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Research Paper

EFFECT OF NaCl AND BORON TOXICITY ON PROLINE BIOSYNTHESIS OF *ORYZA SATIVA* (POKKALI VTL-4)

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Rice has been considered as the staple food of majority of the population of Asia. Soil and irrigating water quality around the globe are deteriorating day by day; hence it is necessary to understand the response of rice crops towards these environmental abuses. The present study was carried out on unique type of rice variety (Pokkali VTL-4) cultivated in acid saline soils of Kerala. *Oryza sativa* (Pokkali VTL-4) plants were cultivated under different boron, proline and NaCl concentration, in order to study their response mechanism under various environmental stresses. There was a considerable decrease in plant boron concentrations after 14 days of proline treatment. Also, reasonable decrease in plant Boron concentration was observed after 7 days of proline treatment. The effect of salinity and boron toxicity on the proline metabolism was also studied by estimating the enzyme Pyroline-5-Carboxylate Synthatase (P5CS). The enzyme P5CS catalyses the first step in the pathway of conversion of glutamate to proline (Glutamate pathway). The enzyme assay indicated that, the P5CS activity increased under low boron and NaCl concentration. There was a considerable decrease in P5CS activity under high boron and NaCl concentration. Hence, the results showed that proline synthesis occurs by Glutamate pathway under less stress condition and by Ornithine pathway under severe stress condition.

Keywords: *Oryza sativa*, Pokkali, NaCl stress, Pyroline-5-Carboxylate synthatase

INTRODUCTION

The Pokkali system of rice (*O. sativa*) cultivation is a unique method of rice production in acid saline soils of Kerala. Plant metabolism is influenced directly or indirectly by various environmental stresses such as high or low temperature, drought and salinity. Proline accumulates in many plant species in response to environmental stress. Although much is now known about proline metabolism, some aspects of its biological functions are still unclear. Proline can act as a signaling molecule to modulate mitochondrial functions, influence cell proliferation or cell death and trigger specific gene expression,

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which can be essential for plant recovery from stress (Bates *et al*., 1973). Proline is a low molecular-weight osmoprotectant which helps to preserve structural integrity and cellular osmotic potential within different compartments of the cell (Iyer and Caplan, 1998).

Verbruggen *et al*., (1993) reported that levels of proline vary among plant organs, highest proline levels are found in flowers, especially in pollen grains, and in seeds, and lowest levels are found in roots. Proline accumulation usually focuses on the plant organs with high metabolism. Rapid catabolism of proline upon relief of stress may provide reducing equivalents that support mitochondrial oxidative phosphorylation and the generation of ATP for recovery from stress and repair of stress-induced damage (Hare and Cress, 1997; Hare *et al*., 1998).

In maize seedlings, the treatments induced proline accumulation by activation of the biosynthetic pathway, including P5CS, δ-OAT and arginase (Yang *et al*., 2009). P5CS catalyses the first step in the pathway of conversion of glutamate to proline. During stress, the expression of P5CS is well correlated with proline content (Yoshiba *et al*., 1995; and Savoure *et al.,* 1995)

Proline accumulation has been suggested to result from: (a) decreased proline degradation (b) increased proline biosynthesis (c) lower proline utilization and (d) protein degradation. In higher plants, proline is synthesized via both glutamate (Glu) and ornithine (Orn) pathways. The glutamate pathway is catalyzed by a single bifunctional enzyme, pyrroline-5-carboxylate synthetase (P5CS) and produces gultamic-semialdehyde (GSA), which is spontaneously converted to pyrroline-5-carboxylate (P5C) and then reduced to proline. Under many stress conditions, especially salinity and drought, proline accumulation is correlated with P5CS activity, as it was suggested to be the key regulatory and rate-limiting enzyme in the biosynthetic pathway (Wang *et al*., 2011).

As the Pokkali rice is cultivated in acid saline soils and directly or indirectly influenced by various environmental stresses, the present study is an attempt to study the effect of NaCl and Boron toxicity on proline biosynthesis in Pokkali (VTL-4) variety of rice.

MATERIALS AND METHODS

Soil samples were collected from different areas of pokkali cultivating fields of Ernakulum and Alapuzha districts of Kerala (Thevara, Kumbalangi, Chellanam, Cheranellur, Vayalar and Nedumudi). The *Oryza sativa* (VTL-4 Pokkali) grains were collected from Vytila Rice Research Station. The grains were sown in different pots consisting of different concentration of Boron, ranging from 2.2 mg/kg to 22.0 mg/kg. Pots were filled with 6 inches of potting soil. The pots were filled with water, up to 2 inches above the top of the soil. Forty VTL-4 rice seeds were sowed in each pot, and seeds were scattered evenly. Pots were labeled and placed in the laboratory, providing necessary lighting

Heat lamps were placed above the pots since it is grown indoors. Water level was maintained in the pots at 2 inches above the soil level until the sprouts reach 5 to 6 inches in height. Water level was increased in all the pots to 4 inches deep to ensure the sprouts have enough water to complete their growth process. Water level was allowed in the container to dissipate gradually. Little or no standing water was left in the pots

when the plants reached their harvest stage, typically in four months. Rice stalks were harvested when they changed from green to gold. Stalks were cut with garden sharp knife, wrapped them in newspaper and allowed them to dry in a warm place for two to three weeks.

Plant Boron Estimation and Application of NaCl Stress

Boron in plant samples were measured by dry ashing and subsequent measurement of Boron was done by Atomic Absorption Spectro-photometer (A.A.S - Perkin Elmer, A ANALYST 200). *O. sativa* (VTL-4 Pokkali) varieties were treated for 24 and 48 hrs with 150 mM NaCl, in order to create a double stress condition. Hence, the plant boron was estimated before the application of NaCl and after the treatment of 150 mM NaCl. The amount of sodium accumulated by the VTL-4 variety was also estimated, both after 24 and 48 hrs NaCl treatment.

Sample Preparation for Boron Estimation by Atomic Spectroscopy

Dry Ashing: 1g dry, ground plant material was taken in porcelain crucible and ignited in a muffle furnace by slowly raising the temperature to 550°C. Ashing was continued for 6 hours after attaining 550°C. Five drops of De-ionized water was added to the ash, and later 10 ml 0.36 N sulfuric acid solution was added into the porcelain crucibles. It was allowed to stand at room temperature for 1 hr and stirred occasionally with a plastic rod to break up ash. Later, the solution was filtered through Whatman No.1 filter paper into a 50 ml polypropylene volumetric flask and brought to volume. Filtrate was used for Boron determination.

Sample Preparation for Sodium (Na) Estimation by Atomic Spectroscopy

The procedure adopted was that of Chapman and Pratt (1986) with slight modifications. 0.5-1.0 g portions of ground plant material is weighed in a 30-50 mL porcelain crucibles or Pyrex glass beakers. Porcelain crucibles were placed into a cool muffle furnace, and temperature was increased gradually to 550°C; ashing was continued for 5 hours after attaining 550°C. Muffle furnace was shut off and door was opened cautiously for rapid cooling. The porcelain crucibles were carefully taken out when cooled. The cooled ash was dissolved in 5 mL portions 2 N hydrochloric acid (HCl) and mixed with a plastic rod; after 15 - 20 minutes, volume was made up to 50 mL using De-ionized water. After mixing well, it was allowed to stand for about 30 minutes, and it was filtered through Whatman No. 42 filter paper, discarding the first portions of the filtrates.

Proline Treatment on *O. Sativa* (VTL-4 Pokkali) Plants

About 1mM Proline solution was added to the pots of 8 weeks old *O. Sativa* (VTL-4 Pokkali) plants. Changes in the growth of the rice plants were monitored for about 14 days. The plant boron concentrations were estimated on 7^{th} day and 14^{th} day of proline treatment by atomic spectroscopy.

Proline Determination

Proline concentration was determined by Bates *et al*., (1973) method. 0.5 g plant material was extracted by homogenizing in 10 ml of 3% aqueous sulphosalicylic acid. Homogenate was filtered through Whatman No.2 filter paper. Later 2 ml of filtrate was taken in the test tube and 2 ml each of glacial acetic acid and acid ninhydrin were added. It was heated in boiling water bath for 1hr and afterwards reaction was terminated by

keeping in ice bath. Then, 4ml toluene was added and stirred well for 20-30 sec. Later, the toluene layer was separated and allowed to retain room temperature. The red color intensity was measured at 520nm. Standard graph was plotted with Proline content on x-axis and Boron concentration on y-axis.

The proline content on fresh-weight-basis was expressed as follows:

m moles per g tissue =

$$\frac{\text{mg proline/mL x mL toluene}}{115.5} \times \frac{5}{\text{G sample}}$$

where, 115.5 is the molecular weight of proline

Pyrroline-5-Carboxylate Synthetase (P5CS) Estimation

Before estimation, Pokkali (VTL-4) plants were treated with 50-500 mM NaCl solutions. Frozen samples (approximately 5 g) were ground in liquid nitrogen and then extracted in 100 mM potassium phosphate buffer (pH 7.4) containing 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM beta-mercaptoethanol, 1% (w/v) polyvinylpolypyrrolidone (PVPP), 5 mM MgCl2 and 0.6 M KCl. The homogenate was centrifuged at 12000 rpm for 20 min at 4°C and the resulting supernatant was kept at –20°C until enzyme assay. All operations were carried out at 4°C (Lutts *et al*., 1999; Chen *et al*., 2001).

The P5CS activity was determined by monitoring the consumption of NADPH and measuring the increase in absorbance at 340 nm. 2 ml reaction mixture containing 75 mM Glutamate, 100 mM Tris-HCl (pH 7.2), 20 mM MgCl2, 5 mM ATP, 0.4 mM NADPH and 0.5 ml enzyme extract was incubated at 37°C for 20 mins, later the absorbance was recorded at 340nm (Stines *et al*., 1999) . P5CS is expressed as unit per mg protein (one unit is defined as an increase in 0.001 A340 per min.)

RESULTS

Symptoms of Boron Toxicity and Deficiency in *O. sativa* (Pokkali VTL-4)

The first symptom of boron toxicity (19.8 mg/kg) was the appearance of a light brown or yellowish white discoloration at the tips and margins of the leaves about six weeks after planting. Gradually the tips and leaf margins turned yellow. Two to four weeks later, elliptical dark brown blotches appeared at the discolored areas in most of the rice samples. Finally the entire leaf blade turned light brown and withered. Vegetative growth was not markedly depressed until the problem turned severe. Elliptical brown blotches were observed on discolored area along the leaf margins.

Boron toxicity symptoms were observed at the leaf tips of the plants of pot-9 (19.8 mg /kg) initially. Later, symptoms were observed in plants of pots-6 (13.2 mg/kg), 7 (15.4 mg/kg), 8 (17.6 mg/kg), and 10 (22.0 mg/kg). After, 7-8 weeks a progression was observed in the boron toxicity symptoms from leaf tips to the whole plants. In pots 1 (2.2 mg/kg) and 2 (4.4 mg/kg), leaf tips and margins turned yellow, also a spindle like appearance was observed at the leaf tips.

Plant Boron Estimation and Application of NaCl Stress

Boron concentration in plants without NaCl stress was analyzed for matured *O. sativa* (Pokkali VTL-4) plants. The peak value was estimated at 165 mg/kg fresh weight for sample-10 (22.0 mg/kg) and the lowest value was 9.0 mg/kg FW for sample-1 (2.2 mg/kg). After, 24 hrs of NaCl stress the peak value of 145 mg/kg FW was obtained in sample-9 (19.8 mg/kg); the previous value of

sample-9 was 127 mg/kg FW. Again the lowest value obtained was from sample-1 (2.2 mg/kg), 15 mg/kg FW. After 48 hrs of NaCl stress, the rate of boron concentration in sample-9 (19.8 mg/kg) was peeked to 176 mg/kg FW, but the boron concentration in sample-1 (2.2 mg/kg) was only 18 mg/kg FW.

Sodium (Na) estimated was almost same, with negligible difference. Sodium concentration in shoot system was 125 and 200 μmol/gm fresh wt after 24 and 48 hrs applications of 150 mM NaCl ; whereas sodium concentration in the root system was 25 and 50 μmol/gm fresh weight.

Proline Treatment on *O. sativa* (VTL-4 Pokkali) Plants

The Boron ions in plants of pot-9 (19.8 mg/kg) were found to be decreased considerably after 7days of proline treatment. Before the proline treatment, the application of NaCl to the plants boosted the boron concentration to 176 mg/kg FW in pot-9 (19.8 mg/kg), but after addition of 1mM Proline solution, it was decreased to 122 mg/kg FW. In other samples also plant boron concentration was found to be decreased (Table 1).

The Boron ions concentrations in plants of pot-9 (19.8 mg/kg) were found to be decreased considerably after 14 days of proline treatment i.e. from 122 mg/kg FW to 115 mg/kg FW. In the other samples also plant Boron concentrations was found to be decreased except in sample-4 (8.8 mg/kg) (Figure 1).

Proline Determination

Proline in *O. sativa* (Pokkali VTL-4) was estimated using a procedure formulated by Bates *et al*., (1973); highest value was estimated to be 2350 μgm/gm fresh weight in pot-6 (13.2 mg/kg). Also, 2190 μgm/gm fresh weight and 2190 μgm/gm fresh weight were estimated in pot-7 (15.4 mg/kg) and pot-8 (17.6 mg/kg) respectively.

Table 1: Boron Concentration in *O. sativa* (Pokkali VTL-4) After 7 Days and 14 Days of Proline Treatment

Sample Numbers	Plant Boron Conc. After 7 days of Proline Treatment (mg/kg) Trial-1	Plant Boron Conc. After 7 days of Proline Treatment (mg/kg) Trial-2	Average (mg/kg)	Plant Boron Conc. After 14 days of Proline Treatment (mg/kg) Trial-1	Plant Boron Conc. After 14 days of Proline Treatment (mg/kg) Trial-2	Average (mg/kg)
Sample 1	15.00	17.00	16.00	15.00	15.00	15.00
Sample 2	23.00	21.00	22.00	19.00	17.00	18.00
Sample 3	29.00	29.00	29.00	28.00	24.00	26.00
Sample 4	32.00	32.00	32.00	33.00	31.00	32.00
Sample 5	58.00	54.00	56.00	49.00	47.00	48.00
Sample 6	60.00	58.00	59.00	50.00	50.00	50.00
Sample 7	63.00	59.00	61.00	58.00	54.00	56.00
Sample 8	80.00	80.00	80.00	78.00	76.00	77.00
Sample 9	121.00	123.00	122.00	114.00	116.00	115.00

Figure 1: Plant Boron Concentration After Addition of Proline on 9 Weeks Old *O. sativa* (Pokkali VTL-4) Plants

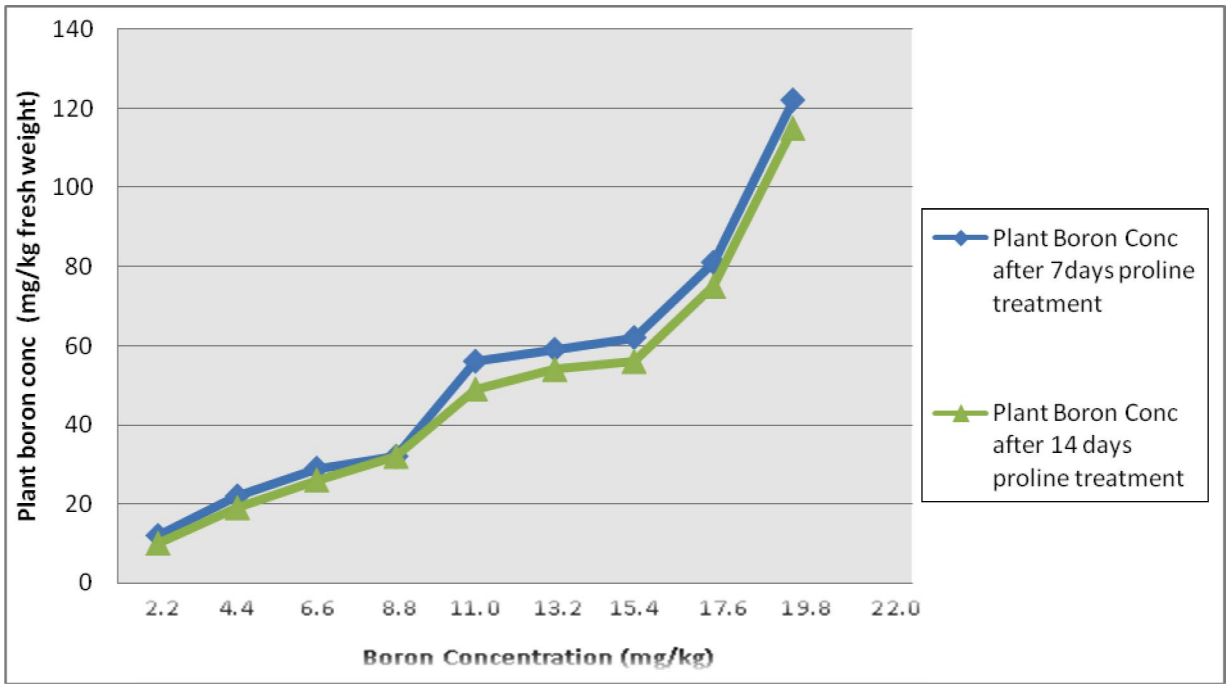
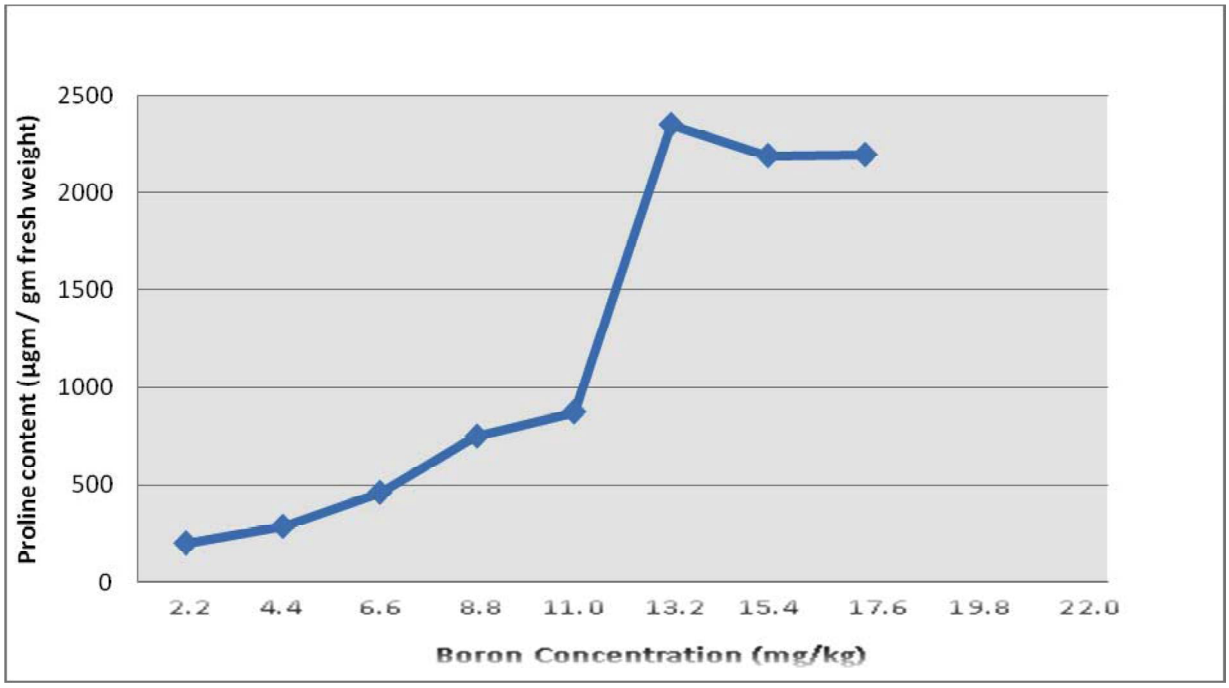


Figure 2: Proline Contents Produced by 15 Weeks old *O. sativa* (Pokkali VTL-4) Plants Under Different Boron Concentration



Lowest value was estimated in sample-1 (2.2 mg/kg) i.e., 200 μgm/gm fresh weight (Figure 2).

Pyrroline-5-Carboxylate Synthetase (P5CS) Estimation

Estimation showed highest value of 370 unit/mg proteins in sample-3 with 6.6 mg/kg boron and 150 mM NaCl. Similarly, 350 unit/mg proteins were estimated in sample-2 (4.4 mg/kg of Boron concentration and 100 mM NaCl concentration). Lowest value of enzyme was observed in sample-9, i.e. 60 unit/mg proteins, having boron concentration of 19.8 mg/kg and 450 mM NaCl concentration (Figure 3).

DISCUSSION

Effects of Proline on Boron Concentrations

In the present study, correlation was noticed between proline accumulation and boron concentrations in *O. sativa* (Pokkali VTL-4). The plants displayed severe Boron toxicity symptoms after 7-8 weeks of cultivation. An initial symptom displayed was wilting at leaf tip and later progressed towards basal area (Figures 4 and 5). There was a considerable decrease in the plant boron concentration after 7 days and 14 days of proline treatment. After 12 weeks of cultivation, samples were estimated for the presence of proline. However, the highest accumulation of proline (2350 μgm/gm fresh weight) was observed at medium concentration of boron (13.2 mg/kg). The proline accumulation was limited initially, and then tends to slowly increase when the boron concentration is above a critical value (11.0 mg/kg). Also, symptoms of boron toxicity were decreased to a reasonable level after 7 days and 14 days of Proline treatment (Figures 6 and 7).

Figure 3: Pyroline-5-Carboxylate Synthatase (P5CS) Activity in *O. sativa* (Pokkali VTL-4) Plants

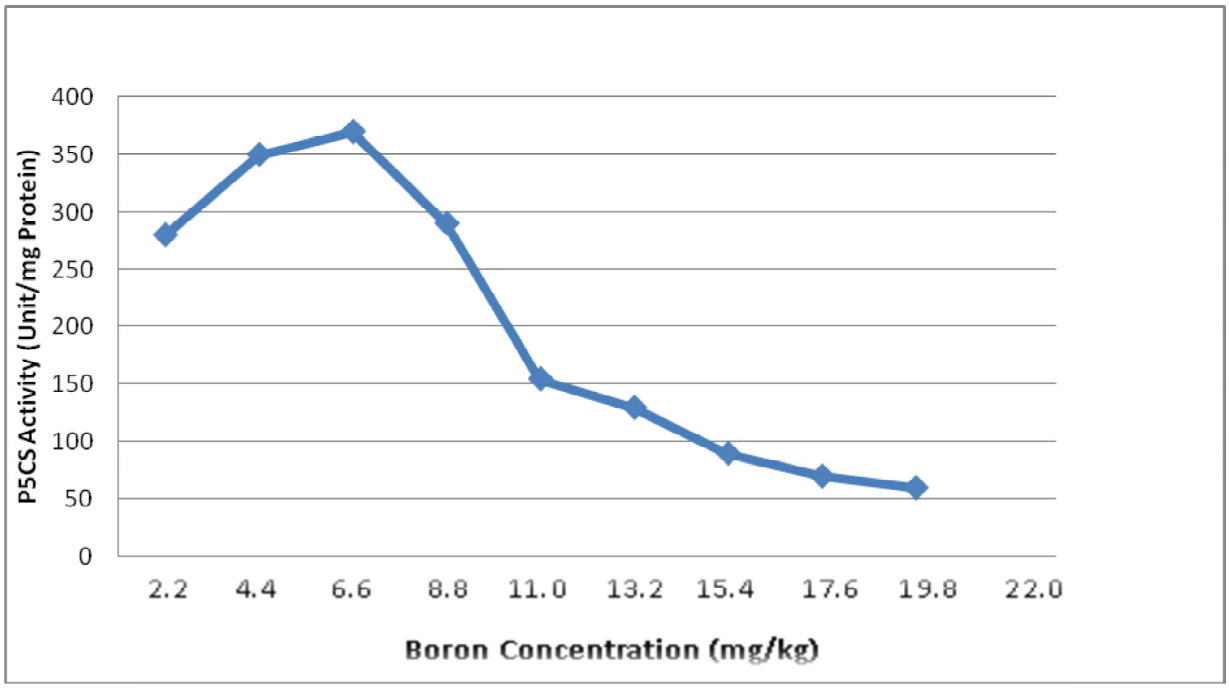


Figure 4: 7-8 Weeks Old Plant, Showing Expansion of Wilting from Leaf Tip to Basal Area

Figure 5: 8-9 Week Old Plant, B Toxicity Effected The Growth

Figure 6: Recovered from Wilting Due to Boron Toxicity After Proline Treatment for 7 days

Figure 7: Recovered from Wilting Due to Boron Toxicity After Proline Treatment for 14 days

The role of proline as an osmoprotectant has been mentioned by Apel and Hirt (2004). The main function of osmoprotectant to preserve structural integrity and cellular osmotic potential within different compartments of the cell was mentioned by Iyer and Caplan, (1998). The work of Apel and Hirt (2004) also stated that proline has been known to be involved in the response to a number of environmental stresses, particularly salt and drought stress. Osmotic stresses are caused by excessive accumulation of salt in the soil, either directly, because of salinization, or indirectly, because of water loss. The decrease in soil water potential led to an alteration of the plant water status which may cause stomatal closure, photosynthesis reduction and thus growth inhibition. Hare and Cress (1997) stated the other consequence of production of reactive oxygen species (ROS) and the accumulation of toxic ions within the cell, causing severe damage to membrane structures, proteins, nucleic acids and lipids. A response to osmotic stress widespread in plants consists of the accumulation of compatible osmolytes which are thought to protect cells against stress damage. Among these plant compatible osmolytes, proline is considered to be of major importance, as it has been reported to accumulate in a large number of species in response to stresses. Hence it is confirmed that under severe environmental stress conditions proline acts as a low molecular weight osmoprotectant/osmolytes.

Effect of NaCl and Boron Toxicity on Proline Metabolism in *O. sativa* (Pokkali VTL-4)

Proline metabolism is a typical biochemical adaptation in living organisms which is subjected to stress condition (Delauney and Verma, 1993). In maize seedlings, the treatments induced proline accumulation by activation of the biosynthetic pathway, including P5CS, δ-OAT and arginase (Yang *et al*., 2009). P5CS catalyses the first step in the pathway of conversion of glutamate to proline. During stress, the expression of P5CS is well correlated with proline content. In *Medicago truncatula* (Armengaud *et al*., 2004) and in young *Arabidopsis* leaves (Roosens *et al*., 1998), induction of δ-OAT mRNA by osmotic stress has been reported, suggesting that both the Glutamate and the Ornithine pathways may contribute to proline accumulation under stress condition. In general, the effect of salt stress on the activities of enzymes was similar in the leaves and roots. In this investigation, the P5CS activity increased at low-concentration of NaCl treatments, and it decreased under high-concentration of NaCl treatments.

In the present study the P5CS activity increased at low-concentration Boron-NaCl (2.2-8.8 mg/kg boron and 50-200 mM NaCl concentration), and it decreased under high Boron-NaCl concentration (13.2-17.6 mg/kg boron and 300-400 mM NaCl concentration). Hence, the results proved that in the proline biosynthesis, the glutamate pathway play leading role under low-concentration boron stresses. Whereas, proline biosynthesis occurs by alternative pathway (Ornithine Pathway) under high stress condition.

CONCLUSION

Rice Plants undergo a series of environmental stress condition in the environment. These stress conditions affects the yield and productivity of the crop. Proline acts as an osmoprotectant on many occasions and helps the crop to overcome the adverse environmental condition. In the present

study it was observed that the proline biosynthesis occurs by alternative pathway (Ornithine Pathway) under high stress condition. Under normal situations proline synthesis occurs by glutamate pathway. It was under the influence of NaCl and Boron toxicity that proline biosynthesis moves into alternative pathway. The effect of salinity and boron toxicity on the proline metabolism was studied by estimating the enzyme Pyroline-5-Carboxylate Synthatase (P5CS). The enzyme P5CS catalyses the first step in the pathway of conversion of glutamate to proline (Glutamate pathway).

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