Title: Response of tree growth and wood structure of *Larix kaempferi*, *Kalopanax septemlobus* and *Betula platyphylla* saplings to elevated CO_2 concentration in a FACE system for 5 years

Running head: RESPONSE OF FOREST TREES TO ELEVATED CO_2

Authors:

YOKO WATANABE[1,2], KEITA WAKABAYASHI[3], SATOSHI KITAOKA[1,4], TAKAMI SATOMURA[1,5], NORIKAZU EGUCHI[1,2], MAKOTO WATANABE[2,3], SATOSHI NAKABA[3], KENTARO TAKAGI[1], YUZOU SANO[2], RYO FUNADA[3] and TAKAYOSHI KOIKE[1,2*]

Authors' affiliations and addresses:

1. Field Science Center for Northern Biosphere, Hokkaido University, Sapporo 060-0809, Japan, youko@for.agr.hokudai.ac.jp

2. Research Faculty of Agriculture, Hokkaido University, Sapporo 060-8589, Japan

3. Faculty of Agriculture, Tokyo University of Agriculture and Technology, Fuchu - Tokyo 183-8509, Japan

4. Forestry and Forest Products Research Institute, Hitsujigaoka-7, Toyohira, Sapporo 062-8516, Japan

5. Faculty of Agriculture, Kagawa University, Kida 761-0795, Japan

* corresponding author

Corresponding author:

Dr. Takayoshi Koike

Tel: +81-11-706--3854

Fax: +81-11-706-2517

tkoike@for.agr.hokudai.ac.jp

Key words: elevated CO_2, FACE, tree growth, water-conducting cells, dye injection

Key Message:

Elevated CO_2 concentration affected biomass partitioning in above-ground biomass, but size and number of water-conducting cells were unchanged in *Larix kaempferi*, *Kalopanax septemlobus* and *Betula platyphylla*.

Abstract

Using a Free Air CO_2 Enrichment (FACE) system, we studied the effect of elevated CO_2 on the growth, leaf gas exchange and xylem anatomy of a conifer, *Larix kaempferi*, and two angiospermous tree species, *Kalopanax septemlobus* and *Betula platyphylla*. Two-year-old seedlings were grown at control sites (ambient; 370 ppm) and FACE sites (elevated; 500 ppm) for 5 years. We measured the lumen area and number of water-conducting cells, as well as biomass and leaf gas exchange, and visualized the functional region of water transport using a dye injection experiment. Elevated CO_2 did not induce any significant changes in growth or in leaf gas exchange or lumen area of earlywood tracheids in *L. kaempferi* relative to ambient CO_2. In two other tree species, elevated CO_2 was found to enhance tree height and total leaf area, with no change in stomatal conductance. In *K. septemlobus* there were no changes in lumen area or number of earlywood vessels, or in the functional region of water transport. *B. platyphylla* also underwent no changes in lumen area or number of vessels, although there was a yearly variation in the size of the vessels. Our results show that five years of CO_2 exposure did not notably affect the anatomical features of water-conducting cells. This finding suggests that, under elevated CO_2, trees respond to changes in water balance due to changes in LA by extending the hydraulically active area of xylem.

Introduction

With increasing atmospheric CO_2 concentration, forest trees comprise an important CO_2 sink (Körner 2003). The stem is a particularly useful carbon (C) sink because trees accumulate new cells with solid cell walls in their stems over long periods (Hyvönen et al. 2007). Changes in tree growth and in the anatomical features of the secondary xylem in the stem under elevated CO_2 concentration might therefore affect the C sink capacity of these trees.

A meta-analysis of Free-air CO_2 enrichment (FACE) experiments found increases in the height, stem diameter, leaf area index and above-ground dry mass of trees grown under elevated CO_2 concentrations (Ainsworth and Long 2005). Ainsworth and Rogers (2007) suggested that there is an increase in light saturated photosynthesis (A_{sat}) and a decrease in stomatal conductance (g_s) in C_3 plants grown under elevated CO_2.

Many studies have found, however, that long-term CO_2 exposure does not induce any change in the lumen size of water-conducting cells of softwoods or hardwoods (e.g. Kilpeläinen et al. 2007; Watanabe et al. 2010). Kilpeläinen et al. (2007) found that the tracheid lumen diameter of latewood of 20-year-old Scots pine grown under elevated CO_2 for six years in a closed chamber was not significantly changed by elevated CO_2, at least during those six years, but that in earlywood an increase in lumen diameter took place in only a single year. Kostiainen et al. (2006) reported that the vessel lumen diameter in 7-year-old *Betula pendula* clones grown in an open-top chamber for three years does not respond to elevated CO_2. The vessel lumen diameter in *Populus tremuloides* clones was not significantly altered from ambient values by three-year CO_2

exposure in FACE experiments, although this parameter tends to increase under elevated CO_2 (Kaakinen et al. 2004). On the other hand, *P. tremuloides* showed no effect of elevated CO_2 on vessel lumen diameter during five-year exposure (Kostiainen et al. 2008).

Elevated CO_2 reportedly increases the size of tracheary elements, however (Kostiainen et al. 2009; Watanabe et al. 2010; Kostiainen et al. 2014). Kostiainen et al. (2009) found an increase in radial diameter of latewood tracheid in *Picea abies* grown under elevated CO_2 in whole-tree chambers. Watanabe et al. (2010) reported that the earlywood vessel lumen area in *Kalopanax septemlobus* saplings grown during 3 years of CO_2 exposure tends to be greater than in control saplings, but no effect of CO_2 on the size of vessels was found in *Quercus mongolica*, *Betula maximowicziana* or *Acer mono* grown in the FACE system. Kostiainen et al. (2014) determined that elevated CO_2 enhanced the vessel diameter in *Populus tremuloides* clones, but not in *Betula papyrifera*, in their FACE experiments. It is clear that the effect of elevated CO_2 on anatomical features of tracheary elements depends on species and on the experimental conditions and that more information is needed to determine the reasons.

The effect of elevated CO_2 on anatomical features of water-conducting cells is believed to be indirect. Kostiainen et al. (2014) stated that the enhanced growth rate of *Populus tremuloides* clones under elevated CO_2 is related to the decrease in cell wall thickness and wood density. Changes due to elevated CO_2 in leaf gas exchange, as measured by parameters such as A_{sat} and g_s, and in biomass allocation, measured by (for instance) the total leaf area, are believed to affect the formation of water-conducting

cells (e.g. Tyree and Alexander 1993; Koike et al. 2015). If g_s decreases without any increase in total leaf area under elevated CO_2 concentrations, we expect a decrease in the size of water-conducting cells so as to maintain the hydraulic balance within the tree. If, on the other hand, the total leaf area increases due to elevated CO_2 concentration, a tree would need more water and the size of water-conducting cells would change in size regardless of changes in g_s. Watanabe et al. (2010) suggested that the increase in vessel lumen area in earlywood in *K. septemlobus* saplings is due to changes in water status and/or the concentration of growth regulators in the stem, via enhancement of the tree height and leaf area. It is therefore necessary to consider the effect of CO_2 on anatomical features of water-conducting cells, together with tree growth and leaf gas exchange traits.

Furthermore, changes in leaf gas exchange and biomass allocation under elevated CO_2 concentrations might alter the functional region for water transport in the stem, because the water transport pathway in the stem is an important component of the soil-plant-atmosphere-continuum (SPAC). Domec et al. (2010) have reported an increase in maximum hydraulic specific conductivity, and a decrease in xylem resistance to embolism, in branches of two diffuse-porous hardwoods, *Liquidambar styraciflua* and *Cornus florida*, but found no correlations between physiological changes and anatomical changes under elevated CO_2 concentrations. A few detailed studies nevertheless connect tree growth and leaf gas exchange with anatomical features of water-conducting cells and water transport pathways in the xylem of forest trees growing under elevated CO_2 concentration (e.g. Norby and Zak 2011; Vaz et al. 2012;

Koike et al. 2015).

The present study sought to determine whether elevated CO_2 affects the wood structure of three tree species through changes in tree growth and leaf gas exchange during five years of CO_2 exposure in a FACE system. We looked at the effect of CO_2 on (1) tree growth, biomass allocation and leaf gas exchange, (2) anatomical features of water-conducting cells, and (3) the functional region of water transport in the stem. Based on the results, we discuss the structural changes in growth and development of tree saplings exposed to elevated CO_2 in relation to water use within the tree.

Materials and Methods

FACE experimental design

Our FACE facilities (three enriched CO_2 sites with CO_2 gas supply system) and control sites (three ambient sites) were set up in the Sapporo Experimental Nursery of Hokkaido University Forests (Sapporo, 43˚06'N, 141˚20'E), in autumn 2002. Each site was a circular plot, 6.5 m in diameter and 5 m in height. Fertile brown forest soil was provided in half of each site, and infertile volcanic ash soil was provided in the other half (Eguchi et al. 2005a, 2008). The CO_2 concentration inside the FACE sites was maintained at around 500 μmol mol^{-1}, corresponding to ambient plus 130 μmol mol^{-1} (Takagi et al. 2004).

In May 2003, two-year-old seedlings of one species of conifer and ten species of deciduous broad-leaved trees that grow in cool temperate forests in northern Japan were planted randomly in each soil type. CO_2 fumigation took place from July 2003 to

August 2007, during the annual growing season (from mid-April to mid-November). These seedlings were grown under natural rainfall. Total precipitation during the growing season from April to September in Sapporo was approximately 460 mm. The experimental design has been described in fuller detail in earlier papers (e.g. Eguchi et al. 2005b; Watanabe et al. 2010).

Materials

We studied three forest tree species. These were one conifer species, *Larix kaempferi*, and two angiospermous tree species: *Kalopanax septemlobus*, a ring-porous species, and *Betula platyphylla* var. *japonica* (abbreviated to *B. platyphylla*), a diffuse-porous species. *L. kaempferi*, which is native to central Japan, is a major conifer species used in planting in Hokkaido (Ryu et al. 2009). *K. septemlobus* and *B. platyphylla* are common tree species in cool temperate forests in Northeast Asia (Lee and Kang 2002; Ryu et al. 2009; Zyryanova et al. 2005). Heights and stem basal diameters (mean ± standard error) of seedlings at both sites were 16.5 ± 0.5 cm and 3.0 ± 0.1 mm for *L. kaempferi* (n = 20), 16.9 ± 0.5 cm and 5.0 ± 0.2 mm for *K. septemlobus* (n = 22), 15.7 ± 0.3 cm and 2.0 ± 0.1 mm for *B. platyphylla* (n = 20) (Watanabe et al. 2010).

For each species we selected from each FACE site and control site a 6-year-old sapling grown in brown forest soil (FACE samples n=3; control samples n=3). Before harvesting these saplings we measured their leaf gas exchange in early July 2007. After measuring the tree height and stem basal diameter we harvested these saplings in late July 2007, and wood samples were taken from the stem base of the saplings. We

measured the fresh weight of these samples and then fixed the samples in FAA (formaldehyde, acetic acid, 50 % ethanol). For measurement of dry mass, we divided each whole sapling into specific organs, as stem, branch and leaves.

For the dye injection experiments (Sano et al. 2005), we selected three saplings of *K. septemlobus* from each control and FACE site. The tree height, stem basal diameter and leaf gas exchange were measured prior to dye injection, in early July 2007.

Methods

Leaf gas exchange measurements

Leaf gas exchange (the light-saturated net photosynthetic rate per leaf area, P_{sat}) and stomatal conductance (g_s) were measured using an open gas exchange system (LiCor-6400; Li-Cor Inc., Lincoln, USA). Measurements were made on mature sun leaves of all saplings of each tree species at a given site in early July 2007, under both ambient and elevated CO_2 concentration (ambient: 370 μmol mol^{-1}, elevated: 500 μmol mol^{-1}). The saturating photosynthetic photon flux (PPF) at the upper leaf surface was 1,500 μmol $m^{-2}s^{-1}$. The leaf temperature was maintained at 25 ˚C (Watanabe et al. 2008).

Measurement of dry mass, leaf mass per area and total leaf area

The organs of the samples were dried in an oven, at a temperature of 75 ˚C for leaves and 105˚C for stem and branch. The dry mass of each organ and the total above-ground dry mass were then measured. The leaf mass per (unit) area (LMA) was measured based

on 20 leaves per sapling, and the total leaf area per sapling (LA) was calculated by dividing the leaf dry mass by the LMA value. Biomass partitioning (the percentage of each organ contributing to the total biomass) was estimated from the dry mass of each organ.

For *K. septemlobus* saplings undergoing dye injection, all leaves were stripped from the plant after dye injection, and the total leaf area was measured with a leaf area meter (LI-3100C; Li-Cor, Lincoln, USA) in early August 2007.

Measurement of ring width

The FAA-fixed samples were washed with fresh water and the wood samples were cut into small pieces. Transverse sections, approximately 16 μm thick, were cut from these samples by a sliding microtome. These sections were stained with 1 % safranin in 30 % ethanol, and were mounted permanently.

Photographs of transverse sections were taken from pith to bark using a light microscope (AxioSkop; Carl Zeiss Inc, Germany) in conjunction with a digital camera (Digital Sight DS-5M; Nikon, Japan). Digital images were synthesized using Adobe Photoshop software (Adobe Systems Inc, USA). We measured the ring width that formed during 2004-2006 from synthesized micrographs, using Image J (Rasband 1997-2012).

Measurement of lumen size in tracheids and vessels

Photographs of transverse sections were obtained from secondary xylem formed in 2006

and 2007 using a light microscope (AxioSkop; Carl Zeiss Inc, Germany) and a digital camera (Digital Sight DS-5M; Nikon, Japan). Digital images were synthesized using Adobe Photoshop software (Adobe Systems Inc, USA). The micrograph areas of each synthesized photograph were approximately 0.19 mm^2 for *L. kaempferi* and 2.62 mm^2 for *K. septemlobus* and *B. platyphylla*. In *L. kaempferi*, earlywood tracheids were selected. In *K. septemlobus*, wide vessel elements were selected from the earlywood. In *B. platyphylla*, vessel elements were selected from the central part of the annual ring. We measured the lumen area of each tracheid and vessel from synthesized micrographs using Image J (Rasband 1997-2012).

For *L. kaempferi*, we calculated the total tracheid lumen area per micrograph (mm^2; TLA), the mean tracheid lumen area of a single tracheid (μm^2; MLA), the percentage of tracheid lumen area per unit micrograph area (%; PLA) and the number of tracheids per square mm (NT). For *K. septemlobus* and *B. platyphylla*, we calculated the total vessel lumen area per micrograph (mm^2; TLA), the mean lumen area of a single vessel (μm^2; MLA), the percentage of vessel lumen area per unit micrograph area (%; PLA), and the number of vessels per square mm (NV); see Watanabe *et al*. (2008, 2010). In all species we also calculated the relative frequency of lumen area comprising water-conducting cells in different size classes. Measured vessels in ring-porous species and diffuse-porous species were distinguished from latewood vessels and from other types of cells (axial parenchyma cells or wood fibres) by means of a threshold value for the vessel lumen area, respectively (5,024 μm^2 for ring-porous woods; 314 μm^2 for diffuse-porous woods). These threshold values were established from a histogram of

porous area in all annual rings of one sample (data not shown).

We calculated the square of the lumen area (squared lumen area) of water-conducting cells as the index of the fourth power of the diameter, using the Hagen-Poiseuille formula to estimate the water transport capability of tracheids and vessels in samples.

Dye injection method

We undertook dye injection according to the method of Sano et al. (2005) in late July and early August 2007. An aqueous solution of 0.5 % (w/v) Acid fuchsin (Wako Chemicals, Tokyo, Japan) was prepared. This solution was filtered through a 0.22-μm filter (GV, Millipore, Billerica, USA). A receptacle was set at the base of each sapling and the aqueous solution was poured into this receptacle. A notch was cut with a chisel below the solution surface in each funnel, and the solution was allowed to flow into the xylem through the notch for about 50 min (see Figure 2 in Sano et al. 2005). After the dye had been introduced, we poured liquid nitrogen into another receptacle located 1m above the first. After 5 min, two discs were cut from each frozen part of each sapling and maintained frozen. Following shaving of the surface of the freeze-dried discs, we observed the dye distribution in the stem and photographed it with a digital camera.

After observation of the dye distribution, the cross-sectional area of all discs was scanned with a flatbed scanner (EPSON GT-X970, SEIKO EPSON Corp., Nagano, Japan). The area of red-colored vessels in all images was quantified using Image J (Rasband 1997-2012) and the proportion of colored vessel area to cross-sectional area

of discs was calculated.

Statistical analysis

To determine the effect of CO_2 on tree growth (tree height, stem basal diameter and biomass), on parameters of leaf gas exchange (P_{sat} and g_s), and on wood properties (ring width, total lumen area, mean lumen area, proportion, number of vessels, squared lumen area and the proportion of colored vessel area to cross-sectional area), we performed t-tests at a marginal probability level $p < 0.10$ (KaleidaGraph 4.1.3, Synergy Software, 2011).

For the vessel frequency, the Mann-Whitney U-test was performed to determine the effect of CO_2 (SPSS 10.0.5. J, SPSS, Tokyo, Japan).

Results

Tree growth and above-ground dry mass

Table 1 shows the tree growth and dry mass of all species. For *L. kaempferi* the tree height and stem basal diameter did not differ significantly between the FACE and control sites. For *K. septemlobus* and *B. platyphylla*, in contrast, the tree height of saplings growing at the FACE site was significantly greater than at the control site, although there were no differences between the sites in stem basal diameter.

The dry mass of each organ and total above-ground dry mass of *L. kaempferi* and *K. septemlobus* did not differ significantly between the FACE and control sites. For *B. platyphylla* the leaf dry mass and stem dry mass were significantly greater at the FACE

site than at the control site, but the branch dry mass did not differ. There was no effect of elevated CO_2 on LMA in any of the three species examined. LA significantly increased at the FACE site in *B. platyphylla*. For *K. septemblobus*, LA was 87% greater at the FACE site than at the control site, although there was no significant difference between control and the FACE site ($P = 0.111$).

Figure 1 shows biomass partitioning in the three species. The percentage of stem dry mass to total above-ground biomass increased in *L. kaempferi* at the FACE site relative to control. In *K. septemlobus* and *B. platyphylla*, the percentage of leaf dry mass increased at the FACE site relative to controls. However, there were no significant differences between FACE and control sites in all organs in the three species.

Leaf gas exchange traits

There were no significant effects of elevated CO_2 on P_{sat} and g_s in the three species examined (Table 1).

Ring width and anatomical features of water-conducting cells

Table 2 shows the width of rings formed from 2004 to 2006. We harvested all saplings before growth ring formation was complete in 2007, so we did not measure the ring width in that year. The growth rings of *L. kaempferi* that formed in 2004 were significantly wider at the FACE site than at controls. There was no significant effect of elevated CO_2 on the width of rings formed in 2005 and 2006 in this species, however. The ring width for *K. septemlobus* and *B. platyphylla* did not differ between FACE and

control sites during 2004-6. The total ring width for the 3 years did not differ significantly between the FACE and control sites in any of the three species studied, although the mean values were all higher at the FACE site.

Table 2 sets out the anatomical features of water-conducting cells produced in 2006 and 2007. For *L. kaempferi*, all anatomical features of tracheids (namely TLA, MLA, PLA and NT in earlywood) exhibited no significantly difference between FACE and control sites in 2006 or 2007. For *K. septemlobus*, the PLA and NV values increased in earlywood at the FACE site in 2006 relative to controls, but the TLA and MLA were not different in that year; in 2007, no parameters of vessels differed significantly between the FACE and control sites. For *B. platyphylla* in 2006, the TLA and PLA values at the FACE site were smaller than at the control site, but MLA and NV did not differ between the sites; in 2007, no vessel parameters differed significantly between FACE and control sites. The squared lumen area of vessels did not differ between the FACE and control sites in *K. septemlobus* or *B. platyphylla*.

Figure 2 shows a histogram of the lumen area of water-conducting cells. In *L. kaempferi*, mid-size tracheids (450 - 600 μm^2) are significantly more common at FACE sites than at control sites, but only in 2007 (Fig. 2a, b). For *K. septemlobus*, in 2006 medium size vessels (10,000 - 14,000 μm^2) were less common at the FACE site than in controls, but no such differences between the FACE site and controls was observed in xylem formed in 2007 (Fig. 2c, d). For *B. platyphylla*, no such differences between the FACE site and controls were observed in xylem formed in 2006 and 2007 (Fig. 2e, f).

Dye distribution

Saplings selected for dye injection were taller at the FACE sites than at control sites. The basal diameter of the stem and total leaf area did not differ significantly between FACE and control sites. Elevated CO_2 did not affect P_{sat} and g_s (Table 3).

Figure 3 shows the dye distribution in saplings grown at the FACE and control sites. In all *K. septemlobus* saplings, at both FACE (Fig. 3b) and control sites (Fig. 3a), vessels in earlywood and whole latewood in the current year and some of the latewood vessels of the previous year were colored red with Acid fuchsin. The average proportion of colored vessel area to cross-sectional area was approximately 1.25 times higher in FACE saplings (10.0 ± 1.2 %) than in control saplings (8.1 ± 0.8 %), although there were no significant differences between control and FACE sites ($P = 0.29$).

Discussion

Tree growth and leaf gas exchange traits under elevated CO_2

In our FACE experiments, an elevated CO_2 concentration significantly enhanced the height of two angiospermous tree species, but did not significantly enhance the basal diameter of the stem (Table 1). This is consistent with a meta-analysis of FACE experiments (Ainsworth and Long 2005; Pinkard et al. 2010).

The meta-analysis of Ainsworth and Long (2005) found a 28 % increase in total above-ground dry matter production due to elevated CO_2 concentration. In the present study, the total above-ground biomass of saplings of *L. kaempferi*, *K. septemlobus* and *B. platyphylla* was respectively 24 %, 18 % and 86 % greater at the FACE site than at the

control site. The significant increase in total above-ground dry mass of *B. platyphylla* under elevated CO_2 could be due to an increase in leaf dry mass and stem dry mass (Table 1). In particular, the leaf dry mass in *B. platyphylla* under elevated CO_2 was approximately three times greater than in control saplings. The increase in leaf dry mass and LA without change in LMA suggests an increase in leaf size at elevated CO_2, and/or an increase in leaf number per sapling (Table 1). This increase in leaf dry mass might be caused by an improvement in growth conditions, such as light, due to the increase in tree height under elevated CO_2.

There were no significant differences in the above-ground biomass partitioning in the three species due to small number of saplings tested in this study (Fig.1). However, our results showed the possibility that the raised CO_2 concentration altered the above-ground biomass partitioning in the three tree species (Fig. 1).

Meta-analyses of tree FACE experiments indicate that elevated CO_2 enhances A_{max} and reduces g_s, improving the water use efficiency (WUE) (Ainsworth and Rogers 2007; Pinkard et al. 2010). In the present study, however, P_{sat} and g_s did not change in the three species examined during 5 years of CO_2 exposure (Table 1). It is possible that long-term CO_2 exposure leads to photosynthetic acclimation to elevated CO_2 (Pinkard et al. 2010). The absence of any significant response in g_s in the present study might be due to the relatively low elevated CO_2 concentration (about 500 ppm) compared to other FACE experiment sites, such as Duke Forest (CO_2 concentration; ambient plus 200 ppm) (Ainsworth and Long 2005).

Ring width after 5 years of CO_2 exposure

In our study, elevated CO_2 significantly enhanced the annual ring width only in one year, 2004 (the second growth season), in saplings of *L. kaempferi* during the period 2003-2007 (Table 2). The review by Yazaki *et al*. (2005) found that elevated CO_2 did enhance growth ring width in softwoods and hardwoods in the short term. Some studies nevertheless found that the response of radial growth to long-term CO_2 fumigation differs from the response to short-term CO_2 fumigation (e.g. Norby et al. 2001; Körner et al. 2005; Kilpeläinen et al. 2007; Koike et al. 2015). Kilpeläinen et al. (2007) found that elevated CO_2 increases growth ring width in four years in 20-year-old Scots pine trees (*Pinus sylvestris*) during the period 1997-2002. Our results also indicate that long-term CO_2 exposure does not stimulate radial growth in trees. Enhancement of the annual ring width which we observed in 2004 might be due to increases in the assimilation rate under elevated CO_2 concentration with light saturation (A_{growth}) in 2003 in saplings of *L. kaempferi* (Eguchi 2008).

Size and number of water-conducting cells after 5 years of CO_2 exposure

Our FACE study determined that the three species did not undergo any significant changes in the size of their water-conducting cells, although saplings of all species showed yearly variation at both control and FACE sites (Table 2; Fig. 2). Kilpeläinen et al. (2007) and Kostiainen et al. (2014) have also found yearly variations in the mean lumen diameter of tracheary elements in Scots pine and in two diffuse-porous woods under elevated CO_2. The formation of tracheary elements is influenced by external and

internal conditions (Denne and Dodds 1981), so it is necessary to consider internal factors such as plant growth regulators and also external factors such as elevated CO_2 concentrations and precipitation.

Functional region of water transport in stem under elevated CO_2

Watanabe et al. (2010) have proposed that the enhanced LA of *K. septemlobus* as a result of elevated CO_2 induced a change in vessel size, although g_s did not change after 3 years of CO_2 exposure. It is also possible that an increase in LA under elevated CO_2 would lead to changes in the functional region for water transport. The dye injection method is capable of visualizing the water-conducting pathway in the stem (Sano et al. 2005; Umebayashi et al. 2008). We therefore investigated whether there are changes in the functional region for water transport through vessels in *K. septemlobus*, using the dye injection method together with measurement of the size of vessels.

The dye distribution revealed that water conducts through earlywood vessels and latewood vessels of *K. septemlobus* in the current year, and through part of the latewood vessels in the previous year, in both controls and under elevated CO_2. This result is consistent with previous studies that visualized the water transport pathway in various tree species using dye injection (Umebayashi et al. 2008; Sano et al. 2011). Our results suggest that the functional region for water transport in the stem of *K. septemlobus* does not change under elevated CO_2.

Do changes in biomass allocation and leaf gas exchange traits due to elevated CO_2

induce changes in anatomical features of water-conducting cells?

Our results indicate that elevated CO_2 does not affect tree growth, leaf gas exchange, anatomical features or water transport ability of *L. kaempferi* saplings. Yazaki et al. (2001, 2004) also found no obvious changes in anatomical features of tracheids in *L. sibirica* and *L. kaempferi* seedlings at elevated CO_2. Domec et al. (2010) suggested that physiological traits in some conifers would respond to elevated CO_2 after the replacement of a large proportion of pre-treatment hydraulic active sapwood by wood produced under elevated CO_2. In our study, *L. kaempferi* saplings produced a large proportion of sapwood under elevated CO_2, but we found no changes in leaf gas exchange traits. It appears that xylem formation of *L. kaempferi* is less sensitive to elevated CO_2.

In *K. septemlobus* and *B. platyphylla*, the elevated CO_2 concentration enhanced the LA value after 5 years of CO_2 exposure, although g_s did not change (Table 1). Enhanced LA would involve more water to leaves, and the anatomical features of water-conducting cells would be influenced by it. No obvious changes in the size or water transport ability of earlywood vessels or in the functional region of water transport were found under elevated CO_2 in *K. septemlobus*, however (Table 2; Fig. 3).

K. septemlobus saplings grown under elevated CO_2 might respond to changes in the water demand of leaves as a result of enhanced LA by increasing the proportion of hydraulically active area. There were also no significant changes in size or water transport ability in *B. platyphylla* after 5 years exposure to CO_2 (Table 2). *B. platyphylla* uses almost all of the vessels in several tree rings for water transport (Utsumi et al.

1998). This species might therefore respond to changes in demand of water to leaves due to enhanced LA by increasing the functional region of water transport.

Our study has shown that enhanced LA did not induce changes in the anatomical features of water-conducting cells in the three tree species examined under elevated CO_2. The wood structure of these trees, including the size of tracheary elements, would be less sensitive to elevated CO_2. Trees might respond to changes in water balance due to changes in LA by extending the hydraulically active area of xylem, rather than by making changes in the vessel size and number of vessels, under elevated CO_2.

Author contribution statement

Yoko Watanabe: Writing of the article, collection and assembly of data, analysis and interpretation of data

Keita Wakabayashi, Satoshi Nakaba, Satoshi Kitaoka, Takami Satomura and Norikazu Eguchi: collection and assembly of data

Makoto Watanabe: Analysis of data

Kentaro Takagi: Conception and design of the study

Yuzou Sano: Collection and assembly of data, analysis and interpretation of data

Ryo Funada: Conception and design of the study

Takayoshi Koike: Conception and design of the study, final approval of the article

Acknowledgements

We thank Dr. A. Sumida for help with the measurement of leaf area using a leaf area

meter (LI-3100C; Li-Cor, Lincoln, USA). Thanks are also due to Mr. T. Ueda of DALTON Co ltd. for maintenance of the FACE system. This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan (nos. 21580170, 21114008, 26292075), a Grant-in-Aid (Research Project for Parameterization of Terrestrial Ecosystem for Integrated Global Modeling, Research Revolution 2002) from the Ministry of Education, Culture, Sports, Science and Technology, Japan and a Grant-in-Aid for Young Scientists (H19-Y-079) from the Northern Advanced Centre for Science and Technology, Japan.

Conflict of interest

None declared.

References

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

Ainsworth EA, Rogers A (2007) The response of photosynthesis and stomatal conductance to rising [CO_2]: mechanisms and environmental interactions. Plant Cell Environ 30:258-270

Denne MP, Dodd RS (1981) The environmental control of xylem differentiation. In: Barnett JR (ed) Xylem Cell Development. Castle House, Kent, pp 236-255

Domec JC, Schäfer K, Oren R, Kim HS, McCarthy HR (2010) Variable conductivity

and embolism in roots and branches of four contrasting tree species and their impacts on whole-plant hydraulic performance under future atmospheric CO_2 concentration. Tree physiol 30:1001-1015

Eguchi N (2008) Study on the changes in CO_2 fixation and storage capacity of deciduous tree species native to cool temperate zone with increasing ambient CO_2 concentration. Doctoral dissertation of The Graduate School of Agriculture of Hokkaido University, Sapporo, Japan. (in Japanese)

Eguchi N, Funada R, Ueda T, Takagi K, Hiura T, Sasa K, Koike T (2005a) Soil moisture condition and growth of deciduous tree seedlings native to northern Japan grown under elevated CO_2 with FACE system. Phyton 45:133-138

Eguchi N, Koike T, Ueda T (2005b) Free Air CO_2 Enrichment Experiment in Northern Japan. Vaisala News 169:15-16

Eguchi N, Karatsu K, Ueda T, Funada R, Takagi K, Hiura T, Sasa K, Koike T (2008) Photosynthetic responses of birch and alder saplings grown in a free air CO_2 enrichment system in northern Japan. Trees 22:437-447

Hyvönen R, Ågren GI, Linder S, Persson T, Cotrufo MF, Ekblad A, Freeman M, Grelle A, Janssens IA, Jarvis PG, Kellomäki S, Lindroth A, Loustau D, Lundmark T, Norby RJ, Oren R, Pilegaard K, Ryan MG, Sigurdsson BD, Strömgren M, van Oijen M, Wallin G. (2007) The likely impact of elevated [CO_2], nitrogen deposition, increased temperature and management on carbon sequestration in temperate and boreal forest ecosystems: a literature review. New Phytol 173:463-480

Kaakinen S, Kostiainen K, Ek F, Saranpää P, Kubiske ME, Sober J, Karnosky DF,

Vapaavuori E (2004) Stem wood properties of *Populus tremuloides*, *Betula papyrifera* and *Acer saccharum* saplings after 3 years of treatments to elevated carbon dioxide and ozone. Global Change Biol 10:1513-1525

Kilpeläinen A, Gerendiain AZ, Luostarinen K, Peltola H, Kellomäki S (2007) Elevated temperature and CO_2 concentration effects on xylem anatomy of Scots pine. Tree Physiol 27:1329-1338

Koike T, Watanabe M, Watanabe Y, Agathokleous E, Mao QZ, Eguchi N, Takagi K, Satoh F, Kitaoka S, Funada R (2015) Ecophysiology of deciduous trees native to Northeast Asia grown under FACE (Free Air CO_2 Enrichment). J Agr Met 71: 174-184.

Körner Ch (2003) Carbon limitation in trees. J Ecol 91:4-17

Körner Ch, Asshoff R, Bignucolo O, Hättenschwiler S, Keel SG, Peláez-Riedl S, Pepin S, Siegwolf RTW, Zotz G (2005) Carbon flux and growth in mature deciduous forest trees exposed to elevated CO_2. Science 309:1360-1362

Kostiainen K, Jalkanen H, Kaakinen S, Saranpää P, Vapaavuori E (2006) Wood properties of two silver birch clones exposed to elevated CO_2 and O_3. Global Change Biol 12:1230-1240

Kostiainen K, Kaakinen S, Warsta E, Kubiske ME, Nelson ND, Sober J, Karnosky DF, Saranpää P, Vapaavuori E (2008) Wood properties of trembling aspen and paper birch after 5 years of exposure to elevated concentrations of CO_2 and O_3. Tree Physiol 28:805-813

Kostiainen K, Kaakinen S, Saranpää P, Sigurdsson BD, Lundqvist SO, Linder S,

Vapaavuori E (2009) Stem wood properties of mature Norway spruce after 3 years of continuous exposure to elevated [CO_2] and temperature. Global Change Biol 15:368-379

Kostiainen K, Saranpää P, Lundqvist SO, Kubiske ME, Vapaavuori E (2014) Wood properties of *Populus* and *Betula* in long-term exposure to elevated CO_2 and O_3. Plant Cell Environ 37:1452-1463

Lee DK, Kang HS (2002) Distribution of *Kalopanax septemlobus* and its growth in Northeast Asia. Eurasian J For Res 5:85-94

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

Norby RJ, Zak DR (2011) Ecological lessons from Free-Air CO_2 Enrichment (FACE) Experiments. Ann Rev Ecol Evol Syst 42:181-203.

Pinkard EA, Beadle CL, Mendham DS, Carter J, Glen M (2010) Determining photosynthetic responses of forest species to elevated [CO_2]: Alternatives to FACE. For Ecol Manag 260:1251-1261

Rasband, W.S. (1997–2012) ImageJ, US National Institutes of Health, Bethesda, MD. http://imagej.nih.gov/ij/.

Ryu K, Watanabe M, Shibata H, Takagi K, Nomura M, Koike T (2009) Ecophysiological responses of the larch species in northern Japan to environmental changes as a basis for afforestation. Landscape Ecol Eng 5:99–106

Sano Y, Okamura Y, Utsumi Y (2005) Visualizing water-conduction pathways of living trees: selection of dyes and tissue preparation methods. Tree Physiol 25:269-275

Sano Y, Morris H, Shimada H, De Craene LPR, Jansen S (2011) Anatomical features associated with water transport in imperforate tracheary elements of vessel-bearing angiosperms. Ann Bot 107:953-964

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

Takagi K, Eguchi N, Ueda T, Sasa K, Koike T (2004) CO_2 control in a FACE system for tree saplings. J Agr Met, Hokkaido 56:9-16 (in Japanese).

Tyree MT, Alexander JD (1993) Plant water relations and the effects of elevated CO_2: a review and suggestions for future research. Vegetatio 104/105:47-62

Umebayashi T, Utsumi Y, Koga S, Inoue S, Fujikawa S, Arakawa K, Matsumura J, Oda K (2008) Conducting pathways in north temperate deciduous broadleaved trees. IAWA J 29:247-263

Utsumi Y, Sano Y, Fujikawa S, Funada R, Ohtani J (1998) Visualization of cavitated vessels in winter and refilled vessels in spring in diffuse-porous trees by cryo-scanning electron microscopy. Plant Physiol 117:1463-1471

Vaz M, Cochard H, Gazarini L, Graça J, Chaves MM, Pereira JS (2012) Cork oak (*Quercus suber* L.) seedlings acclimate to elevated CO_2 and water stress: photosynthesis, growth, wood anatomy and hydraulic conductivity. Trees 26:1145-1157

Watanabe Y, Tobita H, Kitao M, Maruyama Y, Choi DS, Sasa K, Funada R, Koike T (2008) Effects of elevated CO_2 and nitrogen on wood structure related to water transport in seedlings of two deciduous broad-leaved tree species. Trees 22:403-411

Watanabe Y, Satomura T, Sasa K, Funada R, Koike T (2010) Differential anatomical responses to elevated CO_2 in saplings of four hardwood species. Plant Cell Environ 33:1101–1111

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

Yazaki K, Ishida S, Kawagishi T, Fukatsu E, Maruyama Y, Kitao M, Tobita H, Koike T, Funada R (2004) Effects of elevated CO_2 concentration on growth, annual ring structure and photosynthesis in *Larix kaempferi* seedlings. Tree Physiol 24:941-949

Yazaki K, Maruyama Y, Mori S, Koike T, Funada R (2005) Effects of elevated carbon dioxide concentration on wood structure and formation in trees. In: Omasa K, Nouchi I, De Kok LJ (eds) Plant Responses to Air Pollution and Global Change. Springer-Verlag, Tokyo, pp 89-97

Zyryanova OA, Yabarov VT, Abaimov AP, Koike T, Sasa K, Terazawa M (2005) Problems in the maintenance and sustainable use of forest resources in Priamurye in the Russian Far East. Eurasian J For Res 8:53-64

Figure legends

Figure 1. Percentage of stem dry mass, branch dry mass and leaf dry mass to total above-ground biomass in saplings of all species grown at control sites and FACE sites. (a) *Larix kaempferi*, (b) *Kalopanax septemlobus*, (c) *Betula platyphylla*. Statistical results in all organs were not significant between control and FACE sites in the three species.

Figure 2. Distribution of lumen area of water-conducting cells into different size classes in saplings of all species in both 2006 and 2007. (a) *Larix kaempferi* in 2006, (b) *Larix kaempferi* in 2007, (c) *Kalopanax septemlobus* in 2006, (d) *Kalopanax septemlobus* in 2007, (e) *Betula platyphylla* in 2006, (f) *Betula platyphylla* in 2007. Actual *P* values are shown when $P < 0.10$ (n=3).

Figure 3. Micrographs of dye distribution in transverse sections of secondary xylem in saplings of *Kalopanax septemlobus* grown at control site (a) and at FACE site (b) after injection of Acid fuchsin solution. Red-colored vessels are active water conduction. Earlywood vessels formed in the current year and latewood vessels formed in both current year and the previous year were stained by Acid fuchsin, but earlywood vessels formed in the previous year were not stained by Acid fuchsin at both control site and FACE site. White arrowheads; earlywood vessels formed in 2007, black arrowheads; earlywood vessels formed in 2006. Scale bars = 1 mm.

Table 1. Tree growth, dry mass and leaf gas exchange traits of saplings of all species grown under FACE and control sites.

	Larix kaempferi			*Kalopanax septemlobus*			*Betula platyphylla*		
	Control	FACE		Control	FACE		Control	FACE	
Tree height in 2007 (cm)	272 (± 86)	354 (± 35)	n.s.	352 (± 61)	470 (± 20)	$P = 0.033$	522 (± 28)	599 (± 9)	$P = 0.011$
Stem basal diameter in 2007 (mm)	34.3 (± 12.2)	44.4 (± 13.7)	n.s.	59.5 (± 9.8)	69.1 (± 12.7)	n.s.	53.1 (± 6.5)	61.4 (± 7.2)	n.s.
Leaf dry mass (g)	165.8 (± 165.9)	221.0 (± 141.4)	n.s.	436.3 (± 313.5)	755.9 (± 186.5)	n.s.	314.6 (± 160.9)	1170.9 (± 661.8)	$P = 0.095$
Branch dry mass (g)	230.1 (± 95.9)	119.8 (± 190.3)	n.s.	62.2 (± 107.7)	383.4 (± 633.2)	n.s.	438.4 (± 319.9)	655.1 (± 238.0)	n.s.
Stem dry mass (g)	356.0 (± 319.0)	594.0 (± 207.9)	n.s.	2482.5 (± 2044.3)	2382.6 (± 1009.1)	n.s.	1479.5 (± 376.3)	2335.4 (± 560.0)	$P = 0.093$
Total above ground dry mass (g)	751.9 (± 495.6)	934.9 (± 499.7)	n.s.	2981.0 (± 2461.9)	3521.9 (± 831.2)	n.s.	2232.5 (± 847.1)	4161.4 (± 1004.8)	$P = 0.065$
Leaf mass per area (g m^{-2})	91.1 (± 18.2)	68.7 (± 15.7)	n.s.	69.7 (± 24.3)	71.2 (± 7.8)	n.s.	61.1 (± 4.5)	62.9 (± 12.4)	n.s.
Total leaf area per a sapling (m^2)	2.1 (± 2.4)	3.1 (± 1.8)	n.s.	5.8 (± 2.2)	10.8 (± 3.6)	n.s.	5.1 (± 2.3)	18.3 (± 8.2)	$P = 0.056$
P_{sat} (μmol $m^{-2}s^{-1}$)	6.43 (± 1.74)	7.97 (± 1.92)	n.s.	20.06 (± 4.27)	21.22 (± 1.82)	n.s.	11.58 (± 3.11)	14.98 (± 2.01)	n.s.
Stomatal conductance (mol $m^{-2}s^{-1}$)	0.08 (± 0.02)	0.07 (± 0.02)	n.s.	0.34 (± 0.10)	0.26 (± 0.06)	n.s.	0.12 (± 0.04)	0.16 (± 0.02)	n.s.

Values in parentheses are standard errors of the means ($n = 3$). Results of the t-test are shown (n.s., not significant).

Actual P values are shown when $P < 0.10$.

P_{sat}, light saturated net photosynthetic rates

Table 2. Ring width and anatomical features of water-conducting cells of saplings of all species grown under FACE and control sites.

	Larix kaempferi			*Kalopanax septemlobus*			*Betula platyphylla*		
	Control	FACE		Control	FACE		Control	FACE	
Ring width 2004 (mm)	2.38 (± 1.81)	4.95 (± 0.50)	$P = 0.077$	4.31 (± 1.10)	5.98 (± 1.24)	n.s.	3.55 (± 0.77)	5.42 (± 1.59)	n.s.
Ring width 2005 (mm)	3.21 (± 2.05)	3.54 (± 0.66)	n.s.	8.04 (± 2.18)	7.81 (± 2.61)	n.s.	5.82 (± 1.72)	7.85 (± 1.88)	n.s.
Ring width 2006 (mm)	2.74 (± 0.74)	3.35 (± 0.89)	n.s.	5.43 (± 1.03)	7.05 (± 1.22)	n.s.	4.26 (± 1.13)	4.64 (± 0.19)	n.s.
Total ring width (mm)	8.33 (± 2.20)	11.84 (± 0.58)	n.s.	17.79 (± 2.46)	20.83 (± 2.10)	n.s.	13.63 (± 1.72)	17.91 (± 2.02)	n.s.
Total lumen area in 2006 (mm^2)	0.11 (± 0.02)	0.10 (± 0.01)	n.s.	0.22 (± 0.11)	0.31 (± 0.69)	n.s.	0.53 (± 0.06)	0.43 (± 0.03)	$P = 0.086$
Mean lumen area in 2006 (μm^2)	344.5 (± 140.6)	345.2 (± 60.5)	n.s.	9092 (± 664)	11344 (± 3163)	n.s.	2552 (± 629)	2419 (± 142)	n.s.
Proportion in 2006 (%)	64.7 (± 9.7)	62.3 (± 3.1)	n.s.	8.35 (± 2.49)	11.64 (± 1.52)	n.s.	20.2 (± 2.4)	16.3 (± 1.0)	$P = 0.057$
Number in 2006 (number mm^{-2})	2048 (± 639)	1842 (± 357)	n.s.	8.90 (± 2.41)	10.55 (± 1.29)	n.s.	81.5 (± 13.4)	67.5 (± 2.7)	n.s.
Squared lumen area in 2006 (mm^4)	0.16 (± 0.12)	0.15 (± 0.05)	n.s.	96.24 (± 6.86)	163.73 (± 89.80)	n.s.	9.64 (± 4.71)	7.57 (± 0.67)	n.s.
Total lumen area in 2007 (mm^2)	0.11 (± 0.01)	0.12 (± 0.01)	n.s.	0.19 (± 0.08)	0.27 (± 0.11)	n.s.	0.51 (± 0.12)	0.44 (± 0.07)	n.s.
Mean lumen area in 2007 (μm^2)	382.8 (± 175.9)	437.0 (± 57.7)	n.s.	11644 (± 2935)	13918 (± 3819)	n.s.	2895 (± 278)	2791 (± 217)	n.s.
Proportion in 2007 (%)	67.6 (± 4.5)	68.8 (± 4.6)	n.s.	7.27 (± 1.68)	10.41 (± 2.39)	n.s.	19.4 (± 4.6)	16.7 (± 2.6)	n.s.
Number in 2007 (number mm^{-2})	1996 (± 639)	1585 (± 180)	n.s.	6.23 (± 1.29)	7.37 (± 0.67)	n.s.	66.7 (± 13.0)	59.9 (± 7.7)	n.s.
Squared lumen area in 2007 (mm^4)	0.20 (± 0.17)	0.24 (± 0.06)	n.s.	166.44 (± 80.69)	268.77 (± 168.26)	n.s.	11.41 (± 2.02)	9.88 (± 1.33)	n.s.

Values in parentheses are standard errors of the means ($n = 3$). Results of the t-test are shown (n.s., not significant).

Actual P values were shown when $P < 0.10$.

Table 3. Tree height, stem basal diameter and leaf gas exchange traits of *Kalopanax septemlobus* saplings used in the dye injection method.

	Kalopanax septemlobus		
	Control	FACE	
Tree height in 2007 (cm)	405 (± 55)	502 (± 25)	$P = 0.074$
Stem basal diameter in 2007 (mm)	64.8 (± 18.9)	88.8 (± 16.2)	n.s.
Total leaf area per a sapling (m^2)	5.98 (± 4.20)	6.70 (± 0.55)	n.s.
P_{sat} (μmol $m^{-2}s^{-1}$)	20.11 (± 4.23)	25.62 (± 1.78)	n.s.
Stomatal conductance (mol $m^{-2}s^{-1}$)	0.32 (± 0.11)	0.27 (± 0.06)	n.s.

Values in parentheses are standard errors of the means (n = 3). Results of the t-test are shown (n.s., not significant).

Actual P values are shown when $P < 0.10$.

P_{sat}, light saturated net photosynthetic rates

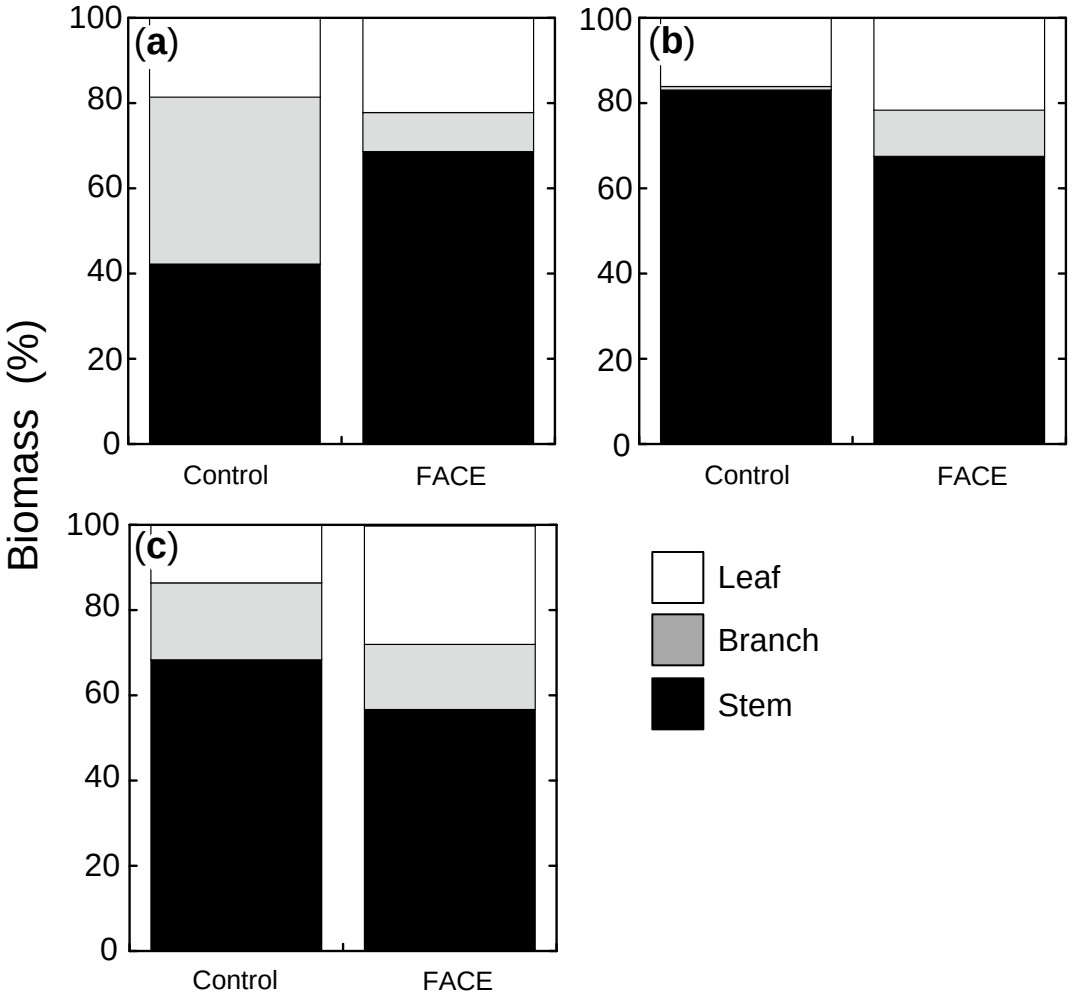

Figure 1

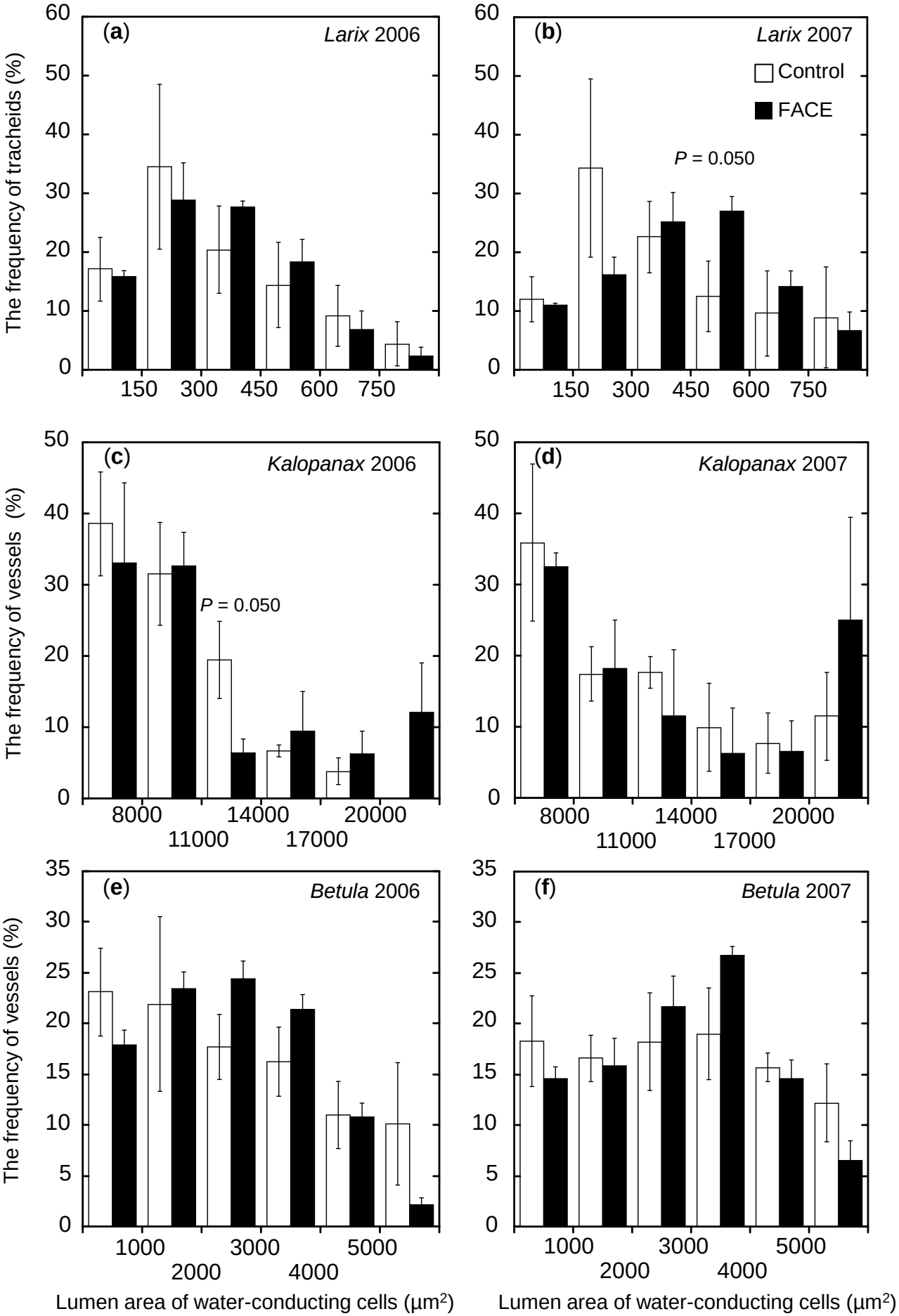
(a) Larix 2006
(b) Larix 2007
Control
FACE
P = 0.050
(c) Kalopanax 2006
P = 0.050
(d) Kalopanax 2007
(e) Betula 2006
(f) Betula 2007
The frequency of tracheids (%)
The frequency of vessels (%)
The frequency of vessels (%)
Lumen area of water-conducting cells (μm²)
Lumen area of water-conducting cells (μm²)

Figure 2

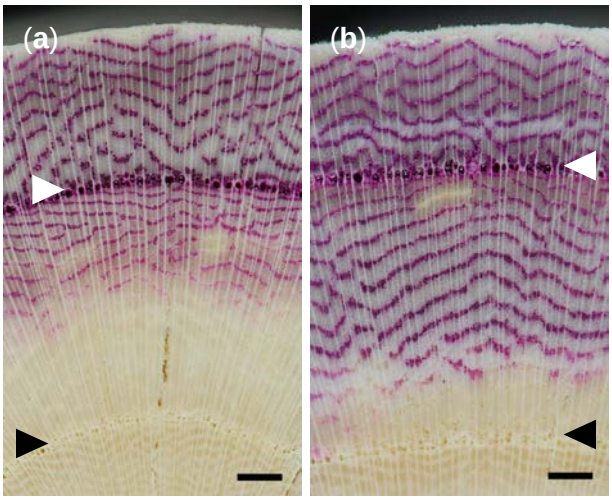

Figure 3