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Seed germination ability and protein profiling of salt marsh plants at different concentration of sodium chloride

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Soil salinity is an increasing problem in agriculture throughout the world. The utilization of halophytic plants for pasture and fodder production in saline soils is the only economic solution presently available. The present study discusses the effects of different concentrations of sodium chloride on seed germination and its impact was determined by protein profiling on *Sesuvium portulacastrum*, *Suaeda maritima* and *Salicornia brachiata*. Seeds germination was substantially delayed and reduced with an increase in NaCl to above threshold level. Changes in the pattern of protein expression were found to be prominent between control and NaCl treated seeds. Proteins at the molecular weight of approximately 42 kDa, 26 kDa and 20 kDa were found to be up-regulated as the concentration of salt increases in *Sesuvium portulacastrum*. Whereas, significant variation in the protein patterns were observed in *Suaeda maritima* such as ~20 to 30 kDa protein bands were not visible and protein band of 55 kDa was particularly increased after 300 mM NaCl treatment. Similarly in *Salicornia brachiata* expression of 45 kDa protein was up regulated and approximately 25 kDa protein expression was down regulated as the concentration of salt increased to about 1.5 M, 2 M and 2.5 M. However, the upper limit for the survival of the seedling was 200 mM, 300 mM and 1 M for *Sesuvium portulacastrum*, *Suaeda maritima* and *Salicornia brachiata*, respectively. On the basis of the present investigation, this study suggests that optimal application of NaCl can benefit plant growth on stress tolerance studies and also helps for further investigation of the salt tolerance networks.

[Keywords: Halophytes, Protein profiling, Seed germination, Salinity, Salt marsh plant]

Introduction

Abiotic stresses are the serious threats to agriculture which severely cause many negative effects and changes to the natural environmental status. Among the many abiotic stresses such as drought, salinity, extreme temperatures and chemical toxicity; increased salinity is expected to cause up to 50 % land loss by the year 2050[1]. Higher concentrations of soluble salt on plant growth are associated with low osmotic potential of soil solution, imbalance of nutrients, specific ion effect or combination of this factors[2-5]. Many researchers highlighted that increased salinity in the arable land is devastating crops growth and productivity worldwide[6-8]. Various studies reported that different concentrations of NaCl negatively affected plant leaves[9-11].

Halophytes are the plants that has the ability to grow and strive their life cycle on extreme saline environment[12]. Halophytic plants naturally have the adaptive mechanism to compete their life in extreme salinity by adjusting plant cell to adjust osmotically and to accumulate organic solutes (proteins, sugar, amino acids, etc). Numerous studies has been undertaken to learn about halophytic seeds germination but there is still much to learn. However, the amount of salinity, temperature, light, life form, habitat, water etc. are considered to be the key factors in halophytic seed germination[13].

Various factors such as salt composition, type of salt, concentration of salt and plant growth stages affect the halophytic plant seed germination. The adaptive mechanism to tolerate salt stress through the various stages of their lifecycle is species specific[14,15]. Especially three promising halophytes of *Sesuvium portulacastrum*, *Salicornia brachiata* and *Suaeda maritima* are widely found in the coastal area of Tamilnadu. These three plants grow well at 100-400 mM especially *Salicornia brachiata* can grow at extreme salinity (1 M) without any toxic symptoms

on the leaves. Meanwhile these plants are useful to study about the salt tolerant mechanism and they are the potential source of salt resistant genes which might be helpful for the molecular biologists and plant breeders to improve the salt tolerance ability of the conventional crops. Hence, studying the seed germination ability of salt marsh plants is much important for our future concern.

The present study was undertaken to evaluate the effects of different concentrations of NaCl on seed germination and protein expression pattern of three different halophytes such as *Sesuvium portulacastrum*, *Salicornia brachiata* and *Suaeda maritima*.

Materials and Methods

Biological material

Seeds of *Sesuvium portulacastrum*, *Suaeda maritima* were collected from adjacent bank of vellar estuary in Parangipettai (lat. 11°29 N; long. 79°46 E), Tamil Nadu and seeds of *Salicornia brachiata* were collected from Mudasalodai (lat. 11°28 N; long. 79°46 E), Tamil Nadu (Fig. 1). Collected seeds were sterilized by agitating the seeds in 400 ml of 70 % v/v ethanol for 5 min and filtered using a Whatman filter paper. Further, the seeds were agitated again for 10 min in 200 ml of aqueous 0.1 % $HgCl_2$ solution. The seeds were filtered and rinsed with sterile water stored in a bottle containing 100 ml of sterile water at

Fig. 1 — Collection area of plant seeds: *Sesuvium portulacastrum* (A & B), *Suaeda maritima* (C & D), *Salicornia brachiata* (E & F). The flower and the seeds of the plants were shown near the respective halophytes

25 °C. After 24 hours, the seeds were filtered and approximately 100 seeds were placed on muslin cloths (wetted with 3 ml of sterile ½ MS media pH 5.8) in petri dishes as replicates. The petri dishes were covered with parafilm and stored in a culture chamber at 25 °C for 72 hrs and later maintained at 25 °C photoperiod for 16 h light.

Application of salinity stress

After 6 days of growing in controlled environment, germinated seeds were placed in fresh petri dishes containing 3 ml of ½ MS media (control) and NaCl solution (100 mM, 200 mM, 300 mM, 400 mM and 500 mM) for *Sesuvium portulacastrum* and *Suaeda maritima*. For *Salicornia brachiata* 3 ml of water (control) and NaCl solution (0.5 mM, 1 M, 1.5 M, 2 M and 2.5 M) were added to all Petri dishes. After 16 days of treatment all the germinated seedlings samples were harvested and stored at -80 °C.

Samples preparation

The seedling samples were individually crushed using a cryogrinding process. Fine powder was utilized for further studies.

Germination test

After treatment every day emerge of radical from the seeds was recorded. The rate of germination was calculated using formula of Germination Percentage: Number of germinated seed / total number of seeds × 100. After 16 days of germination, samples were harvested and immediately stored at -80 °C until use[16].

Total protein extraction

About 100 mg of seeds and 500 mg of leaf powder was homogenised and resuspended in 1 ml of ice cold extraction buffer (100 mM Tris (pH 8.0), 100 mM EDTA, 50 mM borax, 50 mM ascorbic acid, 1 % PVPP w/v, 1 % triton X-100v/v, 2 % of β mercapto ethanol v/v and 30 % of Sucrose w/v). The homogenized sample was vortexed for 5 min at room temperature and two volumes of saturated phenol with Tris (pH 8.0) was added and was further vortexed for 10 min. This mixture was centrifuged for 15,000 x g for 15 min at 4 °C. After centrifugation, aqueous phase was transferred to a fresh centrifuge tube. Equal volume of ice cold extraction buffer was added into the mixture and vortexed for 10 min followed by centrifugation at 15,000 x g for 15 min at 4 °C. The upper phase was carefully taken with micropipette and transferred to a fresh centrifuge tube and the proteins were precipitated by adding five volumes of methanol saturated ammonium acetate and incubating for 6 hours at -20 °C. After incubation the mixture was centrifuged again and the protein pellet was re-suspended and rinsed with ice cold methanol followed by ice cold acetone twice and spun down at 15000 x g for 5 min at 4 °C. After each washing, the mixture was carefully decanted. Finally the washed protein pellets were air dried then recovered with lysis buffer (9 M of urea, 2 % of CHAPS, 13 mM of DTT) and stored at -80 °C.

Total protein quantification

Concentration of total protein was estimated by following both Bicinchoninic acid protein Assay kit (BCA, Sigma) and Bradford assay[18] protocol. Bovine Serum Albumin (BSA) was used as the standard (1 µg/µl) and the absorbance was measured at 562 nm using LAMBDA 25 UV/Vis Spectrophotometer (Perkin Elmer).

Protein profiling (SDS-PAGE)

Extracted proteins from the seedlings were separated in SDS–Polyacrylamide gel. Ten percent (10 %) separating gel was first poured into a sealed glass plate cassette (1.5 mm thickness) after casting of separating gel 5 % stacking gel was poured over the separating gel within the cassette leaving 1 cm gap. Comb was inserted into the cassette[19,20]. The comb was carefully removed after the polymerization of the stacking gel. The desired concentration of protein sample was mixed with 4X sample buffer heated for 3 minutes in a boiling water bath and loaded into the slots. The slots were filled with 1X electrophoresis buffer and a constant current 21 mA/gel was applied. After the completion of electrophoresis, the gel was carefully removed and immersed in Coomassie Blue Staining solution (CBB G-250) and agitated slowly for 2 hours. The gel was then left in destaining solution for 2 to 4 hours with two to three time changing of the solution. After detaining, the gels were stored in 7 % acetic acid[21].

Results and Discussion

Effect of salt concentration on seed germination

For different salt concentration exposed seeds, germination rate was expressed in terms of percentage to relate the rate of germination. *S. portulacastrum* under normal (control) condition showed 57 % of

seed germination. Whereas, at higher salt concentration (500 mM) the rate of seed germination was 21 %, the high salt concentration strongly affected the germination of seeds. Similarly 27 % and 30 % germination observed at 400 mM and 300 mM NaCl concentration, respectively. The optimum concentration of NaCl nearer to the rate of seed germination occurred at control condition was assessed at 100 mM (47 % of seed germination) followed by 40 % in 200 mM NaCl (Fig. 2). The present study agreed with high salinity reduces water uptake essential for the transport of the nutrient needed for germination[22]. Accumulation of more Na^+ and Cl^- ions may reduce the osmotic potential might be the reason of reduced germination under increased salinity treatment.

In *S. maritima*, germination of the seeds was higher in control (96 %) and there was a gradual decrease in seed germination when there was increase in NaCl concentrations, with 100 mM NaCl (55.6 %), 200 mM (40 %), 300 mM (37.8 %), 400 mM (26.9 %) and 500 mM NaCl (20 %). The higher NaCl concentrations, showed substantial reduction of seed germination (Fig. 3). The above result highlights that increasing concentration of NaCl treatment gradually decreases germination percentage. Various reports suggest that *S. maritima* could survive at 100 - 500 mM of NaCl concentration. Concentration of 500 mM of NaCl salinity was reported as the threshold limit for survival of this species. However, we found that flexible or favorable growth response by the seedlings was confined upto 300 mM NaCl, supportive evidence of upper limit of 300 mM NaCl salinity for the favorable growth of *S. maritima* was reported[23]. *S*imilarly as in the halophyte plant, salinity leads to the reduction in germination percentage in some crop plants such as rice and barley seedlings[24,25]. High level of salinity could be attributed to the reduced process of osmosis, ion toxicity and lack of nutrients in the soil which leads to decrease in the rate of seed germination[26].

The determination of seed germination in *S. brachiata* (Fig. 4) revealed that the germination percentage was more in control (65 %) followed by 64 %, 61 %, 54 %, and 44 % in salinized seeds of 0.5 M & 1 M, 1.5 M, 2 M and 2.5 M concentration,

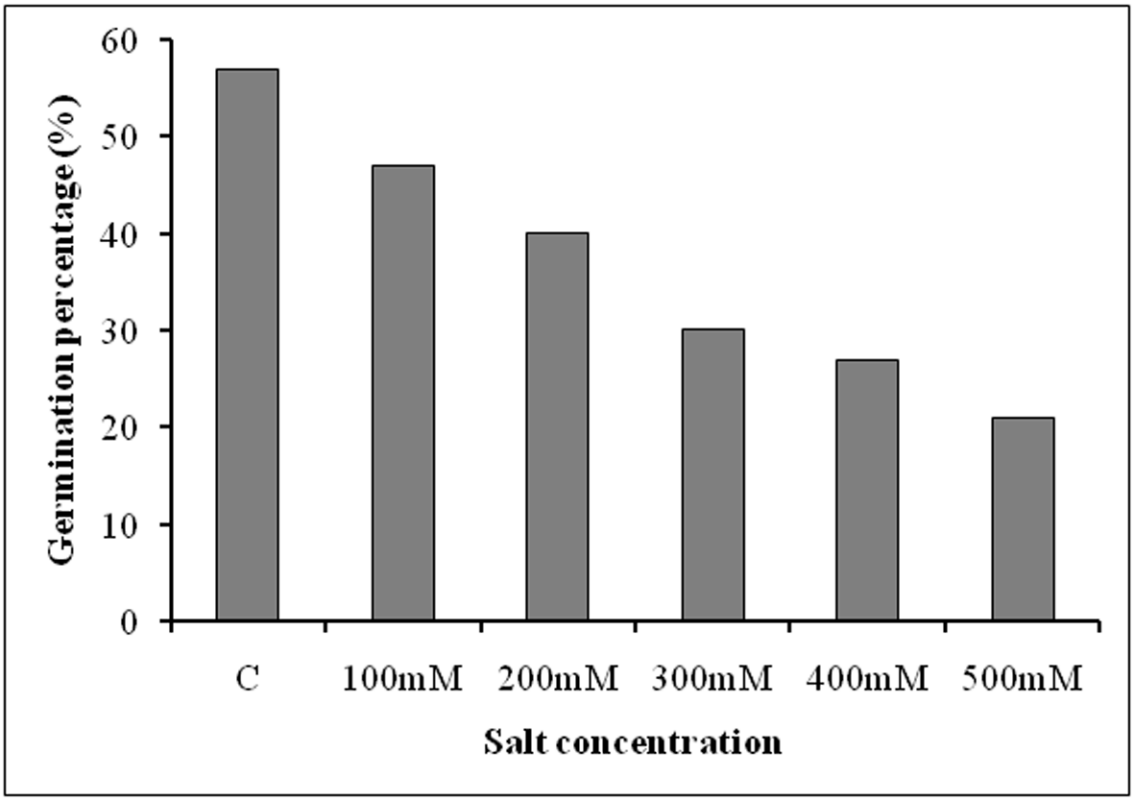

Fig. 2 — Seeds of *Sesuvium portulacastrum* were germinated in petridishes on muslin cloths wetted with ½ MS media. The values shown are the means of germination percentage determined at different concentration of NaCl for replicates with 100 seeds per treatment

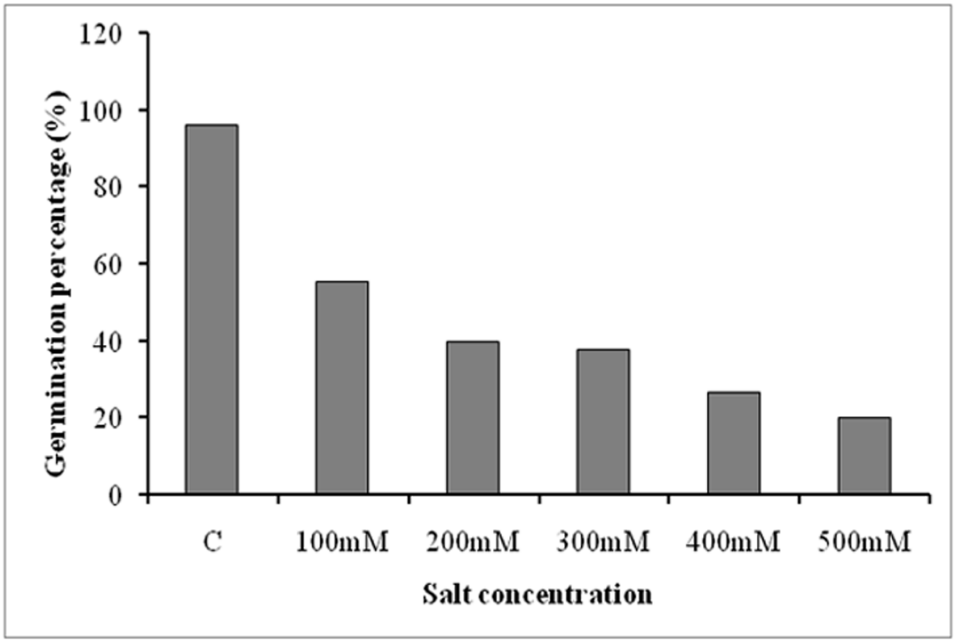

Fig. 3 — Seeds of *Suaeda maritima* were germinated in petridishes on muslin cloths wetted with ½ MS media. The values shown are the means of germination percentage determined at different concentration of NaCl for replicates with 100 seeds per treatment

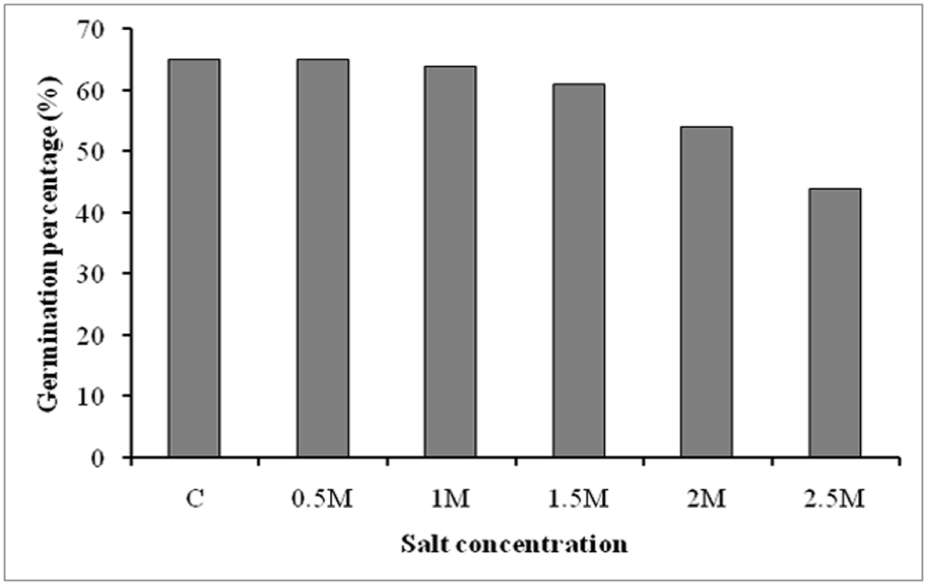

Fig. 4 — Seeds of *S. brachiate* were germinated in petridishes on muslin cloths wetted with ½ MS media. The values shown are the means of germination percentage determined at different concentration of NaCl for replicates with 100 seeds per treatment

respectively. In some cases halophytes such as *Salicornia* and *Sarcocornia* spp, the germination limits occurred only at hypersaline conditions, sometime more than two fold of seawater concentration. Few researchers reported that, seed germination is a multifaceted process playing a tremendous role in the life cycle of plants and the seeds germination get reduced as the level of salt increases[27-30]. In the present study, increased salt concentration negatively affected the germination of seeds which also took more time to germinate while exposed to saline solution than the controlled condition. The presence of cations and anions might have affected the water uptaking potential of the seeds, thereby resulting in the loss of seed germination.

Effect of salinity on seed protein profiling

Changes in protein profile occurred in *S. portulacastrum* during saline exposed conditions were analysed using SDS-PAGE. Clear difference in protein patterns was seen between seeds germinated in control media and seeds germinated saline media on the polyacrylamide gels on the basis of appearance and disappearance of bands to varied intensity of expression. Expression of 42 kDa, 26 kDa and 20 kDa proteins were significantly increased as the concentration of salt increases such as 300 mM, 400 mM and 500 mM of NaCl; but those proteins were not found in control and low salinity level (Fig. 5). High intensive protein polypeptide of 42 kDa constitutes stress specific response like protector against oxidative stress in H_2O_2 stressed wheat seedlings[31]. In rice crop, up-regulated 26 kDa protein band was associated with salt tolerance[32].

Protein extract from germinated seedlings of *S. maritima* under control and different salinity conditions are shown in (Fig. 6). Application of different concentration of salinity conditions induced significant changes in the protein profiles. Seed protein extracts produced two types of proteins patterns, some protein bands were not appeared and other proteins were selectively appeared with high intensity. Especially protein band of 55 kDa has high intensity upto 300 mM NaCl, while at 400 mM and 500 mM NaCl concentrations, protein expression pattern was gradually decreased. It was found that the intensity of bands proteins for 30 to ~20 kDa range was in control and in low salinity (100 and 200 mM) but those proteins were not found in high salinity level of 300 mM, 400 mM and 500 mM NaCl treated seed extracts. However, 300 mM of salinity was considered as the threshold level.

In the present study, 55 kDa protein band intensity of *S. maritima* was being declined after 300 mM NaCl. The protein bands of 20 to 30 kDa were found in control. The bands observed very low in 100 and 200 mM NaCl concentration and absent in the high salinity concentration of 300 mM, 400 mM and 500 mM NaCl treated seed extracts. The presence of major band of 55 kDa seems to be closely related to the major mesophyll protein band of 55 kDa of

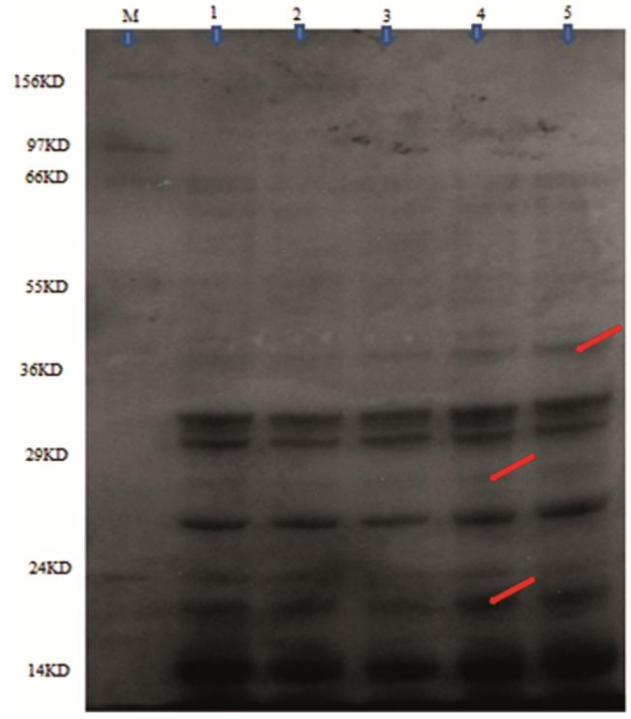

Fig. 5 — Profiling of seed protein from *S. portulacastrum* by SDS-PAGE showed differential expression after treatment with varying concentration of NaCl. Lane M: protein marker; lane 1: control seed maintained in ½ MS media; lane 2-5: Increasing concentration of NaCl (100 mM, 200 mM, 300 mM, and 400 mM) harvested after 16 days. All lanes were loaded with 20 µg protein)

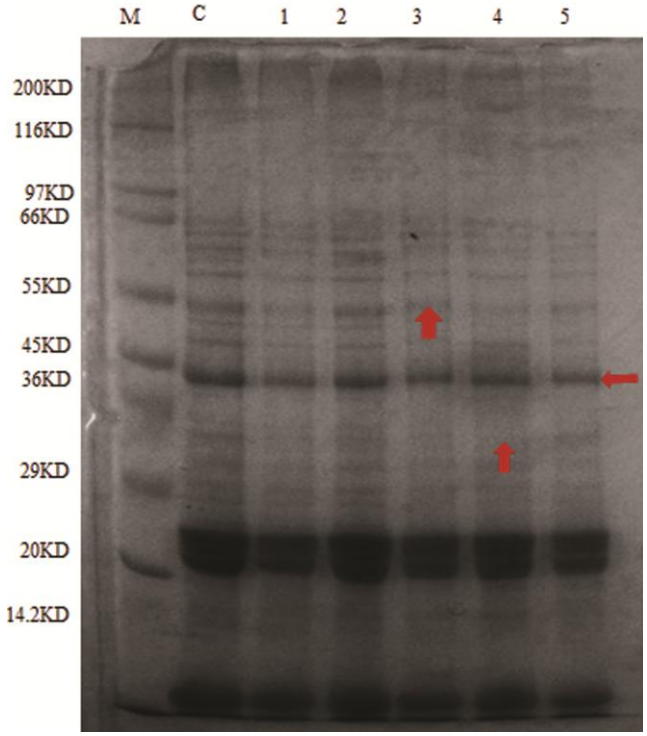

Fig. 6 — SDS-PAGE analysis of seeds protein extracted from *Suaeda maritima* using varying concentration of salt treatment: control (0 mM), 100 mM, 200 mM, 300 mM, 400 mM and 500 mM. CBB G-250 stained 10 % SDS-PAGE gel showed differently expressed proteins after treatment with different concentration of NaCl. Lane M - protein marker, Lane C - control seed, Lane 1-5- NaCl of different concentration (100 mM, 200 mM, 300 mM, 400 mM & 500 mM)

ribulose-1,5-bisphosphate/oxygenase in maize. The enzyme ribulose-1,5-bisphosphate/oxygenase is present in chloroplast and is crucial for the fixation of carbon dioxide[33,34]. Proteins ranging from 45-29 kDa are cytosolic cellular proteins which were involved in gylcolytic pathway necessary for cellular energy conservation[35]. In the present study, increasing concentration of NaCl reduced the expression of some proteins. The results showed that protein expression in tolerant genotype started to decrease after 300 mM NaCl treatment. Changes in the protein profiling of the above study might be the reason for reduced growth of stressed plant under high level of salt concentration. Hence, the above findings considered as a key point to confirm that 300 mM NaCl being considered as the threshold concentration for the favorable growth of seedlings of saltmarsh plants.

In *Salicornia brachiata*, 45 kDa protein was highly expressed at higher NaCl concentration of about 1.5 M, 2 M and 2.5 M, however the same protein was not expressed in control and low salt concentrations such as 0.5 M and 1 M NaCl treated samples (Fig. 7). Therefore, 45 kDa might acted as the inducing agents for the photosynthetic parameters[36]. Same

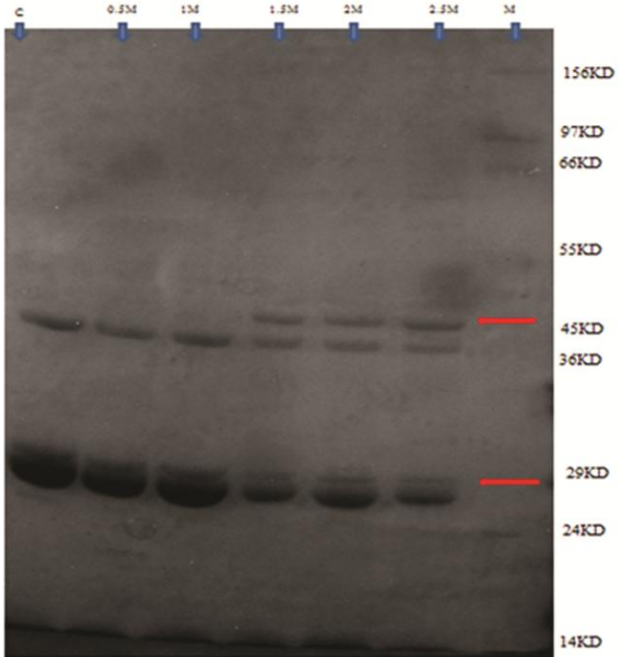

Fig. 7 — SDS-PAGE analysis of seeds protein extracted from *Salicornia brachiata* using varying concentration of salt treatement: control (0 M), 0.5 M, 1 M, 1.5 M, 2 M and 2.5 M. CBB G-250 stained 10 % SDS-PAGE gel showed differently expressed proteins after treatment with different concentration of NaCl. Lane M - protein marker, Lane C-control (0 M), Lane 2-6 – NaCl of different concentration (0.5 M, 1 M, 1.5 M, 2 M and 2.5 M)

polypeptides of 45 kDa were induced by salt stress in *Acanthophyllum sordidum*[37]. Approximately protein band of 26 kDa expression was significantly decreased as the concentration of NaCl increases (1.5 M, 2 M and 2.5 M). In maize plants, under stress condition induction of 26 kDa protein represent osmotin which was involved in the rapid accumulation osmoprotectans such as proline and glycine betaine[38]. Therefore, in many cases concentration of salt treatment regulates the gene which encodes synthesis of osmoprotectans[39]. The formation of low intensity polypeptides indicates the protein degradation during high salinity stress. Therefore 1 M of salinity considered as the threshold level for *Salicornia brachiata*.

Conclusion

Germination and protein profiling of different halophytic varieties were reduced by increasing salt concentration. This study suggests that optimized application of NaCl may enhance the plant growth on stress tolerance studies also the above findings confirmed that upper limit for the survival of the seedling was 200 mM, 300 mM and 1 M for *Sesuvium portulacastrum, Suaeda maritima* and *Salicornia brachiata,* respectively.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Author Contributions

Study conception, design and acquisition of data done by GA, Analysis and interpretation of data by AM, Drafting of manuscript by EK, Critical revision done by TS.

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