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Effects of elevated CO_2 on the vasculature and phenolic secondary metabolism of *Plantago maritima*

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Abstract

We have examined the effect of elevated CO_2 on the vasculature and phenolic secondary metabolism on clones of the maritime plant *Plantago maritima* (L.). Plants were exposed to either ambient (360 μmol CO_2 mol^{-1}) or elevated (600 μmol CO_2 mol^{-1}) atmospheric CO_2 within a Solardome facility and harvested after 12 months' growth. Histochemical analysis of the leaves identified increases in the diameter of the minor leaf vein and associated lignified vessels in plants exposed to elevated CO_2. In the roots the number of lignified root vessels and stele width were also increased, but overall the lignified vessel-wall thickness was reduced in plants exposed to elevated CO_2, compared to those grown under ambient CO_2. To investigate whether or not these subtle changes in lignification were associated with perturbations in phenolic metabolism, aromatic natural products were analysed by HPLC–MS after treatment with cellulase to hydrolyse the respective glycosidic conjugates. The phenylpropanoids *p*-coumaric acid, caffeic acid, ferulic acid and the flavone luteolin were identified, together with the caffeoyl phenylethanoid glycosides, verbascoside and plantamajoside which were resistant to enzymatic digestion. Exposure to enhanced CO_2 resulted in subtle changes in the levels of individual metabolites. In the foliage a one-year exposure to enhanced CO_2 resulted in an increased accumulation of caffeic acid, whilst in the roots *p*-coumaric acid and verbascoside were enhanced. Our results suggest that significant changes in the vasculature of *P. maritima* on exposure to increased CO_2 are associated with only minor changes in the leaves of specific lignin-related metabolites.

Keywords: *Plantago maritima*; Plantaginaceae; Elevated atmospheric CO_2; Phenylpropanoids; Phenylethanoid glycosides; Flavonoids; Lignification

1. Introduction

Atmospheric CO_2 concentrations are predicted to rise from 360 μmol mol^{-1} to between 550 and 1000 μmol mol^{-1} over the next 100 years and this is anticipated to have major effects on the primary and secondary metabolism of plants (Peñuelas and Estiarte, 1998). Plants can use the increased available carbon in sinks of primary and secondary metabolism through enhancing growth and the associated deposition of cellulose and lignin, as well as through the accumulation of defence and storage compounds such as phenolics and starch (Farrar and Williams, 1991; Lindroth et al., 1993). Two major hypotheses predicting the allocation of carbon in plants exposed to elevated CO_2 have been proposed, notably the carbon-nutrient balance model (CBM) (Bryant et al., 1983) and the growth-differentiation balance (GDB) model (Herms and Mattson, 1992). In the case of secondary metabolism, both models predict that if more carbon is incorporated through increased photosynthesis, a greater proportion will be allocated to natural products such as the phenylpropanoids which are of central importance in lignification and the synthesis of bioactive phenolics such as flavonoids (Dixon and Paiva, 1995). Experimental studies examining the

Abbreviations: ESI, electrospray ionisation; FC, field capacity; HPLC, high-performance liquid chromatography; MS, mass spectrometry; PDA, photodiode array; ToF MS, time-of-flight mass spectrometry.

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effects of increased CO_2 concentrations on gross phenolic metabolism in crop plants or trees have given rise to conflicting results, varying from a 14% increase (Peñuelas et al., 1997), to negligible changes or even a reduction in total phenols (Hartley et al., 2000; Bezemer et al., 2000). Similarly contradictory results have been obtained when the effect of elevated CO_2 on total lignin content has been determined (Poorter et al., 1997; Hartley et al., 2000). Instead, it appears that exposure to increased CO_2 results in much more subtle changes in phenolic composition which can require the analysis of individual metabolites when assessing predictive models of C-allocation (Koricheva et al., 1998; Peñuelas and Estiarte, 1998; Hamilton et al., 2001). For example, studies in deciduous trees (Lavola et al., 2000; Veteli et al., 2002) and wheat (Estiarte et al., 1999) have shown that elevated CO_2 elicits changes in only individual secondary metabolites.

In the current study, we have examined the effect of elevated CO_2 on the phenolic secondary metabolism of clonal individuals of the maritime plant *Plantago maritima* (L.). This hardy species has a relatively well-defined metabolic response to abiotic stress at ambient CO_2, accumulating sorbitol as a compatible solute in response to stress treatments (Ahmad et al., 1979; Jefferies et al., 1983). We have been interested in extending the studies on metabolic plasticity in this species to its phenolic metabolism, which relates to lignification and the vascular system, examining the effect of current and elevated concentrations of atmospheric CO_2. To this end, we have carried out detailed ultrastructural analysis of the patterns of lignified tissues in *P. maritima* following a one year long exposure to ambient and elevated CO_2 and then analysed the respective plants for their content of phenylpropanoid-related metabolites.

2. Results

2.1. Effect of long-term enhanced CO_2 exposure on vasculature of P. maritima

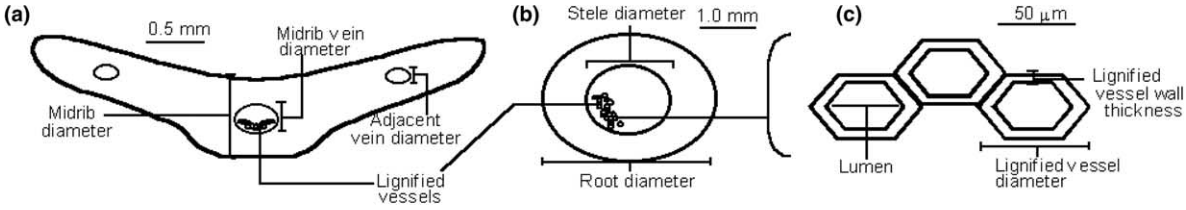
In plants exposed to elevated CO_2 for one year, shoot biomass increased from 1.51 g (±0.31) to 3.37 g (±0.50) and root biomass increased from 1.65 g (±0.17) to 2.28 g (±0.20), an increase of 124% and 38%, respectively ($n = 7$–12), when compared to plants grown at ambient CO_2 for one year. The root and leaf tissue was then sectioned and after staining with calcofluor and acridine orange the vasculature visualised and measured using a micrometer (Figs. 1(f) and (g)). Measurable features were then identified as shown in the schematic (Figs. 1(a), (b) and (c)) and quantified for leaves (Fig. 1(d)) and roots (Fig. 1(e)). From these analyses it was apparent that the vasculature of *P. maritima* had undergone subtle changes after a long term exposure to elevated CO_2. In the leaves, the diameter of the midrib vein was not altered, however, when expressed as a ratio of vein diameter to midrib diameter the vein had increased in size in proportion to the whole leaf width, when compared to plants grown under ambient CO_2 (Fig. 1d). Adjacent veins were wider in those leaves exposed to elevated CO_2, though there was no overall increase in the number of lignified vessels. However, exposure to elevated CO_2 resulted in an increase in the diameters of the lignified vessels in the middle of the leaves ($F_{1,5} = 10.92$; $P = 0.021$). In contrast, within the proximal midrib and middle adjacent veins the lignified vessel wall thickness' were reduced in plants exposed to elevated CO_2 ($F_{1,6} = 29.21$; $P = 0.002$ and $F_{1,5} = 6.60$; $P = 0.050$, respectively) as compared to those exposed to ambient CO_2 for one year.

In the roots, lignification was also visibly affected by elevated CO_2 (Figs. 1(f) and (g)). Thus, stele diameter increased in roots exposed to elevated CO_2 and this enhancement was in proportion to the total width of the root. Such an increase in stele width under elevated CO_2 coincided with an increase in the number of lignified vessel members. There was a minimal increase in the diameter of measured vessels in roots grown under elevated CO_2. However, there was a reduction in the lignified vessel wall thickness to vessel diameter ratio in the middle and distal sections of the root in plants exposed to elevated CO_2 ($F_{1,6} = 8.50$; $P = 0.027$ and $F_{1,6} = 35.02$; $P = 0.001$, respectively), compared to those exposed to ambient CO_2. As well as a lignification of xylem vessel members, an increase in the number of lignified fibre vessels was observed in roots exposed to elevated CO_2 (Figs. 1(e), (f) and (g)).

2.2. Identification of phenylpropanoid metabolites in P. maritima

Having determined significant changes in vascular organisation and lignification in *P. maritima* exposed to elevated CO_2 it was then of interest to determine whether or not there had been perturbations in the levels of phenylpropanoid precursors of lignin and their related metabolites. To establish the range of phenolic metabolites in *P. maritima,* extracts from the foliage and roots of plants grown under ambient and elevated CO_2 conditions were analysed by HPLC with PDA and ESI-ToF MS detection.

Prior to treatment with cellulase to hydrolyse β-*O*-glycosides, a total of 10 UV absorbing peaks with the characteristic spectra of phenolic compounds were determined in the shoots (Fig. 2(b)). Following a treatment with cellulase, a profile of 6 UV absorbing metabolites could be clearly resolved in extracts from both organs (Fig. 2(a)). Since the project was directed to looking at the effect of enhanced CO_2 on the accumulation of the phenolic moieties rather than the conjugating sugars,



Leaf	Proximal		Middle		Distal	
(d)	Atmospheric CO_2 concentration (μmol CO_2 mol^{-1})					
	360	600	360	600	360	600
Midrib vein diameter (μm)	255 ± 6	229 ± 12	186 ± 25	202 ± 16	153 ± 16	145 ± 10
Adjacent vein diameter (μm)	151 ± 8	180 ± 15	120 ± 13	166 ± 11	84 ± 4	117 ± 0
Midrib vein diameter/midrib diameter * 100	24 ± 2	24 ± 2	19 ± 2	25 ± 3	21 ± 2	25 ± 5
No. lignified vessels in midrib vein	21 ± 2	23 ± 1	21 ± 2	20 ± 3	17 ± 4	14 ± 2
No. lignified vessels in adjacent veins	15 ± 1	15 ± 1	11 ± 1	13 ± 1	10 ± 0	10 ± 0
Vessel diameter (μm) in midrib vein	11 ± 0.5	13 ± 0.9	12 ± 0.5	12 ± 0.5	9 ± 2.0	11 ± 0.6
Vessel diameter (μm) in adjacent veins	10 ± 0.1	11 ± 0.7	9 ± 0.5	11 ± 0.4 *	9 ± 1.8	11 ± 0.0
Lignified vessel wall thickness : vessel diameter ratio in midrib vein	0.32 ± 0.01	0.23 ± 0.01 **	0.29 ± 0.02	0.24 ± 0.01	0.30 ± 0.03	0.31 ± 0.04
Lignified vessel wall thickness : vessel diameter ratio in adjacent veins	0.33 ± 0.03	0.26 ± 0.02	0.31 ± 0.01	0.25 ± 0.02 *	0.33 ± 0.01	0.24 ± 0.00

(e) Root	Proximal		Middle		Distal	
	Atmospheric CO_2 concentration (μmol CO_2 mol^{-1})					
	360	600	360	600	360	600
Stele diameter (μm)	1183 ± 255	1618 ± 150	892 ± 180	1288 ± 205	793 ± 238	1298 ± 322
No. lignified vessels	371 ± 95	561 ± 49	211 ± 37	472 ± 122	204 ± 55	487 ± 233
Stele diameter/root diameter * 100	42 ± 4	48 ± 1	41 ± 3	46 ± 2	41 ± 2	46 ± 2
Lignified vessel diameter (μm)	32 ± 2	34 ± 3	35 ± 2	36 ± 2	32 ± 3	37 ± 1
Lignified vessel wall thickness : vessel diameter ratio	0.24 ± 0.01	0.19 ± 0.02	0.23 ± 0.01	0.18 ± 0.02 *	0.24 ± 0.01	0.17 ± 0.01 ***

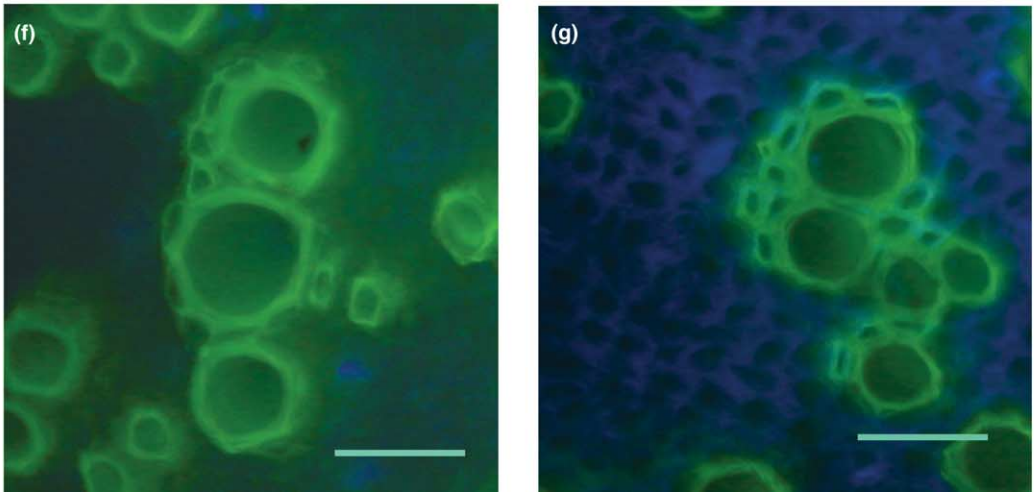


Fig. 1. Key to anatomical measurements made on cross sections of leaf (a); root (b) and lignified vessels (c) transverse cross sections of *P. maritima*. Quantitative changes in leaf (d) and root (e) histochemistry following exposure of *P. maritima* to enhanced CO_2 for 12 months. Data represent mean ± SE. $n = 3$–4 for proximal and middle measurements and $n = 1$–4 for distal measurements. Significant differences between CO_2 treatments are indicated by * ($P < 0.05$); ** ($P \leqslant 0.01$) and *** ($P \leqslant 0.001$). Image of transverse sections of the main root after one year's exposure to either (f) ambient (360 μmol mol^{-1}) or (g) elevated (600 μmol mol^{-1}) atmospheric CO_2 demonstrating visible changes in the width of lignified xylem vessel wall thickness (fluorescent yellow) and increased abundance of lignified fibres adjacent to xylem vessels. Bar length represents 50 μm.

advantage was taken of the increased sensitivity and accuracy in determination afforded following cellulase treatment to identify and subsequently quantify the respective phenolic aglyca. A control HPLC trace has shown that there was no interfering co-eluting absorbing material present in the fungal cellulase preparation. The parent mass ions and UV-spectra of the 6 eluted metabolites (summarised in Table 1) were compared with the data available for phenolic natural products previously identified in *Plantago* species (Moore et al., 1972;

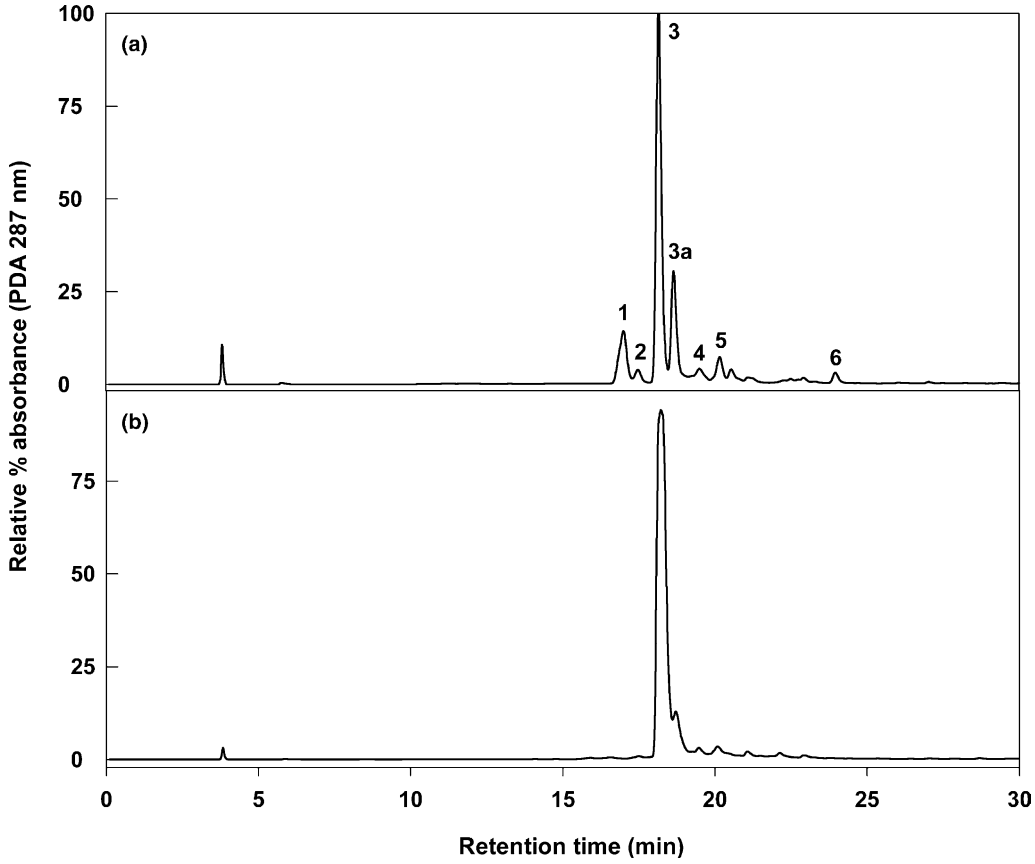


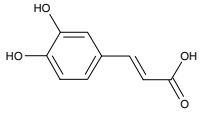
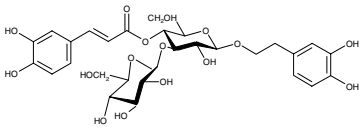
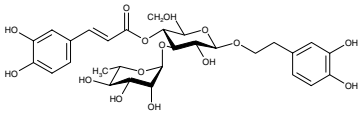
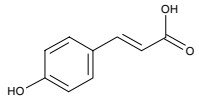
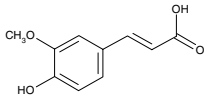
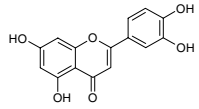
Fig. 2. Typical HPLC chromatograms of shoot extract from *P. maritima* grown under elevated CO_2 for 12 months with (A) and without (B) digestion with cellulase. The eluant was monitored for absorbance at 287 nm with the % scale on the *y*-axis showing the chromatograms scaled to major absorbing peak (= 100%). The relative peak heights in the two chromatograms are therefore not quantitatively comparable. Following cellulase treatment peaks were identified from their UV and mass spectra (see Table 1) as caffeic acid (1), plantamajoside (2), verbascoside (3), verbascoside derivative (3a), *p*-coumaric acid (4), ferulic acid (5) and luteolin (6).

Rønsted et al., 2000). The identities of *p*-coumaric acid, caffeic acid, ferulic acid and luteolin were further confirmed by reference to the chromatographic behaviour and spectral analyses of the respective authentic reference metabolites. By comparison with the HPLC profile obtained with the samples that had not been treated with cellulase, it was clear that the phenylpropanoids and luteolin accumulated in *P. maritima* as multiple conjugates *in planta*. The caffeoyl phenylethanoid glycosides verbascoside and plantamajoside were also identified, being formerly identified in other *Plantago* species (Rønsted et al., 2000). Verbascoside (peak 3) was the major UV absorbing peak present and was consistently associated with a slightly later running peak 3a, which had an identical mass ion to verbascoside. It was concluded that peak 3a was an MS labile derivative of verbascoside, such as an ester. The reason for the caffeoyl phenylpropanoid glycosides being resistant to cellulase treatment was not investigated in detail, but these conjugates have also been shown to be resistant to hydrolysis by β-glucosidases, though susceptible to β-glucuronidase treatment (Ravn and Brimer, 1988). To further confirm the identity of phenolic metabolites, the UV maxima were compared to published UV maxima (Ravn and Brimer, 1988; Grayer and de Kok, 1998). Verbascoside (peaks 3 and 3a) was also ionised at a higher cone voltage (90 V) to initiate the classic fragmentation of the conjugate, producing two ions with a *m/z* of 461 and 161 (Ryan et al., 1999).

2.3. Quantification of phenolics in *P. maritima* grown under ambient and elevated CO_2

Four replicate plants from each treatment were individually extracted and analysed by HPLC–PDA–MS. To monitor recoveries, samples were spiked with a known amount (280 nmol) of the flavonol quercetin at the beginning of the extraction. The mean recovery of the flavonoid spike was determined to be $98 \pm 6\%$ ($n = 3$). Plantamajoside and luteolin was determined to be below the limit of reliable detection in many of the samples and were not routinely quantified. Further analyses were therefore confined to *p*-coumaric acid, caffeic acid, ferulic acid and verbascoside. Irrespective of CO_2 exposure the order of abundance of the phenylpropanoids was verbascoside > caffeic acid > ferulic

Table 1
Identification of phenolic metabolites of *Plantago maritima* by HPLC coupled with PDA and ESI ToF MS detection

Compound number	Compound	Obtained molecular mass ion m/z^-	Reference mass ion m/z^-	PDA UV λ_{max} (nm)	Reference PDA UV λ_{max} (nm)	LC–MS R_t (min)	Compound structure
1	Caffeic acid	179.13	179.16	324	324	17.0	
2	Plantamajoside	639	–	329	332‡	17.5	
3	Verbascoside	623.3	–	331	330†	18.2	
4	*p*-Coumaric acid	163.16	163.15	313	310	19.5	
5	Ferulic acid	193.18	193.18	323	323	20.2	
6	Luteolin	285.18	285.16	349	349	24.0	

† UV λ_{max} from Grayer and de Kok (1998).
‡ UV λ_{max} from Ravn and Brimer (1988).

acid > *p*-coumaric acid in both the foliage and roots (Table 2). Exposure to elevated CO_2 resulted in modest changes the pool sizes of phenolics (Table 2). In the shoot it resulted in a selective doubling of caffeic acid content while in the roots caused a five fold increase in *p*-coumaric together with a 50% increase in verbascoside. No other statistically relevant changes in phenolic content were determined.

3. Discussion

Our results showed an overall reduction in ratio of lignified vessel wall thickness: vessel diameter of leaf and root vessel members in *P. maritima* exposed to elevated CO_2 (Figs. 2(d) and (e)). Since the actual diameters of the lignified vessels were slightly increased throughout the length of the leaf or root, this would imply that the vessel wall surface area available for lignification has actually increased. The increase in the vessel diameter may be caused by increased cell expansion rates commonly observed in plants growing under elevated CO_2 (Pritchard et al., 1999). Therefore, the associated thinner lignified walls may primarily result from a faster cell expansion rate caused by elevated CO_2. In agreement with our results, increases in stem vessel number and lumen area have also been observed in Oak under elevated CO_2 conditions (Atkinson and Taylor, 1996), with increases in main vein diameter and root stele width also observed in *Layia platyglossa* (St. Omer and Horvath, 1984). In the case of *P. maritima* the increases in stele width and associated changes in xylem vessels may be of benefit to the plant, enabling *P. maritima* to translocate water more efficiently (Atkinson and Taylor, 1996).

In addition to the changes in vasculature and associated lignification observed, it might also be anticipated that increased C availability would disrupt the pool sizes of phenylpropanoid lignin precursors and associated metabolites (Peñuelas and Estiarte, 1998). By utilising HPLC–PDA–MS it was possible to identify and quantify the major phenolics in *P. maritima* and determine the subtle effect of exposure to enhanced CO_2 on its

Table 2
Concentrations of the major identified phenolic compounds in *P. maritima* after one year's exposure to either ambient (360 μmol mol^{-1}) or elevated (600 μmol mol^{-1}) atmospheric CO_2

Compound	Atmospheric CO_2 concentration (μmol CO_2 mol^{-1})	
	360	600
p-Coumaric acid		
Shoot	0.41 ± 0.29	0.57 ± 0.23
Root	0.09 ± 0.03	0.52 ± 0.06 (***)
Caffeic acid		
Shoot	1.08 ± 0.22	2.31 ± 0.19 (**)
Root	1.04 ± 0.14	1.25 ± 0.15
Ferulic acid		
Shoot	0.37 ± 0.10	0.51 ± 0.07
Root	0.16 ± 0.02	0.22 ± 0.03
Verbascoside		
Shoot	9.57 ± 1.62	9.24 ± 1.00
Root	17.88 ± 1.70	27.61 ± 4.15

Data are expressed as mg g^{-1} dry weight apart from verbascoside (mg caffeic acid equivalents g^{-1} dry weight) and represent mean ± SE ($n = 4$). Significant differences between CO_2 treatments are indicated by ** ($P \leqslant 0.01$) and *** ($P \leqslant 0.001$).

phenylpropanoid metabolism after a 12 month treatment. Caffeic acid and its derivative verbascoside dominate the phenolic metabolism in *P. maritima* as determined in other *Plantago* species (Rønsted et al., 2000). In contrast, the related compound plantamajoside was only a minor component in *P. maritima* possibly due to its ready interconversion to verbascoside as determined in *P. lanceolata* (Fons et al., 1999).

Our studies showed that elevated CO_2 had a subtle effect on phenylpropanoid metabolism, with the changes being more prominent in the root rather than the shoot. Caffeic acid and its derivative verbascoside were both enhanced in the shoot and root, respectively, together with a selective increase in *p*-coumaric acid content in the roots. Although these changes were modest, the statistical analysis of the results obtained from this clonal material confirmed that they were significant perturbations in phenylpropanoid pool sizes.

Changes in lignification in the vascular system appear to be more likely to be due to altered rates of cell expansion due to more rapid growth as observed in *Plantago media*, *Phaseolas vulgaris* and *Populus euramericana* (Taylor et al., 1994) rather than due to minor changes in the availability of lignin precursors. However, it is intriguing that the pool sizes of caffeic acid, verbascoside and *p*-coumaric acid but not ferulic acid are increased in an organ specific manner after exposure to increased CO_2. These results highlight that contrary to expectations, increased carbon availability has only very subtle effects on plant phenolic metabolism. Analysis of alternative C metabolic sinks of primary and secondary metabolism in *P. maritima* exposed to elevated CO_2 is currently in progress.

4. Experimental

4.1. Plant material and treatments

Clonal shoots of *P. maritima* were collected from a coastal cliff-top in Northumberland, UK (UK Ordnance Survey reference: NU 264 174) and transferred into seed trays containing acid-washed sand. As such, the clonal shoots originating from parent plants were considered to be genetically identical. Plants were maintained in a glass house, with daily watering and fed with 1/4th strength Long-Ashton solution modified to contain 2.8 mg l^{-1} N (as NH_4NO_3) every week. After four weeks, the plants were transferred to the Centre for Ecology and Hydrology (CEH) Solardome facility, Bangor, Gwynedd, UK and individually placed in 500 cm^3 deep pots filled with 800 g of dry, acid-washed sand prior to distributing the plants between four Solardomes (16 plants per dome). The atmosphere of two of the domes was adjusted to ambient CO_2 (360 μmol CO_2 mol^{-1}) while the other two domes were adjusted to 600 μmol CO_2 mol^{-1} as described previously (Rafarel et al., 1995). The study started in July 2001 and the plants watered daily using an automated irrigation system. Once a week each pot was fed with 50 ml 1/4th strength Long Ashton nutrient solution containing 28 mg l^{-1} N (as NH_4NO_3), except in the winter (December 2001 to February 2002) when feeding was reduced to once per month.

In July 2002, all plants were harvested in replicates of up to 12 individuals per CO_2 treatment and the fully expanded leaves and roots detached, weighed and frozen in liquid nitrogen prior to storage at −80 °C. The remaining shoot material from each plant was dried (65 °C, 48 h) for dry weight determination on harvest. For histochemical analysis, fresh leaves and primary roots were immediately fixed in 100% ethanol.

4.2. Lignin histochemical analysis

Hand-cut transverse sections of ethanol-fixed leaf and root tissue were made at the proximal, middle and distal (above 0.5 cm from tip) parts of each sample. Sections were stained sequentially in 0.01% (w/v) calcofluor M2R followed by 0.01% (w/v) acridine orange, each for 1 min, with an intervening wash in distilled water. Sections were then examined using a blue-violet excitation filter and a Nikon Diaphot-TMD-EF fluorescence microscope (Nikon Corporation, Tokyo 100, Japan) as described previously (Gates, 1993). Diameters of the midrib vein and major adjacent shoot veins, root stele diameter and the number of lignified vessel members were recorded using a micrometer. Digital photographs (Nikon Coolpix950, Nikon Corporation, Tokyo 100, Japan) were then taken of the sections and calibration slides. PaintShop Pro. v.6 (Jasc Software Inc., Eden

Prairie, MN 55344, USA) software was used to measure the total diameter and lumen diameter of lignified vessels from the digital photographs.

4.3. Analysis of soluble phenolic metabolites

Frozen plant tissue (0.2 g) was homogenised in ice-cold acetone (5 ml) using a pestle and mortar and acid-washed sand as an abrasive. After decanting the solvent, the residue was re-extracted a further two times each with 5 ml methanol:acetone (1:1 v/v). After filtration, the combined solvent extract was evaporated under vacuum and the residue re-suspended in methanol (2 ml). For the analysis of the phenolics as their respective aglyca, duplicate samples (0.5 ml) of the final methanolic extract were reduced to dryness under vacuum. One sample was resuspended in 0.5 ml 0.15 M citrate-phosphate buffer, pH 5.0, and the other in 0.5 ml of the same buffer containing 1 mg ml^{-1} cellulase from *Trichoderma viride* (Boehringer Mannheim, Germany).

Samples were incubated for 18 h at 30 °C and then successively partitioned against three lots of 0.5 ml water-saturated ethyl acetate. The combined organic phases were evaporated and the residue re-suspended in 0.5 ml methanol. Samples were analysed by high-performance liquid chromatography (HPLC) on a Waters Alliance 2790 HPLC system (Waters Ltd., Hertfordshire, UK) with the eluant analysed by both photodiode array (PDA) spectrometry (Waters 996) and time-of-flight mass spectrometry (ToF MS; Micromass LCT, Micromass UK Ltd., Manchester, UK) the latter using Electro-Spray-Ionisation (ESI). Samples (10 μl) were resolved on a Synergi^{TM} POLAR-RP column (250 × 2.0 mm, Phenomenex, UK) using acetonitrile (solvent A) and 0.5% formic acid (solvent B); with a gradient of increasing B such that initial A:B (95:5 v/v); 2 min, (95:5); 42 min, (0:100); 47 min, (0:100); 48 min, (95:5); 53 min, (95:5) at a flow rate of 0.2 ml min^{-1}. The eluant was monitored for absorbance between 200 and 400 nm with the MS operating in negative ion mode (settings: sample cone voltage = 20 V, extraction cone voltage = 6 V; desolvation temp. = 250 °C, source temp. = 120.0 °C; penning pressure 5.15^{-7} mbar; nebuliser nitrogen gas flow = 4 L h^{-1}; desolvation nitrogen gas flow = 516 L h^{-1}). Mass ions were detected between 100 and 800 *m/z* with raffinose and sodium iodide used to tune and calibrate the MS, respectively. The identification of phenolic metabolites was based on their UV and mass spectral data as compared with published data and supplemented with the use of authentic reference samples of *p*-coumaric acid, caffeic acid, ferulic acid and luteolin (Sigma). To assist in the identification of verbascoside, the sample cone voltage was increased to 90 V to cause characteristic fragmentation of the conjugate (Ryan et al., 1999).

4.4. Statistical analyses

A one-way analysis of variance (ANOVA) was used to test for significant differences between CO_2 treatments. Due to pseudo-replication in the anatomical and lignin analyses, the mean of the measured parameter from all cross sections per shoot or root were taken as one replicate. All statistical analyses were carried out in SPSS v.10.00 (Chicago, IL, USA).

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References

Ahmad, I., Larher, F., Stewart, G.R., 1979. Sorbitol, a compatible osmotic solute in *Plantago maritima*. New Phytol. 82, 671–678.

Atkinson, C.J., Taylor, J.M., 1996. Effects of elevated CO_2 on stem growth, vessel area and hydraulic conductivity of oak and cherry seedlings. New Phytol. 133, 617–626.

Bezemer, T.M., Hefin Jones, T., Newington, J.E., 2000. Effects of carbon dioxide and nitrogen fertilization on phenolic content in *Poa annua* L. Biochem. Syst. Ecol. 28, 839–846.

Bryant, J.P., Chapin, F.S., Klein, D.R., 1983. Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory. Oikos 40, 357–368.

Dixon, R.A., Paiva, N.L., 1995. Stress-induced phenylpropanoid metabolism. Plant Cell 7, 1085–1097.

Estiarte, M., Peñuelas, J., Kimball, B.A., Hendrix, D.L., Pinter, P.J., Wall, G.W., LaMorte, R.L., Hunsaker, D.J., 1999. Free-air CO_2 enrichment of wheat: leaf flavonoid concentration throughout the growth cycle. Physiol. Plant 105, 423–433.

Farrar, J.F., Williams, M.L., 1991. The effects of increased atmospheric carbon dioxide and temperature on carbon partitioning, source-sink relations and respiration. Plant Cell Environ. 14, 819–830.

Fons, F., Tousch, D., Rapior, S., Gueiffier, A., Roussel, J.L., Gargadennec, A., Andary, C., 1999. Phenolic profiles of untransformed and hairy root cultures of *Plantago lanceolata*. Plant Physiol. Biochem. 37, 291–296.

Gates, P., 1993. Plant anatomy, histology and taxonomy for molecular biologists. In: Croy, R.R.D. (Ed.), Plant Molecular Biology Labfax. Bios Scientific Publishers, pp. 1–20.

Grayer, R.J., de Kok, R.P.J., 1998. Flavonoids and verbascoside as chemotaxonomic characters in the genera *Oxera* and *Faradaya* (Labiatae). Biochem. Syst. Ecol. 26, 729–741.

Hamilton, J.G., Zangeri, A.R., DeLucia, E.H., Berenbaum, M.R., 2001. The carbon-nutrient hypothesis: its rise and fall. Ecol. Lett. 4, 86–95.

Hartley, S.E., Jones, C.G., Couper, G.C., Hefin Jones, T., 2000. Biosynthesis of plant phenolic compounds in elevated atmospheric CO_2. Global Change Biol. 6, 497–506.

Herms, D.A., Mattson, W.J., 1992. The dilemma of plants: to grow or defend. Q. Rev. Biol. 67, 283–335.

Jefferies, R.L., Davy, A.J., Rudmik, T., 1983. The growth strategies of coastal halophytes. In: Jefferies, R.L., Davy, A.J. (Eds.), Ecological Processes in Coastal Environments. Blackwell Scientific Publications, Oxford, pp. 243–268.

Koricheva, J., Larsson, S., Haukioja, E., Keinänen, M., 1998. Regulation of woody plant secondary metabolism by resource availability: hypothesis testing by means of meta-analysis. Oikos 83, 212–226.

Lavola, A., Julkunen-Tiitto, R., de la Rosa, T.M., Lehto, T., Aphalo, P.J., 2000. Allocation of carbon to growth and secondary metabolites in birch seedlings under UV-B radiation and CO_2 exposure. Physiol. Plant 109, 260–267.

Lindroth, R.L., Kinney, K.K., Platz, C.L., 1993. Responses of deciduous trees to elevated atmospheric CO_2: productivity, phytochemistry, and insect performance. Ecology 74, 763–777.

Moore, D.M., Williams, C.A., Yates, B., 1972. Studies on bipolar disjunct species II. *Plantago maritima* L. Bot. Not. 125, 261–272.

Peñuelas, J., Estiarte, M., 1998. Can elevated CO_2 affect secondary metabolism and ecosystem function? Trends Ecol. Evol. 13, 20–24.

Peñuelas, J., Estiarte, M., Llusià, J., 1997. Carbon-based secondary compounds at elevated CO_2. Photosynthetica 33, 313–316.

Poorter, H., Van Berkel, Y., Baxter, R., Den Hertog, J., Dijkstra, P., Gifford, R.M., Griffin, K.L., Roumet, C., Roy, J., Wong, S.C., 1997. The effect of elevated CO_2 on the chemical composition and construction costs of leaves of 27 C_3 species. Plant Cell. Environ. 20, 472–482.

Pritchard, S.G., Rogers, H.H., Prior, S.A., Peterson, C.M., 1999. Elevated CO_2 and plant structure: a review. Global Change Biol. 5, 807–837.

Rafarel, C.R., Ashenden, T.W., Roberts, T.M., 1995. An improved Solardome system for exposing plants to elevated CO_2 and temperature. New Phytol. 131, 481–490.

Ravn, H., Brimer, L., 1988. Structure and antibacterial activity of plantamajoside, a caffeic acid sugar ester from *Plantago major* subsp. *major*. Phytochemistry 27, 3433–3437.

Rønsted, N., Göbel, E., Franzyk, H., Jensen, S.R., Olsen, C.E., 2000. Chemotaxonomy of *Plantago*. Iridoid glucosides and caffeoyl phenylethanoid glycosides. Phytochemistry 55, 337–348.

Ryan, D., Robards, K., Prenzler, P., Jardine, D., Herlt, T., Antolovich, M., 1999. Liquid chromatography with electrospray ionisation mass spectrometric detection of phenolic compounds from *Olea europaea*. J. Chromatogr. A 855, 529–537.

St. Omer, L., Horvath, S.M., 1984. Developmental changes in anatomy, morphology and biochemistry of *Layia platyglossa* exposed to elevated carbon dioxide. Am. J. Botany 71, 693–699.

Taylor, G., Ranasinghe, S., Bosac, C., Gardner, S.D.L., Ferris, R., 1994. Elevated CO_2 and plant growth: cellular mechanisms and responses of whole plants. J. Exp. Bot. 45, 1761–1771.

Veteli, T.O., Kuokkanen, K., Julkunen-Tiitto, R., Roininen, H., Tahvanainen, J., 2002. Effects of elevated CO_2 and temperature on plant growth and herbivore defensive chemistry. Global Change Biol. 8, 1240–1252.