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ANALYSIS OF PROTEIN CONTENT AND *IN VITRO* PHENOTYPIC DATA IN SORGHUM LINE FOR SALT TOLERANCE

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ABSTRACT

The capability of crops to grow on saline soils varies among species and depends on the concentration of salts present in the rootzone and on various environmental and cultural conditions. Information on the relative tolerance of different crops is essential to the successful management of salt-affected agricultural lands and waters. Results from over 50 years of research have produced salt tolerance data that relate yield reductions of over 90 different crops to soil salinity. These data are presented in tabular form and give threshold salinity values and percent yield reductions expected at salinities exceeding the threshold. The recommended procedure to acquire reliable data, the yield response function used to quantify salt tolerance data, and factors to consider when evaluating or using these data are also described.

KEYWORDS: Cereal crop, *In vitro*, Phenotype, Rootzone, Sorghum, soil salinity

INTRODUCTION

Sorghum (*Sorghum bicolor* (L.) Moench) is the fifth most important cereal crop of the world and is a major source of food, feed and fodder in the semi-arid tropics (SAT). It is the third most important staple food crop after rice (*Oryza sativa*) and wheat (*Triticum aestivum*) for millions of people in India. The grain molds, shoot fly and prolonged dry spells are main reasons for low productivity in India. Of all the soil mineral stresses or chemical toxicities, acidity, and associated Al_3+ toxicity and salinity are probably the most important constraints to sorghum productivity in tropical environments. Saline and sodic soils cause mineral stresses on approximately 0.9 billion hectors of land (Gourley *et al*.,1997). In addition, the problematic soils that include saline soils which constitute 15% (approx.) of total cultivable area in India, reduce crop productivity leading to food insecurity and rendering crop production non-remunerative.

The increased demand for sorghum, especially for feed uses in SAT regions (Kleih *et al.*, 2000) imposes extension of sorghum cultivation in saline soils. Development of cultivars tolerant to soil salinity along with appropriate management practices is required for enhanced production under saline conditions (Ramesh *et al.,* 2005). Salinity causes reduction in germination (Igartua *et al.*,1994), growth (Maiti *et al.*,1994) and yields of sorghum (Macharia *et al.*,1994) and modifies the physiological and biochemical processes of the plant (Dubey, 1994). Salinity causes more serious damage in the seedling emergence stage than in any other stage in sorghum (Macharia *et al.*,1994). Though sorghum is known to be relatively more tolerant to soil salinity than maize (Zea mays) (Igartua *et al.*,1994; Krishnamurthy *et al.*,2007), genetic enhancement of sorghum for salinity tolerance would further increase sorghum productivity in such soils.

The agricultural potentiality in the salinized areas is directly associated with production of salt resistant cultivars. The intensive crop yield in these salinized areas helps to resolve a great livestock regional problem of forage crop shortage, mainly in dry period of the year. According to the association (Grupo, 2005) in 2004 the Brazilian sorghum area was 1,269,000 hector. According to (Ahloowalia *et al.,* 2004), there were 2,250 new mutants registered by gamma rays from 163 botanical species, from 62 countries. A new genetic material is considered when a change in basic genotype occurs (IAEA, 1977). Bretaudeau and Traore (1990) declared that sorghum mutation induction by gamma rays doses of 200 to 300 Gy presented environmental stress viability tolerance. It is important to define which gamma ray dose is to be utilized to promote favorable changes. Moreover, the confirmation of genetic changes by gamma ray use is possible with microsatellite techniques (Ferreira and Grattapaglia, 1998). In this work sorghum plants were evaluated for high salinity tolerance through electrical conductivity (EC) levels and gamma ray doses. About 7% of the world's total land area is affected by salt, as is a similar percentage of its arable land (Ghassemi *et al*., 1995).

Salinity is often accompanied by other soil properties, such as sodicity and alkalinity, which exert their own specific effects on plant growth. Salinity and waterlogging co-exist in the lower reaches of several river basins throughout the world, affecting agricultural production and the livelihoods of the affected communities (Wichelns and Oster, 2006). Efforts being made to

overcome salinity and water logging problems by consist of engineering solutions such as installation of a drainage system to manage the drainage effluent generated by irrigated agriculture. This is a long term strategy; however drainage installation is expensive. The areas under salt-affected and waterlogged soils are expanding because of inappropriate on-farm water and soil management. Selection and cultivation of high-yielding salt-tolerant varieties of different crops is a potential interim strategy to fulfill the needs of the communities relying on these soils for their livelihoods (Ayers and Westcot, 1989). Many crops show intraspecific variation in response to salinity. Sorghum is moderately salt-tolerant. Generally, substantial genotypic differences exist among sorghum cultivars in response to salinity stress (Sunseri *et al*., 2002; Netondo *et al*., 2004).

Breeding programs for new varieties of sweet sorghum suited to semi arid tropics, temperate areas with rainy summer, Mediterranean areas with dry summer and soil salinity, are under development (Cosentino, 1996). Sorghum is characterized as moderately tolerant to salinity (Almodares and Sharif, 2005; Almodares and Sharif, 2007). Salinity reduces sorghum growth and biomass production . Salinity greatly reduced sorghum growth and this effect was more pronounced at 250 mM than at 125 mM NaCI (Ibrahim, 2004). However it was reported that sorghum growth was significantly reduced at all salinity levels from 50 to 150 mM (El-Sayed et al., 1994). Imposition of salt stress resulted in decreases in the percentage of seeds germinated (Almodares *et al*., 2007), although the strongest decline in germination occurred at the highest salt concentration. Nevertheless, the development of high-yielding salinity tolerant sorghums is the best option to increase the productivity in soils (Igartua *et al.*,1994). Similarly, Gill *et al.*,(2003) observed a great reduction in germination rate due to salt stress, in sorghum seeds at 37°C in NaCl.

The present work has been undertaken to standardize the *in vitro* screening protocols for salt tolerance employing an osmotic agent : Soium Chlorde (NaCl MW - 58.5 mol).

MATERIALS AND METHODS

Seed material: Ten lines of sorghum having different tolerance to salt were used in the study. The material has been procured from the sorghum research centre, Athwalines, Surat (Gujarat State, India).

Seed Germination: Seeds were placed on Petri plates lined with germination paper soaked with water. Petri plates were incubated at room temperature, for 7-8 days. The papers were wetted with 1 ml water, if required. 10-day-old seedlings were used for DNA extraction.

Protein extraction: Shoot of single seedling was grinded in pestle and mortar with 1000 µl of extraction buffer (recipe in the Annexure). Grind the tissue completely. Transfer the extract in 1.5 ml centrifuge tube. Wash the pestle and mortar with another 1000 µl of extraction buffer. Transfer the same to the previous tube. Incubate tubes in refrigerator overnight. After this centrifuge the content at 10000 rpm for 10 min in a refrigerated centrifuge.

SDS-PAGE for proteins: Samples of the same extracts as analysed for total proteins were denaturated according to Laemmli (1970) and O'Farrel (1975). The two glass plates were cleaned thoroughly with 70% alcohol. The spacers and gasket were also cleaned properly. The bigger plant was kept at the bottom on which spacers (1.5 mm) placed at two longitudinal edges. On top of spacers, another glass plate placed. This sandwich placed carefully in casting stand. At first 3 ml of molten agar (1% agar in water) was poured in between the space of two glass plates. This was done to avoid leakage from the base. It was left undisturbed for 30 min. After this leakage checked using water. If leakage is there more agar solution overlaid. Once the leakage stops, water present in between glass plate sandwich was removed by carefully tilting the assembly. 12.5% separating gel solution was prepared using acrylamide, bis-acrylamide, APS and TEMED. 30 ml of this solution was poured in between two glass plates. It was overlaid with 1 ml of DDW. The assembly was left undisturbed for 30-45 min to allow polymerization of gel. Once polymerized, overlaid water was removed from the assembly and 10 ml of stacking gel solution was added. The comb was also placed in this, and left undisturbed till gel formation. The comb was removed carefully without damaging the well. The wells were washed with the tank buffer to remove all salt crystals. After this the gel sandwich was removed from casting stand and loaded in the gel tank. Tank buffer was poured in the assembly and 25-50 µl of samples were loaded in each well. The gel was run at 175V till the samples reach the base of

stacking gel and then it was increased to 200V till the bromophenol blue reaches the end of plate. Gel was then removed and processed for staining. Staining of gels was done with commasie blue.

Comassie Blue Staining: Gel was rinsed briefly with water. Gel was incubated in 0.1% comassie stain for 1 h or overnight on shaker for staining. Stain was collected back in the stock bottle and was distained using distain solution. This step was done overnight or for longer duration, till the background becomes clear.

Recording of data: Data of gels were recorded as the presence and absence of a band in a 0,1 format.

***In vitro* screening experiments:** To assess the role of an osmoticum agent in the growth and development of sorghum following experiments were undertaken. For all experiments, different concentrations of NaCl (Sodium Chloride, MW 50 mM, 100 mM, 150 mM, 200mM) were used as stress inducing agent. It was added to the medium prior to autoclaving and its role in various morphogenic processes was ascertained. All experiments were conducted employing plant tissue culture methods.

Media preparation: There are various formulations available for culture media; we have used the most commonly used one that is MS medium (Murashige and Skoog, 1962). Four stocks are prepared: macronutrient stock, micronutrient stock, organic stock and iron chelate stock. The concentration of stocks varied with laboratory to laboratory (Table 1). All the stock solution stored at low temperatures.

Table 1: Composition of MS medium

Components	Amount (mg/l)
Stock A - Micro salts	
Ammonium nitrate	1650
Potassium nitrate	1900
Calcium chloride	440
Magnesium sulphate	370
Potassium dihydrogen orthophosphate	170
Stock B - Micro nutrient	
Zinc sulphate	8.6
Boric acid	6.2
Sodium molybdate	0.25
Cobalt chloride	25

Magnesium sulphate	0.025
Potassium iodide	0.025
Copper sulphate	0.025
Stock C - Iron chelate	
Ferrous sulphate	27.8
Di sodium EDTA	37.3
Stock D – Vitamine	
Thiamine HCL	0.5
Pyridoxine HCl	0.5
Niacin	0.5
Meso - inositol (dissolve in NaOH)	100

Preparation of stock: Four stock solutions were prepared. For each stock, the components listed above were added initially in a small volume of water. Once all components were dissolved separately, they were mixed and final volume was made up as per the requirement. Care taken to ensure proper dissolving of the individual components. After preparing stocks, they were stored in stock bottles in refrigerator. Stock C stored in brown bottles to prevent its oxidation.

Preparation of media: For preparing a medium appropriate volume of stock was taken. They were added in the beaker having half volume of water initially. After mixing all stock, desired amount of sucrose was added at the rate 3g for 100 ml medium. The solution was kept on magnetic stirrer to ensure complete solubilisation. Antifungal solution was added to medium 200µl for 1 of the medium. Different concentrations of NaCl (MW - 58.5 mol) were added to the medium. Desired amount of NaCl was added to the medium like 50 mM, 100 mM, 150 mM, 200mM per 100 ml medium 50 mM, 100 mM, 150 mM, 200mM. After this, final volume was made using water. pH of the medium was adjusted to 5.8 using 1N NaOH/1N HCl. After this, the medium was dispensed in jam jars at the rate 100 ml/ jar. To each jar 0.9 g of agar at the rate of 0.9% was added. The medium was then autoclaved for sterilization. It was done at 121^{o}C at 105 kPa for 15 minutes. The medium was cooled and swirled the solution gently as it cools. When sufficiently cool, the medium was dispensed into labeled, sterile containers using good aseptic conditions.

Seed sterilization solution: Diluted sodium hypochlorite (1:3) along with a few drops of Tween-20 was used for sterilization of seeds. Seeds were taken in micro centrifuge tubes and sterilizing solution was added to it in such a way that seeds are immersed in the solution. They were kept

on rotator shaker for 10 min. Sterilizing solution was decanted from seeds and seeds were washed with sterile distilled water 3-4 times. Seeds left in water for 1-2 hours, prior to inoculation.

Seed Inoculation: Seeds were either inoculated on Petri plates or jam jars as per the experiment.

Experimental design: An experiment was conducted using 10 genotypes of sorghum and for each experiment 3 seeds were used per experiment. All experiments were repeated at least once maintaining the same number of seeds.

Sub-culturing of explants into a new medium: Explants were transferred to fresh medium after 15 days to same or different media as per requirement.

Incubation of cultures: All cultures were maintained in culture rooms maintained at 25 ± 2ºC with a relative humidity of 80% and photoperiod of 16/8 hours.

Maintaining the cultures: Daily cultures were checked for contamination. All contaminated cultures were removed from culture rooms and healthy explants were rescued from the contaminated medium. In Petri plates, water gets condensed due to transpiration through stomata; this excess water was removed from plate lids on alternate days.

Seed germination experiment: Seeds of different ten lines of sorghum were surface sterilized in as per above protocol. MS medium with different concentrations of NaCl was heated and dispensed in jam jars. Three compartments were made in each jar and in each sector seed (3) of individual line were placed. Jars were incubated in the incubation room and were observed daily. The experiment was terminated after 15 days and then the root length, shoot length, fresh weight of seedling as well as number of leaves were recorded.

Shoot regeneration experiment: Seeds (10) per line surface sterilized as above were inoculated in jars containing MS basal medium. Seeds were incubated for 4-5 days, until the emergence of the plumule and after this were used as a source of explants for further experiment. MS medium containing different concentrations of NaCl (50 mM, 100 mM, 150 mM, 200mM)was melted and were dispensed in a jam jar. The medium was fortified with 1 mg/l BA in all cases. Jam jars were incubated in the incubation room and were observed daily. The experiment was terminated after 15 days and shoot length, fresh weight of seedling as well as number of leaves were recorded. The experiment was repeated twice maintaining the same number of seeds. In addition

to BA, zeatin (0.2 mg/l) was also supplemented to the MS medium having different concentrations of NaCl. For this experiment too, all above mentioned parameters were recorded.

Root development experiment: Seeds (10) per line were taken and treated as above seed treatment then inoculated in jars containing MS medium. 2-week old seedlings were used as a source of explants for further experiment. From seedlings, roots were removed and the left over seedling was inoculated on the half strength MS medium containing different concentrations of NaCl (50 mM, 100 mM, 150 mM, 200mM). All media were fortified with 1 mg/l IBA. The experiment was terminated after 15 days and then the root length, fresh weight of seedling and number of leaves was recorded.

Seedling development experiment: Seeds of ten lines of rice were placed in Petri plates lined with germination paper. The paper was wetted with water. Plates were incubated at the room temperature and were observed daily. 10-day-old seedlings were transferred in Phytajar along with a filter paper bridge. Two seedlings were placed on each bridge and Hoagland medium was used during further experiments. 25 ml of medium as such or supplemented with different concentrations of NaCl (50 mM, 100 mM, 150 mM, 200mM) was added to each phytajar. The medium of each phytajar was changed after 3-4 days. The experiment terminated after one week and all growth parameters were recorded.

Analyses of data: All observations were computed in MS-Excel. They were represented as mean ± standard error.

RESULTS AND DISCUSIONS

SDS Data analysis: Total protein was extracted from seedlings of sorghum for all the treatments for all ten genotypes. Denatured PAGE analyses were carried out for the samples. It was observed that the treatments had a profound effect on the growth of seedling and it was evident through the protein profiling also. We have obtained different banding patterns, which indicate the appearance of new proteins as well as disappearance of existing ones. Although in SDS PAGE, each protein band represents a group of proteins of similar molecular weight as we are not going for any specific protein as such. At this moment of time, we are mainly emphasizing on absence and presence of protein bands only. In that also, we have got a good variations in

banding patterns (Figure 1; Table 2).

Table 2: Effect of altered NaCl content of MS medium, after 10 days of incubation on a single line, based on the protein banding pattern.

Sr. No	Line No.	Treatments									No of bands
		T1	T2	T3	T4	T5	T6	T7	T8	T9	
1	L1	1	1	0	0	0	0	1	1	1	3
		1	1	1	1	1	1	1	1	1	
		1	1	0	0	0	0	1	1	1	
2	L3	1	1	1	1	1	1	1	1	1	1
3	L4	1	1	1	1	1	0	0	0	0	2
		1	1	0	0	0	1	1	1	1	
4	L6	1	0	0	0	0	0	0	0	0	2
		0	1	0	0	1	1	1	1	1	
5	L10	0	1	0	1	1	1	1	1	1	3
		1	0	1	0	0	0	0	0	0	
		0	1	0	1	1	1	1	1	1	

At morphological level: Three different morphogenic modes, *viz.*, Seed germination, shoot regeneration and root development were evaluated in the presence of salt stress created by using different concentrations (50 mM, 100 mM, 150 mM, 200mM) of NaCl (Table 3; Figure 2).

Table 3: Effect of different treatment on sorghum lines after 10 days of incubation based on the protein banding pattern.

Sr. No.	Treatment No.	Genotypes										No. of bands
		1	2	3	4	5	6	7	8	9	10	
1	T1	1	1	1	1	1	1	1	1	1	1	4
		1	1	1	1	1	1	1	1	1	1	
		1	1	1	1	1	1	1	1	1	1	
		1	0	1	0	1	1	1	0	0	0	
2	T3	1	1	1	1	1	1	1	1	1	1	1
3	T4	0	1	1	0	0	0	1	1	1	1	8
		0	1	1	0	1	0	1	1	1	1	
		1	1	1	1	1	1	1	1	1	1	
		0	1	1	0	1	0	1	1	1	1	
		0	1	1	0	1	0	1	1	1	1	

		0	1	1	0	1	0	1	1	1	1	
		1	0	1	0	1	0	1	1	1	1	
		0	1	1	0	1	0	1	1	1	0	
4	T7	1	1	1	1	1	1	1	1	1	1	7
		1	1	1	1	1	1	1	1	1	1	
		1	1	1	1	1	1	1	1	1	1	
		1	1	1	1	1	1	1	1	1	1	
		1	1	0	0	1	0	1	1	1	0	
		1	1	1	1	1	0	1	1	1	0	
		1	1	1	1	1	0	1	1	1	0	

Seed germination: Dehusked seeds were inoculated on MS basal medium supplemented with aforesaid concentrations of NaCl. Seeds started to germinate within 4-5 days of inoculation. Concentrations had a profound affect on seed germination. With the increase in concentrations of NaCl elongation of roots was more comparable to the shoot in almost all genotypes (Table 4; Figure 3). Number of leaves although did not differ much however, the expansion of leaves. The fresh weight of seedling decreased with increase in NaCl. Effect of NaCl was more pronounced in some genotypes compared to others.

Table 4 : Effect of NaCl concentrations on seed Germination.

Sr. No.	Line No.	NaCl conc. (mM)	Number of seeds		Average length (cm)		No. of leaves	Fresh weight (mg)
			Inoculated	Developed	Shoot	Root		
1	1	50	3	3	16.56±1.88	13.38±1.40	1.20±0.44	91.5±2.99
2		100	3	3	18.30±1.70	10.75±1.83	1.12±0.35	90.5±2.84
3		150	3	3	17.40±1.90	9.25±1.88	1.11±0.35	90.8±4.47
4		200	3	3	18.40±1.20	8.45±1.45	1.00±0.00	88.3±5.00
5	2	50	3	3	15.85±1.99	12.78±1.59	1.00±0.00	89.0±3.72
6		100	3	3	16.71±1.93	8.76±1.68	1.33±0.50	80.8±4.01
7		150	3	3	16.75±1.95	9.13±1.35	1.00±0.00	92.8±5.05
8		200	3	3	18.35±1.65	8.15±1.50	1.25±0.40	88.7±5.02
9	3	50	3	3	17.05±1.69	12.50±1.62	1.12±0.36	73.01±3.86
10		100	3	3	15.65±1.89	9.32±2.30	1.33±0.49	68.60±5.21
11		150	3	3	17.45±2.12	8.13±1.35	1.63±0.50	92.01±5.16
12		200	3	3	18.46±1.28	7.65±1.18	1.14±0.37	83.2±5.50
13	4	50	3	3	17.40±1.83	11.80±1.27	1.11±0.33	101.4±4.0
14		100	3	3	18.80±1.27	11.75±1.93	1.11±0.33	114.1±5.3
15		150	3	3	21.20±0.85	7.65±1.18	1.22±0.44	96.0±6.00
16		200	3	3	18.40±1.36	11.83±1.27	1.00±0.00	95.8±5.68
17	5	50	3	3	12.42±1.52	10.69±1.87	1.62±0.51	86.7±4.50

18		100	3	3	12.60±1.40	8.45±2.00	1.33±0.51	83.6±5.02
19		150	3	3	10.10±1.98	5.47±1.64	1.57±0.53	65.8±5.08
20		200	3	3	12.65±1.70	7.46±1.31	1.33±0.50	70.0±6.02
21	6	50	3	3	11.65±1.70	10.03±1.29	1.26±0.44	79.2±3.40
22		100	3	3	11.10±1.65	7.65±1.32	1.25±0.46	75.5±4.56
23		150	3	3	12.55±1.90	7.23±1.33	1.37±0.51	96.3±5.23
24		200	3	3	17.02±1.64	8.93±1.65	1.30±0.55	78.0±5.02
25	7	50	3	3	15.24±1.40	13.32±1.35	1.70±0.44	118.8±4.9
26		100	3	3	15.25±2.04	9.13±1.53	1.77±0.44	102±4.47
27		150	3	3	17.45±2.12	12.50±1.62	1.22±0.44	83.6±5.02
28		200	3	3	18.46±1.28	9.32±2.30	1.00±0.00	65.8±5.08
29	8	50	3	3	17.40±1.83	8.13±1.35	1.62±0.51	70.0±6.02
30		100	3	3	18.80±1.27	7.65±1.18	1.33±0.51	79.2±3.40
31		150	3	3	21.20±0.85	11.80±1.27	1.57±0.53	65.8±5.08
32		200	3	3	12.65±1.70	11.75±1.93	1.33±0.50	70.0±6.02
33	9	50	3	3	11.65±1.70	7.65±1.18	1.57±0.53	79.2±3.40
34		100	3	3	11.10±1.65	10.69±1.87	1.33±0.50	75.5±4.56
35		150	3	3	12.55±1.90	8.45±2.00	1.26±0.44	96.3±5.23
36		200	3	3	17.02±1.64	5.47±1.64	1.25±0.46	78.0±5.02
37	10	50	3	3	15.24±1.40	7.46±1.31	1.37±0.51	83.6±5.02
38		100	3	3	15.25±2.04	10.03±1.29	1.30±0.55	65.8±5.08
39		150	3	3	11.10±1.65	7.65±1.32	1.70±0.44	70.0±6.02
40		200	3	3	12.55±1.90	7.23±1.33	1.26±0.44	79.2±3.40

Shoot regeneration: To assess the affect of NaCl on shoot regeneration, pre-germinated seeds were used. The shoot meristem was excised off and the trimmed seeds were placed on MS medium containing cytokinins (BA or Zeatin) supplemented with different concentrations of NaCl. Average shoot length was affected by the change in concentration of NaCl. With the increase in concentration of NaCl, shoot length reduced gradually (Table 5; Figure 5). In addition to this, the leaf area also changed with increased concentration of NaCl. Some genotypes exhibited a greater reduction in soot length compared to others.

To compare the effect of cytokinins on shoot regeneration, two cytokinins, *viz*., BA and zeatin were used. It was observed that shoots developed in presence of zeatin were much healthier (Figure 6B) and stronger compared to those developed for BA supplemented medium (Figure 6A,7; Table 6). Among these two cytokinins, zeatin exhibited a more consistency in growth compared to BA in the presence of NaCl concentrations.

Table 5 : Effect of NaCl concentrations on shoot regeneration by BA (1.0mg/l) .

Sr. No.	Line No.	NaCl conc. (mM)	Number of seeds		Average length (cm)	No. of leaves
			Inoculated	Devloped		
1	1	50	3	3	10.75±1.83	1.05±0.00
2		100	3	3	9.25±1.88	1.50±0.8
3		150	3	3	8.45±1.45	1.40±0.07
4		200	3	3	12.78±1.59	1.00±0.06
5	2	50	3	3	8.76±1.68	1.00±0.00
6		100	3	3	9.13±1.35	1.06±0.08
7		150	3	3	8.15±1.50	1.50±0.8
8		200	3	3	12.50±1.62	1.00±0.60
9	3	50	3	3	9.32±2.30	1.00±0.00
10		100	3	3	8.13±1.35	1.00±0.00
11		150	3	3	7.65±1.18	2.00±0.50
12		200	3	3	7.70±1.27	1.00±0.00
13	4	50	3	3	8.75±1.93	1.25±0.40
14		100	3	3	7.65±1.18	1.12±0.36
15		150	3	3	11.83±1.27	2.00±0.49
16		200	3	3	10.69±1.87	2.00±0.50
17	5	50	3	3	8.45±2.00	1.50±0.8
18		100	3	3	5.47±1.64	1.40±0.07
19		150	3	3	12.50±1.62	1.00±0.06
20		200	3	3	9.32±2.30	1.00±0.00
21	6	50	3	3	8.13±1.35	1.06±0.08
22		100	3	3	7.65±1.18	1.50±0.8
23		150	3	3	11.80±1.27	1.00±0.60
24		200	3	3	8.75±1.93	1.25±0.40
25	7	50	3	3	8.13±1.35	1.12±0.36
26		100	3	3	7.65±1.18	2.00±0.49
27		150	3	3	7.70±1.27	1.00±0.00
28		200	3	3	8.75±1.93	1.00±0.00
29	8	50	3	3	7.65±1.18	1.00±0.00
30		100	3	3	11.83±1.27	2.00±0.50
31		150	3	3	11.80±1.27	1.00±0.00
32		200	3	3	8.75±1.93	1.25±0.40
33	9	50	3	3	8.13±1.35	1.12±0.36
34		100	3	3	7.65±1.18	2.00±0.49

35		150	3	3	7.70±1.27	1.00±0.00
36		200	3	3	8.75±1.93	1.00±0.00
37	10	50	3	3	7.65±1.18	2.00±0.50
38		100	3	3	11.83±1.27	1.00±0.00
39		150	3	3	7.65±1.18	1.25±0.40
40		200	3	3	7.70±1.27	1.00±0.00

Table - 6: Effect of NaCl concentrations on shoot regeneration by Zeatin (0.2 mg/l).

Sr. No.	Line No.	NaCl conc. (mM)	Number of seeds		Average length (cm)	No. of leaves	Fresh weight (mg)
			Inoculated	Devloped	Shoot		
1	1	50	3	3	10.42±1.52	1.00±0.00	55.8±5.68
2		100	3	3	12.60±1.40	1.00±0.00	46.7±4.50
3		150	3	3	9.10±1.98	1.00±0.00	33.6±5.02
4		200	3	3	12.65±1.70	1.00±0.07	45.8±5.08
5	2	50	3	3	6.65±1.70	1.05±0.06	40.0±6.02
6		100	3	3	7.10±1.65	1.05±0.00	59.2±3.40
7		150	3	3	6.55±1.90	1.50±0.8	45.5±4.56
8		200	3	3	10.42±1.52	1.40±0.07	51.4±4.0
9	3	50	3	3	8.60±1.40	1.00±0.06	55.8±5.68
10		100	3	3	10.10±1.98	1.00±0.00	46.7±4.50
11		150	3	3	9.65±1.70	1.06±0.08	33.6±5.02
12		200	3	3	7.65±1.70	1.50±0.8	45.8±5.08
13	4	50	3	3	8.10±1.65	1.00±0.60	51.4±4.0
14		100	3	3	9.55±1.90	1.00±0.00	44.1±5.3
15		150	3	3	7.65±1.70	1.00±0.00	46.0±6.00
16		200	3	3	10.42±1.52	1.00±0.07	55.8±5.68
17	5	50	3	3	8.60±1.40	1.06±0.0	46.7±4.50
18		100	3	3	10.10±1.98	1.40±0.00	33.6±5.02
19		150	3	3	9.65±1.70	1.50±0.8	45.8±5.08
20		200	3	3	7.65±1.70	1.5±0.05	40.0±6.02
21	6	50	3	3	8.10±1.65	1.00±0.05	59.2±3.40
22		100	3	3	9.55±1.90	1.00±0.06	45.5±4.56
23		150	3	3	12.65±1.70	1.50±0.00	51.4±4.0
24		200	3	3	6.65±1.70	1.5±0.8	44.1±5.3
25	7	50	3	3	7.10±1.65	1.00±0.00	51.5±2.99
26		100	3	3	6.55±1.90	1.05±0.00	40.5±2.84
27		150	3	3	10.42±1.52	1.5±0.8	50.8±4.47
28		200	3	3	8.60±1.40	1.00±0.08	38.3±5.00
29	8	50	3	3	10.10±1.98	1.00±0.08	44.0±3.72
30		100	3	3	9.65±1.70	1.00±0.00	50.8±4.01
31		150	3	3	10.10±1.98	1.40±0.00	32.8±5.05

32		200	3	3	9.65±1.70	1.5±0.8	58.7±5.02
33	9	50	3	3	7.65±1.70	1.00±0.00	63.01±3.86
34		100	3	3	8.10±1.65	1.00±0.00	58.60±5.21
35		150	3	3	9.55±1.90	1.5±0.8	46.7±4.50
36		200	3	3	12.65±1.70	1.00±0.00	33.6±5.02
37	10	50	3	3	6.65±1.70	1.00±0.00	45.8±5.08
38		100	3	3	7.10±1.65	1.00±0.08	40.0±6.02
39		150	3	3	7.65±1.70	1.00±0.00	59.2±3.40
40		200	3	3	8.10±1.65	1.5±0.8	45.55±3.40

Root development: To assess the effect of root development on NaCl concentration, seedlings were used as a source of experiment. 2-week-old seedling was used as a source of explants, roots were excised and inoculated on auxin containing medium. With increased concentration of NaCl, root length increased in almost all genotypes. However, the extent of root elongation varied with genotypes (Table 7; Figure 8, 9). Drying of leaves was observed in all lines at 50 mM, 100 mM, 150 mM, 200mM concentration of NaCl in the medium. However, at 50 mM, 100 mM, 150 mM, 200mM concentration plants had yellowing of leaves in almost all genotypes.

Table 7 : Effect of NaCl concentrations on root development**.**

Sr. No.	Line No.	NaCl conc. (mM)	Number of seeds		Average length (cm)	No. of leaves	Fresh weight (mg)
			Inoculated	Devloped	Root		
1	1	50	3	3	8.38±1.40	1.33±0.49	91.5±2.99
2		100	3	3	7.75±0.83	1.63±0.50	110.5±2.84
3		150	3	3	6.25±1.88	1.14±0.37	90.8±4.47
4		200	3	3	5.45±0.45	1.11±0.33	88.3±5.00
5	2	50	3	3	4.78±0.59	1.11±0.33	89.0±3.72
6		100	3	3	5.76±1.68	1.22±0.44	100.8±4.01
7		150	3	3	4.13±1.35	1.00±0.00	128.8±5.05
8		200	3	3	6.15±0.50	1.22±0.44	88.7±5.02
9	3	50	3	3	4.50±1.62	1.00±0.00	103.01±3.86
10		100	3	3	3.32±0.30	1.62±0.51	68.60±5.21
11		150	3	3	4.78±0.59	1.33±0.51	112.01±5.16
12		200	3	3	5.76±1.68	1.57±0.53	83.2±5.50
13	4	50	3	3	4.13±1.35	1.33±0.50	90.8±4.47
14		100	3	3	6.15±0.50	1.26±0.44	88.3±5.00
15		150	3	3	5.76±1.68	1.25±0.46	89.0±3.72
16		200	3	3	4.13±1.35	1.37±0.51	100.8±4.01
17	5	50	3	3	6.15±0.50	1.30±0.55	128.8±5.05
18		100	3	3	8.38±1.40	1.70±0.44	88.7±5.02

19		150	3	3	7.75±0.83	1.77±0.44	103.01±3.86
20		200	3	3	6.25±1.88	1.22±0.44	68.60±5.21
21	6	50	3	3	5.45±0.45	1.00±0.00	103.01±3.86
22		100	3	3	8.38±1.40	1.33±0.51	68.60±5.21
23		150	3	3	7.75±0.83	1.57±0.53	112.01±5.16
24		200	3	3	6.25±1.88	1.33±0.50	83.2±5.50
25	7	50	3	3	8.38±1.40	1.26±0.44	90.8±4.47
26		100	3	3	7.75±0.83	1.25±0.46	88.3±5.00
27		150	3	3	6.25±1.88	1.37±0.51	89.0±3.72
28		200	3	3	5.45±0.45	1.30±0.55	100.8±4.01
29	8	50	3	3	4.78±0.59	1.70±0.44	128.8±5.05
30		100	3	3	5.76±1.68	1.77±0.44	68.60±5.21
31		150	3	3	4.13±1.35	1.26±0.44	103.01±3.86
32		200	3	3	6.15±0.50	1.25±0.46	68.60±5.21
33	9	50	3	3	4.50±1.62	1.37±0.51	112.01±5.16
34		100	3	3	3.32±0.30	1.30±0.55	83.2±5.50
35		150	3	3	4.78±0.59	1.70±0.44	90.8±4.47
36		200	3	3	5.76±1.68	1.37±0.51	88.3±5.00
37	10	50	3	3	4.13±1.35	1.30±0.55	89.0±3.72
38		100	3	3	6.15±0.50	1.26±0.44	100.8±4.01
39		150	3	3	5.76±1.68	1.25±0.46	83.2±5.50
40		200	3	3	4.13±1.35	1.37±0.51	90.8±4.47

Seedling development: To assess the affect of NaCl on seedling development, development of seedling was studied for a period of 2 weeks. In this experiment, higher concentrations of NaCl were used compared to previous experiments. It was observed that in the presence of NaCl the root length is more compared to control, while the shoot length decreases with the increased concentration. If the length of entire seedling is taken into account, then we might not get the actual affect of NaCl on seedling development as decrease in shoot length would be compensated by increased root length (Figure 10). The fresh weight of seedling also decreased with increased concentration of NaCl due to less availability of water (Table 8). In almost all genotypes, dry leaves were observed in all lines at 50 mM, 100 mM, 150 mM, 200mM and 50 mM, 100 mM, 150 mM, 200mM concentration of NaCl in Hoagland medium, while at 50 mM, 100 mM, 150 mM, 200mM concentration yellowing of leaves was recorded in most of the cases.

Table 8 : Effect of NaCl concentrations of seedling development.

Sr. No.	Line No.	NaCl conc. (mM)	Number of seeds		Average length (cm)		No. of leaves	Fresh weight (mg)
			Inoculated	Devloped	Shoot	Root		

1	1	50	3	3	6.56±1.88	8.38±1.40	1.00±0.00	51.5±2.99
2		100	3	3	8.30±1.70	7.75±0.83	1.00±0.00	40.5±2.84
3		150	3	3	7.40±0.90	6.25±1.88	1.00±0.00	50.8±4.47
4		200	3	3	8.40±0.20	5.45±0.45	1.00±0.00	38.3±5.00
5	2	50	3	3	5.85±0.99	4.78±0.59	2.00±0.00	44.0±3.72
6		100	3	3	6.71±1.93	5.76±1.68	2.00±0.00	50.8±4.01
7		150	3	3	6.75±1.95	4.13±1.35	1.5±0.8	32.8±5.05
8		200	3	3	8.35±1.65	6.15±0.50	2.00±0.00	58.7±5.02
9	3	50	3	3	7.05±0.69	4.50±1.62	1.00±0.00	63.01±3.86
10		100	3	3	5.65±0.89	3.32±0.30	1.00±0.00	58.60±5.21
11		150	3	3	5.85±0.99	4.78±0.59	2.00±0.00	42.01±5.16
12		200	3	3	6.71±1.93	5.76±1.68	1.5±0.8	33.2±5.50
13	4	50	3	3	6.75±1.95	4.13±1.35	1.00±0.00	51.4±4.0
14		100	3	3	8.35±1.65	6.15±0.50	1.00±0.00	44.1±5.3
15		150	3	3	6.71±1.93	5.76±1.68	1.00±0.00	46.0±6.00
16		200	3	3	6.75±1.95	4.13±1.35	1.00±0.00	55.8±5.68
17	5	50	3	3	8.35±1.65	6.15±0.50	2.00±0.00	46.7±4.50
18		100	3	3	6.56±1.88	8.38±1.40	2.00±0.00	33.6±5.02
19		150	3	3	8.30±1.70	7.75±0.83	1.5±0.8	45.8±5.08
20		200	3	3	7.40±0.90	6.25±1.88	2.00±0.00	40.0±6.02
21	6	50	3	3	8.40±0.20	5.45±0.45	1.00±0.00	59.2±3.40
22		100	3	3	6.56±1.88	8.38±1.40	1.00±0.00	45.5±4.56
23		150	3	3	8.30±1.70	7.75±0.83	2.00±0.00	51.4±4.0
24		200	3	3	7.40±0.90	6.25±1.88	1.5±0.8	44.1±5.3
25	7	50	3	3	6.56±1.88	8.38±1.40	2.00±0.00	46.0±6.00
26		100	3	3	8.30±1.70	7.75±0.83	2.00±0.00	55.8±5.68
27		150	3	3	7.40±0.90	6.25±1.88	1.5±0.8	46.7±4.50
28		200	3	3	8.40±0.20	5.45±0.45	2.00±0.00	33.6±5.02
29	8	50	3	3	5.85±0.99	4.78±0.59	1.00±0.00	45.8±5.08
30		100	3	3	6.71±1.93	5.76±1.68	1.00±0.00	33.2±5.50
31		150	3	3	6.75±1.95	4.13±1.35	2.00±0.00	51.4±4.0
32		200	3	3	8.35±1.65	6.15±0.50	1.5±0.8	44.1±5.3
33	9	50	3	3	7.05±0.69	4.50±1.62	2.00±0.00	46.0±6.00
34		100	3	3	5.65±0.89	3.32±0.30	2.00±0.00	55.8±5.68
35		150	3	3	6.56±1.88	8.38±1.40	1.5±0.8	46.7±4.50
36		200	3	3	8.30±1.70	7.75±0.83	2.00±0.00	33.6±5.02
37	10	50	3	3	7.40±0.90	6.25±1.88	1.00±0.00	45.8±5.08
38		100	3	3	8.40±0.20	5.45±0.45	1.00±0.00	40.0±6.02
39		150	3	3	6.56±1.88	8.38±1.40	2.00±0.00	59.2±3.40
40		200	3	3	8.30±1.70	7.75±0.83	1.5±0.8	45.55±3.40

CONCLUSION

Ten genotypes of sorghum were evaluated for the salt tolerance under *in vitro* conditions. For morphogenic data, *in vitro* approaches were employed using NaCl as stress inducing agent. In the presence of NaCl, shoot length decreased with increase in concentration, while the root length exhibited a reverse trend. During shoot development, shoot length also declined with increased concentration of NaCl. Two different cytokinins were used, where zeatin exhibited a much stronger inhibitory effect in presence of NaCl compared to BA. During root development experiment, root length increased with increased in concentration of NaCl in most of genotypes. In the majority of cases, at (50 mM, 100 mM) NaCl, yellowing of leaves was recorded, while at higher concentrations (150 mM) complete drying of the leaves took place. For seedling development, higher concentrations of NaCl used (50 mM, 100 mM, 150 mM, 200mM). The total seedling length increased with increase in 50 mM, 100 mM, 150 mM, 200mM concentration of NaCl as the presence of NaCl induced much elongation of roots. Our four *in vitro* assays gave a clear cut difference between various growth parameters recorded during course of present study to evaluate the effect of salt on overall growth and development of lines. *In vitro* approaches provide a more reliable and repeatable data to evaluate any germplasm for any abiotic stress.

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LIST OF ABBREVIATIONS

µ	**Micro**
$(NH_4)_2SO_4$	Ammonium sulphate
°C	Degree centigrade
APS	Ammonium per sulphate
BA	Benzylaminopurine
C	Carbon
Cm	Centimeter
DDW	Double Distilled Water
dNTP	Deoxyridonucleotide Triphosphates
EDTA	Ethylene Diamine Trichloro Acetate
g	Gram
h	Hour
HCl	Hydrochloric acid
IBA	Indol-3-butyric acid
L	Litre
M	Molar
M.W.	Molecular Weight
mg	Milligram
mL	Milliliter
mm	Millimeter

mM	Millimolar
Na	Sodium
NaCl	Sodium Chloride
NaOH	Sodium hydroxide
NA	Nutrient Agar
NH_4Cl	Ammonium Chloride
nm	Nano meter
OD	Optical Density
SDS	Sodium Dodecyl Sulphate.
SDS- PAGE	Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis
TAE	Tris acetate EDTA
TE	Tris EDTA
TEMED	N,N,N',N'-Tetra methylethylenediamine
Vm	Maximum Velocity of the Reaction
Vol	Volume
YEP	Yeast Extract Pectin

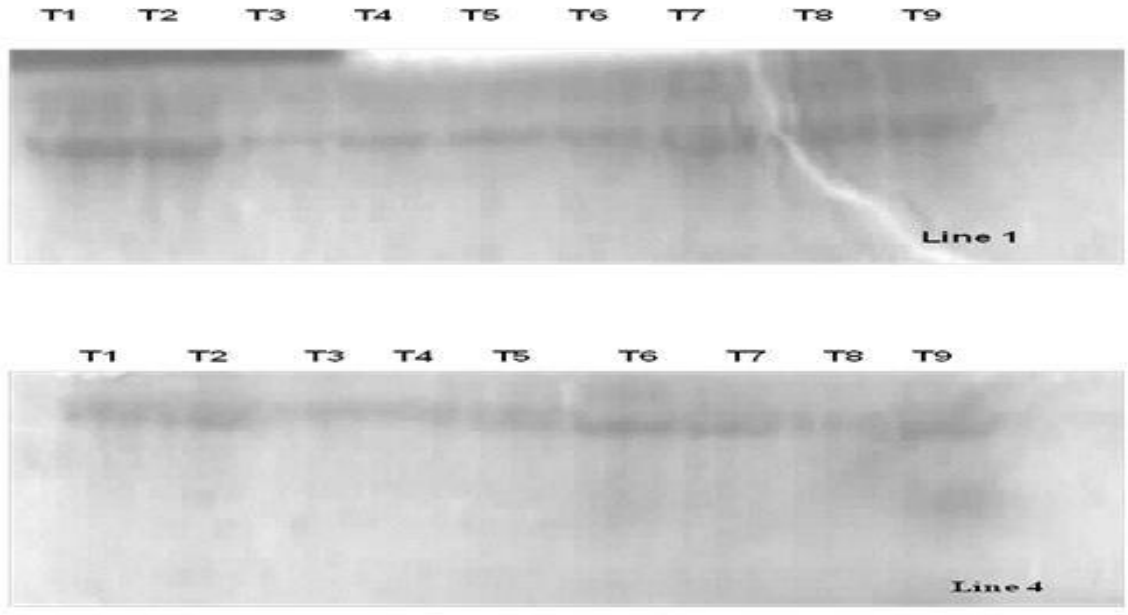


Figure 1: SDS profiling of total protein content of seedling of same line different treatment.

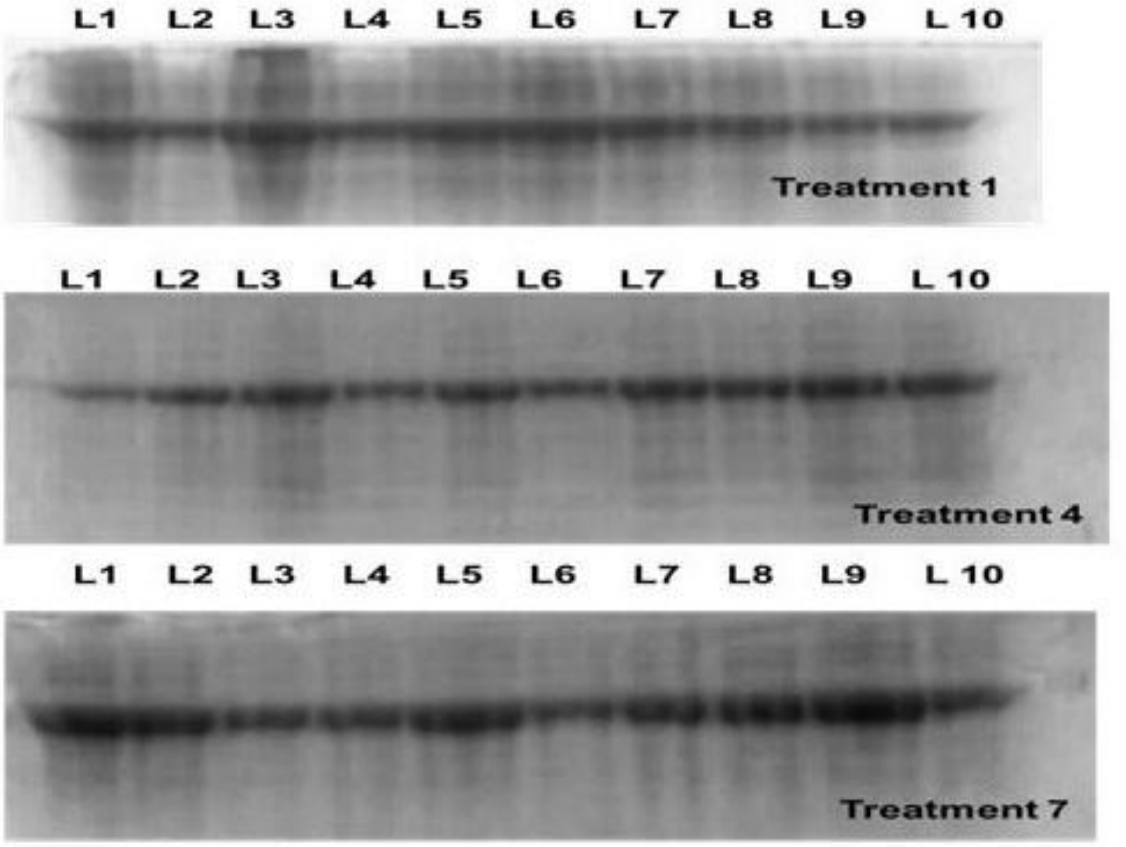
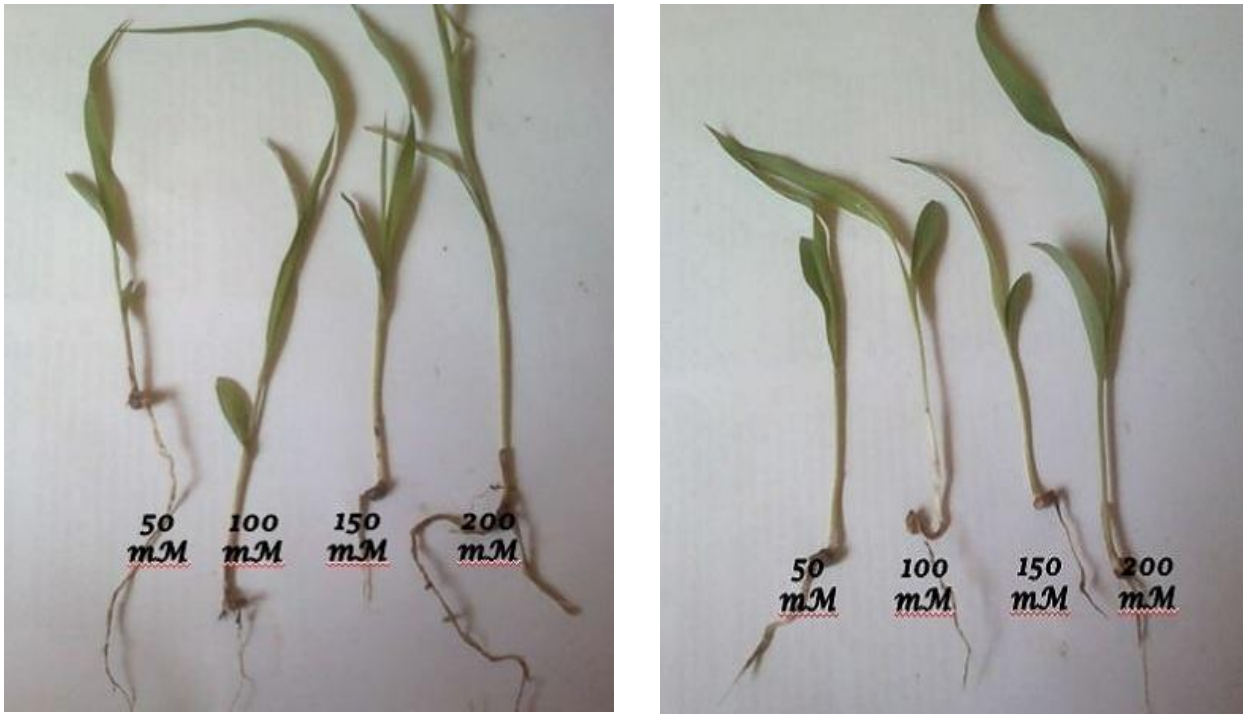


Figure 2: SDS profiling of total protein content of seedling for all test lines under one treatment.



Line 3 **Line 4**

Figure 3: Effect of different concentrations of NaCl on shoot regeneration in Sorghum

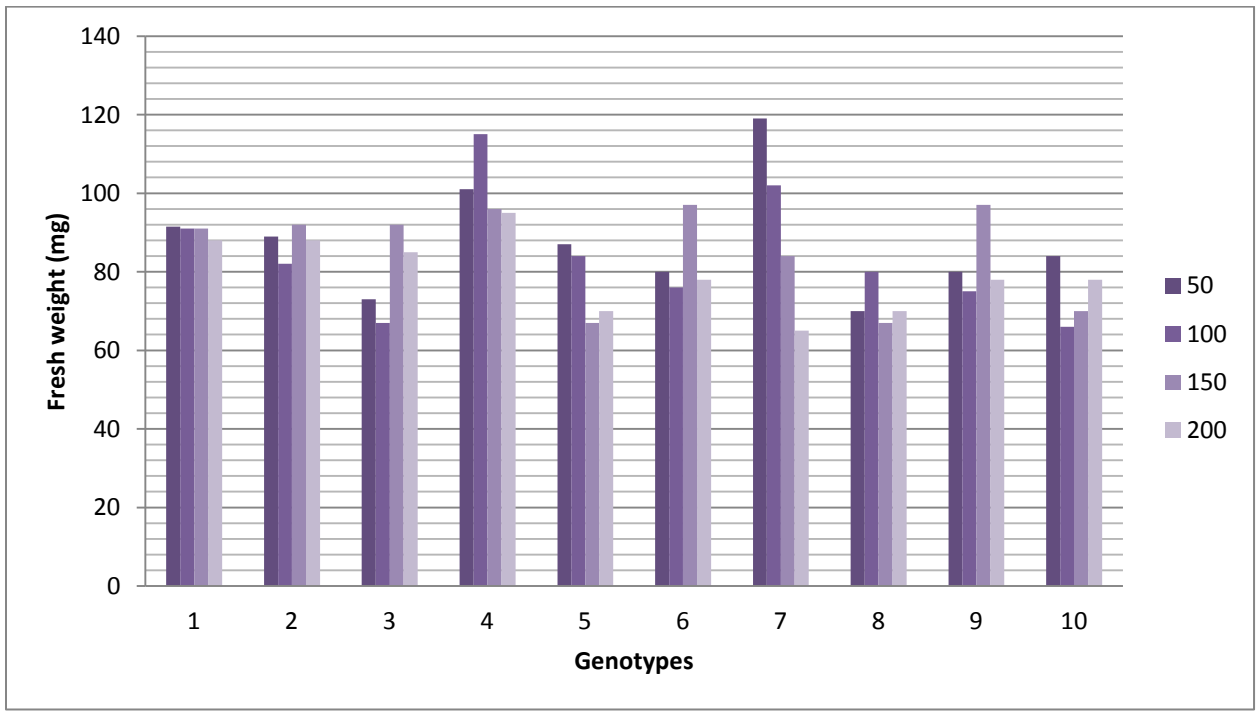
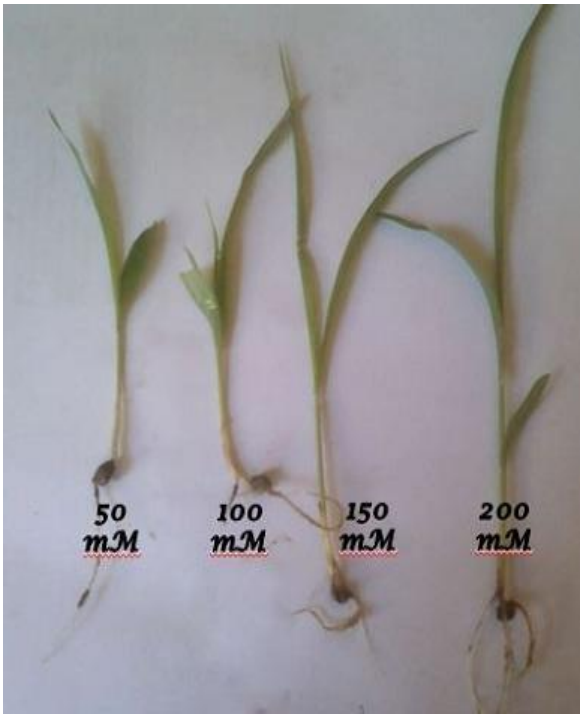
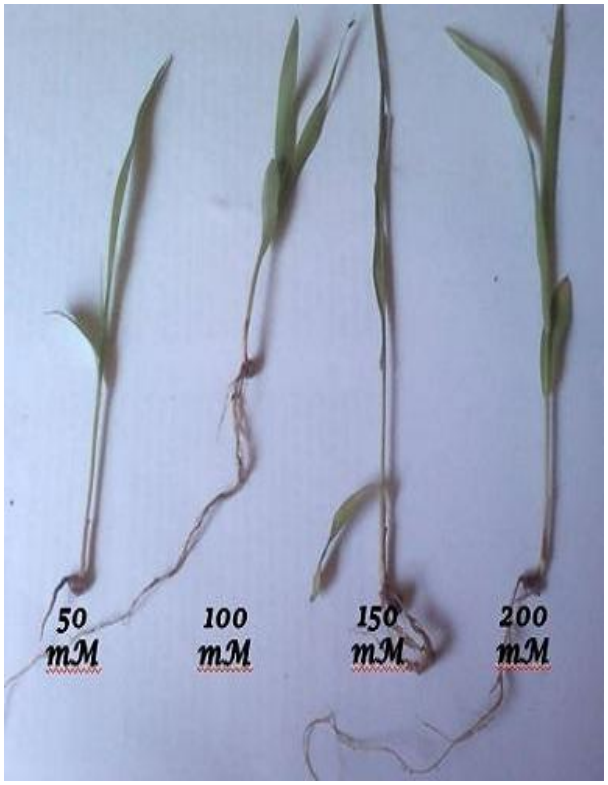


Figure 4: Effect of different concentrations of NaCl on seed germination in Sorghum.

Line 1



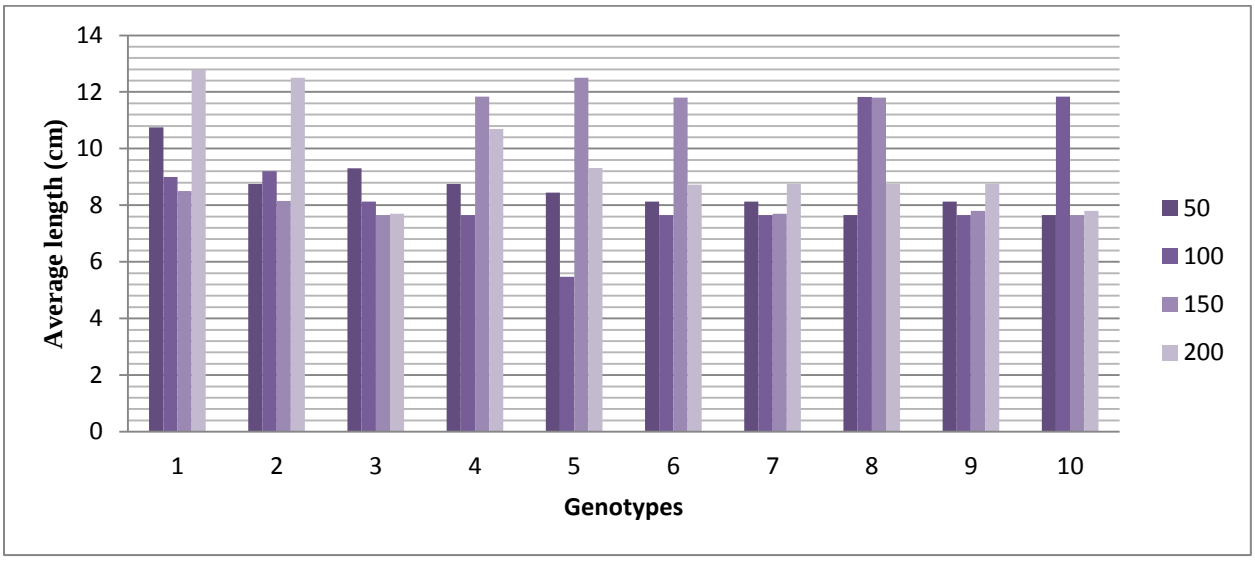
Line 5



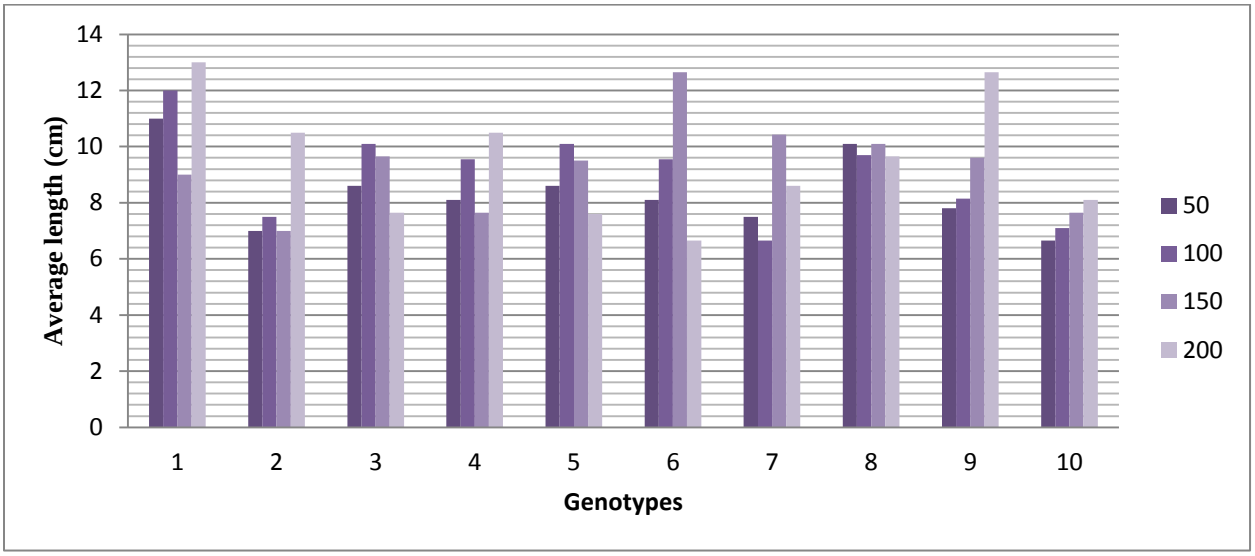
Line 7

Line 9

Figure 5: Effect of different concentrations of NaCl on shoot regeneration in Sorghum.



A: For BA (1.0 mg/l)



B: For Zeatin (0.2 mg/l).

Figure 6: Comparative effect of cytokinins on shoot regeneration in presence of different concentrations of NaCl. **A**: 1 mg/l BA, **B**: 0.5 mg/l Zeatin.

Line 2 **Line 8**

Figure 7: Effect of different concentrations of NaCl on shoot regeneration in Sorghum.

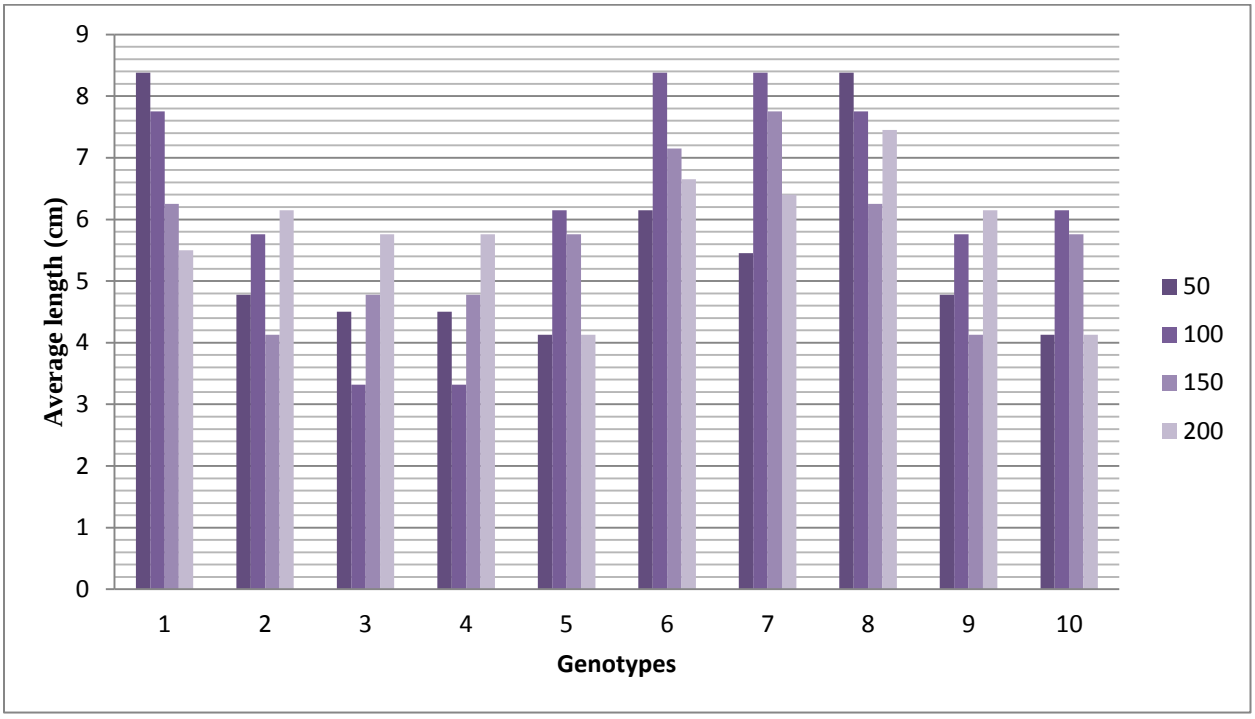


Figure 8: Effect of different concentrations of NaCl on root development in Sorghum.

Line 6 **Line 10**

Figure 9: Effect of different concentrations of NaCl on root development in Sorghum.

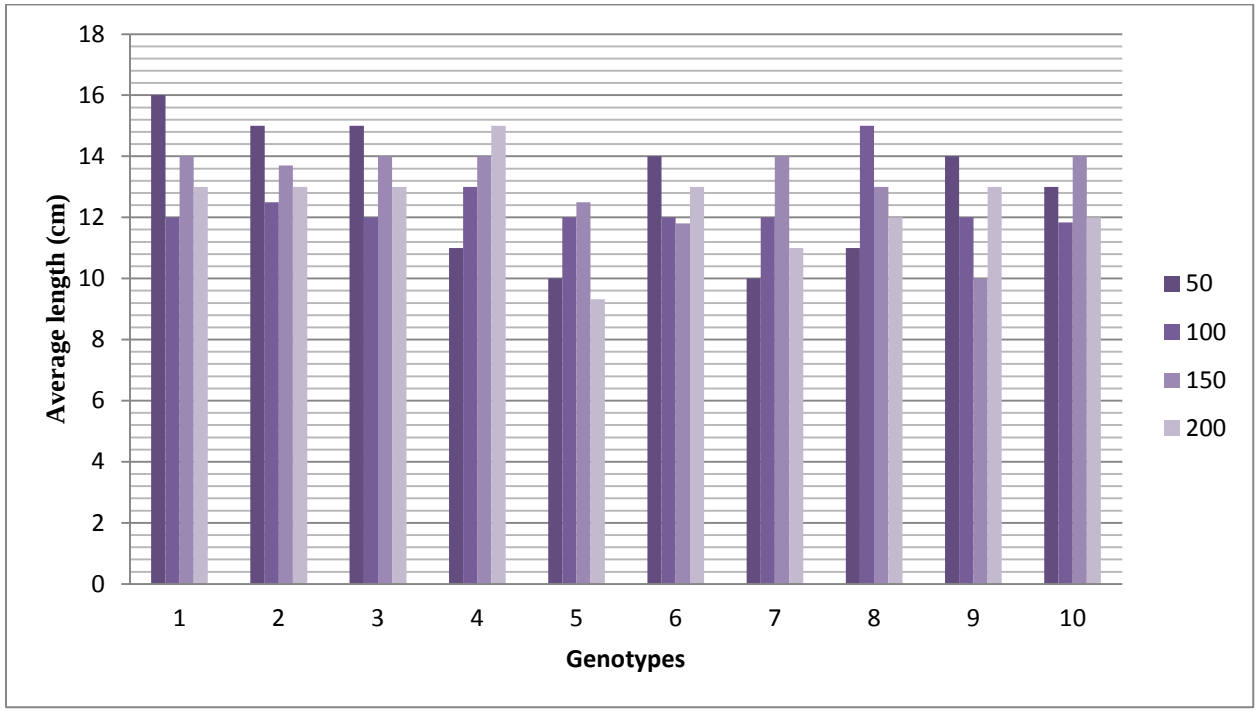


Figure 10: Effect of different concentrations of NaCl on seedling development in Sorghum.