RESEARCH PAPER

N-fertilization has different effects on the growth, carbon and nitrogen physiology, and wood properties of slow- and fast-growing *Populus* species

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Abstract

To investigate how N-fertilization affects the growth, carbon and nitrogen (N) physiology, and wood properties of poplars with contrasting growth characteristics, slow-growing (*Populus* *populus*, Pp) and fast-growing (*P. alba* × *P. glandulosa*, Pg) poplar saplings were exposed to different N levels. Above-ground biomass, leaf area, photosynthetic rates (A), instantaneous photosynthetic nitrogen use efficiency (*PNUE*), chlorophyll and foliar sugar concentrations were higher in Pg than in Pp. Foliar nitrate reductase (NR) activities and root glutamate synthase (GOGAT) activities were higher in Pg than in Pp as were the N amount and *NUE* of new shoots. Lignin contents and calorific values of Pg wood were less than that of Pp wood. N-fertilization reduced root biomass of Pg more than of Pp, but increased leaf biomass, leaf area, A, and *PNUE* of Pg more than of Pp. Among 13 genes involved in the transport of ammonium or nitrate or in N assimilation, transcripts showed more pronounced changes to N-fertilization in Pg than in Pp. Increases in NR activities and N contents due to N-fertilization were larger in Pg than in Pp. In both species, N-fertilization resulted in lower calorific values as well as shorter and wider vessel elements/fibres. These results suggest that growth, carbon and N physiology, and wood properties are more sensitive to increasing N availability in fast-growing poplars than in slow-growing ones, which is probably due to prioritized resource allocation to the leaves and accelerated N physiological processes in fast-growing poplars under higher N levels.

Key words: Amino acids, ammonium transporter, bioenergy, carbohydrates, gene expression, nitrate transporter.

Introduction

Poplar plantations in a short rotation coppicing system have great potential as a bioenergy resource (*Luo and Polle*, 2009). Most poplar plantations grow on marginal lands where N availability is limited (*Finzi et al.*, 2007). Thus, N-fertilization is crucial to ensure high biomass production. Previous studies indicate that high N levels stimulate tree growth and biomass production
(Luo et al., 2006), mainly because N-fertilization induces higher photosynthetic rates, higher leaf chlorophyll concentrations, larger leaves, and greater leaf area (Cooke et al., 2005). High N supply also enhances above-ground biomass production, but inhibits root growth, leading to decreases in the root:shoot ratio (Novaes et al., 2009).

Ammonium (NH$_4^+$) and nitrate (NO$_3^-$) are the two major inorganic N sources for plants. Although abiotic (e.g. soil pH, moisture) and biotic (e.g. mycorrhizal fungi) factors affect inorganic N absorption by plants (Rennenberg et al., 2010; Luo et al., 2011), the uptake of NH$_4^+$ and NO$_3^-$ by woody plants is largely determined by their specific transporters (Rennenberg et al., 2010). The genome of Populus trichocarpa contains 14 putative ammonium transporters (AMTs) (Tuskan et al., 2006; Couturier et al., 2007). By contrast, full family members and their functions of nitrate transporters (NRTs) have not been investigated in Populus species. As a hint, the Arabidopsis genome contains NRT1, NRT2, and NRT3 subfamilies and some members such as AtNRT1;1, AtNRT1;2, AtNRT2;1, and AtNRT3;1 have been functionally characterized (Tsay et al., 2007; Kotur et al., 2012). Among the various putative inorganic N transporters, members of the ammonium transporter family (AMT1;2, AMT1;6, and AMT2;1) and nitrate transporter family (NRT1;1, NRT1;2, NRT2;4B, NRT2;4C, NRT3;1A, NRT3;1B, and NRT3;1C) might play crucial roles in the uptake of NH$_4^+$ and NO$_3^-$ by Populus species (Dluzniewska et al., 2007; Ehling et al., 2007; Plett et al., 2010; Rennenberg et al., 2010). A few studies have indicated that the above-mentioned AMTs are highly expressed in poplar roots/leaves and that their transcript levels respond to the internal N status of plants and external N availability (Selle et al., 2005; Couturier et al., 2007; Ehling et al., 2007; Rennenberg et al., 2010). By contrast, no information is currently available about the expression patterns of NRTs in Populus species.

After uptake, NH$_4^+$ can be directly assimilated into amino acids, whereas NO$_3^-$ needs to be reduced to NH$_4^+$ involving nitrate reductase (NR) before incorporation into amino acids (Nunes-Nesi et al., 2010). NR is a critical enzyme involved in the NO$_3^-$ assimilation pathway and is substrate inducible in poplar (Dluzniewska et al., 2007). After direct uptake or conversion from NO$_3^-$, NH$_4^+$ is assimilated to glutamine and glutamate via a pathway in which the enzyme glutamate synthase (GOGAT) plays an essential role (Dluzniewska et al., 2007). The products of the GOGAT pathway are required for biosynthesis of other nitrogenous compounds (e.g. amino acids, proteins, chlorophylls, and secondary metabolites) (Nunes-Nesi et al., 2010). Total N and amino acid concentrations in plants are frequently found to be elevated in response to higher N supply (Luo et al., 2008; Rennenberg et al., 2010), but the influence of N-fertilization on NUE remains controversial (Good et al., 2004; Finzi et al., 2007).

However, limited information is available about how slow- and fast-growing poplar species respond to different N supply. One possibility is that slow-growing poplars will accumulate N if increased N cannot be used to stimulate growth, thus, decreasing their nitrogen use efficiency (NUE).

With regard to bioenergy resources, not only the quantity but also the quality of plant biomass is of interest because biomass gains may be negatively offset by diminished quality (Canam and Campbell, 2009). This is particularly important for woody biomass because wood quality in terms of chemical and anatomical properties may substantially affect the end use of this material. For instance, both the lignin contents and the composition of syringyl and guaiacyl monomers can influence the suitability of wood for bioethanol production (Studer et al., 2011). N-fertilization can alter the lignin contents and composition (Pitre et al., 2007b; Luo and Polle, 2009). The caloric value of wood, which is determined by its chemical composition (Zhou et al., 2011), is also responsive to N addition (Luo and Polle, 2009). In addition, the anatomical properties of wood (e.g. the dimensions of both vessel elements and fibre cells) are sensitive to N availability (Luo et al., 2005; Pitre et al., 2007a; Hacke et al., 2010). Few studies have addressed the impact of N supply on wood properties in slow- and fast-growing poplar species.

Little is known about the NUE of poplar species with different growth characteristics or how the trees balance growth and carbon physiology in response to increasing N availability. Populus popularis is a slow-growing clone which is often found on nutrient-deficient soils, whereas P. alba×P. glandulosa is a fast-growing species which generally grows on relatively fertile soils (Cao et al., 2012). Both of these poplar species may be selected as woody bioenergy crops. To gain insights into the physiological responses of poplars to N, Populus popularis and P. alba×P. glandulosa were used to address the following questions: (i) How does N availability in soil affect the growth and carbon physiology of slow- and fast-growing poplars? (ii) Do slow- and fast-growing poplars differ in N assimilation and NUE? (iii) Are there N-induced differences in wood properties between slow- and fast-growing poplars?

### Materials and methods

**Plant materials and nitrogen treatment**

Cuttings (c. 15 cm in length, 2 cm in diameter, 1-year-old stem) of Populus popularis (Pp) and P. alba×P. glandulosa (Pg) were obtained from a tree-breeding programme (Cao et al., 2012). The cuttings were rooted and planted in pots (10 L) filled with sandy soil, providing with modified Long-Ashton (LA) nutrient solution (Dluzniewska et al., 2007). After cultivation for 8 weeks in a greenhouse, uniform saplings were selected, transferred to a climate chamber (light intensity, 250 µmol m$^{-2}$ s$^{-1}$, 16/8 h day/night; day/night temperature, 25/20 °C; relative humidity, 75%) and grown for 2 weeks. Plants were carefully irrigated on the morning of every third day with 20 ml LA nutrient solution (Ehling et al., 2007) and daily in the evening with 20 ml distilled water avoiding runoff. Before N treatment, six plants from each species were randomly harvested to determine diameter, height, and biomass. For N treatment, 18 plants from each species were divided into three groups (six plants in each group) and supplied with LA nutrient solution containing, in addition, 0, 5 or 10 mM NH$_4$NO$_3$, respectively. At the beginning of the N treatment, the apex of each plant was marked so that the shoots formed during the N treatment could be distinguished. The N treatment lasted for 7 weeks before harvest.

**Analysis of growth and gas exchange**

To analyse the growth characteristics of poplar plants, the height and basal diameter of the stem of each plant were measured regularly.
A slide caliper was used to determine the basal diameter of each plant 3 cm above the soil surface.

Before harvest, gas exchange of three mature leaves (leaf plastochron index (LP) = 8–10) was determined for each plant. Net photosynthetic rates (A), stomatal conductance (gs), and transpiration rates (E) were determined with a portable photosynthesis system (Li-Cor-6400, Li-Cor Inc, Lincoln, Nebraska, USA) and an attached LED light source (6400–02) as described by He et al. (2011). The instantaneous photosynthetic N use efficiency (PNUE) was calculated as the net CO₂ assimilation per unit of N and time (µmol CO₂ mg⁻¹ N s⁻¹) based on A, the specific leaf area, and the N concentrations of leaves formed during the N treatment (see below).

Harvesting

For each harvested plant, the shoot segment formed during the N treatment (i.e. the shoot above the mark) and the segment below the mark were separated into wood, bark, and leaves. The roots of each plant were also harvested and the length and fresh weight were recorded. Leaves formed during the N treatment were collected to analyse leaf area and specific leaf area as described by Cao et al. (2012). Subsequently, the samples were wrapped with tinfoil and immediately frozen in liquid N. The frozen root, wood, bark, and leaf samples were ground into fine powder in liquid N with a mortar and pestle and stored at −80 °C for further analysis. Frozen powder (c. 50 mg) from each tissue was dried at 60 °C to determine the fresh-to-dry-mass ratio which was used to calculate the dry weight of each tissue. Subsamples from leaves and stem wood formed during the N treatment period were also collected for microscopic and calorific analysis.

Analysis of photosynthetic pigments, soluble sugars, and sugar alcohols

The chlorophyll and carotenoid contents of leaves formed during the N treatment were analysed spectrophotometrically according to the method of He et al. (2011).

Fine powder (c. 50 mg) of fresh roots and leaves formed during the N treatment was extracted with 500 µl of extraction solution (methanol:chloroform:water, 12:5:3, by vol.). Subsequently, soluble sugars and sugar alcohols were determined by gas chromatography-mass spectrometry (GC-MS) as described previously (Luo et al., 2009a,b).

Analysis of transcript levels of representative genes involved in ammonium/nitrate transport or N assimilation

The frozen powder of roots or leaves formed during the N treatment from two plants (c. 500 mg from each plant) in each treatment was combined for total RNA extraction. Total RNA of the tissue was isolated and purified with a plant RNA extraction kit (R6827, Omega). Net photosynthetic rates (A), stomatal conductance (gs), and transpiration rates (E) were determined with a portable photosynthesis system (Li-Cor-6400, Li-Cor Inc, Lincoln, Nebraska, USA) and an attached LED light source (6400–02) as described by He et al. (2011). The instantaneous photosynthetic N use efficiency (PNUE) was calculated as the net CO₂ assimilation per unit of N and time (µmol CO₂ mg⁻¹ N s⁻¹) based on A, the specific leaf area, and the N concentrations of leaves formed during the N treatment (see below).

Analysis of nitrate reductase (NR) and glutamate synthase (GOGAT) activities

NR and GOGAT activities were determined in roots and leaves formed during the N treatment based on the methods of Ehling et al. (2007) and Lin and Kao (1996), respectively.

Determination of amino acids

Free amino acids were quantified in leaves formed during the N treatment, but amino acids could not be measured in roots due to the shortage of material. The extraction of free amino acids from leaves was adapted after the method of Maro et al. (2011). About 50 µl of the extract was analysed by an Amino Acid Analyzer (121MB, Beckman, California, USA).

Determination of N concentrations, N amounts, and N use efficiency (NUE)

To quantify N concentrations in different tissues of the two poplar species, fine powder (c. 0.5 mg DW) from roots and shoots (wood, bark, and leaves) below and above the mark for N treatment was weighed into tin capsules and analysed by an element analyser (Euro EA 3000, Elemental Analyzer, Milan, Italy) as described by Luo et al. (2011). The total N amount of each tissue was estimated by multiplying the N concentration in each tissue by the biomass of that tissue.

Nitrogen use efficiency (NUE) was defined according to Finzi et al. (2007): NUE = biomass/N uptake, where biomass and N uptake are measured in units of dry matter (g DW) production or N uptake (equivalent to N amount in plant) from soil. The NUE of new shoots was calculated based on the dry weight of new shoots formed during the N treatment and the total N amount of the new shoots. Similarly, NUEs were estimated in shoots (above-ground plant part) formed during the whole experimental phase and in plants (roots and above-ground plant part).

Determination of lignin and calorific values in wood

The Klasson lignin content was determined in stem-wood formed during the N treatment according to the method of Luo et al. (2008). The calorific value of wood formed during the N treatment was determined based on the method of Luo and Polle (2009).

Analysis of wood anatomy

Wood samples (c. 1 cm above the mark) formed during the N treatment were used for anatomical analysis. For scanning electron microscopy (SEM), wood samples were prepared according to the method of Pitre et al. (2007a). The SEM observations were made at 13 kV using a scanning electron microscope (JSM-6360LV, Japan Electron Optics Laboratory Co. Ltd, Tokyo, Japan). The characteristics of vessel elements and fibres in wood formed during the N treatment were analysed based on the method of Luo et al. (2005) with minor modifications according to Cao et al. (2012).

Statistical analysis

Statistical tests were performed with Statgraphics (STN, St Louis, MO, USA). The growth variables of the slow- and fast-growing poplar species were compared by one-way ANOVA. To examine the effects of species and N treatment on experimental variables, all variables were analysed by two-way ANOVAs. Data were tested for normality prior to statistical analysis. Differences between means were considered significant when the P-value of the ANOVA F-test was less than 0.05. The Ct values obtained after quantitative PCR were normalized and the fold changes of transcripts were calculated as suggested by Luo et al. (2009a). For principal component analysis (PCA), data were standardized and subsequently computed by the command pcomp() in R (http://www.r-project.org/).
Results and discussion

Prioritized resource allocation to leaves in Pg but not in Pp leads to differences in growth and carbon physiology under N-fertilization

At a given N supply, there were inherent growth differences that resulted in about 2-fold higher biomass in Pg than in Pp (see Supplementary Fig. S2 at JXB online). Growth differences were maintained when poplar plants were fertilized additionally with either 5 or 10 mM NH4NO3 (see Supplementary Fig. S3 and Supplementary Table S2 at JXB online). Growth differences may be associated with the stimulation of photosynthesis and carbohydrate metabolism. Thus, both leaf gas exchange and soluble sugar and sugar alcohol concentrations were measured (see Supplementary Fig. S4 and Supplementary Table S2 at JXB online). Principal component analysis (PCA) was performed on growth, photosynthesis related parameters and carbohydrates (Fig. 1; see Supplementary Table S3 at JXB online). Leaf area, leaf dry weight, and PNUEi were the three most important contributors to PC1, whereas trehalose and sucrose in roots and manitol in leaves were the strongest contributors to PC2 (see Supplementary Table S3 at JXB online). The PCA clearly separated the slow-growing Pp from the fast-growing Pg (Fig. 1). These data indicate that Pg has a stronger responsiveness to N-fertilization than Pp has.

The growth and photosynthetic characteristics of Pp and Pg in this experiment were consistent with results of Pp and Pg from our previous field study (Cao et al., 2012), indicating that the growth of Pp and Pg is stable across a variety of experimental conditions. The leaf chlorophyll contents of Pg were higher than that of Pp, resulting in increases in A, total carbohydrates, and sugar alcohols (see Supplementary Fig. S4 at JXB online). These results indicate that higher A in fast-growing poplars can result in a higher supply of carbon assimilates for plants to develop more and larger leaves, leading to higher leaf biomass and leaf area. Actually, the atmospheric CO2 fixation capacity of a plant depends on whole plant A and leaf area (Cooke et al., 2005; Zhao et al., 2011; Zheng et al., 2011). Current data indicate that higher leaf biomass, A, PNUEi, and larger leaf areas are critical traits for Pg to be a highly productive bioenergy crop. Previous study showed a positive correlation between stem wood formation and whole-plant leaf area (Cooke et al., 2005), but this was not found in the current study. The time scale of this experiment was limited. Therefore, the possibility cannot be excluded that the initial trade-off between leaf and wood biomass will result in increased wood formation in the long run.

The growth data suggest that slow- and fast-growing poplar species have different strategies for biomass allocation under increasing N availability. In Pg, resource allocation was prioritized to leaf production. Higher N supply might have caused an expanded zone of leaf maturation where elevated cell division and/or cell expansion can occur (Cooke et al., 2005). Such processes were apparently more efficient in Pg than in Pp. Although higher N levels resulted in increased photosynthetic rates in both poplar species, the decreased foliar sucrose concentrations suggest that N-fertilization may have resulted in an accelerated export of sucrose to sink tissues and/or conversion to other carbohydrate forms. Consistent with the latter assumption, higher concentrations of fructose, inositol, galactose, and manitol were detected in leaves of fertilized Pg (see Supplementary Fig. S4 at JXB online).
Nitrate uptake and mobilization in plants is mediated by NRTs (Tsay et al., 2007; Wang et al., 2012). Using ‘nitrate transporter’ as a search term in the genome database of *P. trichocarpa* (*Populus trichocarpa* v2.2, http://www.phytozome.org/results.php), 273 hits were found, indicating that the poplar genome might contain a large family of NRTs. However, no information is currently available about the functional characteristics of any NRT in poplar. In *Arabidopsis*, NRT1;1, NRT2;2, and NRT3 contain 53, 7, and 2 subfamily members, respectively, and some of these members have been functionally characterized (Tsay et al., 2007; Wang et al., 2012). In *Arabidopsis*, AtNRT1;1 (At1G12110), AtNRT1;2 (At1G69850), AtNRT2;1 (At1G08090), and AtNRT2;4 (At5G60770) are the closest homologues, respectively, to NRT1;1, NRT1;2, NRT2;4C, and NRT2;4B of poplar species in this study (see Supplementary Fig. S1 at JXB online). In addition, AtNRT3;1 (At5G50200) is the closest homologue to NRT3;1A, NRT3;1B, and NRT3;1C of our studied poplars (see Supplementary Fig. S1 at JXB online). Consistent with the induction of AtNRT1;1 transcript in roots by nitrate (Wang et al., 2012, and references therein), increases in NRT1;1 mRNAs were detected in roots of Pp and Pg after adding 5 or 10 mM NH4NO3 (Fig. 2). In addition to root expression, AtNRT1;1 is expressed in leaf guard cells, promoting stomatal opening in a nitrate-dependent manner (Guo et al., 2003). NRT1;1 was also expressed in leaves of Pp and Pg, but the lack of correlation between NRT1;1 expression and 6177

**Nitrogen uptake and assimilation in Pg compared with Pp cause differences in N physiology under N-fertilization**

Nitrogen uptake and assimilation are essential growth-promoting processes in plants. Based on previous studies in *Populus* species (Selle et al., 2005; Couturier et al., 2007; Ehling et al., 2007; Plett et al., 2010) and our preliminary experiments, genes encoding essential members of transporter families for ammonium (*AMT1;2, AMT1;6, and AMT2;1*) and for nitrate (*NRT1;1, NRT1;2, NRT2;4B, NRT2;4C, NRT3;1A, NRT3;1B, and NRT3;1C*) expressed in both roots and leaves of Pp and Pg were selected for transcript analysis by quantitative RT-PCR. In addition, genes encoding nitrate reductase (NR) and glutamate synthase (*Fd-GOGAT, NADH-GOGAT*) were included since these genes play crucial roles in N assimilation in poplar plants (Dluzniewska et al., 2007). Transcript analysis and PCA showed that mRNA levels of the genes examined in roots and leaves were different between Pp and Pg (Fig. 2; see Supplementary Table S4 at JXB online). For all the transcript levels analysed, PC1 and PC2 accounted for 37% and 23% of the variation, respectively (see Supplementary Table S4 at JXB online). Changes in transcript levels of NRT2;4C and NRT3;1A were the two strongest contributing variables to PC1, whereas AMT1;6 and NR were the strongest contributors to PC2 (see Supplementary Table S4 at JXB online). Transcripts of most genes changed in response to the addition of 5 and/or 10 mM NH4NO3 and, in most cases, transcript levels of the genes examined in Pg were more responsive to N addition than in Pp (Fig. 2; see Supplementary Table S4 at JXB online). These results indicate that fast-growing Pg takes up more N and/or needs to utilize the available N more efficiently compared with slow-growing Pp.

Induction of *AMT1;2* transcripts in roots and leaves of Pp and Pg under a higher N supply is in agreement with previous studies (Selle et al., 2005; Couturier et al., 2007), indicating that *AMT1;2* expression might be ammonium-inducible and that *AMT1;2* functions in ammonium transport in *Populus* species. *AMT1;6* transcripts gradually decreased in leaves of *P. × canescens* during the N starvation period, which was closely correlated with decreases in the asparagine pools (Couturier et al., 2007). Consistently, *AMT1;6* transcripts were induced in roots of Pp and in both roots and leaves of Pg under the higher N supply. This also corresponds well to increases in aspartic acid (a close derivative of asparagine) pools (Fig. 2; see Supplementary Fig. S5 at JXB online). *AMT2;1* was highly expressed in leaves of *P. × canescens* (Couturier et al., 2007), but no information is available on the response of *AMT2;1* transcript to external N changes in poplar plants. The induction of *AMT2;1* transcripts in the roots of Pp and in both roots and leaves of Pg under the higher N supply suggests that *AMT2;1* may play a significant role in ammonium transport in roots and leaves of *Populus* species. These results indicate that the three *AMT* s analysed in this study are involved in ammonium uptake and mobilization in *Populus* species under the higher ammonium supply.

**Accelerated physiological processes of N uptake and assimilation in Pg compared with Pp cause differences in N physiology under N-fertilization**

JXB online). Taken together, these results indicate that prioritized resource allocation to leaves in Pg but not in Pp may lead to growth and carbon physiology differences under N-fertilization. This is a crucial trait for a highly productive bioenergy crop.
Fig. 2. Fold changes of transcripts involved in ammonium (A), nitrate (B, C), transport or N assimilation (D) in roots and leaves of slow-growing *P. popularis* (Pp) and fast-growing *P. alba* × *P. glandulosa* (Pg) exposed to 0, 5 or 10 mM NH₄NO₃ (N). Bars indicate fold changes ±SE (n=3). For each gene, the expression level was set to 1 in roots of Pp exposed to 0 mM NH₄NO₃ and, subsequently, fold changes of transcripts were calculated in roots and leaves of both poplar species. The insert in (C) shows induced mRNA levels of NRT3;1B. (This figure is available in colour at *JXB* online.)
also existed between the expression of NRT3;1C and NRT1;2 and between NRT3;1B and Fd-GOGAT (NADH-GOGAT) in both Pp and Pg. These correlations indicate that NRT3;1B and NRT3;1C may play a pivotal role in the regulation of nitrate uptake and N assimilation in poplar plants.

The expression and enzyme activities of NR and GOGAT are dependent on substrate and on the flux of inorganic N into organic compounds, which is crucial in N assimilation in poplars (Dluzniewska et al., 2007). Although no species difference was detected at the transcript levels of NR between Pp and Pg (Fig. 2), higher N induced greater NR activities in Pg roots than in Pp roots (Fig. 3). At the transcript level, the greater responsiveness of NADH-GOGAT to changes in N (Fig. 2) indicates that this isoform may play a more important role than Fd-GOGAT in both Pp and Pg. Higher activities of GOGAT in Pg roots than in Pp roots and suppression of GOGAT activities in Pp leaves but not in Pg leaves by higher N levels were found (Fig. 3). Lack of correlations between transcript abundance of NR/GOGAT and enzyme activity of NR/GOGAT implies that multiple levels of regulation after transcription occur to modulate glutamate synthesis in Pp and Pg under applied N levels. These results suggest that Pg may require higher NR and/or GOGAT activities than Pp does to efficiently utilize absorbed nitrate and/or ammonium.

Both ammonium and nitrate have to be converted into amino acids after their uptake in plants. Thus, amino acid concentrations may be affected by ammonium and/or nitrate supply. In most cases, higher N supply enhanced the total and individual amino acid concentrations in both species and the increases were greater in Pg than in Pp (see Supplementary Fig. S5 at JXB online). Higher amino acid levels in Pg leaves than in Pp leaves indicate that more N in Pg is channelled to the production of precursors of protein biosynthesis to provide basic compounds for rapid biomass production (Nunes-Nesi et al., 2010).

**Fig. 3.** Nitrate reductase (NR, μM NO$_3^-$ h$^{-1}$ mg$^{-1}$ protein) and glutamate synthase (GOGAT, nkat g$^{-1}$ protein) activities in fine roots and leaves of slow-growing $P$. popularis (Pp) and fast-growing $P$. alba × $P$. glandulosa (Pg) exposed to 0, 5 or 10 mM NH$_4$NO$_3$ (N). Bars indicate means ±SE (n=6). Different letters on the bars indicate significant difference. $P$-values of the ANOVAs of species, N treatment and their interaction are indicated: *$P<0.05$; **$P<0.01$; ns, not significant.
Higher total N in roots, greater increases in the total N of bark and leaves, and higher NUE of Pg in comparison with Pp demonstrate differences in N physiology

To investigate how plant N status was affected by differences in growth physiology and responsiveness to N-fertilization, N concentrations and amounts were quantified in Pp and Pg (Fig. 4; see Supplementary Table S5 at JXB online). In the 0 mM NH4NO3 treatment, N concentration was significantly higher in Pg roots than in Pp roots, but similar results were not found in wood, bark, and leaves (Fig. 4A–D). The addition of 5 mM NH4NO3 increased root and bark N concentrations in both species compared with the controls (Fig. 4A) and greater increases in bark N concentrations were found in Pg than in Pp (Fig. 4C). Supply of 10 mM NH4NO3 enhanced N concentrations in the roots of both species and in the bark and leaves of Pg compared with the controls (Fig. 4A, 4C, 4D). There were huge differences in above- and below-ground N partitioning between Pg and Pp (Fig. 4E–H; see Supplementary Table S5 at JXB online). Specifically, Pg showed strong increases in the amounts of N allocated to wood, bark, and leaves in response to the addition of 5 mM NH4NO3 and further increments in response to 10 mM NH4NO3 addition (Fig. 4F–H). Contrast, the amounts of N allocated to wood and bark were not so pronounced in fertilized Pg and the N amount in Pg roots even showed a strong decrease in response to N fertilization (Fig. 4E–H). The amount of N in leaves was much higher in Pg than in Pp (Fig. 4E–H). The greater allocation of N to leaves of fast-growing Pg under higher N levels again demonstrates prioritized resource allocation to leaves of Pg, rendering Pg more effective on N utilization since more N is allocated to the photosynthetic tissue. This contention is supported by higher PNUE in Pg than in Pp under elevated N levels (see Supplementary Table S2 at JXB online).

In unfertilized poplars, NUE was markedly higher in new shoots of Pg than of Pp (Fig. 5A). N-fertilization decreased NUE in new shoots of both poplar species, but under 5 mM NH4NO3 condition, NUE was higher in new shoots of Pg than of Pp (Fig. 5A). Shoot NUE was similar in both poplar species under the control condition (Fig. 5B). The addition of N reduced shoot NUEs of both species, but under the addition of 5 mM NH4NO3, shoot NUE of Pg was higher than of Pp (Fig. 5B). NUE in plants was similar in both species under 0 mM NH4NO3 conditions (Fig. 5C). Higher N supply decreased NUE in plants of Pp by 18–25% and of Pg by 6–15%, respectively, compared with the controls (Fig. 5C).

Although NUE is an important trait that is often evaluated for agricultural crops (Good et al., 2004; Ju et al., 2009), little information is available about the NUE of woody bioenergy crops such as poplars (Garnett et al., 2009). Over-application of N fertilizers to agricultural land leads to serious environmental threats including the production of the greenhouse gas N2O and nitrate leaching to ground water (Good et al., 2004). Therefore, it is extremely important to select poplars with high NUEs. The NUE of both poplar species in this study decreased as the supply of N increased, suggesting that N-fertilization application to poplar stands should be carefully managed. Under 5 mM NH4NO3 addition, the higher NUEs in Pp than in Pg imply that fast-growing poplars are more N economic than slow-growing poplars in terms of biomass production.

N-fertilization induced differences in wood properties of Pp and Pg

The chemical and anatomical properties are important aspects of wood utilization. Therefore, these properties were characterized (Figs 6, 7; see Supplementary Fig. S6 at JXB online). The lignin content of Pg was lower than that of Pp (Fig. 6). Consistently, previous studies suggest that lignin concentrations are lower in fast-growing species than in slow-growing species (Novaes et al., 2009, 2010). Lignin biosynthesis requires a large amount of carbon which becomes structurally fixed and unavailable for other processes (Luo et al., 2006). Our data indicate that lower lignin concentrations in Pg are likely to be due to more carbon being channelled to active metabolites. N-fertilization tended to reduce lignin contents in wood of both species (Fig. 6), which is consistent with previous studies (Novaes et al., 2009; Pitre et al., 2007a, b). Lower lignin contents in the wood of N-fertilized poplars are advantageous for bioethanol production (Studer et al., 2011) and are probably associated with the formation of a G-layer that is almost devoid of lignin (Pitre et al., 2007b). The calorific value was c. 8% lower in Pg wood than in Pp wood (Fig. 6). The 5 mM NH4NO3 addition resulted in a reduction in calorific values by 0.9 kJ g−1 DW in Pp wood and by 1.3 kJ g−1 DW in Pg wood, respectively, compared with the controls (Fig. 6). Furthermore, the addition of 10 mM NH4NO3 led to reduces in calorific values by 1.6 kJ g−1 DW in Pp wood and by 1.9 kJ g−1 DW in Pg wood, respectively, compared with the controls (Fig. 6). The calorific value of wood is positively correlated with lignin contents in wood cell walls (Amthor, 2003). Thus, the lower calorific values may be ascribed to reduction in lignin in Pg wood compared with Pp wood and in N-fertilized poplars compared with unfertilized ones. N-fertilization increased biomass production but had a negative effect on the heating values of Pg and Pp wood. Using biomass and calorific values of wood formed during the N treatment, wood heating values were calculated as 6.7, 15.3, and 14.4 kJ for Pp and 11.5, 14.8, and 13.8 kJ for Pg in the presence of 0, 5, and 10 mM NH4NO3, respectively. These data reveal a substantial trade-off with respect to wood energetic use after fertilization and indicate that 5 mM NH4NO3 addition may result in optimal energy gain in both poplar species.

Transverse sections of wood in both poplar species were viewed with a scanning electron microscope (Fig. 7). These photographs suggest that wall thickness of fibre cells was greater in Pg wood than in Pp wood under N-fertilization conditions, although no differences in wall thickness of fibre cells were observed under the control conditions (Fig. 7). The cell walls of Pp wood were thicker in the N-fertilized treatment than in the control, indicating that higher N supply stimulated biosynthesis of fibre cell walls in Pp wood (Fig. 7A–C). This increase resulted from the formation of a G-layer in the fibre lumina of Pp wood under N addition (Fig. 7B, 7C). A stimulation of G-layer formation under higher N supply has been observed in other poplar species and is associated with N-induced changes in secondary wall chemical composition and gene expression (Pitre et al., 2010). The dimensions of vessel elements and fibre cells were
Fig. 4. N concentrations and N amounts in fine roots (A, E), wood (B, F), bark (C, G), and leaves (D, H) of slow-growing P. popularis (Pp) and fast-growing P. alba × P. glandulosa (Pg) exposed to 0, 5 or 10 mM NH₄NO₃ (N). Bars indicate means ±SE (n=6). Different letters on the bars indicate significant difference. P-values of the ANOVAs of species, N treatment and their interaction are indicated: *P<0.05; **: P<0.01; ***P<0.001; ****P<0.0001; ns, not significant.
characterized in both poplar species (see Supplementary Fig. S6 at JXB online). Under control conditions, vessel elements were wider and longer in Pg wood than in Pp wood. Higher N levels decreased the length but increased the width of vessel elements and fibres of both species. Similar changes in the dimensions of vessel and fibre cells due to N-fertilization were reported in P. ×euroamericana (Luo et al., 2005) and P. trichocarpa × deltoides (Pitre et al., 2007a). One explanation is that the supply of N may induce changes in cell elongation and expansion (Cooke et al., 2003; Pitre et al., 2010; Plavcová et al., 2012).

In conclusion, above-ground biomass, leaf area, A, and PNUEi were higher in fast-growing Pg than in slow-growing Pp. Leaf glucose, fructose, inositol, and galactose concentrations were also higher in Pg than in Pp. In both species, N-fertilization decreased root length and root biomass and increased leaf biomass, leaf area, A, and PNUEi, and these changes were greater for Pg than for Pp. Among 13 genes involved in ammonium or nitrate transport or N assimilation, transcripts of most genes displayed greater responsiveness to N addition in Pg than in Pp. Leaf NR activity and root GOGAT activity were higher in Pg than in Pp as were N amounts and NUEs in new shoots and in shoots. N-fertilization increased NR activities, N concentrations, and N amounts in both species, but the increases were larger in Pg than in Pp. Lignin contents and calorific values were lower in Pg wood than in Pp wood, but the vessel elements were longer and wider in Pg wood than in Pp wood. N-fertilization not only reduced the calorific values of wood in both species, but also shortened and widened vessel elements and fibres. The supply of 5 mM NH₄NO₃ may result in optimal energy gain in both poplar species. These results suggest that growth, carbon, and N physiology, and wood chemical properties are more sensitive to increasing N availability in fast-growing poplars than in slow-growing ones. This is probably due to prioritized resource allocation to leaves and accelerated N physiological processes in the fast-growing species under higher N levels.

Supplementary data

Supplementary data can be found at JXB online.

Supplementary Table S1. Primers used for qRT-PCR.

Supplementary Table S2. Growth and photosynthetic characteristics.

Supplementary Table S3. PCA of growth, photosynthesis and carbohydrates.

Supplementary Table S4. PCA of transcript changes of representative genes.

Supplementary Table S5. N concentrations and amounts.

Supplementary Fig. S1. Alignments of examined genes at the cDNA and amino acid levels.

Supplementary Fig. S2. Growth characteristics of both poplar species before the N treatment.

Supplementary Fig. S3. Root to shoot biomass ratios.

Supplementary Fig. S4. Soluble sugars and sugar alcohols.

Supplementary Fig. S5. Amino acids.

Supplementary Fig. S6. Characteristics of vessel elements and fibres.

Fig. 5. N use efficiency (NUE, g DW g⁻¹ N) in new shoots (A) formed during the N treatment, in shoots (B) formed during the whole experimental phase and in plants (C) of slow-growing P. × euramericana (Pp) and fast-growing P. × t. deltoides (Pg) exposed to 0, 5 or 10 mM NH₄NO₃ (N). Bars indicate means ±SE (n=6). Different letters on the bars indicate significant difference. P-values of the ANOVAs of species, N treatment and their interaction are indicated: *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; ns, not significant.
Responses of two contrasted poplar species to N-fertilization

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**Fig. 6.** Lignin contents and calorific values in stem wood of slow-growing *P. popularis* (Pp) and fast-growing *P. alba × P. glandulosa* (Pg) exposed to 0, 5 or 10 mM NH$_4$NO$_3$ (N). Bars indicate means ±SE (n=6). Different letters on the bars indicate significant difference. *P*-values of the ANOVAs of species, N treatment and their interaction are indicated: *P*<0.05; **P**<0.01; ***P***<0.001; ****P***<0.0001; ns, not significant.

**Fig. 7.** Scanning electronic microscope photographs of secondary xylem from stem wood of slow-growing *P. popularis* (Pp) and fast-growing *P. alba × P. glandulosa* (Pg) exposed to 0, 5 or 10 mM NH$_4$NO$_3$ (N). (A, D) 0 mM; (B, E) 5 mM; (C, F) 10 mM. Representative sections are presented. The magnification is indicated by the scale bars on the panels.
References


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