

## The effects of phenolic acid on nitrogen metabolism in *Populus × euramericana* ‘Neva’

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**Abstract** The declines in soil fertility and productivity in continuously cropped poplar plantations are related to phenolic acid accumulation in the soil. Nitrogen is a vital life element for poplar and whether the accumulation of phenolic acid could influence nitrogen metabolism in poplar and thereby hinder continuous cropping is not clear. In this study, poplar cuttings of *Populus × euramericana* ‘Neva’ were potted in vermiculite, and phenolic acids at three concentrations (0X, 0.5X and 1.0X) were added according to the actual content (1.0X) in the soil of a second-generation poplar plantation. Each treatment had eight replicates. We measured gas exchange parameters and the activities of key enzymes related to nitrogen metabolism in the leaves. Leaf photosynthetic parameters

varied with the concentration of phenolic acids. The net photosynthetic rate ( $P_N$ ) significantly decreased with increasing phenolic acid concentration, and non-stomatal factors might have been the primary limitation for  $P_N$ . The activities of nitrate reductase (NR), glutamine synthetase (GS) and glutamate synthase (GOGAT), as well as the contents of nitrate nitrogen, ammonium nitrogen, and total nitrogen in the leaves decreased with increasing phenolic acid concentration. This was significantly and positively related to  $P_N$  ( $P < 0.05$ ). The low concentration of phenolic acids mainly affected the transformation process of  $\text{NO}_3^-$  to  $\text{NO}_2^-$ , while the high concentration of phenolic acids affected both processes, where  $\text{NO}_3^-$  was transferred to  $\text{NO}_2^-$  and  $\text{NH}_4^+$  was transferred to glutamine (Gln). Overall, phenolic acid had significant inhibitory effects on the photosynthetic productivity of *Populus × euramericana* ‘Neva’. This was probably due to its influence on the activities of nitrogen assimilation enzymes, which reduced the amount of amino acids that were translated into protein and enzymes. Improving the absorption and utilization of nitrogen by plants could help to overcome the problems caused by continuous cropping.

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## Introduction

China's demand for timber is large and exceeds the domestic supply capacity. Due to the shortage of soil resources, continuous cropping management has typically been used in most forest cultivation (Li et al. 2014, 2016). However, this has resulted in problems such as reductions in productivity and stand volume growth (Zhang and Li 2005; Wu et al. 2014). Poplar is widely planted in the Yangtze-Huaihe river basin to help supply China's demand for timber. However, due to limited suitable land for poplar plantations, continuous cropping is practiced and has caused the problems cited above (Liu et al. 2005; Wang et al. 2010a, 2011). Rlum and Gric (2005) reported that the reductions observed in continuously cropped plantations were related to allelochemical accumulation in the soils. Phenolic acid is an allelochemical with strong allelopathic effects that have been studied in tree plantations (Schutter et al. 2001; Ye et al. 2004; Jeffrey and John 2004; Li et al. 2005; Cao et al. 2005; Wang et al. 2006). Tan et al. (2008) and Wang et al. (2010a) detected phenolic substances in the soils of continuously cropped poplar plantations and reported that allelochemical accumulation increased with each successive generation of trees. Wang et al. (2010a, b) and Yang et al. (2010) studied the mechanisms of plantation productivity decline by quantifying the accumulation patterns of soil chemicals and their effects on rhizosphere soil and the root physiology of poplars. They reported that phenolic acid was the primary cause of reduced productivity in continuously cropped poplar plantations.

The soil concentrations of phenolic acids affect the nitrogen uptake of plants. For example, phenolic acids restrain the  $\text{NO}_3$  uptake by *Cucumis sativus* and lower its productivity (Lü et al. 2002). After generations of continuous cropping, the nitrogen content in continuously cropped poplar trees has declined significantly with increasing soil concentrations of phenolic acids (Liu et al. 2005). Phenolic acids impair the activity of poplar nitrate reductase (NR) and affect the uptake and use of nitrate nitrogen (Wang et al. 2011). The allelopathic effects of phenolic acids are compounded in the field because poplar trees can produce greater quantities of phenolic substances under nitrogen deficient conditions (Wang et al. 2011). To date, the effects of phenolic acids on the photosynthetic physiological and biochemical parameters of poplar and on nitrogen metabolism in poplar have not been reported. Similarly, the relationships between phenolic substances and nitrogen and

ammonia assimilation enzymes have not been reported. Thus, our objectives in this study were to investigate the effects of phenolic acids on poplar photosynthetic physiology and the nitrogen cycle of *Populus × euramericana* in an effort to find solutions to the problems caused by the continuous cropping of *Populus × euramericana*. We studied the effects of phenolic acids on photosynthetic physiology parameters, nitrate nitrogen, ammonium nitrogen, and nitrogen and ammonia assimilation enzymes in the leaves of *Populus × euramericana* 'Neva'.

## Materials and methods

### Experimental plants

In late March 2014, we selected strong, healthy, and similar 1-year-old *Populus × euramericana* 'Neva' seedlings for sampling. Cutting slips at 1.5 cm diameter and 10 cm length were obtained from these seedlings for experiments. These slips were transferred to flower pots filled with vermiculite for culture (3 treatments with 6 plants per treatment). Each pot (38 cm in inner diameter and 45 cm in depth) was filled with 16 L of vermiculite, and pots were irrigated. In early May, the seedlings were treated with phenolic acids every 10 days by the addition of 1.6 L phenolic acids to each pot. In mid-August we measured photosynthetic gas exchange parameters, activities of nitrate reductase (NR), glutamine synthetase (GS), glutamate synthase (GOGAT), and nitrate reductase (NR), and the amounts of nitrate nitrogen, ammonium nitrogen, and total nitrogen in *Populus × euramericana* leaves.

According to the amounts of the five types of phenolic acids ( $X$ ,  $X = 247 \text{ mg L}^{-1}$  *p*-hydroxybenzoic acid + 11  $\text{mg L}^{-1}$  vanillin + 7  $\text{mg L}^{-1}$  ferulic acid + 54  $\text{mg L}^{-1}$  benzoic acid + 2  $\text{mg L}^{-1}$  cinnamic acid) in the soil of a second generation poplar plantation and the absorption rates of these acids (Tan et al. 2008; Wang et al. 2010a), three phenolic acid concentrations (CK (0X), T<sub>1</sub> (0.5X), and T<sub>2</sub> (1.0X)) were used to evaluate the intergenerational cultivation environments during the growth of the poplar seedling root systems (Table 1).

### Photosynthetic gas exchange parameter measurements

We used a portable photosynthesis system (CIRAS-2, Amesbury, MA, USA) to measure the photosynthetic light response parameters. All measurements were conducted from 08:30 to 11:30 a.m. on sunny days. During the measurements, the photosynthetically active radiation ( $\text{PAR} = 1500 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) was supplied by cold Light Emitting Diode (LED) (peak emission wavelength: red

**Table 1** Phenolic acid concentration set in the treatment nutrient solutions

Phenolic acid	Content of phenolic acid in field soil ( $\mu\text{g g}^{-1}$ )	Absorption rate of phenolic acid in soil (%)	Gradient of phenolic acid content ( $\mu\text{g ml}^{-1}$ )		
			CK (0X)	T1 (0.5X)	T2 (1X)
<i>p</i> -hydroxybenzoic acid	152.00	61.66	0	123	247
Vanillin	10.40	93.78	0	6	11
Ferulic acid	6.50	89.30	0	4	7
Benzoic acid	20.60	37.85	0	27	54
Cinnamic acid	1.95	94.41	0	1	2

light (90%) 620–630 nm and white light (10%) 425–625 nm) via the portable photosynthesis system. Three leaves were measured for each treatment, and the measurements were repeated three times for each leaf. For every repetition, PAR was controlled for 120 s to obtain a stable value. The air temperature of the leaf chamber was controlled at approximately  $26 \pm 1.5$  °C, the relative humidity was  $60 \pm 5\%$ , and the  $\text{CO}_2$  concentration was  $370 \pm 6$   $\mu\text{mol m}^{-2}$ , as determined by the photosynthesis system. PAR,  $P_N$ , transpiration rate ( $T_r$ ), stomatal conductance ( $G_s$ ), intercellular  $\text{CO}_2$  concentration ( $C_i$ ), and the air  $\text{CO}_2$  concentration ( $C_a$ ) were recorded automatically through the instrument. The water-use efficiency (WUE) and stomatal limitation ( $L_s$ ) were calculated using Eqs. (1) and (2) (Berry and Downton 1982; Nijs et al. 1997; Li et al. 2015):

$$\text{WUE} = \frac{P_N}{T_r} \quad (1)$$

$$L_s = 1 - \frac{C_i}{C_a} \quad (2)$$

#### Measurement of the activities of enzymes (NR, GS and GOGAT)

According to the method used by Kong and Yi (2008), we measured NR activity. The activity of GS was calculated using the method described by Wang et al. (2008). The measurement of GOGAT was conducted according to the method introduced in the literature (Lin and Kao 1996; Lin et al. 2000; Zhang et al. 1997) with minor modifications.

#### Measurement of the contents of nitrate nitrogen ( $\text{NO}_3^-$ -N), ammonium nitrogen ( $\text{NH}_4^+$ -N), and total nitrogen (total N)

Prior to testing, sample leaves were washed with deionized water, and dried at 85 °C to constant weights. The dried leaves were ground and passed through a 60 mesh screen.

Leaf samples were weighed and mixed with deionized water and activated carbon. Next, they were oscillated in

boiling water for 30 min. Afterwards, the mixtures were cooled and filtered. The filtrates were analyzed by an automated chemistry analyzer (Smartchem200, Italy) to determine the amounts of nitrate nitrogen and ammonium nitrogen.

Leaf samples were weighed and analyzed using a Costech elemental combustion system (Costech ESC4010/4012, Australia) for total nitrogen amount.

#### Statistical analysis

The Statistical Program for the Social Sciences (SPSS, Chicago, IL, USA) software was used for one-way analysis of variance (ANOVA), Least Significant Difference method (LSD) and statistical evaluations, and the test measurements results of different treatments were examined at the 5% level.

## Results and discussion

### The effects of phenolic acid concentration on photosynthetic gas exchange parameters

Under fixed light intensity ( $\text{PAR} = 1500$   $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) and at different concentrations of phenolic acids, the  $P_N$ ,  $E$ ,  $g_s$ ,  $C_i$ ,  $L_s$ , and  $WUE$  of the leaves of poplar changed significantly but with various trends (Table 2).

$P_N$  of poplar leaves was affected by phenolic acid concentration (Table 2). Compared with the control group,  $P_N$  decreased by 11.4 and 22.9% for T1 and T2, respectively, and the differences between treatments T1 and T2 were significant. The photosynthetic rate of leaves decreased with increase in  $C_i$  (Table 2). Farquhar and Sharkey (1982) reported that a non-stomatal factor led to the decrease in  $P_N$ , indicating that phenolic acid affected the internal stomatal photosynthesis of *Populus × euramericana*. For  $G_s$  and  $T_r$ , the same fixed models were found with the decrease in  $P_N$ , and both  $G_s$  and  $T_r$  had similar trends (Table 2). Therefore,  $T_r$  was obtained through stomatal adjustment, indicating that no plasmolysis

occurred in the cells after phenolic acid treatments, and the leaves had normal moisture evaporation function. *WUE* is affected by both  $P_N$  and  $T_r$  (Nijs et al. 1997); therefore, any factors affecting  $P_N$  and  $T_r$  could also affect *WUE*. In this study,  $P_N$  was restrained by non-stomatal factors, while  $T_r$  was affected by stomatal factors; therefore, *WUE* was affected by phenolic acids via both stomatal and non-stomatal factors. Moreover, *WUE* was negatively related to  $G_s$ ; therefore, we expected that the stomatal factor might have a major effect on *WUE*.

Non-stomatal factors resulted in a decline of photosynthetic intensity, possibly due to reduced photosynthetic activities in the mesophyll cells. The might have been due to fact that the fixed photosynthetic enzymes were affected. For example, the photosynthetic rates of leaves at different nitrogen nutrition statuses are closely related to the activity of ribulose diphosphate carboxylase (Xu 2002). Nitrogen is a component substance of all enzymes in plants. Therefore, study of the effects of phenolic acids on nitrogen is important in solving the problems discussed above with the continuous cropping of *Populus × euramericana*.

### The effects of phenolic acid stress on assimilation enzymes

#### *The effects of phenolic acid stress on NR activities*

Plants absorb ammonium salts from the soil, and these are used for composing amino acids. However, if nitrates are absorbed, they can only be used through nitrogen metabolism (Liu et al. 2007; Brandao and Sodek 2009). In general, under field conditions,  $\text{NO}_3^-$  is the primary form absorbed by plants. When  $\text{NO}_3^-$  enters plant cells, it is first reduced to nitrite ( $\text{NO}_2^-$ ) by NR and then to  $\text{NH}_4^+$  by NiR. The activity level of NiR is much higher than that of NR in plants (Nigel et al. 1992; Wei et al. 2008). Our results confirmed these patterns. Therefore, NiR was not the rate-limiting enzyme that influenced the reduction of nitrate in the leaves. NR is a type of inducible enzyme, which is also known as an adaptive enzyme. Inducible enzymes are not from plants but are produced by the inducement from some specific foreign substances, such as the substrate (Meng and Gao 2010). NR is a rate-limiting enzyme that controls the

inorganic assimilation of  $\text{NO}_3^-$  (Campbell 1988). Chaffei et al. (2004) reported that Cd could reduce the activity of NR in tomato (*Lycopersicon esculentum*). Gouia et al. (2003) reported that Cd could reduce NR activity in broad bean (*Phaseolus vulgaris* L. cv. Morgane) seedlings. We found that phenolic acids inhibited the activity of NR, and the inhibiting effect increased with the concentration of phenolic acid. Compared to the control group, the activity of NR in T1 was reduced by 24.2%, while that of NR in T2 was reduced by 71.8% (Table 3). The differences between treatments were significant. Therefore, phenolic acids affected the conversion of  $\text{NO}_3^-$  to  $\text{NH}_4^+$ . NR is a kind of enzyme containing a mercapto group, which can combine with heavy metals to inhibit its activity (Chen et al. 1998; Chang et al. 2005; Chang and Ma 2007). However, further study is needed to evaluate the mechanisms that enable phenolic acids to reduce NR activity.

#### *The effects of phenolic acid stress on the activities of GS and GOGAT*

GS and GOGAT are the key enzymes for nitrogen metabolism (Zhao et al. 1998). Their activities levels directly influence the nitrogen assimilation of plants (Wang et al. 2005; Xiong et al. 2011). The cyclic reaction in which GS and GOGAT are incorporated into the coupling is the main pathway of ammonia assimilation in advanced plants (Singaram and Kamalakumari 1999). In the assimilation of  $\text{NH}_4^+$ , it first combines with glutamic acid to form glutamine (Gln) under the catalysis of GS. Gln then combines with  $\alpha$ -ketoglutaric acid to form glutamic acid under the catalysis of GS (Mo et al. 2001; Meng and Gao 2010).

The activities of GOGAT and GS significantly declined with increasing phenolic acid concentration. The activities of GS in T1 and T2 were reduced by 11.9 and 42.5%, respectively, compared with the control group. The activities of GOGAT in T1 and T2 were reduced by 20.0 and 41.6%, respectively, and the values of T2 (GOGAT and GS) were almost half of those in the control group. Therefore, GOGAT was more susceptible to phenolic acids. Lin et al. (2000) found that the activities of GS and GOGAT declined with increasing NaCl concentration. Our results were similar to those reported by Lin et al. (ibid.)

**Table 2** The responses of *Populus × euramericana* physiological parameters to the treatments under fixed light intensity

Treatment	$P_N$	$C_i$	$T_r$	WUE	$L_s$	$G_s$
CK	20.57 ± 0.896 <sup>a</sup>	203.8 ± 3.45 <sup>c</sup>	3.47 ± 0.315 <sup>a</sup>	5.96 ± 0.408 <sup>b</sup>	0.46 ± 0.009 <sup>a</sup>	554.7 ± 50.43 <sup>a</sup>
T1	18.23 ± 0.878 <sup>b</sup>	226.7 ± 3.62 <sup>b</sup>	2.56 ± 0.296 <sup>b</sup>	7.20 ± 0.555 <sup>a</sup>	0.40 ± 0.008 <sup>b</sup>	457.5 ± 39.62 <sup>b</sup>
T2	15.86 ± 0.896 <sup>c</sup>	243.5 ± 4.98 <sup>a</sup>	2.55 ± 0.313 <sup>b</sup>	6.25 ± 0.501 <sup>b</sup>	0.36 ± 0.013 <sup>c</sup>	437.1 ± 34.69 <sup>b</sup>

Means in the columns within the different concentrations of phenolic acids followed by different letters are significantly different (mean ± SD, LSD-test,  $P < 0.05$ )

**Table 3** The effects of phenolic acid stress on assimilation enzymes

Treatment	NR ( $\mu\text{g g}^{-1}$ FW $\text{h}^{-1}$ )	GS (NADH $\mu\text{mol g}^{-1}$ FW $\text{min}^{-1}$ )	GOGAT ( $\mu\text{mol g}^{-1}$ FW $\text{min}^{-1}$ )
CK	48.52 $\pm$ 1.34 <sup>a</sup>	1.36 $\pm$ 0.030 <sup>a</sup>	0.71 $\pm$ 0.023 <sup>a</sup>
T1	36.78 $\pm$ 1.64 <sup>b</sup>	1.20 $\pm$ 0.034 <sup>b</sup>	0.56 $\pm$ 0.046 <sup>b</sup>
T2	13.70 $\pm$ 2.06 <sup>c</sup>	0.78 $\pm$ 0.041 <sup>c</sup>	0.41 $\pm$ 0.039 <sup>c</sup>

Means in columns within the different concentrations of phenolic acid followed by different letters are significantly different (mean  $\pm$  SD, LSD-test,  $P < 0.05$ )

**Table 4** The effects of phenolic acid stress on the contents of  $\text{NO}_3^-$ -N,  $\text{NH}_4^+$ -N and total N

Treatment	$\text{NO}_3^-$ -N concentration ( $\text{mg g}^{-1}$ DW)	$\text{NH}_4^+$ -N concentration ( $\text{mg g}^{-1}$ DW)	Total N ( $\text{mg g}^{-1}$ DW)
CK	1.22 $\pm$ 0.025 <sup>d</sup>	0.35 $\pm$ 0.031 <sup>a</sup>	35.17 $\pm$ 0.404 <sup>a</sup>
T1	1.11 $\pm$ 0.025 <sup>a</sup>	0.27 $\pm$ 0.031 <sup>b</sup>	34.97 $\pm$ 0.306 <sup>a</sup>
T2	1.03 $\pm$ 0.060 <sup>c</sup>	0.19 $\pm$ 0.038 <sup>c</sup>	33.60 $\pm$ 0.100 <sup>b</sup>

Means in columns within the different concentrations of phenol followed by different letters are significantly different (mean  $\pm$  SD, LSD-test,  $P < 0.05$ )

However, whether the mechanisms of salt stress and phenolic acid stress on these two kinds of enzymes were similar is not clear and needs further study.

Overall, the nitrogen cycle was affected by phenolic acids. Phenolic acids affected both processes in which  $\text{NO}_3^-$  is converted to  $\text{NO}_2^-$  and  $\text{NH}_4^+$  is converted to Gln. However, a low concentration of phenolic acids mainly affected the transformation process of  $\text{NO}_3^-$  to  $\text{NO}_2^-$ , while a high concentration affected both processes in which  $\text{NO}_3^-$  was converted to  $\text{NO}_2^-$  and  $\text{NH}_4^+$  was converted to Gln.

#### *The effects of phenolic acid stress on the contents of $\text{NO}_3^-$ -N, $\text{NH}_4^+$ -N, and total N*

With increasing concentration of phenolic acid, the contents of total nitrogen, ammonium nitrogen and nitrate nitrogen significantly decreased (Table 4). The content of nitrate nitrogen significantly correlated with the activity of NR. The results were consistent with other findings; for example, Lu and Chen (2011) found that the activity of NR in the leaves of *Chinese chive* increased with increasing  $\text{NO}_3^-$ -N content. Chen et al. (2004) reported that  $\text{NO}_3^-$ -N induction significantly increased NR activity in soybean cultivars. Gao et al. (1989) found that nitrate content of spinach and cabbage increased with increasing NR activity in the leaves. Therefore, we hypothesized that the NR activity of *Populus × euramericana* leaves was influenced by  $\text{NO}_3^-$ -N content. For  $\text{NH}_4^+$ -N, it was influenced by both the activity of the enzyme and  $\text{NH}_4^+$  absorptive capacity. The main reason for the decrease in  $\text{NH}_4^+$ -N content in the T1 treatment was the decrease in  $\text{NH}_4^+$  absorption, while the activity of NiR was much higher than that of NR (Nigel et al. 1992). The reason for the decrease

in  $\text{NH}_4^+$ -N content in the T2 treatment was the activity of NR and the  $\text{NH}_4^+$  absorptive capacity. Although glutamate dehydrogenase (GDH) can degrade  $\text{NH}_4^+$ , it has a lower affinity for  $\text{NH}_3$ , and  $K_m$  of GDH was at a  $10^{-3}$  mol  $\text{L}^{-1}$  level; therefore, it could be negligible, as reported by Wei et al. (2008) and Meng and Gao (2010). However, whether the decline of absorbability of  $\text{NH}_4^+$  by *Populus × euramericana* was due to the effect of phenolic acids on the  $\text{NH}_4^+$  carrier protein needs further study.

Overall, photosynthesis of the plants was positively related to the activities of GOGAT and GS and the contents of  $\text{NO}_3^-$ -N,  $\text{NH}_4^+$ -N and total N ( $P < 0.05$ ). The decrease in photosynthesis was affected by non-stomatal factors. The reasons that NR, GS, and GOGAT resulted in decreased photosynthesis needs further investigation because nitrogen is one component of all enzymes in plants, including the enzymes related to the photosynthetic non-stomatal factors. NR, NiR, GS and GOGAT are base enzymes that can transform inorganic nitrogen directly into amino acids (Tischner 2000; Meng and Gao 2010). The activities of these enzymes were directly affected by phenolic acids, which could indirectly affect the nitrogen contents of the plants and thereby impair photosynthesis. Therefore, the absorption and utilization of nitrogen in the continuous cropping of poplar needs to be improved.

## Conclusions

Phenolic acids can significantly impair photosynthesis due to limitations exerted by non-stomatal factors.  $T_r$  also significantly decreased with the increase in the concentration of phenolic acids, but the concentration had no impact on water evaporation in *Populus × euramericana*.

The nitrogen metabolism process was influenced by phenolic acids. The activities of NR, GS and GOGAT significantly decreased with the increase in the concentration of phenolic acid, and the inhibition of nitrogen metabolism increased with the concentration of phenolic acid. A low concentration of phenolic acids mainly affected the transformation process of  $\text{NO}_3^-$  to  $\text{NO}_2^-$ , while a high concentration of phenolic acids affected both processes in which  $\text{NO}_3^-$  was converted to  $\text{NO}_2^-$  and  $\text{NH}_4^+$  was converted to Gln.

The content of  $\text{NO}_3^-$ -N was significantly positively correlated with the activity of NR. The contents of  $\text{NH}_4^+$ -N and total nitrogen were reduced with increasing concentration of phenolic acids.

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