

EVALUATION OF THE RESPONSES OF EIGHT RICE (*Oryza sativa*, L.) GENOTYPES TO VARIOUS CONCENTRATIONS OF NaCl IN A CONTROLLED ENVIRONMENT

James Sulonkwiley Dolo^{1*} Susan Nchimbi-Msolla² John J. Msaky³

1. Department of Crop Science and Horticulture; Sokoine University of Agriculture, Box 3000, Morogoro, Tanzania; Email: dolo.james@gmail.com

2. Department of Crop Science and Horticulture; Sokoine University of Agriculture, Box 3000, Morogoro, Tanzania

3. Department of Soil Science and Geological Studies, Sokoine University of Agriculture, Box 3000, Morogoro, Tanzania

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Abstract

Salinity is an ever increasing problem that reduces rice yield in many rice fields around the world. Developing salt tolerant rice (*Oryza sativa* L.) genotype is one of the solutions to the problem of salinity. This experiment was carried out in the Department of Crop Science and Horticulture at SUA to assess the salinity tolerance of 8 rice genotypes at the seedling stage. Ion accumulation in plants and dry matter content along with molecular markers were used to evaluate the tolerance of each rice genotype. The genotypes were IRRI 112, IRRI 124, FL 478, IRRI 113, IR65912-4B-10-3, IRRI 128, NERICA-L-19 and SUAKOKO-10. In this experiment, the genotypes were exposed to three salinity levels in a randomized complete block design arranged in factorial with three replications. The salinity levels were 100 mM NaCl, 50 mM NaCl and 0 mM NaCl. The homogenous mixture of sand, farm yard manure and rice husk (ratio of 6:2:10 respectively) was used as the planting medium for all rice genotypes. The soil texture was sandy clay-loam. The growth of the genotypes, ion accumulation and dry matter contents were significantly ($p \leq 0.05$) affected by increase in NaCl concentration. Two *Saltol* SSR markers (RM7075 and RM562) were used to determine the presence of salinity tolerance (*saltol*) gene in rice genotypes. Based on the SSR markers, ion accumulation and dry weight of plants, two genotypes (IR65192-4B-10-3, and IRRI112) along with FL478 were selected as salt tolerant while two (IRRI-113 and IRRI-128) were moderately tolerant, and three (NERICA-19, SUAKOKO-10 and IRRI-124) were the most susceptible genotypes. Therefore, two susceptible parents (NERICA-19 and SUAKOKO-10) were selected and two donor parents (FL478 and IR65192-4B-10-3) were selected.

Keywords: salinity stress; NaCl concentration; genotypes; markers; seedling stage; *Oryza sativa*;

1.0 INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important crops used as a source of food in the world, and it accounts for more than 21% of the calorific intakes of the world's population (Ma *et al.*, 2007 and Melissa *et al.*, 2009). Most of the people in rice producing areas of Asia, Africa and South America still depend on rice for their daily caloric intake (SurrIDGE, 2004; Joseph *et al.*, 2010). Nonetheless, rice productivity in many of these areas are seriously affected by salinity stress that affects rice growth at all stages (Maas and Hoffman, 1977; Grover and Pental, 2003). Rice has being characterized as a salt sensitive crop, but there is variation in the extent of its sensitivity. It is known that rice is tolerant to salinity stress during germination and active tillering, whereas it displays more sensitivity during early vegetative and reproductive stages (Lutts *et al.*, 1995; Zhu *et al.*, 2001).

Screening of rice genotypes for salt tolerance at seedling stage is readily acceptable as it is based on a simple criterion of selection, and it also provides rapid screening which is difficult at the vegetative and reproductive stages (Gregorio *et al.*, 1997). Screening of rice genotypes using the conventional method is very difficult because of the large effects of the environment and low narrow sense heritability of salt tolerance (Gregorio, 1997), but the introduction of DNA markers seems to be the best technique for efficient evaluation and selection of plant material (Bhowmik *et al.*, 2009). Recent progress and technical advances in DNA marker technology

permit reduction of time and accuracy of the breeding program where pronounced effects of environment lead to poor selection efficiency (Sultana *et al.*, 2009).

When NaCl is used for screening for salt tolerance, the sodium ion (Na^+) and chloride ion (Cl^-) dissociate from NaCl salts and contaminate the soil medium, because these ions are well known as the toxic ions which damage plant cells in both ionic and osmotic effects. Plant growth and development are directly restrained by these ions which lead to growth reduction and plant death (Lauchli and Grattan, 2007). Sodium and chlorine ions have the ability to restrict the uptake of other essential plant nutrients such as potassium, magnesium and calcium.

Potassium is an essential nutrient that plays a very important role in growth and development of plants. It is actively involved in different cellular and physiological processes including osmotic adjustment, stomata regulation, and cation balance (Marschner, 2012). Regulation of K^+/Na^+ homeostasis within cells is an important indicator of salt tolerance in plants (Zhu, 2003; Siddiqui *et al.*, 2008, 2009). Calcium is another essential element that helps in maintaining structural and functional integrity of membranes, stabilization of cell wall and regulation of ion homeostasis (Arshi *et al.*, 2010; Morgan *et al.*, 2014). These two elements (Potassium and calcium) seem to be readily displaced from binding sites by sodium and chloride ions. Maintaining sufficient concentrations of K^+ and Ca^{2+} in saline soil helps plants in overcoming specific ion toxicities, particularly in susceptible plants, which are more prone to salt damage (Grattan and Grieve, 1999). Also, K^+ and Mg^{2+} have been reported to play an important role in enzyme activation (Barker and Pilbeam, 2007). The role of Mg^{2+} under salt stress has been variable. For instance, Mg^{2+} increases in rice callus (Ahmad *et al.*, 2009) and decreases in soybean callus (Liu and Staden, 2001).

In order to be able to adequately address the problem of soil salinization, the genetic variability of the available genotype needs to be exploited for the identification of tolerant genotype that may sustain a reasonable yield on salt affected soil (Ashraf *et al.*, 2006). Genotyping is done by using selected molecular markers. The microsatellite marker or Simple Sequence Repeat (SSR) has been proved to be ideal for making genetic maps (Islam, 2004; Niones, 2004) and assisting in genotype selection (Bhuiyan, 2005). The SSR marker analysis is a promising mean of identifying genes loci for salt tolerance that can be helpful for plant breeders in the development of new cultivars. Therefore the objective of this study was to assess the salinity tolerance of 8 rice (*Oryza sativa L.*) genotypes at the seedling stage using molecular markers, dry weight and ion accumulation.

1.1 Materials and Methods

Eight (8) rice genotypes (six from IRRI and two from AfricaRice) were tested at different levels of NaCl concentrations at the seedling stage in the screen house at the Sokoine University of Agriculture in 2015 (Table 4.1). IRRI standard protocol (Gregorio *et al.*, 1997) was used to assess the tolerance of the rice genotypes to salinity conditions Table 1.1. Two SSR markers, RM7075 and RM562, which have also been used for salinity tolerance screening, were used to assess the tolerance of the rice genotypes.

Table 1.1: Modified standard evaluation score (SES) of visual salt injury at seedling stage

Scores	Observation	Tolerance
1	Normal growth on 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	Susceptible
9	Almost all plants dead or dying	Highly susceptible

Source: Gregorio *et al.*, 1997, IRRI

The rice genotypes were grown under three conditions of salinity stress using a randomized complete block design with factorial arrangement. The concentrations of NaCl in the irrigation water used were 100 mM NaCl,

50 mM NaCl and 0 mM NaCl respectively. Prior to planting of the genotypes, seeds were germinated and three seedlings sown per pot containing 1.7 kg of homogeneous mixture of planting medium including soil, farm yard manure and rice husk in the ratio 6:2:10. The seedlings were well watered with distilled water for a period of 21 days after sowing and then the salinity treatments were applied. The control pots were irrigated with distilled water up to the end of data collection which was done 43 days.

Plants were removed from pots 22 days after the application of salinity stress; each plant roots were washed with tap water and rinsed with distilled water. The roots were then blotted dry using blotting paper and then were separated from the shoot using scissors. Data were collected on roots and shoots dry weight. All the plant samples were dried at 70 °C for 48 hours in an oven to a constant weight and dry weight (g plant^{-1}) was determined. After drying, shoots and roots were weighed on an electronic beam balance and then ground to powder. The Na^+ , Ca^{2+} , Mg^{2+} , K^+ and K^+/Na^+ were determined. Sodium (Na^+) Ca^{2+} Mg^{2+} and K^+ contents (cmol g^{-1} dry weight) of shoot and root were determined from a 0.5g dried digested sample using a flame photometer.

1.2 Statistical Analysis

Data collected on dry weight and ion accumulation were subjected to two-way analysis of variance for a factorial arrangement in randomized complete block design using the genstat statistical package 14th edition (Goedhart and Thissen, 2011). Treatment (induced salinity) means were compared using Tukey honestly significant test (HSD).

1.3 DNA Extraction and Amplification of Microsatellite Markers

Genomic DNA was isolated from leaves of two-week old plants based on the DNA isolation protocol of Egnin *et al.* (1998). Two selected DNA primers [RM7075 (Bhowmik *et al.*, 2009); and RM562 (Rajendran *et al.*, 2012)] were used for this study. Amplified microsatellite loci were analyzed for polymorphism using 1.5 % Agarose Gel Electrophoresis and the result revealed that the two primers detected clear polymorphism among the rice genotypes analyzed. The primers RM7075 and RM562 were polymorphic and showed clear bands for each rice genotype.

Each PCR reaction was carried out with 21.0 μl reaction mixtures containing DNA premix, 20 μl of primer master mix and 1.0 μl of each template DNA sample. PCR profile was maintained as initial denaturation at 94 °C for 3 minutes, followed by 33 cycles of denaturation at 94 °C for 30 seconds, annealing at 55-62 °C for 30 seconds, and polymerization at 72 °C for 1 minute; and final extension by 5 minutes at 72 °C. A 100 bp DNA ladder was used to determine the band location of the DNA sample.

1.4 Results and Discussion

1.4.1 Screening of salt tolerance by SSR markers

The Banding pattern of the genotypes was scored comparing the banding pattern of FL-478. The genotype that showed similar banding pattern like FL 478, were considered as tolerant and those with different banding pattern were considered susceptible. The selected recurrent parents (NERICA-L-19 and SUAKOKO-10) showed banding patterns different from that of FL-478 and two of the genotypes from IRRI showed similar banding patterns like FL-478 and were therefore tolerant. Two of the tolerant genotypes (IR65912-4B-10-3) and FL478 which also had lower percent reduction in dry weight and lower Na^+ accumulation under saline conditions were selected as the donor parents to be used in the breeding program.

The RM7075 marker identified five tolerant genotypes namely IRR-112, IRRI-113, IRRI-128, IR65192-4B-10-3, FL-478, while three genotypes, IRRI-124, NERICA-L-19 and SUAKOKO-10 were found to be susceptible (Figure 1.1).

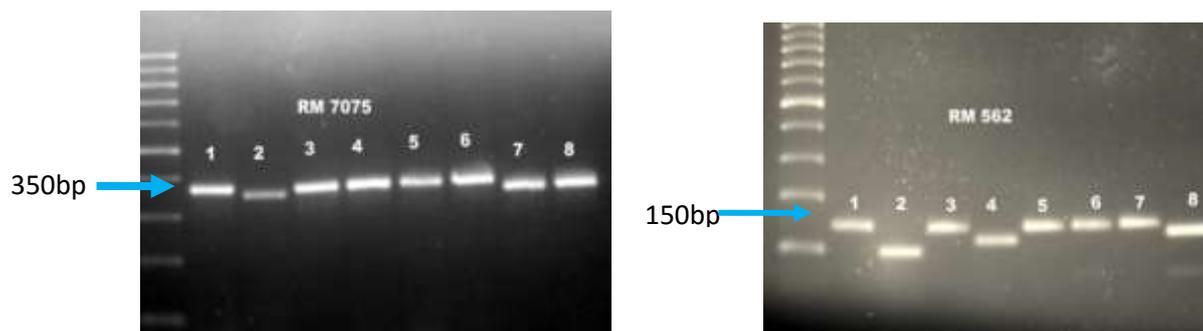


Figure 1.1: Gel images of the foreground selection of donor and recurrent parents using salt tolerance markers RM 7075(A), and RM 562 (B). 1. IRRI 112; 2. IRRI 124; 3. FL 478; 4. IRRI 113; 5. IR65912-4B-10-3; 6. IRRI 128; 7. NERICA-L-19; 8. SUAKOKO-10. Note: The bands of interest are indicated by the arrows.

The marker RM 562 identified five tolerant genotypes namely IRRI 112, IR65192-4B-10-3, FL478, IRRI 128 and NERICA-L-19, and three susceptible genotypes; SUAKOKO-10, IRRI 113 and IRRI 124 (Figure 1.1). The markers showed a clear relationship with the salt tolerance alleles in the rice genotypes. Molecular markers are capable of identifying alleles that are associated with key phenotypic traits (Xu *et al.*, 2004). Nguyen *et al.* (2001) found that microsatellite marker was associated with NaCl tolerant alleles at seedling stage in a crop population and similar results were also reported by Lang *et al.* (2000).

1.4.2 Evaluating the effects of salinity on dry weight of rice genotypes

The mean square values of dry matter weight of 8 rice genotypes are presented in Table 1.2. Significant differences were observed among genotypes after the application of salinity treatments. NaCl concentration effects were significant at ($p < 0.01$). The variety x NaCl concentration interaction was highly significant ($p < 0.05$) for root – shoot ratio. In terms of the root dry weight and the shoot dry weight, there were no significant differences; however, there was more reduction in shoot dry weight than root dry weight. The decrease of shoot and root dry weight might have been due to a reduction in turgor which resulted in lower water potential in plant or a disturbance in mineral supply to root and shoot. These results are similar to the findings of Alam *et al.* (2004); and Mahmood *et al.* (2009).

Table 1.2: Mean square (ANOVA) of rice genotypes under salt stress

Sources of variation	df	SDW	Root/Shoot Ratio	RDW
Salinity levels	2	0.857**	746**	0.874**
Variety	7	0.220**	189.3**	0.044**
Salinity levels x Variety	14	0.013 ^{ns}	28.22**	0.003 ^{ns}

** = significant at (1%)

ns = no significant difference

Note: SDW-shoot dry weight; RDW-root dry weight; plant height (cm) and dry weight (grams).

1.6 Ion accumulation in Rice Genotypes

The mean square effects of NaCl on the ion accumulation in the 8 rice genotypes are presented in Table 1.3 and Appendix 14. For the genotypes aspect, there were significant ($P < 0.01$) differences observed in ion accumulation in all the genotypes after the application of NaCl. Sodium chloride concentration had significant ($P < 0.01$) effects on ion accumulation. The variety x NaCl concentrations interactions were highly significant ($p < 0.05$) for accumulated ions in all the genotypes. One of the mechanisms of salt tolerant plant under high salinity conditions is to accrue and partition Na^+ in the older leaves, but sensitive rice genotypes are not able to do this successfully (Munns and Tester 2008). The current study observed a high potassium-sodium ratio in the shoots of tolerant rice genotypes; this might have been due to the ability of the tolerant rice genotypes to absorb more potassium than sodium in shoots or compartmentalize the sodium ion in the leaves as opposed to the susceptible genotypes. Potassium is considered an essential element in plant growth under saline conditions, because of its role in osmo-regulation and stress mitigation in saline environments (Cakmak, 2010).

Table 1.3: Means square values of NaCl effect on nutrients uptake in rice genotypes

Sources of variation	df	SK ⁺	S Na ⁺	SK ⁺ / S Na ⁺	RCa ²⁺	RK ⁺	RMg ²⁺	RNa ⁺	SCa ²⁺	SMg ²⁺
Salinity levels	2	0.06**	1.7**	0.96**	0.36**	1.5**	0.001**	1.1**	0.03**	0.04**
Variety	7	0.20**	0.07**	0.09**	0.04**	0.12**	0.01**	0.04**	0.02**	0.04**
Salinity level x Variety	14	0.16**	0.04**	0.07**	0.11**	0.05**	0.004**	0.04**	0.02**	0.01**

** = significant at (1%)

Note: SK-potassium on shoot; SNa⁺-sodium in shoot; SCa²⁺-calcium in shoot; SMg²⁺-magnesium in shoot; RCa²⁺-calcium in root; RK⁺-Potassium in root; RMg²⁺-magnesium in root; RNa⁺-sodium in root; SK⁺/SNa⁺-sodium potassium ratio in shoot.

1.7 Ranking of Rice Genotypes based on salinity tolerance

Results show that the differences in performance among the genotypes at high salinity level were much obvious (Tables 1.4 and 1.5). High salinity level adversely influenced the performances of the rice genotypes. It was clear that increasing salinity level from 0mM NaCl to 100 mM NaCl resulted in significant reductions in plant dry weight (root dry weight, shoot dry weight and root and shoot ratio). However, the maximum reduction was obtained at 100 mM, the highest NaCl concentration applied. Reduction in dry matter accumulation has a direct proportion to increased salinity levels. The result of this study agrees with results reported by Majkowska *et al.* (2008). High salinity might have inhibited the root and shoot elongation due to the slowing down of the water absorption by the plant as reported by (Jeannette *et al.*, 2002). Sagi *et al.* (1997) observed that salinity stress adversely effected shoot more than the root growth and Jamil & Rha (2007) reported that the shoot length, root lengths and the dry matter weight of radish plants were decreased with increase in salinity stress. This result is also in agreement with previous reports by Masood *et al.* (2005) which suggested that salt stress reduced the biomass of rice. Essa (2002) reported that shoot dry weight was more sensitive to salinity than root dry weight.

Table 1.4: Percent reduction in physiological traits of rice genotypes

Genotypes	Plant height		RDW		SDW		Root/shoot ratio		Manof traits	Tolerance
	50mM	100mM	100mM	50mM	100mM	50mM	100mM			
	NaCl	NaCl	50mMNa Cl	NaCl	NaCl	NaCl	NaCl			
FL-478	31.2	38.4	42.9	53.6	25.1	41	31.2	38.4	42.9	T
IR65192- 4B-10-3	38.1	44.7	51.4	62.5	20.8	24.5	38.1	49.5	45.3	T
IRRI-112	40	47.2	42.2	63.2	4.1	31.3	40	47.2	47.2	T
IRRI-113	39.6	49.8	49.7	74.9	16.8	49.6	39.6	49.8	56.0	MT
IRRI-124	47.7	54.2	60.6	79	25.7	54.7	47.7	54.2	60.5	S
IRRI-128	39.2	42.1	47.5	66.2	30.3	53.2	39.2	42.1	50.9	MT
NERICA-L-19	40.7	55.2	47.4	73.4	11.2	40.8	40.7	55.1	56.1	S
SUAKOKO-10	46.6	57.8	58.9	73.1	23.1	55.9	46.6	57.8	61.2	S

Note: RDW = root dry weight; SDW = shoot dry weight; plant height (cm) and dry weight (grams).

Table 1.5: Effects of NaCl concentrations on dry matter weight of eight rice genotypes

Genotypes	RDW			SDW			Root/Shoot Ratio		
	0mM	50	100mM	0mM	50	100mM	0mM	50	100mM
	NaCl	mM	NaCl	NaCl	NaCl	NaCl	NaCl	NaCl	NaCl
FL478	0.65J	0.37d	0.30a-f	1.19d	0.90b	0.71a-c	68.60o	47.2	42.2j
IR65192-4B-10-3	0.56h-j	0.27a	0.21a-e	0.90b-d	0.71a	0.68a-d	61.60l	38.1	31.10c
IRRI-112	0.57h-j	0.33c	0.21a-e	0.94cd	0.90b	0.64a-d	61.30l	36.7	32.30d
IRRI-113	0.61ij	0.31a	0.15a-c	0.88b-d	0.73a	0.44a-c	69.60p	42.0	34.90f
IRRI-124	0.45f-j	0.18a	0.09a	0.72a-d	0.54a	0.33ab	63.60m	33.2	29.10b
IRRI-128	0.52g-j	0.27a	0.18a-d	0.99cd	0.69a	0.46a-c	66.30n	40.2	38.40h

		-f			-d			Oi	
NERICA-L-19	0.61ij	0.32b		0.16a-d	0.78a-d	-d	0.46a-c	78.90q	0k 35.40f
SUAKOKO-10	0.39e-i	0.16a		0.11ab	0.61a-c	-c	0.27a	66.00n	0a 27.80f
Salinity level (s.e.)		0.02			0.05			0.06	
Genotpe (s.e.d)		0.03			0.09			0.1	
Salinity x Genotype (s.e.d)		0.06			0.15			0.17	

Tukey ($p \leq 0.05$);

Note: RDW = root dry weight; SDW = shoot dry weight; plant height (cm) and dry weight (grams).

1.8 Conclusions and Recommendations

1.8.1 Conclusion

Salt stress induced changes in ion accumulation in rice and dry matter at the seedling stage. The variations in ion accumulation and dry matter weight of rice genotypes, clearly distinguished the tolerant from susceptible genotypes. The maximum variation was realized when NaCl concentration was increased to 100 mM.

Additionally, there were differences among the genotypes in terms of sodium and potassium absorption, and the molecular markers used showed clear polymorphism among the genotypes. The SSR markers used in this study were able to clearly distinguish tolerant genotypes from susceptible. Further, on the basis of SSR marker analysis, the K^+/Na^+ ratio in the shoots and the interaction among the genotypes at the different levels of salinity, NERICA-L-19 and SUAKOKO-10 were selected as the susceptible parents to be used for the improvement of salt tolerance. The variation among the root and shoot biomass clearly distinguished the susceptible genotypes from the tolerant ones.

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