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Responses of Nitrogen Metabolism to Lead Stress in *Jatropha curcas* Cotyledon

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Abstract

In vitro embryo germination and culture experiments were performed to investigate the responses of nitrogen metabolism to lead stress in *J. curcas* cotyledons at the three developmental stages. Lead (Pb^{2+}) concentrations of 100 and 200 μM in Murashige and Skoog (MS) medium were used. The fresh weights of cotyledons were not affected by Pb treatments; however, the dry weights decreased significantly, while the relative water contents increased as compared to the control. The nitrate, nitrite and ammonium levels also decreased significantly at three developmental stages compared to the control. Nitrate- and nitrite- reductase activities in the treated plants showed significant decline at days 2, 4 and 6. The Pb treatment led to a marked increase in glutamine synthetase activity except during the exposure to 200 μM Pb^{2+} at day 2. Glutamate synthase activity was also considerably enhanced by Pb application. The NADH-GDH and NAD-GDH activities were raised at the three developmental stages after 100 and 200 μM Pb treatments. The alanine- and aspartate- aminotransferase activities were increased after the 100 and 200 μM Pb treatments. The results showed that Pb stress may cause the changes in nitrogen metabolism and relative enzymes during the developmental stages of *J. curcas* cotyledons. © 2013 Friends Science Publishers

Keywords: Embryo germination; Nitrogen level; Nitrogen-assimilating enzymes; *Jatropha curcas*; Toxic element

Introduction

Lead (Pb) is one of the most common heavy metal pollutants. It has a high toxicity to microbes, plants, animals and humans. In plant species, Pb is a non-essential element and may cause direct or indirect response on growth and metabolism when absorbed even at low doses. These responses mainly include the inhibition of seed germination, induction of leaf yellowing, nuclear toxicity, inhibition of root and hypocotyl growth, reduction of photosynthesis and DNA synthesis, inhibition or activation of enzyme activity, etc. (Sengar *et al.*, 2008; Gao *et al.*, 2009). According to previous reports, Pb toxicity on plants may use some biological pathways that can lead to: (1) a reduction of water potential and hyperpolarization of extracellular membrane, resulting in ion imbalance and dehydration of cells; (2) interference with normal uptake, transport and regulation of nutrient ions, which results in metabolic disorders and genetic poisoning in the cell; (3) replacement of the necessary elements in functional proteins and/or enzymes; and (4) also interference with the macromolecules (nucleic acids, carbohydrates and proteins) for the induction of changes in their conformation and activity (Pourrut *et al.*, 2011). Thus, the toxic effects and mechanism of Pb toxicity on plants have attracted the concerns of researchers in the past several years due to which further research is still required to completely analyze these changes.

Nitrogen (N) sources play key roles in plant, and N metabolism may affect different physiological processes in plants. Nitrate (NO_3^-) assimilation is an important biological process in plants. Initially, the NO_3^- is transformed into nitrite under the catalysis of nitrate reductase (NR), followed by conversion into ammonia under the catalysis of nitrite reductase (NiR). Finally, the ammonia is then incorporated into glutamine through the complex metabolism reactions (Stitt *et al.*, 2002). Under Pb stress, N metabolism showed significant changes in some plant species. These changes included the inhibition of uptake and transport of NO_3^-, and the reduction of NR activity (Singh *et al.*, 2003; Xiong *et al.*, 2006). Moreover, glutamine synthetase/glutamate synthase (GS/GOGAT) cycle and glutamate dehydrogenase (GDH) pathways in several plants were significantly induced by Pb treatment (Wu *et al.*, 2008). These findings indicated that Pb stress may induce different responses on N metabolism in plants.

Jatropha curcas L., a member of family Euphorbiaceae plants, mainly grows in tropical and subtropical regions. Different parts of this plant were used to

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develop bio-diesel, bio-pest agents, new drugs, and industrial feeds. In some regions, *J. curcas* is widely distributed near abandoned mines that may have a relatively high content of heavy metals (Makkar and Becker, 2009). Previous studies suggested that *J. curcas* could adjust themselves to tolerate mercury and chromium toxicities by an effective defensive mechanism and hence, allows the plant to maintain its normal growth and metabolic activities (Gao *et al.*, 2010; Yadav *et al.*, 2010). Although there are already many studies on the effects of heavy metal stresses on physiological responses in *J. curcas*, the changes of N metabolism under Pb stress have not been investigated in detail. To investigate the growth and nitrogen metabolism in *J. curcas* cotyledons with Pb application, *in vitro* embryo germination and culture experiments were done under Pb stress during a 6-day period. The N content in various forms and the nitrogen metabolism relative enzyme activities were analyzed with different Pb concentration at the three different developmental stages.

Materials and Methods

Embryo Germination and Seedling Growth

Seeds of *J. curcas* were obtained from Mother Herbs PVT LTD., Delhi, India. The kernel were separated from the seed coats, and surface sterilized with ethanol (70%, v/v) for 30-45 sec, and then with mercuric chloride (0.1%, w/v) for 8-10 min. The kernels were rinsed and soaked with sterilized distilled water for 24 h at room temperature. The embryos were then separated from the kernels, washed several times with distilled water, and planted (three each) on MS medium containing 0 (control), 100 and 200 μM Pb (Pb were supplied as lead acetate) in 100 mL wide-neck bottles. The pH value of the MS medium containing sucrose (30 g/L, w/v) and agar powder (8 g/L, w/v) was adjusted to 5.9±0.1. Finally, the medium was autoclaved at 121 ± 2°C for 15 min. The bottles containing the planted embryos were kept in a plant incubator at 25°C with 12 h photoperiod and 80% relative humidity. Embryos were considered as germinating when they start to turn yellow, which usually occurs after 24 h of incubation. The embryo will be referred to as developed seedling once two cotyledons had developed (6 days). Germinated embryos or developed seedlings were harvested from the three groups every two days, so that cotyledons of different developmental stages were collected. The fresh weights of cotyledons were recorded, and then the cotyledons were freeze dried and stored at 4°C for further analysis.

Determinations of N Content

The NO_3^- contents were measured after 2, 4 and 6 days of Pb treatment. Dry cotyledon samples (about 0.1 g) were ground and homogenized with liquid nitrogen, and then soaked in 2 mL of redistilled water. The extracts were centrifuged (12000 rpm, 10 min) at 4°C, and the supernatants were assayed for N determination. Measurement of NO_3^-content was carried out following the procedure of Agbaria *et al*. (1996). Reaction mixture of 200 μL, containing 40 μL extract and 160 μL 5% salicylic acid in sulfuric acid (98%), was prepared. After 5 min, 3.8 mL of 2 M NaOH was added. The absorbance was measured at 410 nm using a CT-2200 model UV/Vis spectrophotometer (ChromTech Inc., Singapore). NO_3^- contents were calculated using a calibration curve, and expressed as μg NO_3^- g^{-1} dry weight.

The NO_2^- content was analyzed by spectrophotometric method (Werber and Mevarech, 1978). The reaction mixture was prepared by combining 0.5 mL extract, 1 mL 0.2% α-naphthylamine and 1 mL 4% sulfanilic acid in hydrochloric acid. After incubating for 30 min at 35°C, the absorbance of the reaction mixture was measured at 520 nm. NO_2^- contents were estimated and were expressed as μg g^{-1} dry weight.

The measurement of ammonium (NH_4^+) content was performed using Nessler reagent (Molins-Legua *et al.*, 2006). The reaction mixture containing 100 μL extract, 10 μL 10% K-Na tartrate, 100 μL Nessler reagent and 2.4 mL distilled water was prepared. The absorbance was read after 5 min at 425 nm, and the results were calculated and expressed as μg g^{-1} dry weight.

Enzyme Extraction and Assay of NR Activity

Dry samples (about 0.1 g) were homogenized with liquid nitrogen and extracted using 2 mL of 50 mM phosphate buffer (pH 7.5) containing 5 mM cysteine, 0.5 mM EDTA and 0.5% insoluble polyvinylpyrrolidone (PVP). The extracts were obtained by centrifugation at 12000 rpm for 10 min at 4°C, and the activities of NR, NiR and GOGAT were analyzed by following steps. The NR activity assay was measured by the Debouba *et al*. method (2006). The mixture was prepared by combining 100 μL extract, and 1.4 mL of 5 mM EDTA, 7 mM KNO_3 and 0.15 mM NADH in 50 mM phosphate buffer (pH 7.5). After incubation for 30 min at 30°C, 0.1 mL of 0.5 M zinc acetate was added into the mixture. The solution was centrifuged at 5000 rpm for 10 min. The results were expressed as μg NO_2^- per minute per gram dry weight.

Assay of NiR Activity

The assay for NiR activity was carried out by the Losada and Paneque method (1971). The solution included 0.1 mL extract, 0.8 mL of 0.4 mM $NaNO_2$ and 2.3 mM methyl viologen in 50 mM phosphate buffer (pH 7.5) and 0.1 mL of 86.15 mM sodium dithionate in 100 mM $NaHCO_3$. After incubation for 30 min at 30°C, the reaction was terminated using vigorous shaking and boiling for 1 min. The results were expressed as μg NO_2^- reduced per min per g dry weight based on the remaining NO_2^- content.

Assay of GOGAT Activity

The assay for GOGAT activity was performed according to the Rachina and Nicholas method (1985). The mixture consists of 0.1 mL extract and 1.9 mL of 5 mM α-oxoglutaric acid, 10 mM glutamine and 0.15 mM NADH in 50 mM phosphate buffer (pH 7.5). The absorbance was recorded 340 nm for 7-10 min. The results were expressed as μmol NADH per min per g dry weight.

Assay of GS Activity

Dry samples (0.1 g) were ground and homogenized using 2 mL of 0.5 mM EDTA, 1 mM $MgCl_2$, 10 mM β-mercaptoethanol and 0.5% PVP in 50 mM Tris-HCl buffer (pH 7.5). The supernatant was obtained by centrifugation for 10 min at 12000 rpm, and analyzed for GS, GDH, AlaAT and AspAT activities. The assay for glutamine synthetase (GS) activity was measured by the Oaks *et al.* method (1980). The mixture included 100 μL enzyme extract and 1.9 mL of 13 mM hydroxylamine, 1 mM ATP, 50 mM glutamate, 20 mM $MgCl_2$ and 20 mM sodium arsenate in 50 mM Tris-HCl buffer (pH 7.5). The reaction was stopped by adding 1 mL ferric chloride reagent after incubation for 30 min at 37°C, the absorbance was analyzed at 520 nm.

Assay of GDH Activity

GDH activity assay was carried out using the method of Groat and Vance (1981). For NADH-GDH activity, 100 μL extract was added into 1.9 mL of 10 mM α-oxoglutaric acid, 100 mM NH_4Cl and 0.2 mM NADH in 50 mM Tris-HCl buffer (pH 8.0). For NAD-GDH activity, 100 μL extract was added into 1.9 mL of 80 mM L-glutamic acid and 0.2 mM NAD in 50 mM Tris-HCl buffer (pH 8.8). The oxidation/reduction of NADH/NAD was measured by UV-Vis Spectrophotometer at 340 nm for 7-10 min. The activity of GDH in units of μmol of NADH oxidized/NAD reduced per minute per mL was measured at 340 nm.

Assays of AlaAT and AspAT Activity

AlaAT activity was analyzed by the Good and Muench method (1992). When the 100 μL enzyme extract was added, the absorbance was recorded at 340 nm. AspAT activity was assayed by the Griffith and Vance method (1989). AlaAT and AspAT activities were expressed as μmol of product per minute per g dry weight.

Results

Effects of Pb Stress on Growth and Nitrogen Metabolism

Aside from the 10.5% increase from the 200 μM Pb treated samples at day 2, no significant change was observed in the fresh weight of cotyledons after 100 and 200 μM Pb treatments. The dry weight of cotyledons showed significant decrease in response to Pb treatment at the three developmental stages, except after the 100 μM Pb treatment at day 2, where no change was observed. The relative water content in the treated cotyledons was higher than those in the control at the three developmental stages (Table 1). NO_3^- levels in the cotyledons were significantly reduced by Pb exposure at all three stages. At three developmental stages, 100 and 200 μM Pb treatments significantly reduced the levels of NO_2^- in the samples except for the 100 μM Pb application at day 2. In addition to the two N forms mentioned above, NH_4^+ levels in the treated groups also decreased at the three developmental stages; however, there was no significant change observed after treatment of 100 μM Pb at day 4 (Table 1).

Effects of Pb Stress on NR and NiR Activity

As shown in Fig. 1, NR and NiR activities in cotyledons were significantly affected by Pb application at the three developmental stages. At 100 μM Pb treatment, huge decrease in NR activities were observed wherein it reached only 72.2, 76.2 and 49% of the activity in the control at days 2, 4 and 6, respectively. At 200 μM Pb treatment, NR activities only reached 64.8, 63.7 and 41.9% of the control at days 2, 4 and 6, respectively (Fig. 1A). At 100 μM Pb treatment, NiR activity decreased significantly to 50.6% of the control at day 6, but no significant reductions were observed at day 2 and 4. At 200 μM Pb treatment, NiR activities were reduced to 85.1, 37.8 and 40.3% of the control at days 2, 4 and 6, respectively (Fig. 1B).

Effects of Pb Stress on GS and GOGAT Activity

As shown in Fig. 2A, the GS activities at the three developmental stages after 100 μM Pb treatment were significantly higher than those of the controls. At 200 μM Pb treatment, GS activities were significantly increased by 10.8, 160.4 and 136.4% at days 2, 4 and 6, respectively. As shown in Fig. 2B, the increase in GOGAT activities at 100 and 200 μM Pb treatments at three developmental stages ranged from 46.5 to 455.7% and 185.2 to 505.3%, respectively.

Effects of Pb Stress on GDH Activity

The NADH-GDH activity in the cotyledons with 100 μM Pb treatments increased significantly, reaching 160.5, 182.9 and 320.1% of the control at days 2, 4 and 6, respectively (Fig. 3A). The NADH-GDH activity in the cotyledons with 200 μM Pb treatments increased to a greater extent, reaching 131.5, 204.8 and 197.1% of the control at days 2, 4 and 6, respectively. As shown in Fig. 3B, the NAD-GDH activities after 100 μM Pb treatments at days 2, 4 and 6 significantly increased by 9.61, 5.95 and 15.2 folds, respectively. Similarly, the NAD-GDH activities after 200

Table 1: Effects of Pb stress on fresh weight, dry weight, relative water content, NO_3^-, NO_2^- and NH_4^+ in the developing *J. curcas* cotyledon. Data are reported as mean ± standard deviation for three replications

No.	Day	Fresh weight (mg)	Dry weight (mg)	Relative water content (%)	NO_3^- content (μg g^{-1} dw)	NO_2^- content (μg g^{-1}dw)	NH_4^+ content (μg g^{-1} dw)
0	2	52.4 ± 2.12	24.3 ± 1.02	53.7 ± 2.38	734.7 ± 35.4	3.12 ± 0.15	257.7 ± 12.5
	4	63.7± 3.08	24.5 ± 1.15	61.6 ± 2.81	978.4 ± 47.3	2.29 ± 0.11	354.7 ± 16.9
	6	68.4 ± 3.32	29.4 ± 1.37	57 ± 2.15	2427.3±111.9	1.51 ± 0.07	252.3 ± 12.1
100 μM	2	54.9 ± 2.55	24.4 ± 1.19	54.6 ± 2.69	409.3 ± 20.1	3.09 ±0.149	241.3 ± 11.9
	4	63.1 ± 2.87	22.8 ± 1.02	63.9 ± 3.05	916.9 ± 43.8	2.08 ± 0.09	368.7 ± 17.4
	6	69.1 ± 3.26	25.1 ± 1.07	63.7 ± 3.11	1455.2 ± 69.6	0.94 ± 0.04	213.2 ± 10.5
200 μM	2	57.9 ± 2.71	22.6 ± 0.98	61.0 ± 2.89	378.9 ± 18.1	2.33 ± 0.11	213.1 ± 9.75
	4	66.4 ± 3.22	23.8 ± 1.27	64 ± 2.97	831.6 ± 39.2	1.79 ± 0.08	334.6 ± 16.1
	6	69.7 ± 3.38	24.7 ± 1.19	64.5 ± 3.12	1722.4 ± 84.3	0.76 ± 0.03	178.9 ± 8.19

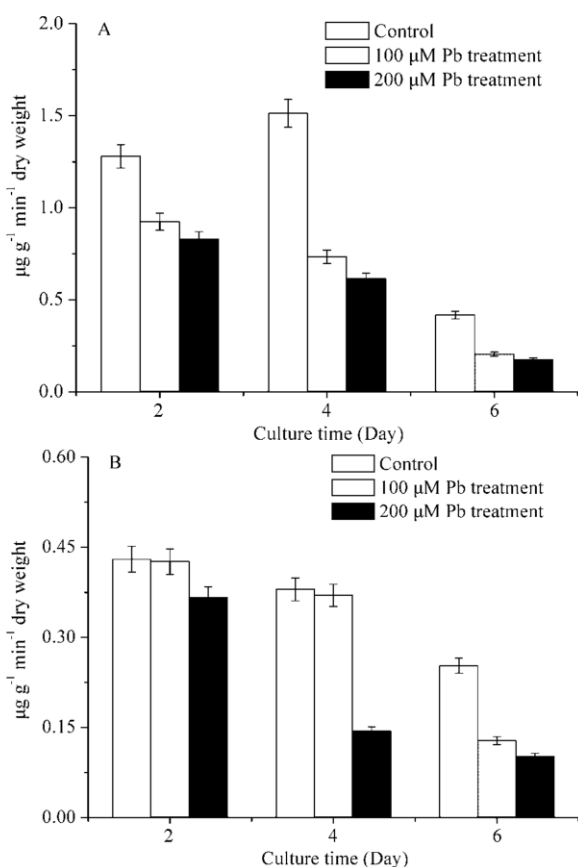

Fig. 1: Nitrate reductase (A) and nitrite reductase activities (B) in the cotyledons of *J. curcas* subjected to 100 and 200 μM Pb treatments. Data are reported as mean ± standard deviation for three replications

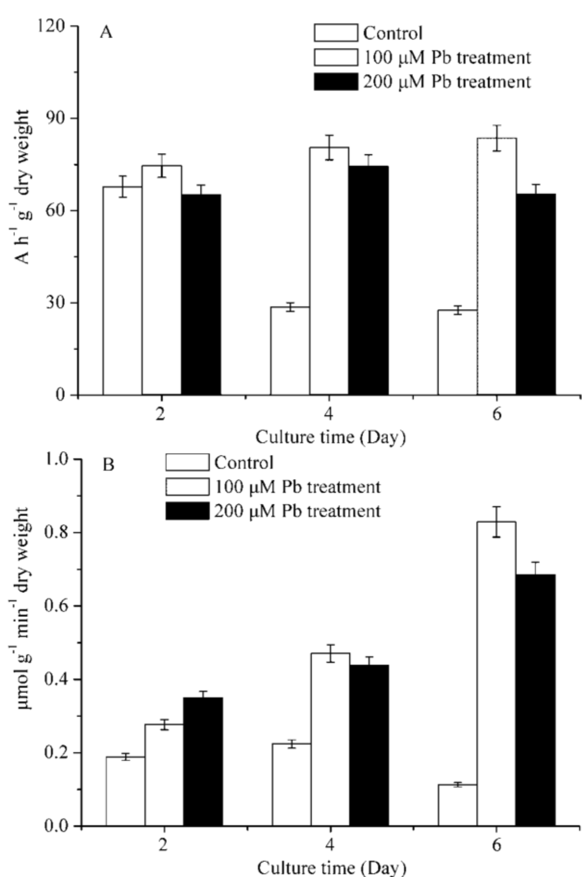

Fig. 2: Glutamine synthetase (A) and glutamate synthase activities (B) in the cotyledons of *J. curcas* subjected to 100 and 200 μM Pb treatments. Data are reported as mean±standard deviation for three replications

μM Pb treatments at days 2, 4 and 6 were higher by 9.99, 5.36 and 10.6 folds, respectively.

Effects of Pb Stress on AlaAT and AspAT Activity

After treatment of 100 μM Pb, AlaAT activities at days 2, 4 and 6 were significantly induced by 2.92, 2.13 and 4.37 folds compared to the control. After treatment of 200 μM Pb, the activities at days 2, 4 and 6 increased by 192.2, 143.7 and 165.9%, respectively (Fig. 4A). The AspAT activities in cotyledons with 100 μM Pb treatments after 2, 4 and 6 days were at least 1.53, 2.24 and 8.49 times higher than those of the controls, respectively. Similarly, the activities in the cotyledons with 200 μM Pb treatments were 1.12, 2.34 and 7.22 times higher than those of the controls.

Discussion

Pb is a non-essential element in plants, and it can seriously

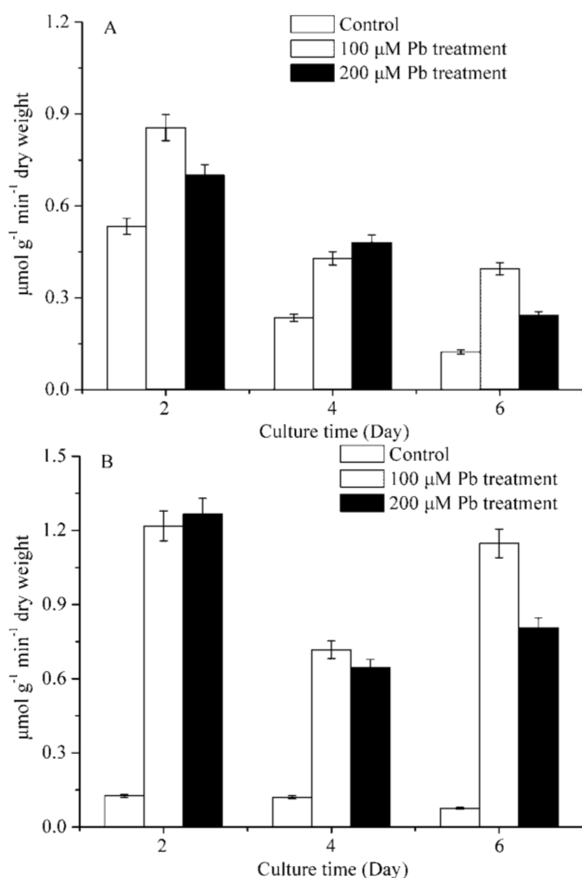

Fig. 3: NADH-glutamate dehydrogenase (A) and NAD-glutamate dehydrogenase activities (B) in the cotyledons of *J. curcas* subjected to 100 and 200 μM Pb treatments. Data are reported as mean ± standard deviation for three replications

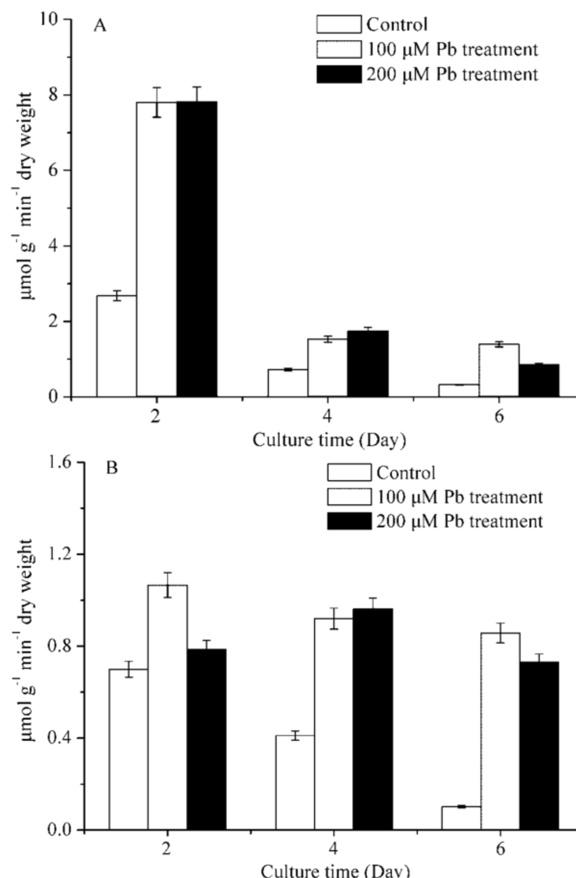

Fig. 4: Alanine aminotransferase (A) and aspartate aminotransferase activities (B) in the cotyledons of *J. curcas* subjected to 100 and 200 μM Pb treatments. Data are reported as mean ± standard deviation for three replications

affect plant growth and metabolism. It can inhibit seed germination, growth of root and hypocotyl, photosynthesis and transpiration, DNA synthesis, and can either inhibit or activate enzyme activity (Pourrut *et al.*, 2011). Our results show no significant increase in fresh weight, but a reduction in dry weight was observed. The reduction of fresh weight, dry weight and relative moisture content (RWC) in leaves as a consequence of Pb stress has been reported in some plant species (Pourrut *et al.*, 2011). These results may be associated with the increase of water content in the cotyledons. The findings from the present study seem to contradict previously reported findings, necessitating the need for further studies.

Leaf nitrogen content shows the tendency of plant to nitrogen uptake and partitioning within the leaf. The tissue N content is well correlated with growth and development in plants (Masclaux-Daubresse *et al.*, 2008). The results from the present study showed significant decrease in the three N forms in *J. curcas* cotyledons when the embryos were germinated and grown in MS medium with Pb. Previously it has been reported that NO_3^-, NO_2^- and NH_4^+ contents in the leaves of tobacco, spinach and sunflower plants were significantly reduced by heavy metal stresses (Ruiz *et al.*, 2007; Wu *et al.*, 2008; Maaroufi Dguimi *et al.*, 2009). Such a reduction might be associated to the inhibition of plant on NO_3^- uptake and transport, also to the changes in NR and NiR activities (Fig. 1).

Several reports indicated a positive relationship between different contents of nitrogen forms and the changes of NR and NiR activity in tobacco and spinach plants under heavy metal stresses (Wu *et al.*, 2008; Maaroufi Dguimi *et al.*, 2009). In fact, the NO_3^- levels in shows the changes of NO_3^- influx, which are corrected to the changes of NR and NiR in plant tissues (Wu *et al.*, 2008; Maaroufi Dguimi *et al.*, 2009). Moreover, the decrease in NR and NiR activities have been observed in plants; this is caused by enzyme breakdown induced by reactive oxygen species generated under heavy metal stresses (Xiong *et al.*, 2006). The current findings suggested that the decreased NR and NiR activities in the treated cotyledons reflected the reduction of NO_3^-,

NO_2^- and NH_4^+ contents (Table 1).

In plants, NH_4^+ may be gradually formed at different metabolic reaction. However, high-level endogenous NH_4^+ is deleterious to plant cell. Therefore, plants have developed mechanisms *via* the GS/GOGAT cycle or GDH pathways to minimize damage by ammonia (Britto and Kronzucker, 2002). The NH_4^+ produced by NiR activity is then incorporated into an organic form primarily by GS/GOGAT cycle(Hodges, 2002). Reports have shown that heavy metal stresses may cause the produce of excessive NH_4^+ in plant tissues by regulating the expression and activity of GS/GOGAT cycle, and further promote the degradation of organic N (Wu *et al.*, 2008). Our results indicated that the increased GS and GOGAT activities in the developing cotyledons seemed to be connected with the balance of NH_4^+ levels exposure to Pb toxicity.

In plants, GDH is involved in condensing NH_4^+ onto carbon skeleton (Dubois *et al.*, 2003). Reports showed that NADH-GDH is significantly induced by heavy metal stress, and might take part in removing excess of NH_4^+ from the plant tissues (Mishra and Dubey, 2011). However, *J. curcas* cotyledons had significantly lower contents of NH_4^+ when treated with 100 and 200 μM. Thus, the activation of GDH may be related to the changes of GS/GOGAT, which maintain the balance of N assimilation under Pb stress.

Aminotransferases, including AlaAT and AspAT, may catalyze the reversible transfer of amino group from glutamate to pyruvate to form 2-oxoglutarate and alanine/aspartate. The regulation of AlaAT and AspAT in several plant species had been studied in response to heavy metal stress (Gajewska *et al.*, 2009; Mishra and Dubey, 2011). As shown in Fig. 4, the addition of Pb may cause significant increase in the activities of AlaAT and AspAT in the developing cotyledon. Thus, the increased activity of AlaAT and AspAT in plant tissues from our experiment may be a detoxification response of the plant to Pb exposure.

In conclusion, Pb toxicity may cause the reduction of different N forms content in the developing cotyledons. It also causes the depression of NR and NiR activity, and the induction of GS/GOGAT and GDH pathways for NH_4^+ assimilation. Moreover, the increased of AlaAT and AspAT activities in the developing cotyledons were also observed exposed to Pb toxicity, suggesting that they are corrected with the detoxification process. These findings suggested the influence of Pb toxicity in *J. curcas* on the plant's nitrogen metabolism. However, a longer period of Pb treatment on *J. curcas* may help understand nitrogen nutrition and metabolism in response to Pb stress.

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