

# The effects of NaCl stress on *Jatropha* cotyledon growth and nitrogen metabolism

S. Gao<sup>1,2</sup>, K.-T. Liu<sup>1</sup>, T.-W. Chung<sup>1</sup>, F. Chen<sup>3</sup>

<sup>1</sup>Institute of Ecological Forestry, Faculty of Forestry, Sichuan Agricultural University, Wenjiang 611130, Chengdu, Sichuan, China.<sup>2</sup>Department of Chemical Engineering/R&D Center for Membrane Technology, Chung-Yuan University, Chungli 320, Taiwan. <sup>3</sup>College of Life Sciences, Sichuan University, 610064, Chengdu, China. \*Corresponding author's email: shun1220@yahoo.com or twchungbill@gmail.com

## Abstract

The effects of treatment with 50 and 100 mM NaCl on cotyledon growth, nitrogen assimilation, and the activities of key nitrogen metabolism enzymes were investigated in developing *Jatropha curcas* cotyledons. Growth inhibition was observed, and relative water content in the developing cotyledons significantly decreased in the 100 mM NaCl treatment. Lower levels of NO<sub>3</sub><sup>-</sup>, NO<sub>2</sub><sup>-</sup>, and NH<sub>4</sub><sup>+</sup> in the cotyledons were recorded when they were supplied with 50 and 100 mM NaCl. Nitrate reductase activities decline by 9.5% to 31.8% with respect to the control groups. Salt treatment also led to a marked decrease in nitrite reductase activity compared with the controls. Glutamine synthase, glutamate synthetase and dehydrogenase, alanine aminotransferase (AlaAT) and aspartate aminotransferase (AspAT) were markedly induced by the addition of NaCl. These findings will be useful for elucidating the roles key enzymes involved in nitrogen assimilation pathway of *J. curcas* plant.

**Keywords:** *Jatropha curcas*, NaCl stress, nitrogen metabolism, growth inhibition, ammonium

## 1. Introduction

Increases in worldwide agricultural and eco-environmental problems related to soil salinity have received considerable attention recently, especially in arid and semi-arid regions. Soil salinity is a major threat to food production because it limits crop yields and restricts the use of previously uncultivated land. At the moment, various factors, such as intensive agronomic practices, poor water management, irrigation without sufficient drainage systems, limited rainfall, high evapo-transpiration,

and high temperature, have affected nearly 20% of the world's cultivated land and nearly half of irrigated lands by salinity (Parvaiz and Satyawati, 2008). Thus, studying salt stress is an important means to elucidate plant biochemical and physiological mechanisms. The direct and indirect effects of salinity on plant tissues may include the following characteristics: 1) reduced water availability as a consequence of high soluble salts in the soil; 2) ion-specific effects that result from an excessive accumulation of toxic ions (Na<sup>+</sup> and Cl<sup>-</sup>)

in plants; and 3) salt-induced secondary stress, e.g., oxidative bursts that are caused by the overproduction of reactive oxygen species (Abogadallah, 2010). Moreover, plant responses to salt stress may also lead to differences in nitrogen (N) metabolism, namely ion uptake, N assimilation, and amino acid and protein synthesis, which all influence each level of plant function from metabolism to resource allocation, growth, and development (Kusano *et al.*, 2011). Plant physiological and biochemical responses to salt stress have been extensively studied, but their underlying mechanisms are still not well understood.

Nitrogen is an essential plant macronutrient, and its availability has a major influence on the growth and development of plants. The most available form of this element in higher plants is nitrate ( $\text{NO}_3^-$ ), which is the most important source of N in the majority of agricultural soils. Compared with other inorganic sources of nitrogen, nitrate must be reduced to ammonium before its incorporation into organic compounds (Näsholm *et al.*, 2009). Nitrate is first reduced by nitrate reductase (NR) to nitrite in the cytosol, and the resulting nitrite is in turn reduced to ammonium by nitrite reductase (NiR) in the plastids/chloroplasts. Ammonium must be rapidly assimilated because it is toxic to plant cells. These processes are carried out by two highly regulated pathways. Ammonium can be directly incorporated into glutamate by the aminating reaction of glutamate dehydrogenase (NADH-GDH). The resulting  $\text{NH}_4\text{N}$  is assimilated into glutamine by glutamine synthetase (GS), which is subsequently converted to glutamate using 2-oxoglutarate by glutamate synthase (GOGAT). The assimilation of ammonium into glutamine and glutamate is vital for plant growth because these two amino acids are precursors for the synthesis of the other amino acids, as well as almost all nitrogenous compounds (Kusano *et al.*, 2011). Alanine aminotransferase (AlaAT) and aspartate aminotransferase (AspAT) are involved in transferring the assimilated nitrogen moieties to organic acids and play an important role in the synthesis of amino acids from glutamate. Aminotransferase-catalyzed reactions are reversible, and alanine and aspartic acid act as

amino donors and may also participate in replenishing the glutamate pool (Hodges, 2002). Changes in the nitrate or ammonium uptake and nitrogen-assimilating enzymes have been observed after NaCl treatment in several plant species, such as the tomato, *Populus x canescens*, and mulberry (Debouba *et al.*, 2006; Dłuzniewska *et al.*, 2007; Surabhi *et al.*, 2008). These investigations have shown that salt stress can affect different plant nitrogen metabolism steps, such as ion uptake, N assimilation, and the synthesis of amino acids and proteins, which may have severe consequences for nitrate assimilation through the inhibition of NR and NiR activity. Salt treatment can also cause significant changes in the activities of GOGAT, GS, and glutamate dehydrogenase. In general, these responses often differ according to the plant species, tissue type, cultivar, and stress conditions (Wang *et al.*, 2007). Therefore, the characterization of N metabolism enzymes is relevant to understanding plant responses to biotic and abiotic stresses in the environment and should lead to a greater understanding of enzyme behavior, nitrogen metabolism, and regulation.

*Jatropha curcas* (*J. curcas*) is cultivated in many tropical and subtropical countries. Different parts of this plant can be used as an energy source and for therapeutic uses, fertilizer, and animal feed. *J. curcas* is also well adapted to environments with constraining conditions, such as gravelly soil, reduced rainfall, high temperatures, and poor soil conditions that are otherwise not suitable for most domestic plant species (Abdulla *et al.*, 2011). A previous report has shown that the capacity of *J. curcas* for NaCl stress is associated with decreased biomass and increased superoxide dismutase, peroxidase, catalase, and phenylalanine ammonia-lyase enzyme activities in seedlings (Gao *et al.*, 2008). Fujimaki and Kikuchi (2010) studied *Jatropha* tolerance-related parameters within a widely used root water uptake model in response to drought and salinity stresses and concluded that *Jatropha* appeared to be moderately tolerant to stress compared with other crops. The photosynthetic machinery is most sensitive to stress. The photosynthetic adaptability of *J. curcas* to increased salinity in liquid culture medium

was reduced with both the the degree of salinity and the period of saline stress (Da Silva *et al.*, 2011). Although many studies have addressed the effects of salt stress on antioxidant responses, photosynthetic adaptability, and salt tolerance, the effects of salt stress on the N metabolism of *J. curcas* have not been studied. Thus, the present study aimed to investigate the effects of NaCl stress on nitrate and ammonium concentration changes, as well as the activity of N metabolism enzymes in *J. curcas* cotyledons. This study will provide a better understanding of nitrogen metabolism actions under NaCl stress in *J. curcas* plants.

## 2. Materials and Methods

### 2.1. Embryo germination and seedling growth

*J. curcas* seeds were purchased from Mother Herbs PVT LTD., Delhi, India, and separated from their seed coats. They were surface sterilized in 70% ethanol for 30-45 sec, followed by a 0.1% mercuric chloride treatment for 8-10 min. Seeds were rinsed with distilled water and soaked for 24-36 h at room temperature, and the embryos were separated from the seeds on a clean bench. These embryos were washed several times in distilled water, and three embryos were planted on Murashige and Skoog medium (MS, ~30 mL) in wide neck bottles (100 mL). The pH of the MS medium was adjusted to  $5.9 \pm 0.1$  prior to autoclaving at  $121 \pm 2^\circ\text{C}$  for 15 min with 30 g/L sucrose and 8 g/L agar powder. These bottles were separated into three groups. One group of 15 bottles (three embryos per bottles) was allowed to grow on basic MS medium as a control. The salt concentrations of the remaining basic media were brought up to 50 or 100 mM NaCl. All groups were labeled with the sampling dates and cultured in a plant incubator at  $25^\circ\text{C}$  with a 12 h photoperiod and 80% relative humidity. Embryos were considered to be germinating when they were yellow, which usually occurred after 24 h of incubation. The stage at which two cotyledons had developed (6 days) was referred to as a developed seedling. Ten germinated embryos

or developed seedlings were harvested from the three groups every two days up to sixth day. Cotyledons at three developmental stages were collected and cotyledon fresh weights were recorded. Cotyledons were then freeze-dried and stored at  $4^\circ\text{C}$  for further analysis.

### 2.2. Determinations of N content

The freeze-dried samples (approximately 100 mg) were ground to a fine powder in a mortar with liquid nitrogen and then homogenized and extracted using 2 mL of double distilled water. The homogenate was centrifuged at 12,000 rpm for 10 min at  $4^\circ\text{C}$ , and the resulting supernatant was used for an N determination assay. The nitrate content was measured by using a slightly modified method from Agbaria *et al.* (1996). A 40  $\mu\text{L}$  extract was taken for the nitrate determination and added to 160  $\mu\text{L}$  of 5% salicylic acid in sulfuric acid (98%), and 3.8 mL of 2 M NaOH was added after 5 min. Mixture absorbances were recorded at 410 nm with a UV/vis spectrophotometer (CT-2200, ChromTech, Singapore), and the nitrate content was calculated using a calibration curve. The results were expressed as  $\mu\text{g}$  of  $\text{NO}_3^- \text{g}^{-1}$  DW.

The  $\text{NO}_2^-$  content was determined with a spectrophotometric method that employed sulfanilic acid and  $\alpha$ -naphthylamine under acidic conditions (Werber and Mevarech, 1978). One-half mL of supernatant was added to the tubes followed by the addition of 1 mL of 0.2%  $\alpha$ -naphthylamine and 1 mL of 4% sulfanilic acid in hydrochloric acid. The mixtures were allowed to stand at  $35^\circ\text{C}$  for 30 min, and their absorbances were measured at 520 nm. The  $\text{NO}_2^-$  content of the samples was estimated by referring to a  $\text{NaNO}_2$  standard curve and expressed in  $\mu\text{g}$   $\text{g}^{-1}$  DW.

The ammonium content was colorimetrically determined with Nessler reagent (Molins-Legua *et al.*, 2006). The reaction mixture consisted of 100  $\mu\text{L}$  of extract, 10  $\mu\text{L}$  of 10% K-Na tartrate, 100  $\mu\text{L}$  of Nessler reagent, and 2.4 mL of distilled water. After 5 min, the absorbance was read at 425 nm, and the ammonium

the absorbance was read at 425 nm, and the ammonium content was calculated in  $\mu\text{g NH}_4^+ \text{g}^{-1}$  DW according a standard calibration curve that had been prepared with  $\text{NH}_4\text{Cl}$ .

### 2.3. Nitrate reductase (NR) and nitrite reductase (NiR) activity assays

Dried cotyledon samples (approximately 100 mg) were ground under liquid nitrogen and homogenized in a 50 mM phosphate buffer (pH 7.5, 1/20, w/v) containing 5 mM cysteine, 0.5 mM EDTA, and 0.5% polyvinylpyrrolidone (PVP). The homogenized suspension was collected by centrifugation at 12,000 rpm for 10 min at 4°C. The supernatant was used to assay the NR, NiR, and GOGAT. The NR activity was measured using a method by Debouban *et al.* (2006). The reaction was initiated by adding 0.1 mL of extract to 1.4 mL of the reaction mixture, which contained 5 mM EDTA, 7 mM  $\text{KNO}_3$ , and 0.15 mM NADH in 50 mM phosphate buffer (pH 7.5). Incubations were carried out at 30°C for 30 min, and the reaction was stopped by adding 0.1 mL of 0.5 M zinc acetate. The solutions were then centrifuged (5000 rpm for 10 min). Nitrite formation was determined by the previously detailed method, and the NR activity was expressed in units, each of which represented the amount of enzyme catalyzing the formation of 1  $\mu\text{g}$  of  $\text{NO}_2^-$  per minute. Nitrite reductase activity was estimated using the method of Losada and Paneque (1971). An extract of 0.1 ml was added to a 0.8 mL solution including 0.4 mM  $\text{NaNO}_2$  and 2.3 mM methyl viologen in 50 mM phosphate buffer (pH 7.5), and the reaction was initiated by adding 0.1 mL of 86.15 mM sodium dithionate in 100 mM  $\text{NaHCO}_3$ . After 30 min of incubation at 30°C, the reaction was terminated by vigorous shaking and boiling for 1 min. The remaining  $\text{NO}_2^-$  concentrations were measured by using the previously detailed method, and the results are expressed as  $\mu\text{g}$  of nitrite reduced  $\text{g}^{-1}$  dry weight  $\text{h}^{-1}$ .

### 2.4. Glutamate synthase (GOGAT) activity assay

GOGAT activity was determined according to the method of Rachina and Nicholas (1985). The reaction buffer (1.9 mL) consisted of 5 mM  $\alpha$ -oxoglutaric acid, 10 mM glutamine, and 0.15 mM NADH in 50 mM phosphate buffer (pH 7.5), and the reaction was started by adding 0.1 mL of enzyme extract (See section 2.3). The oxidation of NADH ( $\epsilon=6.22 \text{ mM}^{-1} \text{ cm}^{-1}$ ) was monitored by reading the absorbance at 340 nm. The results were expressed in units, each of which represented the amount of enzyme catalyzing the oxidation of 1  $\mu\text{mol}$  NADH per min per g of dry weight.

### 2.5. Glutamine synthetase (GS), GDH, AlaAT and AspAT enzyme activity assays

To measure the GS, GDH, AlaAT, and AspAT activities, dried samples were homogenized in a mortar under liquid nitrogen with 50 mM Tris-HCl buffer (pH 7.5) containing 0.5 mM EDTA, 1 mM  $\text{MgCl}_2$ , 10 mM  $\beta$ -mercaptoethanol, and 0.5% PVP. After centrifugation (12 000 rpm for 10 min), the supernatant was retained for further study. Glutamine synthetase (GS) activity was measured by the method of Oaks *et al.* (1980). A quantity of 1.9 mL of reaction buffer included 13 mM hydroxylamine, 1 mM ATP, 50 mM glutamate, 20 mM  $\text{MgCl}_2$ , and 20 mM sodium arsenate in Tris-HCl buffer (pH 7.5), and the reaction was started with the addition of 0.1 mL of enzyme extract. After incubating the reaction for 30 min at 37°C, it was stopped by adding 1 mL of ferric chloride reagent (0.37 M  $\text{FeCl}_3$  and 0.2 M trichloroacetic acid in 0.5 M HCl). After centrifugation (5000 g for 15 min at 4 °C), the absorbance of the supernatant was recorded at 540 nm. The results are expressed as  $\text{A}\cdot\text{h}^{-1} \text{g}^{-1}$  dry weight.

### 2.6. Glutamate dehydrogenase (GDH) enzyme activity assay

GDH activity was measured by recording the oxidation of NADH (aminating GDH activity, NADH-GDH) or

reduction of NAD (deaminating GDH activity, NAD-GDH) at 340 nm by following the method of Groat and Vance (1981). To measure the NADH-GDH activity, a reaction buffer of 1.9 ml was made from 10 mM  $\alpha$ -oxoglutaric acid, 100 mM  $\text{NH}_4\text{Cl}$ , and 0.2 mM NADH in 50 mM Tris-HCl buffer (pH 8.0), and the reaction was started by adding 100  $\mu\text{l}$  of enzyme extract. To measure NAD-GDH activity, a reaction buffer was made from 80 mM L-glutamic acid and 0.2 mM NAD in 50 mM Tris-HCl buffer (pH 8.8), and the reaction was initiated by adding 0.1 ml of enzyme extract. The oxidation/reduction of NADH/NAD was measured by UV-vis spectrophotometer at 340 nm for 450 s, and the activity of GDH was calculated in units of micromoles of NADH oxidized/NAD reduced per minute per ml. The results are expressed as  $\mu\text{mol}$  per minute per g of dry weight.

### 2.7. Alanine aminotransferase (AlaAT) and aspartate aminotransferase (AspAT) enzyme activity assays

Alanine aminotransferase (ALT) transfers the amino group from alanine to  $\alpha$ -oxoglutarate to form pyruvate and glutamate. The pyruvate enters a lactate dehydrogenase-catalyzed reaction with NADH to produce lactate and  $\text{NAD}^+$ . The AlaAT activity was measured as the decrease in absorbance resulting from the consumption of NADH at 340 nm (Griffith and Vance, 1989; Good and Muench, 1992). The reaction buffer consisted of 15 mM  $\alpha$ -oxoglutarate, 0.15 mM NADH, 0.5 M l-alanine, and 5 units of lactate dehydrogenase in 50 mM Tris-HCl buffer (pH 7.5), and the reaction was initiated by adding 100  $\mu\text{L}$  of enzyme extract. AspAT activity was assayed in the aspartate $\rightarrow$ oxaloacetate direction by coupling the reaction with NADH oxidation by malate dehydrogenase. A reaction buffer of 1.4 mL was made of 5 mM EDTA, 0.2 M L-aspartate, 12 mM 2-oxoglutarate, 0.15 mM NADH, and 5 units of malate dehydrogenase in 50 mM Tris-HCl buffer (pH 7.5), and the reaction was started when 100  $\mu\text{l}$  enzyme extract was added to the reaction buffer. AlaAT and

AspAT activities were measured as a function of NADH oxidation at 340 nm, calculated by using the absorption coefficient for NADH ( $\epsilon=6.22 \text{ mM}^{-1} \text{ cm}^{-1}$ ), and expressed in units, each of which represented the amount of enzyme that catalyzes the formation of 1 nmol of product per minute.

### 2.8. Statistical analysis

Figure data are presented as the average of at least three replicates per treatment. Data are reported as the mean  $\pm$  SD.

## 3. Results

### 3.1. Effects of NaCl stress on growth parameters

The changes in the fresh weight, dry weight, and water content in *J. curcas* cotyledons at three developmental stages are shown in Table 1. The fresh weight tended to slightly increase with the developing stage in the 50 mM NaCl treatment. The fresh weight was increased 6.92% and 8.54% at days 4 and 6 compared with the control, respectively. The dry weights increased by 8.94% and 20.1% at days 4 and 6 compared with the control, respectively. The fresh and dry weights showed no significant changes at day 2. Significant decreases in the fresh weights were observed at all three developing stages in the 100 mM NaCl treatment, and the fresh weight decreased by 14.6%, 20.8%, and 27.2% at days 2, 4, and 6 compared with the control, respectively. The dry weight diminished by 9.88% and 6.02% at days 2 and 6, respectively, but there was no significant change comparison with the control. Moreover, the 50 mM NaCl treatment might have caused a slight reduction in the relative water content (RWC) at days 4 and 6 with respect to the control, but the RWC increased by 5.3% on day 2. The RWC decreased in the 100 NaCl mM treatment by 4.7%, 18.3%, and 21.1% at days 2, 4, and 6 compared with the control, respectively.

**Table 1.** Changes in the fresh weight, dry weight, and relative water content of the developing *J. curcas* cotyledon under NaCl stress. Results are presented as the mean  $\pm$  standard deviation ( $n = 3$ ). Significant difference ( $p < 0.05$ ) is denoted as asterisk (\*) between control and lead treatments.

No.	Day	Fresh weight (mg)	Dry weight (mg)	Relative water content (%)
0	2	52.4 $\pm$ 2.12	24.3 $\pm$ 1.02	53.7 $\pm$ 2.38
	4	63.7 $\pm$ 3.08	24.5 $\pm$ 1.15	61.6 $\pm$ 2.81
	6	68.4 $\pm$ 3.32	29.4 $\pm$ 1.37	57 $\pm$ 2.15
50 mM	2	53.4 $\pm$ 2.27	23.2 $\pm$ 0.95	56.5 $\pm$ 2.35
	4	68.1 $\pm$ 3.19*	26.7 $\pm$ 0.98*	60.8 $\pm$ 2.89
	6	74.2 $\pm$ 3.21*	35.3 $\pm$ 1.47*	52.4 $\pm$ 2.45*
100 mM	2	44.8 $\pm$ 1.74*	21.9 $\pm$ 0.89*	51.1 $\pm$ 2.46
	4	50.4 $\pm$ 2.22*	25.1 $\pm$ 1.05	50.3 $\pm$ 2.31*
	6	49.7 $\pm$ 2.19*	27.7 $\pm$ 1.18*	44.4 $\pm$ 1.98*

### 3.2. Effects of NaCl stress on nitrogen metabolism

As shown in Table 2, the  $\text{NO}_3^-$  concentrations in the 50 and 100 mM NaCl treatments decreased by 35.9% and 71.8%, respectively, as early as 2 days after NaCl stress began compared with the controls. At day 4, the  $\text{NO}_3^-$  contents in the 50 and 100 mM NaCl treatments significantly decreased by 42.2% and 61.1% compared with the control, respectively. The  $\text{NO}_3^-$  values significantly decreased after 6 days and were lower than the control by 70.6% and 78.5% in the 50 and 100 mM NaCl treatments. Under all NaCl treatments, the  $\text{NO}_2^-$  contents were always significantly lower than the control. At day 2, the  $\text{NO}_2^-$  content of the 100 mM NaCl treatment decreased by 22.5% compared with the control but showed no significant change in the 50 mM NaCl treatment. At day 4, the decrease in the  $\text{NO}_2^-$  concentration was more severe under the 50 and 100 mM NaCl treatments and decreased by 33.3% and 40.6% relative to the control, respectively. At day 6, the  $\text{NO}_2^-$  contents of the 50 and 100 mM NaCl treatments increased by 87% and 73.4% compared with control, respectively. The  $\text{NH}_4^+$  concentrations decreased by 13.6% in the 100 mM NaCl treatments after 2 days but were not affected by the 50 mM NaCl treatment. After 4 days of exposure, the  $\text{NH}_4^+$  content decreased by

24.6% and 26.3% for the 50 and 100 mM NaCl treatments, respectively. Significant decreases of 9.6% and 36.2% in the  $\text{NH}_4^+$  content were also observed in the 50 and 100 mM NaCl treatments after 6 days, respectively.

### 3.3. Effects of NaCl stress on NR and NiR activity

The effects of NaCl on the activity of NR, which is the enzyme involved in the assimilatory pathway leading from nitrate to nitrite, are shown in Figure 1A. The NR activity generally tended to decrease with increasing NaCl concentrations at the three developmental stages. The NR activity decreased at day 2 by 9.64% and 18.9% in the 50 and 100 mM NaCl treatments, respectively. Similarly, the NR activities at days 4 and 6 were significantly affected by NaCl treatment compared with the control. The NR activity at day 6 was much lower than it was at day 4. As shown in Figure 1B, the NiR activity in the 50 and 100 mM NaCl treatments decreased by 9.6% 90.4% and 23.8% at day 2 comparison with the control, respectively. The NiR activity in the 50 and 100 mM at day 2 comparison with NaCl treatments decreased by 9.6% 90.4% and 23.8% the control, respectively. The NiR activity

**Table 2.** Changes in concentrations of  $\text{NO}_3^-$ ,  $\text{NO}_2^-$ , and  $\text{NH}_4^+$  in developing *J. curcas* cotyledon under NaCl stress. Results are presented as the mean  $\pm$  standard deviation ( $n = 3$ ). Significant difference ( $p < 0.05$ ) is denoted as asterisk (\*) between control and lead treatments.

No.	Day	$\text{NO}_3^-$ content ( $\mu\text{g g}^{-1}$ dw)	$\text{NO}_2^-$ content ( $\mu\text{g g}^{-1}$ dw)	$\text{NH}_4^+$ content ( $\mu\text{g g}^{-1}$ dw)
0	2	734.7 $\pm$ 35.4	3.12 $\pm$ 0.15	257.7 $\pm$ 12.5
	4	978.4 $\pm$ 47.3	2.29 $\pm$ 0.11	354.7 $\pm$ 16.9
	6	2427.3 $\pm$ 111.9	1.51 $\pm$ 0.07	252.3 $\pm$ 12.1
50 mM	2	471.2 $\pm$ 23.6*	3.23 $\pm$ 0.16	257.3 $\pm$ 12.8
	4	565.6 $\pm$ 25.9*	1.53 $\pm$ 0.07*	267.4 $\pm$ 13.2*
	6	712.5 $\pm$ 34.6*	1.31 $\pm$ 0.06*	228.2 $\pm$ 10.9*
100 mM	2	206.9 $\pm$ 10.3*	2.42 $\pm$ 0.12*	222.5 $\pm$ 10.6*
	4	380.8 $\pm$ 18.1*	1.36 $\pm$ 0.06*	261.4 $\pm$ 113.1*
	6	521.9 $\pm$ 25.7*	1.11 $\pm$ 0.05*	161 $\pm$ 7.93*

of the 50 and 100 mM NaCl treatments gradually decreased at day 4 and eventually reached 74.3% and 63.3% of the control value, respectively. After 6 days of treatment, the NiR activity decreased to 82% and 72.4% in the 50 and 100 mM NaCl treatments, respectively.

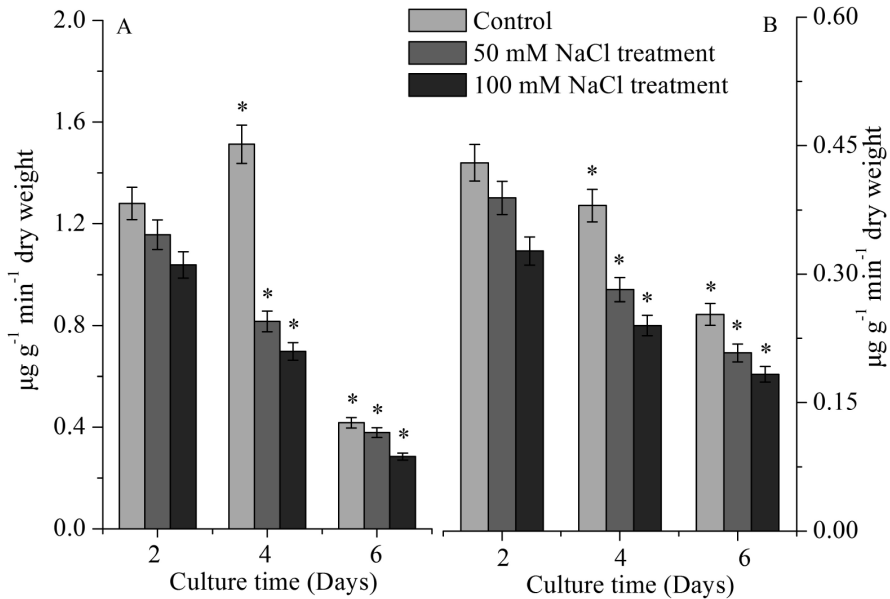
#### 3.4. Effects of NaCl stress on GS and GOGAT activity

The activity of GS at the three germinating stages tended to increase with increasing salt treatments. The GS activity in the 50 and 100 mM NaCl treatments increased by 39.3% and 48.8%, respectively, compared with the control after 2 days of treatment. After 4 days, the 50 and 100 mM NaCl treatments experienced significant increases of 68.2% and 207.8% compared with the control, respectively. The GS activities were 39.9% and 63.9% higher than the control after 6 days of 50 and 100 mM NaCl treatment, respectively (Figure 2A). As shown in Figure 2B, the GOGAT activity increased by 12.1% and 97.5% at day 2 for the 50 and 100 mM NaCl treatments compared with the control value, respectively. The GOGAT activity was increased by 85.8% and 97.5% compared with the controls at day 4 in the 50 and 100 mM NaCl treatments, respectively. The activity was significantly

higher after 6 days of the 50 and 100 mM NaCl treatments, with values 60.5% and 20.4% more than the control groups.

#### 3.5. Effects of NaCl stress on GDH activity

As shown in Figure 3A, the NADH-GDH activity in the 50 mM NaCl treatments increased by 104.3%, 30.1%, and 18.3% compared with the controls at days 2, 4, and 6, respectively. The activities at days 2, 4, and 6 were 90.3%, 268.1%, and 68.9% higher than the controls, respectively, for the 100 mM NaCl treatment. Moreover, the NAD-GDH activities at days 2, 4, and 6 increased by 178%, 46%, and 8.75%, respectively, in the 50 mM NaCl treatments. Similarly, the activities at days 2, 4, and 6 increased by 221.9%, 93.3%, and 25.6%, respectively, in the 100 mM NaCl treatments (Figure 3B).



**Figure 1.** Nitrate reductase (NR, A) and nitrite reductase (NiR, B) activity in the developing cotyledons of *J. curcas* subjected to salt treatment. Results are presented as the mean  $\pm$  standard deviation ( $n = 3$ ). Significant difference ( $p < 0.05$ ) is denoted as asterisk (\*) between control and lead treatments.

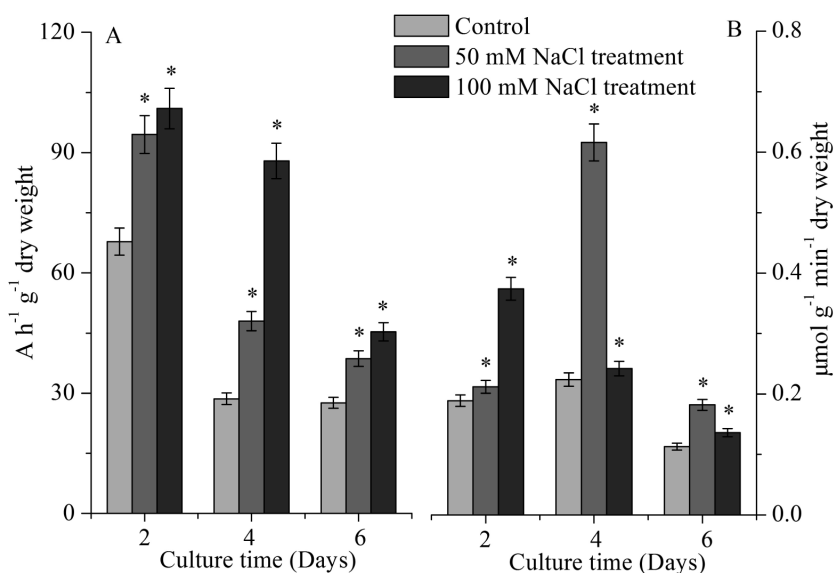
### 3.6 Effects of NaCl stress on AlaAT and AspAT activity

As shown in Figure 4, both the AlaAT and AspAT activities significantly decreased at days 4 and 6 compared to day 2 with NaCl treatment. Under the 50 mM salt treatment, the activities of AlaAT at days 2, 4, and 6 were 125.8%, 56%, and 15% higher than the control groups, respectively. The AspAT activity increased by 60.5%, 15.3%, and 47.3% at 2, 4, and 6 days relative to the control groups, respectively. For the 100 mM NaCl treatment, the AlaAT activities increased by 147.1%, 698.1%, and 112.7% at days 2, 4, and 6, respectively, in relation to the control groups. The AspAT activity also had higher increases than the control groups, with values rising by 67.9%, 177.7%, and 417.1% for days 2, 4, and 6, respectively (Figure 4).

### 4. Discussion

Soil salinity is a prevalent abiotic stress for plants. Growth inhibition is a common response to salinity, and plant growth is one of the most important agricultural indices of salt stress tolerance, as has been indicated by a number of studies (Abogadallah, 2010). Cotyledon fresh and dry weights showed no significant changes when exposed to 50 mM NaCl stress in this study. However, the fresh weight significantly decreased when exposed to 100 mM NaCl, and the dry weight decreased at days 2 and 6 compared with the control. The RWC has been reconsidered to be an indicator of phytotoxicity after salt stress occurred in some plant species. Because the RWC indicates the water status of plant tissues, a reduced RWC seems to indicate disturbed plant homeostasis in response to NaCl stress. Earlier studies have suggested that the RWC is correlated



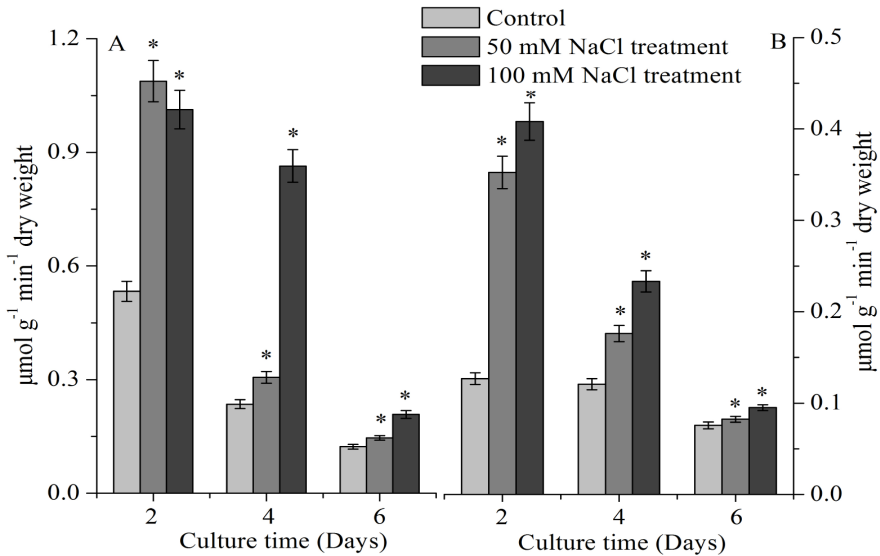


**Figure 2.** Glutamine synthase (GS, A), and glutamate synthetase (GOGAT, B) activity in the developing cotyledons of salt-treated *J. curcas*. Results are presented as the mean  $\pm$  standard deviation ( $n = 3$ ). Significant difference ( $p < 0.05$ ) is denoted as asterisk (\*) between control and lead treatments.

with the concentration and phytotoxicity of salt in plants (del Martínez-Ballesta *et al.* 2006). A decreased RWC may be explained by the changes in leaf fresh weight in response to a salt concentration of 100 mM.

The leaf N concentration not only represents plant N uptake and nutrition status but is also highly correlated with plant growth and biomass (Kusano *et al.*, 2011). Salt stress had different effects on the N concentrations of *J. curcas* cotyledons during the three developmental stages. Decreases were observed in these three N forms in the *J. curcas* cotyledons when embryos were germinated and grown in MS medium in the presence of NaCl stress conditions. The reduction in growth and increased NaCl concentration in plant tissues as a consequence of NaCl stress have been characterized in plants (del Martínez-Ballesta *et al.*, 2006). Data from the present study also show that decreases in leaf

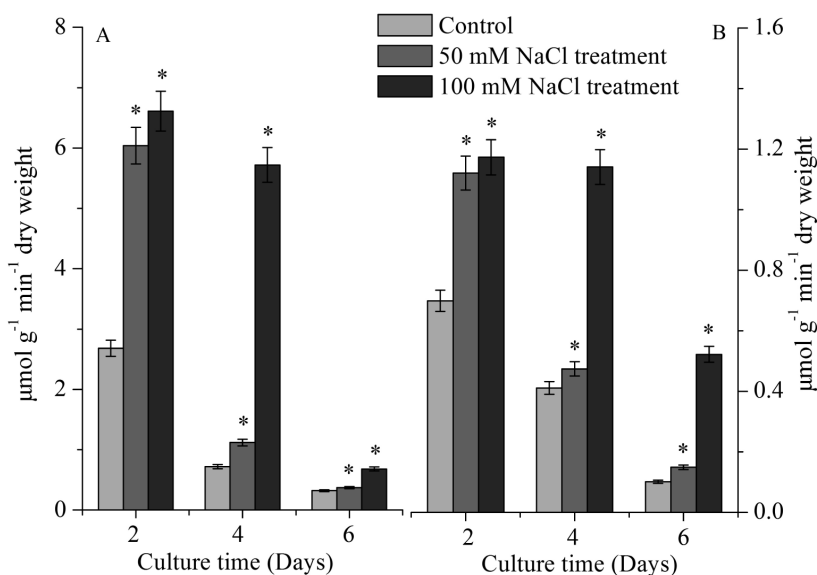
biomass and N content are related to the effects of NaCl stress on the cotyledons. Significant declines of cotyledon  $\text{NH}_4^+$  contents were observed after exposure to 100 mM NaCl. The steep decrease in ammonium content may be responsible for aminating cotyledon GDH activities. Similarly, the contents of cotyledon  $\text{NO}_3^-$  and  $\text{NO}_2^-$  also showed decreases in response to salt stress. In agreement with our results, several studies have reported decreases in N content in plant tissues in response to salt stress. It has also been reported that N metabolism commonly leads to accumulated free amino acids in plant tissues under salt stress conditions (Kusano *et al.*, 2011). Thus, the decrease in cotyledon leaf nitrogen content in our experiment may be a physiological response of *J. curcas* to NaCl stress. The reduction of nitrate to nitrite as catalyzed by NR is considered to be the rate-limiting step in the assimilation of nitrate, which eventually affects the



**Figure 3.** NADH-Glutamate dehydrogenase (NADH-GDH, A) and NAD-Glutamate dehydrogenase (NAD-GDH, B) activity in the developing cotyledons of *J. curcas* after salt treatment. Results are presented as the mean  $\pm$  standard deviation ( $n = 3$ ). Significant difference ( $p < 0.05$ ) is denoted as asterisk (\*) between control and lead treatments.

growth and organic nitrogen status of plants. Nitrate is an important N source for plants in most agricultural soils. The NR catalyzes  $\text{NO}_3^-$  reduction to  $\text{NO}_2^-$  in plant cells, and its activity is inducible by nitrate. For most plant species,  $\text{NO}_3^-$  reduction takes place in the leaves, which consume a large proportion of the energy produced by photosynthesis (Debouba *et al.*, 2007). The first step in nitrate assimilation is its reduction to nitrite, which is catalyzed by NR. The results of the present resent study suggest that decreases in NR and NiR activity occur in the cotyledons under NaCl stress. Two possibilities may be considered to explain these observations. The first may be related to the effect of the salt concentration: the higher the salt concentration in the media, the more serious the inhibition of the

enzyme. Another direct effect of salt treatment on decreased NR activity may be related to an increase in the enzyme breakdown that is induced by toxic oxygen species. Free radicals can cause protein breakdown by oxidative reactions or increased proteolytic activity (Lillo *et al.*, 2004). In our previous salt stress study on *J. curcas*, we observed an increase in plant tissue antioxidant enzyme activity under salt stress. This finding suggested that the salt-mediated generation of reactive oxygen species could have occurred. These radicals could attack NR and cause decreased activity. Our results agree with these findings. In our work, both the  $\text{NO}_3^-$  concentration and NR activity markedly decreased at high salt concentrations at the three developmental stages and



**Figure 4.** Alanine aminotransferase (AlaAT, A) and aspartate aminotransferase (AspAT, B) activity in the developing cotyledons of *J. curcas* following salt treatment. Results are presented as the mean  $\pm$  standard deviation ( $n = 3$ ). Significant difference ( $p < 0.05$ ) is denoted as asterisk (\*) between control and lead treatments.

were consistent with the same patterns during salt treatment. In agreement with some reports in which plant tissue  $\text{NO}_3^-$  content was reduced by the presence of salts in the growing medium, the present findings also showed significant salt-induced reduction of cotyledon nitrate contents (Table 2). Because nitrate is the substrate of NR, inhibition of the latter should consequently lead to an accumulation of nitrate in the plant tissues. However, we did not observe an accumulation of nitrate in our study. One explanation for this result might be that the salt treatment caused a reduction in nitrate uptake, and thus decreased nitrate tissue content. However, the decrease in NR activity diminished the reduction of nitrate (Debouba *et al.*, 2006).

Therefore, the nitrate content pattern shown in Table 2 might be the combined result of nitrate uptake inhibition by salt stress and decreased NR activity. Moreover, these results were in agreement with previous reports showing that the inhibition of NiR activity under saline conditions was reported in several plant species (Debouba *et al.*, 2006; Surabhi *et al.*, 2008). A decrease in the NiR activity of *J. curcas* cotyledons may be considered a direct consequence of the decrease in NR activity and  $\text{NO}_3^-$  content (Table 2). The observed decrease in leaf nitrate contents could affect the subsequent processes that are involved in nitrate reduction and assimilation. In fact, the changes in  $\text{NO}_3^-$  contents may regulate the expression and activities of NR and NiR. The present findings also

showed that changes in NiR and NR activity were correlated with changes in nitrate content in *J. curcas* cotyledons.

The ammonium that is produced by NiR activity is then incorporated into an organic form, primarily by the GS enzyme. Ammonium assimilation is mainly accomplished through the enzymatic activities of the GS/GOGAT cycle. In this pathway, the  $\text{NH}_4^+$  that originates from  $\text{NO}_3^-$  reduction is incorporated as an organic form by GS, which catalyzes the conversion of the amino acid glutamate into the amide glutamine. The GOGAT then catalyzes the reductive transfer of the amide group of glutamine that is formed by GS to 2-oxoglutarate and yields two molecules of glutamate (Mifflin and Habash, 2002; Kusano *et al.*, 2011). The activity of GS at the three germinating stages generally tended to decrease with increased salt treatments. GOGAT is responsible for the reductive transfer of amide N to  $\alpha$ -oxoglutarate for the generation of two molecules of glutamate, one of which is recycled for glutamine biosynthesis (Forde and Lea, 2007). Earlier reports have shown that salt stress may regulate the GS/GOGAT cycle and their activities and promote the degradation of organic N in plant tissues (Wang *et al.*, 2007; Surabhi *et al.*, 2008). Our results indicated that increased GS and GOGAT activities in the developing cotyledons might be involved in ammonium detoxification under NaCl stress.

Ammonium ions are continuously formed during various metabolic processes in the tissues of higher plants. In addition to direct absorption, nitrate reduction, and photorespiration, the catabolism of soluble organic N and the storage of N compounds give rise to substantial quantities of ammonium in plant tissues. Ammonia is rapidly assimilated into organic N via the GS/GOGAT cycle or via the GDH alternative pathway (Mifflin and Habash, 2002). Although the GS/GOGAT cycle is thought to be the major pathway for ammonium assimilation in higher plants, GDH is also able to condense ammonium onto a carbon skeleton (Mifflin and Habash, 2002; Wang *et al.*, 2007). In the present work, NaCl stress was related to a significant

increase in the activities of GS, GOGAT, and GDH. The changes in these enzyme activities might be associated with a significant decrease in the  $\text{NO}_3^-$ ,  $\text{NH}_4^+$ , and organic N content when seedlings were treated with 50 and 100 mM NaCl (Table 2). Some reports have suggested that GS/GOGAT and GDH pathways are activated by salt stress and are the major route of ammonium assimilation (Zhou *et al.*, 2004; Surabhi *et al.*, 2008), which is consistent with our findings. The activation of GS/GOGAT and GDH activity suggests that these enzymes might play important roles in scavenging excessive endogenous ammonium and the replenishment of the glutamate pool during the development of *J. curcas* cotyledons.

Aminotransferases, including AlaAT and AspAT, may catalyze the reversible transfer of an amino group from glutamate to pyruvate to ultimately form 2-oxoglutarate and alanine/aspartate. Aminotransferases play an important role in a diverse variety of pathways in plants, including amino acid biosynthesis and catabolism, photorespiration, and vitamin biosynthesis, as well as carbon and nitrogen shuttles and plant stress responses. These enzymes may therefore be reasonable targets for metabolic engineering to produce crop varieties with enhanced stress resistance and nutrient contents (Liepman and Olsen, 2004). Our results suggest that AlaAT and AspAT levels in *J. curcas* cotyledons significantly increased when treated with NaCl compared with the control. This finding could be related to an increase in the GS/GOGAT cycle and GDH pathway for the treated cotyledons (Figure 2 and Figure 3). It has been shown that increased AlaAT and AspAT activities are observed in some plant species under salt and heavy metal stress (Ramajulu *et al.*, 1994; Surabhi *et al.*, 2008). Increased AlaAT and AspAT may mediate the utilization of ammonia by converting ketoacids into amino acids under these stress conditions, which would further help coordinate N metabolism and amino acid synthesis with the availability of carbon skeletons from the Krebs cycle (Hodges, 2002). These findings may indicate that *J. curcas* induces the activities of AlaAT and AspAT to enable the plant to monitor and

adapt to these changes in its N status and supply that result from salt stress, thereby minimizing the harmful consequences of excess salinity.

## 5. Conclusions

This study is the first to describe the effects of NaCl stress on N metabolism and key enzymes in the nitrogen assimilation pathway of *J. curcas* cotyledons during different developmental stages. Our results showed that NaCl treatment may lead to a significant decrease in  $\text{NO}_3^-$  and N organic concentrations and growth inhibition. The results also suggest that NaCl stress may decrease the activity of NR and NiR, induce the GS/GOGAT cycle and GDH pathways for  $\text{NH}_4^+$  assimilation, and increase aminotransferase activities to meet an increased demand for amino acids under stressful conditions. These findings will be useful for elucidating the roles of key enzymes that might be involved in the nitrogen assimilation pathway of the *J. curcas* plant and will assist in understanding the plant's response to NaCl stress.

## Acknowledgments

This work was supported by the Project Toward Sustainable Green Technology from Chung Yuan Christian University grant CYCU-98-CR-CE.

## References

- Abogadallah, G.M., 2010. Antioxidative defense under salt stress. *Plant Signaling & Behavior*. 5, 369–374.
- Abdulla, R., Chan, E.S., Ravindra, P. 2011. Biodiesel production from *Jatropha curcas*: a critical review. *Critical Reviews in Biotechnology*. 31,53–64.
- Agbaria, H., Heuer, B., Zieslin, N. 1996. Shoot–root interaction effects on nitrate reductase and glutamine synthetase activities in rose (*Rosa* × *hybrida* cvs. Ilseta and Mercedes) graftlings. *Journal of Plant Physiology*. 149, 559–563.
- Da Silva, E.N., Ribeiro, R.V., Ferreira-Silva, S.L., Viégas, R.A., Silveira, J.A.G. 2011. Salt stress induced damages on the photosynthesis of physic nut young plants. *Scientia Agricola*. 68, 62–68.
- Debouba, M., Gouia, H., Suzuki, A., Ghorbel, M.H. 2006. NaCl stress effects on enzymes involved in nitrogen assimilation pathway in tomato "*Lycopersicon esculentum*" seedlings. *Journal of Plant Physiology*. 163, 1247–1258.
- Debouba, M., Maâroufi-Dghimi, H., Suzuki, A., Ghorbel, M.H., Gouia, H. 2007. Changes in growth and activity of enzymes involved in nitrate reduction and ammonium assimilation in tomato seedlings in response to NaCl stress. *Annals of Botany*. 99, 1143–1151.
- del Martínez-Ballesta, M.C., Silva, C., López-Berenguer, C., Cabañero, F.J., Carvajal, M. 2006. Plant aquaporins: new perspectives on water and nutrient uptake in saline environment. *Plant Biology (Stuttgart)*. 8, 535–546.
- Dłuzniewska, P., Gessler, A., Dietrich, H., Schnitzler, J.P., Teuber, M., Rennenberg, H. 2007. Nitrogen uptake and metabolism in *Populus x canescens* as affected by salinity. *New Phytologist*. 173, 279–293.

- Gao, S., Ou-Yang, C., Wang, S., Xu, Y., Tang, L., Chen, F. 2008. Effects of salt stress on growth, antioxidant enzyme and phenylalanine ammonia-lyase activities in *Jatropha curcas* seedlings. *Plant, Soil and Environment* .54, 374–381.
- Good A.G., Muench D.G. 1992. Purification and characterization of an anaerobically induced alanine aminotransferase from barley roots. *Plant Physiology*. 99, 1520–1525.
- Griffith, S.M., Vance, C.P. 1989. Aspartate aminotransferase in alfalfa root nodules. I. Purification and partial characterization. *Plant Physiology*. 90, 1622–1629.
- Groat, R.G., Vance, C.P., 1981. Root nodule enzymes of ammonia assimilation in alfalfa (*Medicago sativa* L.). *Plant Physiology*. 67, 1198–1203.
- Forde, B.G., Lea, P.J., 2007. Glutamate in plants: metabolism, regulation, and signaling. *Journal of Experimental Botany*. 58, 2339–2358.
- Fujimaki, H., Kikuchi, N. 2010. Drought and salinity tolerances of young *Jatropha*. *International* . 24, 121–127.
- Hodges, M. 2002. Enzyme redundancy and the importance of 2-oxoglutarate in plant ammonium assimilation. *Journal of Experimental Botany*. 53, 905–916.
- Jha, A.B., Dubey, R.S. 2004. Arsenic exposure alters activity behaviour of key nitrogen assimilatory enzymes in growing rice plants. *Plant Growth Regulation*. 43, 259–268.
- Kusano, M., Fukushima, A., Redestig, H., Saito, K. 2011. Metabolomic approaches toward understanding nitrogen metabolism in plants. *Journal of Experimental Botany*. 62, 1439–1453.
- Lillo, C., Meyer, C., Lea, U.S., Provan, F., Oltedal, S. 2004. Mechanism and importance of post-translational regulation of nitrate reductase. *Journal of Experimental Botany*. 55, 1275–1282.
- Liepmann, A.H., Olsen, L.J. 2004. Genomic analysis of aminotransferases in *Arabidopsis thaliana*. *Critical Reviews in Plant Sciences*. 23, 73–89.
- Losada, M., Paneque, A. 1971. Nitrite reductase. *Methods in Enzymology*. 23, 487–491.
- Parvaiz, A., Satyawati, S. 2008. Salt stress and phyto-biochemical responses of plants – a review. *Plant Soil and Environment*. 54, 89–99.
- Mifflin, B.J., Habash, D.Z. 2002. The role of glutamine synthetase and glutamate dehydrogenase in nitrogen assimilation and possibilities for improvement in the nitrogen utilization of crops. *Journal of Experimental Botany*. 53, 979–987.
- Molins-Legua, C., Meseguer-Lloret, S., Moliner-Martinez, Y., Campins-Falcó, P. 2006. A guide for selecting the most appropriate method for ammonium determination in water analysis. *Trends in Analytical Chemistry*. 25, 282–290.
- Nåsholm, T., Kielland, K., Ganeteg, U. 2009. Uptake of organic nitrogen by plants. *New Phytologist*. 182, 31–48.
- Oaks, A., Stulen, I., Jones K., Winspear, M.J., Misra, S., Boesel, I.L. 1980. Enzyme of nitrogen assimilation in maize roots. *Planta*. 148, 477–484.
- Rachina, M.A., Nicholas, D.J.D. 1985. Glutamine synthetase and glutamate synthase from *Sclerotinia sclerotiorum*. *Phytochemistry*. 24, 2451–2548.
- Ramajulu, S., Vlerajaniyulu, K., Sudhakar, C. 1994. Short-term shifts in nitrogen metabolism in mulberry *Morus alba* under salt shock. *Phytochemistry*. 35, 991–995.
- Surabhi, G.K., Reddy, A.M., Jyothsnakumari, G., Sudhakar, C. 2008. Modulation of key enzymes of

- nitrogen metabolism in two genotypes of mulberry (*Morus alba* L.) with differential sensitivity to salt stress. *Environmental and Experimental Botany*. 64, 171–179.
- Wang, Z.Q., Yuan, Y.Z., Ou, J.Q., Lin, Q.H., Zhang, C.F. 2007. Glutamine synthetase and glutamate dehydrogenase contribute differentially to proline accumulation in leaves of wheat (*Triticum aestivum*) seedlings exposed to different salinity. *Journal of Plant Physiology*. 164, 695–701.
- Werber, M.M., Mevarech, M. 1978. Purification and characterization of a highly acidic 2Fe-ferredoxin from Halobacterium of the Dead Sea. *Archives of Biochemistry and Biophysics*. 187, 447–456.
- Zhou, W., Sun, Q.J., Zhang, C.F., Yuan, Y.Z., Zhang, J., Lu, B.B. 2004. Effect of salt stress on ammonium assimilation enzymes of the roots of the rice (*Oryza sativa*) cultivars differing in salinity resistance. *Acta Botanica Sinica*. 46, 921–927.