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Full Length Article

Morphological Screening and *SalTol* Region Based SSR Markers Analysis of Rice (*Oryza sativa*) Genotypes for Salinity Tolerance at Seedling Stage

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Abstract

Rice production is greatly limited by high level of soil salinity around the world. To overcome this major abiotic constraint, different strategies have been adopted for the development of salt tolerant rice varieties. In present study, tolerance potential of 63 rice genotypes was evaluated at seedling stage under different levels of salt stress (0, 50, 100 and 150 m*M*). Among screened genotypes, six were found tolerant, 37 moderately tolerant and 20 were sensitive under tested levels of salt stress. Tolerant genotypes showed less reduction in root shoot length, fresh weight and dry weight, compared with FL478 (positive control). Sensitive genotypes showed ~90% reduction in all growth parameters at 150 m*M*. Selected genotypes, representing all three groups, were further assessed for salinity tolerance at molecular level using 21 simple sequence repeat (SSR) markers, residing within *SalTol* QTL region on chromosome 1 and 19 SSR markers were found polymorphic among salt tolerant and sensitive genotypes. Cluster analysis based on SSR markers, grouped genotypes into three clusters as sensitive, tolerant and moderately tolerant. However, population structure analysis combined tolerant and moderately tolerant genotypes in one set, as both had close genetic similarity in response to salinity and placed sensitive genotypes into separate group. Our results revealed that tolerant genotypes can be identified on the basis of growth parameters like root shoot length, root shoot fresh and dry weight at seedling stage under salt stress conditions. Moreover, molecular markers were able in differentiating the identified salt tolerant and sensitive genotypes. The identified salt tolerant genotypes might also serve as potential donors for the introgression salt tolerant genes/QTLs into high yielding, Basmati and non-Basmati rice varieties.

Keywords: Salt concentrations; Growth parameters; Genotyping; Standard evaluation system; UPGMA dendrogram

Introduction

World's climate change has greatly exposed rice production to a range of sever abiotic stresses such a salinity, high temperature, flood, drought and heavy metals (Fusi *et al.*, 2014). Soil salinity is one of the major stresses that adversely affect growth and yield of rice crop throughout the world (Kanawapee *et al.*, 2013). Globally, every day 2,000 hectares of irrigated land is being ruined by high levels of salt (Machado and Serralheiro, 2017). Saline soil and water adversely affect the osmotic potential of plant cells leading to reduced crop production (Yan *et al.*, 2015). High concentration of salt in soil hinders water absorption by plant roots (Munns and Tester, 2008), therefore, plant adopts different physiological and anatomical strategies to resist salt stress damage (Tu *et al.*, 2014).

To fulfill the dietary requirements of ever increasing population, a great challenge to geneticists and breeders is the development of salt resistant crops (Negrão *et al.*, 2017). Rice is a staple food and mostly cultivated in Asia. Rice is prone to different abiotic stresses such as salinity and drought. Current popular rice varieties threshold for salinity tolerance is 3 dS m^{-1}, and beyond this value 12% grain yield reduction was observed per dS m^{-1} (Babu *et al.*, 2014). The response of rice varieties to salt stress varies with different growth stages, *i.e.*, germination, seedling, vegetative and maturation stage. Rice is highly sensitive to salt stress at the seedling stage while moderately sensitive at vegetative stage and again highly sensitive at reproductive stage (Emon *et al.*, 2015). Typical symptoms of saline soil at seedling stage are the burning or whitening of the leaf tips, weak root growth, stunted plant growth and plant death in severe cases

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(Mohammadi *et al.,* 2014). The effects of salt stress on plants are very complex and greatly influenced by environmental factors such as humidity and temperature. Humid regions in Asia are suitable for rice cultivation, but overall productivity is low due to the saline soils (Zeng *et al.,* 2002). The impact of salinity on rice cultivars depends upon the concentration of ions, exposure time, growth stage, variety, physical properties of soil and temperature. Therefore, it is important to develop salt tolerant rice varieties not only to increase the yields but also to remediate the saline soil (Aref and Rad, 2012).

There are some cultivars which have natural tolerance to salinity because of their adaptation to flourish on high salt affected area for generations. However, these cultivars have poor agronomics characteristics (Waziri *et al.,* 2016). Many cultivars like Pokkali, NonaBokra and Hawasi have great potential to survive under high level of soil salinity and therefore used as salt tolerant gene donors (Negrão *et al.,* 2011). Marker assisted backcrossing (MABC) method is useful for introgression of a target region (gene/QTL) from salt tolerant donor to recipient parent by employing tightly linked molecular markers (Hoang *et al.,* 2016). Molecular markers are extensively used for the analysis of genetic diversity and crop improvement (Meti *et al.,* 2013). DNA markers can be utilized for specific chromosome region or the entire genome. Due to their better reproducibility, efficiency, high polymorphism and co-dominant behavior, the SSR markers are extensively used for genetic regions associated with salt tolerance (Islam *et al.,* 2012a). They are widely used for genome wide association mapping, QTL mapping, analysis of mutations and marker assisted selection in rice (Shah *et al.,* 2013). In previous studies several QTLs for salt tolerance have been mapped on rice chromosomes and one major QTL, *SalTol*, mapped on chromosome 1. A gene present in *SalTol* region was isolated by cloning (Ren *et al.,* 2005) and found to be associated with high K^+ uptake and low Na^+ absorption and resulting in low Na/K ratio under salt stress (De Leon *et al.,* 2015).

Green super rice (GSR) is a mega project initiated by Chinese Academy of Agricultural Sciences (CAAS) China, in close collaboration with International Rice Research Institute (IRRI) in 2008. Present study was carried out to evaluate salt tolerance potential of 63 rice genotypes including 55 lines from GSR project, 4 from IRRI and 4 from Pakistan (basmati & non-basmati) under different salinity levels at the seedling stage. The salt tolerant lines were further identified using molecular markers.

Materials and Methods

Experimental Details and Treatments

Plant material: The plant material consisted of 63 rice genotypes, including 55 genotypes from IRRI bred green super rice (GSR), four coarse lines (also received from IRRI) and four genotypes from Pakistan (two basmati and two non-basmati) (Table 1). The seeds were sterilized with fungicide for 8 h. The seeds were pre-germinated in wet filter paper for two days at 36°C. The pre-germinated 15 seeds from each genotypes were transferred into Petri plates (90 × 15 mm) containing distilled water for two days. The experiment was conducted in DNA Markers and Applied Genomics lab at NIBGE, Faisalabad, Pakistan.

Treatments: Four NaCl salinity levels of i.e. 0, 50, 100 150 m*M* were used and saline solution was changed daily. The experiment was conducted in complete randomized design (CRD) with three replications at 28±2ºC temperature. Phenotypic response of different growth parameters was recorded for all treatments including experimental controls (0 m*M* NaCl) after 12^{th} day of stress. The entire experimental setup, comprised of 252 petri plates for all treatments in each replication, was repeated for the confirmation of recorded observations.

Measurement of Plant Biomass Production

Data regarding different growth parameters such as root and shoot length, fresh and dry weight were recoded. Changes in root and shoot length in response to salt stress were measured after 5^{th} and 12^{th} day of salinization, while shoot and root fresh weights were measured after the 12^{th} day of salt stress. Roots and shoots were placed separately in paper bags and dried at 70ºC for 48 h, and then their dry weight was measured using analytical weighing balance.

Scoring of Rice Leaves at Seedling Stage

After 12 days of salt stress, modified Standard Evaluation System (SES) Score (Table 2) was used to evaluate symptoms on leaves under different levels of salt stress. This scoring discriminated the tolerant, moderately tolerant and sensitive genotypes according to their ability to tolerate salt stress (Gregorio *et al.,* 1997). The highly tolerant (HT) genotypes scored "1" if showed no leaf damage, tolerant (T) genotypes scored "3" if it showed minor to no leaf damage, moderately tolerant (MT) scored "5" if rolled leaf having whitish tips, sensitive (S) "7" if leaves showed symptoms from yellowish to dried and highly sensitive (HS) "9" if showed whole plant dead.

Marker Genotyping

On the basis of SES scoring, 34 rice genotypes (highly tolerant, moderately tolerant and sensitive) including IR-29 (sensitive check) and Pokkali (tolerant check) were selected for genotyping. Genomic DNA of the selected rice genotypes was extracted from fresh leaves using CTAB method with minor modifications (Cota-Sánchez *et al.,* 2006). Twenty one SSR markers were used to analyze the genetic diversity and association with salt tolerance and sensitive genotypes. These SSR markers belong to *SalTol* region of rice genome (Thomson *et al*., 2010). Polymerase

Table 1: Visual performance of 63 rice genotypes under salt stress

Genotypes	Source	SES scoring	Tolerance
HHZ5-DT1-DT1	IRRI	5	MT
HHZ5-DT7-Y3-SAL1	IRRI	5	MT
HHZ5-DT20-DT2-DT1	IRRI	5	MT
HHZ5-DT20-DT3-Y2	IRRI	5	MT
HHZ5-SAL8-DT2-SAL1	IRRI	3	T
HHZ5-SAL10-DT3-Y1	IRRI	5	MT
HHZ5-SAL12-DT3-Y2	IRRI	5	MT
HHZ5-SAL14-SAL2-Y1	IRRI	5	MT
HHZ5-SAL14-SAL2-Y2	IRRI	5	MT
HHZ5-Y3-SAL2-SUB1	IRRI	3	T
HHZ5-Y3-Y1-DT1	IRRI	7	S
HHZ5-Y4-SAL1-Y1	IRRI	5	MT
HHZ5-Y7-Y2-SUB1	IRRI	7	S
HHZ8-SAL6-SAL3-SAL1	IRRI	3	T
HHZ8-SAL6-SAL3-Y1	IRRI	3	T
HHZ8-SAL6-SAL3-Y2	IRRI	5	MT
HHZ8-SAL9-DT2-Y2	IRRI	5	MT
HHZ8-SAL12-Y2-DT1	IRRI	5	MT
HHZ8-SAL14-SAL1-SUB1	IRRI	5	MT
HHZ8-SAL14-SAL13-Y2	IRRI	5	MT
HHZ8-Y7-DT2-SAL1	IRRI	5	MT
HHZ9-DT12-DT1-SUB1	IRRI	5	MT
HHZ11-DT7-SAL1-SAL1	IRRI	7	S
HHZ11-Y10-DT3-Y3	IRRI	5	MT
HHZ12-DT10-SAL1-DT1	IRRI	5	MT
HHZ12-SAL2-Y3-Y1	IRRI	5	MT
HHZ12-SAL2-Y3-Y2	IRRI	7	S
HHZ12-SAL8-Y1-SAL1	IRRI	5	MT
HHZ12-SAL8-Y1-Y2	IRRI	5	MT
HHZ12-Y4-DT1-Y1	IRRI	5	MT
HHZ12-Y4-DT1-Y2	IRRI	5	MT
HHZ12-Y4-DT1-Y3	IRRI	5	MT
HHZ12-Y4-Y1-DT1	IRRI	7	S
HHZ12-DT4-DT1-Y1	IRRI	5	MT
HHZ15-DT7-SAL4-SAL1	IRRI	7	S
HHZ17-DT6-Y1-DT1	IRRI	5	MT
HHZ17-Y16-Y3-SAL1	IRRI	5	MT
HHZ17-Y16-Y3-Y1	IRRI	5	MT
HHZ17-Y16-Y3-Y2	IRRI	7	S
HUANG-HUA-ZHAN	CAAS	7	S
IR-64	IRRI	7	S
IR 84675-7-3-2-B-B	IRRI	7	S
IR 84675-25-7-3-B-B	IRRI	5	MT
IR 84675-58-4-1-B-B	IRRI	5	MT
IR 84677-34-1-B	IRRI	5	MT
IR 84677-51-1-B	IRRI	5	MT
IR 84677-132-2-B	IRRI	5	MT
IR 84678-25-5-B	IRRI	5	MT
HHZ 5-SAL9-Y3-Y1	IRRI	3	T
HHZ 5-SAL10-DT1-DT1	IRRI	7	S
HHZ 5-SAL10-DT2-DT1	IRRI	3	T
HHZ 8-SAL9-DT1-Y1	IRRI	5	MT
HHZ 11-SAL6-Y1-Y1	IRRI	7	S
FL478	IRRI	3	T
SB	Pakistan (Basmati)	7	S
Shandar	Coarse	7	S
Shadab	Coarse	7	S
Shaheen Bas	Coarse	7	S
B-2000	Pakistan (Basmati)	7	S
KSK-434	Coarse	5	MT
IR-8	IRRI	7	S
IR-6	Pakistan	7	S
Supri	Pakistan (Non-basmati)	7	S

chain reaction (PCR) was carried out by preparing master mix solution with 10X buffer, 3 µL $MgCl_2$, 0.2 µ*M* primers (forward and reverse), 1 unit Taq DNA polymerase (Fermentas Life Sciences) and 5 µL genomic DNA. Amplified PCR products were resolved on 8% acrylamide gels for 3 h at 100 volts. The gel was stained with ethidium bromide followed by gel documentation using DigiDoc-It®.

Statistical Analysis

Data collected regarding different growth parameters was subjected to different statistical analysis e.g. mean, standard deviation and analysis of variance (ANOVA) for all treatments using the Statistix 8.1 software. Allelic data generated by SSR markers were analyzed using the Power Marker v3.25 software, and different statistical parameters were observed i.e., the number of alleles, major allele frequency and polymorphism information content (PIC). PIC values were calculated according to Anderson *et al*. (1993) formula. Power Marker generated the UPGMA dendrogram and the TREEVIEW to view the tree. Genetic diversity of selected rice lines was analyzed using STRUCTURE v2.3.4 with the set number of subpopulations (k) ranged from 1 to 20. The number of iterations for each K was 10. ΔK graph showed the optimal K is 2 by Evanno. Both Evanno's ΔK and ln P(D) value were used to determine the K-value. If an individual had more than 0.8 memberships, then it was assigned to a particular population, whereas, an individual with less than 0.8 membership assigned to an admixed group.

Results

A significant difference ($P \leq 0.01$) under salt stress was observed in all growth parameters (root shoot length, root shoot fresh and dry weight) by ANOVA. Moreover, ANOVA also identified significant ($P \leq 0.01$) interaction between genotypes, different treatments and growth parameters at seedling stage (Table 3). Different levels of salt-stress showed a variable effect on the studied parameters for all the genotypes (Table 4).

Visual Scoring of Rice Leaves at Seedling Stage

All screened rice genotypes were divided into three groups i.e., tolerant (T), moderately tolerant (MT) and sensitive (S), depending on the visual symptoms of leaves at different levels of salt stress (Table 1). FL478, a highly tolerant variety at the seedling stage, was used as positive control for evaluation of salt treated rice leaves. Tolerant genotypes were scored as 3, moderate tolerant as 5 and sensitive as 7. Under 150 m*M* NaCl stress, the leaves of sensitive rice genotypes showed leaf rolling after 1st to 2nd day, followed by chlorosis on the 3rd to 4th day. By the 8th to 12th day sensitive seedlings were dead. Leaves of tolerant genotypes showed same early response at 150 m*M* stress, but on the 3rd to 4th day they showed the signs of recovery from salt injury, such as leaf greening

Table 2: SES score for visual salt injury of rice genotypes at seedling stage (Gregorio *et al.*, 1997)

Score	Observation	Response category
1	Normal growth with no leaf symptoms	Highly tolerant
3	Nearly normal growth, but leaf tips or few leaves whitish and rolled	Tolerant
5	Growth severely retarded; most leaves rolled; only a few are elongating	Moderately tolerant
7	Complete cessation of growth; most leaves dry; some plants dying	Sensitive
9	Almost all plants dead or dying	Highly sensitive

Table 3: Mean square values of different parameters of 63 rice genotypes

Source of variance	df	SL after 5th day of stress	RL after 5th day of stress	SL after 12th day of stress	RL after 12th day of stress	SFW	RFW	SDW	RDW
Genotypes (G)	62	2.83***	9.94***	2.92***	7.12***	50.23***	60.49***	3.41***	1.20***
Salt stress (S)	3	366.32***	119.17***	689.68***	356.07***	10659.28***	8027.53***	349.66***	158.11***
G x S	189	0.74***	1.59***	1.03***	2.28***	30.45***	26.01***	0.81***	0.34***
Error	510	0.05	0.05	0.04	0.04	1.11	0.29	0.04	0.01
Total	767	-	-	-	-	-	-	-	-
CV	-	4.67%	6.42%	4.08%	4.95%	7.11%	6.91%	5.52%	7.32%

*** High significant at P≤0.01

Table 4: Range of tolerant, moderate tolerant and sensitive genotypes under control and stress condition

Parameters	Range of tolerant genotypes			
	Control	50 m*M*	100 m*M*	150 m*M*
Shoot length after 5th day stress (cm)	5.4–6.9	4.7–5.6	4.1–5	3.5–4.4
Root length after 5th day stress (cm)	3.3–4.3	2.3–4.2	3.27–4.6	3–3.8
Shoot length after 12th day stress (cm)	6.1–7.7	4.1–5.9	3.4–5	3.2–4
Root length after 12th day stress (cm)	4–6.4	2.9–6.2	4–6.03	2.5–3.5
Shoot fresh weight (mg)	2–2.4	1.33–2.2	1.24–1.9	0.84–1.42
Root fresh weight (mg)	0.54–2	0.52–1.72	0.25–1.2	0.27–0.74
Shoot dry weight (mg)	0.39–0.54	0.31–0.45	0.27–0.41	0.24–0.34
Root dry weight (mg)	0.18–0.31	0.13–0.25	0.1–0.2	0.06–0.1
	Range of sensitive genotypes			
Shoot length after 5th day stress (cm)	5–7.6	3–5.4	2.1–3.4	1.2–2.5
Root length after 5th day stress (cm)	2.5–7.3	1.4–3.9	0.9–2.5	0.4–1.9
Shoot length after 12th day stress (cm)	6.2–9.3	3–5.3	1.8–4.2	0.7–3.5
Root length after 12th day stress (cm)	3.1–7.2	2.2–6.5	0.8–4.8	0.8–2.2
Shoot fresh weight (mg)	2.1–3.3	0.6–2.5	0.38–1.14	0.19–1.05
Root fresh weight (mg)	0.82–2.83	0.27–1.47	0.14–0.57	0.03–0.24
Shoot dry weight (mg)	0.38–0.64	0.25–0.46	0.05–0.28	0.02–0.17
Root dry weight (mg)	0.16–0.32	0.02–0.23	0.01–0.1	0–0.06
	Range of moderate tolerant genotypes			
Shoot length after 5th day stress (cm)	4.9–7.5	4.1–5.9	3–4.9	2.9–4.1
Root length after 5th day stress (cm)	2.6–6.2	1.4–5.3	1–5.6	0.4–1.9
Shoot length after 12th day stress (cm)	5.4–8.5	3.9–6.3	3.2–4.9	2.4–4.1
Root length after 12th day stress (cm)	3.4–7.2	2.8–6.2	2.5–5.6	1.8–3.1
Shoot fresh weight (mg)	1.97–3.23	1.14–2.7	0.71–1.9	0.61–1.21
Root fresh weight (mg)	0.82–2.9	0.34–2.1	0.22–1.36	0.19–0.57
Shoot dry weight (mg)	0.03–0.63	0.29–0.55	0.2–0.44	0.15–0.31
Root dry weight (mg)	0.12–0.33	0.08–0.24	0.05–0.11	0.03–0.09

(Fig. 1). Among 63 genotypes, six were identified as tolerant, 37 moderately tolerant and 20 as sensitive. Six rice genotypes i.e. HHZ5-SAL8-DT2-SAL1, HHZ5-Y3-SAL2-SUB1, HHZ8-SAL6-SAL3-SAL1, HHZ8-SAL6-SAL3-Y1, HHZ 5-SAL9-Y3-Y1 and HHZ 5-SAL10-DT2-DT1 were identified as salt tolerant (Table 1).

Salt Screening of Rice Genotypes at Seedling Stage

Root and shoot length: A significant reduction in root shoot length of sensitive genotypes was observed, compared with positive control. The mean values of root length (RL) and shoot length (SL) showed the substantial difference between tolerant, moderately tolerant and sensitive genotypes. Tolerant and MT genotypes maintained their root length up to 100 and 150 m*M* NaCl. In tolerant genotypes, 7–20% reduction while in sensitive genotypes 58–90% reduction in RL was observed (Table 5). RL of tolerant genotypes at 100 m*M* and 150 m*M* stress ranged from 3.3 cm and 3 cm for HHZ5-Y3-SAL2-SUB1 to 4.6 cm and 3.8 cm for HHZ8-SAL6-SAL3-SAL1. RL of sensitive genotypes at 100 m*M* stress ranged from 0.9 cm for Shadab to 2.5 cm for HUANG-HUA-ZHAN and at 150 m*M* stress 0.37 cm for

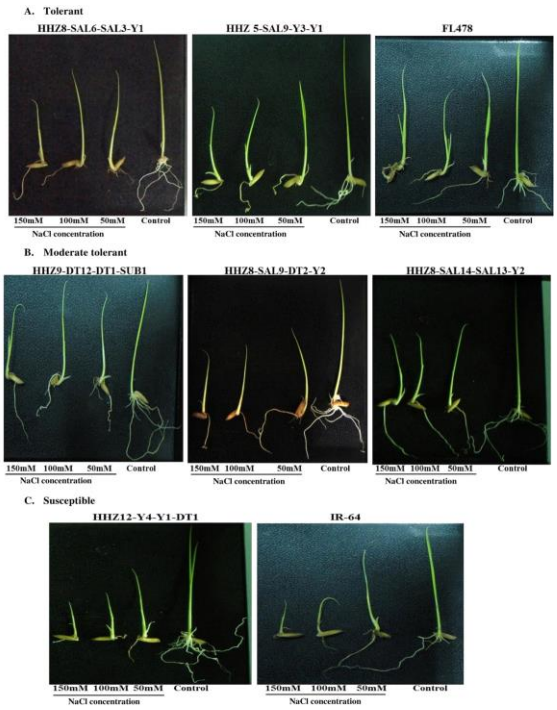

Fig. 1: Visual response of rice genotypes to salinity. A) Tolerant lines, B) Moderate lines and C) Sensitive lines. Seedlings were treated with increased NaCl salinity levels. Visual scoring was recorded after 12th day of stress

Shadab to 1.9 cm for IR-8 (Table 4). At 150 m*M* NaCl sensitive genotypes showed a gradual decrease in the RL.

Tolerant genotypes showed 28–42% reduction in SL at 150 m*M*, while 74–90% reduction was observed in sensitive genotypes (Table 5). SL for tolerant genotypes at 100 m*M* stress ranged between 4.1 cm for HHZ8-SAL6-SAL3-SAL1 and 5 cm for HHZ5-SAL8-DT2-SAL1, whereas at 150 m*M* stress it ranged from 3.5 cm for HHZ 5-SAL10-DT2-DT1 to 4.4 cm HHZ5-SAL8-DT2-SAL1. SL of sensitive genotypes at 100 m*M* stress ranged from 2.1 cm for HHZ11-DT7-SAL1-SAL1 to 3.4 cm for HHZ12-Y4-Y1-DT1 while at 150 m*M* stress it ranged from 1.2 cm for IR 84675-7-3-2-B-B to 2.5 for HHZ5-SAL10-DT1-DT1 (Table 4). Tolerant genotypes maintained their root and shoot length up to 100 m*M* after 12 days of salt stress while stunted length observed at 150 m*M* (Fig. 1). Seedlings of sensitive genotypes were started dying with the passage of time.

Shoot and root fresh and dry weight: Shoot and root fresh weight was significantly affected by high levels of salt stress. Shoot fresh weight (SFW) of tolerant genotypes showed 42–55% reduction in 150 m*M* stress and 23–25% reduction was observed at 100 m*M* stress. Sensitive genotypes showed 82–85% reduction in shoot fresh weight at 150 m*M* stress, while 60–64% reduction observed at 100 m*M* stress. Tolerant genotypes at 100 m*M* and 150 m*M* stress for SFW ranged from 1.24 mg to 0.84 mg for HHZ8-SAL6-SAL3-SAL1 and 1.89 mg and 1.42 mg for HHZ5-SAL8-DT2-SAL1. SFW of sensitive genotypes at 100 m*M* stress ranged from 0.38 mg for IR-6 to 1.14 mg for HHZ5-Y7-Y2-SUB1, while at 150 m*M* stress it ranged from 0.19 mg for Shaheen Basmati and 1.05 mg for IR 84675-7-3-2-B-B (Table 4).

Tolerant genotypes showed a significant reduction in the root fresh weight (RFW) ranging from 47–60% at 150 m*M* while less reduction in RFW i.e. 33–37% observed at 100 m*M* salt stress (Table 5). In sensitive genotypes, percent of reduction at 150 m*M* is 91–97% in case of RFW and 86–89% reduction in 100 m*M* stress. Tolerant genotypes at 100 m*M* and 150 m*M* stress for RFW ranged between 0.35 mg and 0.27 mg for HHZ5-Y3-SAL2-SUB1 and 1.19 mg and 0.74 mg for HHZ5-SAL8-DT2-SAL1. RFW of sensitive genotypes at 100 m*M* stress ranged between 0.14 mg for HHZ5-Y7-Y2-SUB1 and 0.57 mg for HHZ5-SAL10-DT1-DT1, whereas at 150 m*M* stress ranged between 0.03 mg for HHZ5-Y7-Y2-SUB1 and 0.24 mg for IR 84675-7-3-2-B-B (Table 4).

Tolerant genotypes showed 28–57% and 22–36% reduction in shoot dry weight (SDW) at 150 m*M* and 100 m*M* salt stress respectively. Sensitive genotypes showed 75–77% reduction in 150 m*M* salt stress. SDW of tolerant genotypes at 100 m*M* stress ranged between 0.27 mg for HHZ 5-SAL10-DT2-DT1 and 0.41 mg for HHZ8-SAL6-SAL3-SAL1, whereas at 150 m*M* stress ranged between 0.24 mg for HHZ 5-SAL10-DT2-DT1 and 0.34 mg for HHZ8-SAL6-SAL3-Y1. HHZ8-SAL6-SAL3-Y1 was highly tolerant for SDW under salt stress condition. SDW of sensitive genotypes at 100 m*M* stress ranged from 0.05 mg for IR-64 to 0.28 mg for IR-8, whereas at 150 m*M* stress ranged from 0.02 mg for IR-64 to 0.17 mg for IR 84675-7-3-2-B-B (Table 4).

The maximum reduction in root dry weight (RDW), 54–73% and 27–59%, was observed at 150 m*M* and 100 m*M* salt stress respectively. RDW of sensitive genotypes reduced from 91–97% at 150 m*M* salt stress. Tolerant genotypes at 100 m*M* and 150 m*M* stress for RDW ranged from 0.1 mg to 0.06 mg for HHZ8-SAL6-SAL3-SAL1 and 0.2 mg and 0.1 mg for HHZ5-SAL8-DT2-SAL1. HHZ5-SAL8-DT2-SAL1 was tolerant for RDW under salt stress condition. RDW of sensitive genotypes at 100 m*M* stress ranged between 0.01 mg for Shandar, Shaheen Basmati, Basmati 2000 and 0.09 mg for Supri, whereas at 150 m*M* stress ranged from 0 mg for HHZ5-Y7-Y2-SUB1, Basmati 2000 to 0.06 mg for IR 84675-7-3-2-B-B (Table 4).

SSR Markers Analysis

For marker genotyping, 21 SSR markers (from *SalTol* region) were used for selected 34 genotypes. Out of 21 markers, 19 SSR markers showed polymorphism among the 34 genotypes and amplified 53 alleles. The number of alleles ranged from 1 to 6 per locus with the mean of 2.7 alleles (Table 6). Polymorphism level was measured by Polymorphic Information Content (PIC) using formula

Table 5: Percentage reduction of tolerant and sensitive genotypes at 150 m*M* salt stress

Characters	Tolerant genotypes	Sensitive genotypes
Root length	7–20%	58–90%
Shoot length	28–42%	74–90%
Shoot fresh weight	42–55%	82–85%
Root fresh weight	47–60%	91–97%
Shoot dry weight	38–57%	75–77%
Root dry weight	54–63%,	91–97%

Table 6: Number of alleles, PIC value and range of amplicon size (bp) of SSR markers

Marker	Major allele frequency	Allele number	PIC value	Range of amplicon size (bp)
RM1287	0.7059	2	0.329	158–207
RM8094	0.7647	2	0.2951	171–263
RM10720	0.5294	2	0.3741	192–221
RM3412	0.4412	4	0.5911	216–260
RM10748	0.5	3	0.5398	86–97
RM10773	0.5	3	0.4161	407
RM493	0.5294	3	0.4772	212–266
RM140	0.5294	2	0.3741	244–329
RM10793	0.5	2	0.375	130–230
RM10800	1	1	0	140–150
RM10825	0.5882	2	0.3671	80–90
RM10843	0.5294	2	0.3741	151–161
RM10852	0.5	3	0.5253	170–190
RM10864	0.9706	2	0.0555	210–330
RM10871	0.6667	3	0.4122	160–220
RM562	0.3529	6	0.6748	225–284
RM10890	0.6061	3	0.4015	249–262
RM7075	0.6471	2	0.3524	109–130
RM10927	0.5882	3	0.4049	150–160
RM6711	1	1	0	130–150
RM20224	0.7941	3	0.3125	169–204

(Anderson *et al.*, 1993). Significant variation was observed in 19 SSR loci, and PIC values ranged from 0.05 (RM10864) to 0.67 (RM562). UPGMA cluster showed the phylogenetic similarity among 34 rice genotypes. SSR marker based clustering, grouped, salinity tolerant, moderately tolerant and sensitive genotypes into 3 clusters based on their genetic response to salinity stress. Cluster 1 comprised of seven genotypes, of which four, were sensitive, and three are moderately tolerant along with the sensitive check (IR29). However, the second cluster was the largest one which included all tolerant (6) and some moderately tolerant genotypes, while Pokkali and FL478 (tolerant check) was also present in the same cluster. Cluster 3 comprised of moderately tolerant genotypes (Fig. 2). Population structure, divided genotypes into two separate groups. Sharp peak of Evanno's ΔK was observed at K =2 (Fig. 3A). Tolerant and moderately tolerant genotypes (27) were present in group 1 while sensitive genotypes were present in group 2 (Fig. 3B).

Discussion

High concentration of salts in the soil can be very toxic, and it affects the growth of plant at all phenological stages. Soil salinity retards the growth of crops by reducing the water absorption through roots. Rice is sensitive to salinity at all growth stages, but most severe damage is seen at seedling stage (Sakina *et al.,* 2015).

In present study, tolerant genotypes were observed to be less affected by the different levels of salt stress compared with sensitive genotypes for different growth parameters such as root and shoot length, fresh and dry weight. Sensitive genotypes showed yellowing of leaves when exposed to 50 m*M* salt stress for a long time. These genotypes started drying after 3–4 days under increased salt stress of 100 m*M* and 150 m*M*. Sensitive genotypes accumulate salts more quickly than tolerant genotypes and it leads to leaf death, ultimately death of whole plant (Darwish *et al.,* 2009). In breeding strategies, the visual leaf injury scoring is commonly used for overall response of rice varieties under salinity stress (De Leon *et al*., 2015).

In this study, tolerant genotypes showed increased root and shoot length at 100 m*M* and 150 m*M* stress, while moderately tolerant genotypes could maintain their root and shoot growth up to 100 m*M* stress. However, increased concentration of salt caused significant reduction in root and shoot length of sensitive genotypes. It has been reported that high salt concentration inhibit the growth of root shoot length and also reduce the development of new secondary roots with increasing salinized condition (Hosseini *et al.,* 2012; Rubel *et al.,* 2014). Shoot lengths of all genotypes were more affected by all levels of salt stress than root lengths. Exposure for extended time under high salt concentration inhibits the growth of shoot (Ren *et al.,* 2005). It may be possible that tolerant genotypes spread their roots deeper in soil to absorb more water and nutrients during osmotic stress. Under salinity stress, a communication from root to shoot occurs which adjust the whole plant growth as roots are the first part to discover variations in soil water potential (De Leon *et al.,* 2015). Roots have direct interaction with soil for the uptake of water and minerals, therefore root character can successfully be used as selection criteria for salinity tolerance in breeding programme.

Salinity decreases plant biomass with an increase in level of salt stress. Minimum reductions were observed in tolerant genotypes for root and shoot fresh weight but the salinity reduced root and shoot fresh weight of sensitive genotypes with more percent of reduction observed with increasing salt concentration. Sensitive genotypes could not survive in high salt stress environment compared to tolerant genotypes. The long term exposure to high salinity induced osmotic stress and ionic toxicity on sensitive genotypes (Re *et al.,* 1999; Coskun *et al.,* 2013).

In present study, root and shoot total dry weight showed a less reduction in tolerant genotypes. Sensitive genotypes showed ~ 90% reduction under stress condition in root shoot dry weight. It was previously reported that under high salt stress rice leaves become rolled and dried followed by reduced yield and decrease in root and shoot dry weight with increasing salinity (Mansuri *et al.,* 2012).

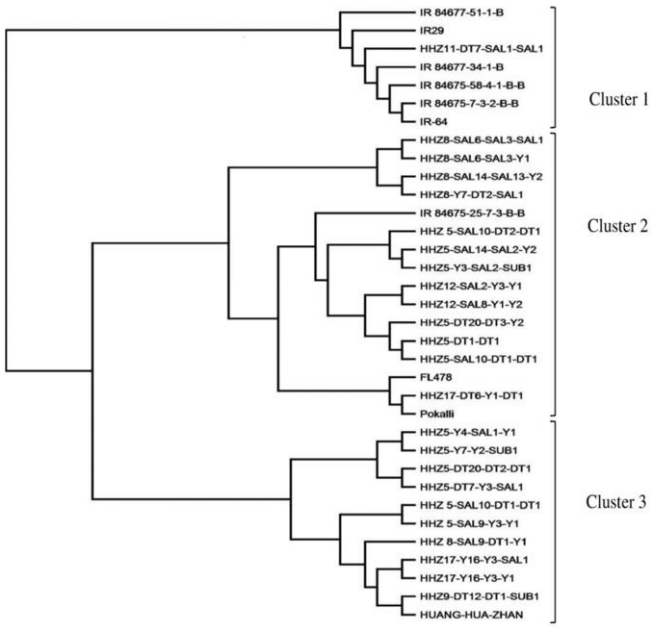

Fig. 2: A UPGMA dendrogram generated from SSR markers is showing the genetic similarity between 34 rice genotypes

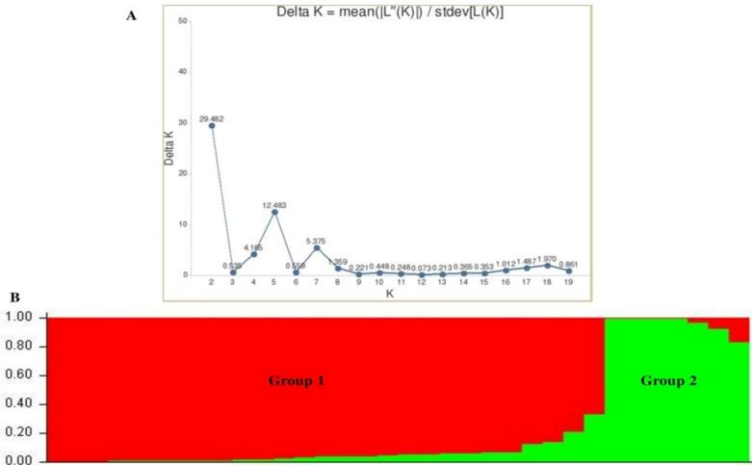

Fig. 3: A) Delta K with K=2-20 and **B)** Population structure of 34 rice genotypes

Overall, root dry weight showed more reduction in comparison to shoot dry weight as the roots are more affected under stress as compared to shoot (Ologundudu *et al.*, 2014). In early growth stages, the growth parameters (shoot fresh and dry weight) are correlated with salt tolerant crop and used as an indicator for salinity tolerance (Haq *et al.*, 2009).

For genotyping, SSR markers were selected due to high coverage for rice genome (Islam *et al.*, 2012b). Cluster analysis for the classification of accessions according to their response to salinity showed that the genotypes were clustered into 3 separate groups. Based on salinity response, the genotypic data separated the tolerant genotypes from sensitive. Population structure analysis placed the tolerant and moderately tolerant into one group and sensitive genotypes into other group. The moderately tolerant genotypes are genetically more close to tolerant one and mostly present in the same group (Zheng *et al.*, 2015). Cluster analysis and population structure revealed the same results, as tolerant and sensitive genotypes were present in different groups.

Out of 21 SSR markers only 19 were found polymorphic among rice genotypes in response to salinity. All 19 SSR markers are associated with *SalTol* QTL, located on chromosome 1 (Babu *et al.*, 2014). In this study, markers showed polymorphism in rice genotypes, and these markers are reported as high PIC values for *SalTol* region (Krishnamurthy *et al.*, 2014). The highest number of alleles

observed in RM562 (6 alleles), followed by RM3412, RM10773, RM493, RM10748, RM10852, RM10890, RM10927, RM20224 and RM10871 (3 alleles). Three SSR markers in the *SalTol* region i.e. RM3412, RM493 and RM10852 differentiate the tolerant genotypes from sensitive genotypes (Krishnamurthy *et al.*, 2015). There is a possibility that different candidate genes and QTLs related to salinity tolerance present in tolerant and moderate tolerant genotypes, so further study is needed to expose the genes and QTLs in these rice genotypes.

Conclusion

High salt concentration (150 m*M*) significantly decrease root shoot length, fresh weigh and dry weight of salt sensitive genotype compared to salt tolerant genotypes. Therefore, growth parameters like root shoot length, root shoot fresh and dry weight are the good indicators for the identification of salt tolerant genotypes at seedling stage along with the SSR markers which also differentiated salt tolerant and sensitive genotypes. Identified germplasm may serve as a source for the development of salt tolerant rice varieties with elite genetic background by employing efficient marker assisted breeding strategies.

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