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Leaf area is stimulated in *Populus* by free air CO_2 enrichment (POPFACE), through increased cell expansion and production

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ABSTRACT

The effects of free-air CO_2 enrichment (FACE) on leaf growth in *Populus*, was studied. For the first time in field conditions, both the production and expansion of leaf cells were shown to be sensitive to atmospheric carbon dioxide. Leaf area expansion rate and final leaf size were stimulated under FACE for three species (*Populus x euramericana* (I-214), *P. nigra* (Jean Pourtet) and *P. alba* (2AS-11), with the largest effect observed for *P. x euramericana* (61%). In this species and in *P. nigra*, both epidermal cell size and cell number were increased, whereas for *P. alba*, only cell production was increased in FACE. Two findings suggest that changes in the cell wall may be important in explaining larger leaf cells in FACE: (i) Leaf cell wall extensibility of rapidly growing leaves increased in all species in FACE; and (ii) an increase in xyloglucan endotransglycosylase activity, a cell wall-loosening enzyme, was increased in FACE and associated with leaf growth rate. The results suggest that the mechanisms by which FACE promotes leaf growth differ, depending on species. Despite this, increases in final leaf size provide an important component driving increased biomass accumulation in POPFACE, during this first year of rapid growth, prior to canopy closure. The question as to whether these effects are the result of a direct response to CO_2, or are driven indirectly through substrate availability remains unresolved, although evidence from the literature suggests that the latter mechanism is most likely.

Key-words: *Populus*; FACE; leaf area; leaf anatomy and biophysics; xyloglucan endotransglycosylase.

INTRODUCTION

The development of leaves and effective display of leaf area is fundamental in determining canopy light interception, carbon gain, water loss and ultimately in driving ecosystem productivity (Lambers & Poorter 1992; Ellsworth *et al.* 1995; Van Volkenburgh 1999). Fast- and slow-growing plant species are often characterized by large and small leaves, respectively, reflecting the importance of leaf area development for rapid plant growth. Leaf growth is also highly responsive to the environment, including the supply of atmospheric CO_2. Carbon dioxide is known, in many instances, to cause a stimulation in the rate of leaf expansion (Ferris & Taylor 1994a) and whole-plant leaf area development (Taylor *et al.* 1994, 1995; Pritchard *et al.* 1999), but to have a lesser effect on leaf initiation. Both processes determining the growth of leaves, cell production and expansion, appear to be sensitive to atmospheric CO_2 (Ferris & Taylor 1994b; Ranasinghe & Taylor 1996; Kinsman *et al.* 1997). For instance, Kinsman *et al.* (1997) showed that in the grass, *Dactylis glomerata* L., the cells appeared to pass through the cell cycle more rapidly when exposed to elevated CO_2. This effect was more pronounced in genotypes originating from low rather than high latitudes. Ranasinghe & Taylor (1996) demonstrated a clear effect of elevated CO_2 in stimulating leaf cell expansion in *Phaseolus vulgaris* L. using a model system where cell production and expansion were temporarily separated. Similar findings were observed in a woody species, *Populus x euramericana*, in controlled environments (Gardner, Taylor & Bosac 1995).

Substantial evidence suggests that this carbon-induced growth effect occurs because of changes in the biophysical properties of the cell wall, particularly enhanced wall extensibility (the coefficient *m*) (Ferris & Taylor 1994a; Taylor *et al.* 1994). A wall-loosening enzyme, xyloglucan endotransglycosylase (XET) (Fry *et al.* 1992) that alters wall structure, has been correlated with increased leaf growth in elevated CO_2 (Ranasinghe & Taylor 1996). An increase in the wall hemicellulose, xyloglucan, and changes in the molecular weight distribution of xyloglucan have also been documented (Ranasinghe 1995). All of these data, however, were collected in controlled conditions, often using the model plant system, *Phaseolus vulgaris* L. where expansion and production are separated temporally using a red light treatment (Van Volkenburgh & Cleland 1979).

There remain some important limitations in this research, since its relevance to plants in the field may be

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questioned. In such highly controlled environments root development and nutrient uptake may be restricted (Arp 1991). The coupling of leaf gas exchange to atmospheric conditions may also be inadequate (Jarvis & McNaughton 1987). There is the problem that for long-lived plants, such as trees, short-term responses may be transitory, with critical feedbacks between plant and soil missing, particularly an altered microbial activity and N cycling rates. Furthermore, the trees in such studies are rarely allowed to reach canopy closure (Norby *et al.* 1999). A second, more intriguing question still remaining, is whether the responses to elevated CO_2 are the result of indirect effects driven by the increased supply of substrates, such as soluble carbohydrates (Taylor *et al.* 1994; Masle 2000), or whether CO_2 also acts directly on the regulation of cell expansion and cell production. There is evidence to suggest that CO_2 may stimulate both cell extension (Summers & Jackson 1996) and cell production (Zhao, Misaghi & Hawes, 2000) in the absence of photosynthesis, but the mechanisms operating remain elusive.

Poplars are rapidly growing woody plants cultivated in short-rotation, where leaf area development is known to be tightly linked to stemwood production. Fast-growing genotypes are characterized by rapid leaf extension rates and the production of large leaves (Ceulemans *et al.* 1990; Gardner *et al.* 1995). These trees grown in intensive, short-rotations, are candidates for sources of renewable, carbon-neutral energy (Armstrong & Claridge, 2000). Quantifying net primary productivity of trees and ecosystems and the responses to future CO_2 concentrations in long-term field based experiments remains therefore an important priority. Such studies provide an opportunity to explore more fundamental aspects of the link between carbon supply and plant growth. By using free air CO_2 enrichment (FACE) technology, the general microclimate of the area under study is unaltered and experimental research can be conducted at the level of the ecosystem (Miglietta *et al.* 1997; Hendrey *et al.* 1999).

The aim of this study was first to determine the influence of FACE on leaf growth of three *Populus* species, suitable for biomass plantation and having different growth rates. We hypothesized that a positive leaf growth response to CO_2 was likely in the first year, where the canopy was open. Second, to identify the cellular mechanisms responsible for leaf growth responses under field exposure to elevated CO_2, assessing the relative importance of cell expansion and production. Future work on dissecting out cell cycle and cell expansion effects, and the link between carbon supply and plant growth, may then be possible.

MATERIALS AND METHODS

Site and growth conditions

The experimental plantation and FACE facility is located in Central Italy, near the city of Tuscania (province of Viterbo, latitude 42°37′04″ N, longitude 11°80′87″ E, altitude 150 m). The 9 ha plantation was planted with *Populus* cuttings of three different species in six experimental plots at a planting density of 10 000 trees per ha (spacing of 1 m × 1 m) during the spring of 1999. The species were: *Populus alba* (clone 2AS-11), *Populus nigra* (clone Jean Pourtet) and the hybrid, *Populus x euramericana* (*Populus deltoides* × *P. nigra,* clone I-214). Within the plantation, six 314 m^2 plots were treated either with atmospheric or enriched (550 μmol mol^{-1} CO_2) CO_2 concentration. Each 314 m^2 plot was divided into six sectors (two sectors per species), with 52 plants per sector. The three enriched plots (rings 1, 4 and 5) were equipped with octagonal-shaped FACE rings enclosing a circular area of 20 m diameter. Each ring had eight vertical telescopic masts which could be erected up to 12 m above the ground, supporting 16 horizontal polyethylene pipes (25 mm diameter) bearing a variable number (245–350 per side) of small holes of 0·3 mm diameter. The directional control of the releasing pipes was activated by 16 on/off solenoid valves and an automated pressure regulator controlled the amounted of CO_2 vented out by the pipes. A computer in the centre of each FACE ring controlled the valves and pressure regulator. Two infrared gas analysers (IRGAs), an anemometer and a wind direction sensor were the monitoring devices used to control the whole FACE system. Details of this system and performance are described by Miglietta, Zaldei & Peressotti (2001).

The measurements in this experiment were made between 16 August and 3 September on young, growing trees. The entire area and all the plants were drip irrigated. The target concentration of the FACE rings was 550 μmol mol^{-1} CO_2. Over the duration of the experiment the seasonal (May–December) average CO_2 concentration (±SD) was 544·2 (±7·1); 541·8 (±67·7); and 544·8 (±68·2) μmol mol^{-1} CO_2 in FACE rings 1, 4 and 5, respectively. Average wind speed (±SD) was 1·8 (±0·53) m s^{-1} reaching a maximum of 5·8 m s^{-1} on five days during this period. Because of the imposed cut-off of CO_2 release under very high wind conditions, the average CO_2 concentration during the period 16 August to 3 September was 541·7 (±63·3), 543·9 (±64·0) and 547·9 (±65) μmol mol^{-1} CO_2 in FACE rings 1, 4 and 5, respectively. The average air temperature (±SD) between 16 August and 3 September was 24 (±2·1) °C reaching a maximum of 36 °C during this period. Precipitation during the measurement period was 23 mm and relative humidity averaged 68 (±9·2)%. Global radiation was 19 029·1 (±3651·9) kJ m^{-2} d^{-1} and the PAR averaged 8686·8 (±1602·9) kJ m^{-2} d^{-1}.

Leaf growth

Measurements of leaf growth were conducted on trees in half of each plot (three sectors). For each species, between 10 and 12 young expanding leaves (one leaf per plant) were selected and labelled on 16 August 1999 in each of six rings (2, 3 and 6 control; 1, 4 and 5 FACE treatment) avoiding guard rows. Leaf length was measured on 17, 20, 23, 25, 27, 30 August and 1 and 3 September 1999. Prior to measurements, the relationship between leaf length and leaf area

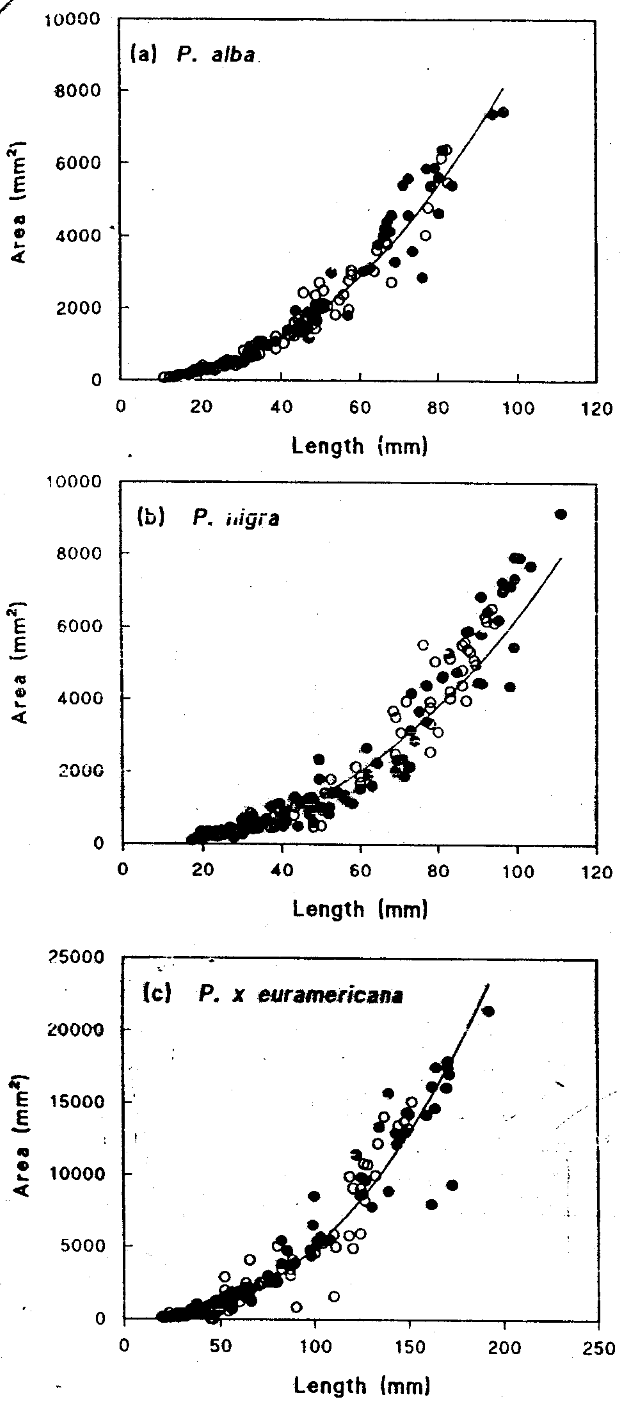


Figure 1. The relationship between leaf area and leaf length of either control (○) or FACE (●) on leaf area (mm^2) of (a) *P. alba*; (b) *P. nigra*; and (c) *P. x euramericana*. The regression equations for the combined treatments are: (a) $y = 0{\cdot}3805x^{2{\cdot}1813}$ ($r^2 = 0{\cdot}98$, 158 d.f.); (b) $y = 0{\cdot}2197x^{2{\cdot}2285}$ ($r^2 = 0{\cdot}94$, 163 d.f.); (c) $y = 0{\cdot}096x^{2{\cdot}3575}$ ($r^2 = 0{\cdot}91$, 189 d.f.).

(Fig. 1) was obtained from both small and large leaves collected in the FACE experiment (approximately $n = 100$). Leaf length, width and area was measured on an image analyser (DELTA-T Devices, Cambridge, UK). There was a close relationship between leaf length and leaf area for all three species ($r^2 = 0{\cdot}99$, 0·98 and 0·99 for *P. x euramericana*, *P. nigra* and *P. alba*, respectively, Fig. 1) and between leaf area and leaf length × leaf breadth (not shown). The former relationships were used to convert leaf length measurements to area in this study. Main stem leaf production was assessed between 16 August and 1 September 1999. Leaf shoot dry mass and the area of mature leaves of each species harvested for epidermal cell imprints was determined and specific leaf area, SLA (g m^2) measured.

Spatial development of leaf area

From the same plants labelled above, six plants were chosen and a younger expanding leaf on each was labelled with a different coloured cotton thread on the 18 August in each of the three species in each of six plots. A total of 18 leaves of each species in each treatment were measured. The leaves were photographed flat against a white background (with marked scale) using a digital camera (Nikon Coolpix 950; Nikon UK Ltd, Kingston-upon-Thames, UK) on 18 August and re-measured on the 24 August 1999. Images were imported into an image processing and analysis program for resizing and format conversion. The program 'Scion Image' (Scion Corporation, Frederick, MD, USA) was used to measure leaf area and spatial development of leaf area by drawing around the same secondary veins on two separate dates to determine growth rate (mm^2 d^{-1}) and the relative spatial growth rate (RSGR, d^{-1}) over different parts of the leaf was calculated by dividing this rate by the initial leaf area. Using this method, for *P. x euramericana*, six new leaves on the same plants as above were labelled on the 26 August in each of six plots and re-measured on the 30 August (18 leaves per treatment). On this occasion, leaves were photographed at a higher resolution. This allowed a more detailed spatial analysis of RSGR over the leaf by drawing around the secondary and tertiary veins on two dates to map RSGR for 22 areas of the leaf. The percentage change from the control was calculated.

Cell wall properties, plasticity and elasticity

Five or six newly expanding leaves (one per plant) were excised on 31 August 1999 from each species in each plot and stored in 70% methanol. The leaves were placed in 3 cm^3 of distilled water for 20 min until fully rehydrated (Ferris & Taylor 1994a; Gardner *et al.* 1995). First, the sample was placed on the lowest setting of a shaker (Orbital Shaker, LH Engineering Co. Ltd, Stoke Pages, UK) for 10 min. The water was replaced with fresh distilled water and the sample left on the shaker for a further 10 min. The leaves were placed on a glass plate and a strip of tissue (3 mm × 5 mm) cut from each leaf either side of the midrib and avoiding major veins. Using a 'homemade' instron apparatus, leaf strips were attached by miniature brass clamps and stretched twice (Ferris *et al.* 1996a). Results are expressed as percentage plasticity (%P, the percentage irreversible extension per 10 g load), and percentage elasticity (%E, the percentage reversible extension per 10 g load).

Xyloglucan endotransglycosylase enzyme activity

The XET activity was assayed by the method of Fry *et al.* (1992). Five newly expanding leaves (one per plant) were excised on the 31 August from each species in each of rings 1, 2, 3 and 4 into liquid nitrogen and stored in a freezer (–70 °C). Frozen leaf material was ground using a pestle and mortar, then extracted in ice-cold buffer (10 mM $CaCl_2$, 50 mM succinic acid/NaOH pH 5·5, 1 mM dithiothreitol (DTT) at 3 cm^3 g^{-1} fresh mass. The homogenate was centrifuged at 4 °C, 10 000 *g* for 4 min in a bench centrifuge and the supernatant was used as the XET preparation. Twenty microlitres of solution B [buffer A containing 0·33% (w/v) chlorobutanol, 0·2% (w/v) tamarind xyloglucan, 1 mM DTT and 1·4 kBq [^{3}H] XLLGol (specific activity 80 mBq μmol^{-1})] was mixed with 10 μL of enzyme solution (supernatant) and incubated at 25 °C for 1 h. The reaction was stopped by the addition of 100 μL of 20% formic acid. This 130 μL mixture was dried on to a 4 cm × 6·6 cm square of Whatman 3 MM chromatography paper (Whatman International Ltd, Maidstone, UK) for more than 2 h at room temperature, and the filters were then washed overnight in running tap water to remove unreacted [^{3}H]XLLGol. The filters were then air-dried at room temperature for more than 2 h, and then at 60 °C for more than 1 h. Filters were rolled up and placed into glass scintillation vials containing 2·0 cm^3 scintillant 'Optiphase safe' (Perkin Elmer, Cambridge, UK) and counted. Assays were performed in triplicate. The XET activity is reported as Bq of [^{3}H] polymer formed per kBq of [^{3}H] oligosaccharide added, per hour per mg wet mass (Fry *et al.* 1992).

Measurement of epidermal cell size and number

A single mature leaf from each of five or six labelled plants of each species in each plot was harvested and its area measured on an image analyser. A small area of the adaxial and abaxial tissue at the base of the leaf was painted with nail varnish and left to dry for 20–25 min. The leaf imprint was removed from the leaf by placing a piece of 'sellotape' over the dried varnish and pressure applied to obtain an imprint (Ferris & Taylor 1994b; Ferris *et al.* 1996b). The 'sellotape' with varnish imprint was peeled from the leaf and placed onto a glass microscope slide. Replicas were examined under the light microscope and epidermal cell density measured. The epidermal cell area (ECA) was obtained using a camera lucida attached to a light microscope which projected cell images onto paper. Twenty cells from five leaves per treatment per species were traced at × 400 magnification. The images were photographed with a digital camera (Nikon View 950) and imported into the image processing and analysis program, 'Scion Image' and mean cell areas per leaf obtained. The number of epidermal cells were counted from three half-fields of view from each of five or six individual leaf surface replicas at × 400 magnification. An eyepiece graticule, precalibrated with a stage micrometer, was used to calculate the area of the half-field of view. Epidermal cell numbers per leaf were estimated by multiplying the average epidermal cell density for each leaf with each leaf area.

Preparation of transverse sections

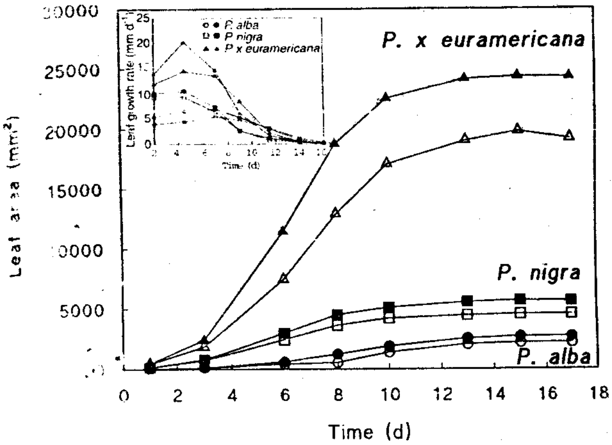
Leaf discs (diameter 6 mm) from the base of five fully expanded mature leaves of each species were placed initially in fixative [formalin : glacial acetic : 70% ethanol (1 : 1 : 18, v/v)]. For light microscopy the discs were cut into 1–2 mm squares and fixed in buffered osmium tetroxide, in order to increase contrast. The specimens were then rinsed in buffer, dehydrated in an ethanol series and embedded in TAAB resin (TAAB Laboratories, Aldermaston, UK) in the normal way. Then 0·5 μm sections were cut on a Leica OMU 3 Ultramicrotome (Leica Microsystems, Milton Keynes, UK) and stained with 1% toluidine blue in 1% borax. Images were captured at × 400 magnification using a camera attached to a Zeiss microscope (Carl Zeiss, Jena, Germany) and then imported into 'Scion Image'. The areas of between five and 10 mesophyll and palisade parenchyma cells from three to five leaves per treatment were measured.

Statistical analysis

Using the Minitab statistical package, the data were analysed using analysis of variance (ANOVA) in a fully randomized block design (mixed model) with plot nested within CO_2 concentration, and CO_2 and species both fixed factors and plot a random factor in the model (Sokal & Rohlf 1981). Post-hoc analysis was applied using a Tukey test where species × plot interactions were significant (not shown). Where there was uneven replication because of missing data, a general linear model was applied. A one-way ANOVA was conducted on each species for the spatial analysis of leaf area development. Data were tested for normality using normal probability plots and homogeneity of variance and percentage data were arcsine transformed (Sokal & Rohlf 1981).

RESULTS

Figure 2 illustrates the effects of FACE on leaf area growth of three species of *Populus*. Leaf area was increased over time in the three species following exposure to FACE and an ANOVA at maturity (d 17) showed a significant CO_2 × species interaction. Leaf length was similarly increased (not shown). The growth rate varied with time (Fig. 2 inset). The growth rate was significantly increased in the linear phase of growth (d 3–d 10) in FACE for all three species (Table 1). Average total leaf area extension rate calculated from initiation until full expansion in *P. x euramericana* was double the rate of *P. nigra* and *P. alba*, irrespective of treatment (Fig. 2, inset; Table 1). In the period d 10–d 17, leaf area extension rate was reduced in FACE in *P. x euramericana* and *P. alba*, whereas in *P. nigra* there was still an



ANOVA	AREA – day 17
Plot (CO_2)	ns
CO_2	***
Species	***
CO_2 × species	**
Species × plot (CO_2)	**

Figure 2. The effects of either control (open symbols) or FACE (solid symbols) on leaf area (mm^2) of *P. alba*, *P. nigra* and *P. x euramericana*. An ANOVA at day 17 is shown below the figure. Inset: the effects of either control (open symbols) or FACE (solid) on leaf extension rate (mm d^{-1}) over time. In each graph, each point is the mean of 28–33 measurements. The statistical analysis on the leaf extension rate at particular points in time is shown in Table 1.

increase in extension rate under FACE (Table 1). Figure 3a–c shows the RSGR over different sections of the leaf from base to tip for each species. Figure 3a–c (inset) shows the characteristic leaf shape of each of these species. For the three species RSGR was increased ($P < 0·05$) at the base of the leaf in FACE. The RSGR (Control; FACE) was higher in *P. x euramericana* irrespective of treatment (2·5; 3·9 d^{-1}), in comparison with *P. nigra* (2·0; 2·8 d^{-1}) and *P. alba* (0·8; 1·1 d^{-1}). The percentage change from ambient in RSGR d^{-1} over 22 regions of the leaf for *P. x euramericana* is shown in Fig. 4. The range in the coefficient of variation for RSGR d^{-1} over the 22 areas of the leaf was 20 to 68% for control leaves and 26 to 67% for FACE leaves (not shown). The highest percentage change occurred at the base of the leaf (52%) and close to the outer margin (53%) (Fig. 4).

Figure 5a illustrates that under FACE, for *P. nigra* and *P. x euramericana*, ECA was increased on both the adaxial and abaxial leaf surfaces but the percentage change in ECA was larger (+48 and +56% on the adaxial and abaxial leaf surface, respectively) in *P. x euramericana* as compared to +19 and +16%, respectively, in *P. nigra*. The ECA of *P. alba* was unaltered (a –2% change) on the adaxial leaf surface but reduced on the abaxial leaf surface under FACE (Fig. 5a). Table 2 shows that there was a significant increase in the palisade parenchyma cell areas under FACE in each species ($P \leq 0·05$), whereas the mesophyll parenchyma cell areas increased in *P. x euramericana* and *P. nigra* but decreased in *P. alba* under FACE ($P \leq 0·001$). Figure 5b shows that the number of epidermal cells per leaf increased significantly under FACE in *P. alba* and *P. x euramericana* and on the abaxial leaf surface of *P. nigra*. There was a significant effect of CO_2 on the area of the mature leaves excised for epidermal cell measurements (Table 1) with a 61% increase under FACE for *P. x euramericana* and 28 and 19% increases under FACE for *P. nigra* and *P. alba*, respectively.

Figure 6a illustrates the influence of FACE on cell wall

Table 1. Summarized analysis of variance table showing *F*-values for the absolute increase in leaf extension rate over time (mm d^{-1}, shown in Fig. 2 inset), in *P. alba*, *P. nigra* and *P. x euramericana* and the mature leaf area of leaves excised for measurements of epidermal cell size and cell numbers per leaf shown in Fig. 5

	Extension rate (mm d^{-1})						Mature leaf area (mm^2)	
	Total		d 3–d 10		d 10–d 17			
Species	Control	FACE	Control	FACE	Control	FACE	Control	FACE
P. alba	3·49	4·19"	4·83	6·27	1·70	1·50	3937·4	4667·0
P. nigra	3·75	4·28	6·38	7·45	0·44	0·51	5218·5	6664·7
P. x euramericana	7·19	8·04	12·28	13·83	3·33	2·48	9417·8	15157·0
ANOVA								
Source of variation	d.f.	*F*-values	d.f.	*F*-values	d.f.	*F*-values	d.f.	*F*-values
Plot (CO_2)	4	1·40 ns	4	1·61 ns	4	0·96 ns	4	2·82*
CO_2	1	11·25***	1	18·85***	1	4·62*	1	41·40***
Species	2	160·45***	2	239·22***	2	84·85***	2	129·64***
CO_2 × species	2	0·28 ns	2	0·21 ns	2	3·50*	2	13·72***
Species × plot (CO_2)	8	3·48***	8	3·29**	8	1·72 ns	8	1·94 ns
Error	162		163		168		69	
Total	179		180		185		86	

***, **, * Significant at the 0·001, 0·01 and 0·05 probability levels, respectively; ns, not significant.

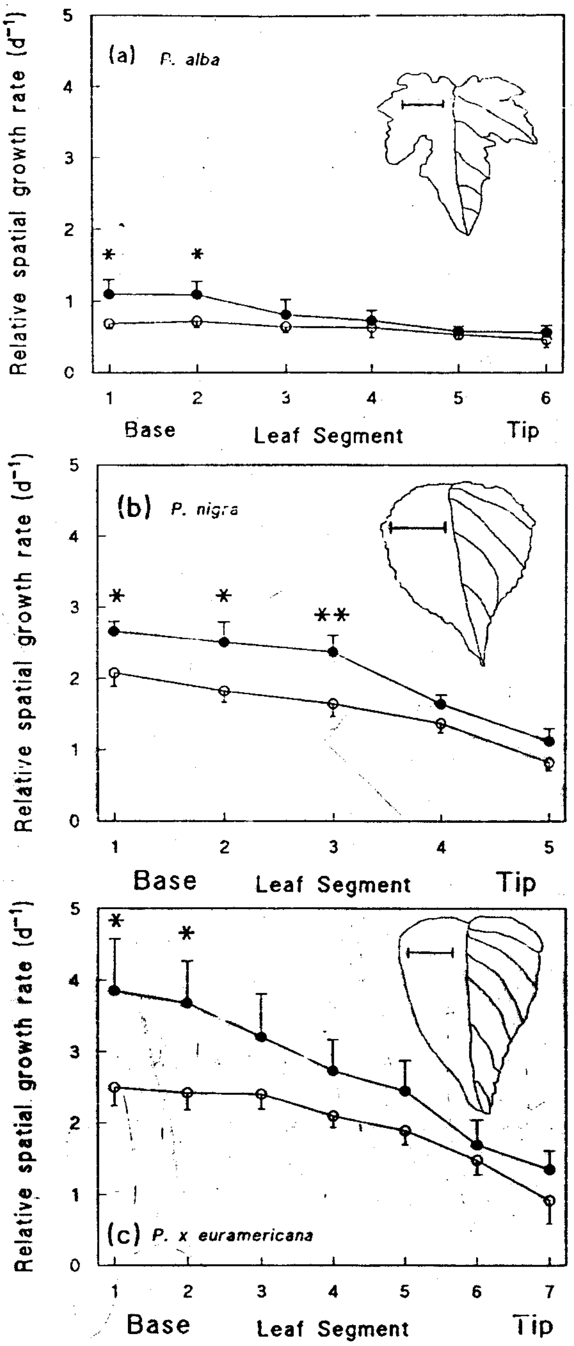


Figure 3. The effects of either control (○) or FACE (●) on mean (±SE) relative segmental growth rate (RSGR; d^{-1}) of (a) *P. alba*; (b) *P. nigra*; and (c) *P. x euramericana*. Each point is the mean of five to six measurements. The results of a one-way ANOVA at each point is shown where significant: *, $P < 0{\cdot}05$; **, $P < 0{\cdot}01$; ***, $P < 0{\cdot}001$. Inset: these plates show the characteristic leaf shape of a young growing leaf of each *Populus* species and the areas between the secondary veins measured to calculate RSGR. Scale bar = 10 mm.

properties of each clone. Cell wall %P (irreversible extensibility) was increased ($P < 0{\cdot}01$) for all species following exposure to FACE, whereas overall %P was higher in *P. alba*, but declining in *P. nigra* and *P. x euramericana*, respectively. For reversible extensibility (%E), there was no significant difference between CO_2 treatments and no difference between species (Fig. 6b). Figure 7 illustrates the influence of FACE on the activity of XET. For young growing leaves in FACE, XET activity was greater in all species ($P < 0{\cdot}01$) compared to controls. There was a significant difference in XET activity between species, with growth rate positively related to XET activity ($P < 0{\cdot}001$; Fig. 7).

Figure 8a shows a significant increase in main stem leaf number under FACE for each species during the measurement period. Figure 8b shows no effect of FACE on SLA of fully expanded leaves. The significant species × plot interaction was largely the result of some variation for *P. nigra* as a result of leaf damage.

DISCUSSION

This research has shown, firstly, that main stem leaf area expansion rate and final leaf area were sensitive to elevated CO_2 in all three species of *Populus* studied here. This is in agreement with previous elevated CO_2 responses for *Populus* grown in open-top chambers (Gardner *et al.* 1995; Ceulemans *et al.* 1996), suggesting that effects on leaf area are important in driving the enhanced biomass production in FACE, again observed in all three species (Gielen *et al.*, personal comm.). Secondly, this research has shown for the first time in field conditions, that leaf cell expansion in asso-

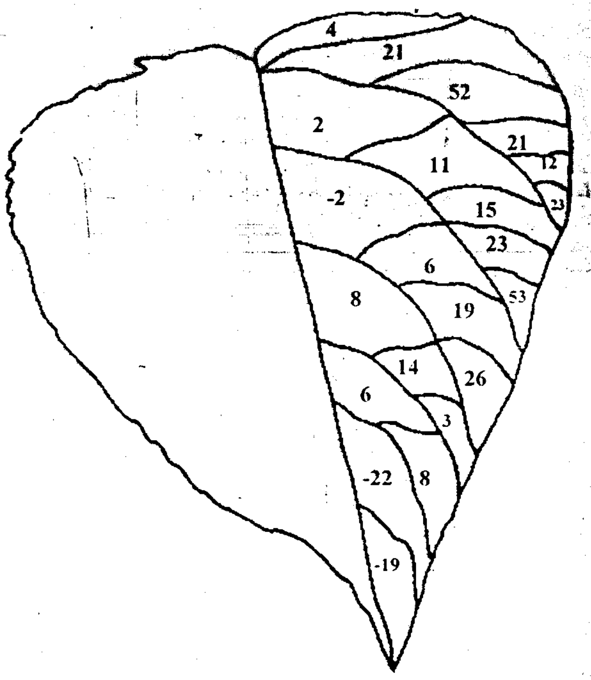
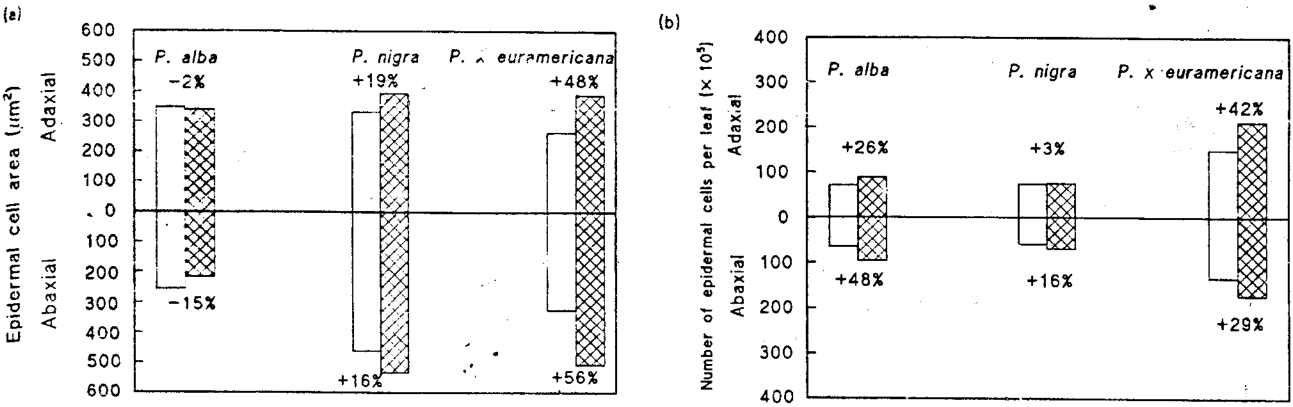


Figure 4. A spatial diagram showing the percentage change from ambient (%) in mean RSGR d^{-1} of growing leaves of *P. x euramericana* calculated as [(FACE – control)/control] × 100. RSGR was measured over 22 areas of the leaf and for five leaves per treatment.



ANOVA	Adaxial	Abaxial
Plot (CO$_2$)	***	ns
CO$_2$	***	***
Species	**	***
CO$_2$ × species	***	***
Species × plot (CO$_2$)	***	***

ANOVA	Adaxial	Abaxial
Plot (CO$_2$)	ns	ns
CO$_2$	***	***
Species	***	***
CO$_2$ × species	***	ns
Species × plot (CO$_2$)	ns	ns

Figure 5. The effects of either control (open bar) or FACE (hatched bar) on leaf (a) epidermal cell area (μm²) and (b) epidermal cell numbers in *P. alba*, *P. nigra* and *P. x euramericana* (adaxial and abaxial surface). In (a) each bar is the mean cell area measured at the base of five leaves (where 20 cells measured per leaf were first averaged). In (b) each bar is the average number of epidermal cells per leaf from five or six leaves. Percentage change is shown in the figures as [(FACE − control)/control] × 100. The ANOVA is shown under the figure: *, $P < 0{\cdot}05$; **, $P < 0{\cdot}01$; ***, $P < 0{\cdot}001$; ns, not significant.

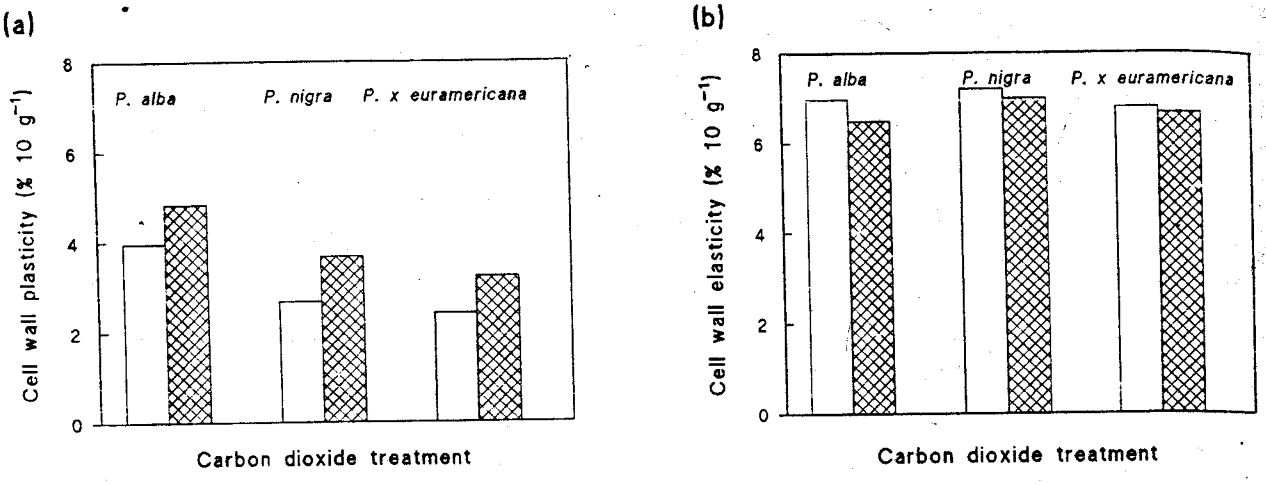
ciation with leaf cell production during early growth, two processes fundamental to plant growth, are sensitive to atmospheric CO$_2$. Interestingly, leaf area expansion rate, irrespective of treatment, was higher in *P. x euramericana* than *P. nigra* and both were higher than *P. alba*. The most responsive species to FACE was also *P. x euramericana*, which was characterized by large increases in epidermal, mesophyll and palisade cell size in FACE (Fig. 5a; Table 2) and a smaller increase in epidermal cell numbers (Fig. 5b). For a *Populus interamericana* hybrid, Radoglou & Jarvis (1990) showed increased leaf size under elevated CO$_2$ was associated with greater leaf cell expansion. This suggests that cell expansion rather than cell production could be considered more important in determining responsiveness to CO$_2$ in *P. x euramericana* and *P. nigra*. It is likely that the more slowly growing *P. alba* cells may remain in the mode of cell expansion and mitosis, whereas the rapidly growing cells of *P. x euramericana* continue to expand after mitosis is complete.

Enhanced leaf growth rate in elevated CO$_2$ has been documented in a number of other studies, but for trees, these have largely been confined to seedlings (Norby *et al.* 1999). In this FACE experiment, during the first year of exposure, with an open canopy and rapid exponential

Table 2. The influence of either control or FACE on the leaf anatomy of mature leaves of *P. alba, P. nigra* and *P. x euramericana*. Measurements were conducted from transverse sections. A summary from a one-way ANOVA is also shown

	Treatment			Source of variation
Leaf cell parameters	Control	FACE	%Δ	*F*-values
P. alba				
Palisade cell area (μm²)	253·90 (11·01)	336·83 (11·81)	+32·7	33·98**
Mesophyll cell area(μm²)	82·50 (1·57)	71·20 (1·67)	−13·7	23·69**
P. nigra				
Palisade cell area (μm²)	360·55 (11·38)	399·84 (3·64)	+10·9	16·67**
Mesophyll cell area(μm²)	171·67 (5·37)	202·30 (2·62)	+17·8	33·98***
P. x euramericana				
Palisade cell area (μm²)	238·4 (13·7)	304·0 (20·3)	+27·5	7·19*
Mesophyll cell area(μm²)	191·7 (7·71)	253·5 (5·07)	+32·2	44·78***

*,**,***significant at the 0·5, 0·01 and 0·001 probability levels, respectively; ns, not significant. Each value is a mean (±SE) from three to five leaves (where five to 10 cells were measured on each leaf and a mean per leaf obtained). % Δ = [(FACE − control)/control] × 100.



(a)

ANOVA	
Plot (CO_2)	ns
CO_2	**
Species	***
CO_2 × species	ns
Species × plot (CO_2)	ns

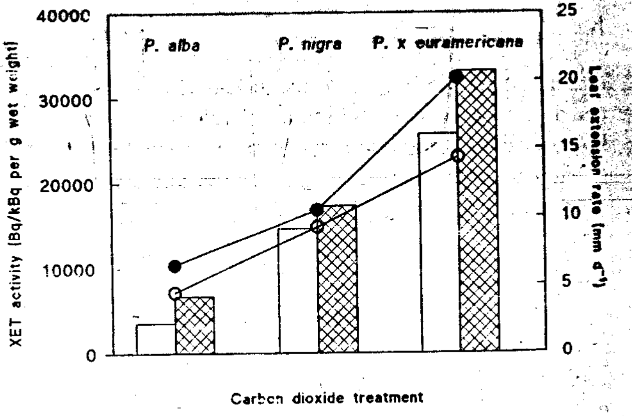
(b)

ANOVA	
Plot (CO_2)	ns
CO_2	ns
Species	ns
CO_2 × species	ns
Species × plot (CO_2)	ns

Figure 6. The effects of either control (open bar) or FACE (hatched bar) on (a) leaf cell wall plasticity per 10 g load and (b) leaf cell wall percentage elasticity per 10 g load of *P. alba*, *P. nigra* and *P. x euramericana*. Each bar is the mean of 15–21 measurements. The ANOVAS are shown under the figure: **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

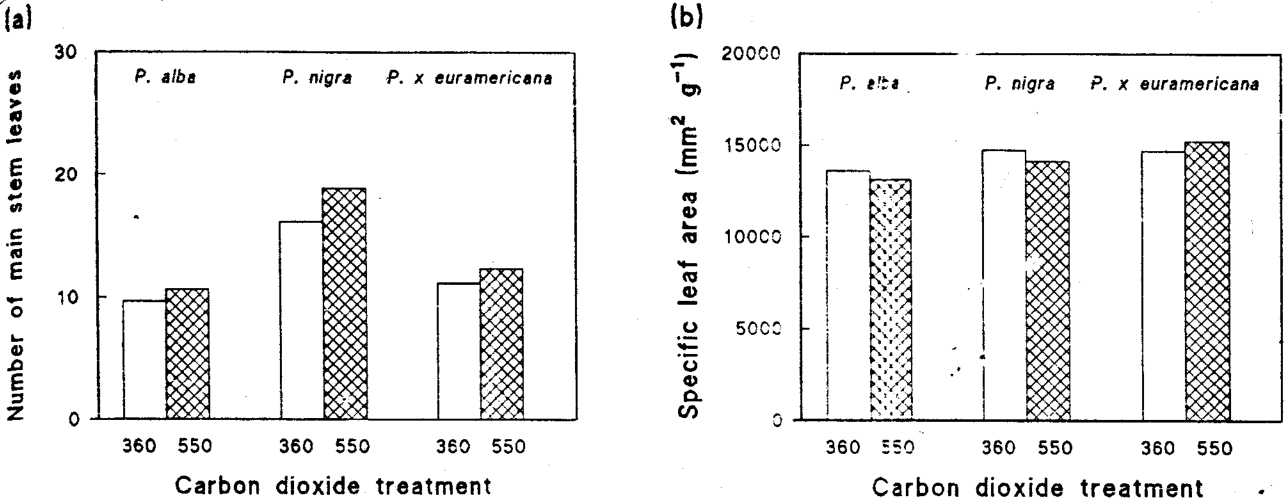
increase in growth, it is perhaps not surprising that enhanced leaf expansion rates were observed. In many respects the trees in this first year of exposure are analogous to the *Populus* in the chamber studies reported by Ceulemans *et al.* (1996) and Gardner *et al.* (1995). Indeed, in both of these studies, increased leaf size was found to be an important determinant of biomass response to elevated CO_2. In such trees it is easy to see that expanding leaves provide a strong sink for fixed carbon, the supply of which will drive further rapid growth. As the canopy develops, and a stable leaf area index (LAI) is achieved, effects of FACE on leaf expansion may diminish since the complexity of the source–sink relationships will be considerably increased. Interestingly, for fully expanded leaves, SLA was unaltered by FACE and although all three species developed more main stem leaves under FACE (Fig. 8a), this response varied as the season progressed and the trees grew bigger (Gielen *et al.*, personal comm.). There are few studies from which to make predictions about the likely future effects on seasonal leaf area development in a closed canopy, although increased branching, leading to indirect effects on leaf production, may override the effects on leaf expansion observed here. It is already known that the production of sylleptic branches (those formed from current year meristems) in *Populus* may depend on available carbohydrate (Scarascia-Mugnozza *et al.* 1997) and that sylleptic branching is increased in elevated CO_2 (Ceulemans *et al.* 1996). Whether this results in the production of a stable but higher LAI is unknown as is the role that leaf expansion will play in determining this canopy structure.

There is good evidence that the effects observed on leaf cell expansion were related to changes in the biochemical and biophysical properties of the cell wall. Leaf cell wall %P, as indicated by Instron measurements, was increased for all three species in FACE, and FACE increased XET



ANOVA	XET
Plot (CO_2)	ns
CO_2	**
Species	***
CO_2 × species	ns
Species × plot (CO_2)	ns

Figure 7. The effects of either control (open bar, ○) or FACE (hatched bar, ●) on mean xyloglucan endotransglycosylase (XET) activity [Bq/kBp per g wet weight (bars, $n = 8$–10)] and mean leaf extension rate [mm d^{-1} (circles, $n = 30$)] at the leaf development stage = 4·5 d after emergence in *P. alba*, *P. nigra* and *P. x euramericana*. The ANOVA on XET activity is shown under the figure: **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.



ANOVA	
Plot (CO_2)	***
CO_2	***
Species	***
CO_2 x species	ns
Species x plot (CO_2)	***

ANOVA	
Plot (CO_2)	ns
CO_2	ns
Species	***
CO_2 x species	ns
Species x plot (CO_2)	ns

Figure 8. The effects of either control (open bar) or FACE (hatched bar) on (a) main stem leaf production between 16 August and 1 September and (b) the specific leaf area (SLA, mm^2 g^{-1}) of fully expanded leaves of *P. alba*, *P. nigra* and *P. x euramericana*. In (a) each bar is the mean of ≈ 30 measurements whereas in (b) each bar is the mean of 15. The ANOVAS are shown under the figures: ***, $P < 0{\cdot}001$; ns, not significant.

activity. Although XET may not be the sole wall-loosening enzyme, it has been found in several tissues assayed and has been correlated closely with growth rates for many cell types (Hetherington & Fry 1993), including bean leaves grown under elevated CO_2 (Ranasinghe & Taylor 1996). It is interesting that along with a slower growth rate, *P. alba* had a lower XET activity per g fresh weight in comparison with the other species. The effects of FACE on XET activity were also largest in this species, but this did not result in larger leaf cells, suggesting other expansion-limiting factors were present. Other agents which directly or indirectly enhance the properties of growing cell walls include expansins, which are proteins on the inner surface of the growing wall; it is suggested that they too modulate extension (Cosgrove 1999) and their role here remains to be elucidated, particularly in *P. alba*. There was also some evidence to show that cell production was stimulated by FACE in all three species. Although speculative, this suggests that FACE affected one of the two 'checkpoints' thought to regulate the plant cell cycle, late G1 (prior to DNA synthesis) and the G2/M transition (De Veylder, Van Montagu & Inze 1997). The interaction of these two responses to FACE – larger leaf cells and increased cell numbers is still unclear as is the exact mechanisms of control. Both processes may be sensitive to the solute or carbon status of the plant cell since cell expansion is driven by cell turgor pressure and increased vacuolar solutes could result in enhanced turgor, wall loosening and growth (Ferris & Taylor 1994a; Taylor *et al.* 1995). It is also probable that FACE had an effect on wall structure, with changes in wall polysaccharides likely. The loosening of the wall could therefore, be altered as a consequence of this in the manner demonstrated here. Evidence from plant and yeast systems exist to support the idea that sucrose may stimulate meristematic activity (Vant Hof, Hoppin & Yagi 1973). More recent studies on light (Granier & Tardieu 1999) and carbon dioxide (Kinsman *et al.* 1997) suggest a link between carbohydrate status and cell cycle in expanding leaves of *Helianthus annuus* and *D. glomerata*, respectively.

In addition to these post-photosynthetic mechanisms and sucrose-regulated gene expression (Stitt & Krapp 1999) it is also possible that direct effects of CO_2 were occurring in expanding and dividing cells. The mechanisms for such effects are not well studied but following exposure to CO_2, cell production may be stimulated in root cells (Zhao *et al.* 2000). Elevated CO_2 may also lead to a reduction in cell pH and a stimulation in 'acid growth' (Cosgrove 1993, 1999). The relevance of these observations to the work reported here on photosynthetic tissue is limited, although these mechanisms warrant further study.

For one point in time, the RSGR was faster between the secondary veins at the base of the leaf and there was a significant increase in FACE for all three species in this region. The high percentage change in relative leaf area expansion at the base of the leaf for the most responsive species, *P. x euramericana* (Fig. 4) suggests that FACE had subtle effects on leaf shape. Ranasinghe & Taylor (1996) showed similar effects on leaf shape of bean (*Phaseolus vulgaris* L. (cv. Tender Green) leaves exposed to elevated CO_2. It also suggests that the spatial pattern of leaf area development in these dicotyledonous leaves was probably accelerated by exposure to FACE. Although dicotyledonous leaf development is more complex than that of monocotyledonous leaves, Granier & Tardieu (1998, 1999) found that in sunflower (*Helianthus annuus* L.), there is nevertheless a distinct basipetal gradient in the sequence of development, with distal, tip areas shown to expand more slowly and to cease expansion first. This is similar to the developmental gradient shown by monocotyledonous leaves, although more complex since meristematic activity occurs in a number of lamina plate meristems. rather than in one single meristematic region. FACE appeared to have the largest effect in areas of the lamina that were growing most rapidly, although a more detailed analysis is required to understand whether these effects were the result of enhanced cell expansion, production or both.

In conclusion, these results show that leaf growth of three *Populus* species is stimulated following exposure to FACE and that mechanistic differences exist between species. For *P. x euramericana* and *P. nigra*, enhanced leaf expansion growth in FACE was largely the result of the production of more larger cells, whereas for *P. alba* leaf expansion growth resulted from the production of more smaller or similar-sized cells. In all species, the increase in leaf area extension rate under FACE, was associated with increased activity of XET, supporting earlier findings that both cell expansion, production and XET activity may be important for leaf growth in elevated CO_2. The information on leaf area development suggests that species with larger leaves will develop larger canopies more quickly and may become more productive in a carbon-rich environment, although it remains to be seen whether the effects reported here persist as the canopy closes.

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