SHORT COMMUNICATION

Stomatal responses of the ‘living fossil’ *Ginkgo biloba* L. to changes in atmospheric CO$_2$ concentrations

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Abstract

Leaf stomatal density and index of *Ginkgo biloba* L. were both significantly ($P < 0.05$) reduced after 3 years growth at elevated CO$_2$ (560 ppm), with values comparable to those of cuticles prepared from Triassic and Jurassic fossil *Ginkgo* leaves thought to have developed in the high CO$_2$ ‘greenhouse world’ of the Mesozoic. A reciprocal transfer experiment indicated that reductions in stomatal density and index irreversibly reduced stomatal conductance, particularly at low leaf-to-air vapour pressure deficits and low internal leaf CO$_2$ concentrations (C). These effects probably contributed to the high water-use efficiency of *Ginkgo* spp. in the Mesozoic relative to those of the present, as determined from carbon isotope measurements of extant and fossil cuticles.

Key words: Stomata, gas exchange, elevated CO$_2$, fossils.

Introduction

The gas exchange of leaves of terrestrial plants is regulated by short-term movements of the stomatal complexes in response to changes in the environment (Cowan, 1977). Further, the direct effects of atmospheric CO$_2$ on stomatal formation (Woodward, 1987) may also play a role in regulating the carbon and water balance of leaves. Studies on leaves from historical archives of material (Woodward, 1987; Peñuelas and Matamala, 1990) and the fossil record (Beerling et al., 1993; Van de Water et al., 1994), as well as from experiments (Woodward and Bazzaz, 1988), have shown a general but not universal inverse relationship between stomatal density (no. of stomatal pores per unit area of leaf) and index (proportion of stomata to epidermal cells) and atmospheric CO$_2$ concentrations.

The impact of a reduction in stomatal density, where it occurs, on leaf gas exchange remains to be determined for temperate deciduous trees growing at CO$_2$ concentrations above present, although two previous studies have indicated the potential for such effects. Woodward and Bazzaz (1988) noted correlations between stomatal density and net photosynthesis and stomatal conductance ($g_s$) for tree and shrub species growing at CO$_2$ concentrations between 225 and 340 ppm, whilst Berryman et al. (1994) reported that at 700 ppm CO$_2$ the tropical trees *Maranthes corymbosa* and *Eucalyptus tetrodonta* showed an irreversible reduction in maximum $g_s$, as a consequence of lower stomatal densities. The results reported here are from a 3 year CO$_2$ enrichment (560 ppm) experiment investigating the stomatal and gas exchange responses of the gymnosperm *Ginkgo biloba* L., an ancient taxon described by Darwin as a ‘living fossil’ and now widely planted as an ornamental species in municipal gardens (Tralau, 1968). Experimental results are compared with stomatal and isotopic (McElwain, 1997) measurements made on fossil leaves of *Ginkgoites troedssonii* Lundblad, *G. marginatus* (Nath.) Florin and *Ginkgo huttonii* (Sternberg) Heer., all with general leaf morphologies close to *G. biloba* (Harris, 1974; Czier, 1998). Following the revision of Czier (1998), the genus *Ginkgoites* is now referred to as *Ginkgo*. The fossil leaves date to the Triassic and Jurassic periods of the Mesozoic era (c. 245–64 millions of years ago), when a high CO$_2$ ‘greenhouse’ climate prevailed (Berner, 1997) and the genus had a more widespread distribution than now (Tralau, 1968; Zhou, 1997).

Materials and methods

One-year-old saplings of *Gingko biloba* of uniform size were obtained from a Sheffield nursery, repotted into 8 l containers and grown at either ambient (350 ppm) or elevated CO$_2$...
concentrations (560 ppm) in four heated greenhouses, mean temperature 25 °C, under natural irradiance throughout the experiment. Irradiance varied between 100–1500 μmol m⁻² s⁻¹, and was typically around 350–450 μmol m⁻² s⁻¹ PAR. Relative humidity within each greenhouse was maintained to a minimum of 75% using atmospheric humidifiers linked to wet and dry bulb psychrometers. The CO₂ concentrations were maintained to within ±5% of the target value by gas flow controllers and monitors (ADC 2000 series). Each of the four greenhouses was divided into two, to provide four replicated ambient and elevated CO₂ environments. Two saplings were grown in each treatment, providing eight replicates per CO₂ concentration. Treatment began in April 1995, and gas exchange measurements were made during August and September 1997. Plants were well-watered throughout to avoid drought effects. In early September a reciprocal transfer experiment was conducted where the eight plants from elevated CO₂ were transferred to the ambient CO₂ greenhouses and the ambient CO₂-grown plants were transferred to the elevated CO₂ greenhouses. Gas exchange measurements were made on both sets of plants 1, 2, 6, and 10 d after transfer to the alternative CO₂ regime.

Instantaneous gas exchange measurements and the responses of photosynthesis (A) to intercellular CO₂ concentration (Cᵢ) were determined using a portable, open gas exchange system (CIRAS-1, PP systems Ltd). The A/ Cᵢ responses were determined in saturating light (900 μmol PAR m⁻² s⁻¹), with a leaf temperature of (mean ± se) 26.3 ± 2.1 °C and a leaf-air vapour pressure deficit (LAVPD) of 0.9 ± 0.2 kPa. Maximum carboxylation efficiency and leaf CO₂ compensation point were estimated as the slope and intercept of the response on the Cᵢ axis. All measurements were made between 08.00 h and 11.00 h to avoid potential diurnal effects (Liang and Maruyama, 1994).

Leaves used for gas exchange measurements were tagged and collected for stomatal density and index counts. Counts were made on acetate impressions of the abaxial (lower) surface of two mature leaves (hypostomatous) per plant and all eight plants (n = 16 leaves per CO₂ treatment). Stomatal and epidermal cell densities were used to calculate stomatal index as [stomatal density] / [stomatal density + epidermal cell density] × 100. Ten counts per leaf were made using a Leica Laborlux microscope linked to a Quantimet 500 image analysis system with a 0.057 mm² field of view. Measurements of stomatal density and index were made using the same microscope system on cuticles prepared from Upper Triassic and Lower Jurassic fossil leaves of G. marginatus and Upper Triassic leaves of G. troedssonii from N.W. Scania (Lundblad, 1959).

Fig. 1. (a) Stomatal densities (mean ± se) and (b) stomatal indices (mean ± se) of Ginkgo biloba leaves from ambient (Amb. 350 ppm) and elevated (Elev. 560 ppm) CO₂ concentrations and those measured on prepared abaxial cuticles of Upper Triassic fossils of G. troedssonii (G.t), Upper Triassic and Lower Jurassic G. marginatus (G.m 1 and G.m 2, respectively) and Middle Jurassic G. huttonii (G.h). A significant reduction in stomatal density and index at elevated CO₂ was detected (P < 0.05) using a two-sample t-test. Stomatal density data for G. huttonii from McElwain and Chaloner (1996), stomatal index data J McElwain (unpublished results). For each set of the fossil leaves, means represent 25–70 counts. Fossil leaves of G. troedssonii and G. marginatus had low stomatal densities (7–30 mm⁻²) and indices (2–4) on the adaxial surface.

Results and discussion

After 3 years’ growth with CO₂ enrichment, leaves of G. biloba showed significant (P < 0.05) reductions in both stomatal density and index compared with their ambient grown counterparts (Fig. 1), indicating a direct effect of CO₂ on stomatal formation in this taxon. The stomatal densities of the Mesozoic fossil Ginkgo spp. leaves were consistently lower than the ambient CO₂-grown plants, but similar to those from elevated CO₂ (Fig. 1a). Furthermore, the stomatal indices of the fossils were lower than those of G. biloba developing with CO₂ enrichment (Fig. 1b). Although the influence of sun versus shade leaf preservation on the results cannot be excluded (Kürschner, 1997), the experimental and fossil data are consistent with the suggestion that the leaves examined from the Mesozoic fossil record developed under CO₂ concentrations higher-than-present (McElwain and Chaloner, 1996) which appeared to influence stomatal formation (Fig. 1b). This suggestion is in agreement with CO₂ estimates from palaeosols and a long-term carbon cycle model for these intervals (reviewed by Berner, 1997) showing concentrations of c. 900–1200 ppm between the Upper Triassic and the Middle Jurassic.

The effect of these reductions in stomatal characters on leaf stomatal conductance was assessed in a reciprocal transfer experiment. Ambient-grown plants, when transferred to elevated CO₂, showed the usual reduction in gs (Fig. 2), through CO₂ inducing partial stomatal closure (Mansfield et al., 1990). Plants grown at 560 ppm CO₂ for 3 years, after transfer to 350 ppm CO₂, showed a transient reduction in gs which increased after 10 d
Stomatal responses of *Ginkgo biloba*

Fig. 2. Time-course of stomatal conductance (mean ± sd) of *G. biloba*, after elevated CO$_2$-grown plants were transferred to ambient CO$_2$ greenhouses (●), and ambient CO$_2$-grown plants transferred to elevated CO$_2$ (○). Measurements are means of eight replicate plants.

(2) but to a lower value than plants from ambient CO$_2$ before transfer to 560 ppm CO$_2$ (Fig. 2; Table 1). These results indicate that the lower stomatal density of plants from elevated CO$_2$ effectively limited the operational $g_s$ of *G. biloba* since the changes in density and index were not associated with any significant changes in stomatal pore length (ambient plants’ pore length = 12.1 ± 0.3 μm, elevated plants’ pore length = 11.5 ± 0.5 μm, values are means ± s.e, n = 48 measurements per treatment). A similar finding has been reported for the tropical trees *M. corymbosa* and *E. tetrodonta* grown under elevated CO$_2$ (Berryman et al., 1994).

Furthermore, measurements of $g_s$ made on both sets of plants after 10 d and across a wide range of LAVPDs and intercellular CO$_2$ concentrations ($C_i$) showed this ‘limiting’ effect of low stomatal density to be most marked at low LAVPDs and low $C_i$ values (Fig. 3) when near-maximal stomatal opening would be expected (Jones, 1992). The reduction in stomatal density and index, with associated effects on $g_s$, contributes a previously unrealized explanation for the marked (200%) increase in leaf water-use efficiency (WUE, $A/E$) of *G. biloba* when grown in the short-term (4 months) with CO$_2$ enrichment and measured across a wide range of leaf water potentials (−1.0 to −0.4 MPa) (Liang et al., 1995).

Measurements of $g_s$ and stomatal density and index made on the same leaves of plants from elevated CO$_2$ were significantly correlated (stomatal density versus $g_s$, n = 8, r = 0.62, P < 0.01; stomatal index versus $g_s$, n = 8, r = 0.75, P < 0.01)—a relationship absent from

![Fig. 3. Stomatal conductance of *G. biloba* leaves grown at (a) ambient (350 ppm) and (b) elevated (560 ppm) CO$_2$ concentrations and measured across a range of leaf-to-air vapour pressure deficits (LAVPD) and internal leaf CO$_2$ concentrations ($C_i$).](https://academic.oup.com/jxb/article-abstract/49/326/1603/532170)

Table 1. Responses of photosynthesis ($A$, μmol m$^{-2}$ s$^{-1}$) and stomatal conductance ($g_s$, mmol m$^{-2}$ s$^{-1}$) of *Gingko biloba* before and 10 d after the reciprocal transfer experiment

<table>
<thead>
<tr>
<th>Measurement</th>
<th>CO$_2$ environment</th>
<th>Growth CO$_2$ environment</th>
<th>Ambient CO$_2$ (350 ppm)</th>
<th>Elevated CO$_2$ (560 ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$A$</td>
<td>$g_s$</td>
<td>$A$</td>
</tr>
<tr>
<td>Ambient CO$_2$</td>
<td>4.9 ± 0.8</td>
<td>116.3 ± 22.0</td>
<td>4.4 ± 1.0</td>
<td>92.0 ± 14.0</td>
</tr>
<tr>
<td>Elevated CO$_2$</td>
<td>5.8 ± 1.0</td>
<td>68.0 ± 9.0</td>
<td>7.8 ± 0.6</td>
<td>105.9 ± 25.4</td>
</tr>
</tbody>
</table>
ambient-grown plants. This correlation suggests that under elevated CO\textsubscript{2}, the total number of stomata on a leaf per se becomes an important regulatory component of leaf gas exchange in G. biloba and, by extension to other taxa showing a reduction in density with CO\textsubscript{2} enrichment, might explain the positive correlation between discrimination against the heavy isotope of carbon, 13C, and stomatal density for Plantago major populations (Griffiths, 1996) and needles of Pinus sylvestris (Beering, 1998).

A/C\textsubscript{4} response curves analyses indicated no significant differences in the photosynthetic characteristics of G. biloba after 3 years' growth at ambient and elevated CO\textsubscript{2} concentrations (Table 2) with no effects of the reduction in stomatal density and index on photosynthetic rates and no down-regulation of the photosynthetic system (Table 2). The lack of down-regulation in G. biloba might be expected since its leaves have high total non-structural carbohydrate (TNC) and starch contents at ambient CO\textsubscript{2} which only show small increases (10–11\%) with CO\textsubscript{2} enrichment (600 ppm) (Körner et al., 1995). Accumulation of TNC in leaves has been suggested as underlying down-regulation of the expression of photosynthetic genes to elevated CO\textsubscript{2} (Drake et al., 1997).

These results suggest that the growth of Ginkgo under the high CO\textsubscript{2} concentrations of the Mesozoic resulted in low stomatal densities and indices (Fig. 1) with inferred lower leaf conductances (Figs 2, 3) and higher water-use efficiencies (WUE). To test this suggestion, estimates of leaf WUE, based on stable carbon isotope analyses, of modern Ginkgo in Egham, Surrey were compared with those of Middle Jurassic Ginkgo from the Ravenscar Group, Yorkshire (McElwain, 1997) (Table 3). The results indicate that Jurassic Ginkgo trees did indeed operate with a much higher WUE than their modern counterparts (Table 3), as expected from the gas exchange data. The estimated proportion of this increase in WUE attributable to a 30\% reduction in stomatal density

### Table 2. Photosynthetic characteristics of leaves of Gingko biloba grown at ambient and elevated CO\textsubscript{2} concentration for 3 years

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Growth CO\textsubscript{2} concentration</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Ambient CO\textsubscript{2}(350 ppm)</td>
</tr>
<tr>
<td>A\textsubscript{max} (\textmu mol m\textsuperscript{-2} s\textsuperscript{-1}) (light- and CO\textsubscript{2}-saturated rate of photosynthesis)</td>
<td>26.2 ± 2.3</td>
</tr>
<tr>
<td>CO\textsubscript{2} compensation point (\textmu mol mol\textsuperscript{-1})</td>
<td>95.6 ± 6.2</td>
</tr>
<tr>
<td>Maximum carboxylation efficiency (m\textsuperscript{-2} s\textsuperscript{-1} mol\textsuperscript{-1})</td>
<td>0.025 ± 0.003</td>
</tr>
<tr>
<td>Dark respiration rate (\textmu mol m\textsuperscript{-2} s\textsuperscript{-1})</td>
<td>2.55 ± 0.4</td>
</tr>
</tbody>
</table>

No significant effects of CO\textsubscript{2} were detected by two-sample t-tests.

### Table 3. Stable carbon isotope composition and inferred water-use efficiencies (WUE in nmol CO\textsubscript{2}mol H\textsubscript{2}O) of fossil and modern leaves of Gingko spp.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>(\delta^{13}C_{p}) (ppm)</th>
<th>(\delta^{13}C_{s}) (ppm)</th>
<th>(\Delta)</th>
<th>CO\textsubscript{2} concentration (ppm)</th>
<th>WUE (1000)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mid-Jurassic fossils</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G. hattori</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole leaves</td>
<td>-23.97 ± 0.22</td>
<td>-5.5</td>
<td>18.9 ± 0.22</td>
<td>1500</td>
<td>335.6 ± 9.6</td>
</tr>
<tr>
<td>Cuticles</td>
<td>-25.57 ± 0.27</td>
<td>-5.5</td>
<td>20.6 ± 0.27</td>
<td>1500</td>
<td>265.3 ± 12.0</td>
</tr>
<tr>
<td>Modern</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G. biloba</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole leaves</td>
<td>-26.44 ± 0.06</td>
<td>-8.0</td>
<td>18.9 ± 0.06</td>
<td>356</td>
<td>79.1 ± 0.7</td>
</tr>
<tr>
<td>Cuticles</td>
<td>-27.67 ± 0.32</td>
<td>-8.0</td>
<td>20.2 ± 0.32</td>
<td>356</td>
<td>66.5 ± 3.2</td>
</tr>
</tbody>
</table>

\(\delta^{13}C_p\): Discrimination against 13CO\textsubscript{2} (\(\Delta\)) calculated as (\(\delta^{13}C_p - \delta^{13}C_p\))(1 + \(\delta^{13}C_p\)/1000) (Farquhar et al., 1989).

\(\delta^{13}C_s\): Modern value from Levin et al. (1994).

\(\Delta\): Discrimination against 13CO\textsubscript{2} (\(\Delta\)) calculated as (\(\delta^{13}C_p - \delta^{13}C_p\))(1 + \(\delta^{13}C_p\)/1000) (Farquhar et al., 1989).
(Fig. 1), calculated using a coupled leaf energy balance-gas exchange model (Beerling and Woodward, 1997), was $c. 25\%$ (at 25°C, 700 µmol m$^{-2}$ s$^{-1}$ PAR, 75% relative humidity and 1500 ppm CO$_2$). It is interesting to note that Ginkgo underwent a striking range contraction after the Pliocene (Tralau, 1968), a time when atmospheric CO$_2$ concentrations had fallen dramatically since the lower Cretaceous when it occupied a more widespread geographical distribution. The majority of this decline is probably attributable to G. biloba being dioecious, a rather frail form of reproductive biology relative to angiosperms and conifers, but the results of this study suggest CO$_2$ effects may also have played a contributory role. Future increases in the concentration of atmospheric CO$_2$ may contribute to restoring the function of this ‘living fossil’ species back to that more representative of its long geological history.

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