DISSERTATIONES BIOLOGICAE UNIVERSITATIS TARTUENSIS
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EVE EENSALU

Acclimation of stomatal structure and function in tree canopy: effect of light and CO_2 concentration

TARTU UNIVERSITY
PRESS

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following papers which are referred to in the text by Roman numerals:

I Eensalu E, Kupper P, Sellin A, Rahi M, Sõber A, Kull O. 2008. Do stomata operate at the same relative opening range along a canopy profile of *Betula pendula*? *Functional Plant Biology* 35: 103–110.

II Tricker PJ, Trewin H, Kull O, Clarkson, GJJ, Eensalu E, Tallis M, Colella A, Doncaster CP, Sabatti M, Taylor G. 2005. Stomatal conductance and not stomatal density determines the long-term reduction in leaf transpiration of poplar in elevated CO_2. *Oecologia* 143: 652–660.

III Calfapietra C, Tulva I, Eensalu E, Perez M, De Angelis P, Scarascia-Mugnozza G, Kull O. 2005. Canopy profiles of photosynthetic parameters under elevated CO_2 and N deposition in a poplar plantation. *Environmental Pollution* 137: 525–535.

IV Merilo E, Tulva I, Eensalu E, Räim O, Calfapietra C, Kull O. Photosynthetic response of two poplar species to elevated CO_2 (EUROFACE) in relation to leaf nitrogen partitioning. Manuscript

The participation of the author in preparing the listed publications is following:

	I	II	III	IV
Original idea	*			
Study design	*		*	*
Data collection	*	*	*	*
Data analyses	*	*	*	
Manuscript preparation	*			*

LIST OF ABBREVIATIONS

A_{leaf}	leaf area (cm^2)
A_{max}	light-saturated net CO_2 assimilation at growth CO_2 ($\mu mol\ m^{-2}\ s^{-1}$)
c_i/c_a	ratio of intercellular to atmospheric CO_2
D	vapour pressure difference between the leaf interior and the bulk air (kPa)
D_e	epidermal cell density (mm^{-2})
D_s	stomatal density (mm^{-2})
g_s	stomatal conductance ($mmol\ m^{-2}\ s^{-1}$)
g_{scalc}	calculated stomatal conductance based on stomatal dimensions ($mmol\ m^{-2}\ s^{-1}$)
g_{smax}	maximum stomatal conductance ($mmol\ m^{-2}\ s^{-1}$)
ISF	indirect site factor (relative units)
l	length of stomatal pore (μm)
l_d	length of guard cell on the dorsal side (μm)
LMA	leaf dry mass per area ($g\ m^{-2}$)
l_s	length of guard cell (μm)
N_a	area-based nitrogen content ($g\ m^{-2}$)
N_m	mass-based nitrogen concentration ($g\ g^{-1}$)
PPFD	photosynthetic photon flux density ($\mu mol\ m^{-2}\ s^{-1}$)
SI	stomatal index (%)
z	guard cell width (μm)
T	air temperature (°C)
WUE	leaf-level instantaneous water-use efficiency ($mmol\ mol^{-1}$)
Ψ_{leaf}	leaf water potential (MPa)

1. INTRODUCTION

Plant stomata constitute the most important control of CO_2 influx and water vapour efflux in leaves. Links between stomatal structure and function reveal how plants are acclimated to their environment along a range of spatial and temporal scales. Sage (1994) defined 'acclimation' as "physiological and morphological responses which improve performance and survival of an individual plant by enhancing growth, resource-use efficiency, reproductive output, stress tolerance, and/or the lifespan of an individual in the modified environment". Stomatal responses to changes in various environmental factors have been studied intensively with most attention given to quantifying the interaction with physiological traits (Hall and Schulze 1980; Kirchbaum *et al.* 1988; Oren *et al.* 1999; Lodge *et al.* 2001). However, acclimation of plant stomata occurs also through morphological adjustments to the prevailing environment. Here, acclimation is defined as a change in stomatal function and structure when plants grow in contrasting CO_2 concentrations or light conditions.

Stomatal control operates on different time-scales and is coordinated by the plant hydraulic system (Williams *et al.* 1996; Aasamaa and Sõber 2001; Tyree and Zimmermann 2002) and by the photosynthetic machinery of leaves (Idso 1991; Uemura *et al.* 2000). Different environmental factors have a long-term effect (lasting days to weeks) on leaf gas-exchange by influencing stomatal development On the other hand, stomatal aperture responds to environmental changes within minutes, representing a short-term response.

The development of stomata is regulated by environmental factors such as light intensity (Tichá 1982), concentration of atmospheric carbon dioxide (Woodward and Kelly 1995) and water supply (Hetherington and Woodward 2003), and by intrinsic signals such as endogenous plant hormones (Franks and Farquhar 2001). The size and distribution of stomata are established during leaf development in the bud and during the early growth stage after bud break. Leaf functioning during the growing season determines the properties of developing buds in the following spring and can act as a signal for young developing leaves (Lake *et al.* 2001; Thomas *et al.* 2003). This hypothetical signal induces an appropriate developmental response to irradiance (Kimura *et al.* 1998; Thomas *et al.* 2003) and to CO_2 concentration (Lake *et al.* 2001). Development of stomata during leaf unfolding and expansion was examined by Tichá (1982), but few studies describe leaf formation under different conditions, particularly at different canopy positions (Thomas *et al.* 2003; Marchi *et al.* 2008).

Light availability in temperate forests varies extensively within the canopy profile leading to structural and physiological changes in leaves that determine the gas exchange performance of the canopy. Increase in maximum stomatal conductance with canopy height has been observed in different woody species (Niinemets *et al.* 1999, Sellin 2001, Sellin and Kupper 2005*a*). Morphologically, this is mostly related to increased stomatal density resulting from higher

light intensity during leaf development and expansion (Tichá 1982), while the length of guard cells – and thus stomatal openness – can decrease (Meidner and Mansfield 1968), increase (Klich 2000) or remain unaffected (Carr 2000). Inconsistent stomatal response was observed also in long-term (years) free air CO_2 enrichment (FACE) experiments on broadleaved species. Depending on the species, plants responded by decreased, increased or unchanged stomatal density with increasing CO_2 concentration (Ferris and Taylor 1994; Ceulemans *et al.* 1995; Woodward and Kelly 1995; Woodward *et al.* 2002) despite a consistent decrease in stomatal conductance (Medlyn *et al.* 2001; Herrick *et al.* 2004).

Spatial patterns in stomatal conductance are strongly related to within-canopy gradients of leaf photosynthetic capacity. Probably the evolutionary pressure to optimize the use of resources together with strong – often a 10–20-fold gradient in photosynthetically active radiation – has lead to great within-canopy gradients in leaf photosynthetic capacity (Woodward 1998; Carswell *et al.* 2000; Kull 2002). Leaf photosynthetic capacity acclimates to micrometeorological conditions in a timescale of a few weeks (Oguchi *et al*. 2005). Although some properties of the leaf photosynthetic machinery may be regulated by longer-term environmental signals (Terashima *et al*. 2006), there seems to exist some uncoupling between timescales of leaf anatomical and physiological acclimation within leaf canopies. Therefore, some leaf properties are determined during leaf development (such as formation of palisades tissue, chloroplast number, stomatal density) and others – such as maximum carboxylation rate, maximum rate of electron transport, stomatal openness – are associated with current environmental conditions (Miyazawa *et al.* 2006; Niinemets *et al.* 2006). The uncoupling among morphological and physiological traits may facilitate adaptation to environmental conditions on a spatial and temporal scale.

Although sensing CO_2 concentration is a stomatal function proposed to be independent of light sensing, the response pathways to light and CO_2 concentration may be linked (Lake *et al.* 2002). Kubiske *et al.* (1997) found that photosynthetic acclimation under elevated CO_2 might be different depending on light availability. It has been hypothesized that elevated CO_2 and shading can interact, inducing a stronger acclimation response than either factor alone (Herrick and Thomas 2001). For example, acclimation response to elevated CO_2 in *Populus tremuloides* was stronger for shade than for sun leaves (Kubiske *et al.* 2002). Therefore, leaf position within the canopy can affect the response of photosynthesis to elevated CO_2, whereas stomatal acclimation to CO_2 may not be driven solely by adjustments in the biochemistry of photosynthesis.

Because intercellular CO_2 concentration is a key variable sensed by guard cells and used to coordinate stomatal opening and photosynthetic rate, many studies have been designed to quantify stomatal responses to rising atmospheric CO_2 concentration (Baker and Allen 1994; Saxe *et al.* 1998; Norby *et al.* 1999). Such coordination operates also on shorter time scales, leading to a relatively unchanging ratio of intercellular to ambient CO_2 concentration (c_i/c_a) (Wong *et al.* 1978; Noormets *et al.* 2001). The c_i/c_a ratio is an index of stomatal acclima-

tion reflecting changes in the relationship between stomatal conductance and net CO_2 fixation (Sage 1994). If stomatal acclimation is more pronounced then stomatal conductance decreases relative to photosynthetic activity, resulting in a lower c_i/c_a. The levels of water stress and light availability increase vertically with canopy height, leading often to increased stomatal limitation of photosynthesis as a result of decreases in c_i (Kull and Niinemets 1998; Niinemets *et al.* 2004).

Short-term stomatal and photosynthetic responses have been studied extensively but less is known on how are the developmental processes of biochemical machinery of photosynthesis and stomatal apparatus interrelated. Photosynthetic rate is often defined as a function of nitrogen content (Evans 1989; Kull and Kruijt 1999; Carswell *et al.* 2000). Area-based nitrogen content (N_a) correlates positively with light availability (Hollinger 1996) but mass-based nitrogen concentration (N_m) does not vary along the light gradient in the canopy (Casella and Ceulemans 2002). Foliar N_m correlates positively with maximum stomatal conductance among plant functional groups (Schulze *et al.* 1994), and within the deficiency range, N_a correlates positively with stomatal pore width (Hunt *et al.* 1985). Such adjustments function to produce enhanced photosynthetic capacity with higher light availability (Niinemets *et al.* 1998, Niinemets *et al.* 1999). In addition to N_a, photosynthetic potential can be affected by the distribution of nitrogen among the photosynthetic apparatus and structural elements in leaves (Kull and Niinemets 1998; Niinemets 2007). Nevertheless, species that show the greatest reductions in leaf N content at elevated CO_2 also exhibit less stimulation in assimilation rates (Nowak *et al.* 2004). Despite of this down-regulation, photosynthetic rate at growth conditions usually remains higher under elevated CO_2 regardless of the duration of the study (Norby *et al.* 1999).

At the moment the degree to which the well known relationship between stomatal conductance and photosynthetic rate is dictated by morphological properties (stomatal density and dimensions) versus functional properties (differential stomatal opening) is unknown.

The aims of my study were:

(1) to determine the most important morphological traits and microclimatic conditions that induce differences in stomatal conductance along the canopy light profile, both in normal growth conditions and under elevated CO_2 concentration;
(2) to determine the relative contributions of stomatal density and stomatal openness to the improvement of leaf-level water-use efficiency under elevated CO_2;
(3) to assess the degree to which stomatal morphological acclimation is consistent with changes in photosynthetic acclimation along canopy gradient,
(4) to examine the relationship between stomatal density and quantity of photosynthetic apparatus and its role in determining the correlation between stomatal conductance and photosynthetic rate.

2. MATERIAL AND METHODS

Paper I

The experimental site is located in Järvselja, Eastern Estonia (58°22′N, 27°20′E), elevation ~40 m a.s.l. The study was carried out on silver birch (*Betula pendula* Roth) trees growing in a temperate mixed forest of a *Vaccinium myrtillus* site type (Paal 1997). Leaf properties were measured on eight neighbouring birch trees (40–50 years old, height 16–20 m) in July-August 2002. Leaves were sampled from the upper (~15–17 m) and lower (~8–9 m) crown position depending on tree height and distance to the crown base from the ground.

Light conditions above the sampled leaves were estimated by hemispherical photography using a COOLPIX 950 digital camera equipped with FC-E8 "fish-eye" lens (Nikon, Japan). Leaf conductance (g_l) was measured *in situ* with a LI–1600M steady-state porometer (Li-Cor, USA) at the upper and lower canopy positions of five trees during 15 days in July 2002. The greatest values of g_l measured across all sampled trees per canopy layer during separate days was used to calculate maximum actual stomatal conductance (g_{smax}), yielding a total of 30 values (15 values of g_{smax} for upper and 15 values for lower canopy). Leaf water potential (Ψ_{leaf}) was measured with a Scholander-type pressure chamber. The long-term and short-term effect of relative humidity (R_H), air temperature (T) and photosynthetic photon flux density (PPFD) to stomatal structure and function were studied.

To study stomatal morphology, ten leaves were collected from each of the 8 examined trees − five from the upper, sun-exposed crown and five from the lower, shaded crown position, totalling 80 leaves. Because birch leaves are hypostomatous, stomatal and epidermal cells were measured only on the abaxial side. Mean stomatal density (D_s) and epidermal cell density (D_e), excluding guard cells, were calculated using a light microscope (PZO; Warsaw, Poland), also stomatal index (SI) was calculated according to Woodward and Kelly (1995).

Pieces of leaves (approx. 4–6 mm^2; half-way from tip to base and half-way from mid-rib to margin) were taken from ten leaves per tree to examine the leaf abaxial side by a scanning electron microscopy (SEM). The leaf fragments (80 samples) were fixed in phosphate buffer containing 4% glutaraldehyde, dehydrated in a 25–100% ethanol gradient and critical point-dried in CO_2. The pieces were then set on a sample stub with conductive tape and were coated with gold (VUP–4; Russia). For details on standard methods, see Hall and Hawes (1991). The leaf samples were photographed by scanning electron microscope (Tesla BS 301; Czech Republic) under 1500–2000× magnification and the digital images were analysed with an image analysis system (Global Lab Image Ver.3.2, USA). Measured stomatal morphological parameters were length of stomatal pore (l), length of guard cell (l_s), guard cell width (z) and length of guard cell on the dorsal side (l_d) (Fig 1 in I).

To parameterize the model of stomatal conductance from Parlange and Waggoner (1970) and Van Gardingen *et al.* (1989), the following formula was used:

$$g_s = \frac{P_a D_w D_s}{RT\left(\frac{d}{\pi a b} + \frac{\ln(4a/b)}{\pi a}\right)}, \quad (1)$$

where P_a is the atmospheric pressure (101000 Pa), D_w is the water diffusivity in air (2.14×10^{-5} m^2 s^{-1}), *a* is the stomatal pore semi-length (m), *b* is the stomatal pore semi-width (m), R is the gas constant (8.31 J mol^{-1} K^{-1}), T is the leaf and air temperature (298 K), and *d* denotes pore depth. Considering that pore depth is difficult to measure and assuming that the cross-section of stomatal guard cell is close to circular, the stomatal depth was considered equal to guard cell width.

Ten additional leaves were collected from the upper and lower canopy positions to determine the maximum width of stomatal pores. The leaves were coated with clear nail varnish and images of the impressions were captured at 400× magnification with a Carl Zeiss microscope (Germany) and photographed using AXIOVISION 3.0 (Carl Zeiss Vision, Germany). The stomatal pore length and width was measured for 80 stomata per canopy position (on 10 leaves) using ImageJ software, ver 1.36b (National Institutes of Health, USA).

Papers II, III, IV

Measurements were carried out at the EUROFACE site in Tuscany, Central Italy (N 42°22', E 11°48'). Three of the six experimental plots were maintained at ambient CO_2, whereas the other three were maintained at elevated CO_2 (550 ppm) using free air carbon dioxide enrichment (FACE) technique as described by Miglietta *et al*. (2001). Research was carried out on three poplar species: *Populus alba* L. (clone 2AS–11), *P. nigra* L. (clone Jean Pourtet) and *P.* × *euramericana* (Dode) Guin. (*P. deltoides* × *P. nigra*, clone I–214) during 2003 (Paper II, III, IV) and 2004 (Paper IV). Stomatal conductance (g_s) was measured *in situ* with an AP–4 porometer (Delta-T Devices, UK) at two (2003) or three (2004) canopy positions. In 2003, g_s was measured only on abaxial surfaces (Paper II, III, IV) and in 2004, on both leaf surfaces (Paper IV). In *P. alba*, stomata exist only on the abaxial leaf side, but two other species are amphistomatous. Maximum photosynthetic rate (A_{max}) was measured as the rate of photosynthesis at PPFD 1000 μmol m^{-2} s^{-1} and at growth CO_2 concentration (Paper II, III, IV) using CIRAS–2 (PP Systems, UK) or LI-COR 6400 (LI-COR Inc., USA) in August-September. The results were used to calculate c_i/c_a ratios and leaf-level instantaneous water-use efficiency (WUE = ratio of photosynthetic assimilation to transpirational water loss).

Leaves used for g_s measurements *in situ* were collected at the end of experiment in order to investigate stomatal morphology (48 leaves were

collected per species = 4 plots × 4 trees × 3 height levels). Stomatal impressions were photographed using a AXIOVISION 3.0 camera attached to a Zeiss microscope. Afterward, stomatal density (D_s), stomatal index (SI), epidermal cell density (D_e), length of stomatal pore (l), length of guard cell (l_s), guard cell width (z), length of guard cell on the dorsal side (l_d) was measured. Later, half of these leaves were used to determine the leaf dry mass per area (LMA) and leaf nitrogen content per area (N_a) (Paper III, IV).

3. RESULTS AND DISCUSSION

3.1. Stomatal structure in canopy vertical profile

3.1.1. Effect of light

Forest canopies are characterized by gradients of environmental conditions along a vertical profile. Leaves at lower crown positions are approximately 2–3 times less illuminated compared to those at the upper position (Table 1 in I; Table 1 in III; Fig. 3 in IV), also leaf attachment height correlated positively with light availability in all studied species ($P<0.05$). Other factors that affect stomatal conductance, such as vapour pressure difference, leaf water potential and temperature also vary along the canopy profile (Table 1 in I). These gradients may cause differences in stomatal morphological and physiological acclimation at various temporal and spatial scales.

Carpenter and Smith (1975) found that fast growing species have more stomata than slowly growing species. This is consistent with my finding that stomatal density (D_s) of the fast growing poplars was 3–5 times that of the slower growing birch (Table 2 in I; Table 1). Both D_s and stomatal index (SI) increased with light availability in the lower epidermis of birch leaves and on both leaf sides of poplar leaves (Table 2 in I; Fig. 2 in I; Fig. 1; Table 1). Because epidermal cell density (D_e) did not change between canopy position, the increases in D_s and SI along vertical gradient indicate that light is primarily responsible for more stomata differentiate during leaf developmental processes. The total number of stomata per leaf increased with light intensity in poplars and did not change in birch ($P>0.05$), due to a compensatory reduction in leaf size with height in birch (Table 2). Smaller leaf area may be beneficial under conditions of water stress and high irradiance occurring in upper canopy (James and Bell 2000). As birch grew in water-limiting conditions, decreasing leaf size with height is consistent with optimization between gas exchange and light interception (Sellin and Kupper 2005). In poplar under well-watered growth conditions, the observed increase in leaf-size with height may achieve the same optimal results (Miglietta *et al.* 2001).

For poplars, stomatal dimensions – l_d, z, l, l_s – generally decreased with increasing irradiance on both leaf sides (Table 1). This is consistent with the negative correlation between D_s and stomatal size described elsewhere (Tichá 1982). High variability in l_d, l, and z for *P.euramericana* resulted in a insignificant effect of canopy position, but the trend was similar to that seen in the other poplar species. In contrast, l_d and z (but not l and l_s) in birch increased marginally with irradiance (Table 2 in I), causing more rounded stomatal complexes in upper canopy than in lower canopy. Tree age (Richardson *et al.* 2000) and soil water availability (Aasamaa *et al.* 2001) can produce differences in stomatal size distribution at similar positions within the canopy, but these factors were not evident in the FACE experiment on even-aged, well-watered

and fertilized poplars trees (Miglietta *et al.* 2001). Increased stomatal size at the lower part of the canopy may represent a potential developmental (long-term) response to high soil water availability (Maherali *et al.* 2002). The decrease in stomatal density with decreasing light and the accompanying increase in stomatal size are a morphological adjustment demonstrating stomatal acclimation to light availability along the canopy profile. Hence, higher stomatal density supports increased CO_2 demand for photosynthesis in upper leaves, and smaller stomata tend to close faster than bigger stomata, therefore, have greater sensitivity to water deficit (Nejad and van Meeteren 2005; Tanaka *et al.* 2005). Some inconsistency between the studied species with respect to observed changes in stomatal density and dimensions with canopy positions may reflect differences in the vertical distribution of irradiance and humidity caused by the much higher leaf area index of the poplar stands (leaf area index at birch stand was 3; and in poplar stand varied 5–6, Liberloo *et al.* 2006).

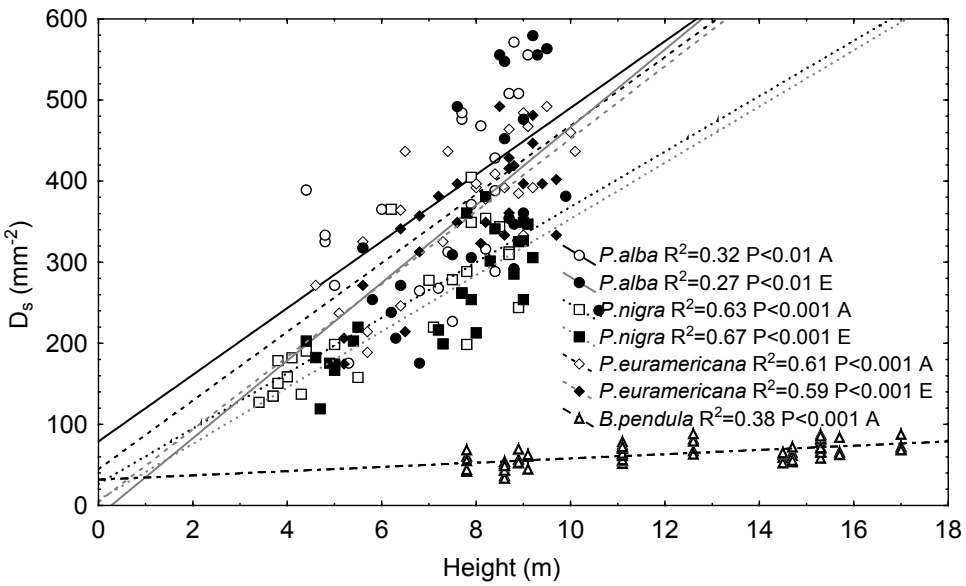

Fig. 1. Relationship between stomatal density (D_s; sum of adaxial and abaxial leaf side) and height from ground in poplar species (data from 2004) and in birch. A, ambient CO_2 and E, elevated CO_2 (about effect of CO_2 see in 3.1.2).

Table 1. Leaf morphological traits of poplar species in the upper, middle and lower canopy positions under ambient CO_2 concentration.
D_s, stomatal density; A_{leaf} , leaf area; D_e, epidermal cell density; SI, stomatal index; l_d, length of guard cell on the dorsal side; z, guard cell width; l, length of stomatal pore; l_s, length of guard cell was measured on both leaf side. Values are mean±SE from 2004.

Species	*P.alba*		
Leaf side	abaxial		
Position	Upper	Middle	Lower
D_s (mm^{-2})	540 ± 12	488 ± 12	304 ± 15
A_{leaf} (cm^2)	79 ± 6.5	57 ± 7.3	15 ± 1.5
l_d (µm)	31.4 ± 0.52	33.8 ± 0.49	32.0 ± 1.05
z (µm)	6.4 ± 0.13	6.9 ± 0.11	6.6 ± 0.27
l (µm)	13.0 ± 0.45	14.7 ± 0.38	13.1 ± 0.70
l_s (µm)	22.5 ± 0.47	24.6 ± 0.42	23.6 ± 0.83

Species	*P.nigra*					
Leaf side	abaxial			adaxial		
Position	Upper	Middle	Lower	Upper	Middle	Lower
D_s (mm^{-2})	224 ± 6	194 ± 7	145 ± 5	95 ± 4	67 ± 4	24 ± 2
D_e (mm^{-2})	3363 ± 114	3043 ± 125	2733 ± 85	2340 ± 86	1883 ± 57	1860 ± 62
A_{leaf} (cm^2)	44 ± 5	30 ± 2.7	18 ± 1.4			
SI (%)	5.9 ± 0.2	5.7 ± 0.3	5.9 ± 0.8	3.9 ± 0.3	3.0 ± 0.5	1.1 ± 0.3
l_d (µm)	32.3 ± 0.66	34.6 ± 1.08	43.8 ± 1.46	34.4 ± 0.56	36.9 ± 1.23	36.8 ± 1.01
z (µm)	7.0 ± 0.21	7.3 ± 0.29	10.8 ± 0.52	7.5 ± 0.18	8.4 ± 0.42	8.3 ± 0.41
l (µm)	13.8 ± 0.48	13.9 ± 0.48	16.8 ± 0.50	14.9 ± 0.35	15.3 ± 0.54	14.4 ± 0.62
l_s (µm)	24.2 ± 0.55	25.7 ± 0.73	32.0 ± 0.88	25.5 ± 0.39	27.1 ± 0.75	26.8 ± 0.78

Species	*P.euramericana*					
Leaf side	abaxial			adaxial		
Position	Upper	Middle	Lower	Upper	Middle	Lower
D_s (mm^{-2})	269 ± 6	248 ± 6	194 ± 8	163 ± 5	131 ± 4	90 ± 5
D_e (mm^{-2})	3347 ± 71	3311 ± 129	2693 ± 102	2955 ± 102	2896 ± 74	2344 ± 99
A_{leaf} (cm^2)	246 ± 21.2	91 ± 16.7	47 ± 10			
SI (%)	9.1 ± 0.8	10.3 ± 1.6	8.0 ± 1.1	5.3 ± 0.2	4.1 ± 0.2	3.9 ± 0.4
l_d (µm)	33.0 ± 0.78	34.0 ± 0.76	33.9 ± 0.77	33.5 ± 0.85	34.2 ± 0.75	34.9 ± 0.95
z (µm)	7.4 ± 0.25	7.5 ± 0.26	7.6 ± 0.31	7.9 ± 0.34	7.4 ± 0.27	8.1 ± 0.45
l (µm)	14.2 ± 0.45	13.9 ± 0.45	14.9 ± 0.52	14.4 ± 0.43	14.6 ± 0.38	14.3 ± 0.42
l_s (µm)	23.7 ± 0.54	25.2 ± 0.60	25.9 ± 0.58	23.2 ± 0.54	24.4 ± 0.49	24.8 ± 0.60

Table 2. Results of analysis of variance for effects of CO_2 and canopy position (Posn) on the morphological leaf traits in poplar species. Abbreviations as in Table 1. ***P<0.001; **P<0.01; *P<0.05; ns – statistically insignificant.

		Abaxial surface								
Species		D_s	D_e	SI	l_d	z	l	l_s	A_{leaf}	$D_s \times A_{leaf}$
P.alba	CO_2	ns	–	–	ns	ns	ns	ns	ns	ns
	Posn	***	–	–	***	ns	**	***	***	***
	CO_2×Posn	ns	–	–	**	ns	**	**	ns	ns
P.nigra	CO_2	ns	ns	**	ns	ns	ns	ns	ns	ns
	Posn	***	ns	ns	***	***	***	***	***	***
	CO_2×Posn	ns	*	ns	**	***	ns	ns	ns	ns
P.euramericana	CO_2	ns	ns	ns	ns	ns	ns	ns	ns	ns
	Posn	***	ns	ns	ns	ns	ns	***	***	***
	CO_2×Posn	ns	ns	ns	ns	ns	ns	ns	ns	ns

		Adaxial surface						
		D_s	D_e	SI	l_d	z	l	l_s
P.nigra	CO_2	ns	ns	ns	ns	ns	ns	ns
	Posn	***	ns	***	***	*	*	***
	CO_2×Posn	ns	ns	ns	ns	ns	ns	ns
P.euramericana	CO_2	ns	ns	ns	ns	ns	ns	ns
	Posn	***	*	ns	*	*	ns	*
	CO_2×Posn	ns	**	ns	ns	ns	ns	*

3.1.2. Effect of CO_2 concentration

Although the effect of CO_2 fumigation on stomatal development has been found to depend on the light regime (Miyazawa *et al.* 2006), the results of my work indicate that D_s in poplars is not always sensitive to CO_2 fumigation (Fig. 1a in II) in 2001–2004. Both SI and D_s were lower under elevated CO_2 during the first (Tricker *et al.*) and second years of the first rotation but there were no differences between the treatments neither during the third growing season nor during any year of the second rotation (Fig. 1a in II; Fig. 2). A lack of CO_2 effect on D_s has been reported earlier by Radoglou and Jarvis 1990; Pearson *et al.* 2006; this research likewise suggests that leaf area did not change under CO_2 fumigation compared to ambient CO_2. In our work, leaf area likewise remained unchanged under CO_2 fumigation (Table 2). Overall, the *P. euramericana* and *P. nigra* had a ratio of adaxial to abaxial D_s of ~0.6, and the ratio was independent of CO_2 treatment (Fig. 2) in contrast to findings of an increased ratio under elevated CO_2 in grasses (Knapp *et al.* 1994). In grassland species stomatal size may decrease, increase, or be unaffected by elevated CO_2 (Maherali *et al.* 2002). This thesis reports slightly smaller stomata (data not

shown) and slightly lower D_s values (Fig. 1) under elevated CO_2, although these effects were not statistically significant (Table 2).

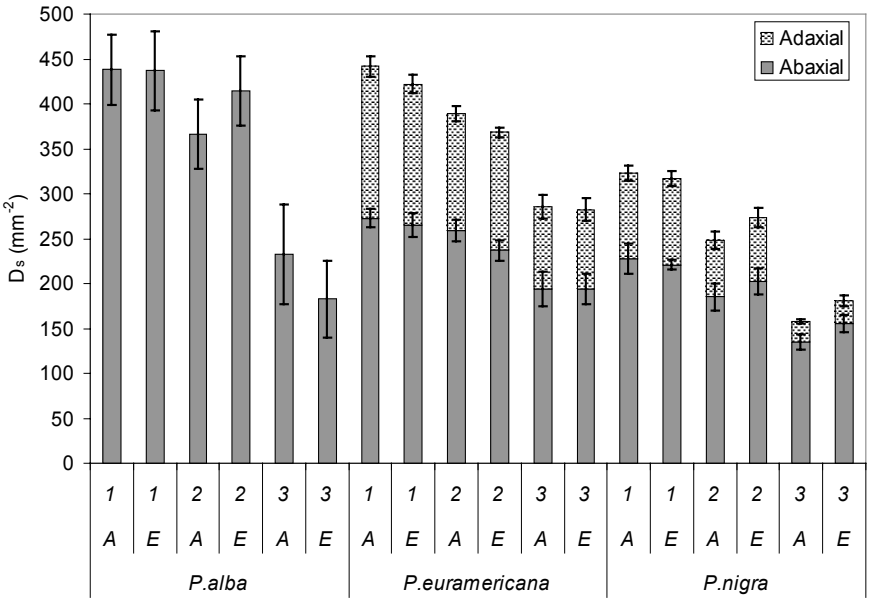

Fig. 2. Abaxial and adaxial stomatal density (D_s) in the leaves of poplar species under different treatments and canopy positions in 2004. Numbers 1–3 on X-axis refer to vertical canopy levels (1-upper, 2-middle, 3-lower). A, ambient CO_2; E, elevated CO_2. Values are mean±SE.

3.2. Stomatal functioning in relation to structural parameters of leaves and stomata

Both short- and long-term responses contribute to the differences in g_s. Changes in stomatal openness are short-term adjustment to environmental variation and alternation of stomatal morphology represents a long-term response. Daily average measured g_s increased with light availability in birch, especially when comparing lower to upper-canopy leaves, but the variability of g_s within each layer was primarily reflected by a negative relationship with vapour pressure difference (D) between intercellular space and atmosphere (Fig. 3 in I). Based on daily average values, the systematic differences in g_s between the crown positions could not be directly related to differences in leaf water potential and photosynthetic photon flux density (PPFD; Fig. 3 in I). The responses observed reflect the combined effects of stomata to light and plant water status. Low vapour pressure difference is associated with low light intensity, but increased vapour pressure difference is usually related to a higher photosynthetic rate. Water potential in the upper canopy is far above the critical value that would

cause cavitation and loss of hydraulic function (Sperry *et al.* 1998). Probably the variation in leaf water potential and PPFD was too small to have any significant direct impact on g_s (Niinemets *et al.* 1999; Flexas *et al.* 2006). In effect, when the buffer between actual water potential and the critical water potential is large, light dominates in stomatal response – when the buffering capacity is small, vapour pressure difference dominates in stomatal response.

Both g_s and g_{smax} increased with light availability in the tree canopy (Fig. 4 in I; Fig 4 in III; Fig. 3 in IV). Moreover, for two of the poplar species, g_s was positively correlated to D_s (Fig. 3). For *P. alba*, this relationship was not significant, D_s was often greater than 437 mm^{-2} (Table 1), whereas the range in which D_s affects g_{smax} is assumed to be below 350 mm^{-2} (Galmés *et al.* 2007). During the 5 experimental years, g_s was significantly reduced by CO_2 enrichment: on average 20% for all studied poplar species (Fig. 2 in II; Fig. 4 in III; Fig. 3 in IV). This is a commonly observed pattern of g_s response to elevated CO_2 concentration (Medlyn *et al.* 2001). Still, g_s decreased less in the lower part of canopy under elevated CO_2 because of wider xylem elements (Kupper *et al.* 2006). This is consistent with the findings for *P. nigra* in this study, in which the g_s reduction under elevated CO_2 was 20% and 9% in upper and lower leaves, respectively, but contradicts the corresponding reductions of 23% and 41% for *P. euramericana*, and 13% and 28% for *P. alba*. Reduction in g_s due to CO_2 enrichment is usually considered to occur via short-term response, evidenced by a reduced stomatal openness, as in this study, and abaxial and adaxial stomata respond similarly to CO_2 (Kamakuru and Furukawa 2008).

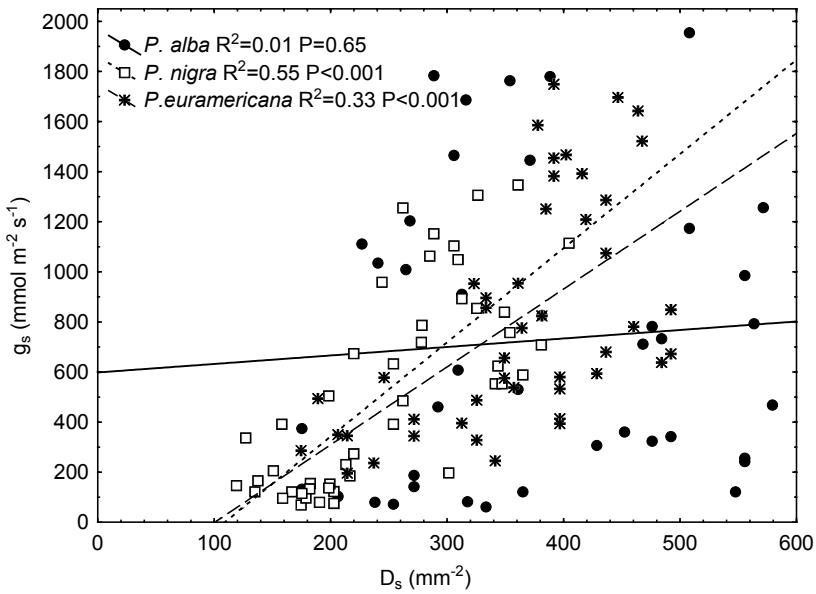

Fig. 3. Relationship between stomatal conductance (g_s) and stomatal density (D_s; both leaf sides summed) on poplar species.

How do changes in light intensity along the canopy vertical profile influence actual stomatal opening relative to the maximum g_s? The variation in the stomatal opening range reflects adaptations to water stress and light intensity (Zheng *et al.* 2007). The relationship between stomatal pore width and stomatal conductance in birch, calculated based on a model (Eqn. 4 in I) is shown in Fig. 5 (in I). Stomatal pore width at measured g_{smax} and g_s (in the final two columns) are shown in figure 5 in I. In upper leaves, stomatal width of approximately 8 μm corresponded to g_{smax} and that of 4 μm corresponded to average g_s. Stomata at the bottom of the canopy were more closed: 4 μm at g_{smax} and 2 μm at average g_s. Modelled stomatal conductance (g_{scalc}), calculated from stomatal dimensions, like g_{smax}, also increased with light availability (Fig. 5 in I). The ratio of g_{smax} for upper to lower leaves averaged 2.1; this ratio for modelled g_{scalc} was only 1.3. Assuming that the ratio of measured g_{smax} between upper and lower-canopy leaves (2.1) is roughly composed of two components, one long-term anatomical (1.3) and one short-term physiological and environmental (2.1/1.3= 1.6), we can conclude that anatomical traits are responsible for a substantial proportion of the differences in g_s between the two *B. pendula* canopy layers.

Experiments with fully open stomata (bagged leaves) showed that the width of stomatal pore is well correlated with the length of stomatal pore: $P<0.05$, $R^2=0.48$ and $P<0.05$, $R^2=0.55$ for the upper and lower canopy, respectively (data not shown). The measured ratio of stomatal pore width/length was similar in upper and lower leaves (0.57 and 0.56), i.e. the ratio of maximally achieved pore width to length was practically the same for both canopy layers (Table 4 in I). Thus, the more easily measurable length of the stomatal pore should describe maximum pore aperture during the experiment. Furthermore, this means that the relative pore aperture compared to its maximum is the same in the leaves of both canopy layers. Using the values of pore width and length respective to maximum measured stomatal conductance, g_{smax} (Fig. 5 in I), the ratio of pore width to length was 0.56 in the upper, but only 0.29 in the lower canopy (Table 4 in I), meaning that stomatal aperture approached a maximum in top leaves (0.56/0.57), whereas stomata were only about half-open in the lower crown (0.29/0.56).

Is the change in stomatal morphology consistent with changes in photosynthesis in canopy profile? In contrast to other studies in which the highest photosynthetic rates were observed in species with amphistomatous leaves (Knapp 1993), the greatest photosynthetic values were measured on hypostomatous leaves of *P. alba* (Fig. 2 in IV). Considering that D_s limits photosynthesis, or develops in balance with photosynthetic capacity, a correlation between D_s and photosynthetic parameters should exist. Indeed, D_s was shown as the main anatomical factor determining the variability in g_{smax} along the canopy because D_s increased consistently with light availability.

Photosynthetic capacity usually increases with light intensity along the canopy vertical profile (Kull and Niinemets 1998; Niinemets *et al.* 2004a).

Therefore, the light-saturated rate of leaf photosynthesis is strongly correlated with anatomical parameters such as leaf thickness, leaf mass per area (LMA) and mesophyll surface area (Oren *et al.* 1986; Oguchi *et al.* 2003; Terashima *et al.* 2006). A 3–15 fold difference in light intensity between the upper and lower canopy resulted in A_{max} and LMA being 2–4 times higher in the upper than lower canopy layer (Table 2 in III, Fig 2. in IV), as was also reported by Oren *et al.* (1986) and Niinemets (2007). In this study, canopy position with high D_s and g_s also exhibited the highest photosynthetic rates and LMA (Fig. 2 in III; Fig. 2 in IV). Another reason for a reduction in net CO_2 assimilation in low D_s conditions (shade leaves) could be a reduced CO_2 supply to the mesophyll caused by the long distance between stomata, i.e long distances to lateral CO_2 diffusion (Morison and Lawson 2007). Interestingly, although A_{max} varies 2–4 fold vertically in the canopy, g_{scalc} (calculated using stomatal dimensions) varied only about half as much in all studied species (1–1.9 fold). It seems that light acclimation of photosynthetic apparatus is greater than that of stomata.

To what degree do stomata acclimate to growth CO_2 concentration? Many studies have observed stomatal acclimation with increasing CO_2 concentration (Sage *et al.* 1989; Morison 1998). The long-term FACE experiment in poplar species revealed no acclimation of stomatal morphology, but differences in stomatal opening under elevated and ambient CO_2 were evident. At the end of the five-year long experiment, the WUE increased approximately 40% under elevated CO_2 (Fig. 3b in II). Over a wide range of species the c_i/c_a ratio is almost constant under ambient CO_2 (Hetherington and Woodward 2003). In this study, the c_i/c_a ratio of upper leaves decreased at high CO_2, indicating stomatal acclimation. The mechanism of stomatal acclimation is not fully known but could be related to a network-based organization of physiological adjustment to light and CO_2. Stomatal conductance is closely related to photosynthetic rate, so stomatal acclimation to growth CO_2 may be a response to photosynthetic acclimation rather than a direct response.

Do stomatal number and content of photosynthetic apparatus correlate? The leaf nitrogen content (N_a) was strongly correlated with photosynthetic rates (data not shown) and also with g_s (Fig. 4). A positive relationship between D_s and N_a was found during a long-term acclimation to irradiance (Fig. 5) and during rise of atmospheric CO_2 concentration over the last 100 years (Peñuelas and Matamala 1990). It is not known which morphological parameters are involved in regulating the relationship between photosynthesis and g_s. Marchi *et al.* (2008) showed that the number of fully developed stomata followed the trend of photosynthesis during leaf expansion. There are a few theories why D_s and N_a are coordinated. g_s and N_a may be both regulated through the hydraulic system of trees because low water transport capacity and low g_s are able to support high photosynthetic rates, only when leaf nitrogen concentration increases (Taylor and Eamus 2008). In addition, a strong correlation between g_s

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and N_a indicates that the content of photosynthetic apparatus and number of stomatal pores for CO_2 supply closely match. Zhao *et al.* (2008) concluded that D_s and N_a could be used as indicators of tree age and height. The present study suggests that gas exchange is strongly related to D_s and N_a along the canopy profile. The photosynthetic rate and leaf nitrogen content do not match exactly the light gradient within a canopy (Kull 2002), but photosynthetic capacity (maximum carboxylation rate) is strongly correlated with N_a (Fig. 5 in IV). The strong correlation between D_s and N_a is most probably caused by light availability along canopy profile, however, development of stomatal number can be also adjusted to CO_2 demand for photosynthesis. Independently of its mechanism, the close correlations between g_s and N_a, and D_s and N_a will serve as tools to simplify the modelling of canopy photosynthetic performance.

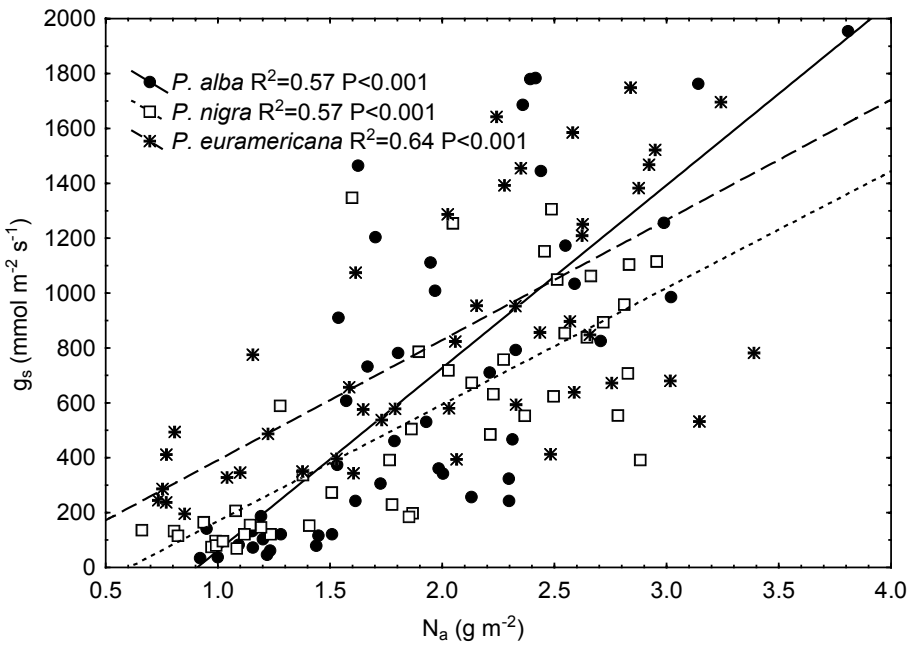

Fig. 4. Stomatal conductance (g_s; sum of abaxial and adaxial side on leaves) versus area-based nitrogen content (N_a) of leaves in poplar species.

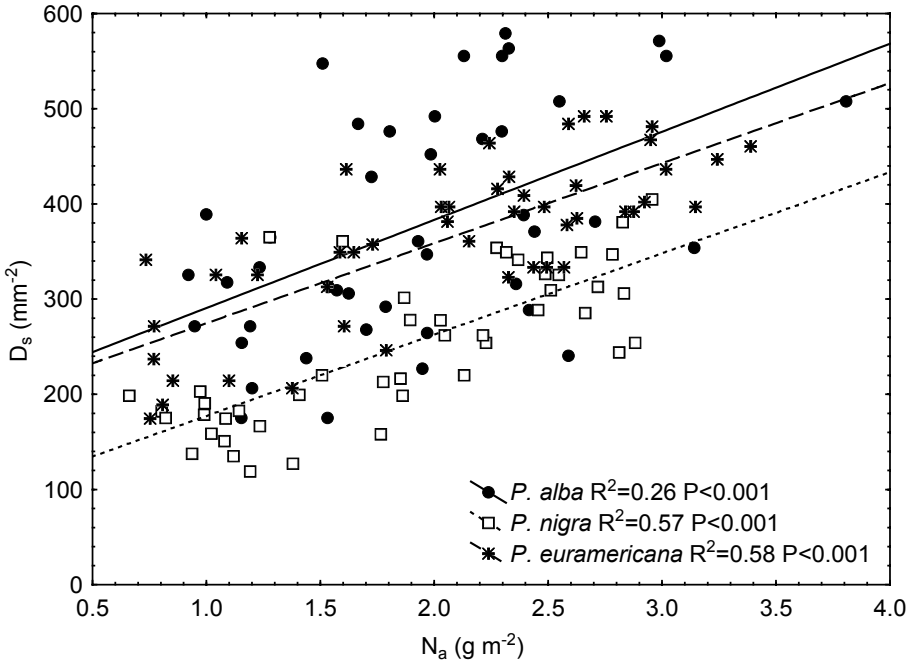

Fig. 5. Stomatal density (D_s; sum of adaxial and abaxial leaf side) versus area-based nitrogen content (N_a) of leaves.

4. CONCLUSIONS

- The stomatal density (D_s) was a stomatal morphological trait that consistently changed along the canopy height profile in broad-leaved tree species. D_s in lower-canopy leaves was about 80% in birch and 55% for poplar species compared to that in the upper canopy, but stomatal dimensions decreased, increased or remained unchanged among canopy positions. Modelled stomatal conductance (g_{scalc}), calculated from stomatal dimensions, like maximum measured stomatal conductance (g_{smax}) also increased acropetally with light availability. Stomatal aperture approached a maximum in top leaves, whereas stomata were only half-open at the crown base. We can conclude that stomatal structure determines approximately 30% of the variation in stomatal conductance.
- In poplar, D_s was lower under elevated CO_2 during the first and second years of the first rotation, but there were no differences between the treatments during the third growing season nor during any year of the second rotation. Higher leaf-level instantaneous water use efficiency (WUE) was related to a significant decrease in stomatal openness, that stomatal conductance (g_s) decreased ~20% under CO_2 fumigation. D_s, in contrast, was not responsible for the changes in higher WUE under CO_2 enrichment.
- A 3–15 fold variation in light intensity along the vertical canopy profile resulted in A_{max} 2–4 times higher for the upper-canopy leaves, while g_{scalc} (calculated using stomatal dimensions) varied only half as much in all studied species (1–1.9 fold), regardless of tree age and height. It seems that photosynthetic rate acclimates to light availability more flexibly than stomatal morphology.
- In this work, the leaf nitrogen content was strongly correlated with D_s and g_s. A positive relationship between D_s and N_a observed in poplar species indicates that leaf morphological parameters are important factors contributing to the functional relationship between photosynthetic rate and stomatal conductance. This study confirms that gas exchange is related to D_s and N_a along the canopy profile – this knowledge will simplify modelling of canopy photosynthetic production.

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SUMMARY IN ESTONIAN

Õhulõhede struktuuri ja funktsiooni kohanemine valgusega ja kõrgendatud CO_2 kontsentratsiooniga lehtpuude võras

Minu doktoritöö eesmärgiks oli uurida õhulõhede ehituse ja funktsioneerimise vahelisi seoseid, ning välja selgitada kuidas valguse intensiivsus ja kõrgendatud CO_2 kontsentratsioon mõjutavad õhulõhede füsioloogilisi ja morfoloogilisi tunnuseid heitlehistel puuliikidel. Eksperimentaalne töö viidi läbi arukasel (*Betula pendula*) ja kolmel papliliigil (*Populus alba*, *P. nigra*, *P. × euramericana*).

Õhulõhed kontrollivad nii CO_2 sisenemist lehte kui ka vee aurumist lehtedest. Uurides õhulõhede struktuuri ja funktsiooni vahelisi seoseid, saame informatsiooni, kuidas taimed on kohanenud oma kasvukeskkonnaga nii ajalises kui ruumilises skaalas. Mitmesugustel keskkonnafaktoritel võib olla pikaajaline mõju õhulõhedele, mille tulemusena toimuvad muutused õhulõhede tiheduses ja mõõtmetes. Teisalt, õhulõhede avatuse muutused kujutavad endast otsest lühiajalist reaktsiooni keskkonnatingimustele. Kohanemisprotsesse, mis toimuvad võrastiku vertikaalprofiilis ei ole seni eriti uuritud, ent samas on need andmed väga vajalikud puistu produktsiooni modelleerimiseks.

Töö tulemused näitasid, et õhulõhede tihedus on kõige olulisem õhulõhede morfoloogiline parameeter, mis võra vertikaalprofiilis muutub. Õhulõhede tihedus suurenes nii valguse intensiivsuse kui ka kõrguse tõustes, samal ajal kui õhulõhede mõõtmed uuritud liikidel kas vähenesid, suurenesid või jäid muutumatuks. Võrreldes võra alumiste ja ülemiste lehtede õhulõhede tihedusi, selgus et arukase võras suurenes õhulõhede tihedus 20% ning papliliikidel keskmiselt 45%. Mõõdetud ja arvutatud õhulõhede juhtivuse võrdlusest selgus, et valguslehtedel töötavad õhulõhed maksimaalsele avatusele väga lähedal, seevastu varjulehtede õhulõhed on vaid pooleldi avatud. Leiti, et õhulõhede struktuur määrab ligikaudu 30% õhulõhede juhtivuse varieeruvusest.

Kõrgendatud CO_2 tingimustes oli papliliikidel õhulõhede juhtivus ~20% madalam, samas kui õhulõhede tihedus vähenes ainult kahel esimesel aastal ning järgnevatel aastatel õhulõhede arvus ega mõõtmetes muutusi ei täheldatud. Fotosünteesi veekasutuse efektiivsus suurenes kõrgendatud CO_2 tingimustes, ja seda ilmselt põhjustatuna õhulõhede väiksemast avatusest suure CO_2 kontsentratsiooni korral.

Valguse intensiivsuse vähenedes lehestikus ~3–15 korda, langes maksimaalse fotosünteesi tase (fotosünteesivõime) 2–4 korda, samas kui maksimaalne õhulõhede juhtivus varieerus kaks korda vähem (vähenemine 1–1.9 korda). Vaatamata puu vanusele (arukask 40–50 a.; paplid 6 a.) ja kõrgusele (arukask ~20 m; paplid ~10 m), kohaneb fotosünteesi kiirus paindlikumalt valguse kättesaadavusega kui õhulõhede morfoloogia.

Taimelehtede üldlämmastiku sisaldus kirjeldab üsna hästi fotosünteesiaparaadi võimsust (Evans 1989). Meie töös oli lehe lämmastikusisaldus tugevalt

korreleeritud õhulõhede tiheduse ja juhtivusega. Positiivne seos õhulõhede tiheduse ja lehe lämmastikusisalduse vahel kinnitab, et morfoloogilised parameetrid on olulised õhulõhede juhtivuse ja maksimaalse fotosünteesikiiruse vahelise seose kujundajad. Sellest tulenevalt võib öelda, et puu gaasivahetus on puistu vertikaalprofiilis seotud nii õhulõhede tiheduse kui lehe lämmastikusisaldusega ning see teadmine lihtsustab suuresti puistu fotosünteetilise produktsiooni modelleerimist.

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Merilo E, Tulva I, Eensalu E, Räim O, Calfapietra C, Kull O.
Photosynthetic response of two poplar species to elevated CO_2 (EUROFACE)
in relation to leaf nitrogen partitioning. Manuscript

Photosynthetic response of two poplar species to elevated CO_2 (EUROFACE) in relation to leaf nitrogen partitioning

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Summary

The effect of long-term CO_2 enrichment on leaf nitrogen partitioning and photosynthesis was studied in two poplar species at the POP-EUROFACE (European FACE experiment with poplars) site during the second and third years following coppice. In *Populus alba*, light-saturated photosynthetic rate at growth CO_2 concentration (A_{max}) increased significantly by 42% under CO_2 enrichment over two years. In *Populus nigra*, a non-significant increase of 29% was observed in A_{max}. Stomatal conductance was significantly reduced by CO_2 enrichment in *P. alba* (22%) and in *P. nigra* (18%). Photosynthetic down-regulation, detected as decreased photosynthetic rate for plants grown in elevated CO_2 when compared with plants grown in ambient CO_2 at a common CO_2 concentration, was not detected in the studied species. In addition, neither maximum carboxylation rate (V_{cmax}) nor maximum rate of electron transport (J_{max}) were significantly reduced in *P. alba* and *P. nigra* due to CO_2 enrichment. Nitrogen re-allocation between leaf photosynthetic and non-photosynthetic pools was detected in *P. nigra* under elevated CO_2: over 2 years the partitioning into non-photosynthetic nitrogen increased by 15% due to CO_2 enrichment. Upper canopy leaves had higher area-based nitrogen content, leaf mass per area, A_{max}, stomatal conductance, V_{cmax} and J_{max} than corresponding leaves from lower canopy layers in both *P. alba* and *P. nigra*. With a few exceptions, the leaves from different canopy positions responded to CO_2 in a similar manner. The differences in photosynthetic response to elevated CO_2 between *P. alba* and *P. nigra* are discussed in relation to changes in leaf N partitioning.

Keywords: light-saturated net CO_2 assimilation, nitrogen partitioning, non-photosynthetic nitrogen, photosynthetic down-regulation, *Populus*, stomatal conductance.

Introduction

The key issues concerning the impact of increasing atmospheric CO_2 concentration on plants are the magnitude and persistence of photosynthetic stimulation and the resulting increment in growth and production. Photosynthetic down-regulation, detected as a lower photosynthetic rate for plants grown in high CO_2 compared with plants grown in ambient CO_2 at a common CO_2 concentration, has been found in many experiments (Medlyn et al. 1999, Norby et al. 1999). Down-regulation may be associated with adjustments at the biochemical level, evidenced as a reduction of V_{cmax} and J_{max} (abbreviations listed in Table 1) in leaves grown in elevated CO_2 (Medlyn et al. 1999, Ellsworth et al. 2004). Long-term studies with woody species have also revealed significantly decreased stomatal conductance in response to growth in elevated CO_2 (Medlyn et al. 2001). Both stomatal limitation and biochemical down-regulation were found to be important in meta-analyses of data from experiments on European forest tree species (Medlyn et al. 1999, Medlyn et al 2001).

Reduced leaf nitrogen content in elevated CO_2 is an important factor in biochemical down-regulation (Medlyn et al. 1999, Ellsworth et al. 2004). Moreover, down-regulation may be related to a down-ward shift in the relationship between photosynthetic parameters (V_{cmax} and J_{max}) and leaf nitrogen, which has been detected in some experiments (Medlyn et al. 1999), but not in others (Medlyn et al. 1999, Herrick and Thomas 2001, Ellsworth et al. 2004). J_{max} and V_{cmax} are often equally affected by elevated CO_2 (Medlyn et al. 1999, Crous and Ellsworth 2004, Ainsworth et al. 2003), indicating no re-allocation of leaf nitrogen within the photosynthetic system. However, a reduction in the ratio of V_{cmax}/J_{max} due to elevated CO_2 has been detected in *Lolium perenne* sward under particular conditions (Ainsworth et al. 2003) and in the recent meta-analysis of data from FACE experiments (Ainsworth and Long 2005).

Not all leaf nitrogen is involved directly in photosynthesis, but is present in non-photosynthetic proteins, leaf structural components, nucleic and amino acids and secondary compounds. This non-photosynthetic nitrogen fraction may reach 50% of the total leaf N (Evans 1989, Grindlay 1997, Evans and Poorter 2001, Takashima et al. 2004, Eichelmann et al. 2005). If the proportion of non-photosynthetic nitrogen increases under elevated CO_2 concentration, as has been shown for *Eucalyptus cladocalyx* (Gleadow et al. 1998), photosynthesis may be biochemically down-regulated even with no reduction in total leaf N. Moreover, sink limitation, accumulation of carbohydrates and resulting reduction in photosynthetic gene expression have been associated with down-regulation (Drake et al. 1997, Moore et al. 1999). Thus, as concluded by Medlyn et al. (1999), apparently several factors are involved in photosynthetic down-regulation under elevated CO_2.

Recent reviews have detected that long-term photosynthetic enhancement is greater in woody rather than herbaceous species (Nowak et al. 2004, Ainsworth and Long 2005). This seems particularly true for poplars, which have indeter-

minate growth patterns associated with high sink strength. It has therefore been suggested that poplars are likely to benefit consistently from elevated CO_2 atmosphere (Gielen and Ceulemans 2001). The poplar FACE experiment (POP-EUROFACE), investigating the effect of CO_2 enrichment on *Populus alba*, *P. x euramericana* and *P. nigra*, started in Italy in 1999 (Miglietta et al. 2001). Poplar is a highly productive species used for biomass production in short rotation forestry. For example, species planted at the EUROFACE site produced up to 18 Mg ha^{-1} y^{-1} of above-ground biomass at ambient CO_2 levels during the first rotation (Calfapietra et al. 2003a). During the second rotation, the annual above-ground biomass production in ambient conditions was even higher (20.9–25.8 Mg ha^{-1} y^{-1}) and with elevated CO_2 increased significantly (28.0–31.0 Mg ha^{-1} y^{-1}) (Liberloo et al. 2006). Published EUROFACE results concerning the magnitude and persistence of photosynthetic response have found high and sustained stimulation (Liberloo et al. 2007) with occasional down-regulation (Bernacchi et al. 2003a, Calfapietra et al. 2005).

The present study aimed to examine more closely the effects of CO_2 enrichment on partitioning of leaf nitrogen among different pools with particular emphasis on the potential role of leaf N in the photosynthetic down-regulation. Considering also that the partitioning between different leaf N pools changes vertically within the foliage (Niinemets and Tenhunen 1997, Evans and Poorter 2001, Eichelmann et al. 2005), measurements in different canopy positions may add further information on the vertical patterns of down-regulation. The vertical profiles of photosynthetic stimulation and potential down-regulation of photosynthesis, being related to nitrogen partitioning within the leaf, add valuable information to those who model the response of canopy processes to elevated CO_2. The following questions were asked: 1) Is photosynthetic stimulation in elevated CO_2 sustained at different canopy levels with no signs of down-regulation? 2) Does elevated CO_2 affect the partitioning of leaf nitrogen between photosynthetic and non-photosynthetic pools? 3) Is there any correlation between photosynthetic response and changes in leaf N partitioning?

Materials and Methods

The experimental site is located in Tuscania, Central Italy (N 42º22´, E 11º48´). Six rings with a diameter of 22 m were established in the experimental area with three rings serving as controls with ambient CO_2 and another three serving as elevated CO_2 treatments (daytime target concentration of 550 μmol mol^{-1}). These elevated CO_2 rings contain pipes releasing extra CO_2 to achieve uniform CO_2 enrichment. Each ring was divided into six equal sectors planted with three poplar species, two sectors per species: *P. alba* L. (genotype 2AS11), *P. nigra* L. (genotype Jean Pourtet) and *P. x euramericana* (genotype I–214). The selected genotypes have been characterized previously (Calfapietra et al. 2001). Planting density within the rings is 10^4 trees ha^{-1}. Outside the rings a poplar

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plantation of similar age was established (*P. x euramericana* genotype I–214, 5000 trees ha^{-1}) to minimize edge effects. The rings were irrigated regularly using drip irrigation system. The design of EUROFACE has been thoroughly described by Miglietta et al. (2001).

CO_2 fumigation started immediately after planting in 1999 and was maintained from bud burst to leaf fall during the first rotation cycle (1999–2001). In winter 2001–2002 the trees were coppiced and CO_2 fumigation was continued in March 2002 for the second rotation. The present study was performed during the second and third growth years of the second rotation in 2003 and 2004, respectively. During the second rotation, additional fertilization was added to three sectors of each experimental ring. Nitrogen fertilization was provided through the drip irrigation system once per week throughout the growing season. The total amount of N supplied was 212 kg ha^{-1} y^{-1} in 2002 and 290 kg ha^{-1} y^{-1} in 2003. Only the results for *P. alba* and *P. nigra* are presented in this report, the results for *P. x euramericana* have been published elsewhere (Calfapietra et al. 2005, Liberloo et al. 2007).

Gas exchange measurements

Gas exchange measurements were made using CIRAS–2 (PP Systems, Hitchin, UK) portable equipment with a halogen light source between September 5–13, 2003 and September 8–22, 2004. In 2003, photosynthesis measurements were performed on the leaves from the uppermost foliage quarter (sun leaves) and from the lowest quarter (shade leaves). In 2004, a third canopy level was added, so that average ISF (indirect site factor) in the second layer was 0.50 and that in the third layer 0.33 times lower than in the uppermost layer. Leaves were cut under degassed water and inserted into the gas exchange system. Leaf temperature was held at 25ºC and photosynthetic photon flux density (PPFD) was maintained at 1000 μmol m^{-2} s^{-1} during the measurement of A/c_i curves. PPFD of 1000 μmol m^{-2} s^{-1} was chosen to maintain a standard procedure throughout the experiment and to avoid photodamage of shade leaves. Measurements of A were performed starting at growth c_a, decreasing stepwise to 50 μmol mol^{-1} and then increasing stepwise to 1200 μmol mol^{-1}. For the leaves of ambient rings, CO_2 concentration in the incoming air thus changed in sequence 400, 250, 125, 50, 400, 600, 800, 1000 and 1200 μmol mol^{-1}. The corresponding sequence for the leaves of FACE rings was 600, 400, 250, 125, 50, 600, 800, 1000 and 1200 μmol mol^{-1}. Due to consumption by photosynthesis, the actual CO_2 concentration within the leaf chamber was about 20–60 μmol mol^{-1} lower than in the incoming air. Photosynthetic rates reached a steady state within 2–3 min following a change in c_a. Maximum photosynthetic rate (A_{max}) was detected as the measured value of photosynthesis (PPFD=1000 μmol m^{-2} s^{-1}) at growth CO_2 concentration (i.e. the first datapoint of A/c_i curve). In order to detect photosynthetic down-regulation (lower photosynthetic rate for plants grown in high CO_2 compared with plants grown in ambient CO_2 at a common CO_2 concentration), photosynthesis at ambient CO_2 concentration

was calculated for the leaves of FACE treatment as the value of CO_2 assimilation rate at a CO_2 concentration of 360 μmol mol^{-1} in the leaf cuvette. Light-saturated carboxylation rate (V_{cmax}) and maximum rate of electron transport (J_{max}) were calculated according to von Caemmerer and Farquhar (1981). The values of parameters K_c, K_o (Michaelis-Menten coefficients of Rubisco activity for CO_2 and O_2, respectively) and Γ* (CO_2 compensation point in the absence of mitochondrial respiration) at 25°C were used to calculate V_{cmax} and J_{max} as in Bernacchi (2003b).

In situ stomatal conductance (g_s) was measured with an AP–4 porometer (Delta-T Devices, UK). The leaves selected from two (in 2003) or three (in 2004) canopy levels were measured at least three times between 10 AM and 5 PM. In 2003, only abaxial leaf surfaces were measured for stomatal conductance, but in 2004, both leaf surfaces were followed and total stomatal conductance was calculated as the sum of adaxial and abaxial conductances. In *P. alba*, stomata exist only on abaxial leaf side and the data represent total g_s in both 2003 and 2004. *P. nigra*, however, is amphistomatous. In 2003, an extra set of measurements on 28 leaves revealed that the ratio of adaxial to abaxial g_s for *P. nigra* was 0.46 (±0.033) and was not significantly influenced by N fertilization. It has been previously found that both abaxial and adaxial leaf surfaces respond to elevated CO_2 similarly (Lake et al. 2001). The value of total leaf conductance for *P. nigra* is thus, on average, 1.46 times higher than the value presented in the Results section for 2003.

Light conditions at every sampled leaf were determined by hemispherical photography using a Nikon CoolPix 950 digital camera equipped with hemispheric lens. ISF, the ratio of diffuse light at leaf level to that above the canopy, was measured with WinSCANOPY software (Regent Instruments Inc., Canada), assuming standard overcast sky.

Analysis of leaf nitrogen and chlorophyll

In 2003, SPAD value was measured in leaves subjected to photosynthesis analysis with a Minolta SPAD–502 chlorophyll meter. The leaf mass per area (LMA) and leaf nitrogen concentration (N_M) were also determined in those leaves. LMA was calculated as the ratio of leaf dry mass (weight of leaves dried at 70ºC for 48 hours) to leaf area and used in the conversions between mass- and area-based expressions of N and Chl. Leaf area was determined using a Li-Cor 3100A leaf area meter (Li-Cor Inc., NE, USA). N_M was measured with PE 2400 Series II element analyser (Perkin Elmer, USA). SPAD values were calibrated against wet-extraction of chlorophyll from a sub-sample of leaves used for stomatal measurements. Circular disks of 10 mm in diameter were cut and chlorophyll from these disks was extracted in 3 ml of N, N`-dimethylformamide (DMF) for 48 hours in darkness. Absorption spectrum was recorded using an Ocean Optics spectrophotometer, and Chl concentrations were calculated according to Porra et al. (1989). Chlorophyll concentration of leaves used in photosynthetic measurements was calculated from SPAD values

using species- and treatment-specific linear regressions between SPAD and chlorophyll concentration. In 2004, circular disks of 10 mm were cut from the leaves of photosynthesis measurements in order to wet-extract the chlorophyll. The remainder of these leaves was used to determine LMA and N_M as described above.

Partitioning of leaf nitrogen into carboxylation (P_R), bioenergetics associated with electron transport (P_B) and thylakoid light-harvesting components (P_L) was calculated according to Niinemets and Tenhunen (1997) using values of V_{cmax}, J_{max} and leaf chlorophyll concentration, respectively. The fraction of leaf non-photosynthetic nitrogen (P_{non}) was calculated as:

$$P_{non}=1-P_R-P_B-P_L$$

Statistical analysis

Statistical analysis was performed using SPSS for Windows. Main treatment effects were tested using multivariate analysis (GLM procedure) with CO_2 level, fertilization level and canopy level as fixed factors. Since the effect of N fertilization was always non-significant, fertilization was excluded from the fixed factors, i.e. the two N levels were pooled, resulting in six measurements for every CO_2 and canopy level combination. A strong chlorosis occurred in plots 5 (elevated CO_2) and 6 (ambient CO_2) in September 2004 and therefore the data of these plots were excluded from further calculations. All effects were considered significant at $P<0.05$. Cases in which $P<0.1$ are also shown in Tables 2–3.

Results

September 2003

Leaf nitrogen concentration (N_M) was significantly lower under elevated CO_2 in *P. alba* sun leaves and in *P. nigra* shade leaves (Figure 1, Table 2). A significant interaction between CO_2 treatment and canopy level for N_M and N_A in *P. nigra* resulted from an opposite response to elevated CO_2 in sun and shade leaves. A significant positive FACE effect on A_{max} was detected only in *P. alba* (Figure 2, Table 2). The effect of CO_2 enrichment on LMA, N_A, A_{360}, v_{cmax} and J_{max} was non-significant in both species (Table 2, Figures 2–3). ISF was not significantly influenced by elevated CO_2, indicating that the corresponding leaves of two treatments were compared under similar light conditions (Table 2, Figure 3). Expressing the photosynthetic characteristics (A_{max}, v_{cmax}, J_{max}) on a mass basis did not affect the results (data not shown). Canopy level strongly affected LMA, N_M, N_A, A_{max}, A_{360}, v_{cmax}, J_{max} and g_s with higher values detected in sun leaves than in the corresponding shade leaves (Table 2, Figures 1–3).

Stomatal conductance was significantly reduced by CO_2 enrichment (Figure 3, Table 2). In *P. alba*, elevated CO_2 resulted in a reduction of *in situ* abaxial g_s

by 13% in sun leaves and 28% in shade leaves. The corresponding reduction for *P. nigra* was 20% and 9%, respectively.

Nitrogen partitioning within the photosynthetic system was influenced by canopy level (Figure 4, Table 2): shade leaves had higher P_L, but lower P_R and P_B than corresponding sun leaves. The partitioning between photosynthetic and non-photosynthetic N pools was not significantly influenced by canopy level. CO_2 enrichment had no statistically significant effect on nitrogen partitioning, though a tendency to partition less nitrogen into carboxylation and more into non-photosynthetic fraction was observed in *P. nigra* leaves grown in elevated CO_2 compared with corresponding leaves grown in ambient CO_2 (Figure 4).

The correlation between N_A-V_{cmax} and N_A-J_{max} was strong (Figures 5a and 5b). In *P. alba*, these relationships were unaffected by CO_2 enrichment, but in *P. nigra*, elevated CO_2 tended to reduce the slopes of N_A-V_{cmax} and N_A-J_{max} regressions. This effect was, however, not statistically significant.

September 2004

A significant positive FACE effect on A_{max} was detected in *P. alba* (Figure 2, Table 2), whereas this stimulative effect of elevated CO_2 on A_{max} was non-significant in *P. nigra*. The effect of CO_2 enrichment on A_{360}, v_{cmax} and J_{max} (expressed either on area or mass basis) was non-significant (Table 2, Figure 2 for A_{360}). Stomatal conductance was significantly reduced by CO_2 enrichment in *P. alba* (4–44% in the leaves from different canopy layers), whereas this reduction (14–30%) was not significant in *P. nigra* (Figure 3, Table 2). On the other hand, a significant FACE effect in *P. nigra* on nitrogen partitioning was observed: less leaf nitrogen was partitioned into Rubisco and more into non-photosynthetic nitrogen compared with leaves grown in ambient CO_2 conditions (Figure 4, Table 2). The effect of canopy level was strong also in 2004, with higher values of LMA, N_A, N_M, A_{max}, A_{360}, v_{cmax}, J_{max} and g_s detected in the upper canopy layers.

The combined data of two years revealed a strong (42%) FACE effect on A_{max} in *P. alba* (Table 3). In *P. nigra*, the photosynthetic stimulation under FACE treatment was 29%, but not statistically significant. Stomatal conductance was significantly reduced due to CO_2 enrichment in *P. alba* and *P. nigra*. Both species showed changes in nitrogen partitioning due to elevated CO_2 (Table 3). In *P. alba*, FACE changed partitioning within the photosynthetic N pool: P_B was significantly higher in the leaves of FACE treatment. The partitioning between photosynthetic and non-photosynthetic N pools was not affected by CO_2 enrichment in *P. alba*. In *P. nigra*, on the other hand, the negative effect of FACE on P_R and the positive effect of FACE on P_{non} were significant. LMA and N_A also tended to be higher in the leaves of elevated CO_2 in *P. nigra*. Though the photosynthesis measured at a common CO_2 concentration of 360 μmol mol^{-1} was lower in the leaves of FACE treatment (Table 3, E/A values 0.89 and 0.84 for *P. alba* and *P. nigra*, respectively), this change was not significant.

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Discussion

A review by Nowak et al. (2004) based on 16 FACE sites detected a mean stimulation in leaf assimilation of 26% due to elevated CO_2, with woody species being more responsive than herbaceous ones. In the recent FACE review by Ainsworth and Rogers (2007), trees were also found to show the greatest photosynthetic response (40–50% increase) and the least changes in V_{cmax} and J_{max} under FACE regime. Liberloo et al (2007) analyzed the combined photosynthetic response of all three EUROFACE poplar species in 2004 and found a significant photosynthetic stimulation of 49% under FACE treatment. We studied *P. alba* and *P. nigra* separately and detected a significant positive effect of elevated CO_2 on A_{max} in *P. alba*, but the photosynthetic stimulation in *P. nigra* during 2003–2004 was non-significant. Our results indicate that the magnitude of photosynthetic response differs in studied species, *P. x euramericana* being the most responsive with stimulation of 54% (Liberloo et al. 2007) and *P. nigra* the least responsive. The stimulation of total biomass production during the second rotation was, in contrast, the highest in *P. nigra* (Liberloo et al. 2006).

Recent EUROFACE articles claim that photosynthetic down-regulation does not occur in poplars, because the sink capacity is large and nitrogen is not limiting (Liberloo et al. 2006, Liberloo et al. 2007). However, sporadic photosynthetic down-regulation associated with particular species, leaf position and timing during the vegetation period has been found in some EUROFACE papers (Bernacchi et al. 2003a, Calfapietra et al. 2005). Davey et al. (2006) discussed that any down-regulation of photosynthetic capacity in these highly productive poplars is short-term and transient — associated more likely with decreased activity rather than decreased levels of photosynthetic enzymes. In order to prevent accumulation of soluble sugars and resulting photosynthetic down-regulation, poplars are able to synthesize more starch under elevated CO_2 conditions (Davey et al. 2006). Our results revealed no significant evidence of photosynthetic down-regulation in *P. alba* and *P. nigra* during 2003–2004. However, a lower photosynthetic rate was detected for plants grown in elevated CO_2 compared with plants grown in ambient CO_2 at a common CO_2 concentration, though this change was non-significant (Table 3). Moreover, the tendency for lower V_{cmax} under elevated CO_2 was evident within the combined data (Table 3). Considering also the non-significant photosynthetic FACE stimulation and altered N partitioning between photosynthetic and non-photosynthetic N pools in *P. nigra*, we cannot maintain that photosynthetic down-regulation did not occur. In *P. x euramericana*, the September measurements took place after bud set, when shade leaves exhibited photosynthetic down-regulation associated with reduced sink size and photosynthate accumulation (Calfapietra et al. 2005). The bud set was observed later in *P. alba* (in October) and in *P. nigra* (late September-October) (Calfapietra et al. 2003b), i.e. subsequent to our measurements. Photosynthetic

down-regulation associated with reduced sink size after bud set was therefore probably not evident in our experiment.

The non-significant photosynthetic FACE response of *P. nigra* could be associated with differences in light availability between CO_2 treatments at common height. We detected no significant differences in light availability between the corresponding leaves of different treatments in either *P. alba* or *P. nigra* (ISF in Table 2). However, the leaves of elevated CO_2 treatment always received less light than the corresponding leaves of ambient CO_2 treatment in *P. nigra* (Figure 3). This difference in leaf growth conditions, even if not significant, could account for — in part — lower stimulation of A_{max} under elevated CO_2 in *P. nigra*. On the other hand, accelerated development did not interfere with the response of A_{max} to elevated CO_2 in either species, since no evidence (lower SPAD/Chl, v_{cmax}, J_{max}) suggested that leaves grown in elevated CO_2 were physiologically older compared to those grown in ambient CO_2. Although accelerated leaf development has been detected in a high CO_2 environment (Sicher 1998, Sild et al. 1999), a delayed autumnal senescence was found in EUROFACE under elevated CO_2 (Tricker et al. 2004, Taylor et al. 2007).

Stomatal conductance was significantly reduced by CO_2 enrichment in both species. This is a general response to elevated CO_2 concentration (Medlyn et al. 2001) and has been found in EUROFACE as well (Calfapietra et al. 2005, Tricker et al. 2005), though the results by Bernacchi et al. (2003a) represent an exception. Reduction in stomatal conductance owing to CO_2 enrichment is usually considered to occur due to reduced stomatal aperture (controlled by the guard cells) and is therefore transient. However, Tricker et al. (2005) found that both stomatal index and stomatal density were reduced by FACE treatment during the first years of the first rotation. Our preliminary results from the second rotation concerning changes in the stomatal density and in the sizes of the guard cells indicate that stomata themselves were smaller under FACE treatment, although stomatal density remained unchanged (unpublished results). If so, the reduction in stomatal conductance due to elevated CO_2 may be, at least partly, a long-term and persistent response, leading to photosynthetic acclimation.

Signs of nitrogen re-allocation in *P. nigra* between photosynthetic and non-photosynthetic leaf N pools were detected: more nitrogen was partitioned into the non-photosynthetic fraction as a result of growth in elevated CO_2. This re-allocation was significant for 2004 and combined data; the same tendency was observed in 2003. Gleadow et al. (1998) suggested that leaf nitrogen could be re-allocated from photosynthesis to other functions such as defence or storage under high CO_2 regimes. Though poplars rely more on carbon-based secondary metabolites (phenolic glycosides and condensed tannins) for protection against herbivores (Harborne 2001), levels of polyamines in the leaves and roots of poplar have been shown to increase under, for example, nutrient deficiency (Houman et al. 1991). Greater allocation of leaf nitrogen into non-

photosynthetic pool is hypothesized to be associated with a downward shift in the relationships between photosynthetic parameters (V_{cmax} and J_{max}) and leaf nitrogen (Medlyn et al. 1999). This downward shift was non-significant in 2003 (Figures 5a and 5b) and in 2004 (Liberloo et al. 2007). Generally, an increase in non-photosynthetic N occurs during leaf senescing, given that N is cycled from leaves to perennial plant parts in the form of N-rich amino acids and other mobile nutrients (Cooke and Weih 2005). We cannot conclude, however, that the higher proportion of non-photosynthetic nitrogen in the leaves of *P. nigra* from FACE treatment is related to accelerated development, since no other evidence supports that conclusion (as discussed above). In *P. alba*, altered N partitioning within the photosynthetic pool was observed for the combined data, with more nitrogen allocated into electron transport components (higher P_B under FACE treatment in Table 3). This response is beneficial in high CO_2 environments, but is rarely detected experimentally (Medlyn et al. 1999).

Light environment strongly affected several leaf parameters (LMA, N_M, N_A, A_{max}, A_{360}, v_{cmax}, J_{max} and g_s) — a typical response from a shade-intolerant species (Kubiske et al. 2002). In addition, P_L was consistently higher in shade leaves, indicating that more N was partitioned into light harvesting components in shade. In CO_2 research, more attention has been paid to the CO_2 response of sunlit leaves growing in the upper canopy levels. Differences in stomatal and photosynthetic responses to high CO_2 between various canopy positions have been found in some studies (Kubiske et al. 1997, Takeuchi et al. 2001, Kubiske et al. 2002, Crous and Ellsworth 2004), but not in others (Gunderson et al. 2002, Herrick and Thomas 2001, Herrick et al. 2004). The differences in CO_2 response between the leaves from different canopy levels in our experiment (Table 2, interaction CO_2*canopy level) were associated with either (1) leaves at different levels responding to elevated CO_2 in a similar direction, but with a different magnitude (e.g. interaction between CO_2 treatment and level for g_s in *P. nigra*) or (2) leaves from different levels responding to elevated CO_2 in an opposite direction (e.g. interaction between CO_2 treatment and canopy level for N_M and N_A in *P. nigra*). These few significant interactions between CO_2 treatment and canopy level did not result in differences in photosynthetic response to elevated CO_2 between sun and shade leaves.

The general insensitivity of leaf characteristics to N fertilization is somewhat surprising. One likely reason for the insensitivity of leaf characteristics to improved nutrition could be that the relatively fertile soil provides sufficient nutrients in the unfertilized plots. Soil N did not decline during the period 1998–2004 even in unfertilized plots, indicating a high mineralization rate (Liberloo et al. 2006). Moreover, Calfapietra et al. (2007) concluded that although elevated CO_2 increased productivity of poplars, N uptake from soil was unaffected and additional N was not needed to support higher productivity in an elevated CO_2 atmosphere. They suggested, however, that N may become limiting during the next rotations (Calfapietra et al. 2007). The second rotation in EUROFACE has produced non-significant nitrogen fertilization effects on photosynthetic

parameters (Calfapietra et al. 2005; Liberloo et al. 2007), but occasionally significant fertilization effects on N_M and LMA (Calfapietra et al. 2005). Biomass production was unaffected by fertilization (Liberloo et al. 2006).

To conclude, the response of *P. alba* to elevated CO_2 was typical of trees with high genetic productivity potential growing in fertile conditions. The photosynthetic stimulation caused by growth in elevated CO_2 was great and sustained over two seasons with no significant biochemical down-regulation in the leaves of FACE treatment. The stomatal conductance was significantly reduced due to elevated CO_2 treatment in *P. alba*. In *P. nigra*, the photosynthetic stimulation was non-significant. *P. nigra* showed nitrogen re-allocation between photosynthetic and non-photosynthetic fractions, with more leaf nitrogen partitioned into non-photosynthetic pool under elevated CO_2. Greater allocation of leaf nitrogen into non-photosynthetic fraction may be one reason for the non-significant photosynthetic stimulation in *P. nigra*.

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Table 1. List of symbols and abbreviations

Symbol	Definition
A_{max}	Light-saturated net CO_2 assimilation at growth c_a (μmol m^{-2} s^{-1})
A_{360}	Light-saturated net CO_2 assimilation at c_a of 360 μmol mol^{-1}(μmol m^{-2} s^{-1})
c_a	Ambient CO_2 concentration (μmol mol^{-1})
c_i	Leaf intercellular CO_2 concentration (μmol mol^{-1})
FACE	Free air CO_2 enrichment
g_s	Stomatal conductance (mmol m^{-2} s^{-1})
ISF	Indirect site factor
J_{max}	Maximum rate of electron transport (μmol electrons m^{-2} s^{-1})
LMA	Leaf mass per area (g m^{-2})
N_A	Leaf area-based nitrogen content (g m^{-2})
N_M	Leaf mass-based nitrogen concentration (%)
PPFD	Photosynthetic photon flux density (μmol photons m^{-2} s^{-1})
P_B	Partitioning of leaf N into bioenergetics associated with electron transport
P_L	Partitioning of leaf N into light harvesting
P_{non}	Partitioning of leaf N into non-photosynthetic nitrogen pools
P_R	Partitioning of leaf N into carboxylation
SPAD	Index of relative chlorophyll content
v_{cmax}	Maximum carboxylation rate (μmol CO_2 m^{-2} s^{-1})

2 3

Table 2. Multivariate analysis of variance for different leaf characteristics (GLM procedure of SPSS, CO_2 concentration and canopy level as fixed factors) in 2003–2004. ns=not significant. All effects were considered significant at $P<0.05$. Cases in which $P<0.1$ are shown.

		P. alba			P. nigra		
		CO_2	canopy level	CO_2*level	CO_2	canopy level	CO_2*level
N_M	2003	0.033	0.004	ns	ns	0.000	0.009
	2004	ns	0.006	ns	ns	0.000	ns
N_A	2003	ns	0.000	ns	ns	0.000	0.028
	2004	ns	0.000	ns	ns	0.000	ns
LMA	2003	ns	0.000	ns	ns	0.000	ns
	2004	0.094	0.000	0.050	ns	0.001	ns
A_{max}	2003	0.036	0.001	ns	ns	0.000	ns
	2004	0.006	0.000	ns	0.061	0.001	ns
A_{360}	2003	ns	0.000	ns	ns	0.001	ns
	2004	ns	0.000	ns	ns	0.001	ns
V_{cmax}	2003	ns	0.000	ns	ns	0.000	ns
	2004	ns	0.000	ns	ns	0.000	ns
J_{max}	2003	ns	0.000	ns	ns	0.000	ns
	2004	ns	0.001	ns	ns	0.000	ns
ISF	2003	ns	0.000	ns	ns	0.000	ns
	2004	0.051	0.000	ns	ns	0.000	ns
g_s	2003	0.021	0.000	ns	0.008	0.000	0.042
	2004	0.049	0.000	ns	ns	0.000	ns
P_R	2003	ns	0.027	ns	ns	ns	ns
	2004	ns	ns	0.031	0.016	ns	ns
P_B	2003	ns	ns	ns	ns	ns	ns
	2004	ns	ns	0.016	ns	ns	ns
P_L	2003	ns	0.000	ns	ns	0.000	ns
	2004	ns	0.000	0.006	ns	0.000	ns
P_{non}	2003	ns	ns	ns	ns	ns	ns
	2004	ns	ns	0.006	0.031	0.009	ns

Table 3. FACE effect (E/A) for combined data (years and light levels together). Significance is indicated as * P<0.1 and ** P<0.01

	P. alba	P. nigra
LMA	1.08	1.09*
N_M	0.92**	1.00
N_A	1.01	1.11*
A_{max}	1.42**	1.29
v_{cmax}	0.95	0.92
J_{max}	1.06	1.09
A_{360}	0.89	0.84
ISF	0.93	0.92*
g_s	0.78**	0.82**
P_R	0.96	0.82**
P_B	1.10**	1.00
P_L	1.05	0.91
P_{non}	1.00	1.15**

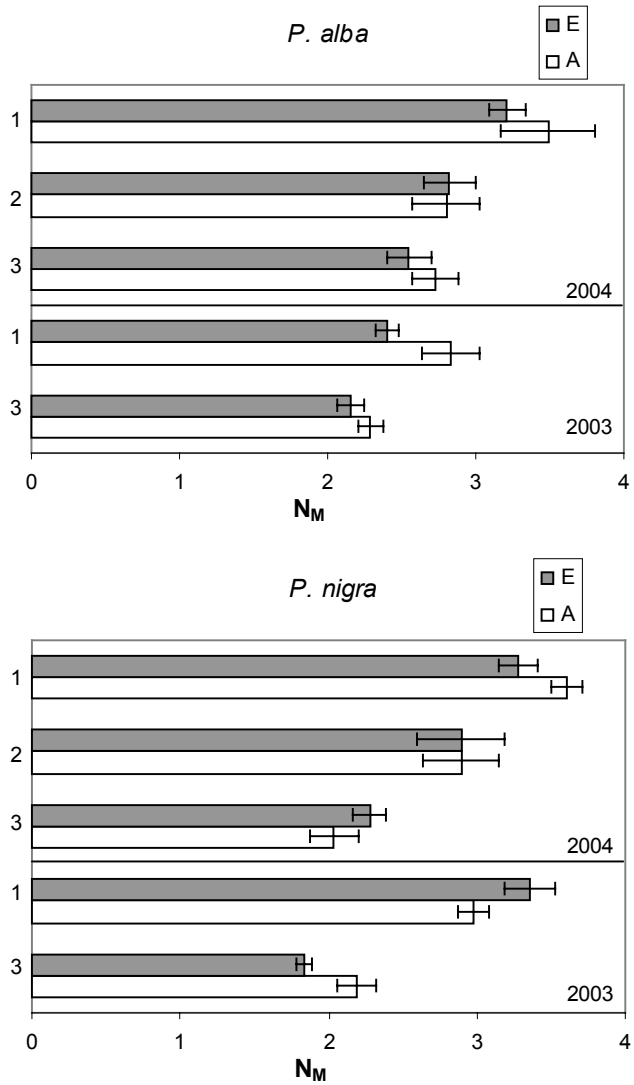

Figure 1. N_M (%; average and SE) in leaves of different treatments and canopy positions. Numbers 1–3 on ordinate axis refer to vertical canopy levels (1=uppermost, 3=lowest level). E=elevated CO_2 ; A=ambient CO_2 .

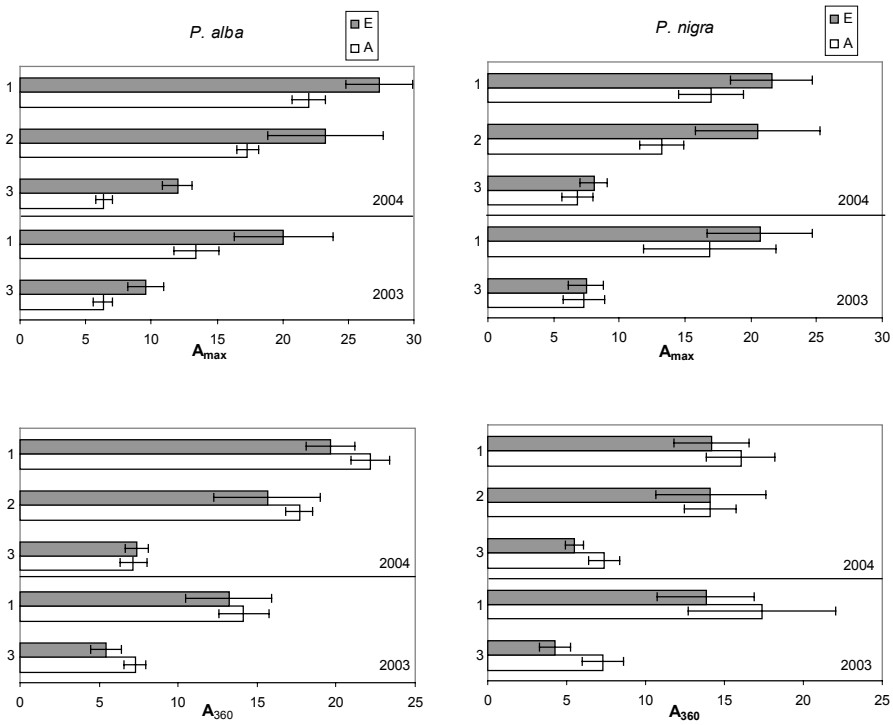

Figure 2. A_{max} (μmol m^{-2} s^{-1}) and A_{360} (μmol m^{-2} s^{-1}) in leaves of different treatments and canopy positions. Otherwise as in Figure 1.

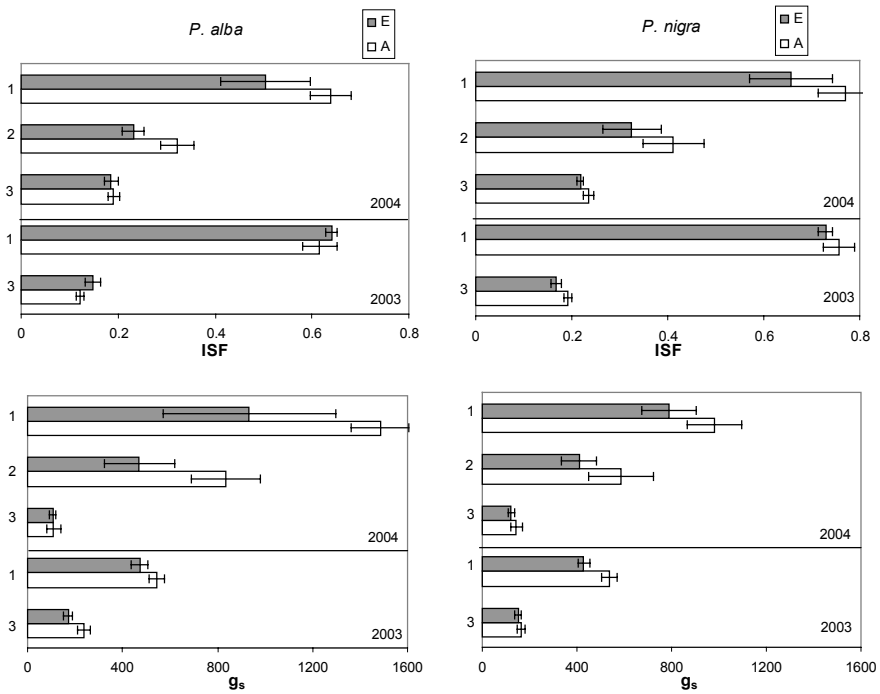

Figure 3. ISF (indirect site factor) and *in situ* stomatal conductance (g_s, mmol m^{-2} s^{-1}) from different treatments and canopy levels. Total stomatal conductance for *P. nigra* in 2003 is on average 1.46 times higher than the value presented in Figure 3. Otherwise as in Figure 1.

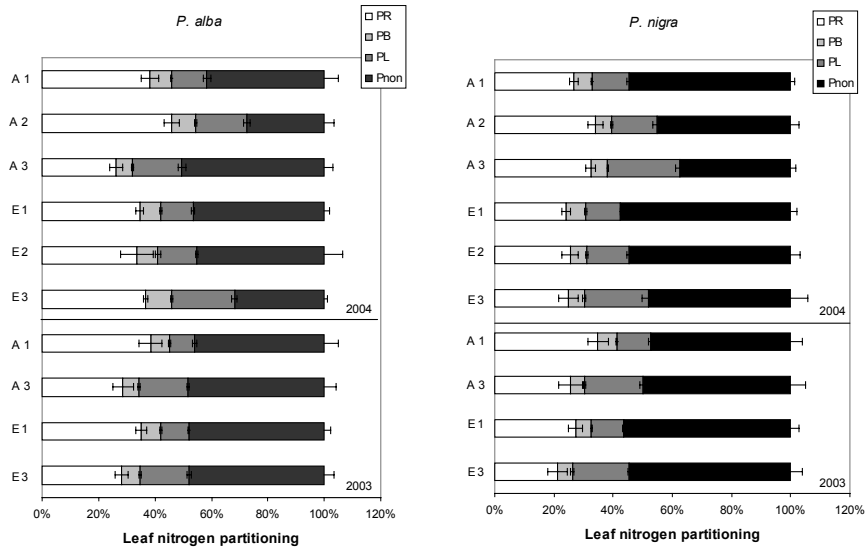

Figure 4. Leaf nitrogen partitioning. E=elevated CO_2; A=ambient CO_2. Numbers 1–3 refer to vertical canopy levels (1=uppermost, 3=lowest level). P_R=partitioning of leaf nitrogen into carboxylation, P_B=partitioning into bioenergetics associated with electron transport, P_L=partitioning into thylakoid light-harvesting components and P_{non}= partitioning into non-photosynthetic pool.

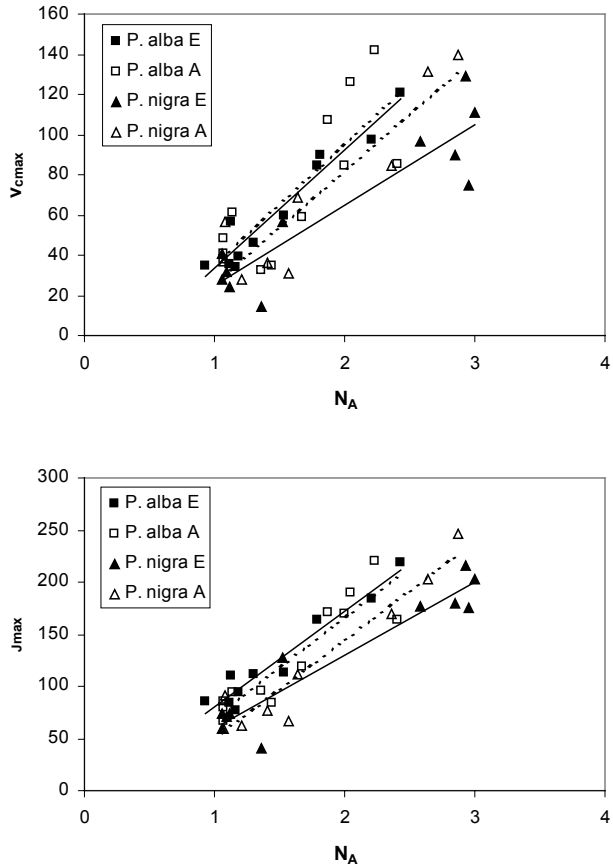

Figure 5a. Relationships between N_A (g m^{-2}) and V_{cmax} (µmol CO_2 m^{-2} s^{-1}) for studied treatments in 2003. Filled symbols and solid lines, elevated CO_2; open symbols and dashed lines, ambient CO_2. All regressions were significant at $P<0.05$ (R^2=0.64–0.93) 5b. Relationships between N_A (g m^{-2}) and J_{max} (µmol electrons m^{-2} s^{-1}) in 2003. Symbols as in Figure 5a. All regressions were significant at $P<0.05$ (R^2=0.83–0.94) Corresponding figures for 2004 are in Liberloo et al. (2007).

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Eensalu E, Kupper P, Sellin A, Rahi M, Sõber A, Kull O. 2008. Do stomata operate at the same relative opening range along a canopy profile of *Betula pendula*? *Functional Plant Biology* 35: 103–110.

Tricker PJ, Trewin H, Kull O, Clarkson, GJJ, Eensalu E, Tallis M, Colella A, Doncaster CP, Sabatti M, Taylor G. 2005. Stomatal conductance and not stomatal density determines the long-term reduction in leaf transpiration of poplar in elevated CO_2. *Oecologia* 143: 652–660.

Calfapietra C, Tulva I, Eensalu E, Perez M, De Angelis P, Scarascia-Mugnozza G, Kull O. 2005. Canopy profiles of photosynthetic parameters under elevated CO_2 and N deposition in a poplar plantation. *Environmental Pollution* 137: 525–535.

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Grants and scholarships:

- Senior personnel in two grants:
  ETF7016 (Robert Szava-Kovats) Stomatal control of photosynthesis acclimation to light.
  ETF6969 (Anu Sõber) Effect of air humidity on interactions between transpiration, photosynthesis and growth rate in fast-growing deciduous trees.
- Scholarship from Estonian Students' Fund USA, 2005
- Scholarship from Marie Curie (HPMT-CT-2001-00259), 2006
- Scholarship from Doctoral School of Ecology and Environmental Sciences, for participation in conference (2006) and for scientistic research in Duke University, USA (2007).

Conference theses:

Eensalu E, Tulva I, Räim O, Calfapietra C, Kull O. Stomatal conductance and stomatal density of three poplar species at the end of and after six years of CO_2 fumigation. Conference "Plant and Environmental Pollution ICPEP-3", Lucknow, India, 29 November–2 December, 2005.

Eensalu E, Tulva I, Räim O, Surovy P, Kull O. Carry-over effects in stomatal conductance after the FACE treatment in poplar species. Conference "International conference in ecophysiology of plants", Olomouc, Czeck Republic, 18–21 September, 2006.

III. Improvement of skills

- Scientistic research in University of Goettingen, Goettingen: April–June, 2006
- Scientistic research in Duke University, Durham, USA: November–December, 2007

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Calfapietra C, Tulva I, Eensalu E, Perez M, De Angelis P, Scarascia-Mugnozza G, Kull O. 2005. Canopy profiles of photosynthetic parameters under elevated CO_2 and N deposition in a poplar plantation. *Environmental Pollution* 137: 525–535.

Saadud uurimistoetused ja stipendiumid:

– Põhitäitja kahes grandis:

ETF7016 (Robert Szava-Kovats) Õhulõhede limitatsioon fotosünteesi valgusele kohanemisel;

ETF6969 (Anu Sõber) Õhuniiskuse mõju kiirekasvuliste lehtpuude transpiratsiooni, fotosünteesi ja kasvukiiruse vahelistele seostele.

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– Marie Curie stipendium (HPMT-CT-2001-00259), 2006
– Stipendium Ökoloogia ja keskkonnateaduste doktorikoolist, osalemiseks konverentsil (2006) ja õppimiseks USAs, Duke Ülikoolis (2007).

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Eensalu E, Tulva I, Räim O, Calfapietra C, Kull O. Stomatal conductance and stomatal density of three poplar species at the end of and after six years of CO_2 fumigation. Konverents"Plant and Environmental Pollution ICPEP-3", Lucknow, India, 29.november-2.detsember, 2005.

Eensalu E, Tulva I, Räim O, Surovy P, Kull O. Carry-over effects in stomatal conductance after the FACE treatment in poplar species. Konverents "International conference in ecophysiology of plants", Olomouc, Tšehhi, 18–21. september, 2006.

III. Erialane enesetäiendus

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