



Title	Response of tree growth and wood structure of <i>Larix kaempferi</i> , <i>Kalopanax septemlobus</i> and <i>Betula platyphylla</i> saplings to elevated CO <sub>2</sub> concentration for 5 years exposure in a FACE system
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1 **Title:** Response of tree growth and wood structure of *Larix kaempferi*, *Kalopanax*  
2 *septemlobus* and *Betula platyphylla* saplings to elevated CO<sub>2</sub> concentration in a FACE  
3 system for 5 years

4

5 **Running head:** RESPONSE OF FOREST TREES TO ELEVATED CO<sub>2</sub>

6

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8 **Key words:** elevated CO<sub>2</sub>, FACE, tree growth, water-conducting cells, dye injection

9

10 **Key Message:**

11 Elevated CO<sub>2</sub> concentration affected biomass partitioning in above-ground biomass, but  
12 size and number of water-conducting cells were unchanged in *Larix kaempferi*,  
13 *Kalopanax septemlobus* and *Betula platyphylla*.

14

15

1 **Abstract**

2 Using a Free Air CO<sub>2</sub> Enrichment (FACE) system, we studied the effect of elevated CO<sub>2</sub>  
3 on the growth, leaf gas exchange and xylem anatomy of a conifer, *Larix kaempferi*, and  
4 two angiospermous tree species, *Kalopanax septemlobus* and *Betula platyphylla*.  
5 Two-year-old seedlings were grown at control sites (ambient; 370 ppm) and FACE sites  
6 (elevated; 500 ppm) for 5 years. We measured the lumen area and number of  
7 water-conducting cells, as well as biomass and leaf gas exchange, and visualized the  
8 functional region of water transport using a dye injection experiment. Elevated CO<sub>2</sub> did  
9 not induce any significant changes in growth or in leaf gas exchange or lumen area of  
10 earlywood tracheids in *L. kaempferi* relative to ambient CO<sub>2</sub>. In two other tree species,  
11 elevated CO<sub>2</sub> was found to enhance tree height and total leaf area, with no change in  
12 stomatal conductance. In *K. septemlobus* there were no changes in lumen area or  
13 number of earlywood vessels, or in the functional region of water transport. *B.*  
14 *platyphylla* also underwent no changes in lumen area or number of vessels, although  
15 there was a yearly variation in the size of the vessels. Our results show that five years of  
16 CO<sub>2</sub> exposure did not notably affect the anatomical features of water-conducting cells.  
17 This finding suggests that, under elevated CO<sub>2</sub>, trees respond to changes in water  
18 balance due to changes in LA by extending the hydraulically active area of xylem.  
19

## 1 **Introduction**

2 With increasing atmospheric CO<sub>2</sub> concentration, forest trees comprise an important CO<sub>2</sub>  
3 sink (Körner 2003). The stem is a particularly useful carbon (C) sink because trees  
4 accumulate new cells with solid cell walls in their stems over long periods (Hyvönen et  
5 al. 2007). Changes in tree growth and in the anatomical features of the secondary xylem  
6 in the stem under elevated CO<sub>2</sub> concentration might therefore affect the C sink capacity  
7 of these trees.

8 A meta-analysis of Free-air CO<sub>2</sub> enrichment (FACE) experiments found increases  
9 in the height, stem diameter, leaf area index and above-ground dry mass of trees grown  
10 under elevated CO<sub>2</sub> concentrations (Ainsworth and Long 2005). Ainsworth and Rogers  
11 (2007) suggested that there is an increase in light saturated photosynthesis ( $A_{\text{sat}}$ ) and a  
12 decrease in stomatal conductance ( $g_s$ ) in C<sub>3</sub> plants grown under elevated CO<sub>2</sub>.

13 Many studies have found, however, that long-term CO<sub>2</sub> exposure does not induce  
14 any change in the lumen size of water-conducting cells of softwoods or hardwoods (e.g.  
15 Kilpeläinen et al. 2007; Watanabe et al. 2010). Kilpeläinen et al. (2007) found that the  
16 tracheid lumen diameter of latewood of 20-year-old Scots pine grown under elevated  
17 CO<sub>2</sub> for six years in a closed chamber was not significantly changed by elevated CO<sub>2</sub>, at  
18 least during those six years, but that in earlywood an increase in lumen diameter took  
19 place in only a single year. Kostianen et al. (2006) reported that the vessel lumen  
20 diameter in 7-year-old *Betula pendula* clones grown in an open-top chamber for three  
21 years does not respond to elevated CO<sub>2</sub>. The vessel lumen diameter in *Populus*  
22 *tremuloides* clones was not significantly altered from ambient values by three-year CO<sub>2</sub>

1 exposure in FACE experiments, although this parameter tends to increase under  
2 elevated CO<sub>2</sub> (Kaakinen et al. 2004). On the other hand, *P. tremuloides* showed no  
3 effect of elevated CO<sub>2</sub> on vessel lumen diameter during five-year exposure (Kostiainen  
4 et al. 2008).

5 Elevated CO<sub>2</sub> reportedly increases the size of tracheary elements, however  
6 (Kostiainen et al. 2009; Watanabe et al. 2010; Kostiainen et al. 2014). Kostiainen et al.  
7 (2009) found an increase in radial diameter of latewood tracheid in *Picea abies* grown  
8 under elevated CO<sub>2</sub> in whole-tree chambers. Watanabe et al. (2010) reported that the  
9 earlywood vessel lumen area in *Kalopanax septemlobus* saplings grown during 3 years  
10 of CO<sub>2</sub> exposure tends to be greater than in control saplings, but no effect of CO<sub>2</sub> on the  
11 size of vessels was found in *Quercus mongolica*, *Betula maximowicziana* or *Acer mono*  
12 grown in the FACE system. Kostiainen et al. (2014) determined that elevated CO<sub>2</sub>  
13 enhanced the vessel diameter in *Populus tremuloides* clones, but not in *Betula*  
14 *papyrifera*, in their FACE experiments. It is clear that the effect of elevated CO<sub>2</sub> on  
15 anatomical features of tracheary elements depends on species and on the experimental  
16 conditions and that more information is needed to determine the reasons.

17 The effect of elevated CO<sub>2</sub> on anatomical features of water-conducting cells is  
18 believed to be indirect. Kostiainen et al. (2014) stated that the enhanced growth rate of  
19 *Populus tremuloides* clones under elevated CO<sub>2</sub> is related to the decrease in cell wall  
20 thickness and wood density. Changes due to elevated CO<sub>2</sub> in leaf gas exchange, as  
21 measured by parameters such as  $A_{\text{sat}}$  and  $g_s$ , and in biomass allocation, measured by (for  
22 instance) the total leaf area, are believed to affect the formation of water-conducting

1 cells (e.g. Tyree and Alexander 1993; Koike et al. 2015). If  $g_s$  decreases without any  
2 increase in total leaf area under elevated CO<sub>2</sub> concentrations, we expect a decrease in  
3 the size of water-conducting cells so as to maintain the hydraulic balance within the tree.  
4 If, on the other hand, the total leaf area increases due to elevated CO<sub>2</sub> concentration, a  
5 tree would need more water and the size of water-conducting cells would change in size  
6 regardless of changes in  $g_s$ . Watanabe et al. (2010) suggested that the increase in vessel  
7 lumen area in earlywood in *K. septemlobus* saplings is due to changes in water status  
8 and/or the concentration of growth regulators in the stem, via enhancement of the tree  
9 height and leaf area. It is therefore necessary to consider the effect of CO<sub>2</sub> on  
10 anatomical features of water-conducting cells, together with tree growth and leaf gas  
11 exchange traits.

12       Furthermore, changes in leaf gas exchange and biomass allocation under elevated  
13 CO<sub>2</sub> concentrations might alter the functional region for water transport in the stem,  
14 because the water transport pathway in the stem is an important component of the  
15 soil-plant-atmosphere-continuum (SPAC). Domec et al. (2010) have reported an  
16 increase in maximum hydraulic specific conductivity, and a decrease in xylem  
17 resistance to embolism, in branches of two diffuse-porous hardwoods, *Liquidambar*  
18 *styraciflua* and *Cornus florida*, but found no correlations between physiological  
19 changes and anatomical changes under elevated CO<sub>2</sub> concentrations. A few detailed  
20 studies nevertheless connect tree growth and leaf gas exchange with anatomical features  
21 of water-conducting cells and water transport pathways in the xylem of forest trees  
22 growing under elevated CO<sub>2</sub> concentration (e.g. Norby and Zak 2011; Vaz et al. 2012;

1 Koike et al. 2015).

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

9

## 10 **Materials and Methods**

### 11 *FACE experimental design*

12 Our FACE facilities (three enriched CO<sub>2</sub> sites with CO<sub>2</sub> gas supply system) and control  
13 sites (three ambient sites) were set up in the Sapporo Experimental Nursery of  
14 Hokkaido University Forests (Sapporo, 43°06'N, 141°20'E), in autumn 2002. Each site  
15 was a circular plot, 6.5 m in diameter and 5 m in height. Fertile brown forest soil was  
16 provided in half of each site, and infertile volcanic ash soil was provided in the other  
17 half (Eguchi et al. 2005a, 2008). The CO<sub>2</sub> concentration inside the FACE sites was  
18 maintained at around 500 μmol mol<sup>-1</sup>, corresponding to ambient plus 130 μmol mol<sup>-1</sup>  
19 (Takagi et al. 2004).

20 In May 2003, two-year-old seedlings of one species of conifer and ten species of  
21 deciduous broad-leaved trees that grow in cool temperate forests in northern Japan were  
22 planted randomly in each soil type. CO<sub>2</sub> fumigation took place from July 2003 to



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

6

### 7 *Materials*

8 We studied three forest tree species. These were one conifer species, *Larix kaempferi*,  
9 and two angiospermous tree species: *Kalopanax septemlobus*, a ring-porous species,  
10 and *Betula platyphylla* var. *japonica* (abbreviated to *B. platyphylla*), a diffuse-porous  
11 species. *L. kaempferi*, which is native to central Japan, is a major conifer species used in  
12 planting in Hokkaido (Ryu et al. 2009). *K. septemlobus* and *B. platyphylla* are common  
13 tree species in cool temperate forests in Northeast Asia (Lee and Kang 2002; Ryu et al.  
14 2009; Zyryanova et al. 2005). Heights and stem basal diameters (mean  $\pm$  standard error)  
15 of seedlings at both sites were  $16.5 \pm 0.5$  cm and  $3.0 \pm 0.1$  mm for *L. kaempferi* (n = 20),  
16  $16.9 \pm 0.5$  cm and  $5.0 \pm 0.2$  mm for *K. septemlobus* (n = 22),  $15.7 \pm 0.3$  cm and  $2.0 \pm$   
17  $0.1$  mm for *B. platyphylla* (n = 20) (Watanabe et al. 2010).

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

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

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

7

## 8 *Methods*

### 9 *Leaf gas exchange measurements*

10 Leaf gas exchange (the light-saturated net photosynthetic rate per leaf area,  $P_{\text{sat}}$ ) and  
11 stomatal conductance ( $g_s$ ) were measured using an open gas exchange system  
12 (LiCor-6400; Li-Cor Inc., Lincoln, USA). Measurements were made on mature sun  
13 leaves of all saplings of each tree species at a given site in early July 2007, under both  
14 ambient and elevated  $\text{CO}_2$  concentration (ambient:  $370 \mu\text{mol mol}^{-1}$ , elevated:  $500 \mu\text{mol}$   
15  $\text{mol}^{-1}$ ). The saturating photosynthetic photon flux (PPF) at the upper leaf surface was  
16  $1,500 \mu\text{mol m}^{-2}\text{s}^{-1}$ . The leaf temperature was maintained at  $25 \text{ }^\circ\text{C}$  (Watanabe et al.  
17 2008).

18

### 19 *Measurement of dry mass, leaf mass per area and total leaf area*

20 The organs of the samples were dried in an oven, at a temperature of  $75 \text{ }^\circ\text{C}$  for leaves  
21 and  $105^\circ\text{C}$  for stem and branch. The dry mass of each organ and the total above-ground  
22 dry mass were then measured. The leaf mass per (unit) area (LMA) was measured based

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

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

8

#### 9 *Measurement of ring width*

10 The FAA-fixed samples were washed with fresh water and the wood samples were cut  
11 into small pieces. Transverse sections, approximately 16  $\mu\text{m}$  thick, were cut from these  
12 samples by a sliding microtome. These sections were stained with 1 % safranin in 30 %  
13 ethanol, and were mounted permanently.

14 Photographs of transverse sections were taken from pith to bark using a light  
15 microscope (AxioSkop; Carl Zeiss Inc, Germany) in conjunction with a digital camera  
16 (Digital Sight DS-5M; Nikon, Japan). Digital images were synthesized using Adobe  
17 Photoshop software (Adobe Systems Inc, USA). We measured the ring width that  
18 formed during 2004-2006 from synthesized micrographs, using Image J (Rasband  
19 1997-2012).

20

#### 21 *Measurement of lumen size in tracheids and vessels*

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

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

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

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

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

6

#### 7 *Dye injection method*

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

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

1 of discs was calculated.

2

### 3 *Statistical analysis*

4 To determine the effect of CO<sub>2</sub> on tree growth (tree height, stem basal diameter and  
5 biomass), on parameters of leaf gas exchange ( $P_{\text{sat}}$  and  $g_s$ ), and on wood properties (ring  
6 width, total lumen area, mean lumen area, proportion, number of vessels, squared lumen  
7 area and the proportion of colored vessel area to cross-sectional area), we performed  
8 t-tests at a marginal probability level  $p < 0.10$  (KaleidaGraph 4.1.3, Synergy Software,  
9 2011).

10 For the vessel frequency, the Mann-Whitney U-test was performed to determine  
11 the effect of CO<sub>2</sub> (SPSS 10.0.5. J, SPSS, Tokyo, Japan).

12

## 13 **Results**

### 14 *Tree growth and above-ground dry mass*

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

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

1 site than at the control site, but the branch dry mass did not differ. There was no effect  
2 of elevated CO<sub>2</sub> on LMA in any of the three species examined. LA significantly  
3 increased at the FACE site in *B. platyphylla*. For *K. septemlobus*, LA was 87% greater  
4 at the FACE site than at the control site, although there was no significant difference  
5 between control and the FACE site ( $P = 0.111$ ).

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

11

#### 12 *Leaf gas exchange traits*

13 There were no significant effects of elevated CO<sub>2</sub> on  $P_{\text{sat}}$  and  $g_s$  in the three species  
14 examined (Table 1).

15

#### 16 *Ring width and anatomical features of water-conducting cells*

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

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

4 Table 2 sets out the anatomical features of water-conducting cells produced in  
5 2006 and 2007. For *L. kaempferi*, all anatomical features of tracheids (namely TLA,  
6 MLA, PLA and NT in earlywood) exhibited no significant difference between FACE  
7 and control sites in 2006 or 2007. For *K. septemlobus*, the PLA and NV values  
8 increased in earlywood at the FACE site in 2006 relative to controls, but the TLA and  
9 MLA were not different in that year; in 2007, no parameters of vessels differed  
10 significantly between the FACE and control sites. For *B. platyphylla* in 2006, the TLA  
11 and PLA values at the FACE site were smaller than at the control site, but MLA and NV  
12 did not differ between the sites; in 2007, no vessel parameters differed significantly  
13 between FACE and control sites. The squared lumen area of vessels did not differ  
14 between the FACE and control sites in *K. septemlobus* or *B. platyphylla*.

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

22



## 1 *Dye distribution*

2 Saplings selected for dye injection were taller at the FACE sites than at control sites.  
3 The basal diameter of the stem and total leaf area did not differ significantly between  
4 FACE and control sites. Elevated CO<sub>2</sub> did not affect  $P_{\text{sat}}$  and  $g_s$  (Table 3).

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

12

## 13 **Discussion**

### 14 *Tree growth and leaf gas exchange traits under elevated CO<sub>2</sub>*

15 In our FACE experiments, an elevated CO<sub>2</sub> concentration significantly enhanced the  
16 height of two angiospermous tree species, but did not significantly enhance the basal  
17 diameter of the stem (Table 1). This is consistent with a meta-analysis of FACE  
18 experiments (Ainsworth and Long 2005; Pinkard et al. 2010).

19 The meta-analysis of Ainsworth and Long (2005) found a 28 % increase in total  
20 above-ground dry matter production due to elevated CO<sub>2</sub> concentration. In the present  
21 study, the total above-ground biomass of saplings of *L. kaempferi*, *K. septemlobus* and *B.*  
22 *platyphylla* was respectively 24 %, 18 % and 86 % greater at the FACE site than at the

1 control site. The significant increase in total above-ground dry mass of *B. platyphylla*  
2 under elevated CO<sub>2</sub> could be due to an increase in leaf dry mass and stem dry mass  
3 (Table 1). In particular, the leaf dry mass in *B. platyphylla* under elevated CO<sub>2</sub> was  
4 approximately three times greater than in control saplings. The increase in leaf dry mass  
5 and LA without change in LMA suggests an increase in leaf size at elevated CO<sub>2</sub>, and/or  
6 an increase in leaf number per sapling (Table 1). This increase in leaf dry mass might be  
7 caused by an improvement in growth conditions, such as light, due to the increase in  
8 tree height under elevated CO<sub>2</sub>.

9       There were no significant differences in the above-ground biomass partitioning in  
10 the three species due to small number of saplings tested in this study (Fig.1). However,  
11 our results showed the possibility that the raised CO<sub>2</sub> concentration altered the  
12 above-ground biomass partitioning in the three tree species (Fig. 1).

13       Meta-analyses of tree FACE experiments indicate that elevated CO<sub>2</sub> enhances  
14  $A_{\max}$  and reduces  $g_s$ , improving the water use efficiency (WUE) (Ainsworth and Rogers  
15 2007; Pinkard et al. 2010). In the present study, however,  $P_{\text{sat}}$  and  $g_s$  did not change in  
16 the three species examined during 5 years of CO<sub>2</sub> exposure (Table 1). It is possible that  
17 long-term CO<sub>2</sub> exposure leads to photosynthetic acclimation to elevated CO<sub>2</sub> (Pinkard et  
18 al. 2010). The absence of any significant response in  $g_s$  in the present study might be  
19 due to the relatively low elevated CO<sub>2</sub> concentration (about 500 ppm) compared to other  
20 FACE experiment sites, such as Duke Forest (CO<sub>2</sub> concentration; ambient plus 200  
21 ppm) (Ainsworth and Long 2005).

22

1 *Ring width after 5 years of CO<sub>2</sub> exposure*

2 In our study, elevated CO<sub>2</sub> significantly enhanced the annual ring width only in one year,  
3 2004 (the second growth season), in saplings of *L. kaempferi* during the period  
4 2003-2007 (Table 2). The review by Yazaki *et al.* (2005) found that elevated CO<sub>2</sub> did  
5 enhance growth ring width in softwoods and hardwoods in the short term. Some studies  
6 nevertheless found that the response of radial growth to long-term CO<sub>2</sub> fumigation  
7 differs from the response to short-term CO<sub>2</sub> fumigation (e.g. Norby *et al.* 2001; Körner  
8 *et al.* 2005; Kilpeläinen *et al.* 2007; Koike *et al.* 2015). Kilpeläinen *et al.* (2007) found  
9 that elevated CO<sub>2</sub> increases growth ring width in four years in 20-year-old Scots pine  
10 trees (*Pinus sylvestris*) during the period 1997-2002. Our results also indicate that  
11 long-term CO<sub>2</sub> exposure does not stimulate radial growth in trees. Enhancement of the  
12 annual ring width which we observed in 2004 might be due to increases in the  
13 assimilation rate under elevated CO<sub>2</sub> concentration with light saturation ( $A_{\text{growth}}$ ) in  
14 2003 in saplings of *L. kaempferi* (Eguchi 2008).

15

16 *Size and number of water-conducting cells after 5 years of CO<sub>2</sub> exposure*

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

1 internal conditions (Denne and Dodds 1981), so it is necessary to consider internal  
2 factors such as plant growth regulators and also external factors such as elevated CO<sub>2</sub>  
3 concentrations and precipitation.

4

#### 5 *Functional region of water transport in stem under elevated CO<sub>2</sub>*

6 Watanabe et al. (2010) have proposed that the enhanced LA of *K. septemlobus* as  
7 a result of elevated CO<sub>2</sub> induced a change in vessel size, although  $g_s$  did not change  
8 after 3 years of CO<sub>2</sub> exposure. It is also possible that an increase in LA under elevated  
9 CO<sub>2</sub> would lead to changes in the functional region for water transport. The dye  
10 injection method is capable of visualizing the water-conducting pathway in the stem  
11 (Sano et al. 2005; Umebayashi et al. 2008). We therefore investigated whether there are  
12 changes in the functional region for water transport through vessels in *K. septemlobus*,  
13 using the dye injection method together with measurement of the size of vessels.

14 The dye distribution revealed that water conducts through earlywood vessels and  
15 latewood vessels of *K. septemlobus* in the current year, and through part of the latewood  
16 vessels in the previous year, in both controls and under elevated CO<sub>2</sub>. This result is  
17 consistent with previous studies that visualized the water transport pathway in various  
18 tree species using dye injection (Umebayashi et al. 2008; Sano et al. 2011). Our results  
19 suggest that the functional region for water transport in the stem of *K. septemlobus* does  
20 not change under elevated CO<sub>2</sub>.

21

22 *Do changes in biomass allocation and leaf gas exchange traits due to elevated CO<sub>2</sub>*

1 *induce changes in anatomical features of water-conducting cells?*

2 Our results indicate that elevated CO<sub>2</sub> does not affect tree growth, leaf gas exchange,  
3 anatomical features or water transport ability of *L. kaempferi* saplings. Yazaki et al.  
4 (2001, 2004) also found no obvious changes in anatomical features of tracheids in *L.*  
5 *sibirica* and *L. kaempferi* seedlings at elevated CO<sub>2</sub>. Domec et al. (2010) suggested that  
6 physiological traits in some conifers would respond to elevated CO<sub>2</sub> after the  
7 replacement of a large proportion of pre-treatment hydraulic active sapwood by wood  
8 produced under elevated CO<sub>2</sub>. In our study, *L. kaempferi* saplings produced a large  
9 proportion of sapwood under elevated CO<sub>2</sub>, but we found no changes in leaf gas  
10 exchange traits. It appears that xylem formation of *L. kaempferi* is less sensitive to  
11 elevated CO<sub>2</sub>.

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

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

1 1998). This species might therefore respond to changes in demand of water to leaves  
2 due to enhanced LA by increasing the functional region of water transport.

3 Our study has shown that enhanced LA did not induce changes in the anatomical  
4 features of water-conducting cells in the three tree species examined under elevated  
5 CO<sub>2</sub>. The wood structure of these trees, including the size of tracheary elements, would  
6 be less sensitive to elevated CO<sub>2</sub>. Trees might respond to changes in water balance due  
7 to changes in LA by extending the hydraulically active area of xylem, rather than by  
8 making changes in the vessel size and number of vessels, under elevated CO<sub>2</sub>.

9

#### 10 **Author contribution statement**

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

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

15 Makoto Watanabe: Analysis of data

16 Kentaro Takagi: Conception and design of the study

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

18 Ryo Funada: Conception and design of the study

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

20

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9

#### 10 **Conflict of interest**

11 None declared.

12

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1 **Figure legends**

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

7

8 Figure 2. Distribution of lumen area of water-conducting cells into different size classes  
9 in saplings of all species in both 2006 and 2007. (a) *Larix kaempferi* in 2006, (b) *Larix*  
10 *kaempferi* in 2007, (c) *Kalopanax septemlobus* in 2006, (d) *Kalopanax septemlobus* in  
11 2007, (e) *Betula platyphylla* in 2006, (f) *Betula platyphylla* in 2007. Actual *P* values are  
12 shown when  $P < 0.10$  (n=3).

13

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

22

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

	<i>Larix kaempferi</i>			<i>Kalopanax septemlobus</i>			<i>Betula platyphylla</i>		
	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 <sup>-2</sup> )	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 <sup>2</sup> )	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)	$P = 0.056$
$P_{\text{sat}}$ (μmol m <sup>-2</sup> s <sup>-1</sup> )	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 <sup>-2</sup> s <sup>-1</sup> )	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  $P < 0.10$ .

$P_{\text{sat}}$ , 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.

	<i>Larix kaempferi</i>			<i>Kalopanax septemlobus</i>			<i>Betula platyphylla</i>		
	Control	FACE		Control	FACE		Control	FACE	
Ring width 2004 (mm)	2.38 (± 1.81)	4.95 (± 0.50)	<i>P</i> = 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 <sup>2</sup> )	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)	<i>P</i> = 0.086
Mean lumen area in 2006 (µm <sup>2</sup> )	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)	<i>P</i> = 0.057
Number in 2006 (number mm <sup>-2</sup> )	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 <sup>4</sup> )	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 <sup>2</sup> )	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 <sup>2</sup> )	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 <sup>-2</sup> )	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 <sup>4</sup> )	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.

	<i>Kalopanax septemlobus</i>		
	Control	FACE	
Tree height in 2007 (cm)	405 ( $\pm$ 55)	502 ( $\pm$ 25)	<i>P</i> = 0.074
Stem basal diameter in 2007 (mm)	64.8 ( $\pm$ 18.9)	88.8 ( $\pm$ 16.2)	n.s.
Total leaf area per a sapling (m <sup>2</sup> )	5.98 ( $\pm$ 4.20)	6.70 ( $\pm$ 0.55)	n.s.
<i>P</i> <sub>sat</sub> ( $\mu$ mol m <sup>-2</sup> s <sup>-1</sup> )	20.11 ( $\pm$ 4.23)	25.62 ( $\pm$ 1.78)	n.s.
Stomatal conductance (mol m <sup>-2</sup> s <sup>-1</sup> )	0.32 ( $\pm$ 0.11)	0.27 ( $\pm$ 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*<sub>sat</sub>, light saturated net photosynthetic rates



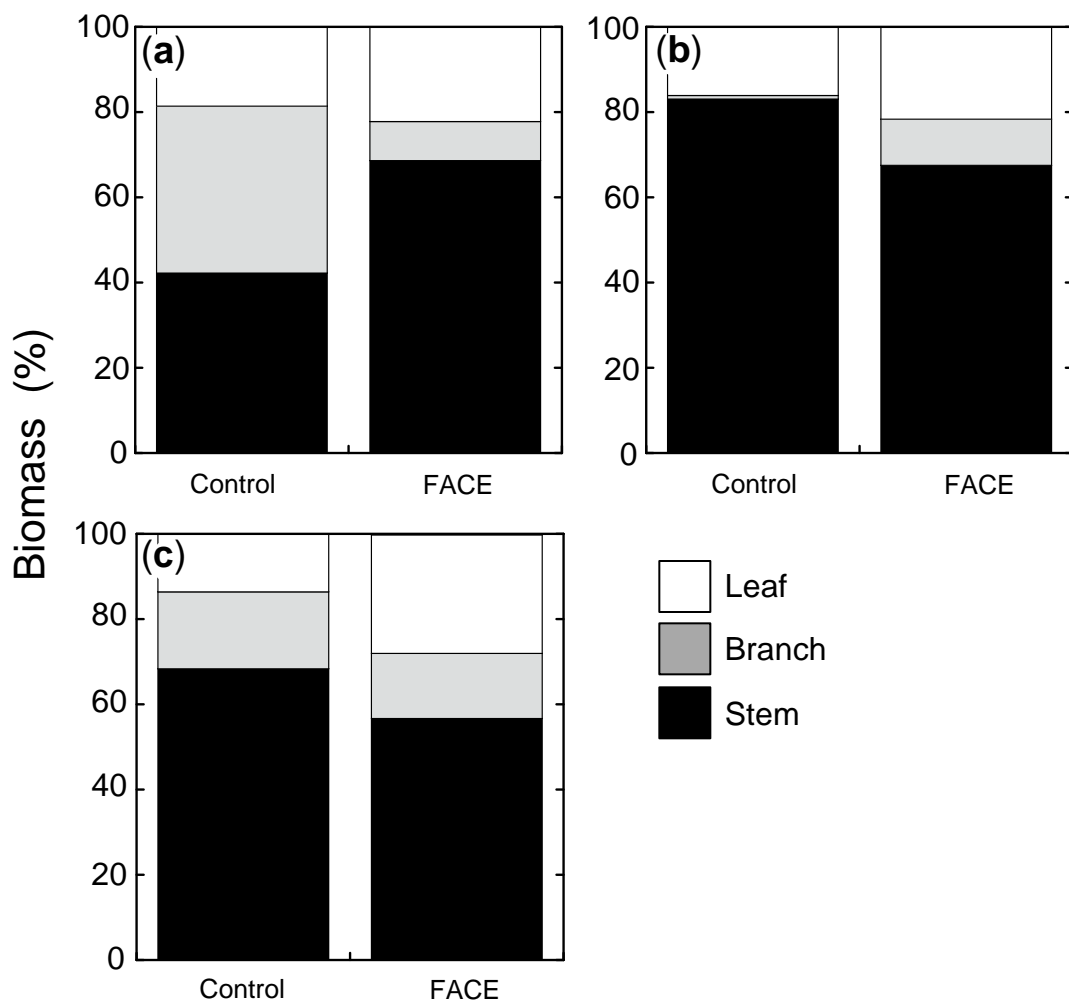


Figure 1

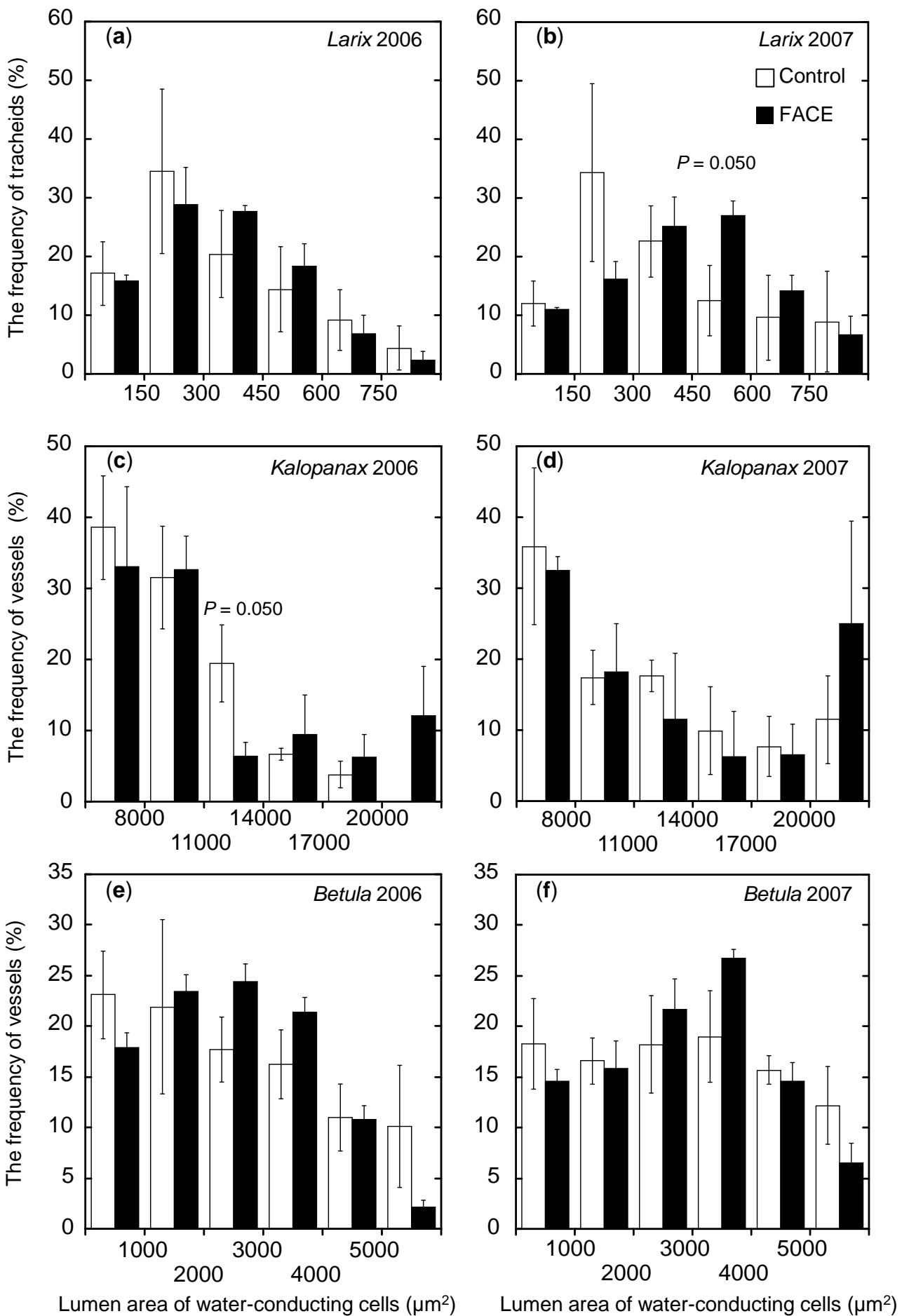


Figure 2

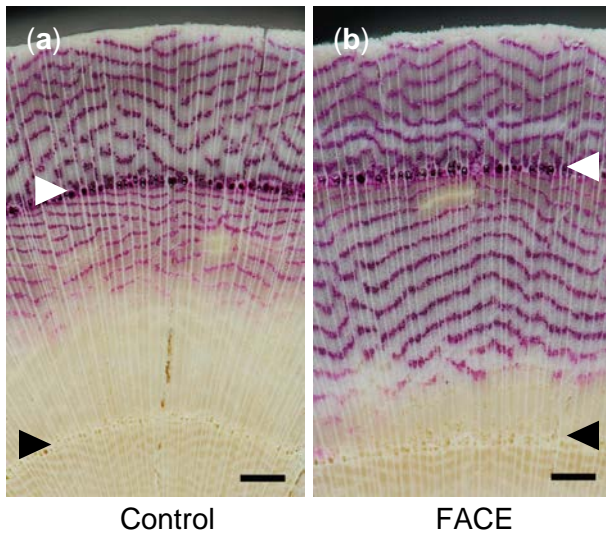


Figure 3