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RESEARCH ARTICLE

Assessment of salt tolerance potential at the germination and seedling stages in pigeonpea (*Cajanus cajan* L.)

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Abstract

Identification of salt-tolerant genotypes and their subsequent utilization in producing salt tolerant varieties would be the most appropriate and cost-effective strategy for improving the yield of pigeonpea (*Cajanus cajan* L.) in salt-affected areas of the country. The present study assessed fifty diverse pigeonpea genotypes for their responses to salt (NaCl) concentrations of 60, 80 and 100 mM at seed germination and seedling stage (21-days-old). The tested genotypes were assessed for their changes in 11 morpho-physiological traits under salt stress and the phenotypic scores of the genotypes were analyzed statistically. Statistically, significant variations were observed among the genotypes for all the morpho-physiological traits under study including the germination percentage and seedling survivability. The genotypic and phenotypic correlation among the traits and the Principal Component Analysis (PCA) revealed that the seedling stage of the crop and 80 mM Nacl concentration are optimum for identifying pigeonpea genotypes tolerant to salt stress under controlled conditions. Out of the 50 genotypes, 10, namely, BDN-708, AKTM 16-41, AKTE 16-09, JKM-7, TV-1, BDN-716, PT 0607-5-1, JKM-189, Phule Rajeshwary, BDN-711 and AKTE-12-04 were found to be tolerant to salt stress and rest were sensitive. The salt tolerant genotypes clustered together under UPGMA, indicating their genetic relatedness for the trait. The salt tolerant genotypes identified in this study would be useful in the development of a mapping population for mapping the salt stress, and breeding for high-yielding pigeonpea varieties with tolerance to salt stress.

Keywords: Pigeonpea, salt stress, parameters, threshold value, salt tolerance

Introduction

Soil salinization is one of the main abiotic stress factors affecting crop yields worldwide. Approximately, 6% of the world's total land area is threatened by salinity, including 20% of arable land and 33% of irrigated land (Shrivastava and Kumar 2015; Kuang et al. 2019; Safdar et al. 2019). Furthermore, land salinization is increasing, with 10 million ha of agricultural land destroyed annually by salt accumulation due to human activity and other factors related to climate change (Smajgl et al. 2015; Isayenkov 2019). Salinity stress significantly decreases plant growth and productivity, reducing crop yield substantially (Munns et al. 2003).

Pigeonpea (*Cajanus cajan* L.), an important legume crop with 4.2 m ha of area under cultivation and an annual production of 3.27 mt (Meshram et al. 2013), is adversely affected by salinity stress. The ability of the plant to survive and complete its life cycle under saline conditions depends on its salt tolerance potential, which varies among different species and growth stages (Zeng et al. 2002; Akbari et al.

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2007). The development of saline tolerant cultivars can be an appropriate approach for minimizing yield losses under salinity stress conditions and thus the salt tolerant lines can be identified by exploitation of genetic variability present in the germplasm. Seed germination and seedling survivability are the initial and critical stages that are adversely affected by salinity (Heenan et al. 1988; Azza et al. 2007; Feizi et al. 2007). It has been reported in several monocot and dicot crops that percent germination, shoot and root length, and dry weight was reduced with increasing levels of NaCl (Jamil et al. 2007; Abbas et al. 2013; Li et al. 2020; Peel et al. 2004; Ahmed et al. 2016). The accumulation of Na+ and Cl- ions in tissues of the plants is the most detrimental effect under high salt concentration. Salinity affects various physiological parameters. and specific reports are available in maize (Khan et al. 2003a), wheat (Khan et al. 2003b), chickpea (Kaya et al. 2008), lentil (Singh 1 et al. 2017), cowpea (Murillo-Amador et al. 2001; Abdel-Haleem and Shaieny 2015), soybean (Kamal et al. 2003) and cotton (Azhar and Ahmad 2000) but the literature available on pigeonpea is replete.

Screening and identifying salt tolerant genotypes at the germination and seedling stages would be the first target for easy crop establishment for higher production under salt stress, particularly in pigeonpea. Therefore, the present study was carried out to investigate the salt tolerance potential by characterizing the pigeonpea genotypes with respect to morphological parameters and anatomical features at different salinity regimes ranging from 0 to 100 mM NaCl concentration at the germination and seedling stage.

Materials and methods

Evaluation of genotypes under salinity stress at the germination stage

A set of 50 diverse genotypes were evaluated for the effect of salinity stress on pigeonpea at the germination stage. Mature seeds were surface sterilized prior to germination with 1 % sodium hypochlorite for 5 minutes, thereafter, they were rinsed three times with sterilized distilled water. Twenty seeds of each genotype were placed between two layers of germination paper arranged in stands. The stands were kept in trays containing 60mM, 80mM and 100mM NaCl solutions. For control conditions, distilled water was used. The trays were kept in a germination chamber at 28 ± 1°C in dark. A completely randomized block design was used for each level of salt treatment with three replications of each genotype. The germination count was started after 72 hours of sowing and continued till the 15th day. A seed was considered to have germinated when both the plumule and the radicle emerged (size >0.5cm). After fifteen days, the germination percentage and the seedlings that emerged were studied in all three stress conditions and compared to the control condition. The normal seedlings bear proper plumule and radical whereas the seedlings lacking either plumule or radical or the albino seedlings were considered as abnormal seedlings. The number of seeds germinated on the 8th day to the total number of seeds placed (Cokkizgin and Cokkizgin 2010) were considered as percent germination.

Evaluation of genotypes under salinity stress at seedling stage

Screening for salt tolerance at the seedling stage was conducted under hydroponic conditions using modified nutrient solution composition as suggested by Samineni et al. (2011) under controlled glasshouse conditions at National Phytotron Facility, ICAR-Indian Agricultural Research Institute, New Delhi, India. The same set of 50 pigeonpea genotypes was used to evaluate the effect of salinity stress at the seedling stage. The sterilized seeds were put on germination in template stand in normal distilled water, and after 12 days of sowing, stress was imposed. The dose of salt stress (NaCl solution) used was 60, 80 and 100 mM along with control condition (0 mM). After fifteen days, the seedling survival under three salinity stress conditions was observed, and all the genotypes' differential behaviour at different salinity conditions was studied. The salt tolerance was determined based on percent seedling survivability (percent ratio of seedlings survived the salt stress to the total number of seedlings treated) and scoring based on visual symptoms on 1 to 5 scale (Table 4) as suggested by Singh et al. 2017 (1 = healthy plants with no visible symptoms of salt stress, 2 = green plants with slight yellowing of leaves, 3 = green plants with yellowing of leaves and necrosis of the margins of older leaves, 4 = necrosis of leaves with green stem, 5 = partial and completely dried leaves and/or stem).

Morphological characterization from germination to seedling stage

At 60 mM NaCl concentration, most genotypes showed no differentiating symptoms. Therefore, a detailed study was conducted at 80 and 100 mM of salt stress to effectively assess salinity tolerance level. Characterization of genotypes based on morphological parameters and anatomical features under induced salinity stress was done from germination to 21 days old seedlings. For determining the critical level of salt stress, 10 traits including germination percentage, shoot length, root length, fresh weight, dry weight, shoot: root ratio, vigour index, germination rate, relative water content and salt tolerance index (STI), were studied at 80mM and 100mM. The Germination rate (GR), i.e., the average number of days needed for plumule or radicle emergence was calculated as per Shaheenuzzamn (2015): Germination rate = (NTn3 NTn6 NTn9 NTn12)/Total number of seeds germinated; where: Tn = number of seeds germinated at day 3, 6, 9, 12; N = days (3, 6, 9, 12).

After 15 days of salt stress, the observations for 10 traits were recorded. Fifteen seedlings from each group (five

seedlings from each replication) were selected randomly to observe the effect of salinity on growth parameters viz., root length (RL), shoot length (SL), shoot to root ratio (S-RR), fresh weight (FW), dry weight (DW), relative water content (RWC) vigour index (VI) and salt tolerance index (STI). The Vigour index was recorded as the product of total seedling length and percent germination (Baki and Anderson 1973).

Root and shoot anatomy

The root and shoot anatomy under control and salt stress (80 mM NaCl) was studied using protocol outlined by Krishnamurthy et al. (2014) with slight modifications. Transverse sections not more than 50 µm of stem (1-cm of the first internodes) and root (1-cm from the root tip) were cut with a razor blade and stained with 2% safranin stain. Five plants per replication for most tolerant and most sensitive genotypes were used and uniform sections were observed for comparison. Observations and photography were done under optical microscope Dewinter OPTIMA DIGI 530.

Root imaging and leaf area parameters

The most tolerant and most sensitive genotypes were selected, root imaging was done using EPSON SCANNER-EXPRESSION 11000 XL, WinRhizo for studying different root parameters and leaves were separated from shoots and leaf area was measured using leaf area meter (LI-COR, LI-3100).

Statistical analysis

The statistical analysis was done using PAST 4.0, and standardized morphological data were subjected to cluster analysis using the Euclidian distance coefficient and the unweighted pair group technique with arithmetic mean (UPGMA).

Results

The results recorded at the germination and seedling stages under different salinity stress are presented below.

Germination and seedling stages

After 15 days of salt stress, the germination percentage (Supplementary Table S1 and Fig. 1a) and type of seedlings (normal/abnormal) emergence were studied in all three stress conditions and compared with the control. It was observed that percentage of normal seedlings germinated under salt stress was higher at 60mM (86.48 ± 2.40% normal seedlings) and 80mM (82.03 ± 2.12% normal seedlings), whereas at 100mM, the reduction in normal seedling was (54.89 ± 3.51%) was noticed (Table 1). The variation in normal seedlings was obviously due to salinity stress. The differential response to salt stress among the genotypes was observed at 80mM and 100mM of salinity stress, which is depicted in Fig. 1a. After fifteen days of salt stress, the seedling survival under three salinity conditions was also observed (Supplementary Table S2). The higher the salinity, lesser the seedling growth was observed. This differential behaviour of all the genotypes at different salinity conditions is depicted in Fig. 1b.

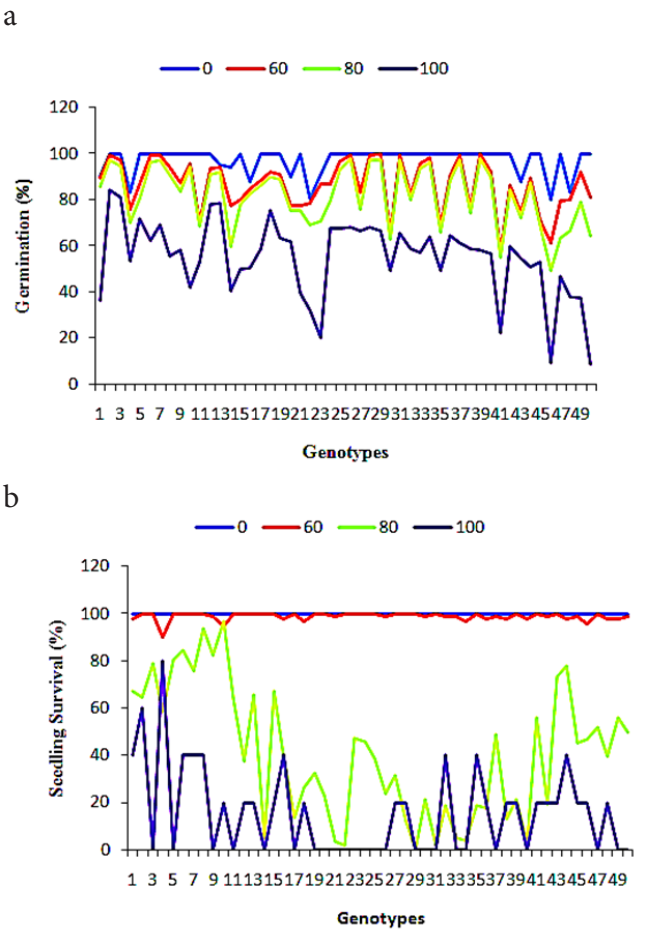


Fig. 1. Differential response of genotypes at different salinity stress levels at the germination stage (a) and seedling stage (b)

Table 1. Per cent normal and abnormal seedlings germinated at different salinity levels

Salinity level (mM)	Germination (%)	
	Normal	Abnormal
0	97.27 ± 0.86	0
60	86.48 ± 2.40	0
80	82.03 ± 2.12	0.4
100	54.89 ± 3.51	46.87 ± 2.56

ANOVA for genotypic response against salt stress at the germination and seedling stages

A comparison of variance components of the G (genotype), S (stress) and G x S for both the germination and seedling survivability showed their contribution to the total variance. Variance component for S was the largest for both the stages under study. The G was found to be significant, suggesting that the genotypes invariably responded differently among each other. There was a significant contribution of G x

S interaction variance on phenotypic expression for the traits under study, indicating an interaction effect of the environment in relation to genotypic performance (Table 2).

Table 2. Analysis of variance indicating the response of genotypes to salt stress and their interaction for germination percentage and seedling survivability

Source of Variation	DF	Germination	Seedling survivability
Salt Stress (S)	3	48,725.52***	#######***
Genotypes (G)	49	1,213.24***	1,093.90***
SXG	147	203.473***	763.21***
Error	398	8.124	0.234***

'***' 0.001(Highly significant), '**'0.01(Highly significant), '*'0.05 (Significant)

At 80mM and 100 mM of salt stress, significant difference was observed among the genotypes at both the germination and seedling stages. At 60mM, almost all the genotypes could bear the salinity stress at both stages. Therefore, it is presumed that it could not be the threshold value of salinity stress to differentiate among the genotypes. The increase in salinity stress from 80 to 100 mM significantly changed the phenotypic difference. The germination percentage reduced from 82.03% under 80mM concentration to 54.89% under 100 mM. Similarly, the seedling survivability also reduced drastically from 40.94 to 15.60% on increasing the salt stress from 80 to 100 mM. Hence, 100 mM was considered as a critical level beyond which the plant could not withstand the stress.

Morphological characterization from germination to seedling stage

Since there was no significant difference observed among the genotypes under control and 60mM salt stress, the detailed study considered other traits such as, germination percentage, shoot length, root length, fresh weight, dry weight, shoot: root ratio, vigour index, germination rate, relative water content and salt tolerance index (STI) was conducted at 80mM and 100mM concentrations for effective assessment of salt tolerance level. The descriptive statistics of all the genotypes at two salinity regimes is depicted along with control condition in Table 3.

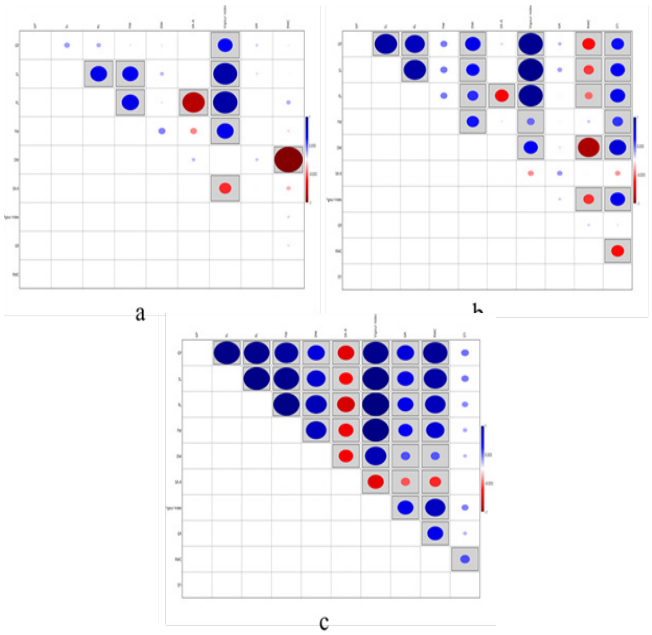


Fig. 2. Correlation among the traits at 0 mM(a); 80 mM (b) & 100 mM (c) *p > 0.05: Crossed/ blank, p < 0.05: boxed blue, Red (-1) to Blue (+1). The higher intensity of the colour reflects the higher value

Table 3. Descriptive statistics of 50 genotypes at two salinity regimes along with control

Trait	GP			SL			RL			FW			DW		
Salt conc, (mM)	0	80	100	0	80	100	0	80	100	0	80	100	0	80	100
Min	80.00	49.33	8.83	15.40	2.30	1.50	8.00	2.30	0.56	0.21	0.10	0.02	0.03	0.01	0.01
Max	100.00	98.00	84.17	28.94	13.50	9.46	21.36	13.50	5.80	0.48	0.17	0.31	0.41	0.03	0.02
Mean	97.27	82.03	54.89	22.87	9.24	6.06	15.11	9.24	2.99	0.33	0.13	0.12	0.05	0.02	0.01
SE	0.81	1.85	2.37	0.42	0.33	0.25	0.46	0.33	0.17	0.01	0.00	0.01	0.01	0.00	0.00
S. D.	5.70	13.08	16.73	2.94	2.35	1.79	3.28	2.35	1.18	0.07	0.02	0.08	0.06	0.00	0.00
CV	5.86	15.94	30.49	12.86	25.40	29.61	21.71	25.40	39.61	20.04	16.31	65.33	117.53	26.52	9.14
Trait	S-RR			VI			GR			RWC			STI		
Salt conc, (mM)	0	80	100	0	80	100	0	80	100	0	80	100	0	80	100
Min	1.13	1.37	1.54	2410.83	175.13	22.80	25.80	21.27	8.50	2.52	76.83	26.36	-	3.29	2.96
Max	3.10	3.09	3.75	5030.00	2087.40	1284.38	40.71	29.36	23.00	92.13	92.82	95.16	-	77.94	52.31
Mean	1.57	2.02	2.14	3699.67	1183.61	543.22	30.18	26.18	15.18	84.31	85.68	83.82	-	44.70	27.99
SE	0.05	0.05	0.06	87.32	64.89	42.08	0.32	0.27	0.47	2.23	0.44	2.20	-	2.11	1.09
S.D.	0.36	0.34	0.43	617.46	458.85	297.58	2.27	1.92	3.35	15.77	3.13	15.52	-	14.91	7.68
CV	23.03	16.64	20.20	16.69	38.77	54.78	7.52	7.33	22.09	18.71	3.66	18.52	-	33.35	27.42

SE = Standard error, SD = Standard deviation, CV = Coefficiant of variance, GP= Germination percentage, SL= Shoot length, RL= Root length, FW= Fresh weight, DW = Dry weight, S-RR = Shoot to root ratio, RWC = Relative water content, VI = Vigour index, STI = Salt tolerance index, Min. = Minimum and Max. = Maximum

Table 4. Classification of genotypes based on their response to salt stress at the seedling and germination stage

S.No.	Genotype	Germination Stage	Seedling stage	SR score	S. no.	Genotype	Germination stage	Seedling stage	SR score
1	AKTM 16-41	Tolerant	Tolerant	4.67	26	AKTM-16-33	Tolerant	Sensitive	2.17
2	AKTE 16-09	Tolerant	Tolerant	4.93	27	AKTE-12-02	Sensitive	Sensitive	2.17
3	JKM-7	Tolerant	Tolerant	4.50	28	AKTM-16-34	Tolerant	Sensitive	3.17
4	BDN-1	Sensitive	Sensitive	3.43	29	AKTE-11-02	Tolerant	Sensitive	3.87
5	TV-1	Tolerant	Tolerant	4.67	30	AKTE-16-03	Sensitive	Sensitive	3.33
6	BDN-716	Tolerant	Tolerant	1.33	31	C-11	Tolerant	Sensitive	4.50
7	PT 0607-5-1	Tolerant	Tolerant	1.67	32	AKTM-12-34	Tolerant	Sensitive	4.83
8	JKM-189	Tolerant	Tolerant	1.33	33	PT-0723-1-2-3	Tolerant	Sensitive	3.90
9	Phule Rajeshwary	Tolerant	Tolerant	2.00	34	BSMR-736	Tolerant	Sensitive	4.50
10	BDN-708	Tolerant	Tolerant	1.33	35	PT-07-04-1-1	Sensitive	Sensitive	4.50
11	AKTM-11-19	Sensitive	Tolerant	1.00	36	BSMR-853	Tolerant	Sensitive	4.33
12	AKTE-16-07	Tolerant	Sensitive	1.33	37	AKTE-16-10	Tolerant	Sensitive	4.33
13	BDN-711	Tolerant	Tolerant	1.33	38	AKTE-16-05	Sensitive	Sensitive	4.33
14	BDN-2013-5	Sensitive	Sensitive	2.00	39	AKT-8811	Tolerant	Sensitive	2.27
15	AKTE-16-05	Sensitive	Tolerant	3.17	40	BDN-2013-41	Tolerant	Sensitive	4.33
16	PKV TARA	Tolerant	Sensitive	1.67	41	BDN-2011-1	Sensitive	Sensitive	3.73
17	AKTM-10-14	Tolerant	Sensitive	4.40	42	AKTE-16-08	Tolerant	Sensitive	4.77
18	AKTM-16-35	Tolerant	Sensitive	1.33	43	PT-0704-1-2	Sensitive	Tolerant	2.17
19	BDN-2014-2	Tolerant	Sensitive	3.17	44	AKTE-12-04	Tolerant	Tolerant	3.97
20	BDN-2013-2	Sensitive	Sensitive	4.30	45	BDN-2013-1	Sensitive	Sensitive	1.33
21	AKTE-16-11	Sensitive	Sensitive	3.93	46	BDN-2	Sensitive	Sensitive	1.00
22	VIPULA	Sensitive	Sensitive	3.33	47	BDN-2008-7	Sensitive	Sensitive	2.43
23	AKTE-16-01	Sensitive	Sensitive	3.67	48	BDN-2010	Sensitive	Sensitive	2.17
24	AKTM-10-16	Sensitive	Sensitive	4.93	49	BDN-2008-1	Sensitive	Sensitive	2.17
25	BDN-2004-3	Tolerant	Sensitive	4.50	50	ICPL 88039	Sensitive	Sensitive	3.17

Correlations

The trait association at all three stress levels (0, 80, and 100 mM) was analyzed to understand the change in the relationship of traits due to salinity stress (Fig. 2a, b and c). Since pigeonpea is directly sown in the field, proper germination and seedling establishment are important for crop stand. Therefore, among all the parameters, germination is the most essential and primary trait to understand the behaviour of genotypes towards salinity stress at the initial stage. The GP showed a highly significant positive correlation with FW (0.67), DW (0.85), SR-R (0.93) and RWC (0.92) at 0mM salinity stress, whereas at 80mM stress, it was also found to be positively significantly associated with SL (0.85), RL (0.75) and vigour index (0.93). At 100mM of salinity stress, germination was positively associated with all the measured traits. The shoot length was positively correlated with SR-R (0.98) and RWC (0.82) at 0 mM, whereas at 80 mM, SL showed a positive association with RL (0.84), DW (0.50) and vigour index (0.95) but at 100 mM, all the traits were found to be associated. The root length exhibited significantly positive association with DW (0.67) at 0mM; vigour index (0.92) and RWC (0.56) at 80 mM. At 100 mM of salinity stress, the root length significantly positively correlated with FW (0.96) and DW (0.78), whereas it was negatively correlated with SR-R (0.65). The germination rate was strongly and positively associated with FW (0.95) and vigour index (0.94) under control conditions. No significant relationship of germination rate with any of the measured traits was found at 80mM, whereas at 100mM of salinity stress, the germination rate was significantly positively correlated with GP (0.64), SL (0.61), FW (0.55) and vigour index (0.58). The vigour index also showed a significant positive correlation with DW at 0mM (0.99) and 100mM (0.78). The salt tolerance index derived from biomass under control and stress conditions highly significantly positively correlated with RL (0.56) and vigour index (0.56) at 80mM of salinity stress.

Table 5. Principal component analysis (PCA) at different salt stress levels

Traits	PC	0 mM		80 mM		100 mM	
		Eigenvalue	%variance	Eigenvalue	%variance	Eigenvalue	%variance
GP	1	3.3532	37.258	4.93419	49.342	6.89298	68.93
SL	2	2.10941	23.438	1.41494	14.149	1.1048	11.048
RL	3	1.19039	13.227	1.28785	12.879	0.783644	7.8364
FW	4	1.09223	12.136	1.09306	10.931	0.563181	5.6318
DW	5	0.795278	8.8364	0.711742	7.1174	0.473049	4.7305
SR-R	6	0.431505	4.7945	0.386473	3.8647	0.140051	1.4005
VI	7	0.020106	0.2234	0.14543	1.4543	0.023095	0.23095
GR	8	0.007157	0.079519	0.012815	0.12815	0.011531	0.11531
RWC	9	0.00073	0.008109	0.008748	0.087482	0.005745	0.057447

GP= Germination percentage, SL= Shoot length, RL= Root length, FW= Fresh weight, DW= Dry weight, S-RR= Shoot to root ratio, RWC= Relative water content, VI= Vigour index and STI= Salt tolerance index.

Table 6. Shifts in the position of tolerant genotypes in clusters

S.No.	Tolerant genotypes	Cluster		
		0	80	100
1	AKTM 16-41	II	II	I
2	AKTE 16-09	II	II	II
3	JKM-7	II	II	II
4	TV-1	II	I	II
5	BDN-716	II	II	I
6	PT 0607-5-1	II	II	II
7	JKM-189	II	II	I
8	Phule Rajeshwary	II	II	I
9	BDN-708	II	II	I
10	BDN-711	I	II	II
11	AKTE-12-04	I	I	II

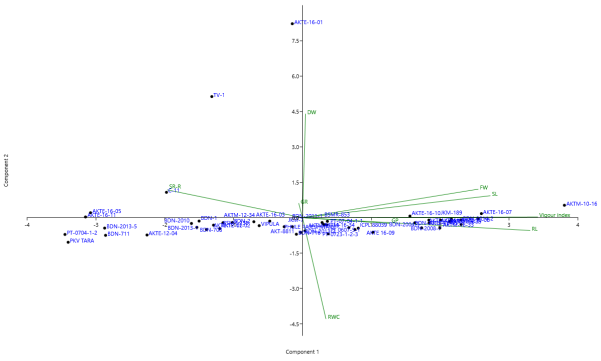


Fig. 3a: PCA biplot at 0mM of salt

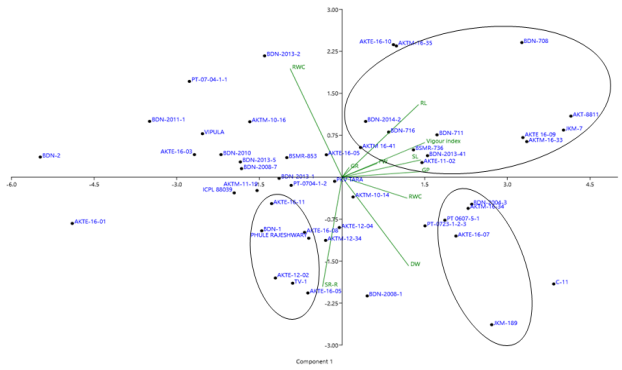


Fig. 3b. PCA biplot at 80 mM of salt

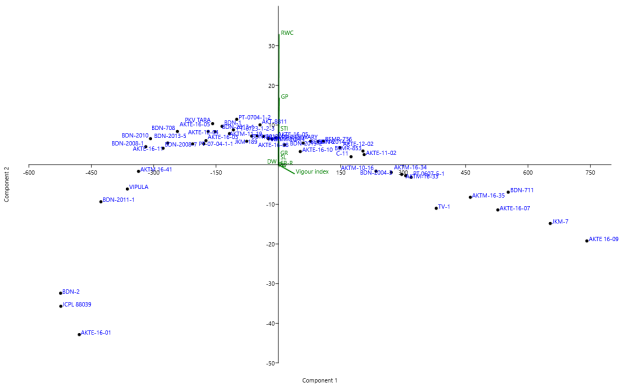


Fig. 3c. PCA biplot at 100mM of salt

Identification of tolerance and critical limit of salinity stress for pigeonpea

At 80 and 100 mM of salt stress, significant difference was observed among the genotypes at both the germination and seedling stages. At 60 mM, almost all the genotypes could bear the salinity stress at both stages. Therefore, it could not be the threshold value of salinity stress to differentiate among the genotypes. The increase in salinity stress from 80 to 100 mM significantly changed the phenotypic difference. The germination percentage reduced from 82.03% (80 mM) to 54.89% (100 mM). Similarly, the seedling survivability also reduced drastically from 40.94 to 15.60% on increasing the salt stress from 80 to 100 mM.

The correlation study among the traits under different

Table 7. Performance of tolerant (BDN 708) and sensitive (BSMR 736) genotypes

Traits	BDN 708		Per cent change	BSMR 736		Per cent change
	Control	Treated		Control	Treated	
Root						
Length (cm)	370.6976	225.2501	-39.2362	333.9898	205.0948	-38.5925
Projected area (cm2)	15.41286	9.62056	-37.581	14.1165	8.51302	-39.6945
Surface area (cm2)	48.42096	30.22388	-37.581	44.34833	26.74442	-39.6946
Average diameter (mm)	0.41508	0.43082	3.79204	0.420867	0.4167	-0.99002
Root volume (cm3)	0.5038	0.3234	-35.8079	0.470667	0.2776	-41.0198
Tips	290.6	382.2	31.52099	358	512.2	43.07263
Shoot						
Length (cm)	24.8	21.1	-14.92	22	18.6	-15.45
Leaf area	104.03	66.59	-35.98	74.4	27.55	-62.96
Leaf area ratio	75.38	61.66		31.93	53.39	

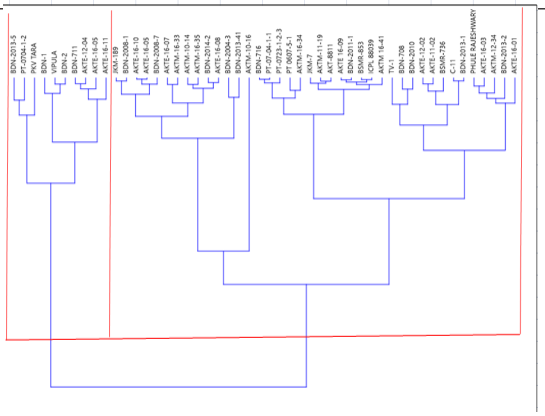


Fig. 4a. UPGMA clustering of genotypes at 0mM NaCl

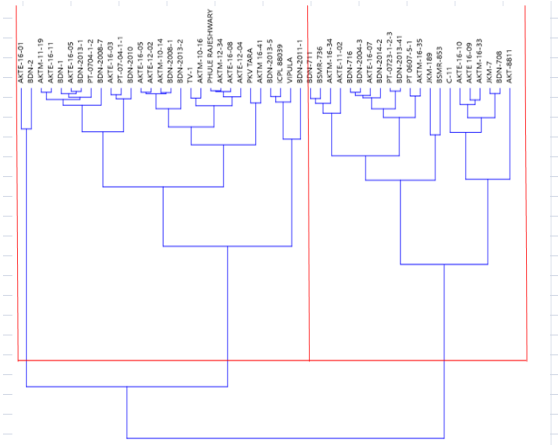


Fig. 4b. UPGMA clustering of genotypes at 80 mM NaCl

stresses also explained the critical changes in phenotypes due to higher stress. From 0 to 80 mM, the correlation among the traits was observed and the relation also changed with the stress level change because of different traits contributing to different levels of salinity tolerance and due to genetic constitution of genotypes. However, at 100 mM, almost all the traits showed association among each other

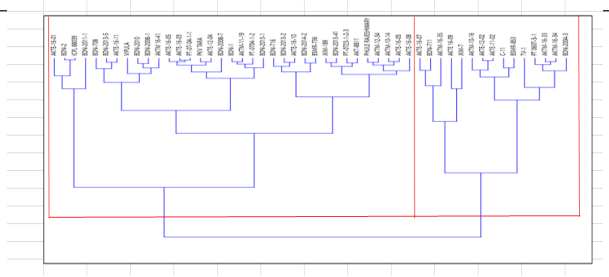


Fig. 4c. UPGMA clustering of genotypes at 100mM NaCl

(Fig. 2c). Thus, at 100 mM, the correlation analysis failed to catch the efficiently associated traits, due to higher level of stress changes the plant performance so adversely that all the traits of plants intended to contribute for plants survivability.

Therefore, upto 80 mM of salinity was considered as the tolerance limit for the pigeonpea genotypes and >80 mM was the critical limit. The salinity stress level ≥100 mM is highly critical, and the genotypic response was abrupt. The differential response of genotypes at salinity tolerance limit (80 mM) at the germination and seedling stage is depicted in Table 4.

Principal Component Analysis

All the 10 traits studied during salt stress from germination to 21 days old seedlings were subjected to Principal Component Analysis (PCA), where salt tolerance index was kept as a dependent variable. PCA was performed for all the traits to investigate the relationships among the traits with respect to salt tolerance index and the factors affecting variation in salt tolerance index. The principal components more than 1 eigenvalue were only considered for further interpretation. Under control condition (0mM NaCl), the PCA explained four components with >1 eigen value viz., PCA1:37.26%, PCA2:23.44%, PCA3:13.23% and PCA4:12.14% with a cumulative phenotypic variance of 86.06% which is at higher side. Thus, the important traits to differentiate among the genotypes at normal control conditions were

germination percentage (GP), shoot length (SL), root length (RL) and fresh weight (FW). At 80 mM of salt stress, 87.30% of phenotypic variance was explained by four components (PCA1:49.34, PCA2:14.15. PCA3:12.88, PCA4:10.93) and the effective traits to identify the salt tolerant genotypes were found to be germination percentage (GP), shoot length (SL), root length (RL), and fresh weight (FW). At 100mM of salt stress, only two factors (PCA1:68.93, PCA2:11.048) explained 79.98% of variation and the traits germination percentage (GP) and shoot length (SL) had higher PCA value. The PC components responsive to salt stress under three conditions are compared in Table 5. It was observed that under normal conditions and 80mM of salt stress, four component were contributing towards major phenotypic variance and the important traits were germination percentage (GP), shoot length (SL), root length (RL) and fresh weight (FW). But, at 100mM of salt stress, the response of the traits towards stress changed. Only two traits, *viz*., germination percentage (GP) and shoot length (SL) were found to be highly responsive towards salt stress, whereas the performance of root length (RL) and fresh weight (FW) was drastically reduced. Thus, it can be established that germination percentage (GP) and shoot length (SL) are the most effective traits for differentiating a wide range of genotypes under salinity stress due to consistency in contribution of traits at all level of stress including control.

PCA biplot analysis

The PCA biplot analysis explains the arrangement of all the genotypes on the basis of their potential to tolerate salt stress. Under control conditions, all the genotypes were found to be concentrated near the axis and were distributed in all the quadrants (Fig. 3a). At 80 mM of salt stress, all genotypes dispersed in all four quadrants and arranged distantly from each other, depicting their differential behaviour under salt stress. The tolerant genotypes selected in both germination and seedling stage majorly falls in first quadrant. In contrast, a few genotypes also lie in third and fourth quadrant (Fig. 3b). At 100mM of salt stress, the response of the genotypes is quite different (Fig. 3c). The performance of the genotypes reduced drastically at high salt stress conditions and most of the genotypes lie in second and first quadrants and a few genotypes were found to be scattered in third and fourth quadrants.

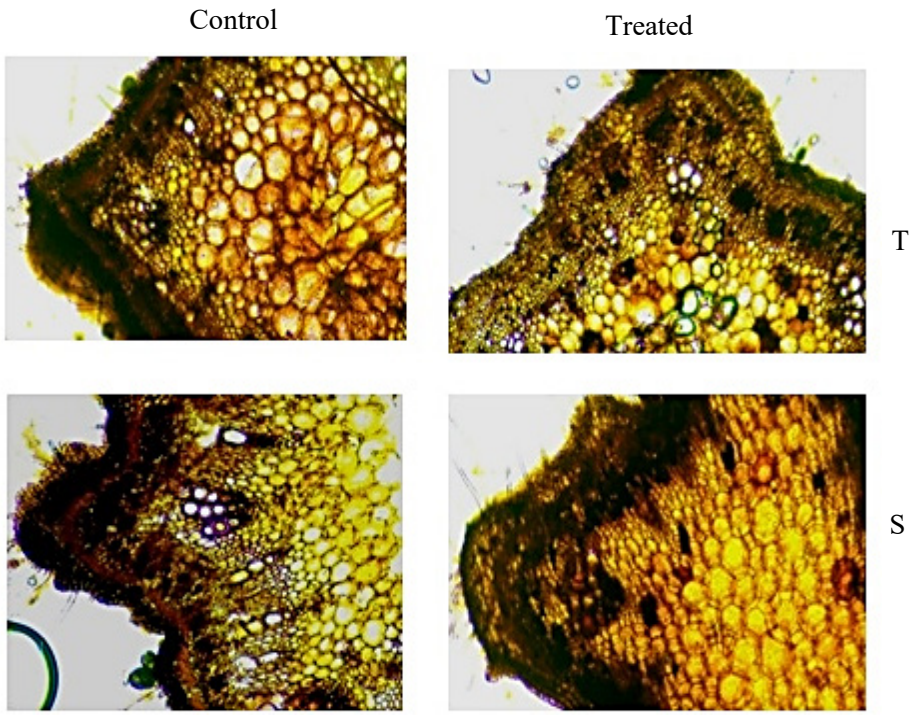
UPGMA clustering

The unweighted pair group method with arithmetic mean (UPGMA) is a simple agglomerative (bottom-up) hierarchical clustering method and is popularly used for classifying sample units based on their pairwise similarities in the traits studied. The present study grouped the most similar genotypes based on their response (mean value) to salt stress. The UPGMA clustering of genotypes at 0, 80 and 100 mM NaCl is depicted in Fig. 4a, b and c, respectively. Under control condition (0 mM NaCl), all the genotypes were arranged in two main clusters. The genotypes falling in cluster I at 0mM were also found in cluster I at 80mM salt stress except BDN-711. It shifted from cluster I to cluster II at both 80 and 100 mM salt concentration. Similarly, AKTE-12-04 was falling in cluster I at 0 and 80 mM NaCl, but it shifted to cluster II in case of 100 mM NaCl. AKTM 10-16 and TV-1 fall in cluster I at 80 mM stress and cluster II in case of control and 100mM of salt stress. Thus, there occurs a reshuffling of genotypes in response to salt stress. This was also indicated in the biplot arrangement of genotypes. The genotypes identified salt tolerance at both stages based on different morphological parameters exhibited shifts in position in UPGMA clusters, as depicted in Table 6 and Fig.4a, b and c.

On studying multiple stress levels at germination and seedling stages, it was found that the performance of all the genotypes reduced drastically at salt stress of >80 mM (Fig. 3c) and most of the genotypes shifted to cluster I depicting the sensitive group. Therefore, 80mM is the threshold value at which the evaluation of genotypes could be done effectively and they can be classified into tolerant and sensitive groups in response to salt stress. Based on morphological parameters and multiple statistical analysis, BDN-708, AKTM 16-41, AKTE 16-09, JKM-7, TV-1, BDN-716, PT 0607-5-1, JKM-189, Phule Rajeshwary, BDN-711, AKTE-12-04 were found to be tolerant whereas BDN-1, AKTE-12-02, AKTE-16-03, PT-07-04-1-1, AKTE-16-05, BDN-2013-5, BDN-2011-1, BDN-2013-2, BDN-2013-1, AKTE-16-11, BDN-2, BDN-2008-7, VIPULA, BDN-2010, BDN-2008-1, AKTE-16-01, AKTM-10-16, ICPL 88039 were classified as sensitive at both the stages. Many genotypes are tolerant to salt stress at the germination stage but exhibit sensitive behaviour at seedling stage. Thus, it can be concluded that the seedling stage was a more sensitive and crucial stage for assessing many genotypes in pigeonpea against salt stress.

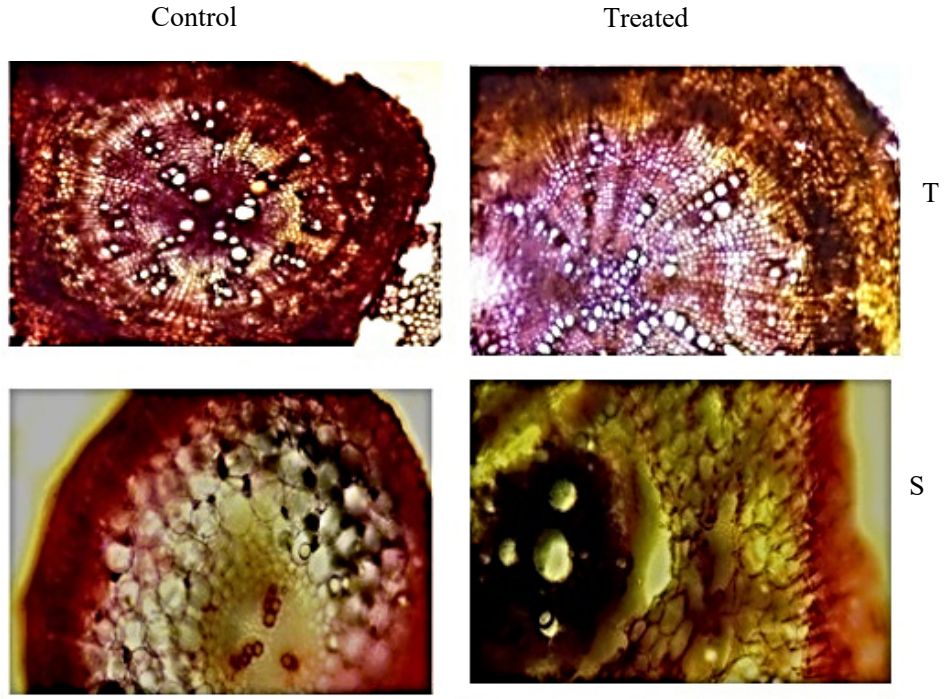
Anatomical studies of root and shoot under salt stress

Salt stress affects not only the overall morphology of the plant but also the anatomy of the plant. Therefore, to examine the effects of salinity stress on pigeonpea, anatomical features were studied at cellular level through dissection of tolerant (BDN-708) and sensitive (BSMR-736) genotypes under 80mM NaCl stress at seedling stage. The changes in vascular bundles and stellar regions due to salt stress were noticed in stem and root sections under optical microscope. The stem and root tissues are altered to make adaptive changes in plants under saline conditions. In case of tolerant genotype (BDN-708) the epidermal cells of stem were larger, well organized and a deeply stained vascular system was observed under salt stress. However, the susceptible genotype (BSMR 736) depicted smaller, shrunken epidermal cells and lightly stained vascular bundles under salt stress. The epidermal rupturing and cortical cell enlargement with rupturing at certain regions was found in sensitive genotype.



T= BDN 708 (salt tolerant), S= BSMR736 (salt sensitive)

Fig. 5a. Anatomical dissection of shoot depicting changes in stellar regions and vascular bundles under salt stress (80mM NaCl)



T- BDN 708 (Tolerant), S- BSMR736 (Sensitive)

Fig. 5b. Anatomical dissection of root depicting changes in stellar regions and vascular bundles under salt stress (80mM NaCl)

In contrast, epidermal layer and cortical bundles were intact. They showed no damage in tolerant genotype (Fig. 5a). Root of tolerant genotype (BDN-708) had thicker epidermis along with three layers of sclerenchymatous cortex and prominent endodermis and pericycle layer in comparison to the sensitive genotype (BSMR 736).

Depositions were found in patches within many layers towards the stele along with cortical cell rupturing at certain regions in sensitive genotype. Shrunken stele area and distorted phloem vessels were observed in sensitive genotype whereas fewer changes were noticed in stellar region of tolerant genotype than control (Fig. 5b).

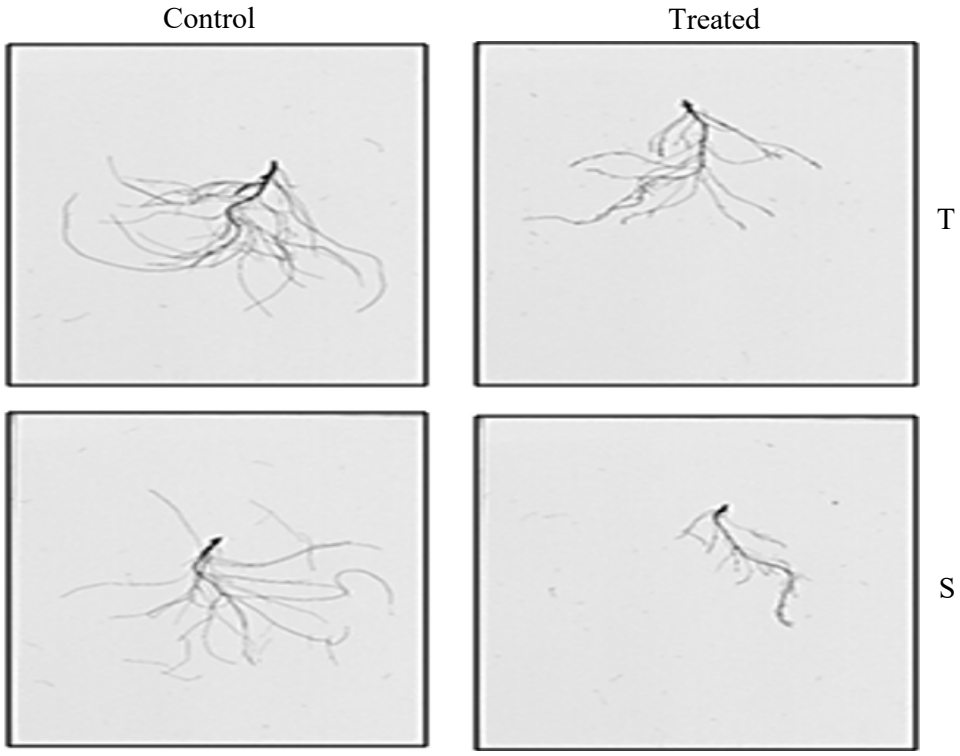
Root imaging and leaf area

The root parameters and leaf area of the tolerant (BDN-708) and sensitive (BSMR-736) genotypes were studied in detail using root scanner and leaf area meter, respectively, to have a clear picture on the morphological changes under salt stress (Table 7). Percent reduction in root length under salinity stress is higher (-39.25) in BDN-708 than in the salt-sensitive genotype (-38.59) in BSMR 736. The reduction in other root parameters viz. projected area, surface area and root volume is higher in BSMR 736 (-39.69, -39.69 and -41.02, respectively as compared to BDN- 708 (-37.58, -37.58 and -35.81, respectively) (Fig. 6). A higher percentage of reduction was recorded in leaf area (-62.96) in BSMR-736 under salt as compared to the salt-tolerant genotype BDN-708. It indicated that the plant's overall performance reduces under salinity conditions and the salt-sensitive genotypes are highly affected by salinity stress. The reduction in root and shoot biomass was higher in BSMR-736 compared to BDN-708.

Discussion

The plants have evolved complex mechanisms for responding to salt stress. However, different genotypes within the same species show different responses to the same stress due to their different genetic constitutions and their ability to tolerate salt stress varies widely among species and varieties (Ashraf and Wu 1994). The pigeonpea, due to its indeterminate growth habit, continues to grow vegetative even after flower initiation for a longer period thus rendering it sensitive to several environmental stresses (factors), such as drought, salinity, heat and cold etc., (Subbarao et al. 1999; Subbarao et al. 2000; Singh et al. 1997; Likoswe and Lawn 2008; Durgesh et al. 2019). These stresses in general have adverse effects on the overall growth and yield potential of crop plants. Salinity stress involves various changes in physiological and metabolic processes depending on the stage of stress, duration of stress, stage of plant growth and environmental conditions. Little has been reported in literature about the salinity stress tolerance in pigeonpea (Ahmed et al. 2016; Banerjee et al. 2018).

Higher germination percentage and seedling survivability is critical for a proper crop stand. Therefore, to explore the salt tolerance potential of pigeonpea, fifty genotypes were studied at different salinity regimes ranging from 0 to 100 mM NaCl concentration at the germination and seedling stage. Based on morphological parameters,



T- BDN 708 (Tolerant), S- BSMR736 (Sensitive)
Fig. 6. Root scan of tolerant (BDN 708) and sensitive (BSMR 736) genotypes under control and salt stress conditions

anatomical features studied under salt stress and multiple statistical analysis, the threshold level of salt stress and critical stage for screening the genotypes was identified along with the classification of genotypes under study into salt tolerant and salt sensitive classes.

The overall mean performance of all the studied genotypes was reduced under salt stress at both stages. The analysis of variance results indicated the presence of significant differences in their performance under salt stress. The differential responses of genotypes to salt stress have previously been reported in many pulse crops viz., chickpea, soybean, lentil, pea etc. (Noreen et al. 2007; Joshi et al. 2021). Li et al. (2020) observed that salinity affects not only seed germination but also seedling growth and development in plants; hence, the germination rate alone cannot accurately evaluate salt tolerance. Therefore, in the present study, the seedling traits were also considered to effectively evaluate genotypes under salt stress. The morphological and anatomical features of the plants under salt stress showed drastic changes in our experiment. The adverse effect of salt stress on root and shoot morphology and anatomy was also reported in lentil by Singh et al. 2017. The salt stress reduced germination percentage and seedling survivability in almost all the pigeonpea genotypes under study. Similar results were reported in mungbean (Mahajan and Tuteja 2005; Nirmala et al. 2013; Mahadavi and Sanavy 2007), pea (Shahid et al. 2012), cowpea (Murillo-Amador et al. 2000 and chickpea). The germination rate, vigour index, relative water content and salt tolerance index showed a differential response in different genotypes. This is attributed to genotypic variability among the pigeonpea genotypes. Shoot and root growth were also reduced by salt stress due to the inhibitory effect of salt on cell division and enlargement (Kaymakanova 2009).

The performance of all the genotypes reduced drastically at salt stress of >80mM (Fig. 3c). Most of the genotypes shifted to cluster I depicting the sensitive group and could not cope up with the stress. The study on the correlation among the traits under consideration revealed that the associations among the traits change as the salt stress is imposed and the stress level increases. Correlation among all the traits was significantly positive at 100mM NaCl due to low performance of all traits at such a higher salinity level. Therefore, it was concluded that 80 mM concentration may be the threshold value at which the classification of genotypes could be done appropriately into tolerant and sensitive groups in response to salt stress. The pronounced effect of salt stress at higher salt levels was also reported in lentil (Singh et al. 2017) and chickpea (Neeraj et al. 2016).

Salt stress damages the plants, leading to various morphological, physiological, biochemical, and anatomical changes. Out of various reasons ascribed to cause damage in the germination of seeds and seedling development, osmotically induced water deficit, specific ion toxicity on embryo viability of the seeds (Houle et al. 2001; Rahnama et al. 2010), homeostasis at cellular level or nutrient imbalance (Ma et al. 2012; Joshi et al. 2021) are the most important. Salt stress impairs seed germination, reduces rhizobium-induced root nodule formation, retards plant development and ultimately reduces crop yield (Greenway and Munns 1980). Genotypic variation for salt tolerance exists in pigeonpea germplasm. Selection for genotypes with tolerance to salt stress will ensure the greater establishment of seedlings in saline soils and better yield under salt stress. However, the lack of an accurate and reliable salt tolerance evaluation parameter is one of the major factors limiting the success rate of conventional breeding for salt tolerance (Zeng et al. 2002). To determine the salt tolerance in pigeonpea more efficiently at the germination and seedling stage, it was necessary to identify some reliable traits as indicators of salt tolerance at these stages. In this study, Principal Component Analysis was performed to identify the most reliable traits determining salt tolerance in pigeonpea. The germination percentage (GP) and shoot length (SL) were the most responsive traits toward salt stress. They could be considered reliable traits for evaluating the salt tolerance in pigeonpea.

However, defining the salt tolerance of one or several pigeonpea genotypes is difficult without comparing large number of genotypes. To evaluate the salt tolerance of pigeonpea genotypes easily and reliably, PCA biplot and UPGMA clustering were done. The genotypes were grouped into two major categories (salt tolerant and salt sensitive) based on their response to salt stress. Based on morphological parameters and multiple statistical analysis, BDN-708, AKTM 16-41, AKTE 16-09, JKM-7, TV-1, BDN-716, PT 0607-5-1, JKM-189, Phule Rajeshwary, BDN-711, AKTE-12-04 were found to be tolerant, whereas BDN-1, AKTE-12-02, AKTE-16-03, PT-07-04-1-1, AKTE-16-05, BDN-2013-5, BDN-2011-1, BDN-2013-2, BDN-2013-1, AKTE-16-11, BDN-2, BDN-2008-7, VIPULA, BDN-2010, BDN-2008-1, AKTE-16-01, AKTM-10-16, ICPL 88039 were classified as sensitive at both the stages of testing.

Several important parameters, such as seed germination, seedling survivability, biomass, leaf necrosis, death and senescence, ion concentrations, osmoregulatory mechanisms and plant growth etc., are usually used for evaluating salt stress response and identification of salt tolerant genotypes in pigeonpea. The present study applied salt stresses by using three different salt concentrations and identified a few pigeonpea genotypes as salt tolerant ones based on their higher seed germination percentage under 80mM NaCl concentration. However, such salt tolerant genotypes did not exhibit similar level of salt tolerance at the seedling stage testing. It reflected the variation in the effectiveness in the selection of salt tolerant genotype based on seed germination and seedling stage testing. Further,

it reflects the complexity of the trait and sensitivity of the testing processes. From this study, it was concluded that accuracy of the selection of salt tolerant pigeonpea is far higher in seedling stage selection than the seed germination stage selection. It may presumably be due to an extra layer of protection provided by seed coat against salt and regulation of water imbibation by the micropyle at the initial stage. Thus the screening at seedling stage can be effectively used in large-scale screening and breeding of salt-tolerant pigeonpea varieties.

Supplementary material

Supplementary Tables S1 and S2 are provided.

Authors' contribution

Conceptualization of research (RJ, KD); Designing of the experiments (RJ,KD, NR); Sharing of experimental materials (ANP, RSR and KD); Execution of field/lab experiments and data collection (RJ, KD, AG, AK,SS); Analysis of data and interpretation (RJ, RPS, KD); Preparation of manuscript (RJ, KD, AT).

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Supplementary Table S1. The data on seed germination under different salt concentrations

S.No.	Genotype	0mM		SD	60mM		SD	80mM		SD	100mM		SD
1	AKTM 16-41	90.33	±	3.06	89.67	±	1.53	86.00	±	2.65	36.50	±	0.87
2	AKTE 16-09	100.00	±	0.00	99.67	±	0.58	97.67	±	0.58	84.17	±	2.93
3	JKM-7	100.00	±	0.00	97.33	±	2.08	94.67	±	2.08	80.83	±	1.76
4	BDN-1	83.33	±	5.77	75.67	±	3.51	70.33	±	2.52	53.67	±	2.08
5	TV-1	100.00	±	0.00	86.67	±	3.21	80.67	±	3.06	71.50	±	6.14
6	BDN-716	100.00	±	0.00	99.33	±	1.15	96.67	±	0.58	62.50	±	2.78
7	PT 0607-5-1	100.00	±	0.00	99.67	±	0.58	97.33	±	1.15	69.17	±	2.75
8	JKM-189	100.00	±	0.00	94.00	±	1.00	91.00	±	1.00	55.50	±	3.28
9	PHULE RAJESHWARY	100.00	±	0.00	87.33	±	5.03	83.67	±	5.13	58.33	±	1.89
10	BDN-708	100.00	±	0.00	95.67	±	1.53	94.13	±	1.63	42.33	±	3.82
11	AKTM-11-19	100.00	±	0.00	70.00	±	7.81	68.67	±	1.53	53.17	±	3.40
12	AKTE-16-07	100.00	±	0.00	93.67	±	2.31	91.33	±	1.53	77.83	±	2.02
13	BDN-711	95.00	±	5.00	94.33	±	3.51	92.00	±	3.61	78.50	±	3.12
14	BDN-2013-5	94.33	±	4.51	77.33	±	5.03	60.00	±	2.65	40.50	±	3.28
15	AKTE-16-05	100.00	±	0.00	80.00	±	2.00	78.00	±	2.00	49.83	±	1.76
16	PKV TARA	87.67	±	2.52	85.00	±	3.00	83.00	±	3.00	50.63	±	4.12
17	AKTM-10-14	100.00	±	0.00	88.33	±	2.08	86.33	±	2.08	58.60	±	3.93
18	AKTM-16-35	100.00	±	0.00	92.00	±	2.00	90.00	±	2.00	75.50	±	4.09
19	BDN-2014-2	100.00	±	0.00	91.33	±	1.53	89.33	±	1.53	63.17	±	3.33
20	BDN-2013-2	90.00	±	5.00	77.67	±	0.58	75.67	±	0.58	61.83	±	4.75
21	AKTE-16-11	100.00	±	0.00	77.33	±	2.52	75.33	±	2.52	39.53	±	2.55
22	VIPULA	80.67	±	5.13	78.33	±	2.08	69.33	±	4.16	32.43	±	4.50
23	AKTE-16-01	91.00	±	3.00	86.67	±	2.52	70.67	±	4.16	20.50	±	3.28
24	AKTM-10-16	100.00	±	0.00	86.67	±	5.86	79.67	±	2.52	67.30	±	1.65
25	BDN-2004-3	100.00	±	0.00	97.00	±	1.00	93.33	±	1.53	67.50	±	1.80
26	AKTM-16-33	100.00	±	0.00	99.67	±	0.58	98.00	±	0.00	68.17	±	2.93
27	AKTE-12-02	100.00	±	0.00	83.33	±	4.04	76.17	±	0.76	66.30	±	3.84
28	AKTM-16-34	100.00	±	0.00	99.33	±	0.58	97.67	±	0.58	68.17	±	7.29
29	AKTE-11-02	100.00	±	0.00	100.00	±	0.00	97.67	±	0.58	66.50	±	4.09
30	AKTE-16-03	100.00	±	0.00	65.00	±	3.00	63.00	±	3.00	49.53	±	2.25
31	C-11	100.00	±	0.00	99.33	±	1.15	97.33	±	1.15	65.57	±	1.50
32	AKTM-12-34	100.00	±	0.00	82.33	±	1.53	80.33	±	1.53	58.50	±	3.12
33	PT-0723-1-2-3	100.00	±	0.00	95.67	±	1.53	93.67	±	1.53	57.33	±	1.53
34	BSMR-736	100.00	±	0.00	98.67	±	1.53	96.67	±	1.53	63.80	±	3.80
35	PT-07-04-1-1	100.00	±	0.00	68.33	±	3.51	66.33	±	3.51	49.50	±	5.50
36	BSMR-853	100.00	±	0.00	90.33	±	2.52	88.33	±	2.52	64.50	±	2.78
37	AKTE-16-10	100.00	±	0.00	99.67	±	0.58	97.67	±	0.58	61.50	±	5.50
38	AKTE-16-05	100.00	±	0.00	76.33	±	3.51	74.33	±	3.51	58.83	±	3.62
39	AKT-8811	100.00	±	0.00	100.00	±	0.00	98.00	±	0.00	58.17	±	6.60
40	BDN-2013-41	100.00	±	0.00	92.33	±	2.52	90.33	±	2.52	56.83	±	4.25
41	BDN-2011-1	100.00	±	0.00	57.00	±	2.65	55.00	±	2.65	22.17	±	4.31
42	AKTE-16-08	100.00	±	0.00	86.33	±	2.08	84.33	±	2.08	59.50	±	7.57
43	PT-0704-1-2	87.67	±	1.15	74.33	±	4.04	72.33	±	4.04	54.50	±	4.82

44	AKTE-12-04	100.00	±	0.00	89.33	±	1.15	87.33	±	1.15	50.83	±	4.54
45	BDN-2013-1	100.00	±	0.00	71.17	±	3.40	69.17	±	3.40	52.83	±	1.26
46	BDN-2	80.00	±	5.00	61.33	±	2.08	49.33	±	3.06	9.50	±	3.77
47	BDN-2008-7	100.00	±	0.00	79.67	±	3.06	63.67	±	4.04	46.83	±	4.31
48	BDN-2010	83.33	±	2.89	80.00	±	5.57	66.67	±	3.21	37.90	±	1.93
49	BDN-2008-1	100.00	±	0.00	92.33	±	2.08	79.00	±	1.73	37.17	±	4.75
50	ICPL 88039	100.00	±	0.00	81.33	±	3.51	64.33	±	1.53	8.83	±	3.75
Mean		97.27	±	0.86	86.48	±	2.40	82.03	±	2.12	54.89	±	3.51
CV		0.884732			2.780501			2.582905			6.395368		

Supplementary Table S2. The data on seedling survivability at different salt concentrations

S. No.	Genotype	0mM	60mM	80mM	100mM	Sr. No.	Genotype	0mM	60mM	80mM	100mM
1	AKTM 16-41	100	98	67	40	27	AKTE-12-02	100	100	31	20
2	AKTE 16-09	100	100	64	60	28	AKTM-16-34	100	100	12	20
3	JKM-7	100	100	79	0	29	AKTE-11-02	100	100	0	0
4	BDN-1	100	90	58	80	30	AKTE-16-03	100	99	21	0
5	TV-1	100	100	80	0	31	C-11	100	100	2	0
6	BDN-716	100	100	85	40	32	AKTM-12-34	100	99	19	40
7	PT 0607-5-1	100	100	76	40	33	PT-0723-1-2-3	100	99	5	0
8	JKM-189	100	100	94	40	34	BSMR-736	100	97	4	0
9	PHULE RAJESHWARY	100	99	82	0	35	PT-07-04-1-1	100	100	19	40
10	BDN-708	100	95	97	20	36	BSMR-853	100	98	18	20
11	AKTM-11-19	100	100	64	0	37	AKTE-16-10	100	99	49	0
12	AKTE-16-07	100	100	38	20	38	AKTE-16-05	100	98	13	20
13	BDN-711	100	100	66	20	39	AKT-8811	100	100	21	20
14	BDN-2013-5	100	100	5	0	40	BDN-2013-41	100	98	2	0
15	AKTE-16-05	100	100	67	20	41	BDN-2011-1	100	100	56	20
16	PKV TARA	100	98	39	40	42	AKTE-16-08	100	99	19	20
17	AKTM-10-14	100	100	14	0	43	PT-0704-1-2	100	100	73	20
18	AKTM-16-35	100	97	26	20	44	AKTE-12-04	100	98	78	40
19	BDN-2014-2	100	100	32	0	45	BDN-2013-1	100	99	45	20
20	BDN-2013-2	100	100	23	0	46	BDN-2	100	96	47	20
21	AKTE-16-11	100	99	3	0	47	BDN-2008-7	100	100	52	0
22	VIPULA	100	100	2	0	48	BDN-2010	100	98	39	20
23	AKTE-16-01	100	100	47	0	49	BDN-2008-1	100	98	56	0
24	AKTM-10-16	100	100	46	0	50	ICPL 88039	100	99	50	0
25	BDN-2004-3	100	100	39	0		Mean	100.00	98.98	40.94	15.60
26	AKTM-16-33	100	99	24	0						