

Short-term physiological and developmental responses to nitrogen availability in hybrid poplar

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Summary

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Received: 15 December 2004
Accepted: 28 February 2005

- Nitrogen fertilization induces dramatic changes in the growth and development of plants, including forest trees. In this study we examined short-term responses of hybrid poplar, *Populus balsamifera* ssp. *trichocarpa* × *deltoides*, to N fertilization.
- Glasshouse-grown saplings subjected to limiting, intermediate, and luxuriant levels of ammonium nitrate over a 28 d time course demonstrated rapid changes to whole-plant architecture and biomass accumulation. Nitrogen-associated shifts in allocation occurred in temporally distinct stages. Nitrogen availability modulated parameters that affect carbon gain, including light-saturated net photosynthesis and leaf area. These parameters were affected by N-induced changes to leaf maturation and senescence. Leaf area was also affected by N-induced sylleptic branch development.
- Genes encoding vegetative storage proteins and starch biosynthetic enzymes exhibited contrasting patterns of expression under differential N availability. A gene encoding a previously uncharacterized putative pectin methylesterase inhibitor displayed expression patterns comparable to the starch biosynthetic genes.
- The results of this study illustrate the phenotypic plasticity that *P. balsamifera* ssp. *trichocarpa* × *deltoides* exhibits in response to differential N availability.

Key words: bark storage protein, carbon–nitrogen balance, leaf development, nitrogen, pectin methylesterase inhibitor, photosynthesis, sylleptic branching.

New Phytologist (2005) **167**: 41–52

© *New Phytologist* (2005) doi: 10.1111/j.1469-8137.2005.01435.x

Introduction

Nitrogen is an important determinant of plant growth and architecture. Rates of growth, patterns of growth and phenology are all affected by N availability (Dickson, 1989; Geiger *et al.*, 1996). Nitrogen availability is often considered to be a limiting factor in plant productivity, including the productivity of forest trees (Dickson, 1989; Oren *et al.*, 2001). Natural temperate and boreal forests are typically N-limited ecosystems if viewed in the context of plant productivity (Vitousek *et al.*, 1997; Oren *et al.*, 2001). As with agricultural crops, N fertilization of forest trees can improve biomass production (Liu & Dickmann, 1992; Heilman & Xie, 1994; Brockley, 1995; Brown *et al.*, 1996). Nitrogen fertilization is increasingly used to improve harvest yields and reduce rotation age in

managed forest tree plantations, particularly in the context of short-rotation woody crops (Tuskan, 1998; Sedjo, 2001).

At the same time, there is accumulating evidence that human activities are increasing N levels in the environment, including forest ecosystems (Vitousek *et al.*, 1997; Tilman *et al.*, 2001). Fertilizers and animal waste resulting from intensive agricultural practices are leading to increased N – mainly nitrates – in groundwater (Nolan *et al.*, 1997), and are predicted to have particularly strong effects on riparian systems and forestlands proximal to agricultural regions (Vitousek *et al.*, 1997). Nitrogen originating from agricultural activities also enters the atmosphere as volatilized ammonia (Tilman *et al.*, 2001). Human activities such as fossil fuel combustion also produce atmospheric N in the form of NO_x species (Nolan *et al.*, 1997; Vitousek *et al.*, 1997). These atmospheric

N pollutants are subsequently deposited on the landscape over large regional scales (Vitousek *et al.*, 1997; Tilman *et al.*, 2001).

Populus spp. and their hybrids provide a pertinent model system for studying the effects of N availability on forest tree growth and development, as *Populus* is used as an intensively managed woody crop throughout many regions of the world (Stettler *et al.*, 1996; Tuskan, 1998; Dickmann *et al.*, 2002). Despite considerable research on the effect of N availability on production physiology in *Populus* across weekly and monthly time scales (Pregitzer *et al.*, 1990; Liu & Dickmann, 1992; Coleman *et al.*, 1998; Ibrahim *et al.*, 1998; Wait *et al.*, 1998), there is comparatively poor understanding of the short-term sequence of physiological events that condition the whole-tree response to N. Obtaining a better grasp of the timing at which phenotypes appear is requisite to defining relationships between physiological, anatomical and molecular phenotypes.

In an earlier paper we demonstrated that N availability mediates diverse changes in gene expression in hybrid poplar, *Populus balsamifera* ssp. *trichocarpa* × *deltoides* (Cooke *et al.*, 2003), and that these changes occur within relatively short time scales (days). Here we describe results of short-term experiments to identify tissue- and organ-level phenotypes associated with N response in *P. trichocarpa* × *deltoides*, to define their temporal and spatial relationships, and to derive a conceptual model of poplar tree response to N. These findings underscore the phenotypic plasticity of *Populus*, and provide insight into the physiological mechanisms invoked by *Populus* in response to altered N availability.

Materials and Methods

Plant material

Investigations were carried out with *Populus balsamifera* ssp. *trichocarpa* (Torr. and Gray) × *deltoides* (Bartr. ex Marsh) hybrid UCC-1, a commercially deployed clone that originated from the University of Washington breeding programme. Experiments were conducted in glasshouses equipped with cooling/heating units to maintain temperatures between 20 and 35°C. Plants were given natural lighting augmented with full-spectrum fluorescent lighting to provide a 15 h light period. Rooted softwood cuttings were produced in 25 cm² pots under mist. Rooted cuttings were transferred to 11.4 l pots after ≈ 1 month, and fertilized twice weekly with Hocking's complete nutrient solution (Hocking, 1971) containing 5 mM NH₄NO₃ for ≈ 2 months, or until plants had at least 15 leaves with a plastochron index (LPI) greater than zero (Larson & Isebrands, 1971). For UCC-1, LPI 0 denotes a leaf blade that is ≈ 3 cm long and is undergoing laminar expansion.

Plants were 60–75 cm tall at the outset of the experiments. Plants were ranked according to height; each cohort was then divided equally, and individuals of a cohort assigned randomly

between N treatments. A randomized complete block design was used for the experiments. Plants were fertigated to runoff daily with Hocking's complete nutrient solution supplemented with 0, 2 or 50 mM NH₄NO₃ for up to 28 d (Cooke *et al.*, 2003). These treatments are designated 0N, 2N, and 50N, respectively. The volume of fertilizer retained by the soil increased as the biomass of plants increased over the course of the experiments.

Carbon and nitrogen analyses

Total C and N content of tissues was determined as a percentage of dry weight using an NCS 2500 automatic elemental analyser (CE Instruments, ThermoQuest Italia SpA, Milan, Italy). The instrument was calibrated with a pine needle standard (National Institute for Standards and Testing, Gaithersburg, MD, USA).

Photosynthesis measurements

Light-saturated net photosynthesis rate was measured on young (LPI 4) and mature (LPI 9–18) leaves with a portable infrared gas analyser (Li 6400P photosynthesis system, Li-Cor, Lincoln, NE, USA). Mature leaves selected for photosynthesis measurements were greater than LPI 6, generally positioned just below the midpoint of the stem, and lacked visible signs of senescence. At day 0, the mean LPI of leaves chosen for mature leaf photosynthesis measurements was LPI 10. The mean LPI selected for mature leaf photosynthesis measurements of 0N, 2N and 50N treatments at day 7 were LPI 11, 11 and 11, respectively; LPI 12, 13 and 13 at day 14; and LPI 13, 14 and 14 at day 28. Photosynthetic photon flux density was maintained at 1000 μmol m⁻² s⁻¹ (determined to be saturating from preliminary measurements), airflow rate at 500 μmol s⁻¹, and leaf temperature between 27 and 30°C. The chamber CO₂ concentration was maintained at ≈ 370 μmol mol⁻¹. All photosynthetic measurements were taken in the early afternoon.

Chlorophyll determination

Leaf discs from young (LPI 3) and mature (LPI 9–18) leaves were extracted in acetone, and chlorophyll content assayed spectrophotometrically according to Strain *et al.* (1971).

Stomatal measurements

Imprints of young leaves (LPI 2) were prepared by spraying the adaxial surface of a leaf with clear acrylic (Krylon). The hardened transparent acrylic layer was peeled from the leaf surface and mounted on a glass microscope slide. A calibrated micrometer was used to calculate field of view and object dimensions. Immature stomata were round and lacked a defined stomatal pore, whereas mature stomata were longer,

ovate and contained a clearly visible stomatal pore. Given the correlation between stomatal maturity and stomatal length, the number of stomata ≥ 0.02 mm long per mm^2 leaf area was used as an index to estimate the relative proportion of mature stomata. Three fields of view were scored per leaf.

Gene expression analyses

A cetyltrimethylammonium bromide (CTAB) method (Chang *et al.*, 1993) was used to prepare total RNA from shoot tips (up to and including LPI 1); young leaves (LPI 3); mature leaves (LPI 15–16); and main stems exhibiting secondary growth (between LPI 14 and 16) from three to five pooled plants per treatment. Formaldehyde–agarose gel electrophoresis of 10 μg total RNA per sample and RNA blot analyses were carried out as described by Cooke *et al.* (2003). Hybridization and washing were carried out at 65°C using standard protocols (Davis *et al.*, 1991). Northern blot analyses were conducted on two independent experiments with identical results. cDNAs used for Northern hybridizations were (GenBank accession, gene product) *BU791161*, ADP glucose pyrophosphorylase large subunit; *BU791129*, starch synthase; *BU791160*, pectin methylesterase inhibitor-like; *AAA16342*, *win4* vegetative storage protein; *CAA49669*, *bsp* bark storage protein.

The putative pectin methylesterase inhibitor sequence was characterized bioinformatically using BLASTX (Altschul *et al.*, 1997); conserved domain database (CDD) (Marchler-Bauer *et al.*, 2003); and SIGNALP (Bendtsen *et al.*, 2004).

Statistics

Whole-plant physiology data were analysed by general linear model in SAS (SAS Institute, Cary, NC, USA) on log-transformed data. Significant differences between treatment means were determined using multiple comparisons of means ($\alpha = 0.05$).

Results

Nitrogen availability and allocation priority

Plants treated with limiting (0N), intermediate (2N) or luxuriant (50N) levels of ammonium nitrate for up to 28 d demonstrated a dramatic N-induced shift in tree architecture (Fig. 1). At 7 d there were significantly more leaves on 2N and 50N plants compared with 0N plants, and by 28 d the 2N and 50N plants had 50 and 75% more leaves, respectively (Fig. 1b). In contrast, the 0N plants maintained a constant leaf number throughout the experiment. At 14 d, the 50N plants exhibited syllepsis (Fig. 1c,d), which was even more pronounced at 28 d. In contrast, sylleptic branches did not elongate from lateral buds in the 0N plants. By 28 d, 2N and 50N trees were significantly taller than 0N trees (Fig. 1a). This morphometric analysis revealed that N

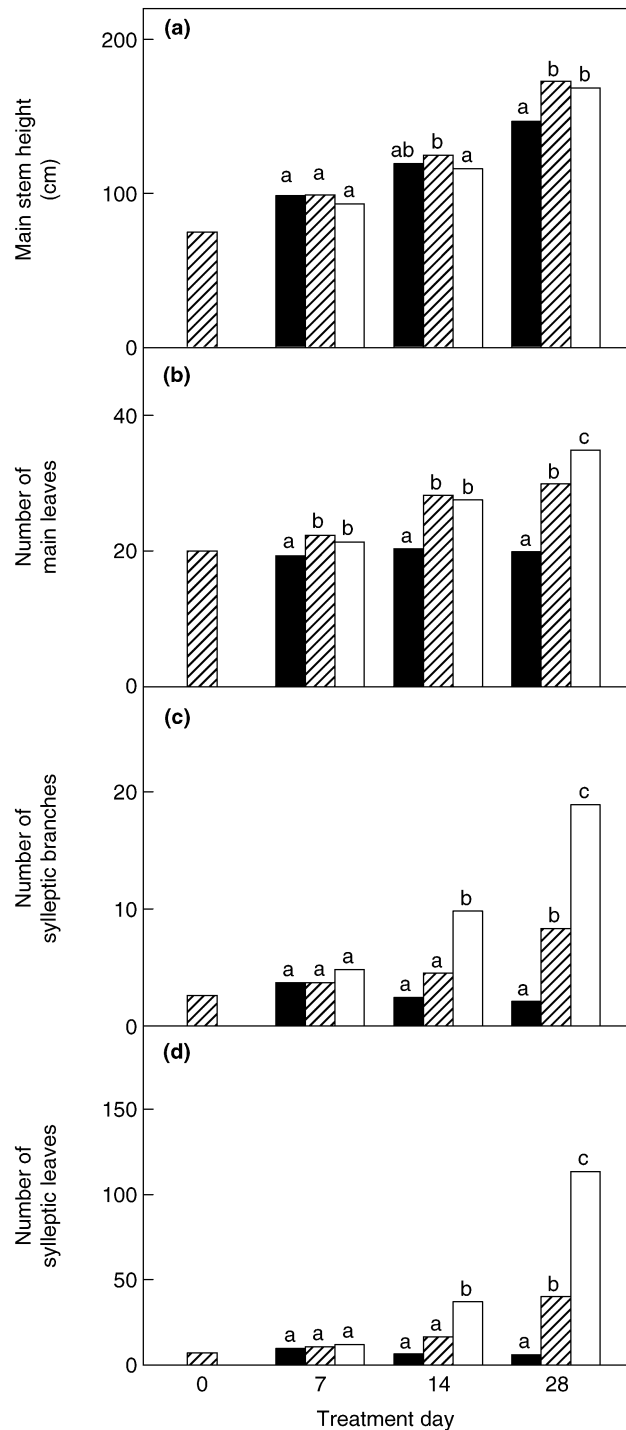


Fig. 1 Effect of differential N availability on plant architecture in *P. trichocarpa* \times *deltoides*. Morphometric measurements of main stems and sylleptic branches of 0N (black bars), 2N (striped bars) and 50N (white bars) treated trees at 0, 7, 14 and 28 d. (a) Height of main stem axes; (b) number of leaves with LPI greater than -1 appended to the main stem; (c) number of sylleptic branches > 1 cm long; (d) number of leaves with LPI greater than -1 appended to sylleptic branches. Data points represent means of at least nine individuals from four independent experiments. Means assigned the same letter within a treatment day are not significantly different ($P \leq 0.05$).

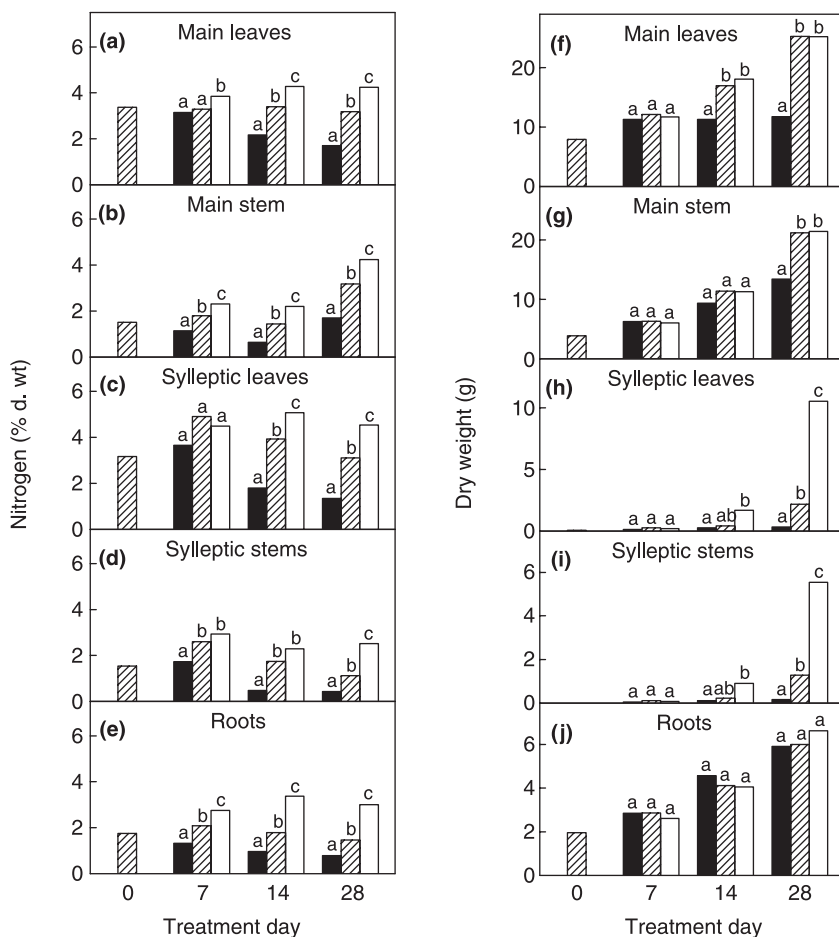


Fig. 2 Effect of differential N availability on percentage N and biomass accumulation. (a–e) Percentage N content; (f–j) dry weight of tissues from 0N (black bars), 2N (striped bars) and 50N (white bars) treated trees at 0, 7, 14 and 28 d. Data points represent means of at least five individuals from four independent experiments. Means assigned the same letter within a treatment day are not significantly different ($P \leq 0.05$).

induces architectural changes in poplars, but the effects of N on leaf number, syllepsis and plant height are temporally discrete.

As expected, 0N, 2N and 50N plants demonstrated accumulation of N in leaves, stems and roots in proportion to the amount supplied (Fig. 2a–e). Significant differences in N concentration in leaves, stems and roots were evident as early as 7 d, with differences between 0N, 2N and 50N treatments more pronounced in woody tissues with roles in nutrient transport/storage (roots and stems) than in leaf tissues. Differences between treatments for all tissues increased progressively through the time course.

Treatment with 0N, 2N and 50N also led to significant differences ($P \leq 0.05$) in most plant biomass components (Fig. 2f–j). As with N-induced changes in architecture, the timing of response varied by component. Biomass of leaves attached to the main stem axis ('main leaves') in 0N plants remained relatively constant over the 28 d time course, while those of 2N and 50N plants increased in parallel, and were significantly greater than 0N main leaf biomass by 14 d (Fig. 2f). Nitrogen addition increased biomass accumulation in sylleptic shoots by 14 d (Fig. 2h,i), while main stem biomass

values were not significantly different among treatments until 28 d (Fig. 2g). Root biomass did not differ between treatments during the course of the study.

The N concentration and biomass measurements revealed effects of N availability on the relative rates at which leaves, stems and roots develop. In addition, we detected gradients of N concentration within tissue cohorts by measuring percentage N in stems and leaves at distinct developmental stages in the same plants. Younger stem and leaf tissues contained higher N concentrations than older tissues collected from the same trees (Fig. 3), indicating substantial heterogeneity of N concentration within a particular organ type.

Closer inspection was made of biomass distribution between the main axis and sylleptic branches of the plants by plotting leaf and stem biomass at 28 d as a function of main leaf N concentration (Fig. 4). Total leaf and total stem biomass correlated well with increasing leaf N concentration (Fig. 4a–d), with P values < 0.0001 and 0.0008 , respectively. While main leaves and main stems account for most of this biomass, and thus showed similar trends (Fig. 4b,e), biomass of sylleptic leaves and sylleptic stems increased sharply at leaf N concentrations $> 4\%$ (Fig. 4c,f).

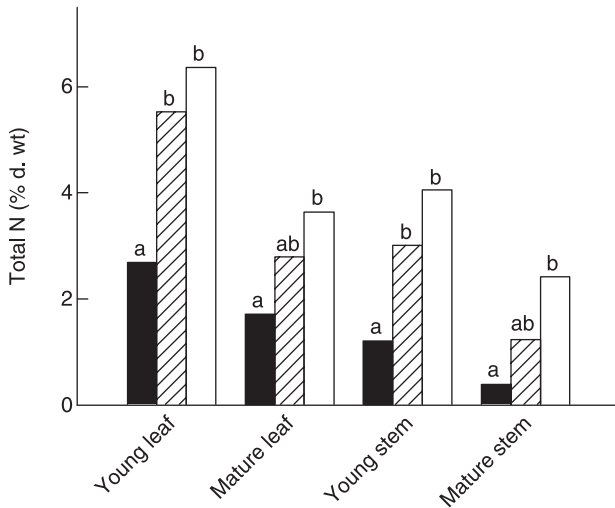


Fig. 3 Effect of differential N availability on percentage N accumulation in developing and mature tissues. Trees were treated with 0N (black bars), 2N (striped bars) or 50N (white bars) for 14 d. Data points represent means of four individuals from two independent experiments. Means assigned the same letter within a treatment day are not significantly different ($P \leq 0.05$).

Nitrogen treatments also altered the distribution of biomass between different plant components (Table 1). In general, increasing N availability resulted in greater allocation of biomass to above-ground components, that is, it increased the slope (m) of the regression line above 1. For example, 2N and 50N plants allocated significantly more biomass to leaves vs roots than did 0N plants. Similarly, 50N plants allocated about twice as much biomass to stems vs roots than did 0N

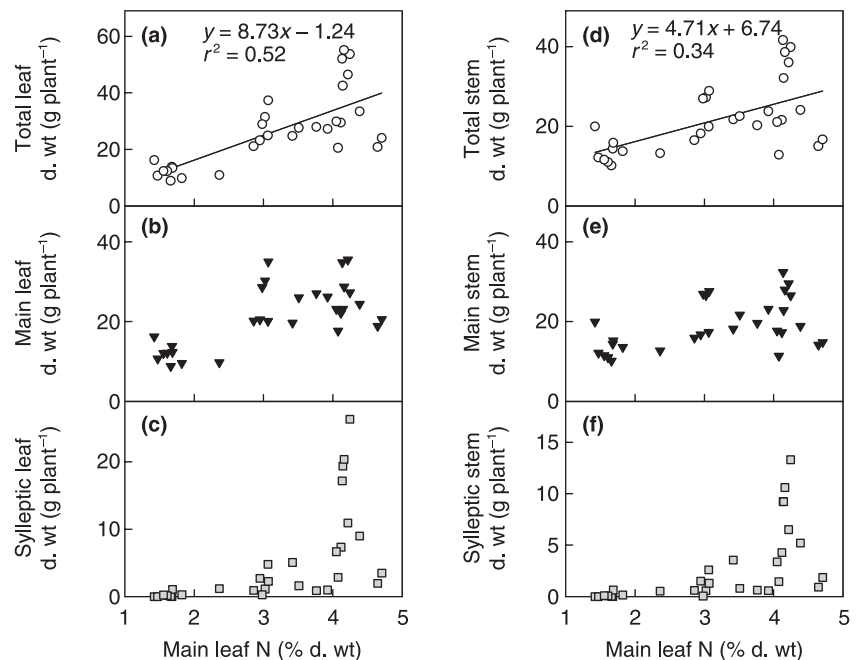
Table 1 Parameters for regression equations relating biomass components in *Populus* seedlings receiving three nitrogen treatments

Comparison (Y vs X)	Treatment	Intercept (b)	Slope (m)	R^2
Leaf vs stem	0N	3.8 a	0.61 a	0.64
	2N	4.6 a	1.02 ab	0.83
	50N	2.6 a	1.25 b	0.97
Leaf vs root	0N	7.2 a	0.81 a	0.58
	2N	13.9 a	2.26 b	0.57
	50N	13.0 a	3.51 b	0.92
Stem vs root	0N	5.7 a	1.32 a	0.89
	2N	10.4 a	2.01 ab	0.56
	50N	8.5 a	2.78 b	0.94

Regressions are of the form $Y = mX + b$. Within a column and comparison type parameters followed by the same lower case letters are not significantly different ($P \leq 0.05$).

plants (2.78 and 1.32 g biomass in stems for each g biomass in roots in 50N and 0N plants, respectively). Above ground, 50N plants allocated 1.25 g biomass into leaves for each g biomass in stems, compared with 0.61 g leaf biomass per g stem biomass in 0N plants. It should be noted that these plants were grown in pots, and while the pots were placed in water-containing saucers that permitted roots to exit the holes in the base of the pot, the constraints exerted on root growth by the pots probably had some effect on the absolute values of the above-ground vs below-ground biomass comparisons, as seen in the compressed y -intercepts (Table 1). Therefore, while the trends obtained in the regressions are important to illustrate shifts in allocation priority, the shoot : root ratios obtained in this study are probably higher than would be observed in field studies.

Fig. 4 Relationship between leaf and stem biomass accumulation and leaf N content. Trees were treated with 0N, 2N or 50N for 28 d. Each data point represents the dry weight of either leaves or stems from an individual plant plotted as a function of percentage N content of the main leaves from the same individual. (a) Sum of main leaf and sylleptic leaf biomass; (b) main leaf biomass; (c) sylleptic leaf biomass; (d) sum of main stem and sylleptic stem biomass; (e) main stem biomass; (f) sylleptic stem biomass. Linear regression equations and coefficients of determination given for (a) and (d).



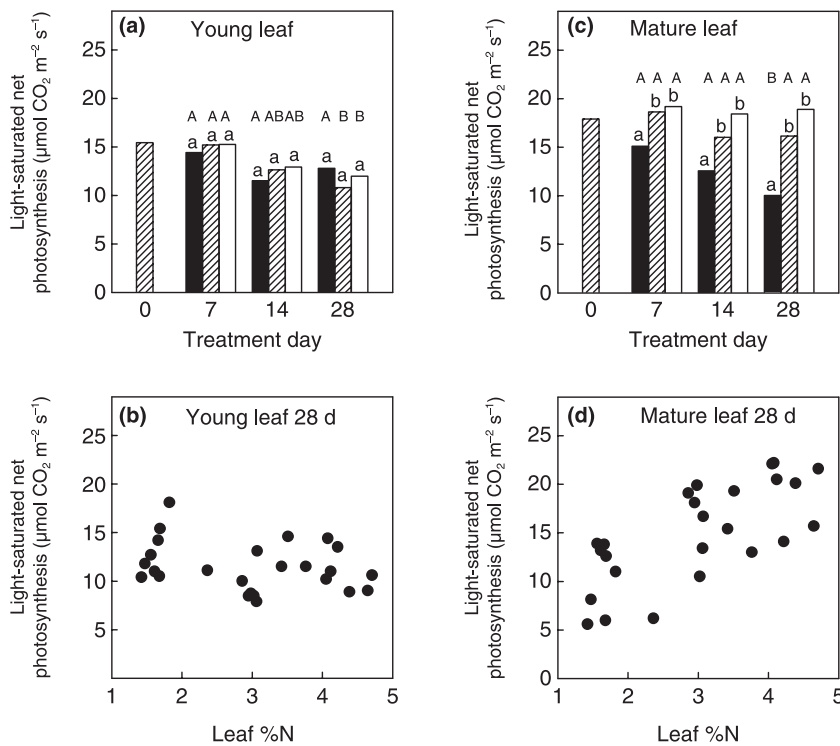


Fig. 5 Effect of differential N availability on photosynthesis in developing and mature leaves. (a) A_{max} for LPI 4 leaves of 0N (black bars), 2N (striped bars) or 50N (white bars) trees treated for 0, 7, 14 or 28 d. (b) Data in (a) plotted as a function of percentage N content of main leaves from the same individual. (c) A_{max} for mature leaves of 0N (black bars), 2N (striped bars) or 50N (white bars) trees treated for 0, 7, 14 or 28 d. See Materials and Methods for details on mature leaf selection criteria. (d) Data in (c) plotted as a function of percentage N content of main leaves from the same individual. Data in (a) and (c) represent means of at least eight individuals from four independent experiments. Means assigned the same lower case letter within a day are not significantly different; means assigned the same upper case letter within an N treatment across days are not significantly different ($P \leq 0.05$).

Nitrogen availability and factors contributing to whole-plant carbon gain

Both light-saturated net photosynthesis rates and chlorophyll content of leaves were influenced by N availability. The patterns of maximum photosynthesis rates (A_{max}) responses to N availability that we observed in young and mature leaves were more complex than a simple 'high N = high A_{max} ' model would suggest (Fig. 5). For young leaves, the 2N and 50N treatments had no impact on A_{max} at any single time point, but showed a gradual decrease in A_{max} from the 7–28 d time points (Fig. 5a). Plotting photosynthetic rates for individual plants at 28 d vs leaf N concentration reinforced this observation (Fig. 5b). In contrast, mature leaves responded strongly to increased N availability, with 2N and 50N treatments showing a 30% increase in A_{max} over the 0N treatment at 7 d (Fig. 5c). These elevated A_{max} rates were maintained in the 2N and 50N treatments, while A_{max} declined from 7 to 28 d in the 0N-treated plants. Furthermore, there was a positive relationship between photosynthesis and leaf N concentration in mature leaves (Fig. 5d).

In contrast to photosynthetic rates, the chlorophyll content of both young and mature leaves was positively correlated with N availability (Fig. 6). The relationship between mature leaf concentration and chlorophyll content of young leaves is described by $y = 4.84x + 6.442$ ($r^2 = 0.435$, $P = 0.0006$), and for mature leaves by $y = 9.13x + 4.31$ ($r^2 = 0.731$, $P < 0.0001$). The differences between 0N plants and 2N and 50N plants were significant by 14 d.

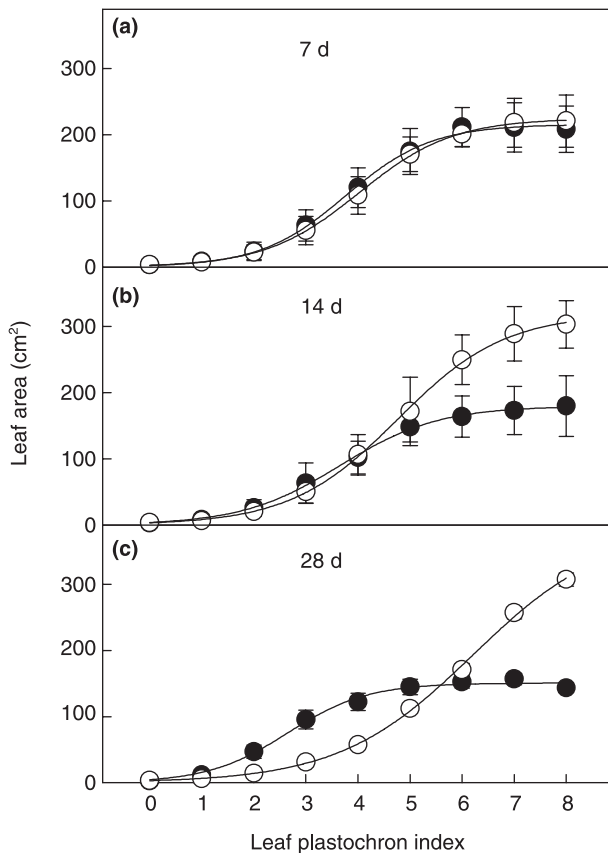
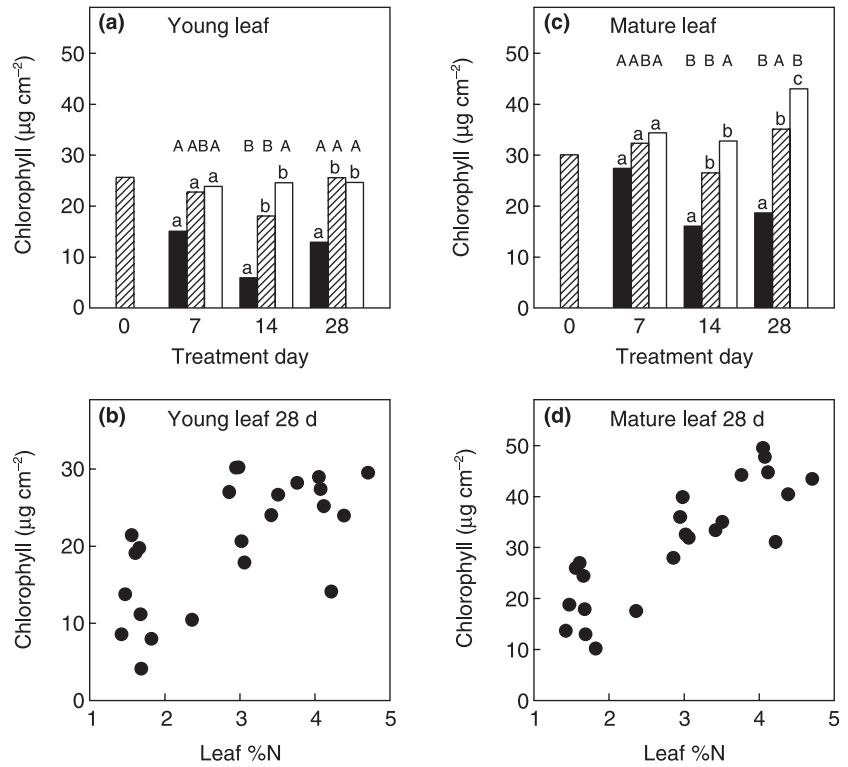
The size of the developing leaf zone (the number of sink leaves on the main stem at a particular time) increased dramatically in response to N fertilization. At 7 d (Fig. 7a), leaf areas at each LPI in the developing leaf zone were comparable for 0N and 50N plants, and leaves had reached full size ($218 \pm 36 \text{ cm}^2$, mean \pm SD) by LPI 7. At 28 d, leaves of 0N plants were fully expanded by LPI 5. In contrast, the zone of leaf expansion increased in 50N plants, such that by 28 d leaves had not yet reached full size at LPI 8 (Fig. 7c). Despite not having reached full size, LPI 8 leaves from 50N plants at 28 d were larger than fully expanded LPI 8 leaves from 50N plants at 14 d (307 ± 30 vs $270 \pm 61 \text{ cm}^2$).

Leaf maturation was further assessed by comparing the proportion of mature (differentiated, presumably functional) stomata per unit leaf area at LPI 2 of 0N and 50N plants over the 28 d time course (Fig. 8). At 7 d the proportion of mature stomata in LPI 2 leaves of 0N and 50N plants was nearly identical. At 14 d the proportion of mature stomata in 0N plants had increased, and by 28 d the difference between the 0N and 50N treatments was statistically significant at $\alpha = 0.05$. Thus LPI 2 leaves of 0N plants were progressively more mature than LPI 2 leaves of 50N plants as the experiment proceeded.

Nitrogen availability and gene-expression patterns associated with nitrogen and carbon utilization

We evaluated the spatial expression of genes associated with N storage and carbon gain in trees treated for 14 d to provide

Fig. 6 Effect of differential N availability on chlorophyll content of developing and mature leaves. (a) Chlorophyll determinations for LPI 3 leaves of 0N (black bars), 2N (striped bars) or 50N (white bars) trees treated for 0, 7, 14 or 28 d. (b) Data in (a) plotted as a function of percentage N content of main leaves from the same individual. (c) Chlorophyll determinations for mature leaves of 0N (black bars), 2N (striped bars) or 50N (white bars) trees treated for 0, 7, 14 or 28 d. See Materials and Methods for details on mature leaf selection criteria. (d) Data in (c) plotted as a function of percentage N content of main leaves from the same individual. Data in (a) and (c) represent means of at least five individuals from four independent experiments. Means assigned the same lower case letter within a day are not significantly different; means assigned the same upper case letter within an N treatment across days are not significantly different ($P \leq 0.05$).



insight into the molecular phenotype underlying the N response (Fig. 9). Transcripts corresponding to two genes encoding vegetative storage proteins (*win4* and *bsp*) were more abundant in shoot tips, stems and roots from 2N and 50N plants compared with 0N plants. Transcript accumulation for *bsp* was higher in 50N than 2N plants, whereas for *win4* transcripts accumulated equally in 2N and 50N plants. Thus transcript abundance was coincident with growth (*win4*) or percentage N (*bsp*).

The starch biosynthetic genes encoding ADP glucose pyrophosphorylase and starch synthase exhibited very similar patterns of transcript abundance: transcripts corresponding to these two genes were detected in LPI 3 leaves of 0N plants, but not in LPI 3 leaves of 2N or 50N plants. In mature leaves, ADP glucose pyrophosphorylase and starch synthase transcripts were detectable in 0N, 2N and 50N plants, with highest transcript levels for both genes found in 0N mature leaves and following a predicted gradient in whole-plant carbon sink demand. Transcripts for these particular ADP glucose pyrophosphorylase and starch synthase genes were not detected in shoot tips, stems or roots.

Fig. 7 Effect of differential N availability on final leaf size. Leaf areas from LPI 0 to LPI 8 for 0N trees (●) and 50N trees (○) after (a) 7; (b) 14; (c) 28 d. Data points represent mean \pm 1 SE of at least 10 individuals from four independent experiments.

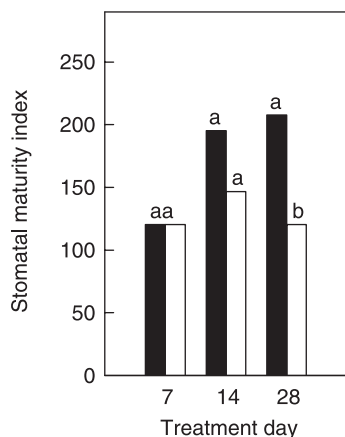


Fig. 8 Effect of differential N availability on leaf development, as determined by relative number of mature leaf stomata for LPI 2 from 0N (black bars) and 50N (white bars) plants after 7, 14 and 28 d. The index was calculated as the number of stomata ≥ 0.02 mm long per mm² leaf area. Data represent means of three separate measurements from LPI 2 leaves sampled from at least four individuals from four independent experiments. Means assigned the same letter indicate no significant difference between treatments within a day ($P \leq 0.05$).

A gene encoding a poplar putative pectin methylesterase inhibitor (PMEI) protein was coregulated with the starch biosynthesis genes. The putative PMEI was most highly expressed in young leaves of 0N plants, and transcript

abundance increased with decreasing N availability in both young and mature leaves (Fig. 9). Transcript levels were below detectable limits in shoot tips and whole stems. The 528 bp 3' cDNA used in this study corresponds to Gene Model ID Poptr1:664439 in version 1 of the sequenced, assembled and annotated genome of *P. trichocarpa* (<http://genome.jgi-psf.org>). Based on an alignment of the gene sequence with the cDNA, the PMEI-like gene appears to lack introns and encodes a protein of 195 amino acids with a single PMEI domain based on CDD similarity (pfam04043, E value $6e-23$) that is most closely related to a PMEI from *Nicotiana tabacum* (40% identical, 60% similar; GenBank accession number BAA95794.1). The encoded protein from poplar appears to encode a cleavable N-terminal signal peptide that is either 21 (SIGNALP-NN) or 23 (SIGNALP-HMM) amino acids in length. The presence of this signal peptide is consistent with its expected extracellular location.

Discussion

Resource allocation patterns change rapidly in response to nitrogen availability

Previous studies have demonstrated that *Populus* allocates a greater proportion of resources to above-ground biomass when pedospheric N is readily available (Ibrahim *et al.*, 1998; Coleman *et al.*, 1998). Together, the N concentration, biomass

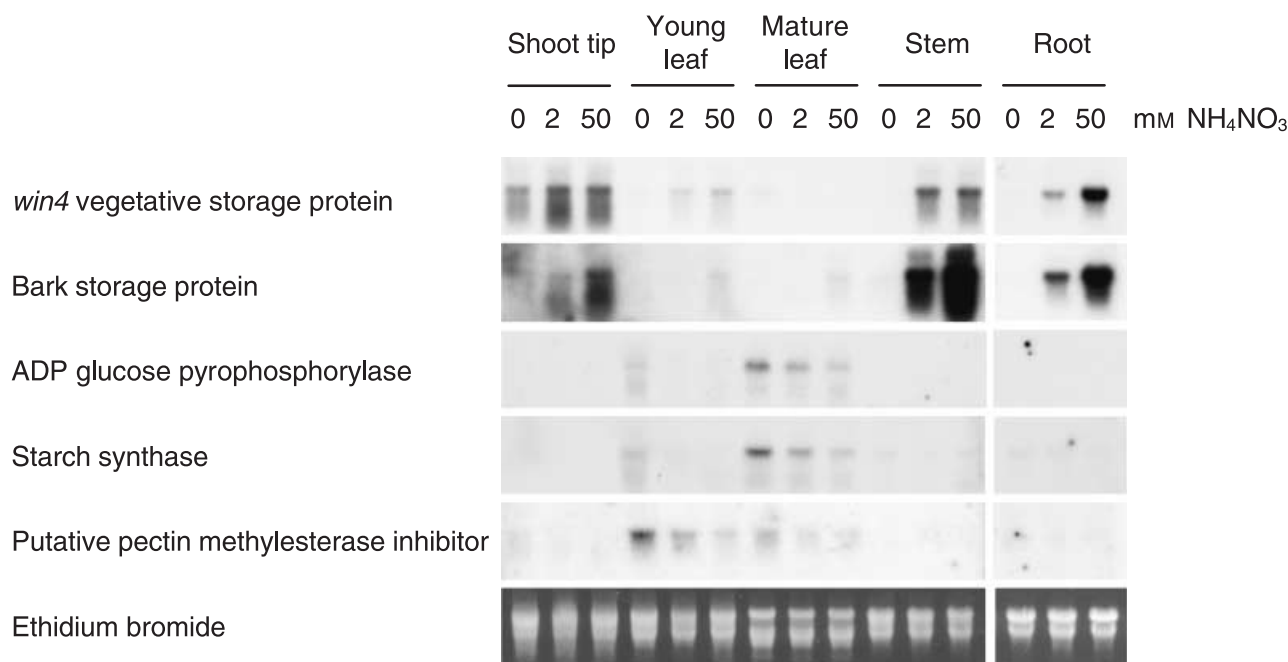


Fig. 9 Effect of differential N availability on the expression of genes associated with N storage, starch biosynthesis and cell-wall properties. Northern blots showing transcript accumulation in shoot tips (up to and including LPI 1), young leaves (LPI 3), mature leaves (generally LPI 15–16), whole stems (sampled between LPI 14 and LPI 17), and roots from 0N, 2N and 50N trees after 14 d. Ethidium bromide staining of the RNA gel is included as a loading comparison.

and allometry data of this study illustrate the temporal relationship between N uptake and shifts in allocation priority for glasshouse-grown *P. trichocarpa* × *deltooides* clone UCC-1 saplings. These N-availability experiments have subsequently been repeated with *P. trichocarpa* × *deltooides* clone H11-11, with very similar results (J.E.K.C. and F. Pitre, unpublished data). The observed time lag between increased leaf production and subsequent elevated stem biomass growth under replete N conditions suggests that resource allocation to leaf area production is prioritized under increased N availability. This supposition is corroborated by the regression analysis.

The observed same-season increase in leaf area production in *P. trichocarpa* × *deltooides* that occurs in response to increased N availability is, in large part, a function of the indeterminate growth habit of *Populus* spp. which, in turn, is a determinant of crown architecture (Wu & Hinckley, 2001). Our results highlight the importance of syllepsis in augmenting above-ground biomass in response to N. Sylleptic branch development responds rapidly to replete N availability: initiation of branch elongation is clearly visible at many axillary buds only 4–6 d after treatment. Interestingly, sylleptic branch development does not respond in a linear fashion to *in planta* N concentrations. Rather, sylleptic branch biomass increases markedly at leaf N concentrations > 4%, which approximately corresponds to the point where the increase in leaf biomass begins to approach the theoretical asymptote.

Once sylleptic branches have attained 10–15 leaves, they become photosynthetically independent (Dickson, 1989) and can contribute notably to the total exported photosynthate, particularly to the main stem (Scarascia-Mugnozza *et al.*, 1999). Previous studies with *Populus* have established that sylleptic branching – and the increased leaf area supported by sylleptics – is correlated with increased biomass productivity, particularly for saplings (Ceulemans *et al.*, 1992; Rae *et al.*, 2004). This relationship between sylleptic branching and biomass productivity in *Populus* is reinforced by the finding that quantitative trait loci (QTLs) controlling stem basal area growth cluster with QTLs for sylleptic branch traits, suggesting pleiotropy of a single QTL governing both traits (Bradshaw & Stettler, 1995).

Carbon acquisition parameters change rapidly in response to nitrogen availability

The C-fixation capacity of a tree is dependent on whole-plant photosynthetic capacity, which is a function of leaf-level net photosynthetic rates and whole-plant leaf area (Host *et al.*, 1990; McGarvey *et al.*, 2004). Previous studies have demonstrated that increased N availability increases photosynthesis (Coleman *et al.*, 1998) and whole-plant leaf area (Liu & Dickmann, 1992; Ibrahim *et al.*, 1998) in *Populus*. Both these component traits responded to N availability in this study, and the responses of these traits were modified by N-induced changes in leaf developmental phenology.

The leaf area and stomatal maturity index data indicate that limiting N accelerates leaf maturation in the developing leaf zone, while luxuriant N prolongs leaf maturation. As a result, the sink-to-source leaf transition zone becomes considerably shorter in N-limited plants than in N-luxuriant plants. These findings are consistent with those of Wait *et al.* (1998). The delayed rate of leaf maturation under replete N conditions was evidenced in the photosynthesis data, where A_{\max} of LPI 4 leaves of 2N and 50N plants declined over the 28 d time course. While limiting N plants clearly exhibited an accelerated rate of leaf maturation in the developing leaf zone, this did not translate to significant differences in A_{\max} . The declining A_{\max} values in mature leaves of limiting N plants over the time course, combined with the observed decrease in chlorophyll content, suggest that limiting N conditions also accelerated the final stage of leaf development – senescence – although leaves were sampled well above the first LPI showing visible signs of senescence. These observations indicate that N-limited trees exhibited leaf senescence at an earlier LPI than adequate or N-luxuriant plants. The earlier transition to senescence presumably allows N-limited plants to remobilize resources from these leaves to support growth in developing regions of the tree. These results highlight the importance of leaf development as a determinant of whole-plant photosynthetic capacity.

Compared with the responses of A_{\max} , the response of leaf area to N availability was relatively straightforward: increased N resulted in greater leaf-area development. Nitrogen-associated increases in leaf area were caused by several factors, including (i) increased main leaf production, a function of the indeterminate growth habit of *Populus*; (ii) increased individual leaf area, probably influenced by the expanded zone of leaf maturation; (iii) delayed leaf senescence; and (iv) sylleptic branch development. Modulation of leaf area on the main axis represents alteration of existing growth patterns by changing the rate of cell division and/or cell expansion, whereas sylleptic branch development represents the initiation of growth at additional meristems. Our results suggest that both these mechanisms are modulated by N availability.

Expression of genes associated with carbon and nitrogen resource utilization are affected by nitrogen availability

Nitrogen-induced changes in developmental processes are accompanied by shifts in gene-expression programs in plants, including hybrid poplars (Cooke *et al.*, 2003). Investigations with *Arabidopsis* and other herbaceous systems have demonstrated the highly integrative nature of N and C metabolism (Koch, 1997; Palanchar *et al.*, 2004). The continual adjustments to C and N metabolism that occur in response to N availability contribute to changes in whole-plant processes that influence overall growth and development (Geiger *et al.*, 1996; Koch, 1997).

Transcript abundance for genes involved in N storage (vegetative storage protein accumulation under luxuriant N

conditions) and C storage (starch accumulation under limiting N conditions) showed opposing trends that highlight the integrative nature of C and N partitioning as a function of resource availability (Schleible *et al.*, 1997). The expression patterns for *win4* and *bsp* are consistent with the results of previous studies, which demonstrated that these vegetative storage proteins respond positively and rapidly to increased N availability (Coleman *et al.*, 1994; Lawrence *et al.*, 1997). The patterns of transcript abundance for the starch biosynthetic genes reflect the photosynthesis trends. The increased transcription of these genes in young leaves of 0N plants relative to 2N and 50N plants provides further evidence that leaves of N-limited plants are undergoing a more rapid transition from sink to source than leaves of N-replete plants. The increased transcription of the starch biosynthetic genes of mature leaves of 0N plants suggests that *Populus* source leaves increase partitioning of C to starch under limiting N conditions, as has been reported in annual plants (Nielsen *et al.*, 1998). This increased C storage under limiting N conditions is probably a function of reduced demand for C skeletons in primary N assimilation, as well as reduced sink strength in developing tissues such as the shoot tip and leaves. Increased starch accumulation is also associated with leaf senescence, possibly resulting from blockage of assimilate export (Jongebloed *et al.*, 2004).

Detailed phenotyping at the whole-plant and tissue levels can provide an experimental framework for assigning putative functions to genes with previously unknown functions. In our previous study aimed at discovering genes associated with the N response in *Populus* (Cooke *et al.*, 2003), we identified a putative PME1 which, in the current study, has been found to be expressed to a greater extent in 0N than in 50N leaves (Fig. 8). The PME1 domain is found in inhibitors of both pectin methylesterases and invertases (Giovane *et al.*, 2004; Rausch & Greiner, 2004). PME1s post-translationally inhibit the activity of their respective target sugar-metabolizing enzymes by forming a noncovalent 1 : 1 complex. The transcript data suggest that expression of a PME1 occurs in conjunction with cell-wall maturation. As such, the target is probably a pectin methylesterase acting to loosen cell walls (Giovane *et al.*, 2004), although there is the possibility that the target could be an invertase expressed in association with leaf sink status (Rausch & Greiner, 2004). Coregulation of this PME1 with an accelerated sink-to-source transition under limiting N – including more rapid acquisition of photosynthetic competence, stomatal maturation and expression of starch biosynthetic enzymes – collectively suggest a role for this protein in leaf development and provide a functional context for more detailed biochemical and molecular studies.

Hybrid poplar exhibits phenotypic plasticity to nitrogen availability

The data from this series of experiments allow us to develop a conceptual model of the early physiological responses of

P. balsamifera ssp. *trichocarpa* × *deltooides* clone UCC-1 to high N fertility. When grown in an environment where N is readily available, *Populus* displays a coordinated suite of responses that drive rapid increases in carbon fixation capacity, and eventually stem growth. The earliest of these responses occurs at 7 d (or earlier), with fertilized plants showing increased tissue N concentration (Figs 2a, 3); vegetative storage protein expression (Coleman *et al.*, 1994; Lawrence *et al.*, 1997); and elevated mature leaf photosynthetic capacity (Fig. 5c). The additional C produced by this greater photosynthetic capacity allows the production of additional leaf area by day 14, both on the main stem and on newly formed sylleptic branches (Fig. 2b). The building of a larger crown is facilitated by shifts in C allocation away from root and stem tissue toward leaves (Table 1). The recently produced foliage on the main stem effectively elongates the developing leaf zone – the newly produced leaves will ultimately produce larger laminae when fully expanded – so molecular markers of the sink-to-source transition indicate slower acquisition of source status. By day 28, the additional C availability resulting from this positive feedback loop enables the production of a greater amount of stem tissue in the N-treated plants (Fig. 2b).

Our findings add to a growing body of literature that illustrate the dramatic capacity of *Populus* spp. and their hybrids to rapidly take up and utilize N from the soil (Liu & Dickmann, 1992; Heilman & Xie, 1994; Ibrahim *et al.*, 1998; Coleman *et al.*, 1998). The results also provide evidence that *P. balsamifera* ssp. *trichocarpa* × *deltooides* hybrids demonstrate phenotypic plasticity in response to N availability, although a single genotype was investigated in this study. This plasticity is evident not only in terms of the considerable changes in morphology and architecture, but also in the short time frame required for these changes to be detectable. The modulation of plant architecture through syllepsis is a striking example of the phenotypic plasticity that *P. balsamifera* ssp. *trichocarpa* × *deltooides* exhibits in response to pedospheric N. Together with the gene-expression studies reported by Cooke *et al.* (2003), the results presented here provide a 'road map' of short-term responses of *Populus* to differential N availability, and lay the foundation for in-depth experimentation on the molecular mechanisms by which N availability acts to increase productivity of forest trees.

Acknowledgements

The authors thank Chris Dervinis, David Noletti and Christina Balangue for technical assistance. This research was supported by the US Department of Energy through Cooperative Agreement Number DE-FC07-97ID13529 (DOE/American Forest and Paper Association's Agenda 2020 Programme) and Grant #DE-AC05-00OR22725 (Office of Science, Office of Biological and Environmental Research). J.E.K.C. was the recipient of a Canadian Sciences and Engineering Research Council postdoctoral fellowship. This

is Florida Agricultural Experiment Station Journal Article No. R-10824.

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