Elevated CO_2 reduces vessel diameter and lignin deposition in some legume plants grown in mini-FACE rings

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Abstract

Studies on stem (and leaf) structure and histology of a semi-natural grassland community, permanently growing in mini-FACE rings under elevated concentrations of atmospheric CO_2 (560 μmol mol^{-1}) are presented. Histochemical analysis of stem sections from legume plants grown under high CO_2 concentration revealed both a reduction of lignin deposition in spring vascular bundles of *Trifolium repens* L., and a decrease in size of the xylem vessels in *Vicia hybrida* L. and *Vicia sativa* L. Thus, the effects of elevated CO_2 on the stem histology of the species investigated are rather species-specific and/or organ-specific, and of major account especially in the early phases of vegetative growth, in particular as regards lignin deposition mechanisms. In leaves, neither differences as to lignification nor any other anatomical structure modification were found under CO_2 enrichment.

Additional key words: lignification, *Trifolium repens*, *Vicia hybrida*, *Vicia sativa*, xylem.

Introduction

The progressive increase of atmospheric levels of carbon dioxide, primary cause of the so-termed "greenhouse effect", will have repercussions on organisms and ecosystems difficult to foresee (Hasselman 1997). Although there have been a large number of publications in which the impact of high atmospheric CO_2 concentrations on plants has been thoroughly investigated (reviewed in Sanità di Toppi *et al*. 2002b), no information is available on influence of elevated concentrations of CO_2 on lignin deposition and vessel diameters in stems and leaves of plants growing in a grassland community. With regard to leaves of several C_3 plants, some authors detected no changes in lignin concentration under elevated CO_2, in comparison with ambient CO_2 (Poorter *et al*. 1997, Blaschke *et al*. 2001); however, other experiments indicated a decreased lignification rate by about 30 % under elevated CO_2 in *Quercus ilex* (Polle *et al*. 2001) and a high CO_2-induced decrease in thickness of the bundle sheath cell walls in the C_4 species *Sorghum bicolor* (Watling *et al*. 2000).

The hypothesis to be tested by this study is that elevated concentrations of atmospheric CO_2 could lead to substantial variations in stem and leaf histology and structure (in particular vessel diameters and lignin deposition) and in protein content of three legume species growing in a semi-natural grassland community.

Materials and methods

Experimental site, plant material and sampling conditions: The experiments were performed on a semi-natural grassland community, mainly composed by *Leguminosae*, *Poaceae* and *Apiaceae*. The experimental field, uniform in soil composition and sun exposure, was located in Rapolano Terme, near Siena, Italy. The field soil had not been fertilized for at least five years before the beginning of the experiments.

Plants grew in mini-FACE rings (six CO_2-enriched rings plus six identical control rings, 2 m in diameter, toroidal distribution), under ambient (360 μmol mol^{-1}) and elevated CO_2 (560 μmol mol^{-1}). The mini-FACE technology was set up and monitored as described in Hendrey *et al.* (1993) and Miglietta *et al.* (1997, 2001). Purified and concentrated CO_2 was supplied by *Messer Italia*, Rapolano Terme, Italy, by means of an underground pipe system.

The experiments presented here focused on three *Leguminosae* species (*Trifolium repens* L., *Vicia hybrida* L., *Vicia sativa* L.). Plants, all homogeneous in their general aspect, dimensions and apparent health conditions, were randomly collected from CO_2-enriched and control rings on May and July 2000, between 10:00 and 12:00 of the same day. For each species, control and CO_2-enriched stems and leaves were as much as possible uniform in their general aspect, age and dimensions.

Histological studies: Two-cm-long stem portions of the three leguminous species, sampled on July 2000, from plants at the same phenological phase, were excised by collecting homogeneous internodes at the height of about 30 cm. At the same time, leaves were collected from neighbouring nodes, and an approx. 5 mm thick portion was excised from the leaf central region. The samples were then fixed in FAA (Ruzin 1999), and, after fixation, dehydrated in absolute ethanol, embedded in *Tecnovit 7100* (*Heraeus*, Wehrheim, Germany), and sectioned in 5 μm thick sections with a semithin microtome (*Reichert-Jung 2040 Autocut Microtome*, Nussloch, Germany) equipped with a tungsten carbide knife. The sections were stained with periodic acid-Schiff reagent and counterstained with Amido Black 10B (Ruzin 1999) or toluidin blue (O'Brien and McCully 1981), as general stains. Some sections of each sample were stained with phloroglucinol for lignin (Ruzin 1999). Histological observations were performed with a *Zeiss Axioskop* (Nussloch, Germany) light microscope. Vessel lumen measurements were taken with a micrometric eyepiece.

Protein determination: Protein concentration in stem and leaf material collected on May and July 2000 was determined by the *Bio-Rad* protein assay (*Bio-Rad*, München, Germany), using bovine serum albumin as a standard, following Bradford (1976).

Statistics: The experimental set consisted of 12 mini-FACE rings (6 fumigated + 6 control ones). All experiments were carried out on at least 6 replicates of stems and leaves collected simultaneously on the same day from different rings (fumigated or control ones). Differences in results were evaluated, where appropriate, by unpaired t-test (*SigmaPlot 5.0*).

Results

In *T. repens* lignification occurs, in the growth period from emergence to sampling time, only in vascular bundles (Fig. 1). The stems contain five vascular bundles, which are capped by a thick collenchymatous structure; the collenchyma gives the bundle a mushroom shape when seen in a transverse section. Between bundle and epidermis there is a 6 - 8 cell layered, photosynthesizing cortex, rich in starch granules. In our samples, the cortex continues into the interfascicular parenchyma, which is formed by larger cells, rich of intercellular spaces, provided with chloroplasts and deposits of starch granules. A large pith tissue is present within the relatively thin bundle layer. At sampling time a layer of summer xylem, rich in fibres and with thinner vessels is being deposited.

In *V. hybrida* and *V. sativa* (Figs. 2, 3), at the sampling time, the situation is of an advanced stem differentiation. The xylem being deposited is of the spring type, and the cambium appears still fairly active.

The stems are winged at 2 or 3 of the four corners, where supplementary cortical vascular bundles can be present. All mechanical tissues, with few exceptions, are concentrated in the corners, *i.e.* collenchyma subepidermically, and sclerenchyma (fibres) closer to the vascular bundle.

The differences between control plants and CO_2-enriched plants were of a different type between *T. repens* on the one hand, and *V. hybrida* and *V. sativa* on the other. In *T. repens* the observation of xylem vessels showed that, apart from the oldest elements (metaxylem) formed in the early stem growth period, vessel differentiation in control plants had an orderly and complete development (Fig. 4*A*). Vascular bundles in CO_2-enriched plants, on the contrary (Fig. 4*B*), revealed a reduced lignification of secondary walls in most of the growth period, achieving a normal degree of differentiation only in the last layers of xylem elements, formed in summer. This feature is constant in all bundles, irrespective of their size.
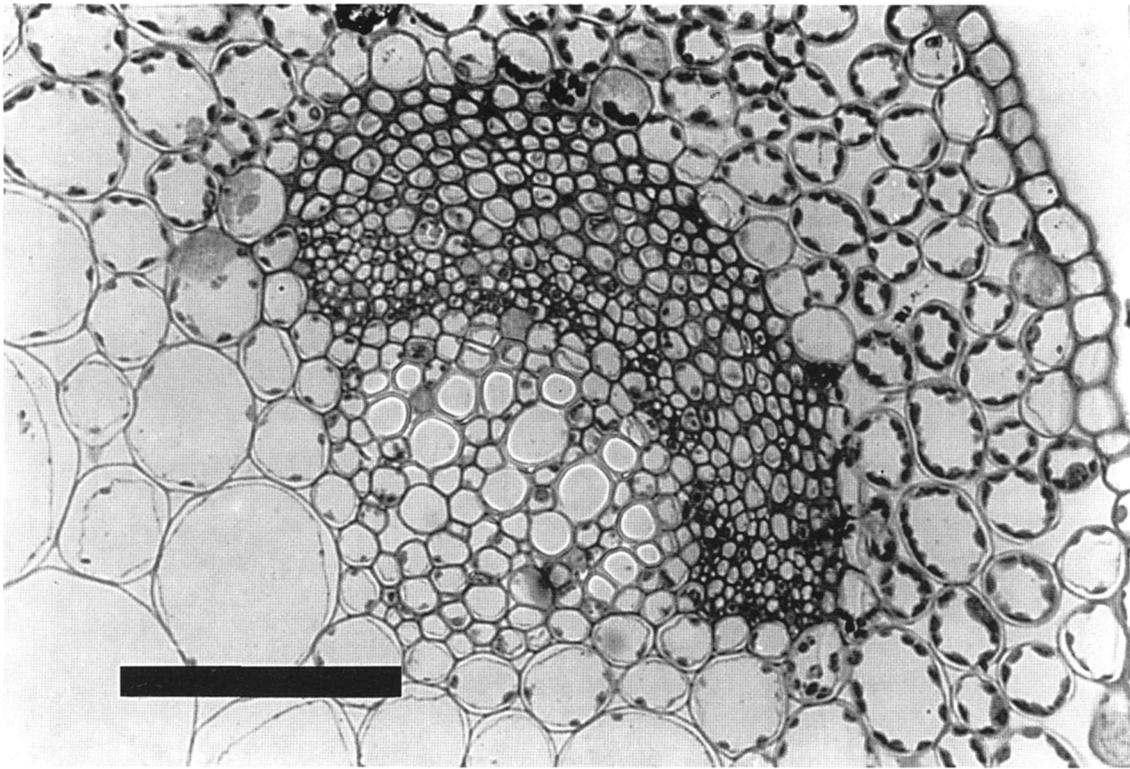
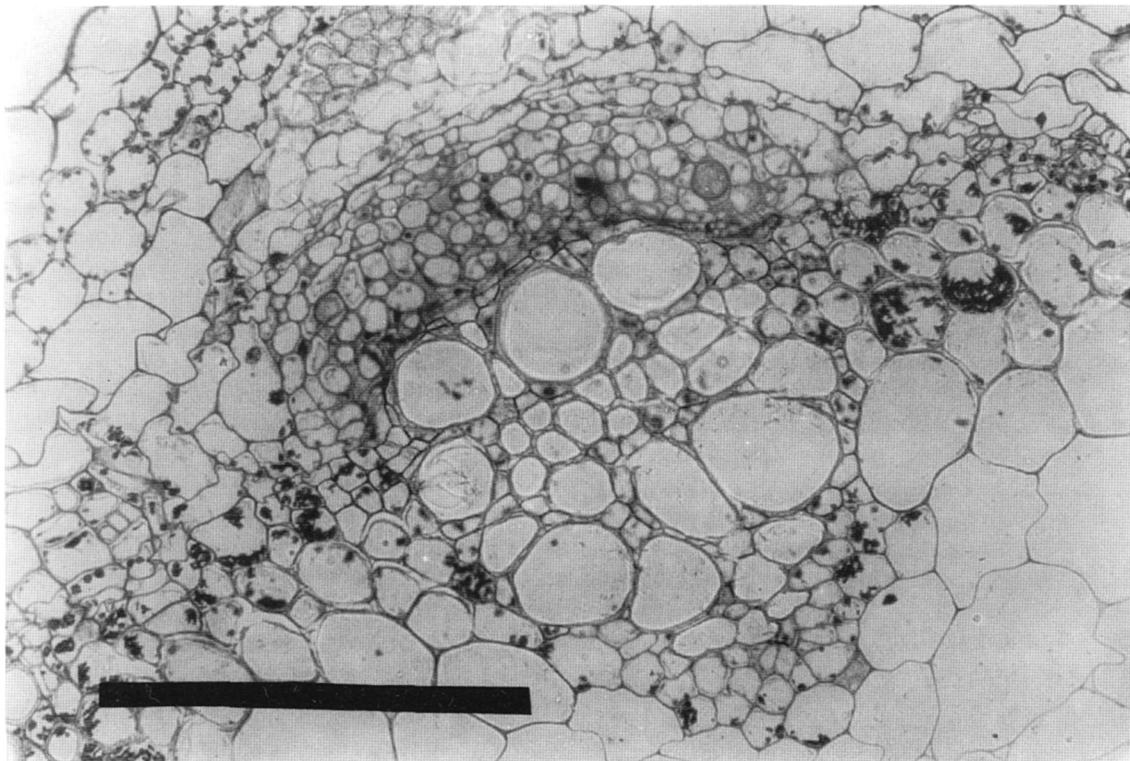Fig. 1. Transverse section of *Trifolium repens* stem. Toluidin blue staining of vascular bundles. *Bar* = 10 μm.

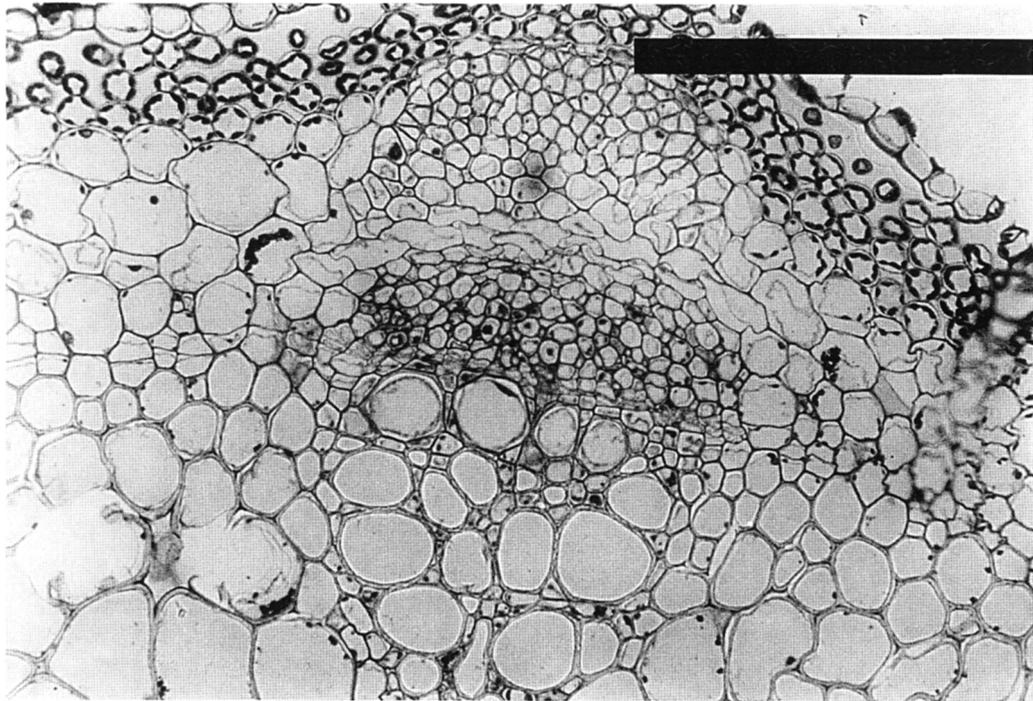Fig. 2. Transverse section of *Vicia hybrida* stem. Toluidin blue staining. *Bar* = 25 μm.

Fig. 3. Transverse section of *Vicia sativa* stem. Toluidin blue staining. *Bar* = 25 μm.

In *V. sativa* and *V. hybrida* no apparent differences existed, as concerns xylem lignification, between controls and CO_2-enriched plants. A marked difference could instead be noticed with reference to vessel lumen: in both species spring xylem produced larger vessels in ambient relative to high CO_2 (Table 1). The differences between ambient and elevated CO_2 appeared to be more marked in *V. hybrida* than in *V. sativa*.

In leaves, no differences as to lignification, nor of any

Table 1. Average size of largest vessels [μm] in bundles from stems grown in mini-FACE rings under ambient (control) and elevated CO_2. Means ± SE. Different letters in rows indicate significant differences at $P \leq 0.01$ (n = 25). No particular differences in vessel size have been noticed in *Trifolium repens* stems.

	Ambient	Elevated
V. hybrida	63.5 ± 3.1a	45.6 ± 3.8b
V. sativa	50.4 ± 2.4a	42.5 ± 3.7b

Table 2. Total protein content [mg cm^{-3}] in stems and leaves of *V. hybrida*, *V. sativa* and *T. repens* growing for two years under ambient CO_2 and elevated CO_2. Plants were sampled on May and July 2000 (Means ± SE, n = 6; all protein concentrations measured in different sampling times have been pooled to obtain the overall data here shown.

	Ambient stems	leaves	Elevated stems	leaves
V. hybrida	1.4 ± 0.1	3.2 ± 0.5	1.4 ± 0.2	2.9 ± 0.6
V. sativa	1.5 ± 0.3	5.2 ± 0.5	1.4 ± 0.2	5.1 ± 0.7
T. repens	2.1 ± 0.1	4.3 ± 0.4	1.9 ± 0.1	4.5 ± 0.4

other important anatomical feature, appeared to exist between control and CO_2-enriched plants in the three species examined (not shown).

Furthermore, no differences were recorded, within the same species, in protein contents of stems and leaves of *T. repens*, *V. hybrida* and *V. sativa*, grown under ambient or elevated CO_2 (Table 2).

Discussion

In our experiments, stem and leaf sections of three legume species were subjected to histological analysis, to directly evaluate possible differences in lignification and xylem vessel development. Lignification is the process of forming lignin, which is composed of monolignols and other phenolic units (Sederoff *et al.* 1999, Hatfield and
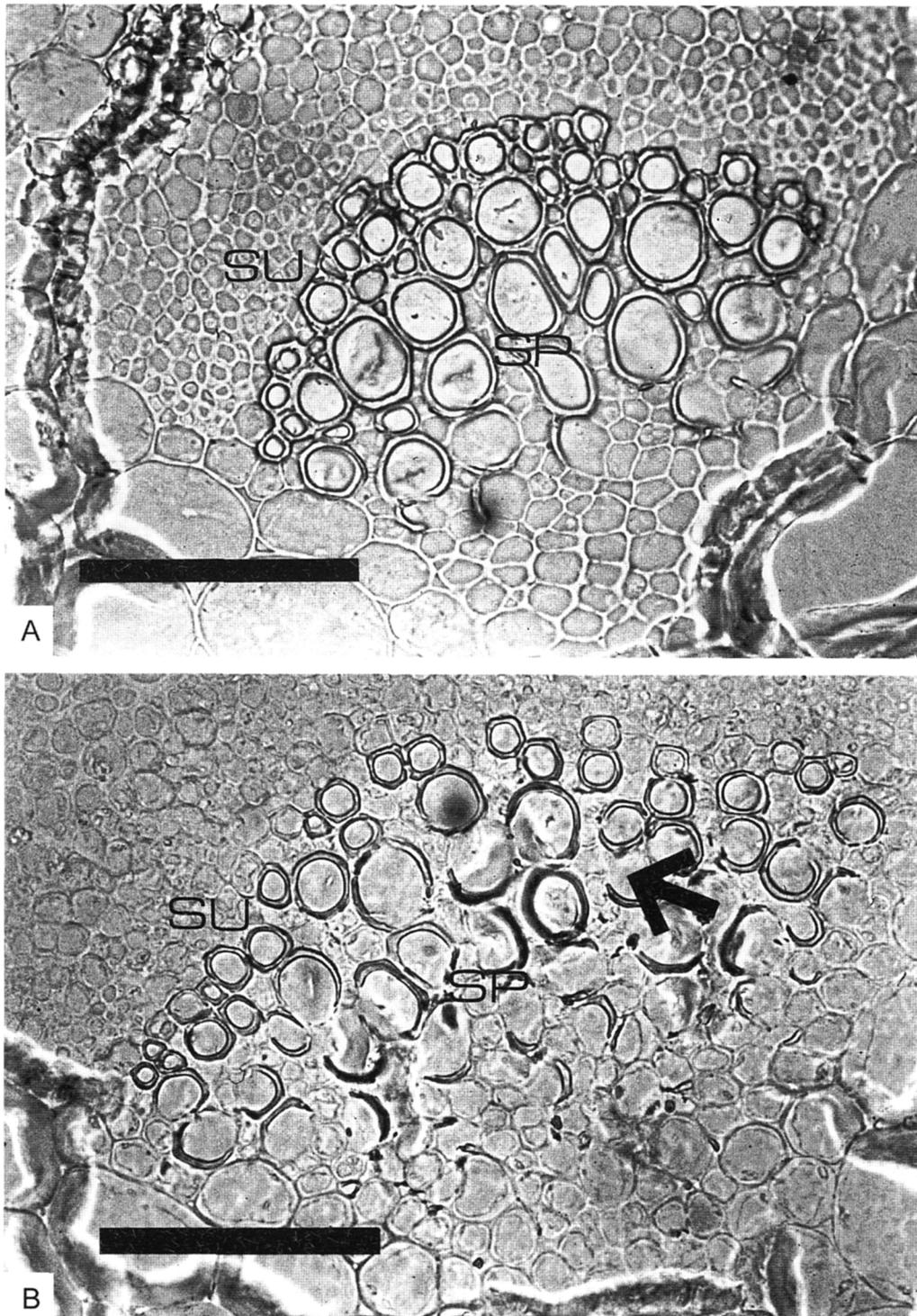
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Fig. 4. Transverse sections of *Trifolium repens* stem, growing under (A) ambient CO_2 and (B) elevated CO_2. The sections shown here, chosen as very representative of other sections, were from stems sampled on July 2000. Detail of a vascular bundle: in (A) phloroglucinol, which stains lignin alone, shows regular deposition of lignin on vessel walls. Only metaxylem elements appear not completely lignified. Vessel lumen decreases as cells develop and differentiate in late spring and early summer. Differently, in (B) reduced and irregular lignin deposition is apparent throughout the spring (the *arrow* gives one example), and only the very last vessel layer appears to have a quasi-normal wall lignification. SU - summer vessels; SP - spring vessels. *Bar* = 100 μm.

Vermerris 2001). Reduction in lignin deposition found in *T. repens* stems grown under elevated CO_2 (Fig. 4*B*) especially in spring, may imply either a CO_2-driven drop in monolignol polymerisation, or perhaps a decrease of monolignol transport from cytosol into the cell wall under high CO_2. Also in bundle sheaths from leaves of sorghum grown at ambient or 700 $\mu mol\ mol^{-1}$ CO_2, despite its C_4 metabolism, the cell walls were about two-fold thinner in elevated CO_2 than in ambient CO_2 (Watling *et al.* 2000). A general limited lignification was noticed in CO_2-enriched soybean plants as well (A. Raschi, unpublished). Furthermore, a significant drop in the lignification rate was found in *Quercus ilex* (young) leaves grown under elevated CO_2: in these experiments, lower rates of lignin production were maintained over a longer period of time under high CO_2, but the final result was an analogous degree of lignification of mature leaves grown in elevated or in ambient CO_2, respectively (Polle *et al.* 2001). This last result seems to be confirmed also by our experiments - at least as regards *T. repens* stems, in which a reduced lignification of secondary walls has been evidenced above all in the spring xylem, and *vice versa* a quasi-normal wall lignification has been found in the last layers of xylem elements, formed in summer (Fig. 4*B*).

The reduced maximum vessel lumen observed in *V. hybrida* and *V. sativa* stems under high CO_2 (Table 1) could imply that the hydraulic conductance of CO_2-enriched plants was possibly reduced, irrespective of the total porosity of the xylem tissue in the sections. The reduction in hydraulic conductance under elevated CO_2 may lead to a decreased transpiration and to a better water usage, possibly prolonging plant performances during periodic drought. However, as discussed also by Tognetti *et al.* (1999) for tree plants, if this reduction in water usage is restricted only to a certain group of species (*i.e. V. hybrida, V. sativa.* and not *T. repens*) or plant organs (stems and not leaves), the beneficial effects of elevated CO_2 on the water balance of a semi-natural grassland community would be relatively minor.

Moreover, from the results presented here, high atmospheric concentrations of CO_2 did not influence the protein content in stems and leaves of the legume species investigated (Table 2), as also noticed in other herbaceous plants grown under high CO_2 (Polle *et al.* 1993, 1997, Rao *et al.* 1995, Schwanz and Polle 1998, Sanità di Toppi *et al.* 2002a).

From the results presented here, it can be concluded that: *a*) elevated CO_2 emitted by mini-FACE rings at environmentally relevant concentrations reduces vessel diameter and lignin deposition in stems of the investigated plant species; *b*) the effects of elevated CO_2 on the stem histology of the three *Leguminosae* are species-specific and/or organ-specific, and of major account especially in the early phases of vegetative growth, in particular as regards lignin deposition mechanisms; *c*) in leaves, neither differences as to lignification nor any other anatomical structure modification were found under CO_2 enrichment.

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