

ASSESSMENT OF SALINITY TOLERANCE IN AN INBRED POPULATION OF RICE (*ORYZA SATIVA* L) DERIVED FROM A *JAPONICA* X *INDICA* CROSS

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ABSTRACT

Development of methodologies for the characterization of parental rice cultivars under different abiotic stress conditions is important to exploit the genetic components of the relevant stress in populations using molecular tools such as QTL analysis. *Japonica* rice cultivar Hyogokithanishiki and *Indica* rice cultivar Hokuriku-142 (Hokuriku) were evaluated for salinity tolerance by EC solution method and MS (Murashige and Skoog) medium method. A plant nutrient solution was used to apply salinity stress for 12-day-old plants in EC solution method. NaCl was used to manipulate the salinity level in both methods. Green shoot length, dry matter weight and root length immediately after the stress and after recovering under normal conditions were measured in replicated experiments. Results showed that green shoot length is the best parameter for evaluation of salinity tolerance using EC solution method at 5 dS/m. This method was therefore applied to an inbred line population (163 F₆ lines) derived from the two cultivars. The frequency distribution of green shoot length in the population showed a nearly normal distribution for surviving lines. Transgressive segregants were observed in both directions indicating the contribution of both parents for salinity tolerance in the population. The bio assay data can be utilized for QTL analysis upon construction of genetic map.

Key words: Bio Assay, Breeding, Electrical Conductivity, Salt Tolerance, Sodium Chloride, Tissue Culture

INTRODUCTION

Salinity affects rice production worldwide, particularly by reducing plant growth and potential yield. Rice is somewhat tolerant to salinity at germination stage (Abeywardhana *et al.* 2004), very sensitive at early seedling stage and gains tolerance during vegetative growth. It becomes sensitive during pollen development stage but increasingly tolerant at maturity stage (Pearson *et al.* 1996). Expansion of salt affected lands resulting in the reduction of cultivable lands is a great challenge with the growing population in the world. Among various problems, irrigation is one of the problems that cause degradation of agricultural land leaving salts in soil after evapotranspiration. Soil salinity has been identified as caused by three different reasons; natural, clearing of native vegetation, and irrigation (Manneh *et al.* 2007). According to the classification of classes of soil salinity, low salinity; EC 2-4 dS/m can be caused by natural salinity and irrigation salinity. Species with low-moderate salt tolerance can be grown successfully under this salt stress. Moderate to high salt tolerant plant spe-

cies are needed when the electrical conductivity falls between 4-8 dS/m. This salinity is caused by irrigation. Under high-salinity conditions with EC exceeding 9 dS/m, only halophytes can be grown (Maas and Hoffman 1977) According to Maas and Hoffman (1977) there exists a threshold salinity level that varies with species and with cultivar within species. Based on some published data, the salinity threshold for rice is around 3dS/m at 25°C (Maas and Hoffman 1977).

Soil salinity constraints rice production and over 30% of the irrigated rice area in the world is affected by saline conditions due to irrational management and defective irrigation practices (Yeo and Flowers 1984). Current research (Maas and Grattan 1999; Hanson *et al.* 1999) indicates that rice yields decrease 12% for every unit (dS/m) increase in above 3.0dS/m. The growth under saline conditions depends on the reducing ability of sodium and chloride uptake while maintaining potassium uptake in to the plant (Koyama *et al.* 2001). The development of appropriate techniques for manage-

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ment of salinity is critical for optimizing rice performance under saline or potentially saline conditions. Mass and Grattan (1999) reported that yields cannot be improved under salt-stressed conditions by increasing the seeding rate. They found that high field-water levels are more growth limiting than shallow water levels. Therefore, the solution for rice growers who are facing salinity problems is, irrigation management strategies that maintain low levels of salinity stress while minimizing high field water levels (Grattan and Grieve 1999). Various methods such as soil reclamation, excessive irrigation, and soil drainage are used to minimize soil salinity; they are always laborious and expensive. Therefore other strategies such as varietal improvement also need to be considered for constant and profitable rice production.

There are several factors influencing salt tolerance in plants. The most important among these are species, temperature, salt composition, growth stage of plant, salinity level, environmental variables, soil fertility and cultivar (Fageria 1985). Physiological characterization of rice cultivars showed that tolerant genotypes maintained a much lower shoot Na⁺ concentration relative to sensitive genotypes under salinity stress (Walia *et al.* 2007).

Though attempts have been made to change the salt tolerance of important crops over many years, the success achieved in producing salt tolerant varieties however has been very limited (Flowers *et al.* 2000). Most of the processes found to be important in plant resistance or tolerance of salinity exhibit quantitative inheritance, hence they show continuous variation and a high degree of environmental interactions (Koyama *et al.* 2001). Evolutionary adaptation to salinity involves complicated physiological and developmental processes (Flowers and Dalmond, 1992) making breeding for salt tolerance a difficult challenge.

Selection of salinity tolerant parental lines considering physiological criteria and pyramiding those traits resulted in the release of two culti-

vars with enhanced resistance to salt (IRRI, 1997). The genetic parameters of rice salt tolerance have been confirmed for a few morphological and physiological traits such as shoot length, root length, shoot fresh weight, shoot dry weight and shoot Na⁺/K⁺ concentration in saline soils (Yeo and Flowers, 1984; Pearson *et al.*, 1996).

Salinity screening studies vary quite drastically at both spatial and temporal levels depending on objectives (Grattan and Grieve, 1999). Some studies examine response at the molecular level while others examine response at the field or regional level (Gu *et al.*, 2005; Kamoshita *et al.*, 2001; Zhang *et al.*, 2005). The entire duration of some experiments may be minutes, as in short-term uptake studies using isotopes, while others may span seasons (Grattan and Grieve, 1999).

Introgression of *Japonica* rice cultivars with *Indica* rice cultivars to overcome the narrow gene pool is well practiced in broad areas of development of stress tolerant rice cultivars. Because of independent domestication events for *Indica* and *Japonica* subspecies for centuries of farmer selection, certain groups of alleles have been brought together in what are known to be traditional varieties. For example, tropical *Japonica* rice varieties can be characterized as having thick, dark green leaves, negative phenol reaction, thick roots, and limited tillering (Saxena, *et al.* 2002). Further, *Indica* rice cultivars named *Pokkali* and *Nona-Bokara* are well-known to be salt tolerant with high heritability values (Gregorio and Senadhira 1993) but it is considered that salt tolerance is co-inherited with other undesirable agronomical characters (Heu and Koh 1991).

In the present study we tested salinity tolerance of 163 inbred lines of F₆ generation derived from a cross between *Indica* and *Japonica* parents. The *Indica* parental cultivar, Hokuriku-142 (Hokuriku) and *Japonica* parental cultivar, Hyogokithanishiki were genetically divergent types, which would provide enough

segregation for both molecular marker alleles and agronomically important genes. None of the parents were reported to be salinity tolerant previously. The objective of this study was to investigate the effectiveness of new breeding materials for rice salt tolerance through genetic recombination between *Indica* and *Japonica* rice.

Currently available screening methodologies for salinity have their own limitations. In the present study two methods and several parameters were used to evaluate salinity tolerance in two parental rice cultivars by laboratory experiments and the methodology was confirmed by applying to the inbred line population derived from them.

MATERIALS AND METHODS

Genotypes used in the study: A recombinant inbred line (RIL) population (F_6 generation) comprising of 163 lines derived from a cross between cold tolerant *Japonica* rice cultivar, Hyogokithanishiki, and a cold susceptible *Indica* rice cultivar, Hokuriku was used. Hokuriku was bred from a cross between a Korean cultivar, Milyang 21 and an IRRI line IR-2061-214-31 at the Hokuriku Agricultural Experimental Station, Japan. Inbred line populations were advanced according to the single seed descent method and were selfed at each generation.

Evaluation of parental cultivars for salinity tolerance: Salinity tolerant levels of the two parental cultivars were evaluated by growing them in solutions with different levels of electrical conductivity (EC) and also by growing plants on MS media (Murashige and Skoog 1962) with different concentrations of NaCl under aseptic conditions.

Growing plants in different levels of EC solutions: Seeds of the parental cultivars were surface sterilized, germinated and grown in 1×10^{-6} Hyponex (N:P:K: 6:10:5, Toyoba, Japan) solution. The experiment was repeated three times in plastic basins, which had upper vessels to keep the plants upright and holes in

the bottom of the upper vessels to get 1ppm Hyponex solution from 2 liter capacity lower basin. Hyponex solution was refreshed every other day for 12 days.

Electrical conductivity calibration curve for NaCl was prepared first using the same source of water and NaCl used in the experiment. Then Hyponex solutions were prepared with a series of EC values from EC 1 to EC 14 by adding NaCl. Twelve-day-old plants were subjected to salinity stress in EC solutions for 8 days. A control experiment was carried out in 1×10^{-6} Hyponex solution. Three replicates were arranged for each EC level and each replicate contained ten plants. During the stress period, Hyponex solution was renewed every other day. After salinity stress, plants were allowed to recover in 1×10^{-6} Hyponex solution for 5 days. On the 5th day of the recovery period, length of green shoot, length of the longest root, dry matter weight of roots and shoots were recorded.

Growing plants on MS media with different concentrations of NaCl: Seeds of parental cultivars were surface sterilized and germinated under aseptic conditions. Germinated seeds were planted in MS basal medium and maintained for 12 days. MS media were prepared with a series of NaCl concentrations from 25 mmol/l to 400 mmol/l, creating 10 different levels of salinity stresses for the plants. Twelve-day-old seedlings were transferred to salinized MS media and maintained for 8 days at $26 \pm 2^\circ\text{C}$ with a 12-h photoperiod ($160 - 170 \text{ mmol m}^{-2} \text{ s}^{-1}$) from daylight fluorescent tubes (Philips TLD 38/54). Ten replicates for each treatment were arranged with 3 plants for each replicate. Then plants were given a 6-day recovery period in tap water (Fig.1). Green shoot length immediately after salinity stress, root length of plant immediately after salinity stress, green shoot length of plant after recovery period, root length of plant after recovery period and dry matter weight of plant after recovery period were collected for comparison of level of salinity tolerance in two parental cultivars, Hyogokithanishiki and Hokuriku.

Evaluation of salinity tolerance of inbred lines derived from Hyogokithanishiki and Hokuriku : Sixty seedlings of each inbred line were planted according to a complete random-

ized design and the experiment was repeated three times in plastic basins, which had upper vessels to keep the plants upright and holes in the bottom of the upper vessels to get 1×10^{-6}

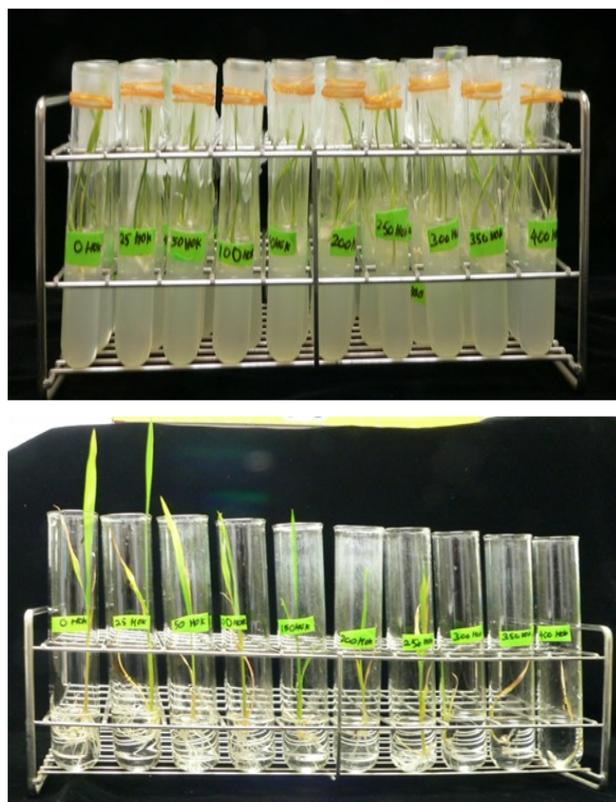


Figure 1: Figure 1 Evaluation of parental cultivars for salinity tolerance on MS medium with different concentrations of NaCl. A: Seedlings under stress conditions on MS medium, B: Seedlings at the end of the 5 day recovery period in water. From left to right, Hyponex solution (1×10^{-6}) supplemented with 0, 25, 50, 100, 150, 200, 250, 300, 350, 400 mmol/l NaCl.

Hyponex solution from 2 liter capacity lower basin. Salinity stress (5 dS/m) was applied over a 8-day period for 12 day old plants. After salinity stress, plants were allowed to recover in 1×10^{-6} Hyponex solution for 5 days. On the 5th day of the recovery period, length of green shoot, length of the longest root, dry matter weight of roots and shoots were recorded.

RESULTS AND DISCUSSION

EC solution method: Hyogokithanishiki and Hokuriku recorded similar green shoot length up to EC 2 dS/m (Fig.2). At salinity levels greater than EC 3 dS/m Hyogokithanishiki recorded significantly higher green shoot length. This difference in the two cultivars was most

evident between EC 5 dS/m and 9 dS/m (Fig. 2).

At different EC solutions, up to EC level 2 dS/m, green shoot length of both Hokuriku and Hyogokithanishiki was almost the same but from EC level 3 dS/m Hokuriku plant height was decreased while Hyogokithanishiki could maintain higher green shoot length (Fig.2). Root length of both Hyogokithanishiki and Hokuriku decreased from 2 dS/m to 14 dS/m

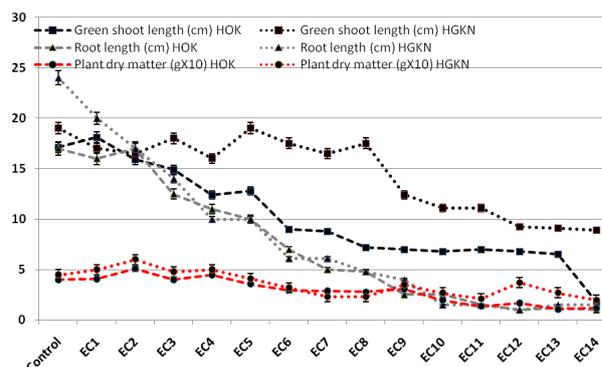


Figure 2 ; Green shoot length (cm); root length (cm); plant dry matter weight (g) of seedlings that survived at the 10th day of recovery after a 8-day salinity stress from 1 dS/m to 14 dS/m (n=20, denoted EC1 – EC14). Bars indicate standard errors. HGKN: Hyogokithanishiki, HOK: Hokuriku.

(Fig.2). Both cultivars maintained relatively high plant dry matter weight up to EC level 4 dS/m. Plant dry matter weight of Hokuriku was drastically reduced from 4 dS/m, whereas in Hyogokithanishiki, a significant dry matter reduction was observed from EC level 5 dS/m. From EC level 6 dS/m, Hyogokithanishiki dry matter production was also decreased and from EC level 9 dS/m onward both cultivars showed low level of dry matter production (Fig. 1). The highest difference between two rice cultivars in dry matter production was recorded at 12 dS/m (Fig. 2). Gregorio and Senadhira (1993) also selected 12 dS/m EC level for salinity tolerance studies in rice for 14 day old seedlings after manipulating EC level by adding a 16:1 mixture of NaCl and CaCl₂ to the nutrient solution which is in agreement with the threshold level achieved in the present study.

Effect of salinity on rice and tolerance levels in rice cultivars have been studied in hydroponic culture systems with 60 mol/m^3 NaCl (Gu *et al.* 2000). They ranked cultivars for salt injury, Na⁺ content in shoot, ratio of shoot fresh

weight and dry matter weight at seedling stage and different agronomic traits in control and salt-treated plants at mature stage (Gu *et al.* 2000). The physiological changes in salt stressed rice are directly impaired on overall growth such as shoot height, fresh weight and dry weight (Aslam *et al.* 1993; Chowdhury *et al.* 1995; Sohn *et al.* 2005; Khan and Panda, 2008).

MS medium method: Hyogokithanishiki recorded significantly higher green shoot length and root length after recovery period in MS basal medium compared to Hokuriku (Fig. 3). When the green shoot length was measured immediately after the stress treatment, Hyogokithanishiki recorded higher green shoot length up to 100 mmol/l NaCl stress, but when the salinity stress was increased further, Hokuriku recorded higher green shoot length than Hyogokithanishiki (Fig. 3). Thach and Pant (1999) also reported a significant decrease in relative growth based on fresh weight when 14-day old seedlings were shifted into test tubes containing the nutrient solutions with different NaCl concentrations. The same has been reported by Cha-um *et al.* (2009) where the less growth reduction percentage in 342 mmol NaCl stressed was observed in salinity tolerant KDML105 compared to salinity susceptible HJ seedlings. Wani and Gosal (2011) reported the existence of the same phenomena in salinity tolerant gene transformation work

In the experiment carried out to evaluate level of salt tolerance of two rice cultivars on MS medium, immediately after NaCl stress, Hokuriku cultivar showed significantly higher green shoot length than Hyogokithanishi at higher NaCl levels (>150mmol/l), but after the recovery period in water, Hyogokithanishiki showed significantly higher green shoot length (Fig. 3). This could not be seen in root length. At both stages, immediately after salt stress and after recovery, Hokuriku reported shorter roots than Hyogokithanishiki (Fig. 4). This could be due to varietal X tissue culture medium interaction. In the control treatment, Hokuriku in MS medium recorded higher dry matter weight than Hyogokithanishiki (data not shown). Therefore, the tissue culture-based approach we tested is not sufficiently reliable to evaluate rice cultivars for salinity stress tolerance due to varietal X medium interactions give false results to-

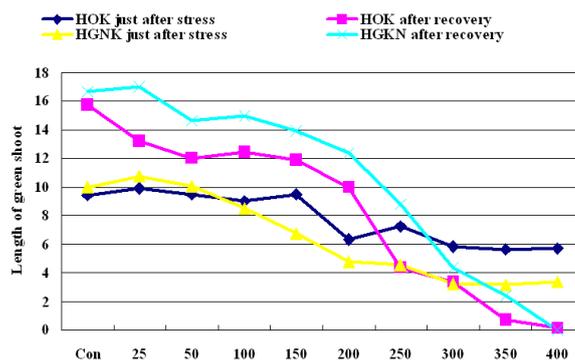


Figure 3. Green shoot length of seedlings that survived after salinity stress imposed using NaCl in MS medium. HGKN: Hyogokithanishiki, HOK: Hokuriku.

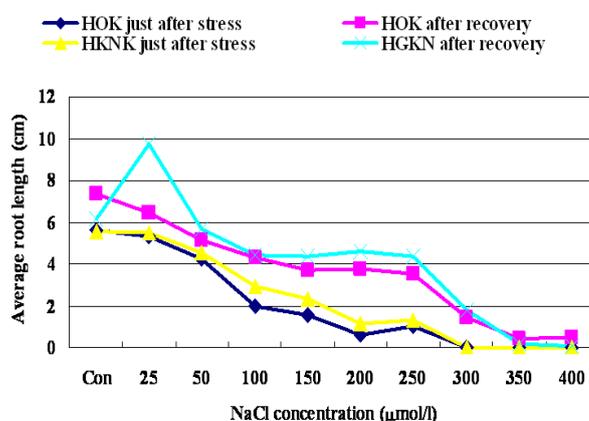


Figure 4. Root length of seedlings that survived the salinity stress at different NaCl concentrations in MS medium. HGKN: Hyogokithanishiki, HOK: Hokuriku.

wards stress tolerance. In the study carried out by Vajrabhaya *et al.* (1989), the salinity tolerant selection was done at 2- 4 week old callus, in the callus induction medium with 2% NaCl concentration. They had not reported a variety X medium interaction. However, rice variety X medium interactions have been reported in many other studies (Rahim *et al.* 1991; Nguyen and Zapata 1993; Islam *et al.* 2005).

According to the ANOVA results, none of the parameters studied using MS medium recorded significant differences between the two rice cultivars (Table 1). The reason why it did not show a significant difference between the parental cultivars for root length under different EC stresses would be that 8 day period in NaCl solutions was not enough to show that much difference. In addition, the initial root system was not damaged before the application of salinity stress to evaluate the length of the long-

est root. Nevertheless, solution culture studies are beneficial since they have advanced our understanding of crop salt-tolerance and have provided insight into physiological mechanisms responsible for nutrient uptake and dis-

Table 1: ANOVA results of all parameters collected from salinity stress treatment

Method	Parameter	P value
EC solution method	Green shoot length	0.006645
	Root length	0.308319
	Plant fresh weight	0.03743
	Plant dry matter weight	0.061928
MS medium method	Shoot length immediately after stress	0.271575
	Shoot length after recovery	0.4338
	Root length just after stress	0.761256
	Root length after recovery	0.6909
	Plant dry matter weight	0.106916

crimination (Gratten and Grieve 1999). Further in solution cultures, concentrations of salts and nutrients are easily controlled over the course of an experiment. Plant responses and interactions observed in artificial media may not necessarily occur as they would under natural conditions. However, solution culture studies are beneficial since they have advanced our understanding of crop salt-tolerance.

Considering literature on solution culture and our own results, salinity level corresponding to EC 5dS/m was decided as the most suitable EC level to apply for the evaluation of inbred line population and green shoot length was used in the bioassay. Under 5 dS/m salinity stress for 8 days, 64 out of 163 inbred lines (39.2 %) dried completely and 21 inbred lines (12.9%) recorded longer green shoot length than salinity tolerant Hyugokithanishiki (Fig. 5). Salinity susceptible parent Hokuriku did not dry completely and scored 12 cm green shoot length (Fig. 6). The most salinity tolerant lines scored 35 cm green shoot length compared to 18 cm in the salinity tolerant parent. Transgressive segregation of the population in both directions was observed indicating that the salinity tolerance of this population is controlled by both parental alleles. Each pair of replicate in salinity tolerant screening of inbred line population showed positive correlations bringing evidence on the consistency of bio assay data.

CONCLUSION

Green shoot length of the salinity stressed Hyugokithanishiki plants showed a significant

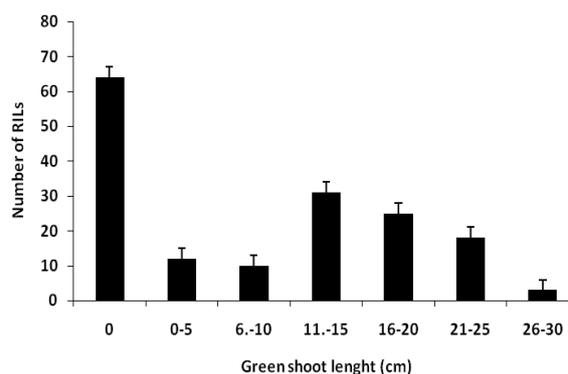


Figure 5 Frequency distribution of the RILs after salinity stress at 5 dS/m. Data was taken on the 10th day of the recovery period after 8-day salinity stress. The experiment was repeated three times.

difference from Hokuriku in EC solution method. EC level 5 dS/m recorded the greatest difference among the parent cultivars, and selecting this level of salinity to screen the inbred line population derived from them enabled us to differentiate the lines according to their tolerance level. Frequency distribution of inbred line population shows that the method applied to evaluate the salinity tolerance is reliable and practically applicable to screen large populations. The results of the bio assay can be directly used for salinity tolerant QTL analysis in future.

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