Wood Quality, Carbon and Nitrogen Partitioning, and Gene Expression Profiling in *Populus* Exposed to Free Air CO₂ Enrichment (FACE) and N-fertilization

Dissertation

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by

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Summary

Atmospheric CO_2 concentrations ($[CO_2]$) are continuously rising since the beginning of the industrialization. At the same time, N-deposition is also increasing. The influences of elevated CO_2 and fertilization on the physiology and development of forest trees have been intensively studied. However, the effects of these environmental factors on wood quality, carbon and nitrogen allocation to long- and short-term C-N pools in wood of forest trees are not clear yet. To shed light on these questions, *Populus × euramericana*, *P. alba*, and *P. nigra* clones were grown in ambient air (about 370 ppm CO_2) and in air with elevated $[CO_2]$ (about 550 ppm CO_2) using Free-Air CO_2 Enrichment (FACE) technology in central Italy. FACE was maintained for five years. After three growing seasons, the plantation was coppiced and one half of each experimental plot was fertilized with nitrogen. In secondary sprouts, this investigation was carried out.

To characterise wood quality in response to elevated CO_2 and N-fertilization, growth and wood anatomy of the three poplar clones were investigated. In the three poplar genotypes, most of anatomical traits showed no uniform response pattern to elevated CO_2 or N-fertilization. In *P. × euramericana*, N-fertilization resulted in significant reductions in fiber lengths. In all three genotypes, N-fertilization caused significant decreases in cell wall thickness. In *P. × euramericana* and *P. alba*, elevated CO_2 also caused decreases in wall thickness, but less pronounced than nitrogen. In *P. nigra* and *P. × euramericana*, elevated CO_2 induced increases in vessel diameters. The combination of elevated CO_2 and N-fertilization resulted in overall losses in cell wall area of 5–12% in all three clones suggesting that in future climate scenarios, the negative effects on wood quality may be anticipated.

To quantify carbon allocation between short- and long-term pools in wood in response to elevated CO_2 and N-fertilization, in *P. nigra*, carbon concentrations and stocks were quantified. Although elevated CO_2, N-fertilization and season had significant tissue-specific effects on carbon partitioning to the fractions of structural carbon, soluble sugars and starch as well as to residual soluble carbon, the overall magnitude of these shifts was small. The major effect of elevated CO_2 and N-fertilization was on biomass production, resulting in about 30% increases in above ground stocks of cell wall mass. Relative C-

partitioning between mobile and immobile C-pools was not significantly affected by elevated CO_2 or N-fertilization. These data demonstrate high metabolic flexibility of *P. nigra* to maintain C-homeostasis under changing environmental conditions.

To characterise secondary metabolites and internal N-pools responding to elevated CO_2 and N-fertilization, carbon-based secondary compounds, concentrations of total N and Klason lignin-bound N were measured in *P. nigra*. Elevated CO_2 had no influence on lignin, cell wall-bound phenolics and soluble condensed tannins. Higher N-supply slightly but markedly stimulated formation of carbon-based secondary compounds. Elevated CO_2 decreased internal N-pools in wood, but external N-supply increased the internal N-pools. In wood, 17—26% of N was bound to Klason lignin forming a resistant N-fraction. Neither elevated CO_2 nor higher N-supply altered N-partitioning between lignin-bound N and other N-containing compounds. Positive correlations existed between the biosynthesis of proteins and secondary compounds in *P. nigra*. These data imply that the growth and defense of forest trees are well orchestrated.

To elucidate the molecular mechanism causing changes in wood properties in response to elevated CO_2 and N-fertilization, wood anatomy, Klason lignin, calorific value, Fourier transform infrared (FT-IR) spectra of wood were analysed and gene expression profiling in the differentiating xylem was performed in *P. × euramericana*. Elevated CO_2 significantly stimulated the annual ring width in the second year after coppicing. However, elevated CO_2 significantly decreased the cell wall, ray parenchyma and vessel lumen fractions, which was mainly due to a significant increase in the fraction of fiber lumina and decreased thickness of fiber walls. Higher N-supply also significantly decreased the cell wall fraction which was due to a marked decrease in the thickness of fiber walls and increases in fiber lumen fraction and fiber lumen diameter. Elevated CO_2 and N-fertilization together stimulated lignin formation. This was also confirmed by mapping of lignin distribution and FT-IR spectra in wood. The calorific value of wood was unaffected by elevated CO_2 or N-fertilization, whereas N-fertilization significantly enhanced the energy potential of the plantation by 16 — 69% due to the stimulation of aboveground biomass.

Gene expression profiling revealed that only few transcripts were markedly affected by elevated CO_2 and/or N-fertilization. Under most conditions, only one transcript was significantly affected on the array containing 3 444 expressed sequence tags (ESTs). When comparing the effect of N-fertilization on gene expression under elevated CO_2, 15

transcripts were significantly up-regulated, including two genes, caffeic acid-3-O-methyltransferase 1 (COMT-1) and ferulate-5-hydroxylase (F5H), which are tightly linked to lignin biosynthesis. This observation corresponds well to the findings in Klason lignin analysis, mapping of lignin distribution and FT-IR spectra. Among the 15 transcripts, the enhanced expression of a tubulin gene associated with the cytoskeleton may be related to the altered anatomical properties (diameters of fiber and vessel lumen) in the developing xylem of *P. × euramericana* under these conditions.

Based on the above results, it is concluded that in future climate scenario, the negative effects on wood quality may be anticipated. However, non-structural carbon compounds can be utilized more rapidly for structural growth under elevated atmospheric CO_2 in fertilized agro-forestry systems. The growth and defense of forest trees were homeostatically balanced even if increases in atmospheric $[CO_2]$ were accompanied by increased N availability.

Keywords: Agroforestry, Biomass, Calorific value, Carbon sequestration, Climate change, Elevated CO_2, FT-IR, Gene expression, Lignin, N-fertilization, Phenolics, Soluble proteins, Starch, Sugar, Tannins, Wood anatomy, Wood properties, *Populus*

Zusammenfassung

Die atmosphärische Kohlenstoffkonzentration (CO_2) ist seit dem Beginn der Industrialisierung kontinuierlich gestiegen. Gleichzeitig hat die Deposition von Stickstoff ebenfalls zugenommen. Die Auswirkungen dieser Umwelteinflüsse auf die Holzqualität und die Verteilung von Kohlenstoff (C)- bzw. Stickstoff (N) in Kurzzeit- und Langzeit-Depots im Holz forstwirtschaftlich genutzter Bäume sind bisher noch nicht klar. Um diese Fragen näher zu beleuchten, wurden Klone der Pappelarten *Populus × euramericana*, *Populus alba* und *Populus nigra* unter ambienten Kohlenstoffkonzentrationen (≈ 370 ppm CO_2) oder erhöhten Kohlenstoffkonzentrationen [CO_2] (≈ 550 ppm CO_2) in Mittelitalien angezogen. Dies fand unter Anwendung der Free-Air CO_2 Anreicherungstechnologie (FACE) statt. Das FACE-Projekt war für sechs Jahre angesetzt. Nach drei Jahren wurde die Anpflanzung auf den Stock zurückgesetzt und nach dem Austrieb die Hälfte der Bäume zusätzlich mit Stickstoff gedüngt. In der vorliegenden Arbeit wurden diese Sekundärtriebe untersucht.

Um die Holzqualität in Bezug auf die erhöhte CO_2-Konzentration und Stickstoffdüngung zu charakterisieren, wurde Wachstum und Holzanatomie der drei Pappelklone untersucht. Die anatomischen Untersuchungen zeigten, daß die Kombination von erhöhtem Kohlendioxid und erhöhter Stickstoffdüngung zu einer einer Verringerung der Zellwände an der Querschnittsfläche in der Höhe 5-12 % in allen drei Klonen führten. Daraus können für die Zukunft negative Effekte auf die Holzqualität abgeleitet werden.

Um die Kohlenstoffverteilung zwischen Kurzzeit- und Langzeitdepots im Holz unter erhöhtem CO_2 und unter erhöhter Stickstoffdüngung zu quantifizieren, wurden in *P. nigra* die Kohlenstoffkonzentration und die Depotgröße bestimmt. Der Haupteffekt von erhöhtem CO_2 und der zusätzlichen Stickstoffdüngung war eine Stimulierung der oberirdischen Biomasse um 30 %. Die relative C-Verteilung zwischen mobilen und immobilen Fraktionen wurde von der erhöhten Kohlendioxid- und Stickstoffkonzentration nicht signifikant beeinflusst.

Um Sekundärmetabolite und die internen Stickstoffdepots unter erhöhtem CO_2 und Stickstoffeinfluss näher zu charakterisieren, wurden kohlenstoffbürtige Produkte wie Phenole und Tannine, die Gesamtstickstoffkonzentration und ligningebundene Stickstoff

1. Introduction

1.1 Evidence of rising atmospheric CO_2 concentrations and changes in N cycle

There is growing evidence that the earth is now in an accelerating era of global change (IPCC 2001). Global change is generally defined as climate change combined with land use change, i.e., changes in atmospheric trace gas concentrations, changes in air temperatures, precipitation patterns and absolute rain amounts combined with deforestation, urbanization and changes in agriculture and forestry (IPCC 2001). Over the past two centuries, atmospheric CO_2 concentrations ($[CO_2]$) have risen at an accelerating pace. Prior to the industrial revolution atmospheric $[CO_2]$ was stable at about 270 ppm (Fig. 1). Today $[CO_2]$ is approximately 38% higher and is predicted to reach 550 ppm in the near future (Fig. 1; IPCC 2001).

The global cycle of N is unique in that it consists of a massive, well-mixed, and (to most organisms) wholly unavailable pool of nitrogen gas (N_2) in the atmosphere; a relatively small and almost wholly biologically mediated conversion of N_2 to chemical forms of N that are available to most organisms; and a pool of N that cycles among plants, animals, microorganisms, soils, solutions, and sediments, and between land, water, and the atmosphere (Delwiche 1970). Human beings have altered the global cycle of N by combustion of fossil fuels, production of nitrogen fertilizers, cultivation of nitrogen-fixing legumes, and other activities (Galloway et al. 1995). The most fundamental change to the global cycle of N was a doubling of the transfer from the vast and un-reactive atmospheric pool to biologically available forms on land (Vitousek et al. 1997; Tilman et al. 2001). Overall, human activity has caused the fixation of about 140 Tg of new N per year in terrestrial ecosystems and mobilized about 70 Tg more (Vitousek et al. 1997). The added N is spread unevenly over Earth's surface. Some areas (e.g. northern Europe) are profoundly altered (Wright and van Breenman 1995), while others (e.g. remote south-temperate regions) receive little direct input (Hedin et al. 1995).
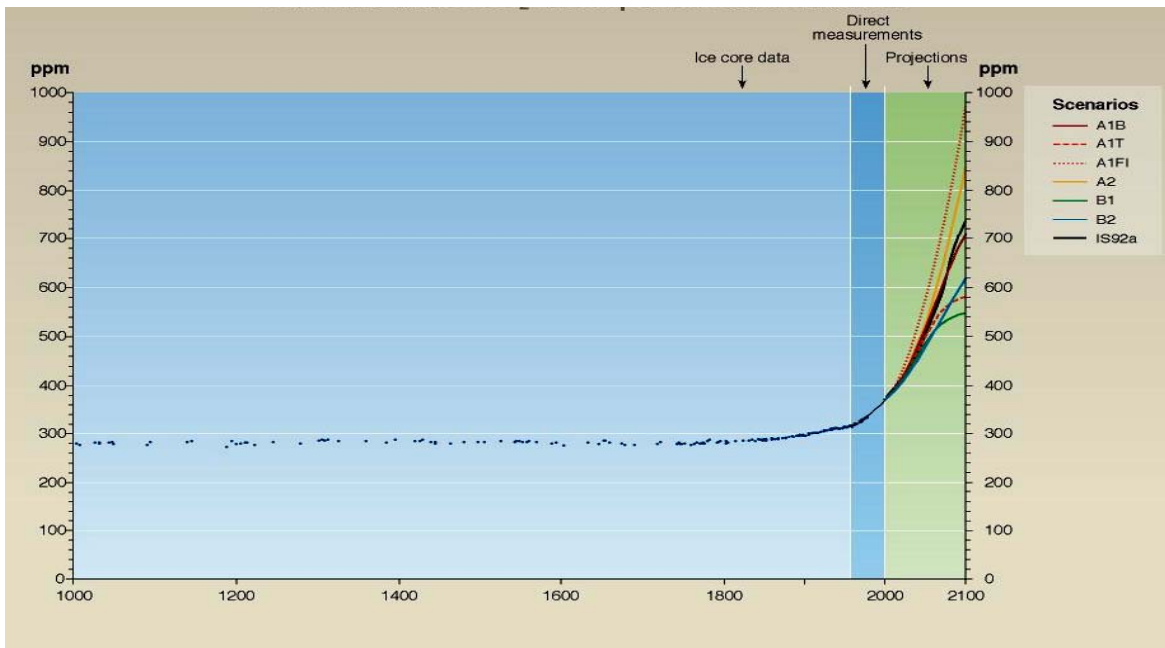
Fig. 1 Past, present and future atmospheric CO_2 concentration (IPCC 2001). The characteristics of different scenarios are as follows:

The scenarios of A1 family are based on very rapid economic growth, low population growth and the rapid introduction of new and more efficient technologies. A1B is balanced in the sense that it does not rely too heavily on a single energy source. A1F1 assumes fossil-intensive technologies, while A1T assumes a non-fossil future.

The A2 scenario describes a very heterogeneous world with high population growth. Economic development is primarily regionally oriented and per capita economic growth and technological changes are fragmented and slow.

The B1 scenario describes a convergent world. It has the same low population growth as A1 with a rapid change in economic structures toward a service and information economy. Other characteristics include a reduction in material intensity, clean and efficient technologies and improved equity.

The B2 scenario emphasizes local solutions to economic, social and environmental sustainability. There is moderate population growth and intermediate levels of economic development. Technological change is less rapid and more diverse than in A1 and B1 scenarios.

The IS92 scenario family was developed by the IPCC in the early 1990s to illustrate a plausible range of future greenhouse gas emissions. IS92a is a member of IS92 scenario family and it combines moderate population and economic growth, with high availability of fossil fuels balanced by reduced solar energy costs. IS92a has been widely used in climate modelling experiments as the standard *business as usual* scenario.

1.2 Basis of plants responding to elevated CO_2 and N-fertilization

Biological principles suggest that higher CO_2 concentration in the atmosphere will result in higher rates of photosynthesis, and in turn, higher yield or higher carbon

accumulation in plants (Lemon 1983). However, nature is not that simple in this case. First, we have better to know how plants respond to elevated $[CO_2]$.

Although many processes in plant metabolism are potentially affected by $[CO_2]$, there is consistent evidence only for effects on ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and stomatal movement in the range of $[CO_2]$ from 270—1000 ppm (Long et al. 2004). Plants can perceive a change in atmospheric $[CO_2]$ via their photosynthetic organs exposed to air. In other words, the inner surfaces of the guard cells of stomata and the mesophyll of higher-plant leaves and other photosynthetic organs may sense a change in atmospheric $[CO_2]$. At the biochemical level, Rubisco is currently $[CO_2]$ substrate limited by atmospheric $[CO_2]$. Therefore, Rubisco responds to rising atmospheric $[CO_2]$ and has the potential to function as a key metabolic step with sufficient regulatory control that a change in reaction rate would alter the flux through a major metabolic pathway (Long et al. 2004). At the physiological level, photosynthesis and stomatal conductance of C3 plants have been reported to respond to the elevated $[CO_2]$ (Drake et al. 1997). The direct increase in photosynthesis of plants grown under elevated $[CO_2]$ results from increased rates of carboxylation and decreased rates of oxygenation of Rubisco in C3 plants. Although most plants, including C4 plants, show a progressive decrease in stomatal conductance in response to rising $[CO_2]$ (Saxe et al. 1998; Medlyn et al. 1999; Gielen and Ceulemans 2001), the mechanism by which stomatal aperture responds to variation in $[CO_2]$ still remains mysterious (Moreson 1998; Long et al. 2004).

Since CO_2 is just one of many inorganic substrates required by plants, obviously, the response of photosynthesis and growth to elevated $[CO_2]$ will depend on the availability of mineral nutrients, especially nitrogen because most forests are standing under nutrient-poor conditions. In agroforestry plantations, ammonia and/or nitrate fertilizers are major forms of N-fertilization applied to the soil to increase the yields. Plants may perceive these N and take them up via specific areas in roots. In plants, inorganic nitrogen must first be reduced to ammonia before incorporation into glutamine and glutamate (Hoff et al. 1994). These amino acids serve to translocate organic nitrogen from sources to sinks in plants. In trees nitrate taken up by roots is mainly assimilated in amino compounds by belowground tissues (Gojon et al. 1994). Thus, these amino compounds and traces of NO_3^- or NH_4^+ are transported from the roots to the shoots in the xylem (Dambrine et al. 1995; Geßler et al. 1998; Schmidt and Stewart 1998). Nitrogen-demanding tissues, e.g., cambial tissues of the stem, leaves, fruits and buds, take N from this xylem-borne pool of amino compounds for

the syntheses of proteins and other organic N-containing compounds (Geßler et al. 2004). Although N-fertilization has complicated effects on C- and N-metabolism in plants (Stitt and Krapp 1999; Stitt 1999), sufficient N-supply is generally observed to raise the yield and increase the period of flourish (Cooke et al. 2005).

1.3 Why do we need FACE to test the responses of plants to elevated CO_2?

In order to elucidate the controversial question of whether the global biota is a source or a sink of carbon, numerous investigations about the interactions between elevated $[CO_2]$ and plants, especially forest trees, were carried out around the world in the past few decades. The influences of elevated $[CO_2]$ on the physiology and development of plants, including forest trees, have been well addressed in many of these studies and intensively reviewed (Stitt 1991; Drake et al. 1997; Norby et al. 1999; Long et al. 2004; Ainsworth and Long 2005). Most information about plant responses to elevated $[CO_2]$ is derived from experimental studies employing greenhouses, artificially illuminated controlled environment chambers, and open top chambers (OTCs). Due to the size limitations of these systems, most of these studies focused on the early stages of plants grown in pots. Arp (1991) showed that in pots rooting volume suppressed the response of plants to elevated $[CO_2]$, essentially demonstrating that loss of a response to increased $[CO_2]$ through acclimation was an artifact of pot size. Even large pots restricted the response of plants to elevated $[CO_2]$ (Ainsworth et al. 2002). To avoid "pot effect", some field studies were based on the use of OTCs. However, there are important differences between the environment within the best-engineered OTCs and the external environment. Thus, the effect of enclosure in the OTC without elevation of $[CO_2]$ may exceed that of any additional effect due to enrichment of $[CO_2]$ (Day et al. 1996). Additionally, small isolation plots in ecological experiments using OTCs are well known to overestimate biomass, production and yields (Roberts et al. 1993). For forests, the situation is even worse. OTCs can only accommodate one or two moderately sized trees, and therefore edge effects are likely extreme and natural canopy closure is prevented (Long et al. 2004). Furthermore, trees and forests are very well coupled to the atmosphere, and this coupling is greatly reduced when trees are enclosed in chambers, introducing an additional artifact (Lee and Jarvis 1996). The greater size of Free Air CO_2 Enrichment (FACE) plots (8-30 m diameter) in comparison with OTCs not only reduces edge effects but also for the first time, enables entire ecosystems to be treated and allows simultaneous study of many plant processes.

FACE systems have already allowed simultaneous study of leaf and canopy gas exchange, biochemical and molecular analysis of photosynthesis, secondary metabolism, leaf area and canopy development, above- and below-ground biomass production, shoot and root development, canopy energy balance, stem water flow, soil moisture, nutrient extraction, and final reproductive yield all within single treatment plots (McLeod and Long 1999). In summary, technical limitations of chamber studies have necessitated the employment of FACE technology to re-evaluate our hypotheses on plant responses to elevated $[CO_2]$.

Currently, full-size FACE systems are about 8-30 m diameter plots although miniFACE systems also exist (Long et al. 2004). Classical FACE plots are approximately circular and surrounded by a ring of pipes that release CO_2, or air enriched with CO_2, at vertical intervals from just above the ground surface to just above the top of the plant canopy. Wind direction, wind velocity, and $[CO_2]$ are measured at the center of each plot and this information is used by a computer-controlled system to adjust CO_2 flow rate, controlled by a mass-flow control valve, to maintain the target elevated $[CO_2]$, typically 550 ppm. With some variations and technical developments FACE systems have been employed in several experiments including studies of cotton, wheat, grassland and desert ecosystems, and forest and plantation trees (Long et al. 2004).

1.4 What is EUROFACE?

To study the functional responses of a cultivated, agroforestry system, namely a plantation of three poplar species, *Populus × euramericana*, *P. alba*, and *P. nigra*, to current and future atmospheric $[CO_2]$, the project—EUROFACE was initiated. The experiment is located in central Italy (Tuscania, 42°22' N, 11°48' E, altitude 150 m) and aims at investigating the responses of a short rotation of poplar coppice to elevated $[CO_2]$ and N-fertilization (Fig. 2). In spring 1999, on 9 ha of land, six experimental plots with homogenous soil and microclimatic conditions were selected and on the surrounding land *P. × euramericana* (Dode) Guinier (clone I-214) was planted at a planting density of 5,000 trees per ha (2 m × 1 m). Each experimental plot was divided into halves by a physical resin-glass barrier (1 m deep in the soil) in each half plot. Each half plot was further divided into three slices (subplots), planted with one of three poplar clones, *P. alba* L. (2AS-11), *P. × euramericana* (Dode) Guinier (I-214), and *P. nigra* L. (Jean Pourtet), at a planting density of 10,000 trees per ha (1 m ×1 m). The main characteristics of the poplar clones used in this experiment are shown in Table 1. In the FACE plots, elevated $[CO_2]$ was

provided by using Free Air CO_2 Enrichment technology. In the year 2001, the trees reached heights of 8.5–9.3 m (Calfapietra et al. 2003) and all trees were cut at the base of the stem at 5–8 cm above the ground. As a result, secondary sprouts developed from the stools in the spring of 2002. Free Air CO_2 Enrichment was continued in FACE plots in subsequent years. In addition, half of each experimental plot was fertilised in subsequent years. The EUROFACE site was managed according to agroforestry practices (Garrett and Buck 1997). A typical plantation management included continuous irrigation, mechanical herb removal and a limited application of insecticides.
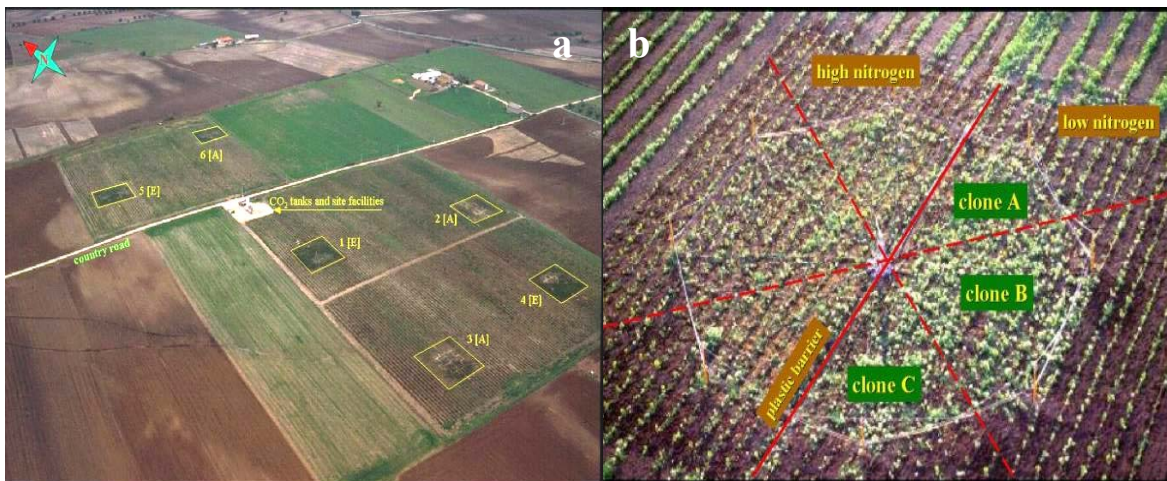
Fig. 2 Six experimental plots (a) which consisted of 3 FACE plots ([E]) and 3 control plots ([A]); with a plastic barrier 1 m deep in the soil, each ring (b) was divided into two subplots treated with high nitrogen and low nitrogen. Each subplot was divided into three slices planted with three poplar clones. Clone A = *P. alba*, clone B = *P. nigra* and clone C = *P. × euramericana*. (Source of photos: http://www.unitus.it/euroface/). In the FACE plots, the horizontal pipes formed an octagon with a diagonal of 22.2 m. The minimum distance between the plots was 120 m. A meteorological station located at the center of each FACE plot was used to control the release of CO_2, whose amount was determined by wind direction and speed and by an algorithm developed for the FACE facility according to a 3-D gas dispersion model. The FACE system was controlled and monitored by a computer to reach the target [CO_2]. The liquid CO_2 was stored in two containers with a maximum capacity of 50 tons.

Table 1 Main characteristics of the poplar genotypes in the EUROFACE experiment. * seed origin, ** origin of the selected hybrid, *** indicative dates for central Italy. Data were modified from Calfapietra et al. (2001).

Genotype name	2AS11	Jean Pourtet	I-214
Species name	*P. alba* L.	*P. nigra* L.	*P. × euramericana* Dode (Guinier)
Sex	Male	Male	Female
Origin	Italy*	France*	Italy**
Rooting	Medium	Very good	Very good
Branching habit	Medium	Very high	Low
Apical control	Good	Good	Very good
Bud burst***	End Mar.	End Mar.	End Mar.
Bud set***	End Oct.	Begin Oct.	Middle Sep.

The overall goals of EUROFACE are (i) to develop remote sensing technology to assess water consumption and energy balance of plantation forestry systems under present and future climatic conditions; (ii) to evaluate how much carbon is being sequestered in the biomass and in the soil of intensive bio-energy forest plantations, in relation to different management regimes (coppicing vs. single-stems, fertilization, species choice); (iii) to discriminate the effect of increasing atmospheric levels of [CO_2], and of N-fertilization from direct-human induced additional C-sequestration in surplus arable land being planted with woody crops.

Pure CO_2 (Messer Griesheim) was released through laser-drilled holes in the polyethylene pipes to achieve the target [CO_2] — 550 ppm inside the FACE plots. Daytime CO_2 enrichment was provided from bud burst to leaf fall (from April 1 to November 15, 228 days, 12 hours per day as mean values over the period). The mean CO_2 concentrations in the FACE plots were: 554 ± 1.6 ppm during the first year after coppice (2002), and 535.9 ± 20.4 ppm during the second year after coppice (2003). The elevated CO_2 concentrations, measured at 1-min intervals, were within 20% deviation from the target concentration for 89.4 and 72.2% of the time for the first and the second year after coppice, respectively. No continuous measurements of CO_2 concentration in the control plots were available for the first and the second year after coppice, but the ambient [CO_2] was about 370 ppm (Calfapietra, personal communication).

The total amount of nitrogen supplied was 212 kg ha^{-1} y^{-1} in 2002 and 290 kg ha^{-1} y^{-1} during 2003. The nitrogen was supplied in constant weekly amounts with a 4:1 NH_4^+:NO_3^-

ratio in 2002, whereas it was supplied in weekly amounts proportional to the growth rate with a 1:1 NH_4^+:NO_3^- ratio in 2003. Each experimental plot was equipped with a plastic 200 L tank where the fertilizer was solubilized and a hydraulic pump (Ferti-injector Amiad, IMAGO srl, Italy) connected to the irrigation system. Only half of each experimental plot was fertilized during the growing season.

At the EUROFACE experimental site, the soil was heavy loam agricultural soil, more than 1 meter deep, originated from a geological substrate derived from sedimentary material of volcanic origin and marine deposits. A soil survey was carried out in November 1998, before the plantation. Sixteen samples were collected in each plot (96 samples as total). Mean values of the top layer (0-20 cm) soil characteristics are shown in Table 2.

At the EUROFACE experimental site, mean daily maximum temperature of the warmest month was 38.8 °C and mean daily minimun temperature of the coldest month was 1.2 °C. Annual average of temperature was 14.1 °C and annual precipitation was 818 mm.

The experimental plantations were drip irrigated during the summer to avoid drought stress. Total water supplied was according to the estimated evapo-tranpiration rates.

Table 2 Mean values (SD) of the top layer (0-20 cm) soil characteristics at the EUROFACE site. Data were provided by Hoosbeek – University of Wageningen.

Soil texture	Silt loam
stone content (%; fraction > 2mm)	1.93 (0.97)
pH (KCl)	5.04 (0.22)
total C (%)	0.98 (0.20)
total N (%)	0.12 (0.02)
C/N ratio	8.00 (1.01)
bulk density (g/cm3)	1.34 (0.09)

1.5 What is known and what is unknown about the responses of plants to elevated CO_2 and N-fertilization?

Our understanding of the effects of increase in [CO_2] on plants has progressed with the development of technologies by which CO_2 enrichment was achieved. Studies on how plants respond to future [CO_2] began about 30 years ago, initially with glasshouses to realize elevated [CO_2], followed by open top chambers (OTCs), natural CO_2 spring and recently by Free Air Carbon dioxide Enrichment (FACE). With the evolution of the technologies by which elevated [CO_2] was achieved, the results obtained in earlier studies

were checked, corrected and the responses of plants to elevated $[CO_2]$ were better understood. Employing a meta-analysis based on data from 120 manuscripts describing physiology and production in the 12 large-scale FACE experiments ($[CO_2]$ range: 475-600 ppm), Ainsworth and Long (2005) found a 31% increase in light-saturated photosynthetic rate and a 28% increase in the diurnal photosynthetic carbon assimilation by FACE. This stimulation was lower than the 53% average increase in photosynthesis across 50 greenhouse and OTC studies reviewed by Curtis and Wang (1998). Ainsworth and Long (2005) also observed that FACE stimulated the overall yield by 12%, again a much lower stimulation compared to the 32% average increase in yield in greenhouse and OTC studies (Curtis and Wang 1998). One largely unanswered question in forest ecosystems is whether biomass production would be increased along with the increase in photosynthesis caused by elevated $[CO_2]$ (Karnosky 2003). This question was partly answered by the meta-analysis of Ainsworth and Long (2005), revealing greater carbon allocation to wood and structure in woody plants and a 28% increase in above-ground dry matter production for trees grown under elevated $[CO_2]$. In an earlier meta-analysis, Long et al. (2004) found that FACE decreased tissue quality with respect to protein, including decreases in Rubisco content and N content of leaves but a near doubling of leaf starch content.

The influences of elevated $[CO_2]$ on the physiology and development of plants, including forest trees, were intensively reviewed (Stitt 1991; Drake et al. 1997; Norby et al. 1999; Long et al. 2004; Ainsworth and Long 2005). However, the effects of CO_2 enrichment on the structure and quality of plants and carbon allocation to long- and short-term carbon pools in plants are not clear yet (Pritchard et al. 1999; Luo et al. 2005a, b). How does elevated $[CO_2]$ affect the wood quality and carbon allocation to long- and short-term pools in the wood of forests? This question is particularly important because forests contained more than 90% of the carbon of earth's living organisms (Geider et al. 2001). Besides the ecological importance of forests, wood is also economically important for human beings. For instance, wood from forest trees has provided structural materials and energy for human beings for a long time. Wood quality is also extremely important for pulp and paper industry because fibre properties, an important aspect of wood quality, affect the quality of final products. With respect to sustainable development, wood quality is also important for forest health because carbon-based secondary compounds, the biochemical aspects of wood quality, such as lignin, phenolics and tannins, affect the ability of forest trees to resist the attacks of pathogen and insects. Unfortunately, in the literature few

studies addressed wood quality in response to elevated [CO_2], especially to FACE. For instance, in a growth chamber experiment, Atkinson and Tayhlor (1996) found that elevated [CO_2] significantly increased both vessel number and mean vessel size of *Quercus* seedlings, but had no influence on the vessel number and size of *Prunus* seedlings. In *Pinus radiata* CO_2 enrichment caused no differences in tracheid length, lumen diameter or wall thickness (Donaldson et al. 1987). In contrast to these results, Conroy et al. (1990) reported that tracheid wall thickness increased by 44% in *Pinus radiata*, whereas Yazaki et al. (2001) found reduced wall thickness in *Larix sibirica* under elevated [CO_2]. Ceulemans et al. (2002) found that the wood of *Pinus sylvestris* grown at elevated [CO_2] contained significantly increased numbers of tracheids, tracheid diameters, and annual ring widths. It is also unclear how carbon-based secondary compounds of wood respond to rising [CO_2]. For instance, in beech with high nutrient supply, lignin concentration decreased in response to elevated [CO_2] (Blaschke et al. 2002), but no significant effects of elevated [CO_2] on wood lignin in other species were observed (Runion et al. 1999; Atwell et al. 2003; Kilpelainen et al. 2003).

The rising atmospheric [CO_2] is resulting in global warming, which causes potentially devastating consequences (IPCC 2001). To alleviate global warming, mankind is making efforts to stabilize the atmospheric [CO_2] by limiting emission of greenhouse gases, conserving carbon stocks in the wood biomass and expanding forest areas to remove carbon from the atmosphere (IPCC 2001; Malhi *et al.* 1999). Among these efforts, to conserve carbon fixed in long-term pools (such as cellulose, hemicelluloses and lignin in the wood of forests) is particularly important. Within a tree, these long-term carbon stocks are the result of complex metabolic pathways. Thus, it is important to evaluate carbon allocation during these metabolic processes and to quantify the carbon finally allocated to these long-term pools (Luo et al. 2005b).

A few reports suggested that N-fertilization increases in the growth rate and results in higher yields and reduces the rotation age in short-rotation woody crops (Brown et al. 1996; Tuskan 1998; Sedjo 2001; Cooke et al. 2005). However, impacts of high nitrogen supply on wood quality and carbon allocation in the wood were questioned (Shupe et al. 1996; Pape 1999). Several studies showed that fertilization enhanced tracheid lumen diameter, decreased cell wall thickness and the basic density of wood (Brolin et al. 1995; Lindström 1996). The results of Yang et al. (1988) and Dutilleul et al. (1998) indicated that

increased nutrient availability might decrease fibre length; however, contradictory results were also reported (Schmidtling 1973; Zobel and Van Buijtenen 1989).

Importantly, most of above results about wood quality responding to rising elevated [CO_2] and N-fertilization were obtained from plants grown under greenhouse and/or chamber conditions. Due to the limitations of these controlled conditions, e.g. change of temperature and microclimate, and limited dimensions, it is hard to scale up these results to the ecosystem level. Furthermore, most of these results were derived from investigations of conifer species. However, it would also be extremely important for us to know how anatomical and biochemical properties might respond to elevated [CO_2] in broadleaf species, which have different properties in comparison with conifers.

Along with the evolution of studies about effects of elevated [CO_2] on plants, the rapid pace of genomics research has energized the entire community of biologists, and genomics techniques and information are being applied in an increasing number of research fields. One emerging area is ecosystem genomics, which promises substantial breakthroughs in ecology and environmental sciences. Although it is still under debate whether forests will be sinks for atmospheric CO_2 in the coming CO_2-enriched world (Phillips et al. 1998; Janssens et al. 2003; Körner et al. 2005), most sceintists agree that forests have already shown their own adaptation to the envrionmentally changing world (e.g. Long et al. 2004; Ainsworth and Long 2005). Recently, Taylor et al. (2005) have shown the genetic adaptation in young and semimature leaves of *Populus* to elevated [CO_2]. In that study, they found some of the most responsive transcripts to elevated [CO_2] and some of these transcripts involved in regulation of C-metabolism and signalling. Wood quality and carbon partitioning in the wood are due to the function of the genes in forest trees. Obviously, these genes need to be investigated to gain knowledge of processes involved in determination of woody quality and carbon partitioning in the wood. The regulation of genes in response to developmental and environmental factors is probable to determine variations in wood quality and channels of C-allocation. At the molecular level, what happens to the wood quality and C-allocation in response to environmental changes? To date, however, little information about this ecological genomics question is available. Transcript profiling holds great promises to unravel the evolutionary and ecological functional genomics because it may provide insights into candidate genes that may be involved in the control of ecological traits (Feder and Mitchell-Olds 2004). At the transcript level, what candidate genes may involve in the processes of the control of wood quality and

carbon allocation in the wood in response to elevated $[CO_2]$ and N-fertilization? Wood is derived from the vascular cambium. Therefore, transcript profiling in cambial tissues may shed light on this question. To our knowledge, however, no data derived from large scale of transcript profiling in cambial materials have been reported previously for tree ecosystems exposed to FACE and N-fertilization.

1.6 What are questions addressed in this study?

To shed light on above questions, in this study, we employ the EUROFACE facilities to achieve the following goals: (i) to characterise wood quality and structure in response to FACE and N-fertilization; (ii) to quantify carbon allocation between short- and long-term pools in the wood in response to FACE and N-fertilization; (iii) to characterise carbon-based secondary compounds responding to FACE and N-fertilization; and (iv) to preliminarily elucidate the molecular mechanism of wood quality responding to FACE and N-fertilization.

To achieve above goals and specifically answer above questions, the following chapters will be treated independently. In the chapter of "*Anatomical properties of secondary xylem in poplar*", wood quality and structure of juvenile wood obtained from EUROFACE site were analysed by microscopic technique and image analysis. In both chapters of "*Carbon partitioning to mobile and structural fractions in poplar*" and "*Carbon-based secondary metabolites and internal N pools in poplar*", by employment of biochemical methods and allometric relationships, carbon allocation and carbon-based secondary compounds in the wood of poplar responding to FACE and N-fertilization were extensively studied. The potentials and implications of forest carbon sequestration were discussed. Finally, in the chapter of "*Wood properties and gene expression profiling in poplar*", gene expression profiling was conducted in the differentiating xylem and a few candidate genes which were closely related to wood quality, carbon allocation and secondary compounds in the xylem of poplar were found to be differentially expressed under FACE and/or N-fertilization conditions.

References

Ainsworth EA, Davey PA, Bernacchi CJ, Dermody OJ, Heaton EA et al. (2002) A meta-analysis of elevated [CO_2] effects on soybean (Glycine max) physiology, growth and yield. Global Change Bioliolgy, 8, 695-709.

Ainsworth EA, Long SP (2005) What have we learned from 15 years of free-air CO_2 enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy properties and plant production to rising CO_2. New Phytologist, 165, 351-372.

Arp WJ (1991) Effects of source-sink relations on photosynthetic acclimation to elevated CO_2. Plant Cell and Environment, 14, 869-875.

Atkinson CJ, Tayhlor JM (1996) Effects of elevated CO_2 on stem growth, vessel area and hydraulic conductivity of oak and cherry seedlings. New Phytologist, 133, 617-626.

Atwell BJ, Henery ML, Whitehead D (2003) Sapwood development in *Pinus radiata* trees grown for three years at ambient and elevated carbon dioxide partial pressures. Tree Physiology, 23, 13-21.

Blaschke L, Forstreuter M, Sheppard LJ et al. (2002) Lignification in beech (*Fagus sylvatica*) grown at elevated CO_2 concentrations: interaction with nutrient availability and leaf maturation. Tree Physiology, 22, 469-477.

Brolin A, Noren A, Stähle E (1995) Wood and pulp characteristics of juvenile Norway spruce: a comparison between a forest and an agricultural stand. Journal of Tappi, 78, 203-214.

Brown KR, Thompson WA, Weetman GF (1996) Effects of N addition rates on the productivity of *Picea sitchensis*, *Thuja plicata*, and *Tsuga heterophylla* seedlings. Trees, 10, 189-197.

Calfapietra C, Gielen B, Sabatti M, Angelis PD, Mugnozza GS, Ceulemans R (2001) Growth performance of *Populus* exposed to "Free Air Carbon Dioxide Enrichment" during the first growing season in the POPFACE experiment. Annals of Forest Science, 58, 819-828.

Calfapietra C, Gielen B, Sabatti M, Angelis PD, Miglietta F, Mugnozza GS, Ceulemans R (2003) Do above-ground growth dynamics of poplar change with time under CO_2 enrichment? New Phytologist, 160, 305-318.

Ceulemans R, Jach ME, Velde RVD, Lin JX, Stevens M (2002) Elevated atmospheric CO_2 alters wood production, wood quality and wood strength of Scots pine (*Pinus sylvestris* L) after three years of enrichment. Global Change Biology, 8, 153-162.

Conroy JP, Milham PJ, Mazur M, Barlow EWR (1990) Growth, dry matter partitioning and wood properties of *Pinus radiata* D. Don. after 2 years of CO_2 enrichment. Plant Cell and Environment, 13, 329-337.

Cooke JEK, Martin TA, Davis JM (2005) Short-term physiological and developmental responses to nitrogen availability in hybrid poplar. New Phytologist, 167, 41-52.

Curtis PS, Wang XZ (1998) A meta-analysis of elevated CO_2 effects on woody plant mass, form, and physiology. Oecologia, 113, 299-313.

Dambrine E, Martin F, Carisey N, Granier A (1995) Xylem sap composition: a tool for investigating mineral uptake in adult spruce. Plant and Soil, 168-169, 233-241.

Day FP, Weber EP, Hinkle CR, Drake BG (1996) Effects of elevated atmospheric CO_2 on fine root length and distribution in an oak-palmetto scrub ecosystem in central Florida. Global Change Biology, 2, 143-148.

Delwiche CC (1970) The nitrogen cycle. Scientific American, 223, 137-146.

Donaldson LA, Hollinger D, Middleton TM, Souter ED (1987) Effect of CO_2 enrichment on wood structure in *Pinus radiata* D.Don. IAWA Bull New Series, 8, 285-289.

Drake BG, Gonzalez-Meler MA, Long SP (1997) More efficient plants: A consequence of rising atmospheric CO_2? Annual Review of Plant Physiology and Plant Molecular Biology, 48, 609-639.

Dutilleul P, Herman M, Avella ST (1998) Growth rate effects on correlations among ring width, wood density, and mean tracheid length in Norway spruce (*Picea abies*). Canadian Journal of Forest Research, 28, 56-68.

Feder ME, Mitchell-Olds T (2004) Evolutionary and ecological functional genomics. Nature Reviews Genetics, 4, 649-655.

Galloway JN, Schlesinger WH, Levy II H, Michaels A, Schnoor JL (1995) Nitrogen fixation: atmospheric enhancement — environmental response. Global Biogeochemical Cycles, 9, 235-252.

Garrett HEG, Buck L (1997) Agroforestry practice and policy in the United States of America. Forest Ecology and Management, 91, 5-15.

Geider RJ, Delucia EH, Falkowski PG, Finzi AC, Grime JP et al. (2001) Primary productivity of planet earth: biological determinants and physical constraints in terrestrial and aquatic habitats. Global Change Biology, 7, 849-882.

Geßler A, Schneider S, Weber P, Hanemann U, Rennenberg H (1998) Soluble N compounds in trees exposed to high loads of N: a comparison between the roots of Norway spruce (*Picea abies*) and beech (*Fagus sylvatica*) trees. New Phytologist, 138, 385-399.

Geßler A, Kopriva S, Rennenberg H (2004) Regulation of nitrate uptake at the whole-tree level: interaction between nitrogen compounds, cytokinins and carbon metabolism. Tree Physiology, 24, 1313-1321.

Gielen B, Ceulemans R (2001) The likely impact of rising atmospheric CO_2 on natural land and managed *Populus*: a literature review. Environment Pollution, 115, 335-358.

Gojon A, Plassard C, Bussi C (1994) Root/shoot distribution of NO_3^- assimilation in herbaceous and woody plants. *In* A Whole Plant Perspective on Carbon-N Interactions. Eds. Roy J and Garnier E. SPB Academic Publishing, The Hague, pp 131-147.

Hedin LO, Armesto JJ, Johnson AH (1995) Patterns of nutrient loss from unpolluted, old-growth temperate forests: evuation of biogechemical theory. Ecology, 76, 493-509.w

Hoff T, Truong HN, Caboche M (1994) The use of mutans and transgenic plants to study nitrate assimilation. Plant Cell and Environment, 17, 489-506.

IPCC (2001) Climate change 2001. Cambridge University Press, Cambridge.

Janssens IA, Freibauer A, Ciais P et al. (2003) Europe's terrestrial biosphere absorbs 7 to 12% of European anthropogenic CO_2 emissions. Science, 300, 1538-1542.

Karnosky DF (2003) Impacts of elevated atmospheric CO_2 on forest trees and forest ecosystems: knowledge gaps. Environmental International, 29, 161-169.

Kilpelainen A, Peltola H, Ryyppo A et al. (2003) Wood properties of Scots pines (*Pinus sylvestris*) grown at elevated temperature and carbon dioxide concentration. Tree Physiology, 23, 889-897.

Körner C, Asshoff R, Bignucolo O et al. (2005) Carbon flux and growth in mature deciduous forest trees exposed to elevated CO2. Science, 309, 1360-1362.

Lee HSJ, Jarvis PG (1996) The effects of tree maturity on some responses to elevated CO_2 in Sitka Spruce (*Picea sitchensis* Bong. Carr.). In *Carbon Dioxide and Terrestrial Ecosystems*, ed. GW Koch, HA Mooney, pp. 5370 San Diego: Academic.

Lemon ER (1983) CO_2 and plants. Boulder, CO, USA: Westview Press.

Lindström H (1996) Basic density in Norway spruce. I. A literature review. Wood and Fiber Science, 28, 15-27.

Long SP, Ainsworth EA, Rogers A, Ort DR (2004) Rising atmospheric carbon dioxide: plants FACE the future. Annual Review of Plant Biology, 55, 591-628.

Luo Z-B, Langenfeld-Heyser R, Calfapietra C, Polle A (2005a) Influence of free air CO_2 enrichment (EUROFACE) and nitrogen fertilization on the anatomy of juvenile wood of three poplar species after coppicing. Trees, 19, 109-118.

Luo Z-B, Calfapietra C, Liberloo M, Scarascia-Mugnozza G, Polle A (2005b) Carbon partitioning to mobile and structural fractions in poplar wood under elevated CO_2 (EUROFACE) and N-fertilization. Global Change Biology (in press)

Malhi Y, Baldocchi DD, Jarvis PG (1999) The carbon balance of tropical, temperate and boreal forests. Plant Cell and Environment, 22, 715-740.

McLeod AR, Long SP (1999) Free-air carbon dioxide enrichment (FACE) in Global Change Research: A review. Advances in Ecological Research, 28, 1-55.

Medlyn BE, Badeck FW, De Pury DGG, Barton CVM, Broadmeadow M, Ceulemans R, De Angelis P, Forstreuter M, Jach ME, Kellomäki S, Laitat E, Marek M, Philippot S, Rey A, Strassemeyer J, Laitinen K, Liozon R, Portier B, Roberntz P, Wang K, Jarvis PG (1999) Effects of elevated [CO_2] on photosynthesis in European forest species: a meta-analysis of model parameters. Plant Cell and Environment, 22, 1475-1495.

Miglietta F, Peressotti A, Vaccari FP, Zaldei A, Angelis PD, Scarascia-Mugnozza G (2001) Free-air CO_2 enrichment (FACE) of a poplar plantation: the POPFACE fumigation system. New Phytologist, 150, 465-476.

Moreson JIL (1998) Stomatal response to increased CO_2 concentration. Journal of Experimental Botany, 49, 443-452.

Norby RJ, Wullschleger SD, Gunderson CA, Johnson DW, Ceulemans R (1999) Tree responses to rising CO_2 in field experiments: implications for the future forest. Plant Cell and Environment, 22, 683-714.

Pape R (1999) Effects of thinning regime on the wood properties and stem quality of *Picea abies*. Scandinavian Journal of Forest Research, 14, 38-50.

Phillips OL, Malhi Y, Higuchi N et al. (1998) Changes in the carbon balance of tropical forest: evidence from long-term plots. Science, 282, 439-442.

Pritchard SG, Rogers HH, Prior SA, Peterson CM (1999) Elevated CO_2 and plant structure: a review. Global Change Biology, 5, 807-837.

Roberts MJ, Long SP, Tieszen LL, Beadle CL (1993) Measurement of plant biomass and net primary production of herbaceous vegetation. In *Photosynthesis and Production in a Changing Environment: A Field and Laboratory Manual*, ed. DO Hall, JMO Scurlock, HR Bolhàr-Nordenkampf, RC Leegood, SP Long, pp. 121 London: Chapman & Hall.

Runion GB, Entry JA, Prior SA et al. (1999) Tissue chemistry and carbon allocation in seedlings of *Pinus palustris* subjected to elevated atmospheric CO_2 and water stress. Tree Physiology, 19, 329-335.

Saxe H, Ellsworth DS, Heath J (1998) Tree and forest functioning in an enriched CO_2 atmosphere. New Phytologist, 139, 395-436.

Schmidt S, Stewart GR (1998) Transport, storage and mobilization of nitrogen by trees and shrubs in wet/dry tropics of northern Australia. Tree Physiology, 18, 403-410.

Schmidtling RC (1973) Intensive culture increases growth without affecting wood quality of young southern pines. Canadian Journal of Forest Research, 3, 565-573.

Sedjo RA (2001) From foraging to cropping: the transition to plantation forestry and implications for wood supply and demand. Unasylva, 52, 24-32.

Shupe TF, Choong ET, Yang CH (1996) The effects of silvicultural treatments on the chemical composition of plantation-grown loblolly pine wood. Wood and Fiber Science, 28, 295-300.

Stitt M (1991) Rising CO_2 levels and their potential significance for carbon flow in photosynthetic cells. Plant Cell and Environment, 14, 741-762.

Stitt M (1999) Nitrate regulation of metabolism and growth. Current Opinion in Plant Biology, 2, 178-186.

Stitt M, Krapp JA (1999) The molecular physiological basis for the interaction between elevated carbon dioxide and nutrients. Plant Cell and Environment, 22, 583-622.

Taylor G, Street NR, Tricker PJ, Sjödin A, Graham Laura, Skogström O, Calfapietra C, Scarascia-Mugnozza G and Jansson S (2005) The transcriptome of *Populus* in elevated CO_2. New Phytologist, 167, 143-154.

Tilman D, Fargione J, Wolff B, D'Antonio C, Dobson A, Howarth R, Schindler D, Schlesinger WH, Simberloff D, Swackhamer D (2001) Forecasting agriculturally driven global environmental change. Science, 292, 281-284.

Tuskan GA (1998) Short-rotation woody crop supply systems in the United States: what do we know and what do we need to know? Biomass and bioenergy, 14, 307-315.

Vitousek PM, Aber JD, Howarth RW, Likens GE, Matson PA, Schindler DW, Schlesinger WH, Tilman DG. (1997) Human alteration of the global nitrogen cycle: sources and consequences. Ecological Application, 7, 737-750.

Wright RF, van Breeman N (1995) The NITREX project: an introduction. Forest Ecology and Management, 71, 1-6.

Yang RC, Wang EIC, Micko MM (1988) Effects of fertilization on wood density and tracheid length of 70-year-old lodgepole pine in west-central Alberta. Can J For Res 18: 954-956

Yazaki K, Funada R, Mori S, Maruyama Y, Abaimov A, Kayama M, Koike T (2001) Growth and annual ring structure of *Larix sibirica* grown at different carbon dioxide concentrations and nutrient supply rates. Tree Physiology, 21, 1223-1229.

Zobel BJ, Van Buijtenen JP (1989) Wood Variation: Its Causes and Control. Springer, Berlin. Heidelberg. New York.

2. Anatomical properties of secondary xylem in poplar

Zhi-Bin Luo, Rosemarie Langenfeld-Heyser, Carlo Calfapietra and Andrea Polle (2005) Influence of free air CO_2 enrichment (EUROFACE) and nitrogen fertilization on the anatomy of juvenile wood of three poplar species after coppicing. **Trees,** 19, 109-118.

Abstract

Populus × euramericana, *P. alba*, and *P. nigra* clones were exposed to ambient or elevated (about 550 ppm) CO_2 concentrations ($[CO_2]$) under field conditions (FACE) in central Italy. After three growing seasons, the plantation was coppiced. FACE was continued and in addition, one-half of each experimental plot was fertilised with nitrogen. Growth and anatomical wood properties were analysed in secondary sprouts. In the three poplar clones, most of the growth and anatomical traits showed no uniform response pattern to elevated $[CO_2]$ or N-fertilization. In cross-sections of young poplar stems, tension wood amounted to 2–10% of the total area and was not affected by elevated $[CO_2]$. In *P. nigra*, N-fertilization caused an about twofold increase in tension wood, but not in the other clones. The formation of tension wood was not related to diameter or height growth of the shoots. In *P. × euramericana*, N-fertilization resulted in significant reductions in fibre lengths. In all three genotypes, N-fertilization caused significant decreases in cell wall thickness. In *P. × euramericana* and *P. alba*, elevated $[CO_2]$ also caused decreases in wall thickness, but less pronounced than nitrogen. In *P. nigra* and *P. × euramericana*, elevated $[CO_2]$ induced increases in vessel diameters. These results show that elevated $[CO_2]$ and N-fertilization affect wood structural development in a clone specific manner. However, the combination of these environmental factors resulted in overall losses in cell wall area of 5–12% in all three clones suggesting that in future climate scenarios negative effects on wood quality are to be anticipated if increases in atmospheric CO_2 concentration were accompanied by increased N availability.

2.1 Introduction

Forest ecosystems cover 43% of the terrestrial biosphere (Melillo et al. 1993). During the past century human activities, such as combustion of fossil fuels, deforestation, wide application of nitrogen-containing fertilisers, etc., have resulted in a dramatic increase in the atmospheric CO_2 concentration and enhanced nitrogen deposition (Huang et al. 1999). Increased $[CO_2]$ is expected to increase biomass accumulation and net primary productivity of forest ecosystems (Melillo et al. 1993; Wullschleger et al. 1995; Gielen and Ceulemans 2001; Calfapietra et al. 2003a). Increasing nitrogen deposition also has been observed to stimulate wood production (Brix 1981; McGuire et al. 1992, 1993).

Despite compelling evidence that these environmental factors can result in enhanced aboveground biomass and increased annual ring width (Hättenschwiler et al. 1996; Telewski et al. 1999; Yazaki et al. 2001; Ceulemans et al. 2002; Mäkinen et al. 2002), there is no clear picture how CO_2 and nitrogen affect the anatomical properties of wood. For example, Atkinson and Taylor (1996) found that elevated $[CO_2]$ significantly increased both vessel number and mean vessel size of *Quercus* seedlings, but had no influence on the vessel number and size of *Prunus* seedlings. In *Pinus radiata* CO_2 enrichment caused no differences in tracheid length, lumen diameter or wall thickness (Donaldson et al. 1987). In contrast to these results, Conroy et al. (1990) reported that tracheid wall thickness increased by 44% in *Pinus radiata*, whereas Yazaki et al. (2001) found reduced wall thickness in *Larix sibirica* under elevated $[CO_2]$. Ceulemans et al. (2002) found that the wood of *Pinus sylvestris* grown at elevated $[CO_2]$ contained significantly increased numbers of tracheids, tracheid diameters, and annual ring widths, whereas wood density remained unchanged and wood strength decreased.

Although high nitrogen increased the growth rate and resulted in higher stem wood production, impacts of high nitrogen supply on wood quality were questioned (Shupe et al. 1996; Pape 1999). Several studies have shown that fertilization enhanced tracheid lumen diameter, decreased cell wall thickness and the basic density of wood (Brolin et al. 1995; Lindström 1996). The results of Yang et al. (1988) and Dutilleul et al. (1998) suggest that increased nutrient availability might decrease fibre length; however, contradictory results were also reported (Schmidtling 1973; Zobel and Van Buijtenen 1989).

Obviously, these contrasting data demand further investigations before general conclusions about the influence of enriched $[CO_2]$ and nitrogen on wood structure and

quality can be drawn (Saxe et al. 1998; Pritchard et al. 1999; Ward and Strain 1999; Ceulemans et al. 2002). With respect to these uncertainties it is important to analyse the wood of trees exposed to elevated [CO_2] and additional nitrogen fertilization under field conditions and to compare the CO_2 responses in different species to find out whether the responsiveness to CO_2 depends strongly on the genetic background or not.

The objective of the present study was to investigate the impact of elevated [CO_2] and N-fertilization on wood structure and quality of field-grown trees. For this purpose the study was conducted at the EUROFACE field site, where poplars have been grown under ambient and elevated [CO_2] of about 550 ppm since 1999. In 2001 the trees had reached heights of 8.5–9.3 m (Calfapietra et al. 2003b) and the plantation was cut. Secondary sprouts developed from the stools in 2002. We used this experimental approach to address the following questions: do elevated [CO_2] and nitrogen as single factors or in combination: (1) affect tension wood formation or is tension wood formation mainly related to growth characteristics (radial growth, height), (2) affect the structure of normal wood, and (3) affect the anatomical properties of wood elements (fibre lumen, vessel lumen, length, etc.)? Since the EUROFACE field site contains three different poplar clones (*P. alba*, *Populus × euramericana* and *P. nigra*) with different growth characteristics, the results of this study can furthermore contribute to elucidate whether the responsiveness of certain traits to [CO_2] and nitrogen enrichment is uniform or is genotype-dependent.

2.2 Materials and methods

2.2.1 *Site description*

The study site is located in central Italy (42°22′ N, 11°48′ E, altitude 150 m) on 9 ha of former agricultural land. In spring 1999, following detailed soil analysis, six 30 m ×30 m experimental areas ("plots") were selected and FACE facilities were installed in three of the plots whereas the other three plots, representing the control treatment, were left under natural conditions. The minimum distance between plots is 120 m to avoid cross-contamination between FACE and control treatments. The CO_2 enrichment was realised through octagonal polyethylene rings (22 m diameter) mounted on telescopic poles. Pure CO_2 (Messer Griesheim) was released through laser-drilled holes in the polyethylene rings to achieve the target [CO_2] (550 µmol mol^{-1}) inside the FACE plots. A meteorological station located at the centre of each FACE plot was used to control the release of CO_2,

whose amount was determined by wind direction and speed and by an algorithm developed for the FACE facility according to a 3-D gas dispersion model. The FACE system was controlled and monitored by a computer to reach the target [CO_2]. In the FACE plots daytime [CO_2] was 554 ± 1.6 µmol mol^{-1} during the growing season of 2002 (from bud burst to leaf fall; F. Miglietta, CNR-IATA, Florence, Italy, unpublished data). A detailed description of FACE facilities was given by Miglietta et al. (2001).

2.2.2 *Plant material and plantation layout*

In spring 1999, on 9 ha of land six experimental plots with homogenous soil and microclimatic conditions were selected and on the surrounding land *P. × euramericana* (Dode) Guinier (clone I-214) was planted at a planting density of 5,000 trees per ha (2 m ×1 m). Each experimental plot was divided into halves by a physical resin-glass barrier (1 m deep in the soil) to provide N-fertilization in each half plot. Each half plot was further divided into three slices, planted with one of three poplar clones, *P. alba* L. (2AS-11), *P. × euramericana* (Dode) Guinier (I-214), and *P. nigra* L. (Jean Pourtet), at a planting density of 10,000 trees per ha (1 m ×1 m). A detailed description of the clone properties was given by Calfapietra et al. (2001). In 2001, the trees reached heights of 8.5–9.3 m (Calfapietra et al. 2003b) and all trees were cut to the base of the stem at 5–8 cm above the ground. As a result, secondary sprouts developed from the stools in the spring of 2002. During the growing season of 2002, Navarson (Amiad, Imago, Italy), a fertiliser with a 10:3:3 NPK content (nitrogen with a 4:1 NH_4^+: NO_3^- ratio) and micro-nutrients, dissolved in 200 l tanks, was applied once per week, starting July 8, for a period of 16 weeks, through hydraulic pumps installed outside plots and a drip-irrigation system. A total amount of 212 kg N ha^{-1} was supplied throughout the growing season.

2.2.3 *Sampling*

In the first week of September 2002, the first harvest was achieved by the following sampling strategy: two stools were marked randomly per subplot; on each marked stool, the shoot with the second thickest diameter, measured at the height of 20 cm above the stool, was harvested. The shoot with the third thickest diameter was marked for the following harvest in March 2003. A total of 72 shoots were harvested from the six experimental plots. Each selected shoot was cut at the stool level. Its height and biomass were determined. An 8 cm long stem segment was removed from the height of 1.92 m to 2.00 m of each shoot

and preserved for anatomical studies in FAE (37% formalin/glacial acetic acid/70% ethyl alcohol =5 parts/5 parts/90 parts) in wide-mouthed jars with lids. The sampling was repeated in March 2003.

2.2.4 *Analysis of wood anatomy*

Stem cross-sections (30 μm) were obtained with a sliding microtome (Reichert-Jung, Heidelberg, Germany) and mounted in 50% glycerol for microscopy. For an overview sections were viewed by fluorescence microscopy (Axioskop, Zeiss, Oberkochen, Germany) using the filter combination G365/FT395/LP420 to document autofluorescence. Sections were stained for 10 min with toluidine blue (pH 7.0, w/v=0.05%). Well-stained sections and a micrometer scale were photographed under a light microscope (Axioskop, Zeiss, Oberkochen, Germany) with a digital camera (Nikon CoolPix 990, Nikon, Tokyo, Japan) with 40× and 400× magnifications. In addition, photographs of stem cross-sections from cambium to pith were taken under a binocular (Stemi SV11, Zeiss, Oberkochen, Germany) with 6× magnification. A 2.5 cm part of the same stem segment was removed and dried at room temperature. Then its cross-sectional plane was polished, stained with zinc chloriodide (Purvis et al. 1966) which stained cellulose indicative for tension wood and photographed under a binocular with 6× magnification.

Microphotographs of normal wood were analysed by an image analysis software (analySIS 3.2, Soft Imaging System, Münster, Germany) for the following parameters: diameter of vessel and fibre lumina, thickness of double fibre wall (the wall between two adjacent fibre cells), areas of ray parenchyma cells, vessel lumina, and fibre lumina as well as the number of vessels per unit area. The unit area was set as a square-shaped area of 598,333 μm^2. The percentage of cell wall area (CWA) was calculated as follows:

CWA (%) = [total area – (vessel lumen area + fibre lumen area + ray parenchyma area)] × 100 / total area.

The xylem width was measured as the distance from the cambium to the pith. For tension wood determination, sections stained purple by zinc chloriodide in whole-stem cross-sections were measured. The percentage of tension wood area (TWA, portion of purple sections) was calculated as follows:

TWA (%) = tension wood area × 100 / (total area – pith area).

To determine the lengths of vessel elements and fibres, the same stem segments as those used for cross-sections were chosen. About 1 mm width of wood next to the cambium

was discarded to avoid young xylem cells and the residual wood was cut longitudinally into pieces for chemical maceration in 65% nitric acid (Merck, Darmstadt, Germany) and traces of sodium chlorate (Merck, Darmstadt, Germany) after Kitin et al. (1999). After 2 h of maceration at room temperature, the materials were transferred into 2mL tubes and centrifuged (3,200g, 2 min, Eppendorf Centrifuge). The pellets were washed with distilled water, centrifuged again, and then preserved in 70% ethanol for further analysis. The prepared cell mixture was stained with toluidine blue (pH 7.0, w/v=0.05%) and mounted in 50% glycerol for microscopy. The short lengths of vessel elements (the short distance between the perforations) were measured after the definition of Chalk and Chattaway (1934).

2.2.5 *Statistical analysis*

To determine the main effects of species (clone), CO_2 treatment (CO_2) and N-fertilization (nitrogen) on all variables, an ANOVA, i.e. a randomised-complete-block design, with species, CO_2 treatment, N-fertilization and their interactions as fixed factors and block as a random factor, was applied. All statistical tests were performed in Statgraphics (STN, St Louis, Mo., USA) using the mixed procedure and plot as a replicate. When interactions were significant, a posteriori comparison of means was done. To reduce the chance on type I errors, all *P*-values of these multi-comparisons were corrected by Tukey method. Data were tested for normality with the Shapiro–Wilk's test. Differences between parameter means were considered significant when the *P*-value of the ANOVA *F*-test was less than 0.05.

2.3 Results

2.3.1 *Growth and tension wood as affected by FACE and N-fertilization*

The heights and diameters of the second and third thickest shoot were measured in September, when the trees were still in the active phase of growth, and in March, when the trees were dormant. There were significant differences between the clones with *P. × euramericana* displaying tallest and thickest shoots and *P. alba* the shortest (Table 1). Exposure to FACE conditions caused increases in height and diameter of the selected shoots (Table 1, *P*-values for CO_2 main effect). Nitrogen fertilization had no significant influence as a main factor, perhaps because of the relatively short duration of the treatment (ca. 2 months before harvest in September). Only *P. × euramericana* shoots responded

positively with respect to diameter and height growth to N-addition (Table 1, significant *P*-value for the interaction of clone × nitrogen).

To find out whether clone-specific growth characteristics or growth stimulation by FACE or N-fertilization affected wood properties, we determined the occurrence of tension wood in stem cross-sections of *P. alba*, *P. nigra* and *P. × euramericana*. Tension wood formation was significantly dependent on the clone and in most cases lower in *P. × euramericana* than in the two other species (Table 1). The main factor CO_2 had no and nitrogen only in March significant effects on the proportion of tension wood formed (Table 1). To test whether correlations existed between tension wood and growth parameters, we performed linear correlation analysis between shoot height or diameter and the portion of tension wood area. No significant relationships were found (R^2=0.0006 for tension wood area vs diameter; R^2=0.0001 for tension wood area vs height). The shoot diameters were measured 0.2 m above ground, whereas the stem sections analysed for tension wood were collected about 2 m above the ground. To investigate the possibility that the tension wood area was related to the radial growth observed 2 m above ground, correlation analysis was also conducted for tension wood area and the xylem width of the wood slice used for anatomical studies (September 2002 samples only). Again no significant correlation was observed (R^2=0.0179).

Table 1 Relative tension wood area (TWA), shoot diameter (D) and shoot height (SH). A: ambient [CO_2], E: elevated [CO_2], L: no N-fertilization, H: N-fertilization. The fractional TWA was determined per whole cross sectional area. The whole cross sectional area (100 %) was defined as the total xylem cross sectional area minus pith area. The shoot diameter was determined at 0.2 m above stool level. 0902: harvest in September 2002; 0303: harvest in March 2003. Data indicate means (± SD, n = 6). The values followed by different letters in the same column indicate significant differences at P ≤ 0.05 for all treatments and species. *P*-values of the ANOVA *F*-test of the main effects of clone, CO_2, nitrogen and their interactions are also indicated.

Clones	CO_2	Nitrogen	TWA 0902 (%)	TWA 0303 (%)	D 0902 (mm)	D 0303 (mm)	SH 0902 (m)	SH 0303 (m)
P. alba	A	L	9.7 ± 4.4 c	2.1 ± 2.0 a	23.3 ± 5.1 abc	20.9 ± 4.3 abc	3.7 ± 0.5 abcd	3.6 ± 0.5 abcd
	E	L	4.3 ± 3.1 a	1.5 ± 1.7 a	23.3 ± 4.4 abc	18.9 ± 1.8 abc	3.5 ± 0.4 a	3.5 ± 0.4 abc
	A	H	8.9 ± 4.1 bc	3.3 ± 5.4 ab	21.5 ± 2.3 ab	17.7 ± 3.9 ab	3.7 ± 0.3 ab	3.3 ± 0.4 ab
	E	H	7.0 ± 3.2 abc	3.6 ± 4.7 ab	21.4 ± 2.5 ab	17.1 ± 3.1 ab	3.6 ± 0.3 a	3.5 ± 0.4 abc
P. x *euramericana*	A	L	5.4 ± 4.3 ab	3.6 ± 3.4 ab	26.8 ± 3.5 cde	21.0 ± 7.7 abc	4.0 ± 0.4 bcdef	3.3 ± 1.0 a
	E	L	3.8 ± 3.4 a	2.4 ± 3.8 a	28.6 ± 3.7 def	22.9 ± 5.2 c	4.1 ± 0.2 def	3.5 ± 0.5 abc
	A	H	3.6 ± 2.2 a	4.9 ± 2.2 ab	30.5 ± 3.6 ef	23.7 ± 5.3 c	4.4 ± 0.3 ef	3.9 ± 0.6 bcd
	E	H	4.4 ± 1.9 a	2.6 ± 1.5 a	32.6 ± 5.0 f	28.9 ± 3.1 d	4.4 ± 0.3 f	4.2 ± 0.3 d
P. nigra	A	L	4.2 ± 2.1 a	3.3 ± 2.5 ab	20.9 ± 5.9 ab	17.4 ± 3.3 ab	3.7 ± 0.6 abc	3.4 ± 0.6 abc
	E	L	5.6 ± 3.9 ab	3.5 ± 2.6 ab	25.5 ± 4.5 bcd	22.1 ± 5.7 bc	4.0 ± 0.2 bcde	3.9 ± 0.5 cd
	A	H	6.5 ± 2.4 abc	7.1 ± 7.3 b	20.4 ± 5.1 a	16.5 ± 3.6 a	3.5 ± 0.5 a	3.3 ± 0.4 a
	E	H	8.9 ± 2.9 bc	7.2 ± 4.9 b	28.1 ± 4.9 cdef	19.3 ± 2.9 abc	4.1 ± 0.2 cdef	3.8 ± 0.5 abcd
P-values (main effects)	Clones		0.0051	0.0625	0.0000	0.0000	0.0000	0.3212
	CO_2		0.3560	0.5216	0.0111	0.0581	0.1616	0.0254
	Nitrogen		0.1675	0.0279	0.3323	0.9768	0.1987	0.3103
P-values (interactions)	Clone × CO_2		0.0169	0.6636	0.0468	0.0939	0.0248	0.2697
	Clone × nitrogen		0.1989	0.4012	0.0802	0.0160	0.1227	0.0099
	CO_2 × nitrogen		0.1455	0.9199	0.5714	0.6473	0.5943	0.5971
	Clone × CO_2 x nitrogen		0.8053	0.8893	0.7750	0.5801	0.8249	0.8024

2.3.2 Influence of FACE and N-fertilization on structural wood composition

The three poplar clones showed significant structural differences in their normal wood composition (Table 2). The secondary xylem of *P. nigra* displayed larger vessel lumina and higher vessel numbers than those of *P. alba* and *P. × euramericana* (Fig. 1a–c). This accumulated to significantly larger vessel lumen areas in *P. nigra* than in the two other genotypes (Fig. 2). In all three poplar species, FACE significantly decreased the percentages of cell wall area compared with those grown at the ambient CO_2 concentration (Fig. 2, Table 2). The decreased portion of cell wall area was mainly caused by increased fibre lumen areas in the three poplar clones (Fig. 2). Furthermore, elevated $[CO_2]$ also resulted in increased percentages of ray parenchyma areas compared with the ambient $[CO_2]$ (Fig. 2, Table 2). N-fertilization also significantly reduced the percentages of cell wall area in comparison with shoots from unfertilised plots (Fig. 2, Table 2). These decreases were accompanied by decreases in vessel lumina but increases in fibre lumina areas (Fig. 2, Table 2). The observation that N-fertilization had these consistent effects on wood properties was surprising since the nitrogen influence on radial or height growth was genotype-specific (Table 1).

Table 2 Statistical results of the percentages of cell wall area (PCWA), the percentages of vessel lumina area (PVA), the percentages of fibre lumina area (PFA), the percentages of ray parenchyma area (PRA), the vessel frequency (VF) and the thickness of double fibre wall (TDFW) of three *Populus* species grown under either ambient (A) or elevated (E) $[CO_2]$ in the presence (H) or absence (L) of N-fertilization. *P*-values of the ANOVA *F*-test of the main effects of clone, CO_2, nitrogen and their interactions are indicated.

	P-values	PCWA	PVA	PFA	PRA	VF	TDFW
	Clone	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Main effects	CO_2	0.0000	0.1554	0.0000	0.0001	0.2851	0.0000
	Nitrogen	0.0000	0.0074	0.0000	0.0078	0.0931	0.0000
	Clone × CO_2	0.0000	0.1960	0.0000	0.0000	0.0020	0.0240
Interactions	Clone × nitrogen	0.0000	0.4350	0.0000	0.5420	0.1890	0.0000
	CO_2 × nitrogen	0.0000	0.8500	0.0020	0.8230	0.0400	0.0000
	Clone × CO_2 × nitrogen	0.7250	0.9380	0.5410	0.0020	0.1430	0.0000
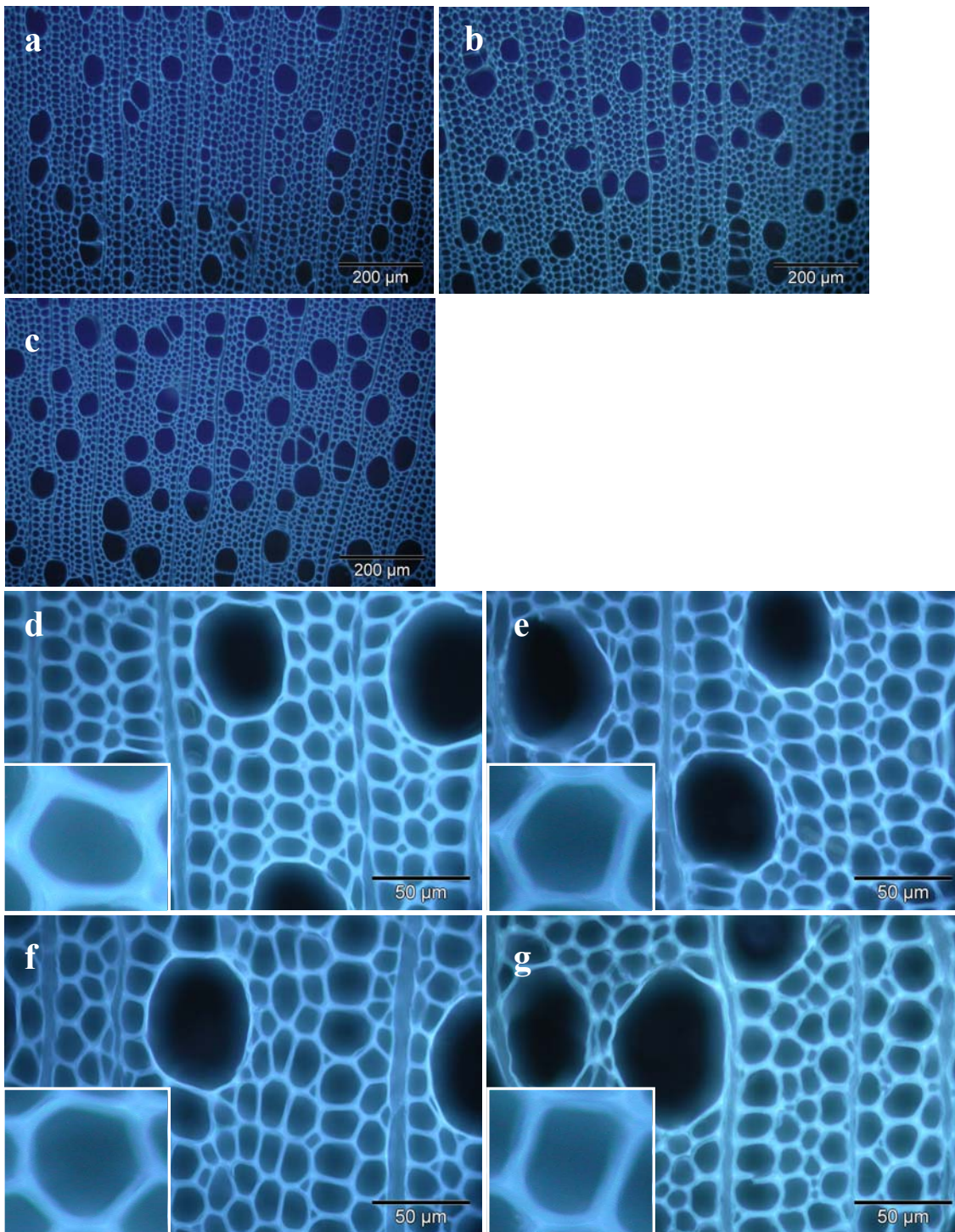
Fig. 1 Typical cross sections of *P. alba* (a), *P. × euramericana* (b) and *P. nigra* (c) grown under ambient [CO_2] in the absence of additional N-fertilization. For more details, cross sections of *P. nigra* (d-g) were photographed with higher magnification and insets in d-g show details of typical thickness of double fibre wall under different conditions (d: under ambient [CO_2] in absence of N-fertilization; e: under ambient [CO_2] in presence of N-fertilization; f: under elevated [CO_2] in absence of N-fertilization; g: under elevated [CO_2] in presence of N-fertilization). The sections were viewed by fluorescence microscopy. Magnifications are indicated by scale bars.

Fig. 2 Relative abundance of cell lumina and wall areas in cross sections of secondary xylem of *P. alba* , *P. × euramericana* and *P. nigra* grown under either ambient (A) or elevated (E) [CO_2] in the presence (H) or absence (L) of N-fertilization. The pies correspond to fractional areas of cell walls (PCWA), vessel lumina (PVA), fibre lumina (PFA), and ray parenchyma (PRA). Data indicate means (n = 18) and different letters indicate significant differences at P ≤ 0.05.

AL

PRA: 5.7% a
PCWA: 44.7% g
PFA: 36.5% b
PVA: 13.1% ab

EL

PRA: 6.7% bcd
PCWA: 37.5% e
PFA: 41.9% ef
PVA: 13.9% b

P. alba

AH

PRA: 6.7% bcd
PCWA: 36.8% e
PFA: 44.6% g
PVA: 11.9% a

EH

PRA: 6.6% bcd
PCWA: 32.3% b
PFA: 48.7% h
PVA: 12.4% a

AL

PRA: 6.1% ab
PCWA: 37.1% e
PFA: 39.6% cd
PVA: 17.2% cd

EL

PRA: 6.2% ab
PCWA: 34.1% d
PFA: 41.6% e
PVA: 18.1% de

P. × euramericana

AH

PRA: 6.5% bcd
PCWA: 32.3% b
PFA: 44.6% g
PVA: 16.6% c

EH

PRA: 6.2% abc
PCWA: 32.5% bc
PFA: 43.7% fg
PVA: 17.6% cd

AL

PRA: 7.2% de
PCWA: 40.4%f
PFA: 32.5% a

EL

PRA: 8.0% e
PCWA: 33.9% cd
PFA: 38.4% bc
PVA: 19.7% f

P. nigra

AH

PRA: 7.0% cd
PCWA: 35.0% d
PFA: 38.4% bc
PVA: 19.6% f

EH

PRA: 9.6% f
PCWA: 30.4% a
PFA: 40.9% de
PVA: 19.1% ef

2.3.3 *Effects of FACE and N-fertilization on anatomical characteristics of wood*

There were significant differences in vessel anatomical traits among the three poplar genotypes (Figs. 1a–c and 3, Tables 2 and 3). Under most conditions, *P. × euramericana* contained the longest vessel elements with diameters intermediate between those of *P. alba* (smaller) and *P. nigra* (larger) (Table 3). The main factors (CO_2) and nitrogen also had significant influence on vessel properties (Table 3). With the exception of *P. alba*, FACE caused decreases in vessel element lengths and increases in vessel element diameters (Table 3). Under most conditions, nitrogen fertilization resulted in the production of shorter vessel elements (Table 3). A notable exception was *P. alba* under ambient CO_2, where nitrogen fertilization resulted in about 12% longer vessels.

Table 3 Anatomical characteristics of vessels and fibres in the secondary xylem. A: ambient [CO_2], E: elevated [CO_2], L: no N-fertilization, H: N-fertilization; ADV: average diameter of vessel lumen, VEL: vessel element length, ADF: average diameter of fibre lumen; FL: fibre length. Data indicate means ($\pm$ SD, n $\geq$ 36). The values followed by different letters in the same column indicate significant differences at P $\leq$ 0.05 for all treatments and species. *P*-values of the ANOVA *F*-test of the main effects of clone, CO_2, nitrogen and their interactions are indicated.

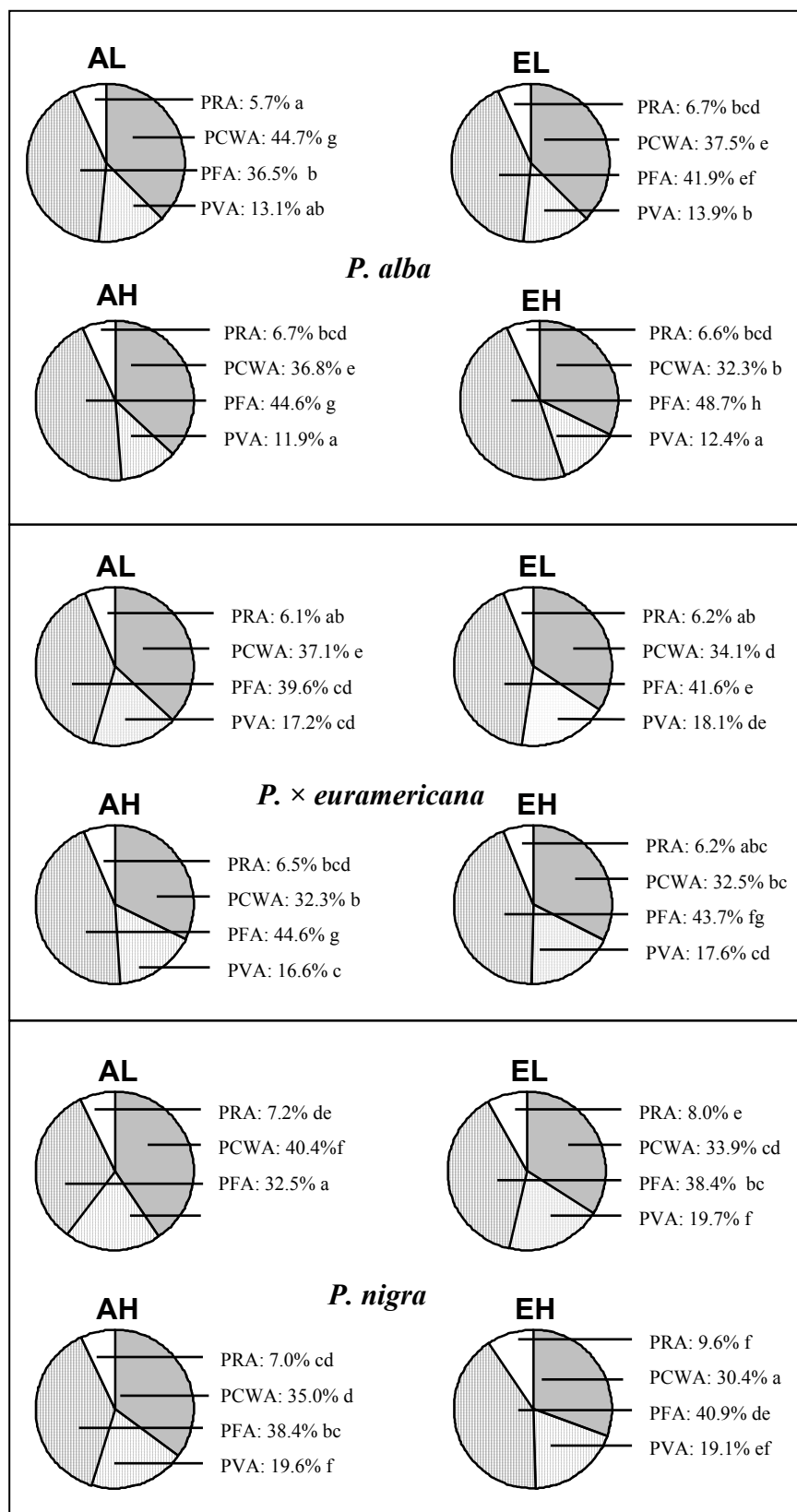| Clones	CO_2	Nitrogen	ADV(µm)	VEL(µm)	ADF(µm)	FL(µm)
P.alba	A	L	67.1 ± 5.9 cd	227.2 ± 52.4 a	20.6 ± 2.7 de	617.8 ± 87.2 e
	E	L	64.1 ± 4.5 b	276.0 ± 45.7de	20.3 ± 2.3 d	669.9 ± 68.4 g
	A	H	65.9 ± 4.6 bcd	268.4 ± 40.5 cde	21.8 ± 2.7 g	677.4 ± 85.9 g
	E	H	65.0 ± 4.1 bc	228.3 ± 43.9 a	21.1 ± 2.6 ef	598.4 ± 71.1 d
P. x euramericana	A	L	61.1 ± 6.2 a	328.5 ± 56.9 g	19.6 ± 2.9 c	641.9 ± 84.0 f
	E	L	68.1 ± 7.2 de	321.1 ± 61.5 g	19.6 ± 2.3 c	702.8 ± 86.5 h
	A	H	67.1 ± 6.5 cd	303.7 ± 65.5 f	19.2 ± 2.5 c	551.7 ± 105.9 a
	E	H	77.7 ± 5.4 i	255.2 ± 62.1 bc	21.4 ± 2.6 fg	569.2 ± 132.6 b
P. nigra	A	L	71.9 ± 6.8 g	264.4 ± 58.9 cd	16.2 ± 2.3 a	578.2 ± 63.9 bc
	E	L	75.0 ± 5.6 h	254.7 ± 39.7 bc	17.5 ± 1.5 b	583.1 ± 72.0 c
	A	H	70.1 ± 5.8 ef	282.1 ± 43.9 e	15.7 ± 1.8 a	609.9 ± 79.3 e
	E	H	73.8 ± 5.2 gh	246.8 ± 43.4 b	17.6 ± 1.9 b	597.9 ± 77.5 d
P-values	Clone		0.0000	0.0000	0.0000	0.0000
	CO_2		0.0000	0.0000	0.0000	0.0012
	Nitrogen		0.0002	0.0001	0.0000	0.0000
	Clone × CO_2		0.0000	0.0010	0.0000	0.0000
	Clone × nitrogen		0.0000	0.0000	0.0000	0.0000
	CO_2 × nitrogen		0.0490	0.0000	0.0000	0.0000
	Clone × CO_2 × nitrogen		0.5350	0.0010	0.0000	0.0000

The vessel frequency in wood of *P. nigra* was significantly higher than in that of *P. ×* *euramericana* or *P. alba* (Figs. 1a–c and 3). The main factors CO_2 and nitrogen had no significant influence on the vessel frequencies (Table 2). Only in *P. nigra* grown under ambient CO_2 nitrogen fertilization caused increased vessel numbers, an effect which disappeared when this clone was grown under FACE conditions (Fig. 3).
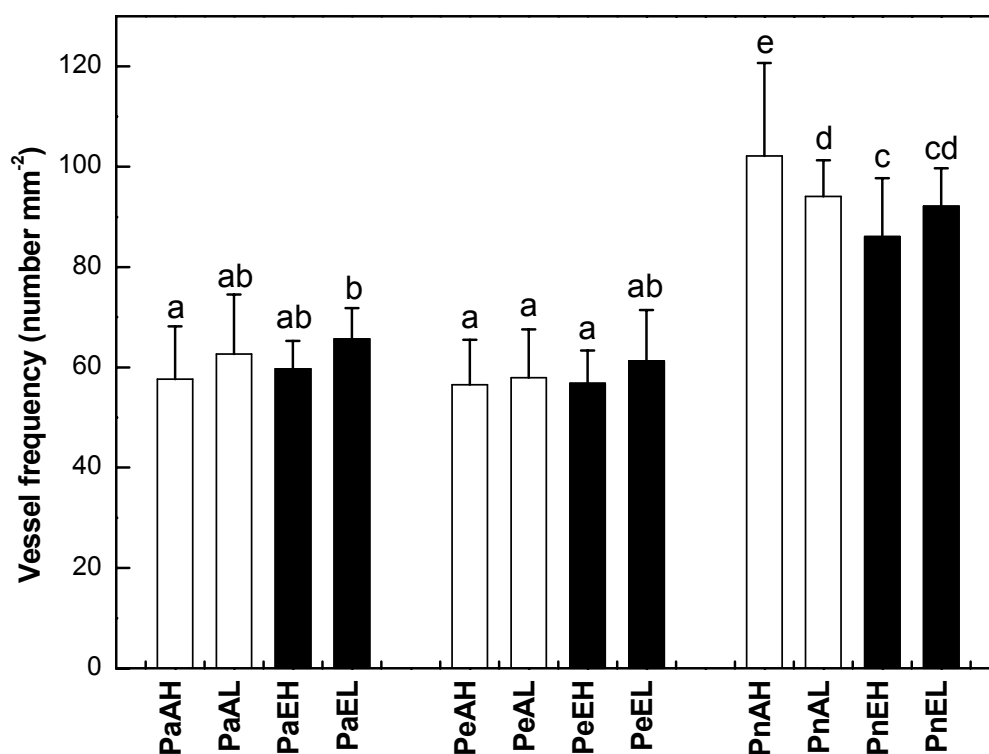
Fig. 3 Vessel frequency (number mm^{-2}) in cross sections of *P. alba* (Pa), *P. × euramericana* (Pe) and *P. nigra* (Pn) grown under either ambient (A) or elevated (E) [CO_2] in the presence (H) or absence (L) of N-fertilization. Bars indicate means (± SD, n = 18). Different letters indicate significant differences at P ≤ 0.05.

The anatomical characteristics of fibres were also significantly affected by the three main factors, clone, CO_2 and nitrogen (Tables 2 and 3). *P. alba* wood generally contained fibres with larger average lumen diameters than that of wood of *P. × euramericana* or *P. nigra* (Table 3). Growth under FACE had no effect on fibre lumen diameter of *P. alba* but caused significant increases in *P. nigra* (Table 3). Nitrogen fertilization caused increased fibre lumina in *P. alba* and *P. × euramericana* under FACE (Table 3).

The influence of FACE or nitrogen fertilization on fibre lengths was also strongly clone-specific. For instance, in *P. × euramericana*, N-fertilization caused strong reductions

in fibre lengths, whereas *P. nigra* showed clear increases (Table 3). In *P. alba*, the response of fibre length to N-fertilization was modulated by FACE (Table 3).

An important feature affecting wood density is the thickness of the fibre wall. For practical purposes, we measured the thickness of the walls between two adjacent fibre cells, denominated as "double fibre wall thickness". This parameter was also significantly affected by clone, CO_2, and nitrogen (Fig. 1d–g, Table 2). The double fibre walls of *P. × euramericana* and *P. nigra* were significantly thicker by 9.4% and 24.9%, respectively, than those of *P. alba* (Fig. 4). In all three poplar genotypes, FACE significantly decreased the thickness of double fibre wall compared with the ambient CO_2 (Figs. 1d–g and 4, Table 2). N-fertilization consistently caused strong decreases of the thickness of double fibre cell walls (Figs. 1d–g and 4, Table 2).
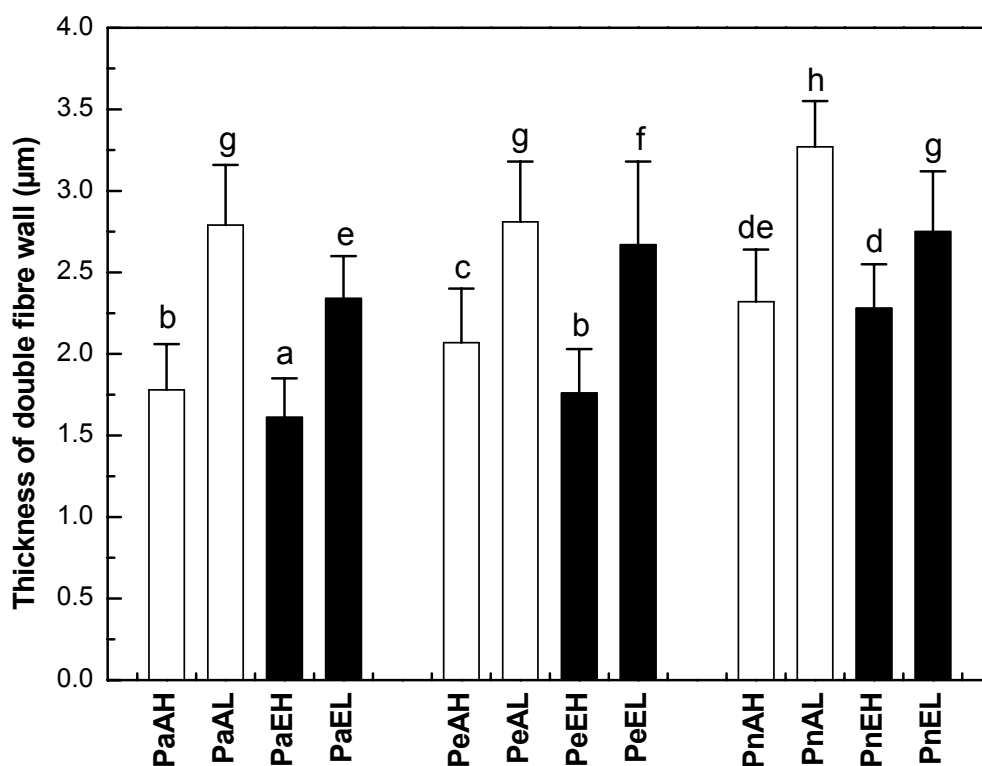

Fig. 4 Thickness of double fibre walls in cross sections of *P. alba* (Pa), *P. × euramericana* (Pe) and *P. nigra* (Pn) grown under either ambient (A) or elevated (E) [CO_2] in the presence (H) or absence (L) of N-fertilization. The bars indicate means (± SD, n = 216). Different letters indicate significant differences at P ≤ 0.05.

2.4 Discussion

2.4.1 *Is tension wood formation affected by FACE or N-fertilization or related to growth characteristics?*

An important difference of our study compared with previous studies on tree responses to elevated [CO_2] was that we analysed newly formed poplar shoots after coppicing. The number of shoots formed on stools differed between the different poplar clones (data not shown). Coppicing increased source-to-sink relationships (Hovenden 2003) probably explaining that in contrast to the previous single-stem system (Calfapietra et al. 2001, 2003a) or to other CO_2-response and N-response studies (Conroy et al. 1990; Prior et al. 1997; Jach and Ceulemans 1999; Peltola et al. 2002; Günthardt-Goerg et al. 1996; Yazaki et al. 2001) FACE and N-fertilization did not always have positive effects on individual shoot heights and diameters (Table 1). This does not imply a lack of nitrogen or FACE effects on biomass because we analysed only selected shoots for the present study.

A major question addressed in this investigation was whether FACE or N-fertilization stimulated tension wood formation or whether tension wood production was mainly related to growth characteristics (radial growth, height) or clone-specific traits. The observation that the clones showed height and radial diameter differences as well as differences in tension wood but no correlation between these parameters (Table 1 and R^2-values under Results), indicates that tension wood formation was clone-specific but not related to their typical growth characteristics. This was corroborated by the finding that the taller and, thus, probably more wind-exposed *P. × euramericana* shoots (2.4–5.4%) produced less tension wood than *P. nigra* (3.3–8.9%). Gartner et al. (2003) induced tension wood formation experimentally by inclining the pots by 30° and found no influence of elevated [CO_2] on tension wood formation in *Quercus ilex*. In their experimental system the highest tension wood formation was found at the stem base. In upright stems the frequency of tension wood formation at the stem base was similar to that in the middle (Gartner et al. 2003). In our study with naturally inclined or upright shoots, tension wood formation in the middle of the stem was quite variable (1.5–9.7%). This suggests that under field conditions tension wood is primarily a response to inclination angles and other forces acting by chance affecting shoots individually. Our data show that the extent of this response was determined by the genetic constitution and stimulated by N-fertilization (Table 1). The latter observation is important because it indicates that an overabundance of nitrogen is likely to have negative effects on wood quality. The mechanisms which lead to the stimulation of tension wood

production are not clear, but it is possible that the decreased wall thickness found here (Fig. 4) may render stems more flexible and, thus, more prone to tension wood formation. In conclusion, tension wood formation in poplar shoots was genotype-dependent and stimulated by nitrogen fertilization but not related to radial and height growth.

2.4.2 *Are the structural properties of normal wood affected by FACE or N-fertilization?*

Environmental factors, such as CO_2-enrichment and N-fertilization, can affect cell division and differentiation finally causing changes in xylem anatomy and wood structural composition. However, as outlined in the introduction, the data found in the literature give no conclusive picture as to whether and how elevated $[CO_2]$ affects xylem anatomy and tissue composition. The present study shows that CO_2-enrichment and N-fertilization under field conditions mainly increased the fraction of fibre lumen area (2–8%) and decreased the cell wall area (−3 to −8%). Decreased cell wall thickness was also found after fertilization of conifers (Brolin et al. 1995; Lindström 1996). These observations indicate that wall thickness is regulated in a yet unknown manner by nitrogen availability in a genotype-independent manner. Cooke et al. (2003) have shown recently that gene expression in phloem versus xylem was altered within few days in response to N-fertilization. It will be interesting to analyse in future studies expression patterns of cambial genes to find out which are regulated by N-fertilization.

The observation that growth under elevated $[CO_2]$ also caused reductions in fibre wall thickness, which were more pronounced in poplars grown on non-fertilised plots than in those on fertilised plots (Fig. 4), is surprising and unexpected because it has been suggested that elevated $[CO_2]$ will increase the internal resources of assimilated carbon for processes such as cell wall formation (Conroy et al. 1990). Thus, in contrast to the results obtained here, increases in wall thickness would be expected. Actually, increased wall thickness was found in *Pinus radiata* seedlings grown under elevated $[CO_2]$ (Conroy et al. 1990). Currently, we can only speculate about the reasons for these contrasting responses to CO_2-enrichment. It is possible that sink/source relationships, unknown environmental factors in the field, species-inherent features, etc., were decisive for wall thickness. But regardless of the reasons, the variable responses indicate that increased photosynthesis, which was generally found under elevated $[CO_2]$ (Medlyn et al. 1999; Hovenden 2003) is not a factor directly stimulating increased cell wall synthesis.

The consistent loss in cell wall area in response to FACE and N-fertilization in all three genotypes is remarkable since other traits showed significant species-specific variations. It should be noted that these structural changes occurred, even though [CO_2] and fertilization effects on height or radial growth were small and not uniform between the three species (Table 1). A wider interpretation of these data may be premature because the results of this study were restricted to juvenile wood, which has anatomical properties different from mature wood (Zobel and Van Buijtenen 1989; Ceulemans et al. 2002). However, if these responses persisted and were also found in other tree species, we predict an aggravation of technological wood properties for trees in future environmental scenarios with increased availability of atmospheric CO_2 and nitrogen in forest ecosystems. In conclusion, independent from the genotype elevated [CO_2] and N-fertilization had negative effects on the structural properties of juvenile poplar wood.

2.4.3 *Is wood anatomy affected by FACE and N-fertilization and what are possible implications?*

In addition to technological wood properties, anatomical features of the wood cells are of interest. For example, vessel lumina determine the capacity for water transport and, thus, contribute to climatic adaptation of trees. In our study, where water was not limiting, CO_2-enrichment resulted in wider vessel lumina. This suggests that poplars grown in the FACE system were more sensitive to drought since wider vessels make trees more prone to cavitation (Hargrave et al. 1994; Tyree et al. 1994). However, a generalisation of these results is not possible since the experimental plots were irrigated and we observed that the anatomy of the secondary xylem in poplar is responsive to water availability (Polle, unpublished data).

Vessel frequency is another important factor for water conduit in the stem. The vessel frequency decreased in *P. nigra* grown on fertilised plots under FACE, but not in the other clones (Fig. 3). In *P. × canescens* grown with CO_2-enrichment in a greenhouse, Gross (2002) also found diminished vessel frequencies. In other studies no influence of elevated [CO_2] on the vessel frequencies (*Q. ilex*, Gartner et al. 2003; *Prunus avium*, Atkinson and Taylor 1996) or increased vessel frequencies (*Q. robur*, Atkinson and Taylor 1996) were found. These contrasting observations suggest that vessel frequency is influenced by strong CO_2 × genotype interactions.

In conclusion, elevated $[CO_2]$ and N-fertilization altered the dimensions of wood cells in a genotype-specific manner, whereas increased N-availability resulted in thinner fibre cell walls independent of the genotype. The combination of both factors led to anatomical alterations in the xylem structure with potentially negative effects on wood quality such as decreased density and decreased mechanical strength in addition to an increased risk of cavitation.

References

Atkinson CJ, Tayhlor JM (1996) Effects of elevated CO_2 on stem growth, vessel area and hydraulic conductivity of oak and cherry seedlings. New Phytol 133: 617-626

Brix H (1981) Effects of nitrogen fertilizer source and application rates on foliar nitrogen concentration, photosynthesis, and growth of Douglas-fir. Can J For Res 11: 775-780

Brolin A, Noren A, Stähle E (1995) Wood and pulp characteristics of juvenile Norway spruce: a comparison between a forest and an agricultural stand. Tappi J 78: 203-214

Calfapietra C, Gielen B, Sabatti M, Angelis PD, Mugnozza GS, Ceulemans R (2001) Growth performance of *Populus* exposed to "Free Air Carbon Dioxide Enrichment" during the first growing season in the POPFACE experiment. Ann For Sci 58: 819-828

Calfapietra C, Gielen B, Galema ANJ, Lukac M, Angelis PD, Moscatelli MC, Ceulemans R, Mugnozza GS (2003a) Free-air CO_2 enrichment (FACE) enhances biomass production in a short-rotation poplar plantation. Tree Physiol 23: 805-814

Calfapietra C, Gielen B, Sabatti M, Angelis PD, Miglietta F, Mugnozza GS, Ceulemans R (2003b) Do above-ground growth dynamics of poplar change with time under CO_2 enrichment? New Phytol 160: 305-318

Ceulemans R, Jach ME, Velde RVD, Lin JX, Stevens M (2002) Elevated atmospheric CO_2 alters wood production, wood quality and wood strength of Scots pine (*Pinus sylvestris* L) after three years of enrichment. Global Change Biol 8: 153-162

Chalk L, Chattaway MM (1934) Measuring the length of vessel members. Tropical Woods 40: 19-26

Conroy JP, Milham PJ, Mazur M, Barlow EWR (1990) Growth, dry matter partitioning and wood properties of *Pinus radiata* D. Don. after 2 years of CO_2 enrichment. Plant Cell Environ 13: 329-337

Cooke JEK, Brown KA, Wu R, Davis JM (2003) Gene expression associated with N-induced shifts in resource allocation in poplar. Plant Cell Environ 26: 757-770

Donaldson LA, Hollinger D, Middleton TM, Souter ED (1987) Effect of CO_2 enrichment on wood structure in *Pinus radiata* D.Don. IAWA Bull New Series 8: 285-289

Dutilleul P, Herman M, Avella ST (1998) Growth rate effects on correlations among ring width, wood density, and mean tracheid length in Norway spruce (*Picea abies*). Can J For Res 28: 56-68

Gartner B, Roy J, Huc R (2003) Effects of tension wood on specific conductivity and vulnerability to embolism of *Quercus ilex* seedlings grown at two atmospheric CO_2 concentrations. Tree Physiol 23: 387-395

Gielen B, Ceulemans R (2001) The likely impact of rising atmospheric CO_2 on natural land and managed *Populus*: a literature review. Environ Pollut 115: 335-358

Gross K (2002) Wachstum und Sekundärstoffwechsel von Wildtyp und transgenen Pappeln (*Populus × canescens* [AIT.] SM.) unter ambienten und erhöhten atmosphärischen CO_2-Konzentrationen. In 6-IV: Cell wall composition under elevated CO_2. Cuvillier Verlag Göttingen, pp 1-16

Günthardt-Goerg MS, Schmutz P, Matyssek R, Bucher JB (1996) Leaf and stem structure of poplar (*P. × euramericana*) as influenced by O_3, NO_3, their combination, and different soil N supplies. Can J For Res 26: 649-657

Hargrave KR, Kolb KJ, Ewers FW, Davis SD (1994) Conduit diameter and drought-induced embolism in *Salvia millifera* Greene (*Labiatae*). New Phytol 126: 695-705

Hättenschwiler S, Schweingruber FH, Körner C (1996) Tree ring responses to elevated CO_2 and increased N deposition in *Picea abies*. Plant Cell Environ 19: 1369-1378

Hovenden MJ (2003) Photosynthesis of coppiced poplar clones in a free-air CO_2 enrichment (FACE) experiment in a short-rotation forest. Func Plant Biol 30: 391-400

Huang Y, Eglinton G, Ineson P, Bol R, Harkness D (1999) The effects of nitrogen fertilization and elevated CO_2 on the lipid biosynthesis and carbon isotopic discrimination in birch seedlings (*Betula pendula*). Plant and Soil 216: 35-45

Jach ME, Ceulemans R (1999) Effects of elevated atmospheric CO_2 on phenology, growth and crown structure of Scots pine (*Pinus sylvestris*) seedlings after two years of exposure in the field. Tree Physiol 19: 289-300

Kitin P, Funada R, Sano Y, Beechman H, Ohtani J (1999) Variations in the lengths of fusiform cambial cells and vessel elements in *Kalopanax pictus*. Anal Bot 84: 621-632

Lindström H (1996) Basic density in Norway spruce. I. A literature review. Wood Fiber Sci 28: 15-27

Mäkinen H, Saranpää P, Linder S (2002) Wood density variation of Norway spruce in relation to nutrient optimisation and fibre dimensions. Can J For Res 32: 185-194

McGuire AD, Joyce LA, Kicklighter DW, Melillo JM, Esser G, Vorosmarty CJ (1993) Productivity response of climax temperate forests to elevated temperature and carbon

dioxide: a North American comparison between two global models. Climate Change 24: 287-310

McGuire AD, Melillo JM, Kicklighter DW, Grace AL, Moore B, Vorosmarty CJ (1992) Interactions between carbon and nitrogen dynamics in estimating net primary productivity for potential vegetation in North America. Global Biogeochemistry Cycles 6: 101-124

Medlyn BE, Badeck FW, De Pury DGG, Barton CVM, Broadmeadow M, Ceulemans R, De Angelis P, Forstreuter M, Jach ME, Kellomäki S, Laitat E, Marek M, Philippot S, Rey A, Strassemeyer J, Laitinen K, Liozon R, Portier B, Roberntz P, Wang K, Jarvis PG (1999) Effects of elevated [CO_2] on photosynthesis in European forest species: a meta-analysis of model parameters. Plant Cell Environ 22: 1475-1495

Melillo JM, McGuire AD, Kicklighter DW, Moore B III, Vorosmarty CJ, Schloss AL (1993) Global climate change and terrestrial net primary production. Nature 363: 234-240

Miglietta F, Peressotti A, Vaccari FP, Zaldei A, DeAngelis P, Mugnozza GS (2001) Free-air CO_2 enrichment (FACE) of a poplar plantation: the POPFACE fumigation system. New Phytol 150: 465-476

Pape R (1999) Effects of thinning regime on the wood properties and stem quality of *Picea abies*. Scand J For Res 14: 38-50

Peltola H, Kilpeläinen A, Kellomäki S (2002) Diameter growth of Scots pine (*Pinus sylvestris*) trees grown at elevated temperature and carbon dioxide concentration under boreal conditions. Tree Physiol 22: 963-972

Prior SA, Runion GB, Mitchell RJ, Rogers HH, Amthor SJ (1997) Effects of atmospheric CO_2 on longleaf pine: productivity and allocation as influenced by nitrogen and water. Tree Physiol 17: 397-405

Pritchard SG, Rogers HH, Prior SA, Peterson CM (1999) Elevated CO_2 and plant structure: a review. Global Change Biol 5: 807-837

Purvis MJ, Collier DC, Walls D (1966) Some useful tests for biologically important substances. In: Laboratory techniques in botany, second edition. Butterworth & Co., London, pp 184

Saxe H, Ellsworth DS, Heath J (1998) Tree and forest functioning in an enriched CO_2 atmosphere. New Phytol 139: 395-436

Schmidtling RC (1973) Intensive culture increases growth without affecting wood quality of young southern pines. Can J For Res 3: 565-573

Shupe TF, Choong ET, Yang CH (1996) The effects of silvicultural treatments on the chemical composition of plantation-grown loblolly pine wood. Wood Fiber Sci 28: 295-300

Telewski FW, Swanson RT, Strain BR, Burns JM (1999) Wood properties and ring width response to long-term atmospheric CO_2 enrichment in field-grown loblolly pine (*Pinus taeda* L.). Plant Cell Environ 22: 213-223

Tyree MT, Davis SD, Cochard H (1994) Biophysical perspectives of xylem evolution: Is there a tradeoff of hydraulic efficiency for vulnerability to dysfunction? IAWA J 15: 335-360

Ward JK, Strain BR (1999) Elevated CO_2 studies: past, present and future. Tree Physiol 19: 211-220

Wullschleger SD, Post WM, King AW (1995) On the potential for a CO_2 fertilization effect in forests: estimates of the biotic growth factor based on 58 controlled-exposure studies. In: Woodwell GM, Mackenzie FT (eds) Biotic feedbacks in the global climatic system. Oxford Universty Press, New York, pp 85-107

Yang RC, Wang EIC, Micko MM (1988) Effects of fertilization on wood density and tracheid length of 70-year-old lodgepole pine in west-central Alberta. Can J For Res 18: 954-956

Yazaki K, Funada R, Mori S, Maruyama Y, Abaimov A, Kayama M, Koike T (2001) Growth and annual ring structure of *Larix sibirica* grown at different carbon dioxide concentrations and nutrient supply rates. Tree Physiol 21: 1223-1229

Zobel BJ, Van Buijtenen JP (1989) Wood Variation: Its Causes and Control. Springer, Berlin. Heidelberg. New York.

3. Carbon partitioning to mobile and structural fractions in poplar

Zhi-Bin Luo, Carlo Calfapietra, Marion Liberloo, Giuseppe Scarascia-Mugnozza and Andrea Polle (2006) Carbon partitioning to mobile and structural fractions in poplar wood under elevated CO_2 (EUROFACE) and N-fertilization. **Global Change Biology,** 12, 272-283

Abstract

To determine whether globally increasing atmospheric CO_2 concentrations can affect carbon partitioning between non-structural and structural carbon pools in agroforestry plantations, *Populus nigra* was grown in ambient air (about 370 μmol mol^{-1} CO_2) and in air with elevated CO_2 concentrations (about 550 μmol mol^{-1} CO_2) using Free-Air CO_2 Enrichment (FACE) technology. FACE was maintained for five years. After three growing seasons, the plantation was coppiced and one half of each experimental plot was fertilized with nitrogen. Carbon concentrations and stocks were measured in secondary sprouts in seasons of active growth and dormancy during two years after coppicing. Although FACE, N-fertilization and season had significant tissue-specific effects on carbon partitioning to the fractions of structural carbon, soluble sugars and starch as well as to residual soluble carbon, the overall magnitude of these shifts was small. The major effect of FACE and N-fertilization was on cell wall biomass production, resulting in about 30 % increased above ground stocks of both mobile and immobile carbon pools compared with fertilized trees under ambient CO_2. Relative C-partitioning between mobile and immobile C-pools was not significantly affected by FACE or N-fertilization. These data demonstrate high metabolic flexibility of *P. nigra* to maintain C-homeostasis under changing environmental conditions and illustrate that non-structural carbon compounds can be utilized more rapidly for structural growth under elevated atmospheric [CO_2] in fertilized agroforestry systems. Thus, structural biomass production on abandoned agricultural land may contribute to achieving the goals of the Kyoto protocol.

3.1 Introduction

Forests are major reservoirs of terrestrial carbon (Schlesinger 1997; Malhi *et al.* 1999) and can serve as long-term carbon storage or as renewable energy source, thereby, limiting burning of fossil fuels. Within a tree, two major functional fractions of carbon can be distinguished: metabolically active carbon pools with a short life-time (a few hours to a few seasons) and structural carbon pools, for example, cellulose, hemicelluloses, and lignin with long life-time (years, decades to centuries). Although these short- and long-term carbon stocks are the result of complex metabolic pathways, the carbon metabolism of a tree may be regarded as an integrated system of sources and sinks (Wareing & Patrick 1975). The carbon sources for growth are pools of reserve carbohydrates and newly produced photosynthate (Kramer & Kozlowski 1979), while carbon sinks are respiration, storage, structural growth and carbon export (e.g. as root exudates, for mycorrhization, and volatile formation). To simplify the interpretation, reserve and storage carbon-bearing compounds are regarded as mobile pools with a short turn-over, whereas carbon stocks in structural components represent long-term structural compounds. Therefore, a straightforward approach to evaluate carbon partitioning between these stocks is to analyse the sizes of carbon pools of non-structural carbohydrates and of structural components in different tissues across different seasons.

In trees, non-structural carbohydrates are mainly starch, glucose, fructose, sucrose, etc. (Hoch *et al.* 2003; Körner 2003), while carbon in cell walls can be regarded as the major structural pool. Non-structural carbohydrate pools change due to alterations in the source-sink balance such as photosynthesis versus respiration and growth in different seasons (Mooney 1972; Chapin *et al.* 1990). In temperate climate, this carbon fraction of trees also underlies significant seasonal dynamics (Sauter & van Cleve 1994; Höll 1997; Sauter & Wellenkamp 1998; Schaberg *et al.* 2000; Hoch *et al.* 2003). To date, little is known about carbon partitioning between short-term and long-term carbon pools in whole trees exposed to a CO_2-enriched atmosphere under field conditions. This question is of interest because small shifts in the pattern of carbon partitioning are expected to have large impacts on the global carbon cycle (Grace 2004).

Soil fertility limits carbon sequestration by trees grown under elevated CO_2 concentrations (Oren *et al.* 2001, Sigurdsson *et al.* 2001). Furthermore, carbon partitioning between mobile and structural C-pools may be changed by N-availability. For example, the concentrations of soluble sugars and starch concentrations decreased in poplar under

elevated CO_2 when N-fertilizers were applied (Curtis *et al.* 2000). Starch accumulated to higher levels in conifer seedlings grown in nutrient deficient soils (Matson & Waring 1984). Currently, most forests grow on nutrient poor sites, but higher nitrogen (N) deposition is expected in the future (Vitousek *et al.* 1997). Thus, the influence of N-fertilization on carbon stocks and partitioning should be considered when managing plantations for carbon-binding or biomass production.

To develop appropriate managing strategies for carbon resources in agroforestry systems, it is necessary to understand the influence of elevated CO_2 and N-availability on carbon partitioning to metabolically active and inactive pools. Towards this goal, we used free air carbon dioxide enrichment (FACE) facilities located in central Italy to investigate changes in different carbon pools and in the total carbon stock in above-ground biomass of poplar (*P. nigra*) in the active and dormant seasons, respectively. The EUROFACE site, which has been planted with different poplar clones, is managed according to agro-forestry practices (Garrett & Buck 1997). When trees reached heights of about 9 m after three years of FACE conditions, the plantation was coppiced (Calfapietra *et al.* 2003). Half of the experimental plots were fertilized in subsequent years. We used secondary sprouts, which developed under these conditions to test the following hypotheses: (i) FACE and N-fertilization have contrasting effects on mobile and physiologically active carbon pools with FACE leading to increases and higher soil fertility leading to decreases in non-structural carbon concentrations; (ii) both FACE and N-fertilization trigger accelerated formation of structural carbon stocks.

3.2 Materials and methods

3.2.1 *Site description*

The EUROFACE facilities are located on 9 ha of former agricultural land in Tuscany (42°22' N, 11°48' E, altitude 150 m) in central Italy. In spring 1999, six 30 m × 30 m experimental plots were selected after detailed soil analysis. In three of the plots FACE facilities were estabilshed, whereas the other three plots that represent the control treatment were left under ambient conditions. CO_2 enrichment was realized through octagonal polyethylene rings (22 m diameter) mounted on telescopic poles. Pure CO_2 (Messer Griesheim) was released through laser-drilled holes in the polyethylene rings. To reach 550 µmol mol^{-1} of the target [CO_2] inside the FACE plots, the system was controlled and monitored by a computer connected with a meteorological station in each FACE plot. In the

FACE plots, daytime $[CO_2]$ were 554 ± 1.6 µmol mol^{-1} and 535.9 ± 20.4 µmol mol^{-1} during the growing seasons from bud break to leaf fall in 2002 and 2003, respectively (Miglietta, CNR-IATA, Florence, Italy, unpublished data). For more information on this FACE system, see a detailed description given by Miglietta *et al.* (2001).

3.2.2 *Plant material and plantation layout*

The six experimental plots are located in a *P. × euramericana* (Dode) Guinier (clone I-214 = *P. deltoides* [Bartr.] Marsch. × *P. nigra* [L.]) plantation. Each plot was divided in halves by a physical resin-glass barrier (1 m depth in the soil) to enable N-fertilization to half of the trees in each plot. Each half-plot was further divided into three triangular sectors. Each sector contained one of the following clones: *P. alba* L. (2AS-11), *P. × euramericana* (Dode) Guinier (I-214) and *P. nigra* L. (Jean Pourtet), at a planting density of 10 000 trees per ha (1 m × 1 m). For a detailed description of the clone properties, see Calfapietra *et al.* (2001). In winter of 2001/2002 the plantation was coppiced. In spring 2002 secondary sprouts developed from the stools. The plantation was drip irrigated. Fertilizers (in 2002 Navarson 20-6-6 and in 2003 Ammonium Nitrate 34-0-0) were applied weekly (in constant amounts in 2002 and in amounts proportional to the growth rate in 2003) through the irrigation system by using a hydraulic pump (Ferti-injector Amiad, IMAGO srl, Italy). Fertilizers were applied in the growing seasons (July 8, 2002—October 20, 2002 and May 6, 2003—September 16, 2003) in total amounts of 212 kg N ha^{-1} in 2002, and 290 kg N ha^{-1} in 2003, respectively.

3.2.3 *Sampling*

In the first week of September 2002 and September 2003 (active seasons, three weeks before bud set) and of March 2003 and March 2004 (dormant seasons), field harvests were carried out as follows: in September, two random stools were marked per sector. On each marked stool, the number of shoots was counted and the diameter of all shoots > 0.5 cm was determined 0.2 m above the stool with a caliper. The shoot with the second thickest diameter was harvested by cutting at stool level. The shoot with the third thickest diameter was marked for harvest in March. In March, before harvest, the diameters were measured again. In September 2003, two new stools per sector were marked and used for measurements and harvests as before. In each field trial, 24 shoots of *P. nigra* were harvested from 9 a.m. to 5 p.m. from the six experimental plots.

Stem disks (about 3 cm thick) were taken at a height of 1.9 m from the sampled shoots. The stem disks were immediately debarked and both wood and bark samples were frozen in liquid nitrogen. In the active seasons, 3 cm^2 discs were excised from vein-free areas of 15 leaves along the stem axis, frozen in liquid nitrogen and stored at $-$ 80°C until biochemical analysis.

3.2.4 *Analysis of non-structural carbohydrates*

Frozen tissues were ground to a fine powder with a ball mill (Retsch, Haan, Germany) pre-cooled in liquid nitrogen. Non-structural carbohydrates were extracted from the plant material (200 mg of wood/bark or 100 mg of leaf) in 2 ml DMSO/HCl (dimethylsulfoxid: 25 % HCl = 80 : 20 (v:v)) at 60 ºC for 30 min. The mixture was centrifuged (5000 g, 10 min, 4 ºC; Mikro 24-48R, Hettich, Tuttlingen, Germany). Two hundred µl supernatant was added to 1.2 mL citrate buffer (0.2 M, pH 10.6), mixed well and centrifuged again (5000 g, 5 min, 4 ºC; Mikro 24-48R, Hettich, Tuttlingen, Germany). Subsequently, 400 µL of supernatant was added to 400 µl citrate buffer (50 µM, pH 4.6), mixed well and used as extract for determination of soluble sugars at 340 nm (Spectrophotometer DU 640, Beckmann, München, Germany) after Schopfer (1989). Five hundred micro-liters reaction mixture (0.75 M triethanolamine-buffer, 4 mM NADP, 10 mM ATP, 9 mM MgSO$_4$, pH 7.6), 200 µL extract and 800 µL double-distilled water were added into a cuvette and mixed well. After 2 min, the background was taken and 20 µL hexokinase (3 mg mL^{-1}, Roche Diognostics GmbH, Mannheim, Germany) were added. When the reaction was accomplished, the absorbance for glucose was measured and 10 µL of phosphoglucose isomerase (10 mg mL^{-1}, Roche Diognostics GmbH, Mannheim, Germany) were added for fructose determination. Finally, 10 µl of ß-fructosidase (5 mg mL^{-1}, Roche Diognostics GmbH, Mannheim, Germany) was added for sucrose measurement. Calibration curves were produced with glucose, fructose and sucrose.

To determine the starch content, 400 µL of the supernatant was hydrolyzed with 400 µL α-amyloglucosidase (66 mg α-amyloglucosidase (EC 3.2.1.3, Sigma, Steinheim, Germany) dissolved in 15 mL 50 µM citrate buffer, pH 4.6), mixed well, incubated at 55 ºC for 20 min and used for the determination of glucose equivalents as described above. Total non-structural carbohydrates (TNC) were calculated as the sum of soluble sugars and starch.

3.2.5 *Analysis of carbon content*

To analyse carbon concentrations of leaves, woody stem and cell wall fractions (see below), aliquots of 0.6-1.0 mg milled materials were weighed into 5×9 mm tin cartouches (Hekatech, Wegberg, Germany) and determined by the Elemental Analyzer EA1108 (Carlo Erba Strumentazione, Rodano, Milan, Italy). Acetanilide (71.09 % C, 10.36 % N; Carlo Erba Strumentazione, Rodano, Milan, Italy) was used as a standard.

3.2.6 *Determination of cell wall fraction*

To determine cell wall fraction, 80 mg fine powder of woody stem sampled for biochemical investigation was extracted in 2 mL 50 % aqueous methanol for 1 h at 40 °C in an ultrasonic bath (Sonorex Super RK 510H, Bandelin electronics, Berlin, Germany) and centrifuged (5000 g, 10 min, 4 °C; Mikro 24-48R, Hettich, Tuttlingen, Germany). The pellet was extracted again with 2 mL 50 % methanol for 10 min in dark at room temperature and centrifuged as above. Subsequently the pellet was washed twice with 2 mL n-hexane (4500 g, 10 min, 4 °C; Mikro 24-48R, Hettich, Tuttlingen, Germany), dried at 60 °C for 48 h and weighed. This fraction represented cell wall biomass (CWB).

3.2.7 *Estimation of cell wall and non-structural biomass*

On the basis of total above-ground woody (stem + branches) biomass production (Liberloo *et al.* 2005), and the measured concentrations of TNC and CWB, the amounts of TNC and of CWB per stool was calculated. The residual mass was calculated as above-ground woody biomass minus TNC and CWB. Based on the carbon content in above-ground woody biomass, cell walls and the molecular C compositions of TNC, different carbon pools per stool were determined.

3.2.8 *Statistical analysis*

To determine the main effects of CO_2 treatment (CO_2), N-fertilization (N) and seasonal changes (Season) on all variables (except variables in leaf tissues where only former two factors were applicable) with ANOVA, a randomized-complete-block design, with CO_2, N, season and their interactions as fixed factors and block as a random factor, was applied. All statistical tests were performed with Statgraphics (STN, St. Louis, Mo, USA) using the mixed procedure and plot as a replicate. When interactions were significant, a posteriori comparison of means was done. To reduce the chance on type I

errors, all *P*-values of these multi-comparisons were corrected by Tukey-HSD method. Data were tested for normality with the Shapiro-Wilkison test. Differences between parameter means were considered significant when the *P*-value of the ANOVA *F*-test was less than 0.05.

3.3 Results

3.3.1 *Carbon partitioning between soluble and immobile fractions in poplar leaves*

FACE and N-fertilization had neither significant influence on the concentrations of glucose and fructose nor on the starch content of leaves randomly sampled over the whole day (Fig. 1, Table 1). In the first year the foliar sucrose concentration was enhanced under FACE (Table 1) but since it contributed less than 1 % to total non-structural carbohydrates, this increase had no effect on C-budget (see below).
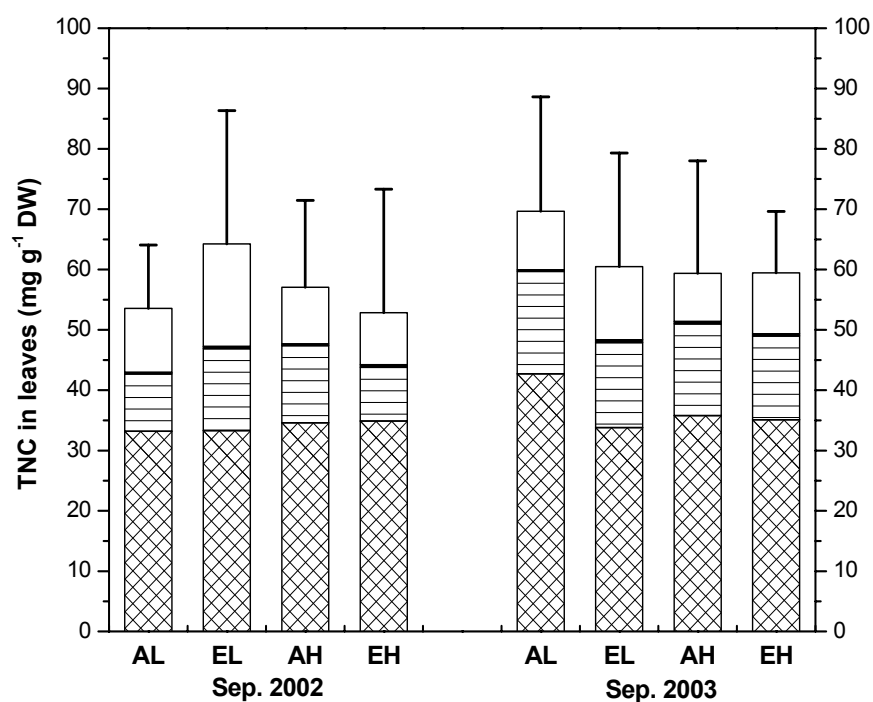
Fig. 1 Non-structural carbohydrate concentrations in leaves of *P. nigra* grown under either FACE (E) or ambient (A) [CO_2] and either fertilized (H) or unfertilized (L) conditions in two years after coppicing. Stacked bars indicate total non-structural carbohydrates (TNC) = glucose (hatched bar) + fructose (lined bar) + sucrose (black bar) + starch (white bar). Bars are means + SD (n = 3) based on leaf dry weight.

Table 1 Statistical results of the influence of FACE and N-fertilization on carbohydrate concentrations in leaves of *P. nigra* after coppicing. Total non-structural carbohydrates (TNC) = glucose + fructose + sucrose + starch. Significance levels (*P*-values of the ANOVAs) of the main factors CO_2 and nitrogen (N) and their interactions are shown.

Seasons	Source	glucose	fructose	sucrose	starch	TNC
	CO_2	0.9426	0.9186	0.0081	0.3978	0.6492
Sep. 2002	N	0.6439	0.5849	0.3071	0.1546	0.5912
	$CO_2 \times N$	0.9820	0.0026	0.3132	0.2772	0.3052
	CO_2	0.1873	0.2300	0.2080	0.3436	0.5244
Sep. 2003	N	0.4301	0.5478	0.8845	0.4353	0.4218
	$CO_2 \times N$	0.2543	0.6248	0.1539	0.9483	0.5080

To determine partitioning to the mobile and immobile C-fractions in leaves, the carbon concentrations of leaves, isolated cell walls and TNC were analysed. Residual soluble carbon was determined as the difference between the total carbon concentration of the leaves and the carbon fractions in TNC and cell walls. In the first year after coppicing, N-fertilization resulted in significant increases in total carbon, which was mainly allocated to the cell wall fraction (Table 2). In the second year, N-effects on preferential C-partitioning to cell walls were no longer observed but total foliar carbon concentrations were significantly affected by C × N interactions with FACE resulting in lower C concentrations in leaves of poplar on non-fertilized and higher C concentrations on fertilized sectors (Table 2). Residual C was higher under elevated than under ambient $[CO_2]$ (Table 2). The mean portion of physiologically active carbon (= residual plus TNC carbon) of total leaf carbon was 33.7 %.

3.3.2 *Non-structural carbohydrates in bark*

Bark plays a fundamental role in transporting assimilated carbon to sink tissues where it is used for growth and/or storage. Consistent effects of N-fertilization or FACE on TNC were not observed, whereas the concentrations of all carbohydrates determined were strongly affected by season (Fig. 2, Table 3). In the first year after coppicing (growth phase 2002 until March 2003), significant effects of nitrogen on glucose and significant interactions between season and N on the carbohydrate concentrations were observed (Fig. 2, Table 3). In the second year after coppicing (growth season 2003 until March 2004), the N-effects disappeared but the starch concentration in bark showed strong interactions of

CO_2 and season resulting only in winter in 18 % higher TNC concentrations in bark of poplar grown under FACE compared with that of trees grown under ambient $[CO_2]$ (Fig. 2, Table 3). Since the contribution of bark to total above-ground biomass is negligible, C-partitioning to different fractions was not determined.

Table 2 Carbon concentrations in different fractions of leaves of *P. nigra* grown under FACE (E) or ambient CO_2 concentrations (A) and either under fertilized (H) or unfertilized (L) conditions. Data indicate means ± SD based on leaf dry weight. Residual-C % = Total-C % − (Wall-bound-C % + TNC-C %). *P*-values of the ANOVAs of CO_2, nitrogen (N) and their interactions are shown.

Season	Treatment	Total-C %	Wall-bound C %	TNC-C %	Residual-C %
Sep. 2002	AL	46.7 ± 0.2	25.4 ± 4.4	2.1 ± 0.4	19.1 ± 4.9
	EL	46.8 ± 0.6	28.5 ± 6.4	2.6 ± 0.9	15.8 ± 7.4
	AH	47.2 ± 0.7	33.6 ± 3.1	2.3 ± 0.6	11.3 ± 3.5
	EH	47.9 ± 0.9	32.5 ± 5.5	2.1 ± 0.8	13.3 ± 5.6
Sep. 2003	AL	45.3 ± 0.8	30.9 ± 2.6	2.8 ± 0.7	11.5 ± 3.0
	EL	44.3 ± 1.1	29.6 ± 1.7	2.4 ± 0.7	12.3 ± 1.8
	AH	44.2 ± 0.6	32.6 ± 1.3	2.4 ± 0.7	9.2 ± 1.8
	EH	45.9 ± 0.9	30.8 ± 2.6	2.4 ± 0.4	12.7 ± 2.8
Sep. 2002	CO_2	0.1368	0.6374	0.6515	0.7663
	N	0.0122	0.0075	0.5910	0.0332
	CO_2 x N	0.2692	0.3299	0.3043	0.2537
Sep. 2003	CO_2	0.2730	0.0897	0.5273	0.0423
	N	0.4469	0.1087	0.4202	0.3544
	CO_2 x N	0.0011	0.8281	0.5084	0.1733
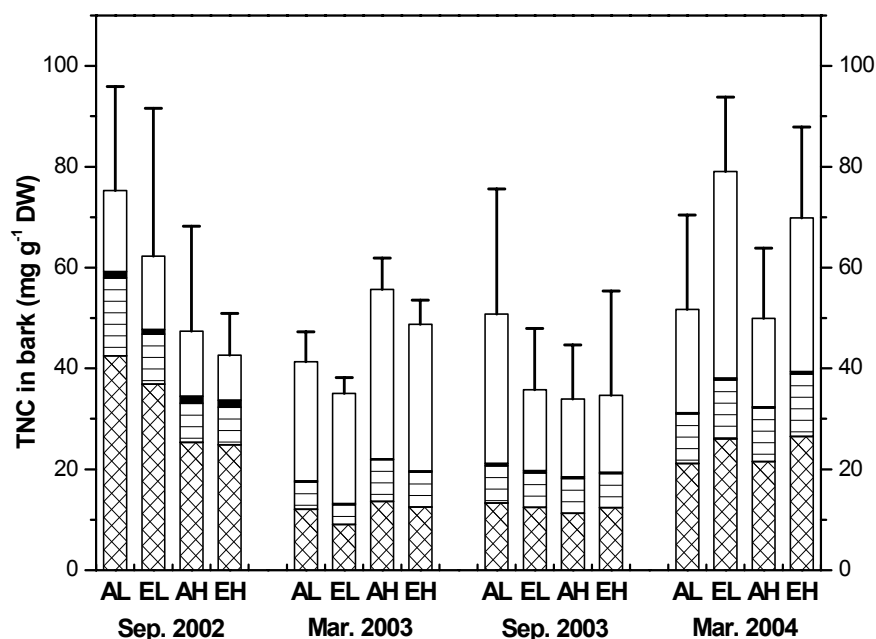
Fig. 2 Non-structural carbohydrate concentrations in bark of *P. nigra* grown under either FACE (E) or ambient (A) [CO_2] and either fertilized (H) or unfertilized (L) conditions in two years after coppicing. Measurements were performed in the active (September) and dormant season (March). Stacked bars indicate total non-structural carbohydrates (TNC) = glucose (hatched bar) + fructose (lined bar) + sucrose (black bar) + starch (white bar). Bars are means + SD (n = 3) based on bark dry weight

Table 3 Statistical results of the influence of FACE and N-fertilization on carbohydrate concentrations in bark of *P. nigra* in two years after coppicing. Measurements were conducted in the active (September) and dormant season (March), respectively. Total non-structural carbohydrates (TNC) = glucose + fructose + sucrose + starch. Significance levels (*P*-values of the ANOVAs) of the main factors CO_2, nitrogen (N), and season and their interactions are shown.

Season	Source	glucose	fructose	sucrose	starch	TNC
Main effects Sep. 2002 – Mar. 2003	CO_2	0.2424	0.0059	0.5961	0.1593	0.0897
	N	0.0071	0.1572	0.0399	0.3082	0.2811
	Season	0.0000	0.0000	0.0000	0.0000	0.0117
Interactions Sep. 2002 – Mar. 2003	CO_2 x N	0.3995	0.0848	0.3031	0.5289	0.6704
	CO_2 x Season	0.8342	0.3057	0.2488	0.9093	0.8104
	N x Season	0.0003	0.0000	0.0236	0.0031	0.0001
Main effects Sep. 2003 – Mar. 2004	CO_2	0.0907	0.3908	0.4537	0.1976	0.1057
	N	0.8280	0.7615	0.3317	0.0669	0.1553
	Season	0.0000	0.0000	0.6620	0.0343	0.0000
Interactions Sep. 2003 – Mar. 2004	CO_2 x N	0.7347	0.9038	0.8663	0.7036	0.6821
	CO_2 x Season	0.1161	0.2018	0.0959	0.0032	0.0038
	N x Season	0.6194	0.5644	0.0731	0.9227	0.7316

3.3.3 *Carbon partitioning between soluble and immobile fractions in woody stems*

Wood formation requires the supply of large amounts of non-structural carbohydrates to produce cellulose, hemicelluloses and lignin. Furthermore, ray and axial parenchyma cells in wood can act as storage sites for carbohydrates (Sauter & van Cleve 1994). Since it is not known whether elevated CO_2 concentrations and nitrogen availability affect mobile carbon pools and carbon storage in wood, we determined the concentrations of glucose, fructose, sucrose, starch, and immobile carbon in poplar xylem in both the active and dormant season (Fig. 3, Table 4). TNC concentrations were strongly affected by season and generally higher in March than in September (Fig. 3, Table 4). In the first year after coppicing, fructose and sucrose concentrations were significantly affected by N-fertilization but not in the second year. Starch was significantly influenced by CO_2 in the first and by nitrogen in the second year resulting in lower starch concentrations in wood in winter on fertilized compared with non-fertilized plots (Fig. 3). However, total TNC concentrations in wood were higher in the second than in the first year after coppicing (Fig. 3) suggesting an age-dependent build-up of C-stores.
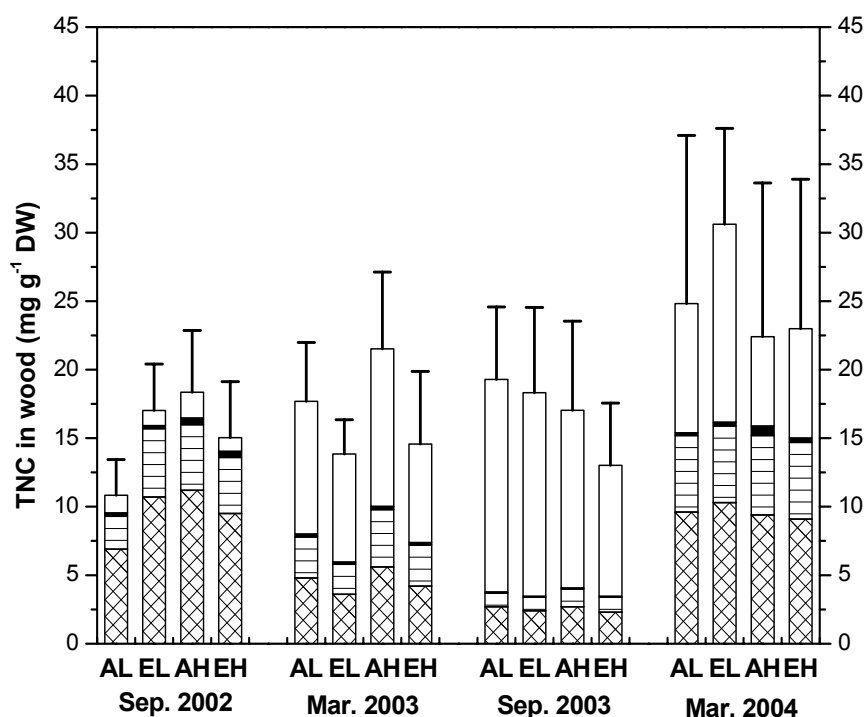
Fig. 3 Non-structural carbohydrate concentrations in xylem of *P. nigra* grown under either FACE (E) or ambient (A) [CO_2] and either fertilized (H) or unfertilized (L) conditions in two years after coppicing. Measurements were performed in the active (September) and dormant season (March). Stacked bars indicate total non-structural carbohydrates (TNC) = glucose (hatched bar) + fructose (lined bar) + sucrose (black bar) + starch (white bar). Bars are means + SD (n = 3) based on wood dry weight.

Table 4 Statistical results of the influence of FACE and N-fertilization on carbohydrate concentrations in xylem of *P. nigra* in two years after coppicing. Measurements were conducted in the active (September) and dormant season (March), respectively. Total non-structural carbohydrates (TNC) = glucose + fructose + sucrose + starch. Significance levels (*P*-values of the ANOVAs) of the main factors CO_2, nitrogen (N), and season and their interactions are shown.

Season	Source	glucose	fructose	sucrose	starch	TNC
Main effects Sep. 2002 – Mar. 2003	CO_2	0.8176	0.9861	0.2541	0.0079	0.1066
	N	0.0769	0.0018	0.0002	0.5224	0.0434
	Season	0.0000	0.0009	0.0000	0.0000	0.2044
Interactions Sep. 2002 – Mar. 2003	CO_2 x N	0.0244	0.0011	0.9128	0.2288	0.0130
	CO_2 x Season	0.0603	0.0006	0.6227	0.0585	0.0070
	N x Season	0.5067	0.5666	0.0029	0.8278	0.8652
Main effects Sep. 2003 – Mar. 2004	CO_2	0.8204	0.6979	0.2481	0.7136	0.7367
	N	0.8440	0.7469	0.0829	0.0135	0.0778
	Season	0.0000	0.0000	0.0001	0.0351	0.0016
Interactions Sep. 2003 – Mar. 2004	CO_2 x N	0.4567	0.3644	0.1274	0.3486	0.3070
	CO_2 x Season	0.4260	0.5202	0.3303	0.1212	0.1795
	N x Season	0.8846	0.4952	0.0686	0.8225	0.8078

The total carbon concentration of the tissue was slightly lower in woody stems of *P. nigra* grown on fertilized plots compared with non-fertilized plots but the effect was only significant in the first year after coppicing (Table 5). In the second year, the influence of CO_2 became dominant resulting in higher C concentrations under FACE compared with ambient [CO_2] (Table 5). These small changes were not caused by changes in carbon allocation to the immobile fraction associated with cell walls (Table 5). Overall, poplar xylem contained 87 to 91 % immobile, about 0.9 to 2.7 % TNC-bound carbon and about 7.3 to 12.0 % carbon in other soluble components (Table 5). To find out whether FACE or N-fertilization caused shifts in the relative partitioning the ratio of metabolically active carbon/structural carbon was calculated (Table 5). However, no significant changes were found (Table 5).

Table 5 Carbon concentrations in different fractions of xylem of *P. nigra* grown under FACE (E) or ambient CO_2 concentrations (A) and either under fertilized (H) or unfertilized (L) conditions. Data indicate means ± SD based on wood dry weight. Residual-C % = Total-C % − (Wall-bound-C % + TNC-C %). *P*-values of the ANOVAs of CO_2, nitrogen (N), season, and their interactions are shown.

Season	Treatment	Total C %	Wall-bound C %	TNC-bound C %	Residual C %	Relative Partitioning
Sep. 2002	AL	48.3 ± 0.3	42.8 ± 2.0	0.44 ± 0.10	5.1 ± 1.9	0.132 ± 0.051
	EL	48.1 ± 0.3	43.4 ± 1.6	0.68 ± 0.13	4.0 ± 1.3	0.109 ± 0.035
	AH	47.8 ± 1.3	43.6 ± 1.0	0.73 ± 0.18	3.5 ± 1.1	0.097 ± 0.027
	EH	47.9 ± 0.7	42.4 ± 2.1	0.60 ± 0.16	4.9 ± 1.9	0.131 ± 0.052
Mar. 2003	AL	47.6 ± 0.3	42.7 ± 2.4	0.71 ± 0.17	4.2 ± 2.3	0.117 ± 0.059
	EL	47.9 ± 0.3	41.5 ± 0.7	0.55 ± 0.10	5.8 ± 0.9	0.152 ± 0.025
	AH	46.9 ± 0.7	41.4 ± 1.5	0.86 ± 0.22	4.7 ± 1.4	0.136 ± 0.042
	EH	47.4 ± 0.3	42.2 ± 1.1	0.58 ± 0.21	4.6 ± 1.3	0.124 ± 0.032
Sep. 2003	AL	47.6 ± 0.5	41.9 ± 0.5	0.77 ± 0.21	4.9 ± 0.7	0.135 ± 0.014
	EL	48.2 ± 0.4	42.6 ± 0.7	0.73 ± 0.25	4.8 ± 0.7	0.131 ± 0.017
	AH	47.1 ± 0.4	41.4 ± 1.0	0.68 ± 0.26	5.0 ± 0.6	0.138 ± 0.012
	EH	48.1 ± 0.2	42.5 ± 1.7	0.52 ± 0.18	5.1 ± 1.7	0.133 ± 0.041
Mar. 2004	AL	47.2 ± 0.5	41.9 ± 0.6	0.96 ± 0.49	4.4 ± 1.1	0.127 ± 0.019
	EL	47.3 ± 0.2	41.2 ± 1.6	1.26 ± 0.28	4.8 ± 1.5	0.149 ± 0.044
	AH	46.9 ± 0.5	41.5 ± 1.2	0.90 ± 0.45	4.5 ± 1.1	0.132 ± 0.034
	EH	47.4 ± 0.5	42.3 ± 0.9	0.92 ± 0.44	4.2 ± 0.7	0.122 ± 0.021
Sep. 2002 - Mar. 2003	CO_2	0.4413	0.6513	0.1062	0.3515	0.4877
	N	0.0138	0.6316	0.0451	0.4772	0.6675
	Season	0.0019	0.0272	0.2107	0.3488	0.2319
	CO_2 x N	0.6048	0.9737	0.0140	0.6621	0.8363
	CO_2 x Season	0.2159	0.9199	0.0076	0.5051	0.7984
	N x Season	0.6774	0.8056	0.8702	0.9180	0.9342
Sep. 2003 - Mar. 2004	CO_2	0.0000	0.1275	0.7447	0.8989	0.9316
	N	0.1190	0.9768	0.0792	0.9499	0.5865
	Season	0.0001	0.1943	0.0016	0.1529	0.8767
	CO_2 x N	0.0904	0.1372	0.3110	0.5816	0.3232
	CO_2 x Season	0.0299	0.1725	0.1793	0.9074	0.5121
	N x Season	0.5460	0.3500	0.8029	0.5230	0.4126

3.3.4 *Above-ground partitioning to metabolically active and structurally bound carbon pools*

Cell wall fractions in woody stems accounted consistently 88.8 ± 2.5 % of dry biomass across all treatments and harvest dates. Since trees show only small growth increments three weeks before bud set, data for above-ground woody biomass in September 03 and 04 from Liberloo *et al.* (2005) were taken to estimate above-ground pools of carbon in the immobile, structurally bound fraction, and physiologically active fractions (Fig. 4, Table 6). In both years after coppicing, FACE significantly increased carbon partitioning to cell walls (Fig. 4, Table 6). N-fertilization decreased carbon partitioning to cell walls under ambient $[CO_2]$ (-9 to -16 %, Fig. 4), but enhanced carbon pools in cell walls under FACE (3 to 12 %, Fig. 4).

Table 6 Statistical results of carbon pools in above-ground woody biomass (total C pool), wall-bound C-pool, TNC and residual C-pool per stool of *P. nigra* grown under either FACE (E) or ambient (A) $[CO_2]$ and either fertilized (H) or unfertilized (L) conditions during active (September) and dormant (March) seasons. Residual C-pool = Total C pool − (wall-bound C-pool + TNC). *P*-values of the ANOVAs of CO_2, nitrogen (N), season and their interactions are indicated.

Season	Source	Total C	Wall-bound C	TNC-C	Residual C
Main effects Sep. 2002 - Mar. 2003	CO_2	0.0000	0.0000	0.8718	0.0636
	N	0.0000	0.0124	0.1233	0.3800
	Season	0.0013	0.0353	0.2349	0.3846
Interactions Sep. 2002 - Mar. 2003	CO_2 x N	0.0000	0.0000	0.1699	0.2432
	CO_2 x Season	0.2679	0.9620	0.0075	0.4615
	N x Season	0.7161	0.9135	0.8140	0.9963
Main effects Sep. 2003 - Mar. 2004	CO_2	0.0000	0.0000	0.2209	0.0724
	N	0.0000	0.0013	0.0425	0.6171
	Season	0.0000	0.1882	0.0013	0.1568
Interactions Sep. 2003 - Mar. 2004	CO_2 x N	0.0000	0.0000	0.9162	0.2191
	CO_2 x Season	0.0175	0.1694	0.1271	0.9955
	N x Season	0.5850	0.3634	0.8156	0.5221
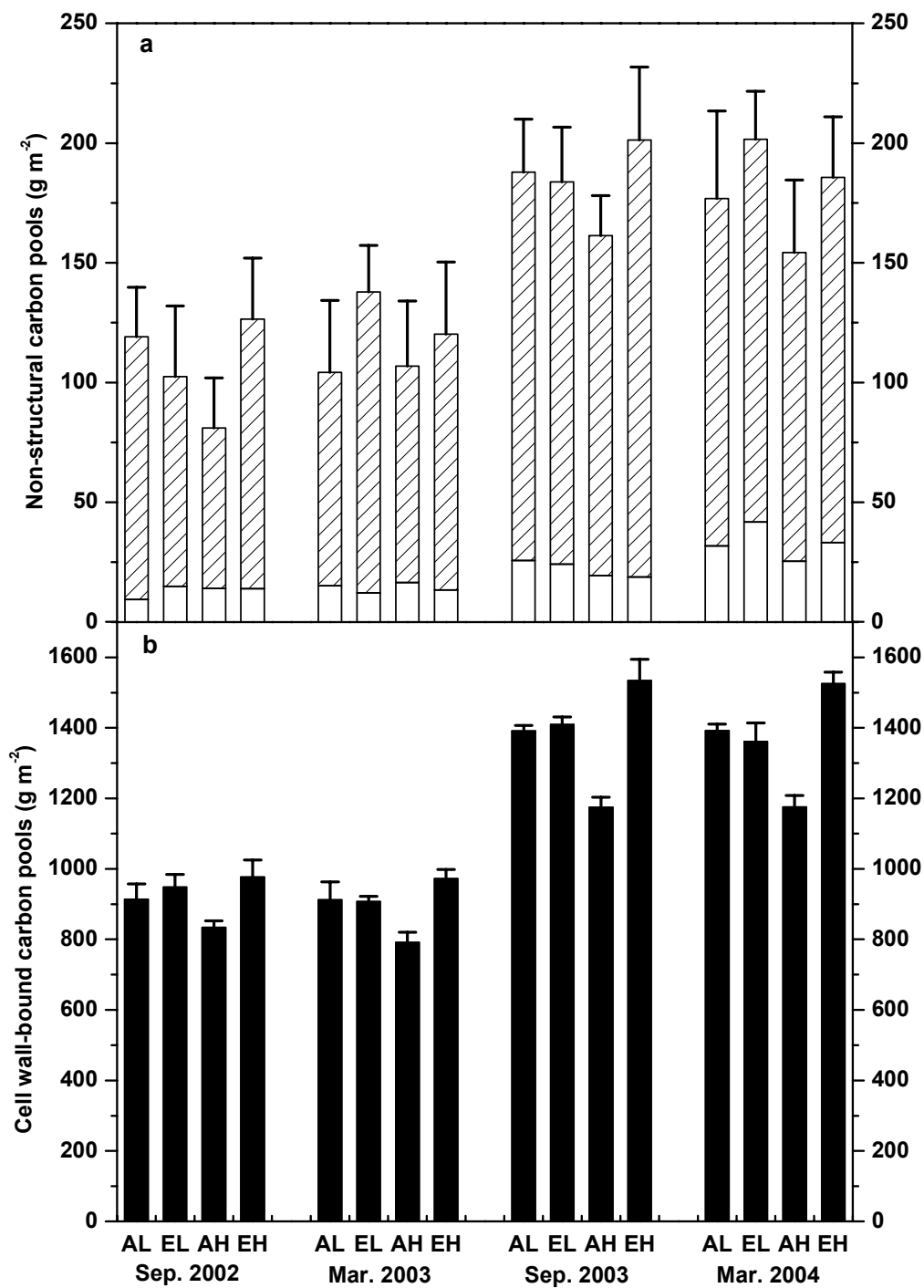
Fig. 4 Non-structural carbon pools of TNC (White bar) and residual soluble carbon (hatched bar) (a) and cell wall bound carbon pools (b) in above-ground woody biomass per stool of *P. nigra* grown under either FACE (E) or ambient (A) [CO_2] and either fertilized (H) or unfertilized (L) conditions. The estimates are based on CWB, TNC and residual mass per stool and the carbon contents in these fractions. Data indicate means + SD (n = 3).

3.4 Discussion

3.4.1 *Is carbon partitioning affected by FACE?*

To optimize carbon binding, a desirable goal would be to maximize growth and C-sequestration in structural biomass and at the same time to maintain the internal storage and mobile C-pools as small as possible. An increment in atmospheric CO_2 generally leads to increased foliar uptake and higher C-conversion rates into carbohydrates by ribulose-1,5-carboxylase/oxygenase (Long *et al.* 2004). In a range of studies performed under controlled environmental conditions, continued exposure to elevated CO_2 concentrations caused accumulation of foliar carbohydrates and down-regulation of leaf-level photosynthesis (Poorter *et al.* 1997; Saxe *et al.* 1998; Medlyn *et al.* 1999; Long *et al.* 2004). In contrast to this, poplars under FACE maintained enhanced net leaf photosynthesis in different seasons for 5 years (Bernacchi *et al.* 2003; Tricker *et al.* 2005). The relative photosynthetic stimulation in poplar leaves was similar to that in sweet gum, *Pinus taeda,* and other tree species under FACE (Norby *et al.* 2002; Hamilton *et al.* 2002, Schäfer *et al.* 2003, Crous & Ellsworth 2004). Among the different clones in the EUROFACE study, *P. nigra* showed the highest carbon assimilation rate under FACE conditions, whereas the day respiration rates in leaves of this species remained unchanged (Hovenden 2003).

Despite FACE-induced increases in photosynthetic rates, corresponding increases in soluble carbohydrate or starch concentrations in leaves were not found (Fig. 1, Table 1). This confirms studies with trees and other species exposed to elevated CO_2 concentrations under field conditions, in which neither photosynthetic acclimation nor carbohydrate accumulation were found (Tognetti *et al.* 1998; Blaschke *et al.* 2001; Rogers *et al.* 2004). Our data show that the influence of FACE on the residual soluble carbon fraction in leaves was small and that cell wall-bound carbon even tended to decrease (Table 2) suggesting that the surplus in assimilated carbon must have been exported to sink tissues or lost by production of volatiles or root exudates. While we have currently no assessment of the latter options, assessment of the carbon pools after three years in this poplar plantation showed that enhanced carbon assimilation fuelled increased above and below-ground growth (Calfapietra *et al.* 2003; Gielen *et al.* 2005). This positive FACE-effect on above-ground woody biomass formation, which has also been observed in a mixed forest FACE experiment (Schäfer *et al.* 2003), persisted for *P. nigra* in two annual growth cycles after coppicing and was also apparent from ca. 25 % increased annual leaf production (Liberloo *et al.* 2005).

The fact that no excess TNC accumulated in transport or storage tissues like bark and woody stem under FACE during the growth phase (Figs. 2 and 3, Tables 3 and 4) is likely to reflect plastic adaptation of sink strength in *P. nigra*. This species is characterised by a high growth rate and continuous production of sylleptic branches, thereby generating high sink strength for carbohydrates. *P. nigra* showed a higher number of surviving secondary shoots and higher leader shoot growth under FACE compared with ambient CO_2 (Liberloo *et al*. 2005). It is, thus, reasonable to assume that a significant portion of carbohydrates formed in *P. nigra* under FACE is promptly converted to structural carbon compounds and that, under FACE, mobile carbon resources are utilized more rapidly for structural growth (production of CWB). In response to this, photosynthates may be produced more quickly to meet the increased demand of mobile carbon. Consequently, carbon partitioned into TNC is relatively stable irrespective of the CO_2 treatment (Fig. 4a, Table 6), but carbon sequestered into CWB is markedly stimulated by FACE, especially in combination with N-fertilization (Fig. 4b, Table 6).

3.4.2 *Is carbon partitioning affected by N-fertilization and seasonal changes?*

In single-stem trees higher nitrogen supply resulted in increased biomass production (e.g. McGuire *et al*. 1993; Hättenschwiler & Körner 1998). Since nitrogen is a limiting factor in most temperate forest ecosystems, one may expect that optimization of agroforestry systems for growth and biomass production would require fertilization. However, it is intriguing that in the EUROFACE coppicing system fertilization caused increased woody biomass production only under FACE and not under ambient CO_2 (Liberloo *et al*. 2005), consequently, resulting in significantly less above-ground carbon sequestration in structural biomass on fertilized than in non-fertilized plots under ambient CO_2 (Fig. 4). The finding that fertilization had negative effects on wood production under ambient CO_2 is unexpected, but in line with an earlier study where we showed that N-fertilization decreased the thickness of cell walls in the xylem of *P. nigra* (Luo *et al.* 2005). Sensitivity of cell wall formation to nitrogen fertilization has also been observed in poplars grown under controlled conditions (Cooke, Martin & Davis 2005). The underlying physiological mechanisms for this response in wood formation are not known. Since the EUROFACE plantation was installed on a former agricultural land, the results imply that at least, in the first years of tree cultivation nitrogen was not a growth-limiting factor. From a practical point of view the results clearly indicate that excess fertilization will not improve

growth but that it may be required in future climate scenarios to maximize carbon binding in woody biomass.

Since increased growth rates enhance the utilization of carbohydrates, one may expect that the tissue concentrations of reserve carbohydrates will decrease. In some coniferous species, N-fertilization was found to lower the carbohydrate reserves in the growing season (Matson & Waring 1984; Birk & Matson 1986; Ludovici *et al.* 2002). In apple trees higher N-supply depleted carbohydrate concentrations, and it was suggested that these decreases might be due to more carbon invested into proteins and amino acids (Cheng *et al.* 2004). Decreases in carbohydrate concentrations in response to fertilization have also been reported for *Populus* (Curtis *et al*. 2000). In *P. nigra* the influence of nitrogen on starch in above-ground tissues became apparent in the second year (Figs. 1-3, Tables 1, 3 and 4) when the influence on growth was also stronger than in the first (Liberloo *et al*. 2005).

Seasonal fluctuations in pools of mobile carbon in ligneous tissues are essential in deciduous tree species to fuel maintenance respiration and provide protection against freezing stress during winter (Ögren 2000), as well as forming a reserve to build new leaves and support new growth in spring (Chapin *et al.* 1990; Fischer & Höll 1991; Canham *et al.* 1999). Detailed analyses of seasonal cycles of mobile carbon fractions in poplar stems showed that starch accumulates from May to October, is converted to soluble carbohydrates as cryoprotectant during winter, reconverted to starch before bud break and mobilized during bud break and growth of new leaves in spring (Guy, Huber & Huber 1992, Sauter & van Cleve 1994). As expected, the mobile carbon pools analysed in the present investigation were strongly affected by seasonal changes (Figs. 2 and 3, Tables 3 and 4). Prior to bud break (March samples), the starch and soluble sugar concentrations were relatively high in bark and wood (Figs. 2 and 3) but differentially affected by nitrogen and FACE-treatments. In bark the influence of FACE dominated in the second year resulting in increased TNC concentrations (Fig. 2); whereas, in the stem N-fertilization caused lower TNC-concentrations (Fig. 3).

Obviously, CO_2 and N influence seasonal partitioning of mobile carbon pools. Interestingly, the total carbon fractions and carbon fractions in wood cell walls were slightly but markedly lower in March than in September (Table 5). Because of this decrease, the overall carbon stocks in above-ground woody biomass, CWB and TNC were lower in the dormant season than in September (Fig. 4, Table 6). Poplars under FACE,

whose biomass production was N-limited, accumulated more carbohydrates in storage tissues than under on fertilized plots (Fig. 3). Since carbohydrates contribute to frost tolerance, we can speculate that under FACE non-fertilized trees, which have higher reserves convertible to cryo-protectants, may be more tolerant to low temperatures than those grown on fertilized plots.

In summary, these analyses show that our initial hypothesis must be rejected: increased structural carbon stocks were found only when FACE-exposed plots were additionally fertilized but not in response to either fertilization or elevated CO_2 as single factors. The mobile carbon fractions fluctuated seasonally in a tissue-specific manner and were also more strongly affected by the combination of N × FACE than by either FACE or N-treatment. Two years after coppicing the estimated structural carbon stocks in above-ground woody biomass ranged from 11 t C ha^{-1} (ambient CO_2, fertilized) to 15 t C ha^{-1} (FACE, fertilized, Fig. 4) corresponding to 28 to 36 t dry biomass ha^{-1}. Average production levels of dry woody biomass, which can be achieved with hybrid poplar clones, are in the range of 20 to 25 t ha^{-1} a^{-1} (Ceulemans & Deraedt 1999). It should be noted that increases in mean temperatures, also anticipated as a result of global change, may have additional synergistic effects, if water is not limiting (Saxe *et al.* 2001, Saxe and Kerstiens 2005). Our results show a potential for significant increases in C-sequestration using managed agroforestry systems with fast-growing species. It is expected that about 5 to 10 % of 178 million ha currently used for agriculture in the 25 member states of the European Union will be taken out of the production. It might be an important step towards utilization of renewable energy, if abandoned agricultural areas were used for woody biomass plantations. Shifting energy production from fossil fuels to C-neutral wood would mitigate current annual anthropogenic CO_2 emissions (6.3 Pg C year^{-1}, Houghton *et al.* 2001) and constitute an important contribution to the goals of the Kyoto protocol (1997).

References

Bernacchi CJ, Calfapietra C, Davey PA, Wittig VE, Scarascia-Mugnozza G, Raines CA, Long SP (2003) Photosynthesis and stomatal conductance responses of poplars to free-air CO_2 enrichment (PopFACE) during the first growth cycle and immediately following coppice. *New Phytologist*, **159**, 609- 621.

Birk EM, Matson PA (1986) Site fertility affects seasonal carbon reserves in loblolly pine. *Tree Physiology*, **2**, 17-27.

Blaschke L, Schulte M, Raschi A, Slee N, Rennenberg H, Polle A (2001) Photosynthesis, soluble and structural carbon compounds in two Mediterranean oak species (*Quercus pubescens* and *Q. ilex*) after lifetime growth at naturally elevated CO_2 concentrations. *Plant Biology*, **3**, 288-297.

Calfapietra C, Gielen B, Sabatti M, Angelis PD, Miglietta F, Mugnozza GS, Ceulemans R (2003) Do above-ground growth dynamics of poplar change with time under CO_2 enrichment? *New Phytologist*, **160**, 305-318.

Calfapietra C, Gielen B, Sabatti M, Angelis PD, Scarascia-Mugnozza G, Ceulemans R (2001) Growth performance of *Populus* exposed to "Free Air Carbon Dioxide Enrichment" during the first growing season in the POPFACE experiment. *Annals Forest Science*, **58**, 819-828.

Canham CD, Kobe RK, Latty EF, Chazdon RL (1999) Interspecific and intraspecific variation in tree seedling survival: effects of allocation to roots versus carbohydrate reserves. *Oecologia*, **121**, 1-11.

Chapin FS, Schulze ED, Mooney HA (1990) The ecology and economics of storage in plants. *Annual Review of Ecology and Systematics*, **21**, 423-447.

Cheng LL, Ma FW, Ranwala D (2004) Nitrogen storage and its interaction with carbohydrates of young apple trees in response to nitrogen supply. *Tree Physiology*, **24**, 91-98.

Ceulemans R, Deraedt W (1999) Production physiology and growth potential of poplars under short-rotation forestry culture. *Forest Ecology and Management,* **121,** 9-23.

Cooke JEK, Martin TA, Davis JM (2005) Short term physiological and developmental responses to nitrogen availability in hybrid poplar. *New Phytologist*, **167:** 41-52.

Crous KY, Ellsworth DS (2004) Canopy position affects photosynthetic adjustments to long-term elevated CO_2 concentration (FACE) in aging needles in a mature *Pinus taeda* forest. *Tree Physiology,* **24:** 961–970

Curtis P, PS, Vogel C, Wang X *et al*. (2000) Gas exchange, leaf nitrogen and growth efficiency of *Populus tremuloides* in a CO_2-enriched atmosphere. *Ecological Applications,* **10,** 3-17.

Fischer C, Höll W (1991) Food reserves of Scots pine (*Pinus sylvestris* L.). I. Seasonal changes in the carbohydrate and fat reserves of pine needles. *Trees*, **5**, 187-195.

Garrett HEG, Buck L (1997) Agroforestry practice and policy in the United States of America. *Forest Ecology and Management*, **91**, 5-15.

Gielen B, Calfapietra C, Lukac M *et al.* (2005) Net carbon storage in a poplar plantation (POPFACE) after three years of free-air CO_2 enrichment. *Tree Physiology*, **25**, 1399–1408.

Grace J (2004) Understanding and managing the global carbon cycle. *Journal of Ecology,* **92,** 189–202.

Guy CL, Huber JL, Huber SC (1992) Sucrose-phosphate synthase and sucrose accumulation at low temperature. *Plant Physiology*, **100**, 502-508.

Hamilton JG, DeLucia EH, George K, Naidu SL, Finzi AC, Schlesinger WH (2002) Forest carbon balance under elevated CO_2. *Oecologia*, **131**, 250-260.

Hättenschwiler S, Körner Ch (1998) Biomass allocation and canopy development in spruce model ecosystems under elevated CO_2 and increased N deposition. *Oecologia*, **113**, 104-114.

Hoch G, Richter A, Körner Ch (2003) Non-structural carbon compounds in temperate forest trees. *Plant, Cell and Environment*, **26**, 1067-1081.

Höll W (1997) Storage and mobilization of carbohydrates and lipids. In: Trees-Contributions to Modern Tree Physiology (eds Rennenberg H, Eschrich W, Ziegler H), pp. 197-211. Backhuys Publisher, Leiden, The Netherlands.

Houghton JT, Ding Y, Griggs DJ *et al.* (2001) IPCC 2001. Climate Change 2001: The Scientific Basis. Contribution of Working Group I to the Third Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, pp. 881.

Hovenden MJ (2003) Photosynthesis of coppicing poplar clones in a free-air CO_2 enrichment (FACE) experiment in a short-rotation forest. *Functional Plant Biology*, **30**, 391-400.

Körner Ch (2003) Carbon limitation in trees. *Journal of Ecology*, **91**, 4-17.

Kramer PJ, Kozlowski TT (1979) Physiology of woody plants. Academic Press, Orlando, Florida, USA.

Kyoto protocol (1997) Kyoto protocol to the United Nations Framework Convention on Climate Change. FCCC/CP/1997/7/Add. 1, Decision 1/CP. 3, Annex 7.

Liberloo M, Dillen SY, Calfapietra C, Marinari S, Luo Z-B, Angelis PD, Ceulemans R (2005) Elevated CO_2 concentration, fertilization and their interaction: growth stimulation in a short-rotation poplar coppice (EUROFACE). *Tree Physiology*, **25**, 179-189.

Long SP, Ainsworth EA, Rogers A, Ort DR (2004) Rising atmospheric carbon dioxide: plants FACE the future. *Annual Review of Plant Biology*, **55**, 591-628.

Ludovici KH, Allen HL, Albaugh TJ, Dougherty PM (2002) The influence of nutrient and water availability on carbohydrate storage in loblolly pine. *Forest Ecology and Management*, **159**, 261-270.

Luo Z-B, Langenfeld-Heyser R, Calfapietra C, Polle A (2005) Influence of free air CO_2 enrichment (EUROFACE) and nitrogen fertilization on the anatomy of juvenile wood of three poplar species after coppicing. *Trees*, 19, 109-118.

Malhi Y, Baldocchi DD, Jarvis PG (1999) The carbon balance of tropical, temperate and boreal forests. *Plant, Cell and Environment*, **22**, 715-740.

Matson PA, Waring RH (1984) Effects of nutrient limitation on mountain hemlock: susceptibility to laminated root rot. *Ecology*, **65**, 1517-1524.

McGuire AD, Joyce LA, Kicklighter DW, Melillo JM, Esser G, Vorosmarty CJ (1993) Productivity response of climax temperate forests to elevated temperature and carbon dioxide: a North American comparison between two global models. *Climate Change*, **24**, 287-310.

Medlyn BE, Badeck FW, De Pury DGG *et al.* (1999) Effects of elevated CO_2 on photosynthesis in European forest species: a meta-analysis of model parameters. *Plant, Cell and Environment*, **22**, 1475-1495.

Miglietta F, Peressotti A, Vaccari FP, Zaldei A, Angelis PD, Scarascia-Mugnozza G (2001) Free-air CO_2 enrichment (FACE) of a poplar plantation: the POPFACE fumigation system. *New Phytologist*, **150**, 465-476.

Mooney HA (1972) The carbon balance of plants. *Annual Review of Ecology and Systematics*, **3**, 315-346.

Norby RJ, Hanson PJ, O'Neill EG *et al.* (2002) Net primary productivity of a CO_2-enriched deciduous forest and the implications for carbon storage. *Ecological Applications*, **12**, 1261-1266.

Oren R, Ellsworth DS, Johnsen KH *et al.* (2001) Soil fertility limits carbon sequestration by forest ecosystems in a CO_2-enriched atmosphere. *Nature*, **411**, 469-472.

Ögren E (2000) Maintenance respiration correlates with sugar but not nitrogen concentration in dormant plants. *Physiologia Plantarum*, **108**, 295-299.

Poorter H, Van Berkel Y, Baxter R (1997) The effect of elevated CO_2 on the chemical composition and construction costs of leaves of 27 C_3 species. *Plant, Cell and Environment*, **20**, 472-482.

Rogers A, Allen DJ, Davey PA *et al.* (2004) Leaf photosynthesis and carbohydrate dynamics of soybean grown throughout their life-cycle under Free-Air Carbon dioxide Enrichment. *Plant, Cell and Environment*, **27**, 449-458.

Sauter JJ, van Cleve B (1994) Storage, mobilization and interrelations of starch, sugars, protein and fat in the ray storage tissue of poplar trees. *Trees*, **8**, 297-304.

Sauter JJ, Wellenkamp S (1998) Seasonal changes in content of starch, protein and sugars in the wood of *Salix caprea* L. *Holzforschung*, **52**, 255-262.

Saxe H, Ellsworth DS, Heath J (1998) Tree and forest functioning in an enriched CO_2 atmosphere. *New Phytologist*, **139**, 395-436.

Saxe H, Cannell MGR, Johnsen Ø, Ryan MG, Vourlitis G (2001) Tree and forest functioning in response to global warming *New Phytologist,* **149**: 369–400.

Saxe H, Kerstiens G (2005) Climate change reverses the competitive balance of ash and beech seedlings under simulated forest conditions. *Plant Biology*, **7:** 375–386

Schaberg PG, Snyder MC, Shane JB, Donnelly JR (2000) Seasonal patterns of carbohydrate reserves in red spruce seedlings. *Tree Physiology*, **20**, 549-555.

Schäfer KV, Oren R, Ellsworth DS, Lai CT, Herrick DJ, Finzi AC, Richter DD, Katul GG (2003) Exposure to an enriched CO_2 atmosphere alters carbon assimilation and allocation in a pine forest ecosystem. *Global Change Biology,* **9:** 1378–1400.

Schlesinger WH (1997) Biogeochemistry: an analysis of global change, 2nd edn. Academic Press, New York, USA.

Schopfer P (1989) Experimentelle Pflanzenphysiologie, Band 2, Einführung in die Anwendungen, pp. 39-50.

Sigurdsson BD, Thorgeirsson H, Linder S (2001) Growth and dry-matter partitioning of young *Populus trichocarpa* in response to carbon dioxide concentration and mineral nutrient availability. *Tree Physiology*, **21**, 941-950.

Tognetti R, Johnson JD, Michelozzi M, Raschi A (1998) Response of foliar metabolism in mature trees of *Quercus pubescens* and *Quercus ilex* to long-term elevated CO_2. *Environmental and experimental Botany*, **39**, 233-245.

Tricker PJ, Trewin H, Kull O *et al.* (2005) Stomatal conductance and not stomatal density determines the long-term reduction in leaf transpiration of poplar in elevated CO_2. *Oecologia*, **143**, 652-660.

Vitousek PM, Aber JD, Howarth RW *et al.* (1997) Human alteration of the global nitrogen cycle: sources and consequences. *Ecological Applications*, **7**, 737-750.

Wareing PF, Patrick J (1975) Source-sink relations and the partition of assimilates in the plant. In: Photosynthesis and Productivity in Different Environments (ed Cooper JP), pp. 481-499. Cambridge University Press, UK.

4. Carbon-based secondary metabolites and internal N pools in poplar

Zhi-Bin Luo, Carlo Calfapietra, Andrea Polle (2005) Effects of Free Air CO_2 Enrichment (FACE) and N-fertilization on carbon-based secondary metabolites and internal N-pools in wood of *Populus nigra*. **(Manuscript)**

Abstract

To study carbon-based secondary compounds and the internal N-pools in woody stem in response to changing environmental conditions, *Populus nigra* was exposed to ambient [CO_2] (about 370 µmol mol^{-1} CO_2) and elevated [CO_2] (about 550 µmol mol^{-1} CO_2) concentrations using Free-Air CO_2 Enrichment (FACE) technology. FACE was maintained for five years. After three growing seasons, the plantation was coppiced and one half of each experimental plot was fertilized with nitrogen. Carbon-based secondary compounds and concentrations of total N and Klason lignin-bound N were measured in secondary sprouts in seasons of active growth and dormancy during two years after coppicing. Unexpectedly, FACE had no influences on lignin, cell wall-bound phenolics and soluble condensed tannins. Higher N-supply slightly but markedly stimulated formation of carbon-based secondary compounds. *P. nigra* grown under FACE had lower internal N-pools in comparison with those grown under ambient atmospheric [CO_2], but external N-supply enhanced the internal N-pools. Seasonal changes markedly affected the internal N-pools. In wood, 17 — 26% of N was bound to Klason lignin forming a resistant N-fraction. Neither FACE nor higher N-supply altered N-partitioning between lignin-bound N and other N-containing compounds. Positive correlations existed between the biosyntheses of proteins and secondary compounds in *P. nigra*. These data imply that the growth and defense of forest trees are well orchestrated and that increases in protein biosynthesis through increased fertilization have no negative effects on formation of secondary metabolites.

4.1 Introduction

Human activities have perturbed the global environment, which is currently characterised by increasing concentrations of the atmospheric carbon dioxide ($[CO_2]$) and increasing nitrogen (N) deposition via combustion of fossil fuels, wide application of nitrogen-containing fertilizers, etc. (IPCC 2001; Vitousek et al. 1997; Tilman et al. 2001). To assess the responses of the terrestrial vegetation to rising in atmospheric CO_2 and N deposition have been major goals in plant sciences and ecology in recent decades. Much is known about plant responses to both elevated $[CO_2]$ and N-fertilization. For instance, elevated atmospheric $[CO_2]$ enhances tree growth and yield, photosynthetic rates, and water use efficiency (Smith et al. 2000; Norby et al. 2002; Long *et al.* 2004; Ainsworth & Long 2005). N-fertilization increases the growth rate and results in higher wood production (McGuire et al. 1995; Cooke et al. 2005). Low soil nitrogen availability may limit the response of trees to enriched atmospheric $[CO_2]$ (Oren *et al.* 2001; Sigurdsson *et al.* 2001). However, in trees, particularly in deciduous species, growth depends not only on an external N supply but also on internal N reserves (Dickson 1989; Millard 1996) because N storage in trees may uncouple growth from N uptake and allow growth to occur when external N supply is limited (Chapin et al. 1990). Although many studies conducted under greenhouse/growth chamber conditions have shown that elevated CO_2 and N-availability affect plant N-metabolism (Cotrufo et al. 1998; Stitt & Krapp 1999; Norby et al. 2000; Kruse et al. 2003), much is still unknown about plant internal N reserves responding to elevated atmospheric $[CO_2]$ and external N-resources, especially in plants grown under a more realistic natural conditions than controlled growth conditions.

To date, evidence suggests that many plants respond to increasing atmospheric $[CO_2]$ by changes in secondary metabolism (Bezemer & Jones 1998). Secondary metabolism is referred to compounds present in cells that are not necessary for the survival of cells but are thought to be required for plant resistance in the environment exposed to abiotic and biotic stresses (Kliebenstein 2004). Phenolic compounds, condensed tannins and lignin are major and ubiquitous secondary metabolites in terrestrial higher plants (Harborne 1997). These carbon-based secondary metabolites serve numerous important functions, such as wound sealing, defense against herbivores and pathogens (Dixon & Paiva 1995; Hartley & Jones 1997), controlling the rates of plant decomposition (Cotrufo et al. 2005), mediating interactions among plants, and between plants and soil biota (Harborne 1997; Northrup et al. 1998). The first step in the shikimate pathway of phenolic biosynthesis in higher plants

is the conversion of phenylalanine (PHE) to trans-cinnamic acid by the enzyme phenylalanine ammonia-lyase (PAL) (Herrman 1995), and obviously, protein synthesis and phenolics synthesis use the same amino acid precursor—PHE. Therefore, PHE incorporated into proteins can not be simultaneously deaminated to phenolics. PHE in cells is thought to be a limiting metabolite (Lambers 1993) that prevents cells from having simultaneously high rates of both protein and phenolic synthesis (Coronado et al. 1995; Yeoman & Yeoman 1996). Substantial evidence is found that there are tradeoffs between plant processes that require high rates of protein (e.g., structural growth) versus phenolic synthesis (Jones & Hartley 1999). Elevated CO_2 and N-fertilization are frequently reported to affect plant growth, development and carbon-based secondary compounds, including phenolics (Hartley et al. 2000; Long et al. 2004; Ainsworth & Long 2005; Cooke et al. 2005). Most of these studies focused only on leaves of plants. However, information about how secondary metabolites and internal N-pools of the whole plant (especially wood of forest trees) respond to these changing environmental factors under field condition is needed to validate this balance.

Here we use free air carbon dioxide enrichment (FACE) facilities located in central Italy to investigate how internal N-pools and carbon-based secondary compounds in poplar (*P. nigra*) were affected by elevated atmospheric $[CO_2]$ and N-fertilization in 2 years after coppicing. In this study, our aims are (i) to identify carbon-based secondary metabolites and examine how these compounds were affected by FACE, N-fertilization and seasonal changes; (ii) to distinguish N-pools in soluble proteins and structurally bound N resources (Klason lignin bound N) and how FACE, N-fertilization and seasonal changes affect these N-pools; and (iii) to test whether there is a negative balance between protein biosynthesis and production of secondary compounds.

4.2 Materials and methods

4.2.1 *Site description and FACE facilities*

The experimental site was located on a former agricultural land in central Italy (Tuscania, 42°22' N, 11°48' E, altitude 150 m). In spring 1999, six 30 m × 30 m experimental plots were selected after detailed soil analysis. Three of the plots were installed with FACE facilities, whereas the other three plots were left under ambient $[CO_2]$ conditions (370 μmol mol^{-1} CO_2). Pure CO_2 (Messer Griesheim) was released through laser-drilled holes in polyethylene tubes to reach 550 μmol mol^{-1} of the target $[CO_2]$ inside

the FACE plots. A meteorological station was applied to control the release of CO_2 at each FACE plot. The FACE system was monitored by a computer to reach the target [CO_2]. A three-year-old poplar plantation exposed to FACE with trees reaching heights of about 9 m was coppiced in winter of 2001/2002 (Calfapietra et al. 2003). In subsequent years secondary sprouts developed from the stools and FACE was maintained. Additionally, half of the experimental plots were fertilised. In the FACE plots daytime [CO_2] was 554 ± 1.6 μmol mol^{-1} and 535.9 ± 20.4 μmol mol^{-1} during the growing seasons from bud break to leaf fall in the year 2002 and 2003, respectively (Miglietta, CNR-IATA, Florence, Italy, unpublished data). A detailed description about the FACE facilities was given by Miglietta et al. (2001).

4.2.2 *Plant material and N-fertilization*

Each experimental plot was divided in halves by a physical resin-glass barrier (1 m deep in the soil) to enable N-fertilization in each half plot in the post-coppice phase from year 2002 onwards. Each half plot was further divided into three triangular areas. Each triangular section was planted with one of poplar species, i.e. *P. alba* L. (2AS-11), *P. × euramericana* (Dode) Guinier (I-214) and *P. nigra* L. (Jean Pourtet), at a planting density of 10 000 trees per ha (1 m × 1 m). A detailed description of the clone properties was given by Calfapietra et al. (2001). Fertilisers (in 2002 Navarson 20-6-6 (N-P-K) and in 2003 Ammonium Nitrate 34-0-0 (N-P-K)) were applied weekly (in constant amount in 2002 and in amounts proportional to the growth rate in 2003) by using a hydraulic pump (Ferti-injector Amiad, IMAGO srl, Italy) per plot connected to the irrigation system. Fertilisers were applied in the growing seasons (July 8, 2002—October 20, 2002 and May 6, 2003—September 16, 2003) in total amounts of 212 kg N ha^{-1} in 2002, and 290 kg N ha^{-1} in 2003, respectively.

4.2.3 *Sampling*

Since bud break of these poplar species in central Italy is the end of March and bud set is the middle of September or later (Calfapietra et al. 2001), field samplings were done according to following strategy. In the first weeks of September (active seasons) 2002 and 2003, and March (dormant seasons) 2003 and 2004, field harvests were conducted. In September 2002, two stools were marked randomly per experimental variable, and on each marked stool, the diameters of all shoots with diameters more than 0.5 cm were determined

and the shoot with the second thickest diameter measured at the height of 20 cm above the stool, was harvested. The third thickest one was marked for next harvest in March 2003. In September 2003, other two stools were marked randomly per experimental variable and the same harvesting strategy was applied. In each field campaign, 24 shoots of *P. nigra* were harvested from six experimental plots. The harvested shoot was cut at the stool level.

Stem segments (about 3 cm long) were taken at the height of 1.9 m from the sampled shoots, debarked and immediately frozen in liquid nitrogen. In harvests of the active seasons, 3 cm^2 leaf discs were sampled. All samples were wrapped in foil and plunged immediately into liquid nitrogen and transferred into the laboratory and stored at − 80 °C until biochemical analysis.

4.2.4 *Analysis of soluble- and cell wall bound-phenolics*

Soluble phenolics were extracted from 80 mg fine powder of leaf, bark or woody stem with 2 mL of 50% methanol in an ultrasonic bath (60 min, 40 ºC; Sonorex Super RK 510H, Bandelin electronics, Berlin, Germany) after Pritchard et al. (1997). The mixture was centrifuged (4500 rpm, 10 min, 4 ºC). The supernatant was collected. The pellet was extracted again with 2 mL of 50% methanol for 10 min in dark at room temperature and centrifuged as above, and the supernatant was collected. The two supernatants were combined and adjusted to a volume of 25 mL with double-distilled water. Soluble phenolics were determined by employing Folin-Ciocalteus method as described below.

One mL of methanol extract was mixed with 5 mL of Folin-Ciocalteus-Phenolreagent (1:10 diluted with distilled water; Merck, Darmstadt, Germany). After 3 min at room temperature, 4 mL of 7.5% Na_2CO_3 solution was added and mixed well. After 30 min incubation at room temperature in dark, absorbance was determined spectrophotometrically at 765 nm (Spectrophotometer DU 640, Beckmann, München, Germany) using catechin (Sigma-Aldrich, Deisenhofen, Germany) as the standard. The results were expressed as catechin equivalents.

To determine the composition of soluble phenolic compounds, methanol extracts (20 mL) were evaporated (Rotavapor EL 130, Büchl, Switzerland) at 45 ºC and dissolved in 2 × 500 μL 50% methanol. Subsequently, the solution was centrifuged twice (10 min, 15000g, 4ºC). The supernatant was used for a high-performance liquid chromatograph (HPLC; Beckmann-Coulter, München, Germany) analysis. Fifty μL of the solution was separated on a reversed-phase ODS column (5 μm size of particles, 250 × 4.6 mm, Ultrasphere,

Beckmann, München, Germany) with the following gradient of solvent A (1.5% phosphoric acid (v/v)) and solvent B (water/methanol/acetonitrile Far UV (1:1:1; v/v/v), pH 2.5) at a flow of 1 mL min^{-1}: 1 min 20% B, 25 min 60% B, 35 min 100% B and 40 min 20% B (modified after Strack et al., 1989). Soluble phenolic compounds were detected at 280 nm (Diodenarray-detector 168, Beckmann-Coulter, München, Germany) by co-elution of internal standards.

To determine cell wall-bound phenolic compounds, the pellet left after extraction of soluble phenolics was washed twice (10 min, 4500 rpm, 4 ºC) with 2 mL n-hexane, dried at 60 ºC for two days and subsequently weighed. This fraction represented "cell walls". Cell walls were homogenised in 2 mL of 1 M NaOH. The suspension was incubated in the ultrasonic bath (60 min, 40 ºC) and centrifuged (10 min, 4500 rpm, 4 ºC), and the supernatant was collected. The pellet was extracted again with 2 mL of 1 M NaOH for 10 min in dark at room temperature. The supernatants were combined and adjusted to 10 mL with distilled water. Cell wall-bound phenolics were measured with Folin-Ciocalteus-Phenolreagent (Merck, Darmstadt, Germany), using *p*-coumaric acid (Fluka, Buchs, Switzerland) as a reference. The results were expressed as *p*-coumaric acid equivalents.

To determine the composition of cell wall-bound phenolic compounds, NaOH extracts were adjusted to pH 1 and extracted twice in 10 mL ethylacetate. The upper phases were combined, evaporated at 45 ºC (Rotavapor EL 130, Büchl, Switzerland), and dissolved in 2 × 500 µL 50% methanol. The extract was centrifuged twice (10 min, 15000 g, 4 ºC) and used for HPLC analysis as stated before.

4.2.5 *Analysis of condensed tannins*

Condensed tannins (proanthocyanidin) were determined after Booker et al. (1996). Fine ground materials were extracted twice with 50% methanol, the extracted solution was combined by sample and final volume was adjusted to 10 mL. Nine hundred µL of soluble extracts were mixed with 0.1 mL of 100% methanol and 6 mL of acid butanol (5% concentrated HCl in butanol (v/v)) and 0.2 mL of 2% $FeNH_4(SO_4)_2 \cdot 12H_2O$ in 2 M HCl ($FeNH_4(SO_4)_2 \cdot 12H_2O$/HCl=w/v). The solutions were incubated in sealed polypropylene tubes in the water bath at 95 °C for 50 min and then cooled in dark. Absorbance of the solution and the supernatant of the mixture were determined spectrophotometrically at 550 nm (Porter et al., 1986; Booker et al., 1996). A standard curve was constructed using

condensed tannins purified from wood of *P. nigra* grown under natural conditions (see below), and the results were expressed as condensed tannin equivalents.

The procedures for the purification of condensed tannins from poplar wood were adapted from Warren et al.(1999), Booker et al. (1996) and Czochanska et al. (1980). Two 5-g freeze-dried fine wood powder of *P. nigra* grown under natural conditions were extracted three times with 25 mL of 70% acetone containing 0.1% (w/v) ascorbic acid, and the supernatants were combined. After filtering (0.4 μm, Springfield Mill, Kent, England), the acetone was evaporated from the soluble fraction at 30 ºC under reduced pressure in a rotary evaporator (Rotavapor EL 130, Büchl, Switzerland). The remaining aqueous solution was then extracted twice with 50 mL of diethyl ether and three times with ethyl acetate. The aqueous fraction was reduced in volume in a rotary evaporator (34 ºC, Rotavapor EL 130, Büchl, Switzerland) and freeze-dried. The crude product was dissolved in 25 mL of 50% methanol and 10 mL of the solution was applied to a 1.5 x 10 cm column (Sephadex LH-20, Phamacia, Sweden) previously equilibrated with 50% methanol. The adsorbed condensed tannins were washed with 1.9 L of 50% methanol and the polymers were eluted with 70 mL of 50% acetone. The acetone was evaporated in a rotary evaporator (33 ºC, Rotavapor EL 130, Büchl, Switzerland) and the aqueous solution was freeze-dried. The final product was a fluffy tan powder. Purified poplar wood condensed tannins were confirmed to be similar to the criteria (UV spectrum and $E_{1\%}$ = 260—280 at 500 nm for the vanillin addition product in HCl) used by Czochanska et al. (1980).

4.2.6 *Analysis of Klason lignin*

The determination of acid-insoluble (Klason) lignin was adapted from Dence (1992). About 500 mg (W1) fine powder of wood or leaves were extracted with 40 mL 0.5 M phosphate buffer (pH 7.8, 0.5% Triton). The mixture was incubated on a shaker for 30 min, centrifuged (5000 g, 10 min, 4 ºC). The precipitate was kept and resuspended in 0.5 M phosphate buffer as above. Subsequently, the pellet was washed 4 times (30 min for each washing) in 100% methanol. The resulting pellet was consisted mainly of structural biomass. The pellet was immersed in 40 mL of an 96% ethanol/cyclohexan (1/2, V/V), incubated in a water bath (6 h, 50 ºC) and shaken every 30 min. Afterwards, the mixture was centrifuged (4500 g, 10 min, 4 ºC). The precipitate was washed again with 40 mL ethanol/cyclohexan mixture as above. Then, the pellet was washed with 20 mL 100% acetone and centrifuged. This washing step was repeated three times. The precipitate was

dried overnight under the fume hood. The pellet was mixed well with 8 mL 72% H_2SO_4 and incubated for 60 min at room temperature. Subsequently, the mixture was suspended in 200 mL distilled H_2O, and mixed well. The mixture was autoclaved (1 h, 121 ºC). The mixture was cooled down to room temperature and filtered with a pre-weighed (W2) filter (Filterpaper 1, Springfield Mill, Kent, England) and washed with distilled water. The filter containing the lignin was dried in an oven (24 – 48 h, 70 – 80 ºC) and stored in a desiccator. Finally, the filter containing the lignin was weighed (W3) and Klason lignin was calculated as (W3-W2) x 100/W1.

4.2.7 *Analyses of soluble proteins and nitrogen pools*

To analyse soluble protein concentrations, the tissues were ground into fine powder with a ball mill cooled in liquid nitrogen (Retsch, Haan, Germany). Soluble proteins were extracted from 100 mg fine powder in 2 mL 0.1 M phosphate buffer (pH 7.8) with 100 mg polyvinyl polypyrrolidone (Sigma, St. Louis, USA) for 15 min incubation in the ice. The mixture was centrifuged (15000 rpm, 30 min, 4 ºC) and the supernatant was taken. Subsequently, five hundred µL of supernatant was eluted using the NAP 10 columns (Sephadex G-25 DNA Grade, Amersham Biosciences, Uppsala, Sweden) with 1 mL 0.1 M phosphate buffer (pH 7.0). Soluble proteins were determined according to Bradford (1976), using bovine serum albumin (BSA, protein standard, Interchim, Montlucom, France) as a standard.

To determine nitrogen concentrations in leaves, wood and Klason lignin, aliquots of 0.6-1.0 mg dry and fine powder were weighed into 5×9 mm tin cartouches (Hekatech, Wegberg, Germany) and analysed by the Elemental Analyzer EA1108 (Carlo Erba Strumentazione, Rodano, Italy). Acetanilide (71.09% C, 10.36% N; Carlo Erba Strumentazione, Rodano, Italy) was used as a standard.

4.2.8 *Statistical analysis*

To determine the main effects of CO_2 treatment (CO_2), N-fertilization (N) and seasonal changes (Season) on all analysed parameters (except parameters in leaf tissues where only former two factors were applicable) with ANOVA, a randomized-complete-block design, with CO_2, N, Season and their interactions as fixed factors and block as a random factor, was applied. All statistical tests were performed with Statgraphics (STN, St. Louis, Mo, USA) using the mixed procedure and plot as a replicate. When interactions

were significant, a posteriori comparison of means was done. To reduce the chance on type I errors, all *P*-values of these multi-comparisons were corrected by Tukey-HSD method. Data were tested for normality with the Shapiro-Wilk's test. Differences between parameter means were considered significant when the *P*-value of the ANOVA *F*-test was less than 0.05.

4.3 Results

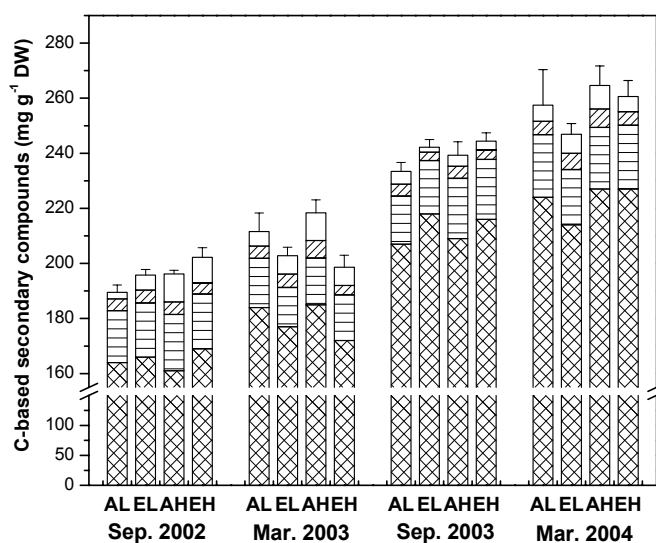### 4.3.1 *Carbon-based secondary compounds*

Carbon-based secondary compounds, such as lignin, phenolics and tannins, are important for forest trees as the defence against abiotic and biotic factors. Therefore, the abundance of these components in tissues implies certain tissue qualities for resistance to attacks of pathogens and insects. In many plant species, these compounds have often shown responses to rising $[CO_2]$ and N-fertilization (Penuelas & Estiarte 1998). Our data demonstrate that both factors N-fertilization and season markedly enhanced the biosyntheses of carbon-based secondary compounds indicated as the sum of lignin, wall-bound phenolics, soluble phenolics and condensed tannins (Table 1, Fig. 1). There was one exception, namely that N-fertilization slightly but statistically markedly caused decreases in the cell wall-bound phenolics. As a major component of carbon-based secondary compounds, lignin contents in woody stem of *P. nigra* were 161-227 mg g^{-1}, which increased as the shoots got older after coppicing (Table 1, Fig. 1). Neither FACE nor N-fertilization had effects on lignin concentrations in the woody stem of *P. nigra* (Table 1, Fig. 1). Season also markedly affected the wall-bound phenolics. Unexpectedly, FACE slightly but statistically significantly reduced soluble phenolics biosynthesis in woody stem of *P. nigra* (Table 1, Fig. 1). N-fertilization stimulated the biosynthesis of condensed tannins, this trend was more pronounced under ambient CO_2 (interaction of CO_2 x N). Condensed tannins also accumulated with increasing seasons although this finding was limited to low nitrogen conditions (interaction N x Season, Table 1, Fig. 1).

To investigate whether FACE and/or N-fertilization affected the composition of soluble phenolics and cell wall bound phenolics, the HPLC profiles were conducted. By co-elution with internal standards, the following compounds in soluble phenolics were identified: 3,4-dihydroxybenzoic acid, chlorogen acid, ferulic acid and cinnamic acid (Fig. 2). FACE and N-fertilization had no influence on the composition of soluble phenolics in woody stem of *P. nigra* although the relative abundance of individual component was

altered by FACE and/or N-fertilization (Fig. 2). In contrast to soluble phenolics, wall bound phenolics were mainly composed of hydroxybenzoic acid (Fig. 3). The composition of wall bound phenolics were unchanged by FACE and/or N-fertilization (Fig. 3).

Table 1 Statistical results of concentrations of Klason lignin, cell wall bound phenolics, soluble phenolics and soluble condensed tannins in woody stem of *P. nigra* grown under either FACE (E) or ambient (A) [CO_2] and either fertilised (H) or unfertilised (L) conditions and harvested in different seasons. Significance levels (*P*-values of the ANOVAs) of CO_2, nitrogen (N), season and their interactions are indicated.

Treatments	Lignin	Wall-bound phenolics	Soluble phenolics	CT in soluble part	Sum
CO_2	0.9114	0.2319	0.0449	0.3430	0.4149
N	0.5631	0.0004	0.5980	0.0013	0.0374
Season	0.0000	0.0000	0.0028	0.0005	0.0000
CO_2 x N	0.7832	0.3290	0.0153	0.0467	0.6727
CO_2 x Season	0.0403	0.0899	0.5325	0.4851	0.0112
N x Season	0.4991	0.1077	0.9517	0.0278	0.6210
CO_2 x N x Season	0.6462	0.1107	0.1758	0.2860	0.7109

Fig. 1 Carbon based secondary compounds in wood of *P. nigra* grown under either FACE (E) or ambient (A) [CO_2] and either fertilised (H) or unfertilised (L) conditions. Crossed bar: Klason lignin; horizontally lined bar: cell wall bound phenolics; hatched bar: soluble phenolics; blank bar: soluble condensed tannins. The data shown indicate means ± SE of six measurements for each treatment based on dry weight.
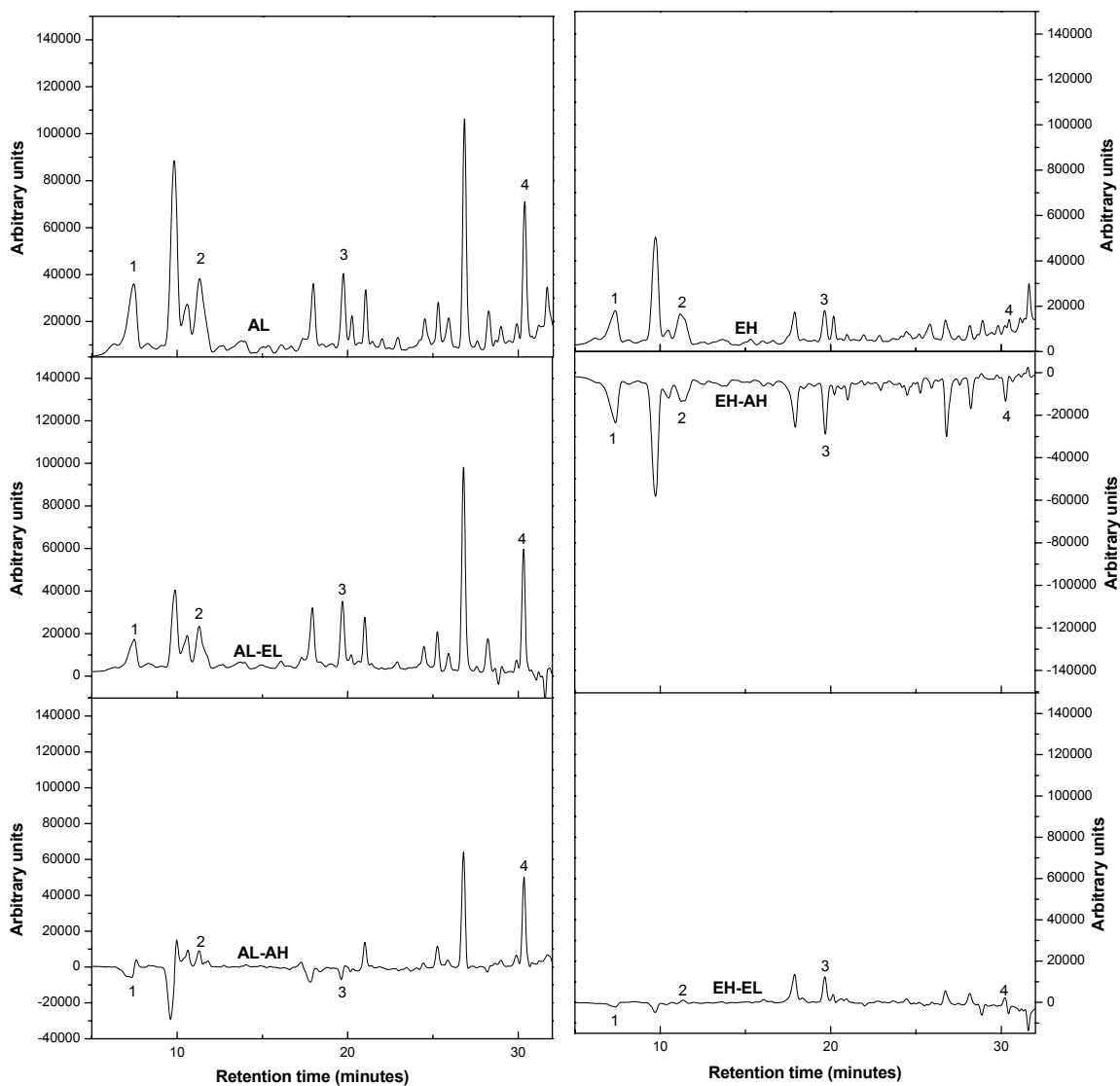
Fig. 2 Soluble phenolics in woody stem of *P. nigra* grown under either FACE (E) or ambient (A) [CO_2] and either fertilised (H) or unfertilised (L) conditions. Typical HPLC chromatographs are shown. To compare the difference of chromatographs between the treatments, the subtracted chromatographs are also shown. The following compounds are indicated by co-elution of internal standards: 1 = 3,4-dihydroxybenzoic acid, 2 = chlorogen acid, 3 = ferulic acid, and 4 = cinnamic acid.
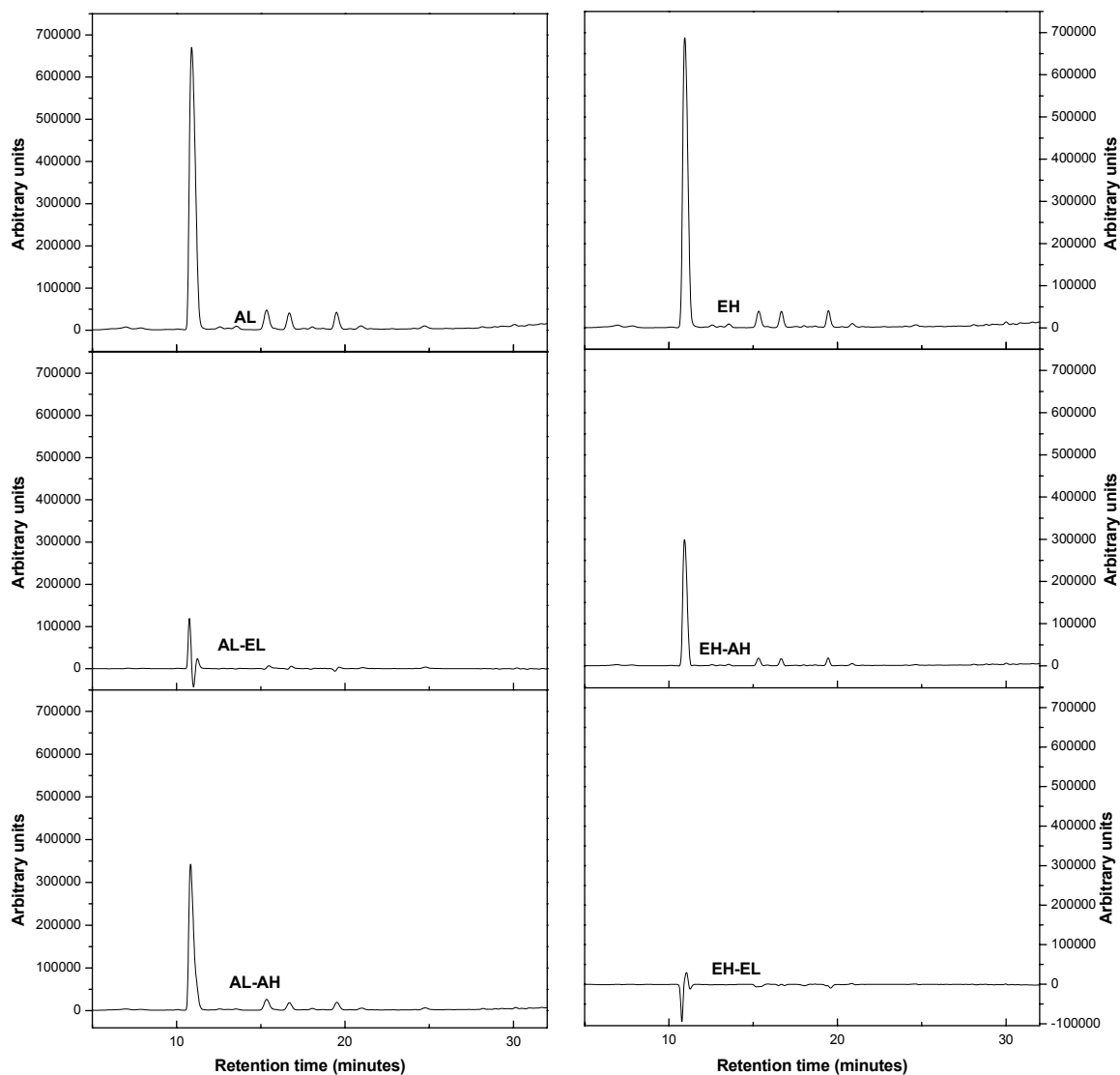
Fig. 3 Cell wall bound phenolics in woody stem of *P. nigra* grown under either FACE (E) or ambient (A) [CO_2] and either fertilised (H) or unfertilised (L) conditions. Typical HPLC chromatographs are shown. To compare the difference of chromatographs between the treatments, the subtracted chromatographs are also shown. By co-elution of internal standards, the major compound (4-hydroxybenzoic acid) is detected.

4.3.2 *Soluble proteins and internal N-pools*

Since leaves and bark play important roles in synthesis and/or storage of N-containing compounds in plants, soluble proteins and N status in these tissues were investigated (Table 2, Fig. 4). FACE caused decreases in soluble proteins and total N concentrations in leaves in the second year after coppicing, whereas N-fertilization increased these compounds (Table 2). However, in bark, neither FACE nor N-fertilization

had effects on soluble proteins. Concentrations of soluble proteins of bark in the dormant seasons were 31 — 53% higher than in the growing seasons (Fig. 4).

Table 2 Soluble proteins and nitrogen fractions in different components of leaves of *P. nigra* grown under either FACE (E) or ambient (A) [CO_2] and either fertilised (H) or unfertilised (L) conditions during two active (September) seasons. The values shown indicate means (± SE, n = 6) based on dry weight. *P*-values of the ANOVAs of CO_2, nitrogen (N) and their interactions are shown.

| Seasons	Treatments	Proteins (mg g^{-1} DW)	Lignin-bound N %	Total N %
Sep. 2002	AL	89.1 ± 10.2	no data	2.96 ± 0.14
	EL	75.4 ± 5.9	no data	2.45 ± 0.12
	AH	95.9 ± 8.2	no data	2.97 ± 0.24
	EH	91.8 ± 7.4	no data	3.36 ± 0.15
Sep. 2003	AL	109.5 ± 7.4	0.83 ± 0.05	2.36 ± 0.03
	EL	101.7 ± 7.2	0.85 ± 0.04	2.00 ± 0.10
	AH	139.3 ± 5.5	0.91 ± 0.04	2.36 ± 0.03
	EH	118.2 ± 4.9	0.84 ± 0.03	2.37 ± 0.07
Sep. 2002	CO_2	0.2821	no data	0.7202
	N	0.1672	no data	0.0129
	CO_2 x N	0.5565	no data	0.0157
Sep. 2003	CO_2	0.0334	0.5000	0.0112
	N	0.0016	0.4529	0.0074
	CO_2 x N	0.3076	0.3288	0.0070
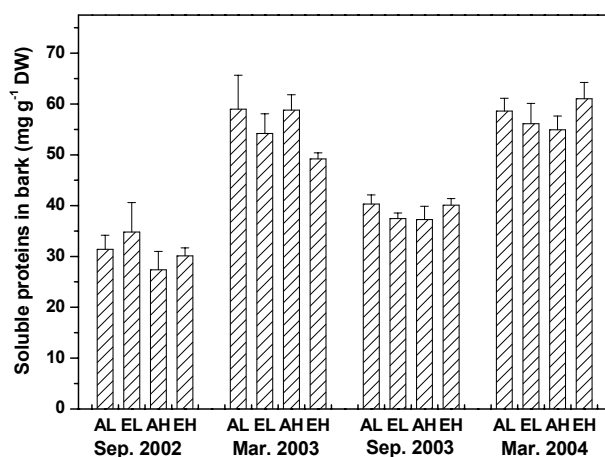
Fig. 4 Soluble proteins in bark of *P. nigra* grown under either FACE (E) or ambient (A) [CO_2] and either fertilised (H) or unfertilised (L) conditions. The data shown indicate means ± SE of six measurements for each treatment based on dry weight.

Wood occupies a large fraction of biomass in a mature tree. Woody stem is an important part in a growing tree. Furthermore, ray and axial parenchyma cells in wood can act as storage sites for proteins (Sauter & van Cleve 1994). Plants grown under elevated [CO_2] conditions were frequently observed to have lower protein and N concentrations in comparison with those grown under ambient [CO_2] (Poorter et al. 1997; Long et al. 2004). Our data show that the concentrations of soluble proteins in woody stem ranged from 7.8 to 21.6 mg g^{-1} depending on treatments (Fig. 5a). FACE decreased the concentrations of soluble proteins in woody stem of *P. nigra*, but this effect was very weak or even going to the opposite in the growing seasons (Table 3, Fig. 5a). In contrast, higher N-supply significantly enhanced concentrations of soluble proteins in the stem of *P. nigra* (Table 3, Fig. 5a). The concentrations of soluble proteins in the stem showed significant seasonal-variation. The concentrations of soluble proteins in the dormant seasons (March) were markedly higher (52 ― 143%) than in the growing (September) seasons (Table 3, Fig. 5a).

Table 3 Statistical results of concentrations of soluble proteins, nitrogen bound to lignin and total nitrogen in woody stem of *P. nigra* grown under either FACE (E) or ambient (A) [CO_2] and either fertilised (H) or unfertilised (L) conditions and harvested in different seasons. Significance levels (*P*-values of the ANOVAs) of CO_2, nitrogen (N), season and their interactions are indicated.

Treatments	Soluble proteins	Lignin bound N	Total N
CO_2	0.0255	0.0052	0.0005
N	0.0042	0.0001	0.1962
Season	0.0000	0.0000	0.0000
CO_2 x N	0.1826	0.0201	0.2042
CO_2 x Season	0.3766	0.0282	0.0130
N x Season	0.1344	0.0964	0.9297
CO_2 x N x Season	0.6872	0.1265	0.9277

Total N and Klason lignin-bound N concentrations in woody stem of *P. nigra* ranged from 2.2 to 3.9 mg g^{-1} and from 0.37 to 1.01 mg g^{-1} based on dry mass, respectively (Fig. 5b). FACE significantly decreased concentrations of total N and lignin-bound N in woody stem of *P. nigra*, whereas N-fertilization enhanced lignin-bound N concentrations (Table 3, Fig. 5b). Similar to soluble proteins, total N and lignin-bound N concentrations showed significant seasonal-variation. Total N and lignin-bound N concentrations in woody stem of *P. nigra* in the dormant seasons were markedly higher than in the growing seasons (Table 3, Fig. 5b).
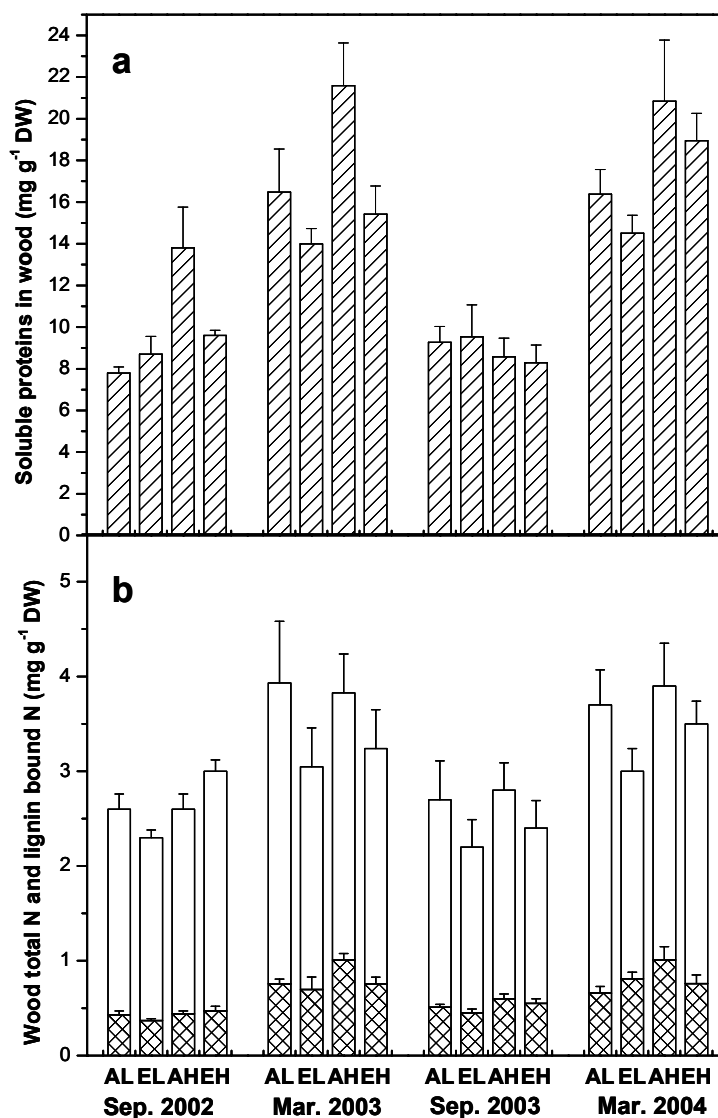
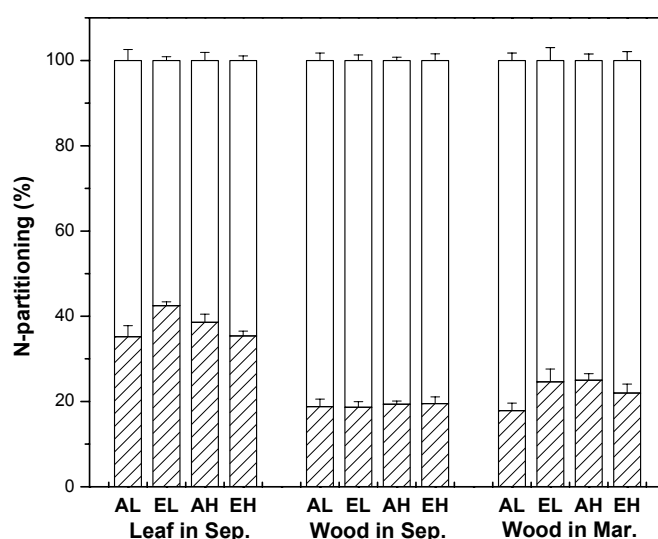**Fig. 5** Soluble proteins in wood (a) and N concentrations in wood and Klason lignin (b) of *P. nigra* grown under either FACE (E) or ambient (A) [CO$_2$] and either fertilised (H) or unfertilised (L) conditions. Crossed bar: N-bound to Klason lignin; blank bar: total nitrogen minus nitrogen bound to Klason lignin. The data shown indicate means ± SE of six measurements for each treatment based on dry weight.

To check the relative nitrogen partitioning, the percentage of nitrogen bound to lignin and percentage of total nitrogen minus nitrogen bound to lignin in woody stem of *P. nigra* were calculated (Table 4, Fig. 6). Our data show that 17 — 26% of total nitrogen was bound to lignin in woody stem of *P. nigra*. Neither FACE nor N-fertilization affected the relative nitrogen partitioning (Table 4, Fig. 6). Interestingly, in the leaf tissue of *P. nigra*, 35.3 — 42.3% of total nitrogen was bound to lignin. This finding was unexpected since it shows a higher sequestration of N in leaves than in wood (Fig. 6). Neither FACE nor N-fertilization affected the percentage of nitrogen bound to lignin in the leaf tissue of *P. nigra* (Table 4, Fig. 6).

Table 4 Statistical results of relative nitrogen partitioning — percentage of nitrogen bound to lignin and percentage of total nitrogen minus nitrogen bound to lignin in *P. nigra* grown under either FACE (E) or ambient (A) [CO_2] and either fertilised (H) or unfertilised (L) conditions. Data from wood september 2002 and wood september 2003, wood march 2003 and wood march 2004, are combined, respectively. Significance levels (*P*-values of the ANOVAs) of CO_2, nitrogen (N) and their interactions are indicated.

Treatments	Leaf Sep.	Wood Sep.	Wood Mar.
CO_2	0.2980	0.9936	0.2669
N	0.3269	0.4708	0.1780
CO_2 x N	0.0086	0.8913	0.0055

Fig. 6 Relative N-partitionin in leaf and wood of *P. nigra* grown under either FACE (E) or ambient (A) [CO_2] and either fertilised (H) or unfertilised (L) conditions. Hatched bars indicate percentage of nitrogen bound to Klason lignin; blank bars indicate total nitrogen subtracts percentage of nitrogen bound to Klason lignin. The data shown indicate means ± SE of six measurements for each treatment.

4.3.3 *Balance between carbon-based secondary compounds and soluble proteins*

To check whether there was a negative balance between soluble proteins and carbon-based secondary compounds, correlations between soluble proteins and soluble phenolics, cell-wall bound phenolics, soluble condensed tannins and the sum of these carbon-based secondary compounds were evaluated (Fig. 7). Our data clearly demonstrate that positive correlations were always observed between soluble proteins and carbon-based secondary compounds in the examined tissues. Furthermore, these positive correlations were statistically significant. This observation indicates that there were no tradeoffs between the formations of soluble proteins and these carbon-based secondary compounds.
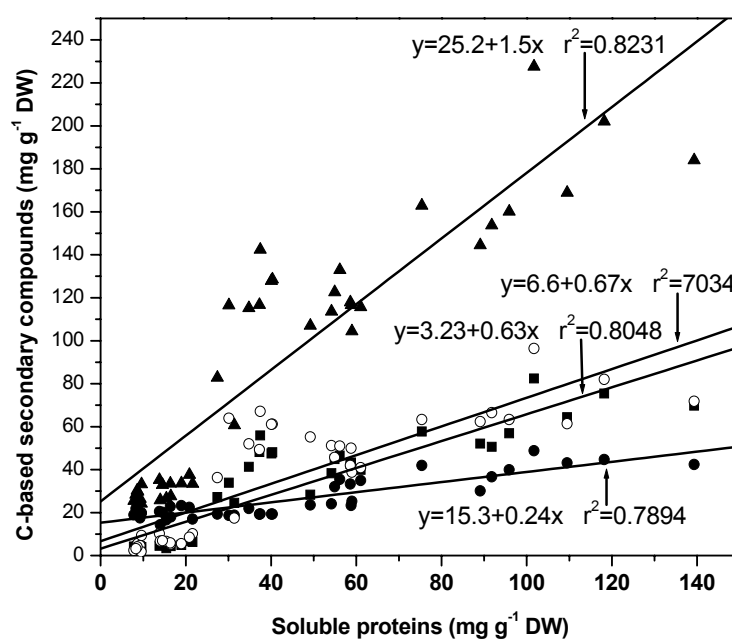
Fig. 7 Correlations of soluble proteins and soluble phenolics (squares), cell-wall bound phenolics (closed circles), soluble condensed tannins (open circles) and the sum of these carbon-based secondary compounds (triangles) in tested tissues (data points from leaf, bark and woody stem were pooled) of *P. nigra* grown under either FACE (E) or ambient (A) $[CO_2]$ and either fertilised (H) or unfertilised (L) conditions. All correlations are statistically significant ($P < 0.0001$).

4.4 Discussion

4.4.1 *Are secondary metabolites affected by FACE, N-fertilization or season?*

Carbon-based secondary metabolites play important roles in ecological interactions. Hence, it is essential to understand the responses of these metabolites to rising global $[CO_2]$ and N-deposition in order to predict forest resistance to insects and pathogens in the future environments. Numerous studies about this topic were conducted in the greenhouses and

growth chambers (reviewed by Lindroth 1996a, b; Coley 1998; Koricheva et al. 1998; Penuelas & Estiarte 1998). To our knowledge, no studies, however, have determined chemical defense compounds in wood of forest trees grown in the field under FACE and N-deposition conditions. Our results show that FACE had negligible effects on the examined carbon-based secondary compounds in woody stem of *P. nigra* (Table 1, Figs. 1-3). Leaves of plants grown under elevated [CO_2] were often observed increases in concentrations of phenolics and condensed tannins (Lavola & Julkunen-Tiitto 1994; Roth & Lindroth 1994), but in some species this response was lacking (Johnson & Lincoln 1991; Fajer et al. 1992; Kuokkanen et al. 2001). According to the carbon nutrient balance (CNB) hypothesis (Bryant et al. 1983), plants increase photosynthesis and carbon gain under elevated [CO_2] and low N conditions. The "excess" carbon may be allocated to carbon-based defenses. Under FACE, "excess" carbon that might be invested into secondary compounds was not found (Luo et al. 2005) although net leaf photosynthesis was stimulated under FACE (Bernacchi *et al.* 2003; Tricker *et al.* 2005). Where did the carbon go? A higher proportion of carbon under FACE was allocated to the formation of biomass (Luo et al. 2005) while Klason lignin, an important structure component, was unaffected in woody stem of *P. nigra* under FACE (Table 1). In the literature, only a few studies have addressed the effects of elevated [CO_2] on lignin content in plants with varying findings. For example, depending on the nutrient level, the lignin concentrations were unaffected or diminished by elevated [CO_2] in beech seedlings (Blaschke et al., 2002). Recently, Kostiainen et al. (2004) observed that elevated [CO_2] decreased the concentration of acid-soluble lignin in stem wood of 41-year-old Norway spruce trees. Elevated [CO_2] had no effects on lignin concentrations of mature xylem tissues of tabacoo although the growth was stimulated (Schlimme et al., 2002; Blaschke et al., 2004). These observations suggest that plant growth and lignin formation display homeostasis.

On the other hand, N-fertilization may affect the C/N ratio of the plant and thus, the allocation of resources to carbon-based secondary compounds. With N-fertilization, carbon may be shunted into growth so that other carbon pools, such as phenolics and tannins, should decrease (Coley et al. 2002). However, in the present study, carbon-based secondary metabolites in woody stem of *P. nigra* showed no response or increases as an unexpected response to N-fertilization (Table 1, Figs. 1-3). It has been suggested that synthesis of phenolics and condensed tannins might be regulated by the availability of phenylalanine and its demand for protein synthesis (Lambers 1993; Coronado et al. 1995; Yeoman &

Yeoman 1996) rather than, as predicted by the CNB hypothesis, by the plant's carbohydrate status. Current data seems to support the idea that N-fertilization enhances the biosynthesis of phenylalanine which further simultaneously stimulates the biosyntheses of carbon based secondary compounds and proteins.

It is interesting to note that seasonal changes had a marked influence on the abundance of carbon based secondary compounds in *P. nigra* (Table 1, Fig. 1). It is not surprised that lignin concentrations in the second year were higher than in the first year after coppicing since histochemical methods detected that lignification was continuing in the juvenile wood of *P. nigra* (data not shown). However, it is hard to understand that lignin concentrations in March were slightly but markedly higher than in September during 2 years after coppicing because it is unlikely that lignification takes place in the dormant seasons. Harvests made at the beginning of Septembers, ca. 4 weeks prior to bud set, may cause this observation since it is probable that lignification happened when poplar trees were growing vigorously at the end of the growing season on this experimental site.

In this study, the observation that neither FACE nor N-fertilization altered the composition of soluble- and wall bound-phenolics (Figs. 2 and 3) may indicate that all these components in specific tissues were indispensable and important to resist insects and pathogens.

4.4.2 *Are the internal N-pools affected by FACE, N-fertilization or season?*

The primary effects of elevated $[CO_2]$ on forest ecosystems is well characterised at the level of photosynthesis, but this response may be modified by secondary responses, such as feedback of altered plant C-N allocation (Norby et al. 2000). Plant internal N-pools may be particularly important for plants responding to the changing environmental conditions given that external N was frequently reported a limiting resource in many forest ecosystems and there were many points of interaction between C-N metabolism in plants (Stitt & Krapp 1999). Plant growth, in particular, growth of deciduous species, depends not only on external N supply but also on internal N-pools (Millard 1996). As *Populus* is a fast growing deciduous species, its growth might strongly depend on these N resources. Therefore, we investigated the internal N-pools of field-grown poplars exposed to FACE and N-fertilization. The finding that FACE led to decreases in N concentrations in leaves, bark and woody stem (Tables 2 and 3, Figs. 4 and 5) is consistent with other studies (Poorter et al. 1997; Cotrufo et al. 1998; Long et al. 2004). Frequently, plants under

elevated [CO_2] might accumulate more soluble sugars and/or starch in leaves, bark and woody stem, which could "dilute" N-pools in these tissues (Stitt & Krapp 1999). However, this was not the case in the present study because no increases in total non-structural carbohydrates were found in these tissues under FACE (Luo et al. 2005). Therefore, as proposed by Stitt & Krapp (1999), decreases in concentrations of N and soluble proteins under FACE may be due to one or more of following reasons: first, a higher nitrogen use efficiency because of reallocation of protein under FACE; second, ontogenetic drift leading to accelerated senescence as a result of the faster growth under FACE; third, inadequate N-fertilization under ambient N conditions; fourth, inadequate rates of N-uptake and assimilation under FACE. The lower nitrogen concentrations in tissues (leaf, bark and wood) under elevated [CO_2] accompanying a considerable fraction of N bound to lignin which is not easily degradable (Fig. 6) will have an important ecological significance. Lower levels of N in tissues can decrease rates of colonization by decomposing fungi (Chapin & Kedrowski 1983) and result in slowing the release of N from tissues and the conversion of organic N to inorganic N available for plant uptake (Strain & Bazzaz 1983). Finally, these slowing processes might limit fast growing forests, such as poplar, to grow vigorously under elevated [CO_2]. Furthermore, lower levels of N in woody stem may limit the re-growth of forest at the beginning of the growing seasons when the growth is mainly depending on internal N-resources (Millard 1996; Geßler et al. 2004; Cooke & Weih 2005).

In forest trees, nitrate taken up by roots is mainly assimilated in amino compounds by belowground tissues (Gojon et al. 1994). Thus, these amino compounds and traces of NO_3^- or NH_4^+ are transported from the roots to the shoots in the xylem (Dambrine et al. 1995; Geßler et al. 1998; Schmidt & Stewart 1998). Nitrogen-demanding tissues, e.g., cambial tissues of the stem, leaves, fruits and buds, take N from this xylem-borne pool of amino compounds for the synthesis of proteins and other organic N-containing compounds (Geßler et al. 2004). Therefore, external N-supply is expected to increase internal N-pools in trees. Our results are consistent with this expectation (Tables 2 and 3, Figs. 4 and 5). Previous studies showed that application of nitrate fertilizer resulted in increased rates of nitrate uptake (Clarkson & Lüttge 1991) and higher rates of nitrate reduction to ammonia (Lam et al. 1996). Enhanced rates of ammonia assimilation led to higher levels of amino acids, proteins and other nitrogen-containing compounds in plants (Marschner 1995). Furthermore, it has been proposed that nitrate in fertilizers is not only a resource but also acts, directly or indirectly, to trigger signals that modulate gene expression, metabolism and

development in plants (Stitt & Krapp 1999; Cook et al. 2003, 2005). It is expected that changes in these processes under elevated [CO_2] and/or N-fertilization conditions might have profound effects on C-N metabolism and growth (Stitt & Krapp 1999). This may in part explain that N-fertilization had no effects on N-partitioning although positive effects of N-fertilization on internal N-pools were detected (Tables 2-4, Figs. 4-6).

Seasonal fluctuations in the internal N-pools in the deciduous tree species are essential to support new growth at the beginning of the growing season (Sauter & van Cleve 1994; Norby et al. 2000; Geßler et al. 2004). Therefore, we investigated the seasonal effects on the internal N-pools (Table 3, Figs. 4 and 5). The seasonal pattern that tissues harvested in March (dormant season) contained markedly higher concentrations of internal N and/or soluble proteins than the samples harvested in September (active season) is consistent with a more detailed study (Sauter & van Cleve 1994). These authors demonstrated that the parenchyma cells in wood served as primary vegetative storage tissues for seasonal accumulated protein and/or N-containing constituents. Seasonal changes in the internal N-pools of tissues are a characteristic of the perennial habit and are resulted from physiological and molecular events associated with leaf senescence and N remobilization (Andersson et al. 2004; Cooke & Weih 2005). During leaf senescence, amino acids resulting from the hydrolysis of proteins and other N-containing constituents are loaded into phloem and transported into the parenchyma cells in bark, wood and root (Norby et al. 2000; Hörtensteiner & Feller 2002; Geßler et al. 2004). Rubisco breakdown during leaf senescence accounts for a notable proportion of the N exported from leaves (Brendley & Pell 1998; Millard & Thomson 1989), and probably contributes significant N resources for the synthesis of bark storage proteins in perennial organs, e.g., bark and wood (Cooke & Weih 2005). In the spring, N reserves in bark and woody perennial tissues are broken and amino acids are transported to the growing sites to supply N for early season development of expanding buds (Bollmark et al. 1999). Apparently, significant seasonal fluctuations in the internal N-pools in the present study correspond well to these physiological and molecular events.

4.4.3 *Is there a negative balance between protein biosynthesis and production of secondary metabolites*?

Due to the common precursor — phenylalanine in the biosynthetic pathways of proteins and secondary compounds, it is reasonable to assume that a negative balance exists

between the biosyntheses of these two categories of compounds since internal resources are limited in plants. However, our data do not support this plausible explanation (Fig. 7). The positive correlations between these pathways indicate that it is important for the whole plant to maintain simultaneously biosynthesis of both classes of compounds. From this point of view, the dilemma — growth or defense seems not to exist in poplar trees under elevated $[CO_2]$ and/or high N-availability. At least, the present data suggest that at the whole tree level, growth and defense orchestrate each other quite well.

In summary, FACE had no influence on lignin, cell wall-bound phenolics and soluble condensed tannins. Higher N-supply markedly stimulated carbon based secondary compounds. *P. nigra* grown under FACE had lower internal N-pools in leaf, bark and wood in comparison with those grown under ambient $[CO_2]$, but external N-supply caused increases in the internal N-pools. Neither FACE nor higher N-supply altered the N-partitioning between lignin-bound N and other N-containing compounds. The seasonal patterns of the internal N-pools were observed. Under the current experimental conditions, positive correlations existed between the biosyntheses of proteins and secondary compounds in *P. nigra*. These results imply that the growth and defense of forest trees may orchestrate each other quite well and forest trees will benefit from the coincidence of elevated atmospheric $[CO_2]$ and N-deposition in the future environmental scenarios.

References

Ainsworth EA, Long SP (2005) What have we learned from 15 years of free-air CO_2 enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy properties and plant production to rising CO_2. New Phytologist, 165, 351-372.

Andersson A, Keskitalo J, Sjödin A, Bhalerao R, Sterky F, Wissel K, Tandre K, Aspeborg H, Moyle R, Ohmiya Y, Brunner A, Gustafsson P, Karlsson J, Lundeberg J, Nilsson O, Sandberg G, Strauss S, Sundberg B, Uhlen M, Jansson S, Nilsson P (2004) A transcriptional timetable of autumn senescence. Genome Biology, 5, R24.

Bernacchi CJ, Calfapietra C, Davey PA, Wittig VE, Scarascia-Mugnozza G, Raines CA, Long SP (2003) Photosynthesis and stomatal conductance responses of poplars to free-air CO_2 enrichment (PopFACE) during the first growth cycle and immediately following coppice. New Phytologist, 159, 609- 621.

Bezemer TM, Jones TH (1998) Plant-insect interactions in elevated atmospheric CO_2: quantitative analyses and guild effects. Oikos, 82, 212-222.

Blaschke L, Forstreuter M, Sheppard LJ et al. (2002) Lignification in beech (*Fagus sylvatica*) grown at elevated CO_2 concentrations: interaction with nutrient availability and leaf maturation. Tree Physiology, 22, 469-477.

Blaschke L, Legrand M, Mai C, Polle A (2004) Lignification and structural biomass production in tobacco with suppressed caffeic/5-hydroxy ferulic acid-*O*-methyl transferase activity under ambient and elevated CO_2 concentrations. Physiologia Plantarum, 121, 75-83.

Bollmark L, Sennerby-Forsse L, Ericsson T (1999) Seasonal dynamics and effects of nitrogen supply rate on nitrogen and carbohydrate reserves in cutting-derived *Salix viminalis* plants. Canadian Journal of Forest Research, 29, 85-94.

Booker FL, Anttonen S, Heagle AS (1996) Catechin, proanthocyanidin and lignin concentrations of loblolly pine (*Pinus taeda*) needles after chronic exposure to ozone. New Phytologist, 132, 483-492.

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of proteins utilizing the principle of protein-dye binding. Analytical Biochemistry, 72, 248-254.

Brendley BW, Pell EJ (1998) Ozone-induced changes in biosynthesis of Rubisco and associated compensation to stress in foliage of hybrid poplar. Tree Physiology, 18, 81-90.

Bryant JP, Chapin FS III, Klein DR (1983) Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory. Oikos, 40, 357-368

Calfapietra C, Gielen B, Sabatti M, Angelis PD, Scarascia-Mugnozza G, Ceulemans R (2001) Growth performance of *Populus* exposed to "Free Air Carbon Dioxide Enrichment" during the first growing season in the POPFACE experiment. Annals Forest Science, 58, 819-828.

Calfapietra C, Gielen B, Sabatti M, Angelis PD, Miglietta F, Mugnozza GS, Ceulemans R (2003) Do above-ground growth dynamics of poplar change with time under CO_2 enrichment? New Phytologist, 160, 305-318.

Chapin FS, Kedrowski RA (1983) Seasonal changes in nitrogen and phosphorus fractions and autumn retranslocation in evergreen and deciduous taiga trees. Ecology, 64, 376-391.

Chapin FS, Schulze ED and Mooney HA (1990) The ecology and economics of storage in plants. Annual Review of Ecology and Systematics, 21, 423-447.

Clarkson DT, Lüttge U (1991) Mineral nutrition: inducible and repressible transport systems. Progress in Botany, 52, 61-63.

Coley PD (1998) Possible effects of climate change on plant/herbivore interactions in moist tropical forests. Climate Change, 39, 455-472.

Coley PD, Massa M, Lovelock CE and Winter K (2002) Effects of elevated CO_2 on foliar chemistry of saplings of nine species of tropical tree. Oecologia, 133, 62-69.

Cooke JEK, Brown KA, Wu R, Davis JM (2003) Gene expression associated with N-induced shifts in resource allocation in poplar. Plant Cell and Environment, 26, 757-770.

Cooke JEK, Martin TA, Davis JM (2005) Short-term physiological and developmental responses to nitrogen availability in hybrid poplar. New Phytologist, 167, 41-52.

Cooke JEK, Weih M (2005) Nitrogen storage and seasonal nitrogen cycling in *Populus*: bridging molecular physiology and ecophysiology. New Phytologist, 167, 19-30.

Coronado C, Zuanazzi JAS, Salland C, Quirion JC, Esnault R, Husson HP, Kondorosi A, Ratet P (1995) Alfalfa root flavonoid production is nitrogen regulated? Plant Physiology, 108, 533-542.

Cotrufo MF, Ineson P, Scott AY (1998) Elevated CO_2 reduces the nitrogen concentration of plant tissues. Global Change Biology 4, 43-54.

Cotrufo MF, Angelis PD and Polle A (2005) Leaf litter production and decomposition in a poplar short-rotation coppice exposed to free air CO_2 enrichment (POPFACE). Global Change Biology, 11, 971-982.

Czochanska Z, Foo LY, Newman RH, Porter LJ (1980) Polymeric proanthocyanidins. Stereochemistry, structural units, and molecular weight. Journal of the Chemical Society. Perkin Transactions I: 2278-2286.

Dambrine E, Martin F, Carisey N, Granier A (1995) Xylem sap composition: a tool for investigating mineral uptake in adult spruce. Plant and Soil, 168-169, 233-241.

Dence CW (1992) Lignin determination. *In* Methods in Lignin Chemistry (Stephen YL ed), Springer-Verlag, Berlin, pp 33-61.

Dickson RE (1989) Carbon and nitrogen allocation in trees. Ann. Sci. For. 46: 631-647.

Dixon RA, Paiva NL (1995) Stress-induced phenylpropanoid metabolism. Plant Cell, 7, 1085-1097.

Fajer ED, Bowers MD, Bazzaz F (1992) The effects of nutrients and enriched CO_2 environments on production of carbon-based allelochemicals in *Plantago*: a test of the carbon/nutrient balance hypothesis. American Naturalist, 140, 707-723.

Geßler A, Schneider S, Weber P, Hanemann U, Rennenberg H (1998) Soluble N compounds in trees exposed to high loads of N: a comparison between the roots of Norway spruce (*Picea abies*) and beech (*Fagus sylvatica*) trees. New Phytologist, 138, 385-399.

Geßler A, Kopriva S, Rennenberg H (2004) Regulation of nitrate uptake at the whole-tree level: interaction between nitrogen compounds, cytokinins and carbon metabolism. Tree Physiology, 24, 1313-1321.

Gojon A, Plassard C, Bussi C (1994) Root/shoot distribution of NO_3^- assimilation in herbaceous and woody plants. *In* A Whole Plant Perspective on Carbon-N Interactions. Eds. Roy J and Garnier E. SPB Academic Publishing, The Hague, pp 131-147.

Harborne JB (1997) Plant secondary metabolism. In: Plant Ecology, Second Edition. Crawley, MJ (ed.), pp. 132-155. Blackwell Science, Oxford.

Hartley SE, Jones CG (1997) Plant chemistry and herbivory: or why the world is green. In: Plant Ecology, Second Edition. Crawley MJ (ed.), pp. 284-324. Blackwell Science, Oxford.

Hartley SE, Jones CG, Couper GC, Jones TH (2000) Biosynthesis of plant phenolic compounds in elevated atmospheric CO_2. Global Change Biology, 6, 497-506.

Hermann KM (1995) The shikimate pathway as an entry to aromatic secondary metabolism. Plant Physiology, 107, 7-12.

Hörtensteiner S, Feller U (2002) Nitrogen metabolism and remobilization during senescence. Journal of Experimental Botany, 53, 927-937.

IPCC (2001) Climate Change 2001: The Scientific Basis. Contribution of Working Group I to the Third Assessment Report of the Intergovernmental Panel on Climate Change (eds Houghton JT, Ding Y, Griggs DJ, Noguer M, van der Linden PJ, Dai X, Maskell K, Johnson CA). Cambridge University Press, Cambridge, UK.

Jones CG, Hartley SE (1999) A protein competition model of phenolic allocation. Oikos, 86, 27-44.

Johnson RH, Lincoln DE (1991) Sagebrush allocation patterns and grasshopper nutrition: the influence of CO_2 enrichment and soil mineral limitation. Oecologia, 87, 127-134.

Kliebenstein DJ (2004) Secondary metabolites and plant/environment interactions: a view through *Arobidopsis thaliana* tinged glasses. Plant, Cell and Environment, 27, 675-684.

Koricheva J, Larsson S, Haukioja E, Keinanen M (1998) Regulation of woody plant metabolism by resource availability: hypothesis testing by means of a meta-analysis. Oikos, 83, 212-226.

Kostiainen K, Kaakinen S, Saranpaa P, Sigurdsson B, Linder S, Vapaavuori E (2004) Effect of elevated [CO_2] on stem wood properties of mature Norway spruce grown at different soil nutrient availability. Global Change Biology, 10, 1526-1538.

Kruse J, Hetzger I, Mai C, Polle A and Rennenberg H (2003) Elevated pCO_2 affects N-metabolism of young poplar plants (*Populus tremula × P. alba*) differently at deficient and sufficient N-supply. New Phytologist, 157, 65-81.

Kuokkanen K, Julkunen-Tiitto R, Keinänen M, Niemelä P and Tahvanainen J (2001) The effect of elevated CO_2 and temperature on the secondary chemistry of *Betula pendula* seedlings. Trees, 15, 378-384.

Lam HM, Coschigano KT, Oliveira IC, Melo-Oliveira R and Coruzzi GM (1996) The molecular-genetics of nitrogen assimilation into amino acids in higher plants. Annual Review of Plant Physiology and Plant Molecular Biology, 47, 569-593.

Lambers H (1993) Rising CO_2, secondary plant metabolism, plant-herbivore interactions and litter decomposition. Vegetatio, 104/105, 263-271.

Lavola A, Julkunen-Tiitto R (1994) The effect of elevated carbon dioxide and fertilization on primary and secondary metabolites in birch, Betula pendula (Roth). Oecologia, 99, 315-321.

Lindroth RL (1996a) Consequences of elevated atmospheric CO_2 for forest insects. In: Körner C, Bazzaz FA (eds) Carbon dioxide, populations, and communities. Physiological ecology series. Academic Press, San Diego, pp 347-361.

Lindroth RL (1996b) CO_2-mediated changes in tree chemistry and tree-Lepidoptera interactions. In: Koch GW, Mooney HA (eds) Carbon dioxide and terrestrial ecosystems. Physiological ecology series. Academic Press, San Diego, pp 105-120.

Long SP, Ainsworth EA, Rogers A, Ort DR (2004) Rising atmospheric carbon dioxide: plants FACE the future. Annual Review of Plant Biology, 55, 591-628.

Luo Z-B, Calfapietra C, Liberloo M, Scarascia-Mugnozza G, Polle A (2005) Carbon partitioning to mobile and structural fractions in poplar wood under elevated CO_2 (EUROFACE) and N-fertilization. Global Change Biology (in press)

Marschner M (1995) Mineral Nutrition of Higher Plants. 2nd edn. Academic Press Limited, London, UK.

McGuire AD, Melillo JM and Joyce LA (1995) The role of nitrogen in the response of forest net primary production to elevated atmoshperic carbon dioxide. Annual Review of Ecological Systems, 26: 473-503.

Miglietta F, Peressotti A, Vaccari FP, Zaldei A, Angelis PD, Scarascia-Mugnozza G (2001) Free-air CO_2 enrichment (FACE) of a poplar plantation: the POPFACE fumigation system. New Phytologist, 150, 465-476.

Millard P (1996) Ecophysiology of the internal cycling of nitrogen for tree growth. Z. Pflanzenernaehr. Bodenkd. 159: 1-10.

Millard P, Thomson CM (1989) The effect of the autumn senescence of leaves on the internal cycling of nitrogen for the spring growth of apple trees. Journal of Experimental Botany, 40, 1285-1289.

Norby RJ, Long TM, Hartz-Rubin JS, O'Neill EG (2000) Nitrogen resorption in senescing tree leaves in a warmer, CO_2-enriched atmosphere. Plant and Soil, 224, 15-29.

Norby RJ, Hanson PJ, O'Neill EG, Tschaplinski TJ, Weltzin JF, Hansen RT, Cheng W, Wullschleger SD, Gunderson CA, Edwards NT, Johnson DW (2002) Net primary productivity of a CO2-enriched deciduous forest and the implications for carbon storage. Ecological Applications, 12, 1261-1266.

Northrup RR, Dahlgren RA, McColl JG (1998) Polyphenols as regulators of plant-litter-soil interactions: a positive feedback. Biogeochemistry, 42, 189-220.

Oren R, Ellsworth DS, Johnsen KH *et al.* (2001) Soil fertility limits carbon sequestration by forest ecosystems in a CO_2-enriched atmosphere. Nature, 411, 469-472.

Penuelas J, Estiarte M (1998) Can elevated CO_2 affect secondary metabolism and ecosystem function? Trends in Ecological Evolution, 13, 20-24.

Poorter H, Van Berkel Y, Baxter R (1997) The effect of elevated CO_2 on the chemical composition and construction costs of leaves of 27 C_3 species. Plant, Cell and Environment, 20, 472-482.

Porter LJ, Hrstich L and Chan BG (1986) The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. Phytochemistry, 25, 223-230.

Pritchard S, Peterson C, Runion GB, Prior S and Rogers H (1997) Atmospheric CO_2 concentration, N availability, and water status affect patterns of ergastic substance deposition in longleaf pine (*Pinus palustris* Mill.) foliage. Trees, 11, 494-503.

Roth SK, Lindroth RL (1994) Effects of CO_2-mediated changes in paper birch and white pine chemistry on gypsy moth performance. Oecologia, 98, 133-138.

Sauter JJ, van Cleve B (1994) Storage, mobilization and interrelations of starch, sugars, protein and fat in the ray storage tissue of poplar trees. Trees, 8, 297-304.

Schlimme M, Blaschke L, Lagrimini M, Polle A (2002) Growth performance and lignification in tobacco with suppressed apoplastic anionic peroxidase activity under ambient and elevated CO_2 concentrations. International Journal of plant sciences, 163, 749-754.

Schmidt S, Stewart GR (1998) Transport, storage and mobilization of nitrogen by trees and shrubs in wet/dry tropics of northern Australia. Tree Physiology, 18, 403-410.

Sigurdsson BD, Thorgeirsson H, Linder S (2001) Growth and dry-matter partitioning of young *Populus trichocarpa* in response to carbon dioxide concentration and mineral nutrient availability. Tree Physiology, 21, 941-950.

Smith SD, Huxman TE, Zitzer SF, Charlet TN, Housman DC, Coleman JS, Fenstermaker LK, Seemann JR and Nowak RS (2000) Elevated CO_2 increases productivity and invasive species success in an arid ecosystem. Nature, 408, 79 – 82.

Stitt M, Krapp JA (1999) The molecular physiological basis for the interaction between elevated carbon dioxide and nutrients. Plant Cell and Environment 22(583-622).

Strack D, Heilmann J, Wray V, Dirks H (1989) Structures and accumulation patterns of soluble and insoluble phenolics from Norway spruce needles. Phytochemistry, 28, 2071-2078.

Strain BR, Bazzaz FA (1983) Terrestrial plant communities. *In* CO_2 and Plants. Ed. Lemon ER. Pp 177-222. Westview Press, Boulder, CO.

Tilman D, Fargione J, Wolff B, D'Antoio C, Dobson A, Howarth R, Schindler D, Schlesinger WH, Simberloff D and Swackhamer D (2001) Forecasting agriculturally driven global environmental change. Science, 292, 281-284.

Tricker PJ, Trewin H, Kull O *et al.* (2005) Stomatal conductance and not stomatal density determines the long-term reduction in leaf transpiration of poplar in elevated CO_2. *Oecologia*, In press.

Vitousek PM, Aber JD, Howarth RW *et al.* (1997) Human alteration of the global nitrogen cycle: sources and consequences. Ecological Applications, **7**, 737-750.

Warren JM, Allen HL and Booker FL (1999) Mineral nutrition, resin flow and phloem phytochemistry in loblolly pine. Tree Physiology, 19, 655-663.

Yeoman MM, Yeoman CL (1996) Tansley Review No. 90. Manipulating secondary metabolism in cultured plant cells. New Phytologist, 134, 553-569.

5. Wood properties and gene expression profiling in poplar

Zhi-Bin Luo, Janice Cooke, John Mackay, Andrea Polle (2005) Wood properties and gene expression profiling in *Populus × euramericana* exposed to Free Air CO_2 Enrichment (FACE) and N-fertilization. (**Manuscript**)

Abstract

To study wood properties and the underlying molecular mechanisms of their variations, *Populus × euramericana* trees were exposed to ambient [CO_2] (about 370 μmol mol^{-1} CO_2) or elevated [CO_2] (about 550 μmol mol^{-1} CO_2) using Free-Air CO_2 Enrichment (FACE) technology in combination with two N-levels. In total FACE was maintained for five years. After three growing seasons, the plantation was coppiced and one half of each experimental plot was fertilized with nitrogen. Wood anatomy, fibre properties, Klason lignin, calorific value and Fourier transform infrared (FT-IR) spectra of wood were analysed in secondary sprouts during the first two years after coppicing. FACE significantly stimulated the annual ring width in the second year after coppicing. However, FACE significantly decreased the cell wall, ray parenchyma and vessel lumen fractions, which was mainly due to a significant increase in the fraction of fibre lumina and decreased thickness of fibre walls. Higher N-supply also significantly decreased the cell wall fraction which was due to a marked decrease in the thickness of fibre walls and increase in fibre lumen fraction and fibre lumen diameter. FACE and N-fertilization together stimulated lignin formation. This was also confirmed by maps of lignin distribution and FT-IR spectra in wood. N-fertilization significantly enhanced the energy potential by 16 — 69%, which was mainly due to its stimulation of aboveground biomass.

Gene expression profiling revealed that only few transcripts were markedly affected by FACE and/or N-fertilization. In the developing xylem of poplar grown under FACE and N-fertilization condition compared to FACE and low N supply condition, the transcripts of two genes, caffeic acid-3-O-methyltransferase 1 (COMT-1) and ferulate-5-hydroxylase (F5H), which tightly linked to lignin biosynthesis, were increased. This observation corresponds well to the findings in Klason lignin analysis, mapping of lignin distribution and FT-IR spectra. The expression of a tubulin gene associated with the cytoskeleton was enhanced under FACE and N-fertilization condition in comparison with FACE and low N supply condition. A change in the expression of this gene may be related to the altered anatomical properties (diameters of fibre and vessel lumen) in the developing xylem of *P. × euramericana* under these conditions.

In summary, poplar wood grown under FACE and/or N-fertilization condition, wood properties were altered. This is probably caused by the alteration of molecular composition, which is further caused by changes in gene expression associated with FACE and/or N-fertilization induced shifts in resource allocation.

5.1 Introduction

Atmospheric CO_2 concentrations ($[CO_2]$) have been steadily rising since the beginning of industrialization (IPCC, 2001). Increases in $[CO_2]$ enhance photosynthesis and growth of plants, including forest trees, but these physiological responses to elevated $[CO_2]$ depend on both the functional groups (C3 vs. C4) of plants and the nutrient conditions of the soil (Drake et al., 1997; Long et al., 2004). At the same time, N-deposition is also increasing because of wide application of N-containing fertilizers to improve yield (Vitousek et al., 1997; Tilman et al., 2001). Since forests contain more than 90% of the carbon of terrestrial living organisms (Geider et al. 2001), forests are essential for our understanding of carbon fluxes between atmospheric C sinks and land-based C sinks (Grace, 2004). In short-term controlled studies, it has been shown that elevated $[CO_2]$ stimulated C capture by forest trees by the process of photosynthesis (Saxe et al., 1998; Gielen and Ceulemans, 2001). However, on long-term large scale, many questions still need to be answered. For instance, how do elevated $[CO_2]$ and N-deposition affect wood properties? And if so, what are the underlying molecular mechanisms? Wood properties are referred to properties such as the size, shape and arrangement of cells in wood, as well as the structure and chemistry of the cell walls (Whetten et al., 2001). Since the majority of carbon in wood of forest trees is held in the cell walls (Luo et al., 2005a), understanding the responses of the structure and chemistry of cell walls in wood to elevated $[CO_2]$ and N-deposition is particularly important. Besides the ecological importance of wood in forest trees, wood is also economically important for human beings, e.g. as structural material and energy resources.

In the current literature, contrasting effects of elevated $[CO_2]$ on wood quality and structure have been reported. Cell wall thickness of tracheids increased in response to elevated $[CO_2]$ in *Pinus radiata* D. Don seedlings (Conroy et al., 1990; Atwell et al., 2003), but decreased in Siberian larch (Yazaki et al., 2001). In *P. × euramericana* and *P. alba*, cell wall thickness was also decreased by elevated $[CO_2]$ (Luo et al. 2005b). Tracheid diameter in Scots pine (Ceulemans et al., 2002) and tracheid lumen diameter in Siberian larch (Yazaki et al., 2001) increased in elevated $[CO_2]$, whereas no changes were found in radiata pine (Atwell et al., 2003). Few studies addressed the responses of wood chemical composition to elevated $[CO_2]$. Under higher nutrient supply, lignin concentrations in beech decreased in response to elevated $[CO_2]$ (Blaschke et al., 2002), but no significant effects of elevated $[CO_2]$ on lignin concentrations in wood of other species were observed (Runion et

al., 1999; Kilpelainen et al., 2003; Atwell et al., 2003). It needs to be noted that most of these results were obtained from plants grown under controlled conditions. Due to the limitations of controlled conditions, it is hard to scale up these results to the ecosystem level. Furthermore, most of these results were derived from investigations of conifer species. However, it is also important for us to know how anatomical and biochemical properties respond to elevated $[CO_2]$ in broadleaf species, which have different structural features compared to conifers.

The process of wood formation and wood properties are due to the function of genes and proteins in the cambium and differentiating xylem. The regulation of genes in response not only to developmental but also to environmental factors is probably crucial in determining the variation in wood properties. The fibre length, diameter and cell wall thickness all affect the strength and density of wood, and in turn affect the quality of wood-based products (Whetten et al., 2001). With the development of genomic tools, analysis of networks of genes involved in certain biological process is feasible. Microarrays have been applied successfully to examine gene expression patterns in plants under different experimental conditions because transcript profiling holds a tremendous promise to dissecting the regulatory mechanisms and transcriptional networks underlying biological processes (Alba et al., 2004; Taylor et al., 2005). Using this resource to examine the gene responses to changing environmental conditions may help us to understand the altered growth, development and wood properties of forest trees grown under elevated $[CO_2]$ and/or N-fertilization. To investigate the influence of environmental conditions (elevated $[CO_2]$ and N-fertilization) on wood properties, FACE technology was used, i.e. a system enabling open-air fumigation of a large number of trees with elevated atmospheric $[CO_2]$. The advantage of the FACE approach is that whole ecosystems may be studied during long-term exposure.

To analyse wood properties, *Populus × euramericana* planted on a previous agriculture land in central Italy, was used and combined with cDNA microarray analysis. Our objectives are (i) to determine wood properties in response to elevated $[CO_2]$ and N-fertilization; and (ii) to preliminarily characterise the molecular mechanisms underlying these responses.

5.2 Materials and Methods

5.2.1 *FACE facilities*

The experimental site was located on a former agricultural land in central Italy (Tuscania, 42°22' N, 11°48' E, altitude 150 m). In spring 1999, six 30 m × 30 m experimental plots were selected after detailed soil analysis. Three of the plots were equipped with FACE facilities, whereas the other three plots were left under ambient [CO_2] conditions. Pure CO_2 (Messer Griesheim) was released through laser-drilled holes in the polyethylene rings to reach 550 µmol mol^{-1} of the target [CO_2] inside the FACE plots. A meteorological station was applied to control the release of CO_2 at each FACE plot. The FACE system was monitored by a computer to reach the target [CO_2] (550 µmol mol^{-1}). A three-year-old poplar plantation exposed to FACE with trees reaching heights of about 9 m was coppiced in winter of 2001/2002 (Calfapietra et al. 2003). In subsequent years the secondary sprouts growing from the stools were exposed to FACE, and additionally, half of each experimental plot was fertilised. In the FACE plots daytime [CO_2] were 554 ± 1.6 µmol mol^{-1} and 535.9 ± 20.4 µmol mol^{-1} during the growing seasons (from bud break to leaf fall) of 2002 and 2003, respectively (Miglietta, CNR-IATA, Florence, Italy, unpublished data). In the control plots, ambient [CO_2] was about 370 µmol mol^{-1}. A detailed description about the FACE facilities was given by Miglietta et al. (2001).

5.2.2 *Plant material and N-fertilization*

Each experimental plot was divided in halves by a physical resin-glass barrier (1 m deep in the soil). Each half plot was further divided into three triangular areas. Each triangular section was planted with one of the following poplar species, *P. alba* L. (2AS-11), *P. × euramericana* (Dode) Guinier (I-214) and *P. nigra* L. (Jean Pourtet), at a planting density of 10 000 trees per ha (1 m × 1 m). A detailed description of the clone properties was given by Calfapietra et al. (2001). N-fertilization was provided in half of each plot in the post-coppice phase (from the year 2002 afterwards). Fertilisers (in 2002 Navarson 20-6-6 (N-P-K) and in 2003 Ammonium Nitrate 34-0-0 (N-P-K)) were applied weekly (in constant amount in 2002 and in amounts proportional to the growth rate in 2003) by using a hydraulic pump (Ferti-injector Amiad, IMAGO srl, Italy) per plot connected to the irrigation system. Fertilisers were applied in the growing seasons (July 8, 2002—October 20, 2002 and May 6, 2003—September 16, 2003) in total amounts of 212 kg N ha^{-1} in 2002, and 290 kg N ha^{-1} in 2003, respectively.

5.2.3 *Sampling*

Since budbreak of these *Populus* species in central Italy is at the end of March and bud set in the middle of September or later (Calfapietra et al. 2001), field samplings were performed according to following strategy: in the first week of September (active season) 2002 and 2003, and March (dormant season) 2003 and 2004, respectively, field harvests were conducted. In September 2002, two stools were marked randomly per experimental variable, and on each marked stool, the diameters of all shoots with diameters more than 0.5 cm were determined and the shoot with the second thickest diameter measured at the height of 20 cm above the stool, was harvested. The third thickest one was marked for next harvest in March 2003. In September 2003, other two stools were marked randomly per experimental variable and the same harvesting strategy was applied. In each field campaign 24 shoots of *P. × euramericana* were harvested from six experimental plots. The harvested shoot was cut at the stool level.

A stem portion (about 50 cm length) was taken at a height of 1.5 m from the sampled shoot. The stem portion was immediately debarked and the differentiating xylem was scraped off the stem using a razor blade. The stem disk (debarked, about 3cm in thickness) was taken at a height of 1.9 m. The differentiating xylem and wood disk were immediately frozen in liquid nitrogen and stored at − 80°C. An 8 cm long stem segment was removed from the height of 1.92 m to 2.00 m of each shoot and preserved for anatomical studies in FAE (37% formalin/glacial acetic acid/70% ethyl alcohol =5 parts/5 parts/90 parts).

Samples harvested in September 2002 and 2003 were used for analyses of wood anatomy, carbon and nitrogen contents, and Klason lignin. Samples harvested in September 2003 were used for Fourier transform infrared (FT-IR) spectra analyses. Samples harvested in September 2003 and March 2004 were used for investigation of calorific value in wood. Differentiating xylem samples harvested in September 2002 were used for the transcript profiling.

5.2.4 *Wood anatomical analyses*

Stem cross-sections (30 µm in thickness) were obtained with a sliding microtome (Reichert-Jung, Heidelberg, Germany) and mounted in 50% glycerol for microscopic analyses. A detailed description of the preparation and measurement has been given elsewhere (Luo et al., 2005b).

To analyse fibre lengths, stem wood harvested in March 2004 was separated into two parts (wood formed in the year 2002 and 2003, respectively) according to the growth ring under a stereomicroscope (Wildleitz Canada, Ottawa, Canada). This sample was further cut into small chips of approximately 2 x 2 x 30 mm dimension. Half gram of this wood chips were placed in test tubes with Franklin solution (glacial acetic acid : 30% peroxide = 1:1 (v:v)) for 72 hours at 65 °C. Subsequently, the maceration solution was removed by emptying the mixture into coarse crucibles and rinsing with de-ionized water 5 times. The pulped wood was re-suspended in 20 ml Tris buffer (pH 8.0) and stored in cold room (4 °C) until analysis. Prior to analysis in the Fibre Quality Analyzer (FQA; OpTest Equipment Inc., Hawkesbury, Ontario, Canada), the pulped wood was stirred by glass balls (0.6 cm in diameter) to get fibres well-separated. Two hundred µL of stirred pulped wood solution was further diluted with 600 mL de-ionized water, which was injected to the FQA machine. The reading frequency of fibres was 30-40 fibres per second. Each measurement occurred automatically yielding the mean of 8000 fibre lengths. The average contour length of detected fibres (Ln), the length weighted average of detected fibres (Lw) and the weight weighted average of detected fibres (Lww) were defined as follows:

Arithmetic length (Ln) = $\Sigma n_i L_i / \Sigma n_i$;

Length weight length (Lw) = $\Sigma n_i L_i^2 / \Sigma n_i L_i$;

Weight weighted length (Lww) = $\Sigma n_i L_i^3 / \Sigma n_i L_i^2$;

Where: i = 1, 2, …n reading, n = fibre count in the "i th" reading, L = contour length – histogram class center length in the "i th" reading.

5.2.5 *Determination of carbon and nitrogen concentrations*

To determine carbon and nitrogen concentrations, aliquots of 0.6-1.0 mg dry and fine powder of stem wood were weighed into 5 × 9 mm tin cartouches (Hekatech, Wegberg, Germany) and analysed by an Elemental Analyzer EA1108 (Carlo Erba Strumentazione, Rodano, Italy). Acetanilide (71.09% C, 10.36% N; Carlo Erba Strumentazione, Rodano, Italy) was used as the standard.

5.2.6 *Determination of Klason lignin,*

The determination of acid-insoluble (Klason) lignin was adapted from Dence (1992). About 500 mg (W1) fine powder of wood or leaves were extracted with 40 ml 0.5 M

phosphate buffer (pH 7.8, 0.5% Triton). The mixture was incubated on a shaker for 30 min and centrifuged (5000 g, 10 min, 4 ºC). The precipitate was kept and resuspended in 0.5 M phosphate buffer as above. Subsequently, the pellet was washed 4 times (30 min for each washing) in 100% methanol. The resulting pellet consisted mainly of structural biomass. The pellet was immersed in 40 ml of 96% ethanol/cyclohexan (1/2, V/V), incubated in a water bath (6 h, 50 ºC) and shaken every 30 min. Afterwards, the mixture was centrifuged (4500 g, 10 min, 4 ºC). The precipitate was washed again with 40 ml ethanol/cyclohexan mixture as above. Then, the pellet was washed with 20 ml 100% acetone and centrifuged. This washing step was repeated three times. The precipitate was dried overnight under a fume hood. The pellet was mixed well with 8 ml 72% H_2SO_4 and incubated for 60 min at room temperature. Subsequently, the mixture was suspended in 200 ml distilled H_2O, and mixed well. The mixture was autoclaved (1 h, 121 ºC). The mixture was cooled down to room temperature, filtered over with a pre-weighed (W2) filter (Filterpaper 1, Springfield Mill, Kent, England) and washed with distilled water. The filter containing the lignin was dried in an oven (24 – 48 h, 70 – 80 ºC) and stored in a desiccator. The filter containing the lignin was weighed (W3) and Klason lignin was calculated as (W3-W2) x 100/W1.

5.2.7 *Fourier transform infrared (FT-IR) spectra analyses in wood*

FT-IR spectroscopy (Mercury Cadmium Telluride (MCT) detector) combined with a light microscope, provides a technique for measuring vibrational spectra of biological samples (e.g. microbial cells, plant cell walls) or individual morphological components in distinct positions of a tissue. The FT-IR microscopy enables the acquisition of spectra with a spatial resolution in the micrometer range, i.e. spectra from single fibre to tissues consisted of multi-cells can be obtained, representing the overall molecular composition of the tissue.

Stem-wood samples harvested in September 2003 were used for this analysis. Wood cross sections (20 µm in thickness) were prepared with a sliding microtome (Reichert-Jung, Heidelberg, Germany) and mounted in a drop of distilled water and dried at room temperature for FT-IR microscope (Bruker EQUINOX 55 spectrometer + Bruker Hyperion 3000 microscope, Bruker, Ettlingin, Germany).

All spectra were obtained at a resolution of 4 cm^{-1} and 30 scans per measurement were recorded. Well-dried stem-wood transverse sections were applied to a KBr microscope window (13 mm diameter by 2 mm thickness). An area of the sample (only

fibre cell walls) of 50 x 50 µm was selected. The spectrum of fibre cell walls was recorded in wood formed in year 2002 and in that of 2003, respectively. A background spectrum of the clear KBr window was recorded prior to acquisition of sample spectra. The spectrum of the background was subtracted by spectrum of the sample before conversion into an absorbance spectrum. Sample homogeneity was assessed by obtaining spectra from different areas of the same cross section and from different stem cross sections of the same treatment.

Fourier transform infrared (FT-IR) imaging, employing a focal plane array (FPA) detector, is a relatively new technique for rapid analyses of biological samples. IR absorbance spectra (via FPA detector) were recorded on a Bruker EQUINOX 55 spectrometer interfaced to a Bruker Hyperion 3000 microscope. All FT-IR imaging spectra were obtained at a resolution of 12 cm^{-1} and 16 scans were recorded. Similar to the MCT detector, well-dried stem transverse sections were applied to a KBr microscope window (13 mm diameter by 2 mm thickness). An area of the sample of 256 x 256 µm was selected for the analyses of spectra acquired by the FPA detector.

5.2.8 *Calorific value of wood*

The calorific value of stem-wood was analysed with a calorimeter (IKA-Kalorimetersystem C 7000, IKA-Werke Gmbh & CO.KG, Staufen, Germany). Poplar wood was dried and pressed to fine powder with a mill (Retsch, Haan, Germany). About 500 mg powder was weighed and pressed into pellets using a presser attached to the calorimeter. The pellet was hanged in a bomb containing 5 ml distilled water and O_2 (30 mbar). The bomb was set into the calorimeter. The software led the procedure of the burning. The calorific value was measured according to the following principle: when the sample is completely burned in oxygen, the released energy heats the water in the bomb and the degree of increase in temperature of the water is a direct measure for the internal energy of the burning reaction. Using benzoic acid (pellets; IKA-Werke Gmbh & CO.KG, Staufen, Germany) as a standard (calorific value: 26457 ± 20 J g^{-1}), the calorific values of samples were calculated.

To calculate the energy potential of *P. × euramericana* in the coppicing system, aboveground woody biomass production per stool was estimated as follows: After removing the leaves in the active season, shoots were oven-dried for four days at 70 °C, and weighed. To estimate aboveground woody (stem + branches) biomass production per

stool, allometric relationships were established between weight and volume of the harvested shoots (Table 1). For this purpose the shape of the shoot was considered as a cone, whose volume was calculated based on its diameter and height. To determine the total volume of all shoots per stool, the heights of non-harvested shoots were estimated based on the relationships of diameters and heights of harvested shoots (Table 1). Analysis of covariance indicated no effects of FACE and N-fertilization on these allometric relationships (data not shown). Thus, data from different treatments of each harvest were pooled to establish these relationships. Using the total volume of all shoots per stool, aboveground woody biomass per stool was estimated for each harvest. Using aboveground woody biomass production and the calorific values of wood samples, the energy potential of *P. × euramericana* in this coppicing system was estimated.

Table 1 Allometric relationships to estimate aboveground woody biomass. According to Norby et al. (2001) aboveground woody biomass (BM [g]) was estimated as $BM = a_1 * \Sigma_1^n V_n$, with V_n = volume per shoot and n = number of shoots per stool. The volume was calculated with $V = 1/3 * \pi * r^2 * h$, with r = radius of the shoot at 0.2 m above stool level and h = total height of the shoot. To obtain estimates for V of non-harvested shoots, the relationship between height and diameter (d) was modeled with: $\lg h_n = a_2 * \lg d_n$. Each relationship was established with n = 24 harvested shoots.

	BM = a_1*V			lg h = a_2 * lg d		
Season	a_1	r^2	P	a_2	r^2	P
Sep. 2003	451527	0.9012	< 0.0001	0.5027	0.5682	<0.0001
Mar. 2004	778523	0.9077	< 0.0001	0.4541	0.6860	< 0.0001

5.2.9 *RNA preparation*

To extract total RNA, the frozen differentiating xylem was milled in a ball mill (Retsch, Haan, Germany), which was pre-cooled in liquid nitrogen. Total RNA was extracted as described by Chang et al. (1993) with the following modification: no spermidine was applied in the extraction buffer, and 2% ß-mercaptoethanol was used. An additional extraction step was performed after precipitation with 2.5 M LiCl. Total RNA was purified using Microcon columns according to the manufacturer's specification (Y30 columns, Millipore, MA, USA). The purity and integrity of RNA can influence cDNA synthesis, incorporation of fluorescent dyes, dye stability, and probe-target hybridization (Alba et al. 2004). Therefore, the purity of RNA was assessed by multiscan spectrum

(Thermo Labsystems, Vantaa, Finland). RNA integrity was assayed using a bioanalyzer (Agilent 2100 Bioanalyzer, Agilent Technologies Inc., Waldbronn, Germany).

5.2.10 *cDNA synthesis and purification*

For the synthesis of complementary DNA (cDNA), the Invitrogen kit (Catalog no. L1014-02, Invitrogen Corp., Carlsbad, CA, USA) was applied. Forty µg of total RNA was mixed with 5 µg anchored Oligo $(dT)_{20}$ Primer (from the kit), with 5 ng controlling mRNA and DEPC-treated water (from the kit) was added to a final volume of 25 µL. After denaturation for 5 min at 65 °C and chilling on ice for at least 1 min, 8 µL of 5× first-strand buffer (from the kit), 2 µL of 0.1 M DTT (from the kit), 2 µL of 10 mM dNTP mix (dATP, dGTP, dCTP and dTTP, including one aminoallyl-modified nucleotide, and one aminohexyl-modified nucleotide, from the kit), 1 µL of 40 U/µL RNaseOUT (from the kit) and 2 µL of 400 U/µL SuperScript III Reverse Transcriptase (from the kit) were added. The mixture was mixed well and incubated at 46 °C for 3 hrs.

After reverse transcription, RNA was degraded by adding 15 µL of 1 M NaOH and incubation for 15 min at 65 °C. The sample was chilled on ice before mixing with 15 µL of 1 M HCl. Afterwards, 20 µL of sodium acetate (3 M, pH 5.2) was added. To purify the cDNA, the QIAquick® purification kit (Catalog no. 28104, QIAGEN Inc., Ontario, Canada) was used. Five hundred µL of binding buffer (from the purification kit) was added, mixed well and transferred to the column (from the purification kit). The column was centrifuged (1 min, 13 000 rpm, room temperature) and the flow-through was discarded. Six hundred fifty µL of phosphate wash buffer (5 mM KH_2PO_4/K_2HPO_4 buffer, pH 7.3, 81% ethanol) was added to the column, centrifuged (1 min, 13 000 rpm, room temperature) and the flow-through was discarded. The washing procedure was repeated. Then the column was transferred to a new 1.5 mL tube. Fifty µL of phosphate elution buffer (4 mM K_2HPO_4/KH_2PO_4 buffer, pH 8.5) was added directly on the center of the membrane, incubated for 1 min and centrifuged (1 min, 13 000 rpm, room temperature). Another 50 µL of phosphate elution buffer was added to elute cDNA. Afterwards, the cDNA was precipitated in an ethanol mixture (10 µL of 3 M pH 5.2 sodium acetate, 2 µL of 20 mg/mL glycogen and 300 µL of ice-cold 100% ethanol) overnight before centrifugation (13 000 rpm, 4 °C, 20 min). The cDNA pellet was dried.

5.2.11 *Labeling of cDNA targets and purification*

The labeling protocol is based on the Superscript Indirect cDNA Labeling System kit from Invitrogen (Catalog no. L1014-02, Invitrogen Corp., Carlsbad, CA, USA). In this labeling system two types of modified nucleotides (dUTP) are used, one containing an aminoallyl modification and the other an aminohexyl modification. After incorporation of these nucleotides during cDNA synthesis, the modified cDNA was labeled using an N-hydroxy-succinimide ester form of cyanine 3 (Cy^{TM}3, Catalog no. PA23001) and cyanine 5 (Cy^{TM}5, Catalog no. PA25001), respectively (Amersham Biosciences UK Limited, Amersham Place Little Chalfont Buckinghamshire, UK).

The cDNA pellet (from chapter 5.2.9) was re-suspended in 5 µL of 2× coupling buffer (from the kit). The mixture was incubated at 46 °C for 1 hr with thorough mixing at 15 min interval to ensure that the cDNA pellet was completely re-suspended prior to the labeling reaction. Five µL of DMSO (from the kit) was added to the dyes (Cy^{TM} 3/Cy^{TM} 5), mixed thoroughly, and centrifuged briefly. Subsequently, the mixture was added to the cDNA solution and incubation for 1 hr at room temperature in the dark.

To purify the labeled cDNA targets, the QIAquick$^®$ purification kit (Catalog no. 28104, QIAGEN Inc., Ontario, Canada) was used. Forty µL of 100 mM sodium acetate (pH 5.2) was added to the coupling reaction before 250 µL of binding buffer (from the purification kit) was applied, mixed well and transferred to a column (from the purification kit). The column was centrifuged (1 min, 13000 rpm, room temperature) and the flow-through was discarded. Six-hundred-fifty µL of wash buffer (from the purification kit) was added. The column was centrifuged (1 min, 13 000 rpm, room temperature) and flow-through was discard. The washing procedure was repeated and the column was transferred to a new 1.5 µL tube. Fifty µL of elution buffer (from the purification kit) was applied to the center of the membrane of the column to elute the labeled cDNA. After incubation for 1 min and centrifugation (1 min, 13 000 rpm, room temperature), 50 µL of elution buffer was added to the membrane and the column was centrifuged (1 min, 13 000 rpm, room temperature).

For the quality control of cDNA synthesis and dye labeling reactions, 1 µL of labeled cDNA targets was diluted in 75 µL of elution buffer (from the purification kit), mixed well and transferred to a 96-well plate. Fluorescence intensity was read in a scanner (Typhoon 9400, Amersham Biosciences, Sunnyvale, CA, USA) employing the software Typhoon Scanner Control 4.0 and was quantified with the software (ImageQuant TL, version

2003.03, Amersham Biosciences, Sunnyvale, CA, USA). Due to the different labeling efficiency of two dyes, fluorescence intensities of $Cy^{TM}3$ two times higher than the background and of $Cy^{TM}5$ ten times higher than the background are regarded as successful cDNA synthesis and labeling.

The cDNA targets from successful cDNA synthesis and labeling reactions, which were to be hybridized to the same array, were combined according to the experimental design prior to ethanol precipitation. The combined labeled-cDNAs were precipitated in an ethanol mixture (20 µL of 3M pH 5.2 sodium acetate, 4 µL of 20 mg/mL glycogen, 600 µL of ice-cold 100% ethanol) for 1 hr at − 30 °C, centrifuged (13 000 rpm, 4 °C, 20 min) and dried. The dried cDNA targets were then suspended in 3.5 µL of 10 mM EDTA and incubated at 95 °C for 3 min and chilled on ice for at least 1 min prior to the addition of 30 µL of pre-warmed (42 °C) hybridization solution (50% formamide, 5× SSC (Sodium chloride/Sodium Citrate), 0.1% SDS (Sodium Dodecylsulfate), 0.1 mg/ml Herring Sperm DNA (Catalog no. 9605-5-D, Trevigen, Helgerman Court, Gaithersburg, USA)).

5.2.12 *Experimental design of hybridizations*

The cDNAs from the developing xylem in *P. × euramericana* trees exposed to either low (L) or high (H) N-fertilization in combination with either ambient (A) or elevated (E) [CO_2] were hybridized. Four to six randomly chosen biological replicates for each nitrogen and [CO_2] combination (AL, AH, EL, EH) were used with dye swaps. A loop design for this experiment was chosen (Fig. 1).
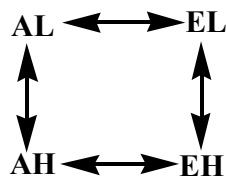
Fig. 1 Experimental design for gene expression profiling using a 3.4K POP cDNA microarray. cDNAs from the developing xylem of *P. × euramericana* sampled in September 2002 from trees exposed to either low (L) or high (H) N-fertilization in combination with either ambient (A) or elevated (E) [CO_2] were hybridized in a loop design. The cDNAs were derived independently from 6 biological replicates within each condition. In a direct comparison (AL vs. AH; AL vs. EL; EH vs. AH; EH vs. EL), the differential expression of the genes in corresponding samples was measured directly on the same slide. Dye-swap experiments involved two hybridizations for two cDNA samples, in which dye assignment was reversed in the second hybridization.

5.2.13 *Microarray hybridization*

3.4K poplar glass-spotted cDNA microarrays were used for this study. The poplar array contained 3444 ESTs (Mackay & Cooke, unpublished data). Array details and sequence details for all ESTs are available from a database (http://www.ccgb.umn.edu/biodata/poplar/).

Microarray slides were pre-hybridized in pre-hybridization solution (50% formamide, $5\times$ SSC, 0.1% SDS, 0.1 mg/ml BSA) for 1—2 hrs at 42 °C before incubating twice in $0.1 \times$ SSC for 5 min at room temperature. Afterwards the slides were washed twice in water for 30 seconds at room temperature, dipped briefly in isopropanol and dried quickly by centrifugation (1600 rpm, room temperature, 3 min). The cover slips (lifterslip, Erie Scientific Company, Portsmouth, NH, USA) were also washed in water followed by washing in 100% ethanol and dried by centrifugation (1600 rpm, room temperature, 3 min).

Subsequently, the cDNA targets solution (33.5 µL) was applied to a pre-hybridized array and covered with a clean cover glass. Hybridization was conducted in Corning hybridization chambers (Sigma-Aldrich, Sigma-Aldrich Corp. St. Louis, MO, USA) at 42 °C overnight in darkness. Post-hybridization washes were carried out in Coplin jars (MarketLab, Kentwood, MI, USA), including washing once in buffer A ($2\times$ SSC, 0.5% SDS) at 42 °C for 15 min, and washing twice in buffer B ($0.5\times$ SSC, 0.5% SDS) at 42 °C for 15 min. Afterwards, the slides were washed twice in buffer C ($0.1\times$ SSC) at room temperature for 1 min and quickly dried by centrifugation (1600 rpm, room temperature, 5 min). The dry arrays were stored in the darkness until scanning.

5.2.14 *Acquisition, transformation, and processing of microarray data*

The arrays were scanned using a two-channel confocal microarray scanner (ScanArray Express 2.0, PerkinElmer Inc. Boston, USA) and the associated ScanArray Express software (version 2.0, Packard BioScience, BioChip Technologies, Boston, USA). Scans were conducted at a resolution of 10 µm with the laser power set at 90% of maximum and the photon multiplier tube typically set between 65 and 80%. Excitation/emission settings were 543/570 nm and 633/670 nm for the $Cy^{TM}3$ and $Cy^{TM}5$ fluorescence dyes, respectively. A typical scanned image is shown in Fig. 2. Raw fluorescence image data were saved as .tif files, which were subsequently converted to numerical signal data (.txt files) using QuantArray software (version 3.0, Packard BioScience, BioChip Technologies, Billerica, USA).

Processing of microarray data (.txt files) involved data filtration, log transformation, data normalization, and statistical analysis to identify high quality data.
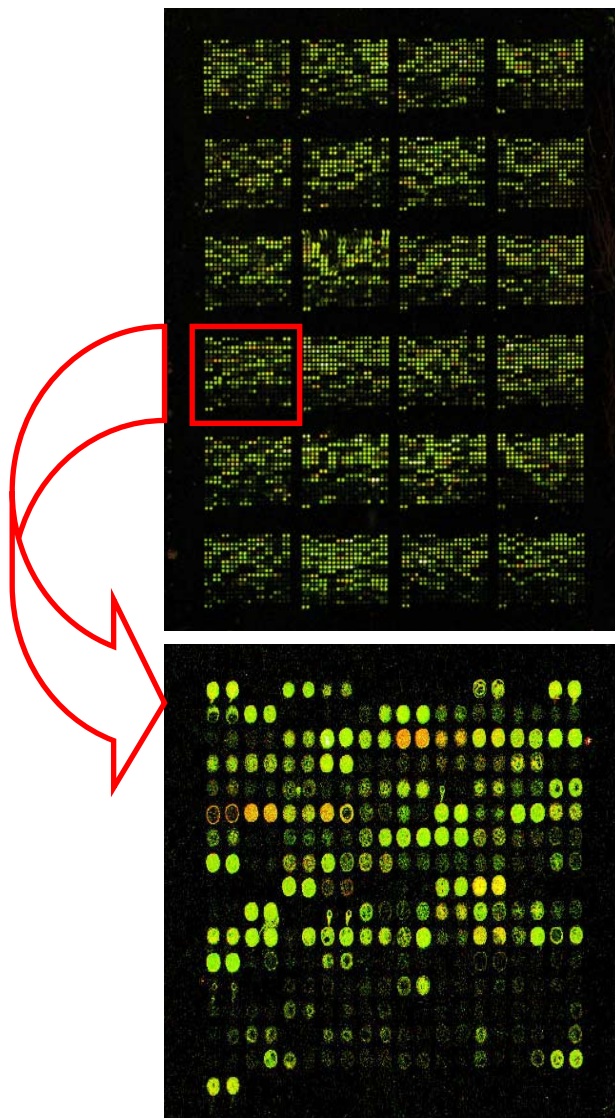
Fig. 2 A typical image after scanning the Cy^{TM} dye labeled cDNA hybridisation with 3.4K POP cDNA microarray. The section in the red rectangle is also shown in detail. 3.4K poplar glass-spotted cDNA microarray contains 7680 spots per slide and 3 444 ESTs printed in duplicate.

5.2.15 *Statistical analysis*

To determine the main effects of CO_2 treatment (CO_2) and N-fertilization (N) on all variables with ANOVA, a randomized-complete-block design, with CO_2, N and their interactions as fixed factors and block as a random factor, was applied. All statistical tests were performed with Statgraphics (STN, St. Louis, Mo, USA) using the mixed procedure and plot as a replicate. When interactions were significant, a posteriori comparison of

means was done. To reduce the chance on type I errors, all *P*-values of these multi-comparisons were corrected by Tukey-HSD method. Data were tested for normality with the Shapiro-Wilkison test. Differences between parameter means were considered significant when the *P*-value of the ANOVA *F*-test was less than 0.05.

For the microarray data, the analysis was done using LIMMA package in Bioconductor (http://www.bioconductor.org/) in R computing environment (http://www.r-project.org). Data were global loess normalized. Linear models with B statistics implemented in the LIMMA package were used to identify genes that might be differentially expressed.

5.3 Results

5.3.1 *Anatomical properties of stem-wood*

For anatomical characteristics of the stem-wood of *P. × euramericana*, annual xylem width, fractions of vessel lumina, ray parenchyma, fibre lumina and cell walls, vessel frequency, thickness of fibre walls, vessel lumen diameter and fibre lumen diameter were determined (Table 2). Compared with ambient condition, FACE significantly stimulated annual ring width in year 2003 but not in 2002, the first year after coppicing. By contrast, N-fertilization had a stimulating effect on annual ring width in 2002 and not in 2003. FACE caused significant decreases in the relative fractions of cell walls, ray parenchyma and vessel lumina, which was mainly due to significant increases in fibre lumina resulting from enhanced fibre lumen diameters (Table 2). Furthermore, FACE decreased thickness of fibre walls and vessel lumen diameters (Table 2). These effects in 2003 were more pronounced than in 2002.

In contrast to FACE, N-fertilization affected wood properties more significantly in 2002 than in 2003. Higher N-supply significantly decreased the cell wall fraction which was due to a marked decrease in the thickness of double fibre walls and increase in the fraction of fibre lumina (Table 2).

Since the fibre properties are important for pulp and paper production, fibre lengths of *P. × euramericana* were also investigated (Table 3). Neither FACE nor N-fertilization effects were observed.

Table 2 Anatomical properties of stem-wood in *P. × euramericana* grown under either FACE (E) or ambient (A) [CO_2] and either with (H) or without (L) fertilization in two years. The values shown indicate means (± SD; n > 18). *P*-values of the ANOVAs of CO_2, nitrogen (N) and their interactions are shown. XW: annual xylem ring width; VLF: vessel luminal fraction; RF: ray parenchyma fraction; FLF: fibre lumen fraction; CWF: cell wall fraction; VF: vessel frequency; TFW: thickness of fibre double walls; VLD: vessel lumen diameter; FLD: fibre lumen diameter.

Harvest Season	Treatments	XW (mm)	VLF (%)	RF (%)	FLF (%)	CWF (%)	VF (number mm^{-2})	TFW (µm)	VLD (µm)	FLD (µm)
Sep 02 *	AL	5.8 ± 1.2	17.3 ± 3.2	6.1 ± 1.1	39.6 ± 4.2	37.1 ± 2.7	57.9 ± 5.8	2.8 ± 0.4	61.1 ± 6.2	19.6 ± 2.9
	EL	6.3 ± 1.1	18.1 ± 2.0	6.2 ± 1.4	41.6 ± 2.8	34.1 ± 1.9	61.3 ± 6.1	2.7 ± 0.5	68.1 ± 7.2	19.6 ± 2.3
	AH	7.5 ± 0.9	16.6 ± 2.3	6.5 ± 1.3	44.6 ± 2.9	32.3 ± 1.9	56.5 ± 5.4	2.1 ± 0.3	67.1 ± 6.5	19.2 ± 2.5
	EH	7.4 ± 1.3	17.6 ± 1.8	6.2 ± 1.3	43.7 ± 3.7	32.5 ± 2.8	56.8 ± 3.9	1.8 ± 0.3	77.7 ± 5.4	21.4 ± 2.6
Sep 03	AL	4.7 ± 1.9	18.1 ± 2.1	6.2 ± 1.4	40.5 ± 2.1	35.2 ± 2.2	51.5 ± 11.4	3.6 ± 0.6	67.2 ± 13.5	15.4 ± 1.2
	EL	5.3 ± 1.8	17.5 ± 2.3	4.9 ± 1.5	43.9 ± 2.2	33.6 ± 2.1	61.3 ± 10.9	2.6 ± 0.5	58.4 ± 13.6	17.4 ± 1.8
	AH	2.8 ± 2.6	18.8 ± 1.5	6.7 ± 2.4	37.5 ± 3.5	36.9 ± 3.2	57.1 ± 10.4	3.4 ± 0.5	63.3 ± 11.4	16.9 ± 1.2
	EH	5.7 ± 2.6	16.5 ± 2.9	5.5 ± 1.2	45.6 ± 4.2	32.5 ± 2.7	55.2 ± 11.8	2.7 ± 0.5	58.6 ± 14.6	17.0 ± 1.2
Sep 02	CO_2	0.3501	0.0932	0.7567	0.5400	0.0162	0.3919	0.0000	0.0000	0.0000
	N	0.0000	0.3135	0.4723	0.0000	0.0000	0.1685	0.0000	0.0000	0.0001
	CO_2 x N	0.0876	0.8698	0.5753	0.0726	0.0068	0.4684	0.0008	0.0839	0.0000
Sep 03	CO_2	0.0018	0.0058	0.0025	0.0000	0.0000	0.1290	0.0000	0.0000	0.0047
	N	0.1785	0.7680	0.1910	0.3388	0.5719	0.9194	0.1711	0.0511	0.0000
	CO_2 x N	0.0400	0.1164	0.9577	0.0029	0.0229	0.0285	0.0002	0.0356	0.0010

* Data are modified from Luo et al. (2005b).

Table 3 Fibre lengths of stem-wood in *P. × euramericana* grown under either FACE (E) or ambient (A) [CO_2] and either fertilised (H) or unfertilised (L) conditions. Samples harvested in March 2004 were separated into two parts (wood formed in the year 2002 and 2003, respectively) according to the growth ring. The values indicate means ± SD of six measurements for each treatment. *P*-values of the ANOVAs of CO_2, nitrogen (N) and their interactions are shown. Ln: the average contour length of detected fibres; Lw: the length weighted average of detected fibres; Lww: the weight weighted average of detected fibres. Due to the different preferences of these fibre lengths in the industry, here we present all these values.

| Year	Treatments	Ln (µm)	Lw (µm)	Lww (µm)
2002	AL	437.8 ± 35.5	503.1 ± 39.0	560.4 ± 41.4
	EL	444.5 ± 44.2	504.8 ± 55.1	557.6 ± 65.3
	AH	417.8 ± 41.5	473.9 ± 45.6	522.1 ± 52.9
	EH	428.7 ± 33.0	481.7 ± 37.6	524.1 ± 43.1
2003	AL	517.6 ± 39.5	591.7 ± 46.2	654.3 ± 58.6
	EL	498.2 ± 75.3	580.5 ± 88.1	659.1 ± 94.4
	AH	453.5 ± 63.7	514.5 ± 71.7	563.4 ± 78.4
	EH	509.2 ± 79.8	598.8 ± 105.3	684.4 ± 132.3
2002	CO_2	0.5846	0.7988	0.9859
	N	0.2733	0.1689	0.1034
	CO_2 x N	0.8969	0.8693	0.9105
2003	CO_2	0.5113	0.2822	0.1201
	N	0.3403	0.3830	0.4077
	CO_2 x N	0.1817	0.1632	0.1489

5.3.2 *Chemical wood properties*

In the first year after coppicing, the carbon content of wood was not affected by FACE or N-fertilization (Table 4). After two years, N-fertilization caused small decrease in the carbon concentration, whereas FACE tended to counteract this influence (Table 4). In the first year after coppicing, N-fertilization caused 19 — 40% increases in nitrogen concentration in wood, whereas this effect disappeared in the second year after coppicing (Table 4).

To get better understanding of wood properties, we also analysed the lignin contents in stem-wood of *P. × euramericana* (Table 4). FACE slightly but significantly enhanced lignin concentrations in comparison with ambient [CO_2] (Table 4). Higher N-supply increased in Klason lignin concentrations only under FACE conditions compared to low N-supply (Table 4).

Table 4 Carbon, nitrogen and Klason lignin concentrations in stem-wood of *P. × euramericana* grown under either FACE (E) or ambient (A) [CO_2] and either fertilised (H) or unfertilised (L) conditions. The values shown indicate means (± SD) of six measurements based on dry weight. *P*-values of the ANOVAs of CO_2, nitrogen (N) and their interactions are shown.

Harvest season	Treatments	C%	N%	Lignin%
Sep 02	AL	47.9 ± 0.3	0.233 ± 0.023	18.3 ± 1.2
	EL	47.7 ± 0.5	0.230 ± 0.047	18.6 ± 0.6
	AH	47.8 ± 0.4	0.277 ± 0.105	17.9 ± 0.9
	EH	47.6 ± 0.6	0.323 ± 0.043	19.9 ± 0.7
Sep 03	AL	47.9 ± 0.9	0.233 ± 0.065	20.0 ± 0.5
	EL	48.2 ± 0.7	0.175 ± 0.045	20.3 ± 0.5
	AH	46.8 ± 0.8	0.268 ± 0.073	19.9 ± 0.4
	EH	47.7 ± 0.7	0.232 ± 0.099	21.9 ± 0.7
Sep 02	CO_2	0.2442	0.4061	0.0051
	N	0.5626	0.0145	0.1932
	$CO_2 \times N$	0.7868	0.3391	0.0283
Sep 03	CO_2	0.0805	0.1274	0.0000
	N	0.0201	0.1406	0.0014
	$CO_2 \times N$	0.3608	0.7206	0.0009

To find out whether the chemical composition of poplar wood was altered by FACE and/or N-fertilization, the stem-wood cross section was investigated by FT-IR. A typical stem-wood FT-IR spectrum of poplar wood is shown in Fig. 3. A strong hydrogen bond (O-H) stretching absorption was detected at about 3400 cm^{-1} and a prominent C-H stretching absorption around 2900 cm^{-1}. In addition, there are many peaks in the fingerprint region between 1800 and 900 cm^{-1} which were previously identified (Harrington et al., 1964; Hergert, 1971; Schultz and Glasser, 1986; Faix, 1992; Collier et al., 1992; Pandey and Theagarajan, 1997): 1738 cm^{-1} for unconjugated C = O in xylans (hemicellulose), 1648 cm^{-1} for absorbed O-H and conjugated C-O, 1596 cm^{-1} and 1505 cm^{-1} for the aromatic skeleton in lignin, 1457 cm^{-1} and 1422 cm^{-1} for C-H deformation in lignin and carbohydrates, 1367 cm^{-1} for C-H deformation in cellulose and hemicellulose, 1319 cm^{-1} for C-H vibration in cellulose and C_1-O vibration in syringyl derivatives, 1234 cm^{-1} for syringyl ring and C-O stretch in lignin and xylan, 1154 cm^{-1} for C-O-C vibration in cellulose and hemicellulose, 1100 cm^{-1} for aromatic skeleton and C-O stretch, 1029 cm^{-1} for C-O stretch in cellulose and hemicellulose.
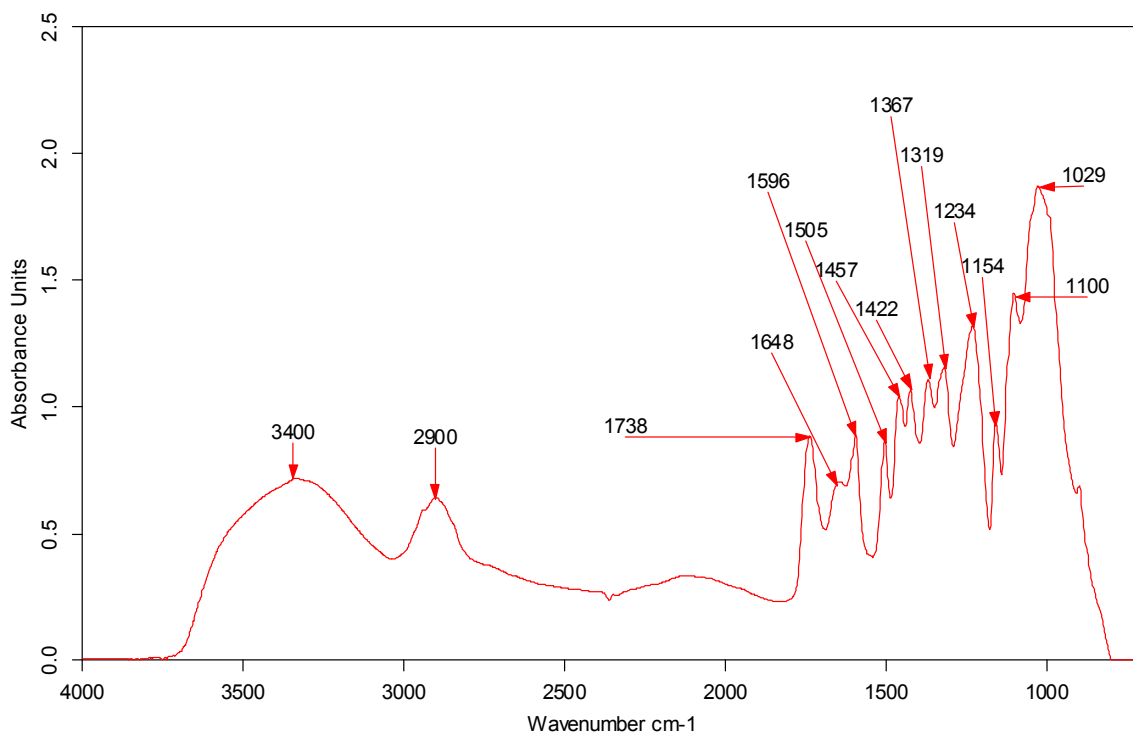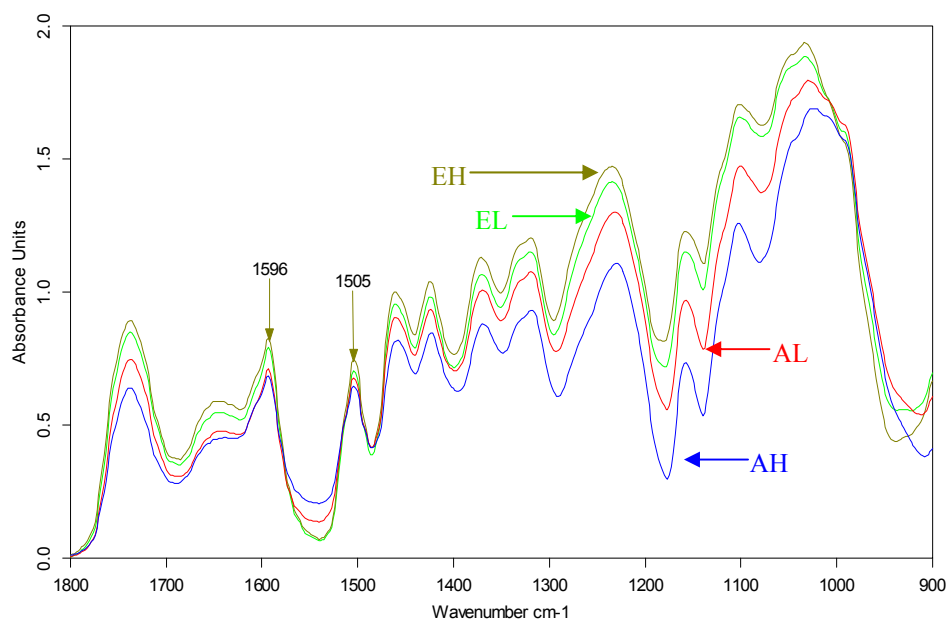
Fig. 3 FT-IR spectrum of stem wood of *P.* × *euramericana* grown under ambient CO_2 and low nitrogen conditions in the growing season 2003. The spectrum was recorded on poplar cross sections with MCT detector. A mean of (n = 18) spectra is shown. Number on the arrow-end indicates the wavenumber of absorbance peak as explained in the text.

The fingerprint regions of spectra of poplar wood formed under different growth conditions are shown in Fig. 4. The relative intensities of peaks 1596 cm^{-1} and 1505 cm^{-1} are higher in wood cell walls formed under FACE and N-fertilization than in wood cell walls formed under FACE and low nitrogen condition or under ambient [CO_2] conditions. These data indicate that the aromatic skeleton in lignin responsible for these peaks was more abundant under FACE and N-fertilization condition than that under other conditions. These results are also consistent with Klason lignin (Table 4).

By using a focal plane array (FPA) detector, the lignin distribution was mapped in cross sections of poplar wood (Fig. 5). These maps show that lignin was concentrated in the corners of fibre cell walls, middle lamellae and walls of ray parenchyma cells. These maps also suggest that lignin abundance in the wood was similar to the results obtained by Klason lignin method (Table 4).

Fig. 4 FT-IR spectra of stem wood of *P. × euramericana* grown under either FACE (E) or ambient (A) $[CO_2]$ and either fertilized (H) or unfertilized (L) conditions in the growing season 2003. Spectra were obtained by the MCT detector. Data show mean of (n = 18) spectra in the range of 900 to 1800 cm^{-1}.
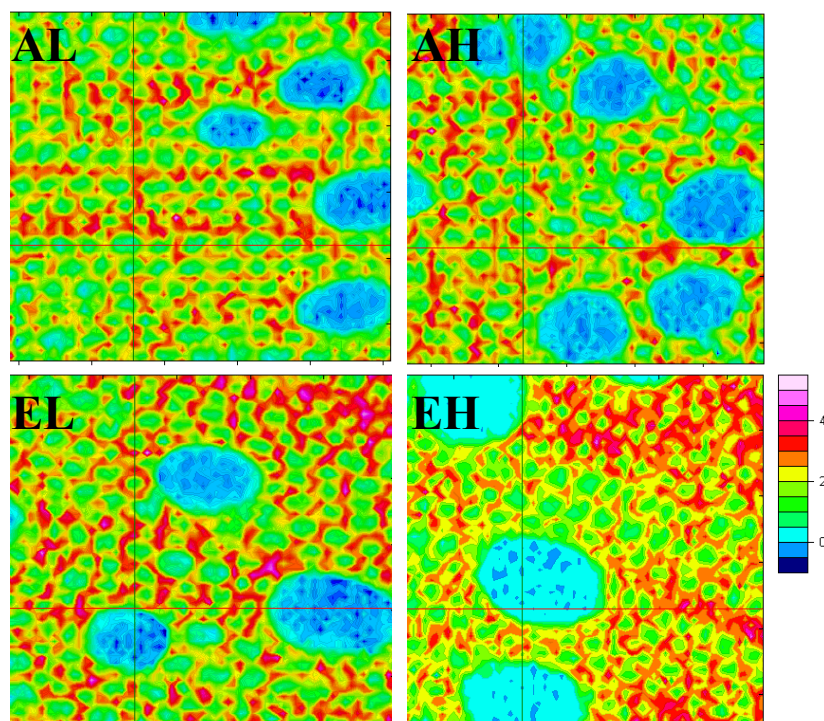
Fig. 5 Typical lignin distribution in the stem wood of *P. × euramericana* grown under ambient CO_2 and low nitrogen (AL); ambient CO_2 and high nitrogen (AH); FACE and low nitrogen (EL); FACE and high nitrogen (EH) conditions. The colour scale indicates the relative abundance of lignin. The distribution mapping was done by employing FPA detector in combination with a microscope and the associated software.

5.3.3 *Energy content of wood*

To find out whether the chemical composition affected the energy content of wood, the calorific value of stem-wood was investigated (Table 5). FACE tended to increase the calorific value of stem-wood in samples harvested in March 2004. No N-fertilization effects were found. Based on the aboveground woody biomass production (Table 1), the energy potential of the *P. × euramericana* coppice at this site was estimated to range from 70 — 111 MJ m^{-2} after 2 years of growth. Although no fertilization effects on the calorific value were detected, N-fertilization significantly stimulated the energy production by 16 — 69% (Table 5), which was mainly due to the stimulation of aboveground woody biomass (Table 1).

Table 5 Calorific values of stem-wood of *P. × euramericana* grown under either FACE (E) or ambient (A) [CO_2] and either fertilised (H) or unfertilised (L) conditions. The energy potential per stool is estimated by calorific value of wood and the aboveground woody (stem + branches) biomass production that is based on the allometric relationships (see Table 1). The values shown indicate means ± SD (n = 3). *P*-values of the ANOVAs of CO_2, nitrogen (N) and their interactions are shown.

| Season	Treatments	Calorific value (J g^{-1})	Energy per stool (MJ m^{-2})
Sep. 2003	AL	19281 ± 126	73.7 ± 13.8
	EL	19388 ± 97	59.9 ± 10.1
	AH	19306 ± 146	90.4 ± 20.4
	EH	19344 ± 117	100.9 ± 20.2
Mar. 2004	AL	19121 ± 40	92.3 ± 10.7
	EL	19148 ± 101	70.1 ± 24.8
	AH	19115 ± 121	106.7 ± 20.8
	EH	19283 ± 52	111.3 ± 13.5
Sep. 2003	CO_2	0.3369	0.8682
	N	0.8960	0.0173
	CO_2 x N	0.6348	0.2424
Mar. 2004	CO_2	0.0860	0.4277
	N	0.2303	0.0304
	CO_2 x N	0.1907	0.2409

5.3.4 *Gene expression patterns in developing xylem*

Through many processes in woody plants, cells originating from the vascular cambium finally differentiate into functional cells in xylem and phloem tissues (Larson 1994). These processes involve several fundamental steps including cell division, cell expansion, deposition of secondary cell wall materials and programmed cell death. During these developmental processes, wood properties are determined by the function of many genes. Since genes respond not only to developmental but also to environmental factors, the regulation of genes in response to environmental factors, such as elevated $[CO_2]$ and N-fertilization, probably plays an essential role in determining the variation in wood properties. In order to understand the molecular mechanisms underlying the variation in wood properties as affected by FACE and/or N-fertilization, transcriptional profiling was performed.

Since experimental materials came from a field study, the variability of the samples was higher and consequently it was difficult to detect significant changes in transcripts with only a low number of replicates. Therefore, genes with significant changes in transcripts were identified based on both statistical indicators (*B*-values and *P*-values) in order to achieve a high probability of differential expression. Employing this strategy, a few transcripts that display changes were identified (Table 6), including genes related to cell-wall biosynthesis. Most changes in transcript expression occurred under FACE and N-fertilization condition compared with FACE and low N-fertilization (Table 6, EH-EL). Importantly, all identified transcripts under these comparison conditions were up-regulated by N-fertilization, including two transcripts involved in the lignin biosynthesis i.e. caffeic acid 3-O-methytransferase 1 (COMT-1) and ferulate-5-hydroxylase (F5H) (Table 6, Fig. 6). Additionally, under N-fertilization conditions, FACE caused decreases in a transcript for a ripening regulated protein in comparison with ambient $[CO_2]$ (Table 6, EH-AH). Interestingly, this transcript was up-regulated by N-fertilization under ambient $[CO_2]$ conditions (Table 6, AH-AL).

Table 6. Annotation and statistics of expressed sequence tags (ESTs) for differentiating xylem of *P. × euramericana* grown under either FACE (E) or ambient (A) [CO_2] and either fertilised (H) or unfertilised (L) conditions. A positive M value indicates up-regulated gene expression in one combination of treatments to another. In contrast, a negative M value suggests down-regulated gene expression in one combination of treatment to another.

Treatments	Identifier	Annotation	*M*	*P*	*B*
AH-AL	MN5188443	Ripening regulated protein DDTFR10	2.02	0.055	1.14
EL-AL	MN5184294	No annotation available	-0.69	0.080	2.56
EH-AH	MN5188443	Ripening regulated protein DDTFR10	-2.02	0.020	1.16
EH-EL	MN5183717	Isoflavone reductase HOMOLOGUE (PILH gene)	0.99	0.001	5.46
	MN5189945	Putative senescence-associated protein (Fragment)	2.24	0.002	4.38
	MN5183875	Unknown protein	1.61	0.002	3.84
	MN5183149	Heat shock 70 kDa protein, mitochondrial precursor	1.59	0.003	3.48
	MN5189108	Hypothetical protein	2.09	0.003	3.72
	MN5184204	Ferulate-5-hydroxylase	0.86	0.007	2.86
	MN5189892	No annotation available	1.29	0.014	2.06
	MN5188098	Tubulin alpha-2/alpha-4 chain	0.80	0.024	1.50
	MN5183865	Glyceraldehyde 3-phosphate dehydrogenase, cytosolic (EC 1.2.1.12)	0.63	0.024	1.49
	MN5184441	Plasma membrane intrinsic protein	0.65	0.033	0.99
	MN5190264	Hypothetical protein	1.03	0.033	0.95
	MN5183608	Caffeic acid 3-O-methyltransferase 1 COMT 1 (EC 2.1.1.68)	0.74	0.037	0.68
	MN5184294	No annotation available	0.60	0.037	0.64
	MN5183126	AT5g17920/MPI7_60	0.69	0.037	0.62
	MN5185095	Hypothetical protein	0.74	0.049	0.29
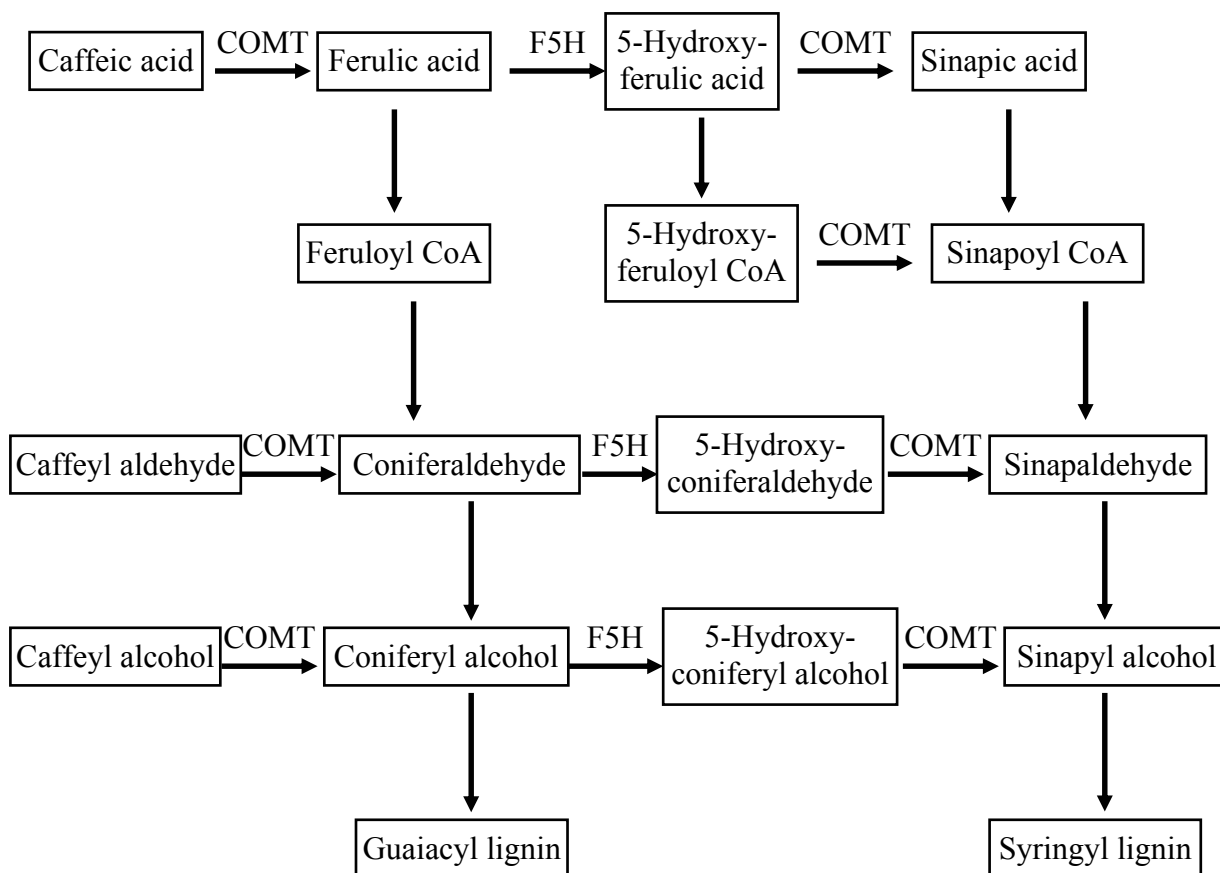
Fig. 6 A simplified scheme of functions of both genes (F5H and COMT) in the pathway of lignin biosynthesis after Humphreys and Chapple (2002).

5.4 Discussion

5.4.1 *Wood properties*

The effects of elevated [CO_2] and N-fertilization on wood anatomical properties have been tested only in few species (e.g. Telewski et al., 1999; Yazaki et al., 2001; Atwell et al., 2003; Kostiainen et al., 2004; Luo et al., 2005b). Here we investigated wood anatomy in the two annual rings of juvenile wood formed under FACE and N-fertilization conditions. Our data show that both FACE and N-fertilization altered annual ring width and xylem cell dimensions in a year-specific manner (Table 2). The finding that FACE continuously caused the losses in cell wall area and decreases in thickness of cell walls in 2 years after coppicing suggests that in future climate scenarios, negative effects on wood properties are to be anticipated (Luo et al. 2005b). Our data may indicate that FACE and N-fertilization affected cell differentiation because after cell division in the cambial region, the xylem cells

undergo three major processes of differentiation: growth in the radial and longitudinal directions, secondary wall formation and lignification of the cell wall (Gindl et al., 2000). The effects of FACE and N-fertilization on wood anatomy found here may be explained by increased activity of cambium and/or faster growth rate in radial direction during the first step of differentiation under FACE and/or N-fertilization conditions. Since *P. × euramericana* is growing vigorously in 2 years after coppicing (data not shown), and the stem-wood is juvenile and its anatomical characteristics are still developing, annual variations in wood anatomy may occur. Thus, it is not surprise that FACE and N-fertilization affected wood anatomy in a year-specific manner. Anyway, the anatomical changes of wood found here may also influence the mechanical strength of the stem wood.

The majority of carbon in the phenylpropanoid pathway is channeled toward the synthesis of lignin, a complex three-dimensional polymer which is a principally structural component of wood cell walls (Humphreys and Chapple, 2002). To gain information whether carbon and nitrogen resources affecting lignin biosynthesis resulting in altered wood properties, concentrations of carbon, nitrogen and lignin in wood were investigated (Table 4). Changes in concentrations of carbon, nitrogen and lignin under changing environmental conditions suggest that FACE and N-fertilization may affect wood properties by altering resource partitioning. As the second most abundant polymer in wood cell walls, eclipsed only by cellulose, lignin is a major carbon sink in wood (Polle et al., 1997; Boerjan et al., 2003). During the completely burning reaction, oxidation of lignin-bound carbon is expected to release much energy. To find out whether the lignin abundance in cell walls affected energy content in wood, the calorific value in wood was also analysed (Table 5). Our data show that under FACE and N-fertilization conditions the calorific values of stem-wood of *P. × euramericana* were ca. 19.0 to 19.4 MJ/kg (Table 5), which is in the range (15.8 to 24.3 MJ/kg) of other studies (Ciria et al., 1996; Klasnja et al., 2002). The energy content of wood is affected by the proportion of biochemical components present in it (Kataki and Konwer, 2001). The calorific value of lignin is about 25.0 MJ/kg (Klasnja et al. 2002) and thus almost twice that of cellulose (Chaffey, 2000). Therefore, higher lignin content in wood may lead to higher calorific value. This may partly explain the observation that the calorific values of poplar wood were higher under FACE conditions than those under ambient [CO_2] conditions (Table 5) since FACE slightly enhanced the lignin biosynthesis (Table 4). It is worthwhile to note that two-year-old poplar wood as fuelwood, 70.1—111.3 MJ m^{-2} of energy production (Table 5) at the EUROFACE

site indicates that fast growing tree species on short rotations, such as *P. × euramericana*, are attractively alternate fuel sources.

To gain information on the biochemical composition of wood cell walls formed under FACE and N-fertilization conditions, FT-IR spectra of stem wood were acquired (Figs. 3 and 4). Higher intensities of peaks (1596 cm^{-1} and 1505 cm^{-1}) in wood cell walls formed under FACE and N-fertilization condition in comparison with FACE and low N-supply imply a relatively higher lignin abundance in wood under these conditions because these peaks respond to the abundance of aromatic skeleton in lignin (Collier et al., 1992). This observation corresponds well to Klason lignin concentration and the mapping of lignin distribution in wood (Fig. 5, Table 4). Current data demonstrate that FACE and N-fertilization altered the biochemical composition of wood cell walls, including lignin, by shifting partitioning of carbon and nitrogen resources. In the literature, only a few studies have addressed the effects of elevated $[CO_2]$ and N-fertilization on lignin content in woody plants with varying findings (e.g. Blaschke et al., 2002; Kostiainen et al., 2004). Depending on the nutrient level, the lignin concentrations were unaffected or diminished by elevated $[CO_2]$ in beech seedlings (Blaschke et al., 2002). Recently, Kostiainen et al. (2004) observed that elevated $[CO_2]$ decreased the concentration of acid-soluble lignin in stem wood of 41-year-old Norway spruce trees. Our finding about N-fertilization effects on lignin concentration is consistent with the results of Kostiainen et al. (2004) who found that fertilization increased the concentrations of gravimetric lignin in stem wood of 41-year-old Norway spruce trees. Increases in lignin abundance in wood cell wall may be resulted from the up-regulation of relevant genes under FACE and N-fertilization condition in comparison with FACE and low N-supply.

5.4.2 *Wood properties and gene expression pattern in wood-forming tissues*

Although two reports have provided some information on gene expression in leaves of *Populus* species exposed to FACE (Taylor et al., 2005; Gupta et al., 2005), the transcriptional profiling studied here provides an initial insight into how gene expression is likely to be altered in developing xylem following the exposure of a forest ecosystem to elevated $[CO_2]$ and N-fertilization. *P. × euramericana* tree growth was stimulated by FACE and/or N-fertilization in 2 years after coppicing (Liberloo et al., 2005), and this was likely to be associated with altered wood properties (Tables 2-5; Luo et al., 2005b). Therefore, a

major aim of this investigation is to use transcriptional profiling to deduce patterns of gene expression and candidate genes involving the determination of wood properties.

Perhaps the most impressive finding of this study is that only few transcripts were markedly affected by FACE and/or N-fertilization (Table 6). This finding is likely to be associated with the array used in this study since genes printed on the array were collected from primary and secondary stem, shoot tips and leaves of *Populus trichocarpa × P. deltoides* plants with altered N status (N fertilization and girdling). However, it is also probable that these experimental results reflected the realistic situation of poplar trees grown under the field conditions. Nevertheless, the differentially expressed genes in response to FACE and/or N-fertilization may provide some clues to the responses of wood properties to these changing environmental factors at the molecular level. Since the function of COMT-1 is to catalyze the conversion of caffeic acid to ferulic acid and of 5-hydroxyferulic acid to sinapic acid and the resulting products may subsequently be converted to the corresponding alcohols that are incorporated into lignins (Bugos et al., 1992), up-regulation of COMT-1 in the differentiating xylem may point to increased lignin biosynthesis in woody stem of poplar grown under FACE and N-fertilization condition in comparison with wood formed under FACE and low N supply (Fig. 6). This observation corresponds well to the findings obtained by Klason lignin method (Table 4), FT-IR spectra and mapping (Figs. 4 and 5). Another gene encoding ferulate-5-hydroxylase (F5H) is also up-regulated in woody stem of poplar grown under FACE and N-fertilization condition compared to FACE and low N supply condition (Table 6). Ferulate-5-hydroxylase (F5H) is a cytochrome-P450-dependent monooxygenase required for the biosynthesis of syringyl monomers (Humphreys and Chapple, 2002). When F5H was overexpressed in *Arobidopsis*, tobacco and poplar plants, they were found to deposit lignin composed almost entirely of syringyl monomers (Meyer et al., 1996; Franke et al., 2000). This suggests that the up-regulation of F5H may enhance the biosynthesis of syringyl lignin. Another interesting finding is that the expression of a tubulin gene associated with the cytoskeleton (Hertzberg et al., 2001) was increased under FACE and N-fertilization condition in comparison with FACE and low N supply condition (Table 6). A change in the expression of this gene may be related to the altered anatomical properties (diameters of fibre and vessel lumen) in woody stem of *P. × euramericana* under FACE and N-fertilization condition in comparison with FACE and low N-supply (Table 2). However, these differentially expressed genes can not help us to understand why the fibre cell walls were diminished under FACE and N-

fertilization condition in comparison with FACE and low N-supply. Clearly, to explain the diminishing fibre cell walls under FACE and N-fertilization condition in comparison with FACE and low N-supply, more studies are needed at the molecular level. Additionally, cautions should be applied to the interpretations of cDNA microarray results since these results are not yet confirmed by other independent tests, such as quantitative RT-PCR.

In conclusion, anatomical and biochemical properties in woody stem of *P. × euramericana* were altered along with changes in gene expression when poplar trees were exposed to FACE and/or N-fertilization. FACE and/or N-fertilization altered annual ring width and stimulated the lumina of vessels and fibres, but both factors diminished the double fibre walls. FACE and/or N-fertilization changed in the FT-IR spectra in woody stem of *P. × euramericana*. Although no fertilization effects on the calorific value of poplar wood were detected, the energy production of *P. × euramericana* coppice was stimulated by N-fertilization. These changes may be caused by the alteration of the molecular composition, which is further caused from the changes in gene expression associated with FACE and/or N-fertilization induced shifts in resource allocation in *P. × euramericana*.

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References

Alba R, Fei Z, Payton P, Liu Y, Moore SL, Debbie P, Cohn J, Ascenzo M, Gordon JS, Rose JKC, Martin G, Tanksley SD, Bouzayen M, Jahn MM, Giovannoni J (2004) ESTs, cDNA microarrays, and gene expression profiling: tools for dissecting plant physiology and development. The Plant Journal, 39, 697-714.

Atwell BJ, Henery ML, Whitehead D (2003) Sapwood development in *Pinus radiata* trees grown for three years at ambient and elevated carbon dioxide partial pressures. Tree Physiology, 23, 13-21.

Blaschke L, Forstreuter M, Sheppard LJ et al. (2002) Lignification in beech (*Fagus sylvatica*) grown at elevated CO_2 concentrations: interaction with nutrient availability and leaf maturation. Tree Physiology, 22, 469-477.

Boerjan W, Ralph J, Baucher M (2003) Lignin biosynthesis. Annual Review of Plant Biology, 54, 519-546.

Bugos RC, Chiang VL, Campbell WH (1992) Characterization of bispecific caffeic acid/5-hydroxyferulic acid O-methyltransferase from aspen. Phytochemistry, 31, 1495-1498.

Calfapietra C, Gielen B, Sabatti M, Angelis PD, Scarascia-Mugnozza G, Ceulemans R (2001) Growth performance of *Populus* exposed to "Free Air Carbon Dioxide Enrichment" during the first growing season in the POPFACE experiment. Annals Forest Science, 58, 819-828.

Calfapietra C, Gielen B, Sabatti M, Angelis PD, Miglietta F, Mugnozza GS, Ceulemans R (2003) Do aboveground growth dynamics of poplar change with time under CO_2 enrichment? New Phytologist, 160, 305-318.

Ceulemans R, Jach ME, van de Velde R et al. (2002) Elevated atmospheric CO_2 alters wood production, wood quality and wood strength of scots pine (*Pinus sylvestris L.*) after three years of enrichment. Global Change Biology, 8, 153-162.

Chaffey NJ (2000) Cytoskeleton, cell walls and cambium: new insights into secondary xylem differentiation. In: Savidge R, Barnett J, Napier R (eds) Cell and molecular biology of wood formation. Oxford, Bios Scientific Publishers, pp. 31-42.

Chang S, Puryear J, Cairney J (1993) A simple and efficient method for isolating RNA from pine trees. Plant Molecular Biology Reporter, 11, 113-116.

Ciria MP, Gonzales E, Mazon P, Carrasco J (1996) Influence of the rotation age and plant density on the composition and quality of poplar biomass. Biomass for energy and the

environment. Proceedings of the Ninth European Bioenergy Conference, Copenhagen, Denmark, vol. 2, pp. 968-973.

Collier W, Schultz T, Kalasinsky V (1992) Infrared study of lignin: reexamination of aryl-alkyl ether C-O stretching peak assignments. Holzforschung, 46, 523-528.

Conroy JP, Milham PJ, Mazur M et al. (1990) Growth, dry weight partitioning and wood properties of *Pinus radiata* D. Don after 2 years of CO_2 enrichment. Plant, Cell and Envrionment, 13, 329-337.

Dence CW (1992) Lignin determination. *In* Methods in Lignin Chemistry (Stephen YL ed), Springer-Verlag, Berlin, pp 33-61.

Drake BG, Gonzalez-Meler MA, Long SP (1997) More efficient plants: A consequence of rising atmospheric CO_2? Annual Review of Plant Physiology and Plant Molecular Biology, 48, 609-639.

Faix O (1992) Fourier transform infrared spectroscopy. In: Lin SY, Dence CW (Eds.), Methods in Lignin Chemistry. Springer, Berlin, pp. 83-109

Franke R, McMichichael CM, Meyer K, Shirley AM, Cusumano JC and Chapple C (2000) Modified lignin in tobacco and poplar plants overexpressing the *Arabidopsis* gene encoding ferulate-5-hydroxylase. The Plant Journal, 22, 223-234.

Geider RJ, Delucia EH, Falkowski PG, Finzi AC, Grime JP et al. (2001) Primary productivity of planet earth: biological determinants and physical constraints in terrestrial and aquatic habitats. Global Change Biology, 7, 849-882.

Gielen B, Ceulemans R (2001) The likely impact of rising atmospheric CO_2 on natural and managed *Populus*: a literature review. Environmental Pollution, 115, 335-358.

Gindl W, Grabner M, Wimmer R (2000) The influence of temperature on latewood lignin content in treeline Norway spruce compared with maximum density and ring width. Trees, 14, 409-414.

Grace J (2004) Understanding and managing the global carbon cycle. Journal of Ecology, 92, 189-202.

Gupta P, Duplessis S, White H, Karnosky DF, Martin F, Podila GK (2005) Gene expression patterns of trembling aspen trees following long-term exposure to interacting elevated CO_2 and tropospheric O_3. New Phytologist, 167, 129-142.

Harrington KJ, Higgins HG, Michell AJ (1964) Infrared spectra of *Eucalypus regnans* F. Muell. And *Pinus radiata* D. Don. Holzforschung, 18, 108-113.

Hergert HL (1971) Infrared spectra. In: Sarkanen KV, Ludwig CH (Eds.), Lignins: Occurrence, Formation, Structure and Reactions. Wiley interscience, New York, pp. 267-297

Hertzberg M, Aspeborg H, Schrader J, Andersson A, Erlandsson R, Blomqvist K, Bhalerao R, Uhlen M, Teeri T, Lundeberg J et al. (2001) A transcriptional roadmap to wood formation. Proc Natl Acad Sci USA, 98, 14732-14737.

Humphreys JM, Chapple C (2002) Rewriting the lignin roadmap. Current Opinion in Plant Biology, 5, 224-229.

IPCC (2001) Climate change 2001. Cambridge University Press, Cambridge, pp 881.

Kataki R, Konwer D (2001) Fuelwood characteristics of some indigenous woody species of north-east India. Biomass and Bioenergy, 20, 17-23.

Kilpelainen A, Peltola H, Ryyppo A et al. (2003) Wood properties of Scots pines (*Pinus sylvestris*) grown at elevated temperature and carbon dioxide concentration. Tree Physiology, 23, 889-897.

Klasnja B, Kopitovic S, Orlovic S (2002) Wood and bark of some poplar and willow clones as fuelwood. Biomass and Bioenergy, 23, 427-432

Kostiainen K, Kaakinen S, Saranpaa P, Sigurdsson B, Linder S, Vapaavuori E (2004) Effect of elevated [CO_2] on stem wood properties of mature Norway spruce grown at different soil nutrient availability. Global Change Biology, 10, 1526-1538.

Larson PR (1994) The vascular cambium: Development and Structure, Springer Series in Wood Science (Springer, Berlin).

Liberloo M, Dillen SY, Calfapietra C, Marinari S, Luo Z-B, Angelis PD, Ceulemans R (2005) Elevated CO_2 concentration, fertilization and their interaction: growth stimulation in a short-rotation poplar coppice (EUROFACE). Tree Physiology, 25, 179-189.

Long SP, Ainsworth EA, Rogers A, Ort DR (2004) Rising atmospheric carbon dioxide: plants FACE the future. Annual Review of Plant Biology, 55, 591-628.

Luo Z-B, Calfapietra C, Liberloo M, Scarascia-Mugnozza G, Polle A (2005a) Carbon partitioning to mobile and structural fractions in poplar wood under elevated CO_2 (EUROFACE) and N-fertilization. Global Change Biology (in press)

Luo Z-B, Langenfeld-Heyser R, Calfapietra C, Polle A (2005b) Influence of free air CO_2 enrichment (EUROFACE) and nitrogen fertilization on the anatomy of juvenile wood of three poplar species after coppicing. Trees, 19, 109-118.

Meyer K, Cusumano JC, Somerville C, Chapple CCS (1996) Ferulate-5-hydroxylase from *Arabidopsis thaliana* defines a new family of cytochrome P450-dependent monooxygenases. Proc Natl Acad Sci USA, 93, 6869-6874.

Miglietta F, Peressotti A, Vaccari FP, Zaldei A, Angelis PD, Scarascia-Mugnozza G (2001) Free-air CO_2 enrichment (FACE) of a poplar plantation: the POPFACE fumigation system. New Phytologist, 150, 465-476.

Norby RJ, Todd DE, Fults J, Johnson DW (2001) Allometric determination of tree growth in a CO_2-enriched sweetgum stand. *New Phytologist*, 150, 477-487.

Pandey KK, Theagarajan KS (1997) Analysis of wood surfaces by diffuse reflectance (DRIRT) and photoacoustic (PAS) Fourier transform infrared spectroscopic techniques. Holz als Roh- und Werkstoff 55, 383-390

Polle A, Otter T, Sandermann JH (1997) Biochemistry and physiology of lignin synthesis. In: Trees-Contributions to Modern Tree Physiology (eds. Rennenberg H; Eschrich W; Ziegler H), Backhuys Publishers, Leiden, Netherlands, pp. 455-475.

Runion GB, Entry JA, Prior SA et al. (1999) Tissue chemistry and carbon allocation in seedlings of *Pinus palustris* subjected to elevated atmospheric CO_2 and water stress. Tree Physiology, 19, 329-335.

Saxe H, Ellsworth DS, Heath J (1998) Tree and forest functioning in an enriched CO_2 atmosphere. New Phytologist, 139, 395-436.

Schultz TP, Glasser WG (1986) Quantitative structural analysis of lignin by diffuse reflectance Fourier transform spectrometry. Holzforschung, 40, 37-44.

Taylor G, Street NR, Tricker PJ, Sjödin A, Graham Laura, Skogström O, Calfapietra C, Scarascia-Mugnozza G and Jansson S (2005) The transcriptome of *Populus* in elevated CO_2. New Phytologist, 167, 143-154.

Telewski FW, Swanson RT, Strain BR, Burns JM (1999) Wood properties and ring width response to long-term atmospheric CO_2 enrichment in field-grown loblolly pine (*Pinus taeda* L.). Plant Cell and Environment, 22, 213-223.

Tilman D, Fargione J, Wolff B, D'Antonio C, Dobson A, Howarth R, Schindler D, Schlesinger WH, Simberloff D, Swackhamer D (2001) Forecasting agriculturally driven global environmental change. Science, 292, 281-284.

Vitousek PM, Aber JD, Howarth RW, Likens GE, Matson PA, Schindler DW, Schlesinger WH, Tilman DG. (1997) Human alteration of the global nitrogen cycle: sources and consequences. Ecological Application, 7, 737-750.

Whetten R, Sun Y-H, Zhang Y, Sederoff R (2001) Functional genomics and cell wall biosynthesis in loblolly pine. Plant Molecular Biology, 47, 275-291.

Yazaki K, Funada R, Mori S et al. (2001) Growth and annual ring structure of *Larix sibirica* grown at different carbon dioxide concentrations and nutrient supply rates. Tree Physiology, 21, 1223-1229.

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7. Curriculum Vitae

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07/1997 – 09/2000 Institute of Forest Survey of Guangxi, Nanning, China (gained knowledge and skills on forest inventory)

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10/2004 – 11/2004 Pfleiderer AG, Werk Arnsberg, Germany (gained knowledge and skills on analytical chemistry)

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Publications

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1. **Zhi-Bin Luo**, Rosemarie Langenfeld-Heyser, Carlo Calfapietra, and Andrea Polle (2005) Influence of free air CO_2 enrichment (EUROFACE) and nitrogen fertilization on the anatomy of juvenile wood of three poplar species after coppicing. **Trees** 19: 109-118

2. **Zhi-Bin Luo**, Carlo Calfapietra, Marion Liberloo, Giuseppe Scarascia-Mugnozza, Andrea Polle (2006) Carbon partitioning to mobile and structural fractions in poplar wood under elevated CO_2 (EUROFACE) and N-fertilization. **Global Change Biology** 12: 272-283

3. Marion Liberloo, Sophie Dillen, Carlo Calfapietra, Sara Marinari, **Zhi-Bin Luo**, Paolo De Angelis, Reinhart Ceulemans (2005) Elevated CO_2 concentration, fertilization and their interaction: growth stimulation in a short-rotation poplar coppice (EUROFACE). **Tree Physiology** 25: 179-189

4. Marion Liberloo, Carlo Calfapietra, Martin Lukac, Douglas Godbold, **Zhi-Bin Luo**, Andrea Polle, Olevi Kull, Michal Marek, Christine Raines, Gail Taylor, Giuseppe Scarascia-Mugnozza and Reinhart Ceulemans (2006) Woody biomass production during second rotation of a bio-energy *Populus* plantation increases in a future high CO_2 world. **Global Change Biology** (In press)

5. **Zhi-Bin Luo,** Huancheng Ma, Longbing Rao (2002) Water retaining agent and its application in forestry. **Forest Research** 15: 620-627 (in Chinese with English Abstract)

Posters

1. **Zhi-Bin Luo**, Carlo Calfapietra, Andrea Polle (2004) Seasonal changes of carbohydrates and nitrogen in *Populus nigra* exposed to free air carbon dioxide enrichment (EUROFACE) and N-fertilization after coppicing. International FACE Workshop: Short- and long-term effects of elevated atmospheric CO_2 on managed ecosystems. Ascona, Switzerland, 20–25 March 2004

2. **Zhi-Bin Luo**, Carlo Calfapietra, Andrea Polle (2004) Non-structural carbohydrate dynamics in *Populus nigra* exposed to Free Air Carbon dioxide Enrichment (FACE) and N-fertilization. Understanding Poplar: From Genes to Functions. Poplar-Symposium, 13 and 14 May, 2004, Goettingen, Germany.

Oral Presentation

Effects of Free Air CO_2 Enrichment (FACE) and N-fertilization on carbon-based secondary metabolites of *Populus nigra*. Botany Conference, 5-10 Sep., 2004, Braunschweig, Germany.