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A study on effect of NaCl stress on Kodomillet (*Paspalum scrobiculatum*) during germination stage

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Abstract: Kodomillet (*Paspalum scrobiculatum*) is one of the important and neglected minor millet with high reserves of protein. The cultivation of this crop declined year by year, this situation may be due to the different abiotic stresses pertaining to low yields. Salinity stress is one of the important abiotic factor influences the growth of the crop species at different stages of the life cycle. Seeds are very sensitive to salinity stress at germination and seedling stage and kodomillet is not an exceptional one. As there is no previous reports available regarding the response of the kodomillet germplasm against salt stress this work was taken up to identify the salt tolerant accessions of kodomillet at germination and early seedling stage. The kodomillet germplasm was obtained from ICRISAT and NBPGR. All the test varieties were treated with different NaCl levels such as 50 mM, 100 mM, 150 mM and 200 mM for about 144 hrs. The effect of salt stress was measured in terms of percent germination, germination energy, germination index, seed vigour index, relative water content, length of radicle and plumule, fresh and dry weights of the seedlings. In addition with these the biochemical activity was calculated by estimating the proline, peroxidase (POX) and polyphenol oxidase (PPO). Among all the test varieties the accessions IC 76 and IPS 583 found to be tolerant against change in NaCl stress, followed by the other genotypes. These findings might be useful in further breeding experiments to develop the salt tolerant kodomillet genotypes.

Keywords: Germination Energy, Germination Index, Kodomillet, Peroxidase, Proline, Seed Vigour Index.

Introduction

Kodo millet (*Paspalum scrobiculatum*) is a minor grain crop of India, grows on a fairly large scale for its food and fodder in India and Africa. Kodo millet contains 11% protein and nutritional value of protein has been found to be slightly better than that of other minor millets. Kodo millet is grown in an area of about 907,800 ha with annual production of about 310,710 tones (H. S. Yadava & A. K. Jain, 2006). Among the small millets excluding finger millet, kodo millet shares around 36.61 and 31.52% of total area and production of the country and the national productivity of kodo millet is about 342 kg/ha (H. S. Yadava & A. K. Jain, 2006).

Even though kodomillet is useful to human beings in many ways, its production rate is at alarming stage from fast few years, as the crop is subjected to salinity stress at germination, flowering and grain filling stages pertaining to low yields. Moreover there are no previous reports available about the response of kodomillet to the salt stress during germination, this situation aware us to identify the available germplasm for their salt stress tolerance.

The study of germination and seedling characteristics might give a clear idea to screen the plants against salinity stress. Germination percentage, germination energy, seedling vigour, RWC, germination index and seedling growth in terms of radicle and plumule length are the significant parameters in identifying salt tolerant genotypes especially at times of germination. The analysis of activity of proline, peroxidase, polyphenol oxidase and catalase will provide adequate knowledge in search of determining the potent genotypes to combat the salinity stress.

Seed response to salinity can be simulated by NaCl induced ionic stress in the germination experiments. Ionic stress is induced by a toxic accumulation of NaCl in plant tissues (Murillo-Amador *et al.,* 2002). Seed germination is a crucial and vulnerable stage in the life history of terrestrial angiosperms and decides seedling establishment and plant growth. The success of seedling vigour depends on formation of radicle and plumule.

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Accumulation of Na^+ and Cl^- causes an imbalance in nutrient uptake and results in toxicity. Salt stress along with low moisture content leads to inhibition of certain metabolic activities during germination (Younis *et al.,* 1991). The activity of some enzymes changes due to the uptake of toxic ions results in inhibited seed germination and leads to mortality (Smith *et al.,* 1991, Neumann, 1997). As the seed germination is the first step towards the growth of plant, it is necessary to know the stability of germplasm against salinity stress.

Materials and Methods

Kodomillet (*Paspalum scrobiculatum*) germplasm was procured from International Crop Research Institute for the Semi-Arid Tropics (ICRISAT) Patancheru, Hyderabad and National Bureau of Plant Genetic Resources (NBPGR) Rajendranagar, Hyderabad, A.P., INDIA.

Seeds were surface sterilized by soaking them in a solution of 2.0% aqueous sodium hypochlorite for 15 minutes at room temperature and then rinsed thoroughly with distilled water. A total of 25 seeds per plate were spread evenly on moist two fold Whatmann No. 1 filter paper kept in petri plates of 12 cm in diameter. Seeds were treated with 50, 100, 150 and 200 mM concentrations of NaCl solutions and double distilled water was used to treat the controls. In each petri plate 5 ml of the appropriate solutions were added on alternate days or when ever required, the entire setup was kept in an incubator and maintained at 25°C.

Data was collected after 144 hrs of the experiment, the percentage of germination and germination count was taken at every 24 hrs interval. The following parameters were studied and analysed.

The seeds having a radical length of more than 2 mm were considered as germinated (Mackay *et al.,* 1995). The seedlings were weighed immediately after harvest and considered it as fresh weight. These samples were kept at 70°C for three days and were considered as dry weight (Afzal *et al.,* 2005). Germination percentage was calculated according Mackay *et al.,* (1995).

$$\text{Germination\%} = \frac{\text{Number of seeds germinated}}{\text{Total Number of seeds}} \times 100$$

A measure of the rapidity of germination expressed as the percentage of seeds germinating within a given time under defined conditions, it can be considered as Germination Energy. Germination energy was measured according Yan Li (2008).

$$GE = \frac{\text{Total germinated seeds in NaCl conc. in 3 days}}{\text{Total Number of seeds for germination}}$$

Germination index is a measure of seed vigour and defined it as a function of total germination and mean germination rate as shown in the equation Czabator (1962).

GI = Total germination × Mean germination rate

Seedling vigour index (SVI) was calculated using the modified formula of Abdul-Baki & Anderson (1973).

SVI = Seedling length × Germination percentage

Relative water content was estimated according Fletcher *et al.,* (1988). RWC (%) was calculated by the formula given by Kramer (1983).

$$RWC = \frac{\text{Fresh weight – Dry weight}}{\text{Turgid weight - Dry weight}} \times 100$$

The extraction and estimation of free proline was done in the seedlings according to Bates *et al.,* (1973).

Fresh plant material of 500 mg was homogenized using of 3% aqueous sulfosalicylic acid. The homogenate was filtered through four layered muslin cloths and the filtrate was collected and made up to known volume. Two milliliter of filtrate was taken into a test tube and 2 ml of acid ninhydrin and 2 ml of glacial acetic acid was added. The tubes were incubated at 100°C for 1 hour in a boiling water bath. After incubation the tubes were transferred to an ice bath to terminate the reaction. Four milliliter toluene was added to the contents of the tubes and mixed thoroughly using a test tube stirrer for 15 seconds; chromophore containing toluene was aspirated from aqueous phase. Then the absorbance of the solution was measured at 520 nm using a UV-Vis spectrophotometer (Elico *SL 159*) and toluene is used as a blank. Proline was measured from the standard curve prepared with authentic proline and its amount was calculated on dry weight basis.

The activity of peroxidase and polyphenol oxidase was assayed according Manoranjan Kar *et al.,* (1975). 500 mg plant material was grinded using pre chilled pestle and mortar by adding 30 - 40 ml phosphate buffer (0.02 M). The contents were filtered through cheese cloth and centrifuged at 2000 rpm for 10 min. To determine the activity of polyphenol oxidase, the reaction mix was prepared by adding 2 ml of buffer, 1ml of enzyme extract. The mixture was incubated for 5 min and the reaction was stopped by adding 1ml of 2.5 N H_2SO_4 .Optical densities was measured at 420 nm against a blank containing 1ml of H_2SO_4, 2 ml of buffer 1ml of pyrogallol and 1ml of boiled enzyme extract.

Enzyme activity was calculated by subtracting the absorbance value of blank from the sample and expresses the enzyme activity as absorbing units per 1 gram fresh weight per 5 min. Peroxidase was measured by making a reaction mixture 3 ml of pyrogallol phosphate buffer and 0.1 ml of enzyme extract into a cuvette. To the reaction mixture 0.5 ml of H_2O_2 was added and gently shaken. The absorbance was measured after 3 min at 420 nm. The same procedure was continued to know the control value by using boiled enzyme extract. The enzyme activity was measured by subtracting the absorbance value of the blank from the sample and expressed the enzyme activity as absorbing units per 1 gram fresh weight per 3 minutes.

Results

The per cent germination was varied significantly among the selected cultivars between controls and treatments. The ability of germination was declined as the concentration increases from 50 mM to 200 mM (Table 1). Among the treatments all the genotypes showed highest germination percent at 50 mM NaCl concentration. Except IPS 145 and IPS 610 all the genotypes reported significant percent germination at 100 mM NaCl concentration (Table 1). There is no significant percent germination at 150 mM, 200 mM concentration. Anova results have revealed that germination energy and germination index varied significantly between the genotypes and the treatments, and it ranges from 1.01 to 0.01. Highest germination energy observed in control of IC 76 (1.01) and lowest was reported at 200mM concentration (0.01) in all cultivars (Table 1). Germination index also effected by NaCl concentration and it varies from 4.16 (IPS 583, IC 76) in controls to (0.01) at 200 mM in all accessions except IPS 145 (0.41) at 200 mM, NaCl concentration. Germination index noted significant at control, 50 mM, and 100 mM (Table 1).

Among the six varieties studied, all varieties showed significant difference with other genotypes in terms of their radicle length (Table 1). Varieties, IC 76 and IPS 145 showed maximum (2.56 cm) and minimum (1.20 cm) radicle length respectively in case of control. Radicle length decreased in seedlings in all the treatments. When compare the levels of significance, accession IC 76 is the only cultivar recorded significantly even at 150 mM NaCl treatment, followed by IPS 583 at 100 mM concentration. The plumule length of control seedlings in petri plate varied from 4.53 to 7.53 cm. Varieties IC 76 showed a maximum (7.53 cm) and IPS 145 showed minimum (4.53 cm) plumule lengths, respectively. It was observed that the plumule length decreased with increasing level of NaCl concentration (Table 1).

Relative water content was observed among all the treatments and there is a significant variation among the varieties. IC 76 showed highest relative water content (93.70) followed by IPS 583 (92.20) and IPS 351 (91.70) in controls. But among the treatments IC 76, IPS 583 and IPS 351, IC 88 recorded high (Table 1). Under non saline conditions accessions IC 76 followed IPS 583 produced maximum Seedling Vigour index, IPS 145 and IPS 610 produced minimum Seedling Vigour Index (Table 1). All the varieties showed significant variation between the treatments up to 50 mM concentration, while the accession IC 76 reported significant even at 100 mM NaCl concentration (Table 1).

Plants usually produce more amount of proline during stress periods and it is one of the important parameter to screen the salt tolerant varieties. Among all the genotypes the concentration of proline is significant at 150 mM and 200 mM and seems to be recorded high in IC 76 and IPS 583, where as in case of IC 76 it is significant at 200 mM and in case of IPS 145, proline production is not yet significant at any concentration of NaCl (Figure 1).

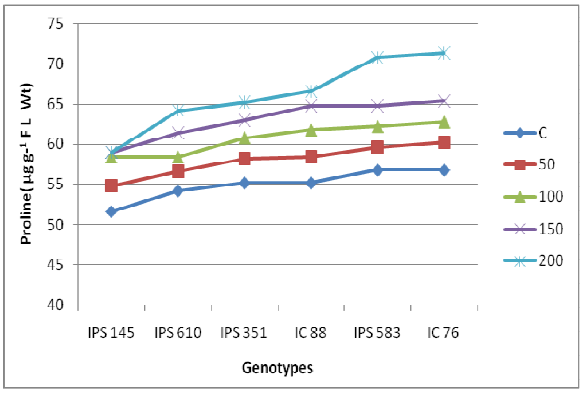

Figure.1: Proline activity against change in NaCl concentrations in test genotypes

Polyphenol oxidase activity was determined in control as well as in NaCl treatment (Figure 2). The level of enzyme activity in the germination stage was higher at 200 mM in IC 76, IPS 583 in IPS 145 found to be lower in both control and NaCl treatment. In all the accessions, the enzyme activity increased with the progress of concentration from control to 200 mM. The level of peroxidase activity was higher in IC 76 and lower in IPS 145. Accessions IPS 583, IC 76 and IPS 145, IPS 610 have shown maximum and minimum enzyme activity during germination at 200 mM concentration of NaCl (Figure 3).

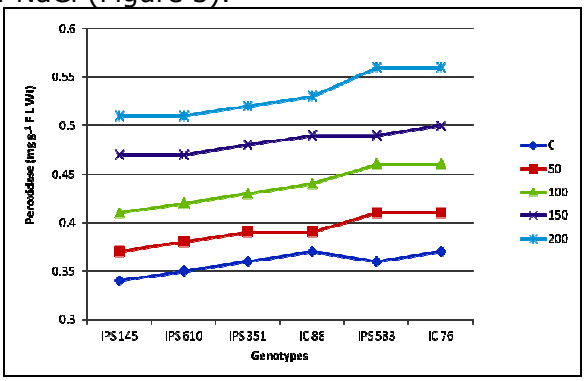

Figure.2: Increase in peroxidase activity with increase in NaCl

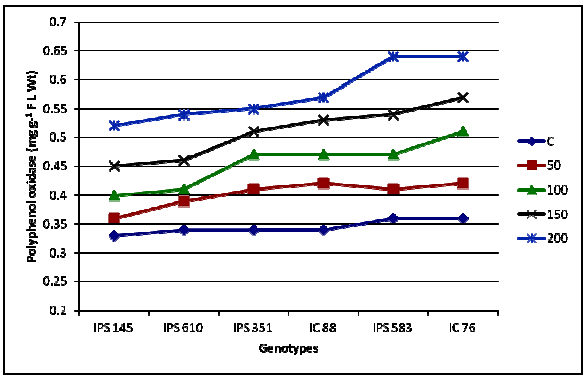

Figure.3 Increase in polyphenol oxidase activity with increase in NaCl

The fresh and dry weights were decreased among all the accessions as the concentration of the NaCl increases. The weights were decreased thrice when all the samples were dried, and more decrease in dry weights was observed in the accession IPS 145. The genotype IC 76 and IPS 583 showed less decrease in the dry weights even at high salt stress treatments. IC 76 and IPS 583 found to be recorded high fresh and dry weights in all the salt treatments (Table 2).

Discussion

The percent seed germination was in coincide with Basalah (1991), who revealed that high levels of soil salinity can significantly reduce seed germination. According to Waisel (1972), increasing salinity concentrations in germination often cause osmotic toxicity which may inhibit germination percentage. Low germinative energy is sometimes associated with chemical treatment (Dendy, 1995) and inability to uptake water (Hofmeyr, 1970 & Daiber, 1975). This situation results in the lengthening the time needed to complete germination (Ayers, 1952). Germination index can be considered as a perfect measure of plant response to salinity because the formula takes into consideration the speed and total germinated seeds**.** The germination index decreased in all the varieties it is may be due to increase in salinity. Munns and Termaat (1986) have suggested that salinity decrease radicle and plumule growth and if we increase salinity level, the amount of reduction will increase. This reduction is due to osmotic pressure which leads to inability in water absorbance. Hence cell division and differentiation reduce and reduction of plumule and radicle length have takes place.

The present results were in line with NaCl (Murillo *et al.,* 2002 & Szigeti, 1991) regarding to depletion in relative water content due to hike in NaCl concentration. It is because of reduce in water supply to the cells by increasing in Na^+ ions in cytoplasm which compete with K^+ ions results in decrease in osmotic potential in cell cytoplasm of all the cultivars. Seed vigour index is related to special impact of ions and reduction of environmental water potential in the presence of salinity. Result showed that if salinity increases, seed characteristics will decrease these results are in support to the findings of Kader & Jutzi (2004).

The production of reactive oxygen species (ROS) is one of the common effects of salt on crop species which directly effects the growth of the plants and the rate of production of anti oxidant enzymes may vary based on the salt concentration (Munns & Tester, 2008; Ashraf, 2009). It is now widely confirmed that ROS results in cellular damage (Mittler, 2002; Apel & Hirt, 2004). Accumulation of proline leads to protection of some enzymes (Sreenivasulu *et al.,* 1999; Taffouo *et al.,* 2009) by acting as an osmoregulator, which in turn results in salinity stress tolerance in the test genotypes. In contrast, salt tolerant accessions showed increase in the peroxidase activity when compared with other accessions. These results are in line with some earlier studies (Gosset *et al.,* 1994; 1996; Lopez *et al.,* 1996) in which it was found that raise in the activity of POX was related to induces salt tolerance in different crop species.

The PPO activity was enhanced with increase in NaCl concentration. High polyphenol oxidase activity under stress suggests that its ability to oxidize and to reduce the toxic substances such as phenolic compounds which are generally described to be accumulated during salt stress (Subhasini & Reddy, 1990; Gasper *et al.,* 1985; Ashish & Anath, 2005). The same increase in PPO against salt stress was reported in large number of plant species (Agastin *et al.,* 2000).

The fresh and dry weights were reduced drastically as the concentration of NaCl increases and these results are supported by Bernstein *et al.,* 1993; deLacerda *et al.,* 2003). Reduction in shoot and root dry weights could perhaps be used as one of the good parameters to characterize salinity tolerance in kodomillet.

Conclusion

Our results concluded that lines of kodomillet IC 76 and IPS 583 tolerant, IC 88 and IPS 351 moderately tolerant and IPS 145, IPS 610 sensitive to salinity differed significantly in all of the observed parameters, including reduction in shoot lengths, fresh weights and dry weights of shoots and roots along with biochemical characters in response to salinity stress. The tolerant varieties were found be effective for cultivating against salt accumulated soils.

Table.1: Effect of different concentrations of NaCl on the following parameters

Variety	IPS 145					IPS 610					IPS 351				
Character	C	50mM	100mM	150mM	200mM	C	50mM	100mM	150mM	200mM	C	50mM	100mM	150mM	200mM
% G	86.00*	84.40*	24.80	8.80	1.20	95.00*	91.00*	50.60	12.80	1.20	96.20*	93.80*	61.00*	22.40	1.20
GE (%)	0.56*	0.37*	0.02	0.01	0.01	0.59*	0.38*	0.03	0.01	0.01	0.75*	0.44*	0.14	0.02	0.01
GI (%)	3.57*	3.50*	1.01	0.01	0.01	4.00*	3.83*	2.08	0.33	0.01	4.03*	3.91*	2.50*	0.50	0.01
RL(cm)	1.48*	0.81*	0.06	0.04	0.01	1.20*	0.91*	0.63*	0.35	0.01	1.79*	1.46	0.66	0.48	0.01
PL(cm)	4.53*	2.40*	0.42	0.02	0.01	4.85*	3.46*	0.57	0.26	0.01	5.97*	3.73*	0.71	0.36	0.01
RWC (%)	80.40*	72.20*	70.10*	50.30	48.80	87.40*	80.10*	70.20*	50.60	49.80	90.10*	80.20*	72.80	65.10	52.50
SVI	523.00*	376.50*	19.00	0.30	0.10	630.30*	389.10*	60.80	8.70	0.10	716.10*	425.80*	84.50	19.40	0.10
FW (g)	0.061*	0.046*	0.043	0.039	0.027	0.062*	0.055*	0.048	0.039	0.028	0.066*	0.065*	0.049	0.044	0.031
DW (g)	0.021*	0.016	0.016	0.012	0.008	0.023	0.017	0.017	0.013	0.011	0.024*	0.022*	0.019	0.013	0.013

Table.2: Effect of different concentrations of NaCl on the following parameters

Variety	IC 88					IPS 583					IC 76					
Character	C	50mM	100mM	150mM	200mM	C	50mM	100mM	150mM	200mM	C	50mM	100mM	150mM	200mM	CD at 5%
% G	96.40*	94.00*	65.00*	38.20	1.40	99.20*	94.60*	71.20*	41.00	4.00	99.20*	98.80*	73.20*	67.00	11.00	**0.60**
GE (%)	0.83*	0.44*	0.18	0.04	0.01	0.92*	0.46*	0.24	0.04	0.01	1.01*	0.50*	0.30	0.05	0.01	**0.01**
GI (%)	4.04*	3.91*	2.65*	0.91	0.01	4.16*	3.92*	3.05*	1.58	0.01	4.16*	4.12*	3.07*	2.75	0.41	**0.01**
RL (cm)	1.90*	1.64*	1.00	0.80	0.01	2.09*	1.67*	1.01	0.97	0.10	2.56*	1.73*	1.23	1.09	0.12	**0.01**
PL (cm)	7.23*	3.85*	0.73	0.55	0.01	7.53*	4.04*	0.93	0.62	0.03	7.56*	4.45*	1.23	0.91	0.08	**0.01**
RWC (%)	91.70*	81.10*	75.40	68.40	64.40	92.20*	85.40*	80.60*	70.60	68.00	93.70*	87.50*	83.60*	74.80	73.60	**0.79**
SVI	873.70*	517.40*	86.00	47.80	0.10	926.30*	520.90*	159.70	60.50	0.70	998.80*	571.40*	159.90	133.50	1.20	**0.39**
FW (g)	0.072*	0.066*	0.060*	0.044	0.034	0.076*	0.073*	0.065*	0.053	0.037	0.082*	0.081*	0.065	0.058	0.043	**0.002**
DW (g)	0.026*	0.025*	0.022	0.015	0.013	0.065*	0.028	0.025	0.023	0.015	0.077*	0.066*	0.061*	0.028	0.024	**0.002**

*Significant at 5% level

Germination percentage (%G), Germination energy (GE), Germination index (GI), Radicle length (RL), Plumule length (PL), Relative water content (RWC), Seedling vigour index (SVI), Seedling fresh weight (FW), Seedling dry weight (DW).

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