

RESEARCH PAPER

Elevated CO₂ and/or ozone modify lignification in the wood of poplars (*Populus tremula* × *alba*)

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Abstract

Trees will have to cope with increasing levels of CO₂ and ozone in the atmosphere. The purpose of this work was to assess whether the lignification process could be altered in the wood of poplars under elevated CO₂ and/or ozone. Young poplars were exposed either to charcoal-filtered air (control), to elevated CO₂ (800 µl l⁻¹), to ozone (200 nl l⁻¹) or to a combination of elevated CO₂ and ozone in controlled chambers. Lignification was analysed at different levels: biosynthesis pathway activities (enzyme and transcript), lignin content, and capacity to incorporate new assimilates by using ¹³C labelling. Elevated CO₂ and ozone had opposite effects on many parameters (growth, biomass, cambial activity, wood cell wall thickness) except on lignin content which was increased by elevated CO₂ and/or ozone. However, this increased lignification was due to different response mechanisms. Under elevated CO₂, carbon supply to the stem and effective lignin synthesis were enhanced, leading to increased lignin content, although there was a reduction in the level of some enzyme and transcript involved in the lignin pathway. Ozone treatment induced a reduction in carbon supply and effective lignin synthesis as well as transcripts from all steps of the lignin pathway and some corresponding enzyme activities. However, lignin content was increased under ozone probably due to variations in other major components of the cell wall. Both mechanisms seemed to coexist under combined treatment and resulted in a high increase in lignin content.

Key words: ¹³C labelling, elevated CO₂, lignin, ozone, poplar, wood.

Introduction

Climate change has been attributed to greenhouse gas emissions due to human activities since the industrial era. CO₂ and tropospheric ozone are two of the most important greenhouse gases in our atmosphere that are predicted to continue increasing during this century (IPCC, 2007).

CO₂ is the first greenhouse gas of anthropogenic origin involved in global warming. The concentration of CO₂ has increased from 280 µl l⁻¹ before the industrial era to

380 µl l⁻¹ at the present time and could double in 2100 according to IPCC scenarios (IPCC, 2007). The effects of elevated CO₂ on forest trees have been studied extensively. Usually, an increase in growth and biomass has been observed in tree species (Ainsworth and Long, 2005). Stimulation of growth and biomass is often due to higher carbon assimilation (Curtis and Wang, 1998; Ainsworth and Rogers, 2007).

Abbreviations: OW, opposite wood; TW, tension wood.

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The average concentration of ozone in the troposphere before the industrial era was about 10 nl l^{-1} . Since the intensification of industrial activities and transport in the 20th century, the ozone content in the atmosphere has reached 20 nl l^{-1} to 45 nl l^{-1} in different regions of the world (Vingarzan, 2004). Models predict an average ozone concentration of up to 80 nl l^{-1} in 2100 (IPCC, 2007). The increase in ground-level ozone can be accompanied by episodes of ozone peaks, sometimes over 200 nl l^{-1} . Tropospheric ozone, as well as being a greenhouse gas, is now considered to be the most important air pollutant affecting vegetation (Karnosky *et al.*, 2007). Ozone affects tree growth and biomass (Wittig *et al.*, 2009) by decreasing photosynthesis and stomatal conductance (Wittig *et al.*, 2007).

The opposite effects of elevated CO_2 and ozone as single factors on plant growth and biomass have often been investigated, whereas their combined effects are less well documented. Previous studies showed that elevated CO_2 compensated the negative effects of ozone (Isebrands *et al.*, 2001; Karnosky, 2003; Riikonen *et al.*, 2004; King *et al.*, 2005). The protection provided by CO_2 enrichment could result from the stimulation of photosynthesis, which would support the detoxification process (Gillespie *et al.*, 2011). However, other results reported negative effects of the interaction between the two gases (Wustman *et al.*, 2001). The beneficial effect of high CO_2 is dependent on species, soil fertility, and forest age (Karnosky *et al.*, 2003).

Wood is of primary importance in various industries such as paper manufacture and construction or as a renewal energy source for biofuel (Carroll and Somerville, 2009). Changes in tree growth and biomass resulting from modifications of atmospheric composition, could affect biofuel yields from lignocellulosic biomass. Besides the amount of biomass produced, wood composition is important in bioenergy production. The saccharification process used in biofuel production, is strongly inhibited by the presence of lignins (Weng *et al.*, 2008). A current challenge is thus to reduce significantly the presence of lignins in the lignocellulosic biomass or to remove lignin by physical treatment with acid or steam (Hamelinck *et al.*, 2005). Lignins are the second most abundant component of wood after cellulose and account for 17–30% of the cell wall. Lignins provide the rigidity and structural support that allow water transport in the vascular system. These polymers are mostly derived from three hydroxycinnamyl alcohol monomer precursors (monolignols) which differ in their degree of methoxylation, namely *p*-coumaryl, coniferyl, and sinapyl alcohols (Boerjan *et al.*, 2003). When incorporated into the lignin polymer, these monolignols produce *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units, respectively. However, lignin content and composition vary between species, cell types, and cell wall layers, and may be influenced by developmental and environmental factors (Campbell and Sederoff, 1996).

Although wood composition is important for the production of bioenergy, our knowledge of the effects of elevated CO_2 and ozone on lignification is still limited. Most studies addressed lignin content and various responses were

reported. Elevated CO_2 decreased lignin content in beech (Blaschke *et al.*, 2002), birch (Kostiainen *et al.*, 2006), and Scots pine (Overdieck and Fenselau, 2009). Lignin content was increased in poplar subjected to elevated CO_2 (Luo *et al.*, 2008; Luo and Polle, 2009). Other studies showed that lignin content remained unchanged under elevated CO_2 (Runion *et al.*, 1999; Atwell *et al.*, 2003; Kilpelainen *et al.*, 2003; Kaakinen *et al.*, 2004; Kostiainen *et al.*, 2008, 2009). The results varied greatly depending on the species and experimental devices used. In particular, soil nitrogen content seemed to affect the response of lignification to elevated CO_2 (Luo and Polle, 2009). Tropospheric ozone increased lignin content (Kaakinen *et al.*, 2004) in trembling aspen and silver birch after exposure for three years, but this was no longer true after five years (Kostiainen *et al.*, 2008). Increased lignin content was found in young poplars subjected to ozone (Richet *et al.*, 2011). Finally, the combined effects of elevated CO_2 and ozone on wood composition are not well documented. The increase in lignin concentration in response to ozone in aspen and birch was counteracted by elevated CO_2 (Kaakinen *et al.*, 2004), but this was no longer observed at the same site two years later (Kostiainen *et al.*, 2008).

Most studies were performed in free air experiments which have certain ecological relevance (Matyssek *et al.*, 2010). Nevertheless, they provide limited information about the underlying mechanisms because of interactions with other stresses (biotic or abiotic) or specific site conditions. Indeed, very few reports have dealt with the response of lignin metabolism. One study performed in controlled chambers showed that lignin biosynthesis activities were decreased by ozone in poplar whereas the lignin content increased in the cell wall (Richet *et al.*, 2011). This increase was explained by a strong decrease in cellulose content, the major component of wood.

The purpose of this work was to determine the effect of elevated CO_2 and/or ozone on the lignification process and carbon allocation to the wood. Young poplars (*Populus tremula* × *alba* clone INRA 717-1-B4) were grown for 60 d under different conditions, charcoal-filtered air (control), elevated CO_2 ($800 \mu\text{l l}^{-1}$), ozone (200 nl l^{-1}) or a combination of elevated CO_2 and ozone. The experiment was performed in controlled chambers in order to control various parameters (light intensity, photoperiod, humidity, gas concentration, and soil fertility). Previous work had demonstrated that upright poplars (*Populus tremula* × *alba* clone INRA 717-1-B4) growing in controlled chambers produced tension wood (TW) which was not distributed in any set pattern (Richet *et al.*, 2011). The trees were therefore bent to limit TW formation to the upper side of the stem and normal or opposite wood (OW) to the lower side. Since these two kinds of wood have different lignin contents, their responses were analysed separately. The activities and transcript levels of enzymes of lignin metabolism were monitored in both woods for each culture condition. Lignin content was also analysed. Finally, ^{13}C labelling was used to measure carbon allocation of new assimilates to wood and lignin.

Materials and methods

Plant material and growth conditions

Plants of hybrid poplar (*Populus tremula* × *alba*, clone INRA 717-1-B4) obtained by micropropagation were potted in 5.0 l containers filled with compost (N/P/K 14/16/18, 1.2 kg m⁻³, Gramoflor SP1 Universel) and 20 g of slow release 13:13:13 N:P:K fertilizer (Nutricot T 100, Fertil, Boulogne-Billancourt, France). Plants were grown in controlled chambers under the following climatic conditions: 14 h light period, PAR (photosynthetically active radiation) 250–300 μmol m⁻² s⁻¹ supplied by high pressure sodium vapour lamps (SON-T AGRO, 400W, Philips, Eindhoven, The Netherlands), relative humidity 75/85% (day/night) and temperature 22/18 °C (day/night). The young tree shoots were artificially stacked and tilted at 42° from the vertical in order to limit TW formation to the upper side of the stem (Richet *et al.*, 2011).

Elevated CO₂ and ozone treatments

Elevated CO₂ and ozone fumigation experiments were performed in the controlled chambers used for plant acclimation under the same climatic conditions as described before. The young trees (seven fully expanded leaves, 3 months old) were exposed either to charcoal-filtered air (control), to elevated CO₂ (800 μl l⁻¹) (24 h d⁻¹), to ozone fumigation (200 ± 20 nl l⁻¹) during the light period or to a combination of elevated CO₂ and ozone. Eight chambers were used, that is two chambers per condition. The fumigation experiment ran for 60 d. Additional CO₂ was provided directly by CO₂ gas cylinder (CO₂ B50, >99.5%; AIR LIQUIDE, SA, Paris, France). Ozone was generated by electrical discharge (CMG3-3; Innovatec II, Rheinbach, Germany) using pure oxygen. Air from all controlled chambers was continuously monitored for CO₂ by infrared spectrometer (Beryl 100 Cosma®, Cosma®, Igny, France) and for ozone by UV analyser (O341M, Environment SA, Paris, France).

Growth and biomass measurements

The time-course of growth was monitored throughout the experiment. Growth (height and diameter) was measured on six plants per treatment (randomly selected in two chambers per treatment). Radial growth was measured 10 cm above the root collar with a Vernier calliper. At the end of the experiment (day 60), six plants per treatment were harvested and dried at 60 °C for shoot (leaves and stems) biomass analysis. The number of leaves lost during the experiment was recorded.

Leaf gas-exchange

Net CO₂ assimilation (μmol CO₂ m⁻² s⁻¹) and stomatal conductance to water vapour (*g*_w, mol H₂O m⁻² s⁻¹) were monitored using a portable gas-exchange system Li-6200 (Li-Cor, Inc., Lincoln, NE, USA) after 35 d of fumigation as described in Bagard *et al.* (2008). Measurements were performed inside the fumigation chambers on the first fully developed leaf 2 h after the beginning of the light period at 22 °C, 75% of relative air humidity, and under 200 ± 20 μmol PAR m⁻² s⁻¹. Measurements were performed on four trees randomly selected in two chambers per treatment.

Cell wall thickness and cambial activity

Three poplars were randomly selected in two chambers per condition after 60 d of treatment for measurements of cell wall thickness and cambial activity. Samples for cell wall thickness were saturated in distilled water. Images were obtained by an Environmental Scanning Electron Microscopy (ESEM) (FEI Quanta 200 ESEM). The wood samples in the ESEM chamber were kept at a pressure higher than 8 Torr (several purge cycles ensured that the gas in the chamber was mostly water vapour), to maintain full hydration of the cell walls. Cell wall thickness was measured on approximately 100 fibres per image and six images per plant (3 OW and 3 TW) using the custom software MeshPore (Perré, 2005).

Cambial activity was assessed as previously described by Richet *et al.* (2011). The number of cambium cell layers was recorded on three trees per treatment.

Enzyme extraction

Three trees were randomly selected in two chambers per condition for each enzyme activity measurement. Samples were harvested in the middle of the photoperiod, frozen in liquid nitrogen, and stored at -80 °C until analysis. Two stem levels, corresponding to two different developmental stages, were sampled. The lower stem was located 10 cm above the root collar and corresponded to wood which had developed before and during the exposure experiment. The middle stem had developed only during the fumigation experiment. The bark was removed from each sample and the opposite wood (OW) on the lower quarter side was kept separate from the tension wood (TW) on the upper quarter side. Frozen stems (about 300 mg) were ground in a mortar chilled with liquid nitrogen. Extracts were obtained from the powdered stems, as described by Cabané *et al.* (2004).

Enzyme activities

The time-course of enzyme activities involved in lignin biosynthesis was analysed in OW and TW from the lower and middle stems from each treatment. Shikimate dehydrogenase (SHDH) (EC 1.1.1.25), phenylalanine ammonia-lyase (PAL) (EC 4.3.1.5), and cinnamyl alcohol dehydrogenase (CAD) (EC 1.1.1.195) activities were assayed as previously described by Richet *et al.* (2011). Enzyme activities were reported as the mean value for three trees per time and per treatment. The protein content of the enzyme source was determined with Bio-Rad protein reagent dye (Bio-Rad) using bovine serum albumin as standard.

RNA extraction and cDNA synthesis

Three trees were randomly chosen in two chambers per treatment for RNA analysis. Samples (about 100 mg) of OW and TW from lower stem were placed in teflon jars chilled with liquid nitrogen and ground to a fine powder for 2 min using a mixer mill MM301 (Retsch, France). Total RNA was extracted with TRIzol (Invitrogen) following the manufacturer's instructions, treated with DNase I, Amp Grade (Invitrogen), and cleaned with RNeasy MinElute CleanUp columns (Qiagen). cDNA was generated from 1 μg of total RNA using the iScript™ cDNA Synthesis Kit (Bio-Rad).

Quantitative real-time PCR

Quantitative real-time PCR reactions were prepared using iQTM SYBR® Green Supermix (Biorad) and performed in a MyiQ Single-Color Real-Time PCR Detection System ICycler (Biorad). Conditions for real-time PCR were as follows: denaturation by hot start at 95 °C for 3 min, followed by 40 cycles of a two-step programme consisting of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 45 s. Dissociation curves were verified for each reaction. The relative transcript abundance of genes involved in lignin synthesis was quantified. All genes from the phenylpropanoid and monolignol pathways showing high expression levels in poplar xylem were selected (Shi *et al.*, 2010), namely, phenylalanine ammonia-lyase (PAL), *PtPAL113*, *PtPAL2*, *PtPAL4*, *PtPAL5*; cinnamate-4-hydroxylase (C4H), *PtC4H1*, *PtC4H2*, 4-coumarate:CoA ligase (4CL), *Pt4CL3*, *Pt4CL5*; cinnamoyl-CoA reductase (CCR), *PtCCR2*; cinnamyl alcohol dehydrogenase (CAD), *PtCAD1*; *p*-hydroxycinnamoyl-CoA:quinic acid shikimate *p*-hydroxycinnamoyltransferase (HCT), *PtHCT1*; 4-coumarate 3-hydroxylase (C3H), *PtC3H3*; caffeoyl-CoA *O*-methyltransferase (CCoAOMT), *PtCCoAOMT1*, *PtCCoAOMT2*; coniferyl aldehyde 5-hydroxylase/ferulate 5-hydroxylase (Cald5H), *PtCald5H1*, *PtCald5H2*; caffeic acid/5-hydroxyconiferaldehyde *O*-methyltransferase (COMT), *PtCOMT2*. Primers used for amplification are listed in Supplementary Table S1 at JXB online.

Critical thresholds (Ct) for all genes were quantified in triplicate and normalized with GeNorm software (Vandesompele *et al.*, 2002). Four genes were used as endogenous controls: Ubiquitin-conjugated enzyme E2-17 kDa 10/12 (*UBC10*) (primers according to Richet *et al.* (2011)), polyubiquitin (*UBQ11*), actin 2/7 (*ACT2*), and cyclophylin (*CYP*) (primers according to Brunner *et al.* (2004)). Transcript relative abundance was calculated as the mean of three biological replicates (three trees per condition) and three analytical replicates.

Lignin determination

Dry wood samples (300 mg) were ground and subjected to an exhaustive solvent extraction in a Soxhlet apparatus (toluene:ethanol, 1:1 v:v, then ethanol, and finally water) to obtain extractive-free samples namely cell wall residues. The lignin content was determined by the Klason method from extractive-free powder according to the standard procedure (Dence, 1992). The Klason lignin content was expressed as a percentage of cell wall residue (extractive-free material) or dry mass. All Klason analyses were run in duplicate (technical repeat) with three trees randomly chosen in two chambers per treatment. In addition, the acid-soluble lignin was determined according to the standard procedure and showed no differences between treatments.

¹³C labelling and sampling

After 35 d of the experiment, five hybrid poplars were randomly selected in two chambers per treatment and placed in a VTPH 5/1000 controlled environment chamber (Vötsch Industrietechnik GmbH, Reiskirchen-Lindenstruth, Germany) operated as a semi-closed system designed for ¹³C labelling (Maillard *et al.*, 2001). Plants were then exposed for 4 h during the light period to ¹³C-enriched air (4.4 atom% ¹³C) with a CO₂ concentration of 400 μl l⁻¹ or 800 μl l⁻¹. Climatic conditions (light intensity, humidity, and temperature) were similar to those of the controlled chambers during the exposure treatments. At the end of the labelling period, trees were replaced in the appropriate controlled chambers for 72 h and then harvested in the middle of the light period. The plant material was quickly frozen in liquid nitrogen, freeze-dried, weighed, and ground to a fine powder with a laboratory mill (Tecator, Cyclotec 1093, Höganäs, Sweden) for ¹³C analyses in wood and lignin. At the same time, two unlabelled plants from each treatment were harvested for measurement of natural ¹³C abundance.

¹³C analyses and calculations

The ¹³C/¹²C isotopic ratio of wood and lignin was measured with an elemental analyser (NA 1500 NCS, Carlo Erba, Milan, Italy) coupled to a Delta-S isotopic ratio mass spectrometer (Finnigan, Mat, Thermoquest Corp., San Jose, CA).

Carbon isotopic abundance (*A_C*%) was computed for labelled (*A_C* labelled%) and unlabelled (*A_C* unlabelled%) plant components as:

$$A_C\% = {}^{13}\text{C}/({}^{13}\text{C}+{}^{12}\text{C})\times 100$$

Relative specific allocation of C (*RSAC*%) is defined as the proportion of newly incorporated C atoms relative to the total C atoms in the plant component:

$$RSAC\% = \frac{(A_{C\text{ labelled component}} - A_{C\text{ unlabelled component}})}{(A_{C\text{ labelled atmosphere}} - A_{C\text{ unlabelled atmosphere}})}\times 100$$

with *A_C* labelled atmosphere of 4.4%.

To calculate *A_C* unlabelled atmosphere for each treatment, the time-integrated δ¹³C of air in the chambers was assessed by measuring δ of leaf dry matter of *Zea mays* L. according to Marino and McElroy (1991).

Statistical analysis

Student's *t* test or the non-parametric Mann–Whitney U-test for independent samples, in accordance with the preliminary test for

equality of variances (*P* < 0.05), were used to test the trends between the replicate chambers for each treatment. No significant effect was observed for height and radial growth of trees in the replicate chambers. The experiments were then considered to be completely randomized designs for tree selection and sampling. An analysis of variance (ANOVA) followed by Fisher's LSD test was used to reveal any significant differences (*P* < 0.05 or *P* < 0.1) between the control and other treatments. In all figures and tables, values are mean ± SE.

Results

Young poplars (*Populus tremula* × *alba*, clone INRA 717-1-B4) growing upright (staked or not) in controlled chambers developed TW without any defined pattern (Richet *et al.*, 2011) so the trees were bent to limit the development of TW to the upper side of the stem and OW to the lower side. Young poplar trees were grown for 60 d in controlled chambers under four different conditions, control (filtered air), elevated CO₂ (800 μl l⁻¹), ozone (200 nl l⁻¹), and a combination of elevated CO₂ and ozone.

Growth and biomass

Growth (height and diameter) was increased under elevated CO₂ treatment and decreased under ozone exposure (Fig. 1). The profiles of height growth for the elevated CO₂/ozone combination and the control were similar and the profile of radial growth was intermediate between the control and ozone treatments.

The elevated CO₂ treatment enhanced the biomass of stem and leaves by about 20% without modifying leaf loss. Under ozone treatment, shoot biomass was reduced by 33% in the stem, up to 55% in the leaves, and leaf loss was dramatically increased (Table 1). The combined treatment induced the same effects in leaves (leaf biomass and leaf loss) as the ozone treatment but these were less pronounced. However, the reduction of stem biomass was similar to that observed with the ozone treatment.

Gas exchange measurements

Net CO₂ assimilation and stomatal conductance to water vapour (*g_w*) were measured after 35 d of fumigation. Net CO₂ assimilation was increased by 30% under elevated CO₂ and decreased by 45% under ozone treatment (Table 2). However, net CO₂ assimilation remained unchanged under the combined treatment. Stomatal conductance (*g_w*) was decreased by 40–50% compared with the control, whatever the treatment (Table 2).

Wood anatomy parameters

Cambial activity (number of cell layers) and fibre cell wall thickness were measured at the end of exposure (day 60) in OW and TW of lower stems from each treatment (Table 3). Under elevated CO₂, cambial activity was increased (1.4-fold) only in TW. The number of cell layers was reduced (2-fold) under ozone exposure compared with the control whatever the wood tissue. Cambial activity in the combined treatment remained unchanged compared with the control whatever the tissue.

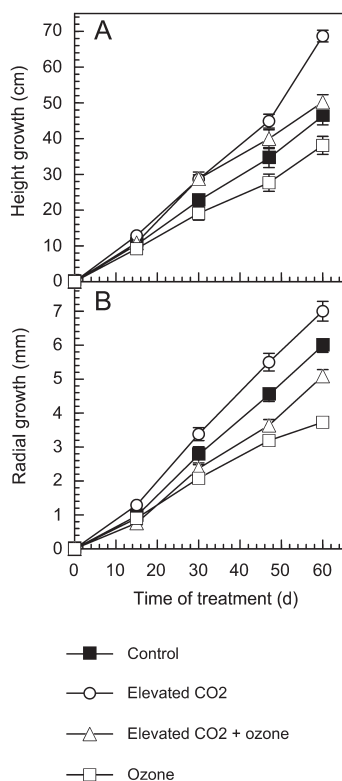


Fig. 1. Height (A) and radial (B) growth of poplars since the beginning of the treatment, i.e. height and diameter differences compared with the height and diameter at time zero. Trees were grown for 60 d under control (filtered air), ozone (200 nl l⁻¹), elevated CO₂ (800 μl l⁻¹) or a combination of ozone and elevated CO₂ conditions. Values are mean ±SE (n=6).

Table 1. Shoot biomass and leaf loss of hybrid poplar grown under different treatments for 60 d

Treatment	Dry mass (g)		Leaf loss (% total leaves)
	Stem	Leaves	
Control	19.1±1.6 a	17.5±1.5 a	10.6±2.4 a
Elevated CO ₂	23.3±2.3 b	21.9±1.6 b	14.8±4.4 a
Elevated CO ₂ and ozone	14.3±1.4 c	14.1±1.1 c	44.2±3.9 b
Ozone	12.7±0.5 c	8.0±1.0 d	66.4±3.3 c

Values are mean ±SE (n=6). Mean values not sharing the same letter are significantly different at $P < 0.05$.

Cell wall thickness was generally higher in TW than in OW (Table 3). Cell wall thickness of fibres from the stem of trees subjected to elevated CO₂ remained unchanged whatever the wood tissue compared with the control. Ozone treatment reduced fibre cell wall thickness in both tissues compared with the control, the decrease being more pronounced in TW than in OW. After the combined treatment, cell wall thickness was not affected in OW but was reduced in TW.

Enzyme activities involved in lignification

The response of the lignin pathway during the experiment was assessed by monitoring the time-course of activity of

Table 2. Net CO₂ assimilation and stomatal conductance to water vapour (g_w) expressed on a leaf area basis in hybrid poplar after 35 d of treatments

	Net CO ₂ assimilation (μmol CO ₂ m ⁻² s ⁻¹)	g_w (mol H ₂ O m ⁻² s ⁻¹)
Control	7.63±0.18 a	0.073±0.010 a
Elevated CO ₂	10.03±1.02 b	0.044±0.001 b
Elevated CO ₂ and ozone	8.72±0.93 a,b	0.038±0.007 b
Ozone	4.19±0.05 c	0.035±0.001 b

Values are mean ±SE (n=4). Mean values not sharing a same letter are significantly different at $P < 0.05$.

the enzymes involved in lignin biosynthesis (SHDH, PAL, CAD) in the OW and TW of lower and middle stems of young trees subjected to different treatments for 60 d (Fig. 2; see Supplementary Fig. S1 at *JXB* online).

Under elevated CO₂ exposure, SHDH, PAL, and CAD activities remained unchanged in the OW of lower stems. These activities were not affected in TW before 40 d of fumigation. However a reduction, compared with the control, was observed after 60 d. CAD activity was reduced to a greater extent than SHDH and PAL (Fig. 2). The enzyme activities in middle stems were similar to those in the control treatment (see Supplementary Fig. S1 at *JXB* online).

The activities of SHDH, PAL, and CAD were decreased in plants exposed to ozone compared with control plants, whatever the wood tissues (OW and TW) in both lower and middle stems (Fig. 2; see Supplementary Fig. S1 at *JXB* online). The decline of PAL and CAD activities was more pronounced (4–6-fold), than that of SHDH in the lower stems after 60 d of treatment. By comparison, the effects were less pronounced in the middle stems in both wood tissues (see Supplementary Fig. S1 at *JXB* online).

Enzyme activities in the combined treatment, compared with the control, were decreased in both wood tissues from the lower stems (Fig. 2). The reduction was moderate in OW and similar to the ozone-induced reduction in TW. CAD activity was generally more affected than SHDH and PAL activities. The effects were similar but less pronounced in both wood tissues of middle stems than in those of lower stems (see Supplementary Fig. S1 at *JXB* online).

Transcript levels in the lignin pathway

Transcripts were analysed at the end of the experiment (day 60) in OW and TW of lower stems. All genes from the lignin pathway showing high expression levels in poplar xylem were examined (Shi *et al.*, 2010). Under control conditions, the lignin-related gene levels were similar in OW and TW except for *PAL1/3*, *PAL2* (lower in TW), and *CAD1* (higher in TW) (Fig. 3; see Supplementary Table S2 at *JXB* online).

Under elevated CO₂, the RNA levels of all genes remained unchanged in OW compared with the control treatment. A similar result was observed in TW except for *PAL4*, *PAL5*, *C4H2*, *4CL3*, and *Cald5H2* which decreased

Table 3. Wood anatomy in lower stems of hybrid poplar grown for 60 d under different conditions

Treatment	Cambium cell layers (number)		Fibre cell wall thickness (μm)	
	Opposite wood	Tension wood	Opposite wood	Tension wood
Control	6.14 \pm 0.25 a	6.36 \pm 0.51 a	3.59 \pm 0.16 a,b	5.17 \pm 0.24 a
Elevated CO ₂	6.31 \pm 0.45 a	8.56 \pm 0.80 b	3.63 \pm 0.23 a	5.35 \pm 0.10 a
Elevated CO ₂ and ozone	5.48 \pm 0.28 a	5.68 \pm 0.24 a	2.99 \pm 0.22 b,c	4.32 \pm 0.13 b
Ozone	3.06 \pm 0.51 b	3.38 \pm 0.32 c	2.73 \pm 0.21 c	3.74 \pm 0.10 c

Values are mean \pm SE ($n=3$ biological replicates). Mean values not sharing the same letter are significantly different at $P < 0.05$.

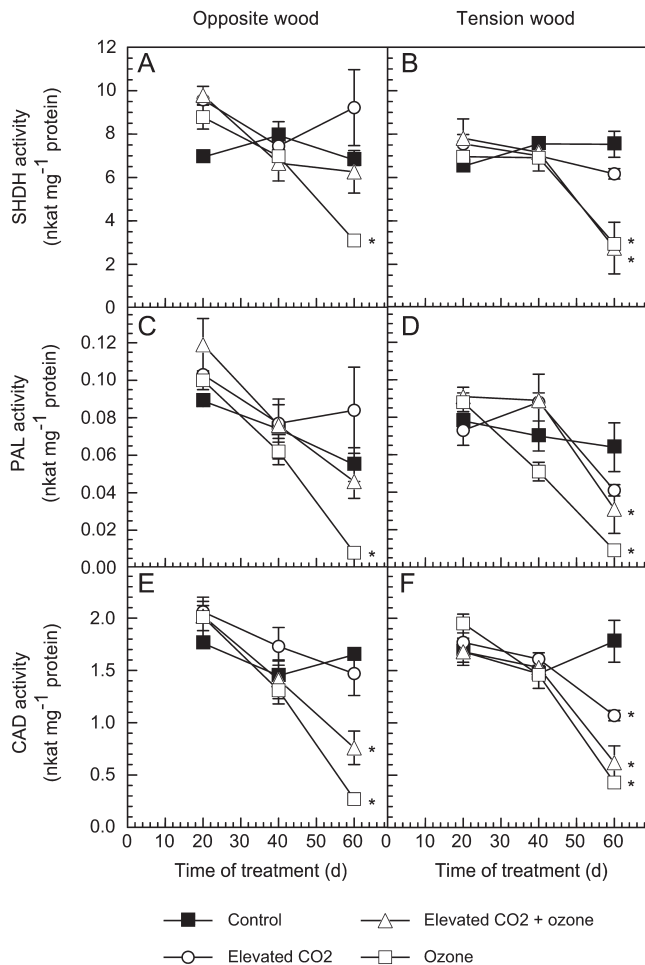


Fig. 2. Time-course of activities of SHDH (A, B), PAL (C, D), and CAD (E, F) enzymes in opposite wood (OW) (A, C, E) and tension wood (TW) (B, D, F) of the lower stem of hybrid poplar subjected to different treatments. Values are mean \pm SE ($n=3$). Difference between control and treatments at day 60 was statistically significant at $P < 0.05$ (*).

(Fig. 3; see Supplementary Table S2 at *JXB* online). Ozone treatment induced a decrease in transcript levels of all genes involved in the lignin pathway whatever the tissue, OW or TW. In the combined treatment, many genes showed a reduction in transcript levels particularly in TW. The profile of the reduction was intermediate between the reductions observed in the ozone and control treatments.

Lignin content

Lignin content in relation to cell wall residue (CWR) was determined at day 60 in the OW and TW of lower and middle stems from each treatment (Fig. 4). The lignin content was higher in OW than in TW whatever the treatment or stem level. Lignin content was increased in elevated CO₂, ozone, and the combined treatment whatever the wood tissue or stem level. The highest increase was observed under the combined ozone and elevated CO₂ treatment. Lignin content was enhanced by 14.5% compared with the control.

Relative specific allocation of carbon ($RSAC$) in wood and lignin

The capacity of wood and lignin to concentrate newly acquired C was assessed by performing a ¹³C pulse-chase experiment on young poplar trees grown under different conditions and estimating $RSAC$ in the OW and TW of lower and middle stems.

The carbon newly incorporated in wood (wood $RSAC$) was higher in the middle stem than in the lower stem under control conditions (Fig. 5A, B). Plants subjected to elevated CO₂ showed a significant increase in the allocation of newly incorporated carbon only in the TW of lower and middle stems. Under ozone treatment, $RSAC$ was significantly decreased (up to 2-fold) whatever the wood tissue or stem level. No changes in carbon allocation were observed in the combined treatment except in TW of the middle stem (Fig. 5B).

The carbon newly incorporated in lignin was decreased (1.5–2-fold) under ozone treatment in both wood tissues and at both stem levels (Fig. 5C, D). Few changes were observed under the other treatments. Nevertheless, carbon allocation to lignin was significantly increased under elevated CO₂ in the TW of lower stem (Fig. 5C), and in OW of middle stem of plants subjected to the combined treatment (Fig. 5D).

Discussion

The effect of elevated CO₂ on lignification

As soil fertility has been shown to be very important in the response of trees to elevated CO₂ (Oren *et al.*, 2001; Long *et al.*, 2004), the young poplars in our experiment were fertilized to avoid N limitation. Poplar growth and biomass

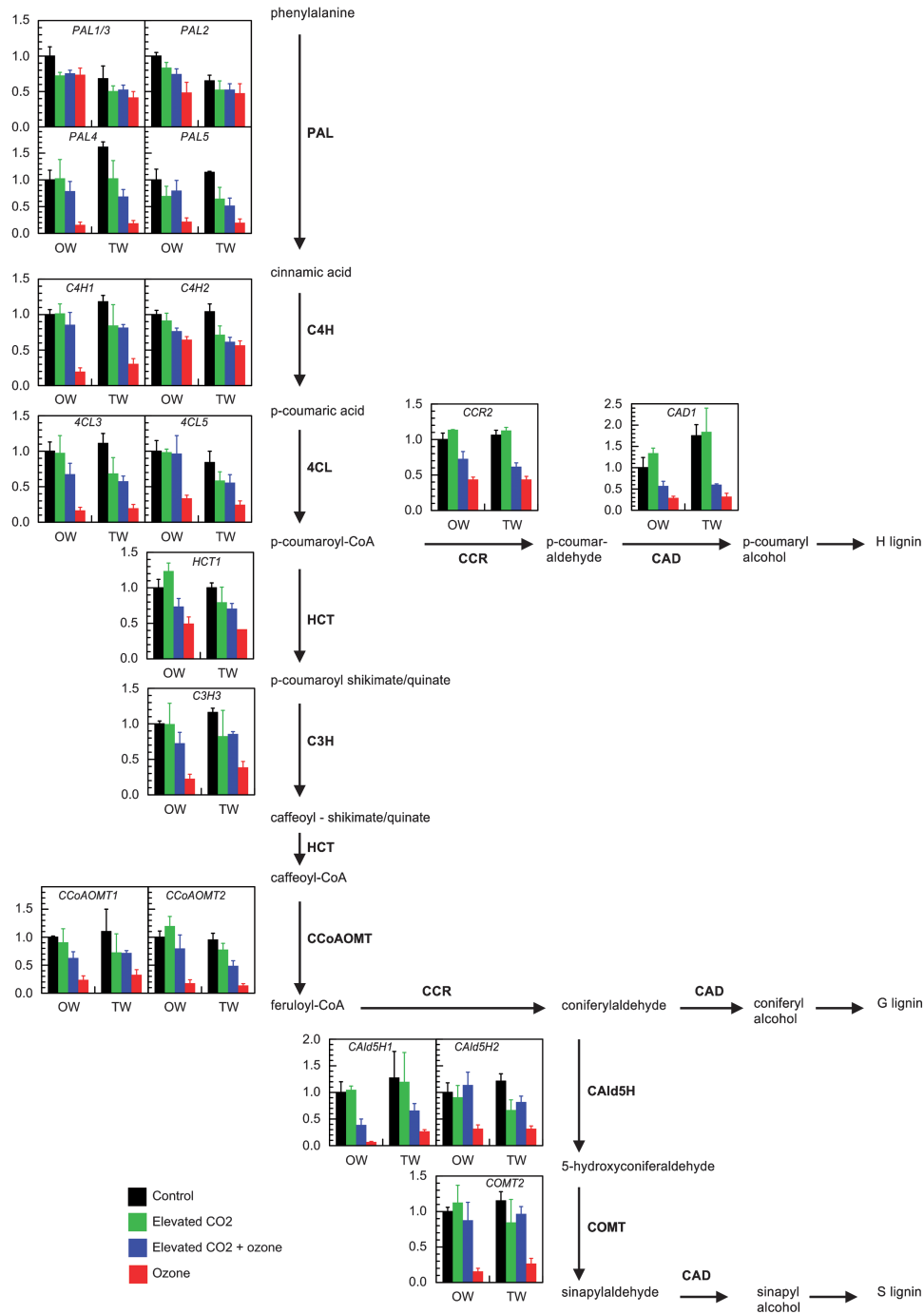


Fig. 3. Transcript abundance of genes involved in the lignin biosynthesis pathway. Analyses were performed on opposite wood (OW) and tension wood (TW) of lower stem of hybrid poplar subjected for 60 d to different treatments. Transcript levels were normalized and expressed as fold changes compared with values of opposite wood in control conditions. y axis is in arbitrary units. Values are mean \pm SE ($n=3$ biological replicates). For statistical differences refer to Supplementary Table S2 at *JXB* online. The most favoured route in angiosperms lignin pathway is shown. Phenylalanine ammonia-lyase (PAL), cinnamate-4-hydroxylase (C4H), 4-coumarate:CoA ligase (4CL), *p*-hydroxycinnamoyl-CoA:quinic/shikimate *p*-hydroxycinnamoyltransferase (HCT), 4-coumarate 3-hydroxylase (C3H), caffeoyl-CoA *O*-methyltransferase (CCoAOMT), cinnamoyl-CoA reductase (CCR), coniferyl aldehyde 5-hydroxylase (CAld5H), caffeic acid/5-hydroxyconiferylaldehyde *O*-methyltransferase (COMT), cinnamyl alcohol dehydrogenase (CAD).

increased under elevated CO₂ in our experiment as is usually found with N fertilization.

Cell wall lignin content was increased in the wood of young poplars grown in elevated CO₂ (Fig. 4). A similar result was found in coppices of *Populus* \times *euramericana* and

Populus nigra in a free air CO₂ enrichment experiment (Luo *et al.*, 2008; Luo and Polle, 2009) and in birch (Mattson *et al.*, 2005). However, different responses were observed in other experiments. Lignin content was reduced in beech (Blaschke *et al.*, 2002) whereas no modification was

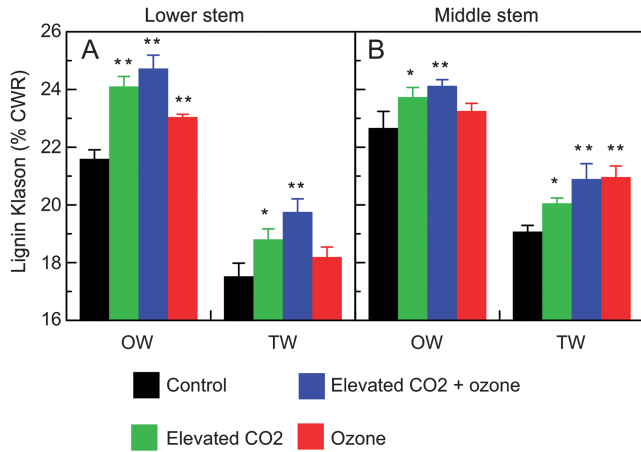


Fig. 4. Lignin content in opposite wood (OW) and tension wood (TW) of lower (A) and middle (B) stems of hybrid poplar after 60 d of growth under different conditions control (filtered air), ozone (200 nl l^{-1}), elevated CO_2 (800 $\mu\text{l l}^{-1}$) or the combination of ozone and elevated CO_2 . Values are mean \pm SE ($n=3$). Difference between control and treatments was statistically significant at $P < 0.05$ (**) or $P < 0.1$ (*).

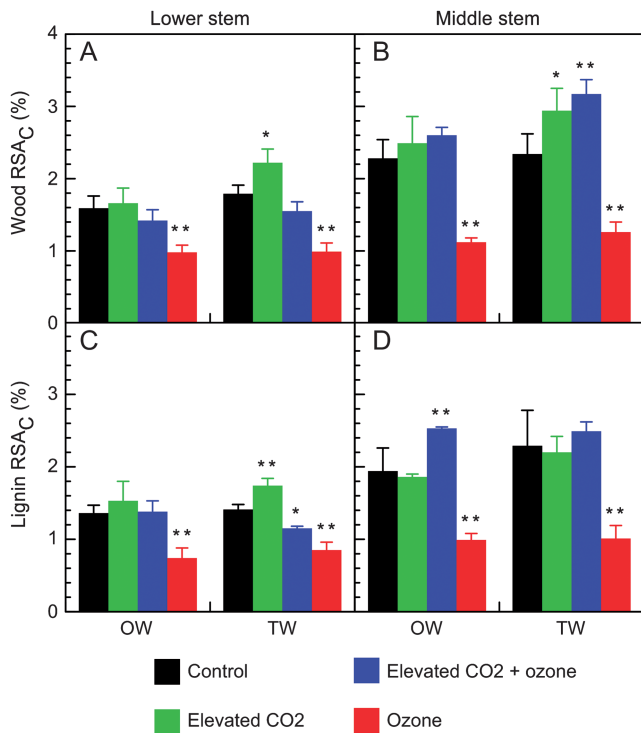


Fig. 5. Changes in relative specific allocation of carbon (RSA_C %) for wood (A, B) or lignin (C, D) in opposite wood (OW) and tension wood (TW) of lower (A, C) and middle (B, D) stems of hybrid poplar grown for 35 d under different treatments. Values are mean \pm SE ($n=5$ for wood and $n=3$ for lignin analyses). Difference between control and treatments was statistically significant at $P < 0.05$ (**) or $P < 0.1$ (*).

reported in birch, maple or aspen clones in a free air CO_2 enrichment experiment (Kaakinen *et al.*, 2004; Kostianen *et al.*, 2008). Responses may depend on the species, the season, and N supply. Cell wall lignification was assessed by

expressing the lignin content as a percentage of the cell wall residue or extractive-free material. In our experiment, the wood of poplars grown in elevated CO_2 was enriched in extractives (see Supplementary Table S3 at *JXB* online) so the lignin content expressed on a dry mass basis remained unchanged under elevated CO_2 . Since lignin content is often expressed on a dry mass basis, variations in cell wall lignin content could have been masked in some cases by the accumulation of extractives, as suggested by Penuelas and Estiarte (1998).

The allocation of new assimilates to wood or lignin was assessed by performing a ^{13}C pulse-chase experiment. The labelling experiments revealed more pronounced effects in TW than in OW (Fig. 5). TW was probably more responsive since it represents a strong sink for carbon especially under elevated CO_2 . Larger amounts of new assimilates were allocated to wood, as indicated by the increased wood RSA_C levels in poplars under elevated CO_2 (Fig. 5). This result is consistent with the increased carbon assimilation, radial growth, and cambial activity (Tables 2, 3). As a consequence, new assimilates were more utilized for lignin biosynthesis since the lignin RSA_C was higher in wood of poplars grown under elevated CO_2 . The continuous labelling experiment in beech also showed an increased utilization of new assimilates in lignin under elevated CO_2 (Dyckmans *et al.*, 2000). All these results, together with the increased lignin content, strongly suggest that effective lignin biosynthesis was enhanced under elevated CO_2 . Wood cell walls were more lignified under elevated CO_2 due to the increased synthesis of lignin without any modifications of cell wall thickness (Table 3).

Lignin metabolism activities (enzyme activities and RNA levels) were unchanged or slightly decreased in TW of poplars grown under elevated CO_2 (Figs 2, 3). It can be hypothesized that enzyme activities decreased to limit an increase of lignin synthesis due to substrate excess under elevated CO_2 . This hypothesis presupposes that the substrate supply was limiting and not the enzyme activities, under control conditions. Indeed, in transgenic trees, enzyme activities had to be strongly reduced for any effect on lignin biosynthesis to be observed under control conditions (Baucher *et al.*, 2003). Six of the 17 genes analysed in this study were significantly down-regulated under elevated CO_2 . The regulation by elevated CO_2 seemed to affect only a few genes and only some members within a given gene family in the lignin pathway.

Ozone effect on lignification

Young poplars subjected to ozone fumigation displayed a higher wood lignin content than control poplars (Fig. 4). These findings are in accordance with our previous results obtained under controlled conditions (Richet *et al.*, 2011).

The pathways involved in lignin biosynthesis (enzyme activities and RNA levels) were strongly reduced in wood under ozone treatment (Figs 2, 3). A decrease in *PALI*, *CCR2*, and *CAD1* had previously been recorded in poplars after 46 d of ozone fumigation. It is shown here for the first

time that all genes from the phenylpropanoid and monolignol pathways highly expressed in wood (Shi *et al.*, 2010) were reduced at transcript level under ozone treatment. These results suggest a tight co-ordination of the response to ozone and that all these genes could share regulatory elements and associated transcription factors for their regulation in the presence of ozone. Shi *et al.* (2010) found common putative regulatory elements in these genes. However, it is shown here that, within a gene family, some transcripts were more altered than others suggesting that the corresponding promoters differed in their responsiveness to ozone.

The reduction of lignin pathway activities was consistent with the decreased cambial activity (Table 3) of poplars subjected to ozone. Wood formation was slowed down so lignin biosynthesis activities were reduced. The reductions in radial growth (Fig. 1) and stem biomass (Table 1) were a consequence of decreased cambial activity. It is also shown that photosynthesis was reduced in leaves (Table 2) which suggested that less carbon was supplied to the stems. The allocation of new assimilates to wood or lignin was assessed for the first time, to our knowledge, by performing a ¹³C pulse-chase experiment. The labelling experiment which showed that wood *RSAC* was reduced under ozone confirmed that less new carbon was allocated to wood under ozone exposure (Fig. 5A, B). These results are consistent with a decreased phloem loading induced by ozone (Andersen, 2003). Interestingly, the labelling experiment also showed a reduced use of assimilates for lignin biosynthesis since lignin *RSAC* was decreased by ozone (Fig. 5C, D). The results obtained by ¹³C labelling, together with the response of enzymes and transcripts involved in lignification, reinforce the hypothesis that ozone reduces effective lignin biosynthesis. It can also be hypothesized that the lignin pathway (enzyme activities and RNA levels) is controlled by carbon supply in poplars subjected to ozone. A control by sugars has also been suggested in normal growth conditions (Rogers *et al.*, 2005).

Lignin biosynthesis was repressed under ozone whereas the lignin content of the cell wall was increased (Fig. 4). Since lignin, cellulose, and hemicellulose are the main components of the cell wall, a modification in the abundance of one component would affect the relative contents of the others. Our results strongly support the notion that lignin biosynthesis was reduced under ozone and that lignin content was increased, probably because one or both of the other components was also affected. Indeed, both cellulose content and cellulose biosynthesis activities were shown to decrease in poplars subjected to ozone treatments (Richet *et al.*, 2011) which is consistent with the thinner cell walls observed in this experiment (Table 3). It can therefore be hypothesized that the synthesis of the cell wall components was reduced and that lignin was less reduced than the other components leading to more lignified cell walls.

Effect of elevated CO₂ and ozone on lignification

In our experiment, elevated CO₂ and ozone applied separately had opposite effects on many parameters: growth, biomass,

photosynthesis, cambial activity, and wood cell wall thickness. Poplar grown under combined elevated CO₂ and ozone displayed intermediary effects for all these parameters. The negative effects of ozone were generally limited by elevated CO₂. This compensatory effect has been observed in many cases (Karnosky *et al.*, 2003).

Nevertheless, the labelling experiments showed more subtle changes in plants exposed to a combination of elevated CO₂ and ozone. Indeed, lower and middle stems displayed different responses. Incorporation of new assimilates into the wood and lignin of the lower stems was moderately reduced compared with the control suggesting that elevated CO₂ had a compensatory effect. By contrast, incorporation of new assimilates into the wood and lignin of the middle stems was similar or higher than that observed under elevated CO₂. The lower stems responded like the ozone-treated stems and the behaviour of the middle stems resembled that of stems under elevated CO₂.

In contrast to other parameters, the lignin content was similarly affected by elevated CO₂ and ozone applied separately in poplar wood. The compensatory effect of elevated CO₂ was not recorded here under the combined treatment since the lignin content in the wood of poplars grown under elevated CO₂ and ozone was higher than in the wood of poplars grown under each treatment (Fig. 4). Thus the effects on lignin content were additive when elevated CO₂ was combined with ozone. It is shown that different mechanisms leading to increased lignin content occurred under elevated CO₂ and under ozone treatments. Both mechanisms may be involved in the response to combined treatment resulting in an additive effect.

In conclusion, the first detailed analysis of the lignification process in wood of young poplars subjected to elevated CO₂ and/or ozone is reported here. Both constraints induced increased lignification of the cell wall but the underlying mechanisms were different. Under elevated CO₂, modifications in lignin content were under the control of carbon supply to the stem. Under ozone treatment, changes in lignin content were controlled by the balance between lignin and other cell wall components. Elucidation of these steps should help in identifying key genes involved in the wood response to elevated CO₂ and/or ozone. These candidate genes should be validated on mature trees growing in natural conditions before they could be used in tree improvement for a better adaptation to future climate.

Supplementary data

Supplementary data can be found at *JXB* online.

Supplementary Table S1. Primers used for quantitative real-time PCR.

Supplementary Table S2. Statistical analyses of transcript levels of different genes involved in lignin biosynthesis.

Supplementary Table S3. Extractives and lignin content on a dry mass basis in opposite wood and tension wood of hybrid poplar grown under different conditions.

Supplementary Fig. S1. Time-course of the activities of SHDH, PAL, and CAD enzymes in opposite wood and tension wood of middle stem from hybrid poplar subjected to different treatments.

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