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Asian Journal of Plant Science and Research, 2013, 3(6):20-25

ISSN : 2249-7412
CODEN (USA): AJPSKY

A study on effect of salt stress in the seed germination and biochemical parameters of rice (*Oryza sativa* l.) under *in vitro* condition

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ABSTRACT

In the present study deals with the Oryza sativa .L was selected for callus induction and regeneration in in vitro condition and also the physiological and biochemical studies were carried under the different salinity levels. The different concentration of 2, 4-D were used to induce callus in the mature seeds and the callus was developed from the region of the seeds and was visible within 7-10 days. The salt stress significantly influenced the germination percentage was reduced gradually with increasing salt stress from 0 to 300 mM NaCl. The rate of proline accumulation was observed much higher (205.4 mg/g) at 300 mM NaCl stress as compared to control (70.15). A continuous decrease in protein content with increase in salt stress was observed, where around 64% reduction was observed in plants under 300 mM NaCl stress as compared to the control plants. The starch content was higher in control (617.2) than sample (345.5) with salinity stress. Among this study investigated that the overall showed better tolerance to salt stress with a lesser extent of antagonistic effect of NaCl on germination and biomass production at seedling stage.

Key words: Salt stress, Seed germination, *Oryza sativa*. L, Proline and Callus

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INTRODUCTION

Rice (*Oryza sativa L*) is the most important food crop in the world and feeds over half of the global population. In Asia where it covers half of the arable land used for agriculture in many countries. More than 90% of rice is produced and consumed in Asia and it is the only major cereal crop that is consumed almost exclusively by human. India is the largest rice growing country accounting for about one-third of the world acreage under the crop. About 84% of rice production growth has been attributed to the use of modern technologies.

Rice is one of the most important world food crops, serving as the staple food for over one-third of the world's population (Khush, 1997). It is one of the most widely grown crops in coastal areas frequently inundated with saline sea water during high tidal period (Mori and Kinoshita, 1987). Salinity is considered as one of important physical factors influencing rice production. At the present, salinity is the second most widespread soil problem in rice growing countries after drought and is considered as a serious constraint to increased rice production worldwide (Gregorio, 1997). There exists tremendous variation for salt tolerance within species in rice, providing opportunities to improve crop salt-stress tolerance through genetic means.

Various abiotic stresses including high or low temperature, water scarcity, high salinity and heavy metals exert drastic antagonistic effects on crop metabolism and thereby plant growth, development and ultimately crop productivity. Amongst these, soil salinity is a major factor limiting the crop production globally (Kumar *et al.*, 2010). Soil salinity affects large areas of the world cultivated land causing significant reductions in crop yield (Tavakkoli *et al.*, 2011). In Asia alone, 21.5 million ha of land area is thought to be salt-affected, with India having 8.6 million ha of such area which constitutes a major part of problem soils in India (Sahi *et al.*, 2006). However, improvement in salt tolerance of crop plants remains elusive, due to the fact that salinity affects almost every aspect

of the physiology and biochemistry of plants at both whole plant and cellular levels. Generally, soil salinity affects plants through osmotic effects, ion-specific effects, and oxidative stress (Pitman and Lauchli, 2002).

In India and especially in coastal rice fields of Tamil Nadu state, soil salinity is a major stress that reduces the rice productivity to a great extent (Kumar *et al.*, 2008). Salinity is detrimental to the various processes of crops such as seed germination, seedling growth and vigor, vegetative growth, flowering and fruit set and ultimately it causes diminished economic yield and also quality of produce (Sairam and Tyagi 2004). In response, plants have developed a number of mechanisms to counteract high salt stress such as mineral ions homeostasis and accumulation of compatible solutes such as proline. Moreover, salt stress responses of plants may depend upon salt type, concentration, and genotype. Therefore, screening for salt-stress tolerant genotypes in important crops such as rice will help in ensuring future crop production. In addition, studying differential responses of genotypes with contrasting stress tolerances will help reveal the underlying salt stress tolerance mechanisms (Kumar *et al.*, 2008).
Soil salinity is one of the main obstacles to increasing rice production. Since rice (*Oryza sativa*) is rated as an especially salt sensitive crop (Shannon *et al.* 1998), most of the modern high-yielding rice cultivars perform poorly in saline environments. In addition, as saline soils are usually waterlogged, it is not feasible to grow crops other than rice in such areas. The rice germ plasm has genetic variability for salt tolerance (Xie *et al.* 2000), Plants are exposed to many types of environmental stresses such as salt, drought, and freezing. Among these stresses, salinity is one of the major factors that limit crop production world-wide. Salt tolerance of plants is a complex trait that is controlled by a number of salt stress-responsive genes (Zhu 2000; Ueda *et al*. 2002).

The present investigation was aimed to study the comparative effects of NaCl stress towards germination, plant growth and various biochemical parameters including total proteins, sugars and carbohydrates, starch and proline accumulation in rice genotypes and also determine the most suitable concentration and combination of growth regulators for improvement in calli induction and *in vitro* plant regeneration efficiency in rice.

MATERIALS AND METHODS

Collection seeds

The local (Tamil Nadu, India) scented rice (*Oryza sativa* L.) was selected for this study. Certified seeds were collected from local market Thanjavur, Tamil Nadu, India.

Callus induction

Collected seeds were surface sterilized with 70 % (v/v) ethanol for 1 min and 0.1% (w/v) mercuric chloride for 10 min and then washed several times with sterile distilled water (Ramesh and Gupta, 2005). The seeds were induced on Petri dishes (10 cm) containing MS (Murashige and Skooge, 1962) media containing basal salts, 3% sucrose (30 g L-1) as a source of carbon, 0.6% (6 g L-1) agar, different concentration of 2,4-D (2,4- Dichlorophenoxy acetic acid) and the NAA:BAP. The combination of these hormones produced the shoots and roots. The pH of the media was adjusted to 5.8 with 1N NaOH and 1N HCl using electronic pH indicator. The media was autoclaved at a temperature of 121°C and pressure of 15 Ibs psi for 20 min. The medium filled plates were kept in laminar airflow cabinet and were subjected to UV light for 20 min.

Salt treatments

The experiment was conducted in laboratory at the room temperature with 12 h daylight. Every day 5 ml of distilled water (with or without varying NaCl levels, i.e. 50, 100, 150, 200 and 300 mM) was applied per Petri dish and all the observations were recorded on the 21st day after germination. The physiological and biochemical studies were carried under the different salinity levels and all the experiments were carried out in triplicate.

Plant growth analysis

Total germination was expressed as a percentage of the control for each variety. The germination percentage, root length, shoot length, and root/shoot ratio of seedling were recorded on the 21st day of germination.

Biochemical analyses

Determination of proline content

Free proline content was estimated by following the method of Bates *et al.* (1973). Fresh 0.5 gm root and shoot samples were homogenized in 5 ml of 3% sulpho salicylic acid using a mortar and pestle. About 2 ml of extract was taken in test tube and to it 2 ml of glacial acetic acid and 2 ml of ninhydrin reagent were added. The reaction mixture was boiled in a water bath at 100°C for 30 min. After cooling the reaction mixture, 4 ml of toluene was added. After thorough mixing, the chromophore containing toluene was separated and absorbance of red color developed was read at 520 nm against toluene black on UV-visible spectrophotometer (Chemito, UV- 2600). The proline concentration was determined using calibration curve and expressed as mg proline per g fresh weight of tissue.

Determination of total protein content

Proteins were estimated using Lowry *et al.* (1951) method. Fresh samples (250 mg) were homogenized in 2.5 ml of phosphate buffer (pH 7.0).The extract was centrifuged at 5000 g for 15 min at 4°C and the supernatant was transferred to a tube containing a mixture of 20 ml acetone and 14 ml β- Mercaptoethanol for precipitation of protein. The sample tubes were stored at 0°C for 5 h and then centrifuged at 10000 *g* for 20 min. The supernatant was discarded and the pellet was dissolved in 2.5 ml 1 N sodium hydroxide solution. Aliquot of 0.2 ml from this sample was used to prepare the reaction mixture. The intensity of blue color developed was recorded at 660 nm and protein concentration was measured using bovine serum albumin as standard.

Determination of Total Phenols

Total phenol contents were estimated by following Malick and Singh (1980). Total phenols were extracted from 500 mg of fresh roots and shoot tissues separately in 80% (v/v) ethanol and estimated by Folin-Ciocalteau reagent. The absorbance of the reaction was measured at 650 nm wavelength on UV-visible spectrophotometer (Chemito, UV-2600). Total phenols were calculated by using standard graph of catechol.

Determination of reducing and non-reducing sugar levels

Reducing sugars were estimated using Dinitrosalicylic acid (DNSA) reagent by following the method of Miller (1972). Fresh shoots (0.1 g) of plants with or without salt concentrations (0-200 mM NaCl) were homogenized in hot 80% ethanol. The extract was centrifuged at 5000 *g* for 15 min at room temperature and the supernatant was evaporated by keeping in water bath at 80°C and sugars were dissolved by adding 10 ml distilled water. Reducing sugars were estimated by using DNSA reagent colorimetrically at 530 nm wavelength and calculated from graph plotted using glucose as a standard. Non-reducing sugars were estimated using anthrone reagent. Fresh shoots (0.25 g) were hydrolysed separately by keeping in boiling water bath for 3 h with 2.5 N HCl (5 ml) and was neutralized with Na2CO3 after cooling it to room temperature. Volume was made up to 100 ml and centrifuged at 5000 *g* for 15 min at room temperature. Non-reducing sugars were estimated spectrophotometrically at 630 nm wavelength on UV-visible spectrophotometer (Chemito, UV-2600) and calculated from graph plotted using glucose as a standard.

Determination of Starch

Starch was estimated using anthrone reagent by following the method given by Thayumanavan and Sadasivam (1984). Fresh roots and shoots (250 mg each) were separately homogenized in hot 80% ethanol (v/v) to remove sugars. Residue was retained after centrifugation at 5000 x *g* for 15 min at room temperature. The starch was extracted by 52% perchloric acid at 0°C for 20 min. Starch was estimated by using anthrone reagent spectrophotometrically at 630 nm wavelength on UV-visible spectrophotometer (Chemito, UV-2600) and calculated from graph plotted using glucose as a standard.

Statistical analyses

Each Petri dish was considered as replicate and all the treatments were repeated three times and data are expressed as mean and standard error (S.E.). All the statistical analyses were done using SPSS statistical software package.

RESULTS

Callus induction

The morphology of callus is taken as a criterion for selection of callus for regeneration under stress condition. Experiments for callus induction and regeneration were conducted in rice (*Oryza sativa* L). Callus was developed from the region of the seeds and was visible within 7-10 days. The different concentration of 2, 4-D were used to induce callus in the mature seeds. The NAA and BAP were used to the regeneration of shoot and root development (Table.1).

Table 1. Effects of different concentration of NaCl on seed germination and growth parameters in *Oryza sativa. L*

S. No.	NaCl stress (mM)	Germination percentage Mean ±S.E	Root length (cm) Mean ±S.E	Shoot length (cm) Mean ±S.E	Root/Shoot ratio(cm)
1	0 (Control)	100± 1.0	14.5 ±1.2	10.5 ±2.1	1.38
2	50	100± 2.3	12.0 ±1.3	8.1 ±1.2	1.48
3	100	96 ±1.8	11.5± 0.9	7.5 ±1.0	1.62
4	150	88 ±2.2	6.8 ±0.6	3.5 ±0.3	1.75
5	200	68 ±1.7	4.5± 0.3	2.2± 0.2	2.05
6	300	48 ±1.6	1.8± 0.1	0.8 ±0.1	2.20

Table 2. Effects of different concentration of NaCl on proline, protein and phenol content in *Oryza sativa. L*

S. No.	NaCl stress (mM)	Total proline content (mg/g fresh weight) Mean ± S.E	Total protein content (mg/g fresh weight) Mean ± S.E	Total phenol content (mg/g fresh weight) Mean ± S.E
1	0 (Control)	70.15± 2.3	461.7± 3.8	11.5 ±0.4
2	50	77.5 ±1.6	349.4 ±7.6	13.6 ±1.5
3	100	110.7± 3.9	298.5± 5.7	16.4± 2.3
4	150	125.6± 5.4	248.6± 6.5	19.4 ±2.6
5	200	155.8 ±9.4	212.4 ±7.5	28.3 ±2.8
6	300	205.4 ±13.8	184.3± 4.5	32.5 ±2.9

Table 3. Effects of different concentration of NaCl stress on reducing and non-reducing sugars and starch content in *Oryza sativa. L*

S. No.	NaCl stress (mM)	Reducing sugar content (mg/g fresh weight) Mean ± S.E	Non-reducing sugar content (mg/g fresh weight) Mean ± S.E	Starch content (mg/g fresh weight) Mean ± S.E
1	0 (Control)	120.5 ±4.3	124.5 ±5.9	617.2± 25.4
2	50	170.8±7.5	103.5± 3.5	345.5± 16.4
3	100	200.5 ±12.4	67.8 ±4.5	176.3± 12.3
4	150	270.4 ±10.5	34.5 ±3.2	102.5 ±8.5
5	200	283.4 ±10.5	11.4± 0.5	49.5 ±2.4
6	300	290.3 ±10.8	10.3 ±0.6	30.4 ±2.3

Effect of NaCl stress on shoot development

The most critical stage in seedling establishment is usually considered as seed germination which consequently determines the successful crop production. Understanding the responses of plants at these stages is particularly important for elucidating the mechanisms of salt resistance or sensitivity in plants and their survival.

In the present report, NaCl induced salt stress significantly influenced the germination percentage was reduced gradually with increasing salt stress from 0 – 300 mM NaCl (Table1). The results clearly showed that the percentage emergence was reduced by increasing salt levels. However, noticeable difference was evidenced of germination rate. Up to 50 mM NaCl, 100% germination was observed. Increasing salt stress resulted in gradual decrease in shoot and root length with more adverse effects on shoots growth (Table 1).

Effect of NaCl stress on proline content:

Similar responses were observed in the present investigation and salinity stress resulted into a sharp increase in proline content. However, the rate of salt stress-induced proline accumulation was considerably higher. In control plants the proline content was almost similar, however, the rate of proline accumulation was observed much higher (205.4 mg/g) at 300 mM NaCl stress as compared to control (70.15) (Table 2).

Effect of NaCl stress on protein content:

Under control (non-saline) conditions, showed higher protein content than sample (Table 2). However, a continuous decrease in protein content with increase in salt stress was observed, where around 64% reduction was observed in plants under 300 mM NaCl stress as compared to the control plants. On the other hand, the protein content was increased up to 50 mM and even at 300 mM NaCl stress level the reduction in protein content was 64% (Table 2).

Effect of NaCl stress on total phenol content:

The results presented in Table. 3 makes it clear that total phenol content was gradually increased with progressing salt stress, however contrasting results were evidenced lower magnitude of increase in phenol contents in the later (Table. 2).

Effect of NaCl stress on sugars and starch content:

Reducing sugar content was higher in control than sample with or without salinity stress. On the other hand sample showed higher non-reducing sugar level than control, irrespective of the salinity stress level. Reducing sugar content was increased with continuous progression of NaCl stress, without much striking difference amongst them (Table 3). However considerable amount of differential response was observed in terms of non-reducing sugars. Most striking results were seen in starch content where a sharp decrease was observed in plants with increasing salt stress from 50 – 300 mM NaCl, whereas starch content was increased significantly under the influence of salt stress in plants. The starch content was higher in control (617.2) than sample (345.5) with salinity stress.

DISCUSSION

In the present study the efficiency of seed germination in *Oryza sativa L* under the different salt concentration. In addition the callus induction and regeneration were used by the different concentration of 2, 4-D containing MS media and also assess the various biochemical parameters from the regenerated plants. Results of the previous studies by Kumar *et al.* (2009) demonstrated that the salt tolerant cultivars produce greater biomass than salt sensitive mungbean and rice cultivars respectively, when irrigated with NaCl dominated waters. Rapid accumulation of free proline is a typical response to salt stress. Similar responses were observed in the present investigation and salinity stress resulted into a sharp increase in proline content irrespective of the cultivars. In control plants the proline content was almost similar in both the varieties, however, as the magnitude of salinity stress increased, the rate of proline accumulation was observed much higher in (305 % of control) and at 200 mM NaCl stress. When exposed to high salt content in soil, many plants have been observed to accumulate high amounts of proline, in some cases several times the sum of all other amino acids (Kumar *et al.*, 2009). Proline is a known osmo-protectant, and plays an important role in osmotic balancing, protection of sub-cellular structures, enzymes and in increasing cellular osmolarity (turgor pressure) that provide the turgor necessary for cell expansion under stress conditions (Sairam and Tyagi 2004). In the present study the rate of proline accumulation was observed much higher (205.4 mg/g) at 300 mM NaCl stress as compared to control (70.15) (Table 2).

Similar to the results of present investigation, Kafi *et al.* (2003) observed higher starch content in salt tolerant wheat genotype at 300 mM NaCl stress. Dubey and Singh (1999) obtained the similar kind of results and concluded that the starch content was reduced with much higher magnitude in salt sensitive rice cultivars than that of salt tolerant ones up to moderate salinity stress (150 mM). Starch is an important component of plant tissues and accumulates in leaves as a temporary reserve form of carbon and is the principal component of dry mass accumulated in mature leaves, hence the accumulation of more starch in Ambemohar may be seen as the protective mechanism during stress conditions.

Besides the highest average number of shoots (3.5±0.5) was observed on the callus derived from 2.0 mg L- 1 2, 4-D (Table .1). Pandey *et al*. (1994) reported that 2.0 and 3.0 mg L-1 IAA and kinetin produced the most shoots. These findings are fully consistent to the present study. Jiahua *et al.* (1995) also reported that 0.5 and 2.0 mg L–1 IAA and kinetin in regeneration medium were beneficial for green plantlet differentiation of japonica rice (*Oryza sativa*). The present study revealed that the different concentration of 2, 4-D were used to induce callus in the mature seeds. The NAA and BAP were used to the regeneration of shoot and root development.

Some reports implicate on the positive correlation of proline accumulation to drought and salt tolerance in several species including rice (Ahmad *et al.* 2007), while other reports suggest that proline accumulation might mainly be a consequence of salt stress rather than being involved in its alleviation. In this study, proline accumulation was highest in the salt sensitive cultivar under 50 and 100 mM NaCl, so it might be a consequence of injury rather than being involved in the stress tolerance.

CONCLUSION

From the results obtained in the present investigation, we can conclude that overall showed better tolerance to salt stress with a lesser extent of antagonistic effect of NaCl on germination and biomass production at seedling stage. In addition showed higher proline, protein and starch content with lesser poly phenol levels under varying salt stress condition and all these biochemical parameters might have played an important role in its salt tolerance nature. In further research should be carried out to find out the produce the salt tolerance rice (*Oryza sativa L*) at genetic level.

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