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Evaluation of Salinity Stress Effects on Seed Germination and Seedling Growth and Estimation of Protein Contents in Kodo Millet (*Paspalum scrobiculatum* L.)

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Cereals in general and millets in particular have been identified as major food crops globally and increase in crop production is required to meet the demands of the ever increasing global population. However, continuous climatic variations or abiotic stresses have led the drastic reduction in food grain yields. In view of affects of abiotic stresses on food crops plants, present study was undertaken to analyze the comparative responses of salinity stresses induced by various concentrations of NaCl (50mM, 100mM, 250mM and 500mM) and sea water (5%, 10%, 15%, 20%, 25%, 50%, 75% and 100%) during seed germination and seedling growth under ex-vitro conditions in kodo millet (*Paspalum scrobiculatum* L.). After 6-days and 12-days of salinity stress treatments, observations were recorded and after 12-days of treatments, results reveal that salinity stress caused by NaCl-salt concentration (250mM) proves to be very lethal causing strong seed germination and therefore, the mean germination frequency (33% ±0.530 was recorded while further increase in NaCl concentration (500mM) was found to be fully toxic and seed germination frequency was obtained as zero in comparison to control experiment (94% ±0.35). Similarly, in case of salinity stress induced by sea water treatments, 25% of the sea water concentration was found to cause strong promotion instead of inhibition and (71% ±0.17) of the seeds could exhibit promotion in germination frequency and further increase in sea water concentration (50%) and above was turned out to be fully toxic. Furthermore, during biochemical studies, protein contents in the tissues growing under NaCl-salinity stresses at (50mM, 100mM and 250mM) were estimated and significantly it was found to decline with the increase in concentration of NaCl-salt stress solutions. After 12th days of treatments, protein contents were found to be minimum (198.2 mg/g) in the tissues that were growing in high concentration of NaCl (250mM) solution than the control solution (476.4mg/g) grown tissues.

Key words: Abiotic Stress, Kodo Millet, Protein, Salinity, Sea Water, Seed Germination, Seedling.

Small millets have been always significant for their various benefits and sometimes, millets are considered nutritionally superior to other carbohydrate sources like rice and wheat (Hegde *et al*., 2005). It is documented that kodo millet (*Paspalum scrobiculatum* L.) is rich in glutamic acid (mainly glutamine), alanine, leucine, and serine but lacking in lysine (Sridhar *and* Lakshminarayana, 1994). Moreover, it is suggested that in comparison to other millets crops like finger millet, barnyard millet and pearl millets, kodo millet has the highest free radical quenching potential, indicating possible useful antioxidant activity. Unfortunately, it is argued that the radical quenching activity generally decreases when the grain is decorticated or heated by roasting and boiling (Hedge *and* Chandra, 2005).

Today, our food crops are globally affected by various types of biotic and abiotic stresses causing huge damage to crops and yields. Thus in order to increase crop productivity, production of stress tolerant plants and genotypes could be always meaningful approach. Amongst abiotic stresses, salinity nature of soil and water is one of the most deciding stress factors that are limiting the crop production. The composition of salts in large amounts mostly includes calcium, sodium, magnesium, chloride and sulphate ions and in relatively small amounts are potassium, carbonates, bicarbonates, borate and lithium salts (Zhu, 2001). Moreover, it is argued that during accumulation of these salts, osmotic pressure of the soil solution increases due to restricted water intake by plants (Cramer *et al*., 1999)

Available literature reveals that salinity leads to many adverse effects on the morphology, anatomy and physiology of pearl millet (Hussain *et al*., 2010). It is documented that seed germination is a crucial phase in plant life that plays important roles in seedling establishment and subsequent growth (Bewley, 1997). Germination is regulated by multiple endogenous factors, such as plant hormones, and by environmental conditions, including temperature and light (Liu *et al*., 2018).

Moreover, negative effects of salinity stress have been observed on percent germination, height, grain and straw yield of pearl millet with increasing concentration of salinity (Hussain *et al*., 2008). In general, plants adapt their metabolism during exposure to stress conditions in order to cope up with the changed environment. However, survival frequency of plants under these stressful conditions depends on the plant's ability to perceive the stimulus, generate and transmit signals and initiate biochemical changes that adjust the metabolism accordingly (Hasegawa *et al*., 2000; Johnsi Rani, 2011).

Interestingly, most of the studies on the effect of salinity on seed germination have been conducted using NaCl solutions. Such investigations may not provide information on germination under field conditions, because soils contain different salts, which may collectively influence germination in different ways from their individual effects (Ungar, 1996). However, sea water salt mimics the composition of saline soil solutions and has been used to study the synergistic effect of different salts during seed germination (Liu *et al*., 2006; Panuccio *et al*., 2014).

Additionally, it has been observed that the sea water salinity delays seed germination and reduces germination percentage especially with increasing salinity level. On increasing sea water salinity in tissue culture medium up to 75% (v/v) level, seed germination and callus initiation was found to be inhibited. Moreover, shoots were regenerated with 50% (v/v) of sea water but were failed to grow in to plants (Raveendar *at al*., 2008). Salinity in general is known to impair seed germination, reduces nodule formation retards plant development and reduces crop yield (Greenway *and* Munns, 1980).

Present study was undertaken to evaluate the effects of salinity stresses caused by NaCl and sea water treatments during seed germination and seedling growth in kodo millet crop. This study also involves analyzing the effects of salinity stress on the protein contents in the seeds that were grown under NaCl- salinity stress conditions.

MATERIALS AND METHODS

Seed Collection and Sterilization

Seeds of kodo millet (*Paspalum scrobiculatum* L.) wild cultivar were collected from PASIC, Puducherry (India). Healthy and uniform seeds of kodo millet were selected and washed thoroughly with teepol-20 and further were surface sterilized with ethanol (70%) for a minute followed by $HgCl_2$ (0.1%) treatments for 10 minutes. Later sterilized seeds were washed 3-4 times with distilled water

Salinity Stress Treatments

In present study, two different salinity stress inducing agents (NaCl and Sea Water) were employed in various concentrations. Moreover, sterilized seeds were treated with various NaCl salt concentrations (50mM, 100mM, 250mM and 500mM) and sea water (v/v) concentrations (5%, 10%, 15%, 20%, 25%, 50%, 75% and 100%) for causing the salinity stresses during seed germination and seedling growths.

Furthermore, sterilized seed were initially soaked in the respective concentrations of NaCl salt solutions and sea water solutions for 3hrs followed by soaked seeds were then placed in sterile petridishes (9 cm diameter) lined with two sterile filter papers added with either 5 ml of distilled water as control experiment or the respective salinity solutions for stress treatments.

Determination of Total Protein Content

For endogenous protein estimation, sterilized distilled water (SDW)-treated seed samples were considered as control experiment while various concentrations of NaCl-salt treated samples as stress treatments. Moreover, protein contents were estimated by using Lowry *et al.* (1951) method. To begin with, fresh samples (250 mg) were homogenized in 2.5 ml of phosphate buffer (pH-7.0) then the extract was centrifuged at 5000 *g* for 15 min at 4°C and the supernatant was transferred to a tube containing a mixture of 20 ml acetone and 14 ml β- mercaptoethanol for precipitation of protein.

The sample tubes were stored at 0°C for 5 h and then centrifuged at 10000 *g* for 20 min. The supernatant was discarded and the pellet was dissolved in 2.5 ml 1 N sodium hydroxide solution. Aliquot of 0.2 ml from this sample was used to prepare the reaction mixture. The intensity of blue color developed was recorded at 660 nm and protein concentration was measured using bovine serum albumin protein (BSA) solution as standard solution.

Statistical Analysis

Statistical data were generated after first count (6th day after treatments) and final count (12th day after treatments). Moreover, germination percentage (GP) and germination rate (GR) was calculated by the following formulae (Ruan *et al.,* 2002);

GP = Number of total germinated seeds/Total number of seeds tested × 100

$$GR = \frac{\text{Number of Germinated seeds}}{6^{th}\text{ Day of Count}} + \frac{\text{Number of Germinated Seeds}}{12^{th}\text{ Day of Count}}$$

RESULTS

During present study, all the observations for salinity stress treatments were recorded at the end of 6th day of the treatments for the partial or incomplete germination and 12th day of the treatments for the full and complete germination. Moreover, germination and seedling growth of kodo millet seeds were significantly affected by salinity stress induced by various concentrations of NaCl and sea water and responses were obtained based on the concentration of stressors and also on duration of the treatments.

Effect of NaCl –Salinity Stress on Seed Germination

Prior to initiate the stress treatments, healthy and sterilized seeds were soaked in the respective NaCl solutions (50mM, 100mM, 250mM and 500mM) for salinity stress experiments and in distilled water as control experiment for three hours.

In control experiment, seeds were found to exhibit the little indication of germination at the end of 6th day of the treatments whereas in NaCl-treated experiments (50mM, 100mM and 250mM), treated seeds were apparent with merely the emergence of proliferating scutellar region of the mature embryos of the seeds. However, at the end of 12th day of treatments, the full germination with complete developments of seedlings was observed in control experiment (Fig. 1A) whereas complete seedlings developments were also seen in NaCl treated seeds.

During this study, the lowest concentration of NaCl (50mM) was proved to be slightly effective to cause salinity stress inhibition and therefore, seedlings were found to be shorter than the control seedlings (Fig. 1B). Moreover, further increase in NaCl concentration (100mM) was found to be comparatively more inhibitory for seed germination and seedlings developments (Fig. 1C) in comparison to the control treatments. Significantly, the higher concentration of NaCl (250mM) was turned out to be strongly inhibitory for kodo millet seed germination and only few seeds could be able to germinate and grow into poor seedlings (Fig. 1D). Furthermore, with very high NaCl concentration (500mM), kodo seed germination was not visible even after 12-days of treatments (Fig. 1E**)**, but few seeds could show only the emergence of coleoptiles and coleorhizae after 15-days of treatments thus indicating that NaCl-salt concentration (500mM) was proved to be fully toxic and lethal level of salinity stress.

Effect of Sea Water- Salinity Stress on Seed Germination

In order to evaluate the effects of sea water salinity stress on kodo millet seed germination, further experiments were undertaken to treat the seeds with the various concentrations of sea water. Significantly, lower concentrations of sea water (5%, 10%, 15%, 20% and 25%) were proved to be slightly inhibitory and seeds were observed to germinate partially at the end of 6-days of treatments in comparison to control treatments. In contrast, seeds treated with higher concentrations of sea water (50%, 75% and 100%), were failed to show germination symptoms.

Significantly, at the end of 12th days of treatments, sea water treated seeds exhibited equally good germination response in comparison to control seed treatments (Fig. 1F-J) and significantly, the lengths of the seedlings were observed to be more elongated in sea water treated seedlings than the NaCl-salt treated seedlings. However, the tolerance ability of seedlings with sea water salinity stress was more pronounced with the seeds that were treated with (20% and 25%) of sea water concentrations (Fig. 1I & J) respectively than the seeds that were treated with the lower concentrations (5%, 10% and 15%) of sea water (Fig. 1F, G & H) respectively.

Significantly, kodo millet seeds that were treated with (50%, 75%, and 100%) of sea water concentrations were found to be totally suppressed to germinate even after 12th day of sea water treatments (Fig. 1 K & L) respectively and therefore, during present study, above 25% of sea water concentrations were proved to show salinity stress inhibition caused by sea water.

Rate of Seed Germination under NaCl-Salinity Stress

After 6th day of treatments in control experiment, frequency of incomplete seed germination was found to be 49% whereas in case of NaCl-stress treatments, the incomplete germination frequency (37% and 29%) were recorded with lower NaCl-concentrations (50mM and 100mM) of solutions respectively (Table 1). However, with further increase in NaCl-concentration (250mM) solution, only 18% seeds were found to exhibit incomplete germination. Furthermore, after 12th day of treatments, the frequency of full seed germination was gradually affected by NaCl salinity stress and (81%, 67% and 33%) frequencies were recorded with the concentrations of NaCl (50mM, 100mM and 250mM) solutions respectively in comparison to control frequency (94%). Moreover, very high concentration of NaCl

(500mM) was proved to be fully toxic and therefore frequency of complete seed germination was recorded to be zero.

Rate of Seed Germination under Sea Water (SW)-Salinity Stress

In comparison to NaCl-salinity stress treatments after 6th day of treatments, minimum inhibitory effects caused by sea water salinity stress was found with low concentration (5%) of sea water and therefore, seed germination frequency was recorded as (57%). However, further increase in sea water concentration (10%, 15%, 20% and 25%), the seed germination frequency was gradually declined (44%, 36%, 27% and 24%) respectively at the 6th day of treatments (Table 2).

Significantly, at the end of 12th day of sea water treatments, treated seeds were found to exhibit fast germination and thus frequency of seed germination was recorded as (79%, 58% and 47%) with the sea water concentrations (5%, 10% and 15%) respectively in comparison to control (94%). Interestingly, high frequency of seed germination (64% and 71%) was observed in the seeds that were treated with even higher concentrations (20% and 25%) respectively of sea water. However, further increase in sea water concentration (50%) and above were proved to be fully inhibitory and frequencies were recorded as zero,

Results revealed that the salt sensitivity response could show high significant difference between the two modes of salinity stress (NaCl and sea water) treatments. In case of NaCl salinity treatments, high concentration of NaCl (500mM) was found to be toxic level and complete germination frequency was recorded to be zero whereas salinity caused by sea water beyond 25% proved to be lethal.

Table 1. Effects of NaCl-salt salinity stress on seed germination and protein contents in Kodo Millet (*Paspalum scrobiculatum* L.)

S. No.	6th Day		12th Day	
	Concentration of NaCl (mM)	**Germination Percentage (%) Mean ± S.E.**	**Germination Percentage (%) Mean ± S.E.**	**Total Protein Content Mean (mg/g fresh weight)**
1	0 (Control)	49±0.17	94±0.35	476.4
2	50	37±0.88	81±0.53	359.9
3	100	29±0.88	67±0.53	307.0
4	250	18±0.35	33±0.53	198.2
5	500	0	0	-

Table 2. Effects of Sea Water salinity stress on seed germination in Kodo Millet (*Paspalum scrobiculatum* L.)

S. No.	6 th Day		12th Day
	Concentration of Sea Water (%)	**Germination Percentage (%) Mean ±S.E.**	**Germination Percentage (%) Mean ±S.E.**
1	0 (Control)	49±0.17	94±0.35
2	5	57±0.17	79±1.2
3	10	44±0.7	58±0.35
4	15	36±0.17	47±0.7
5	20	27±0.35	64±0.5
6	25	24±1.4	71±0.17
7	50	0	0
8	75	0	0
9	100	0	0

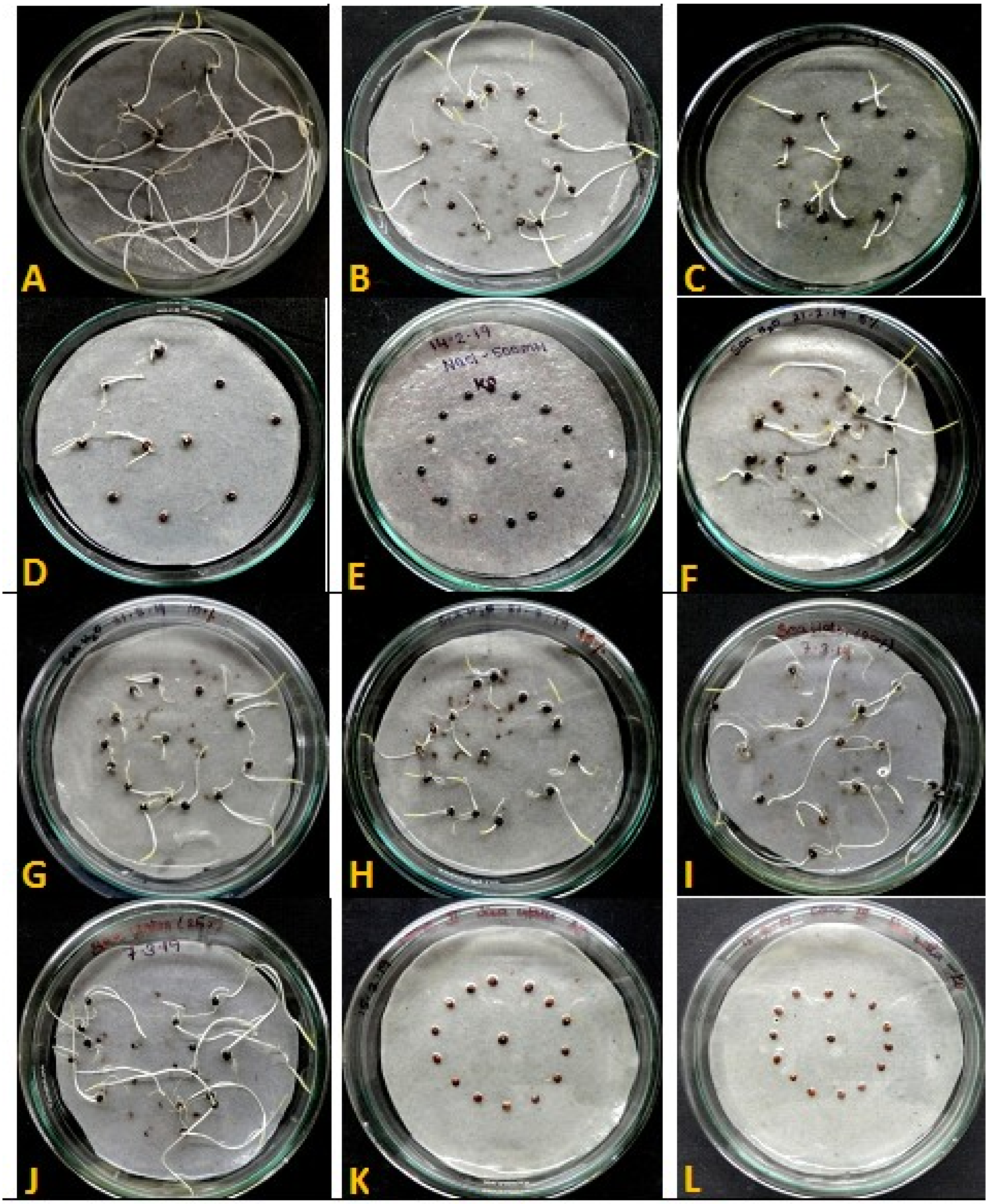

Figure 1. *Paspalum scrobiculatum* L., showing effects of salinity stress during seed germination after 12-days of treatments;

(A) Control Seedlings **(B)** NaCl-50mM **(C)** NaCl-100mM **(D)** NaCl-250mM **(E)** NaCl-500mM **(F)** Sea Water-5% **(G)** Sea Water-10% **(H)** Sea Water-15% **(I)** Sea Water-20% **(J)** Sea Water-25% **(K)** Sea Water-50% **(L)** Sea Water-75%

Figure 2. *Paspalum scrobiculatum* L., showing effects of salinity Stress during seedling growth induced by NaCl- salt Treatments;

(A) Control+50mM **(B)** Control+100mM **(C)** Control+500mM of NaCl (Seedlings after 12-days of treatments) **(D)** Control+50mM **(E)** Control+100mM **(F)** Control+250mM of NaCl (Seedlings in plastic cups after 15-days of treatments).

Effect of Salinity Stress on Seedlings

Seedling height, shoot and root elongation were found to decrease with the increase in salinity stress caused by various concentrations of NaCl-salt. At lower concentrations of NaCl (50mM and 100mM) salt, seedlings were exhibited the maximum susceptibility to salinity stress and seedlings were observed to be little shorter than the control seedlings (Fig. 2 A & B) respectively. However, seedlings grown at high NaCl-concentration (250mM) showed maximum difference in all aspects of seedlings growth while at 500mM of NaCl salt treatment, poorly germinated seeds were failed to grow into seedlings (Fig. 2 C**)** even after 12-days of treatments. Interestingly, root formation in general was observed to be inhibited substantially in NaCl-stress treated seedlings. On transfer to cup soil, seedlings in NaCl-salt solution (50mM and 100mM) treated soil cups were seen almost similar to the control seedlings (Fig. 2 D & E) respectively while seedlings that were growing with high concentration of NaCl (250mM) were found to exhibit a significant reduction in growth (Fig. 2 F) after 15-days of treatments.

During this study, salinity stress caused with NaCl treatments were reflected greater stress inhibitory effects in comparison to sea water-salinity stress. Moreover, with increase in salinity stress caused by both modes (NaCl and sea water), stress inhibition was clearly observed in terms of root formation and growth in seedlings. In case of sea water treatments with concentrations (5% to 25%), root formation was significantly suppressed while shoot lengths of the seedlings were found to be more elongated even than the control seedlings. However, there was no indication of inhibition response caused by sea water stress in the shoot growth of the seedlings.

Effect of Salinity Stress on Protein Contents

Under control (non-saline) conditions, germinated seeds were found to contain the maximum protein level than NaCl-treated sample solutions and further gradual decrease in protein content was observed with increase in salt stress. During present study, the control treated samples were found to exhibit the highest protein content (476.4mg/g) whereas the samples collected from NaCl treated germinated seeds (50mM, 100mM and 250mM) showed gradual reduction in protein content (Table 1). Results also revealed that the minimum amount of protein (198.2 mg/g) content was estimated in the samples that were extracted from the higher concentration of NaCl (250mM) treated seeds than the other lower concentrations of NaCl treated samples.

DISCUSSION

Millets have been considered as the main plant genetic resources for the agriculture that contribute food security to the poor people inhabiting arid, infertile, marginal and poor lands at global level. The fast maturation and all season growth characteristics offer millets desirable crops for more intensive cropping systems and these could also be utilized as a catch or relay crop in combination with other crops that are slow in maturation (Lata, 2015).

Unfortunately, today abiotic stresses also have emerged as a major cause of crop failure leading to decrease in average yield for most food crops by more than 50% and restricting the sustainability of the agricultural industry (Mahajan *and* Tuteja, 2005). Moreover, it is suggested that abiotic stresses can directly or indirectly affect the physiological status of an organism by altering its metabolism, growth and development (Chutia *and* Borah, 2012; Vibhuti Shahi *et al*., 2015) and adversely affect agricultural productivity.

It is documented that salt stress in general is a major ecological factor which is commonly known to limit the food crops potential to survive and yield. Moreover, it is reported that salinity stress affects development processes such as seed germination, seedling growth and vigour, vegetative growth, flowering and fruit set (Sairam *and* Tyagi, 2004; Johnsi Rani, 2011).

Moreover, it is suggested that high salinity is a consequence of the excess accumulation of various ions such as sodium, calcium, magnesium, chloride and sulphate in the soil and sodium chloride is the most harmful for plant growth and development. Several classical methods are available for screening salt tolerance of plants because the majority of the physiological activities such as germination, K^+ uptake, photosynthesis, biomass production and also of biochemical processes, such as electrolyte leakage or chlorophyll content is highly sensitive to salt stress (Tari *et al*., 2013).

During present study, the rate of seed germination and seedling growths were dependent on the concentrations of NaCl and sea water. Moreover, with the increase in their concentrations frequency of germination and seedlings growths were found to gradually decline and in comparison to control (94%), the frequency of seed germination was observed gradually in declining order (81%, 67% and 33%) with NaCl-salt solutions (50mM, 100mM and 250mM) respectively. However, very high concentration of NaCl (500mM) was proved to be almost lethal level for this millet variety. Similar study in sweet sorghum, salt stress causes gradual reduction in the percentage (Almodares *et al*., 2007b) and also increase in the duration of germination (Gill *et al*., 2003). Furthermore, significant differences among cultivars have also been found in the sensitivity of germination to high salinity (Samadani *et al*., 1994).

In contrast, during this study salinity stress caused by sea water was proved to be less inhibitory and lethal than stress inhibition caused by NaCl-salt and moreover, 50% (v/v) of sea water was observed to cause full salinity stress inhibition. Interestingly, seed germination and seedling growth during sea water treatments of kodo millet seed was found to be very slow at the end of 6^{th} day of treatments but later sea water treatment was proved to be stimulatory for this millet variety. Moreover, other study also suggests that the isolated cells are much more tolerant to salt than the whole plant in rice (Watad *et al*., 1991). In additional study, salt tolerant callus could also be obtained from high yielding salt sensitive rice cultivars with 50% (v/v) of sea water treatment (Raveendar *et al*., 2008)

In addition to toxic effects of certain ions, higher concentration of salt is known to reduce the water potential in the medium which hinders water absorption by germinating seeds and leads to reduction in germination (Hakim *et al.,* 2010). It was also suggested that osmotic effect due to salinity proves to be the main inhibitory factor that reduces seed germination (Akbar *and* Ponnamperumal, 1982).

Reduction in seedling height is common phenomenon of many crop plants grown under saline conditions (Javed *and* Khan, 1975). Shoot and root length provides an important clue to the response of plants to salt stress (Jamil *and* Rha, 2007). Significantly, during present study, the shoot and root length of seedlings that were grown in various NaCl salt solutions also showed decreasing trend, indicating that the salt stress not only affects germination but also the growth of seedlings, and thus affects the dry matter production of the seedlings.

However, during this study, salinity stress caused by sea water was failed to inhibit shoot growth and therefore shoot length was found to be more elongated than the control seedlings. In contrast, root length was found to be inhibited. This result is in conformity with the findings of other studies where shoot and root lengths were conspicuously affected by salt (Djanaguiraman *et al.,* 2003; Hakim *et al.,* 2010).

Effects of Salinity Stress on Protein Contents

It is argued that protein content and composition is an important determinant of cereal grain quality and moreover, it is sensitive to drought and heat stress as well as atmospheric CO_2 concentration. Heat stress in general is known to reduce starch deposition in wheat grain and results in an increase in grain protein content (Wardlaw *et al.*, 2002; Gooding *et al.*, 2003). Furthermore, it is also documented that stress also affects protein composition and thus, wheat dough strength is adversely affected by even a short period of high temperature (> 35°C) during grain filling (Wrigley *et al.,* 1994; Halford *et al*., 2015).

During present study on *P. scrobiculatum* L., protein contents were found to gradually decrease with the increase in NaCl concentrations in the stress solutions. Moreover, protein content was found to be the minimum in the samples that were extracted from the germinating seeds growing with high NaCl (250mM) salt solution while it was found to be the maximum in control treatments. Similar results of gradual reduction in protein contents have been reported in rice during *in vitro* NaCl-stress treatments where germinated seedlings exhibited reduction in protein contents with the increase in NaCl-stress level (Rajakumar, 2013).

In other studies also, drought stress has also been shown to cause a small reduction in total protein content in two maize cultivars (Ali *et al.*, 2010) but in contrast it was found to be increased by up to a five times in rice (Crusciol *et al.*, 2008; Fofana *et al.*, 2010). Moreover, temperature stress, on the other hand, also causes a reduction in protein content in rice (Ziska *et al.*, 1997).

CONCLUSION

During this study on Kodo millet (*Paspalum scrobiculatum* L.), results reveal a significant difference between the two modes of salinity stress (NaCl and sea water) treatments. In case of NaCl stress treatments, high concentration beyond (250mM) was found to be toxic level to inhibit germination completely whereas salinity stress caused by sea water proved to be stimulatory for seedling growths with (20% -25%) of sea water and (50%) of sea water was turned out to be toxic concentration of sea water. These results indicate that sea water plays some stimulatory roles to influence millet seed germination at particular concentration. During estimation of protein contents, a gradual decrease in protein level was observed in germinated seeds with increasing NaCl concentration in the treatment solution.

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