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RESEARCH ARTICLE

Responses of nitrogen metabolism to copper stress in *Luffa cylindrica* roots

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Abstract

Pot experiments were performed to investigate the responses of nitrogen metabolism to copper stress in Luffa cylindrica roots. Four treatments were used, in which varying copper concentrations (25, 50, 75 and 100 μM) were added to MS medium. The fresh weights of the roots decreased gradually with the increasing copper concentrations. At the lower concentrations, the ammonium and nitrite levels increased compared with the control, but the nitrate concentrations significantly decreased with 75 and 100 μM copper. Compared with the control, nitrate reductase activity levels gradually increased with increasing copper concentrations of up to 50 μM, and nitrite reductase activity levels significantly decreased. Copper stress led to variable increases in the activities of glutamine synthetase and glutamate synthase compared with the controls. The NADH- glutamate dehydrogenase (NADH-GDH) and NAD-glutamate dehydrogenase (NAD-GDH) activities were affected by the copper treatments, but that of NAD-GDH was significantly reduced by 100 μM copper. The activities of alanine aminotransferase (AlaAT) and aspartate aminotransferase (AspAT) showed varying changes following the copper treatments. The present results indicate that Luffa cylindrica shows altered activities of enzymes associated with nitrogen metabolism during copper stress, enabling it to monitor and adapt to changes in its N status and supply, thereby minimizing the harmful effects of the stress.

Keywords: Nitrogen metabolism, *Luffa cylindrica*, copper stress, nitrogen-assimilating enzymes

1. Introduction

Copper (Cu) is an essential element for plant growth that plays a significant role in many physiological processes, including photosynthesis, respiration, carbohydrate distribution, nitrogen fixation, protein metabolism, cell wall metabolism and hormone perception. Plants typically find ample supplies of copper in the soil, but high concentrations of copper can be a stress factor triggering physiological responses including the inhibition of plant growth, the production of reactive oxygen species (ROS) and alterations in enzymatic and non-enzymatic antioxidant activities (Gao *et al*., 2008; Yruela, 2009). Moreover, copper stress may result in altered nitrogen (N) metabolism-related activities, including ion uptake, N assimilation, and amino acid and protein synthesis (Xiong *et al.*, 2006; Azmat and Khan, 2011). In plants, the response of N metabolism to copper stress has been extensively studied because of the importance of understanding the behavior of N

metabolism-associated enzymes in the presence of highly toxic metals.

N plays important roles in plant growth and development, and its metabolism affects all levels of plant function. However, N metabolism requires a complex series of biochemical reactions. For example, in nitrate assimilation, the nitrate is converted to NO_2^- by nitrate reductase (NR), and NO_2^- is converted to NH_4-N by nitrite reductase (NiR). The resulting NH_4-N is then assimilated into amino acids and other metabolites through the combined actions of glutamine synthetase (GS) and glutamate synthase (GOGAT) in addition to glutamate dehydrogenase (GDH), alanine aminotransferase (AlaAT) and aspartate aminotransferase (AspAT) (Stitt *et al*., 2002; Mokhele *et al*., 2012). Previous studies have shown that high concentrations of copper in plants lead to significant changes in N metabolism, including the inhibition of the uptake and transportation of nitrate and the regulation of NR and NiR activities (Xiong *et al*., 2006; Azmat and Khan, 2011). Moreover, the GS-GOGAT cycle and GDH pathways in some plant species have been shown to be significantly induced by copper treatment, reflecting a general disorder of N assimilation (Ludewig *et al.*, 2007). These findings have increased the understanding of the behavior of N metabolism-associated enzymes and the regulation of N metabolism under copper stress.

Luffa cylindrica L., which is commonly known as the sponge gourd, is cultivated in tropical and subtropical Asia regions and consumed daily by the population. The seeds and sponges of the old fruits are also used in traditional Chinese medicine as anthelmintics, stomachics, and antipyretic phytomedicinal drugs (Yang *et al*., 1999). Although there have been many studies assessing the effects of heavy metal stresses on physiological and defense responses in *Luffa cylindrica* (He and Li, 2010), the effects of copper on N metabolism have not been studied in this plant. Therefore, to assess the copper stress-induced effects on N metabolism in *Luffa cylindrica* roots, in vitro cultures were established using embryos, and the germination patterns and culture characteristics were observed and analyzed under copper stress. Furthermore, the concentrations of various forms of N and the activities of N-assimilating enzymes were evaluated in relation to varying copper concentrations.

2. Materials and Methods

2.1. Embryo germination and seedling growth

Luffa cylindrica seeds were obtained from a traditional Chinese medicine market in Chengdu, China. The seeds were separated into seed coats and were surface sterilized in 70% ethanol for 30-45 sec followed by 0.1% mercuric chloride for 8-10 min. The seeds were then rinsed with distilled water and soaked for 24 h at 25 °C, and the embryos were separated from the seeds on a clean bench. The embryos were washed several times using distilled water, and three embryos each were planted in 100-mL wide-neck bottles on aliquots of MS medium containing 0 (control), 25, 50, 75 and 100 μM Copper, supplied as $CuSO_4$. The pH value of the medium was adjusted to 5.9 ± 0.1 prior to autoclaving at 121 °C for 15 min with 30 g/L sucrose and 7.5 g/L agar powder. The cultures were placed in a plant incubator at 25 °C with a 12 h photoperiod and 80% relative humidity. The embryos were considered to be germinating when they turned yellow, which usually occurred after 24 h of incubation. Those that developed two cotyledons (at 7 days) were referred to as developed seedlings. The fresh weights of the roots were recorded, and they were stored at 4 °C for further analyses.

2.2. Determination of N concentrations

The root samples were ground and homogenized in a mortar with liquid nitrogen and then extracted using 2 mL of redistilled water. The extracts were centrifuged (12,000 rpm, 10 min) at 4 °C, and the supernatants were used to determine the N concentrations. Measurements of nitrate concentrations were conducted according

to Agbaria *et al*. (1996). Determinations of NO_2^- concentrations were performed with a spectrophotometric method (Werber and Mevarech, 1978). Ammonium concentrations were assayed using the Nessler reagent (Molins-Legua *et al*., 2006). These results were expressed as μg per g fresh weight.

2.3. Protein extractions and estimations of NR, NiR and GOGAT activities

To estimate the NR, NiR and GOGAT activities, the samples were ground using liquid nitrogen in a mortar and homogenized and extracted in 50 mM phosphate buffer (pH 7.5, 1/20 w/v) containing 5 mM cysteine, 0.5 mM EDTA and 0.5% insoluble polyvinylpyrrolidone. The extracted suspensions were collected by centrifuging at 12,000 rpm for 10 min at 4 °C and used for further studies.

NR activity assays were performed according to Debouban *et al*. (2006) with slight modifications. A 1.5-mL reaction mixture contained 0.1 mL of the extraction and 1.4 mL of 50 mM phosphate buffer (pH 7.5) with 5 mM EDTA, 7 mM KNO_3 and 0.15 mM NADH. The reaction was stopped by the addition of 0.1 mL of 0.5 M zinc acetate after incubation for 30 min at 30 °C, and the mixture was then centrifuged at 5000 rpm for 10 min. The results were expressed as the amount of enzyme required to form 1 μg NO_2^- per min per g of fresh weight.

Nitrite reductase activity assays were carried out according to Losada and Paneque (1971). Each reaction tube contained 0.1 mL of the extraction, 0.8 mL of 50 mM phosphate buffer (pH 7.5) with 0.4 mL of 2.3 mM methyl viologen and 0.1 mL of 100 mM $NaHCO_3$ with 86.15 mM sodium dithionate. After incubation for 30 min at 30 °C, the reaction was terminated by vigorous shaking and boiling for 1 min. The results were expressed as μg nitrite reduced per min per g of fresh weight.

GOGAT activity assays were performed according to Rachina and Nicholas (1985). A 2-mL reaction mixture contained 0.1 mL of the extraction and 1.9 mL of 50 mM phosphate buffer (pH 7.5) with 5 mM α-oxoglutaric acid, 10 mM glutamine and 0.15 mM NADH. The absorbance was recorded by monitoring the oxidation of NADH (ε = 6.22 mM^{-1} cm^{-1}) at 340 nm for 7-10 min. The results are expressed as the enzyme required to oxidize 1 μmol NADH per min per g of fresh weight.

2.4. Assays for GS, GDH, AlaAT and AspAT activities

The fresh samples were ground with liquid nitrogen and homogenized in a mortar containing 50 mM Tris-HCl buffer (pH 7.5) with 0.5 mM EDTA, 1 mM $MgCl_2$, 10 mM β-mercaptoethanol and 0.5% PVP. After centrifugation (12,000 rpm, 10 min), the extracted suspensions were used for the measurements of the GS, GDH, AlaAT and AspAT activity levels.

GS activity assays were performed according to Oaks *et al*. (1980). The reaction mixture contained 0.1 mL of the enzyme extract and 1.9 mL of Tris-HCl buffer (50 mM, pH 7.5) with 13 mM hydroxylamine, 1 mM ATP, 50 mM glutamate, 20 mM $MgCl_2$ and 20 mM sodium arsenate. The mixture was incubated for 30 min at 37 °C, and 1 mL of ferric chloride reagent was added (0.37 M $FeCl_3$ and 0.2 M trichloroacetic acid in 0.5 M HCl). After centrifugation (5000 g for 15 min at 4 °C), the absorbance was recorded at 520 nm. The results were expressed as A per h per g fresh weight.

GDH activity assays were carried out according to Groat and Vance (1981). For the NADH-GDH activity measurements, the 1.9 mL reaction buffer contained 10 mM α-oxoglutaric acid, 100 mM NH_4Cl and 0.2 mM NADH in 50 mM Tris-HCl buffer (pH 8.0), and the reaction started when 100 μL of extract was added. For the NAD-GDH activity measurements, the reaction buffer consisted of 80 mM L-glutamic acid and 0.2 mM NAD in 50 mM Tris-HCl buffer (pH 8.8), and the reaction was initiated when 100 μL of extract was added. The oxidation/reduction of NADH/NAD was measured by a UV-Vis Spectrophotometer (TU-1901 UV-Vis Spectrophotometer, Purkinje General, Beijin, China) at 340 nm for 7-10 min, and the

activity of GDH in units of μmol of NADH oxidized/ NAD reduced per min per mL was calculated using an extinction coefficient for NADH at 340 nm. The results were expressed as μmol per min per g fresh weight.

AlaAT activity assays were conducted according to Good and Muench (1992). The reaction buffer consisted of 15 mM α-oxoglutarate, 0.15 mM NADH, 0.5 M L-alanine and 5 units lactate dehydrogenase in 50 mM Tris-HCl buffer (pH 7.5), and the reaction was initiated by adding 100 μL of the enzyme extract. AspAT activity levels were assessed according to Griffith and Vance (1989). The 1.4 mL reaction buffer included 5 mM EDTA, 0.2 M L-aspartate, 12 mM 2-oxoglutarate, 0.15 mM NADH and 5 units malate dehydrogenase in 50 mM Tris-HCl buffer (pH 7.5), and the reaction started when 100 μL of the enzyme extract was added to the reaction buffer. AlaAT and AspAT activities were calculated using an absorption coefficient for NADH ($\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$) and were expressed as the amount of enzyme required to form 1 μmol of product per min per g of fresh weight.

2.5. Statistical analysis

The data are presented in the figures as the averages of at least three replicates per treatment and reported as the means ± SD. Statistical significance was evaluated with Student's t-test, and considered to be significant when the P value was less than 0.05.

3. Results

3.1. Effects of copper stress on fresh weights and N concentrations

The effects of copper stress on the fresh weights and NH_4^+, NO_3^- and NO_2^- concentrations of the *Luffa cylindrica* roots are shown in Figure 1. The fresh weights of the roots gradually decreased with increasing copper concentrations, showing measurements of 72.4%, 53.8%, 48.8% and 10.9% relative to the control at copper concentrations of 25, 50, 75 and 100 μM, respectively (Figure 1A).

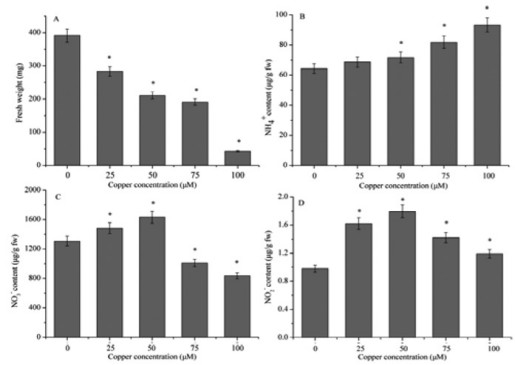

Figure 1. Effects of copper stress on fresh weights (A) and NH_4^+ (B), NO_3^- (C) and NO_2^- (D) concentrations in *Luffa cylindrica* roots. Data are displayed as means ± standard deviations for three replications. Significant difference ($p< 0.05$) is denoted as asterisk (*) between control and copper treatments.

Ammonium levels in the treated groups increased by 6.73%, 11.4%, 27.2 and 45% relative to the control at copper concentrations of 25, 50, 75 and 100 μM, respectively (Figure 1B). Nitrate concentrations in the roots were significantly induced by the 25 and 50 μM copper treatments, but the values were inhibited by 75 and 100 μM of copper (Figure 1C). NO_2^- concentrations showed increases of 165.5%, 183.2%, 145%, and 121.6% relative to the control at copper concentrations of 25, 50, 75 and 100 μM, respectively (Figure 1D).

3.2. Effects of copper stress on NR and NiR activities

The effects of copper stress on the NR and NiR activities in the *Luffa cylindrica* roots are shown in Figure 2. NR activities were induced by 39.5%, 49.5% and 11.7% at copper concentrations of 25, 50, and 75 μM; treatment with 100 μM copper inhibited NR by 21% relative to the control (Figure 2A). As shown in Figure 2B, NiR activity levels gradually decreased with increasing copper concentrations, showing reductions of 15.6%, 36%, 44.3% and 49.2% relative to the control, respectively.

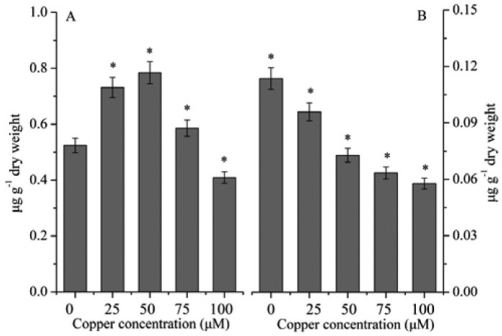

Figure 2. Effects of copper stress on nitrate reductase (A) and nitrite reductase (B) activities in *Luffa cylindrica* roots. Data are displayed as means ± standard deviations for three replications. Significant difference ($p< 0.05$) is denoted as asterisk (*) between control and copper treatments.

3.3. Effects of copper stress on GS and GOGAT activities

The effects of copper stress on the GS and GOGAT activities in the *Luffa cylindrica* roots are shown in Figure 3. As shown in Figure 3A, GS activity levels significantly increased by 105.7%, 90.3%, 54.6% and 52.7% relative to the control at copper concentrations of 25, 50, 75 and 100 μM, respectively. As shown in Figure 3B, GOGAT activity levels increased by 102.1%, 157.8%, 92.6% and 31.9% relative to the control at copper concentrations of 25, 50, 75 and 100 μM, respectively.

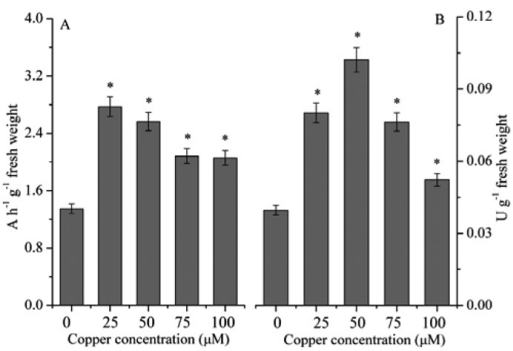

Figure 3. Effects of copper stress on glutamine synthetase (A) and glutamate synthase (B) activities in *Luffa cylindrica* roots. Data are displayed as means ± standard deviations for three replications. Significant difference ($p< 0.05$) is denoted as asterisk (*) between control and copper treatments.

3.4. Effects of copper stress on GDH activities

The effects of copper stress on GDH activities in the *Luffa cylindrica* roots are shown in Figure 4. As shown in Figure 4A, NADH-GDH activity levels in the roots increased significantly following the copper treatments, with values reaching 203.8%, 193.5% 178.6% and 161.1% of the control value at copper concentrations of 25, 50, 75 and 100 μM, respectively. As shown in Figure 4B, NAD-GDH activity levels were significantly induced by copper concentrations of 25, 50, and 75 μM but inhibited by 100 μM copper.

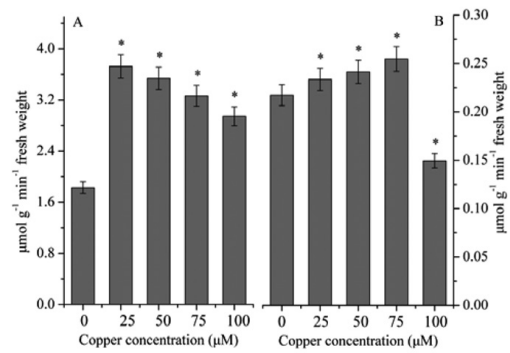

Figure 4. Effects of copper stress on NADH-glutamate dehydrogenase (A) and NAD-glutamate dehydrogenase (B) activities in *Luffa cylindrica* roots. Data are displayed as means ± standard deviations for three replications. Significant difference ($p< 0.05$) is denoted as asterisk (*) between control and copper treatments.

3.5. Effects of copper stress on AlaAT and AspAT activities

The effects of copper stress on AlaAT and AspAT activities in the *Luffa cylindrica* roots are shown in Figure 5. As shown in Figure 5A, AlaAT activity levels at copper concentrations of 25, 50, and 75 μM increased by 29.9%, 35.8% and 30.6% relative to the control values, but activity levels at the concentration of 100 μM showed no change. As shown in Figure 5B, AspAT activity levels increased gradually with rising copper concentrations up to 75 μM, with the peak value being approximately 130.5% higher than that of the control.

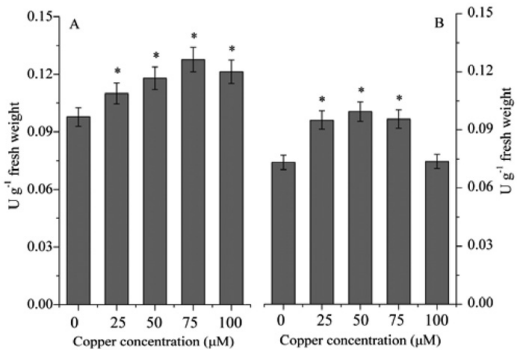

Figure 5. Effects of copper stress on alanine aminotransferase (A) and aspartate aminotransferase (B) activities in *Luffa cylindrica* roots. Data are displayed as means ± standard deviations for three replications. Significant difference ($p< 0.05$) is denoted as asterisk (*) between control and copper treatments.

4. Discussion

Heavy metals are prevalent abiotic stressors affecting plant growth and development. Growth inhibition is a common response to this type of stress, and plant growth is one of the most important agricultural indices of heavy metal stress tolerance, as has been indicated by numerous studies. Copper is an essential micronutrient for normal plant growth and metabolism, but it can be toxic to plants at higher concentrations (Yruela 2009). Excess copper has been reported to lead to the inhibition of young seedling growth and root elongation and to damage root epidermal cells and root cell membranes (Xiong and Wang, 2006; Lequeux *et al*., 2010). The present results suggest that there is a correlation between increasing copper concentrations and reduced root fresh weight (Figure 1A). N metabolism is a prerequisite for normal plant growth and development because almost all 'vital' biomolecules are nitrogenous substances (Mokhele *et al.*, 2012). The forms and concentrations of N within a plant vary widely according to the organ, developmental stage, and environmental conditions. The root is obviously the predominant organ where many exchanges of a variety of N forms occur between root cells and the soil solution (Stitt *et al.*, 2002). We show that the NO_2^- and NH_4^+ concentrations significantly increased in the *Luffa cylindrica* roots following the copper treatments, but the NO_3^- concentrations were inhibited at 75 and 100 μM copper (Figure 1). The present findings seem to contradict those from previous reports, which have suggested that NO_3^-, NO_2^- and NH_4^+ concentrations in leaves are significantly reduced by heavy metal stresses in tobacco, and spinach plants (Wu *et al*., 2008; Maaroufi Dguimi *et al*. 2009). These changes may be a combined result of the inhibition of the uptake and transport of NO_3^- along with the decreased activities of NR and NiR (Figure 2).

In plant cells, nitrate is reduced to ammonium through a two-step process. The first is the reduction of nitrate to nitrite, which is catalyzed by NR, and the second is the reduction of nitrite to ammonium by NiR (Stitt *et al*., 2002). Several studies have shown that changes in the concentrations of different N forms are correlated with the activities of NR and NiR in tobacco and spinach plants that have been exposed to heavy metal stress. Moreover, decreases in NR and NiR activities have been observed in plants due to their breakdown as induced by the ROS that are generated following heavy metal stress (Xiong *et al*., 2006). The present findings suggest that the higher copper concentrations of 75

and 100 μM result in decreased levels of NR activity, and concentrations of 25–100 μM also lead to reduced levels of NiR activity. These changes may be related to the reductions in the NO_3^- and NO_2^- concentrations that occurred at copper concentrations of 75 and 100 μM (Figure 1).

The primary pathway for the entry of ammonium into amino acids is the GS/GOGAT cycle, which involves the sequential actions of GS and GOGAT (Figure 3). Biochemical, genetic and molecular evidences have shown that the major pathway for ammonia assimilation into organic molecules is the GS/GOGAT cycle in higher plants (Stitt *et al*., 2002). There is also evidence that alternative routes for assimilating ammonium may be triggered when GS activity is impaired (Skopelitis *et al*. 2006). The present results indicated that the activities of GS and GOGAT increased to varying degrees relative to those of the controls. These findings appear to differ from those of other studies addressing the GS/GOGAT cycle. Thus, additional studies are required to examine these conflicting results. An alternative pathway for N assimilation may involve the direct reductive amination of α-ketoglutarate by the GDH. This enzyme catalyzes a reversible reaction that synthesizes (NADH-GDH) or deaminates (NAD-GDH) glutamate in vitro (Stitt *et al*., 2002). GDH is ubiquitous in plants and is often present at high levels in plant tissues, including both senescing tissues and root tissues (Ludewig *et al*., 2007). This enzyme can operate in either the assimilation or the reassimilation of ammonium, thereby complementing the enzymes of the GS/GOGAT cycle in glutamate synthesis, especially under stressful conditions such as elevated temperatures, water, salinity, pollution and heavy metals (Skopelitis *et al*. 2006). Recent data from studies of transgenic tobacco plants with altered GDH genes revealed that all GDH isoenzymes strongly deaminate glutamate, and only the anionic GDH isoforms exhibit very low aminating activities under normal trophic conditions (Skopelitis *et al*. 2007). The findings from the present work suggested that varying increases in the activities of NADH-GDH and NAD-GDH occurred following all copper treatments with the exception of 10 μM copper. Thus, this study has provided insight into the tissues that show variable GDH activity levels in response to elevated copper concentrations. Aminotransferases, including AlaAT and AspAT, can catalyze the reversible transfer of an amino group from glutamate to pyruvate to form 2-oxoglutarate and alanine/aspartate. Their regulation in several plant species has been studied in response to heavy metal stress (Gajewska *et al*., 2009; Mishra and Dubey, 2011). Thus, these two enzymes may be reasonable targets for metabolic engineering to produce crop varieties with enhanced stress resistance capabilities and nutrient concentrations. As shown in Figure 5, the activities of AlaAT and AspAT in the roots were significantly induced by the copper treatments. Thus, in our experiment, increases in AlaAT and AspAT activities in the plant tissues may indicate a detoxification response to copper exposure.

5. Conclusions

In conclusion, the results of this study support the hypothesis that copper is toxic to *Luffa cylindrica*, which is at least partially due to the influences of copper on N metabolism and on enzymes associated with N metabolism. We hope that these findings will contribute to the improved understanding of the response mechanisms of *Luffa cylindrica* to copper stress and lend further insight into metal-microbe interactions in natural environments. We believe that future studies using copper treatments for longer exposure times will aid in the elucidation of the N nutrition- and metabolism-related changes that occur following exposure to copper toxicity in *Luffa cylindrica.*

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