   | 0.04a ± 0.02                      | 0.04a ± 0.01    | 0.05a ± 0.01   |
| APX (units/mg protein)                 |                |                                   |                 |                |
| <i>M. sativa</i>                       | 0.039b ± 0.007 | 0.067a ± 0.008                    | 0.035b ± 0.011  | 0.031b ± 0.010 |
| <i>M. officinalis</i>                  | 0.008c ± 0.002 | 0.022a ± 0.005                    | 0.016ab ± 0.003 | 0.015b ± 0.001 |
| <i>A. adsurgens</i>                    | 0.011b ± 0.00  | 0.013ab ± 0.003                   | 0.014a ± 0.002  | 0.014a ± 0.003 |
| GR (units/mg protein)                  |                |                                   |                 |                |
| <i>M. sativa</i>                       | 0.08a ± 0.01   | 0.05ab ± 0.04                     | 0.07a ± 0.01    | 0.04b ± 0.01   |
| <i>M. officinalis</i>                  | 0.14b ± 0.04   | 0.19ab ± 0.02                     | 0.18ab ± 0.01   | 0.20a ± 0.01   |
| <i>A. adsurgens</i>                    | 0.11d ± 0.02   | 0.15c ± 0.01                      | 0.23b ± 0.03    | 0.31a ± 0.00   |
| MDA (units/mg protein)                 |                |                                   |                 |                |
| <i>M. sativa</i>                       | 0.21b ± 0.02   | 0.26ab ± 0.03                     | 0.29a ± 0.03    | 0.29a ± 0.04   |
| <i>M. officinalis</i>                  | 0.15b ± 0.01   | 0.20a ± 0.01                      | 0.19ab ± 0.03   | 0.21a ± 0.03   |
| <i>A. adsurgens</i>                    | 0.24a ± 0.01   | 0.25a ± 0.04                      | 0.25a ± 0.04    | 0.27a ± 0.05   |

Data represent mean values ± S.D. of analyses of 4 replicates.

Means followed by the same letter are not significantly different at  $P = 0.05$  within the same species.

under salt stress is related to a higher production of highly toxic AOS. Determining the MDA concentration and hence, the extent of membrane lipid peroxidation, has often been used as a tool to assess the severity of the oxidative stress (Amor et al., 2005). Our data show that, after 1 day treatment of the three species seeds with 300 mM NaCl, the membrane lipid peroxidation was induced. After 2 or 4 days salt stress for *M. sativa* and 1, 2 or 4 days salt stress for *M. officinalis*, the MDA contents in the seeds were much higher than in those of the control, while no significant increase was found in *A. adsurgens* seeds after salt stress (Table 1).

Salt stress can cause plant membrane damage and stimulate formation of AOS such as superoxide, hydrogen peroxide and hydroxyl radicals. Among the AOS, superoxide is converted by SOD enzyme into  $H_2O_2$ , which is further scavenged by CAT and various peroxidases. APOX and GR also play a key role by reducing  $H_2O_2$  to water through the Halliwell-Asada pathway (Noctor and Foyer, 1998). In the present study, the diverse responses of SOD, CAT, POD, APX and GR

enzyme activities to NaCl stress on 1, 2 or 4 days salt stressed seeds of *M. sativa*, *M. officinalis* and *A. adsurgens* suggest that oxidative stress could be an influential component of environmental stresses on plant seeds.

Salinity led to an increase of SOD activity of *M. sativa* seeds but it decreased that in the *M. officinalis* and *A. adsurgens* seeds after being treated with salt for 1 or 2 days (Table 1), which suggested that salinity induced more production of  $O_2^-$  in species of *M. sativa* which was counterbalanced by increasing activity of SOD. With the lengthening of the salt stress time, the SOD activities decreased in the three species seeds. Since SOD was inactivated by singlet oxygen and peroxy radicals (Escobar et al., 1996), it could have been deactivated in the three species seeds by the increased levels of AOS. Under salt stress treatments, no great differences were found among the three species in the average values of SOD, which indicates that SOD is indispensable to scavenging AOS in seeds of the three species.

CAT, POD, APX and GR, as antioxidative enzymes,

play a key role in scavenging  $H_2O_2$  in the cells (Noctor and Foyer, 1998). In our study, according to the active diversity of these enzymes in seeds of *M. sativa*, *M. officinalis* and *A. adsurgens*, we could conclude that the mechanisms in scavenging  $H_2O_2$  were different in the three species under stress conditions. In *M. officinalis*, the CAT activities of seeds were much weaker than in the *M. sativa* and *A. adsurgens* seeds both in the control and with salt treatments and were significantly inhibited in salt treatments. The POD and APX activities in seeds of *M. officinalis* were also feeble than in seeds of *M. sativa* at control and with salt treatments; however, they were significantly induced by salt. These results suggested that POD and APX could be more important than CAT in scavenging  $H_2O_2$  in seeds of *M. officinalis* under salt stress conditions. In *M. sativa* seed, POD and APX are also more important in scavenging  $H_2O_2$  under salt stress conditions due to the intenser activities at control and with salt treatments and induced the POD and APX activities to increase after short period salt stress. After 300 mM NaCl treatment for 4 days, the activities of CAT, POD and APX became weaker than those of the controls of the three species, resulting in the accumulation of  $H_2O_2$  and a higher lipid peroxidation level in the seeds. POD activity in seeds of *A. adsurgens* was much weaker than that in seeds of *M. officinalis* and *M. sativa* in control and under salt treatments, witnessing that POD might not play the key role of reducing  $H_2O_2$  at stress conditions in seeds of *A. adsurgens*.

In the antioxidant defense system pathway, monodehydroascorbate that is spontaneously dismutated to dehydroascorbate can react with glutathione to produce ascorbate and oxidized glutathione (GSSG) in a reaction catalyzed by dehydroascorbate reductase. GSSG is reduced by GR, requiring the consumption of NADPH. Singlet oxygen and hydroxyl ions are eliminated in the glutathione pathway (Bray et al., 2000). Bor et al., (2003) reported that Increased GR activity in leaves of sugar beet plants was closely related with salt tolerant capacity of these plants. In our study, the GR activity decreased in seeds of *M. sativa* after salt stress and increased in seeds of *M. officinalis* and *A. adsurgens*. This indicated that seeds of *M. sativa* could suffer from much severer toxicity of singlet oxygen and hydroxyl ions under salt stress condition.

In conclusion, the effects of NaCl on seed germination of the three species are due to osmotic stress, ion toxicity and oxidative stress. However, the adaptive mechanisms of the three species are variant. The seeds of *M. sativa* are susceptible to low level NaCl concentrations and suffer from much severer ion toxicity at high NaCl concentrations. As a salt tolerant plant, seeds of *M. officinalis* sustain salt stress of a higher concentration. Delaying initiation of germination process is the important mechanism of *A. adsurgens* to adapt to the saline environment. The membrane lipid peroxidation is induced by NaCl in seeds of the three species. The diverse responses of

antioxidant enzyme activities to NaCl stress in seeds of *M. sativa*, *M. officinalis* and *A. adsurgens* suggest that oxidative stress could be an influential component of environmental stresses on plant seeds.

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