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
Zhibin Luo
Born in Hunan, China

Goettingen, November 2005
Supervisor:    Prof. Dr. Andrea Polle

Referee:          Prof. Dr. Andrea Polle

Co-referee:     Prof. Dr. Holger Militz

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Table of contents

Table of contents ................................................................. I
Summary .................................................................................. V

1. Introduction ........................................................................ 1
   1.1 Evidence of rising atmospheric CO₂ concentrations and changes in N cycle ............. 1
   1.2 Basis of plants responding to elevated CO₂ and N-fertilization ............................... 2
   1.3 Why do we need FACE to test the responses of plants to elevated CO₂? ............... 4
   1.4 What is EUROFACE? .............................................................................. 5
   1.5 What is known and what is unknown about the responses of plants to elevated CO₂ and N-fertilization? ......................................................... 8
   1.6 What questions are addressed in this study? .................................................... 12

2. Anatomical properties of secondary xylem in poplar ........................................ 19
   2.1 Introduction .................................................................................. 21
   2.2 Materials and methods .................................................................. 22
       2.2.1 Site description ........................................................................ 22
       2.2.2 Plant material and plantation layout ........................................... 23
       2.2.3 Sampling .................................................................................. 23
       2.2.4 Analysis of wood anatomy .......................................................... 24
       2.2.5 Statistical analysis .................................................................. 25
   2.3 Results ...................................................................................... 25
       2.3.1 Growth and tension wood as affected by FACE and N-fertilization ............. 25
       2.3.2 Influence of FACE and N-fertilization on structural wood composition ....... 28
       2.3.3 Effects of FACE and N-fertilization on anatomical characteristics of wood ...... 31
   2.4 Discussion .................................................................................. 34
       2.4.1 Is tension wood formation affected by FACE or N-fertilization or related to growth characteristics? .............................................................. 34
       2.4.2 Are the structural properties of normal wood affected by FACE or N-fertilization? ........................................................................ 35
       2.4.3 Is wood anatomy affected by FACE and N-fertilization and what are possible implications? ................................................................. 36
3. Carbon partitioning to mobile and structural fractions in poplar .......................... 42
  3.1 Introduction ................................................................................................. 44
  3.2 Materials and methods ................................................................................ 45
    3.2.1 Site description ....................................................................................... 45
    3.2.2 Plant material and plantation layout ...................................................... 46
    3.2.3 Sampling ................................................................................................. 46
    3.2.4 Analysis of non-structural carbohydrates .............................................. 47
    3.2.5 Analysis of carbon content ..................................................................... 48
    3.2.6 Determination of cell wall fraction ....................................................... 48
    3.2.7 Estimation of cell wall and non-structural biomass ............................... 48
    3.2.8 Statistical analysis .................................................................................. 48
  3.3 Results ......................................................................................................... 49
    3.3.1 Carbon partitioning between soluble and immobile fractions in poplar leaves 49
    3.3.2 Non-structural carbohydrates in bark ................................................. 50
    3.3.3 Carbon partitioning between soluble and immobile fractions in woody stems 53
    3.3.4 Aboveground partitioning to metabolically active and structurally bound carbon pools 56
  3.4 Discussion ................................................................................................... 58
    3.4.1 Is carbon partitioning affected by FACE? .............................................. 58
    3.4.2 Is carbon partitioning affected by N-fertilization and seasonal changes? 59

4. Carbon-based secondary metabolites and internal N pools in poplar ....................... 67
  4.1 Introduction .................................................................................................. 69
  4.2 Materials and Methods ................................................................................ 70
    4.2.1 Site description and FACE facilities .................................................... 70
    4.2.2 Plant material and N-fertilization ......................................................... 71
    4.2.3 Sampling ................................................................................................. 71
    4.2.4 Analysis of soluble- and cell wall bound-phenolics .............................. 72
    4.2.5 Analysis of condensed tannins .............................................................. 73
    4.2.6 Analysis of Klason lignin .................................................................... 74
    4.2.7 Analyses of soluble proteins and nitrogen pools ................................. 75
    4.2.8 Statistical analysis .................................................................................. 75
4.3 Results ................................................................................................................................. 76
  4.3.1 Carbon-based secondary compounds ............................................................................. 76
  4.3.2 Soluble proteins and internal N-pools ............................................................................ 79
  4.3.3 Balance between carbon-based secondary compounds and soluble proteins .......... 84
4.4 Discussion ............................................................................................................................ 84
  4.4.1 Are secondary metabolites affected by FACE, N-fertilization or season? ...................... 84
  4.4.2 Are the internal N-pools affected by FACE, N-fertilization or season? ......................... 86
  4.4.3 Is there a negative balance between protein biosynthesis and production of secondary metabolites? .................................................................................................................. 88

5. Wood properties and gene expression profiling in poplar ................................................. 97
  5.1 Introduction ......................................................................................................................... 100
  5.2 Materials and Methods ......................................................................................................... 102
    5.2.1 FACE facilities .............................................................................................................. 102
    5.2.2 Plant material and N-fertilization ................................................................................ 102
    5.2.3 Sampling ..................................................................................................................... 103
    5.2.4 Wood anatomical analyses .......................................................................................... 103
    5.2.5 Determination of carbon and nitrogen concentrations .................................................. 104
    5.2.6 Determination of Klason lignin ..................................................................................... 104
    5.2.7 Fourier transform infrared (FT-IR) spectra analyses in wood ...................................... 105
    5.2.8 Calorific value of wood ............................................................................................... 106
    5.2.9 RNA preparation ......................................................................................................... 107
    5.2.10 cDNA synthesis and purification ............................................................................... 108
    5.2.11 Labeling of cDNA targets and purification ............................................................... 109
    5.2.12 Experimental design of hybridizations ...................................................................... 110
    5.2.13 Microarray hybridization ............................................................................................. 111
    5.2.14 Acquisition, transformation, and processing of microarray data ............................... 111
    5.2.15 Statistical analysis .................................................................................................... 112
  5.3 Results ................................................................................................................................ 113
    5.3.1 Anatomical properties of stem-wood .......................................................................... 113
    5.3.2 Chemical wood properties ......................................................................................... 115
5.3.3 Energy content of wood ................................................................. 119
5.3.4 Gene expression patterns in developing xylem ................................ 120
5.4 Discussion .................................................................................. 122
  5.4.1 Wood properties .................................................................... 122
  5.4.2 Wood properties and gene expression pattern in wood-forming tissues 124
6. Acknowledgements ........................................................................ 132
7. Curriculum Vitae ........................................................................ 134
Summary

Atmospheric CO₂ concentrations ([CO₂]) are continuously rising since the beginning of the industrialization. At the same time, N-deposition is also increasing. The influences of elevated CO₂ 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 × euramerica*, *P. alba*, and *P. nigra* clones were grown in ambient air (about 370 ppm CO₂) and in air with elevated [CO₂] (about 550 ppm CO₂) using Free-Air CO₂ 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 CO₂ 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 CO₂ or N-fertilization. In *P. × euramerica*, N-fertilization resulted in significant reductions in fiber lengths. In all three genotypes, N-fertilization caused significant decreases in cell wall thickness. In *P. × euramerica* and *P. alba*, elevated CO₂ also caused decreases in wall thickness, but less pronounced than nitrogen. In *P. nigra* and *P. × euramerica*, elevated CO₂ induced increases in vessel diameters. The combination of elevated CO₂ 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 CO₂ and N-fertilization, in *P. nigra*, carbon concentrations and stocks were quantified. Although elevated CO₂, 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 CO₂ 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 CO₂ 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 CO₂ and N-fertilization, carbon-based secondary compounds, concentrations of total N and Klason lignin-bound N were measured in *P. nigra*. Elevated CO₂ 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 CO₂ 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 CO₂ 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 CO₂ 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. × eurameriana*. Elevated CO₂ significantly stimulated the annual ring width in the second year after coppicing. However, elevated CO₂ 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 CO₂ 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 CO₂ 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 CO₂ 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 CO₂, 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 CO₂ in fertilized agro-forestry systems. The growth and defense of forest trees were homeostatically balanced even if increases in atmospheric [CO₂] were accompanied by increased N availability.

**Keywords:** Agroforestry, Biomass, Calorific value, Carbon sequestration, Climate change, Elevated CO₂, FT-IR, Gene expression, Lignin, N-fertilization, Phenolics, Soluble proteins, Starch, Sugar, Tannins, Wood anatomy, Wood properties, *Populus*
Zusammenfassung


Um die Holzqualität in Bezug auf die erhöhte CO₂-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 CO₂ und unter erhöhter Stickstoffdüngung zu quantifizieren, wurden in *P. nigra* die Kohlenstoffkonzentration und die Depotgröße bestimmt. Der Haupeffekt von erhöhtem CO₂ 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 CO₂ und Stickstoffeinfluss näher zu charakterisieren, wurden kohlenstoffbürtige Produkte wie Phenole und Tannine, die Gesamtstickstoffkonzentration und ligningebundene Stickstoff
bei P. nigra gemessen. Weder die erhöhte CO₂-Konzentration noch die erhöhte Stickstoffzufuhr veränderten die Stickstoffverteilung zwischen ligningebundenem Stickstoff und anderen stickstoffhaltigen Verbindungen. In P. nigra existierte eine positive Korrelation zwischen der Proteinbiosynthese und der Produktion von Sekundärmetaboliten.

Um einen Einblick in die molekularen Mechanismen zu gewinnen, die für die Veränderungen der Eigenschaften des Holzes unter erhöhtem Kohlendioxydgehalt und erhöhter Stickstoffdüngung verantwortlich sind, wurden Holzanatomie, Ligningehalt, Brennwert und chemische Holzzusammensetzung (mittels Fourier-Transform-Infrarotspektroskopie, FTIR) analysiert und in Bezug zum Genexpressionprofil des sich differenzierenden Xylem gesetzt. Gemeinsam verursachen die CO₂-Begasung und die Stickstoffdüngung eine erhöhte Ligninbildung; der Brennwert des Holzes wurde durch diese Behandlungen nicht beeinflusst. Die Transkriptionsanalyse mit Hilfe eines microarrays, der ca. 3 444 verschiedene Genfragmente der Pappel trug, ergab, dass nur wenige Transkripte durch die erhöhte CO₂-Konzentration und/oder die vermehrte Stickstoffzugabe signifikant verändert waren. Dies beinhaltet zwei Gene, die bei der Ligninbiosynthese eine Rolle spielen, d.h. eine Kaffeésäure-O-Methyltransferase 1 (COMT-1) und eine Ferulat-5-Hydroxylase (F5H). Daß erhöhtes CO₂ in Kombination mit N-Düngung den Ligninstoffwechsel antreibt, wird auch durch die Ergebnisse der Klasonligninanalyse und der FTIR-Analyse gestützt.

Auf der Basis dieser Ergebnisse kann vermutet werden, dass unter den für die Zukunft prognostizierten Klimabedingungen negative Auswirkungen auf die Holzqualität zu erwarten sind. Assimilierter Kohlenstoff kann vermehrt für das strukturelle Wachstum eingesetzt werden, insbesondere wenn die für die Plantagenwirtschaft genutzten Flächen unter erhöhtem CO₂ zusätzlich gedüngt werden. Das Abwehrvermögen in Form phenolischer Verbindungen war trotz vermehrten Wachstums unter diesen Bedingungen nicht vermindert.

1. Introduction

1.1 Evidence of rising atmospheric CO₂ 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 CO₂ concentrations ([CO₂]) have risen at an accelerating pace. Prior to the industrial revolution atmospheric [CO₂] was stable at about 270 ppm (Fig. 1). Today [CO₂] 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 (N₂) in the atmosphere; a relatively small and almost wholly biologically mediated conversion of N₂ 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 [CO₂].

Although many processes in plant metabolism are potentially affected by [CO₂], there is consistent evidence only for effects on ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and stomatal movement in the range of [CO₂] from 270—1000 ppm (Long et al. 2004). Plants can perceive a change in atmospheric [CO₂] 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 [CO₂]. At the biochemical level, Rubisco is currently [CO₂] substrate limited by atmospheric [CO₂]. Therefore, Rubisco responds to rising atmospheric [CO₂] 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 [CO₂] (Drake et al. 1997). The direct increase in photosynthesis of plants grown under elevated [CO₂] 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 [CO₂] (Saxe et al. 1998; Medlyn et al. 1999; Gielen and Ceulemans 2001), the mechanism by which stomatal aperture responds to variation in [CO₂] still remains mysterious (Moreson 1998; Long et al. 2004).

Since CO₂ is just one of many inorganic substrates required by plants, obviously, the response of photosynthesis and growth to elevated [CO₂] 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 NO₃⁻ or NH₄⁺ 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 CO₂?

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 [CO₂] and plants, especially forest trees, were carried out around the world in the past few decades. The influences of elevated [CO₂] 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 [CO₂] 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 [CO₂], essentially demonstrating that loss of a response to increased [CO₂] through acclimation was an artifact of pot size. Even large pots restricted the response of plants to elevated [CO₂] (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 [CO₂] may exceed that of any additional effect due to enrichment of [CO₂] (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 CO₂ 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 [CO2].

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 CO₂, or air enriched with CO₂, at vertical intervals from just above the ground surface to just above the top of the plant canopy. Wind direction, wind velocity, and [CO₂] are measured at the center of each plot and this information is used by a computer-controlled system to adjust CO₂ flow rate, controlled by a mass-flow control valve, to maintain the target elevated [CO₂], 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 [CO₂], 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 [CO₂] 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 [CO₂] was
provided by using Free Air CO₂ 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 CO₂ 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 CO₂, 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 [CO₂]. The liquid CO₂ 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).

<table>
<thead>
<tr>
<th>Genotype name</th>
<th>2AS11</th>
<th>Jean Pourtet I-214</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species name</td>
<td><em>P. alba</em> L.</td>
<td><em>P. nigra</em> L.</td>
</tr>
<tr>
<td>Sex</td>
<td>Male</td>
<td>Male</td>
</tr>
<tr>
<td>Origin</td>
<td>Italy*</td>
<td>France*</td>
</tr>
<tr>
<td>Rooting</td>
<td>Medium</td>
<td>Very good</td>
</tr>
<tr>
<td>Branching habit</td>
<td>Medium</td>
<td>Very high</td>
</tr>
<tr>
<td>Apical control</td>
<td>Good</td>
<td>Good</td>
</tr>
</tbody>
</table>

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: $554 \pm 1.6$ ppm during the first year after coppice (2002), and $535.9 \pm 20.4$ 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 NH₄⁺:NO₃⁻ 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 minimum 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.

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

1.5 What is known and what is unknown about the responses of plants to elevated CO₂ and N-fertilization?

Our understanding of the effects of increase in [CO₂] on plants has progressed with the development of technologies by which CO₂ enrichment was achieved. Studies on how plants respond to future [CO₂] began about 30 years ago, initially with glasshouses to realize elevated [CO₂], followed by open top chambers (OTCs), natural CO₂ spring and recently by Free Air Carbon dioxide Enrichment (FACE). With the evolution of the technologies by which elevated [CO₂] was achieved, the results obtained in earlier studies
were checked, corrected and the responses of plants to elevated [CO₂] were better understood. Employing a meta-analysis based on data from 120 manuscripts describing physiology and production in the 12 large-scale FACE experiments ([CO₂] 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 [CO₂] (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 [CO₂]. 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 [CO₂] 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 CO₂ 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 [CO₂] 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 [CO₂], especially to FACE. For instance, in a growth chamber experiment, Atkinson and Tayhlor (1996) found that elevated [CO₂] 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 CO₂ 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 [CO₂]. Ceulemans et al. (2002) found that the wood of Pinus sylvestris grown at elevated [CO₂] 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 [CO₂]. For instance, in beech with high nutrient supply, lignin concentration decreased in response to elevated [CO₂] (Blaschke et al. 2002), but no significant effects of elevated [CO₂] on wood lignin in other species were observed (Runion et al. 1999; Atwell et al. 2003; Kilpelainen et al. 2003).

The rising atmospheric [CO₂] is resulting in global warming, which causes potentially devastating consequences (IPCC 2001). To alleviate global warming, mankind is making efforts to stabilize the atmospheric [CO₂] 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 [CO₂] 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 [CO₂] in broadleaf species, which have different properties in comparison with conifers.

Along with the evolution of studies about effects of elevated [CO₂] 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 CO₂ in the coming CO₂-enriched world (Phillips et al. 1998; Janssens et al. 2003; Körner et al. 2005), most scientists agree that forests have already shown their own adaptation to the environmentally 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 [CO₂]. In that study, they found some of the most responsive transcripts to elevated [CO₂] 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 \([\text{CO}_2]\) 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


2. Anatomical properties of secondary xylem in poplar

Abstract

*Populus × eurameriana*, *P. alba*, and *P. nigra* clones were exposed to ambient or elevated (about 550 ppm) CO₂ concentrations ([CO₂]) 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 [CO₂] 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 [CO₂]. 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. × eurameriana*, N-fertilization resulted in significant reductions in fibre lengths. In all three genotypes, N-fertilization caused significant decreases in cell wall thickness. In *P. × eurameriana* and *P. alba*, elevated [CO₂] also caused decreases in wall thickness, but less pronounced than nitrogen. In *P. nigra* and *P. × eurameriana*, elevated [CO₂] induced increases in vessel diameters. These results show that elevated [CO₂] 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 CO₂ concentration were accompanied by increased N availability.

**Keywords** Climate change, Elevated CO₂, N-fertilization, Wood anatomy, *Populus*
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 [CO₂] and additional nitrogen fertilization under field conditions and to compare the CO₂ responses in different species to find out whether the responsiveness to CO₂ depends strongly on the genetic background or not.

The objective of the present study was to investigate the impact of elevated [CO₂] 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 [CO₂] 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 [CO₂] 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 [CO₂] 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 CO₂ enrichment was realised through octagonal polyethylene rings (22 m diameter) mounted on telescopic poles. Pure CO₂ (Messer Griesheim) was released through laser-drilled holes in the polyethylene rings to achieve the target [CO₂] (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 CO₂,
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 [CO₂]. In the FACE plots daytime [CO₂] 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 NH₄⁺: NO₃⁻ 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 µm². The percentage of cell wall area (CWA) was calculated as follows:

\[ \text{CWA} \text{ (%)} = \frac{\text{total area} - (\text{vessel lumen area} + \text{fibre lumen area} + \text{ray parenchyma area})}{\text{total area}} \times 100 \]

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:

\[ \text{TWA} \text{ (%)} = \frac{\text{tension wood area}}{\text{total area} - \text{pith area}} \times 100 \]

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), CO₂ treatment (CO₂) and N-fertilization (nitrogen) on all variables, an ANOVA, i.e. a randomised-complete-block design, with species, CO₂ 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 CO₂ 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. × euramerica*. Tension wood formation was significantly dependent on the clone and in most cases lower in *P. × euramerica* 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 [CO₂], E: elevated [CO₂], 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, CO₂, nitrogen and their interactions are also indicated.

<table>
<thead>
<tr>
<th>Clones</th>
<th>CO₂</th>
<th>Nitrogen</th>
<th>TWA 0902 (%)</th>
<th>TWA 0303 (%)</th>
<th>D 0902 (mm)</th>
<th>D 0303 (mm)</th>
<th>SH 0902 (m)</th>
<th>SH 0303 (m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. alba</td>
<td>A</td>
<td>L</td>
<td>9.7 ± 4.4 c</td>
<td>2.1 ± 2.0 a</td>
<td>23.3 ± 5.1 abc</td>
<td>20.9 ± 4.3 abc</td>
<td>3.7 ± 0.5 abcd</td>
<td>3.6 ± 0.5 abcd</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>L</td>
<td>4.3 ± 3.1 a</td>
<td>1.5 ± 1.7 a</td>
<td>23.3 ± 4.4 abc</td>
<td>18.9 ± 1.8 abc</td>
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<td>H</td>
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<td>3.3 ± 5.4 ab</td>
<td>21.5 ± 2.3 ab</td>
<td>17.7 ± 3.9 ab</td>
<td>3.7 ± 0.3 ab</td>
<td>3.3 ± 0.4 ab</td>
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<tr>
<td></td>
<td>E</td>
<td>H</td>
<td>7.0 ± 3.2 abc</td>
<td>3.6 ± 4.7 ab</td>
<td>21.4 ± 2.5 ab</td>
<td>17.1 ± 3.1 ab</td>
<td>3.6 ± 0.3 a</td>
<td>3.5 ± 0.4 abc</td>
</tr>
<tr>
<td>P. x eurameriana</td>
<td>A</td>
<td>L</td>
<td>5.4 ± 4.3 ab</td>
<td>3.6 ± 3.4 ab</td>
<td>26.8 ± 3.5 cde</td>
<td>21.0 ± 7.7 abc</td>
<td>4.0 ± 0.4 bcdef</td>
<td>3.3 ± 1.0 a</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>L</td>
<td>3.8 ± 3.4 a</td>
<td>2.4 ± 3.8 a</td>
<td>28.6 ± 3.7 def</td>
<td>22.9 ± 5.2 c</td>
<td>4.1 ± 0.2 def</td>
<td>3.5 ± 0.5 abc</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>H</td>
<td>3.6 ± 2.2 a</td>
<td>4.9 ± 2.2 ab</td>
<td>30.5 ± 3.6 ef</td>
<td>23.7 ± 5.3 c</td>
<td>4.4 ± 0.3 ef</td>
<td>3.9 ± 0.6 bcd</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>H</td>
<td>4.4 ± 1.9 a</td>
<td>2.6 ± 1.5 a</td>
<td>32.6 ± 5.0 f</td>
<td>28.9 ± 3.1 d</td>
<td>4.4 ± 0.3 f</td>
<td>4.2 ± 0.3 d</td>
</tr>
<tr>
<td>P. nigra</td>
<td>A</td>
<td>L</td>
<td>4.2 ± 2.1 a</td>
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<td>20.9 ± 5.9 ab</td>
<td>17.4 ± 3.3 ab</td>
<td>3.7 ± 0.6 abc</td>
<td>3.4 ± 0.6 abc</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>L</td>
<td>5.6 ± 3.9 ab</td>
<td>3.5 ± 2.6 ab</td>
<td>25.5 ± 4.5 bcd</td>
<td>22.1 ± 5.7 bc</td>
<td>4.0 ± 0.2 bcde</td>
<td>3.9 ± 0.5 cd</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>H</td>
<td>6.5 ± 2.4 abc</td>
<td>7.1 ± 7.3 b</td>
<td>20.4 ± 5.1 a</td>
<td>16.5 ± 3.6 a</td>
<td>3.5 ± 0.5 a</td>
<td>3.3 ± 0.4 a</td>
</tr>
<tr>
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<td>E</td>
<td>H</td>
<td>8.9 ± 2.9 bc</td>
<td>7.2 ± 4.9 b</td>
<td>28.1 ± 4.9 cdef</td>
<td>19.3 ± 2.9 abc</td>
<td>4.1 ± 0.2 cdef</td>
<td>3.8 ± 0.5 abcd</td>
</tr>
</tbody>
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P-values (main effects)

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<tr>
<th>Clones</th>
<th>CO₂</th>
<th>Nitrogen</th>
<th>0.0051</th>
<th>0.0625</th>
<th>0.0000</th>
<th>0.0000</th>
<th>0.0000</th>
<th>0.3212</th>
</tr>
</thead>
</table>
| P-values (interactions)
| Clone × CO₂   | 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 |
| CO₂ × nitrogen  | 0.1455 | 0.9199  | 0.5714 | 0.6473 | 0.5943 | 0.5971 |
| Clone × CO₂ × 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 CO2 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 [CO2] also resulted in increased percentages of ray parenchyma areas compared with the ambient [CO2] (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) [CO2] in the presence (H) or absence (L) of N-fertilization. *P*-values of the ANOVA *F*-test of the main effects of clone, CO2, nitrogen and their interactions are indicated.

<table>
<thead>
<tr>
<th></th>
<th>PCWA</th>
<th>PVA</th>
<th>PFA</th>
<th>PRA</th>
<th>VF</th>
<th>TDFW</th>
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<tbody>
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<td><strong>Clone</strong></td>
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<td>0.0000</td>
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<td>0.0000</td>
</tr>
<tr>
<td><strong>CO2</strong></td>
<td>0.0000</td>
<td>0.1554</td>
<td>0.0000</td>
<td>0.0001</td>
<td>0.2851</td>
<td>0.0000</td>
</tr>
<tr>
<td><strong>Nitrogen</strong></td>
<td>0.0000</td>
<td>0.0074</td>
<td>0.0000</td>
<td>0.0078</td>
<td>0.0931</td>
<td>0.0000</td>
</tr>
<tr>
<td><strong>Clone × CO2</strong></td>
<td>0.0000</td>
<td>0.1960</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.0200</td>
<td>0.0240</td>
</tr>
<tr>
<td><strong>Clone × nitrogen</strong></td>
<td>0.0000</td>
<td>0.4350</td>
<td>0.0000</td>
<td>0.5420</td>
<td>0.1890</td>
<td>0.0000</td>
</tr>
<tr>
<td><strong>CO2 × nitrogen</strong></td>
<td>0.0000</td>
<td>0.8500</td>
<td>0.0020</td>
<td>0.8230</td>
<td>0.0400</td>
<td>0.0000</td>
</tr>
<tr>
<td><strong>Clone × CO2 × nitrogen</strong></td>
<td>0.7250</td>
<td>0.9380</td>
<td>0.5410</td>
<td>0.0020</td>
<td>0.1430</td>
<td>0.0000</td>
</tr>
</tbody>
</table>
Fig. 1 Typical cross sections of *P. alba* (a), *P. × eurameriana* (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.
### 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 (± SD, n ≥ 36). 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 indicated.

<table>
<thead>
<tr>
<th>Clones</th>
<th>CO$_2$</th>
<th>Nitrogen</th>
<th>ADV(µm)</th>
<th>VEL(µm)</th>
<th>ADF(µm)</th>
<th>FL(µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. alba</em></td>
<td>A L</td>
<td>67.1 ± 5.9 cd</td>
<td>227.2 ± 52.4 a</td>
<td>20.6 ± 2.7 de</td>
<td>617.8 ± 87.2 e</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E L</td>
<td>64.1 ± 4.5 b</td>
<td>276.0 ± 45.7 de</td>
<td>20.3 ± 2.3 d</td>
<td>669.9 ± 68.4 g</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A H</td>
<td>65.9 ± 4.6 bcd</td>
<td>268.4 ± 40.5 cde</td>
<td>21.8 ± 2.7 g</td>
<td>677.4 ± 85.9 g</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E H</td>
<td>65.0 ± 4.1 bc</td>
<td>228.3 ± 43.9 a</td>
<td>21.1 ± 2.6 ef</td>
<td>598.4 ± 71.1 d</td>
<td></td>
</tr>
<tr>
<td><em>P. x euramericana</em></td>
<td>A L</td>
<td>61.1 ± 6.2 a</td>
<td>328.5 ± 56.9 g</td>
<td>19.6 ± 2.9 c</td>
<td>641.9 ± 84.0 f</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E L</td>
<td>68.1 ± 7.2 de</td>
<td>321.1 ± 61.5 g</td>
<td>19.6 ± 2.3 c</td>
<td>702.8 ± 86.5 h</td>
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<td>A H</td>
<td>67.1 ± 6.5 cd</td>
<td>303.7 ± 65.5 f</td>
<td>19.2 ± 2.5 c</td>
<td>551.7 ± 105.9 a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E H</td>
<td>77.7 ± 5.4 i</td>
<td>255.2 ± 62.1 bc</td>
<td>21.4 ± 2.6 fg</td>
<td>569.2 ± 132.6 b</td>
<td></td>
</tr>
<tr>
<td><em>P. nigra</em></td>
<td>A L</td>
<td>71.9 ± 6.8 g</td>
<td>264.4 ± 58.9 cd</td>
<td>16.2 ± 2.3 a</td>
<td>578.2 ± 63.9 bc</td>
<td></td>
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<tr>
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<td>E L</td>
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<td>17.5 ± 1.5 b</td>
<td>583.1 ± 72.0 c</td>
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</tr>
<tr>
<td></td>
<td>A H</td>
<td>70.1 ± 5.8 ef</td>
<td>282.1 ± 43.9 e</td>
<td>15.7 ± 1.8 a</td>
<td>609.9 ± 79.3 e</td>
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</tr>
<tr>
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<td>E H</td>
<td>73.8 ± 5.2 gh</td>
<td>246.8 ± 43.4 b</td>
<td>17.6 ± 1.9 b</td>
<td>597.9 ± 77.5 d</td>
<td></td>
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</tbody>
</table>

**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 CO₂ and nitrogen had no significant influence on the vessel frequencies (Table 2). Only in *P. nigra* grown under ambient CO₂ 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) [CO₂] 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, CO₂ 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, CO₂, 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 CO₂ (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) [CO₂] 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 [CO₂] 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 CO₂-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 R²-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 [CO₂] 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 CO2-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 [CO2] affects xylem anatomy and tissue composition. The present study shows that CO2-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 [CO2] 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 [CO2] 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 [CO2] (Conroy et al. 1990). Currently, we can only speculate about the reasons for these contrasting responses to CO2-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 [CO2] (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 [CO₂] 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 CO₂ and nitrogen in forest ecosystems. In conclusion, independent from the genotype elevated [CO₂] 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, CO₂-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 CO₂-enrichment in a greenhouse, Gross (2002) also found diminished vessel frequencies. In other studies no influence of elevated [CO₂] 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 CO₂ × genotype interactions.
In conclusion, elevated [CO₂] 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.

Acknowledgements

We are grateful to the European Union (contract number: EVR1-CT-2002-40027) and the Programme "Nachwuchswissenschaftler aus außereuropäischen Ländern nach Niedersachsen" for financial support. Christine Kettner, Gisbert Langer-Kettner, Michael Reichel, Rainer Schulz and Thomas Klein are acknowledged for their assistance with sample collection in the field.
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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 CO₂ (EUROFACE) and N-fertilization. Global Change Biology, 12, 272-283
Abstract

To determine whether globally increasing atmospheric CO₂ 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 CO₂) and in air with elevated CO₂ concentrations (about 550 µmol mol⁻¹ CO₂) using Free-Air CO₂ 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 CO₂. 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 [CO₂] in fertilized agroforestry systems. Thus, structural biomass production on abandoned agricultural land may contribute to achieving the goals of the Kyoto protocol.

**Keywords** Agroforestry, Bark, Biomass, Carbon sequestration, Global Change, Kyoto protocol, *Populus*, Sugar, Starch, Xylem
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 Cleeve 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 CO₂-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 CO₂ 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 CO₂ 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 CO₂ 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 established, whereas the other three plots that represent the control treatment were left under ambient conditions. CO₂ enrichment was realized through octagonal polyethylene rings (22 m diameter) mounted on telescopic poles. Pure CO₂ (Messer Griesheim) was released through laser-drilled holes in the polyethylene rings. To reach 550 µmol mol⁻¹ of the target [CO₂] 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 [CO₂] 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 cm² 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 °C for 30 min. The mixture was centrifuged (5000 g, 10 min, 4 °C; 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 °C; 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 MgSO₄, 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 Diagnostics GmbH, Mannheim, Germany) were added. When the reaction was accomplished, the absorbance for glucose was measured and 10 µL of phosphoglucone isomerase (10 mg mL⁻¹, Roche Diagnostics GmbH, Mannheim, Germany) were added for fructose determination. Finally, 10 µl of β-fructosidase (5 mg mL⁻¹, Roche Diagnostics 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-amylolaccosidase (66 mg α-amylolaccosidase (EC 3.2.1.3, Sigma, Steinheim, Germany) dissolved in 15 mL 50 µM citrate buffer, pH 4.6), mixed well, incubated at 55 °C 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 CO₂ treatment (CO₂), 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 CO₂, 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 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.](image-url)
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 CO₂ and nitrogen (N) and their interactions are shown.

<table>
<thead>
<tr>
<th>Seasons</th>
<th>Source</th>
<th>glucose</th>
<th>fructose</th>
<th>sucrose</th>
<th>starch</th>
<th>TNC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sep. 2002</td>
<td>CO₂</td>
<td>0.9426</td>
<td>0.9186</td>
<td>0.0081</td>
<td>0.3978</td>
<td>0.6492</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>0.6439</td>
<td>0.5849</td>
<td>0.3071</td>
<td>0.1546</td>
<td>0.5912</td>
</tr>
<tr>
<td></td>
<td>CO₂ × N</td>
<td>0.9820</td>
<td>0.0026</td>
<td>0.3132</td>
<td>0.2772</td>
<td>0.3052</td>
</tr>
<tr>
<td>Sep. 2003</td>
<td>CO₂</td>
<td>0.1873</td>
<td>0.2300</td>
<td>0.2080</td>
<td>0.3436</td>
<td>0.5244</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>0.4301</td>
<td>0.5478</td>
<td>0.8845</td>
<td>0.4353</td>
<td>0.4218</td>
</tr>
<tr>
<td></td>
<td>CO₂ × N</td>
<td>0.2543</td>
<td>0.6248</td>
<td>0.1539</td>
<td>0.9483</td>
<td>0.5080</td>
</tr>
</tbody>
</table>

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 [CO₂] (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
CO₂ 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 [CO₂] (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 CO₂ 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 CO₂, nitrogen (N) and their interactions are shown.

<table>
<thead>
<tr>
<th>Season</th>
<th>Treatment</th>
<th>Total-C %</th>
<th>Wall-bound C %</th>
<th>TNC-C %</th>
<th>Residual-C %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sep. 2002</td>
<td>AL</td>
<td>46.7 ± 0.2</td>
<td>25.4 ± 4.4</td>
<td>2.1 ± 0.4</td>
<td>19.1 ± 4.9</td>
</tr>
<tr>
<td></td>
<td>EL</td>
<td>46.8 ± 0.6</td>
<td>28.5 ± 6.4</td>
<td>2.6 ± 0.9</td>
<td>15.8 ± 7.4</td>
</tr>
<tr>
<td></td>
<td>AH</td>
<td>47.2 ± 0.7</td>
<td>33.6 ± 3.1</td>
<td>2.3 ± 0.6</td>
<td>11.3 ± 3.5</td>
</tr>
<tr>
<td></td>
<td>EH</td>
<td>47.9 ± 0.9</td>
<td>32.5 ± 5.5</td>
<td>2.1 ± 0.8</td>
<td>13.3 ± 5.6</td>
</tr>
<tr>
<td>Sep. 2003</td>
<td>AL</td>
<td>45.3 ± 0.8</td>
<td>30.9 ± 2.6</td>
<td>2.8 ± 0.7</td>
<td>11.5 ± 3.0</td>
</tr>
<tr>
<td></td>
<td>EL</td>
<td>44.3 ± 1.1</td>
<td>29.6 ± 1.7</td>
<td>2.4 ± 0.7</td>
<td>12.3 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>AH</td>
<td>44.2 ± 0.6</td>
<td>32.6 ± 1.3</td>
<td>2.4 ± 0.7</td>
<td>9.2 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>EH</td>
<td>45.9 ± 0.9</td>
<td>30.8 ± 2.6</td>
<td>2.4 ± 0.4</td>
<td>12.7 ± 2.8</td>
</tr>
<tr>
<td>Sep. 2002</td>
<td>CO₂</td>
<td>0.1368</td>
<td>0.6374</td>
<td>0.6515</td>
<td>0.7663</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>0.0122</td>
<td>0.0075</td>
<td>0.5910</td>
<td>0.0332</td>
</tr>
<tr>
<td></td>
<td>CO₂ x N</td>
<td>0.2692</td>
<td>0.3299</td>
<td>0.3043</td>
<td>0.2537</td>
</tr>
<tr>
<td>Sep. 2003</td>
<td>CO₂</td>
<td>0.2730</td>
<td>0.0897</td>
<td>0.5273</td>
<td>0.0423</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>0.4469</td>
<td>0.1087</td>
<td>0.4202</td>
<td>0.3544</td>
</tr>
<tr>
<td></td>
<td>CO₂ x N</td>
<td>0.0011</td>
<td>0.8281</td>
<td>0.5084</td>
<td>0.1733</td>
</tr>
</tbody>
</table>
Fig. 2 Non-structural carbohydrate concentrations in bark of P. nigra grown under either FACE (E) or ambient (A) [CO₂] 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 CO₂, nitrogen (N), and season and their interactions are shown.

<table>
<thead>
<tr>
<th>Season</th>
<th>Source</th>
<th>glucose</th>
<th>fructose</th>
<th>sucrose</th>
<th>starch</th>
<th>TNC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Main effects</td>
<td>CO₂</td>
<td>0.2424</td>
<td>0.0059</td>
<td>0.5961</td>
<td>0.1593</td>
<td>0.0897</td>
</tr>
<tr>
<td>Sep. 2002 – Mar. 2003</td>
<td>N</td>
<td>0.0071</td>
<td>0.1572</td>
<td>0.0399</td>
<td>0.3082</td>
<td>0.2811</td>
</tr>
<tr>
<td></td>
<td>Season</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.0117</td>
</tr>
<tr>
<td>Interactions</td>
<td>CO₂ x N</td>
<td>0.3995</td>
<td>0.0848</td>
<td>0.3031</td>
<td>0.5289</td>
<td>0.6704</td>
</tr>
<tr>
<td>Sep. 2002 – Mar. 2003</td>
<td>CO₂ x Season</td>
<td>0.8342</td>
<td>0.3057</td>
<td>0.2488</td>
<td>0.9093</td>
<td>0.8104</td>
</tr>
<tr>
<td></td>
<td>N x Season</td>
<td>0.0003</td>
<td>0.0000</td>
<td>0.0236</td>
<td>0.0031</td>
<td>0.0001</td>
</tr>
<tr>
<td>Main effects</td>
<td>CO₂</td>
<td>0.0907</td>
<td>0.3908</td>
<td>0.4537</td>
<td>0.1976</td>
<td>0.1057</td>
</tr>
<tr>
<td>Sep. 2003 – Mar. 2004</td>
<td>N</td>
<td>0.8280</td>
<td>0.7615</td>
<td>0.3317</td>
<td>0.0669</td>
<td>0.1553</td>
</tr>
<tr>
<td></td>
<td>Season</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.6620</td>
<td>0.0343</td>
<td>0.0000</td>
</tr>
<tr>
<td>Interactions</td>
<td>CO₂ x N</td>
<td>0.7347</td>
<td>0.9038</td>
<td>0.8663</td>
<td>0.7036</td>
<td>0.6821</td>
</tr>
<tr>
<td>Sep. 2003 – Mar. 2004</td>
<td>CO₂ x Season</td>
<td>0.1161</td>
<td>0.2018</td>
<td>0.0959</td>
<td>0.0032</td>
<td>0.0038</td>
</tr>
<tr>
<td></td>
<td>N x Season</td>
<td>0.6194</td>
<td>0.5644</td>
<td>0.0731</td>
<td>0.9227</td>
<td>0.7316</td>
</tr>
</tbody>
</table>
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 CO₂ 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 CO₂ 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) [CO₂] 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.](image-url)
**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 CO₂, nitrogen (N), and season and their interactions are shown.

<table>
<thead>
<tr>
<th>Source</th>
<th>glucose</th>
<th>fructose</th>
<th>sucrose</th>
<th>starch</th>
<th>TNC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Main effects</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sep. 2002 – Mar. 2003</td>
<td>CO₂</td>
<td>0.8176</td>
<td>0.9861</td>
<td>0.2541</td>
<td>0.0079</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>0.0769</td>
<td>0.0018</td>
<td>0.0002</td>
<td>0.5224</td>
</tr>
<tr>
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<td>Season</td>
<td>0.0000</td>
<td>0.0009</td>
<td>0.0000</td>
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</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sep. 2002 – Mar. 2003</td>
<td>CO₂ x N</td>
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<td>CO₂ x Season</td>
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<td>N x Season</td>
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<td>0.5666</td>
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<td>0.8278</td>
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<tr>
<td>Sep. 2003 – Mar. 2004</td>
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<td>0.0351</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sep. 2003 – Mar. 2004</td>
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<td>0.3644</td>
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</tr>
<tr>
<td></td>
<td>CO₂ x Season</td>
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<td>0.5202</td>
<td>0.3303</td>
<td>0.1212</td>
</tr>
<tr>
<td></td>
<td>N x Season</td>
<td>0.8846</td>
<td>0.4952</td>
<td>0.0686</td>
<td>0.8225</td>
</tr>
</tbody>
</table>

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 CO₂ became dominant resulting in higher C concentrations under FACE compared with ambient [CO₂] (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.

<table>
<thead>
<tr>
<th>Season</th>
<th>Treatment</th>
<th>Total C %</th>
<th>Wall-bound C %</th>
<th>TNC-bound C %</th>
<th>Residual C %</th>
<th>Relative Partitioning</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sep. 2002</td>
<td>AL</td>
<td>48.3 ± 0.3</td>
<td>42.8 ± 2.0</td>
<td>0.44 ± 0.10</td>
<td>5.1 ± 1.9</td>
<td>0.132 ± 0.051</td>
</tr>
<tr>
<td></td>
<td>EL</td>
<td>48.1 ± 0.3</td>
<td>43.4 ± 1.6</td>
<td>0.68 ± 0.13</td>
<td>4.0 ± 1.3</td>
<td>0.109 ± 0.035</td>
</tr>
<tr>
<td></td>
<td>AH</td>
<td>47.8 ± 1.3</td>
<td>43.6 ± 1.0</td>
<td>0.73 ± 0.18</td>
<td>3.5 ± 1.1</td>
<td>0.097 ± 0.027</td>
</tr>
<tr>
<td></td>
<td>EH</td>
<td>47.9 ± 0.7</td>
<td>42.4 ± 2.1</td>
<td>0.60 ± 0.16</td>
<td>4.9 ± 1.9</td>
<td>0.131 ± 0.052</td>
</tr>
<tr>
<td>Mar. 2003</td>
<td>AL</td>
<td>47.6 ± 0.3</td>
<td>42.7 ± 2.4</td>
<td>0.71 ± 0.17</td>
<td>4.2 ± 2.3</td>
<td>0.117 ± 0.059</td>
</tr>
<tr>
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<td>EL</td>
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<td>41.5 ± 0.7</td>
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<td>0.152 ± 0.025</td>
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<tr>
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<td>AH</td>
<td>46.9 ± 0.7</td>
<td>41.4 ± 1.5</td>
<td>0.86 ± 0.22</td>
<td>4.7 ± 1.4</td>
<td>0.136 ± 0.042</td>
</tr>
<tr>
<td></td>
<td>EH</td>
<td>47.4 ± 0.3</td>
<td>42.2 ± 1.1</td>
<td>0.58 ± 0.21</td>
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<td>0.124 ± 0.032</td>
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<td>Sep. 2003</td>
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<td>0.138 ± 0.012</td>
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<td>EH</td>
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<td>4.4 ± 1.1</td>
<td>0.127 ± 0.019</td>
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<td>AH</td>
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<table>
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<th>Season</th>
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<th>N</th>
<th>CO$_2$ x N</th>
<th>CO$_2$ x Season</th>
<th>N x Season</th>
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<td>0.6316</td>
<td>0.9737</td>
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</table>
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 [CO₂] (-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) [CO₂] 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 CO₂, nitrogen (N), season and their interactions are indicated.

<table>
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<th>Season</th>
<th>Source</th>
<th>Total C</th>
<th>Wall-bound C</th>
<th>TNC-C</th>
<th>Residual C</th>
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<td>CO₂ x Season</td>
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<td>N x Season</td>
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<td>CO₂ x Season</td>
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</tr>
<tr>
<td>N x Season</td>
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<td>0.3634</td>
<td>0.8156</td>
<td>0.5221</td>
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</tbody>
</table>
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) [CO₂] 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, CO₂ 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 CO₂ 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 CO₂, 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 CO₂ emissions (6.3 Pg C year⁻¹, Houghton et al. 2001) and constitute an important contribution to the goals of the Kyoto protocol (1997).

Acknowledgements

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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


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

Zhi-Bin Luo, Carlo Calfapietra, Andrea Polle (2005) Effects of Free Air CO₂ 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 [CO₂] (about 370 µmol mol⁻¹ CO₂) and elevated [CO₂] (about 550 µmol mol⁻¹ CO₂) concentrations using Free-Air CO₂ 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 [CO₂], 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.

Keywords Lignin, Phenolics, Tannins, Soluble proteins, Internal N-pools, Populus
4.1 Introduction

Human activities have perturbed the global environment, which is currently characterised by increasing concentrations of the atmospheric carbon dioxide ([CO₂]) 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 CO₂ and N deposition have been major goals in plant sciences and ecology in recent decades. Much is known about plant responses to both elevated [CO₂] and N-fertilization. For instance, elevated atmospheric [CO₂] 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 [CO₂] (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 CO₂ 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 [CO₂] 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 [CO₂] 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 CO₂ 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 [CO₂] 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 [CO₂] conditions (370 μmol mol⁻¹ CO₂). Pure CO₂ (Messer Griesheim) was released through laser-drilled holes in polyethylene tubes to reach 550 μmol mol⁻¹ of the target [CO₂] inside
the FACE plots. A meteorological station was applied to control the release of CO₂ at each FACE plot. The FACE system was monitored by a computer to reach the target [CO₂]. 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 [CO₂] 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 cm² 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 °C; Sonorex Super RK 510H, Bandelin electronics, Berlin, Germany) after Pritchard et al. (1997). The mixture was centrifuged (4500 rpm, 10 min, 4 °C). 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% Na₂CO₃ 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, Büchel, Switzerland) at 45 °C and dissolved in 2 × 500 µL 50% methanol. Subsequently, the solution was centrifuged twice (10 min, 15000g, 4°C). 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 °C) with 2 mL n-hexane, dried at 60 °C 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 °C) and centrifuged (10 min, 4500 rpm, 4 °C), 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 °C (Rotavapor EL 130, Büchl, Switzerland), and dissolved in 2 × 500 µL 50% methanol. The extract was centrifuged twice (10 min, 15000 g, 4 °C) 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% \(\text{FeNH}_4(\text{SO}_4)_2\cdot12\text{H}_2\text{O}\) in 2 M HCl (\(\text{FeNH}_4(\text{SO}_4)_2\cdot12\text{H}_2\text{O}/\text{HCl}=\text{w/v}\)). 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, Büchel, 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, Büchel, 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, Pharmacia, 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, Büchel, 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% H₂SO₄ and incubated for 60 min at room temperature. Subsequently, the mixture was suspended in 200 mL distilled H₂O, and mixed well. The mixture was autoclaved (1 h, 121 °C). 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 °C) 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 °C) 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 x 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 CO₂ treatment (CO₂), 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 CO₂, 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 pathogen 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, chlorogenic 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) [CO₂] and either fertilised (H) or unfertilised (L) conditions and harvested in different seasons. Significance levels (*P*-values of the ANOVAs) of CO₂, nitrogen (N), season and their interactions are indicated.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Lignin</th>
<th>Wall-bound phenolics</th>
<th>Soluble phenolics</th>
<th>CT in soluble part</th>
<th>Sum</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO₂</td>
<td>0.9114</td>
<td>0.2319</td>
<td>0.0449</td>
<td>0.3430</td>
<td>0.4149</td>
</tr>
<tr>
<td>N</td>
<td>0.5631</td>
<td>0.0004</td>
<td>0.5980</td>
<td>0.0013</td>
<td>0.0374</td>
</tr>
<tr>
<td>Season</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.0028</td>
<td>0.0005</td>
<td>0.0000</td>
</tr>
<tr>
<td>CO₂ x N</td>
<td>0.7832</td>
<td>0.3290</td>
<td>0.0153</td>
<td>0.0467</td>
<td>0.6727</td>
</tr>
<tr>
<td>CO₂ x Season</td>
<td>0.0403</td>
<td>0.0899</td>
<td>0.5325</td>
<td>0.4851</td>
<td>0.0112</td>
</tr>
<tr>
<td>N x Season</td>
<td>0.4991</td>
<td>0.1077</td>
<td>0.9517</td>
<td>0.0278</td>
<td>0.6210</td>
</tr>
<tr>
<td>CO₂ x N x Season</td>
<td>0.6462</td>
<td>0.1107</td>
<td>0.1758</td>
<td>0.2860</td>
<td>0.7109</td>
</tr>
</tbody>
</table>

Fig. 1 Carbon based secondary compounds in wood of *P. nigra* grown under either FACE (E) or ambient (A) [CO₂] 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) [CO₂] 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) [CO₂] 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) [CO₂] 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 CO₂, nitrogen (N) and their interactions are shown.

<table>
<thead>
<tr>
<th>Seasons</th>
<th>Treatments</th>
<th>Proteins (mg g⁻¹ DW)</th>
<th>Lignin-bound N %</th>
<th>Total N %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sep. 2002</td>
<td>AL</td>
<td>89.1 ± 10.2</td>
<td>no data</td>
<td>2.96 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>EL</td>
<td>75.4 ± 5.9</td>
<td>no data</td>
<td>2.45 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>AH</td>
<td>95.9 ± 8.2</td>
<td>no data</td>
<td>2.97 ± 0.24</td>
</tr>
<tr>
<td></td>
<td>EH</td>
<td>91.8 ± 7.4</td>
<td>no data</td>
<td>3.36 ± 0.15</td>
</tr>
<tr>
<td>Sep. 2003</td>
<td>AL</td>
<td>109.5 ± 7.4</td>
<td>0.83 ± 0.05</td>
<td>2.36 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>EL</td>
<td>101.7 ± 7.2</td>
<td>0.85 ± 0.04</td>
<td>2.00 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>AH</td>
<td>139.3 ± 5.5</td>
<td>0.91 ± 0.04</td>
<td>2.36 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>EH</td>
<td>118.2 ± 4.9</td>
<td>0.84 ± 0.03</td>
<td>2.37 ± 0.07</td>
</tr>
<tr>
<td>Sep. 2002</td>
<td>CO₂</td>
<td>0.2821</td>
<td>no data</td>
<td>0.7202</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>0.1672</td>
<td>no data</td>
<td>0.0129</td>
</tr>
<tr>
<td></td>
<td>CO₂ x N</td>
<td>0.5565</td>
<td>no data</td>
<td>0.0157</td>
</tr>
<tr>
<td>Sep. 2003</td>
<td>CO₂</td>
<td>0.0334</td>
<td>0.5000</td>
<td>0.0112</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>0.0016</td>
<td>0.4529</td>
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</tr>
<tr>
<td></td>
<td>CO₂ x N</td>
<td>0.3076</td>
<td>0.3288</td>
<td>0.0070</td>
</tr>
</tbody>
</table>

**Fig. 4** Soluble proteins in bark of *P. nigra* grown under either FACE (E) or ambient (A) [CO₂] 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 [CO₂] conditions were frequently observed to have lower protein and N concentrations in comparison with those grown under ambient [CO₂] (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) [CO₂] and either fertilised (H) or unfertilised (L) conditions and harvested in different seasons. Significance levels (P-values of the ANOVAs) of CO₂, nitrogen (N), season and their interactions are indicated.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Soluble proteins</th>
<th>Lignin bound N</th>
<th>Total N</th>
</tr>
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<tbody>
<tr>
<td>CO₂</td>
<td>0.0255</td>
<td>0.0052</td>
<td>0.0005</td>
</tr>
<tr>
<td>N</td>
<td>0.0042</td>
<td>0.0001</td>
<td>0.1962</td>
</tr>
<tr>
<td>Season</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.0000</td>
</tr>
<tr>
<td>CO₂ x N</td>
<td>0.1826</td>
<td>0.0201</td>
<td>0.2042</td>
</tr>
<tr>
<td>CO₂ x Season</td>
<td>0.3766</td>
<td>0.0282</td>
<td>0.0130</td>
</tr>
<tr>
<td>N x Season</td>
<td>0.1344</td>
<td>0.0964</td>
<td>0.9297</td>
</tr>
<tr>
<td>CO₂ x N x Season</td>
<td>0.6872</td>
<td>0.1265</td>
<td>0.9277</td>
</tr>
</tbody>
</table>
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) [CO₂] 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 CO₂, nitrogen (N) and their interactions are indicated.

<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td>CO₂</td>
<td>0.2980</td>
<td>0.9936</td>
<td>0.2669</td>
</tr>
<tr>
<td>N</td>
<td>0.3269</td>
<td>0.4708</td>
<td>0.1780</td>
</tr>
<tr>
<td>CO₂ x N</td>
<td>0.0086</td>
<td>0.8913</td>
<td>0.0055</td>
</tr>
</tbody>
</table>

**Fig. 6** Relative N-partitioning in leaf and wood of *P. nigra* grown under either FACE (E) or ambient (A) [CO₂] 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.

![Graph showing correlations](image)

**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) [CO₂] 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 [CO₂] 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 tobacco 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 \textit{P. nigra} (Table 1, Fig. 1). It is not surprising 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 \textit{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 \textit{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 \textit{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 [CO₂] 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 [CO₂] 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 [CO₂]. 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 NO₃⁻
or NH₄⁺ 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 [CO₂] 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 [CO₂], 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 [CO₂] and N-deposition in the future environmental scenarios.

**Acknowledgements**

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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 CO₂ Enrichment (FACE) and N-fertilization. (Manuscript)
Abstract

To study wood properties and the underlying molecular mechanisms of their variations, *Populus × euramerica*na 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. × euramerica*na 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.

**Keywords** Calorific value, FT-IR, Gene expression, Global change, Lignin, Wood properties
5.1 Introduction

Atmospheric CO₂ concentrations ([CO₂]) have been steadily rising since the beginning of industrialization (IPCC, 2001). Increases in [CO₂] enhance photosynthesis and growth of plants, including forest trees, but these physiological responses to elevated [CO₂] 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 [CO₂] 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 [CO₂] 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 [CO₂] 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 [CO₂] on wood quality and structure have been reported. Cell wall thickness of tracheids increased in response to elevated [CO₂] 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 [CO₂] (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 [CO₂], whereas no changes were found in radiata pine (Atwell et al., 2003). Few studies addressed the responses of wood chemical composition to elevated [CO₂]. Under higher nutrient supply, lignin concentrations in beech decreased in response to elevated [CO₂] (Blaschke et al., 2002), but no significant effects of elevated [CO₂] 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 [CO₂] 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 [CO₂] and/or N-fertilization. To investigate the influence of environmental conditions (elevated [CO₂] 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 [CO₂]. 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 [CO₂] 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 [CO₂] conditions. Pure CO₂ (Messer Griesheim) was released through laser-drilled holes in the polyethylene rings to reach 550 µmol mol⁻¹ of the target [CO₂] inside the FACE plots. A meteorological station was applied to control the release of CO₂ at each FACE plot. The FACE system was monitored by a computer to reach the target [CO₂] (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 [CO₂] 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 [CO₂] 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 3 cm 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:

\[
\text{Arithmetic length (Ln)} = \frac{\sum n_i L_i}{\sum n_i}; \\
\text{Length weight length (Lw)} = \frac{\sum n_i L_i^2}{\sum n_i L_i}; \\
\text{Weight weighted length (Lww)} = \frac{\sum n_i L_i^3}{\sum n_i L_i^2};
\]

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 °C). 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 °C) and shaken every 30 min. Afterwards, the mixture was centrifuged (4500 g, 10 min, 4 °C). 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% H₂SO₄ and incubated for 60 min at room temperature. Subsequently, the mixture was suspended in 200 ml distilled H₂O, and mixed well. The mixture was autoclaved (1 h, 121 °C). 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 °C) and stored in a desiccator. The filter containing the lignin was weighed (W₃) and Klason lignin was calculated as \((W₃ - W₂) \times \frac{100}{W₁}\).

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, Ettingen, 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 = a1*ΣVn, with Vn = volume per shoot and n = number of shoots per stool. The volume was calculated with V = 1/3 *π* r²*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 hₙ = a₂ * lg dₙ. Each relationship was established with n = 24 harvested shoots.

<table>
<thead>
<tr>
<th>Season</th>
<th>a₁</th>
<th>r²</th>
<th>P</th>
<th>a₂</th>
<th>r²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sep. 2003</td>
<td>451527</td>
<td>0.9012</td>
<td>&lt; 0.0001</td>
<td>0.5027</td>
<td>0.5682</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Mar. 2004</td>
<td>778523</td>
<td>0.9077</td>
<td>&lt; 0.0001</td>
<td>0.4541</td>
<td>0.6860</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

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% β-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 KH₂PO₄/K₂HPO₄ 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 K₂HPO₄/KH₂PO₄ 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™3, Catalog no. PA23001) and cyanine 5 (Cy™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™3/Cy™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 discarded. 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\textsuperscript{TM3} two times higher than the background and of Cy\textsuperscript{TM5} 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\textsubscript{2}] were hybridized. Four to six randomly chosen biological replicates for each nitrogen and [CO\textsubscript{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\textsubscript{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× SSC, 0.1% SDS, 0.1 mg/ml BSA) for 1—2 hrs at 42 °C before incubating twice in 0.1× 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× SSC, 0.5% SDS) at 42 °C for 15 min, and washing twice in buffer B (0.5× SSC, 0.5% SDS) at 42 °C for 15 min. Afterwards, the slides were washed twice in buffer C (0.1× 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™3 and Cy™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.

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 $P. \times$ euramerica, 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 $P. \times$ euramerica 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.

<table>
<thead>
<tr>
<th>Harvest Season</th>
<th>Treatments</th>
<th>XW (mm)</th>
<th>VLF (%)</th>
<th>RF (%)</th>
<th>FLF (%)</th>
<th>CWF (%)</th>
<th>VF (number mm$^{-2}$)</th>
<th>TFW (µm)</th>
<th>VLD (µm)</th>
<th>FLD (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sep 02 *</td>
<td>AL</td>
<td>5.8 ± 1.2</td>
<td>17.3 ± 3.2</td>
<td>6.1 ± 1.1</td>
<td>39.6 ± 4.2</td>
<td>37.1 ± 2.7</td>
<td>57.9 ± 5.8</td>
<td>2.8 ± 0.4</td>
<td>61.1 ± 6.2</td>
<td>19.6 ± 2.9</td>
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<tr>
<td></td>
<td>EL</td>
<td>6.3 ± 1.1</td>
<td>18.1 ± 2.0</td>
<td>6.2 ± 1.4</td>
<td>41.6 ± 2.8</td>
<td>34.1 ± 1.9</td>
<td>61.3 ± 6.1</td>
<td>2.7 ± 0.5</td>
<td>68.1 ± 7.2</td>
<td>19.6 ± 2.3</td>
</tr>
<tr>
<td></td>
<td>AH</td>
<td>7.5 ± 0.9</td>
<td>16.6 ± 2.3</td>
<td>6.5 ± 1.3</td>
<td>44.6 ± 2.9</td>
<td>32.3 ± 1.9</td>
<td>56.5 ± 5.4</td>
<td>2.1 ± 0.3</td>
<td>67.1 ± 6.5</td>
<td>19.2 ± 2.5</td>
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<tr>
<td></td>
<td>EH</td>
<td>7.4 ± 1.3</td>
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<td>43.7 ± 3.7</td>
<td>32.5 ± 2.8</td>
<td>56.8 ± 3.9</td>
<td>1.8 ± 0.3</td>
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<td>AL</td>
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<td>18.1 ± 2.1</td>
<td>6.2 ± 1.4</td>
<td>40.5 ± 2.1</td>
<td>35.2 ± 2.2</td>
<td>51.5 ± 11.4</td>
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</tr>
<tr>
<td></td>
<td>EL</td>
<td>5.3 ± 1.8</td>
<td>17.5 ± 2.3</td>
<td>4.9 ± 1.5</td>
<td>43.9 ± 2.2</td>
<td>33.6 ± 2.1</td>
<td>61.3 ± 10.9</td>
<td>2.6 ± 0.5</td>
<td>58.4 ± 13.6</td>
<td>17.4 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>AH</td>
<td>2.8 ± 2.6</td>
<td>18.8 ± 1.5</td>
<td>6.7 ± 2.4</td>
<td>37.5 ± 3.5</td>
<td>36.9 ± 3.2</td>
<td>57.1 ± 10.4</td>
<td>3.4 ± 0.5</td>
<td>63.3 ± 11.4</td>
<td>16.9 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>EH</td>
<td>5.7 ± 2.6</td>
<td>16.5 ± 2.9</td>
<td>5.5 ± 1.2</td>
<td>45.6 ± 4.2</td>
<td>32.5 ± 2.7</td>
<td>55.2 ± 11.8</td>
<td>2.7 ± 0.5</td>
<td>58.6 ± 14.6</td>
<td>17.0 ± 1.2</td>
</tr>
</tbody>
</table>

* Data are modified from Luo et al. (2005b).
Table 3  Fibre lengths of stem-wood in *P. × euramerica*na grown under either FACE (E) or ambient (A) [CO₂] 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 CO₂, 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.

<table>
<thead>
<tr>
<th>Year</th>
<th>Treatments</th>
<th>Ln (µm)</th>
<th>Lw (µm)</th>
<th>Lww (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2002</td>
<td>AL</td>
<td>437.8 ± 35.5</td>
<td>503.1 ± 39.0</td>
<td>560.4 ± 41.4</td>
</tr>
<tr>
<td></td>
<td>EL</td>
<td>444.5 ± 44.2</td>
<td>504.8 ± 55.1</td>
<td>557.6 ± 65.3</td>
</tr>
<tr>
<td></td>
<td>AH</td>
<td>417.8 ± 41.5</td>
<td>473.9 ± 45.6</td>
<td>522.1 ± 52.9</td>
</tr>
<tr>
<td></td>
<td>EH</td>
<td>428.7 ± 33.0</td>
<td>481.7 ± 37.6</td>
<td>524.1 ± 43.1</td>
</tr>
<tr>
<td>2003</td>
<td>AL</td>
<td>517.6 ± 39.5</td>
<td>591.7 ± 46.2</td>
<td>654.3 ± 58.6</td>
</tr>
<tr>
<td></td>
<td>EL</td>
<td>498.2 ± 75.3</td>
<td>580.5 ± 88.1</td>
<td>659.1 ± 94.4</td>
</tr>
<tr>
<td></td>
<td>AH</td>
<td>453.5 ± 63.7</td>
<td>514.5 ± 71.7</td>
<td>563.4 ± 78.4</td>
</tr>
<tr>
<td></td>
<td>EH</td>
<td>509.2 ± 79.8</td>
<td>598.8 ± 105.3</td>
<td>684.4 ± 132.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Year</th>
<th>Treatments</th>
<th>Ln (µm)</th>
<th>Lw (µm)</th>
<th>Lww (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2002</td>
<td>CO₂</td>
<td>0.5846</td>
<td>0.7988</td>
<td>0.9859</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>0.2733</td>
<td>0.1689</td>
<td>0.1034</td>
</tr>
<tr>
<td></td>
<td>CO₂ x N</td>
<td>0.8969</td>
<td>0.8693</td>
<td>0.9105</td>
</tr>
<tr>
<td>2003</td>
<td>CO₂</td>
<td>0.5113</td>
<td>0.2822</td>
<td>0.1201</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>0.3403</td>
<td>0.3830</td>
<td>0.4077</td>
</tr>
<tr>
<td></td>
<td>CO₂ x N</td>
<td>0.1817</td>
<td>0.1632</td>
<td>0.1489</td>
</tr>
</tbody>
</table>

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. × euramerica*na (Table 4). FACE slightly but significantly enhanced lignin concentrations in comparison with ambient [CO₂] (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) [CO₂] 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 CO₂, nitrogen (N) and their interactions are shown.

<table>
<thead>
<tr>
<th>Harvest season</th>
<th>Treatments</th>
<th>C%</th>
<th>N%</th>
<th>Lignin%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sep 02</td>
<td>AL</td>
<td>47.9 ± 0.3</td>
<td>0.233 ± 0.023</td>
<td>18.3 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>EL</td>
<td>47.7 ± 0.5</td>
<td>0.230 ± 0.047</td>
<td>18.6 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>AH</td>
<td>47.8 ± 0.4</td>
<td>0.277 ± 0.105</td>
<td>17.9 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>EH</td>
<td>47.6 ± 0.6</td>
<td>0.323 ± 0.043</td>
<td>19.9 ± 0.7</td>
</tr>
<tr>
<td>Sep 03</td>
<td>AL</td>
<td>47.9 ± 0.9</td>
<td>0.233 ± 0.065</td>
<td>20.0 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>EL</td>
<td>48.2 ± 0.7</td>
<td>0.175 ± 0.045</td>
<td>20.3 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>AH</td>
<td>46.8 ± 0.8</td>
<td>0.268 ± 0.073</td>
<td>19.9 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>EH</td>
<td>47.7 ± 0.7</td>
<td>0.232 ± 0.099</td>
<td>21.9 ± 0.7</td>
</tr>
<tr>
<td>Sep 02</td>
<td>CO₂</td>
<td>0.2442</td>
<td>0.4061</td>
<td>0.0051</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>0.5626</td>
<td>0.0145</td>
<td>0.1932</td>
</tr>
<tr>
<td></td>
<td>CO₂ × N</td>
<td>0.7868</td>
<td>0.3391</td>
<td>0.0283</td>
</tr>
<tr>
<td>Sep 03</td>
<td>CO₂</td>
<td>0.0805</td>
<td>0.1274</td>
<td>0.0000</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>0.0201</td>
<td>0.1406</td>
<td>0.0014</td>
</tr>
<tr>
<td></td>
<td>CO₂ × N</td>
<td>0.3608</td>
<td>0.7206</td>
<td>0.0009</td>
</tr>
</tbody>
</table>

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 C₁-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.
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) [CO₂] 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 CO₂ and low nitrogen (AL); ambient CO₂ 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 \( P. \times euramerica \) 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 \( P. \times euramerica \) 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.

<table>
<thead>
<tr>
<th>Season</th>
<th>Treatments</th>
<th>Calorific value (J g(^{-1}))</th>
<th>Energy per stool (MJ m(^{-2}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sep. 2003</td>
<td>AL</td>
<td>19281 ± 126</td>
<td>73.7 ± 13.8</td>
</tr>
<tr>
<td></td>
<td>EL</td>
<td>19388 ± 97</td>
<td>59.9 ± 10.1</td>
</tr>
<tr>
<td></td>
<td>AH</td>
<td>19306 ± 146</td>
<td>90.4 ± 20.4</td>
</tr>
<tr>
<td></td>
<td>EH</td>
<td>19344 ± 117</td>
<td>100.9 ± 20.2</td>
</tr>
<tr>
<td>Mar. 2004</td>
<td>AL</td>
<td>19121 ± 40</td>
<td>92.3 ± 10.7</td>
</tr>
<tr>
<td></td>
<td>EL</td>
<td>19148 ± 101</td>
<td>70.1 ± 24.8</td>
</tr>
<tr>
<td></td>
<td>AH</td>
<td>19115 ± 121</td>
<td>106.7 ± 20.8</td>
</tr>
<tr>
<td></td>
<td>EH</td>
<td>19283 ± 52</td>
<td>111.3 ± 13.5</td>
</tr>
<tr>
<td></td>
<td>CO(_2)</td>
<td>0.3369</td>
<td>0.8682</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>0.8960</td>
<td>0.0173</td>
</tr>
<tr>
<td></td>
<td>CO(_2) x N</td>
<td>0.6348</td>
<td>0.2424</td>
</tr>
<tr>
<td></td>
<td>CO(_2)</td>
<td>0.0860</td>
<td>0.4277</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>0.2303</td>
<td>0.0304</td>
</tr>
<tr>
<td></td>
<td>CO(_2) x N</td>
<td>0.1907</td>
<td>0.2409</td>
</tr>
</tbody>
</table>
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 [CO₂] 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-methyltransferase 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 [CO₂] (Table 6, EH-AH). Interestingly, this transcript was up-regulated by N-fertilization under ambient [CO₂] 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.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Identifier</th>
<th>Annotation</th>
<th>M</th>
<th>P</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>AH-AL</td>
<td>MN5188443</td>
<td>Ripening regulated protein DDTFR10</td>
<td>2.02</td>
<td>0.055</td>
<td>1.14</td>
</tr>
<tr>
<td>EL-AL</td>
<td>MN5184294</td>
<td>No annotation available</td>
<td>-0.69</td>
<td>0.080</td>
<td>2.56</td>
</tr>
<tr>
<td>EH-AH</td>
<td>MN5188443</td>
<td>Ripening regulated protein DDTFR10</td>
<td>-2.02</td>
<td>0.020</td>
<td>1.16</td>
</tr>
<tr>
<td>EH-EL</td>
<td>MN5183717</td>
<td>Isoflavone reductase HOMOLOGUE (PILH gene)</td>
<td>0.99</td>
<td>0.001</td>
<td>5.46</td>
</tr>
<tr>
<td></td>
<td>MN5189945</td>
<td>Putative senescence-associated protein (Fragment)</td>
<td>2.24</td>
<td>0.002</td>
<td>4.38</td>
</tr>
<tr>
<td></td>
<td>MN5183875</td>
<td>Unknown protein</td>
<td>1.61</td>
<td>0.002</td>
<td>3.84</td>
</tr>
<tr>
<td></td>
<td>MN5183149</td>
<td>Heat shock 70 kDa protein, mitochondrial precursor</td>
<td>1.59</td>
<td>0.003</td>
<td>3.48</td>
</tr>
<tr>
<td></td>
<td>MN5189108</td>
<td>Hypothetical protein</td>
<td>2.09</td>
<td>0.003</td>
<td>3.72</td>
</tr>
<tr>
<td></td>
<td>MN5184204</td>
<td>Ferulate-5-hydroxylase</td>
<td>0.86</td>
<td>0.007</td>
<td>2.86</td>
</tr>
<tr>
<td></td>
<td>MN5189892</td>
<td>No annotation available</td>
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<td>0.014</td>
<td>2.06</td>
</tr>
<tr>
<td></td>
<td>MN5188098</td>
<td>Tubulin alpha-2/alpha-4 chain</td>
<td>0.80</td>
<td>0.024</td>
<td>1.50</td>
</tr>
<tr>
<td></td>
<td>MN5183865</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase, cytosolic (EC 1.2.1.12)</td>
<td>0.63</td>
<td>0.024</td>
<td>1.49</td>
</tr>
<tr>
<td></td>
<td>MN5184441</td>
<td>Plasma membrane intrinsic protein</td>
<td>0.65</td>
<td>0.033</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>MN5190264</td>
<td>Hypothetical protein</td>
<td>1.03</td>
<td>0.033</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td>MN5183608</td>
<td>Caffeic acid 3-O-methyltransferase 1 COMT 1 (EC 2.1.1.68)</td>
<td>0.74</td>
<td>0.037</td>
<td>0.68</td>
</tr>
<tr>
<td></td>
<td>MN5184294</td>
<td>No annotation available</td>
<td>0.60</td>
<td>0.037</td>
<td>0.64</td>
</tr>
<tr>
<td></td>
<td>MN5183126</td>
<td>AT5g17920/MPI7_60</td>
<td>0.69</td>
<td>0.037</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td>MN5185095</td>
<td>Hypothetical protein</td>
<td>0.74</td>
<td>0.049</td>
<td>0.29</td>
</tr>
</tbody>
</table>
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 [CO₂] 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. × eurameriana* 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. × eurameriana* 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 [CO₂] 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 monoxygenase 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. × euramerica* 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


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7. Curriculum Vitae

Zhibin Luo

Personal data

Born: November 22, 1973 in Hunan, China
Nationality: Chinese
Marital Status: Married, No children

Education

09/1981 – 07/1986 Primary school, Xiaodong, Xiangxiang county, Hunan, China
09/1986 – 07/1989 Secondary school, Dasha, Xiangxiang county, Hunan, China
09/1989 – 07/1993 High school, Dongshan, Xiangxiang county, Hunan, China

09/1993 – 07/1997 Central-south Forestry University, Zhuzhou, China (earned B.S. in Forestry)
09/2000 – 07/2002 Southwest Forestry University, Kunming, China (earned M.S. in Plant Ecophysiology)
Thesis: Application of Hydro-gel to Afforestation in Dry-hot Valleys in Southwest China
Advisor: Prof. Dr. Huancheng Ma

Thesis: Wood Quality, Carbon and Nitrogen Partitioning, and Gene Expression Profiling in Populus Exposed to Free Air CO2 Enrichment (FACE) and N-fertilization
Supervisor: Prof. Dr. Andrea Polle

Experiences

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

07/2002 – 10/2002 Chinese Forestry Publishing House, Beijing, China (gained knowledge and skills on publication of environment sciences)

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

03/2005 – 05/2005 Functional Genomics Lab, Forest Biology Research Center, Laval University, Quebec, Canada (gained knowledge and skills on cDNA microarray)
Publications

Original papers


Posters


Oral Presentation

Effects of Free Air CO₂ Enrichment (FACE) and N-fertilization on carbon-based secondary metabolites of *Populus nigra*. Botany Conference, 5-10 Sep., 2004, Braunschweig, Germany.