



Analysis of proline metabolic enzymes in *Oryza sativa* under NaCl stress

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Abstract

The regulation of proline accumulation in seedlings of rice (*Oryza sativa* L. cv. Badami) was investigated. The increasing concentration of NaCl from 0.5 to 2.5 % progressively increased the proline content in rice. Proline accumulation caused by NaCl was related to protein proteolysis, an increase in OAT, P5CS, P5CR activity, a decrease in PDH activity. The maximum increase in proline content was recorded at 2.5 % NaCl concentration as compared to control and other concentrations of NaCl. The highest significant activity of proline synthesizing enzymes, Δ^1 -Pyrroline-5-carboxylate synthetase, Δ^1 -Pyrroline-5-carboxylate reductase and Ornithine- δ -aminotransferase with a lowest activity of proline hydrolysis enzymes; Proline dehydrogenase were also recorded at 2.5 % salinity over control and other concentrations of NaCl with a in-significant increase in the activity of Δ^1 -Pyrroline-5-carboxylate synthetase and Ornithine- δ -aminotransferase at 0.5 % concentration of NaCl over control. Externally the addition of 300mg $MnCl_2$, 220 ml⁻¹ ½ strength Hoagland solution, having 1% NaCl, was seen to increase a 893.9 % in proline content of this variety as compared to control.

Key words

Protein proteolysis, NaCl Stress, Proline, *Oryza sativa*

Introduction

Rice (*Oryza sativa* L.) is the most important food crop of the developing world. Asian farmers produce about 90 % of the total, with two countries, China and India, growing more than half the total crop (USDA, 2008). Plants are exposed to various types of environmental stress. Among these stresses, osmotic stress in particular that due to drought and salinity is the most serious problem that limits plant growth and crop productivity in agriculture. Proline accumulation in plant cells exposed to salt or water stress is a wide spread phenomenon (Wang *et al.*, 2011; Bakht *et al.*, 2012; Mattioni *et al.*, 1997). However, the actual role of proline accumulation remains unclear (Rhodes *et al.*, 1999), but it has been speculated that it can serve as an osmotic regulator, a protector of enzyme denaturation, a stabilizer of some macro molecules or molecular assemblies, are reservoir of nitrogen and carbon sources or a hydroxyl radical scavenger. However, some reports indicate no correlation between proline accumulation and stress resistance (Turan *et al.*, 2009). Proline accumulation in plant tissues has been suggested to result from (a) a decrease in

proline degradation, (b) an increase in proline biosynthesis, (c) a decrease in protein synthesis or proline utilization, and (d) hydrolysis of proteins (Yoshiba *et al.*, 1997). It has been shown that proline accumulation in response to NaCl could be attributed to an increase in P5CR activity, an increase in OAT activity or a decrease in PDH activity (Mattioni *et al.*, 1997; Bakht *et al.*, 2012). In this regard, the present study was undertaken to know the different enzymes involved in proline metabolism under NaCl stress and by adding $MnCl_2$ to rice seedling.

Materials and Methods

Plant materials and growth conditions : This experiment was conducted in plastic glass of 250 ml capacity in the growth chamber during Kharif season of 2008 at Institute of Life Science, Bhubaneswar, Orissa. Rice (*Oryza sativa* L.) cv. non-salt tolerant variety 'Badami' was used in this study. Seeds of Badami were obtained from Orissa University of Agriculture and Technology, Bhubaneswar. The surface-sterilized seeds were soaked in de-ionized (MilliQ) water overnight, transferred over wet filter paper in a Petri plate

and kept at 25°C for germination. Rice seeds germinated fully in 24 hr. Germinated rice seeds were grown (in a growth chamber) hydroponically over nylon nets in 250 ml capacity plastic glass containing half-strength Hoagland's solution. After 6 days, the seedlings in individual glass were treated with 0.5 (85 mM), 1.5 (255 mM) and 2.5% (425 mM) NaCl. For this, initially NaCl, sufficient to make the concentration of NaCl was dissolved in a few ml of half-strength Hoagland's solution and was poured into individual beakers except into that meant for control. After incubation for 1 hr in dark, NaCl was added further to raise its concentration to desired levels. Volume of the solution was maintained at the 200 ml mark by adding half-strength Hoagland's solution. Plants were kept in dark for another hour after application of the final treatment and then exposed to source of light (200 nmol m⁻² s⁻¹). The growth chamber was maintained at 24±3°C, relative humidity 70–75%, with 14 hr light and 10 hr dark periodicity. The seedlings were harvested after 12 hr of treatment for the enzyme activity study.

Determination of proline and protein : Leaves of seedlings were homogenized with 3% sulfosalicylic acid and the homogenate was centrifuged at 3,000 g for 20 min. The supernatant was treated with acetic acid and acid ninhydrin, boiled for 1 hr and then absorbance at 520 nm was determined. Contents of proline are expressed as µg g⁻¹ f. wt. of leaf (Bates *et al.*, 1973). For protein estimation, leaf segments were homogenized in 50 mM sodium phosphate buffer (pH 6.8). The extracts were centrifuged at 17,600 g for 20 min, and the supernatants were used for determination of protein by the method of Bradford (1976). Protein content was expressed as mg g⁻¹ f. wt. of leaf.

Enzyme assays : A similar extraction procedure was used for Pyrroline-5-carboxylate synthetase (P5CS) and Proline de hydrogenase (PDH), which is mainly based on the procedure described by Lutts *et al.* (1999). Seedlings were homogenized in a prechilled mortar and pestle with 50 mM Tris-HCl buffer (pH 7.4) containing 7 mM MgCl₂, 0.6 M KCl and 3 mM EDTA. The homogenate was centrifuged at 15,000 g for 20 min. The solution used for extraction of Ornithine-5-aminotransferase (OAT) was 100 mM potassium phosphate buffer (pH 7.4) containing 1 mM pyridoxal-5-phosphate, 1 mM EDTA and 10 mM 2-mercaptoethanol. The extract was centrifuged at 12,000 g. All the extraction procedures were conducted at 4°C. Pyrroline-5-carboxylate reductase (P5CR) was assayed by a NADH dependent P5CR reaction (Madan *et al.*, 1995). The assay mixture contained 0.06 mM NADH, 0.15 mM p5c, 120 mM potassium phosphate buffer, 2 mM dithiothreitol and the enzyme extract. The reaction was started by the addition of P5C and the decrease in absorbance was followed at 340 nm. P5CR is expressed as units m g⁻¹ protein (1U=Amount of enzyme required to produce 1 µ mol NADH per minute). OAT activity was assayed according to Vogel and Kopac (1960). The assay mixture contained 0.2 ml enzyme extract and 0.8 ml 100 mM

potassium phosphate buffer (pH 8.0) containing 50 mM L-ornithine, 20 mM α-ketoglutarate and 1 mM pyridoxal-5-phosphate. The reaction medium was incubated at 37°C for 30 min. The reaction was stopped by adding 0.5 ml trichloro acetic acid (10%) and the colour was developed by incubating the reaction mixture with 0.5 ml o-aminobenzaldehyde (0.5%) in ethanol (95%) for 1 hr. After centrifugation at 12,000 g for 10 min, the clear supernatant fraction was collected to measure the absorbance at 440 nm. OAT was expressed as unit's mg⁻¹ protein (1U=Amount of enzyme required to produce 1 nmol p5c per minute). PDH was assayed by following the NAD⁺ reduction at 340 nm in a 0.15 M Na₂CO₃-HCl buffer (pH 10.3) containing 13 mM L-proline and 1.5 mM NAD⁺ (Lutts *et al.*, 1999). PDH was expressed as units mg⁻¹ protein (1U=Amount of enzyme required to produce 1 µ mol NADH per minute).

Statistical analysis : All data were analyzed with three replication for analysis of variance by CRD method and the standard error of each mean value was also calculated for presentation with bar diagram.

Results and Discussion

In the present study it was observed that Badami species showed increase in the level of proline in their leaf tissues upon salinity treatment significantly over control (Table 1). The content of proline was found to be maximum at 2.5% NaCl concentration (160.15% more than control) followed by 1.5% NaCl concentration (106.94% more than control) 0.5% NaCl concentration (31.98 % more than control). Most of the suggestions regarding proline in salinity tolerance in plants is based on its enhanced accumulations in response to the stress.

Proline accumulation in leaf tissues has been suggested to result from a decrease in proline degradation, an increase in proline biosynthesis, a decrease in protein synthesis or proline utilization, and hydrolysis of protein (Charest and Phan, 1999; Yoshida *et al.*, 1997)

The activity of Δ¹-Pyrroline-5-carboxylate synthetase in the control leaf sample was recorded 0.2116 U mg⁻¹ protein min⁻¹, which increased significantly 6.21%, 12.25

Table 1 : Effect of NaCl salinity on proline content of rice cv. Badami grown in ½ strength Hoagland solution after 12 hrs of treatment

NaCl treatment	Proline (µg g ⁻¹ f.wt.)
Control	28.23 ± 2.82
0.5 %	37.52 ± 2.12
1.5 %	58.43 ± 1.98
2.5 %	73.44 ± 1.48

Values are mean of three replicates ± SE

% and 18.8% at 0.5%, 12.25% and 2.5 % NaCl concentration, respectively (Fig.1).The maximum increase in Δ^1 -Pyrroline-5-carboxylate synthetase activity was recorded at 2.5 % NaCl concentration. Although an increase in P5CS activity at 0.5 % NaCl concentration was observed insignificant.

The control activity of Δ^1 -Pyrroline-5-carboxylate reductase was observed 0.1736 U mg⁻¹ protein min⁻¹. A significant increase in Δ^1 -Pyrroline-5-carboxylate reductase activity was noticed with increasing level of NaCl concentration up to 2.5 % NaCl concentration (Fig. 2).The maximum increase in Δ^1 -Pyrroline-5-carboxylate reductase activity was recorded at 2.5 % NaCl concentration that is 0.238 U mg⁻¹ protein min⁻¹ (37.04 % more than control) followed by 0.2243 U mg⁻¹ protein min⁻¹. At 1.5 % NaCl concentration (29.17 % more than control) 0.202 U mg⁻¹ protein min⁻¹. at 0.5 % NaCl concentration (16.31 % more than control). These works collaborate with the work of Madan *et al.* (1995), Mattioni *et al.* (1997) and Sudhakar *et al.* (1993).

Similar to Δ^1 -Pyrroline-5-carboxylate synthetase, the control activity of Ornithine- δ -aminotransferase was 0.2569 U mg⁻¹ protein min⁻¹. A significantly improving trend was also seen in the Ornithine- δ -aminotransferase activity up to

2.5% NaCl concentration except at 0.5% NaCl concentration where a non significant increase in Ornithine- δ -aminotransferase activity was recorded (Fig.3). A 48.02 % more activity of Ornithine- δ -aminotransferase was observed at 2.5 % NaCl concentration than the control level activity of Ornithine- δ -aminotransferase followed by 30.64 % more Ornithine- δ -aminotransferase activity at 1.5 % NaCl concentration and 20.91% more Ornithine- δ -amino transferase activity at 0.5 % NaCl concentration.

Similar results were also reported by Turan *et al.* (2009) and Bakht *et al.* (2012). The increase in proline content in plants under salt stress is due to a hypothesis that proline is synthesized from glutamic acid (Glu) via Pyrroline-5-carboxylate (p5c) by two enzymes, Δ^1 -Pyrroline-5-carboxylate synthetase and Δ^1 -Pyrroline-5-carboxylate reductase (P5CR).It has been shown from labeling experiment that ornithin (orn) can also serves as a precursors to proline biosynthesis in higher plants (Chiang and Dardekar, 1995).The isolation of cDNA encoding Ornithine- δ -aminotransferase (OAT) in higher plants (Delauney and Verma, 1993; Roosens *et al.*, 1998) suggests that Ornithine- δ -aminotransferase participates in proline biosynthesis by reducing P5C from ornithin and α -ketoglutarate.

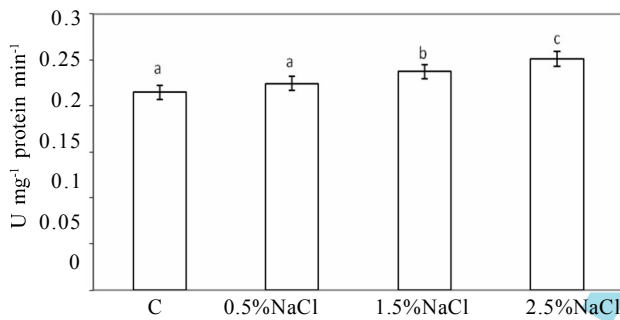


Fig. 1 : Effect of salinity levels on P5CS activity in Badami. Vertical bars indicate \pm S.E. of mean followed by different alphabets are significantly different at P=0.05 for treatments. Values are mean of three replicates

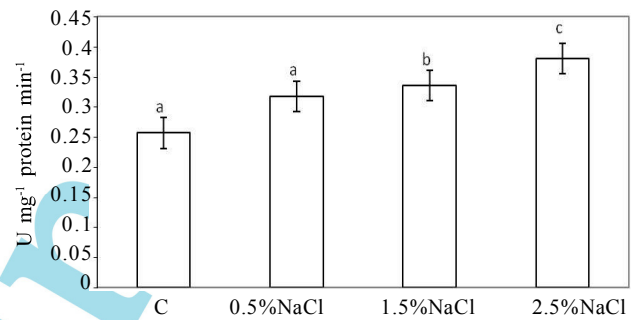


Fig. 3 : Effect of salinity levels on OAT activity in Badami. Vertical bars indicate \pm S.E. of mean followed by different alphabets are significantly different at P=0.05 for treatments. Values are mean of three replicates

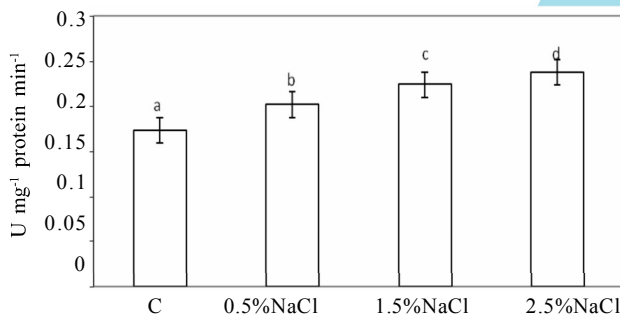


Fig. 2 : Effect of salinity levels on P5CR activity in Badami. Vertical bars indicate \pm S.E. of mean followed by different alphabets are significantly different at P=0.05 for treatments. Values are mean of three replicates

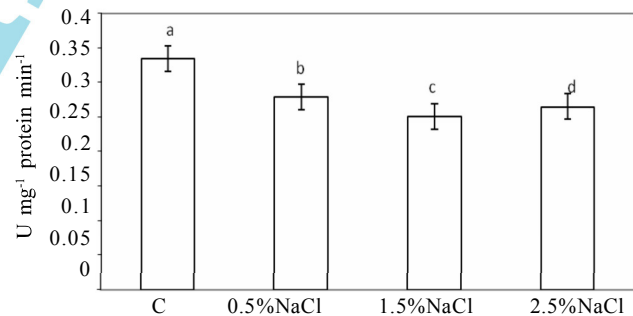


Fig. 4 : Effect of salinity levels on PDH activity in Badami. Vertical bars indicate \pm S.E. of mean followed by different alphabets are significantly different at P=0.05 for treatments. Values are mean of three replicates

The trend of increase in enzyme activity of Δ^1 -Pyrroline-5-carboxylate synthetase was recorded more than the activity of Δ^1 -Pyrroline-5-carboxylate reductase activity and Ornithine- δ -aminotransferase activity.

The activity of proline dehydrogenase at control level was recorded $0.3346 \text{ U mg}^{-1} \text{ protein min}^{-1}$. A significant decrease was recorded in proline dehydrogenase activity with an increasing level of NaCl concentration up to 2.5 % (Fig.4). The maximum decrease in proline dehydrogenase activity was recorded at 2.5 % NaCl concentration (30.67 %) followed by 1.5 % (25.0 %) and 0.5 % NaCl concentration (16.63 %), respectively. Such types of similar results have been reported (Mattioni *et al.*, 1997; Chuan *et al.*, 2002).

The increase in proline content in plants under salt stress is because of a hypothesis; many plants accumulate proline far in excess of protein synthesis demand. Proline catabolism is repressed under osmotic stress, but once the stress is withdrawn, proline is oxidised to P5C by proline dehydrogenase (PDH), also known as proline oxidase, the first enzyme in the proline degradation pathway. P5C is then converted back to glutamate by the enzyme P5C dehydrogenase (P5CDH). Thus, both PDH and P5CDH form two important enzymes in the degradation of proline to glutamate in higher plants (Kavikishore *et al.*, 2005).

In higher plants, proline accumulation appears to be essential due to catabolism rate minimization. In plants, proline is synthesized from glutamic acid (Glu) via Δ^1 -pyrroline-5-carboxylate (P5C) by two enzymes, P5C synthetase (P5CS) and P5C reductase (P5CR). It has been shown from labelling experiments that ornithine(Orn) can also serve as a precursor to proline biosynthesis in higher plants (Chuan *et al.*, 2002). The isolation of cDNA encoding ornithine- δ -aminotransferase (OAT) in higher plants (Roosens *et al.*, 1998) suggests that OAT participates in proline biosynthesis by producing P5C from ornithine and α -ketoglutarate. Arginine can also contribute to proline biosynthesis, and the pathway from arginine proceeds via ornithine as a result of catalytic activity of arginase (Lingnowski and Splittstoesser, 1971). Proline is metabolized to glutamic acid via P5C by two enzymes, proline dehydrogenase (PDH) and P5C dehydrogenase, (Yoshida *et al.*, 1997).

In the present study, the proline accumulation although increased significantly in all the treatment over control values (Table 2). Ornithine is a precursor of proline biosynthesis, by using 75 mM ornithine in $\frac{1}{2}$ strength Hoagland solution $18.77 \mu\text{g g}^{-1}$ proline was recorded (554.5% more than control) whether we are getting $26.36 \mu\text{g g}^{-1}$ proline by the addition of 75 mM ornithine in 250 mM NaCl stress (821.6 % more than control) so it is clearly seen by the data that NaCl is positively involved in proline biosynthesis.

In the present study proline biosynthesis in rice cv badami was mainly due to NaCl. Thus it is of great interest to know whether proline accumulation caused by NaCl is also being increase due to other inorganic salts. Results (Table 2) show that MnCl_2 at 300 mg concentration was observed to increase proline content highest than other concentration of it and other salts over control significantly. This increase in proline content was seen 621.84 % more at MnCl_2 of 300 mg concentration followed by CaCl_2 500 mg(130.36 %), KCl 200 mg (123.78 %), NH_4Cl_2 200 mg (99.6%), KCl 400 mg (85.57 %), MnCl_2 600 mg (69.36%) and CaCl_2 200 mg (28.35 %) over control.

Results (Table 3) indicated a linear decrease in proline content was recorded in plants down to 50 mg concentration of MnCl_2 via MnCl_2 200 mg and MnCl_2 100 mg significantly. Now it is proved that 300 mg concentration of MnCl_2 is highly contributing in increasing proline accumulation in rice cv badami under 1 % NaCl stress condition. Most likely the synthesis of proline and its consequent accumulation in badami is a consequence of intracellular ionic adjustment that takes place under salt stress to keep the metabolic activities going on (Rout and Shaw, 1998).

The addition of MnCl_2 was seen to enhance at proline content in plants under 1% NaCl stress. Thus, the role of proline as osmotic regulator; protector of enzyme

Table 2 : Proline accumulation by adding ornithin(precursor of Proline synthesis) with and without NaCl in $\frac{1}{2}$ strength 220 ml Hoagland solution at 1 DAT

Treatment	Proline content ($\mu\text{g g}^{-1}$ f.wt.)
Control	2.86 ± 0.65
Control+75 mM Ornithine	18.77 ± 0.49
NaCl 250 mM	5.13 ± 0.44
NaCl 250 Mm+75 mM Ornithine	26.36 ± 0.36

HAT=Hours after treatment

Table 3 : Increased proline accumulation in plants under NaCl salt stress condition by adding different inorganic salts in $\frac{1}{2}$ strength 220 ml Hoagland solution having 1.0% NaCl at 1 DAT

Treatment	Proline content ($\mu\text{g g}^{-1}$ f.wt.)
Control	58.43 ± 2.01
200 mg CaCl_2	75.03 ± 1.63
500 mg CaCl_2	134.6 ± 1.74
200 mg KCl	130.76 ± 1.56
400 mg KCl	108.43 ± 1.52
300 mg MnCl_2	421.76 ± 1.63
600 mg MnCl_2	98.96 ± 1.0
200 mg NH_4Cl_2	116.63 ± 0.87
400 mg NH_4Cl_2	67.6 ± 0.76

DAT=Days after treatment

Table 4 : Proline accumulation in plants under NaCl salt stress condition by adding more effective different concentration of MnCl₂ in ½ strength 220 ml Hoagland solution having 1.0 % NaCl at 1 DAT

Treatment	Proline content (µg g ⁻¹ f.wt.)
Control	49.96 ± 2.22
50 mg MnCl ₂	79.43 ± 2.03
100 mg MnCl ₂	118.43 ± 1.63
200 mg MnCl ₂	144.86 ± 1.24
300 mg MnCl ₂	366.03 ± 2.13
400 mg MnCl ₂	193.6 ± 1.49
600 mg MnCl ₂	115.3 ± 1.98

DAT=Days after treatment; Values are mean of replicates

denaturation; reservoir of nitrogen and carbon source for post growth; stabilizer of membranes and machinery of protein synthesis; hydroxyl radical scavenger and to provide salinity tolerance to plants may be promoted by addition of MnCl₂ under 1% salt stress condition.

In conclusion, our results suggest that proline accumulation in rice leaves caused by NaCl is related to enzyme activity during proline metabolism which could provide valuable information on the physiological significance of its accumulation.

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