Antioxidant Enzymes and Germination Pattern: Upshot of High Salinity on Soluble Protein and Average Weight of *Spinacia oleracea* (Spinach) Seedlings

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Authors' contributions

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ABSTRACT

Spinach (*Spinacia oleracea L.*) is widely considered as a functional food mainly due to its various beneficial components including vitamins, minerals, phytochemicals, bioactive and antioxidant compounds that stimulate health beyond basic nutrition. The overwhelming agricultural crop at salinity conditions faced several abiotic and biotic stresses that unfortunately reduced the potential applicability of highly nutritious plants. Therefore, in this study the response of antioxidant enzymes

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were studied at different germination stages of the seedlings and results suggested that all antioxidant enzymes play a crucial role during oxidative stress. Spinach (*Spinacia oleracea L.*) seedlings had the potential to germinate remarkably well up to 800 mM NaCl concentration and the average weight of seedlings and soluble protein content was efficiently increased at high salinity. During oxidative stress, catalase, ascorbate peroxidase, guaiacol peroxidase, superoxide dismutase, and glutathione reductase significantly showed tolerances to salinity stress conditions. Among them, Ascorbate Peroxidase presented significant activity even at 600 mM of NaCl in germination stage 1. The antioxidant defense mechanism of *Spinacia oleracea* is activated at the very early stage of germination which perhaps helps the plant to survive under harsh conditions thus maintaining the nutritional components of the plant.

ABBREVIATIONS

ROS   *: reactive oxygen species;*
CAT   *: catalase; APX - ascorbate peroxidase;*
GPX   *: guaiacol peroxidase;*
SOD   *: superoxide dismutase;*
GR   *: glutathione reductase;*
H_2O_2 *: hydrogen peroxide;*
NADPH *:nicotinamide adenine dinucleotide phosphate hydrogen,*
EDTA   *: ethylene diamine tetra acetic acid,*
NBT   *: nitroblue tetrazolium.*

1. INTRODUCTION

Salinity is one of the major abiotic stress affecting plant growth and severely strike agricultural productivity worldwide. Salinity shock affects the metabolism of plant cells leading to severe crop injury and decrease production yield. Oxidative stress is one consequence of salinity that may be responsible for much of the damage. Furthermore, the high light intensity, extreme temperatures drought, herbicide treatment are also the causes of activation of oxygen species. The reactive oxygen species (ROS) produce in plants due to the high salinity causes the disruption of lipids, proteins, and nucleic acid [1]. The plant defense mechanism contains several ROS scavenging enzymes for the control of ROS generation and to protect cells under stress conditions. The ROS produced within the cells is detoxified by non-enzymatic and enzymatic antioxidant systems. The major enzymes which play an important role in defense mechanisms against the oxidative damage are catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), superoxide dismutase (SOD) and glutathione reductase (GR) [2,3]. The increase in ROS begins a chain reaction, SOD responsible for the dismutation of O_2- radicals to molecule O_2 and H_2O_2 [4]. The ascorbate-glutathione cycle detoxified H_2O_2, which involves the oxidation and re-reduction of ascorbate and glutathione through APX and GR action [5,6,7,8]. Spinach (*Spinacia oleracea*) is a plant that usually cultivates all over the world as a cool-seasoned annual green leafy vegetable [9]. It encompasses beneficial ingredients such as an excellent source of protein, minerals and fiber. Spinach is an ironic source of main micronutrients including zinc, iron, manganese and magnesium with trace quantities of vitamin E, A, C, K, folate, thiamine (B1), pyridoxine (B6) and riboflavin (B2). The bioactive components and antioxidantenzymes of spinach specifically help in the reduction of oxidative stress. These biological activities contribute as anti-cancer, anti-obesity, hypoglycemic, and hypolipidemic properties of spinach [10]. Not much data is available for the expression of antioxidant activity in *S. oleracea* seedlings at high salinity levels and at different germination stages. The current research was designed to explore the effects of salinity on different germination stages and the antioxidant enzymes profiling of seeds of *S. oleracea* variety available in Pakistan.

2. MATERIALS AND METHODS

2.1 Plant Material

Spinach belongs to cool-seasoned vegetable that germinate at 10-25ºC. In this study, seeds of *Spinacia oleracea* var. prickly heat (Spinach) were purchased from Sindh Agriculture University (Tandojam, Sindh, Pakistan). The seeds were used for experimental work, stored at $4°C$ to $8°C$. The spinach seeds were washed manually to remove all foreign matter including dust, dirt, stones and chaff. All the experiments were conducted at Plant Tissue Culture Laboratory of PCSIR Laboratories Complex, Karachi.

2.2 Seed Germination Conditions

The experimental procedures were conducted between 20°C to 25°C. The pots were purely designed with 15 cm in diameter arranged in a completely random design (CRD). In 5 pots, different treatments of NaCl solutions were supplemented with washed sandy loam. Different range of NaCl solutions (200, 400, 600, 800, and 1000 mM) were incorporated in the soil separately and the electrical conductivity of the soil was maintained throughout the experimentation to retain the respective salinity levels. The seeds of *S. oleracea* (n = 100) were sown in a depth of 2.5 cm in each pot. The germination of seeds started after fourth day, the seedlings were collected at 0, 4, 8 and 24 hours later than germination. The term seedling used throughout the study expresses the early developmental stages of the *S. oleracea* seeds and the term germination is referred to as the end of the germination after the appearance of radical. The maintained normal conditions were used for control pots and sampled at the same time as the stressed seedlings. In control pot, only water was added instead of NaCl solution.

Germination stages are described as below;

Germination stage 1: Seedlings collected at 0 hour later than germination (just after emergence of radical).
Germination stage 2: Seedlings collected at 4 hours later than germination.
Germination stage 3: Seedlings collected at 8 hours later than germination.
Germination stage 4: Seedlings collected at 24 hours later than germination.

2.3 Enzyme Extracts Preparation

Germinated seedlings (0.5 g) were removed from the soil and thoroughly washed with distilled water. Seedlings were homogenized in chilled mortar and pestle in 2.0 ml buffer (50 mM phosphate buffer, pH-7.0). The insoluble material was removed by centrifugation at 35000×g for 15 minutes at 4°C and the supernatant was used for the determination of antioxidant enzyme activities. The extracts were kept at -20°C until further use in analysis.

2.4 Determination of Catalase (EC 1.11.1.6)

The CAT activity was estimated spectrophotometrically by the method as described previously with some modifications [11]. The H_2O_2 decomposition was measured at 240 nm taking $\Delta\varepsilon$ at 240 nm as 43.6 mM cm-1 [12]. The reaction mixture (3.0 mL) contained 10.5 mM H_2O_2 in 50 mM potassium phosphate buffer (pH-7.0) and 0.1 ml of enzyme extract was added to initiate the reaction at 25°C. The CAT activity was calculated by the decrease in absorbance at 240 nm. The amount of enzyme catalyzing the conversion of 1.0 mM of H_2O_2 min-1 at 25°C is equal to the one unit of CAT activity.

2.5 Determination of Ascorbate Peroxidase (EC 1.11.1.11)

APX activity was determined according to method of Nakano and Asada [13]. The reaction mixture (2.0 mL) contained 50 mM potassium phosphate buffer (pH-7.0), 0.2 mM EDTA, 0.5 mM ascorbic acid and 0.25 mM H_2O_2. The enzyme extract (0.1 ml) was added to initiate the reaction at 25°C. The amount of ascorbate oxidized was calculated from the extinction coefficient 2.8 mM cm^{-1} by recording the decrease in absorbance at 290 nm for one minute. The unit of ascorbate peroxidase activity at 25°C is expressed as one micromole of ascorbic acid oxidized min^{-1}.

2.6 Determination of Guaiacol Peroxidase (EC 1.11.1.7)

GPX activity was measured spectrophotometrically at 25°C [14]. The reaction mixture (2.0 ml) consisted of 50 mM potassium phosphate buffer (pH-7.0), 2 mM H_2O_2 and 2.7 mM guaiacol. The enzyme extract (0.1 ml) was added to initiate the reaction at 25°C. The rate of formation of tetraguaiacol at 470 nm ($\Delta\varepsilon$ = 26.6 mM cm^{-1}) used to measure the initial rate of guaiacol oxidation. The amount of enzyme required to catalyze the conversion of one micromole of hydrogen peroxide with guaiacol as hydrogen donor per minute under specified conditions is defined as one unit activity.

2.7 Determination of Glutathione Reductase (EC 1.6.4.2)

GR activity was determined by measuring the decrease in absorbance at 340 nm (ε = 6.2 mM cm^{-1}) as a rate of NADPH oxidation at 25°C [15]. The reaction mixture (1.0 mL) consisted 100 mM Tris-HCl buffer (pH-7.8), 21 mM EDTA, 0.005 mM NADPH, 0.5 mM oxidized glutathione (GSSG) and the enzyme extract. The reaction

was initiated by the addition of NADPH. One Unit activity is defined as amount of enzyme which reduced 1.0 µmol of oxidized glutathione per minute under standard assay conditions.

2.8 Determination of Superoxide Dismutase (EC 1.15.1.1)

The SOD activity was performed by using the method of Beyer [16]. The assay mixture contains 27.0 mL of 50 mM potassium phosphate buffer (pH-7.8), 1.5 mL of L-methionine (300 mg 10 mL-1), 1.0 mL of 14.4 mg 10 mL-1 nitroblue tetrazolium salt (NBT), and 0.75 mL of Triton X-100. The 1.0 mL aliquots of the above mixture were dispensed into small glass tubes, and the procedure was followed by the addition of enzyme extract (0.02 ml) and (0.01 ml) riboflavin (4.4 mg dL-1). The assay solution was mixed and then illuminated for 15 minutes in an aluminum foil-lined box in 25 W fluorescent light. A sample in control tube was replaced by 0.02 ml of buffer and the absorbance was measured at 560 nm. The light was switched off and tubes were placed in the dark to stop the reaction. Increasing in absorbance was measured at 560 nm due to the formation of formazan in control was taken as 100%. The enzyme activities of the samples were calculated by determining the percentage inhibition per minute. One unit of SOD is that amount of enzyme which causes a 50% inhibition of the rate for reduction of NBT under the specific assay conditions.

2.9 Total Protein

Total protein concentrations of the enzyme samples were estimated by using the method of Lowry [17] with bovine serum albumin used as a standard. All measurements were carried out in triplicates and the average value was taken.

3. RESULTS AND DISCUSSION

3.1 Effect of Salinity on Different Stages of Germination

Inhibition in growth of *S. oleracea* seeds over a range of salinity had a pronounced effect on germination potential. The rate of germination slowed down at high salinity levels (Fig. 1a and 1b). Similar findings were also observed in the germination of salinity treated tobacco and wheat seeds [18,19]. Germination percentage was calculated as ratio of number of germinated seeds to total number of seeds tested.

Germination in *S. oleracea* seeds was started 4 days later from seed sowing and all the seeds germinated up to 8th day. Germination in control was taken as 100%, as all seeds germinated properly up to the germination stage 4. By comparing different levels of salinity stress, it was concluded that higher concentration of salt resulted in lower levels of germination. This highlights the adverse effects of increased salinity levels on the germination of this *S.oleracea* seeds. This is mainly due to the salinity stress caused by high level of Na^+ and Cl^- concentrations, which generate nonspecific osmotic stress causing water deficiency and specific accumulation of toxic ions which might contributes in disturbing nutrient acquisition resulting cytotoxicity in plant [20].

3.2 Effect of Various Concentration of NaCl on Seed Germination

It was observed that *S. oleracea* seeds tolerated salt stress up to 800 mM concentration, however rate of germination dropped drastically to 48.2%, 37.6%, 11.7% and 7.0% in 200, 400, 600 and 800 mM NaCl respectively, when compared to control. No germination was observed at 1000 mM NaCl concentration (Fig. 2A). It has been reported that the growth of *S. oleracea* ceases above 300 mM NaCl level [21]. Likewise, when average fresh weight of spinach seedling was observed in the current study, there was a slight increase in average weight at 400 and 600 mM concentration as compared to control, while above 600 mM, a decline in the pattern was noticed (Fig. 2B). Similar pattern was observed in a previous study carried out by Coughlan and they also reported that there was a decrease in fresh weight of the seedling with the increase in salinity [21]. The increase in average seedling weight of spinach seeds at 400 mM and 600 mM concentration of NaCl was might be due to the voltage-independent channels that basically established during the main pathway for Na^+ uptake in high salt conditions through plasma membrane [22]. In similar studies, the levels of Na^+ ion augmented in response to increasing levels of salinity in the wheat seedlings [23]. In this experiment, the cells of the plant roots could uptake Na^+ from the saline substrates through passive diffusion across the plasma membrane and transfer the absorbed Na^+ to the shoots [24]. Thus, increased salt-stress resulted in a significant increase in average weight of seedling by establishing appropriate level of voltage independent channel of sodium ions.

Total protein value showed a gradual increase along with increasing in salinity level (Fig. 2C), however the soluble protein content decreased at 800 mM. It has also been reported an increase in soluble protein content and this may be due to stress induced synthesis of proteins [25]. Different concentrations of NaCl also increased protein content in seedlings of *Acanthophyllum* species, though this pattern was only observed in salt tolerant species of some plants such as barley, sunflower, finger millet and rice [26,27].

3.3 Effect of Catalase Activity on Different Stages of Seed Germination

It was observed that when the seeds were exposed to salt stress, a gradual decline in catalase activity was observed at all germination stages (Fig. 3). In control, the activity started increasing up to germination stage 2 and sequentially declined up to germination stage 4, however it was higher as compared to the other stages exposed to stress. The highest CAT activity was detected at 600 mM during germination stage 1. It is prominent that as the germination stages proceeds, catalase activity declined gradually in control as well as in all other samples (Fig. 3A). A sharp decline in CAT activity was observed as the salinity increased and this might be due to the enhancement levels of H_2O_2 [28]. It has been reported that ROS including superoxide radical and H_2O_2 are elevated with increased salinity due to imbalance in the production and destruction of ROS [29]. The CAT activity in plants such as pea and rice seedling get reduce due to the salt stress, while in seedling of *Cicer arietinum,* salt stress enhanced the activity [30,31,32].

3.4 Effect of Ascorbate Peroxidase Activity on Different Stages of Seed Germination

A similar pattern was observed for APX activities in all samples of four germination stages. Maximum APX activity was detected at 600 mM NaCl concentration in stage 1. It is noticeable that APX activity in all stress samples at various germination stages is higher than control. It means that in stress conditions, this enzyme has a positive impact at all stages and helps in survival of *S. oleracea* seedlings even at high salinity levels (Fig. 4). In fact, the increase in APX activity in all stressed seedling samples may be due to the pre-existing APX activation or due to salt treatment caused the synthesis of

new APX. This may also be associated with the deactivation of CAT and H_2O_2 over production [28,33]. So, APX was likely to be more important than CAT in the detoxification [34].

3.5 Effect of Guaiacol Peroxidase Activity on Different Stages of Seed Germination

Since H_2O_2 involved in peroxidase mediated oxidative polymerization, which results in cell wall strengthening. The highest GPX activities were detected only during germination stage 1 and in all NaCl concentrations; the values were approximately similar as compared to control. With a gradual increase in germination stages, GPX activities declined (Fig. 5). Importantly, the GPX activity has been used as an indicator for different stresses such as high temperature, salinity and drought in foxtail millet and rice varieties [35,9].

3.6 Effect of Glutathione Reductase Activity on Different Stages of Seed Germination

Particularly, GR was expressed in very low quantities as compared to other antioxidant enzymes. The activities in all stress conditions and in all four stages remain almost the same except for germination stage 1. Although, GR was the most abundantly produced at 200 mM NaCl concentration as compared to control and all other germination stages (Fig. 6). The increasing content of GR may be driven by enhanced H_2O_2 formation in salt stress [36]. So, the oxido-reduction cycle involving glutathione and ascorbate plays an important role in control of endogenous H_2O_2 content and GR play an important role in this process [37]. The reason of this is that GR has the ability to regenerate another powerful mechanism for water-soluble antioxidant known as ascorbic acid through the ascorbate-glutathione cycle [7]. As a consequence, it triggers the mechanism so the activities of APX have been expressed higher as compared to GR (Fig. 6).

## 3.7 Effect of Superoxide Dismutase Activity on Different Stages of Seed Germination

Data for SOD activities shows that as germination stage precedes forward, the activities increases in all stress conditions (Fig. 7). As a matter of fact, in all stages, the

SOD activity in each NaCl concentration is less as compared to control. The highest level of SOD enzyme activity was observed in control at germination stage 4 as compared to other stages. SOD is the only antioxidant enzyme which is prominently expressed under stress conditions. Enhanced formation of an upstream SOD results in scavenging of free oxygen radical and catalyze the dismutation of superoxide to H_2O_2. In other plant species, such as citrus and pea it has been reported that salt induces a significant increase in SOD activity [37]. Increased activity of SOD under saline conditions was also reported by other researchers as well for different plant species [38,25,39,40]. In the same manner, sodium chloride provoked a dose dependent increase in SOD activity in *J. curcas* callus which could represent a defense mechanism against NaCl induced generation of O_2^-, OH and O_2. On the other hand, under salt stress, the SOD activity decreased in sunflower [41].

Fig (a)

Fig (b)
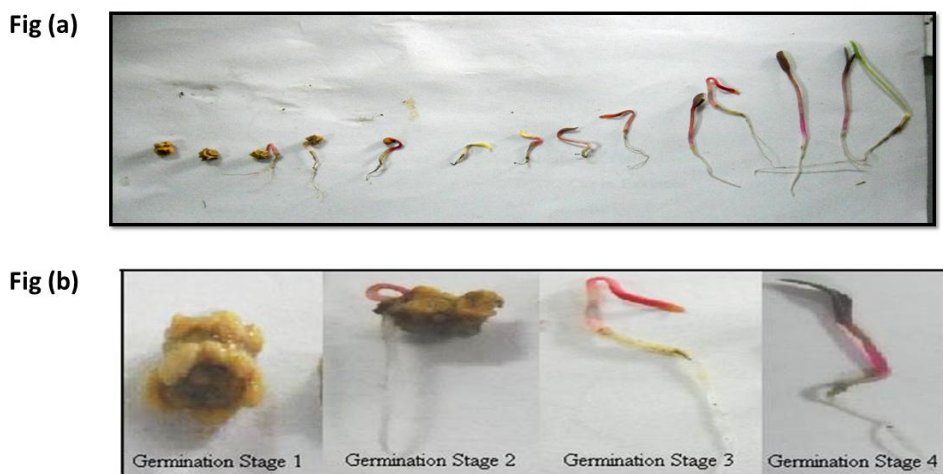
Fig. 1. a. Different stages of germination of *Spinacia oleracea*; b. germination stage 1: seedlings collected just after emergence of radical (0 hour); germination stage 2: seedlings collected 4 hours later from germination; Germination stage 3: seedlings collected 8 hours later from germination; germination stage 4: seedlings collected 24 hours later from germination
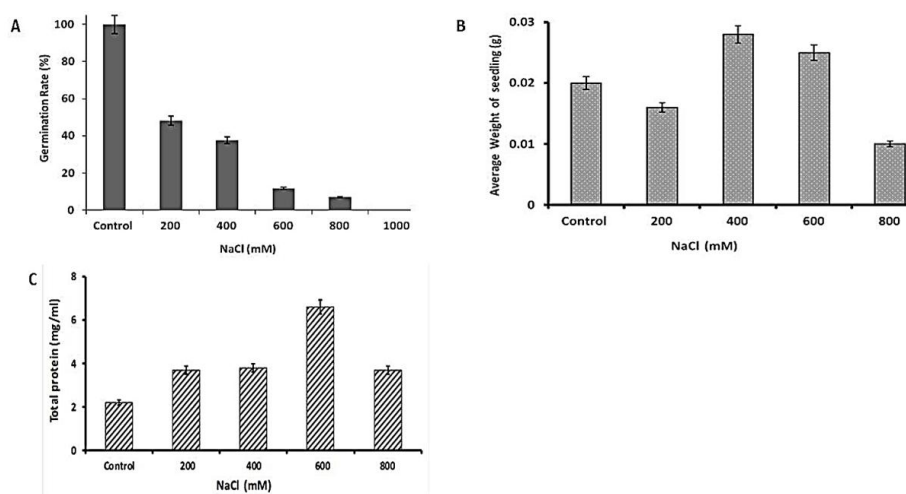

Fig. 2. Effect of salt stress on (A) germination rate; (B) fresh weight of seedlings and (C) total protein content of *Spinacia oleracea* seedlings (n=3; standard deviation ± SD: 2%; p-value < 0.005)
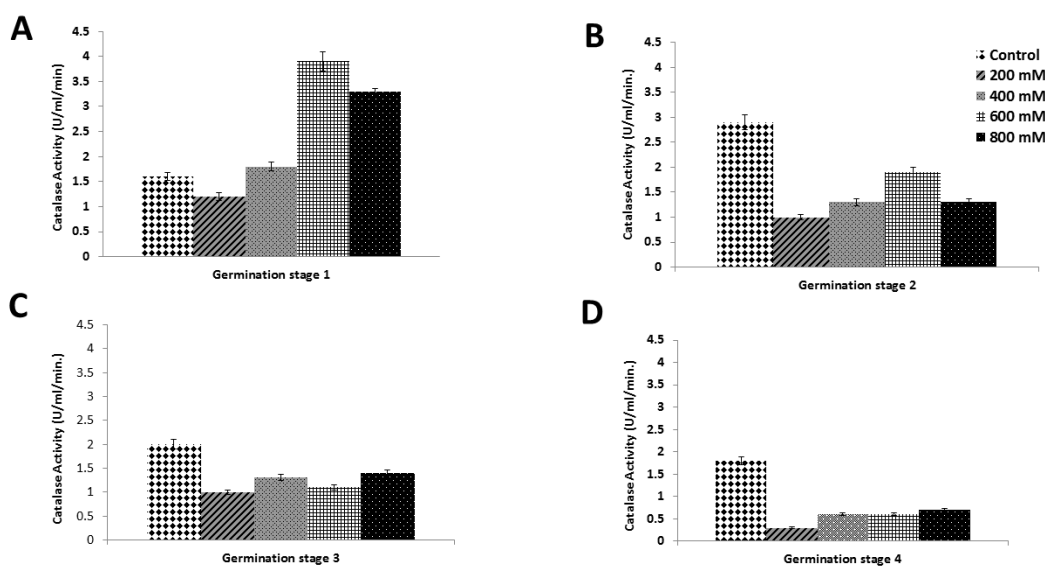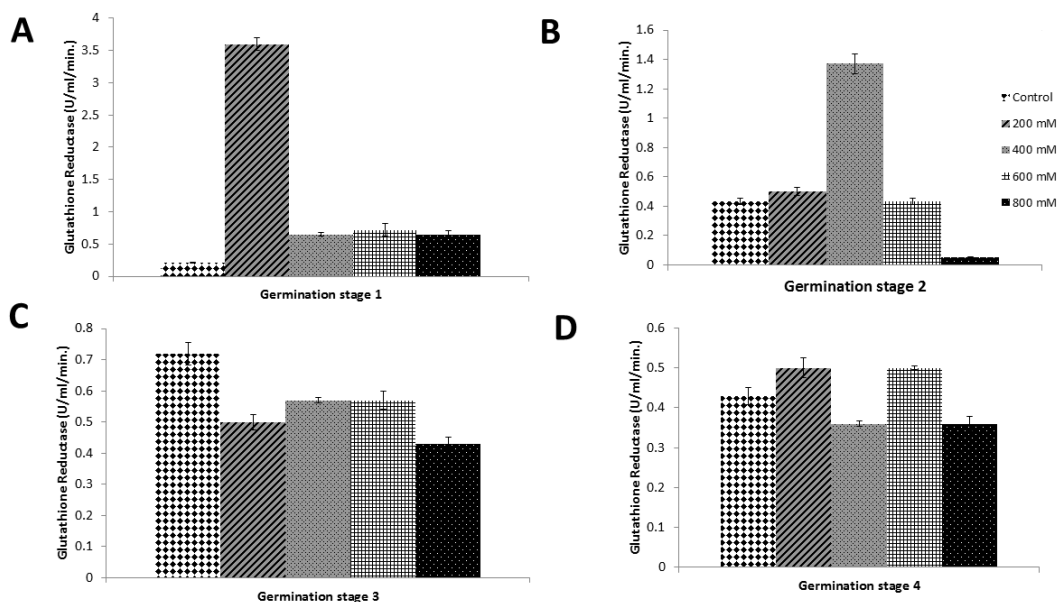
Fig. 3. Effect of catalase activity on different stages of germination at various concentration of NaCl; (A): Effect of catalase activity at germination stage 1 under different concentration of NaCl; (B): Effect of catalase activity at germination stage 2 under different concentration of NaCl; (C): Effect of catalase activity at germination stage 3 under different concentration of NaCl; (D): Effect of catalase activity at germination stage 4 under different concentration of NaCl. (n=3; Standard deviation ± SD: 2%; p-value < 0.005)
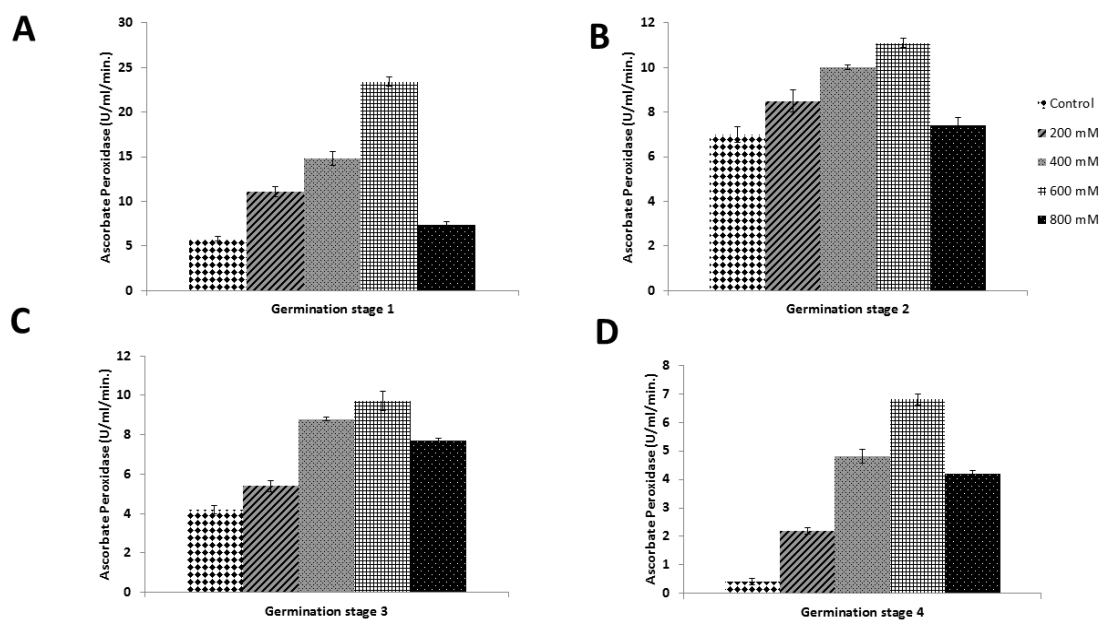
Fig. 4. Effect of ascorbate peroxidase activity on different stages of germination at various concentration of NaCl; (A): effect of ascorbate peroxidase activity at germination stage 1 under different concentration of NaCl; (B): effect of ascorbate peroxidase activity at germination stage 2 under different concentration of NaCl; (C): effect of ascorbate peroxidase activity at germination stage 3 under different concentration of NaCl; (D): effect of ascorbate peroxidase activity at germination stage 4 under different concentration of NaCl. (n=3; standard deviation ± SD: 2%; p-value < 0.005)
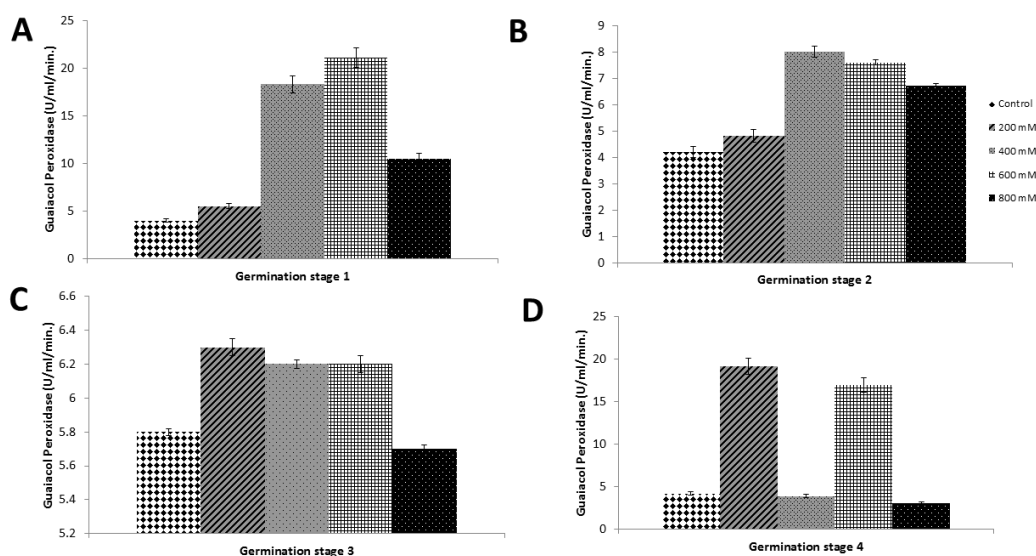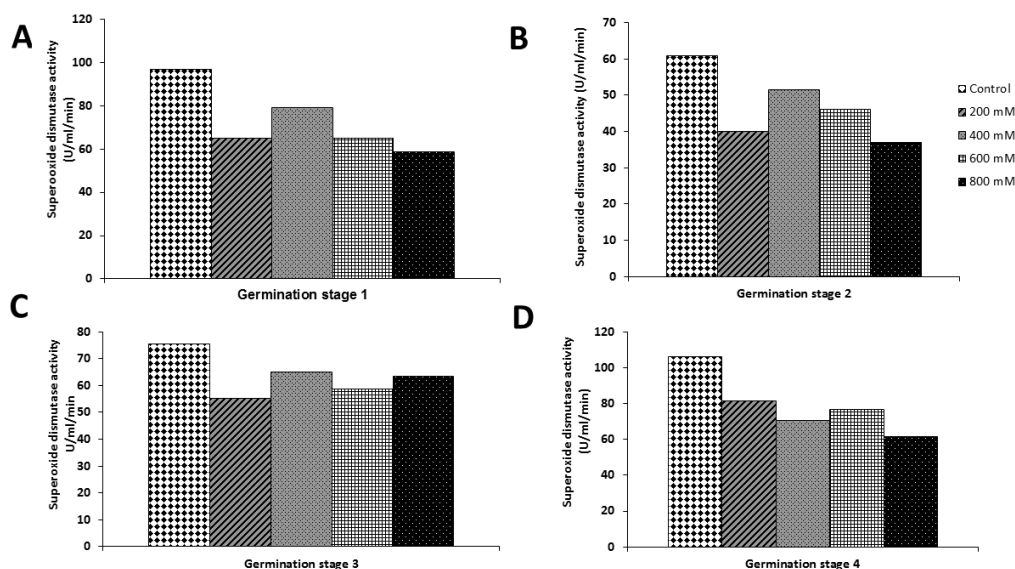
Fig. 5. Effect of guaiacol peroxidase activity on different stages of germination at various concentration of NaCl; (A): effect of guaiacol peroxidase activity at germination stage 1 under different concentration of NaCl; (B): effect of guaiacol peroxidase activity at germination stage 2 under different concentration of NaCl; (C): effect of guaiacol peroxidase activity at germination stage 3 under different concentration of NaCl; (D): effect of guaiacol peroxidase activity at germination stage 4 under different concentration of NaCl. (n=3; standard deviation ± SD: 2%; p-value < 0.005)

Fig. 6. Effect of glutathione reductase activity on different stages of germination at various concentration of NaCl; (A): effect of glutathione reductase activity at germination stage 1 under different concentration of NaCl; (B): effect of glutathione reductase activity at germination stage 2 under different concentration of NaCl; (C): effect of glutathione reductase activity at germination stage 3 under different concentration of NaCl; (D): effect of glutathione reductase activity at germination stage 4 under different concentration of NaCl. (n=3; standard deviation ± sd: 2%; p-value < 0.005)

Fig. 7. Effect of superoxide dismutase activity on different stages of germination at various concentration of NaCl; (A): effect of superoxide dismutase activity at germination stage 1 under different concentration of NaCl; (B): effect of superoxide dismutase activity at germination stage 2 under different concentration of NaCl; (C): effect of superoxide dismutase activity at germination stage 3 under different concentration of NaCl; (D): effect of superoxide dismutase activity at germination stage 4 under different concentration of NaCl. (n=3; standard deviation ± SD: 2%; p-value < 0.005)

4. CONCLUSION

It is concluded that each antioxidant enzyme investigated at various stages of germination in *Spinacia oleracea* var. Prickly heat seedlings plays a crucial role for survival of enzymes even at over stressed saline conditions. Superoxide dismutase was the most abundant enzyme expressed at very high levels of NaCl, at every germination stage. Meanwhile, other antioxidant enzymes were also expressed at the same time with varying titer. All of these five antioxidant enzymes work simultaneously which play an essential protective role in scavenging processes in *Spinacia oleracea* seedlings when exposed to high salinity levels.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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