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Full Length Research Paper

Growth responses of NaCl stressed rice (*Oryza sativa* L.) plants germinated from seed in aseptic nutrient cultures supplemented with proline

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Negative impact of salinity on plant germination is significant because of abundance of Na^+ in culture medium, which causes growth inhibition. Effect of salinity (NaCl) in the presence of proline was assessed in rice (*Oryza sativa* L.) variety Khushbo-95 at seedling stage. Seeds were cultured on MS_0 (MS basal medium), MS_1 (MS_0 + 100 mM NaCl) and MS_2 (MS_1 + 5 mM proline) for 20 days. Seedlings and its biomass decreased in saline culture. Similarly, total protein and sugar contents also decreased, while reducing sugars and proline contents increased. These parameters were observed to be slightly adverse in cultures supplemented with proline (MS_2) and NaCl (MS_2). Among cultures, leaf demography (cell size) was affected significantly; this may be the reflection of accumulation of proline, Na^+ and Cl^- and exclusion of K^+ in developed rice seedlings.

Key words: *Oryza sativa* L., seedling biomass, epidermal cells, proline content.

INTRODUCTION

The agricultural land productivity is mainly limited by soil salinity. More than 6% world's land area is salt affected with most of its solution containing $30gL^{-1}$ sodium chloride. About 15 out of 20% area is salt affected, which produces one third world's food. This affected area is higher than non-irrigated lands (FAO, 2007; Munns and Tester, 2008), which is a real threat to human's food security. Existed situation may be tackled by cultivation of salt tolerant wild plants (Qadir et al., 1998; Zhu, 2001; Kim et al., 2004). Rice is a third most important cereal crop after wheat and cotton, especially in Asia. Today, its production is not good because of increasing numbers of biotic and abiotic stresses such as various diseases and shortage of water and salinity, respectively (Oerke et al., 1994; Ferrero et al., 2001; Ferrero et al., 2002). Meanwhile, rice is evolved in glycophytic habitat and moderately sensitive to saline soil than other cereal crops. Excess of salinity has been adversely affecting the potential yield of crop plants (Ashraf, 2004; Flowers and Flowers, 2005). Attempts to reduce the soil salinity, using mechanical methods (such as reclamation, irrigation and drainage) are not always practical or economical. New salt tolerant varieties are increasingly needed for rice production in salt affected areas (Qadir et al., 1998; Munns, 2002; Yamamoto et al., 2003; Kim et al., 2004).

Cell culture system has been a very useful tool in finding mechanisms involved in developing salt tolerance operating at cell level. Meanwhile, crop improvement strategies are based on rapid selection on a mass scale. Plant growth under salinity stressed conditions leads to development of a specific characteristic like compatible osmolytes such as proline, sugars and protein. These traits provide potential biological markers to develop selection criteria for salt resistant phenomena (Hasegawa et al., 2000; Shonjani, 2002; Cherian and Reddy, 2003; Elavumoottil et al., 2003). Meanwhile, seedling stage is

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often thought to be most sensitive to salinity (Jones and Jones, 1989).

In vitro culture system is useful in studying stress responses, providing possibilities to generate conditions with more or less similar intensity and regime of stress, in which plants may have evolved in the past, or exposed along ontogenesis. Therefore, it is a dire need to take immediate action and use modern technologies along with agronomical practices in order to uplift ongoing agriculture system.

The objective of this study was to observe the effect of salinity (NaCl) under *in-vitro* cultured seeds of rice *(Oryza sativa* L.) variety Khushbo-95 in the presence of proline along with NaCl. Such aseptic culturing may provide a system for assessing salinity induced changes in growth-related metabolic contents in developing seedling. For instance, role of proline in improvement of salt tolerance ability in rice may also be judged.

MATERIALS AND METHODS

Healthy seeds of rice (*O. sativa* L.) variety Khushbo-95 were dehusked and washed with tap-water. They were surface sterilized with 50% commercially available Robin Bleach® containing 5% sodium hypochlorite (NaOCl) for 1 h, with continuous stirring and then rinsed with autoclaved distilled water 3 times.

Sterilized seeds were cultured on three MS (Murashige and Skoog, 1962) media; a. MS_0 (MS basal medium), b. MS_1 (MS0 + 100mM NaCl), c. MS_2 (MS1+ 5 mM proline) aseptically. Cultures were incubated in growth chamber at 25 ± 2°C in dark for three days and then grown under light conditions (1600 lux, 16/8 h day and night) for 20 days.

After 1 week of culturing, seed germination rate was measured in each culture and finally the seedlings at 20 days were removed from cultures and washed with tap water. The plantlets were measured and fresh weight was obtained after drying the biomass with Whatman No 40 filter papers and dry biomass drying at 72ºC for 3 days and weighted. Biochemical contents like proteins were determined using Lowry et al. (1951) method; sugars content by Montgomery (1961) method; reducing sugars by Miller (1959) and Malavolta et al. (1989) methods. Relative water contents were also measured in accordance to Conroy et al. (1988). Different anatomical studies of developed seedlings were also performed by the following: Gielwanowska et al. (2005) and Johansen (1940) methods. The significance of the collected data from each culture was computed by using COSTAT computer package (CoHort software, Berkeley, USA).

RESULTS AND DISCUSSION

Plant growth is inhibited by a number of environmental factors, with salinity being considered as a major constraint (Ashraf, 2004; Flowers, 2004; Munns et al., 2006). Plants as well as stages of their growth greatly differ under saline conditions among cereal crops (Ashraf et al., 2006; Ulfat et al., 2007), where rice crop is most sensitive. As seed germination in rice (*O. sativa* L.) variety (Khushbo-95) was 100% in control (MS_0), it decreased in MS_1 nutrient cultures and increased slightly in MS_2 medium (Table 1a). Salt-stressed conditions reduce germination rate and also its seedling growth. Proline has played positive role (MS_2) through an increase in seed germination (Naheed et al., 2007, 2008; Munns and Tester, 2008).

Under optimal supply level of nutrients, the normal plant growth is induced and below them a reduction in growth occurs. The increase in optimal concentration of nutrients may increase the growth rate while single salt (NaCl) added reduces growth of cultured plantlets supplement with secondary metabolites, making plants that grow under environmental stressed conditions (e.g proline-containing mineral cultures under saline conditions) to increase the plant growth rate than saline cultures alone. In present study, plant biomass decreased considerably in saline stressed cultures than control, while stressed condition was alleviated by supplying proline in salt stressed nutrient cultures (Table 1a). Such differential growth has been developed because of the supplementation of various types of nutrient cultures (Marschner, 1995; Grattan and Grieve, 1999; Raptan et al., 2001; Flowers and Flowers, 2005; Niknam et al., 2006).

A number of bio-compounds are required in coordinate actions that regulate an array of physiological processes that lead to their specific adaptations; these processes are not exclusively involved in regulation of plant growth including photosynthesis, osmotic adjustment, osmo protectants and anti oxidative defense (Paul and Foyer, 2001; Friecke and Peters, 2002; Khedr et al., 2003; Apel and Hirt, 2004; Foyer and Noctor, 2005; Munns, 2005; Munns and Tester, 2008). Application of NaCl that significantly increases Na^+ and Cl^- in growing plantlets may be due to higher concentration of Na^+ in mineral medium. Ultimately, it resulted in increased uptake of Na^+ and Cl^- by plants (Table 1c). Such ionic or nutrient imbalance conditions are resulted in salt stressed plants due to relative competition for salty ions than balanced nutrients (Kaya et al., 2001; Munns, 2002; Munns et al., 2006). A significant reducing effect (NaCl) is observed on K^+ concentration among cultures. Under saline stressed conditions, a passive selective uptake of Na^+ and K^+ occurs. An increased uptake of Na^+ at the cost of K^+ or decline in K^+ concentration occurs in stressed cultures (Table 1c). The abundance of Na^+ significantly inhibited the growth of shoots (Table 1a). According to previous reports, ionic imbalance causes reduction in seedling growth under NaCl stressed conditions (Kuiper, 1984; Aslam et al., 1996; Malik et al., 1999; Abid et al., 2002; Hussain et al., 2003). In the present experiment, the proline added to the nutrient medium significantly developed a reverse situation of the ionic accumulation (Table 1d).

Proline contents are common features of *in vitro* growing seedlings or cell cultures in response to salt stressed with reduction in growth (Al-Khayri, 2002). Proline accumulates at higher amounts than other amino acids in salt stressed plants (Table 1d). This increment under saline stressed conditions non-significantly reduces in cultures supplemented with proline along with NaCl. Similar behavior

Table 1. Different bio-parameters of aseptically developed seedlings of rice (*O. sativa* L.) variety Khushbo-95 in the presence of NaCl and proline (20-days culture).

Medium	a. Morphometrics of seedlings				
	Germination (%)	**Plant height (cm)**	**# of leaflets**	**F Wt (g)**	**D Wt (g)**
MS$_0$	^{a}97.3±2.50	^{a}4.6±0.150	^{a}2.65±0.10	^{a}0.604±0.02	^{a}0.312±0.03
MS$_1$	^{c}78.4±1.50	^{c}2.8±0.05	^{b}2.04±0.05	^{c}0.217±0.03	^{c}0.073±0.02
MS$_2$	^{b}88.9±1.84	^{b}3.4±0.04	^{a}2.46±0.11	^{b}0.237±0.04	^{b}0.093±0.02
Significance	***	***	*	***	***

	b. Anatomical characters of seedlings		
	Epidermal (µm)	**Cortex cell (µm)**	**Aerenchyma cells (µm)**
S$_0$	^{b}8.25±0.10	^{b}17.52±0.03	^{a}23.12±0.03
MS$_1$	^{a}11.12±0.03	^{a}26.31±0.09	^{b}10.21±0.09
MS$_2$	^{b}6.79±0.12	^{c}14.92±0.08	^{b}8.91±0.10
Significance	***	***	***

	c. Different inorganic contents in seedlings		
	K^+	**Na^+**	**Cl^-**
MS$_0$	^{a}12.21±0.32	^{b}3.80±0.15	^{b}6.32±0.13
MS$_1$	^{b}8.56±0.19	^{a}5.42±0.14	^{a}9.68±0.19
MS$_2$	^{b}9.84±0.15	^{a}5.26±0.24	^{a}8.75±0.23
Significance	**	**	**

	d. Different organic contents in seedlings				
	Proteins contents (g)	**Sugars contents (g)**	**Reducing sugars (g)**	**Proline (g)**	**H_2O contents (%)**
MS$_0$	^{b}0.31±0.02	^{a}0.23±0.03	^{b}0.12±0.03	^{b}1.25±0.05	^{c}48.34±0.61
MS$_1$	^{c}0.29±0.03	^{b}0.19±0.02	^{a}0.13±0.03	^{a}1.34±0.08	^{a}66.37±0.84
MS$_2$	^{a}0.41±0.07	^{a}0.23±0.05	^{b}0.12±0.03	^{a}1.38±0.09	^{b}60.78±0.49
Significance	***	***	*	***	***

of reducing sugars' accumulation has also been observed. The protein contents among the rice seedling cultures also decreased under NaCl stressed cultures, but increased in the presence of proline (Table 1d). The decrease or increase in protein contents under saline or saline supplemented with proline cultures may be because of release of some proteins to the medium due to osmoticum or decrease in protein synthesis rate (Hall and Flowers, 1973; Mass et al., 1979; Fedina et al., 2002; Cherian and Reddy, 2003; Nikam et al., 2006).

A somewhat delay in seed germination was also observed that correlated directly or indirectly with salt stress. Some seeds are not germinated in saline cultures; probably seeds containing embryos could be damaged due to an accumulation of Na^+ and Cl^-. Delay in germination is a physiological disturbance because of an alteration in K^+ and Na^+ contents. So ionic ratios are very important in determinations of relative toxicities that could provide relative biological processes' rates under specific ionic antagonisms (Mirza and Mahmood, 1986; Wilson et al., 2000; Rahman et al., 2008).

The developed ionic situations in seedlings enhanced specific internal structural modifications (Table 1d). Some of the cell types collapsed, while others expanded for the prevention of toxic affects of salt stress but observed to be normalized in the presence of proline in salt stressed cultures. The saline cultures prevent the availability of the absorption of water by roots or are unable to absorb saline water. In both cases, seedlings growth is affected. Generally, salinity causes slow or less mobilization of reserve food, cell division, cell enlargement and enhances hypocotyls injury (Khan et al., 1984; Assadian and Miyamoto, 1987; Mer et al., 2000; Tezara et al., 2003).

In this experiment, it is concluded that abiotic stress, like salinity, behaves destructively in a complex phenotypic and physiological phenomena in growing plantlets and that could ultimately reduce character yields of crops. At the same time, proline behaves positively for seedling growth in salt stressed cultures. Proline gradually increased germination rate (percent) and favored the plant growth and physiological characters under salinity stress. Growth characters such as plant biomass decreased because of accumulation of Na^+, Cl^- and decrease of K^+. Reducing sugars and proline contents increased while proline decreased non-significantly and reducing sugars increased further when proline is supplied in salt stressed cultures. So, plant growth enhancement occurs in saline culture when proline is used as a supplement agent.

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