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Research article

Heteromorphic seeds of coastal halophytes *Arthrocnemum macrostachyum* and *A. indicum* display differential patterns of hydrogen peroxide accumulation, lipid peroxidation and antioxidant activities under increasing salinity

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ABSTRACT

Reactive oxygen species homeostasis during germination of heteromorphic seeds is not fully understood. This study elucidates changes in levels of hydrogen peroxide (H_2O_2), malondialdehyde (MDA) and enzymatic antioxidants in heteromorphic seeds of contrasting congeneric halophytes *Arthrocnemum macrostachyum* (C_3 perennial) and *A. indicum* (C_4 perennial) during germination under increasing salinity. There was no dormancy in *A. macrostachyum* (black and brown) and *A. indicum* (large and small) seeds. Seeds of *A. macrostachyum* displayed greater salinity tolerance compared to *A. indicum* seeds. Under non-saline conditions, large *A. indicum* seeds and brown *A. macrostachyum* seeds showed slightly higher germination than their respective counterparts. H_2O_2 content of black compared to brown *A. macrostachyum* seeds increased with salinity and that of small compared to large *A. indicum* seeds increased only in 400 mM NaCl. High catalase and ascorbate peroxidase with constitutive superoxide dismutase levels coincided with unaltered MDA in black *A. macrostachyum* seeds under salinity. Whereas, there was a decline in most antioxidant enzyme activities alongside low/unchanged H_2O_2 in the brown *A. macrostachyum* seeds under salinity. Unaltered H_2O_2 and MDA with low/unchanged antioxidant enzyme activities in large *A. indicum* seeds under salinity occurred. Unchanged enzyme activities alongside a rise in H_2O_2 and MDA levels were observed in the small *A. indicum* seeds under salinity. These data hence highlight differential H_2O_2 homeostasis strategies in the heteromorphic seeds of the test species.

1. Introduction

Seed germination holds a key position between the resistant seed bank and vegetative stages in the life cycle of plants (Bewley and Black, 1994). It ensures continuity of race and establishment of a population in suitable community gaps when conditions are favourable. Therefore, it is of particular importance for the plants of stressful habitats such as halophytes, which inhabit coastal and inland saline areas (Gul et al., 2013). A number of studies have indicated that salinity of the soil and/or water plays a key role in determining seed germination responses of the halophytes (Gul et al., 2013). Similar to most plants, salinity is also inhibitory to the germination of halophytes, nevertheless the threshold levels causing germination cessation and/or viability loss are substantially higher (Gul et al., 2013; Melendo and Giménez, 2018). However, biochemical basis of the high salinity tolerance of halophyte seeds is not fully understood.

Salinity induces excessive production of reactive oxygen species (ROS), which results in oxidative injury to vital cell constituents such as nucleic acids, membrane lipids and proteins (Hameed et al., 2014; Demidchik, 2015). Since seeds of halophytes experience large variations in soil/water salinity, the success of their seed germination and seedling establishment hence would depend on the efficient quenching of ROS by the seeds' antioxidant machinery, which consists of enzymatic and non-enzymatic components (Demidchik, 2015). Superoxide dismutase, ascorbate peroxidase and catalase are common antioxidant enzymes, while ascorbic acid and glutathione are commonly found antioxidant substances (Bailly, 2004). Nevertheless, many antioxidants such as ascorbate might be absence in quiescent seeds (Hameed et al., 2014). ROS quenching and components of antioxidant machinery have mostly been studied in crop seeds and this knowledge on halophyte seeds is scarce (Kranner and Seal, 2013; Hameed et al., 2014).

Many halophytes of the arid and semiarid areas produce heteromorphic seeds varying in morpho-physiological attributes as an adaptation to survive in extreme and fluctuating environments (Gul et al.,

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2013; Nisar et al., 2019). Dormancy status, germination requirements, relative contribution to population and stress tolerance of the heteromorphic seeds have commonly been studied (Imbert, 2002; Yang et al., 2017; Wang et al., 2012; Liu et al., 2018). However, biochemical aspects of the seed heteromorphism have been studied by only few (Liu et al., 2018; Rasheed et al., 2019). In addition, comparative studies on antioxidant machinery involved in ROS homeostasis during germination of heteromorphic seeds, especially of halophytes, are generally missing.

Arthrocnemum macrostachyum (Moric) C. Koch and *A. indicum* (Willd.) Moq., are two succulent Salicornioideae halophytes of the salt marshes along the coastal area of Pakistan and many other countries (Freitag et al., 2001; Nisar et al., 2019). *Arthrocnemum macrostachyum* (a.k.a. Glaucous Glasswort) is a C_3 perennial euhalophyte (Redondo-Gómez et al., 2010; Khan and Qaiser, 2006), which holds immense potential to become an edible crop (Barreira et al., 2017) for saline lands. Whereas, *A. indicum* (Syn. *Tecticornia indica*) is a C_4 perennial shrub, which is less common than the *A. macrostachyum* (Khan and Qaiser, 2006) and used by local people to cure kidney diseases and fever (Ravindran et al., 2005). Test species produce heteromorphic seeds differing in color (*A. macrostachyum*: black and brown) and size (*A. indicum*: distinct large and small) (Nisar et al., 2019). Recently, Nisar et al. (2019) reported numerous resemblances and differences in germination ecology of the heteromorphic seeds of these halophytes. In addition, some information regarding salt-tolerance mechanisms in mature plants of these species also exist (Redondo-Gómez et al., 2010). However, comparative details about the biochemical basis of tolerance, especially ROS production and quenching during germination of heteromorphic seeds in response to salt exposure is absent. To fill in this knowledge gap, this study aimed to answer following questions: 1) Do heteromorphic seeds of the test species vary in germination attributes under non-saline and saline conditions? 2) Does salinity affect ROS (e.g. H_2O_2) content of the heteromorphic seeds of the test species differently? 3) What are the differences/similarities in the antioxidant enzyme activities of the heteromorphic seeds? and 4) Does the level of malondialdehyde (a common oxidative damage marker) under high salinity differ in the two seed morphs of the test halophytes?

2. Materials and methods

2.1. Study site and seed acquisition

Seeds of *A. indicum* and *A. macrostachyum* were gathered in June 2015 from a dry-marsh basin located along Gadani ship-breaking yard (25° 4′36.62"N and 66°42′35.91"E) Balochistan, Pakistan. This area is characterized by harsh weather conditions with mean annual precipitation of < 350 mm and summer temperature often exceeding 40 °C. Randomly handpicked seeds from large number of plants were taken to laboratory where they were manually separated from inflorescence husk Fig. 1. Cleaned seeds were surface sterilized with 1% (v/v) sodium hypochlorite for 1 min, rinsed with distilled water, air dried and stored briefly (i.e. ~6 weeks; which did not affect their germination; data not given) in plastic petri plates at room temperature (25–30 °C) until use.

2.2. Germination assay of heteromorphic seeds

Seed germination was tested within 6 weeks of collection. For seed germination assay a programmed incubator was used, which was set at 15/25 °C (dark/light) temperature cycle. Small (i.e. five-centimetre diameter) petri-plates with transparent lids were used. Each petri-plate contained twenty-five seeds soaked in a NaCl solution (0, 200, and 400 mM, prepared in distilled water). There were four replications for each NaCl treatment. Germination was scored as protrusion of embryonic axis on every alternate day for 20 days. Germination rate was calculated by using the Timson Index given by Khan and Ungar (1984)

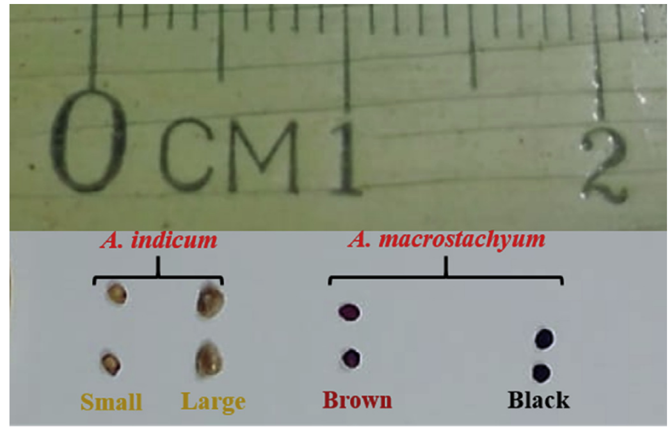

Fig. 1. Comparison of heteromorphic seeds of test species.

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2.3. Levels of oxidative damage markers

Contents of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA), commonly used oxidative damage markers, in seeds from above treatments after 3 (*A. macrostachyum*) and 7 (*A. indicum*) d (i.e. time for germination completion in the unstressed condition) respectively were quantified in TCA extracts with the help of methods of Loreto and Velikova (2001) and Heath and Packer (1968) respectively.

2.4. Activities of antioxidant enzymes

Seeds from above treatments after 3 (*A. macrostachyum*) and 7 (*A. indicum*) d (i.e. time for germination completion in the unstressed condition) respectively were finely ground in liquid nitrogen and mixed in an extraction medium (2% of polyvinylpolypyrrolidone, 1 mM ascorbic acid and 5 mM disodium EDTA dissolved in potassium phosphate buffer pH 7.0) followed by centrifugation at 12000 ×g for 20 min at 4 °C. The supernatant was used for the assays of catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR) and ascorbate peroxidase (APX) by using methods described in Hameed et al. (2012).

2.5. Statistical analyses

Arcsine transformed germination data were used for the analysis of variance (ANOVA) to check whether morphology (M), salinity (S) and their interaction (M × S) significantly influenced germination and biochemical parameters of the seeds. Bonferroni test highlighted the significant ($P < 0.05$) differences among mean values. Mean values of two seed morphs of each species within a salinity level were compared using a *T*-test. Statistical analyses were carried out in SPSS version 20.

3. Results

3.1. Germination of heteromorphic seeds

Heteromorphic *A. macrostachyum* seeds showed optimal germination in water (Fig. 2). In general, both seed morphs of *A. macrostachyum* exhibited comparable final germination and rates under increasing salinity (Fig. 2; Table 1). Germination rate of either seed type declined significantly ($P < 0.05$) in high salinity (Fig. 2). Germination of large compared to small *A. indicum* seeds was significantly ($P < 0.05$) higher in distilled water (Fig. 2, Table 2). Final and rate of germination decreased with increasing salt concentrations in both morphs and there was no germination at 400 mM NaCl (Fig. 2). Generally, seeds of *A. macrostachyum* displayed better germinablity in 400 mM NaCl solution than those of *A. indicum*.

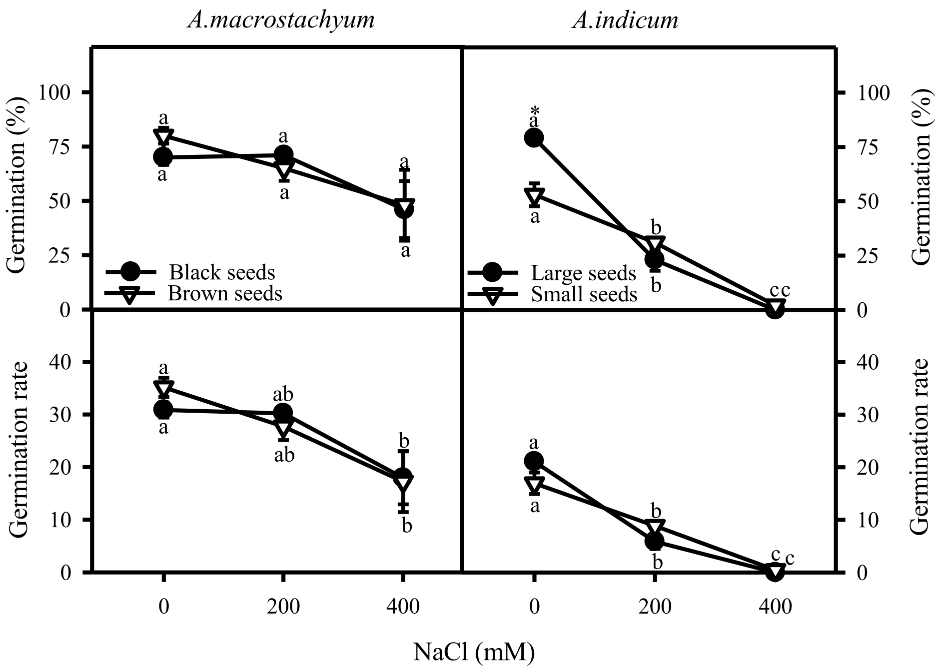

Fig. 2. Effects of salinity on germination and rate of germination of the heteromorphic seeds of *A. macrostachyum* and *A. indicum*. Line values are mean ± standard error. Asterisks (*) indicate significant difference between two morphs within a salinity level (T-test, $P < 0.05$). Different letters across salinity are significantly different (Bonferroni test, $P < 0.05$).

Table 1
Effects of seed morphology, salinity and their interaction on germination, rate of germination and biochemical parameters of the heteromorphic seeds of *Arthrocnemum macrostachyum* indicated by ANOVA.

Parameters	Morphology (M)		Salinity (S)		M × S	
	F	*P*	*F*	*P*	*F*	*P*
Germination	0.072	0.792	5.062	0.018	0.381	0.688
Rate of Germination	0.017	0.898	10.651	0.001	0.519	0.604
H_2O_2	90.915	0.00	7.831	0.007	2.488	0.125
MDA	0.693	0.421	0.545	0.593	1.212	0.332
SOD	32.306	0.00	6.829	0.012	4.923	0.03
CAT	0.899	0.364	1.58	0.249	19.977	0.00
APX	634.25	0.00	14.242	0.001	237.584	0.00
GR	73.386	0.00	14.804	0.001	17.801	0.00

Table 2
Effects of seed morphology, salinity and their interaction on germination, rate of germination and biochemical parameters of the heteromorphic seeds of *Arthrocnemum indicum* indicated by ANOVA.

Parameters	Morphology (M)		Salinity (S)		M × S	
	F	P	F	P	F	P
Germination	4.364	0.051	218.932	0.00	16.841	0.00
Rate of Germination	0.061	0.808	155.796	0.00	5.609	0.013
H_2O_2	10.73	0.007	13.176	0.001	19.872	0.00
MDA	220.479	0.00	11.884	0.001	37.372	0.00
SOD	49.752	0.00	2.82	0.112	2.862	0.109
CAT	0.109	0.749	1.882	0.202	1.975	0.189
APX	26.39	0.001	18.989	0.001	69.367	0.00
GR	1.786	0.223	15.321	0.003	0.093	0.912

3.2. Levels of oxidative damage markers

Hydrogen peroxide (H_2O_2) content of black *A. macrostachyum* seeds were higher compared to brown seeds and also increased with increases in salinity. However, those of brown seeds remained unaltered across salinity treatments (Fig. 3). MDA levels did not increase with increases in salinity in either seed type (Fig. 3).

H_2O_2 content of small seeds of *A. indicum* increased at 400 mM NaCl, while those of large seeds remained unaffected by salinity (Fig. 3). Likewise, levels of MDA of small seeds increased with increasing salinity and those of large seeds remained unaltered across salinity treatments (Fig. 3).

3.3. Activities of antioxidant enzymes

Salinity did not affect superoxide dismutase (SOD) activity of black seeds of *A. macrostachyum*, whereas a decline in SOD activity of brown seeds was observed across salinity treatments (Fig. 4). Catalase (CAT) activity in germinating black seeds increased, but that of brown seeds declined in 400 mM NaCl (Fig. 4). Ascorbate peroxidase (APX) activity of the black seeds was significantly ($P < 0.05$) higher under saline than non-saline condition, while that of brown seeds declined only transiently at 200 mM NaCl than other treatments. Activity of GR remained unaffected by salinity in black seeds, but that of brown seeds increased with salinity increases (Fig. 4).

In heteromorphic seeds of *A. indicum*, activities of CAT and GR were generally comparable with little or no influence of salinity (Table 2; Fig. 4). A two-way ANOVA indicated a significant effect of seed morphology on the SOD and APX activities (Table 2), which however remained unaffected by salinity increments (Fig. 4).

4. Discussion

Large and small seeds of *A. indicum*, while black and brown colored seeds of *A. macrostachyum* were found (Fig. 1). Similarly, a number of halophytes such as *Suaeda aralocaspica* (Wang et al., 2017), *S. acuminata* (Wang et al., 2012) and *S. splendens* (Redondo-Gómez et al., 2008) showed seed color heteromorphism. While, many halophytes including *A. tringularis* and *Salicornia europea* displayed seed heteromorphism of size (Philipupillai and Ungar, 1984; Khan and Ungar, 1984a,). Seed heteromorphism is a bet-hedging strategy of the plants inhabiting unpredictable environments, where a single phenotype cannot be successful (Imbert, 2002; Lenser et al., 2016). As a result, many plants of such habitats form heteromorphic seeds with different longevity, germination time and requirements (Imbert, 2002; Yao et al., 2010; Liu et al., 2018). Hence, production of heteromorphic seeds in the test species appears an important adaptation for survival in unpredictable salt -marshes (Khan and Gul, 1998).

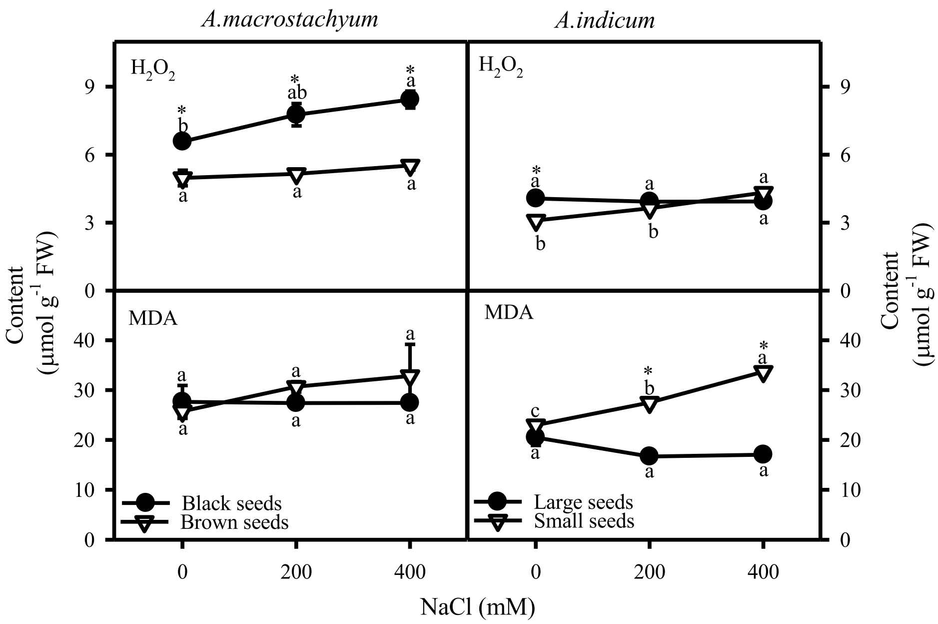

Fig. 3. Effects of salinity on H_2O_2 and MDA contents of the germinating heteromorphic seeds of *A. macrostachyum* and *A. indicum*. Line values are mean ± standard error. Asterisks (*) indicate significant difference between two morphs within a salinity level (T-test, $P < 0.05$). Different letters across salinity are significantly different (Bonferroni test, $P < 0.05$).

Heteromorphic seeds of *A. indicum* and *A. macrostachyum* germinated readily in distilled water, which is a common adaptation of subtropical perennial halophytes to take advantage of short monsoon period (Gul et al., 2013). The seed morphs of each species did not differ in salinity tolerance from each other but generally heteromorphic *A. macrostachyum* seeds had higher germination in high salinity than those of *A. indicum*. Under non-saline conditions large compared to small *A. indicum* seeds showed higher germination. In water, brown seeds also showed slightly (although statistically non-significant) higher germination than black *A. macrostachyum* seeds. Brown *Suaeda aralocaspica* (Wang et al., 2008) seeds were non-dormant but its black seeds had dormancy. This difference might be linked to differential phenolic make up of the seeds, as some phenolics are germination inhibitors (Muscolo et al., 2001). Similarly, large *Salsola komarovii* (Takeno and Yamaguchi, 1991) seeds had greater germination in comparison to small ones, which could be related to comparatively higher nutrient content in large seeds (Yao et al., 2010). Hence, higher and/or faster germination of at least one seed morph of each test species would be competitively advantageous after monsoon rain when marsh-sediments' salinity is low, thereby increasing chances of their survival.

Hydrogen peroxide (H_2O_2) was detected in the germinating heteromorphic seeds of both tested halophytes. Similarly, H_2O_2 was also detected in un-stressed germinating seeds of other halophytes such as *Suaeda fruticosa* and *Limonium stocksii* (Hameed et al., 2014). Reactive oxygen species (ROS) such as H_2O_2 are usually generated in germinating seeds during imbibitional reactivation of mitochondrial oxygen metabolism (Pergo and Ishii-Iwamoto, 2011) and NADPH oxidase (NOX) activity (Ishibashi et al., 2010). Low levels of ROS especially H_2O_2 play important roles in various germination-related processes such as cell wall loosening, signalling and decreasing abscisic acid levels (Bailly et al., 2008; El-Maarouf-Bouteau and Bailly, 2008), thereby creating an "oxidative window" (*Sensu* Bailly et al., 2008) for seed germination completion. The H_2O_2 content of black as compared to brown *A. macrostachyum* seeds increased with increases in salinity and that of small compared to large *A. indicum* seeds increased at 400 mM NaCl. Information about ROS dynamics in heteromorphic seeds especially of halophytes is largely absent. However, higher H_2O_2 production in seedlings derived from brown as compared to those from yellow seeds of *Atriplex centralasiatica* following 4 days of NaCl treatment was observed (Xu et al., 2011). Hence, it appears that heteromorphic seeds may differ in H_2O_2 levels during germination, which could be related to the composition of antioxidant systems.

Malondialdehyde (MDA; a common lipid peroxidation marker) content of small but not large seeds of *A. indicum* increased with increases in salinity, while those of heteromorphic seeds of *A. macrostachyum* were comparable with no change across salinity treatments. Changes in MDA content of germinating seeds of two dicot halophytes (Hameed et al., 2014) and 7d old seedlings of model halophyte *Thellungiella halophila* (Guo et al., 2012) have also been studied. Whereas, similar to the large seeds of *A. indicum*, MDA content of *Salsola drummondii* germinating seeds did not increase in response to salinity increments (Rasheed et al., 2015). MDA content of *S. ikonnikovii* seedlings also remained unaltered in up to 300 mM NaCl (Xing et al., 2013).

Antioxidant enzymes are important for seed germination under stressful environments (Bailly, 2004; Kranner and Seal, 2013). Salinity caused a surge in activities of CAT (400 mM NaCl only) and APX (both 200 and 400 mM NaCl) with no effect on SOD and GR activities in black seeds, while an increase in GR (both 200 and 400 mM NaCl) activity along with a decline in activities of SOD (both 200 and 400 mM NaCl), CAT (400 mM NaCl) and APX (200 mM NaCl) in brown *A. macrostachyum* seeds. CAT activity in the seeds (which are of black color) of *Suaeda fruticosa* also increased under saline conditions; while in *Gypsophila oblanceolata* seeds decreased under salinity (Sekmen et al., 2012). High activities of CAT and APX with constitutive SOD levels in black seeds of *A. macrostachyum* under saline conditions might be involved in quenching of H_2O_2 content, which is supported by unaltered MDA levels under increasing salinity. A decline in the activities of most antioxidant enzymes in brown seeds of *A. macrostachyum* under saline conditions could be explained in light of low and unaltered levels of H_2O_2, indicating there was no need of higher activities of these enzymes. Activities of most antioxidant enzymes (i.e. CAT, GR and GPX) of heteromorphic seeds of *A. indicum* were generally comparable and insensitive to salinity. Likewise, salinity increments did not affect the activities of SOD, APX and GPX in germinating seeds of *Salsola drummondii* (Rasheed et al., 2015).Unaltered levels of H_2O_2 and MDA levels in the large seeds of *A. indicum* also reflect that there was no need of enhanced antioxidant enzyme activities, while unchanged enzyme activities alongside rise in H_2O_2 and MDA levels under saline conditions in small seeds indicate that perhaps due to low food reserves small seeds couldn't strengthen the antioxidant defense to prevent oxidative damage. Low recovery of germination of small compared to large seeds of *A. indicum* from (200 and 400 mM NaCl) salinity in a recent study (Nisar et al., 2019) also supports aforementioned finding of this study.

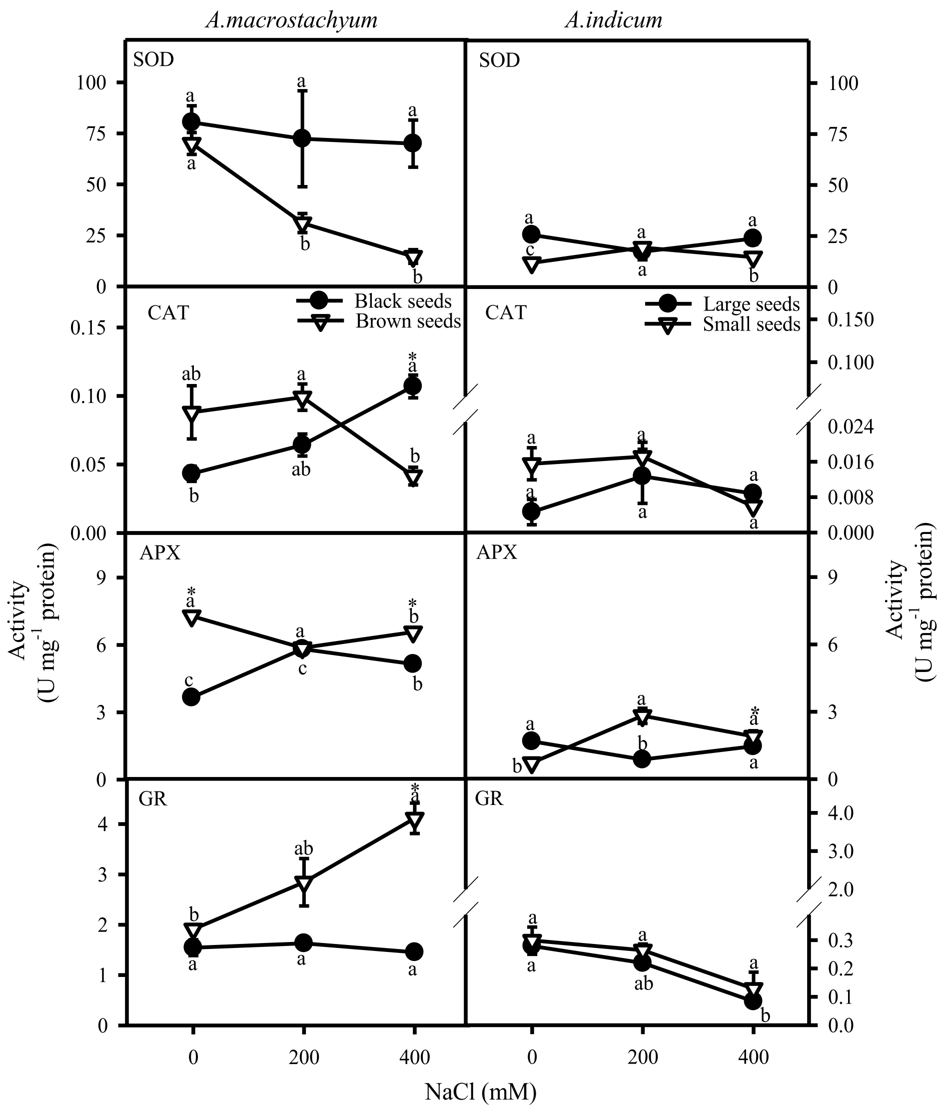

Fig. 4. Effects of salinity on antioxidant enzyme activities of the germinating heteromorphic seeds of *A. macrostachyum* and *A. indicum*. Line values are mean ± standard error. Asterisks (*) indicate significant difference between two morphs within a salinity level (T-test, $P < 0.05$). Different letters across salinity are significantly different (Bonferroni test, $P < 0.05$).

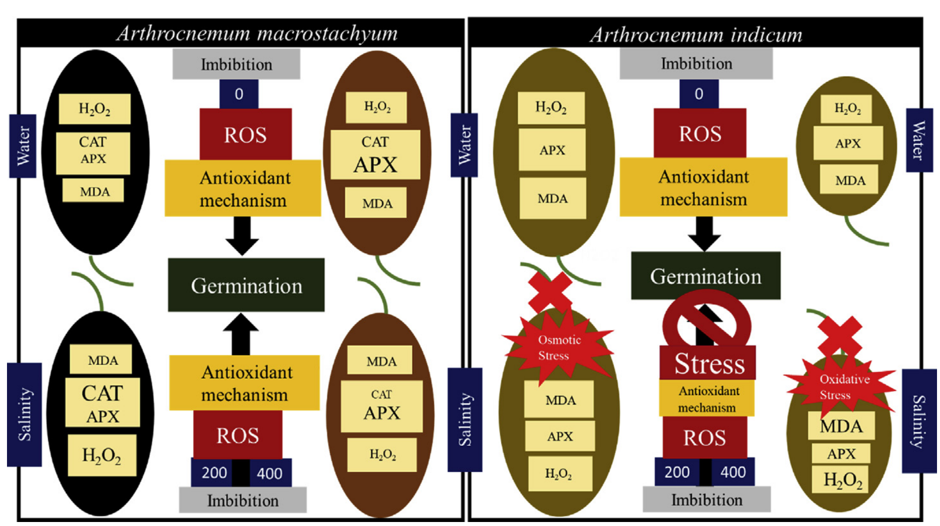

Fig. 5. Summary of the ROS homeostasis operative in the germinating seeds of test species under non-saline and saline conditions.

5. Conclusions

Arthrocnemum macrostachyum seeds were more tolerant to high NaCl dose than those of *A. indicum*. In absence of NaCl, large *A. indicum* seeds and brown *A. macrostachyum* seeds showed slightly higher germination than their respective counterparts. In addition, our data also indicates differential H_2O_2 homeostasis mechanisms, as discussed above, in the heteromorphic seeds of test species (Fig. 5).

Author contributions

Farah Nisar and Abdul Hameed designed and executed the experiment, analyzed the data and prepared the manuscript. Bilquees Gul and M. Ajmal Khan contributed in interpretation of the results and manuscript writing.

Conflicts of interest

All authors declare no conflict of interest.

Acknowledgments

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