ARTICLE

Responses of rice (*Oryza sativa* line LX278) calli and seedlings to salinity treatment: Towards the development for salt tolerance

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Saline soil is a major problem that affects growth and yield of rice, the staple food in the Philippines; hence, there is a need to develop a local variety that can thrive under this condition for sustainable production. The goal of the study is to obtain baseline information regarding the responses of rice (*Oryza sativa* line LX278) calli and seedlings to salt. This is important prior to genetic transformation so that any improvement in growth performance, after transformation, could be attributed to the introduced salinity tolerance gene. Results showed that the mean fresh weight of LX278 rice calli exposed to various concentrations of NaCl, ranging from 50mM to 175mM, significantly decreased as the concentrations of NaCl increased compared to the control. Prolonged exposure of up to a month with 150 mM NaCl and 175mM NaCl restricted and inhibited, respectively, the growth of calli. Two-way ANOVA (analysis of variance) showed that the effect of the interaction of salt concentration and the duration of exposure on mean fresh weight of calli was highly significant. Meanwhile, LX278 rice seedlings in hydroponics, exposed to NaCl of EC=12 dSm^{-1}, exhibited a salt sensitive response, relative to Pokkali, the tolerant control, and IR 29, the highly susceptible control, 16 days after initial salinization using the rating system based on visual symptoms of salt toxicity. Results of the present study agree with earlier findings that rice tolerates salinities of up to 30mM ~ 3dS m^{-1} and, beyond this, growth is compromised because of osmotic and ionic effects.

KEYWORDS

salinity tolerance rating based on visual symptoms of salt toxicity, *Oryza sativa* line LX278, mean fresh weight of calli, saline soil, seedlings in hydroponics with NaCl of EC=12dS m^{-1}

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INTRODUCTION

Saline soil is one abiotic factor that decreases the yield of rice, the staple food of almost half of the world's population. In the Philippines, about 500,000-600,000 hectares of coastal land, which is an integral component of agricultural area, are saline-prone and are distributed as follows: Luzon 180,000 ha, Visayas 220,000 ha and Mindanao 160,000 ha (FAO AGL 2000). Of these, 200,000 hectares are seriously salt-affected producing only 20 - 40 cavans of rice/ha in extreme cases compared to 100 cavans/ha in favorable areas (Bonilla et al 1998).

Among cereals, corn and millet are considered moderately salt-sensitive, oat, sorghum and wheat are moderately tolerant while barley, rye and triticale are salt- tolerant (USDA-ARS 2005). Rice has been reported in the literature as salt-sensitive (Lafitte et al 2004, Seetharam et al 2009) although within the rice species, the level of salt resistance varies. For example, the indica variety, Pokkali, is classified as highly salt-tolerant (Djanaguiraman et al 2003, Mohammadi-Nejad et al 2008), IR64 is tolerant (Mohammadi-Nejad et al 2008), IR74 is moderately tolerant (Gregorio et al 1997), while IR28 (Mansuri et al 2012) and the majority of high-yielding cultivars such as IR29, IR36 and IR72 are salt-sensitive (Moons et al 1995).

To increase harvest in salinity-affected areas, there is a need to develop a variety of rice with enhanced tolerance to salt stress. Several strategies have been proposed to achieve this, including conventional breeding programmes, the use of in vitro selection, pooling physiological traits, interspecific hybridization, using halophytes as alternative crops, the use of marker-aided selection, and the use of transgenic plants (Flowers 2004). The long-term goal of this research is to develop a salt-tolerant rice by genetic transformation specifically by particle bombardment. Line LX278 rice, which is capable of callus formation and plant regeneration (Evangelista et al 2009), will serve as the future recipient of the *hva*1 gene, a salt tolerance gene from barley. Once transformed, LX278 rice, an elite line from a japonica/indica cross, could serve as a breeding parent to transfer the *hva*1 gene either to an indica or japonica rice by conventional breeding. Through this approach the effect of salinity on rice productivity will be lessened.

This paper is the first report on the responses to salinity of LX278 rice calli and seedlings in hydroponic system prior to transfer of the *hva*1 gene. Specifically, the effects of varying concentrations of NaCl on the fresh weight of LX278 calli, different lengths of exposure to NaCl, and the interactions of these 2 factors were evaluated. The response of LX278 rice seedlings to NaCl of EC (Electrical Conductivity) = 12 deciSiemens per meter (dS m^{-1}) was also assessed. Results of the present study will provide baseline information regarding the susceptibility or tolerance of LX278 rice calli and seedlings to NaCl so that any improvement after transformation could be attributed to the effect of the introduced foreign gene.

MATERIALS AND METHODS

Callus induction, proliferation and NaCl treatment

Mature seeds of LX278 were dehulled and surface sterilized for 1 min with 70% ethyl alcohol then twice with 50% sodium hypochlorite for 30 min each. Afterwards the seeds were rinsed thrice with sterile distilled water and plated in culture bottles. The set-ups were kept in the dark at 20°C to induce and proliferate callus. Calli were subcultured 3 times. Following this, all the calli were pooled and randomly distributed with 10 callus clumps per bottle. Calli were grown in RS medium (Philippine Rice Research Institute Laboratory Manual, 1999) with or without NaCl supplement. Concentrations of NaCl used were 50mM, 100mM, 150mM and 175mM. Every treatment consisted of 20 replicates. For each treatment, the total fresh weight of 10 calli contained in a culture bottle was taken each week for 5 weeks and the mean weight of 20 replicates was determined and recorded. Mean fresh weight of calli at different salt concentrations was analyzed and compared by One-

Table 1. Salinity tolerance scores based on visual symptoms of salt injury (Gregorio et al 1997)

Score	Observations	Tolerance
1	Normal growth, no leaf symptoms	Highly tolerant
3	Nearly normal growth, but leaf tips or few leaves whitish or 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

way ANOVA (analysis of variance) and Duncan's Multiple Range Test at α=0.05 using the SPSS program. The effect of the different salt concentrations and duration of exposure to salt on mean fresh weight of calli was also determined by two-way ANOVA at α=0.05.

Salinity screening of LX278 seedlings

The response to NaCl (EC=12 dSm^{-1}) of LX278 seedlings was evaluated according to the Standard Evaluation System developed by IRRI, using the procedure of Gregorio et al (1997). Test seeds were heat-treated for 5 days in a convection oven set at 50°C to break seed dormancy. The seeds were then surface sterilized and rinsed well with water. The sterilized seeds were germinated in a Petri dish lined with moist filter paper at 30°C. After 48 hrs, the radicle of each pre-germinated seed was placed in each hole of a styrofoam seedling float. The seedling floats were placed inside the greenhouse under natural environmental conditions. Initially, the seedlings were grown in water. Four days later, the water in the trays was replaced with salt-amended Yoshida nutrient solution (Yoshida et al 1976). The culture solution was maintained at pH 5.0 daily. Approximately 3g of NaCl was added per liter of nutrient solution to obtain an initial salinity of EC=6 dS m^{-1}. After three days, salinity was increased to 12dSm^{-1} by adding NaCl to the nutrient solution and was replenished with a fresh solution of the same salinity 8 days afterwards. The initial and final salinity tolerance ratings were performed on the 10th and 16th day, respectively, after initial salinity treatment.

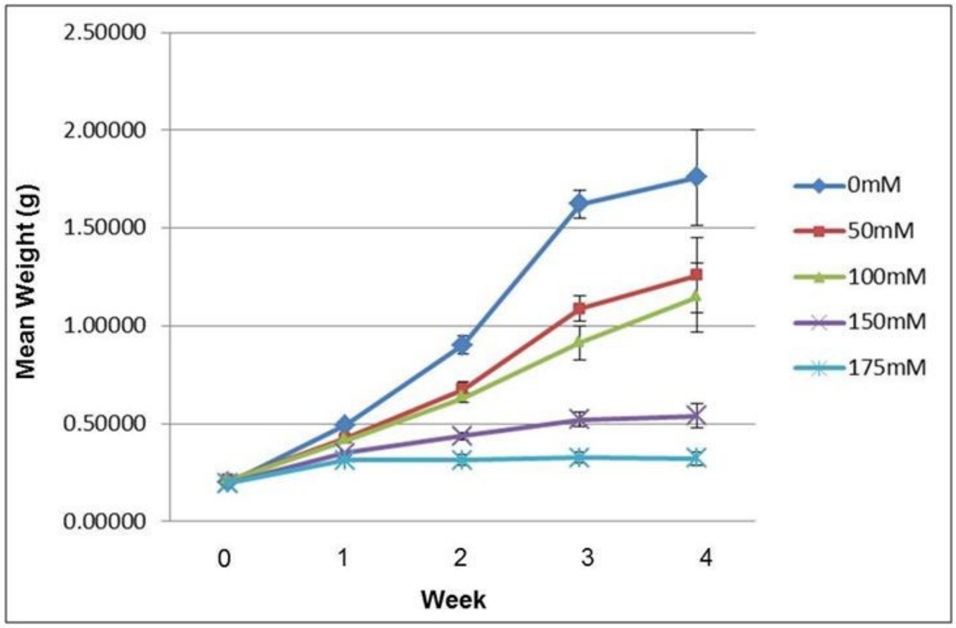

Figure 1. Weekly changes on mean fresh weight of LX278 calli exposed to different salt concentrations (NaCl). Vertical bars represent standard error of the mean.

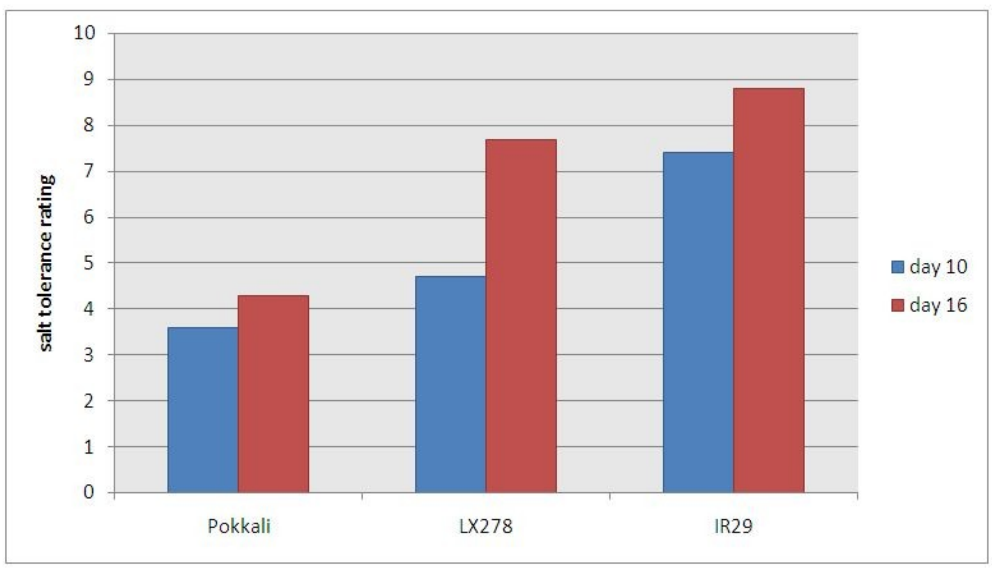

Figure 2. Salt tolerance rating based on visual symptoms of toxicity of LX278 rice seedlings on days 10 and 16 after initial salt treatment in comparison to Pokkali, the salt-tolerant control, and IR29, the salt-sensitive control

Two check varieties were used as a guide in rating the visual symptoms of LX278 to salinity stress: IR29, salt-sensitive control, and Pokkali, tolerant control. The check varieties were placed on both ends while LX278 were placed in between them in a seedling float that had 10 rows with 10 holes each. Ten holes were used for each check variety and LX278. The pH of the solution was also maintained daily at 5.0.

LX278 and each kind of check variety were given a rating using the ten seedlings in a column contained in a seedling float. The mean score for 32 replicates was taken as their salinity tolerance rating. The scoring system is given in Table 1.

Figure 3. Morphological response of LX278 rice seedlings to NaCl at EC= 12 dS m^{-1} 16 days after initial salt treatment in comparison to control checks Pokkali (PK) and IR29.

RESULTS

Response of rice calli to different concentrations of NaCl

Growth of LX278 rice calli was affected by different concentrations of NaCl and resulted in lower mean weight of calli compared to the control. As shown in Figure 1, the mean fresh weight taken at weekly intervals was higher at lower salt concentrations and vice versa. In particular, the mean fresh weight of the calli for the duration of the study was greatest in those exposed to 50mM NaCl, the lowest concentration used, and least in those under 175mM NaCl, the highest concentration used. One-way ANOVA at α=0.05 with Duncan's Multiple Range Test as post-hoc showed no significant difference in the initial weights of the calli so subsequent differences noted could only be attributed to NaCl treatments. In a span of one week the mean weight of calli grown in 50mM and 100mM NaCl were significantly lower than those of the control and were significantly higher than those calli exposed to 150mM NaCl or 175mM NaCl. From the second week onwards the mean weights of the calli were significantly different from the control and from each other except for those in the 50mM and 100mM NaCl which did not vary significantly from one another. Compared to the control, longer contact with salt (NaCl) of any concentration resulted in lower mean weight. One-month exposure to 150mM NaCl concentration restricted growth of calli. Specifically, weekly gain in mean weight of calli decreased from 79.6% on the 1st week of exposure to 150mM NaCl, to 24.2% on the 2nd week, 19.2% on the 3rd week and only 3.1% on the 4th week. Prolonged exposure with 175mM of NaCl inhibited growth of the calli. Initially, a 60% increase in mean fresh weight was observed on the 1st week of exposure to salt but growth slowed down to 1% and 3%, after the 2nd week and 3rd week, respectively. Growth totally stopped (-1.5%) after one month's exposure because of hypertonic medium. Necrosis was evident in calli that experienced prolonged exposure to 150mM NaCl and 175mM NaCl. Two-way ANOVA results showed a p value of 0.000 indicating that the effect of the interaction of salt concentration and the duration of exposure on mean fresh weight of calli was highly significant (α=0.05).

Response of LX278 rice seedlings to NaCl of EC=12 dS m^{-1}

Figures 2 and 3 summarize the effect of NaCl of EC=12 dS m^{-1} on LX278 rice seedlings and the 2 controls. The IR29 seedlings, the susceptible control, were the most affected by NaCl. On day 10, IR29 seedlings had a mean tolerance rating of 7.4, a susceptible response. The seedlings were short, 5-6 out of 10 seedlings on each float had dry leaves and were dying. By day 16, 9-10 of the seedlings on each float were dead (mean score of 8.8), the roots were underdeveloped and were brown in color, a highly susceptible response. In comparison, Pokkali seedlings showed tolerant response to salt on day 10 (mean tolerance rating of 3.6); the 10 seedlings on each float exhibited normal height, although 3-5 of them had rolled leaves and/ or scorched tips or margins. This response was maintained by Pokkali on day 16, but more than half of the seedlings (6-8) had affected leaves (average score of 4.3). On day 10, LX278 seedlings exhibited moderately tolerant response with 6-8 of them demonstrating reduced growth, rolled leaves and bleached tips (mean score 4.7) on each seedling float. By day 16, the seedlings displayed a susceptible response with 6–8 of them dying and most of their leaves have dried (mean score of 7.7).

DISCUSSION

Salinity tolerance within the rice species is genotype-dependent, thus, it is important to determine the level of tolerance to salt of LX278 rice prior to implementation of crop improvement program. The seedlings of LX278 were salt-sensitive based on a rating system using visual symptoms of salt injury 16 days after initial salinity treatment as shown in Figure 2. They were susceptible to NaCl of EC=12 dS m^{-1} (mean score of 7.7) while Pokkali, one of the controls, maintained tolerant reaction to salt (average score of 4.3). The response to salt of LX278 seedlings was more similar to IR29, the other control, although IR29 was relatively more salt-sensitive (mean score of 8.8). Seedlings of LX278 displayed stunted growth, and most of them were dying 16 days after initial salinity treatment as reflected in Figure 3.

The response of rice whole plants to salinity changes during its life cycle. It is comparatively tolerant of salt stress during germination, active tillering, and towards maturity and is sensitive during early seedling and reproductive stages (Lafitte et al 2004). Seedlings were used to determine salinity tolerance level of LX278 rice, in the present study, because any difference in level easily becomes apparent during this stage.

The results demonstrate that mean fresh weight of LX278 rice calli was affected by increasing NaCl concentrations. From the first week onwards, compared with the control, mean fresh weight of the calli was significantly lower upon exposure to 50, 100, 150 and 175 mM of NaCl. Longer duration of exposure to 50mM and 100mM NaCl decreased growth of calli relative to the control. Pronounced decrease in growth of calli was observed with prolonged exposure with 150mM NaCl and inhibited those in 175mM NaCl.

Both rice calli and seedlings were negatively affected by NaCl, the calli of LX278 at 50mM (roughly equivalent to 5 dS m^{-1}) up to 175mM (roughly equivalent to 17.5 dS m^{-1}) and whole plant at 12dS m^{-1}. These results are consistent with earlier findings that rice is highly susceptible to saline soil, i.e., soil with high concentrations of soluble salts and has an EC= 4 dS m-1 or more (USDA-ARS 2008), and generally only tolerates salinities ranging from 1.9–3 dS m^{-1} (Grattan et al 2002), comparable to a concentration of around 20–30 mM NaCl. Beyond this range, growth of rice is compromised. Under natural conditions, areas with salinities of 12 dS m^{-1} or higher are unsuitable for rice production (Karim et al 1990). At 8-16 dS m^{-1}, considered highly saline region, only tolerant plants grow satisfactorily and above 16 dS m^{-1}, regarded as extremely saline area, only a few very tolerant plants grow adequately (Mitin 2009).

Salinity is said to decrease growth through osmotic effects, toxic levels of Na^+ and Cl^- in the cell and negative effects on K^+, Ca^{2+}, and NO_3^- nutrition (Flowers and Colmer 2008). The response of plants to salinity occurs in 2 phases through time (Munns and Tester 2008). Osmotic stress has an immediate effect on growth while ionic stress impacts on growth much later. In the first phase, LX278 rice experienced water stress as a result of higher osmotic concentration in the medium leading to lower fresh weight of calli and shorter seedlings. In the second phase, toxic levels of Na^+ and Cl^- in the calli led to necrosis and in seedlings resulted in premature senescence of leaves.

Salinity could rise to 100 mM NaCl, about 10 dS m^{-1} in the field (Munns et al 2006). Thus, there is a need to develop a rice variety that could thrive under this condition if the country is to sustain rice production. However, as shown in the results, LX278 rice calli and seedlings showed poor growth performance in salty condition. In the next study, *hva*1, a salt tolerance gene from barley will be introduced by particle bombardment into LX278 rice to improve its growth and yield in saline soil.

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CONFLICTS OF INTEREST

No conflict of interest

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

Lead author: Fredeslinda C. Evangelista- did the experiment, wrote the manuscript Second author: Rhodora Aldemita- served as co-adviser of the lead author Third author: Lilian B. Ungson- served as adviser of the lead author

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