



Regulation of ammonium-induced proline accumulation in detached rice leaves

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Abstract

Accumulation of proline in response to NH_4Cl was studied in detached leaves of rice (*Oryza sativa* cv. Taichung Native 1). Increasing concentrations of NH_4Cl from 50 to 200 mM progressively increased proline content and this was correlated with the increase in ammonium content. Proline accumulation induced by NH_4Cl was related to proteolysis, an increase in ornithine- δ -aminotransferase activity, a decrease in proline dehydrogenase activity, and a decrease in proline utilisation and could not be explained by NH_4Cl -induced modification in Δ^1 -pyrroline-5-carboxylate reductase activity. The content of glutamic acid was decreased by NH_4Cl , whereas the increase in arginine and ornithine contents was found to be associated with the increase in proline content in NH_4Cl -treated detached rice leaves.

Abbreviations: FW – fresh weight, GABA – γ -aminobutyric acid, OAT – ornithine- δ -aminotransferase, P5C – Δ^1 -pyrroline-5-carboxylate, P5CR – Δ^1 -pyrroline-5-carboxylate reductase, PDH – proline dehydrogenase

Introduction

The ammonium ion is a central intermediate in the metabolism of plants. Ammonium has been shown to accumulate in leaves subjected to water stress, when exposed to excess Cu or excess Cd, and during dark-induced leaf senescence (Chen and Kao 1998; Chen et al. 1997; Chien and Kao 2000; Lin and Kao 1998; Postitus and Jacobi 1976; Thomas 1978). Proline has been shown to accumulate in plants subjected to various types of stress (Aziz et al. 1998; Bassi and Sharma 1993; Charest and Phan 1990; Chen and Kao 1995; Guerrier 1995; Lutts et al. 1999; Madan et al. 1995; Mattioni et al. 1997; Savoure et al. 1997; Wu et al. 1995; Yang et al. 1999, 2000) and during dark-induced leaf senescence (Chou et al. 1990; Wang et al. 1982). Proline accumulation is often considered to be involved in stress resistance mechanisms, although its precise role continues to be controversial (Hare et al. 1999; Yoshiba et al. 1997). Based on data in the literature, Rabe (1990) postulated that any stress con-

dition causing reduced growth or impaired plant health will result in ammonium accumulation early in the stress period and suggested that the detoxification process in which excess ammonium in the cells is removed results in the accumulation of nitrogen-containing compounds, such as putrescine and proline. In previous studies, we demonstrated that exogenous NH_4Cl and methionine sulfoximine, which caused an accumulation of ammonium in detached rice leaves, increased proline content (Yang and Kao 1999).

Proline accumulation in plant tissues has been suggested to be the result of (a) a decrease in proline degradation, (b) an increase in proline biosynthesis, (c) a decrease in protein synthesis or proline utilisation, and (d) hydrolysis of proteins (Charest and Phan 1990; Hare et al. 1999; Yoshiba et al. 1997). In plants, proline is synthesised from glutamic acid via Δ^1 -pyrroline-5-carboxylate (P5C) by two enzymes, P5C synthetase and P5C reductase (P5CR) (Yoshiba et al. 1997). It has been shown from labelling experiments that ornithine can also serve as a precursor to proline

biosynthesis in higher plants (Brown and Fowden 1966; Chiang and Dandekar 1995; Coleman and Hegarty 1957). The isolation of cDNA encoding ornithine- δ -aminotransferase (OAT) in higher plants (De-launey and Verma 1993; 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 (Brown and Fowden 1966; Lingnowski and Splittstoesser 1971). Proline is metabolised to glutamic acid via P5C by two enzymes, proline dehydrogenase (PDH) and P5C dehydrogenase (Yoshiba et al. 1997).

Studying the effect of ammonium on enzyme activities involved in proline biosynthesis and degradation could provide valuable information on the physiological significance of its accumulation. However, to our knowledge, no such study has been undertaken. Neither do we know whether three amino acids (glutamic acid, ornithine and arginine) involved in the proline biosynthesis pathways are limiting factors for proline induced by excess ammonium. This paper reports the results of an investigation into the regulation of proline accumulation in detached rice leaves exposed to NH_4Cl .

Materials and methods

Plant material

Rice (*Oryza sativa* L., cv. Taichung Native 1) was cultured as described previously (Lin et al. 1999). Briefly, rice seedlings were planted on a stainless net floating on half-strength Johnson's modified nutrient solution (Johnson et al. 1957) in a 500 ml beaker. The nutrient solution (pH 4.8) was replaced every three days. Rice plants were grown for 12 days in a greenhouse, where natural light was provided and the temperature was controlled at 30 °C during the day and at 25 °C at night. The apical 3 cm of the third leaf was used for the experiment. A group of 10 segments was floated in a Petri dish containing 10 ml of test solution. Incubation was at 27 °C in the light ($40 \mu\text{mol m}^{-2} \text{s}^{-1}$). All the experiments were repeated four times and yielded results consistent with the data presented here.

Determinations of proline and other amino acids

Proline was extracted and its concentration determined by the method of Bates et al. (1973). Leaf segments were homogenised 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 h and then absorbance at 520 nm was determined. Contents of proline are expressed as $\mu\text{mol g}^{-1}$ FW. For determination of glutamic acid, glutamine, arginine, ornithine, γ -aminobutyric acid (GABA), and total amino acids, leaf samples were extracted with 2% sulfosalicylic acid and the homogenate was centrifuged at 15,000 g for 20 min. The supernatant was used directly for amino acid analysis. Amino acid analysis was done using an amino acid analyzer (Beckman 6300, California, USA) and contents of amino acids are expressed as nmol g^{-1} FW. For protein determination, leaf segments were homogenised 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 is expressed as mg g^{-1} FW.

Enzyme assay

A similar extraction procedure was used for P5CR and PDH, which is mainly based on the procedure described by Lutts et al. (1999). Detached rice leaves were homogenised in a prechilled mortar and pestle with 50 mM Tris-HCl buffer (pH 7.4) containing 7 mM MgCl_2 , 0.6 M KCl and 3 mM EDTA. The homogenate was centrifuged at 15,000 g for 20 min. The supernatant was desalted by Sephadex G-25 column before the assay of P5CR and PDH. The solution used for extraction of 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. The supernatant was desalted by Sephadex G-25 column before the assay of OAT. All the extraction procedures were done at 4 °C.

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 g^{-1}

FW (one unit is defined as a decrease in 1 A_{340} per min) or units mg^{-1} protein (one unit is defined as a decrease in 0.01 A_{340} per min). 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 trichloroacetic 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 h. After centrifugation at 12,000 g for 10 min, the clear supernatant fraction was taken to measure the absorbance at 440 nm. OAT is expressed as units g^{-1} FW (one unit is defined as an increase in 1 A_{440} per h) or units mg^{-1} protein (one unit is defined as an increase in 0.01 A_{440} per h). PDH was assayed by following the NAD^+ reduction at 340 nm in a 0.15 M Na_2CO_3 -HCl buffer (pH 10.3) containing 13 mM L-proline and 1.5 mM NAD^+ (Lutts et al. 1999). PDH is expressed as units g^{-1} FW (one unit is defined as an increase in 1 A_{340} per min) or units mg^{-1} protein (one unit is defined as an increase in 0.01 A_{340} per min).

Determination of proline utilisation

For proline utilisation, detached rice leaves were pre-treated with 50 mM ornithine for 3 h (since addition of ornithine has been observed to be more effective than that of glutamic acid or arginine in increasing proline content in rice leaves (Yang et al. 1999)) to increase the endogenous proline content and then transferred to distilled water and 100 mM NH_4Cl for 8 h in the light. Proline content was then determined. The decline in proline content was considered as a measure of proline utilisation (Yang et al. 1999).

Determination of ammonium

Ammonium was extracted by homogenising leaf segments in 0.3 mM sulphuric acid (pH 3.5). The homogenate was centrifuged for 10 min at 39,000 g and the supernatant was used for the determination of ammonium as described previously (Lin and Kao 1998).

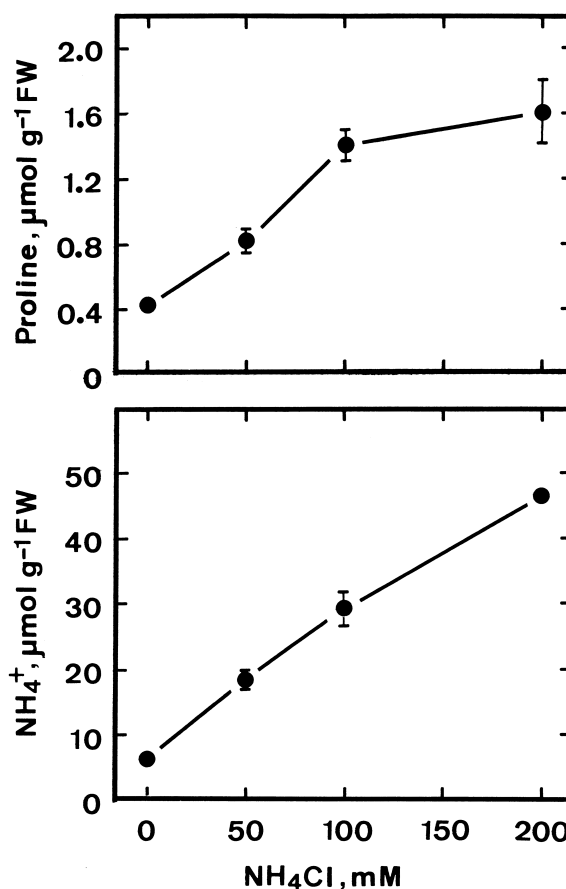


Figure 1. Effect of NH_4Cl on ammonium and proline contents in detached rice leaves. All measurements were made 12 h after treatment in the light. Vertical bars represent SE ($n=4$).

Results and discussion

Proline content in detached rice leaves increased with the increase of NH_4Cl concentrations (Figure 1). Proline content increased 4- to 5-fold in detached leaves treated with 100 mM NH_4Cl for 12 h (Figures 1 and 2). To be sure that the proline accumulation described was related to leaf ammonium content, ammonium concentrations were determined in detached rice leaves treated with various concentrations of NH_4Cl . Ammonium content in NH_4Cl -treated detached rice leaves increased with increasing concentrations of NH_4Cl (Figure 1). Since treatment of 100 mM KCl only resulted in a slight increase in ammonium and proline contents (Figure 2), it seemed that the increase in proline content in NH_4Cl -treated detached rice leaves was mainly associated with the increase in ammonium content rather than of Cl^- .

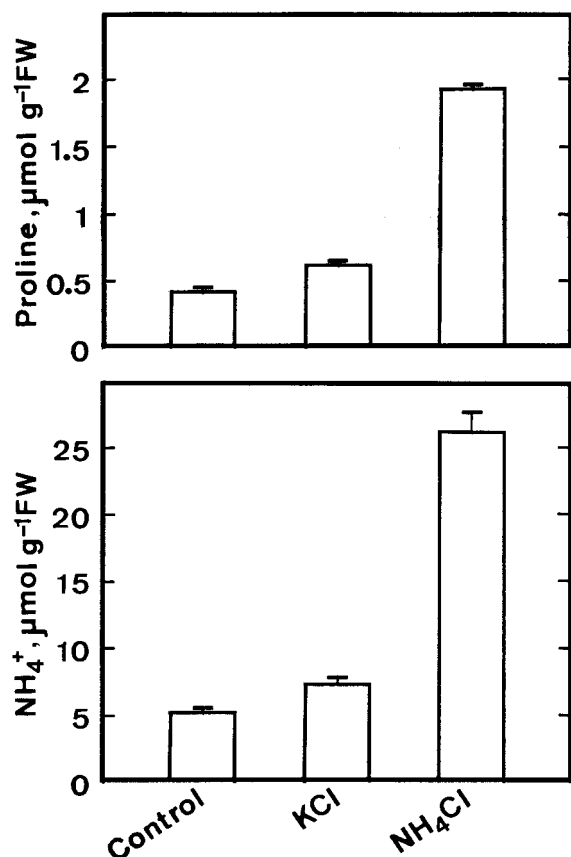


Figure 2. Effect of KCl and NH₄Cl on ammonium and proline contents in detached rice leaves. Detached rice leaves were treated with distilled water, 100 mM KCl or 100 mM NH₄Cl for 12 h in the light. Vertical bars represent SE (n=4).

It is well established that glutamic acid, ornithine and arginine can all contribute to the accumulation of proline in plant tissues (Chiang and Dandekar 1995). In previous studies, we reported that exogenous application of ornithine or arginine was effective in increasing the proline content in detached rice leaves (Yang et al. 1999). In contrast, proline content could not be increased in detached rice leaves by the addition of glutamic acid up to 50 mM (Yang et al. 1999). It seems that detached rice leaves contained sufficient amounts of glutamic acid. Thus, it is of great interest to know the effect of NH₄Cl on the content of ornithine, arginine and glutamic acid contents in detached rice leaves. Table 1 shows that NH₄Cl treatment resulted in an increase in ornithine and arginine contents in detached rice leaves and a decrease in glutamic acid content. These observations suggest that high contents of endogenous ornithine and arginine are associated with NH₄Cl-induced proline ac-

cumulation in detached rice leaves. Also shown in Table 1 is that NH₄Cl treatment increases glutamine and GABA contents. It seems that the decreased content of glutamic acid by NH₄Cl may result from it being metabolised to GABA (Ireland and Lea 1999). The increase in glutamine content and the decrease in glutamic acid in NH₄Cl-treated detached rice leaves also suggest that NH₄Cl inhibits the conversion of glutamine to glutamic acid, a step catalysed by glutamic acid synthase (Ireland and Lea 1999). The results of Table 1 show that protein content decreases and total amino acids increases in NH₄Cl-treated detached rice leaves, indicating that hydrolysis of proteins in NH₄Cl-treated detached rice leaves is also responsible for the accumulation of proline.

To determine the role of biosynthetic pathways for proline accumulation caused by NH₄Cl, the effect of NH₄Cl on OAT and P5CR activities was examined. The results are shown in Figure 1. OAT activity, expressed either on per g FW or per mg protein basis, increased with increase of concentrations of NH₄Cl. However, NH₄Cl had no effect on P5CR activity in detached rice leaves. Thus, the increase in OAT activity may have contributed, to some extent, to the elevated content of proline by NH₄Cl. Increase in OAT activity, along with an increase in the content of proline has also been reported in wheat under cold stress (Charest and Phan 1990), and in *Brassica juncea* under salt stress (Madan et al. 1995). NH₄Cl could also have an effect on the bifunctional enzyme P5C synthetase involved in the synthesis of P5C from glutamic acid (Hare et al. 1999; Yoshiba et al. 1997). However, for unknown reasons, we were unable to detect any P5C synthetase activity in crude extracts. Therefore, this enzyme was not considered in the present work.

The enzyme PDH is reported to catalyse proline oxidation (Hare et al. 1999; Yoshiba et al. 1997). In the present investigation, NH₄Cl treatment resulted in a decrease in PDH activity in detached rice leaves (Figure 3). This result suggests that proline oxidation (or degradation) contributes to proline accumulation in detached rice leaves exposed to NH₄Cl.

Recently, we demonstrated that proline in detached rice leaves exposed to water stress is less utilised than in the water controls (Yang et al. 2000). It is possible that less utilisation of proline may also contribute to the accumulation of proline in detached rice leaves caused by NH₄Cl. The results reported in Table 2 demonstrated that this is, in fact, correct.

Table 1. Contents of glutamate, ornithine, arginine, GABA, protein and total amino acids in detached rice leaves incubated in distilled water or NH_4Cl .

Treatment	Protein (mg g^{-1} FW)	Amino acids (nmol g^{-1} FW)					
		Total amino acids	Glutamine	Glutamate	Ornithine	Arginine	GABA
H_2O	60.55 ± 1.05	20120 ± 775	1383 ± 88	6898 ± 215	17 ± 1	287 ± 50	382 ± 142
NH_4Cl	50.29 ± 0.74	78533 ± 1169	32877 ± 1183	3179 ± 110	55 ± 8	1088 ± 149	2653 ± 933

Detached rice leaves were treated with distilled water or NH_4Cl (100 mM). All measurements were made 12 h after treatment. Means \pm SE (n=4).

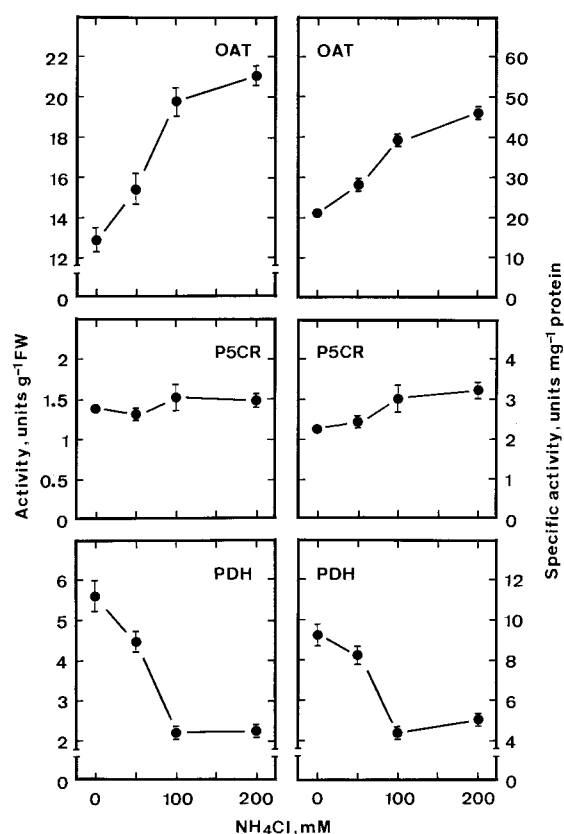


Figure 3. Effect of NH_4Cl on the activities of OAT, P5CR and PDH in detached rice leaves. All measurements were made 12 h after treatment in the light. Vertical bars represent SE (n=4).

In conclusion, proline accumulation, in detached leaves, caused by ammonium is related to protein hydrolysis, an increase in ornithine and arginine contents, an increase in OAT activity, a decrease in PDH activity, and a decrease in proline utilisation. In the present investigation, the regulation of proline accumulation caused by ammonium was studied using detached rice leaves. It is not known whether similar mechanism is operated in intact rice leaves. Thus, ammonium-regulated proline accumulation in intact leaves of rice plants merits future investigation.

Table 2. Proline content in ornithine-pretreated detached rice leaves incubated in distilled water and NH_4Cl

Treatment	Proline, $\mu\text{mol g}^{-1}$ FW
H_2O , 3 h	0.77 ± 0.17
Ornithine, 3 h	27.13 ± 0.68
Ornithine, 3 h \rightarrow H_2O , 8 h	13.78 ± 0.45
Ornithine, 3 h \rightarrow NH_4Cl , 8 h	19.12 ± 0.66

Detached rice leaves were pretreated with 50 mM ornithine for 3 h in the light and then incubated in distilled water or NH_4Cl (100 mM) for 8 h in the light. Means \pm SE (n=4).

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References

- Aziz A., Martin-Tanguy J. and Larher F. 1998. Stress-induced changes in polyamine and tyramine levels can regulate proline accumulation in tomato leaf discs treated with sodium chloride. *Physiol. Plant.* 104: 195–202.
- Bassi R. and Sharma S.S. 1993. Changes in proline content accompanying the uptake of zinc and copper by *Lemma minor*. *Ann. Bot.* 72: 151–154.
- Bates L.S., Waldren S.P. and Teare I.D. 1973. Rapid determination of free proline for water-stress studies. *Plant Soil.* 39: 205–207.
- Bradford M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Anal. Biochem.* 72: 248–254.
- Brown D.H. and Fowden L. 1966. Metabolism of δ -acetylornithine in two Leguminous species. *Phytochemistry.* 5: 887–892.
- Charest C. and Phan C.T. 1990. Cold acclimation of wheat: Properties of enzymes involved in proline metabolism. *Physiol. Plant.* 80: 159–168.
- Chen L.-M. and Kao C.H. 1998. Relationship between ammonium accumulation and senescence of detached rice leaves caused by excess copper. *Plant Soil.* 200: 169–173.
- Chen S.L. and Kao C.H. 1995. Cd induced changes in proline content and peroxidase activity in roots of rice seedlings. *Plant Growth Regul.* 17: 67–71.

- Chen S.J., Hung K.T. and Kao C.H. 1997. Ammonium accumulation is associated with senescence of rice leaves. *Plant Growth Regul.* 21: 195–201.
- Chiang H.-H. and Dandekar A.M. 1995. Regulation of proline accumulation in *Arabidopsis thaliana* (L.) Heynh during development and in response to desiccation. *Plant Cell Environ.* 18: 1280–1290.
- Chien H.-F. and Kao C.H. 2000. Accumulation of ammonium in rice leaves in response to excess cadmium. *Plant Sci.* 156: 111–115.
- Chou I.T., Chen C.T. and Kao C.H. 1990. Regulation of proline accumulation in detached rice leaves. *Plant. Sci.* 70: 43–48.
- Coleman R.G. and Hegarty M.P. 1957. Metabolism of D, L-ornithine- 2^{14}C in normal and potassium-deficient barley. *Nature.* 179: 376–377.
- Delauney A.J. and Verma D.P.S. 1993. Proline biosynthesis and osmoregulation in plants. *Plant. J.* 4: 215–223.
- Guerrier G. 1995. Effect of salt-stress on proline metabolism in calli of *Lycopersicon esculentum*, *Lycopersicon pennellii*, and their interspecific hybrid. *Can. J. Bot.* 73: 1939–1946.
- Hare P.D., Cress W.A. and van Staden J. 1999. Proline synthesis and degradation: a model system for elucidating stress-related signal transduction. *J. Exp. Bot.* 333: 413–434.
- Ireland A.J. and Lea P.J. 1999. The enzymes of glutamine, glutamate, asparagine, and aspartate metabolism. In: Singh B.K. (ed.), *Plant Amino Acids*. MerceL Dekker Inc, New York, pp. 49–109.
- Johnson C.M., Stout P.R., Broyer T.C. and Carlton A.B. 1957. Comparative choline requirements of different plant species. *Plant. Soil.* 8: 337–353.
- Lin J.-N. and Kao C.H. 1998. Water stress, ammonium, and leaf senescence in detached rice leaves. *Plant Growth Regul.* 26: 165–169.
- Lin J.-N., Wang J.-W. and Kao C.H. 1999. Effect of abscisic acid and water stress on the senescence of detached rice leaves. *Biol. Plant.* 42: 313–316.
- Lingnowski E.M. and Splittstoesser W.E. 1971. The change in arginine levels and metabolism of urea and ornithine in *Cucubita moschatta* seedlings. *Physiol. Plant.* 25: 225–229.
- Lutts S., Majerus V. and Kinet J.M. 1999. NaCl effects on proline metabolism in rice (*Oryza sativa* L.) seedlings. *Physiol. Plant.* 105: 450–458.
- Madan S., Nainawatee H.S., Jain R.K. and Chowdhury J.B. 1995. Proline and proline metabolising enzymes in *in-vitro* selected NaCl-tolerant *Brassica juncea* L. under salt stress. *Ann. Bot.* 76: 51–57.
- Mattioni C., Lacerenza N.G., Troccoli A., De Leonardi A.M. and Di Fonzo N. 1997. Water and salt stress-induced alterations in proline metabolism of *Triticum durum* seedlings. *Physiol. Plant.* 101: 787–792.
- Postitus C. and Jacobi G. 1976. Dark starvation and plant metabolism. VI. Biosynthesis of glutamic acid dehydrogenase in detached leaves of *Cucurbita maxima*. *Z. Pflanzenphysiol.* 78: 133–140.
- Rabe E. 1990. Stress physiology: The functional significance of the accumulation of nitrogen-containing compounds. *J. Hort. Sci.* 65: 231–243.
- Rena A.B. and Splittstoesser W.E. 1975. Proline dehydrogenase and pyrroline-5-carboxylate reductase from pumpkin cotyledons. *Phytochemistry.* 14: 657–661.
- Roosens N.H.C.J., Thu T.T., Iskandar H.M. and Jacobs M. 1998. Isolation of the ornithine- δ -aminotransferase cDNA and effect of salt stress on its expression in *Arabidopsis thaliana*. *Plant. Physiol.* 117: 263–271.
- Savoure A., Hua X.-J., Bertauche N., van Montagu M. and Verbruggen N. 1997. Abscisic acid-independent and abscisic acid-dependent regulation of proline biosynthesis following cold and osmotic stresses in *Arabidopsis thaliana*. *Mol. Gen. Genet.* 254: 104–109.
- Thomas H. 1978. Enzymes of nitrogen mobilization in detached leaves of *Lolium temulentum* during senescence. *Planta.* 142: 161–169.
- Vogel R.H. and Kopac M.J. 1960. Some properties of ornithine- δ -transaminase from Neurospore. *Biochem. Biophys. Acta.* 37: 539–540.
- Wang C.Y., Cheng S.H. and Kao C.H. 1982. Senescence of leaves VII. Proline accumulation in senescing excised leaves. *Plant. Physiol.* 69: 1348–1349.
- Wu J.-T., Chang S.J. and Chou T.-L. 1995. Intracellular proline accumulation in algae exposed to copper and cadmium. *Bot. Bull. Acad. Sin.* 36: 69–93.
- Yang C.-W. and Kao C.H. 1999. Ammonium in relation to proline accumulation in detached rice leaves. *Plant Growth Regul.* 30: 139–144.
- Yang C.-W., Lin C.C. and Kao C.H. 1999. Endogenous ornithine and arginine contents and dark-induced proline accumulation in detached rice leaves. *J. Plant. Physiol.* 155: 665–668.
- Yang C.-W., Wang J.-W. and Kao C.H. 2000. The relation between accumulation of abscisic acid and proline in detached rice leaves. *Biol. Plant.* 43: 301–304.
- Yoshida Y., Kiyosue T., Nakashima K., Yamaguchi-Shinozaki K. and Shinozaki K. 1997. Regulation of levels of proline as an osmolyte in plants under water stress. *Plant. Cell Physiol.* 38: 1095–1102.