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The Detoxification Effect of Nitrogen on Cadmium Stress in *Populus yunnanensis*

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Abstract: In this study, researchers used *Populus yunnanensis*, a native precious poplar species which is only distributed in Southwestern China as a model species to study its morphological, physiological and biochemical responses to the interaction of different concentrations of nitrogen and Cd, discussing the detoxification effect of nitrogen to Cd stress in *Populus yunnanensis*. The treatments were consisted of three Cd levels (0, 2 and 4 mg kg^{-1}) and three N levels (0, 20 and 40 mg kg^{-1}). After 40 days treatment, the results indicated that at the same N level, Cd treatment had significant effect on all measured parameters, including plant growth, chlorophyll concentration, Malondialdehyde (MDA) concentration, antioxidant enzymes activities (SOD, POD, CAT and APX) and free proline. Meanwhile, study also showed there was significant difference among the three levels of N treatment. With the increasing concentration of N supplement, it showed more significant effect of alleviation to Cd induced damage in *Populus yunnanensis*.

Key words: Detoxification, nitrogen, cadmium, chlorophyll, antioxidant enzymes, free proline

INTRODUCTION

With the development of urbanization and industrialization, the contamination of heavy metals which are directly produced and released by human activities directly implicated in the generation of oxidative stress in the plant surrounding environment which make ecological environment became unbalanced and deteriorated. Cadmium (Cd) is one of the major industrial pollutants because its highly toxicity and migration which shows its phytotoxicity even at low doses (Chakravarty and Srivastava, 1992; Das *et al*., 1997). This metal is especially active in the soil so it can be absorbed and accumulated by plant which may cause obviously damage to plant growth and development. When Cd is over accumulated by plant, it will influence plant physiology and biochemistry activity such as photosynthesis, respiration, transpiration or even relative gene expression then performed by plant appearance such as leaves chlorosis, growth inhibition and even plant death.

In addition, recently reports showed that after adding nitrogen to plant, the damage caused by Cd stress can be efficiently alleviated. Moreover, plant even present better growth than those of plants which did not under stress. Some research indicate that adding appropriate nitrogen in nutrient solution can obviously enhance the development of root system and increase accumulation of Cd in Sedum alfredii Hance (Li *et al*., 2007). Further, under the same concentration of Cd stress, the biomass of *Kandelia candel* dramatically increased with the increasing nitrogen. Refer to these different research, researchers agreed that adding nitrogen can evidently alleviate the damage from Cd stress and this effect can be differential along with the different plant species as well as the different concentration of both Cd and nitrogen (Hassan *et al*., 2005).

Populus yunnanensis is one of the most valuable and unique poplar species which is distributed in high elevation regions of low latitudes and occurring only in southwestern China. *P. yunnanensis* is horizontal distributed in middle and northern Yunnan province and southwestern Sichuan province and vertical distributed in altitude 800-3200 m. *P. yunnanensis* is one of the fast growing tree species which plays an important role in forestry production and environmental protection. It is famous for fast growth, ornamental value and high adaptability which is widely used as afforestation and timber tree species. However, with the development of industrialization in southwestern China, the soil suffers increasing heavy metals pollution which had detrimental influence upon the growth and development of *P. yunnanensis* in this region. In this study, we comprehensively investigated the morphological, physiological and biochemical responses of *P. yunnanensis* under the interaction of different concentrations of nitrogen and Cd treatment. The aims were to test the detoxification effect of nitrogen to Cd stress in *P. yunnanensis* as well as to study the

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interaction effect of heavy metal and nutrient element on *P. yunnanensis*. Therefore, the differential influence of different concentrations of Cd and N treatments on *P. yunnanensis* including the growth of height and DBH (Diameter at Breast Height), chlorophyll content, antioxidant enzymes activities and lipid peroxidation, free proline content were observed after 40 days exposure to different concentrations of N and Cd treatments. Hoping the results of this study can provide us theoretical and practical experiences to the protection of *P. yunnanensis* as well as other naive plants suffered from heavy metals pollution, thus promoting the development of methodology of plant remediation.

MATERIALS AND METHODS

Plant material and treatments: About 81 healthy cuttings with an average of 5 nodes and about 15 cm of height were sampled from 81 different trees from their natural habitat (Meigu, Sichuan province). All trees were collected from the same population shared similar conditions of water and soil nutrients. After sampled, these 81 cuttings were replanted into 27 plastic pots (3 cuttings per pot, each pot is 20* 20* and 30 cm which can contain 30 kg homogenized soil (pH = 7.0±0.12). The cuttings were grown in a naturally lit greenhouse which provided shelter from rainfall at the Sichuan Agricultural University. Cuttings were watered with tap water every 2 days to maintain 100% field capacity. In this study there were 3 treatments of cadmiun including control (0 mg kg^{-1}), low Cd (2 mg kg^{-1}) and high Cd (4 mg kg^{-1}) and the 3 treatments of nitrogen comprising control (0 mg kg^{-1}), low N (20 mg kg^{-1}) and high N (40 mg kg^{-1}). So, there were totally 9 treatments and for each treatment it was set up in triplicate to ensure the reproducibility of results. When the cuttings grew at 8-10 leaf stage (2-3 weeks old, 20 cm in height) they were subjected to the above 9 treatments and each 3 pots as one group which was treated by the same treatment. The treatments started on 20 April 2010 and the plants were harvested on 1 June 2010. The height and DBH (diameter at breast height) of each cutting was measured before treatments, leaves samples (about 8 mm diameter) were cut with a scissor from the third pair of leaves (counting from the bottom of the plant) after harvested and stored immediately at -80°C for later analysis.

Measurement of chlorophyll content: Leaf samples were taken from the third leaf from the bottom of plant right after harvested and used for determinations. After extraction with 80% acetone for 48 h, the absorbance of chlorophyll was measured by spectrophotometer at 645 and 663 nm according to Lichtenthaler (1987).

Measurements and assays of antioxidant enzymes activities: For SOD, CAT and POD extraction, leaf samples (0.5 g fresh leaves) were ground in liquid nitrogen and extracted with 50 mM potassium phosphate buffer (pH 7.8) which contained 0.5 mM EDTA with pre-chilled pestle and mortar. Each homogenate was transferred to centrifuge tubes and was centrifuged at 15000 g, 4°C for 15 min.

The supernatant was used for enzyme activity assay. For APX extraction, leaf samples (0.5 g) were homogenized in 50 mM ice cold potassium phosphate buffer (pH 7.5) which contained 0.5 mM EDTA, 2 mM Ascorbate (AsA) and 5% Poly Vinyl Pyrrolidin (PVP) with pre-chilled pestle and mortar. Other stages were similar to extraction of other enzymes (Esfandiari *et al*., 2007). All operations were performed at 0-4°C. SOD activity was estimated according to Sairam *et al*. (2002), CAT activity was measured according to Aebi (1984) and the activity of POD were assayed according to the method of Guo *et al*. (2004) by monitoring the rate of guaiacol oxidation at 470 nm. In addition, APX activity was measured according to Yoshimura *et al*. (2000) by monitoring the rate of ascorbate oxidation at 290 nm. A unit of antioxidant enzymes activities was expressed as the change in absorbance per minute and specific activities as enzyme units per g of Fresh Weight (FW).

Measurement of lipid peroxidation: The level of lipid peroxidation was expressed by Malondialdehyde (MDA) concentration which was measured by colorimetric method according to Stewart and Bewley (1980). The 0.5 g of leaf samples were homogenized in 6 mL of 50 mM potassium phosphate buffer (pH 7.8). To each 1 mL aliquot of the supernatant, 2 mL of 0.6% Thiobarbituric Acid (TBA) in 10% TCA was added. The mixtures were incubated at 95°C for 15 min then quickly cooled the reaction tubes with an ice bath. After that the mixtures were centrifuged at 10,000 g for 15 min. The absorbance of the supernatant was determined at 600, 532 and 450 nm by spectrophotometer. Lipid peroxidation was expressed as the MDA content in nM per g of Fresh Weight (FW).

Measurement of free proline: Free proline was extracted and its concentration was measured by using the method of Bates *et al*. (1973). Leaf samples (0.5 g) were homogenized with 5 mL 3% sulfosalicylic acid and then the homogenate was centrifuged at 3000 g for 10 min. After that the supernatant was heated (80°C) for 1 h and then the absorbance of the supernatant was determined at 520 nm.

Statistical analysis: All data presented were means± standard value and the measurements were done with three replicates for statistical validity. One-way Analysis of Variance (ANOVA) was performed to check the variability of data and validity of the results. The data were analyzed with the software Statistical Package for the Social Sciences (SPSS, Chicago, IL, USA) version 13.0 and the results were considered significant if $p<0.05$.

RESULTS AND DISCUSSION

Growth: The growth of height and DBH of *P. yunnanensis* for 40 days exposure to treatments were shown in Fig. 1. It showed that under the same N treatment, different concentrations of Cd stress on *P. yunnanensis* resulted in a significant inhibition on the growth of both height and DBH and this inhibition expressed more significantly with the increasing Cd concentrations. In addition, under the same Cd treatment, the height and DBH growth shows a dramatically increase along with the concentration of N increased. Results of univariate analysis (Table 1) indicated that Cd and N treatment both have extremely significant influence on the height and DBH growth of *P. yunnanensis* ($p<0.01$) and the interaction effect of Cd and N is significant ($p<0.05$). The results of multiple comparison (Table 2) shows that under the same Cd treatment, the increased concentration of N can significantly improve plant growth and the higher concentration of N (40 mg kg^{-1}) plays more significant effect compared to the lower concentration of N (20 mg kg^{-1}) which indicated the difference between different N level is significant ($p<0.05$).

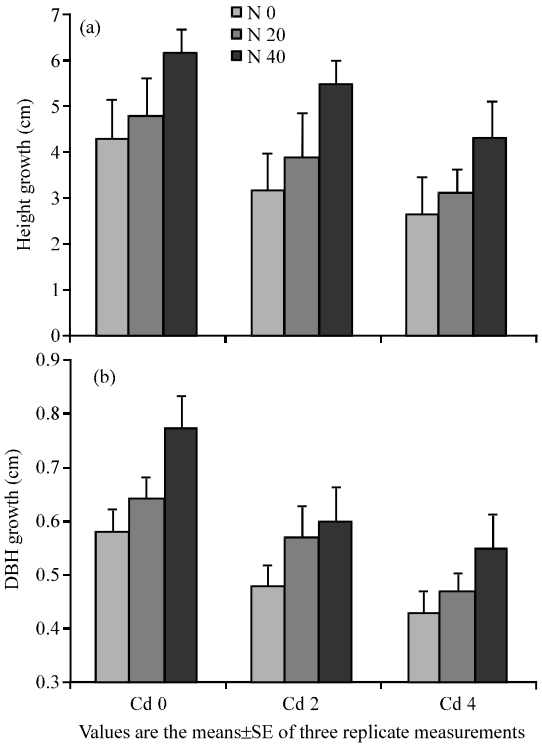

Fig. 1: Interactive effects of Cd and N treatment on growth of height and DBH in *P. yunnanensis*. (a) Height growth, (b) DBH growth

The concentrations of chlorophyll: Chlorophyll is the main pigment which can help plant to photosynthesize. When plant exposure to stress, their photosynthesis will be inhibited and the concentrations of chlorophyll can directly indicate the extent of stress-induced damage in plant. The concentrations of chlorophyll in the leaves of *P. yunnanensis* for 40 days exposure were shown in Fig. 2. All Cd treatments indicated a significant decrease in chlorophyll contents. It is obvious that under the same N level, the concentrations of chlorophyll in the leaves

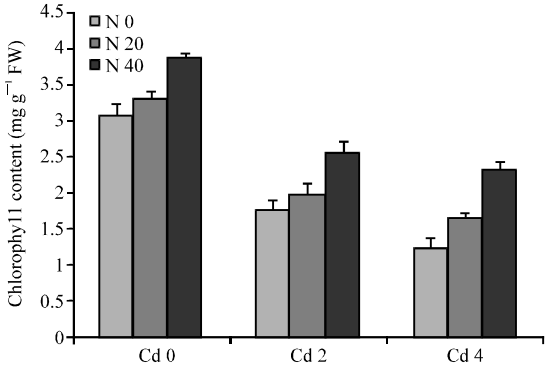

Fig. 2: Interactive effects of Cd and N treatment on chlorophyll contents of *P. yunnanensis*

Table 1: Analysis of variance significance for the interaction effects of Cd and N on height and DBH growth in *P. yunnanensis*

	Height		DBH	
Univariate	F	p	F	p
Cd	72.834	0.0007<0.01	31.273	0.0036<0.01
N	97.375	0.0004<0.01	19.483	0.0087<0.01

Table 2: Multiple comparison of height and DBH growth on N treatment under different Cd concentrations in *P. yunnanensis*

Treatment (mg kg^{-1})		Average growth (cm)	
Cd	N	Height	DBH
0	0	4.33^{b}	0.58^{b}
	20	4.83^{b}	0.64^{ab}
	40	6.17^{a}	0.77^{a}
2	0	3.17^{b}	0.48^{b}
	20	3.90^{b}	0.57^{ab}
	40	5.50^{a}	0.60^{a}
4	0	2.67^{b}	0.43^{b}
	20	3.15^{b}	0.47^{ab}
	40	4.33^{a}	0.55^{a}

Different letters in the same column indicate a significant difference between the different N treatment under the same Cd stress ($p<0.05$)

Table 3: Analysis of variance significance for the interaction effects of Cd and N on chlorophyll content in *P. yunnanensis*

	Chlorophyll content	
Univariate	F	p
Cd	334.090	0.0001<0.01
N	87.756	0.0005<0.01

Table 4: Multiple comparison of chlorophyll content on N treatment under different Cd concentrations in *P. yunnanensis*

Treatment (mg kg^{-1})		
Cd	N	Chlorophyll content (mg g^{-1} FW)
0	0	3.085^{b}
	20	3.301^{b}
	40	3.865^{a}
2	0	1.757^{b}
	20	1.988^{b}
	40	2.557^{a}
4	0	1.250^{b}
	20	1.660^{b}
	40	2.324^{a}

treated with Cd significantly decreased compared with that of control which indicated that Cd treatment had significant effect on the synthesis of chlorophyll and the inhibition effect was more significant along with the increased Cd level. On the other hand, the N treatment represented a significant improvement on the chlorophyll contents. The concentrations of chlorophyll witnessed a gradual rise along with the increased N level under the same concentration of Cd stress. According to the multiple comparison (Table 3), Cd and N treatment both had extremely significant influence on the chlorophyll content of *P. yunnanensis* ($p<0.01$) and the interaction effect of Cd and N was significant ($p<0.05$). According to the results of multiple comparison (Table 4) we can see clearly that under the same level of Cd stress, the concentrations of chlorophyll showed a positive correlation to that of N level and the higher concentration of N (40 mg kg^{-1}) played more significant effect compared to the lower concentration of N (20 mg kg^{-1}) ($p<0.05$) which indicated nitrogen can effectively alleviated Cd induced inhabitation on chlorophyll synthesize.

The activities of antioxidant enzymes: Enzymatic antioxidant system is one of the protective mechanisms including SOD, POD, CAT and APX which can eliminate Active Oxygen Species (AOS) in plant cell and alleviate stress-induced oxidative damage (Beak and Skinner, 2003). The activities of 4 antioxidant enzymes in the leaves of *P. yunnanensis* for 40 days exposure were shown in Fig. 3.

According to Fig. 3a, it is shown that under the same concentration of N treatment, Cd stress induced a significant increase in SOD compared with control which indicated that *P. yunnanensis* has a certain degree of resistance to Cd stress. Even when the Cd stress level

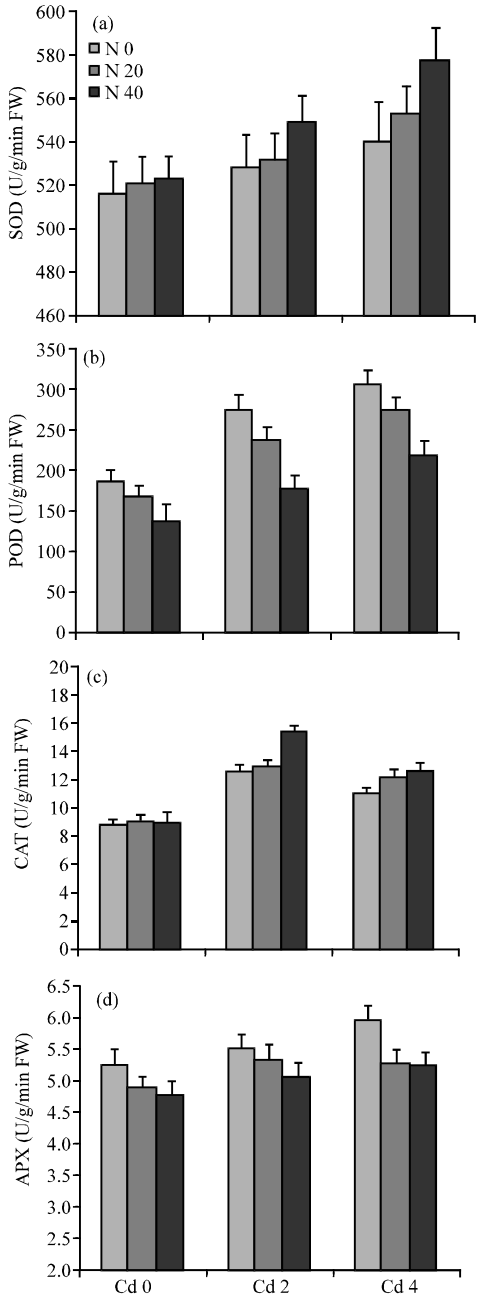

Fig. 3: Interactive effect of Cd and N treatment on antioxidant enzymes activities of *P. yunnanensis*; (a) SOD activities, (b) POD activities, (c) CAT activities, (d) APX activities

reached at 2 mg kg^{-1}, the stress didn't exceed the maximum resistance of plants. Additional, SOD activities represented a significant increase with the increasing N concentration under the same Cd level. From the results of multiple comparison (Table 5), it is indicated that Cd and N treatment both had significant influence on SOD

Table 5: Analysis of variance significance for the interaction effects of Cd and N on antioxidant enzymes activities in *P. yunnanensis*

	SOD		POD		CAT		APX	
Univariate	F	P	F	p	F	p	F	p
Cd	15.162	0.0136<0.05	48.581	0.0016<0.05	27.129	0.0047<0.05	13.393	0.0169<0.05
N	11.407	0.0429<0.05	28.399	0.0043<0.05	2.777	0.1753>0.05	16.233	0.012<0.05

Table 6: Multiple comparison of antioxidant enzymes activities on N treatment under different Cd concentrations in *P. yunnanensis*

Treatment (mg kg^{-1})		Enzymes activities (U/g/min FW)			
Cd	N	SOD	POD	CAT	APX
0	0	517^{b}	188^{a}	8.83^{a}	5.26^{a}
	20	521^{ab}	169^{b}	9.08^{a}	4.89^{b}
	40	523^{a}	138^{c}	9.00^{a}	4.77^{b}
2	0	528^{b}	278^{a}	12.64^{a}	5.51^{a}
	20	532^{ab}	239^{b}	12.96^{a}	5.32^{b}
	40	549^{a}	179^{c}	15.40^{a}	5.05^{b}
4	0	540^{b}	308^{a}	11.04^{a}	5.95^{a}
	20	553^{ab}	275^{b}	12.24^{a}	5.28^{b}
	40	577^{a}	220^{c}	12.64^{a}	5.24^{b}

activities ($p<0.05$). Furthermore, the concentrations of N were positively correlated to the activities of SOD (Table 6) and there also was significant difference of effects between higher N (40 mg kg^{-1}) and lower N (20 mg kg^{-1}).

POD activities were significant influenced by the interaction of Cd and N treatment. According to the Fig. 3b, it is shown that under the same N level the POD activities gradually increased compared to that of control. On the contrary, at the same Cd treatment, POD activities witnessed a significant decrease along with the increased N level. From the results of multiple comparison (Table 5), both Cd and N treatment had significant influence on POD activities ($p<0.05$). Furthermore, the concentrations of N was positively correlated to the activities of SOD (Table 6) and the difference between different N level was significant ($p<0.05$).

For the CAT activities, there was a significant difference induced by different Cd treatment under the same N level ($p<0.05$). The activities witnessed a significant growth followed by a decline (Fig. 3c) which indicated that with the increasing concentration of Cd stress, plant generated more CAT to eliminate active oxygen. However, when the Cd stress reached to a certain degree, CAT activities were inhibited. Additional, there was no significant difference among three N concentrations under the same Cd level (Table 5).

Both Cd and N treatment have significant influence on APX activities ($p<0.05$). According to Fig. 3d, the APX activities showed a gradual rise with the increasing concentrations of Cd treatments under the same N level which indicated that Cd treatment had significant effect on the APX activities. On the contrary at the same Cd treatment, APX activities witnessed a slight decrease along with the increased N level. Although, the concentrations of N were positively correlated to the activities of APX (Table 6) and N treatment could largely decreased APX activities, there were no significant difference of effects between higher N (40 mg kg^{-1}) and lower N (20 mg kg^{-1}).

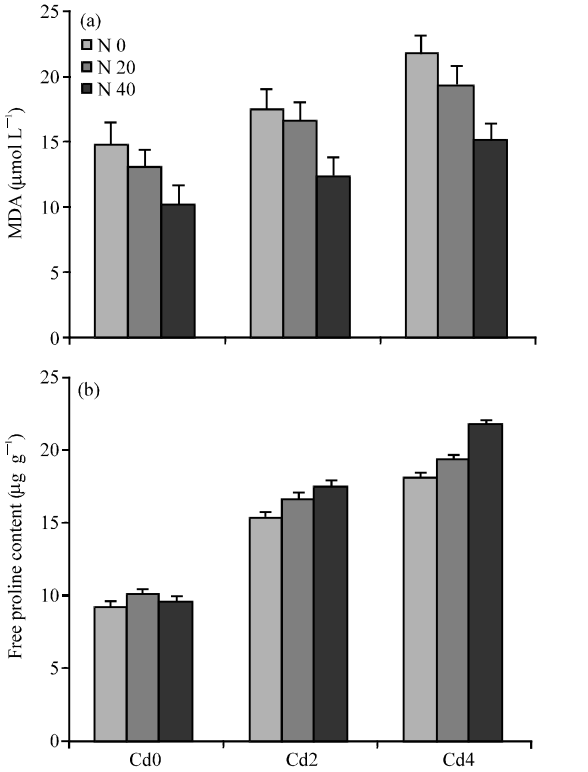

Fig. 4: Interactive effect of Cd and N treatment on MDA and free proline content in *P. yunnanensis*, (a) MDA content, (b) Free proline content

The concentrations of MDA and free proline: Malondialdehyde (MDA) is the main product of membrane lipid peroxidation which can indicate the extent of stress-induced damage in plant. The results in Fig. 4a showed that the MDA concentration in the leaves of *P. yunnanensis* is extremely increased under the Cd treatment compared with that of control which suggested that the Cd treatments had a significant positive correlation to the MDA accumulation ($p<0.01$) (Table 7). On the contrary, under the same Cd level, the MDA content witnessed a significant decrease with the increasing concentration of N and the difference between different N level was significant (Table 8) which indicated N treatment can significant alleviated the lipid peroxidation in *P. yunnanensis* ($p<0.01$).

Table 7: Analysis of variance significance for the interaction effects of Cd and N on MDA and free proline content in *P. yunnanensis*

	MDA		Free proline	
Univariate	F	p	F	p
Cd	69.768	0.0008<0.01	95.642	0.0004<0.01
N	61.172	0.001<0.01	3.844	0.1171>0.05

Table 8: Multiple comparison of MDA and free proline content on N treatment under different Cd concentrations in *P. yunnanensis*

	Treatment (mg kg^{-1})		
Cd	N	MDA	Free proline
0	0	14.9^{a}	9.2^{a}
	20	13.1^{b}	10.1^{a}
	40	10.2^{c}	9.6^{a}
2	0	17.5^{a}	15.4^{a}
	20	16.6^{b}	16.6^{a}
	40	12.4^{c}	17.5^{a}
4	0	21.8^{a}	18.1^{a}
	20	19.3^{b}	19.3^{a}
	40	15.1^{c}	21.8^{a}

Free proline plays an important role in osmoregulation and its accumulation was considered significant for the adaptation to stress in plant (Tang, 1984). The free proline concentrations in leaves of *P. yunnanensis* showed a significant difference under Cd stress ($p<0.01$). With the increasing Cd level, free proline content had a rapid rise followed by a slowly increase which indicated that Cd stress improved the accumulation of free proline and played a protective role in *P. yunnanensis*. However, when the Cd level reached to a certain degree, the synthesis of proline was inhibited and influenced the increase of proline. Additional, N treatment had no significant influenced on the concentration of free proline under the same Cd level and there was also no significant difference among three N concentrations (Table 8) which indicated that the concentrations of free proline were only affected by Cd stress.

Cd contaminated has detrimental influence on plant growth and development. Over-accumulation of Cd concentration in plants shows differential symptoms of toxicity including the growth inhibition, leaves chlorosis and biomass decrease. The results showed that Cd stress obviously inhibited the growth of *P. yunnanensis* slowed down the synthesis process of chlorophyll which decreased its concentration in plants.

Over-accumulated Cd injury to plants is mainly because of altered oxidant levels which causing the occurrence of oxidative stress due to accumulation of Active Oxygen Species (AOS) including superoxide radical (O^{2-}), Hydroxyl radical (OH^{-}) and hydrogen peroxide (H_2O_2). In normal circumstances, the speed of generation and elimination of AOS is balanced. However, when plants exposed to Cd stress, plants generate more AOS than elimination which cause over accumulation of AOS in plants which lead to significant damage to membrane system in plants and induce lipid peroxidation, increase permeability and conductivity (Gallego *et al*., 1996; Chaoui *et al*., 1997). The concentration of Malondialdehyde (MDA) which is a general indicator of lipid peroxidation can demonstrate the extent of oxidative stress in plants (Chaoui *et al*., 1997). On the contrary, plant cells have their own defense mechanisms against AOS. One of the protective mechanisms is the enzymatic antioxidant system which involves the sequential and simultaneous action of a number of enzymes such as SOD, POD, CAT, APX (Bowler *et al*., 1992). The results showed that under Cd stress, MDA and free proline concentration in plants was significant increased with the increasing Cd level. However, *P. yunnanensis* showed a certain degree of resistance to Cd stress and the concentration of Cd treatment didn't exceed its maximum capacity of resistance. Thus the oxidative stress caused by Cd addition was moderate which allowing the plants to adapt it actively by increasing their own antioxidant enzymes activity. Meanwhile, elevated level of these antioxidative enzymes in plants following the Cd treatment in this study demonstrated that these enzymes acted together to alleviate the impact of Cd stress. Furthermore, it is worth noting that the influenced of Cd stress on plant has significant difference among those three Cd concentrations.

Nitrogen is one of the essential nutrient elements. The deficiency of nitrogen will lead to metabolic disturbance in plants thus causing the decrease of plant growth and productivities. With pollution of heavy metals being serious day by day, the heavy metals stress may become the main reason of the deficiency or inefficiency of nutrient element. Meanwhile, supplement of nitrogen for plants can obviously alleviate heavy metal-reduced damage. Williams *et al*. (1967) suggested that adding nitrogen to soil, nitrification will occur and obviously decreased the pH of soil thus significant increasing the solubility of heavy metals in soil which decrease the adsorption amount of heavy metals.

CONCLUSION

In this study, the researchers discovered that nitrogen could effectively alleviated Cd induced damage in *P. yunnanensis* and there was a significant difference of effects on plants between lower N concentration (20 mg kg^{-1}) and higher N concentration (40 mg kg^{-1}). Supplement of high-concentration nitrogen to *P. yunnanensis* under Cd stress obviously enhanced plants growth, promoted the synthesis of chlorophyll as well as

the activities of antioxidant enzymes which partially alleviated the accumulation of AOS associated with Cd exposure which indicated that the increasing concentration of N supplement had more significant effect of alleviation to Cd induced damage in *Populus yunnanensis*.

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